Chapter 9
Learning and Memory

Makoto Mizunami and Yukihisa Matsumoto

Abstract Crickets have excellent capabilities for olfactory and visual learning and thus are useful organisms in which to study the mechanisms of learning and memory. Our studies on crickets have revealed detailed information about signaling cascades underlying long-term memory (LTM) formation, namely, that the serial activation of the NO-cGMP system, cyclic nucleotide-gated (CNG) channel, the calcium/calmodulin system, and cAMP-protein kinase A (PKA) underlies LTM formation. Our studies also suggest that octopaminergic (OA-ergic) and dopaminergic (DA-ergic) neurons convey information about appetitive or aversive unconditioned stimuli (US), respectively, in conditioning of odors, visual patterns, and color cues. Our studies also suggest that activation of OA-ergic and DA-ergic neurons is needed for retrieval of appetitive and aversive memory, respectively, in olfactory learning and visual learning. Many of these findings differ from those reported for the fruit fly *Drosophila*, suggesting unexpected diversity in the mechanisms of learning and memory in different species of insects. Studies of the functional significance and underlying evolutionary history of such diversity should emerge as important areas of research. Recently, new techniques such as RNA interference and transgenesis have been successfully applied to crickets, which should help deepen the study of the cellular and molecular mechanisms of learning and memory in crickets.

Keywords *Gryllus bimaculatus* • Olfactory learning • Visual learning • NO signaling • Octopamine • Dopamine • Classical conditioning • Long-term memory

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9.1 Introduction

Insects are useful organisms for the study of the neural mechanisms of learning and memory. This is because they exhibit a rich variety of learning, and because their brains, which we refer to as microbrains (Mizunami et al. 1999, 2004), are accessible to detailed experimental analysis. Previously, most studies on the mechanisms of learning and memory in insects have been performed on only two species of insects, namely, the fruit fly *Drosophila melanogaster* (Davis 2005, 2011) and the honeybee *Apis mellifera* (Menzel and Giurfa 2006). Recently, we demonstrated that the cricket *Gryllus bimaculatus* and the cockroach *Periplaneta americana* (Mizunami et al. 1998b; Watanabe et al. 2011) are also useful for exploring the mechanisms of learning and memory. In this chapter we review recent progress from our studies on olfactory and visual learning in crickets.

9.2 Olfactory Learning in Crickets

We used a "classical conditioning and operant testing procedure" in our conditioning experiments (Matsumoto and Mizunami 2002a; Mizunami and Matsumoto 2010; see Chap. 17). For olfactory conditioning, a filter paper soaked with an odor (conditioned stimulus, CS) was brought near the antennae of the cricket, and then a drop of water or sodium chloride solution (appetitive or aversive unconditioned stimulus, US) was applied to the mouth. In the operant odor preference test, crickets were individually placed in a test chamber and allowed to visit two odor sources on the floor (e.g., banana and apple odor sources). The time that the crickets explored each odor source with the mouth or palpi was measured for evaluating relative odor preference of the crickets. Similar procedures were employed for conditioning of visual patterns (Unoki et al. 2006) and color cues (Nakatani et al. 2009).

The first form of learning we studied in crickets was olfactory learning. We found that one conditioning trial was sufficient to establish a memory lasting for several hours (midterm memory, MTM) in appetitive olfactory conditioning (Fig. 9.1a). Two conditioning trials (with an intertrial interval (ITI) of 5 min) induced memory that lasted for at least 1 day (Unoki et al. 2005), which was characterized as protein-synthesis-dependent long-term memory (LTM) (Matsumoto et al. 2003). In aversive olfactory conditioning, two trials were sufficient to establish 30-min retention, but six trials (with a 5-min ITI) were needed to establish 1-day retention (Unoki et al. 2005). Based on the results of subsequent studies, we concluded that memory after aversive learning is less durable than after appetitive learning when learning odors, visual patterns (Unoki et al. 2006), or color cues (Nakatani et al. 2009).

Subsequently, we showed that crickets are capable of (1) retaining memory for life (Matsumoto and Mizunami 2002b), (2) simultaneously memorizing seven odor

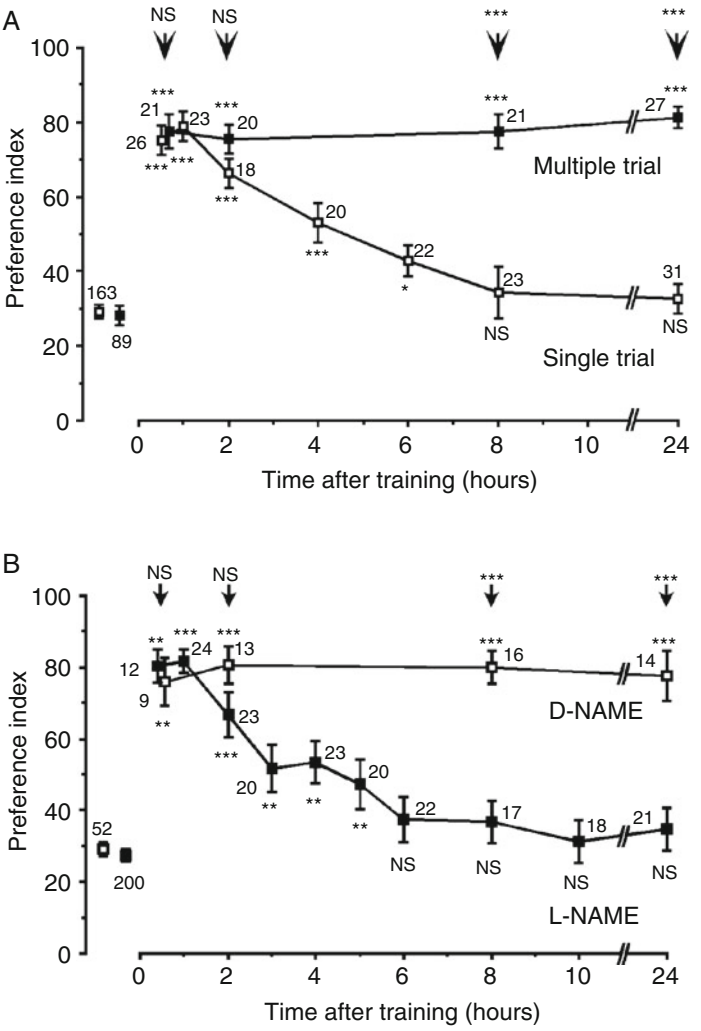

Fig. 9.1 (**a**) Memory retention after single- and multiple-trial appetitive olfactory conditioning. Seven groups of animals were subjected to single-trial conditioning (*open squares*) and another four groups were subjected to four-trial conditioning, with an ITI of 5 min (*black squares*). (**b**) Effects of L-NAME, an inhibitor of NO synthase, or D-NAME, a noneffective isomer, on LTM formation. Prior to the four-trial conditioning, animals in ten groups were each injected with 3 μl saline containing 400 μM L-NAME (*black squares*), and animals in another four control groups were each injected with 3 μl saline containing 400 μM D-NAME (*open squares*). Odor preference tests were given to animals before and at various times after conditioning. Preference indices (PIs) for the rewarded odor are shown as means ± SEM. PIs before conditioning are shown as pooled data for each category of animal groups. Statistical comparisons of odor preferences were made before and after conditioning for each group (Wilcoxon's test) and between single- and multiple-trial groups at each time after conditioning (Mann-Whitney test). The results are shown at each data point and above the *arrow*, respectively (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS $p > 0.05$). The number of animals is shown at each data point (Modified from Matsumoto et al. 2006)

pairs (Matsumoto and Mizunami 2006), and (3) performing context-dependent discrimination learning, i.e., selecting one of a pair of odors and avoiding the other in one context and the opposite pairing in another context (Matsumoto and Mizunami 2004). Moreover, we found that crickets exhibit second-order conditioning, i.e., crickets that had been subjected to pairing of a stimulus (CS1) and a US and then subjected to pairing of another stimulus (CS2) and the CS1 exhibited conditioned responses to CS2, although they had never experienced pairing of the CS2 with the US (Mizunami et al. 2009). Therefore, although our current research focuses on the mechanisms of elemental associative learning between CS and US, crickets may emerge as organisms to study the mechanisms of sophisticated forms of associative learning.

9.3 Role of the NO-cGMP System in Formation of LTM

Nitric oxide (NO) is a membrane-permeable molecule that functions in intercellular signaling. It is produced by NO synthase (NOS), diffuses into neighboring cells, and stimulates soluble guanylyl cyclase (sGC) to produce cyclic GMP (cGMP). Studies on honeybees have suggested that the NO-cGMP signaling system and cAMP system act in parallel and complementarily for the formation of LTM (Müller 2000). Our studies on crickets, however, brought us to a different conclusion (Matsumoto et al. 2006, 2009). Multiple (two or more) appetitive olfactory conditioning trials led to LTM that lasted for at least 1 day in crickets. On the other hand, memory induced by single-trial appetitive conditioning decayed within several hours (Fig. 9.1a). Injection of inhibitors of the enzyme catalyzing the formation of NO, cGMP, or cAMP into the hemolymph prior to multiple-trial conditioning blocked formation of LTM, as is shown in the example in Fig. 9.1b. In contrast, injection of an NO donor, a cGMP analog, or a cAMP analog prior to single-trial conditioning induced LTM, suggesting participation of the NO-cGMP system and cAMP system in LTM formation. Induction of LTM by injection of an NO donor or a cGMP analog paired with single-trial conditioning was blocked by inhibition of the cAMP system. However, induction of LTM by a cAMP analog was unaffected by inhibition of the NO-cGMP system. The results suggest that the cAMP pathway is a downstream target of the NO-cGMP pathway for LTM formation. We also obtained evidence suggesting that cyclic nucleotide-gated (CNG) channels and calcium-calmodulin intervene between the NO-cGMP system and the cAMP system. We have thus proposed that serial activation of the NO-cGMP system, CNG channel, and calcium-calmodulin and cAMP systems underlies formation of LTM in crickets (Fig. 9.2).

Further, we have found that RNA interference (RNAi) of the NOS gene impairs LTM formation in crickets (Takahashi et al. 2009). Crickets injected with double-stranded RNA (dsRNA) into the hemolymph 2 days before conditioning exhibited impairment of 1-day memory retention, although 30-min retention was intact. In

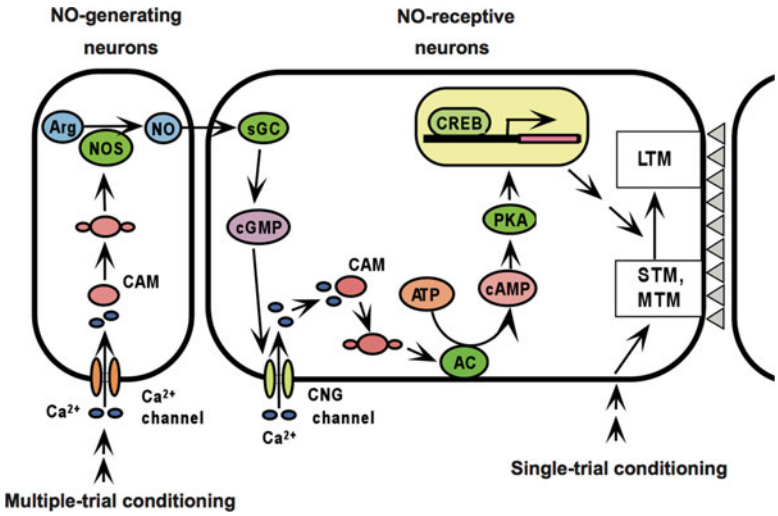

Fig. 9.2 A model of signaling cascades underlying LTM formation in crickets, proposing a serial arrangement of the NO-cGMP system and the cAMP system for LTM formation. Single-trial conditioning induces synaptic plasticity of limited durability, which is thought to underlie short-term memory and midterm memory. Multiple-trial conditioning activates the NO-cGMP system, and this in turn activates adenylyl cyclase (*AC*) and then PKA, via the cyclic nucleotide-gated (*CNG*) channel and calcium-calmodulin (*CAM*) system. Activation of PKA is assumed to activate a transcription factor, cAMP-responsive element-binding protein (*CREB*), which leads to protein synthesis that is necessary to achieve long-term plasticity of synaptic connection upon other neurons. *Arg* arginine, *NOS* NO synthase, *sGC* soluble guanylyl cyclase (Modified from Matsumoto et al. 2009)

situ hybridization demonstrated a high level of expression of NOS mRNA in one class of Kenyon cells (intrinsic neurons) of the mushroom body, in addition to some neurons around the antennal lobe (primary olfactory center) and the optic lobe (visual center). The mushroom body is a multisensory association center of the insect brain (Mizunami et al. 1998a, b) and participates in olfactory learning in honeybees (Menzel and Giurfa 2006), fruit flies (Davis 2011), and cockroaches (Watanabe et al. 2011). RNAi will likely become a useful method for study of the mechanisms of learning and memory in crickets.

Interestingly, despite the accumulation of information on the molecular and neuronal mechanisms of LTM formation in *Drosophila*, there have been no reports suggesting participation of NO in LTM formation in this species (Davis 2005, 2011). Moreover, we also obtained evidence showing that the cAMP system does not participate in the formation of short-term memory (STM) in olfactory learning in crickets (Matsumoto et al. 2006), in contrast to the well-established fact that the cAMP system plays critical roles in STM formation in olfactory learning in *Drosophila* (Davis 2005). We thus suggest that there is a diversity in the molecular mechanisms of learning and memory in different insects.

9.4 Roles of OA-ergic and DA-ergic Neurons in Olfactory Memory Formation

We studied the roles of octopaminergic (OA-ergic) neurons and dopaminergic (DA-ergic) neurons in appetitive and aversive olfactory conditioning in crickets (Unoki et al. 2005). In mammals, midbrain DA-ergic neurons convey appetitive and aversive signals in various forms of learning (Schultz 2006). In insects, earlier studies suggested that OA- and DA-ergic neurons play roles in appetitive and aversive olfactory conditioning, respectively, in honeybees (Hammer and Menzel 1998; Farooqui et al. 2003) and the fruit fly *Drosophila* (Schwaerzel et al. 2003), although recent studies on *Drosophila* have suggested that DA-ergic neurons participate in both appetitive and aversive learning, as will be discussed later.

We found that crickets injected with an OA receptor antagonist (epinastine or mianserin) into the hemolymph before conditioning exhibited an impairment of appetitive conditioning to an odor with water reward. In contrast, these animals exhibited no impairment of aversive conditioning to an odor with sodium chloride punishment. The latter finding indicates that OA receptor antagonists do not impair sensory function, motor function or the motivation necessary for learning. We thus conclude that OA-ergic neurons are specifically involved in conveying water reward. We also found that injection of a DA receptor antagonist (fluphenazine, chlorpromazine, or spiperone) impaired aversive learning with sodium chloride punishment but not appetitive learning with a water reward. We thus conclude that DA-ergic neurons are specifically involved in conveying sodium chloride punishment. Overall, we can conclude that OA- and DA-ergic neurons convey information about appetitive and aversive US, respectively, in olfactory conditioning in crickets.

9.5 Roles of OA-ergic and DA-ergic Neurons in Formation of Memory for Visual Patterns and Color Cues

We next studied the roles of OA-ergic and DA-ergic neurons in appetitive and aversive conditioning of a visual pattern (Unoki et al. 2006) and a color cue (Nakatani et al. 2009). Crickets injected with an OA receptor antagonist (epinastine or mianserin) into the hemolymph before visual pattern conditioning exhibited an impairment of appetitive learning, whereas aversive learning of a visual pattern was unaffected (Unoki et al. 2006). In contrast, a DA receptor antagonist (fluphenazine, chlorpromazine, or spiperone) impaired aversive learning but not appetitive learning. Similarly, crickets injected with an OA receptor antagonist into the hemolymph before color conditioning exhibited an impairment of appetitive learning without any effect on aversive color learning (Nakatani et al. 2009). In contrast, injection of a DA receptor antagonist into the hemolymph impaired aversive color learning but had no effect on appetitive color learning. These results indicate that the roles of

OA-ergic and DA-ergic neurons in conveying information about appetitive and aversive US, respectively, are ubiquitous in learning of odor, visual pattern, and color stimuli. OA-ergic and DA-ergic neurons may serve as general reward and punishment systems, respectively, for learning in crickets.

Recent studies on *Drosophila*, on the other hand, have suggested that different classes of DA-ergic neurons mediate reward and punishment in olfactory conditioning (Liu et al. 2012; Burke et al. 2012), a finding fundamentally different from that in crickets. This strengthens our suggestion that there is fundamental diversity in the mechanisms of learning and memory in insects.

9.6 Participation of OA-ergic Neurons and DA-ergic Neurons in Appetitive and Aversive Memory Retrieval

We then studied the roles of OA-ergic and DA-ergic neurons in appetitive and aversive memory retrieval (Mizunami et al. 2009). Crickets were subjected to appetitive or aversive olfactory conditioning. Then they were injected with OA or DA receptor antagonists before a retention test. Injection of an OA receptor antagonist completely impaired appetitive olfactory memory retrieval but had no effect on aversive olfactory memory retrieval (Fig. 9.3a). In contrast, injection of a DA receptor antagonist completely impaired aversive memory retrieval but had no effect on appetitive memory retrieval (Fig. 9.3b). Moreover, we observed that injection of an OA and DA receptor antagonist before the retention test impaired appetitive and aversive memory retrieval, respectively, in visual pattern learning. Therefore, we concluded that participation of OA- and DA-ergic neurons in the retrieval of appetitive memory and aversive memory, respectively, is ubiquitous in learning of odors and visual patterns. This differs from reports on *Drosophila* that impairment of OA- or DA-ergic transmission had no effect on memory retrieval (Schwaerzel et al. 2003; Liu et al. 2012; Burka et al. 2012).

9.7 Proposal of a New Model of Classical Conditioning in Insects

Findings in crickets described above were not consistent with conventional neural models of classical conditioning in *Drosophila*. Figure 9.4a illustrates a model proposed by Schwaerzel et al. (2003) to account for the roles of extrinsic and intrinsic neurons of the mushroom body in appetitive or aversive olfactory conditioning in *Drosophila*. The model assumes, at first, that the "CS" neurons (Kenyon cells of the mushroom body) that convey information of the CS make synaptic connections with dendrites of "CR" neurons (efferent (output) neurons in the lobes of the mushroom body). A conditioned response (CR) that mimics an

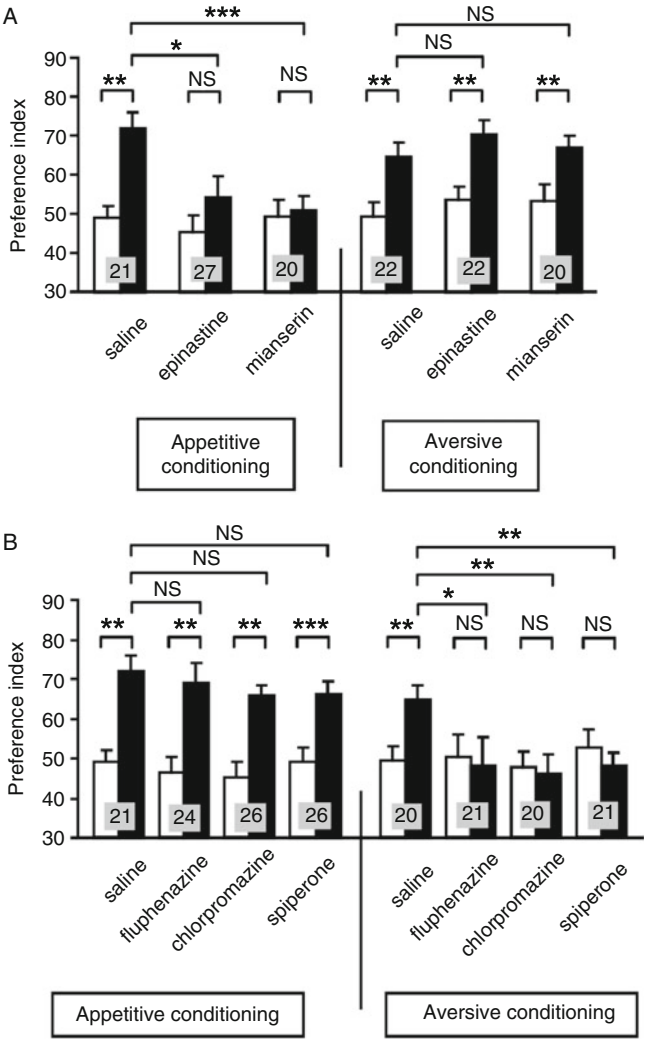

Fig. 9.3 OA and DA receptor antagonists impair appetitive and aversive olfactory memory retrieval, respectively. Effects of OA (**a**) and DA (**b**) receptor antagonists on olfactory memory retrieval. Fourteen groups of crickets were subjected to appetitive (*left*) or aversive (*right*) olfactory conditioning trials. The next day, each group was injected with 3 μl of saline or saline containing 1 μM epinastine, 1 μM mianserin, 500 μM fluphenazine, 500 μM chlorpromazine, or 500 μM spiperone before the final test. Preference indices for the rewarded odor (in the case of appetitive conditioning) or unpunished control odor (in the case of aversive conditioning) before (*white bars*) and 1 day after (*black bars*) conditioning are shown with means + SEM. The results of statistical comparison before and after conditioning (Wilcoxon's test) and between experimental and saline-injected control groups (Mann-Whitney test) are shown as *asterisks* (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, NS $p > 0.05$). The number of crickets is shown at each data point (Modified from Mizunami et al. 2009)

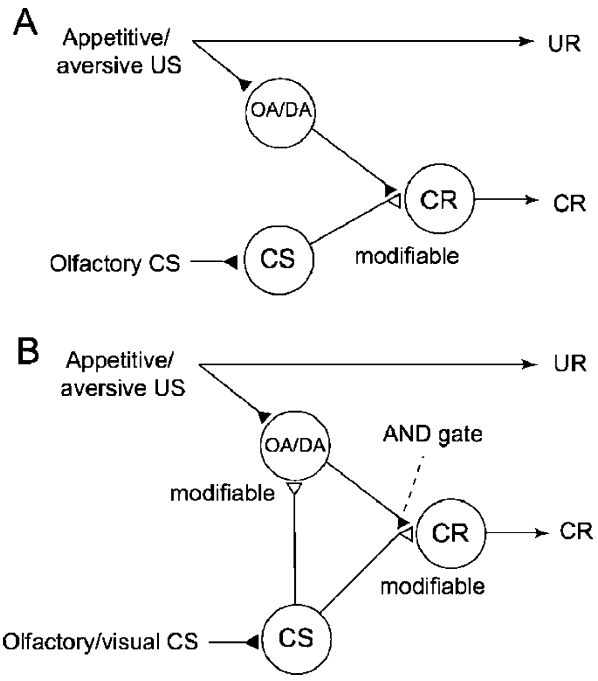

Fig. 9.4 Conventional and new models of classical conditioning in insects. (**a**) A model proposed by Schwaerzel et al. (2003) to account for the roles of intrinsic and extrinsic neurons of the mushroom body in olfactory conditioning in *Drosophila*. OA-ergic and DA-ergic neurons ("OA/DA" neurons) project to the lobes of the mushroom body and convey information about appetitive and aversive US, respectively. "CS" neurons, which are Kenyon cells of the mushroom body and convey information about the CS, make synaptic connections with "CR" neurons, which are efferent neurons of the lobes. "CR" neurons are assumed to induce a conditioned response (CR), the efficacy of the connection being strengthened by conditioning. "OA/DA" neurons make synaptic connections with axon terminals of "CS" neurons. (**b**) Our new model of classical conditioning. In the model, it is assumed that coincident activation of "OA/DA" neurons and "CS" neurons is needed to activate "CR" neurons to lead to a CR (AND gate). It is also assumed that conditioning strengthens the efficacy of synaptic transmission from "CS" neurons to "OA/DA" neurons (Modified from Mizunami et al. 2009)

unconditioned response (UR) can activate these output neurons, but these synaptic connections are weak or silent before conditioning. Secondly, it is assumed that OA- and DA-ergic efferent neurons projecting to the lobes ("OA/DA" neurons), which convey information about appetitive and aversive US, respectively, make synaptic connections with axon terminals of "CS" neurons. Thirdly, it is assumed that the efficacy of synaptic transmission from "CS" neurons to "CR" neurons, which induces a CR, is strengthened by coincident activation of "CS" neurons and "OA/DA" neurons in conditioning. This model matches our finding that activation of OA- or DA-ergic neurons is needed for memory acquisition. However, it does not account for our finding that activation of these neurons is needed for memory retrieval.

We have, therefore, proposed a new model (Fig. 9.4b, Mizunami et al. 2009), which minimally modifies the conventional model. In our model, it is assumed, at first, that activation of "OA/DA" neurons is needed to "gate" the synaptic pathway from "CS" neurons to "CR" neurons after conditioning. Secondly, it is assumed that synaptic connections from "CS" neurons to "OA/DA" neurons, which encode appetitive/aversive US, are strengthened by coincident activation of "CS" neurons and "OA/DA" neurons by pairing of a CS with a US. In short, this model assumes formation of two kinds of memory traces by conditioning. Results of our pharmacological analysis coupled with a second-order conditioning procedure confirmed predictions from the model (Mizunami et al. 2009). Moreover, this model provides a framework to explain neural mechanisms of sensory preconditioning, a higher-order learning phenomenon (Matsumoto et al. 2013).

9.8 Diversity in the Mechanisms of Learning and Memory in Insects

Our studies on crickets suggest that there are some fundamental differences in the basic mechanisms of learning and memory between the cricket and the fruit fly. Such differences are summarized in Table 9.1. It could be argued that some of these differences might be due to differences in experimental approach (i.e., pharmacology in crickets, genetic manipulation in flies), but it is difficult to believe that methodological differences could account for all of the distinctions noted. One of our research goals is to confirm such diversity and to evaluate its functional significance and underlying evolutionary history. In conclusion, studies on crickets, as well as other species of insects such as fruit flies, honeybees, moths, and cockroaches, promise to yield a better understanding of the diversity and evolution of brain mechanisms underlying learning and memory in insects.

Table 9.1 Proposed differences in the mechanisms of learning and memory in crickets and fruit flies

	Cricket *Gryllus bimaculatus*	Fruit fly *Drosophila melanogaster*
Conditioning	OA or DA neurons participate in appetitive or aversive conditioning, respectively	DA neurons participate in both appetitive and aversive conditioning
Memory retrieval	OA or DA neurons participate in appetitive or aversive memory retrieval, respectively	OA or DA neurons do not participate in memory retrieval
STM formation	The cAMP system does not participate in STM formation	The cAMP system participates in STM formation
LTM formation	The NO-cGMP system and cAMP system participate in LTM formation	The cAMP system, but not NO-cGMP system, participates in LTM formation

STM short-term memory, *LTM* long-term memory. For references, see text

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