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Biogenic amines, caffeine and tonic immobility in *Tribolium castaneum*

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ABSTRACT

Biogenic amines are physiologically neuroactive substances that affect behavioural and physiological traits in invertebrates. In the present study, the effects of dopamine, octopamine, tyramine and serotonin on tonic immobility, or death-feigning, were investigated in *Tribolium castaneum*. These amines were injected into the abdomens of beetles artificially selected for long or short duration of tonic immobility. In beetles of the long strains, the durations of tonic immobility were shortened by injection of dopamine, octopamine and tyramine, and the effects of these amines were dose-dependent. On the other hand, serotonin injection did not affect the duration of tonic immobility. In the short-strain beetles that rarely feign death, no significant effects of the amines were found on the duration of tonic immobility. Brain expression levels of octopamine, tyramine and serotonin did not differ between long- and short-strain beetles, in contrast to the higher dopamine levels in short strains previously reported. Caffeine decreased the duration of death-feigning in both oral absorption and injection experiments. It is known that caffeine activates dopamine. Therefore, the present results suggest that the duration of tonic immobility is affected by dopamine via the dopamine receptor in *T. castaneum*.

1. Introduction

Biogenic amines are physiologically neuroactive substances that affect behavioural and physiological traits in invertebrate and vertebrate animals, and they act as neurotransmitters, neuromodulators and neurohormone in the central and peripheral nervous systems (Evans, 1980; Kravitz, 1988; Blenau and Baumann, 2001). Many behaviours in arthropods are controlled by neuroactive substances or biogenic amines, including octopamine, dopamine and serotonin, that are derived from the amino acids tyrosine or tryptophan (Evans, 1980; Bicker and Menzel, 1989; Stevenson et al., 2000; Libersat and Pflüger, 2004). There are extensive literatures on the roles of octopamine/serotonin in controlling posture and agonistic behaviours in crustacea (e.g., Kravitz, 1988, 2000). These biogenic amines bind particular receptors with different subtypes expressed in neurons or other tissues, act on several target tissues simultaneously and cause complicated behavioural and physiological modulation.

Biogenic amines may modulate behavioural activities to regulate expressions of particular behaviours. Generally, dopamine mainly acts on central nervous system to increase basic activity, and octopamine acts on both central and peripheral systems to activate particular behaviours. Several studies have been conducted on the relationship between behavioural activity and dopamine in *Drosophila melanogaster* (Meehan and Wilson, 1987; Pendleton et al., 2000, 2002; Kume et al., 2005). In crickets, dopamine and octopamine are required for a successful aggressive encounter (Adamo et al., 1995; Stevenson et al., 2000), and flying and fighting abilities are also regulated by biogenic amines (Hofmann and Stevenson, 2000; Stevenson et al., 2005). In honeybees, dopamine enhances the activity level of the motor response in worker bees (Bozic and Woodring, 1998; Menzel et al., 1999) and queen bees (Harano et al., 2005, 2008). In the cockroach, dopamine and octopamine enhance escape behaviour (Goldstein and Camhi, 1991; Casagrand and Ritzmann, 1992). Roeder et al. (2003) showed that octopamine activates many behaviours and physiological patterns. Wicker-Thomas and Hamann (2008) showed that dopamine activate many varieties of behaviours of *Drosophila* flies including locomotion, sexual behaviour, and pheromone production or secretion, and they stated that dopamine control mainly fly's activity which is a major role for insect behaviours.

When insects are attacked by natural enemies, they often freeze. This behaviour is known as tonic immobility; it is also called death-feigning or playing possum (Fabre, 1900; Frost, 1959; Edmunds, 1974; Ruxton et al., 2004), and is observed in mammals (Francq, 1969), birds (Sargeant and Eberhardt, 1975), reptiles (Gehlbach, 1970) and insects (Fabre, 1900; Frost, 1959; Edmunds, 1974; Miyatake et al., 2004). Recently, the number of studies that focus on the adaptive significance of this behaviour has increased (Miyatake et al., 2004; Honma et al., 2006; Ruxton, 2006; Ohno and

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Miyatake, 2007; Cassill et al., 2008; Hansen et al., 2008). Tonic immobility is well studied in beetles (e.g., Allen, 1990; Acheampong and Mitchell, 1981; Prohammer and Wade, 1981; Miyatake, 2001a,b; Ohno and Miyatake, 2007). In particular, geographical genetic variation is known in the duration of tonic immobility in the red flour beetle *Tribolium castaneum* (Herbst) (Prohammer and Wade, 1981), and strains selected for shorter and longer duration of death-feigning have been established (Miyatake et al., 2004). We previously compared locomotor activity levels of two strains: S strains with shorter duration and lower frequency of death-feigning and L strains with longer duration and higher frequency of death-feigning (Miyatake et al., 2008). We showed that S strains traveled further had higher dopamine expression levels in their brains (Miyatake et al., 2008). This finding suggests that the level of biogenic amines in an insect body affects tonic immobility at the genetic level. However, it has not been demonstrated that dopamine and other possible biogenic amines could cause the shorter tonic immobility in the red flour beetle. To determine the amine reaction systems on tonic immobility may give a hint to clarify the evolutionary process of surviving strategy and provide a model for evolution of behaviours mediating biogenic amine systems.

Generally, neuromodulators or biogenic amines have a collective effect on behaviours, meaning that each monoamine does not correspond to one behaviour (Libersat and Pflüger, 2004). For example, in aggressive behaviours in lobsters, serotonin and octopamine act as similar effects on neuromuscular levels, but these also act on the central nervous systems simultaneously and cause opposing postures on behavioural levels (Kravitz, 1988, 2000). Such multi-level effects of several biogenic amines on a particular behaviour have been reported in insects (Bicker and Menzel, 1989; Blenau and Baumann, 2001). Therefore, it is also possible that other amines besides dopamine affect positively or negatively tonic immobility in insects. In the present study, we investigated the potential effects of dopamine, octopamine, tyramine and serotonin on tonic immobility. First, we measured the duration of tonic immobility of beetles injected with dopamine, octopamine, tyramine or serotonin. The beetles used were derived from strains selected for death-feigning duration: long strains freeze more than 10 min when they are attacked by spiders or when they are stimulated artificially, while short strains never freeze (Miyatake et al., 2004, 2008). The relationships between the frequency of tonic immobility, duration of death-feigning and injection of amines were examined quantitatively in order to clarify the effect of biogenic amines on tonic immobility.

Second, we compared the levels of brain biogenic amine expression in adult beetles of the selected strains. A previous study (Miyatake et al., 2008) revealed that brain dopamine expression was higher in the short-strain beetles than in the long-strain beetles. Therefore, in this study, we compared the brain expressions of octopamine, tyramine and serotonin in the short and long strains.

Lastly, we also examined the effect of caffeine, which is known to enhance dopamine receptor activities (Fredholm et al., 1999), on tonic immobility. The activating effect of caffeine on dopamine is well known in vertebrates (Garrett and Griffiths, 1997). In invertebrates, Ho and Sehgal (2005) showed that caffeine affects sleep in *D. melanogaster*. To confirm the effect of dopamine on tonic immobility, we examined the effect of caffeine on tonic immobility using two methods, oral absorption and injection, of administering caffeine.

2. Materials and methods

2.1. Insects and culture

The *T. castaneum* beetle culture used in this study has been maintained in laboratories for more than 25 years. The beetles were fed wholemeal (Yoshikura Shokai, Tokyo, Japan) enriched with brewer's yeast (Asahi Beer, Tokyo, Japan) as the rearing medium and kept in a chamber (Sanyo, Tokyo, Japan) maintained at 25 ± 1 °C and 60% RH under a photoperiod of 16:8 (L:D) h (lights on at 0700, light off at 2300).

2.2. Observation of tonic immobility and artificial selection

One day before observation, each beetle was placed in a well of a 48-well tissue culture plate (Falcon, Becton Dickinson and Co., Lincoln Park, NJ, USA) to prevent disturbance by other beetles, which usually reduces the duration of tonic immobility (Miyatake, 2001a). The next day, each beetle was gently placed on its back in a white china saucer (140 mm diameter, 15 mm deep). Tonic immobility was induced by touching the abdomen of the beetle with a wooden stick. A trial consisted of provoking tonic immobility and recording its duration with a stopwatch. The behaviour duration was defined as the length of time between the stick touching the beetle and detection of its first visible movement. If the beetle did not freeze, the touch was repeated. All the trials were conducted between 13:00 and 17:00 in the chamber described above. Details of the methods of artificial selection for the duration of tonic immobility have been published by Miyatake et al. (2004, 2008). Two selection replicates each of the short and long strains initiated at the same time were tested and maintained in the chamber. The selection regimes were continued for more than 20 generations for all strains.

2.3. Injection of biogenic amines

Beetles aged 1–2 weeks after emergence from replicate 1 of strains S and L were fixed to an agarose medium by fine forceps. One of the diluted amines, 101.2 nl of 10% octopamine, tyramine or dopamine or 2% serotonin was injected into the body cavity from the genital part of the ventral abdomen with a fine glass capillary whose tip was as thin as possible connected to an oil pressure injection machine (Nanoject Auto-nanoliter injector, Drummond Scientific Company, Broomall, PA, USA) under a microscope. Because serotonin does not dissolve in water at 10%, we used 2% serotonin. The same amount of Milli-Q water was injected as the control. Injection was performed in a laboratory kept at 25 °C. One hour after the injection, the beetles were placed on a white china saucer kept in the chamber described above and tonic immobility was observed. Mann–Whitney *U*-tests were used to compare duration of tonic immobility between controls and each treatment.

Different dilutions of substances were also injected to beetles from the long strain: 1%, 5% or 10% octopamine, tyramine or dopamine, or 0.2%, 1% or 2% serotonin. The Bonferroni method (Rice, 1989) was used after Mann–Whitney *U*-tests to compare the duration of tonic immobility among concentrations. In these experiments, we did not determine the beetle's sex, but chose beetles randomly from each strain.

2.4. Brain expression levels of biogenic amines

Adult *T. castaneum* males, who are walking, not be immobilized, were killed with liquid nitrogen and stored in a 80 °C freezer until HPLC-electrochemical detection (ECD) analysis. Ages of beetles used were about 2 weeks. Brains of *T. castaneum* were removed in an ice-cold phosphate buffer solution (pH 6.7) on a dissecting dish cooled by ice under a dissecting microscope. Each dissected brain was homogenized in a micro-glass homogenizer in 50 μl of ice-cold 0.1 M perchloric acid containing 12.5 ng/ml 3,4-dihydroxybenzylamine (DHBA) as the internal standard and centrifuged at 20,000 × *g* for 30 min at 0 °C. Supernatants were transferred to micro-vials for analysis by HPLC-ECD.