Effects of Caffeine on Olfactory Learning in Crickets

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Caffeine is a plant-derived alkaloid that is generally known as a central nervous system (CNS) stimulant. In order to examine the effects of caffeine on higher CNS functions in insects, we used an appetitive olfactory learning paradigm for the cricket Gryllus bimaculatus. Crickets can form significant long-term memories (LTM) after repetitive training sessions, during which they associate a conditioned stimulus (CS: odor) with an unconditioned stimulus (US: reward). Administration of hemolymphal injections of caffeine established LTM after only single-trial conditioning over a wide range of caffeine dosages (1.6 μg/kg to 39 mg/kg). We investigated the physiological mechanisms underlying this enhancement of olfactory learning performance pharmacologically, focusing on three major physiological roles of caffeine: 1) inhibition of phosphodiesterase (PDE), 2) agonism of ryanodine receptors, and 3) antagonism of adenosine receptors. Application of drugs relevant to these actions resulted in significant effects on LTM formation. These results suggest that externally applied caffeine enhances LTM formation in insect olfactory learning via multiple cellular mechanisms.

Key words: caffeine, olfactory learning, cricket, phosphodiesterase, ryanodine receptor, adenosine receptor

INTRODUCTION

Long-lived animals with complex lifestyles depend heavily on learning and memory. The capacity for learning and memory directly affects the extent to which an animal refers to empirically obtained information to survive in its environment. Vertebrates, especially mammals, are useful models for exploring the neural mechanisms of learning and memory under a variety of approaches (Johansen et al., 2011; Kandel et al., 2013; Gasbarri et al., 2014; Kandel et al., 2014). Insects, the most diverse group of invertebrates, also serve as useful models for studying higher CNS functions because of the simplicity of their nervous system, their amenability to genetic approaches, and the availability of established learning paradigms (Heisenberg, 2003; Mizunami et al., 2014; Giurfa, 2013). The best-known experimental paradigm for learning and memory in insects is olfactory associative conditioning in honeybees, in which bees associate a specific odor presented as a CS in a training session, with a reward (usually sucrose solution) presented as a US (Takeda, 1961; Bitterman et al., 1983; Menzel, 2012; Geddes et al., 2013; Scheiner et al., 2013). Since honeybees have the most prominent learning and memory capacity among insect species, this paradigm has frequently been used as a model system to explore their behavioral and physiological mechanisms (Sandoz, 2011; Menzel, 2012; Scheiner et al., 2013).

Crickets are also used for a variety of neurobiological studies (Huber et al., 1989). Matsumoto and Mizunami (2002) developed an olfactory learning paradigm in the field cricket, wherein preference for a specific odor is evaluated before and after a training session during which the crickets associate the CS (odor) with the US (reward/punishment). Using this paradigm, a series of pharmacological studies proposed a biochemical model explaining the formation of long-term memory (LTM), involving NO-cGMP and cAMP pathways (Matsumoto et al., 2006, 2009; Mizunami et al., 2014). Caffeine is a purine alkaloid found in some plant groups, such as Camelina, Coffea, Theobroma, and Citrus (Ashihara et al., 2008). Since caffeine is a bitter tastant, it is considered a defensive substance that minimizes damage to caffeine-containing plants caused by herbivores (Mustard et al., 2012; Wright et al., 2013; Swarup et al., 2014). The ingestion of caffeine-containing plants may also alter animal behavior. For example, caffeine administration affects locomotor activity in rats (Marin et al., 2011), circadian rhythm in mice (Oike et al., 2011), and a variety of physical and mental activities in humans (Glade, 2010). The major physiological effects of caffeine are as follows: 1) inhibition of phosphodiesterase (PDE) increases the concentration of cyclic nucleotides such as cAMP and cGMP, 2) activation of ryanodine receptors increases intracellular calcium (Ca\(^{2+}\)) concentrations, 3) antagonism of adenosine receptors induces a variety of physiological processes in the relevant organs/tissues (Fredholm et al., 1999; O’Brien, 2011; Mustard, 2014).
These targets of caffeine are distributed widely throughout the body, but possibly most densely in the CNS.

The effect of caffeine on learning and memory in insects has been documented in honeybees and fruit flies. Si et al. (2005) reported that topically applied caffeine produces improved memory retention in honeybees in olfactory and visual associative learning. Using a conventional olfactory associative learning paradigm, Perisse et al. (2009) demonstrated that injection of caffeine into the honeybee brain enhances memory formation after single-trial conditioning. Caffeine injection induced a transient Ca$^{2+}$ increase in the brain and LTM formed by multiple-trial conditioning was impaired by an intracellular Ca$^{2+}$ chelator; they therefore concluded that caffeine may increase intracellular Ca$^{2+}$ release from the cytoplasmic Ca$^{2+}$ stores and lead to LTM formation via de novo protein synthesis. Wright et al. (2013) recently reported that honeybees that consumed sucrose solution (US) containing a realistic dose of caffeine exhibited superior memory retention in olfactory learning. Pharmacological and electrophysiological analysis revealed that caffeine may potentiate synaptic plasticity in the mushroom body via inhibition of the presynaptic adenosine receptors (Wright et al., 2013). In Drosophila, caffeine application resulted in the impairment of visual learning performance (Folkes and Spatz, 1984) and an increase in cAMP levels in the brain (Wang et al., 1998). However, our understanding of the effects of caffeine on learning and memory is still insufficient.

In this study, we examined whether caffeine affects olfactory learning performance in crickets. We also investigated the possible physiological roles of caffeine using relevant drugs to alter specific cellular processes.

**MATERIALS AND METHODS**

**Insects**

Adult male crickets (Gryllus bimaculatus) at one week after imaginal molting were used. They were reared in a 12 h:12 h light:dark cycle (photophase 9:00–21:00) at 28 ± 2°C and provided a diet of insect pellets (Oriental Yeast Co., Tokyo) and drinking water ad libitum. Three days before start of the experiment, crickets were individually placed in plastic petri plates (85 mm in diameter) and deprived of water to enhance their motivation to search for it.

**Olfactory conditioning**

We followed a procedure for appetitive olfactory conditioning developed by Matsumoto and Mizunami (2002), in which peppermint odor, as a CS, is associated with water as a US. Briefly, the animal was put in a plastic container (60 mm in diameter, 75 mm in depth) and received conditioning trials either once or four times. A small piece of filter paper (5 × 5 mm) attached at the shaft of a syringe needle was soaked with peppermint essence (Asaoka Spice Inc., Tokyo), and was used as an odor source. In each conditioning trial, the odor source was held close to the antennae of the insect for 3 s, and a drop of water from the tip of the needle was transferred to the mouth. In the case of multiple-trial conditioning, the inter-trial interval was 5 min.

**Odor preference test**

Odor preference was tested both before and after the conditioning session, following the procedure developed by Balderrama (1980) and modified for crickets by Matsumoto and Mizunami (2002). In brief, an animal was put in a plastic chamber (width: 15.5 × depth: 33 × height: 7 cm) with two openings in the bottom for odor source pots containing peppermint and vanilla essences (Asaoka Spice Inc., Tokyo), respectively. After a 5 min acclimation period in the waiting chamber next to the test arena, the animal was gently guided into the test arena and was allowed to search inside it for 4 min. After 2 min from the beginning of the test, the positions of the two odor source pots were reversed to eliminate the effect of place memory. The time spent visiting each odor source was manually measured by the experimenter, and the cumulative time was recorded.

**Data analysis**

Odor preferences of individual crickets were quantified by calculating the peppermint preference index (PPI), defined as follows (Matsumoto and Mizunami, 2000):

\[
\text{PPI} (%) = \frac{tP}{(tP + tV)} \times 100
\]

where tP and tV are the times spent visiting the peppermint and vanilla odor sources, respectively.

To verify the establishment of LTM, the PPI 24 h after conditioning was compared with the PPI before conditioning. Memory retention more than 24 h after conditioning has been characterized as protein synthesis-dependent LTM (Matsumoto et al., 2003). For statistical comparison, Wilcoxon’s matched-pairs signed-rank test was used. The difference was considered significant when the probability (P) was less than 0.05.

**Application of chemicals**

Chemicals dissolved in 3 μl of cricket saline (Usherwood and Grundfest, 1965) were injected into the head capsule of the animals, using a microsyringe. The following chemicals were used for injection: caffeine monohydrate (Wako), 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), cyclolheximide (CHX) (Sigma-Aldrich), BAPTA-AM (Sigma-Aldrich), dantrolene sodium (Wako), ruthenium red (Sigma-Aldrich), 8-cyclopentyl-1, and 3-dipropylxanthine (DPCPX) (Sigma-Aldrich). The physiological actions of these chemicals are summarized in Table 1.

Dimethyl sulfoxide (DMSO) was used as a solvent for insoluble drugs; the final concentration of DMSO in the injected solution was 1 or 2%. Drugs were usually applied 30 min prior to the conditioning session, except for BAPTA-AM and CHX. BAPTA-AM was injected 30 min prior to the routine caffeine application. CHX was injected 60 min after the conditioning session.

The dose and injection timing of drugs, except for caffeine, were chosen on the basis of previous studies as follows: IBMX, Matsumoto et al. (2006); CHX, Matsumoto et al. (2003); BAPTA-AM, Perisse et al. (2009); ruthenium red and dantrolene, Vázquez-Martínez et al. (2003); DPCPX, Wright et al. (2013).

**RESULTS**

**Effects of caffeine on cricket olfactory memory formation**

We first confirmed that animals injected with saline and received 4-trial conditioning established protein synthesis-dependent LTM 24 h after training. The median PPI was sig-

<table>
<thead>
<tr>
<th>Drug</th>
<th>Action</th>
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<tbody>
<tr>
<td>Caffeine</td>
<td>CNS stimulant (multi-functional)</td>
</tr>
<tr>
<td>IBMX</td>
<td>Phosphodiesterase inhibitor</td>
</tr>
<tr>
<td>CHX</td>
<td>Protein synthesis inhibitor</td>
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<tr>
<td>BAPTA-AM</td>
<td>Intracellular Ca$^{2+}$ chelator</td>
</tr>
<tr>
<td>Dantrolene</td>
<td>Ryanodine receptor antagonist</td>
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<tr>
<td>Ruthenium red</td>
<td>Ryanodine receptor antagonist</td>
</tr>
<tr>
<td>DPCPX</td>
<td>Adenosine receptor antagonist</td>
</tr>
</tbody>
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nificantly increased from 13.5% (pre-test) to 51.3% (post-test) ($P < 0.005, n = 20$, Wilcoxon test), strongly suggesting the establishment of olfactory LTM after multiple-training sessions in the present experimental conditions.

Various concentrations of caffeine (0.5 μM to 50 mM) were applied to crickets before single-trial conditioning, and their effects were examined to characterize the dose-response relationship (Fig. 1). Based upon the mean body weight of adult male crickets reared in our laboratory colony (742 mg, $n = 30$), the dosages (drug mass per body weight) were calculated and are indicated in Fig. 1. The pre- and post-test PPIs of crickets injected with saline or with 0.5 μM or 1 μM caffeine were not significantly different. In contrast, crickets injected with more than 2 μM caffeine exhibited significantly higher PPI scores 24 h after single-trial conditioning than before conditioning. These results indicate that caffeine facilitates olfactory LTM formation over a wide range of concentrations.

Caffeine is a CNS stimulant and changes animal behavior (Fredholm et al., 1999; Mustard, 2014), which opens the possibility that it might affect odor preferences as well. We compared PPI scores before and 24 h after caffeine injection, without conducting any conditioning trials. This complementary investigation was performed for relatively low (5 μM) and high (50 mM) concentrations of caffeine (Fig. 2A). The injected volume was 3 μl throughout this experiment. In both cases, PPI scores were not significantly changed following caffeine injection, suggesting that administration of caffeine itself does not affect the innate odor preference of crickets. For another control, we tested whether caffeine-injected crickets exhibited odor preference 24 h after CS/US unpaired training (Fig. 2B). Crickets were injected with high-concentration (50 mM) caffeine and separately received CS and US once, so that the inter-stimulus interval was 2.5 min (Matsumoto and Mizunami, 2002). This procedure did not produce significant changes in the PPI score between the pre- and post-tests. These results confirm that the elevation of PPI score 24 h after single-trial conditioning in caffeine-injected crickets reflects enhancement of olfactory LTM formation.

Involvement of novel protein synthesis

Does the caffeine-enhanced memory formed by single-trial conditioning actually involve de novo protein synthesis? To answer this question, crickets were first injected with 5 μM caffeine 30 min before single-trial conditioning, and 20 mM CHX, a protein synthesis inhibitor, was additionally injected 60 min after conditioning. In order to reconfirm inhibition of de novo protein synthesis using the same dose of CHX, another group of animals received 4-trial conditioning and then underwent application of 20 mM CHX. In both conditions, PPIs did not significantly change after the training session (Fig. 3). Thus, the olfactory memory established by single-trial conditioning with caffeine application may be associated with de novo protein synthesis, as is the case for normal LTM formation after multiple-trial conditioning.

Participation of intracellular Ca$^{2+}$

Early intracellular Ca$^{2+}$ concentration increases are thought to be critical for LTM formation in honeybee olfactory learning (Perisse et al., 2009). In cricket olfactory learning, an increase in intracellular Ca$^{2+}$ concentration is also considered to be a critical process related to Ca$^{2+}$-calmodulin pathways for LTM formation (Matsumoto et al., 2006; Mizunami et al., 2014; Fig. 7). We therefore examined the
effect of an intracellular Ca\(^{2+}\) chelator BAPTA-AM on caffeine-enhanced olfactory LTM formation. Animals in the control group that were injected with vehicle (2% DMSO) and 5 \(\mu\)M caffeine exhibited a significant increase in PPI after single-trial conditioning. In contrast, animals injected with 500 \(\mu\)M BAPTA-AM before caffeine injection (5 \(\mu\)M) and subsequent single-trial conditioning also failed to induce significant 24 h memory formation.

Involvement of PDE, ryanodine receptors and adenosine receptors

Since caffeine acts as a competitive inhibitor of PDE, its administration may lead to an increase in the level of cyclic nucleotides (Horrigan et al., 2006; O’Brien, 2011; Mustard, 2014). Both cGMP- and cAMP-mediated pathways for LTM formation have been proposed in our model of olfactory learning in crickets (Matsumoto et al., 2006; Fig. 7). To confirm the involvement of PDE, we first examined the effect of the PDE inhibitor IBMX in the present experimental conditions. Crickets were injected with IBMX (200 \(\mu\)M) 30 min before single-trial conditioning, and their PPIs in the pre- and post-tests were compared. The median PPI was significantly increased from 30.0% (pre-test) to 49.1% (post-test) \((P < 0.01, n = 22)\), as was the case with caffeine application.

It is known that caffeine activates ryanodine receptors in the endoplasmic reticulum (ER) and releases Ca\(^{2+}\) from the ER into the cytoplasm (Garcia et al., 2006; Mustard, 2014). Two kinds of ryanodine receptor inhibitors, ruthenium red and dantrolene, were used to verify involvement of the relevant mechanisms (Fig. 5). Crickets were injected with caffeine (5 \(\mu\)M) plus either ruthenium red (5 \(\mu\)M) or dantrolene (50 \(\mu\)M), after which they underwent single-trial conditioning. In both treatments, no significant difference was observed between the pre- and post-test PPIs. These results suggest that caffeine increases intracellular Ca\(^{2+}\) concentration via
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Fig. 7. Possible targets of caffeine in a model of LTM formation in cricket olfactory learning. Multiple-trial conditioning (MTC) activates a series of molecular pathways including NO-cGMP and cAMP systems, which ultimately leads to de novo protein synthesis, eliciting long-term synaptic plasticity (modified from Matsumoto et al., 2009; Mizunami et al., 2014; 2015). Single-trial conditioning (STC) is usually insufficient to activate the LTM formation pathway, but treatment with caffeine makes it possible to activate this pathway even by STC. Putative targets of caffeine are phosphodiesterase (PDE), n-type calcium channels (CaM), and adenine nucleotide-gated channel (CNG). Calcium/calmodulin-dependent protein kinase II (CaMKII), Calcium/calmodulin-dependent protein kinase II; AC, adenyl cyclase; PKA, protein kinase A; CREB, cAMP-responsive element-binding protein.

the ER during LTM formation.

Another possible physiological action is that caffeine antagonizes adenosine receptors (O’Brien, 2011; Mustard, 2014). To test this, crickets were injected with the adenosine receptor inhibitor DPCPX (1 μM) 30 min before conditioning and then received single-trial conditioning. The administration of DPCPX resulted in a significant increase in PPI after the training (Fig. 6), as was the case with caffeine administration.

DISCUSSION

Since the drugs used in the present study were injected into the hemolymph, they may have been delivered not only to the CNS but also to the organs/tissues of the whole body. It is therefore a concern that application of these drugs, specifically blockers or antagonists, may impair cellular functions and lead to abnormal behaviors or even death. Based on visual inspection, however, animals injected with these agents did not exhibit abnormal behaviors in the preference tests. Moreover, for the entirety of our daily observations, no side effects of injected drugs were minimal within the dosages adopted in this study.

In this study, we found that hemolymphal injection of caffeine facilitated appetitive olfactory memory formation in crickets. In the present paradigm, protein synthesis-dependent LTM maintained for more than 24 h is formed by multiple-trial conditioning, but not by single-trial conditioning (Matsumoto et al., 2006). However, caffeine administration enabled stable LTM formation even after single-trial conditioning (Fig. 1). The least effective concentration of injected caffeine solution was 2 μM, which corresponds to a dosage of 1.6 μg/kg. The positive effect of caffeine was observed with a considerably higher concentration of up to 50 mM (39 mg/kg dosage). The latter value is comparable to that used in a previous study for olfactory LTM formation in honeybees (Perisse et al., 2009). Given that the average body weight of worker honeybees (Apis mellifera) is 110 mg (Hrassnigg and Crailsheim, 2005), injection of 1 μl of 20 mM caffeine (Perisse et al., 2009) is estimated to correspond to a dosage of 35 mg/kg.

Based on our previous studies, we have proposed a molecular model of LTM formation in cricket olfactory learning (Matsumoto et al., 2009; Mizunami et al., 2014, 2015) (Fig. 7). The main site of the model pathway is currently hypothesized to lie in the mushroom body, a critical region for LTM formation (Mizunami et al., 2015). Multiple-trial conditioning (MTC), but not single-trial conditioning (STC), may sequentially activate NO-cGMP and cAMP signaling pathways and lead to the de novo protein synthesis required for LTM formation. Since the hydrophobic nature of caffeine allows its passage through cellular membranes, externally applied caffeine may be smoothly delivered into the intracellular targets and affect a variety of molecular mechanisms (Fredholm et al., 1999).

Caffeine acts as an inhibitor of PDE and induces subsequent upregulation of cyclic nucleotides such as cGMP and cAMP (Carson and Lue, 2005; Horrigan et al., 2006; O’Brien, 2011). Since these second messengers play critical roles in our model (Fig. 7), caffeine appears to activate the olfactory LTM formation pathway. However, a contradictory possibility presents itself, given the effective concentration: it is known that a millimolar level of caffeine is usually required for inhibition of PDE (Fredholm et al., 1999; Nehlig, 1999; Mustard, 2014). From the body weight of the crickets used (742 mg in average) and the least effective concentration of injected caffeine solution and its volume (2 μM and 3 μl), it can be calculated that the final caffeine concentration would decline to sub-micromolar levels in the body fluid. The accelerator effect of caffeine at such low concentrations thus might not be due to inhibition of PDE. In contrast, application of relatively higher concentrations (possibly 5 mM and 50 mM) of caffeine would steadily suppress PDE, and thus, lead to increase in the concentration of cyclic nucleotides.

An increase in intracellular Ca^2+ concentration may play a significant role in LTM formation, as the Ca^2+-calmodulin signaling pathway is implicated in our model (Matsumoto et al., 2009; Mizunami et al., 2014). Indeed, in the present study, an intracellular Ca^2+ chelator, BAPTA-AM, impaired...
caffeine-enhanced LTM formation. Plausible explanations for the origin of Ca\(^{2+}\) influx are influx through ion channels and/or release from the ER in the relevant neurons. BAPTA-AM is a non-specific chelator for intracellular Ca\(^{2+}\); therefore, its application may affect both of these possible mechanisms. In many animals, including insects, caffeine activates ryanodine receptors, which may cause an increase in the intracellular Ca\(^{2+}\) via its release from the ER (Kato et al., 2009; Mustard, 2014). Perisse et al. (2009) showed that injection of caffeine prior to single-trial olfactory conditioning causes an early increase in Ca\(^{2+}\) concentration in the honeybee brain and facilitates LTM formation. Here, we demonstrated that two kinds of ryanodine receptor antagonists ( ruthenium red and dantrolene) effectively blocked LTM formation induced by the application of a micromolar amount (5 \(\mu M\)) of caffeine (Fig. 5). Thus, intracellular Ca\(^{2+}\) increases mediated by ryanodine receptors appear to play a key role in caffeine-enhanced LTM formation. However, this hypothesis again has the same problem regarding caffeine concentration, since the activation of ryanodine receptors usually requires millimolar levels of caffeine (Fredholm et al., 1999; Nehlig, 1999; Mustard, 2014).

Caffeine acts as an antagonist of adenosine receptors in mammals. These receptors are effectively inhibited by biologically relevant concentrations of caffeine at the micromolar range (Fredholm et al., 1999; Nehlig, 1999; Rivera-Oliver and Diaz-Rios, 2014). Adenosine receptors are widely distributed in the brain, in which they are involved in various neuronal functions. Notably, activation of adenosine receptors located at nerve terminals inhibits transmitter release (Fredholm et al., 1999; Dunwiddie and Masino, 2001; Tomáš et al., 2014). In insects, adenosine receptors have been identified in *Drosophila* (Dolezelova et al., 2007); however, their amino acid sequences are somewhat different from those in mammals. The physiological function of insect adenosine receptors remains unclear (Mustard, 2014). Recently, Wright et al. (2013) proposed an intriguing hypothesis for the mechanism of caffeine-enhanced LTM formation in honeybee olfactory learning. In this model, caffeine in the floral nectar and pollen of certain plant species is ingested by foraging honeybees in the natural environment. The ingested caffeine modulates adenosine receptors located at the terminals of the antennal lobe projection neurons, sending sensory signals to Kenyon cells in the mushroom body. This causes disinhibition of transmitter release from the presynaptic neurons, which may induce synaptic plasticity in components of the mushroom body such as Kenyon cells and the subsequent extrinsic neurons. Following the experiment described in Wright et al. (2013), we also examined whether the adenosine receptor antagonist DPCPX mimics the effects of caffeine, and obtained results consistent with those for honeybees (Fig. 6). Given these findings, we suspect that synaptic plasticity mediated by adenosine receptors is a critical mechanism in the caffeine-enhanced LTM formation in crickets.

Caffeine affects dopamine signaling in vertebrates (Fredholm et al., 1999; Ferré, 2008) and possibly in invertebrates (Mustard, 2014). For example, in honeybees, expression of a dopamine receptor mRNA is triggered after treatment with caffeine (Kucharski and Ma leszka, 2005). In the cricket olfactory learning paradigm, we demonstrated that dopamine signaling would be involved in the formation and retrieval of the “aversive” memory instead of the “appetitive” one adopted in this study (Mizunami et al., 2009). Further study will be required to verify this putative interaction between caffeine and the dopaminergic system.

How does caffeine change the outcome of STC to stable LTM, causing STC to function as MTC? We hypothesize three targets of caffeine specifically in Kenyon cells in the mushroom body, as follows (Fig. 7). When considering the underlying mechanisms, the concentration of caffeine is an important factor. The LTM formed by relatively low concentrations of caffeine, in the micromolar range, may involve both ryanodine and adenosine receptors. The increase of intracellular Ca\(^{2+}\) mediated by ryanodine receptor activation would prime the relevant neurons (possibly Kenyon cells) to form LTM even by STC. Meanwhile, adenosine receptors at the presynaptic terminal involved in the mushroom body can be inhibited to produce a long-term synaptic plasticity via disinhibition of transmitter release. Relatively high concentrations of caffeine, at the millimolar range, may inhibit PDE and produce cyclic nucleotides, leading to further activation of the LTM formation pathway.

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