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弘前大学リポジトリ
GENETIC DISSECTION OF AGE-RELATED MEMORY IMPAIRMENT IN DROSOPHILA

Daisuke Yamazaki$^{1,2}$, Junjiro Horiuchi$^{2}$ and Minoru Saitoe$^{1}$

Abstract Age-related memory impairment (AMI) is an important phenotype of brain aging. Understanding the molecular mechanisms underlying AMI is important not only from a scientific viewpoint but also for the development of therapeutics that may eventually lead to developing drugs to combat memory loss. AMI has been generally considered to be an overall or nonspecific decay of memory processes that results from dysfunction of neural networks. However, extensive behavioral genetic characterization of AMI with Drosophila demonstrated that AMI results from disruption in specific memory process. In Drosophila, memory acquired after a single olfactory conditioning paradigm has three distinct phases: short-term memory (STM), middle-term memory (MTM), and longer-lasting anesthesia-resistant memory (ARM). Significantly, AMI results from the specific decay of only one memory component, ann-dependent MTM, and not other components. Since amnesiac encodes peptides that regulate adenyl cyclase activity, these studies suggest the importance of the cAMP signaling pathway in AMI in Drosophila, a finding consistent with several models of AMI in mammals. In fact, hypomorphic mutations in PKA catalytic subunit significantly suppress AMI. As cAMP signaling is an essential signaling for learning and memory, these studies suggest antagonistic pleiotropic effect of cAMP signaling. Due to its short lifespan, powerful genetics, and well-characterized and conserved pathways involved in memory and lifespan, Drosophila will be a useful model system for studying the molecular mechanisms underlying this process.

Key words: Age-related memory impairment; Drosophila; genetics; cAMP; PKA

Introduction

Even without the characteristic symptoms of Alzheimer’s and other neurodegenerative diseases, most people experience a characteristic decrease in memory with advancing age referred to as age-related memory impairment (AMI). The frequency and severity of AMI increases significantly for people over the age of eighty. Consequently, an increase in average lifespan leads to an increase in the number of people who suffer from severe AMI. Although many research groups have reported anatomical and physiological changes associated with AMI$^{1,2}$, neither its underlying molecular mechanisms nor its genetic relationship with aging are clearly understood. A major obstacle in performing genetic and behavioral analyses of AMI has been the long life span of animal models. Jiang et al reported a number of genes, involved in neuronal function in the hypothalamus and cortex of mice, with altered expression upon aging$^{3}$. However, it is difficult to carry out behavioral genetics to test whether mutations in these genes affect AMI because the lifespan of mice is about two years. Drosophila has numerous advantages in studying AMI, including a relatively short lifespan, 30 to 40 days, powerful genetics, and a quantifiable and well-characterized assay for memory. Hence, Drosophila offers a unique opportunity for understanding the molecular mechanisms of AMI.

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**Genetic dissection of AMI with Drosophila**

A large number of *Drosophila* memory mutants have been identified using a Pavlovian olfactory conditioning paradigm that was first developed by Quinn and colleagues in 1974, and modified by Tully and Quinn in 1985 (Fig. 1). In combination with pharmacological interventions, behavioral genetic studies of olfactory memory have identified genes that function at specific memory phases (Table 1): learning acquisition (LRN), short-term memory (STM), middle-term memory (MTM), anesthesia-resistant memory (ARM) and long-term memory (LTM). As shown in Figure 2, mutants in each memory phase show characteristic memory retention curves.

Using a courtship conditioning paradigm, Savvateeva et al., showed a decay in memory upon aging for mutants in the kynurenine pathway. However, they did not find significant memory impairment in aged wild-type flies. In contrast, AMI in wild-type flies can clearly be observed using a Pavlovian olfactory conditioning paradigm. When memory retention curves are made for flies of various ages, old flies show a significant memory impairment that increases with age (Fig. 3A). Significantly, as flies age, their memory retention curves become similar to those of *ann* mutants, drastic decrease in memory 1 hr after training. This suggests that AMI is not a general loss of memory but rather a specific decay in *ann*-dependent MTM. In support this idea, *ann* mutants do not show further memory decay upon aging, in contrast to other memory mutants such as *lat* and *vol* (Fig. 3B).

The *ann* gene encodes a putative secreted protein that is thought to be cleaved into three neuropeptides, two of which have homologies to mammalian pituitary adenyl cyclase activating

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**Table 1** Drosophila memory mutants, affected memory phases, and encoded genes (n.d. not defined in olfactory conditioning)

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<tr>
<th>Affected memory phase</th>
<th>Mutant name</th>
<th>Gene</th>
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<tr>
<td>LRN</td>
<td>leucine</td>
<td>DPC subunit</td>
</tr>
<tr>
<td></td>
<td>serine</td>
<td>PKA catalytic subunit</td>
</tr>
<tr>
<td></td>
<td>dopamine</td>
<td>PKA regulatory subunit</td>
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<td></td>
<td>Gαs</td>
<td>Stimulatory Go</td>
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<tr>
<td></td>
<td>fast</td>
<td>Adhesion molecule Fast</td>
</tr>
<tr>
<td>STM</td>
<td>dunce</td>
<td>cAMP-Phosphodiesterase</td>
</tr>
<tr>
<td></td>
<td>melagoga</td>
<td>Ca²⁺/CaM-dependent adenylyl cyclase</td>
</tr>
<tr>
<td></td>
<td>voldio</td>
<td>Adhesion molecule integrin</td>
</tr>
<tr>
<td>MTM</td>
<td>anamnesia</td>
<td>Neuropeptide homologous to PACAP, OHRH</td>
</tr>
<tr>
<td></td>
<td>DOP-</td>
<td>PKA catalytic subunit</td>
</tr>
<tr>
<td>ARM</td>
<td>radish</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td></td>
<td>PRK</td>
<td>Atypical PKC</td>
</tr>
<tr>
<td>LTM</td>
<td>for-CREB</td>
<td>Repressor isoform of transcription factor CREB</td>
</tr>
<tr>
<td></td>
<td>c-class</td>
<td>Inhibitor of cyscine proteinase, cathepsin</td>
</tr>
<tr>
<td>wild</td>
<td>ANN</td>
<td>Protein inhibitor for CaMII</td>
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**Figure 1** Pavlovian olfactory conditioning

In standard single-cycle olfactory conditioning, approximately 100 flies are exposed sequentially to two conditioned stimuli (CS), consisting of two different odors. During exposure to the first (CS+) odor, flies are also exposed to the unconditioned stimulus (US), mild electrical shocks. During exposure to the second (CS-) odor, flies are not shocked. Flies learn to associate the CS+ with shock and avoid this odor. To test for memory retention of this association, trained flies are placed at a choice point of a T-maze in which they are exposed simultaneously to the CS+ and CS-. Memory is quantified as a performance index (PI) where a PI of 100 is obtained when all flies choose the CS+ and a PI of 0 is obtained when flies distribute evenly between the odors.
peptide (PACAP) and growth hormone releasing hormone (GHRH)\(^7\). The amn gene products are highly expressed in a pair of cells, the dorsal paired medial (DPM) cells (see Fig. 4A, B). DPM cells innervate the output lobes of the mushroom body (MB), the neural center for olfactory memory. Blocking synaptic transmission between DPM cells and MB also decreases 1 hr memory without significantly affecting LRN\(^8\). Significantly, many genes essential for memory are highly expressed in MB. These are including Ca\(^{2+}\)/CaM-dependent adenyl cyclase (AC) encoded by rut\(^9\), cAMP-specific phosphodiesterase encoded by dnc\(^{10}\), and the catalytic subunit of cAMP-dependent protein kinase (PKA) encoded by DC\(0^{11}\). In particular, a temperature sensitive allele of DC\(0\) (DC\(0^{\text{ts}}\)) is defective for MTM, similar to amn mutants. A possible role for amn function is that Drosophila PACAP is released from DPM cells to regulate the activity of the rut-AC in the mushroom bodies.

Figure 2 Characteristic retention curves of memory mutants
Memory retention curves can be generated by measuring memory at various time intervals after training. Shown are memory retention curves of a LRN mutant, linotte (A), STM mutants, rutabaga and dune (B), an MTM mutant, amnesiac (C), and an ARM mutant, radish (D). (Adapted from Dubnau and Tully, 2001 [42])

Figure 3 Memory retention in aged flies are similar to that of the MTM mutant, amnesiac
A. Significant AMI is observed in 20-day and older flies as a severe impairment in memory one hour after training. Memory retention characteristics of aged flies are highly reminiscent of that of the middle-term memory mutant amnesiac. While there are minor differences in 0 hr and 7 hr memory, there is a prominent reduction in memory between these timepoints (especially in 1 hr memory). B. Age-related changes in 1 hr memory in flu, vol, latP, rut, amn\(^{28A}\), amn\(^{X8}\) and amn\(^{1}\) mutants. In contrast to other memory mutants, 1 hr memory is not reduced upon aging in amn mutants, even at 50 days of age. (Adapted from Tamura et al.[12])

Possible roles of the cAMP/PKA pathway in AMI
Since AMI consists of a decrease in amn-dependent memory, it is possible that aging results in downregulation of a putative amn signaling pathway. However, amn expression does not change upon aging and overexpression of an amn transgene does not suppress AMI\(^{12}\). Thus it is likely that either a step downstream from amn is disrupted or that some post-transcriptional regulation of amn is altered upon aging.

Given that DPM cells innervate the MBs,
AMI may arise in the MBs and thus screening of AMI mutants was performed with mutations in genes expressed predominantly in MBs. Through this screening, heterozygous mutations in *DC0* (also called Pka-C1), the gene encoding the catalytic subunit of PKA, was found as strong AMI suppressors, delay AMI more than twofold (Fig. 5A and B)\(^{13}\). Significantly, activity of PKA in these *DC0/* flies was reduced to approximately 60% that in wild-type controls and AMI is restored when a *DC0* transgene is expressed in MBs. Therefore, these studies suggest that activity of cAMP/PKA pathway impairs memory at old age.

In mammalian systems, it has been widely reported that increases in the cAMP/PKA pathway activity can ameliorate AMI. Analogs of cAMP or agonists of dopamine D1/D5 receptors, which are positively coupled with adenylate cyclase increase both the protein synthesis-dependent phase of hippocampal LTP (L-LTP) and hippocampus-dependent LTM in aged mice\(^{10}\). Also, concentrations of rolipram, a cAMP phosphodiesterase inhibitor, that increase stimulated levels of cAMP without affecting basal levels, improves hippocampus-dependent LTM in aged mice\(^{10}\). These results suggest that aging may lead to a reduction of cAMP/PKA activity in the brain resulting in AMI. However, there is a caveat to this model. It is likely that the improvement of memory and LTP by stimulation of PKA is not specific to aged animals. In many cases, memory is also improved in young animals, raising the possibility that decreasing PKA activity is not the cause of AMI\(^{16}\). Rather, increasing PKA activity improves memory in general.

While decreases in cAMP/PKA activity may be responsible for AMI in the hippocampus, increases in cAMP/PKA activity seems to be responsible for AMI in the prefrontal cortex (PFC). In aged rats, inhibition of the cAMP/PKA pathway by addition of the PKA

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**Figure 4** Morphology of DPM cells

A-B. Overall morphology of DPM cells in young (A) and aged (B) wild-type flies. Volume rendering images from stacks of confocal images of mCD8-GFP reporter expression in DPM cells (driven by c316-GAL4). Arrowheads indicate DPM cell soma. Axonal terminals of DPM cells clearly depict the lobes of MBs. Scale bar equals 50 μm. (Adapted from Tamura et al.\(^{12}\))

**Figure 5** Isolation of *DC0* hypomorphic mutation as a suppressor of AMI

A. Age-related changes in 1 hr memory in 14 of 54 MB mutants assayed. Line 1, wild-type; lines 2-10, enhancer GAL4 lines; line 11, fasII\(^{22}\); line 12, *DC0*\(^{13}\)/+; line 13, vol\(^{2}\); line 14, dnc\(^{2}\); line 15, raf\(^{1}\} \text{N = 4-6 for all groups). All MB mutants except } DC0^{13}/+ \text{ are homozygotes. B. The } DC0^{13}/+ \text{ heterozygous mutation delays the onset of AMI.}
antagonist, Rp-cAMP, in the PFC ameliorates AMI of working memory\textsuperscript{17}. Significantly, the improvement of working memory is greater in aged rats with more severe cognitive deficits, while memory enhancements are not observed in young adult rats. These observations support the idea that AMI is induced by activity of cAMP/PKA signaling. Although basal levels of adenyl cyclase isoforms, AC2 and AC3, and phosphodiesterase isoforms, PDE4a, PDE4b and PDE4d do not show age-related changes, CRE binding activity, which is likely to be a downstream of cAMP/PKA signaling\textsuperscript{18}, significantly increases in the PFC and higher number of cells show immunostaining with anti-phospho-CREB antibody in aged rats\textsuperscript{17}. This suggests that aging results in increase PKA activity in the PFC. Inconsistent with these studies, however, PKA activity does not changed upon aging in \textit{Drosophila} brain\textsuperscript{18}.

\section*{cAMP/PKA signaling for calcium dysregulation model for AMI}

PKA activity has also been linked to L-type voltage gated Ca\textsuperscript{2+} channel (LVGCC) conductance. PKA dependent phosphorylation of the Ca\textsubscript{1.2} LVGCC strongly enhances its activity and there is a greater than 2-fold increase in phosphorylation in the hippocampus of aged rats\textsuperscript{19}. In addition, protein levels of LVGCCs (Ca\textsubscript{1.3}) are increased in the hippocampus of aged rats\textsuperscript{20}. These results are significant because a predominant model for AMI is the Ca\textsuperscript{2+} dysregulation model in which aging results in increased Ca\textsuperscript{2+} influx through LVGCCs, resulting in alternations in Ca\textsuperscript{2+}-dependent synaptic plasticity and AMI\textsuperscript{15}. In the hippocampus of aged rats, the threshold for LTD induction is increased, while the threshold for LTP induction is reduced\textsuperscript{15}. Increased Ca\textsuperscript{2+} influx through LVGCCs enhances Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel activity which in turn increases afterhyperpolarization (AHP) amplitude and duration\textsuperscript{21}. An increase in AHP, the hyperpolarization following action potentials, should decrease the probability of LTP induction, requiring higher frequency stimuli. Evidence that an increased Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel activity suppresses LTP induction comes from a study demonstrating that the Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel blocker apamin reduces the threshold for LTP in aged rats\textsuperscript{21}. Moreover, there is an elevated expression of small-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (SK channels) in the hippocampus of aged mice, and the injection of an antisense oligomer for SK channels both ameliorates AMI of a hippocampus-dependent memory task and increases the probability of LTP induction\textsuperscript{22}. Significantly, the LVGCC blocker, nimodipine, ameliorates AMI in rabbits\textsuperscript{22,23}. Also, the LVGCC blocker nifedipine facilitates LTP and reverses the increased probability of LTD induction in aged hippocampal slices\textsuperscript{23}. 
The linkage between AMI and aging

The molecular mechanisms affecting AMI and organismal aging (longevity) are thought to be tightly lined. For example, a well known model for aging is the free-radical or oxidative-damage model. In this hypothesis, normal metabolism produces reactive oxygen species which cause molecular damage to DNA, proteins and lipids. Oxidative damage accumulates over time, eventually causing aging and death. Supporting this idea, mutations that extend lifespan tend to have increased tolerance to dietary paraquat, a toxic free radical generator. Furthermore, overexpression of antioxidant genes such as SOD1 and catalase as well as feeding of SOD/catalase mimetics have been shown to increase lifespan in Drosophila and C. elegans. It has been reported that a diet rich in antioxidants delays AMI in canines. In rats, feeding mitochondrial metabolites decreases oxidative damage of nucleic acids in the brain and improves performance in memory tasks in aged animals. Also, infusion of catalytic scavengers of reactive oxygen species into the brains of rats both ameliorates oxidative damage and reverses cognitive defects associated with age. Thus it seems that oxidative damage may be a cause of both aging and AMI. Consistent with this model, there is a large overlap in genes with altered expression upon aging and upon exposure to oxidative stress. Recently, it has been shown that upon human aging, expression of a set of genes important in synaptic function and plasticity, vesicular transport and mitochondrial function are reduced while expression of genes involved in stress response and repair are upregulated. Downregulated genes include, glutamate receptors, adenylyl cyclase associated proteins, CaM kinases, PKC, and MAP kinase. Strikingly, the promoters of these down regulated genes are preferentially damaged in the brains of aged humans and by oxidative stress in cultured human neurons.

Another theoretical way to reduce oxidative damage in organisms is to reduce the rate of metabolism. This should increase lifespan and possibly delay AMI. Raising Drosophila at 18°C, to lower metabolic rate, extends lifespan and delays the onset of AMI (Tamura and Saitoe unpublished observations). In addition, virgin or female sterile flies have extended lifespan, again likely due to a reduction in the metabolic activity required for reproduction. Calorie or dietary restriction (CR), which may also reduce metabolic activity, has been well characterized as a life extending mechanism and works in organisms as diverse as yeast, Drosophila, C. elegans, and mammals. In certain conditions, CR also improves performance of various memory tasks in aged mice, rats and Drosophila (Ito and Saitoe unpublished observations). Although CR extends lifespan and ameliorates AMI in some cases, there are some reports that CR can extend lifespan without affecting or facilitating cognitive aging. Aged rats maintained at only 35% of normal weight show extended lifespan. However, their cognitive function is worse than ad lib control aged rats. These data suggest that optimal levels of CR for lifespan extension and for amelioration of AMI may not be the same. Notably, hypomorphic mutation in DC0 delay AMI without affecting lifespan. Although linkage between oxidative stress and cAMP/ PKA signaling for AMI is not yet clear, these results suggest that AMI is regulated by signaling pathway distinct from organismal aging.

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