The oxytocin/vasopressin-like peptide inotocin regulates cuticular hydrocarbon synthesis and water balancing in ants

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Oxytocin/vasopressin-like peptides are important regulators of physiology and social behaviour in vertebrates. However, the function of inotocin, the homologous peptide in arthropods, remains largely unknown. Here we show that the level of expression of *inotocin* and *inotocin receptor* are correlated with task allocation in the ant *Camponotus fellah*. Both genes are up-regulated when workers age and switch task from nursing to foraging. *in situ* hybridisation revealed that *inotocin receptor* is specifically expressed in oenocytes which are specialised cells synthesising cuticular hydrocarbons which function as desiccation barriers in insects and for social recognition in ants. dsRNA injection targeting *inotocin receptor,* together with pharmacological treatments using three newly-identified antagonists blocking inotocin signalling, revealed that inotocin signalling regulates the expression of *cytochrome P450 4G1*(*CYP4G1*) and the synthesis of cuticular hydrocarbons, which play an important role in desiccation resistance once workers initiate foraging.

Social insect | oxytocin/vasopressin-like peptide | division of labour | behaviour tracking | cuticular hydrocarbon

Introduction

In humans and mammals, the neuropeptide hormones oxytocin and vasopressin are involved in a number of physiological processes and are increasingly implicated in a variety of social behaviours. In particular, oxytocin signalling regulates uterine contraction, lactation and energy metabolism, and has been demonstrated to increase levels of parental care, pair bonding and cooperation (1-4). Vasopressin is important for water balancing via the control of anti-diuresis, regulation of blood pressure, and plays a role in influencing social behaviours such as territorial defense, aggression and also pair bonding (5-8). Across vertebrates, oxytocin/vasopressin-like peptide homologues appear to have conserved physiological (9, 10) and behavioural functions (11-15).

An increasing number of genomic and transcriptomic analyses have revealed that a further homologue, inotocin, is specific to invertebrates where it is conserved across at least 100 species (16). This places the origin of the oxytocin/vasopressin-like peptide family prior to the split of the Protostomia and Deuterostomia, more than 600 million years ago (17). Studies on inotocin signalling in insects (16, 18-22) suggest a possible role in diuresis in *Tribolium castaneum* (18) and locomotor activity in ants (21). However, the molecular and cellular functions of inotocin signalling remain largely unknown. Given the important role of oxytocin/vasopressin-like peptides in regulating social behaviour in vertebrates (23-25), we conducted a study using ants as a model organism to investigate the physiological role of inotocin signalling and its potential implication in social behaviour.

Ants live in large and complex societies consisting of one or more reproductive queens and many non-reproductive workers (26). Workers exhibit division of labour with individuals performing specific tasks within a colony (27-29). Individual task preference is generally correlated with age, with younger workers remaining inside the nest to nurse the developing offspring, and older workers foraging outside the nest. This age-based division of labour is often referred to as task polyethism, although individuals may also flexibly switch their role according to colony demands (30-32).

As workers transit from nursing to foraging, they experience new environmental challenges such as fluctuating temperatures and low humidity, both significant threats in terms of water loss and desiccation. Previous work on various insects showed that cuticular hydrocarbons (CHCs) on the body surface play an important role in protecting against desiccation (33-35). CHCs also play an important role in social recognition (36), especially to distinguish nestmates from non-nestmates in ants (37-39). It has been reported that workers change their CHC profiles before initiating foraging (40-43), possibly to better cope with the drier environment that they will encounter outside of the nest. However, the mechanisms regulating these changes remain largely unknown.

In this study, we first compare the expression of inotocin signalling in different castes and body parts of two ant species of the genus *Camponotus* and then use genetic and pharmacological manipulations to investigate the possible physiological function of this pathway. We found that *inotocin* and its receptor are most highly expressed in workers, particularly foragers, whereas queens and males show low expression levels. Histological analyses revealed that *inotocin receptor* is specifically expressed in

Significance

Inotocin, the oxytocin/vasopressin-like peptide is widely conserved in arthropods, however little is known about its molecular function. Here we show that, in ants, the expression levels of *inotocin* and its receptor are correlated with the age of workers and their behavior. We also demonstrate that inotocin signalling is involved in desiccation resistence by regulating the synthesis of cuticular hydrocarbons. We propose that the up-regulation of *inotocin* and its receptor as workers age and switch task from nursing to foraging is a key physiological adaption to survive drier environments outside of the nest.

Reserved for Publication Footnotes

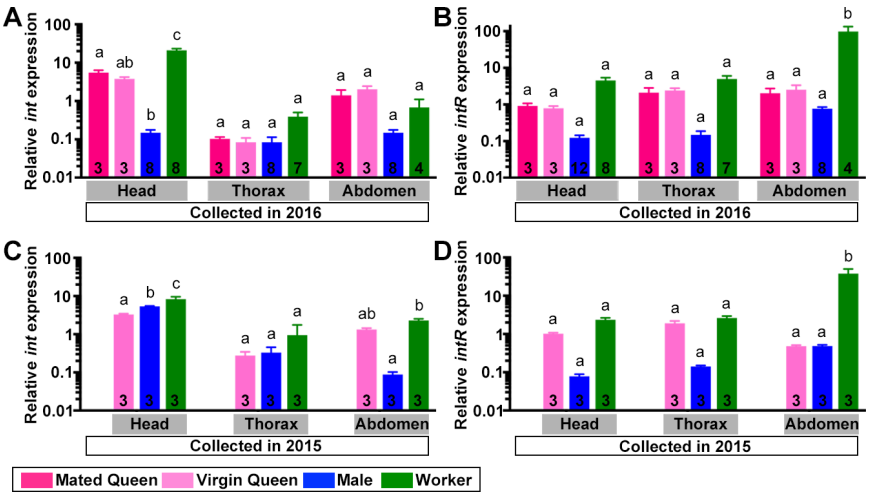

Fig. 1. Relative expression of *inotocin* and *inotocin receptor* in different castes and tissues of *Camponotus japonicus* Relative expression (mean ± SEM) of *inotocin* (*int* in A and C) and *inotocin receptor* (*intR* in B and D) in the head, thorax and abdomen of virgin queens (pink), mated queen (red), males (blue) and workers (green). The expression of each gene was scaled by the average value in each graph. Values are therefore not comparable between graphs.Number of individuals are shown in each box. The relative expression levels of each gene were tested with two-way ANOVA. Groups differing significantly (p <0.05) are marked with different letters. Samples shown in panel A and B were collected in 2016 and samples in panels C and D in 2015. We did not collect any mated queen in 2015.

the oenocytes, a type of specialised cell that produces CHCs in insects (44-46). We also show that inotocin signalling regulates the expression of CHCs via *CYP4G1,* the key enzyme for hydrocarbon synthesis (45, 46). Based on these results, we propose that inotocin signalling is a key regulator of hydrocarbon metabolism, which in turn may allow an adaptive plastic response as workers transition from the relatively safe environment of the nest to a hostile foraging environment.

Results

Expression profiles of int/intR in reproductive castes

To compare the expression levels of *inotocin* (*int*) and *inotocin receptor* (*intR*) among castes, we collected young virgin queens, mated queens, males, and workers of the carpenter ant *Camponotus japonicus* in 2016. There was substantial variation in levels of *int* mRNA among body parts (head, thorax and abdomen; F_{49}=47.3, p<0.0001) and castes (virgin queens, mated queens, males and workers; F_{49}=30.2, p<0.0001) as well as a significant interaction between body parts and castes (F_{49}=31.4, p<0.0001, Fig. 1A and SI Appendix, Table S1). In the head, the level of expression was high in workers, intermediate in the two types of queens and low in males. In the thorax, the levels of expression were very low in each of the four castes while in the abdomen, the levels of expression were intermediate with no significant differences among castes.

The expression of the *intR* was significantly different between body parts (F_{53}=11, p=0.0001) and castes (F_{53}=18.3, p<0.0001). There was also a significant interaction between body parts and castes (F_{53}=12.2, p<0.0001, Fig. 1B and SI Appendix, Table S1). In the head and thorax, *intR* expression was very high in workers, intermediate in queens and very low in males. In the abdomen, the level of expression was significantly higher in workers than males and the two types of queens. Similar results were obtained in *C. japonicus* samples collected in 2015 (Fig. 1C and 1D, and SI Appendix, Table S1).

To investigate the role of inotocin signalling on social behaviour, we conducted a detailed analysis of the expression of *int* and *intR* in workers. Because it is difficult to control for worker age and precisely determine worker task in the field, we used *Camponotus fellah* workers from colonies established from founding queens collected in 2003 and 2007 throughout all of the following experiments. We firstly confirmed that the expression profiles in reproductive castes of *C. fellah* are similar with those of *C. japonicus* (SI Appendix, Fig. S1 and Table S1), with the difference that the expression of *int* mRNA is higher in the heads of males than queens and workers.

Correlation between *int*/*intR* expression and task allocation in workers

To determine the approximate age of each worker in the laboratory-reared *C. fellah* colonies, we colour-marked newly-eclosed workers every month. Task allocation was defined by the spatial location of the ants in the rearing box, 'nurses' being workers collected in the nest and 'foragers' those in the foraging arena (Fig. 2A). There was a significant association between age and foraging propensity (χ^2=255.2, p<0.0001, Fig. 2B). Individuals started foraging after they were 4-months old with a significant increase in the proportion of foraging with increased age (Fig. 2B and SI Appendix, Fig. S2A-C).

Given that *int* is predominantly expressed in the heads (Fig. 1A, 1C and SI Appendix, Fig. S1A), we compared the expression profiles of *int* in heads of nurses and foragers of different age classes. *int* expression was significantly associated with age (F_{184}=3.6, p=0.0023) and task (F_{186}=4.4, p=0.037; age x task interaction: F_{185}=0.056, p=0.94). The level of expression of *int* was lowest in young nurses and highest in old foragers (Fig. 2C). The level of *int* expression was also significantly higher in foragers than nurses within one of the three age classes containing both types of workers (5-month old individuals: F_{30}=4.8, p=0.037, SI Appendix, Table S2). Given that *intR* is predominantly expressed in the abdomen (Fig. 1B, 1D and SI Appendix, Fig. S1B), we compared the expression profiles of *intR* in the abdomens of nurses and foragers of different age classes. Similar to *int* in the head, there was a significant effect of age on *intR* expression (F_{178}=26.5, p<0.0001, Fig. 2D) and an interaction effect between age and task (F_{178}=4.2, p=0.017) but *intR* expression was not significantly associated with task (F_{178}=0.37, p=0.54). There was, however, a significant difference in *intR* expression between nurses and foragers within two of the three age classes containing both types of workers (5-month old: F_{34}=10.8, p=0.0024, and 6-month old: F_{31}=5.3, p=0.028, SI Appendix, Table S2). These results suggest that *int* and *intR* expression are associated not only with age, but also with the task performed by workers.

To further investigate the role of *int* and *intR* on division of labour and to control for age-dependent effects, we set up nine groups each containing 10 4-month old nurses and monitored their behaviour as division of labour was re-organised with those that remained as nurses, and others who started foraging. We measured activity level, time in the food region and time in the nest (Fig. 2E and SI Appendix, Fig. S2D) with an automated video tracking system (47) during six days. The level of *int* expression in the whole body was positively correlated with the overall level of activity (F_{71} =48.9, p<0.0001, Fig. 2G) but not the amount of

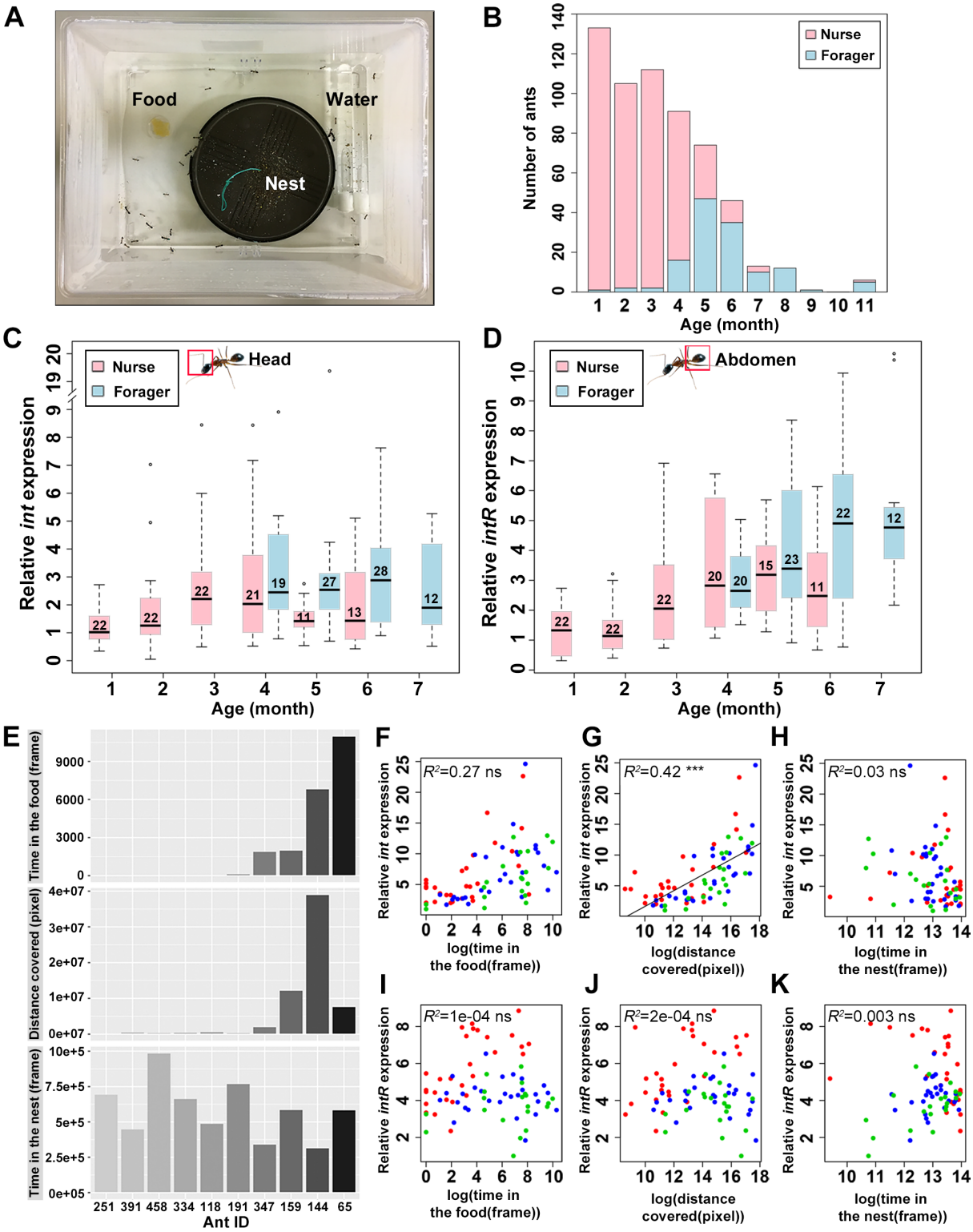

Fig. 2. Correlation between the expression of inotocin signalling and task allocation (A)Setup of the *Camponotus fellah* colonies. (B) Age-dependent division of labour in one representative colony. Nurses are shown in pink, and foragers in the light blue. (C and D) Box-plots of the relative expression levels of *int* in the head (C) and *intR* in the abdomen (D) for each age class. Number of individuals is shown in each box. (E) The time spent in the food region and in the nest and distance moved was quantified over six days in the tracking system for one representative box. (F-H) Relationship between the behavioural parameters and *int* expression. (I-K) Relationship between the behavioural parameters and *intR* expression. Different colours indicate the colony of origin of workers. R^2 and *p*-values are shown on the top left of the graphs. The correlation between the expression of *int* or *intR* and behavioural parameters were tested with GLMM. ns $p>0.05$; ***$p<0.001$.

time spent in the food region (F_{71} =2.3, p=0.13, Fig. 2F) nor time spent in the nest (F_{71} =0.25, p=0.62, Fig. 2H). The level of expression of *intR* was not significantly correlated with any of these three behavioural measures (time in the food region:

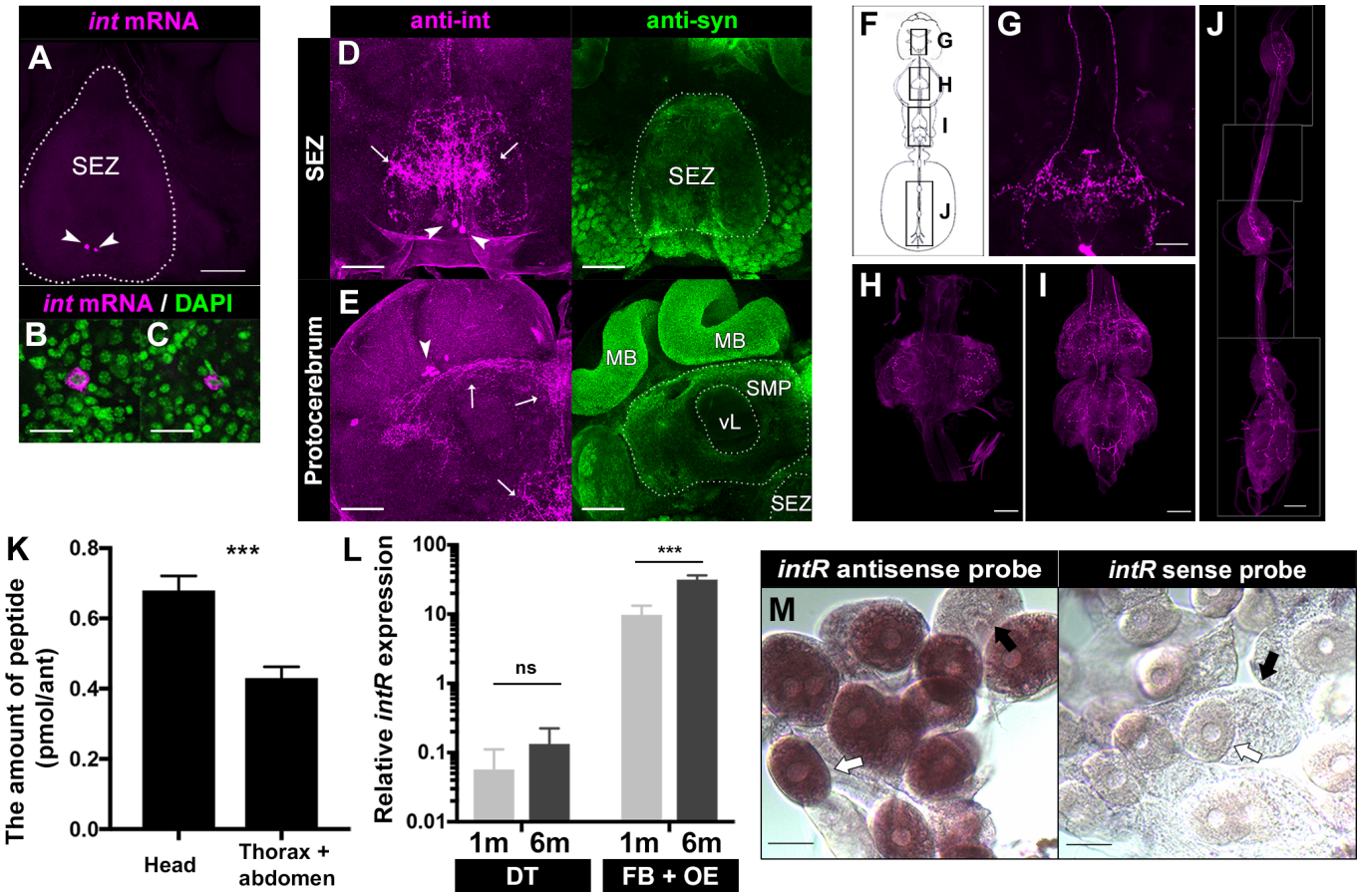

Fig. 3. The expression of *inotocin* in neural tissues and *inotocin receptor* in the fat body plus oenocytes (A) Fluorescent *in situ* hybridisation using specific *int* antisense probe (shown in magenta). Arrowheads indicate the pair of neurons labelled with *int* mRNA in subesophageal zone (SEZ, outlined with white dotted line). (B and C) Magnified image of each *int* mRNA positive neuron (shown in magenta in A) with stained nucleus using DAPI (shown in green). (D and E) Immunohistochemistry for anti-int antibody (shown in magenta) and anti-synapsin antibody (shown in green) in SEZ (D) and protocerebrum (E). Arrowheads indicate cytoplasmic expression of anti-int immunoreactivity. Arrows indicate the neurons positive with anti-int antibody in SEZ (D) and protocerebrum (E), in which brain compartments are labelled as mushroom body (MB), medial superior protocerebrum (SMP), and vertical lobe (vL). (F) Schematic presentation from central to peripheral nervous system in workers. (G-J) One pair of neurons labelled with anti-int antibody from subesophageal zone to ventral nerve cord (G), the prothoracic (H), mesothoracic and metathoracic (I), and abdominal ganglia (J). (K) Distribution of endogenous inotocin peptide tested with unpaired two-tailed *t*-test. ***$p<0.001$. (L) qRT-PCR for the *intR* in the digestive tract (DT) and fat body plus oenocytes (FB+OE). The expression of *intR* mRNA was tested with GLMM with Benjamini-Hochberg *post-hoc* test. ns $p>0.05$; ***$p<0.001$. (M) *in situ* hybridisation using *intR* antisense probe (upper panel) and sense probe (bottom panel) in the dissected fat body plus oenocytes. Oenocytes are indicated with open arrows and trophocytes (the lipid storing cells of the fat body) with filled arrows. Scale bars, 100 µm (A, D, E, G-J), 20 µm (B, C, and M).

$F_{70}=0.14$, $p=0.71$, Fig. 2I; distance covered: $F_{70}=1.24$, $p=0.27$, Fig. 2J; time in the nest: $F_{70}=3.79$, $p=0.06$, Fig. 2K). A similar experiment conducted with younger (1 to 2-month old) workers (SI Appendix, Fig. S3) revealed a similar pattern for *int*, but the level of expression of *intR* was also significantly associated with the overall level of activity (*int*; $F_{43}=12.3$, $p=0.001$, SI Appendix, Fig. S3F, *intR*; $F_{44}=21.2$, $p<0.0001$, SI Appendix, Fig. S3I). These results further support the view that the expression levels of *int* and *intR* are both associated with the age and the behaviour of workers.

Cellular localisation of int and *intR* in workers

To better understand the function of inotocin signalling, we next looked for cellular localisation of *int* in the head and *intR* in the abdomen of workers. Fluorescent *in situ* hybridisation with an *int* gene probe revealed highly specific expression of *int* mRNA in one pair of neurons of the subesophageal zone (indicated with arrowheads in Fig. 3A and magnified in Fig. 3B and 3C). This expression pattern is similar to that of anti-vasopressin antibody staining in locusts (18) and ant *Lasius neglectus* (21). To further clarify the localisation of inotocin proprotein we created an antibody against the inotocin proprotein (anti-int) targeting the C-terminus region of the inotocin proprotein (SI Appendix, Fig. S4A-C). To examine whether the expression of *int* mRNA and its proprotein were in the same cells of the subesophageal zone, we performed sequential staining with immunohistochemistry and fluorescent *in situ* hybridisation which confirmed that the two neurons of the subesophageal zone expressing *int* mRNA were also labelled with anti-int antibody (shown in the upper panel in SI Appendix, Fig. S4D). By contrast, no signal was found when performing a negative control immunostaining with an anti-int antibody pre-absorbed with the antigen peptide (bottom panel in SI Appendix, Fig. S4D), consistent with specificity of the immunoreactive signals labelled with anti-int antibody.

In addition to a pair of neurons in subesophageal zone (indicated with arrowheads in Fig. 3D), we identified a cluster of neurons labelled with anti-int antibody in protocerebrum (Fig. 3E). They spread from the subesophageal zone to the lateralis of medial superior protocerebrum (indicated with arrows in Fig. 3D and 3E). Cytoplasmic immunoreactivity of anti-int antibody on the lateralis of protocerebrum (indicated with arrowheads in Fig. 3E) was very similar to the neurosecretory neurons expressing

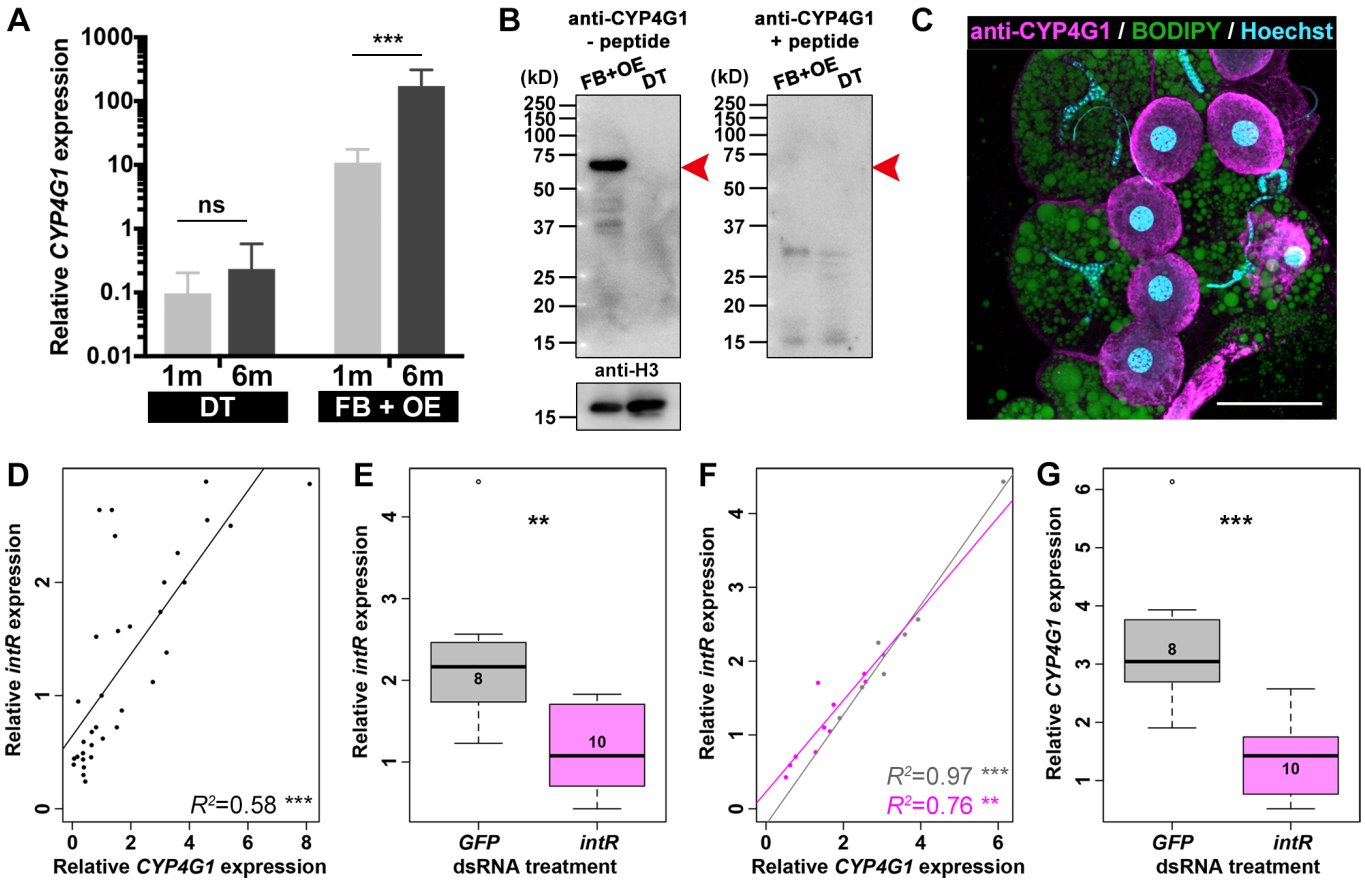

Fig. 4. Expression of CYP4G1 correlating with that of *inotocin receptor* (A) qRT-PCR data for *CYP4G1* expression in the digestive tract (DT) and fat body plus oenocytes (FB+OE) for 1-and 6-month old individuals.**$p<0.01$; ***$p<0.001$ (GLMM with Benjamini-Hochberg *post-hoc* tests). (B) The CYP4G1 protein is expressed in the fat body plus oenocytes (FB+OE) but not in the digestive tract (DT). The band at 63kD (shown with red arrowhead in the left panel) was not detected with the anti-CYP4G1 antibody after the absorption with its antigen peptide (right panel). (C) The CYP4G1 protein (magenta) is specifically present in the oenocytes (round-shaped nucleus stained with Hoechst, light blue), but not in the trophocytes (irregular shaped nucleus and lipid droplets labelled with BODIPY, green). Scale bar, 50µm. (D) Correlation between the expression of *CYP4G1* and *intR* in the abdomen. R^2 and *p*-values are shown on the bottom right of the graph. ***$p<0.001$. (E) Feeding of dsRNA targeting *intR* leads to a significant decrease in the level of expression of *intR* (magenta, unpaired two-tailed *t*-test, **$p<0.01$) compared to feeding of dsRNA targeting *GFP* (grey). Number of individuals is shown in each box. (F) Relationship between the expression levels of *intR* and *CYP4G1* in the treatment with dsRNA targeting *GFP* (grey) and *intR* (magenta). R^2 and *p*-values for each treatment are shown on the bottom right of the graph. (G) *CYP4G1* expression is significantly down-regulated with feeding dsRNA targeting *intR* (magenta, unpaired two-tailed *t*-test)compared to feeding of dsRNA targeting *GFP* (grey). Number of individuals is shown in each box.

PERIOD in honeybees (48), suggesting that these immunoreactive neurons may also have a neurosecretory role.

Additional immunohistochemistry with anti-int antibody revealed a pair of descending axons coming from the cell bodies in subesophageal zone, which are spanning the length of the ventral nerve cord (Fig. 3F and 3G) and extending through the prothoracic (Fig. 3H), mesothoracic, metathoracic (Fig. 3I) and abdominal ganglia (Fig. 3J).

We next examined the expression profile of endogenous inotocin peptide between the head, thorax and abdomen of 6-month old foragers. LC-MS/MS revealed the distribution of inotocin peptide between tissues, with the highest level of inotocin peptide in the head ($t_{10}=4.8$, $p=0.0007$, Fig. 3K). This result is consistent with the highest expression of *int* mRNA in the head, suggesting that the inotocin peptide is mainly expressed in the central nervous system, as is the case with oxytocin and vasopressin in mammals. In addition, the distribution of inotocin peptide in the thorax and abdomen suggests that inotocin peptide could activate its receptor which is highly expressed in the abdomen (Fig. 1B, 1D, and 2D).

Next, we examined in which specific tissues of the abdomen *intR* was expressed. We focused on the digestive tract, fat body and oenocytes which are interspersed within the fat body in ants (49, 50). Because it is technically very difficult to separate oenocytes from the fat body we extracted RNA from the fat body plus oenocytes. We found that *intR* mRNA was expressed at significantly greater levels in the fat body plus oenocytes than in the digestive tract (age effect, $F_{30}=148$, $p<0.0001$, tissue effect, $F_{30}=525$, $p<0.0001$, tissue x age interaction, $F_{30}=145$, $p<0.0001$, Fig. 3L), implicating the fat body or oenocytes as a likely tissue expressing *intR* in the abdomen. In addition, there was a strong age effect ($p<0.0001$, Fig. 3L) with 6-month old workers expressing significantly higher levels of *intR* in the fat body plus oenocytes than 1-month old workers. This result is consistent with expression data from the whole abdomen (Fig. 2D).

To determine the source of *intR* expression at the cellular level we conducted *in situ* hybridisation. These analyses revealed specific expression in the oenocytes which can be recognised by their round-shaped nucleus (see open arrows, upper panel Fig. 3M). By contrast there was no signal in the trophocytes which are the main cells of the fat body and are recognisable by their irregular nucleus (see filled arrows, upper panel Fig. 3M). Taken together these data indicate that the inotocin proprotein is expressed in the central and peripheral nervous systems and then transferred through to the abdomen where it likely activates inotocin receptor sites specifically expressed in the oenocytes.

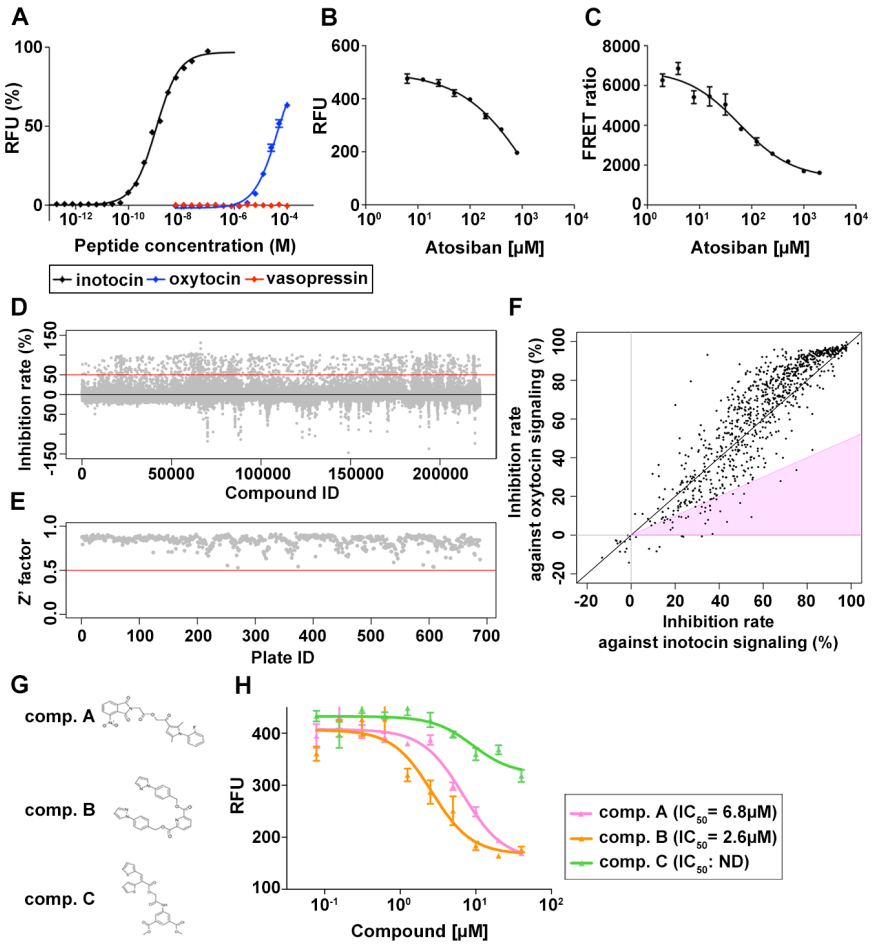

Fig. 5. Chemical screening to identify the inhibitor against inotocin signalling (A) Relative fluorescent intensity (RFU) of Fluo-4 probe in response to inotocin, oxytocin and vasopressin in CHO cells expressing inotocin receptor. Inotocin receptor is activated by inotocin (black line, EC_{50}=1.1nM), oxytocin (blue line, EC_{50}=41.6μM), but not by vasopressin (red line). (B) Atosiban blocks the activation of inotocin signalling (IC_{50}=599μM). 5nM inotocin was used as an approximate EC_{80} concentration. Data represents the mean ± SEM (n=3). (C) Competition curve of specific binding of 10nM labelled-inotocin ligand with increasing concentrations of atosiban (1.95μM-2mM) measured by TR-FRET. Data represent the mean ± SEM of a representative experiment (n=3). TR-FRET ratio=(665 nm acceptor signal/620 nm donor signal) × 10,000. (D) Scatter plot showing the inhibition rate of each compound in the 1st screening. The red line indicates a 50% inhibition. (E) Scatter plot showing the Z' factor for each plate in the 1st screening. All Z' factor in each plate are more than 0.5 indicated by the red line. (F) Inhibition rate against inotocin and oxytocin signalling are plotted for each of the 902 compounds showing >50% inhibition rate in the 1st screening. Compounds showing <50% inhibition rate for inotocin than oxytocin are in the pink shadowed region. (G) Structure of three compounds A, B and C. (H) Dose-dependent inhibition against inotocin signalling of comp. A (IC_{50}=6.8μM, pink line), comp. B (IC_{50}=2.6μM, orange line), and comp. C (IC_{50}; no data, green line). Data represent the mean ± SEM of a representative experiment (n=4). Relative fluorescent unit (RFU) indicates the activation of inotocin receptor calculated from Fluo-4 intensity.

Correlation between *intR* and *CYP4G1* expression

Given that *intR* is expressed in oenocytes, we investigated the relationship between inotocin signalling and the CHC synthesis pathway to which the cells primary function is linked. We firstly focused on the *CYP4G1* gene, which is an aldehyde oxidative decarbonylase P450. This gene is highly conserved in insects and is known to be expressed in the oenocytes (44-46), where it regulates the final step of CHC synthesis by converting aldehyde to hydrocarbons (45). We found higher expression of *CYP4G1* in the fat body plus oenocytes than in the digestive tract (age effect, F_{30}=12.4, p=0.0014, tissue effect, F_{30}=220, p<0.0001, tissue x age interaction, F_{30}=9.8, p=0.0038, Fig. 4A). The expression of *CYP4G1* was higher in the fat body plus oenocytes of 6-month old than 1-month old workers (p<0.0001, Fig. 4A). A similar pattern was found by using a specific antibody against CYP4G1 protein, with CYP4G1 expressed in the fat body plus oenocytes but not in the digestive tract (Fig. 4B). Immunohistochemistry with this antibody further revealed that the CYP4G1 protein is specifically expressed in the oenocytes but not in the trophocytes (Fig. 4C). In addition to the similarity between the histological expression of CYP4G1 (Fig. 4C) and *intR* (Fig. 3M), the expression of *CYP4G1* mRNA is up-regulated in an age-dependent manner both in the head (SI Appendix, Fig. S5A) and abdomen (SI Appendix, Fig. S5B), and there was also a positive correlation between the expression of these two genes in the abdomen (R^2=0.58, t_{32}=6.6, p<0.0001, Fig. 4D).

To study the relationship between inotocin signalling and *CYP4G1* expression, we performed a RNA interference assay by feeding 5-month old workers with dsRNA targeting *intR* with the previously described protocol (51). Workers fed with dsRNA of *intR* showed a lower level of expression of *intR* in the abdomen than control ants (t_{16}=3.3, p=0.0044, Fig. 4E). Moreover, the expression of the *intR* was significantly correlated with the expression of *CYP4G1* in both ants treated with dsRNA of *intR* (R^2=0.76 t_8=5.04, p=0.001, Fig. 4F) and control ants treated with dsRNA of *GFP* (R^2=0.97, t_6=14.36, p<0.0001, Fig. 4F), resulting in the down-regulation of *CYP4G1* expression in the dsRNA of *intR* treated ants (t_{16}=4.06, p=0.0009, Fig. 4G).

Identification of antagonists that block inotocin signalling

We next developed a pharmacological protocol to inhibit inotocin signalling. We established a Chinese hamster ovarian (CHO) cell line which stably expressed the inotocin receptor and monitored the activation of the receptor against inotocin peptide by measuring the increase in cytosolic calcium concentration with the fluorescent calcium probe Fluo-4. We confirmed that the inotocin receptor is activated by the synthesised inotocin peptide in a dose-dependent manner (EC_{50}=1.09nM, Fig. 5A) as previously described (21, 22, 52). We also showed that the inotocin receptor is activated by oxytocin (EC_{50}=41.6μM, Fig. 5A) but not vasopressin (Fig. 5A), suggesting the possibility that oxytocin antagonists may also inhibit inotocin signalling. To test this, we used atosiban, an oxytocin analogue with the modification of amino acids at 1, 2, 4 and 8, which is medically used for the treatment of preterm labour (53). The activation of the inotocin receptor by inotocin was inhibited by atosiban in a dose-dependent manner (--IC_{50}=599μM, Fig. 5B), although this effect was weaker than against oxytocin signalling (IC_{50}=0.65μM, SI Appendix, Fig. S6) (54, 55). To examine whether atosiban

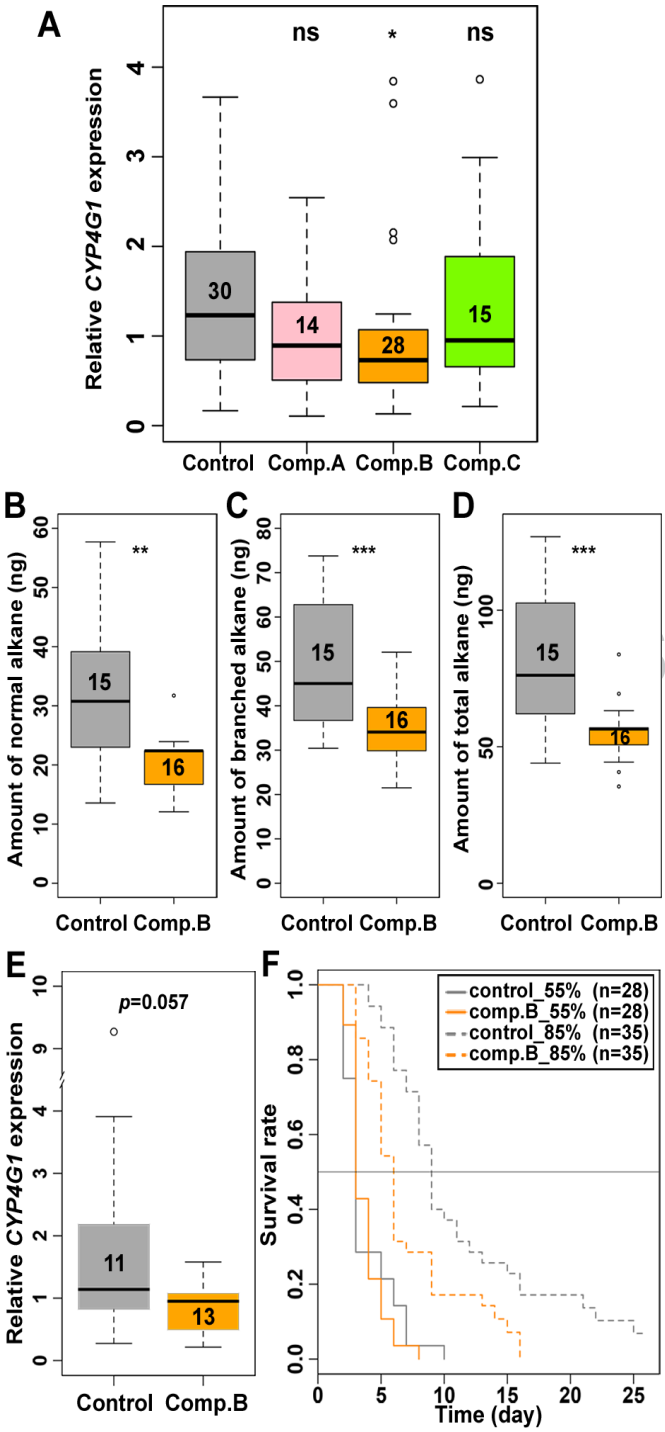

Fig. 6. Relationship between inotocin signalling, hydrocarbon synthesis and desiccation resistance(A) *CYP4G1* expression (qRT-PCR) for comp. A-treated, comp. B-treated, comp. C-treated and control 4-month old workers. ns $p>0.05$; *$p<0.05$ (GLMM with Dunnet *post-hoc* tests). (B-D) The quantities of normal (B), branched (C) and total alkanes (D) in comp. B-treated and control >5-month old workers. (E) qRT-PCR for *CYP4G1* in comp. B-fed and control 4-month old workers. Number of individuals is shown in each box. The data were analysed with GLMMs. **$p<0.01$; ***$p<0.001$. (F) Survival curves for control (grey) and comp. B-treated (orange) ants in 55% RH (solid line) or 85% RH (dashed line). Sample sizes are as follows; n=28 for ctrl_55% and comp. B_55%, and n=35 for ctrl_85% and comp. B_85%).

prevents binding of inotocin to inotocin receptor, as is the case with oxytocin (56), we performed a time-resolved fluorescence resonance energy transfer (TR-FRET) assay to measure the binding efficiency between inotocin and inotocin receptor. Atosiban treatment resulted in a dose-dependent decrease in the FRET ratio (IC_{50}=58.8μM, Fig. 5C), indicating that it directly inhibits binding between inotocin and its receptor, thereby functioning as an antagonist of inotocin signalling. Because the inhibition effect of atosiban against inotocin signalling was not as strong as against oxytocin signalling, we performed a high throughput chemical screening to identify novel chemical compounds acting as antagonists to inotocin signalling.

In the first screening with 224,400 chemical compounds library, we obtained 902 compounds whose inhibition rate against inotocin signalling was higher than 50% (Fig. 5D). The Z' value (a parameter assessing the robustness of an assay for high throughput screening) was more than 0.5 in all assay plates, indicating that the system was adequately optimised for the screening (Fig. 5E). Here it is expected that these candidate chemicals contain nonspecific compounds blocking not only inotocin signalling but also other signalling pathways, for example by targeting factors common with the G protein coupled-receptor (GPCR) pathway. To exclude such non-specific inhibition, we performed a second screening in which we examined the inhibitory effects of each compound against both inotocin and oxytocin signalling (Fig. 5F). If the effector site of these candidates is inotocin receptor itself, the inhibition rate against oxytocin signalling should be weaker than against inotocin signalling. Twenty-three compounds showed higher inhibition rate against inotocin signalling than oxytocin signalling (pink-shaded region in Fig. 5F). Among these 23 candidates, three chemicals (named comp. A, comp. B, or comp. C in Fig. 5G) showed an inhibition rate that was less than half on oxytocin signalling than on inotocin signalling even in tests with the higher dosage (20μM) of each compound. These three chemicals also showed a dose-dependent inhibition against inotocin signalling (Fig. 5H) and were therefore selected for studying the role of inotocin signalling on CHC synthesis.

Regulation of cuticular hydrocarbon synthesis via inotocin signalling

To study the effect of antagonists *in vivo*, we injected compounds A, B and C into 4-month old workers and examined the expression of *CYP4G1* with qRT-PCR. If these compounds inhibit inotocin signalling, the injection of each compound should, similar to the *intR* dsRNA treatment, lead to a down-regulation of *CYP4G1* expression. Consistent with this reasoning, *CYP4G1* expression was lower in workers injected with compound A, B and C, although the difference was only significant for compound B (p=0.042, Fig. 6A). In addition, we found that *CYP4G1* expression was also significantly down-regulated by atosiban (1mM: p=0.026, 2mM: p=0.0002, SI Appendix, Fig. S7A). These results, together with those of the dsRNA treatment, indicate that inotocin signalling positively regulates *CYP4G1* expression.

We next examined whether down-regulation of *CYP4G1* expression has a quantitative effect on the expression of CHCs by testing compound B, the antagonist that most effectively inhibited inotocin signalling *in vitro* (Fig. 5H), and significantly down-regulated the expression of *CYP4G1* (Fig. 6A). In *C. fellah*, and broadly speaking in ants, CHCs are mostly composed of long chain saturated (normal) and methyl-branched (branched) alkanes (37). We found that the quantity of normal alkanes was higher in foragers than nurses (F_{22}=9.4, p=0.006, SI Appendix, Fig. S8A), though neither the quantities of branched alkanes (F_{22}=1.1, p=0.3, SI Appendix, Fig. S8B), nor total alkanes (F_{22}=0.6, p=0.45, SI Appendix, Fig. S8C) was significantly different between the two types of workers. By comparing the quantity of CHCs between control and compound B-injected >5-month old workers, we found that compound B-treated ants had reduced quantities of normal alkanes (F_{28}=13.5, p=0.001, Fig. 6B) and branched alkanes (F_{27}=16.5, p=0.0004, Fig. 6C). Ac-

cordingly, the total quantity of alkanes was also significantly lower (F_{27}=16.7, p=0.0004, Fig. 6D). Injection of 2mM atosiban also led to reduced quantities of normal alkanes (F_{40}=7.75, p=0.008, SI Appendix, Fig. S7B) and a tendency for reduced branched alkanes (F_{40}=3.1, p=0.088, SI Appendix, Fig. S7C), with the effect that the total quantity of alkanes was also significantly lower (F_{40}=6.87, p=0.012, SI Appendix, Fig. S7D).

If down-regulation of *CYP4G1* decreases production of CHCs, we predicted that ants treated with compound B should have lower resistance to desiccation and survive less well in an environment where water intake is restricted. To test this, we compared the survivability of workers fed with 1μM compound B (treatment) with those treated with 1% DMSO (control) when given no access to water. We first confirmed that ants fed with compound B had a tendency for lower expression of *CYP4G1* than control ants (F_{22}=4.03, p=0.057, Fig. 6E), as previously observed in the injection assays (Fig. 6A). To manipulate desiccation rates, we placed seven workers in a box at either 55% relative humidity (RH) or 85% RH. Under 55% RH both treatment and control workers died rapidly and there was no difference in survival (p=0.76), suggesting that these experimental conditions were too severe for ants, regardless of treatment. Under the 85% RH condition both treatment and control groups survived significantly longer (p<0.0001, Fig. 6F) and there was a significant lower survival for ants treated with compound B compared to control ants (p=0.026, Fig. 6F). This suggests that in conditions where water is absent ants are capable of surviving longer with intact *CYP4G1* and CHC function.

Discussion

Our study reveals that the levels of *int* and *intR* mRNA are very high in workers, and positively correlated with age and foraging behaviour. Moreover, when ants of the same age were placed into a new artificial nest and allowed to re-establish division of labour, those initiating foraging had higher levels of *int* or *intR* expression than those staying within the nest, suggesting that inotocin signalling may be linked to the onset of foraging behaviour in ants. The levels of expression of oxytocin and vasopressin is known to also influence social behaviour in mammals. For example, the level of social investigation is associated with the level of expression of *oxytocin* and *vasopressin* mRNA in mice (57). In mice, the level of expression of *vasopressin* is also correlated with aggressive behaviour (58, 59) while oxytocin signalling is involved with social bonding and affiliation (4, 60, 61) .

CHCs are known to prevent desiccation in insects (33-35) and recent work on flies supports the hypothesis that increased CHC production improves desiccation resistance (62). In ants, dessication is an important challenge when workers switch task and initiate foraging. We found that *C. fellah* workers switch task when they are 4-months old. Similar results were obtained in other studies (32, 47). As workers age, they experience a drastic change in their environment as they switch from brood care and other within-nest tasks to foraging outside of the colony. Factors such as humidity and temperature can vary greatly over time and space outside the nest while they are relatively stable within the nest. Similar to findings in the ants *Pogonomyrmex barbatus* and Formica exsecta (40-43), we found task-associated variation in CHCs, with foragers exhibiting the highest proportion of normal alkanes. Moreover, experimental increments of temperature and decreases in humidity also triggered an increase in the proportion of normal alkanes in harvester ants (41). Given that we found that *intR* is expressed in the oenocytes, we investigated the relationship between inotocin signalling and the CHC synthesis pathway. We focused on the *CYP4G1* gene, which is known to be expressed in oenocytes (44-46), where it regulates the final step of CHC synthesis by converting aldehyde to hydrocarbons (45). Using immunohistochemistry, we first confirmed that the CYP4G1 protein is specifically expressed in the oenocytes in workers. Next, we showed a strong positive association between the levels of expression of *intR* and *CYP4G1*, suggesting that increased levels of *intR* expression might be associated with increased level of *CYP4G1* when workers start foraging. These lines of evidence suggest foraging ants are able to reduce the risk of desiccation through up-regulation of the inotocin signalling pathway, generating elevated quantities of protective CHCs.

Because variation in task allocation is associated with variation in CHCs not only in *C. fellah* (this study) but also in the two other ants that have been investigated so far (i.e., *P. barbatus* and F. exsecta, (40-43)), it would be interesting to test whether inotocin signalling does also regulate the production of CHCs in these species. In the ants *Lasius niger* and *Lasius neglectus*, the level of expression of *intR* has also been shown to be higher in the abdomen than head and thorax (21), suggesting that this gene might also be implicated in the production of CHCs. Moreover, as in *C. fellah,* the expression of *int* is also higher in the head of foragers than nurses in the ant *Monomorium pharanonis* (63). The similarity in the expression profiles of *inotocin* and *inotocin receptor* in the ant species that have been studied so far suggests that inotocin signaling may, as in *C. fellah,* also function to regulate the expression of CHCs in other ant species.

To provide functional evidence for a role of *intR* on the level of expression of *CYP4G1* we fed 5-month old workers with dsRNA targeting *intR*. These treated workers exhibited a lower level of expression of both *intR* and *CYP4G1* compared to control ants. Next, we conducted pharmacological studies to identify new compounds acting as antagonists of the inotocin signalling pathway. These studies identified three effective antagonist compounds (named A, B and C). Injection of these antagonists led to decreased expression of *CYP4G1.* Finally, *CYP4G1* expression was also significantly down-regulated by atosiban, an inhibitor of oxytocin in vertebrates (64, 65), which we showed to also be effective in decreasing the activation of inotocin receptor by inotocin *in vitro*. Together, these results show that inotocin signalling positively regulates *CYP4G1* expression.

Our experiments also showed that workers treated with compound B had reduced quantities of normal and branched alkanes and a lower survival than control workers under water-limited conditions. These findings are interesting because *int* and its receptor are both up-regulated when workers age and switch task from nursing to foraging. Since workers are more likely to encounter drier environments when they forage, this suggests that inotocin signalling may be implicated in regulating physiological adaptations via increased CHC synthesis when workers initiate foraging.

Our study provides the first evidence that the inotocin signalling pathway regulates CHC synthesis via *CYP4G1* expression in insects, and this pathway is up-regulated with age and task preference in ants. Interestingly, genomic studies revealed that the inotocin signalling pathway has been lost in the honeybee and some other species of aphids and flies (16). As these species have the *CYP4G* gene (45) and expresses CHCs on their body surface, it is possible that other signalling pathways have taken over the functions of inotocin signalling in these species, as discussed previously (16, 22). It will therefore be of interest to compare the mechanisms of regulation of *CYP4G* gene and CHCs in these species that have lost the inotocin signalling pathway. Such comparative studies may also shed light on the ancestral role of the oxytocin/vasopressin family peptide in insects and vertebrates.

Methods

More details are provided in SI Appendix.

Ants

C. fellah colonies were initiated from queens collected after a mating flight in March 2003 and 2007 in Tel Aviv, Israel. The ants were reared in an incubator (NIPPON MEDICAL & CHEMICAL INSTRUMENTS CO., LTD) under controlled conditions (12:12 LD, 30°C, 55∼60% RH). For all experiments, we

used workers from four queenright colonies that each had one queen and approximately 500 workers. We used only minor workers with a body size <8 mm. To determine their age, we painted every month all newly-eclosed workers with a unique colour code. Young virgin queens, males and workers were collected at the beginning of mating flights in May 2015 and 2016 in Tokyo and Ibaraki, Japan (*C. japonicus*) and March in 2018 in Rehovot, Israel (*C. fellah*). Wingless mated queens of both species were collected on the ground just after the mating flight.

Quantitative RT-PCR

RNA was extracted with Trizol (Invitrogen) from whole body (Fig. 2F-2K), body parts (Fig. 1, 2C, 2D, 4D-4G, 6A, 6E, SI Appendix, Fig. S1, S5, and S7A), or dissected tissues (Fig. 3L and 4A). RNA from queens was sequentially purified with RNA Plus micro Kit (Qiagen). cDNA was synthesised from 200ng total RNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara). qRT-PCR was performed with Takara SYBR Premix Ex Taq II (Tli RNaseH Plus) using 7900HT (Applied Biosystems), a LightCycler 480 (Roche) or MX3000P (Agilent). Primers for qRT-PCR are listed in Table S2. All primers had similar PCR amplification efficiency (~2.0). The most stable reference genes and gene normalisation factors were determined by the software geNORM with Biogazelle software (qbase). We used *ef1a* and *rp2* for whole body, head and abdomen analyses, and *gapdh* and *rp2* for the fat body plus oenocyte and digestive tract analyses. The relative expressions of target genes were scaled against average values in each experiment with the Biogazelle software (qbase).

Quantification of peptide with LC-MS/MS

The detailed procedure for peptide extraction is shown in SI Appendix. The amount of inotocin peptide was quantified by ultra-performance liquid chromatography (ACQUITY System, Waters), equipped with a tandem mass spectrometer, TQD (Waters). Separation was achieved on an AccQ-Tag Ultra RP Column (2.1×100mm, 1.7μm particles, Waters) at 40°C. The two mobile phases consisted of (A) 20mM formic acid and (B) acetonitrile. Linear gradients from 99.9% (A) to 50% (A) were used for the separation at a flow rate of 0.3 ml/min. The effluent was then introduced into TQD, ionised by electrospray ionization with positive ion mode (ESI+), and detected by multiple reaction monitoring (MRM) using the transition of m/z 328.4 to m/z 86.38. Sample concentrations were calculated from the standard curve obtained from serial dilution of synthesised inotocin peptide (Biogate Co., Ltd).

DNA constructs

The cDNA of the *int* and *intR* was obtained by reverse transcription using Prime script™ RT Reagent Kit with gDNA eraser (Takara) and RNA isolated from whole body of *C. fellah* and *C. japonicus* worker from laboratory colony. The oxytocin receptor (OXTR) and Gα15 were amplified from the following vectors respectively: Human OXTR in pcDNA3.1+ (cDNA Resource Center #OXTR00000), Human Gα15 in pcDNA3.1+ (cDNA Resource Center #GNA1500000). Detailed procedure to construct *pPB-intR-mcherry* and *pPB-OXTR-mcherry* is shown in SI Appendix.

Generation of antibodies

Polyclonal rabbit antibody to inotocin proprotein was generated with a synthetic peptide CKISDVADREITDLYMVLSGNEVSNEILP corresponding to the C terminus of inotocin proprotein (MBL Co., Ltd). Polyclonal rabbit antibody to CYP4G1 was generated with a synthetic peptide RDDLDDIDENDRGEKRRLA corresponding from 301 to 319 amino acid sequence of CYP4G1 (Hokudo Co., Ltd). The antibodies were used for immunostaining (Fig. 3D-3J, 4C, and SI Appendix, Fig. S4D) and western blot (Fig. 4B, SI Appendix, Fig. S4B and S4C).

Western blot analysis

We subjected tissue lysates to 12.5% SDS-PAGE analysis and immunoblotting. Rabbit polyclonal antibody to inotocin (1:50), rabbit polyclonal antibody to CYP4G1 (1:500), anti-HistoneH3 antibody (1:500, Cell signaling), anti-myc (1:1000, Invitrogen), anti-βtubulin (1:2000, Invitrogen), anti-gapdh (1:2000, Cell signaling) were used as primary antibodies, whereas horseradish peroxidase–conjugated antibody to rabbit (1:1000, Cell Signaling), antibody to mouse (1:1000, Promega) were used as secondary antibodies. Immobilon Western (Millipore) were used for detection. As negative control, we mixed in a ratio of 1:20 (mol) the antibodies for inotocin and CYP4G1 with their synthesised antigen peptides incubated at 4°C overnight. After the overnight incubation they were stored in 50% glycerol at -20°C. Images were captured with the LAS4000 (GE Healthcare ImageQuant) or Chemidoc™ XRS+ systems (BIO-RAD).

Immunohistochemistry

Whole brain immumohistochemistry was performed as described (66) with some modifications in SI Appendix. Fluorescent images were captured with TCS-SP5 laser scanning confocal microscopy (Leica) with a HC PL Fluotar 10x/0.30 objective (Leica, 506505) or HC PL APO 20x/0.70 objective (Leica, 506513) and the Application Suite Advanced Fluorescence software (Leica) or LSM700 laser scanning confocal microscopy (Carl Zeiss) with a Plan-APOCHROMAT 10X/0.45 or 20X/0.8 objective (Carl Zeiss) and ZEN 2011 software.

***in situ* hybridisation**

To examine *int* mRNA expression in the brain, fluorescent *in situ* hybridisation was performed using RNAscope® Multiplex Fluorescent Reagent Kit 2.0 according to the manufacturer's instructions (Advanced Cell Diagnostics). Detailed protocol is shown in SI Appendix.

dsRNA generation and treatment

Gene-specific 520bp sequences for *intR* (1-520bp) and control *EGFP* (104-623bp) with T7 RNA polymerase sequence at both ends were amplified by PCR with primers listed in Table S3, and *in vitro* transcription was performed using T7 RiboMAX Express RNAi System (Promega) according to the manufacturer's protocol. The concentration and the size of dsRNA were determined with a nanodrop spectrophotometer and electrophoresis. dsRNA was adjusted to 4μg/μl with 300mM sucrose water. The feeding treatments with dsRNA were performed as previously described (51). For first 10 days of the treatment, ants were fed daily with 20μl of dsRNA-sucrose water, then with 20μl sucrose water with no dsRNA during the last 5 days. After these 15 days treatment, RNA was extracted from each body part for qRT-PCR analyses.

Calcium assay and chemical screening

The protocol to generate stable cell lines and cell preparation for calcium assay is shown in SI Appendix. We used Functional Drug Screening System (FDSS7000, Hamamatsu Photonics) to measure the fluorescent level of Fluo-4 with the excitation and emission wavelength bands centered at 480 and 540 nm, respectively. The ligand was diluted with the buffer (20mM HEPES in HBSS) and dispensed to the non-binding 384 well plate (Greiner). 5μl of ligand solution was dispensed to each well with plastic tip (Hamamatsu Photonics) 10 seconds after the measurement started. The fluorescent intensity was measured for 2 min in each plate. The ligands were washed away between each experiment with a cleaning solution (10mM citric acid (Nakarai) in 50% ethanol). The fluorescent intensity from 1 to 10 second, or from 11 to 120 second in the measurement was used in the following formula. Response to the ligand (R) was given by

$$R = \mathrm{Max}\,(\mathrm{Intensity}_{11-120}) - \mathrm{Average}(\mathrm{Intensity}_{1-10})$$

We performed the chemical screening with 222,400 compounds obtained from the Drug Discovery Initiative (DDI, The University of Tokyo). Detailed procedures for 1st and 2nd chemical screening are shown in SI Appendix.

Binding assay with TR-FRET

Inotocin receptor ligand binding assays were performed using the Tag-lite® assay (cisbio) according to the manufacturer's instructions shown in SI Appendix.

Antagonist injection

To select the appropriate dosage of each compound for injection, we first examined the effect of DMSO on ant survivability, because compound A, B, and C were stocked in DMSO (10mM concentration). We found that 1% of DMSO showed no effect on survivability while 10% of DMSO resulted in high lethality. Therefore, we injected 100μM of compound A (PB03670558, U.O.S.), B (PB153374836, U.O.S.) or C (T5275621, Enamine) in 1% DMSO to avoid negative effects of DMSO. Because atosiban (Sigma-Aldrich) had a weaker inhibition rate (Fig. 5B) than compound A, B or C (Fig. 5H) *in vitro* and because it is soluble in water we used higher concentrations (1mM and 2mM). Ants were anesthetised on ice and injected into the abdomen through the intersegmental membrane with 1.5 μl of the different compound solution with a IM-300 microinjector (Narishige). Control saline was prepared as the mixture of ant saline with the solvent of each chemical compounds (with water for atosiban (1:10 for 1mM, and 1:5 for 2mM), and with DMSO for compound A, B, and C (1:100)). For the expression analysis of *CYP4G1*, RNA was extracted at 24 hours after the injection of each compounds.

Behaviour tracking system and tracking data processing

Behavioural tracking was performed as described previously (47). Ants were tagged with unique matrix codes (1.6mm side length) after immobilisation on ice. Tracking experiments were performed under controlled conditions (12:12 LD, 30 °C, 55∼60% RH). The tracking data were post-processed as described previously (47). Parameters for time spent in the food, distance ant moved, and time spent in the nest were obtained as described previously (67).

Cuticular hydrocarbon analyses

The extraction and quantification of CHCs was performed as described previously (68) with some modifications. Detailed protocol is shown in SI Appendix.

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collection and analysis, decision to publish, or preparation of the manuscript. **AUTHOR CONTRIBUTIONS** AK planned and directed this study, performed experiments, and wrote the manuscript with LK. NM, HT, and TO conducted the pharmacological screening and *in vitro* experiment. NM and MM did the LC-MS/MS experiments. SM conducted behavioural experiments and conducted the statistical analyses. MM and LK contributed to the planning of the study. All authors contributed to editing the manuscript. **DECLARATION OF INTERESTS** The authors declare no conflict of interest.

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