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RESEARCH ARTICLE

Serotonergic control in initiating defensive responses to unexpected tactile stimuli in the trap-jaw ant *Odontomachus kuroiwae*

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ABSTRACT

The decision to express either a defensive response or an escape response to a potential threat is crucial for insects to survive. This study investigated an aminergic mechanism underlying defensive responses to unexpected touch in an ant that has powerful mandibles, the so-called trap-jaw. The mandibles close extremely quickly and are used as a weapon during hunting. Tactile stimulation to the abdomen elicited quick forward movements in a dart escape in 90% of the ants in a colony. Less than 10% of the ants responded with a quick defensive turn towards the source of stimulation. To reveal the neuronal mechanisms underlying this defensive behavior, the effect of brain biogenic amines on the responses to tactile stimuli were investigated. The levels of octopamine (OA), dopamine (DA) and serotonin (5HT) in the brain were significantly elevated in ants that responded with a defensive turn to the unexpected stimulus compared with ants that responded with a dart escape. Oral administration of DA and 5HT demonstrated that both amines contributed to the initiation of a defensive response. Oral administration of L-DOPA weakly affected the initiation of the defensive turn, while 5-hydroxy-L-tryptophan (5HTP) strongly affected the initiation of defensive behavior. Oral administration of ketanserin, a 5HT antagonist, inhibited the initiation of the defensive turn in aggressive workers, abolishing the effects of both 5HT and 5HTP on the initiation of turn responses. These results indicate that 5HTergic control in the nervous system is a key for the initiation of defensive behavior in the trap-jaw ant.

KEY WORDS: Biogenic amines, Serotonin, Dopamine, Defensive behavior, Escape behavior

INTRODUCTION

Ponerine ants hunt and consume small arthropods, which represent the major component of their diet (Peeters, 1997). During hunting, forager ants must increase their levels of aggressiveness to attack and capture prey, and to defend against unexpected encounters with an enemy. Defensive behavior can escalate to violent attacks against opponents, which also increase the risk of damage. Therefore, the choice to escape may in some cases increase the survival by avoiding such risks. Similar to individual behavior, social decisions to defend or escape are crucial in the survival of a colony (Holway et al., 1998).

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The ants in the genus *Odontomachus* have long and powerful mandibles, the so-called trap-jaw, that functions as a weapon when hunting (De la Mora et al., 2008). The trap-jaw ant captures prey by closing the mandibles extremely quickly (Movie 1) (Gronenberg, 1996a; Just and Gronenberg, 1999). If the ant encounters a potential threat, however, the ant responds rapidly with a defensive turn response or with a dart escape response (Movie 2). Behavioral responses to unexpected tactile stimulation with either defensive turns or dart escapes are common in other arthropods such as crayfish (Nagayama et al., 1986) and crickets (Alexander, 1961). It is believed that social experience influences the behavioral response to an unexpected tactile stimulus (Song et al., 2006).

Biogenic amines function as neurotransmitters, neuromodulators and neurohormones in the brain, and play key roles in behavior (Evans, 1980; Baumann et al., 2003; Roeder, 2005). The octopaminergic (OAergic) system may be associated with nest-mate recognition in social insects such as honeybees (Robinson et al., 1999) and ants (Vander Meer et al., 2008). Aminergic systems in the brain are closely associated with aggressive behavior in arthropods (Edwards and Kravitzt, 1997; Kravitz, 2000; Stevenson et al., 2005; Hoyer et al., 2008; Johnson et al., 2009; Rillich et al., 2011). The effect of biogenic amines on aggressive behavior has also been demonstrated in ants [e.g. octopamine (OA) (Aonuma and Watanabe, 2012b; Yakovlev, 2018), tyramine (TA) (Szczuka et al., 2013), dopamine (DA) (Vander Meer et al., 2008; Ohkawara and Aonuma, 2016; Shimoji et al., 2017) and serotonin (5HT) (Kostowski et al., 1975)]. To gain an understanding of the neuronal mechanisms underlying the initiation of defensive behavior in the trap-jaw ant, this study focused on the behavioral responses to unexpected tactile stimuli. Moreover, the study investigated how biogenic amines contribute to the initiation of behavioral responses to such unexpected tactile stimuli, because social interactions between nest-mates influence aminergic homeostasis in the brain (Wada-Katsumata et al., 2011).

The levels of amines in the brains of workers were measured and compared between those that responded to tactile stimulation with a defensive turn and those that responded with a dart escape. The effects of amine oral administration on initiation of defensive behavior was then examined to confirm whether biogenic amines are associated with the initiation of defensive behavior. This work provides insights into the understanding of how trap-jaw ants initiate defensive behavior against an unexpected encounter with an enemy.

MATERIALS AND METHODS

Animals

Workers of the trap-jaw ants *Odontomachus kuroiwae* (Matsumura 1912) were used throughout this study. Colonies of ants were collected in Okinawa, Japan. They were mostly polygynous and contained three to four queens and 200–300 workers and broods.

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Each colony was installed into an artificial plaster nest (200×200×40 mm) in a plastic case (600×400×200 mm) on a 14 h:10 h light:dark cycle (lights on at 06:00 h) at 25±2°C. Ants were fed a diet consisting of insect jelly (Marukan Co., Ltd, Osaka, Japan), cricket nymphs, and water *ad libitum*. Notably, there are no distinctive differences between major and minor workers in *O. kuroiwae*.

Behavioral experiments

Odontomachus kuroiwae workers were randomly collected inside the plaster nests and kept isolated in a plastic Petri dish (50 mm diameter) for 60 min before behavioral experiments. To elicit defensive turn or dart escape behavior, the abdomen of an ant was touched gently using the tip of a fine paintbrush. A similar behavioral assay was used in previous studies on crayfish (Nagayama et al., 1986; Aonuma et al., 1994). Behavioral responses to the tactile stimulation of the abdomen were observed and recorded using a digital video camera (JVC, GC-P100, Tokyo Japan) for later analysis. The defensive levels were classified into four types (Fig. 1, Movie 2). An ant that responded with a quick forward movement, the so-called dart escape, to a tactile stimulus was scored as 'level −1'. If an ant did not respond to the stimulus, it was scored as 'level 0'. Defensive turn responses were divided into two types so that if an ant turned towards the stimulus without opening its mandibles it was scored as 'level 1', while if it turned with its mandibles open it was scored as 'level 2'. Either a defensive turn or a dart escape is usually elicited as soon as the tip of the paintbrush makes contact with the abdomen (Fig. S2). If the ant was pinched between the brush and floor, it tried to bite the paintbrush. This aggressive attack was judged as a different response from the defensive turn and omitted from data analysis.

Measurement of brain biogenic amines

The levels of biogenic amines in the brain were measured using high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) (Wada-Katsumata et al., 2011; Aonuma and Watanabe, 2012a). Workers of *O. kuroiwae* with a body mass of 9.9±1.1 mg (N=60, mean±s.d.) were randomly collected from the inside of the nest in the colonies. Because the levels and content of brain amines vary with age (Seid and Traniello, 2005, 2006; Aonuma and Watanabe, 2012a), aged workers with a dark brown body color were used in this study. The body color of aged workers in the trap-jaw ant is much darker than that of newly emerged individuals (Fig. S1). Test animals were sampled between 10:00 and 12:00 h to avoid circadian effects (Tomioka et al., 1993). This study compared the levels of brain amines of ants that showed dart responses (level −1) with those that showed turn responses (level 2) to tactile stimulation of the abdomen. Thirty level −1 workers and another 30 level 2 workers were collected from three different colonies (10 ants from each colony). Ants were then quickly frozen in liquid N_2 to inhibit enzyme activity. The brains were dissected out in ice-cold normal saline (128.3 mmol l^{-1} NaCl, 4.7 mmol l^{-1} KCl, 1.63 mmol l^{-1} $CaCl_2$, 6 mmol l^{-1} $NaHCO_3$, 0.32 mmol l^{-1} NaH_2PO_4, 82.8 mmol l^{-1} trehalose, pH 7.4). Each brain was placed into a micro glass homogenizer and homogenized with 50 µl of ice-cold 0.1 mol l^{-1} perchloric acid containing 5 ng of 3,4-dihydroxybenzylamine (DHBA, Sigma-Aldrich, St Louis, MO, USA) as an internal standard. After centrifugation of the homogenate (0°C, 15,000 ***g***, 30 min), 35 µl of supernatant was collected.

The HPLC-ECD system comprised a pump (EP-300, EICOM Co., Kyoto, Japan), an auto-sample injector (M-504, EICOM Co.) and a C18 reversed-phase column (250×4.6 mm internal diameter, 5 µm average particle size, CAPCELL PAK C18MG, Shiseido, Tokyo, Japan) heated to 30°C in the column oven. A glass carbon electrode (WE-GC, EICOM Co.) was used for electrochemical detection (ECD-100, EICOM Co.). The detector potential was set at 890 mV versus an Ag/AgCl reference electrode, which was also maintained at 30°C in a column oven. The mobile phase, containing 0.18 mol l^{-1} chloroacetic acid and 16 µmol l^{-1} disodium EDTA, was adjusted to pH 3.6 with NaOH. Sodium-1-octanesulfonate at 1.85 mmol l^{-1} as an ion-pair reagent and CH_3CN at 8.40% (v/v) as an organic modifier were added into the mobile phase solution. The flow rate was kept at 0.7 ml min^{-1}. The chromatographs were acquired using the computer program PowerChrom (eDAQ Pty Ltd, Denistone East, NSW, Australia). The supernatants from the samples were injected directly onto the HPLC column. After acquisition, they were processed to determine the amount of biogenic amines in the same sample by the ratio of the peak area of substances to that of the internal standard DHBA. A standard mixture containing amines, precursors and metabolites was used for quantitative determination. The 20 compounds at 100 ng ml^{-1} each were DL-3,4-dihydroxy mandelic acid (DOMA), L-β-3,4-dihydroxyphenylalanine (DOPA), L-tyrosin (Tyr), *N*-acetyloctopamine (Nac-OA), (-)-noradrenaline (NA), 5-hydroxy-L-tryptophan (5-HTP), (-)-adrenaline (A), DL-octopamine (OA), 3,4-dihydroxybenzylamine (DHBA, as an internal standard), 3,4-dihydroxyphenylacetic acid (DOPAC), *N*-acetyldopamine (Nac-DA), 3,4-dihydroxyphenethylamine (DA), 5-hydroxyindole-3-acetic acid (5HIAA), *N*-acetyltyramine (Nac-TA), *N*-acetyl-5-hydroxytryptamine (Nac-5HT), tyramine (TA), L-tryptophan (Trp), 3-methoxytyramine (3MTA), 5-hydroxytryptamine (5HT) and 6-hydroxymelatonin (6HM). Nac-OA, Nac-DA, and Nac-TA were synthesized by Dr Matsuo (Keio University, Japan). All other substances were purchased from Sigma-Aldrich.

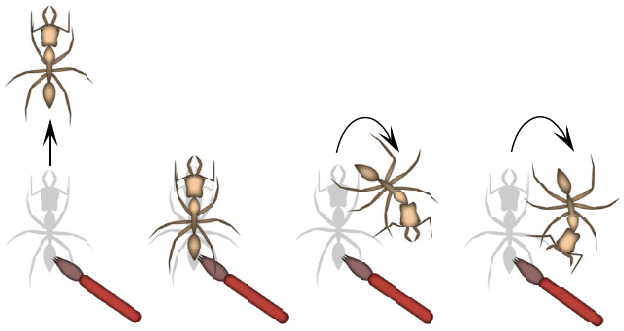

Fig. 1. Pictograms illustrating the responses of ants to tactile stimulation of the abdomen of the ants. The response was classified into four levels (levels −1 to 2). Level −1, dart response: the ant quickly moved forward. Level 0, no response: the ant shows no obvious response to a tactile stimulus. Level 1, turn responses without opening of the mandibles: the ant orientated towards the source of the stimulus without opening the mandibles. Level 2, turn responses with open mandibles: the ant orientated towards the source of the stimulus and opened the mandibles widely.

Pharmacological experiments

To investigate the effect of biogenic amines on the initiation of defensive responses to the tactile stimuli, pharmacological experiments were performed. For insect pharmacological studies, one of the following three different pharmacological treatment

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methods is typically used to increase biogenic amine levels in the nervous system: injection, topical or oral application. Similar to drug injection, the oral application of either biogenic amines (Barron et al., 2007) or their precursors (Sasaki et al., 2012; Bubak et al., 2020) effectively increases biogenic amine levels in the brain, head capsule, thorax and abdomen. In the present study, oral drug application was employed to avoid potential damage and stress associated with head injection or topical application.

Ants were collected from colonies and placed in plastic Petri dishes. Each ant was left to rest for 60 min after isolation, and then response to the tactile stimulus was examined. Ants that respond with a dart response to the tactile stimulus (less aggressive workers) were used for pharmacological experiments to examine the effects of biogenic amines on the behavior. Pharmacological agents were dissolved in a 20% sucrose solution and 5 µl of the solutions was fed to the ants. For the control, a 20% sucrose solution was used. To manipulate the levels of biogenic amines, serotonin (5HT), octopamine (OA), dopamine (DA), precursor of serotonin 5-hydroxy-L-tryptophan (5HTP) and precursor of dopamine L-β-3,4-dihydroxyphenylalanine (L-DOPA) were used. After oral administration of each agent, the responses of the ant to the tactile stimulus were examined. To confirm the effects of amine and precursor oral application, inhibitors of either DA receptors or 5HT receptors were orally applied. To inhibit DA receptors, chlorpromazine was used (Degen et al., 2000; Mizunami and Matsumoto, 2010), and to inhibit 5HT receptors, ketanserin was used (Vleugels et al., 2015). The effects of the inhibitors on the responses to the tactile stimulus were investigated in less aggressive workers (level −1) and more aggressive workers (levels 1 and 2). All substances were purchased from Sigma-Aldrich.

Statistical analyses

Statistical analyses were performed using Graphpad Prism (Graphpad, v.8.4.2). A Kruskal–Wallis test was used to analyze the difference in behavioral responses among colonies. Differences in the levels of biogenic amines were analyzed using unpaired *t*-tests with Welch's correction. Differences were considered significant at $P<0.05$ level (two-tailed). ANOVA with Tukey's multiple comparison test was used to analyze the pharmacological experiments.

RESULTS

Dart escape and defensive turns

In total, 580 *O. kuroiwae* workers were randomly collected from seven different colonies to examine their behavioral responses to tactile stimuli. There was no significant difference in the responses of the ants to the stimulus among the different colonies (Kruskal–Wallis test). Most of the workers (523 in total out of 580 ants from seven colonies, 90.2±5.2%, mean±s.d.) responded to the stimulus with a dart escape (level −1). As soon as the tip of the paintbrush made contact with their abdomen, the ants quickly moved forward away from the stimulus source. In contrast, 2.4±1.4% (14 in total out of 580) of the ants showed no obvious response to the stimulus (level 0). The behavior of level 0 ants was clearly different from that of the level −1 ants, as the ants did not move away from the stimulus source. Even when the ants walked after the tactile stimulus, their walking speed was much slower than that of level −1 ants (Movie 2). Approximately 10% of the ants responded to the tactile stimulus with a defensive turn (including levels 1 and 2). Fourteen out of 580 ants (2.4±2.2%) responded with a level 1 turn, and most of them showed drumming-like antennal movements to probe the source of the stimulus using antennae. In contrast, 5.0±3.7% (29 in total out of 580 ants) of the ants responded with a level 2 turn to the stimulus. These ants also probed the stimulus source using their antennae. Because further stimulation to the mandibles of the ant was not performed in this study, the ants did not attack the source of the stimulus.

Levels of biogenic amines in the brain

Thirty level 2 workers showing a turn response and 30 level −1 workers showing a dart response were collected from three different colonies (10 ants per level from each colony), and the biogenic amine concentrations in their brains were analyzed using HPLC-ECD.

OA is generated from L-tyrosine through a synthetic pathway different from that for DA generation. Tyrosine decarboxylase generates TA, and then tyramine β-hydroxylase generates OA. Both TA and OA were detected in the brains of all ants that responded to the stimulus. There was no significant difference in the amount of TA in the brains between the ants that showed level 2 turn responses (0.42±0.37 pmol brain^{-1}, N=30, mean±s.d.) and those that showed level −1 dart responses (0.36±0.19 pmol brain^{-1}, N=30) (Fig. 2A). In contrast, the amount of OA in the brains of ants that showed level 2 turn responses (0.94±0.28 pmol brain^{-1}, N=30) was significantly

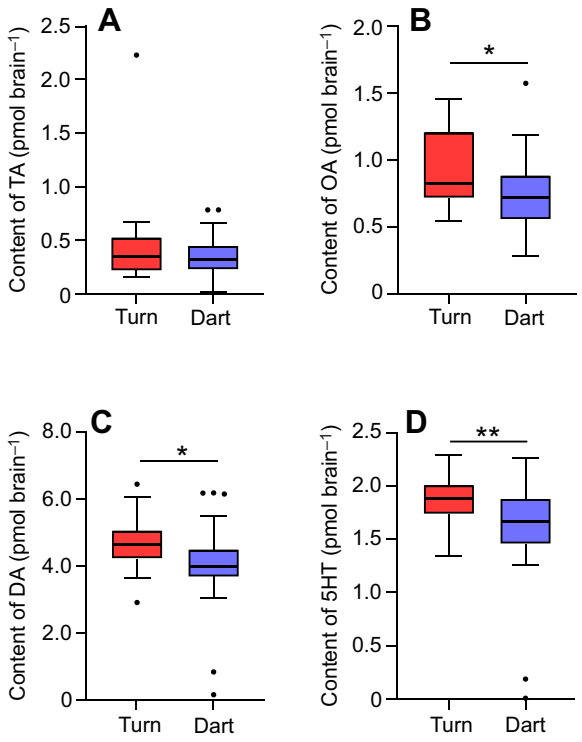

Fig. 2. Contents of biogenic amines in the brains of ants that showed level 2 turn responses and those that showed level −1 dart responses, to tactile stimulation. Box-and-whisker graphs indicate minimum, median, maximum, and 25th and 75th percentiles. (A) Content of TA in the brain. There was no significant difference in the TA contents between the ants that showed level 2 turn responses and those that showed level −1 dart responses (P=0.394). (B) OA content in the brain. Brain OA levels in the ants that showed level 2 turn response were significantly higher than in the ants that showed level −1 dart responses (P=0.0226). (C) DA content in the brain. Brain DA levels in the ants that showed level 2 turn responses were significantly higher than in the ants that showed level −1 dart responses (P=0.0329). (D) 5HT content in the brain. Brain 5HT levels in the ants that showed level 2 turn responses were significantly higher than in the ants that showed level −1 dart responses (P=0.0086).

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greater (P=0.0226) than in the ants that showed level −1 dart response (0.76±0.29 pmol brain^{-1}, N=30) (Fig. 2B). Nac-OA and Nac-TA are catabolites of OA and TA, respectively, which are generated through the activation of arylalkylamine N-acetyltransferase. This study failed to detect Nac-OA, but Nac-TA was detected in the brains of all ants. There was also no significant difference in the levels of brain Nac-TA between ants that showed dart and turn responses (Fig. S2A).

DA is generated from DOPA thorugh the activation of aromatic L-amino acid decarboxylase, and catabolized to Nac-DA through the activation of arylalkylamine N-acetyltransferase. DA and Nac-DA were detected in all samples. The DA content in level 2 ants was 4.67±0.73 pmol brain^{-1} (N=30), while that in level −1 ants was 4.07±1.30 pmol brain^{-1} (N=30). The DA content in the brains of ants showing turn responses was significantly elevated compared with that in the brains of ants showing dart responses (P=0.0329) (Fig. 2C). DOPA is generated from tyrosine by the activation of tyrosine hydroxylase. This study failed to measure both tyrosine and DOPA, because the peaks of these two substances on the chromatogram appeared with unknown front peaks. In contrast, the catabolite of DA (Nac-DA) was detected in the brain. The amount of Nac-DA in the brains of level 2 ants was 0.96±0.43 pmol brain^{-1} (N=30), while that of level −1 ants was 0.93±0.36 pmol brain^{-1} (N=30). There was no significant difference in Nac-DA content between these ants (Fig. S2B).

5HT was also detected in the brain of each ant. The amount of 5HT in the brains of level 2 ants was 1.87±0.23 pmol brain^{-1} (N=30), while that in the brain of level −1 ants was 1.60±0.48 pmol brain^{-1} (N=30) (Fig. 2D). The amount of 5HT in the brain was significantly elevated in level 2 ants compared with level −1 ants (P=0.0086). 5HT is generated from 5HTP by the activation of aromatic L-amino acid decarboxylase. 5HTP was also detected in the brains of each ant. The content of 5HTP in the brains of level 2 ants was 0.34±0.33 pmol brain^{-1} (N=30) and slightly higher than that in the brains of level −1 ants (0.23±0.34 pmol brain^{-1}, N=30), although there was no statistically significant difference (Fig. S2C). Endogenous 5HT is catabolized to Nac-5HT by arylalkylamine N-acetyltransferase. Nac-5HT was detected at a level of 0.36±0.22 pmol brain^{-1} (N=30) in the brains of level 2 ants, while that in the brains of level −1 ants was 0.30±0.13 pmol brain^{-1} (N=30) (Fig. S2D).

Effects of biogenic amine oral administration

Measurements of brain amines demonstrated that the levels of OA, DA and 5HT in ants showing a turn response (level 2) to tactile stimulation were significantly higher than those in ants showing a dart response (level −1). To investigate which brain amines were associated with the initiation of defensive behavior, oral administration of the agonists was performed.

For control experiments, the effect of orally administrating a 20% sucrose solution was examined first (Fig. 3A). Sixty workers were randomly collected from three colonies (20 workers from each colony) and their responses to the tactile stimulus were observed. Fifty-four out of 60 ants responded with a dart escape, and these 54 ants were used to examine the effect of 20% sucrose solution on the response to the tactile stimulus. There was no significant change in the behavioral level scores after administration of the 20% sucrose solution, with most ants still responding with a dart escape after 1 and 2 h.

Oral administration of OA did not significantly change the defensiveness level of the ants. Twenty workers were randomly collected from a colony and their responses to tactile stimulation were observed prior to the application of 1 mmol l^{-1} OA solution.

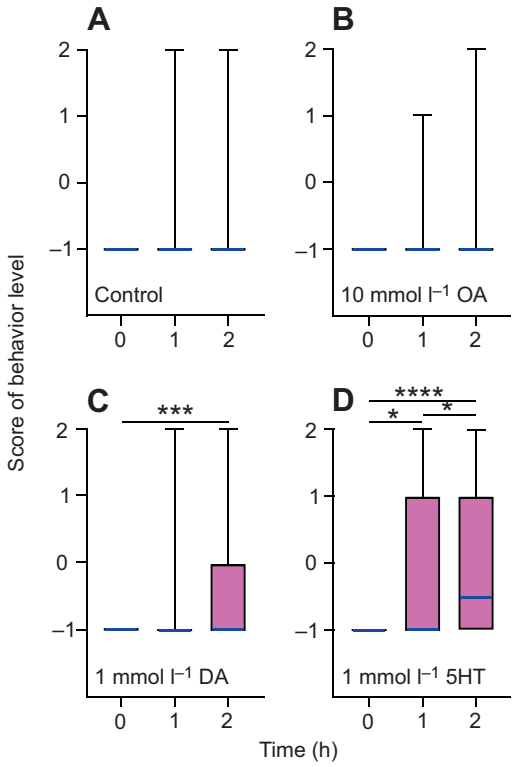

Fig. 3. Pharmacological manipulation of biogenic amines in the brain. Box-and-whisker graphs indicate minimum, median (indicated by a blue bar), maximum, and 25th and 75th percentiles. (A) Effects of oral application of a 20% sucrose solution on defensive behavior. The responses of the ants that showed dart responses to a tactile stimulus were examined (N=54, collected from three different colonies). No significant changes in response to the tactile stimulus were observed after 1 and 2 h after application. (B) Oral administration of 10 mmol l^{-1} OA (N=35, collected from two different colonies). The ants that showed dart responses to the tactile stimulus did not initiate a turn response to the tactile stimulus after 1 and 2 h. (C) Oral administration of 1 mmol l^{-1} DA (N=30 collected from two different colonies). Defensiveness score levels were significantly increased after 2 h. (D) Oral administration of 1 mmol l^{-1} 5HT (N=38, collected from two different colonies). Defensiveness score levels increased significantly after 1 and 2 h. *P<0.05, ***P<0.001, ****P<0.0001.

All ants responded to the stimulus with a dart escape. Oral administration of 1 mmol l^{-1} OA did not change the response to the stimulus even after 2 h. The effect of a 10 mmol l^{-1} OA solution on the behavior was then examined. Forty workers were randomly collected from two different colonies (20 ants from each) and the response to the tactile stimuli prior to oral administration of OA was observed. Thirty-four out of the 40 ants receiving a 10 mmol l^{-1} OA solution orally responded with a dart escape in response to the tactile stimulus (Fig. 3B). Most of these ants did not change their response to the stimulus. Three out of 34 ants showed no obvious response to the stimulus and only one ant showed a turn response after 1 h. Four out of 34 ants showed a turn response to the stimulus 2 h after administration (level 0: N=1; level 1: N=2; level 2: N=1).

Oral administration of DA significantly increased the behavioral level scores of the ants (Fig. 3C). Thirty ants were randomly collected from two colonies (15 ants from each of two colonies) and responses to the tactile stimulus prior to the administration was observed. All of them responded with a dart escape to the stimulus. Oral administration of 1 mmol l^{-1} DA significantly increased the behavioral level scores after 2 h (P=0.0006), although there was no significant difference to that after 1 h (Fig. 3C). Three ants out of 30 responded with a turn behavior after 1 h following oral

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administration of DA (level 0: *N*=1; level 1: *N*=2; level 2: *N*=1). The number of ants that showed a turn response increased to 5 (level 1: *N*=2; level 2: *N*=3) and the number of the ants that did not respond to the stimulus (level 0) was 9.

Oral administration of 1 mmol l^{-1} 5HT solution significantly increased the behavioral level scores after 1 and 2 h (Fig. 3D). Forty workers were randomly collected from two colonies (20 ants from each) and responses to the tactile stimulus were observed. Thirty-eight out of 40 ants showed a dart response to the stimulus and were then used to examine the effect of 5HT administration on the behavior. Ten out of 38 ants responded with a turn response to the tactile stimulus 1 h after administration of 1 mmol l^{-1} 5HT (level 1: *N*=7; level 2: *N*=3). The number of the ants that responded with a turn response increased after 2 h (level 0: *N*=3; level 1: *N*=8; level 2: *N*=8).

Effects of L-DOPA oral administration

To increase endogenous DA levels in the brain, its precursor L-DOPA was orally applied to the ants. Oral administration of 10 mmol l^{-1} L-DOPA solution significantly increased the behavioral level scores after 2 h (Fig. 4A). Before oral administration of 10 mmol l^{-1} L-DOPA, 60 workers were collected from three colonies (20 ants from each of three colonies) and their responses to the tactile stimulus were observed. Fifty-three out of 60 ants responded with a dart response to the stimulus. Then the ants were given a 10 mmol l^{-1} L-DOPA solution orally. Although no obvious behavioral change was observed after 1 h, the number of ants that showed a turn response increased to 19 out of 53 ants after 2 h (level 1: *N*=11; level 2: *N*=8). There were nine ants that showed no obvious response to the stimulus (level 0).

Oral administration of 1 mmol l^{-1} L-DOPA solution also significantly increased the behavioral level scores after 2 h (*P*=0.0007) (Fig. 4B). Sixty ants were randomly collected from three colonies (20 ants from each) and their responses to the tactile stimulus prior to the oral application of 1 mmol l^{-1} L-DOPA solution were observed. Forty-seven out of 60 ants responded with a dart response to the tactile stimulus and were then given 1 mmol l^{-1} L-DOPA solution orally. There was no obvious change in their responses to the stimulus, but the number of ants that responded with a turn response increased after 2 h. Six out of 47 ants responded with a turn response (level 1: *N*=3; level 2: *N*=3) and three out of 47 ants did not respond to the stimulus (level 0). There was a significant difference between 1 and 10 mmol l^{-1} L-DOPA 2 h after oral administration (*P*=0.006).

Oral administration of 0.1 mmol l^{-1} L-DOPA significantly increased the behavioral level scores after 3 h (0 h versus 3 h: *P*<0.0001, 1 h versus 3 h: *P*=0.0036, 2 h versus 3 h: *P*=0.0022) (Fig. 4C). Forty-five ants collected from three colonies (15 ants from each) were used. Forty-one out of 45 ants showed a dart response to the stimulus before oral administration. There was no obvious effect on the responses to the tactile stimulus after 1 and 2 h. However, the number of the ants that responded with a turn increased to 13 out of 41 ants after 3 h (level 0: *N*=3; level 1: *N*=9; level 2: *N*=4). Note that there were not as many ants representing the level 2 defensive turn response to the tactile stimulus.

Effects of chlorpromazine oral administration

To confirm that DA contributes to the initiation of defensive turn responses, the effect of chlorpromazine, a DA receptor antagonist, on the responses was examined (Fig. 5). Twenty level −1 workers were collected from two colonies. They responded with a dart response to the tactile stimulus even after oral administration of 1 mmol l^{-1} chlorpromazine (Fig. 5A). In contrast, workers that showed level 1 or level 2 turn responses to the tactile stimuli (Fig. 5B) significantly decreased their behavioral scores after oral application of 1 mmol l^{-1} chlorpromazine. The inhibitory effect was maximal after approximately 3 h (*N*=16, 0 h versus 1 h: *P*=0.0006, 0 h versus 2 h: *P*<0.0001, 0 h versus 3 h: *P*<0.0001, 1 h versus 3 h: *P*=0.004; for more details, see Fig. 5 legend). Oral administration of either DA (Fig. 3C) or L-DOPA (Fig. 4) initiated defensive turns in less aggressive workers. To confirm the contribution of the DAergic system to the initiation of the defensive turn, a cocktail of chlorpromazine and either DA (Fig. 5C) or L-DOPA (Fig. 5D) was orally applied. Chlorpromazine abolished the effects of DA and L-DOPA. Less aggressive workers (level −1) did not initiate a defensive turn even 3 h after oral application.

Effects of 5HTP oral administration

To increase endogenous 5HT in the ants, 5HTP was orally applied (Fig. 6). Oral administration of 10 mmol l^{-1} 5HTP significantly increased the defensiveness score level after 1 h (0 h versus 1 h: *P*=0.0014) (Fig. 6A). Forty workers were randomly collected (20 ants from each colony) and the response to the tactile stimulus before oral administration was observed. Since all of them responded with a dart response, they were all given 10 mmol l^{-1} 5HTP. The number of ants that responded with a defensive turn to the stimulus increased to 11 out of 40 ants after 1 h (level 1: *N*=6; level 2: *N*=5). The behavioral level scores significantly increased after 90 min (0 h versus 1.5 h: *P*<0.0001, 1 h versus 1.5 h: *P*=0.0002). Twenty-three out of 40 ants responded with a turn response to the stimulus after 90 min (level 0: *N*=1; level 1: *N*=4; level 2: *N*=19).

The effect of 1 mmol l^{-1} 5HTP on behavior was then examined. Oral administration of 1 mmol l^{-1} 5HTP significantly increased the

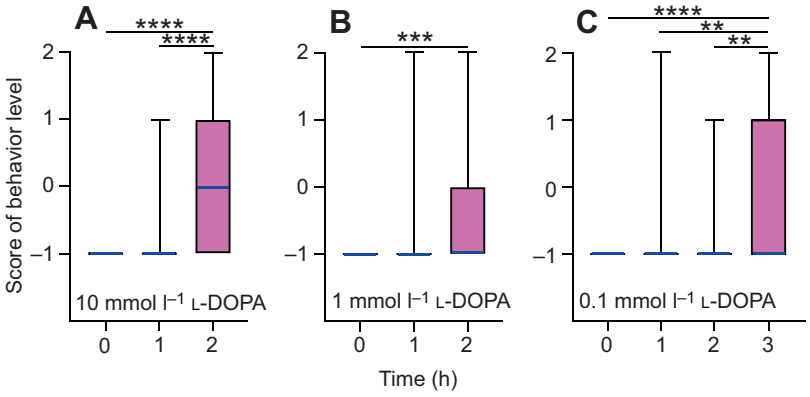

Fig. 4. Pharmacological manipulation of endogenous DA in the brain using L-DOPA. Box-and-whisker graphs indicate minimum, median (indicated by a blue bar), maximum, and 25th and 75th percentiles. (A) Effect of oral administration of 10 mmol l^{-1} L-DOPA, a precursor of DA (*N*=53). Defensiveness score levels did not change after 1 h, however significantly increased after 2 h. (B) Effect of oral administration of 1 mmol l^{-1} L-DOPA (*N*=47). Defensiveness score levels did not change after 1 h but increased significantly after 2 h. (C) Effect of oral application of 0.1 mmol l^{-1} L-DOPA (*N*=41). Defensiveness score levels did not change after 1 and 2 h but increased significantly after 3 h. ***P*<0.01, ****P*<0.001, *****P*<0.0001.

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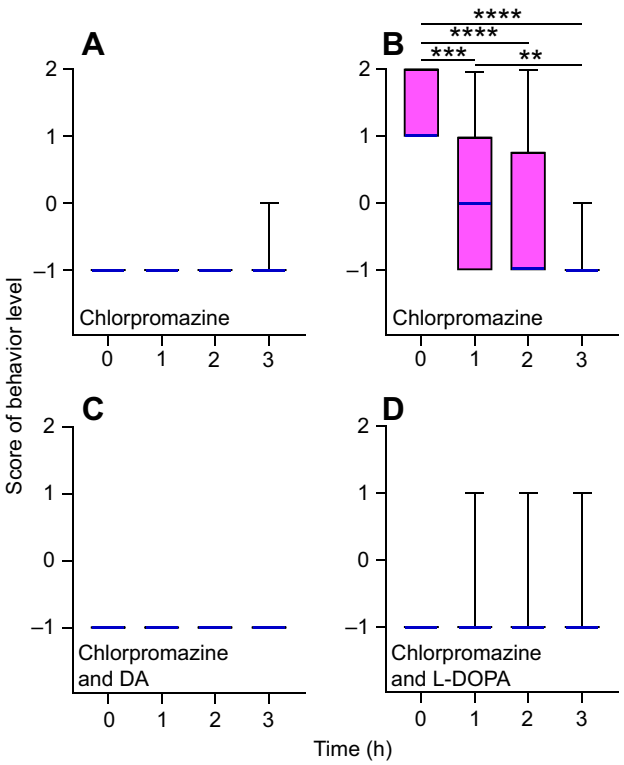

Fig. 5. Effect of oral administration of the DA inhibitor chlorpromazine. Box-and-whisker graphs indicate minimum, median (indicated by a blue bar), maximum, and 25th and 75th percentiles. (A) Effect of 1 mmol l^{-1} chlorpromazine on the responses to the tactile stimuli in less aggressive workers (level −1 workers, *N*=20). No workers responded with defensive turns even after oral administration of chlorpromazine. (B) Effect of 1 mmol l^{-1} chlorpromazine on the responses to the tactile stimuli in more aggressive workers (10 level 1 and six level 2 workers). The workers that responded with defensive turns shift to a dart response after oral administration of chlorpromazine. The behavioral level scores of most workers decreased 1 h after oral administration of chlorpromazine, although two level 2 workers and four level 1 workers still showed their original aggressiveness. In contrast, only two level 2 workers retained aggressiveness 2 h after oral administration. Ten workers decreased their score to level −1. After 3 h from the oral administration of chlorpromazine, most workers responded with dart escape to the tactile stimuli and only one worker (original aggressiveness was level 1) did not respond to the stimulus. (C) Effects of oral administration of a cocktail of 1 mmol l^{-1} chlorpromazine and 1 mmol l^{-1} DA on the responses to the tactile stimuli in less aggressive workers (*N*=20). Oral administration of the cocktail did not change the behavioral level scores. (D) Effects of oral administration of a cocktail of 1 mmol l^{-1} chlorpromazine and 1 mmol l^{-1} DOPA on the responses to the tactile stimuli in less aggressive workers (*N*=19). Oral administration of the cocktail did not change the behavioral level scores. ***P*<0.005, ****P*<0.0005, *****P*<0.0001.

behavioral level scores after 1 h (0 h versus 1 h: P=0.0086) and after 2 h (0 h versus 2 h: P<0.0001, 1 h versus 2 h: P<0.0001) (Fig. 6B). Sixty ants were randomly collected from three colonies (20 ants from each colony) and responses to the tactile stimulus prior to oral application were observed. Fifty-three out of 60 ants responded with a dart escape to the tactile stimulus and were used for subsequent tests. Administration of 1 mmol l^{-1} 5HTP solution increased the number of ants that responded with a turn response to nine out of 53 ants after 1 h (level 1: N=4; level 2: N=5). The number of ants that responded with a turn increased to 20 out of 53 after 2 h (level 1: N=5; level 2: N=15).

Oral administration of 0.1 mmol l^{-1} 5HTP did not increase the behavioral level scores after 1 h (Fig. 6C). However, it increased the behavioral level scores significantly after 2 h (P<0.0001) and 3 h (P<0.0001). Sixty workers from three colonies were collected (20 ants each from each colony) and the response to the tactile stimulus before 5HTP administration was observed. Forty-two ants responded with a dart response and were subsequently used to examine the effect of 0.1 mmol l^{-1} 5HTP. The number of ants that responded with a turn response to the stimulus increased to 13 out of 42 ants after 2 h (level 0: N=5; level 1: N=11; level 2: N=2). The number of the ants that responded with a turn response increased to 36 out of 42 ants after 3 h (level 0: N=1; level 1: N=24; level 2: N=12). The effect of 5HTP oral administration on initiating the turn response was both dose and time dependent. Note that the oral application of 5HTP increased the number of level 2 defensive turns more than L-DOPA.

Effects of ketanserin oral administration

To confirm that 5HT contributes to the initiation of defensive turn responses, the effect of ketanserin, a 5HT receptor antagonist, on the responses was examined (Fig. 7). Twenty level −1 workers were collected from two colonies and their responses to the tactile stimulus were examined. All workers responded with a dart response to the tactile stimulus even after oral administration of 0.1 mmol l^{-1} ketanserin (Fig. 7A). In contrast, workers that showed level 1 or level 2 turn responses to the tactile stimuli (Fig. 7B) significantly decreased their behavioral level scores after oral application of 0.1 mmol l^{-1} ketanserin. The inhibitory effect was maximal after approximately 3 h (0 h versus 1 h: P<0.0001, 0 h versus 2 h: P<0.0001, 0 h versus 3 h: P<0.0001; for more details, see Fig. 7 legend). Oral administration of either 5HT (Fig. 3D) or 5HTP (Fig. 6) initiated defensive turn in less aggressive workers. To confirm the contribution of 5HTergic system to the initiation of defensive turn behavior, a cocktail of ketanserin and either 5HT (Fig. 7C) or 5HTP (Fig. 7D) was orally applied. Ketanserin abolished the effects of 5HT and 5HTP. Less aggressive workers (level −1) did not initiate a defensive turn even 3 h after oral application.

DISCUSSION

An increase in aggressiveness during hunting and defensive behavior is closely linked to the generation of ultra-fast movements of the mandible in the trap-jaw ant. Many studies have focused on the rapid movement of the trap-jaw, including its sensory-motor control and neuroanatomy (Gronenberg, 1995, 1996b; Gronenberg et al., 1998, 1993), kinetics (Patek et al., 2006) and ecological relevance (Larabee and Suarez, 2015). However, it remains unclear how the nervous system modulates aggressiveness to initiate defensive behavior in the trap-jaw ant. This study aimed to gain an understanding of the neuronal mechanism underlying the initiation of defensive movements.

The predominant response to unexpected touch in the trap-jaw ant was a dart escape. Less than 10% of the workers in the colony made defensive turns. Aggressiveness is crucial not only to initiate defensive behavior against enemies but also to establish sociality in ants (Cuvillier-Hot et al., 2002; Tanner and Adler, 2009). The ants that responded with defensive turns to the tactile stimulus probed the source (paintbrush) using their antennae. In this study, few workers bit the paintbrush by quickly closing the mandibles, indicating that the ants might not identify the paintbrush as either a threat or prey. The colony size of ponerine ants may be associated with a hunting strategy (Beckers et al., 1989). Ponerine ants whose colony size is 200–300 individuals, such as *O. kuroiwae*, are thought to go hunting alone or in tandem. The results of this study demonstrate

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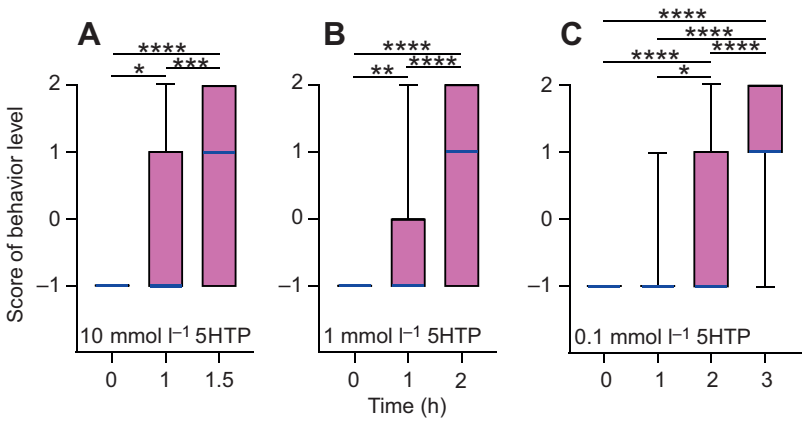

Fig. 6. Effect of 5HTP oral administration. Box-and-whisker graphs indicate minimum, median (indicated by a blue bar), maximum, and 25th and 75th percentiles. (A) Effect of 10 mmol l^{-1} 5HTP oral administration (*N*=20). The behavior level scores significantly increased after 1 and 1.5 h. (B) Effect of 1 mmol l^{-1} 5HTP oral administration on the responses to the tactile stimuli in less aggressive workers (*N*=53). The behavior level scores increased after 1 and 2 h. (C) Effect of 0.1 mmol l^{-1} 5HTP oral administration (*N*=42). The behavioral level scores did not change after 1 h. However, the scores increased significantly after 2 and 3 h. The effect of 0.1 mmol l^{-1} 5HTP was time dependent and the score at 3 h was significantly higher than that at 2 h. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

that the aggressive behavior of the workers in the nest of *O. kuroiwae* is mostly suppressed. Indeed, less than 10% of workers from a colony developed aggressiveness. Considering the hunting strategy of the trap-jaw ants, the workers that developed their aggressiveness may potentially play the tasks such as foraging or guarding the nest.

The brain biogenic amines OA, DA and 5HT are candidate neuromodulators regulating aggressiveness to initiate defensive behavior in the trap-jaw ant. The levels of these amines in the brain were significantly elevated in the ants that responded to the tactile stimulus with defensive turns compared with those in the ants that responded with a dart escape to the stimulus (Fig. 2). In this study, oral administration of pharmacological agents was performed to manipulate biogenic amine concentrations in the brain. One of the great advantages of a non-invasive method for pharmacological experiments is that it is less stressful for the animals. In contrast, other kinds of physical stimuli such as injection and topical application could result in stress for the ants. Barron et al. (2007) indicated that molecules smaller than 500 Da such as biogenic amines may pass from the hemolymph into the brain and other nervous tissues. Previous studies have demonstrated the effectiveness of oral application of precursors in the manipulation of brain amine concentrations in insects (honeybee: Božič and Woodring, 1998; Sasaki et al., 2012). Through biosynthesis in the brain, biogenic amine concentration is increased by applying the appropriate precursor. The present study examined the effects of DA and 5HT precursor oral administration on the defensive responses to the touch stimulus.

Oral administration of OA did not affect the initiation of defensive behavior in the trap-jaw ant. The OAergic system in the ant may be involved in behaviors other than initiating defensive mechanisms, although the elevation of brain OA is associated with an increase in aggressiveness in insects [i.e. cricket (Stevenson et al., 2005; Rillich et al., 2011), *Drosophila* (Zhou et al., 2008) and the ant *Formica japonica* (Aonuma and Watanabe, 2012b)]. The actions of the OAergic and TAergic systems in insects are thought to be homologous with the noradrenergic system in vertebrates (Roeder, 1999). OA acts as a multifunctional mediator in insects. OA and its precursor TA itself mediates the defensive behavior of soldiers in termites (Ishikawa et al., 2016). Brain OA increases pheromone sensitivity in the silkmoth (Pophof, 2000, 2002; Gatellier et al., 2004). The OAergic system in the brains of social insects is associated with nest-mate recognition [honeybee (Robinson et al., 1999); fire ant (Vander Meer et al., 2008)]. During predator–prey encounters, the trap-jaw ant expresses defensive or threatening postures that include opening the mandibles. The mandibles have sensory hairs to detect prey or predators and close extremely rapidly (Gronenberg, 1995; Gronenberg et al., 1993). Elevation of OA in the brain could be associated with increasing sensitivity to sensory signals. Further studies investigating the roles of OA in the trap-jaw ant are necessary to determine the precise role that OA plays in aggressive behavior.

Oral administration of DA and its precursor L-DOPA increased the initiation of turn responses to unexpected touch, although the ants rarely opened their mandible. Furthermore, oral administration of either DA or L-DOPA affected the initiation of defensive turn responses at rather high concentrations compared to 5HT and 5HTP. Oral administration of the DA receptor inhibitor chlorpromazine decreased the aggressiveness of workers, as a larger number of aggressive workers (level 1 and level 2 workers) responded with dart escapes to the tactile stimuli. Chlorpromazine also abolished the effects of DA and L-DOPA on initiation of defensive turn responses. These results suggest that the DAergic system is weakly involved in the initiation of defensive responses to unexpected tactile stimuli. A pharmacological increase in DA levels elevates aggressiveness towards prey in the ant *Formica polyctena* (Szczuka et al., 2013). The DAergic system is closely associated with nest-mate hierarchy and ovarian activity in the ponerine ant *H. saltator* (Penick et al., 2014). The results of the present study support the modulatory role of DA on the elevation of aggressiveness.

The DAergic system is thought to be multifunctional in insects. DA may regulate arousal level in the brain, which in turn initiates a variety of behaviors in *Drosophila* (Andretic et al., 2005). The behaviors modulated by DA in *Drosophila* are sleep, locomotion, courtship, learning, etc. (Van Swinderen and Andretic, 2011). Many of the workers in a colony are inactive in social insects [e.g. ant (Charbonneau and Dornhaus, 2015; Charbonneau et al., 2017); honeybee (Moore et al., 1998)]. The arousal level of the brain is one of the crucial factors for social insects to initiate a variety of behaviors. Brain DA levels in the dancer honeybee are higher than in the followers, and feeding L-DOPA increases the number of dancers in the colony (Božič and Woodring, 1998). Regulating the arousal level in the brain is crucial for ants to increase their aggressive level, initiating locomotion for foraging and so on. Further investigation is necessary to reveal the roles of DA in regulating of the arousal level in the brain and, thus, in the initiation of a variety of behaviors in the ants.

DA is also closely associated with reproductive behavior in insects (Gruntenko et al., 2005; Sasaki and Harano, 2010). Increases in brain DA levels are associated with the initiation of egg-laying behavior in the eusocial wasp (Sasaki et al., 2007). DA may be

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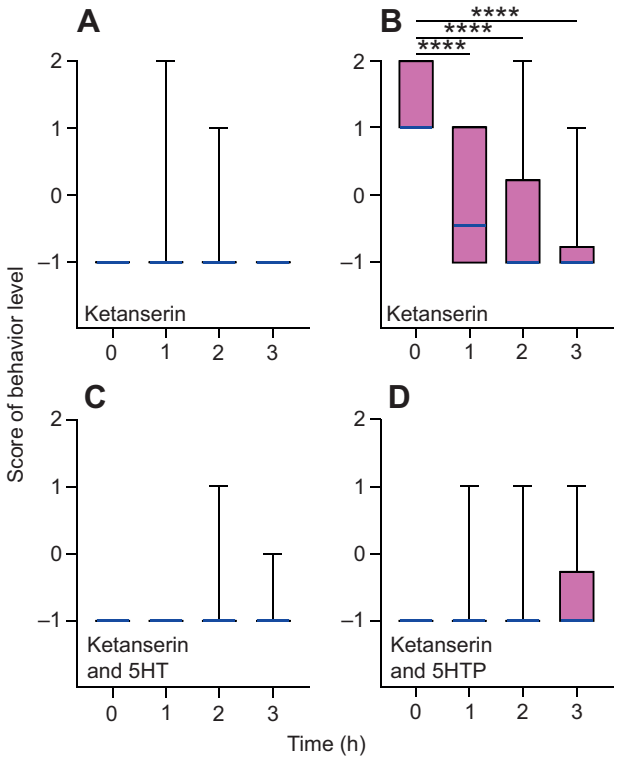

Fig. 7. Effect of oral administration on the 5HT inhibitor ketanserin. Box-and-whisker graphs indicate minimum, median (indicated by a blue bar), maximum, and 25th and 75th percentiles. (A) Effect of 0.1 mmol l^{-1} ketanserin oral administration on the responses to the tactile stimuli in less aggressive workers (level −1 workers, *N*=20). No workers responded with defensive turns even after oral administration of ketanserin. (B) Effect of 0.1 mmol l^{-1} ketanserin oral administration on the responses to the tactile stimuli in more aggressive workers (14 level 1 workers and eight level 2 workers). After 1 h from the oral administration of 0.1 mmol l^{-1} ketanserin, 11 workers showed level −1 dart response. Three level 2 workers showed level 1 after 1 h. Five level 1 *w*orkers still showed level 1 turn responses after 1 h. The other three workers did not respond to the stimulus. After 2 and 3 h from the oral administration, most of all workers showed dart response to the tactile stimuli. The behavioral level scores were significantly decreased. (C) Effects of oral administration of a cocktail of 0.1 mmol l^{-1} ketanserin and 0.1 mmol l^{-1} 5HT on the responses to the tactile stimuli in less aggressive workers (level −1 workers, *N*=20). Oral administration of the cocktail did not change the behavioral level scores. Only one worker showed a level 1 turn response 2 h after oral administration, and it did not respond to the tactile stimuli after 3 h. (D) Effects of oral administration of a cocktail of 0.1 mmol l^{-1} ketanserin and 0.1 mmol l^{-1} 5HTP on the responses to the tactile stimuli in less aggressive workers (level −1 workers, *N*=40). Oral administration of the cocktail did not change the behavioral level scores. After 1 h from oral administration, most of the workers responded with a dart response (32 workers showed level −1, six workers showed level 0 and two workers showed level 1). After 2 h, most workers still responded with a dart response to the stimulus (33 workers showed level −1, three workers showed level 0 and four workers showed level 1). Oral administration of the cocktail slightly increased the defensive level after 3 h. Thirty out of 40 workers showed level −1 dart responses, four workers did not respond to the stimuli and 6 workers showed level 1 turn responses. However, the increase was not statistically significant. ****P<0.0001.

linked with copulation behavior in female *Drosophila*, (Neckameyer, 1998). In the ant *Diacamma* sp., the workers decrease the amount of DA in the brain by making contact with the queen, which in turn suppresses their aggressiveness towards their nest mates (Shimoji et al., 2017). In honeybees, the mandibular gland pheromone of the queen modulates the action of DA in the brain of workers (Beggs et al., 2007; Beggs and Mercer, 2009), and increases in the levels of DA in the brains of workers are closely associated with ovary development (Sasaki and Nagao, 2001). These previous findings suggest that there is only a weak involvement of the DAergic system in the defensive behavior of the trap-jaw ant, and that instead, the system serves to suppress the activation of reproduction in the workers and to avoid conflicts among nest-mates.

Oral administration of 5HT and its precursor 5HTP increased the number of defensive turn responses to unexpected tactile stimulation in the trap-jaw ant. 5HT may contribute to the modulation of aggressive behavior in crustaceans (Kravitz and Huber, 2003) as well as vertebrates (Olivier, 2004; Montoya et al., 2012). This study shows that, in contrast to the administration of DA and L-DOPA, the administration of 5HT or 5HTP made the ants open their mandibles widely and probe the source of stimulus with their antennae. Oral administration of ketanserin, an inhibitor of the $5HT_2$ receptor, decreased the aggressiveness of workers, as more aggressive workers (level 1 and level 2 workers) responded with dart escape to the tactile stimuli. Furthermore, administration of ketanserin inhibited the effects of 5HT and 5HTP, which indicates that endogenous 5HT release modulates the initiation of defensive behavior in trap-jaw ants. A tonic increase in $5HT_2$ receptor escalates aggression, and ketanserin strikingly abolishes aggressive behavior in mice (Shih et al., 1999; Takahashi et al., 2011). The 5HTergic system in the trap-jaw ant could be similar to the mammalian system in regulating aggressive behavior. The regulation of aggressiveness by the action of 5HT in the brain varies among insects [e.g. 5HT increases aggressiveness in ants (Kostowski and Tarchalska, 1972) but suppress aggressiveness in crickets (Rillich and Stevenson, 2018)]. The elevation of brain 5HT is closely linked to aggression between interspecies and intraspecies in the ant *Formica rufa* (Tarchalska et al., 1975), and enhances aggressive behavior in *Drosophila* (Dierick and Greenspan, 2007). Oral administration of 5HTP and 5HT enhances the expression of high-intensity aggressive behaviors and increases the winning probability of agonistic contests in the stalk-eyed fly *Teleopsis dalmanni* (Bubak et al., 2014), while 5HT increases the aggressiveness of subordinates in crayfish (Huber et al., 1997; Kravitz, 2000). The results of this study support these previous studies. However, the opposite effects of 5HT on aggressiveness in insects has also been reported. The serotonin 5-HT_2 receptor suppresses aggressive behavior in *Drosophila* (Johnson et al., 2009), and 5HT depresses aggressiveness in subordinate crickets after agonistic interactions (Rillich and Stevenson, 2018). Synaptic responsiveness to 5HT changes with social status in crayfish (Yeh et al., 1997). Social interactions are one of the important factors maintaining homeostasis of aminergic control in ants, and contact with nest-mate workers rescues depressed DA and OA levels in the ant (Wada-Katsumata et al., 2011). The sting alarm pheromone isoamyl acetate upregulates brain 5HT and DA, which elevates workers' aggressiveness to enhance social defensive behavior in honeybees (Bubak et al., 2020). Social insects change the titer of brain amines according to social experience. Therefore, the co-effect of brain amines needs to be unveiled in order to understand aggressiveness and the initiation of defensive behavior in the trap-jaw ant.

This study demonstrates that the 5HTergic system contributes to the initiation of defensive responses to unexpected tactile stimuli and that DA can weakly contribute to the initiation of defensive responses in the trap-jaw ant. Further investigation of the co-effects of 5HT and DA on initiation of defensive behavior in the trap-jaw ant would help us to unveil the neuronal mechanisms underlying social escape and social defense in social insects.

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Competing interests
The author declares no competing or financial interests.

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Supplementary information
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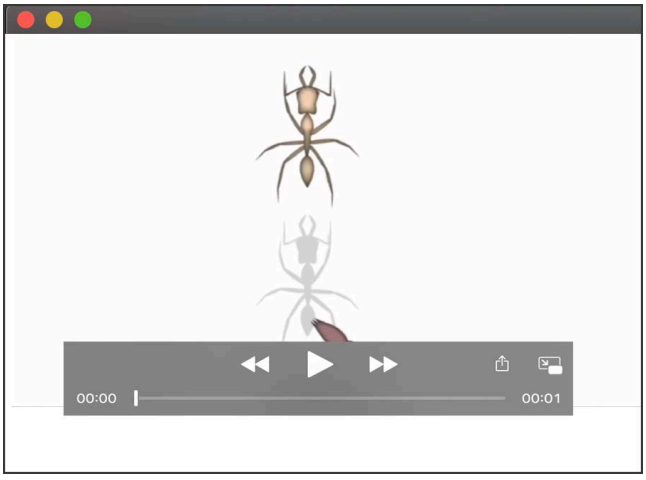
Supplementary Information

Movie 1. Hunting behavior of the trap-jaw ant *Odontomachus kuroiwae*. The ant orients to a small insect while opening the mandibles widely. After it detects and identifies the prey using the antennae, the mandibles close extremely fast, to bite, and then it stings to inject venom into the prey.



Movie 2. Behavioral responses of the trap-jaw ant to unexpected tactile stimuli to the abdomen. The movie indicates the *Level -1* dart response, *Level 0* no response, *Level 1* turn response without open mandibles, and *Level 2* turn response with open mandibles.

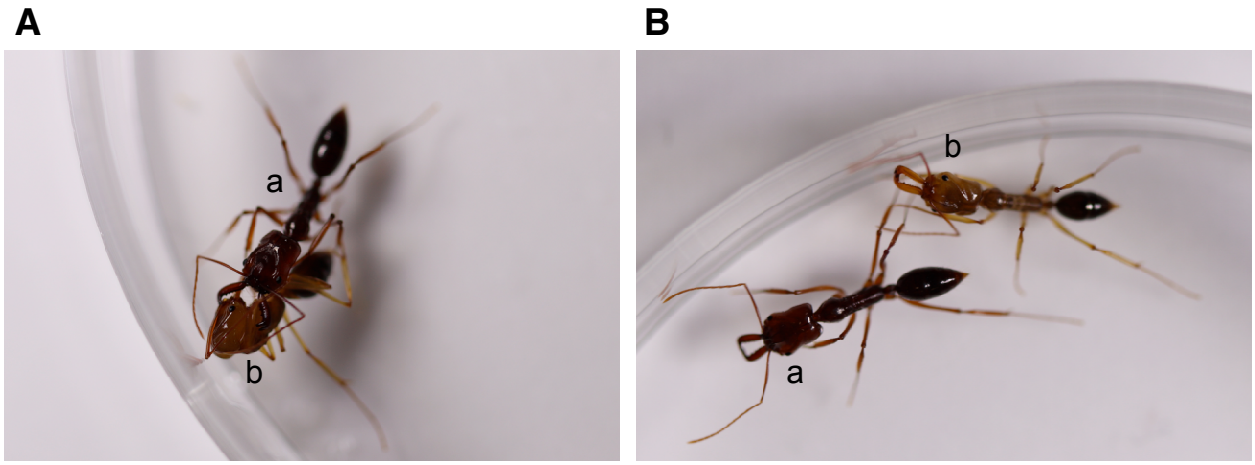


Fig S1. Aged worker and newly emerged worker. **A**: An aged worker (a) hold a newly emerged worker (b) using mandibles. **B**: The body color of aged worker (a) is much darker than that of newly emerged worker (b).

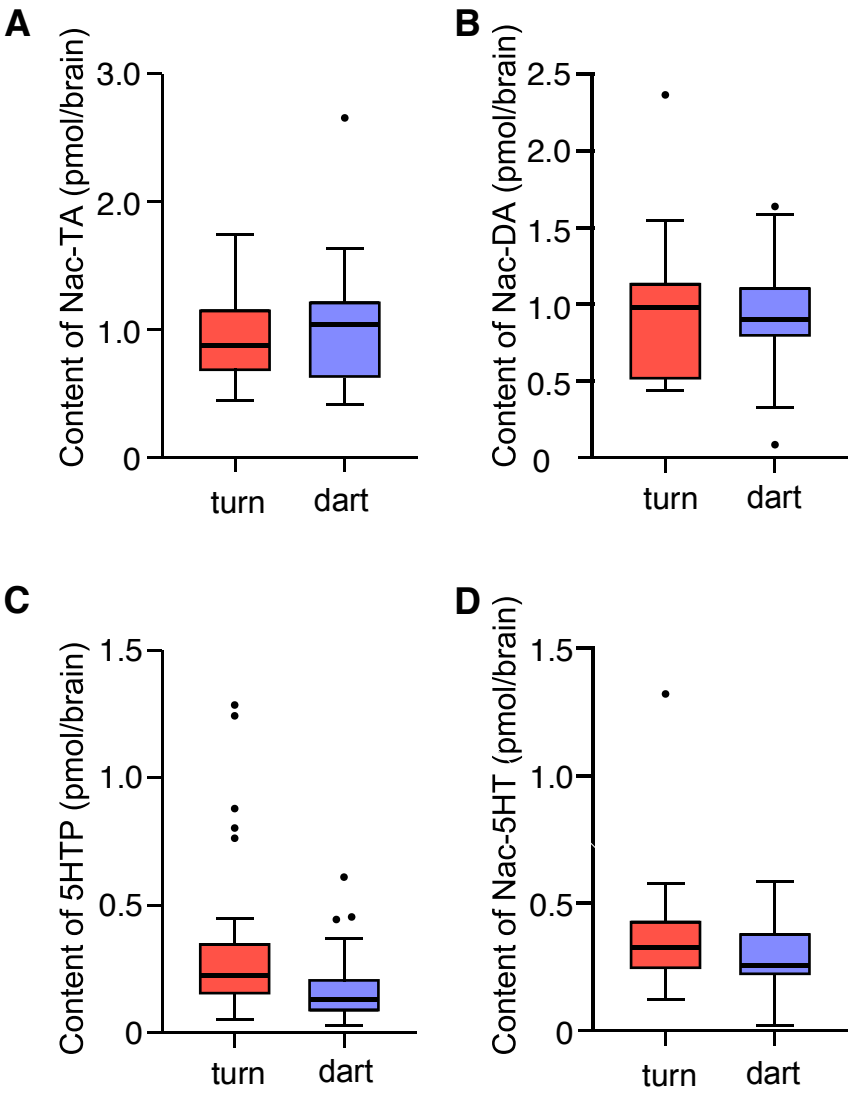


Fig S2. Amount of precursor and catabolites of biogenic amines in the brain. Box-and-whisker graphs indicate minimum, median, maximum, 25% percentile and 75% percentile. **A:** Amount of 5HTP in the brain. There was no significant difference between turn and dart (Unpaired t test with Welch's correction: $p = 0.21$). **B:** Amount of Nac-5HT in the brain. There was no significantly difference between turn and dart (Unpaired t test with Welch's correction: $p = 0.18$). **C:** Amount of Nac-DA in the brain. There was no significant difference between turn and dart (Unpaired t test with Welch's correction: $p = 0.80$). **D:** Amount of Nac-TA in the brain. There was no significant difference between turn and dart (Unpaired t test with Welch's correction: $p = 0.75$).