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

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ABSTRACT

Decision to express defensive response or escape response to a potential threat is crucial for insects to survive. This study investigated an aminergic mechanism underlying defensive response to unexpected touch in the ant that has powerful mandible so-called trap-jaw as a weapon that moves extremely fast when hunting. Tactile stimulus onto the abdomen elicited quick forward-movement so-called as “dart escape” in 90% of the ants in a colony. Less than 10% of the ants responded with quick orientation toward the source of stimulus that is so-called “defensive turn”. To unveil the neuronal mechanisms underlying defensive behavior, effect of brain biogenic amines on the response to the tactile stimulus was investigated. Contents of octopamine (OA), dopamine (DA) and serotonin (5HT) in the brain are significantly elevated in the ants that respond with defensive turn to unexpected touch than the ants that responded with dart escape. Oral administration of DA and 5HT demonstrated that both amines contribute initiation of defensive response to tactile stimulus. Oral administration of L-DOPA weakly affected to initiate defensive turn in the ant. On the other hand, 5HTP strongly affected to initiate defensive behavior. Oral administration of antagonist of 5HT ketanserin abolished effect of 5HTP. These results indicate that endogenous 5HT in the brain mainly modulate initiation of defensive behavior in the trap-jaw ant.

INTRODUCTION

Ponerine ants hunt small arthropod animals as their major diet (Choe and Crespi, 1997). It must be necessary for foragers to elevate aggressiveness to attack and capture a prey and to defend against unexpected encounter with an enemy. Defensive behavior sometime escalates to violent attack against an opponent, which increases risks of their damages. To avoid the risks, choice of escape could increase survivability. Similar to individual behavior, social decision to defend or to escape must be crucial for colony to survive (Holway et al., 1998).

The ant genus *Odontomachus* have long and powerful mandible so-called trap-jaw that functions as a weapon when hunting (De la Mora et al., 2008). The trap-jaw ant capture a prey with closing the mandible extremely fast (S. 1) (Gronenberg, 1996a) (Just and Gronenberg, 1999). On the other hand, if the ant encounters a potential threat, the ant responds urgently with defensive turn response or with dart escape response (S. 2). Behavioral responses with both defensive turn and dart escape to unexpected touch are common to arthropod animals (crayfish: (Nagayama et al., 1986), cricket: (Alexander, 1961)). It is believed that social experience influences the behavioral response to unexpected touches (Song et al., 2006). This study hypothesize that biogenic amines contribute to initiate behavioral response to unexpected tactile stimuli, since social interaction between nest-mates influences aminergic homeostasis in the brain of the ant (Wada-Katsumata et al., 2011).

Biogenic amines function as neurotransmitter, neuromodulator and neurohormone in the brain and play principal roles in behavior (Evans, 1980) (Baumann et al., 2003) (Roeder, 2005). Octopaminergic (OAergic) system is thought to 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 arthropod animals (Edwards and Kravitzt, 1997) (Kravitz, 2000) (Stevenson et al., 2005) (Hoyer et al., 2008) (Johnson et al., 2009) (Rillich et al., 2011a). Effects of biogenic amines on aggressive behavior were also demonstrated in ants (OA: (Aonuma and Watanabe, 2012b) (Yakovlev, 2018), TA: (Szczuka et al., 2013), DA: (Ohkawara and Aonuma, 2016; Vander Meer et al., 2008) (Shimoji et al., 2017), 5HT: (Kostowski et al., 1975). This study focuses on the behavioral responses to unexpected tactile stimulus to gain understandings of neuronal mechanism underlying initiation of defensive behavior in the trap-jaw ant. The levels of brain amines of the workers were measured and compared between those who respond with defensive turn and with escape dart to tactile stimulus. Effects of oral administration of amines on initiating defensive behavior was then examined to confirm brain amines are associated with initiating defensive behavior. This work gains understanding how trap-jaw ant initiates defensive behavior against unexpected encounter of an enemy.

MATERIALS AND METHODS

Animals

Workers of the trap jaw ants *Odontomachus kuroiwae* were used in this study. Colonies of the ants were collected in Okinawa, Japan. They are mostly polygynous and

contained 3-4 queens, 200-300 of workers and broods. Each colony was installed into an artificial plaster nest (2000mm ×2000mm×40mm) in a plastic case (6000mm ×4000mm×2000mm) on a 14h:10h light and dark cycle (lights on at 6:00) at 25±2˚C. Ants were fed a diet of insect jelly (Marukan Co., Ltd, Osaka, Japan), cricket nymphs and water *ad libitum*. *O. kuroiwae* has no distinction between major and minor workers.

Behavior experiments

Workers of the ants *O. kuroiwae* were randomly collected from colonies and kept isolated in a plastic petri dish (φ50mm) for 60mim before behavior experiments. To elicit defensive turn or dart escape behavior, the abdomen of the trap-jaw ant was touched softly by using the tip of a fine paint brush. Similar behavior assay was described in the previous studies of crayfish (Nagayama et al., 1986) (Aonuma et al., 1994). Behavioral response to the tactile stimulus onto the abdomen was observed and recorded using digital video cam (JVC, GC-P100, Tokyo Japan) for latter analysis. The defensiveness levels were classified in to 4 types (Fig. 1, S. 2). An ant that respond with quick forward moving so called *dart escape* to the tactile stimulus is scored as "*Level -1*". If an ant does not respond to the tactile stimulus, it is scored as "*Level 0*". Defensive turn responses are divided into 2 types. The ant that turns toward the stimulus source without opening mandible was scored as "*Level 1*". If it turns with opening mandible, it is scored as "*Level 2*".

Measurement of brain biogenic amines

Contents of brain biogenic amines of the ant 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* whose body mass were 9.9±1.1mg (N=60, mean±SD) were randomly collected from colonies. Since contents of brain amines change depending on age (Seid and Traniello, 2005) (Seid and Traniello, 2006) (Aonuma and Watanabe, 2012a), newly emerged workers were not used in this study. Test animals were sampled between 10:00 -12:00 to avoid circadian effects. Indeed, it is reported that amount of 5HT in insect brain changes dependent on circadian cycle (Tomioka et al., 1993). This study compared the contents of brain amines of the ants that showed dart response (*level 0*) and turn response (*level 2*) to the tactile stimulus onto the abdomen. Thirty of *level 0* workers and other 30 of *level 2* workers were collected from 3 different colonies (10 ants from each colony). Ants were then quickly frozen in liquid N_2 to inhibit enzyme activities. A brain of ant was dissected out in ice-cold normal saline (128.3 mM NaCl, 4.7 mM KCl, 1.63 mM $CaCl_2$, 6 mM $NaHCO_3$, 0.32 mM NaH_2PO_4, 82.8 mM trehalose, pH 7.4). Each brain was collected into a micro glass homogenizer and homogenized with 50µl of ice-cold 0.1M perchloric acid containing 5ng of 3, 4-dihydroxybenzylamine (DHBA, SIGMA, St Louis, MO, USA) as an internal standard. After centrifugation of the homogenate (0°C, 15000g, 30min), 35µl of supernatant was collected.

The HPLC-ECD system was composed of a pump (EP-300, EICOM Co., Kyoto, Japan), an auto-sample injector (M-504, EICOM Co., Kyoto, Japan) and a C18 reversed-phase column (250 mm ×4.6 mm internal diameter, 5µm 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 890mV versus an Ag/AgCl reference electrode, which was also maintained at 30°C in a column oven. The mobile phase containing 0.18M chloroacetic acid and 16μM disodium EDTA was adjusted to pH 3.6 with NaOH. Sodium-1-octanesulfonate at 1.85mM 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 0.7ml/min. The chromatographs were acquired using a computer program PowerChrom (eDAQ Pty Ltd, Denistone East, NSW, Australia). The supernatants of samples were injected directly onto the HPLC column. After acquisition, they were processed to obtain the amount of biogenic amines in the same sample by the ratio of the peak area of substances to the internal standard DHBA. We used a standard mixture for quantitative determination that contained amines, precursors and metabolites. Twenty compounds at 100ng/ml 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-Dihydroxy phenylacetic 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), 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.

Pharmacological experiments

The ants were randomly collected from colonies and placed in plastic petri dishes. After each ant were kept isolated for 60min for a rest, the response to the tactile stimuli was observed. The ants that showed dart response to the tactile stimuli were used for pharmacological experiments. Pharmacological agents were dissolved in 20% sucrose solution and orally applied to the ants. For control, 20% sucrose solution was used. To manipulate the levels of biogenic amines in the brain, 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, response of the ant to the tactile stimulus was observed. To inhibit 5HT receptor, ketanserin was used (Vleugels et al., 2015). All substances were purchased from SIGMA.

Statistical analysis

Statistical analysis of deference in behavioral responses among colonies used was Kruskal-Wallis test. Differences in the levels of biogenic mines were tested using unpaired t test with Whelch's correction. Differences were considered significant at $p<0.05$ level (two-tailed). Statistical analysis of pharmacological experiments used was ANOVA with Tukey's multiple comparison test.

RESULTS

Dart escape and defensive turn

The workers of the trap-jaw ant *O. kuroiwae* were collected from 7 different colonies and examined behavioral responses to the tactile stimulus. In total 580 of workers were randomly collected from different colonies and observed the responses to the tactile stimulus. There was no significant difference among colonies in response of the ant to the stimulus (Kruskal-Wallis test). Most of workers (523 in total out of 580 ants from 7 colonies, 90.2±5.2%, Mean±SD) showed dart escape response (*level -1*) to the stimulus. As soon as the tip of fine drawing brush was touched on the abdomen, the ants quickly moved forward to leave away from the stimulus source. The ants that represented no obvious response to the stimuli (*level 0*) were 2.4 ±1.4% (14 in total out of 580). The behavior of *level 0* was obviously different from *level -1* dart response. The ants did not leave away from the stimulus source. Even if the ants walked after the tactile stimulus, walking speed was much slower than that of dart escape response (S. 2). The ants that responded with defensive turn to the tactile stimulus was about 10% in total. The ants that responded with *level 1* turn was 2.4±2.2% (14 in total out of 580 ants). Most of them showed antennae boxing toward stimulus source after they turned toward the source,. The ants that responded with *level 2* turn to the stimulus were 7.4±4.8% (29 in total out of 580 ants). These ants also showed antennae boxing toward the stimulus source. Antennae boxing functions to detect and identify the source of unexpected touch. It is reported in ants that antennae boxing is observed in the dominant workers during agonistic interaction (Gobin et al., 2001). This indicates that aggressive workers showed turn responses to the tactile stimulus. However, there were few ants that escalated aggressiveness to initiate violent attack against the stimulus source in this study.

Contents of brain biogenic amines

Thirty of *level 2* workers that showed turn response and 30 of *level -1* workers that showed dart response were collected from 3 different colonies (10 ants each from each colony), and biogenic amines in the brain were by using HPLC-ECD.

OA is generated from L-tyrosine through different synthetic pathway from DA generation. Tyrosine decarboxylase generates TA and then tyramine ß-hydroxylase generate OA. Both TA and OA were detected in all brains of the ant that showed both turn and dart response. There was no significant difference in the amount of brain TA between the ants that showed *level 2* turn response (0.42±0.37pmol/brain, N=30, Mean±SD) and *level -1* dart response (0.36±0.19pmol/brain, N=30) (Fig. 2A). On the other hand, the amount of brain OA of the ant that showed *level 2* turn response (0.94±0.28pmol/brain, N=30) was significantly more (p=0.0226) than the ant that showed *level -1* dart response (0.76±0.29pmol/brain, N=30) (Fig. 2B). Nac-OA and NacTA are catabolite of OA and TA respectively by the activation of arylalkylamine N-acetyltransferase. This study failed to detect Nac-OA, since the peak of Nac-OA appeared at front peaks of the chromatogram. Nac-TA was detected in all brains of the ants. There was also no significant difference in the levels of brain Nac-TA between the ant that showed dart and turn responses (S. 3A).

DA is generated from DOPA by activation of aromatic L-amino acid decarboxylase and catabolized to Nac-DA by activation of arylalkylamine N-acetyltransferase. DA and Nac-DA were detected in all samples. The content of brain DA of the ant that showed *level 2* turn response was 4.67±0.73 pmol/brain (N=30). The

content of brain DA of the ant that showed *level -1* dart response was 4.07±1.30 pmol/brain (N=30). The content of brain DA in the ants that showed turn response was significantly elevated than that in the ants that showed dart response (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 peaks of these two substances on the chromatogram appeared as front peaks. The catabolite of DA was detected in the brain. The amount of Nac-DA in the brain of *level 2* ant was 0.96±0.43pmol/brain (N=30) and that of *level -1 ant* was 0.93±0.36pmol/brain (N=30). There was no significant difference between them (S. 3B).

5HT was detected in each brain of the ants. The content of brain 5HT in the *level 2* ant was 1.87±0.23 pmol/brain (N=30). The content of brain 5HT in *level -1* ant was 1.60±0.48 pmol/brain (N=30) (Fig. 2D). Brain 5HT was elevated significantly in *level 2* ants than that in *level -1* ant (p=0.0086). 5HT is generated from 5HTP by the activation of aromatic L-amino acid decarboxylase. 5HTP was also detected in each brain of the ant. The content of 5HTP in *level 2* ant was 0.34±0.33pmol/brain (N=30) and slightly more than that in *level -1* ant (0.23±0.34pmol/brain, N=30), although there was no significance (S. 3C). Endogenous 5HT is catabolized to Nac-5HT by arylalkylamine N-acetyltransferase. Nac-5HT was detected 0.36±0.22pmol/brain (N=30) in the brain of *level 2* ant. Brain Nac-5HT was also detected in *level -1* ant (0.30±0.13pmol/brain, N=30) (S. 3D).

Effects of oral administration of biogenic amines

Measurements of brain amines demonstrated that the contents of OA, DA and 5HT in

the ants that showed turn response (*level 2*) to the tactile stimulus were significantly higher than the ants that showed dart response (*level -1*). To investigate which brain amines are associates with initiating defensive behavior, oral administration of the agonists was performed.

For control experiment, effect of oral administration of 20% sucrose solution was examined first (Fig. 3A). Sixty workers were randomly collected from 3 colonies (20 workers from each colony) and observed response to the tactile stimulus. Fifty-four out of 60 ants responded with dart escape, and these 54 ants were used to examine the effect of 20% sucrose solution on the response to the tactile stimuli. There was no significant change in the score of defensiveness level after administration of 20% sucrose solution. Most of them still responded with dart escape after 1hr and after 2hr.

Oral administration of OA did not significantly change in the score of the defensiveness level of the ants. Twenty workers were randomly collected from a colony and response to the tactile stimuli was observed prior to the application of 1mM OA solution. All of them responded dart escape to the stimuli. Oral administration of 1mM OA did not change response to the stimulus even after 2hr. Then effect of 10 mM OA solution on the behavior was examined. Forty workers were randomly collected from 2 different colonies (20 ants each) and observed the response to the tactile stimuli prior to oral administration of OA. Thirty-four out of 40 ants responded with dart to the stimulus, and they were orally applied 10mM OA solution (Fig. 3B). Most of them neither changed behavior response to the stimulus. Three ants out of 34 did not show obvious response to the stimulus, and only 1 ant showed turn response after 1hr. Four ants out of 34 showed turn response to the stimulus after 2hr of administration (*level 0*: N=1, *level*

1: 2, *level 2*: N=1).

Oral administration of the DA significantly increased the score of defensiveness level of the ants (Fig. 3C). Thirty ants were randomly collected from 2 colonies (15 ants each from 2 colonies) and observed the responses to the tactile stimuli prior to the administration. All of them responded with dart escape to the stimulus. Oral administration of 1mM DA significantly increased the score of defensiveness level after 2hr (p=0.0006), although there was no significance between after 1hr (Fig. 3C). Three ants out of 30 responded turn behavior after 1hr of oral administration of DA (*level 0*: N=1, *level 1*: N=2, *level 2*: N=1). Number of ants that showed turn response increased to 5 (*level 1*: N=2, *level 2*: N=3) and number of the ant that ignored the stimulus (*level 0*) was 9.

Oral administration of 1mM 5HT solution significantly increased the score of defensiveness level after 1hr and 2hr (Fig. 3D). Forty workers were randomly collected from colonies (20 ants each from 2 colonies) and observed the responses to the tactile stimuli. Thirty-eight out of 40 ants showed dart response to the stimuli and then used to examine the effect of administration of 5HT on the behavior. Ten out of 38 ants responded with turn to the tactile stimulus after 1hr of 1mM 5HT administration (*level 1*: N=7, *level 2*: N=3). Number of the ants that respond with turn increased more after 2hr (*level 0*: N=3, *level 1*: N=8, *level 2*: N=8).

Effects of oral administration of L-DOPA

To increase endogenous DA in the brain, its precursor L-DOPA was orally

applied to the ants. Oral administration of 10mM L-DOPA solution significantly increased the score of defensiveness level after 2hr (Fig. 4A). Before the oral administration of 10mM L-DOPA, 60 workers were collected from 3 colonies (20 ants each from each colony) and the response to the tactile stimulus was observed. Fifty-three out of 60 ants responded with dart to the stimulus and they were orally applied 10mM L-DOPA solution. Although no obvious behavior change was observed after 1hr, number of ants that showed turn response increased 19 out of 53 ants after 2hr (*level 1*: N=11, *level 2*: N=8). There were 9 ants that did not show obvious response to the stimuli (*level 0*).

Oral administration of 1mM L-DOPA solution also significantly increased the score of defensiveness level after 2hr ($p=0.0007$) (Fig. 4B). Sixty ants were randomly collected from 3 colonies (20 ants each) and observed response to the tactile stimuli prior to oral application of 1mM L-DOPA solution. Forty-seven out of 60 ants responded with dart to the tactile stimuli and were applied 1mM L-DOPA solution. There was no obvious change in response to the stimuli, number of ants that responded with turn increased after 2hr. Six out of 47 ants responded with turn (*level 1*: N=3, *level 2*: N=3) and 3 out of 47 ants ignored the stimulus (*level 0*). There was significant difference between 1mM L-DOPA and 10mM L-DOPA after 2hr of the oral administration (1mM L-DOPA 2hr vs.10mM L-DOPA 2hr: $p=0.006$).

Oral administration of 0.1mM L-DOPA significantly increase the score of defensiveness level after 3hr (0hr vs. 3hr: $p<0.0001$, 1hr vs. 3hr: $p=0.0036$, 2hr vs 3hr: $p=0.0022$) (Fig. 4C). Forty-five ants collected from 3 colonies (15 ants each from each colony) were used. Forty-one out of 45 ants showed dart response to the stimulus before

the oral administration. There was no obvious effect on the responses to the tactile stimulus after 1hr and 2hr. However, number of the ants that responded with turn increased to 13 out of 41 ants after 3hr (*level 0*: N=3, *level 1*: N=9, *level 2*: N=4). Note that there were not many ants representing level 2 defensive turn response to the tactile stimulus.

Effects of oral administration of 5HTP

To increase endogenous 5HT in the ants, 5HTP was orally applied (Fig. 5). Oral administration of 10mM 5HTP significantly increased the score of defensiveness level after 1hr (0hr vs. 1hr: $p = 0.0014$) (Fig. 5A). Forty workers were randomly collected (20 ants each from each colony) and the response to the tactile stimulus was observed before oral administration. Since all of them responded with dart, they were orally applied 10mM 5HTP. Number of ants that respond with defensive turn to the stimulus increased to 11 out of 40 ants after (*level 1*: N=6, *level 2*: N=5) after 1hr. The score of the level more significantly increased after 90min (0hr vs. 1.5hr: $p<0.0001$, 1hr vs. 1.5hr: $p=0.0002$). Twenty three out of 40 ants responded with turn to the stimulus after 90min (*level 0*: 1, *level 1*: N=4, *level 2*: N=19).

Effect of 1 mM 5HTP on the behavior was then examined. Oral administration of 1mM 5HTP significantly increased the score of defensiveness level after 1hr (0hr vs. 1hr: $p=0.0086$) and after 2hr (0hr vs. 2hr: $p<0.0001$, 1hr vs. 2hr: $p<0.0001$) (Fig. 5B). Sixty ants were randomly collected from 3 colonies (20 ants each from each colony) and observed response to the tactile stimuli prior to oral application. Fifty-three out of 60 ants responded with dart escape to the tactile stimuli and used. Administration of 1mM

5HTP solution increased number of ants that respond with turn to 9 out of 53 ants after 1hr (*level 1*: N=4, *level 2*: N=5). Number of ants that responded with turn increased to 20 out of 53 after 2hr (*level 1*: N=5, *level 2*: N=15).

Oral administration of 0.1mM 5HTP did not increased the score of the defensiveness level after 1hr (Fig. 5C). However, it increased the score of the defensiveness level significantly after 2hr ($p<0.0001$) and after 3hr ($p<0.0001$). Sixty workers from 3 colonies were collected (20 ants each from each colony) and observed response to the tactile stimulus before administration of 5HTP. Forty-two ants responded with dart and used them to examine the effect of 0.1mM 5HTP. Number of the ants that responded with turn to the stimulus increased to 13 out of 42 ants after 2hr (*level 0*: N=5, *level 1*: N=11, *level 2*: N=2). Number of the ants that responded with turn more increased to 36 out of 42 ants after 3hr (*level 0*: N=1, *level 1*: N=24, *level 2*: N=12). The effect of oral administration of 5HTP on initiating turn response was both dose dependent and time dependent. Note that oral application of 5HTP increased more ants that represented *level 2* defensive turn than that of L-DOPA.

To confirm that increase in endogenous 5HT contribute to initiating defensive turn response to the tactile stimuli, effect of 5HT receptor antagonist ketanserin on behavior was examined (Fig.5 D). Since less than 10% of the workers in the colony of *O. kuroiwae*, cocktail of 5HTP and ketanserin was orally applied to the ant that showed dart response to the tactile stimulus. Forty-five ants were randomly collected from 3 colonies (15 ants each from each colony) and observed the response to the tactile stimuli prior to oral application. Forty out of 45 ants responded with dart to the tactile stimulus and orally applied cocktail of 0.1mM ketanserin and 0.1mM 5HTP diluted in 20%

sucrose solution. After 1hr of the administration, most of ants responded with dart (*level -1*: N=32, *level 0*: N= 6, *level 1*: N= 2). After 2hr, most of ants still responded with dart to the stimulus (*level -1*: N=33, *level 0*: N= 3, *level 1*: N= 4). After 3hr, 6 out of 40 ants that responded with turn (*level 1*) to the tactile stimulus (*level -1*: N=30, *level 0*: N= 4, *level 1*: N= 6). There was no ant that responded with turn with opening mandible (*level 2*), when the cocktail of ketanserin and 5HTP. This indicates that ketanserin inhibited effect of 5HT that is generated by administration of 5HTP.

DISCUSSION

Increase in aggressiveness during hunting behavior and defensive behavior must closely likn to generate ultra-fast movement of the mandible in the trap-jaw ant. Many studies have so-far focused on ultra-fast movement of the trap-jaw i.e. sensory-motor control and neuroanatomy (Gronenberg, 1995; Gronenberg, 1996b; Gronenberg et al., 1998; Gronenberg et al., 1993), kinetics (Patek et al., 2006) and ecological meanings (Larabee and Suarez, 2015). However, it still remains unclear how nervous system modulates aggressiveness to initiate defensive behavior in the trap-jaw ant. This study gains understanding neuronal mechanism underlying initiation of defensive movement that is represented prior to hunting and mandible jump.

The major response to unexpected touch in the trap-jaw ant was dart escape. Less than 10% of workers in the colony represented defensive turn. Aggressiveness is crucial process not only to initiate defensive behavior against enemies but also to

establish sociality in ants. The ants that responded with defensive turn to the touch represented antennae boxing. Antennae boxing in the ant could function to identify the source of stimulation and is associated with aggression during agonistic behavior and hunting in ponerine ants (Gobin et al., 1998). Few workers escalated to violent attack against the drawing brush used for stimulation in this study indicating that the ants might not identify the drawing brush as an urgent threat nor a prey. It is thought that colony size of ponerine ants is associates with hunting strategy (Beckers et al., 1989). Ponerine ants whose colony size are 200-300 such as *O. kuroiwae* is thought to go hunting alone or tandem. This suggest that social aggression in the ant *O. kuroiwae* is mostly suppressed and less than 10% of workers in a colony maintain aggressive potentially to play role of foraging or grading of the colony.

Brain biogenic amines OA, DA and 5HT are candidate neuromodulators that regulate aggressiveness to initiate defensive behavior in the trap-jaw ant. The contents of these amines in the brain were significantly elevated in the ants that responded with defensive turn to the tactile stimulus than those in the ants that respond with dart escape to the stimulus (Fig. 2).

Oral administration of OA did not affect initiating defensive behavior in the trap-jaw ant. OAergic system in the trap-jaw ant might be involved in other than initiating defensive behavior, although elevation of brain OA is associated with increase in aggressiveness in insects (i.e. cricket: (Stevenson et al., 2005) (Rillich et al., 2011b), *Drosophira*: (Zhou et al., 2008), and the ant *Formica japonica*, (Aonuma and Watanabe, 2012b). Actions of OAergic system and TAergic system in insects are thought to be homologous of noradrenergic system in vertebrates (Roeder, 1999). OA play

multifunctional mediator in insects. OA and its precursor TA itself mediate defensive behavior of solders in termite (Ishikawa et al., 2016). Brain OA increase pheromone sensitivity in the silkmoth (Pophof, 2000, 2002; Gatellier et al., 2004). OAergic system in the brain of social insects associate with nest-mate recognition (honeybee: (Robinson et al., 1999); fire ant: (Vander Meer et al., 2008)). It is also demonstrated that OA functions to generate energy for muscle activity via activation of trehalase (Candy, 1978) (Jahagirdar et al., 1984) (Vaandrager et al., 1988) (Orchard et al., 1993). During predator-prey encounters, the trap-jaw ant expresses defensive or threatening posture that is opening the mandible and antennae boxing. The mandible has sensory hairs to detect a prey or predator to close it extremely fast (Gronenberg, 1995; Gronenberg et al., 1993). Furthermore, strong contraction of adductor muscles before closing the mandible contribute to generate ultra-fast movement. Elevation of brain OA could be associated with increasing sensitivity of chemotactile signals and/or muscle metabolism to generate ultra-fast movement of the mandible. Further study to investigate roles of OA in the trap-jaw ant is necessary.

Oral administration of DA and its precursor L-DOPA increased initiation of turn response to unexpected touch in the trap-jaw ants, although they rarely open the mandible. This suggests that DAergic system is weakly involved in initiation of defensive response to unexpected touch in the trap-jaw ant. It was demonstrated that pharmacological increase in DA elevates aggressiveness toward a prey in the ant *Formica polyctena* (Szczuka et al., 2013). 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 this study support modulatory effect of DA on elevation of aggressiveness.

DAergic system is thought to be multifunctional. DA is closely associated with reproductive behavior in insects. It was demonstrated that increase in brain DA is associated with initiation of egg-laying behavior in the eusocial wasp (Sasaki et al., 2007). DA is thought to relate with copulation behavior in female *Drosophila,* (Neckameyer, 1998). The workers decrease the amount DA in the brain by contact with queen, which in turn suppresses aggressiveness against the nest mates in the ant *Diacamma* sp. (Shimoji et al., 2017). In honeybee, mandibular gland pheromone of the queen modulates action of DA in the brain of workers (Beggs et al., 2007) (Beggs and Mercer, 2009) and increase in brain DA of workers develop their ovary (Sasaki and Nagao, 2001). These previous findings suggest to us that weak involvement of DAergic system in defensive behavior of the trap-jaw ant links to a DAergic function to avoid activating reproduction of workers and to avoid conflicts among nest-mates.

Oral administration of 5HT and its precursor 5HTP increased defensive turn response to unexpected touch in the trap-jaw ant. It is believed that 5HT contributes modulation of aggressive behavior in invertebrates as well as vertebrates. This study demonstrated that different from administration of DA and L-DOPA, the ants represented opening the mandible widely and antennae boxing. Furthermore, administration of ketanserin that is inhibitor of $5HT_2$ receptor inhibited effect of 5HTP, which indicate endogenous 5HT release modulates initiation of defensive behavior in the trap-jaw ants. It was demonstrated that tonic increase in $5HT_2$ receptor escalate aggression and ketanserin strikingly abolished aggressive behavior in mice (Shih et al., 1999) (Takahashi et al., 2011). 5HTergic system in the trap-jaw ant could be similar to mammalian system in regulating aggressive behavior. Brain 5HT is demonstrated to regulate aggressiveness multifunctionally in invertebrate animals. It was reported that

elevation of brain 5HT is closely linked to aggression toward interspecies and intraspecies in the ant *Formica rufa* (Tarchalska et al., 1975). Elevation of brain 5HT enhances aggressive behavior in *Drosophila* (Dierick and Greenspan, 2007). Oral administration of 5HTP and 5HT enhances expression of high-intensity aggressive behaviors and increases wining probability of agonistic contest in stalk-eyed fly *Teleopsis dalmanni* (Bubak et al., 2014). 5HT increases aggressiveness of subordinate crayfish (Huber et al., 1997) (Kravitz, 2000). The results in this study support these previous studies. On the other hand, opposite effect of 5HT on aggressiveness in insect was also reported. Serotonin 5-HT_2 receptor suppresses aggressive behavior in *Drosophila* (Johnson et al., 2009). It is demonstrated that 5HT depresses aggressiveness in subordinate cricket after agonistic interaction. (Rillich and Stevenson, 2018). It is demonstrated that synaptic responsiveness to 5HT changes with social status in crayfish (Yeh et al., 1997). Social interaction is of one of the important factor to maintain homeostasis of aminergic control in the ant (Wada-Katsumata et al., 2011). Contact with nest-mate workers rescued depressed DA and OA level in the ant. It is demonstrated that sting alarm pheromone, isoamyl acetate upregulates brain 5HT and DA, which elevate workers' aggressiveness to enhance social defensive behavior in honeybee (Bubak et al., 2020). Social insects change the titer of brain amines according to social experience. It must be important to unveil co-effects of brain amines to understand aggressiveness to initiate defensive behavior in the trap-jaw ant.

This study concludes that increase in brain 5HT elevates aggressiveness to initiate defensive response to unexpected tactile stimulus and that brain DA weakly contributes to initiate defensive behavior in the trap-jaw ant. Further investigation of co-effect of 5HT and DA on initiating defensive behavior in the trap-jaw ant would help

us to unveil neuronal mechanisms underlying social escape and social defense in social insects.

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Competing interests

The authors declare no competing financial interests.

Author contribution

H.A. conceived and designed the experiment; H.A. performed the experiment and analyzed the data; H.A. wrote the paper.

Figure legends

Fig. 1. Pictogram illustrating the responses to tactile stimulus onto the abdomen of the ant. The response was classified into 4 levels (level -1 to 2). *Level -1* dart response: the ant quickly moves forward. *Level 0* no response: the ant shows no obvious response to tactile stimulus. *Level 1* turn response without opening mandible: the ant orients toward the source of the stimulus without opening the mandible. *Level 2* turn response with opening mandible: the ant orients toward the source of the stimulus and opens the mandible widely.

Frig. 2. Contents of biogenic amines in the brain of the ant that showed *Level 2* turn response and that showed *level -1* dart response to tactile stimulus. Box-and-whisker graphs indicates minimum, median, maximum, 25% percentile and 75% percentile. **A:** Content of TA in the brain. There was no significant difference in the contents of TA between the ants that showed *level 2* turn response and that showed *level -1* dart response (p= 0.394). **B:** Content of OA in the brain. Brain OA level in the ants that showed *level 2* turn response was significantly higher than the ants that showed *level -1* dart response (p= 0.0226). **C:** Content of DA in the brain. Brain DA levels in the ants that showed *level 2* turn response was significantly higher than the ants that showed *level -1* dart response (p= 0.0329). **D:** Content of 5HT in the brain. Brain 5HT level in the ants that showed *level 2* turn response was significantly higher than the ants that showed *level -1* dart response (p= 0.0086).

Fig. 3. Pharmacological manipulation of biogenic amines in the brain. Box-and-whisker graphs indicates minimum, median, maximum, 25% percentile and 75% percentile. **A:** Effect of oral application of 20% sucrose solution on defensive behavior. The responses of the ants that showed dart response to tactile stimulus were examined (N=54, collected from 3 different colonies). No significant changes in response to the tactile stimulus were observed after 1hr and after 2hr of the application. **B:** Oral administration of 10mM OA (N=35, collected from 2 different colonies). The ants that showed dart response to the tactile stimulus did not initiate turn response to the tactile stimulus after 1hr and 2hr. **C:** Oral administration of 1mM DA (N=30 collected from 2 different colonies). Score of defensiveness level was significantly increased after 2hr. **D:** Oral administration of 1mM 5HT (N=38, collected from 2 different colonies). Score of defensiveness level increased significantly after 1hr and 2hr. *: $p<0.05$, ***: $p<0.001$, ****: $p<0.0001$

Fig. 4. Pharmacological manipulation of endogenous DA in the brain by using L-DOPA. Box-and-whisker graphs indicates minimum, median, maximum, 25% percentile and 75% percentile. **A:** Effect of oral administration of 10mM L-DOPA that is a precursor of DA. Score of defensiveness level did not changed after 1hr, however significantly increased after 2hr. **B:** Effect of oral administration of 1mM L-DOPA. Score of defensiveness level did not changed after 1hr but increased significantly increased after 2hr. **C:** Effect of oral application of 0.1mM L-DOPA. Score of defensiveness level did not changed after 1hr and 2hr. It increased significantly after 3hr. *: $p<0.05$, **<0.01, ***: $p<0.001$, ****: $p<0.0001$

Fig. 5. Effect of oral administration of 5HTP and ketanserin. Box-and-whisker graphs indicates minimum, median, maximum, 25% percentile and 75% percentile. **A:** Effect of oral administration of 10mM 5HTP (N=20). Score of defensiveness level significantly increased after 1hr and 1.5hr. **B:** Effect of oral administration of 1mM 5HTP (N=53). Score of defensiveness level increased after 1hr and 2hr. **C:** Effect of oral administration of 0.1mM 5HTP (N=42). Score of defensiveness level did not changed after 1hr. However, score of defensiveness level increased significantly after 2hr and 3hr. The effect of 0.1mM 5HTP was time dependent and the score of 3hr was significantly higher than that of 2hr. **D:** Effect of oral administration of a cocktail of 0.1mM ketanserin and 0.1mM 5HTP (N=40). Oral administration of the cocktail did not change the score of defensiveness level. Oral administration of the cocktail slightly increased after 3hr, but it was not significant. *: $p<0.05$, **<0.01, ***: $p<0.001$, ****: $p<0.0001$

Supplementary Information

S1. Hunting behavior of the trap-jaw ant *Odontomachus kuroiwae*. The ant orients to a small insect with opening the mandible widely. After it detects and identifies the prey using antennae, it closes the mandible extremely fast, bits with the mandible, and stings to inject venom into the prey.

S2. Behavioral responses of the trap-jaw ant to unexpected tactile stimuli onto the

abdomen. The movie indicates *Level -1* dart response, *Level 0* no response, *Level 1* turn response without opening mandible, and *Level 2* turn response with opening mandible.

S3. Amount of precursor and catabolites of biogenic amines in the brain. Box-and-whisker graphs indicates minimum, median, maximum, 25% percentile and 75% percentile. **A:** Amount of 5HTP in the brain. There was no significantly 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 significantly 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 significantly difference between turn and dart (Unpaired t test with Welch's correction: p=0.75).

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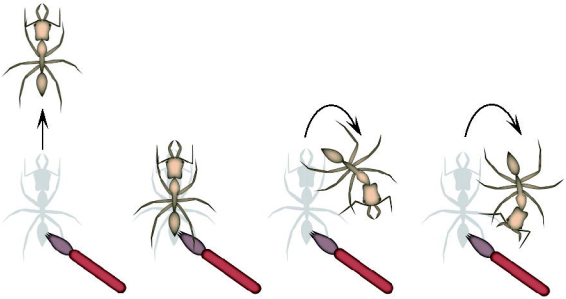
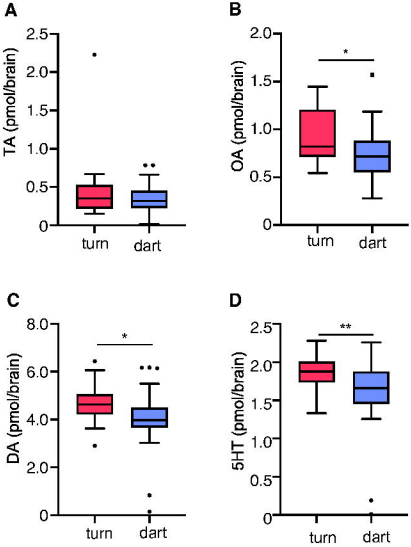

level -1
Dart
level 0
No response
level 1
Turn without opening mandible
level 2
Turn with opening mandible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TA (pmol/brain)
2.5
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1.5
1.0
0.5
0
turn
dart
B
OA (pmol/brain)
2.0
1.5
1.0
0.5
0
*
turn
dart
C
DA (pmol/brain)
8.0
6.0
4.0
2.0
0
*
turn
dart
D
5HT (pmol/brain)
2.5
2.0
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1.0
0.5
0
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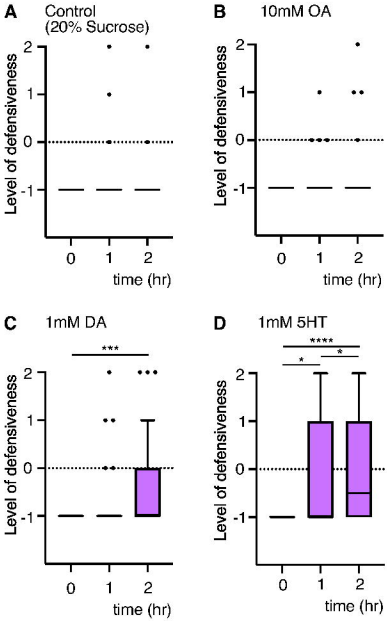
turn
dart


A
Control
(20% Sucrose)
B
10mM OA
C
1mM DA
D
1mM 5HT
Level of defensiveness
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-1
0
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2
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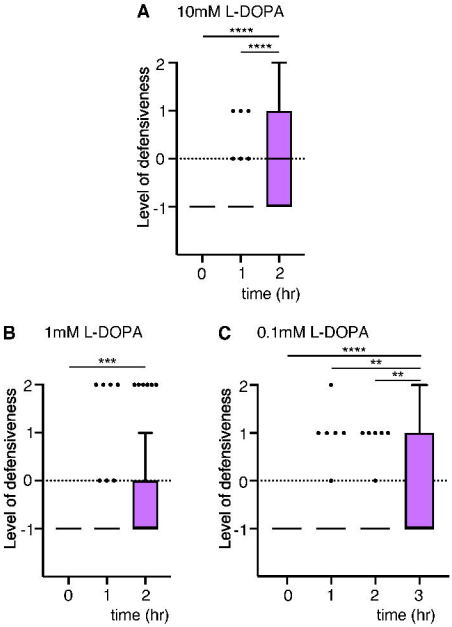
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10mM L-DOPA
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Level of defensiveness
2
1
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-1
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1
2
time (hr)
B
1mM L-DOPA
***
Level of defensiveness
2
1
0
-1
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1
2
time (hr)
C
0.1mM L-DOPA
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Level of defensiveness
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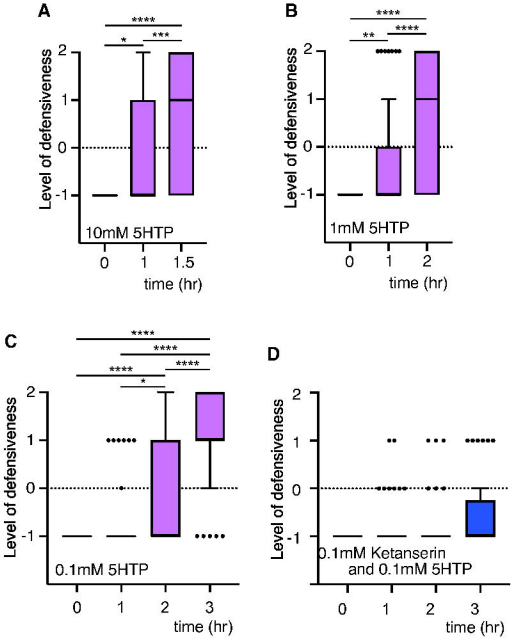
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Level of defensiveness
2
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10mM 5HTP
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time (hr)
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Level of defensiveness
2
1
0
-1
1mM 5HTP
0
1
2
time (hr)
C
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****
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*
Level of defensiveness
2
1
0
-1
0.1mM 5HTP
0
1
2
3
time (hr)
D
Level of defensiveness
2
1
0
-1
0.1mM Ketanserin
and 0.1mM 5HTP
0
1
2
3
time (hr)