Relationship between visual learning/memory ability and brain cAMP level in *Drosophila* *

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Abstract Using the flight simulator system, the operant conditioned visual flight orientation behavior in *Drosophila* was studied. It was demonstrated that the visual learning performance is associated with age; flies learn more reliably at 3—4 days than at 1—2 days of age; the cAMP level of brain is also increasing with age; the brain cAMP content of nonlearner flies of wild type is much higher than that of normal flies; the cAMP level of brain increased abnormally after being fed with caffeine, and the learning performance decreased. These results imply that a moderate range of cAMP level is necessary for the visual learning and memory process. Abnormally high or low level of cAMP causes defects of learning and memory ability.

Keywords: ***Drosophila*, operant conditioned learning and memory, cAMP, neural development.**

Drosophila has become an important experimental animal model because of its two advantages, i.e. its relatively clear genetic background and a variety of associative learning abilities. Single-gene olfactory learning mutants have been isolated. Some of them are related to the cAMP signal transduction cascade, such as dunce (dnc), rutabaga (rut) and dco[1,2].

Up to now, a few reports on the visual learning and memory have been published. In Folker's system, the color discrimination ability of dnc and rut decreases soon after training, but the mutants restore the same performance as that in wild-type flies 2 h after training. So they concluded that the products of duc and rut genes are not crucial for the process of visual learning /memory[3]. We used the flight simulator system to study the visual learning behavior[4], and found that cAMP system also takes part in the visual learning and memory.

Neurophysiological studies have found that cAMP system can cause influence on the synaptic plasticity of neurons at cellular level, and the involvement of cAMP system in learning process may be accomplished through regulating the synaptic plasticity[5]. On the other hand, synaptic plasticity is also the important physiological basis of development of nervous system. These indicate that there may exist some relations between learning and nervous system development. In this paper, we use the flight simulator to study the relation between learning performance and the cAMP level in the view of development.

1 Materials and methods

1.1 Fly stocks and feeding

Wild-type Berlin flies (*Drosophila melanagaster*) were used in this study. Flies were

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grown at 25℃ and a relative humidity of 40% in a 14 h:10 h light/dark cycle with lights on at 7 am, and raised on a Wuerzburg food medium[6]. Adult flies were transferred to a fresh bottle after hatching. Single flies were selected, attached to a small hook of copper wire glued to their head and thorax, and put into transparent chambers ($\pi \times 8^2 \times 30$ mm^3) individually, where they stayed overnight so that they became accustomed to the situation with the triangle hooks on their backs. 5% (w/v) glucose solution was given. After a certain time, these flies were trained and tested individually[6].

1.2 Apparatus

The experimental set-up was a flight simulator under the closed loop condition[6]. The flight simulator established normal negative feedback between the fly's yaw torque and angular velocity of the visual panorama surrounding it. The torque meter transduced the fly's yaw torque into a DC voltage that was stored in the computer continuously. The negative angular velocity for driving the panorama and its angular position could be derived directly from the on-line torque signal of the torque meter. With help of a DC servomotor, the test fly was allowed to control, by its intended turns, the rotation of the surrounding panorama. There were four patterns on the panorama periphery: two upright and two inverted T-shaped figures in the four quadrants with identical patterns facing each other. For conditioning, one of the two patterns (upright or inverted T) was paired with the negative reinforcement (heat punishment), i.e. heat punishment was switched on by computer whenever the fly flied toward the patterns paired with heat punishment. The flies learned quickly to avoid the patterns paired with heat punishment and tried to fix the other patterns. They established the association between the visual patterns and heat punishment. At the test session the heat was switched off in order to evaluate the associative memory retention according to the pattern preferences.

1.3 Methods

1.3.1 Training. Training procedure was composed of the pre-training session, two training sessions and the test session; each session lasted 6 min, and was further divided into 3 phases; each phase continued for 2 min (table 1). The pre-training session comprised three consecutive test periods, during which the flies became accustomed to the situation without heat reinforcement. The training session comprised two consecutive training periods and one test period. During the training period, the computer-controlled infrared beam, focused onto the fly, and was switched on whenever the fly flied toward the punished patterns on the two quadrants facing each other. The test fly tried to drive the panorama by its intended turns to select no punished patterns. When another non-punished pattern entered the frontal quadrant, the beam was intercepted by the computer-controlled shutter. The last phase was test period, during which the memory performances were tested. The test session comprised three consecutive test periods, during which the fly was tested for learning acquisition or memory retention without being heated. All the above experiments were performed at 24—25℃ and a relative humidity of 40%.

Table 1

Test	Test	Test	Training	Training	Test	Training	Training	Test	Test	Test	Test
Pretraining session			Training session			Training session			Test session		

1.3.2 Evaluation of learning/memory ability. A performance index (PI) was used to evaluate the ability of learning and memory. The performance index was classified into three categories: (i) during the pretraining session it is defined as pattern preference index; (ii) during the training session it is defined as avoidance index; (iii) during the test session it is defined as learning index. PI is calculated as $(t1 - t2)/(t1 + t2)$, where t1 and t2 indicate the time that the fly spends fixating the no-heat and heat-associated quadrants, respectively. An index value of 1 indicates complete avoidance of heat-associated pattern, and -1 indicates complete fixation of heat-associated pattern. At the end of each period, PI of this period was given by the computer; data statistics was performed with all the flies being trained to ensure the learning and memory abilities.

1.3.3 The method of feeding fly with caffeine. Newly hatching adult flies were transferred to a fresh food bottle, then transferred to an empty bottle after 2 h with nothing to eat. These flies were divided into two groups randomly, and transferred separately to two bottles with a piece of paper on the bottom. The paper of one bottle was soaked in 5% glucose solution (w/v) containing 50 mmol/L caffeine; the paper of control bottle was soaked only in 5% glucose solution. Single fly was prepared with a small hook of copper wire glued to its head and thorax and put into transparent chambers individually. There was a piece of paper soaked in the same solution on the bottom, so that the fly had drunk solution over 24 h before it was trained.

1.3.4 Radioimmunoassay of brain cAMP level. The kit for cAMP assay was purchased from the Chinese Academy of Atom Energy. The flies were killed by being transferred to the liquid nitrogen quickly. After shaking, the heads were collected, 300 μL 1 mol/L $HClO_4$ was added and homogenized, then centrifuged at 25 000 g for 15 min. The supernatant was adjusted to pH 7.0, centrifuged at 25 000 g for 15 min again, then evaporated to dry at 75℃ via water bath. The pellet was dissolved in 100 μL Tris-HCl buffer (pH 7.5). To 40 μL sample solution, H^3-cAMP (30 μL) and binding protein (60 μL) were added, and incubated at 4℃ for 2 h. Then adsorbing reagent was added and mixed by gently vortexing, centrifuged for 5 min at 4 000 g. 120 μL supernatant was transferred to a scintillation bottle. 1.5 mL 100% alcohol and 3.5 mL oscillation solution were added, mixed, and counted using a Beckman scintillation counter.

1.4 Data processing

All the data were expressed in the form of $+/-$ standard errors. The relatively significant differences of the data were assessed with T-test.

2 Results

2.1 Relation between learning performance and age of *Drosophila*

To study the relation between learning and age, newly hatching adult flies were transferred to a fresh bottle. Some flies were selected for analysis of visual learning and memory after 14—24 h (1—2 days group), while others were kept in the bottle for 48—72 h (3—4 days group). The performance indices of 1—2 days group and 3—4 days group flies are shown in fig. 1. In both groups, the flies can avoid the visual patterns paired with heat-punishment well. In the first training session, after the two training phases, two groups had lower learning index in the test phases, but they were not significantly different from each other. In the second training session, after the two training phases, in the test phase, 3—4-day-old flies possessed high learning

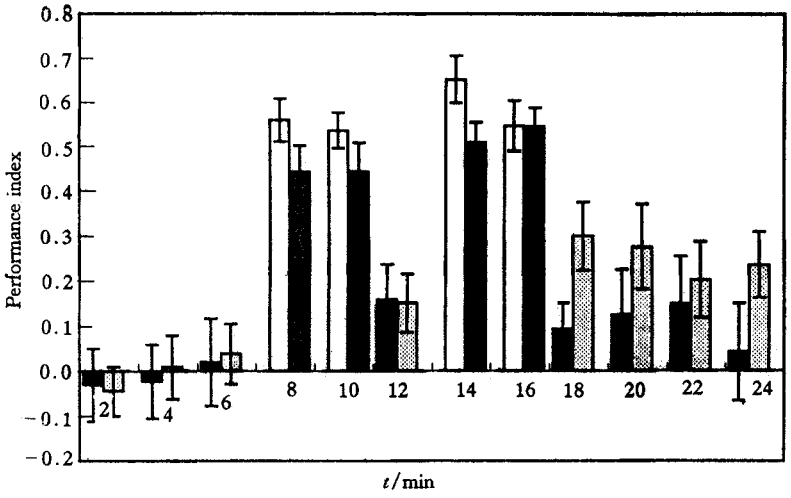

Fig. 1. Performance index of flies with different ages in training and test periods. □, The avoidance index of the 1—2 days group flies ($n=23$); ■, the learning index of the 1—2 days group flies; ■, the avoidance index of the 3—4 days group flies ($n=20$); ▨, the learning index of the 3—4 days group flies.

indices. The PIs of 3—4 days group flies ($PI=0.30\pm0.08$) were significantly different from that of 1—2 days group flies ($PI=0.09\pm0.10$, $P<0.05$). In addition, in the test session, the PI of 1—2 days group flies was a little bit different from zero, whereas that of 3—4 days group flies was significantly different from zero. Therefore, the learning and memory ability of 3—4 days group flies is significantly better than that of 1—2 days group flies. It is shown that with the increasing age, the abilities of learning/memory increased. But after 4 d, the learning/memory ability does not increase any more.

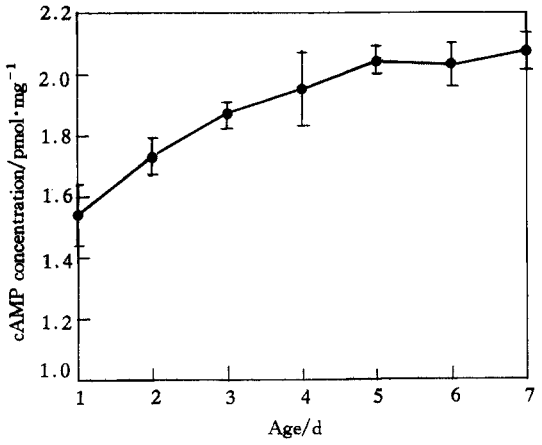

Fig. 2. The relationship between brain cAMP level and age in *Drosophila*. The brain cAMP level increases with the age.

2.2 Relation between brain cAMP level and the age of *Drosophila*

The flies in the bottles hatching for 4 h were collected. 210 individual flies were randomly sampled and seven groups were transferred to the fresh bottle with 30 flies in each group. Every 24 h, one group was chilled in liquid N_2 and kept; the seven groups represented flies with different ages ranging from 1 to 7 d. The brain cAMP level of each group was analyzed (fig. 2). With the increasing age, the brain cAMP levels increase, but the tendency slows down from the 5th day. This indicates that both the learning /memory ability and brain cAMP level are certainly related with the age.

2.3 Screening of nonlearner individuals

The 3-day-old flies were selected to determine whether the flies were nonlearner individuals.

Fig. 3 shows the PIs of 18 individual flies having the normal learning/memory abilities and 15 nonlearner individuals. The nonlearner flies demonstrated the normal flight and avoidance response, but could not establish the association between heat-punishment and visual patterns; their learning PIs were close to zero. It can be sure that these flies whose PI are not significantly different from zero ($p > 0.05$) in training and during test periods may have not established the associative memory and cannot make correct selection, and show learning/memory defect in this learning paradigm.

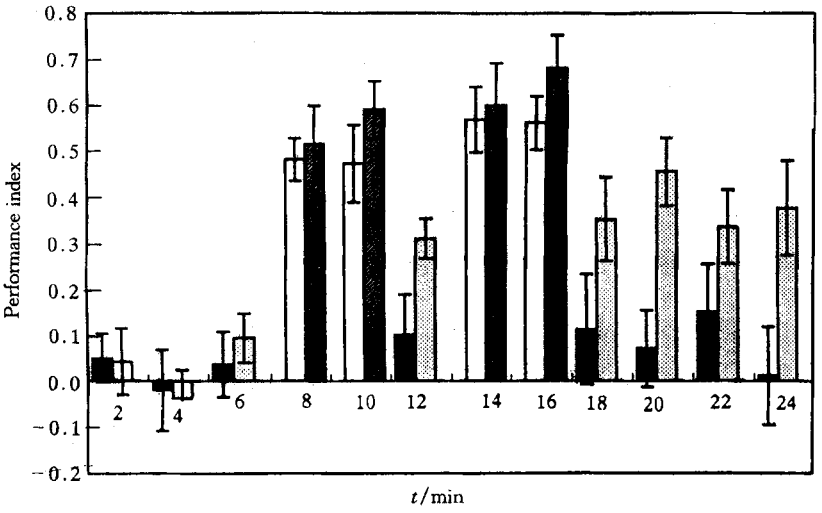

Fig. 3. Performance index of 3-day-old normal flies and nonlearners in the training and test periods. ■, The avoidance index of the normal group flies ($n = 20$); ▨, the learning index of the normal group flies ($n = 18$); □, the avoidance index of the nonlearners ($n = 18$); ■, the learning index of the nonlearners, which has little difference from zero.

2.4 Brain cAMP level analysis of normal and nonlearner flies

The cAMP levels in the brain of normal flies and nonlearner flies were determined. The results are shown in fig. 4. The brain cAMP levels of learning/memory nonlearner flies are much higher than that of normal flies of the same age. So there may be some relation between learning/memory and cAMP level.

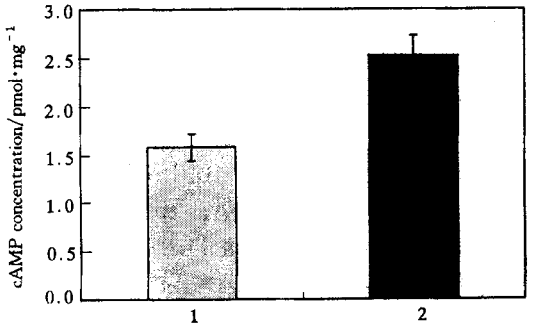

Fig. 4. Comparison of the brain cAMP levels between 3-day-old normal flies and nonlearner. 1, The brain cAMP content of the normal flies; 2, the brain cAMP content of the nonlearners.

In fig. 2, the brain cAMP level of 3-day-old flies is (1.87 ± 0.04) pmol/mg. But in fig. 4, the brain cAMP level of 3-day-old flies as the control group is (1.58 ± 0.14) pmol/mg. One of the reasons which make the difference between two groups may be the different sampling methods used. In the age-related experiment, the random sampling method was used; both the normal and nonlearner flies were sampled. In this flight simulator paradigm, the nonlearner flies consisted of about 10% of the whole tested flies. In fig. 4, the flies used as control group were selected as normal learning flies. Since there is a higher cAMP level in the nonlearner flies brain, this mixture causes the low-

er cAMP level of the control group in fig. 4 than that of random sampling group.

2.5 The learning ability of *Drosophila* can be destroyed by caffeine

To study the learning ability of flies fed with caffeine, the flies fed with caffeine were randomly sampled for experiment. The performance indices of the group fed with caffeine and the controlled group flies are shown in fig. 5. During the two training sessions, the flies fed with caffeine had normal flight and avoidance response, but could not establish the association between heat-punishment and visual patterns. In the test session, the PI of these flies was not a little bit different from zero ($P>0.05$), but that of control group flies was significantly different from zero ($P<0.05$).

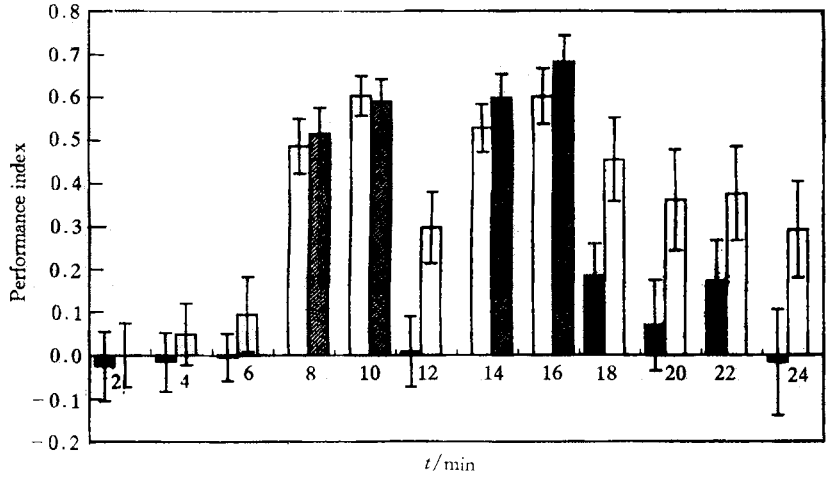

Fig. 5. Performance index of the flies fed with caffeine and control group in training and test periods. □, The avoidance index of the flies fed with caffeine ($n=15$); ■, the learning index of the flies fed with caffeine ($n=15$) which has little difference with zero; ■, the avoidance index of the control group flies ($n=20$); ▨, the learning index of the control group.

It can be seen that in fig. 5, the flight orientation and avoidance response are normal, but the learning index shows that this kind of flies is not able to establish associative memory, so they cannot make correct choice between the punished and non-punished patterns in the test period. The reason for the decline of learning and memory ability is that caffeine induces inhibitory effect on the activity of PDEase, as the result of this effect, the cAMP level raises abnormally (the brain cAMP level of the flies fed with caffeine is (2.99 ± 0.12) pmol/mg, that of control group is (1.88 ± 0.14) pmol/mg), the normal roles of cAMP system are impaired, and the learning/memory ability is defected.

3 Discussion

The above described results indicate that in the flight simulator system: (i) the visual learning and memory abilities of *Drosophila* of wild-type increase with the age, and accordingly the brain cAMP levels in *Drosophila* have the same tendency. Therefore, there may be a certain relation among the visual learning/memory ability, growth age and brain cAMP level; (ii) there are some nonlearners flies which have abnormally high cAMP content in brain; (iii) cAMP

metabolism is disturbed by feeding the flies with caffeine, so that the brain cAMP level increases abnormally. This causes a substantial decline of learning index.

In the olfactory learning and memory paradigm, the larvae of both wild and dnc type have much lower memory abilities than those of adults[7]. The learning and memory ability is increasing with the development of *Drosophila*. Our result here reveals that visual learning/memory is also related to the development process, just as that in olfactory learning and memory behavior.

The involvement of cAMP signaling cascade in the learning and memory has been studied intensively so far in olfactory learning paradigm. By now, it has been proved that the cAMP system involving PDEase plays important roles in the learning and memory of *Aplysia*, fly and rat[2]. Perhaps the defect of memory retention through metamorphosis in dnc is the result of abnormal cAMP metabolism. We use the flight simulator system to study the visual learning and memory behavior of *Drosophila*, and find that there do exist relations between learning and memory ability, the age of flies and brain cAMP level.

The phenomenon of increasing cAMP levels with the development of *Drosophila* is found not only in the stage of eclosion, more significant tendency is found in the metamorphosis process. For example, the cAMP level in pupa is higher than that of larva, and a larger enhancement can be found in the process from larva to adult (data not shown). In fact, this kind of phenomenon is not restricted to *Drosophila*, in rat, for example, the cAMP level of neonatal rat rises rapidly in a certain period after birth[8].

In the stage of 3-day-old larva, the flies were fed with caffeine in order to enhance the cAMP level temporarily, then the food without caffeine was fed, and the cAMP level restored to its normal level. When these flies grew up, the cAMP level and activity of PDEase were normal in the training period, and the flight and avoidance response to the patterns paired with heat seem to be normal, but they could not establish the association between visual patterns and heat-punishment; the learning ability of this kind of flies is defective. We propose that the exposure to caffeine in the medium during larva stage results in the abnormal enhancement of cAMP level, the aberrant development of neural system, and the defect of learning and memory in adult. On the other hand, the exposure of normal adults to caffeine in the medium leads to the decline in the learning and memory ability, therefore the abnormal increase of cAMP level by caffeine can influence the learning and memory behavior directly.

The results from neurophysiology provide some clues to the detailed mechanisms of cAMP system in regulation of learning and memory behavior. Nazif et al.[9] found that the injection of cAMP into *Aplysia* sensory neurons can lead to the change of neuronal morphology. Wu and Kim[10] found that the growth cone motility in cultured larval CNS neurons from *Drosophila* is related closely to cAMP level. The synaptic plasticity of mushroom body neurons in dnc and rut mutants has been altered, and this may be the reason for the defect of learning and memory in dnc and rut[7]. The abnormal enhancement of cAMP may hamper the formation of long term potentiation (LTP)[11]. It has been proved that the involvement of cAMP in learning and memory process may be realized through regulating the synaptic plasticity. This kind of effects includes the short term effect on the ion channel and other protein modification, and the long term effect on the change of gene expression. Other signal transduction systems (such as Ca^{2+}, NO, etc.) are also involved in the regulating of synaptic plasticity. These systems may play roles in regulating synaptic plasticity through interactions[12]. The memory retention detected 24 min after training

belongs to short term memory (STM), and it has been shown that the regulation effect of cAMP on synaptic plasticity is mainly through the STM, thus influencing the learning and memory performance.

Hesenberg et al.[13] indicated that even in the adult fly, the process of reorganization still takes place in most regions of its brain, so that the fly can adapt to specific survival circumstance. It is widely believed that the modification of synaptic strength underlies the learning and memory, and development of neural system in flies as well[14]. It is revealed that cAMP system can influence the synaptic plasticity of neurons[7,9,11,15], and the involvement of cAMP system in learning process may be accomplished through regulating the synaptic plasticity[14]. On the other hand, synaptic plasticity is also the important physiological basis of nervous development. We can conclude that when fly grows elder, the neural system becomes more matured, the ability of cells to regulate cAMP system is enhanced, and the neurons have greater ability to deal with the stimulator and therefore the learning and memory behavior is more reliable.

Our another interesting finding is that there are some nonlearner individuals in every generation, and the brain cAMP level of which is much higher than that in the normal flies, and it is very close to that of dnc. From the results of correlations analysis among age, cAMP level, and learning and memory ability, we find that a relatively high cAMP level corresponds to a better degree of development. But the brain cAMP level of nonlearner flies exceeds the average range of normal cAMP level, and they do not show high learning and memory ability, but a disrupted learning and memory. Therefore, if the cAMP level is abnormally higher than the normal values, it will affect the learning and memory ability. To confirm this notion, we fed caffeine to the adult flies, and found that the learning and memory ability decreased significantly with the abnormal enhancement of cAMP level. So we conclude that a suitable range of cAMP level is necessary for the stability and reliability of learning and memory. Abnormally high, or abnormally low level of cAMP will affect the learning and memory ability, and the memory mutant dnc and rut represent the two extremes, respectively.

The cytosol cAMP level is regulated by a complicated system. We still do not know why there exist some nonlearners in each generation, which factors lead to the abnormal enhancement of cAMP level in certain propotion of these flies, and how the enhancement of cAMP level affects the behavior of learning and memory. The study on these problems is in progress. Exploring deeply into the clues from cAMP system to synaptic plasticity, to neural development, and to learning and memory behavior will help us to elucidate the molecular mechanisms underlying learning and memory processes.

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