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Oxytocin-like signaling in ants influences metabolic gene expression and locomotor activity

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Abstract

Ants are emerging model systems to study cellular signaling because distinct castes possess different physiologic phenotypes within the same colony. Here we studied the functionality of inotocin signaling, an insect ortholog of mammalian oxytocin (OT), which was recently discovered in ants. In *Lasius* ants, we determined that specialization within the colony, seasonal factors, and physiologic conditions down-regulated the expression of the OT-like signaling system. Given this natural variation, we interrogated its function using RNAi knockdowns. Next-generation RNA sequencing of OT-like precursor knock-down ants highlighted its role in the regulation of genes involved in metabolism. Knock-down ants exhibited higher walking activity and increased self-grooming in the brood chamber. We propose that OT-like signaling in ants is important for regulating metabolic processes and locomotion.

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Author Contributions

Z. Liutkevičiūtė performed RNA extraction, qPCR, knock-down, and behavior experiments; E. Gil-Mansilla performed dissections and immunostaining experiments; T. Eder and T. Rattei performed the transcriptome analysis; T. Eder and F. Grebien performed transcript/GO analyses; B. Casillas-Pérez analyzed recordings of the walking assay and performed statistical analyses together with Z. Liutkevičiūtė; M. G. Di Giglio and E. Muratspahić performed the pharmacological assays; Z. Liutkevičiūtė, E. Gil-Mansilla, T. Eder, M. G. Di Giglio, and E. Muratspahić prepared the figures; M. Muttenthaler synthesized the peptides; Z. Liutkevičiūtė, M. Muttenthaler, S. Cremer, and C. W. Gruber designed the experiments; Z. Liutkevičiūtė and C. W. Gruber wrote the manuscript, with contributions of E. Gil-Mansilla, T. Eder, B. Casillas-Pérez, and S. Cremer; and all authors commented and approved the submitted version of the manuscript.

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Keywords

vasopressin; vasotocin; inotocin; insect; G protein–coupled receptor

Neuropeptides and regulatory peptide hormones control many important developmental, physiologic, and behavioral processes in animals (1). Oxytocin (OT) and vasopressin (VP) neuropeptides and their GPCRs comprise one of the oldest and best-studied peptidergic signaling systems. It has been present across a range of distantly related species throughout the evolution of animals, dating back more than 600 million years (2, 3). In humans and other mammalian species, OT and VP mediate a range of peripheral and central physiologic functions by signaling *via* 1 OT and 3 VP receptors (4, 5). In contrast, fish can contain a greater number of OT/VP-like receptors and peptides, and invertebrates generally express only 1 peptide and 1 receptor. All vertebrate OT- and VP-like peptides are considered to have evolved from the ancestral nonapeptide vasotocin by gene duplication (6, 7).

In mammals, the hormone OT in the periphery is important for reproduction by triggering myometrial muscle contraction during childbirth, for milk ejection during breast feeding, and for male ejaculation. OT acts centrally as a neurotransmitter and is involved in complex social behaviors such as trust, empathy, maternal care, and bonding as well as stress and anxiety. VP regulates fluid balance and blood pressure in the periphery and is centrally implicated in memory and learning but also in stress-related and aggressive behavior (5). There has been an increasing amount of evidence that OT signaling regulates feeding behavior and appetite in animals and is involved in the maintenance of energy homeostasis and metabolism. For instance, OT inhibits food intake, increases energy expenditure and lipolysis, regulates gastric motility, and improves glucose tolerance by effects on the pancreas in rodents (8). Due to this physiologic importance, ligands targeting the OT and VP receptors have potential therapeutic applications (8–12).

OT/VP-like peptides have been identified and studied in several invertebrate species, including molluscs, annelids, nematodes, and arthropods (13). It has been suggested that OT/VP-like signaling in invertebrates resembles its role in humans and other vertebrates (13, 14). In the periphery, OT/VP-like peptides act as myoactive compounds, stimulating contractions in reproductive and cardiovascular tissues and regulating fluid homeostasis. In the CNS, they act as neuromodulators and are involved in many aspects of behavior as well as learning and memory. However, information of their biologic functions in invertebrates is limited due to great biodiversity and a lack of well-established model systems. Most previous studies of the OT/VP signaling system in invertebrates were based on transcript analysis and immune-localization of the receptors or peptides, *in vitro* cell culture assays, or observations of behavioral changes upon artificial peptide administration (13). The most comprehensive genetic and behavioral studies were performed in the model nematode *Caenorhabditis elegans*. In these worms, OT/VP-like (nematocin) signaling controls mate searching and mating behaviors in males as well as gustatory learning processes that regulate food preference (15, 16). In addition, nematocin appears to regulate parental–offspring social behavior (17).

Despite the global importance and enormous variety of insect species, the OT/VP-like signaling system has been studied in very few insects, mainly the red flour beetle *Tribolium castaneum* (18, 19) and the migratory locust *Locusta migratoria* (20, 21). It was suggested that the inotocin (insect OT/VP-like) signaling system may be involved in larval development and water homeostasis. Recently it has become evident that most insect orders contain components of this peptidergic signaling system (3, 7). We identified transcripts encoding the inotocin receptor (IR) and the inotocin precursor (IP) in 20 ant species (3, 22). Furthermore, inotocin was shown to activate its cognate receptor *in vitro* in the ant *Lasius niger* (23), and expression of the receptor was confirmed in the heads of queens (24).

Ants are eusocial insects characterized by reproductive division of labor, cooperative brood care, and overlapping generations (25). Since the sequences of several ant genomes became available, tremendous efforts have been taken to understand these fundamental processes of ant biology at the molecular level (26, 27). Therefore, the discovery of the OT/VP-like inotocin signaling system in ants allows studies of its role in individual physiology but also of its involvement in social organization in ant colonies. Hence, we were particularly interested to study the function of this complex neuropeptide signaling pathway in *Lasius* ants. We analyzed the expression and diversity of the inotocin signaling system throughout the different castes, developmental stages, and parts of the ant body. Most importantly, we studied the functionality of inotocin signaling *in vitro* and determined its role in individual ant physiology *in vivo* using IP knock-down (IP-KD) worker ants, comparative transcriptomics, behavioral experiments, and physiologic measurements.

Materials and Methods

Ants

L. niger queens (mated and virgin) and males were collected during their mating flight in the United Kingdom (Egham, Surrey, 2010) (28) and Austria (Vienna area, 2015) and reared in the laboratory to establish colonies. Only workers from >1-yr-old colonies were used for experiments. *L. neglectus* colonies were collected from the Jena Botanical Garden (Jena, Germany) in 2015 and 2016, as reported by Ugelvig *et al.* (29), and reared in the laboratory in nests containing 5–10 queens and 500–2000 workers. Virgin queens and males were collected from the Jena Botanical Garden, Germany, in 2015. Males were also obtained from *L. neglectus* colonies and reared in the laboratory in 2016 and 2017. Virgin queens were kept in the laboratory for at least 1 d before the experiments to be sure they were not mated and remained winged. Ants were fed *ad libitum* 30% sucrose solution and minced cockroaches. Ants were kept under a day/night light and temperature cycle. For all the experiments, if not specified otherwise, ants were collected from foraging areas during summer-day conditions. Summer conditions were maintained for 7 mo (d: 14 h at 27°C; night: 10 h at 21°C), autumn conditions for 1 mo (d: 10 h at 15°C; night: 14 h at 10°C), winter conditions for 3 mo (d: 10 h at 8°C; night: 14 h at 5°C), and spring conditions for 1 mo (d: 10 h at 15°C; night: 14 h at 10°C). For developmental analysis of gene expression, larvae were separated into 5 groups based on their length as follows: *L. neglectus* first group: 0.9–1.2 mm, second group: 1.3–1.7 mm, third group: 1.8–2.1 mm, fourth group: 2.2–2.6 mm, fifth group: 2.7–3.3 mm; *L. niger* first group: 0.8–1.2 mm, second group: 1.3–1.6 mm, third group: 1.7–2.3 mm, fourth

group: 2.4–2.9 mm, fifth group: 3 mm. Ants from summer and winter conditions were collected at the same time from 2 colonies. Summer and winter colonies were obtained from the same year of collection (2014), divided into 2 colonies after collection, and kept in the laboratory for 2 yr before the experiments and for at least 1 mo in winter or summer conditions.

Molecular cloning and *in vitro* pharmacology

The IP and IR of *L. neglectus* were identified by transcriptome mining and have been deposited in GenBank (National Center for Biotechnology Information, Bethesda, MD, USA; <https://www.ncbi.nlm.nih.gov/genbank/>; MG210941, MG210942). They were cloned into expression constructs according to a recent publication for *L. niger* (23). Similarly, synthesis of native and tritiated peptides, functional receptor activation assays, saturation bindings, and cellular receptor imaging were performed as previously described (23, 30, 31).

Dissection of organs

Before dissections, ants were cooled on ice (immunostaining experiments) or killed by freezing at -80°C or immersion in liquid nitrogen [dissections for quantitative PCR (qPCR)]. Organs were dissected on ice in moth solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 5 mM CaCl_2 , 4 mM NaHCO_3 , 5 mM Hepes, pH 7.2) for immunostaining or in PBS for qPCR.

Whole mount immunostaining and confocal microscopy

Dissected organs were fixed in fresh 4% paraformaldehyde in PBS for 1 h at room temperature. Tissues were washed 3 times for 5 min with 1% Triton in PBS at room temperature and blocked with 10% normal goat serum in 1% Triton in PBS for 45–60 min at room temperature. Then organs were incubated with the primary rabbit anti-rat VP antibody (PC234L; Calbiochem, San Diego, CA, USA), which was shown to recognize insect inotocin (18), and diluted 1:700–1:1000 in PBS containing 0.1% Tween and 2% normal goat serum for 2 d at 4°C . To test the specificity of the primary antibody, absorption controls were incubated with a primary antibody solution preabsorbed with 10 $\mu\text{g}/\text{ml}$ synthetic VP for 1 d at 4°C . Subsequently, tissues were washed 3 times 5 min with 0.1% Tween in PBS and incubated with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (A-11008; Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:800–1:1000 with 0.1% Tween in PBS overnight at 4°C . After rinsing the secondary antibody 3 times for 5 min with 0.1% Tween in PBS at room temperature, nuclei were stained with 1 $\mu\text{g}/\text{ml}$ DAPI in PBS for 10 min in the darkness followed by washing 3 times with PBS for 5 min. Finally, organs were mounted with permafluor or 100% glycerol and stored at 4°C or -20°C , respectively. Preparations were analyzed with a confocal microscope (A1; Nikon, Tokyo, Japan) using excitation wavelengths of 405 nm for DAPI, and 488 nm for inotocin staining, respectively. Captured images were processed with NIS-Elements software (Nikon).

RNA isolation, reverse transcription and quantitative PCR

RNA extractions of single ants or organs in pools of 2–10 were carried out using the Quick-RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA). Homogenization of the samples was

performed in 350 µl of lysis buffer with 2–4 BashingBeads (Zymo Research) using the Precellys 24 Homogenizer (PepLab, Erlangen, Germany) at 6000 rpm (2–3 times, 30 s). DNaseI treatment was performed after eluting RNA from columns prior to reverse transcription accordingly: samples (50 µl) in 0.5 times DNaseI buffer (B43; Thermo Fisher Scientific) were incubated with 1 U of DNaseI for 20 min at room temperature following the inactivation of DNaseI by adding EDTA (2.5 mM final concentration) and heat denaturation at 70°C for 10 min. The purity and concentration of RNA were determined by optical density measurements on a Nanodrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Gene expression analyses were performed using Fast SYBR Green Master Mix (Applied Biosystems) or SSoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) together with 0.2 µM of each specific primer (Supplemental Table 1) (Merck, Darmstadt, Germany) with a StepOne real-time PCR machine (Applied Biosystems) operating in Fast mode. The program used for amplification was the following: 95°C for 20 s, followed by 40 cycles of 3 s of 95°C denaturation and 30 s of 62°C annealing/extension in 15 µl reaction volume for Fast SYBR Green Master Mix (Applied Biosystems) or 95°C for 30 s, followed by 40 cycles of 5 s of 95°C denaturation and 30 s of 62°C annealing/extension in 10–15 µl reaction volume for SSoAdvanced Universal SYBR Green Supermix (Bio-Rad). Initially, qPCR samples were analyzed using 2 housekeeping genes: transcription elongation factor 1 (EF1) and glyceraldehyde-3-phosphate dehydrogenase. After determining the expression ratio per nanogram of RNA of those 2 genes, we excluded glyceraldehyde-3-phosphate dehydrogenase from further analyses because it yielded unusual and higher (>30-fold) expression ratios in some samples (e.g., in the thorax of males and workers). On the other hand, expression of EF1 was comparably stable across all tested samples (variation was <3.5-fold). This was considered acceptable and sufficient for our analysis because all conclusions made are based on large variation (10-fold or higher). Primer efficiency was found to be between 93 and 106% for all primer sets and both Master Mix using standard curves of 10-fold dilutions. Primer specificity was monitored based on melting curve analysis after each run. Relative gene expression was calculated using the 2^{-C_t} method (32). Expression was normalized to the housekeeping gene EF1 and is presented as the fold-difference between the lowest expressed sample (IP of *L. neglectus* worker abdomen). Additionally, the results from the 2^{-C_t} method were confirmed by calculations using the LinRegPCR program (33). All primer sequences are shown in Supplemental Table 1.

Starvation experiments

L. neglectus workers from the same nest were reared in groups of ~140 individuals each. After 3 d, they were treated under either control conditions or starvation. We collected and froze 20 ants per group before treatment (i.e., after the 3 d in their respective box; baseline, d 0 of the experiment). Food (30% sucrose solution) was removed from the starvation treatment group after the collection of the baseline sample, and the ants were left only with water for 2 d. We then collected 20 ants from each group (d 2). We then fed both groups with sucrose and collected 20 ants from each group after another 1 and 6 d (d 3 and 9, respectively). Ants were collected at the same time (12 PM). RNA extraction, RT-PCR, and

qPCR were performed as described above. For both IP and IR, we performed 2-way ANOVAs with treatment, day (0, 2, 3, 6), and their interaction. In case of significant interaction (IP), we performed Tukey's HSD *post hoc* tests with Benjamini-Hochberg correction to determine on which days the 2 treatments differed significantly from one another. Statistical analyses were carried out in R v.3.3.2 (34).

Transcript knock-down experiments

Double-stranded RNA (dsRNA) probes were prepared using the TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific) and specific PCR products for IP and control [green fluorescent protein (GFP)]. PCR products, with T7 RNA polymerase sequences at both ends, were prepared with specific primers (Supplemental Table 1) from DNA templates (pEGFP-N1 plasmid for GFP control and PCR fragment of full-length IP) using standard PCR conditions (DreamTaq Green DNA Polymerase; Thermo Fisher Scientific). PCR products were used directly without any purification step to synthesize RNA. After RNA synthesis and treatment with DNaseI (included in the transcription kit), RNA probes were purified using the MEGA-clear Transcription Clean-Up Kit (Thermo Fisher Scientific). The concentration and quality of the dsRNA was determined using a Nanodrop spectrophotometer and gel electrophoresis. At the final preparation step, dsRNA was annealed by heating the probes at 85–90°C and letting them cool slowly to room temperature. Annealed dsRNA was mixed with sucrose solution to obtain the desired (1–2 µg/µl) concentration of probes in 10% (w/w) sucrose, which were measured in “ready to feed” aliquots, stored at –20°C, and thawed not more than twice. Feeding experiments were carried out similar to Ratzka *et al.* (35). Briefly, ants were reared in groups of 3–15 and left without food for 5 h to 3 d. Then, every second day ants were supplied 7–10 µl of 1–2 µg/µl dsRNA in 10% sucrose solution for a period of 4–15 d. dsRNA probes against GFP were used to feed the control group. The change in expression was analyzed by qPCR after RNA extraction of whole animals as previously described. We did not observe differences operating distinct dsRNA concentrations (1–2 µg/µl) and starvation periods (5 h up to 3 d) prior to dsRNA probe feeding. For behavioral and mRNA-seq experiments we used 5 h for starvation before starting feeding with 1 µg/µl dsRNA in 10% sucrose solution.

Quantitative sequencing of mRNA, differential gene expression, and gene ontology enrichment analyses

Three samples of IP-KD and 4 control (CTRL) samples fed with dsRNA against GFP were sequenced (Supplemental Table 2). Each sample comprised a pool of 3–5 ants. RNA was extracted from a single ant, expression of precursor was analyzed by qPCR, and RNA was repurified in a pool. RNA was enriched for polyA sequences using the Dynabeads mRNA Purification Kit for mRNA from total RNA (Ambion, Austin, TX, USA). cDNA and dsDNA libraries were prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) and were quantified by Kapa qPCR (Kapa Biosystems, Charlestown, MA, USA). Libraries were sequenced on an Illumina HiSeq2500 Sequencer, applying PE50 rapid mode. Raw sequencing reads were processed with FastQC (v.0.11.4) (36) for quality assessment (Supplemental Table 2). Quality trimming and filtering, as well as length filtering after trimming, was done with Prinseq-lite (v.0.20-4) (37). Remaining high-quality sequence reads were mapped against an *L. neglectus*

transcriptome assembly (Cremer, Eder, and Rattei, unpublished data) using BWA (v.0.7.12) (Supplemental Table 2) (38). Post-processing and counting of aligned reads per transcript were done with SAMtools (v.0.7.12) (39). For the annotation of the *L. neglectus* transcripts we used blast+ (v. 2.6.0) (40, 41) and InterProScan (v.5.22-61) (42) in combination with Blast2GO (v.4.1.5) (43). Differential expression analysis was done with edgeR (44), and the gene ontology (GO) enrichment analysis for the differential expressed transcripts was carried out *via* topGO (45). PCA plot of IP-KD *vs.* CTRL transcriptomes was generated based on edgeR normalized expression data with factoextra (46) in R (34).

Behavioral experiments

The test for changes in the ants' behavior in a group and in isolation after IP knockdowns were recorded using StreamPix digital video recording software (NorPix, Montreal, QC, Canada) with 4 cameras in parallel. To observe behavior in the group, 4 dishes were recorded simultaneously (diameter, 5.5 cm; 1 dish per camera; *i.e.*, 2 GFP controls and 2 IP-KD dishes per replicate time point). For the individual ant activity assay, 16 dishes were recorded simultaneously (diameter, 3.5 cm; 4 dishes per camera; *i.e.*, 2 controls and 2 IP-KD dishes on the same camera, 8 GFP controls and 8 IP-KD dishes per replicate time point). During the experiments, including days of feeding, ants were kept at 27°C and on a 12-h light/dark cycle. Video recording was performed at 28°C (behavior in the group experiment) or 25°C (walking assay).

Behavior in the group experiment

Nine nestmate workers were reared in a group in plastered Petri dishes (diameter, 9 cm) and fed dsRNA against the IP or GFP for 7 d. On d 7, 5 ants were transferred to a new dish (diameter, 5.5 cm) containing a brood chamber with 5–10 middle-size larvae covered with transparent foil, a cricket (protein source for larvae), and sucrose solution with dsRNA. On the following day, videos were recorded for 2 h. Afterward, ants were frozen immediately at –80°C, and each ant was analyzed by qPCR to check for the success of the IP-KD. Recordings of 24 independent replicates were performed in the mornings or evenings: 12 replicates at the last hour of darkness (the whole Petri dish was covered with a red foil) and at the first hour of light and 12 replicates at the last hour of light and the first hour of darkness. qPCR analyses confirmed statistically significant knockdown in 5 replicates from the morning, and 7 replicates from the evening ($P < 0.03$, Mann-Whitney *U* test). Only these significant knockdowns were analyzed for their behavioral changes. We observed the ants for 10 s every 5 min. During each observation, each ant was assigned a score of 1 for its location (outside or brood chamber) and for performing a certain type of behavior, namely trophallaxis, allogrooming, antennation (moving antenna, but no change of position), self-grooming, walking (changing position in the dish), feeding (both sugar solution and cricket), and resting (not active: sitting still and not moving at all). If the ant changed behavior or its location during the 10 s observation period, it received multiple scores; a change of behavior in the observation time window was quite rare and accounts only for 6% of all scores. The sum of scores was analyzed separately for dark and light periods as well as for morning and evening recordings. Because we could not detect differences between light and dark or between morning and evening ($P > 0.5$, Mann-Whitney *U* test), all data were presented together (morning, 5 replicates; evenings, 7 replicates), and statistical analyses were carried

out in R v.3.3.2. For each behavior (with the exception of feeding, which only occurred outside the brood chamber), we tested the main effects (predictors) of location (inside/outside the brood chamber) and treatment (GFP control/IP-KD treatment) and their interaction. We included time of day as random intercept. All models were compared with their null model (containing only the intercept and random effect) to obtain an overall significance value using likelihood ratio tests (47). Next, we tested whether the interaction was significant by comparing the full model with a reduced model without the interaction. When the interaction was not significant, the model was rerun without the interaction to receive better estimates of the main effects. In cases where the interaction was significant (self-grooming and trophallaxis), the main effect *P* values cannot be interpreted reliably; therefore, we followed with 2 separate tests for the effect of treatment separately for the ants inside. The number of statistical tests performed per experiment was controlled to protect against a false-discovery rate using the Benjamini-Hochberg procedure ($\alpha = 0.05$), and adjusted *P* values are reported. We checked the distribution of model residuals, linearity of residuals against each variable, collinearity between fixed and random effects, and influential data points and found no evidence of violated assumptions. For mixed effects modeling, we used the packages “lme4” (48). All logistic regressions were performed using either generalized linear models or generalized linear mixed models, which had binomial error terms and logit-link function. One replicate was removed of IP-KD group (only in location “inside brood chamber”) due to obstruction of the brood chamber during the observation period. Statistical results of each behavior are shown in Supplemental Table 3.

Walking assay

Six ants were isolated from a colony and fed dsRNA against IP or GFP for 9–13 d in a plastered Petri dish (diameter, 5.5 cm). Then 1 ant was isolated onto a new plastered dish (diameter, 3.5 cm) and recorded for 60 min. Motion trajectories were obtained from video using Ferda software v.2.2.10 (Naiser and Matas, unpublished results). Behavioral parameters were computed using custom scripts written in MatLab R2015a (MathWorks, Natick, MA, USA): 1) episode number (number of walking episodes), 2) episode duration [average time between onset and offset of walking episode (ms)], 3) walking speed [mean instantaneous velocity per episode (mm/s)], 4) walking time [number of frames between episode onset and offset divided by the frame rate (s)], and 5) distance moved [sum of displacements during all walking episodes (mm)]. Statistical analysis was carried out using R v.3.3.2. We analyzed the effect of knockdowns on each behavioral parameter (episode number, duration, walking speed, walking time, and distance moved) using generalized linear mixed models “lme4” R package (49) with feeding treatment (IP-KD or GFP control) as predictor and including day as a random intercept to control for the time ants have been isolated from the colony and fed the dsRNA probes. We ensured all data fit the assumptions of the model (*i.e.*, normality of residuals, Cook’s distance, dffits, dfbetas, and leverage). We square-root transformed response variables to reach normality, except for speed, which was already normally distributed. We tested the effect of treatment by comparing each model with a null model with a likelihood ratio test. To control for multiple testing, we corrected the resulting *P* values using the Benjamini-Hochberg procedure to protect against a false-discovery rate of 0.05% (50). Adjusted *P* values are reported in Supplemental Table 4.

Feeding experiment

Six nestmate workers were taken from their nest and jointly fed dsRNA against IP or GFP for 10 d in a plastered Petri dish (diameter, 5.5 cm). Afterwards, individual ants were isolated onto a new nonplastered dish (diameter, 3.5 cm) and left without food or water for 3.5 h at 27°C. Then food was introduced [5 µl of 10% sucrose and 5% erioglaucine (FD&C Blue No. 1; MilliporeSigma Burlington, MA, USA) in a 200-µl tube], and the following parameters were monitored: 1) how much time the ants need to find the food (the food was considered detected when the ant started feeding or touched the food with the antenna), 2) the amount of food eaten, measured similar to the method described in Koto *et al.* (51) (see below), and 3) number of ants (replicates) eating or being near the food 0.5 and 1.5 h after the food was introduced. An ant was considered to be “near the food” when it was in the tube with the feeding solution but not feeding. Ten IP-KD ants and 10 GFP controls were monitored in parallel. Ants observed once for feeding received a score of 1, ants found to be feeding twice (both time points) received a score 2, and ants that were not feeding received a score of 0 [n (control) = 88, score 1 (control) = 7, score 0 (control) = 81; n (IP-KD) = 89, score 1 (IP-KD) = 12, score 2 (IP) = 3, score 0 (IP-KD) = 74]. Statistical analyses were done with GraphPad Prism (GraphPad Software, La Jolla, CA, USA) using a Mann-Whitney *U* test. To calculate the percentage of ants feeding or being near the food, the same ant behaving equally (feeding or being near the food) during both measurements was counted only once.

Physiologic measurements

The amount of food eaten was measured similarly to the method described in Koto *et al.* (51). Briefly, ants were fed 10% sucrose and 5% erioglaucine (FD&C Blue No. 1), which enabled us to measure calorimetrically the volume eaten by ants. Ants were killed by freezing at −20°C, and each ant was homogenized in 350 µl Lyses buffer [Quick-RNA MiniPrep Kit (Zymo Research) with 2 BashingBead (Zymo Research) using a Precellys 24 Homogenizer at 6000 rpm (3 times 30 s)]. After the centrifugation (16,000 *g*, 15 min, room temperature), 300 µl was transferred to measure the amount of blue dye at 633 nm using a spectrophotometer. The amount eaten (nl) was calculated using the calibration curve, which was prepared by adding the known amounts of feeding solution and using ants that were not exposed to the dye. Statistical analyses were done using GraphPad Prism using a Mann-Whitney *U* test.

Statistical analysis

Statistical analyses were performed using GraphPad Prism or R v.3.3.2. Details of the tests are provided in the section of each respective experiment. Two-tailed *P* values are reported throughout (Supplemental Tables 3–5).

Results

Discovery and pharmacology of the inotocin neuropeptide receptor signaling system in *L. neglectus*

In earlier work, we discovered the genes and transcripts encoding the IP and IR in insects and particularly in ants (3, 22). We validated the expression of the receptor by molecular cloning and confirmed the functionality of the peptide/receptor pair by pharmacological characterization of the inotocin signaling system of the black garden ant (*L. niger*) (23). Here, we report the coding sequences of the IP and IR in the closely related species *L. neglectus*, elucidated by transcriptome mining. The translated amino acid sequences of precursor and receptor from both species are very similar, and their overall architectures appeared to be conserved within ants, other insects, and vertebrate animals, including humans (Supplemental Fig. 1). The precursor encodes for a nonapeptide with the sequence CLITNCPRG preceding the typical GKR processing signal, which results in a C-terminal amide (Fig. 1A). The receptor sequence aligns well with the typical 7-transmembrane architecture of GPCRs (Fig. 1B). Because there was only 1 significant hit in the *L. neglectus* transcriptome for each (IR and IP), we suggested that the OT/VP-like signaling system in *L. neglectus* is composed of a single peptide and receptor, as is observed in other insect species (3). After cloning of the *L. neglectus* receptor into a suitable expression vector and synthesis of the inotocin peptide, we performed second messenger analysis and binding studies of this signaling system *in vitro*. Inotocin is a potent agonist of its cognate receptor in *L. neglectus* for the Gq- and Gs-signaling pathways ($EC_{50} IP_1 = 0.063$ nM, Fig. 1C; $EC_{50} CRE = 0.14$ nM, Fig. 1D). A tritiated inotocin peptide binds to receptor-expressing membranes with an affinity of $K_d = 0.7$ nM (Fig. 1E), and the GFP-tagged version of the receptor localizes mainly at the plasma membrane (Fig. 1F). These results suggest that *L. neglectus* contain a functional cognate inotocin peptide–receptor signaling pair.

Quantitative expression of IP and IR genes in *Lasius* ants

We used qPCR to investigate the expression of IP and IR in different developmental stages, castes, parts of the body, and organs of *L. neglectus* (Fig. 2) and *L. niger* (Supplemental Fig. 2). The expression of IP and IR was lower by a factor of 7–174 as compared with adult workers in all early development stages (egg, larvae) and was intermediate in pupae in both species. There were no major differences in expression of both genes in the different castes of pupa (workers, queens, males) of *L. neglectus*. Both virgin and mated *L. niger* queens showed at least a 10-fold decrease in expression of IP as compared with workers, whereas in *L. neglectus* the expression was high in virgin queens but was decreased by 17-fold in mated queens. On the other hand, the differences in IR expression in various castes were much smaller, and the highest expression was observed in *L. neglectus* males (3-fold higher as compared with workers) (Fig. 2A).

Next, we dissected workers, queens, and males into their 3 body parts (head, thorax, and abdomen) and checked the expression level of IP and IR (Fig. 2B and Supplemental Fig. 2B). The precursor was mainly expressed in heads of all 3 castes of both species (1–3 orders of magnitude difference between heads and thorax or abdomen). We observed that IP expression in heads of mated queens of both species (and virgin queens of *L. niger*) was on

average 1 order of magnitude lower as compared with workers. In workers and queens of *L. niger*, the expression of the receptor gradually increased from head to thorax and to abdomen, whereas males exhibited similar high expression throughout their body. *L. neglectus* displayed similar levels of IR expression throughout the body in workers and queens, whereas IR expression was at least 1 order of magnitude higher in the heads of males as compared with other sampled body parts.

After recognizing that IR was expressed throughout the ant body, we dissected various organs and analyzed IR expression levels in different castes in *L. neglectus* (Fig. 2C) and *L. niger* (Supplemental Fig. 2C). We found a high IR expression in most organs, the highest being in nervous system, parts of the digestive system (crop-proventriculus and rectum-gut), fat body, and respiratory system; midgut, Malpighian tubules, and ovaries showed considerably lower expression levels. Very high IR expression levels were detected in all parts of the male head, especially the mandibular glands (17- to 300-fold higher compared with workers), and in the reproductive system (8-fold higher compared with workers' ovaries). *L. neglectus* virgin and mated queens exhibited similar (up to 2-fold differences) IR expression level in most organs; the only marked differences were observed in Malpighian tubules and glands of the abdomen, where IR expression was 4- to 6-fold higher in mated queens as compared with virgin queens, whereas ovaries and crop-proventriculus exhibited ~3-fold higher IR expression in virgin queens as compared with mated queens. On the other hand, while comparing virgin and mated *L. niger* queens, we observed a 3- to 7-fold higher expression of IR in most of the organs (rectum-gut, fat body, Malpighian tubules, ovaries, and spermatheca) in mated queens, with the highest increase of expression observed in the nervous system (12-fold). To confirm these observations, we designed a second pair of qPCR primers to reanalyze expression levels of IR transcripts in certain organs. Two primer pairs, which anneal to different parts of the receptor mRNA (the first set of primers amplifies the region of 157–162 bp, and the second set of primers amplifies the region of 412–558 bp of the receptor) yielded similar results (Supplemental Fig. 3A), confirming our initial findings.

The qPCR results indicated that the highest expression of IP was observed in ant heads, but detectable (low) expression was found in thorax and abdomen (Fig. 2B); nevertheless, the inotocin peptide was identified by immunostaining only in 2 cells of the sub-esophageal ganglion (SEG), which distribute to positive-stained projections into the brain and the ventral nerve cord (see below). In addition, we analyzed the expression of IP in whole heads, brains, and the remaining parts of heads (excluding brain and postpharyngeal glands of workers or brain and mandibular glands of males). In most of these samples, we detected very similar expression levels in all 3 head preparations (Supplemental Fig. 3B). This indicated that inotocin-positive projections, which were present in the rest of heads, thorax, and abdomen, also contain the mRNA of IP.

In summary, we showed that in both *Lasius* species: 1) expression of the IR and IP was low in larvae and increased in pupa, with the highest level observed in adult ants; 2) mated queens exhibited a ~10-fold lower expression of the precursor as compared with workers; 3) the precursor was mainly expressed in heads, whereas the receptor was expressed throughout

the body of ants in all castes; and 4) the highest level of expression of the receptor was found in the heads of males.

Cellular localization of the inotocin peptide in *Lasius* ants by immunostaining

Immunostaining revealed the presence of 2 VP-immunoreactive neurons in the CNS of *L. neglectus* workers (12 out of 13 brains were positive), mated queens (5 out of 5 brains were positive), and males (7 out of 12 brains were positive) (Fig. 3A, B and Supplemental Fig. 4B, C). Similarly, 2 positive cells were identified in brains of *L. niger* workers (Supplemental Fig. 4D). These cells are presumably responsible for the synthesis and storage of inotocin. The soma of these cells (10–11 μm in diameter, 12 μm in length) were in the midline of the anteroventral surface of the SEG, and each cell presented a unique axon that entered the SEG and bifurcated consecutively. The projections generated from these axons reached the ventral surface of the antennal lobe in the brain and both thoracic and abdominal ganglia. Notably, the contact area of the SEG and esophagus contained very dense branches. The branches in all ganglia of the ventral nerve cord were located on the surface, from where they presumably release inotocin into the hemolymph. No differences in axon branching in the different castes were observed. In addition, we analyzed all organs of the abdomen and head (excluding fat of the head), and none of the other organs was immunoreactive to the VP antibody. Consistent with these immunostaining results, the highest expression of the precursor using qPCR was found in heads of all castes (Fig. 2B and Supplemental Fig. 2B). Antibodies raised against the IR were not specific, and therefore no localization data of the receptor at the protein level are available.

Previously immunostaining of CNS tissue of grasshoppers (52), cockroaches, and a mantis species (53) revealed that neuronal projections from the 2 inotocin-positive neurosecretory cell bodies in SEG differ in some insects, allowing us to classify them into 2 groups: 1) “Schistocerca-type” grasshoppers and cockroaches that show no significant VP-like immune-reactive (VPLI) neuron branching in peripheral nerves or optic lobes and 2) “Locusta-type” grasshoppers that contain extensive branching by the VPLI neurons in the peripheral nerves and optic lobes. Here, we identified the same 2 inotocin-positive cells in the SEG in all castes of ants and detected neuronal projections. Therefore, with respect to localization of inotocin, we added *Lasius* to the first group of insects because we did not see branching of VPLI neurons into optic lobes or into peripheral nerves. Expression analyses of the inotocin signaling system and localization of the mature peptide (Figs. 2 and 3, and Supplemental Figs. 3 and 4) showed only minor differences between *L. niger* and *L. neglectus*; thus, we selected *L. neglectus* as the representative species for further experiments.

Expression profile of IP and IR genes in *L. neglectus* under various physiologic conditions

Previously, in fish it was shown that expression of the OT/VP precursors is affected by seasonal changes (54); thus, we analyzed similar effects in ants. We observed statistically significant (2- to 3-fold) increases in the expression of both receptor and precursor in summer as compared with winter conditions ($P = 0.0003$, Student's *t* test) (Fig. 4A and Supplemental Table 5). Because ants feed very little in winter and because the expression of the IR in both ant species is high in organs involved in feeding and metabolism (crop-

proventriculus, rectum-gut, and fat body) (Fig. 2; Supplemental Fig. 2), we hypothesized that IP and/or IR expression could be affected by starvation. We observed a 3-fold decrease of mean values in IP expression after a 2-d starvation period followed by 1 d with reintroduced food ($P < 0.001$, Tukey HSD *post hoc* test after significant interaction in 2-way ANOVA) (Fig. 4C; Supplemental Table 5). This effect was reversible because continuous feeding for 6 d after starvation yielded approximately the initial expression level of the precursor (Fig. 4C). The expression of the receptor was not significantly affected during the experiment (Supplemental Fig. 5). Because foragers contain lower amounts of lipids as compared with nurses (55) and because nurses and foragers exhibit differences in feeding behavior (56), we collected 20 ants from the foraging area and brood chamber and determined the expression of the IP and IR in the respective individuals. We found that expression of the IP and IR of nurses was significantly reduced (by ~70%; $P = 0.0243$, Student's *t* test) (Fig. 4B and Supplemental Table 5). In summary, expression of both IP and IR is lower in winter vs. summer conditions and in nurses vs. foragers, and mRNA of the IP is down-regulated after a starvation period.

Transcriptional changes after IP-KO in *L. neglectus*

Given the observed differences in the expression levels of the inotocin signaling components, we interrogated this natural variation using functional genomics. To obtain IP-KD ants we used a dsRNA probe against IP to feed ants (35), inducing significant RNAi-mediated knockdown of IP mRNA as compared with controls (ants fed dsRNA probe against GFP) (Supplemental Fig. 6). The designed dsRNA probe against IP was 317 bp in length, which aligns with the C-terminus of the precursor. Different concentrations of the probe (1–2 $\mu\text{g}/\mu\text{l}$) in the feeding solution were tested, and each condition yielded a decrease in expression (~10 times) in most ants (Supplemental Fig. 6A). We also analyzed the stability of dsRNA probes and found the dsRNA to be resistant to degradation: after exposing the probe for 2–3 d to ants, we observed degradation only in 1 dsRNA sample (Supplemental Fig. 6B).

Next, we performed mRNA sequencing of *L. neglectus* to understand how reduced IP levels would affect the ants' gene expression profile (transcriptome). We mapped the sequenced reads against 56,754 *L. neglectus* transcript contigs (Cremer, Eder, and Rattei, unpublished results) (Supplemental Table 2), of which 36.9% ($n = 20,945$) were annotated (at least 1 GO term was assigned). Principal component analysis of IP-KD and CTRL transcriptomes showed that IP-KD samples cluster together separately from CTRL (Supplemental Fig. 7). Differential expression analysis of mRNA-seq data of IP-KD vs. GFP-controls yielded a list of 105 differentially expressed genes, of which 69 were down-regulated and 36 up-regulated (Supplemental Fig. 8 and Supplemental Table 6). It was possible to annotate 76% of these differentially expressed transcripts ($n = 80$ transcripts; 57 down-regulated and 23 up-regulated), which were subsequently used for gene ontology enrichment analyses (Fig. 5; and Supplemental Table 7). Additionally, differences in the expression levels of 11 transcripts were confirmed by qPCR (Supplemental Fig. 9). Among up-regulated differentially expressed transcripts, we found 5 ribosomal transcripts, 2 amino-peptidase N, 2 DNA mismatch repair proteins, and others. These transcripts are related to GO terms such as peptide biosynthetic processes, various metabolic processes, regulation of DNA

biosynthetic processes, and others. This indicates that peptide/protein and DNA synthesis is increased in IP-KD ants. Among the down-regulated transcripts, 15 could be connected to fat metabolism: 10 cytochrome P450 4C1 (CYP450-4C1), 3 fatty acid synthases, 1 lipid storage droplets surface-binding protein, and 1 fatty acid-binding homolog protein. These genes account for 26% of all annotated down-regulated transcripts in our differential expression analyses. In *Blaberus discoidalis* CYP450-4C1 is involved in fatty acid oxidation during starvation to synthesize trehalose, the main circulating carbohydrate of insects, but is not needed for vitellogenin synthesis, which is up-regulated under high-feeding conditions (57–59). In summary, ~100 differentially expressed genes were identified in IP-KD ants, many of which can be linked to various metabolic processes, such as fat, protein, and DNA metabolism.

Analysis of behavioral changes in IP-KD ants of *L. neglectus*

We performed experiments with IP-KD ants to determine whether the inotocin signaling system is involved in (social) behavior similar to humans and other mammals (60). During the experiments, ants were kept in small groups ($n = 5$ workers) in a dish comprising a brood chamber, larvae, and food (sucrose and a small cricket). qPCR analyses confirmed statistically significant knockdown in 12 replicates (Supplemental Fig. 10A; each $P < 0.03$, Mann-Whitney U test), which were analyzed for behavioral changes. Common types of ant behavior, such as the individual behaviors of self-grooming, walking, resting, and feeding, as well as the social group behaviors allogrooming, trophallaxis (sharing food by regurgitation), and antennating the other individual, were monitored for 2 h and counted every 5 min. None of the social interactions (antennating, trophallaxis, and allogrooming) exhibited significant differences (Supplemental Fig. 10 and Supplemental Table 3), but IP-KD ants exhibited statistically significant higher scores for self-grooming inside the brood chamber and lower scores outside the brood chamber (Fig. 6A). Additionally, IP-KD ants had statistically significant higher scores for walking ($P < 0.001$; generalized linear mixed models) (Fig. 6A and Supplemental Table 3), and, consistent with this observation, the number of inactive (*i.e.*, resting) ants was significantly reduced in the IP-KD group ($P = 0.024$, generalized linear mixed models) (Supplemental Fig. 10C and Supplemental Table 3). Trophallaxis occurred mostly inside the brood chamber, with no significant difference in frequency between the IP-KD and the control group. Outside the nest, there was a higher occurrence of trophallaxis in the control group, which could not be considered as statistically significant (Supplemental Fig. 10C and Supplemental Table 3).

Given these observations in small groups of animals, we focused on specific activities and monitored individual ants. Similarly, the walking activity of individual ants was significantly different: IP-KD ants spent more time walking and walked longer distances, and their walking episodes occurred more often as compared with GFP controls ($P = 0.021$, generalized linear mixed models) (Fig. 6B–D and Supplemental Table 4). However, their walking speed and the duration of the walking episodes did not change between the treatment groups (Supplemental Fig. 11A, B and Supplemental Table 4). On the other hand, feeding was observed rarely in the small group experiment (score 0 in ~60% of replicates) (Supplemental Fig. 10D); thus, we performed feeding activity studies with single ants. After a starvation period of 3.5 h, we analyzed the time the ants needed to find food, the amount

they ate, and the percentage of ants that were feeding and near the food. Although there were no statistically significant differences between the IP-KD and GFP control groups in the amount of food eaten after 3 and 6 h (Supplemental Fig. 11D), we detected a clear trend that twice as many IP-KD ants were feeding as compared with controls ($P = 0.066$, Mann-Whitney U test) (Fig. 6E and Supplemental Table 5). Nevertheless, there were no significant differences between the number of ants near the food (Fig. 6E) and the time it took them to find the food (Supplemental Fig. 11C), suggesting that locomotor activity and feeding are independently regulated events.

In summary, IP-KD ants exhibited higher locomotor activity as compared with controls (~3 times), they preferred to perform self-grooming inside the brood chamber, and there was a trend of more IP-KD ants feeding (~2 times).

Discussion

OT/VP-like peptides and their receptors have been identified in many orders of insects (inotocin), but information about their biologic function is limited. In this work, we used ants as a model to characterize the role of this important and evolutionary highly conserved peptidergic signaling system. We performed a comprehensive analysis of the inotocin signaling system in *Lasius* ants comprising pharmacological second messenger analysis, mRNA quantification of IP and IR, immunolocalization of the peptide, and mRNA-seq and behavioral analysis of IP-KD ants.

The inotocin signaling system in *L. neglectus* was composed of 1 peptide and 1 receptor, in agreement with other ant species (7). Our findings that the receptor was expressed throughout the body and the peptide was produced in the brain and presumably released into the periphery allowed us to speculate that inotocin function is not limited to a specific organ but may hold a more general physiologic role, such as feeding, energy conservation, or metabolic homeostasis. To test this hypothesis, we determined the changes in the expression levels of the precursor and receptor under different physiologic conditions. We discovered that expression of the precursor was down-regulated when feeding started after fasting and that both receptor and precursor were down-regulated in winter conditions and in nurses responsible for brood care. Furthermore, mRNA-seq analysis of IP-KD ants revealed several differentially expressed genes that suggested a correlation between inotocin signaling to fat, protein, and DNA metabolism. Our results clearly indicated that IP-KD ants were more active in walking and performed more self-grooming inside the brood chamber (and less outside) and that there was a clear trend of more IP-KD ants that were feeding as compared with controls (Fig. 7).

Growing evidence suggests that OT is an important regulator of energy homeostasis in mammals (8). Several comprehensive studies have shown that increased OT levels in the brain lead to restrictions in food uptake and catabolic regulatory effects, which are thought to contribute to long-term weight loss in mice (61) and rats (62). OT is involved in the regulation of appetite (63, 64). Additionally, the eating disorder anorexia nervosa (65) and hyperphagia during pregnancy (66) are related to imbalanced OT levels. Here we determined that the OT/VP signaling system in ants is involved in locomotor activity, feeding, and

metabolism: 1) high expression of the receptor was monitored throughout the body and especially in organs linked to food metabolism and energy storage (crop-proventriculus, rectum-gut, and fat body) in all castes; 2) dense, VP-positive branches were present in the contact area of the SEG and esophagus as well as in thoracic and abdominal ganglia, and presumably this allowed inotocin to be released into the hemolymph, acting as an endocrine hormone being capable of reaching all organs; 3) starvation and winter conditions led to down-regulation of IP expression; 4) mRNA-seq of IP-KD ants yielded information about several differentially expressed genes involved in fat, protein, and DNA metabolism; and 5) more IP-KD ants were found to be feeding as compared with controls. In addition, the observed higher walking frequency of IP-KD could indicate higher foraging activity potentially driven by a higher interest to eat. Supporting this, previously it was shown that starvation induces foraging behavior (67), and higher activity in ants correlates with lower trehalose levels (*i.e.*, the main circulating carbohydrate in insects) and fructose titers in hemolymph, which induces ants to search for food (68).

The lowest expression of the precursor and/or receptor was correlated to situations where energy is accumulated or economized (*i.e.*, during winter, during larval development, and in mated queens, which require more energy for egg-laying). In contrast, males, which mate and die very soon after the mating flight and hence do not need to preserve energy resources, have the highest expression level of the receptor. This is further supported by the ~10-fold reduction in expression of the precursor in *L. neglectus* mated queens as compared with virgin queens, whereas *L. niger* virgin and mated queens displayed low expression in all cases. These differences between the species could reflect diverse life histories: during a long period of starvation, *L. niger* queens establish a colony by themselves, and this is dependent on their body reserves, whereas *L. neglectus* queens start a colony with workers and hence constantly receive food from the workers. One could speculate that mated queens of both species are preserving energy for producing more eggs, as well as virgin queens of *L. niger* (low expression of IP), which must establish the colony by itself. In contrast, virgin queens of *L. neglectus* can still afford higher energy consumption (high expression of IP).

IP-KD *L. neglectus* exhibited a nurse-like phenotype: 1) self-grooming behavior was performed more often inside the brood chamber (*i.e.*, the nursing area) as compared with the foraging area; 2) in nurses of *Solenopsis invicta* 9 cytochrome P450 4C1 transcripts were found to be down-regulated (69), similar to what we found in IP-KD ants (Supplemental Figs. 8 and 9); and 3) isolated, single nurses of *Camponotus rufiper* exhibit higher locomotor activity as compared with foragers (56). Additionally, in many ant species nurses contain higher fat (or other nutrient) stores as compared with foragers (55), which agrees with our results and interpretation that nurses had a lower expression of the inotocin signaling system components, which correlates to higher fat reserves.

Besides a functional role in feeding, appetite, and metabolism, OT/VP-like peptides are mainly known for their involvement in social and reproductive behavior as well as for maintenance of water homeostasis. Although we did interrogate the possible effects of inotocin signaling system on typical social behaviors (*e.g.*, allogrooming, antennation, and trophallaxis) of IP-KD ants vs. controls (Supplemental Fig. 10E–G), overall our

observations suggest that this peptide signaling system is not involved in the regulation of behavior in a social context.

Very high receptor expression in heads and the reproductive organs of males indicate a potential role of the inotocin signaling system in male reproduction. The highest expression of the receptor was found in mandibular glands (15-fold higher as compared with brains), which is hypertrophied in males and might be the source of the sex pheromone (70). This agrees with the literature because OT/VP-like signaling is linked to reproduction in males in many invertebrates and vertebrate species (4, 5, 13).

In earlier studies, an antiparallel inotocin homodimer was shown to have diuretic activity in *Locusta migratoria* (21) and to stimulate cyclic AMP (71) on isolated Malpighian tubules/midgut preparations. However, some facts contradict the diuretic action of inotocin in insects: 1) the pseudo-cyclic inotocin monomer was not active in these studies, 2) the results with *L. migratoria* Malpighian tubules could not be reproduced using the antiparallel inotocin homodimer (20), and 3) low IR expression and no direct inotocin activity on diuresis in Malpighian tubules was found in beetles (18). In ants, the expression level of the IR in Malpighian tubules was low, limiting the possibility of an exclusive function of inotocin signaling for water homeostasis.

The well-known functions of OT/VP are related to reproductive and social behavior, but accumulating evidence suggests it links its role in reproduction to energy status and feeding behavior in vertebrates (8). This is not surprising because reproduction depends on the availability of food and the energy balance of the organism. Previous studies of the OT/VP-like signaling system in invertebrates mainly focused on reproductive behavior, although not much is known regarding feeding. However, it was shown that in *C. elegans* nematocin can modulate the gustatory plasticity circuit that directs food preference (15) as well as male mating circuits (16). Working with ants provides the advantage to separate reproductive individuals (males and queens) from nonreproductive (workers), which makes it possible to discriminate the 2 functions of the OT/VP-like signaling system. The presence of both receptor and precursor in all castes and developmental stages already indicates broader functions than only reproduction.

Based on these considerations and our data, we conclude that inotocin signaling in ants is involved in protein, fat, and DNA metabolism; walking activity; and potentially feeding behavior. We propose that OT/VP-like peptides in ants are important for regulation of energy status, associated with locomotion and appetite, which may be an ancient function of the OT/VP-like signaling system in invertebrates. Together with a recent study about the function of the peptide hormone corazonin (27), our results will fundamentally aid understanding of neuropeptidergic signaling in ants. Because the ability to move is important for survival—at least during nonsedentary and mobile life stages of animals—understanding how ant colonies, and insects in general, regulate metabolic activity and locomotion will trigger further studies about the biologic regulation of how insects find food, fend off enemies, or escape from predators.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CTRL	control
dsRNA	double-stranded RNA
EF1	transcription elongation factor 1
GFP	green fluorescent protein
GO	gene ontology
IP	inotocin precursor
IP-KD	inotocin precursor knockdown
IR	inotocin receptor
OT	oxytocin
qPCR	quantitative PCR
SEG	sub-esophageal ganglion
VP	vasopressin
VPLI	vasopressin-like immune reactive

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Figure 1.

Structure and pharmacology of the *L. neglectus* inotocin signaling system. *A*) Chemical structure of the *Lasius* inotocin peptide (CLITNCPRG-NH₂). *B*) Illustration of the typical 7-transmembrane (TM) GPCR architecture of the IR from *L. neglectus*. Amino acid sequence differences to the *L. niger* receptor (GenBank: A0A1L2FSX9) are presented via 1-letter code, residue number, and approximate position (P₁₃S, M₁₅₅V, T₂₀₅M, I₂₃₄V, N₂₅₁V, and V₂₆₉I), and receptor domains are labeled (N-terminal domain; ICL, intracellular loops; ECL, extracellular loops; C-terminal domain). *C*, *D*) Concentration-dependent receptor activation was measured by inositol-phosphate 1 (IP₁) (*C*) and luciferase reporter (*D*) assay via

activation of the cAMP-response element to determine Gq- and Gs-dependent signaling of inotocin, respectively, in HEK293 cells transiently expressing the *L. neglectus* IR. The error bars represent the mean $\pm$ SEM from 3 independent experiments. Potency (EC₅₀) was determined by nonlinear regression (sigmoidal, 3 parameters, fixed Hill slope of 1) and normalized to the percentage of maximal activation. *E*) Saturation radioligand binding was performed on membrane preparations (10 μ g) transiently expressing IR from *L. neglectus* and specific binding (pmol of [³H]inotocin/mg of membrane protein) was determined as difference between nonspecific and total binding ($n = 2$). Affinity (K_d) was determined by nonlinear regression using a 1-site binding equation. *F*) HEK293 cells transiently expressing a GFP-tagged version of the *L. neglectus* IR localizes mainly at the plasma membrane (green). Cell nuclei were stained with DAPI (blue).

Figure 2.

Quantification of mRNA transcripts of the inotocin signaling system in *L. neglectus*.

Expression of IR and IP in different development stages and castes (A), parts of the body (B), and organs (only receptor) (C) was determined by qPCR. At least 3 biologic replicates are represented as a dot plot with a mean $\pm$ SEM. A) Eggs ($n = 10-30$) and larvae ($n = 3-6$) were pooled, and pupa and different adults were analyzed as 1 animal per biologic replicate. B) Workers ($n = 8-15$) and males ($n = 5-9$) were pooled, whereas only 1 queen was used per biologic sample of head, thorax, and abdomen. C) Organs ($n = 4-8$) of different animals for workers and males and queens ($n = 1-2$) were pooled together in every biologic sample. “Mated queen” corresponds to queens that mated at least 2 mo prior to sampling. Dufour’s gland and poison gland of the abdomen were analyzed together (termed “gland”). The gland of the head corresponds to postpharyngeal gland in workers and mandibular glands in males, respectively, which is a predominant gland in each case. The rest of the head is defined as head without brain and postpharyngeal (workers) or mandibular (males) gland. The reproductive system of males corresponds to the testis, seminal vesicles, and ejaculatory

duct; the reproductive system of queens and workers corresponds only to ovaries. M, mated; v, virgin.

Figure 3.

Localization of the inotocin peptide in *L. neglectus* by immunostaining. *A*) Ant brain with the 2 immunoreactive somata in the SEG. A, anterior; L, lateral. *B*) Magnification of SEG. *C*) Abdominal ganglia of the ventral nerve cord (from top starting with the petiole ganglion). Scale bars, 100 μm (*A*, *C*) and 50 μm (*B*). DAPI nuclear staining is shown in blue; inotocin peptide staining is shown in green. For further information, refer to Supplemental Fig. 4.

Figure 4.

Expression profiles of the IP and IR of *L. neglectus* during different seasons (A), in foragers vs. nurses (B), and under starvation (only precursor) (C). Results are represented as dot blots with means $\pm$ SEM. A) Ants from summer- and winter-conditioned colonies were collected at the same time ($n = 13-21$, Student's *t* test). B) Ants were collected from foraging area (foragers) and brood chamber (nurses) from the same nest ($n = 20$, Student's *t* test). C) Relative expression ratio of a precursor before (d 0), during (d 2), and after (d 3 and 9) the starvation period of 48 h ($n = 17-20$). See detailed statistical analyses in Supplemental Table 5.

Figure 5.

Altered gene expression of IP-KD *L. neglectus* workers. mRNA sequencing: GO term enrichment analyses of differentially expressed transcripts. Statistically significant GO terms ($P < 0.01$) are shown, which were reduced using the Revigo online tool (semantic similarity was measured using the Lin method; only GO terms covering 2 or more transcripts were used) (72). A full list of statistically significant GO terms ($P < 0.01$) is shown in Supplemental Table 7. Black bars correspond to biologic process, gray bars correspond to molecular functions, and white bars correspond to cellular components. [#]The full GO term is “oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen,” A, activity; B, binding; MP, metabolic process; str, structural.

Figure 6.

Physiology of IP-KD *L. neglectus* workers. **A**) Behavior in a group experiment: walking and self-grooming scores of 5 ants in a group were measured every 5 min during 2 h outside and inside brood chamber ($n = 12$). **B–D**) Walking assays. Single ant walking activity on a dish was monitored for 60 min and is presented as walking time (**B**), distance (**C**), and number of walking episodes (**D**) [control (ctrl), $n = 20$; IP-KD, $n = 19$]. **E**) Eating experiment. After a starvation period of 3.5 h, food was introduced, and the percentage of feeding ants and ants being near the food was calculated (ctrl, $n = 88$; IP-KD, $n = 89$). Data are presented as dot plots and median with interquartile range. Generalized linear mixed models (**A–D**) or Mann-Whitney U test (**E**) were used. A detailed description of statistical analyses is provided in Supplemental Table 3. * P value reflects treatment effect over all data.

Figure 7.
Summary of the functional role of inotocin signaling in *Lasius* ants. * $P = 0.066$.