

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

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21 **ABSTRACT**

22 Decision to express defensive response or escape response to a potential threat is crucial
23 for insects to survive. This study investigated an aminergic mechanism underlying
24 defensive response to unexpected touch in the ant that has powerful mandible so-called
25 trap-jaw as a weapon that moves extremely fast when hunting. Tactile stimulus onto the
26 abdomen elicited quick forward-movement so-called as “dart escape” in 90% of the ants
27 in a colony. Less than 10% of the ants responded with quick orientation toward the
28 source of stimulus that is so-called “defensive turn”. To unveil the neuronal
29 mechanisms underlying defensive behavior, effect of brain biogenic amines on the
30 response to the tactile stimulus was investigated. Contents of octopamine (OA),
31 dopamine (DA) and serotonin (5HT) in the brain are significantly elevated in the ants
32 that respond with defensive turn to unexpected touch than the ants that responded with
33 dart escape. Oral administration of DA and 5HT demonstrated that both amines
34 contribute initiation of defensive response to tactile stimulus. Oral administration of
35 L-DOPA weakly affected to initiate defensive turn in the ant. On the other hand, 5HTP
36 strongly affected to initiate defensive behavior. Oral administration of antagonist of
37 5HT ketanserin abolished effect of 5HTP. These results indicate that endogenous 5HT
38 in the brain mainly modulate initiation of defensive behavior in the trap-jaw ant.

39

40 INTRODUCTION

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

48 The ant genus *Odontomachus* have long and powerful mandible so-called
49 trap-jaw that functions as a weapon when hunting (De la Mora et al., 2008). The
50 trap-jaw ant capture a prey with closing the mandible extremely fast (S. 1) (Gronenberg,
51 1996a) (Just and Gronenberg, 1999). On the other hand, if the ant encounters a potential
52 threat, the ant responds urgently with defensive turn response or with dart escape
53 response (S. 2). Behavioral responses with both defensive turn and dart escape to
54 unexpected touch are common to arthropod animals (crayfish: (Nagayama et al., 1986),
55 cricket: (Alexander, 1961)). It is believed that social experience influences the
56 behavioral response to unexpected touches (Song et al., 2006). This study hypothesize
57 that biogenic amines contribute to initiate behavioral response to unexpected tactile
58 stimuli, since social interaction between nest-mates influences aminergic homeostasis in
59 the brain of the ant (Wada-Katsumata et al., 2011).

60 Biogenic amines function as neurotransmitter, neuromodulator and
61 neurohormone in the brain and play principal roles in behavior (Evans, 1980) (Baumann
62 et al., 2003) (Roeder, 2005). Octopaminergic (OAergic) system is thought to be

63 associated with nest-mate recognition in social insects such as honeybees (Robinson et
64 al., 1999) and ants (Vander Meer et al., 2008). Aminergic systems in the brain are
65 closely associated with aggressive behavior in arthropod animals (Edwards and Kravitz,
66 1997) (Kravitz, 2000) (Stevenson et al., 2005) (Hoyer et al., 2008) (Johnson et al.,
67 2009) (Rillich et al., 2011a). Effects of biogenic amines on aggressive behavior were
68 also demonstrated in ants (OA: (Aonuma and Watanabe, 2012b) (Yakovlev, 2018), TA:
69 (Szczuka et al., 2013), DA: (Ohkawara and Aonuma, 2016; Vander Meer et al., 2008)
70 (Shimoji et al., 2017), 5HT: (Kostowski et al., 1975). This study focuses on the
71 behavioral responses to unexpected tactile stimulus to gain understandings of neuronal
72 mechanism underlying initiation of defensive behavior in the trap-jaw ant. The levels of
73 brain amines of the workers were measured and compared between those who respond
74 with defensive turn and with escape dart to tactile stimulus. Effects of oral
75 administration of amines on initiating defensive behavior was then examined to confirm
76 brain amines are associated with initiating defensive behavior. This work gains
77 understanding how trap-jaw ant initiates defensive behavior against unexpected
78 encounter of an enemy.

79

80

81 **MATERIALS AND METHODS**

82 **Animals**

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

85 contained 3-4 queens, 200-300 of workers and broods. Each colony was installed into
86 an artificial plaster nest (2000mm ×2000mm×40mm) in a plastic case (6000mm
87 ×4000mm×2000mm) on a 14h:10h light and dark cycle (lights on at 6:00) at 25±2°C.
88 Ants were fed a diet of insect jelly (Marukan Co., Ltd, Osaka, Japan), cricket nymphs
89 and water *ad libitum*. *O. kuroiwae* has no distinction between major and minor workers.

90

91 **Behavior experiments**

92 Workers of the ants *O. kuroiwae* were randomly collected from colonies and kept
93 isolated in a plastic petri dish (φ50mm) for 60min before behavior experiments. To
94 elicit defensive turn or dart escape behavior, the abdomen of the trap-jaw ant was
95 touched softly by using the tip of a fine paint brush. Similar behavior assay was
96 described in the previous studies of crayfish (Nagayama et al., 1986) (Aonuma et al.,
97 1994). Behavioral response to the tactile stimulus onto the abdomen was observed and
98 recorded using digital video cam (JVC, GC-P100, Tokyo Japan) for latter analysis. The
99 defensiveness levels were classified in to 4 types (Fig. 1, S. 2). An ant that respond with
100 quick forward moving so called *dart escape* to the tactile stimulus is scored as “*Level*
101 *-1*”. If an ant does not respond to the tactile stimulus, it is scored as “*Level 0*”.
102 Defensive turn responses are divided into 2 types. The ant that turns toward the stimulus
103 source without opening mandible was scored as “*Level 1*”. If it turns with opening
104 mandible, it is scored as “*Level 2*”.

105

106 **Measurement of brain biogenic amines**

107 Contents of brain biogenic amines of the ant were measured using high-performance
108 liquid chromatography (HPLC) with electrochemical detection (ECD)
109 (Wada-Katsumata et al., 2011) (Aonuma and Watanabe, 2012a). Workers of *O.*
110 *kuroiwae* whose body mass were 9.9 ± 1.1 mg (N=60, mean \pm SD) were randomly
111 collected from colonies. Since contents of brain amines change depending on age (Seid
112 and Traniello, 2005) (Seid and Traniello, 2006) (Aonuma and Watanabe, 2012a), newly
113 emerged workers were not used in this study. Test animals were sampled between 10:00
114 -12:00 to avoid circadian effects. Indeed, it is reported that amount of 5HT in insect
115 brain changes dependent on circadian cycle (Tomioka et al., 1993). This study
116 compared the contents of brain amines of the ants that showed dart response (*level 0*)
117 and turn response (*level 2*) to the tactile stimulus onto the abdomen. Thirty of *level 0*
118 workers and other 30 of *level 2* workers were collected from 3 different colonies (10
119 ants from each colony). Ants were then quickly frozen in liquid N₂ to inhibit enzyme
120 activities. A brain of ant was dissected out in ice-cold normal saline (128.3 mM NaCl,
121 4.7 mM KCl, 1.63 mM CaCl₂, 6 mM NaHCO₃, 0.32 mM NaH₂PO₄, 82.8 mM trehalose,
122 pH 7.4). Each brain was collected into a micro glass homogenizer and homogenized
123 with 50 μ l of ice-cold 0.1M perchloric acid containing 5ng of 3,
124 4-dihydroxybenzylamine (DHBA, SIGMA, St Louis, MO, USA) as an internal standard.
125 After centrifugation of the homogenate (0°C, 15000g, 30min), 35 μ l of supernatant was
126 collected.

127 The HPLC-ECD system was composed of a pump (EP-300, EICOM Co., Kyoto,
128 Japan), an auto-sample injector (M-504, EICOM Co., Kyoto, Japan) and a C18
129 reversed-phase column (250 mm \times 4.6 mm internal diameter, 5 μ m average particle size,
130 CAPCELL PAK C18MG, Shiseido, Tokyo, Japan) heated to 30 °C in the column oven.

131 A glass carbon electrode (WE-GC, EICOM Co.) was used for electrochemical detection
132 (ECD-100, EICOM Co.). The detector potential was set at 890mV versus an Ag/AgCl
133 reference electrode, which was also maintained at 30°C in a column oven. The mobile
134 phase containing 0.18M chloroacetic acid and 16µM disodium EDTA was adjusted to
135 pH 3.6 with NaOH. Sodium-1-octanesulfonate at 1.85mM as an ion-pair reagent and
136 CH₃CN at 8.40% (v/v) as an organic modifier were added into the mobile phase
137 solution. The flow rate was kept at 0.7ml/min. The chromatographs were acquired using
138 a computer program PowerChrom (eDAQ Pty Ltd, Denistone East, NSW, Australia).
139 The supernatants of samples were injected directly onto the HPLC column. After
140 acquisition, they were processed to obtain the amount of biogenic amines in the same
141 sample by the ratio of the peak area of substances to the internal standard DHBA. We
142 used a standard mixture for quantitative determination that contained amines, precursors
143 and metabolites. Twenty compounds at 100ng/ml each were DL-3,4-Dihydroxy
144 mandelic acid (DOMA), L-β-3,4-Dihydroxyphenylalanine (DOPA), L-Tyrosin (Tyr),
145 N-Acetyloctopamine (Nac-OA), (-)-noradrenaline (NA), 5-Hydroxy-L-tryptophan
146 (5-HTP), (-)-adrenaline (A), DL-Octopamine (OA), 3,4-Dihydroxybenzylamine (DHBA,
147 as an internal standard), 3,4-Dihydroxy phenylacetic acid (DOPAC), N-Acetyldopamine
148 (Nac-DA), 3,4-Dihydroxyphenethylamine (DA), 5-Hydroxyindole-3-acetic acid
149 (5HIAA), N-Acetyltyramine (Nac-TA), N-Acetyl-5-hydroxytryptamine (Nac-5HT),
150 Tyramine (TA), L-Tryptophan (Trp), 3-Methoxytyramine (3MTA),
151 5-Hydroxytryptamine (5HT), 6-Hydroxymelatonin (6HM). Nac-OA Nac-DA and
152 Nac-TA were synthesized by Dr. Matsuo (Keio University, Japan). All other substances
153 were purchased from SIGMA.

154

155 **Pharmacological experiments**

156 The ants were randomly collected from colonies and placed in plastic petri dishes. After
157 each ant were kept isolated for 60min for a rest, the response to the tactile stimuli was
158 observed. The ants that showed dart response to the tactile stimuli were used for
159 pharmacological experiments. Pharmacological agents were dissolved in 20% sucrose
160 solution and orally applied to the ants. For control, 20% sucrose solution was used. To
161 manipulate the levels of biogenic amines in the brain, serotonin (5HT), octopamine
162 (OA), dopamine (DA), precursor of serotonin 5-Hydroxy-L-tryptophan (5HTP) and
163 precursor of dopamine L- β -3,4-Dihydroxyphenylalanine (L-DOPA) were used. After
164 oral administration of each agent, response of the ant to the tactile stimulus was
165 observed. To inhibit 5HT receptor, ketanserin was used (Vleugels et al., 2015). All
166 substances were purchased from SIGMA.

167

168 **Statistical analysis**

169 Statistical analysis of deference in behavioral responses among colonies used was
170 Kruskal-Wallis test. Differences in the levels of biogenic mines were tested using
171 unpaired t test with Welch's correction. Differences were considered significant at
172 $p < 0.05$ level (two-tailed). Statistical analysis of pharmacological experiments used was
173 ANOVA with Tukey's multiple comparison test.

174

175

176 **RESULTS**

177 **Dart escape and defensive turn**

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

200

201 ***Contents of brain biogenic amines***

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

205 OA is generated from L-tyrosine through different synthetic pathway from DA
206 generation. Tyrosine decarboxylase generates TA and then tyramine β -hydroxylase
207 generate OA. Both TA and OA were detected in all brains of the ant that showed both
208 turn and dart response. There was no significant difference in the amount of brain TA
209 between the ants that showed *level 2* turn response (0.42 ± 0.37 pmol/brain, N=30,
210 Mean \pm SD) and *level -1* dart response (0.36 ± 0.19 pmol/brain, N=30) (Fig. 2A). On the
211 other hand, the amount of brain OA of the ant that showed *level 2* turn response
212 (0.94 ± 0.28 pmol/brain, N=30) was significantly more ($p=0.0226$) than the ant that
213 showed *level -1* dart response (0.76 ± 0.29 pmol/brain, N=30) (Fig. 2B). Nac-OA and
214 NacTA are catabolite of OA and TA respectively by the activation of arylalkylamine
215 N-acetyltransferase. This study failed to detect Nac-OA, since the peak of Nac-OA
216 appeared at front peaks of the chromatogram. Nac-TA was detected in all brains of the
217 ants. There was also no significant difference in the levels of brain Nac-TA between the
218 ant that showed dart and turn responses (S. 3A).

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

223 content of brain DA of the ant that showed *level -1* dart response was 4.07 ± 1.30
224 pmol/brain (N=30). The content of brain DA in the ants that showed turn response was
225 significantly elevated than that in the ants that showed dart response ($p=0.0329$) (Fig.
226 2C). DOPA is generated from tyrosine by the activation of tyrosine hydroxylase. This
227 study failed to measure both tyrosine and DOPA because peaks of these two substances
228 on the chromatogram appeared as front peaks. The catabolite of DA was detected in the
229 brain. The amount of Nac-DA in the brain of *level 2* ant was 0.96 ± 0.43 pmol/brain
230 (N=30) and that of *level -1 ant* was 0.93 ± 0.36 pmol/brain (N=30). There was no
231 significant difference between them (S. 3B).

232 5HT was detected in each brain of the ants. The content of brain 5HT in the
233 *level 2* ant was 1.87 ± 0.23 pmol/brain (N=30). The content of brain 5HT in *level -1* ant
234 was 1.60 ± 0.48 pmol/brain (N=30) (Fig. 2D). Brain 5HT was elevated significantly in
235 *level 2* ants than that in *level -1* ant ($p=0.0086$). 5HT is generated from 5HTP by the
236 activation of aromatic L-amino acid decarboxylase. 5HTP was also detected in each
237 brain of the ant. The content of 5HTP in *level 2* ant was 0.34 ± 0.33 pmol/brain (N=30)
238 and slightly more than that in *level -1* ant (0.23 ± 0.34 pmol/brain, N=30), although there
239 was no significance (S. 3C). Endogenous 5HT is catabolized to Nac-5HT by
240 arylalkylamine N-acetyltransferase. Nac-5HT was detected 0.36 ± 0.22 pmol/brain
241 (N=30) in the brain of *level 2* ant. Brain Nac-5HT was also detected in *level -1* ant
242 (0.30 ± 0.13 pmol/brain, N=30) (S. 3D).

243

244 ***Effects of oral administration of biogenic amines***

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

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

250 For control experiment, effect of oral administration of 20% sucrose solution
251 was examined first (Fig. 3A). Sixty workers were randomly collected from 3 colonies
252 (20 workers from each colony) and observed response to the tactile stimulus. Fifty-four
253 out of 60 ants responded with dart escape, and these 54 ants were used to examine the
254 effect of 20% sucrose solution on the response to the tactile stimuli. There was no
255 significant change in the score of defensiveness level after administration of 20%
256 sucrose solution. Most of them still responded with dart escape after 1hr and after 2hr.

257 Oral administration of OA did not significantly change in the score of the
258 defensiveness level of the ants. Twenty workers were randomly collected from a colony
259 and response to the tactile stimuli was observed prior to the application of 1mM OA
260 solution. All of them responded dart escape to the stimuli. Oral administration of 1mM
261 OA did not change response to the stimulus even after 2hr. Then effect of 10 mM OA
262 solution on the behavior was examined. Forty workers were randomly collected from 2
263 different colonies (20 ants each) and observed the response to the tactile stimuli prior to
264 oral administration of OA. Thirty-four out of 40 ants responded with dart to the stimulus,
265 and they were orally applied 10mM OA solution (Fig. 3B). Most of them neither
266 changed behavior response to the stimulus. Three ants out of 34 did not show obvious
267 response to the stimulus, and only 1 ant showed turn response after 1hr. Four ants out of
268 34 showed turn response to the stimulus after 2hr of administration (*level 0*: N=1, *level*

269 *1: 2, level 2: N=1*).

270 Oral administration of the DA significantly increased the score of
271 defensiveness level of the ants (Fig. 3C). Thirty ants were randomly collected from 2
272 colonies (15 ants each from 2 colonies) and observed the responses to the tactile stimuli
273 prior to the administration. All of them responded with dart escape to the stimulus. Oral
274 administration of 1mM DA significantly increased the score of defensiveness level after
275 2hr ($p=0.0006$), although there was no significance between after 1hr (Fig. 3C). Three
276 ants out of 30 responded turn behavior after 1hr of oral administration of DA (*level 0:*
277 *N=1, level 1: N=2, level 2: N=1*). Number of ants that showed turn response increased
278 to 5 (*level 1: N=2, level 2: N=3*) and number of the ant that ignored the stimulus (*level*
279 *0*) was 9.

280 Oral administration of 1mM 5HT solution significantly increased the score of
281 defensiveness level after 1hr and 2hr (Fig. 3D). Forty workers were randomly collected
282 from colonies (20 ants each from 2 colonies) and observed the responses to the tactile
283 stimuli. Thirty-eight out of 40 ants showed dart response to the stimuli and then used to
284 examine the effect of administration of 5HT on the behavior. Ten out of 38 ants
285 responded with turn to the tactile stimulus after 1hr of 1mM 5HT administration (*level*
286 *1: N=7, level 2: N=3*). Number of the ants that respond with turn increased more after
287 2hr (*level 0: N=3, level 1: N=8, level 2: N=8*).

288

289 ***Effects of oral administration of L-DOPA***

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

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

300 Oral administration of 1mM L-DOPA solution also significantly increased the
301 score of defensiveness level after 2hr ($p=0.0007$) (Fig. 4B). Sixty ants were randomly
302 collected from 3 colonies (20 ants each) and observed response to the tactile stimuli
303 prior to oral application of 1mM L-DOPA solution. Forty-seven out of 60 ants
304 responded with dart to the tactile stimuli and were applied 1mM L-DOPA solution.
305 There was no obvious change in response to the stimuli, number of ants that responded
306 with turn increased after 2hr. Six out of 47 ants responded with turn (*level 1*: N=3, *level*
307 *2*: N=3) and 3 out of 47 ants ignored the stimulus (*level 0*). There was significant
308 difference between 1mM L-DOPA and 10mM L-DOPA after 2hr of the oral
309 administration (1mM L-DOPA 2hr vs. 10mM L-DOPA 2hr: $p=0.006$).

310 Oral administration of 0.1mM L-DOPA significantly increase the score of
311 defensiveness level after 3hr (0hr vs. 3hr: $p<0.0001$, 1hr vs. 3hr: $p=0.0036$, 2hr vs 3hr:
312 $p=0.0022$) (Fig. 4C). Forty-five ants collected from 3 colonies (15 ants each from each
313 colony) were used. Forty-one out of 45 ants showed dart response to the stimulus before

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

319

320 ***Effects of oral administration of 5HTP***

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

331 Effect of 1 mM 5HTP on the behavior was then examined. Oral administration
332 of 1mM 5HTP significantly increased the score of defensiveness level after 1hr (0hr vs.
333 1hr: $p = 0.0086$) and after 2hr (0hr vs. 2hr: $p < 0.0001$, 1hr vs. 2hr: $p < 0.0001$) (Fig. 5B).
334 Sixty ants were randomly collected from 3 colonies (20 ants each from each colony) and
335 observed response to the tactile stimuli prior to oral application. Fifty-three out of 60
336 ants responded with dart escape to the tactile stimuli and used. Administration of 1mM

337 5HTP solution increased number of ants that respond with turn to 9 out of 53 ants after
338 1hr (*level 1*: N=4, *level 2*: N=5). Number of ants that responded with turn increased to
339 20 out of 53 after 2hr (*level 1*: N=5, *level 2*: N=15).

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

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

360 sucrose solution. After 1hr of the administration, most of ants responded with dart (*level*
361 *-1*: N=32, *level 0*: N= 6, *level 1*: N= 2). After 2hr, most of ants still responded with dart
362 to the stimulus (*level -1*: N=33, *level 0*: N= 3, *level 1*: N= 4). After 3hr, 6 out of 40 ants
363 that responded with turn (*level 1*) to the tactile stimulus (*level -1*: N=30, *level 0*: N= 4,
364 *level 1*: N= 6). There was no ant that responded with turn with opening mandible (*level*
365 *2*), when the cocktail of ketanserin and 5HTP. This indicates that ketanserin inhibited
366 effect of 5HT that is generated by administration of 5HTP.

367

368

369 **DISCUSSION**

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

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

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

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

398 Oral administration of OA did not affect initiating defensive behavior in the
399 trap-jaw ant. OAergic system in the trap-jaw ant might be involved in other than
400 initiating defensive behavior, although elevation of brain OA is associated with increase
401 in aggressiveness in insects (i.e. cricket: (Stevenson et al., 2005) (Rillich et al., 2011b),
402 *Drosophira*: (Zhou et al., 2008), and the ant *Formica japonica*, (Aonuma and Watanabe,
403 2012b). Actions of OAergic system and TAergic system in insects are thought to be
404 homologous of noradrenergic system in vertebrates (Roeder, 1999). OA play

405 multifunctional mediator in insects. OA and its precursor TA itself mediate defensive
406 behavior of soldiers in termite (Ishikawa et al., 2016). Brain OA increase pheromone
407 sensitivity in the silkworm (Pophof, 2000, 2002; Gatellier et al., 2004). OAergic system
408 in the brain of social insects associate with nest-mate recognition (honeybee: (Robinson
409 et al., 1999); fire ant: (Vander Meer et al., 2008)). It is also demonstrated that OA
410 functions to generate energy for muscle activity via activation of trehalase (Candy,
411 1978) (Jahagirdar et al., 1984) (Vaandrager et al., 1988) (Orchard et al., 1993). During
412 predator-prey encounters, the trap-jaw ant expresses defensive or threatening posture
413 that is opening the mandible and antennae boxing. The mandible has sensory hairs to
414 detect a prey or predator to close it extremely fast (Gronenberg, 1995; Gronenberg et al.,
415 1993). Furthermore, strong contraction of adductor muscles before closing the mandible
416 contribute to generate ultra-fast movement. Elevation of brain OA could be associated
417 with increasing sensitivity of chemotactile signals and/or muscle metabolism to
418 generate ultra-fast movement of the mandible. Further study to investigate roles of OA
419 in the trap-jaw ant is necessary.

420 Oral administration of DA and its precursor L-DOPA increased initiation of
421 turn response to unexpected touch in the trap-jaw ants, although they rarely open the
422 mandible. This suggests that DAergic system is weakly involved in initiation of
423 defensive response to unexpected touch in the trap-jaw ant. It was demonstrated that
424 pharmacological increase in DA elevates aggressiveness toward a prey in the ant
425 *Formica polyctena* (Szczuka et al., 2013). DAergic system is closely associated with
426 nest-mate hierarchy and ovarian activity in the ponerine ant *H. saltator* (Penick et al.,
427 2014). The results of this study support modulatory effect of DA on elevation of
428 aggressiveness.

429 DAergic system is thought to be multifunctional. DA is closely associated with
430 reproductive behavior in insects. It was demonstrated that increase in brain DA is
431 associated with initiation of egg-laying behavior in the eusocial wasp (Sasaki et al.,
432 2007). DA is thought to relate with copulation behavior in female *Drosophila*,
433 (Neckameyer, 1998). The workers decrease the amount DA in the brain by contact with
434 queen, which in turn suppresses aggressiveness against the nest mates in the ant
435 *Diacamma* sp. (Shimoji et al., 2017). In honeybee, mandibular gland pheromone of the
436 queen modulates action of DA in the brain of workers (Beggs et al., 2007) (Beggs and
437 Mercer, 2009) and increase in brain DA of workers develop their ovary (Sasaki and
438 Nagao, 2001). These previous findings suggest to us that weak involvement of DAergic
439 system in defensive behavior of the trap-jaw ant links to a DAergic function to avoid
440 activating reproduction of workers and to avoid conflicts among nest-mates.

441 Oral administration of 5HT and its precursor 5HTP increased defensive turn
442 response to unexpected touch in the trap-jaw ant. It is believed that 5HT contributes
443 modulation of aggressive behavior in invertebrates as well as vertebrates. This study
444 demonstrated that different from administration of DA and L-DOPA, the ants
445 represented opening the mandible widely and antennae boxing. Furthermore,
446 administration of ketanserin that is inhibitor of 5HT₂ receptor inhibited effect of 5HTP,
447 which indicate endogenous 5HT release modulates initiation of defensive behavior in
448 the trap-jaw ants. It was demonstrated that tonic increase in 5HT₂ receptor escalate
449 aggression and ketanserin strikingly abolished aggressive behavior in mice (Shih et al.,
450 1999) (Takahashi et al., 2011). 5HTergic system in the trap-jaw ant could be similar to
451 mammalian system in regulating aggressive behavior. Brain 5HT is demonstrated to
452 regulate aggressiveness multifunctionally in invertebrate animals. It was reported that

453 elevation of brain 5HT is closely linked to aggression toward interspecies and
454 intraspecies in the ant *Formica rufa* (Tarchalska et al., 1975). Elevation of brain 5HT
455 enhances aggressive behavior in *Drosophila* (Dierick and Greenspan, 2007). Oral
456 administration of 5HTP and 5HT enhances expression of high-intensity aggressive
457 behaviors and increases winning probability of agonistic contest in stalk-eyed fly
458 *Teleopsis dalmanni* (Bubak et al., 2014). 5HT increases aggressiveness of subordinate
459 crayfish (Huber et al., 1997) (Kravitz, 2000). The results in this study support these
460 previous studies. On the other hand, opposite effect of 5HT on aggressiveness in insect
461 was also reported. Serotonin 5-HT₂ receptor suppresses aggressive behavior in
462 *Drosophila* (Johnson et al., 2009). It is demonstrated that 5HT depresses aggressiveness
463 in subordinate cricket after agonistic interaction. (Rillich and Stevenson, 2018). It is
464 demonstrated that synaptic responsiveness to 5HT changes with social status in crayfish
465 (Yeh et al., 1997). Social interaction is one of the important factors to maintain
466 homeostasis of aminergic control in the ant (Wada-Katsumata et al., 2011). Contact with
467 nest-mate workers rescued depressed DA and OA level in the ant. It is demonstrated
468 that sting alarm pheromone, isoamyl acetate upregulates brain 5HT and DA, which
469 elevate workers' aggressiveness to enhance social defensive behavior in honeybee
470 (Bubak et al., 2020). Social insects change the titer of brain amines according to social
471 experience. It must be important to unveil co-effects of brain amines to understand
472 aggressiveness to initiate defensive behavior in the trap-jaw ant.

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

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

479

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486

487

488 **Competing interests**

489 The authors declare no competing financial interests.

490

491 **Author contribution**

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

494

495

496 **Figure legends**

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

504

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

517

518 **Fig. 3.** Pharmacological manipulation of biogenic amines in the brain. Box-and-whisker
519 graphs indicates minimum, median, maximum, 25% percentile and 75% percentile. **A:**
520 Effect of oral application of 20% sucrose solution on defensive behavior. The responses
521 of the ants that showed dart response to tactile stimulus were examined (N=54,
522 collected from 3 different colonies). No significant changes in response to the tactile
523 stimulus were observed after 1hr and after 2hr of the application. **B:** Oral administration
524 of 10mM OA (N=35, collected from 2 different colonies). The ants that showed dart
525 response to the tactile stimulus did not initiate turn response to the tactile stimulus after
526 1hr and 2hr. **C:** Oral administration of 1mM DA (N=30 collected from 2 different
527 colonies). Score of defensiveness level was significantly increased after 2hr. **D:** Oral
528 administration of 1mM 5HT (N=38, collected from 2 different colonies). Score of
529 defensiveness level increased significantly after 1hr and 2hr. *: $p<0.05$, ***: $p<0.001$,
530 ****: $p<0.0001$

531

532 **Fig. 4.** Pharmacological manipulation of endogenous DA in the brain by using L-DOPA.
533 Box-and-whisker graphs indicates minimum, median, maximum, 25% percentile and
534 75% percentile. **A:** Effect of oral administration of 10mM L-DOPA that is a precursor of
535 DA. Score of defensiveness level did not changed after 1hr, however significantly
536 increased after 2hr. **B:** Effect of oral administration of 1mM L-DOPA. Score of
537 defensiveness level did not changed after 1hr but increased significantly increased after
538 2hr. **C:** Effect of oral application of 0.1mM L-DOPA. Score of defensiveness level did
539 not changed after 1hr and 2hr. It increased significantly after 3hr. *: $p<0.05$, ** <0.01 ,
540 ***: $p<0.001$, ****: $p<0.0001$

541

542 **Fig. 5.** Effect of oral administration of 5HTP and ketanserin. Box-and-whisker graphs
543 indicates minimum, median, maximum, 25% percentile and 75% percentile. **A:** Effect
544 of oral administration of 10mM 5HTP (N=20). Score of defensiveness level
545 significantly increased after 1hr and 1.5hr. **B:** Effect of oral administration of 1mM
546 5HTP (N=53). Score of defensiveness level increased after 1hr and 2hr. **C:** Effect of
547 oral administration of 0.1mM 5HTP (N=42). Score of defensiveness level did not
548 changed after 1hr. However, score of defensiveness level increased significantly after
549 2hr and 3hr. The effect of 0.1mM 5HTP was time dependent and the score of 3hr was
550 significantly higher than that of 2hr. **D:** Effect of oral administration of a cocktail of
551 0.1mM ketanserin and 0.1mM 5HTP (N=40). Oral administration of the cocktail did not
552 change the score of defensiveness level. Oral administration of the cocktail slightly
553 increased after 3hr, but it was not significant. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****:
554 $p<0.0001$

555

556 **Supplementary Information**

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

561

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

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

565

566 **S3.** Amount of precursor and catabolites of biogenic amines in the brain.
567 Box-and-whisker graphs indicates minimum, median, maximum, 25% percentile and
568 75% percentile. **A:** Amount of 5HTP in the brain. There was no significantly difference
569 between turn and dart (Unpaired t test with Welch's correction: $p=0.21$). **B:** Amount of
570 Nac-5HT in the brain. There was no significantly difference between turn and dart
571 (Unpaired t test with Welch's correction: $p=0.18$). **C:** Amount of Nac-DA in the brain.
572 There was no significantly difference between turn and dart (Unpaired t test with
573 Welch's correction: $p=0.80$). **D:** Amount of Nac-TA in the brain. There was no
574 significantly difference between turn and dart (Unpaired t test with Welch's correction:
575 $p=0.75$).

576

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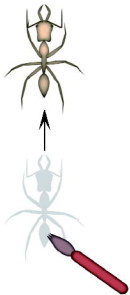
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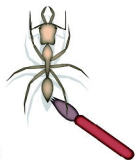
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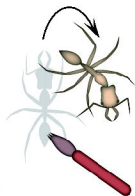
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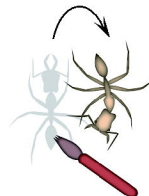
level -1
Dart



level 0
No response



level 1
Turn without
opening mandible



level 2
Turn with
opening mandible

