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Depolymerization of actin facilitates memory formation in an insect

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In mammals, memory formation and stabilization requires polymerization of actin. Here, we show that, in the honeybee, inhibition of actin polymerization within the brain centres involved in memory formation, the mushroom bodies (MBs), enhances associative olfactory memory. Local application of inhibitors of actin polymerization (Cytochalasin D or Latrunculin A) to the MBs 1 h before induction of long-term memory increased memory retention 2 and 24 h after the onset of training. Post-training application of Cytochalasin D also enhanced retention, indicating that memory consolidation is facilitated by actin depolymerization. We conclude that certain aspects of memory mechanisms could have been established independently in mammals and insects.

Keywords: memory; olfactory conditioning; actin; honeybee

1. INTRODUCTION

Insects are often used to understand cellular and molecular foundations of learning and memory [1,2]. However, the mechanisms of memory formation in insects may differ from those of mammals. In mammals, actin polymerization is essential for stable long-term potentiation (LTP) [3–5] and stabilization of newly acquired memory [6]. Abundant actin filaments have been found in insect mushroom bodies (MBs), the areas of brain that demonstrate experience-dependent plasticity, and it has been suggested that rearrangement of filamentous (f)-actin is required for learning in insects [7]. Here, we investigate the role of dynamic actin in memory formation in the honeybee.

2. METHODS

(a) *Rhodamine–phalloidin staining*

Bees were anaesthetized by cooling, the brains were dissected free and fixed in 4 per cent paraformaldehyde in 0.1 M phosphate buffer. Vibrotome sections were permeabilized with 0.1 per cent Triton-X100 and stained with rhodamine–phalloidine (Molecular Probes, 1 : 100). Digital images were obtained using a confocal microscope Leica TCS SP2.

(b) *Training and testing*

Honeybee foragers (*Apis mellifera*) were trained using a three trial olfactory conditioning technique with 10 min inter-trial intervals (ITIs). Each bee was tested in the 2 and 24 h memory retention tests. The odour (conditional stimulus (CS), clove) was blown on to the antennae for 5 s; 3 s after the onset of odour presentation, 50 per cent of sucrose (unconditional stimulus, US) was first delivered to antennae and then to the proboscis for 5 s. In the memory retention tests, CS alone was presented for 5 s without reinforcement by the US. In backward pairing, the onset of US presentation preceded the onset of CS presentation with the same time overlap between stimuli and the same ITIs. In sensitization controls, US was presented alone once for 5 s.

(c) *Drug application*

Microinjections of 260 nl of Cytochalasin D (Sigma) or Latrunculin A (Molecular Probes) into the MB region were performed via the median ocellar tract using a microinjector (Picrospritzer II, General Valve Corporation). In each experiment, the vehicle control group received 260 nl of dimethyl sulfoxide (DMSO) at the concentration in Ringer identical to those used in the inhibitor group. Cytochalasin D was injected at concentrations 8.3 or 0.83 ng nl^{-1} in 33.3 per cent or 3.3 per cent DMSO in Ringer, respectively. Latrunculin A was injected at a concentration of 0.25 ng nl^{-1} in 10 per cent DMSO. Pre-training injections were performed 1 h before training. Post-training Cytochalasin D injections were performed either between 45 min and 1 h after training or 3 h before the 24 h memory retention test.

(d) *Statistics*

Fisher exact tests were used to evaluate statistical significance and χ^2 tests were used to find out whether the effect of drug affected the acquisition in second and third trials, the binomial distribution was used to calculate confidence intervals.

3. RESULTS

Rhodamine–phalloidin staining of MBs revealed abundant actin filaments within microglomeruli, the synaptic complexes where MB input neurons synapse with the intrinsic MB neurons (figure 1) [7]. To modify the ratio of f-actin to the globular actin, we injected inhibitors of actin polymerization (Cytochalasin D or Latrunculin A) into MBs. The performance of bees injected with inhibitors of actin polymerization and those injected with the vehicle (DMSO) alone was compared.

A standard olfactory reward conditioning of the proboscis extension response (PER) was employed by forward pairing of an odour (CS) and sugar solution (US) in three trial training [8]. Memory retention tests were performed 2 and 24 h later by presenting the CS alone. Cytochalasin D or Latrunculin A was locally delivered to the MB region before or after training. Injections 1 h before training resulted in lowering acquisition both in drug-treated and in control animals (compare figure 2*a*,*b*,*h* with *e*,*f*), which is likely to be a consequence of incomplete recovery of bees from injection. Cytochalasin D injected 1 h before training at a concentration of 8.3 ng nl^{-1} significantly increased the percentage of positive responses to CS (PER rate) in both memory retention tests, 2 h ($p_{\text{one-tailed}} = 0.0012$; $p_{\text{two-tailed}} = 0.0017$) and 24 h ($p_{\text{one-tailed}} = 0.0085$; $p_{\text{two-tailed}} = 0.015$) and showed the same trend during acquisition ($p = 0.15$) (figure 2*a*). A 10-fold lower concentration of Cytochalasin D did not elicit any significant effect (figure 2*b*), indicating that Cytochalasin D improved memory retention in a concentration-dependent manner. To rule out non-associative effects of Cytochalasin D, we performed control experiments, in which both CS and US were presented, but the sequence of their presentation was reversed. Such backward pairing does not elicit excitatory associations between the US and CS [9]. No significant effect of Cytochalasin D

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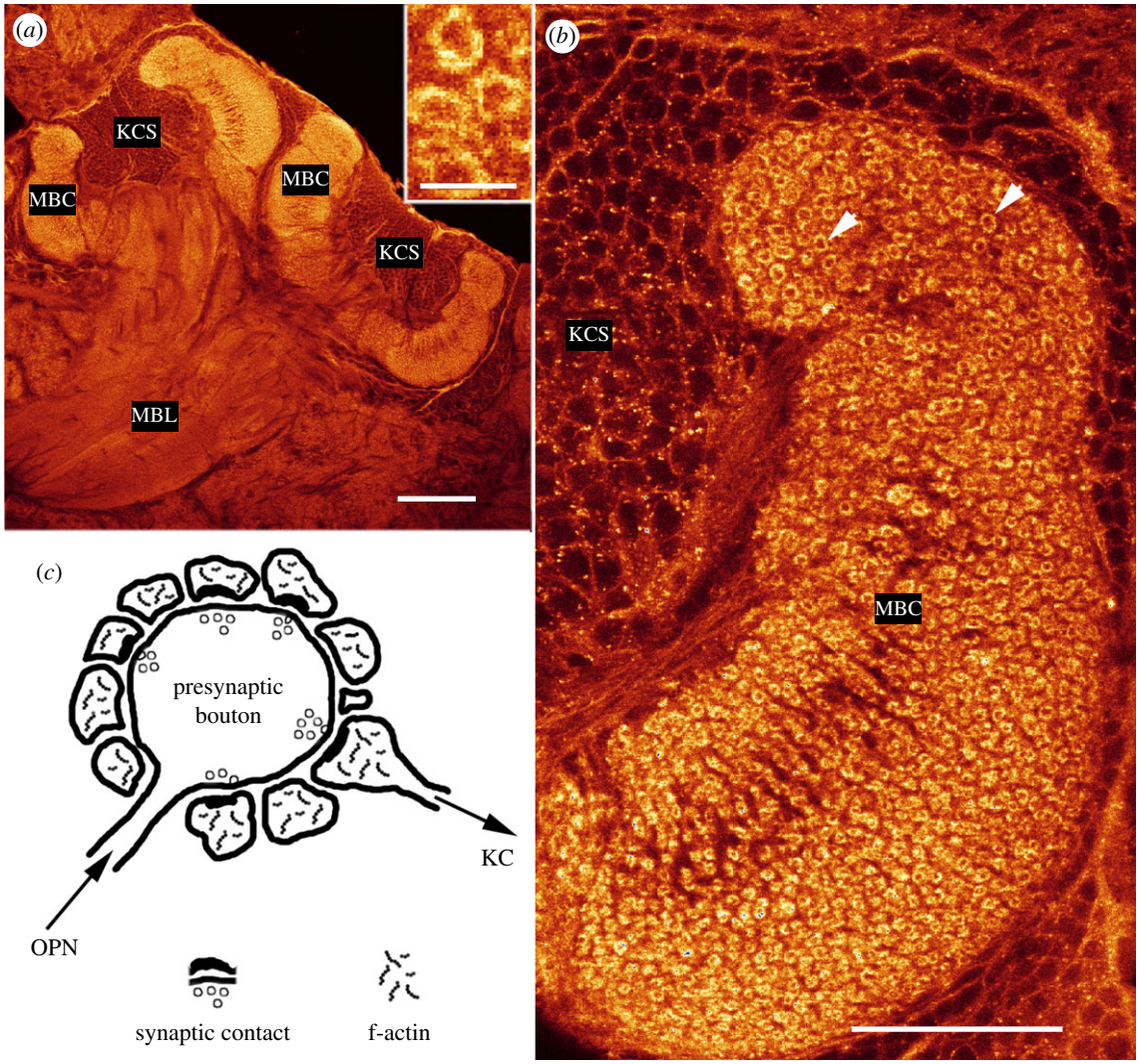

Figure 1. Expression of f-actin in the MBs. (*a*) The MB sensory input neuropiles, the calyces (MBC), are enriched in f-actin; microglomeruli (*b*, arrowheads) accumulate f-actin in the peripheral postsynaptic rings (inset); (*c*) a simplified diagram of a microglomerulus receiving an olfactory input. Each microglomerulus is composed of a centrally located presynaptic bouton of an olfactory projection neuron (OPN) surrounded by multiple actin-rich postsynaptic dendritic spines of the Kenyon cells. MBL, MB lobes; KCS, Kenyon cell somata; KC, Kenyon cell. Scale bar: (*a*) 100 µm (5 µm in inset) and (*b*) 50 µm.

on PER rate was seen either in 2 or 24 h tests (figure 2*c*).

A trend of higher acquisition in Cytochalasin-injected bees compared with the vehicle control may indicate a facilitatory effect of Cytochalasin D on weak visual context-CS association [10]. Prolonged non-associative sensitization by US [11] could account for the possible early effect of Cytochalasin D (figure 2*a*,*b*). To rule out this possibility, we presented the US alone 1 h after Cytochalasin D injection. Cytochalasin D had no effect on PER rate 30 s after US presentation (figure 2*d*), when sensitization is mostly expressed [11]. Also, Cytochalasin D itself did not produce enhanced responsiveness to the CS, because 2 and 24 h tests showed no differences in PER rate between the Cytochalasin D and vehicle groups. To test whether Cytochalasin D specifically improves memory retrieval, we injected Cytochalasin D on the next day after training, 3 h before the 24 h memory retention test. No effect on PER rate was obtained in the test following Cytochalasin D application (figure 2*e*). Thus, Cytochalasin D facilitates formation of associative memory rather than retrieval function. Post-training Cytochalasin D application performed between 45 min and 1 h after conditioning significantly increased PER rate in the 24 h memory retention test ($p_{\text{one-tailed}} = 0.029$; $p_{\text{two-tailed}} = 0.055$; figure 2*f*).

Because associative olfactory memory in bees consolidates for approximately 2.5 h after conditioning [12], the memory consolidation was not yet completed by the injection time. This means that memory consolidation does not require actin polymerization, but instead is facilitated by inhibition of actin polymerization. We further confirmed that vehicle alone did not elicit any significant effect on PER rate (figure 2*g*), and both Cytochalasin D and vehicle alone did not affect survival rate of the bees (not shown). Finally, Latrunculin A injected before training at a concentration of 0.25 ng nl^{-1} had a similar enhancing effect on retention (figure 2*h*), both during memory acquisition ($p = 0.05$ for both second and

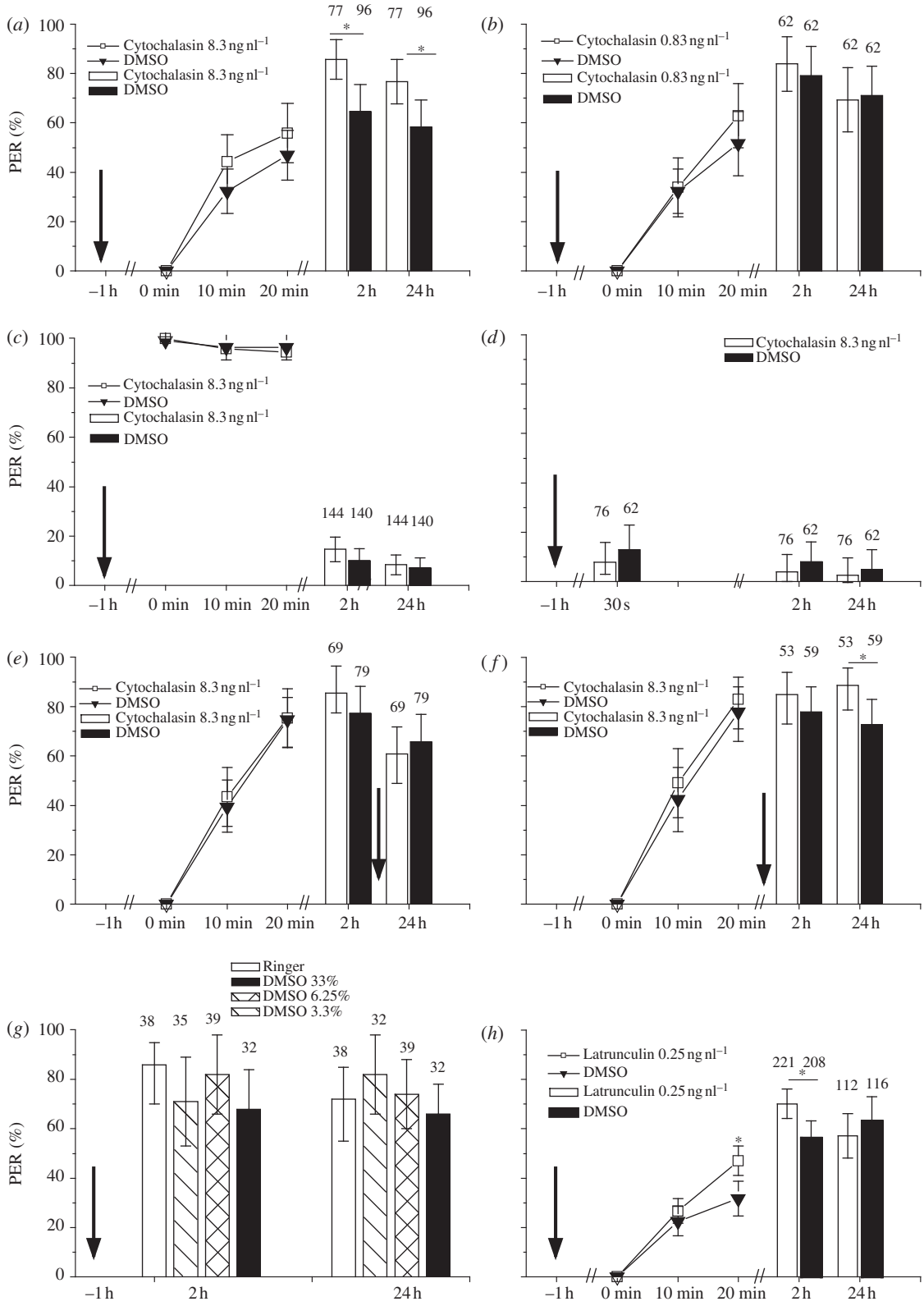

Figure 2. Effect of actin polymerization inhibitors on PER rate after induction of long-term memory (LTM). (*a*) Pre-training application of 8.3 ng nl^{-1} Cytochalasin D and (*b*) 0.83 ng nl^{-1} Cytochalasin D; (*c*) application of 8.3 ng nl^{-1} Cytochalasin D 1 h before backward pairing and (*d*) single US presentation; (*e*,*f*) post-training applications of 8.3 ng nl^{-1} Cytochalasin D 3 h before 24 h memory retention test (*e*) and between 45 min and 1 h after training (*f*); (*g*) pre-training application of DMSO alone; (*h*) pre-training application of Latrunculin A. Number of animals per group is given on the top of the histograms. Arrows indicate injection time.

third trials; third training trial, $p_{\text{one-tailed}} = 0.0008$; $p_{\text{two-tailed}} = 0.0015$) and the 2 h test ($p_{\text{one-tailed}} = 0.0019$; $p_{\text{two-tailed}} = 0.0036$). Because Cytochalasin D and Latrunculin A inhibit actin polymerization by different mechanisms [13], the fact that they have similar effects strongly suggests that the drugs enhanced memory retention specifically via inhibition of actin polymerization.

4. DISCUSSION

Our data show that actin depolymerization enhances associative olfactory memory in the honeybee, which is in conflict with the current view concerning a role of actin dynamics in synaptic plasticity and memory formation in mammals. The enhancing effect of actin depolymerization on memory formation in the honeybee is likely to reflect the fact that actin-dependent cellular mechanisms involved in formation of long-term memory (LTM) in insects and mammals are not identical. Cofilin is an actin severing protein whose constitutive activity is regulated by RAC, a small GTPase, which suppresses cofilin activity by phosphorylation. In *Drosophila*, inhibition of the RAC-cofilin signalling pathway attenuates passive memory decay while RAC activation accelerates this process [14,15]. By contrast, in mammals, RAC activation promotes the stabilization of LTP [16]. Because RAC activation leads to actin polymerization, inhibition of RAC in *Drosophila* would lead to actin depolymerization-driven memory stabilization, which is in line with our data.

How may actin depolymerization improve LTM? In mammals, addition and elimination of dendritic spines and synapses occur both spontaneously [17,18] and in response to LTP [19], long-term depresssion [20] and LTM induction [21,22]. In insects, a transition to new behavioural tasks is accompanied by enforcements of specific synaptic connections and elimination of others within the MB calyx [23]. In addition, the MB calyx expands over the adult life cycle, and this expansion is at least partly independent of experience [24]. We speculate that a persistent production of dendritic spines incorporated into synaptic circuitry in a 'memory-independent' manner may underlie the memory decay mediated by RAC/cofilin [14]. Since the outgrowth of new neuronal protrusions, including dendritic filopodia and spines, requires actin polymerization [25,26], a shift of actin dynamics to a depolymerizing mode is expected to inhibit the formation of new spines. Inhibition of actin polymerization before or shortly after induction of LTM would then prevent the 'non-specific' synaptogenesis, facilitate rewiring of existing synaptic connections and thereby enforce specific synaptic circuits associated with newly acquired memory. In addition, actin-dependent changes in synaptic efficiency at individual synapses may account for LTM in the honeybee.

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