Piracetam Improves Olfactory Learning and Memory in *Drosophila melanogaster*

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Abstract

Piracetam is a nootropic compound reported to enhance α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function and increase neuronal membrane fluidity, but its effects on associative learning are not well characterized. This study tested whether piracetam improves olfactory associative learning and memory in wild-type *Drosophila melanogaster* using an adapted version of Tully and Quinn's (1985) classical conditioning paradigm in which vortexing served as the aversive stimulus. Flies were tested for baseline odor preference, conditioned, or conditioned after 48 hours of exposure to 0.5 mM piracetam, with four trials per group. Flies initially preferred 1-octanol (OC) over 4-methylcyclohexanol (MCH). Conditioning shifted preference towards MCH, and piracetam-fed flies showed a higher learning index than conditioned flies that were not given piracetam. These results suggest that piracetam may enhance performance in olfactory associative learning. Because learning and memory decline in conditions such as neurodegeneration, these findings support further study of piracetam as a cognitive enhancer, including its bioavailability, dosing, and underlying mechanisms in neural plasticity.

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Introduction

Mammalian memory formation and olfactory associative learning in *Drosophila melanogaster* both inform on neural mechanisms behind learning, memory, and behavior. In mammals, declarative memories form in the trisynaptic hippocampal circuit, which contains projections that are key supporters of memory encoding, with three synaptic sites beginning with sensory input processing in the entorhinal cortex (EC) (Basu and Siegelbaum 2015). First, the EC excites granule cells of the dentate gyrus (DG) through the perforant pathway; next, mossy fiber projections of the DG excite cornu ammonis 3 (CA3) pyramidal neurons (PyNs), which in turn excite cornu ammonis 1 (CA1) PyNs through the Schaffer collateral (SC) pathway (Basu and Siegelbaum 2015). CA1 PyNs also receive glutamatergic excitatory input from PyNs in the EC, as well as gamma-aminobutyric acid (GABA)ergic signaling, feedforward inhibition, and feedback excitation (Basu and Siegelbaum 2015).

Learning is informed by long-term potentiation (LTP). LTP relies on depolarization, where the inside of a neuron becomes less negative and is able to fire action potentials, the foundation of neuronal signaling (Kennedy 2016). Synaptic strengthening occurs after strong, repeated stimulation, and presents as transformations of neurons, formation of new synaptic connections, increased neurotransmission and neuronal firing rate, or a longer lasting signal (Purves et al. 2001). Due to feedforward excitation, signaling can spread farther and faster, allowing for more widespread synaptic activation and LTP induction (Basu and Siegelbaum 2015). More active glutamate receptors, including α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptors, are then available due to the coincidence detection of presynaptic activity and strong postsynaptic depolarization (Basu and Siegelbaum 2015). This results in a rise in intracellular calcium (Ca^{2+}) levels and increased activation of Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII), another key player in LTP induction (Basu and Siegelbaum 2015). LTP is an excellent proxy for studying learning and memory because it discusses the electrical properties that these processes rely on.

Olfactory learning studies in *Drosophila* have provided key advancements in learning and memory. *Drosophila* are skilled in learning about odors in laboratory tasks and share similar olfactory system and neuronal mechanisms of learning with vertebrates (Busto, Cervantes-Sandoval, and Davis 2010). Studies indicate that the mushroom body (MB) neurons are the critical site for olfactory learning in *Drosophila* (Busto, Cervantes-Sandoval, and Davis 2010). Odor molecules bind to olfactory receptor neurons (ORNs), which project to the antennal lobe (AL) and form synapses with interneurons and projection neurons (PNs): PNs synapse onto Kenyon cells (KCs) which finally converge onto MB outputs as shown in *Figure 1* (Busto et al. 2010). KCs represent odor information, with learning largely attributed to KC firing, dopamine (DA) driven plasticity, and downstream signaling including the insertion and removal of receptors from cell membranes (Busto, Cervantes-Sandoval, and Davis 2010).

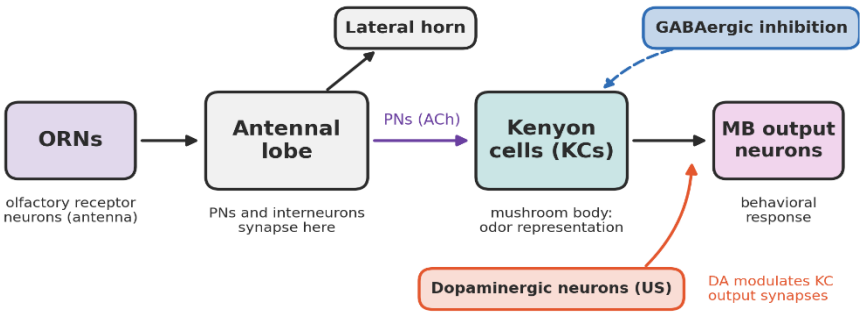


Figure 1. Schematic of the *Drosophila* olfactory circuit (derived from Busto et al. 2010).

Olfactory classical (Pavlovian) conditioning refers to the formation of an association between an odor and a reinforcer: flies are required to associate an odor used as a conditioned stimulus (CS) with a negative (aversive) or positive (appetitive) stimulus used as an unconditioned stimulus (US) (Busto, Cervantes-Sandoval, and Davis 2010). Memory of this association is then tested in a T-maze where flies are required to make a choice between two odors (Tully and Quinn 1985). KC signaling is mediated by nicotinic acetylcholine receptors (nAChRs) and GABAergic signaling, allowing for flexibility (Su and O'Dowd 2003).

Olfactory associative conditioning relies heavily on key neurotransmitters (NTs) including DA, GABA, and acetylcholine (ACh). DA is critically involved in learning, memory, and behavioral processes, and dopaminergic neurons are thought to communicate with MB neurons, transmitting information about aversive USs (Busto, Cervantes-Sandoval, and Davis 2010). In aversive conditioning, PNs release ACh at MB synapses, causing calcium (Ca^{2+}) channels to open: this increased intracellular Ca^{2+} concentration allows Ca^{2+} to bind calmodulin (CaM). Ca^{2+}-bound CaM activates adenylyl cyclase (AC), which acts as a coincidence detector for DA and ACh and increases levels of cyclic adenosine monophosphate (cAMP) in response, a molecule that integrates the signals from the odor and the stimulus (Busto, Cervantes-Sandoval, and Davis 2010). This mechanism is illustrated in *Figure 2.* In appetitive conditioning, the same pathway is induced in MB neurons (Busto, Cervantes-Sandoval, and Davis 2010), but more research is required to uncover the role of DA in appetitive conditioning.

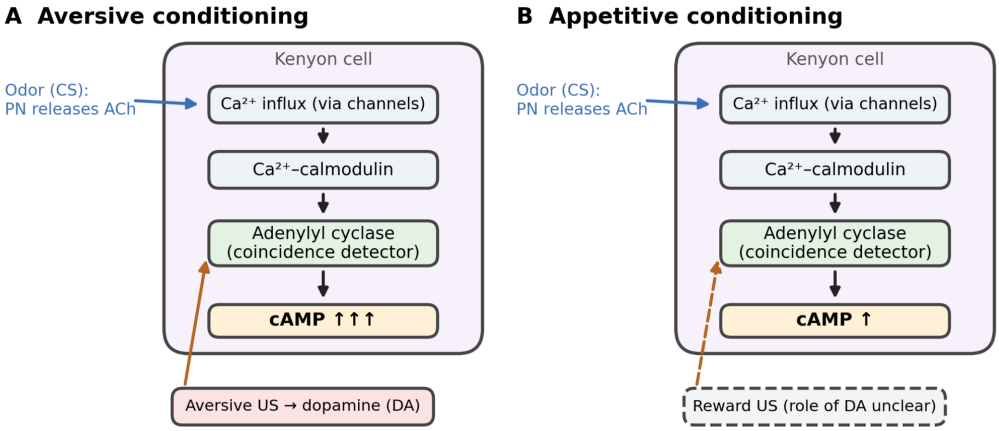


Figure 2. Schematic of molecular learning processes in a Kenyon cell during aversive (A) versus appetitive (B) conditioning (derived from Busto, Cervantes-Sandoval and Davis 2010). Odor-driven acetylcholine (ACh) release by projection neurons (PNs) opens calcium channels (Ca^{2+}) and Ca^{2+}-bound calmodulin (CaM) activates adenylyl cyclase, which detects the coincidence of ACh and DA to raise cyclic adenosine monophosphate (cAMP). The role of DA in appetitive conditioning is not yet known.

Another important transmitter is GABA, the main inhibitory NT: olfactory learning circuits are densely innervated with GABAergic neurons, including MB neurons which produce GABA and an ionotropic GABA-A receptor known as *Rdl* (Busto, Cervantes-Sandoval, and Davis 2010). Inhibition from GABAergic systems modulates the signal strength of information about the CS being transmitted to MB neurons, in addition to controlling the integration of the reward and pain circuits with the odorant processing pathways (Busto, Cervantes-Sandoval, and Davis 2010). This olfactory circuitry relies on both ionotropic (electrical) signaling and metabotropic (chemical) signaling, aiding in learning and memory.

Piracetam is scientifically relevant to studies on learning and memory. Molecularly, piracetam is a nootropic drug, as it functions without causing stimulation or sedation, and it is a cyclic derivative of GABA with different mechanisms (National Center for Biotechnology Information 2024). It has reported neuroprotective and anticonvulsant properties, as well as improving cardiovascular health and increasing neuronal membrane fluidity (Winblad 2005). A placebo-controlled double-blind study tested piracetam as a treatment for cortical myoclonus, a disorder commonly caused by abnormal discharges from the sensorimotor cortex (Brown 1993). Brown's (1993) study demonstrated that the total myoclonus score of patients improved by as much as 98% with piracetam as a treatment, indicating that piracetam has a wide variety of clinical benefits on the brain. Piracetam is more effective in the aged brain, and it has various effects on glutamate neurotransmission, such as enhancing the efficacy of AMPA-induced Ca^{2+} influx (Ahmed and Oswald 2010). Piracetam also increases the maximal density of specific binding sites for AMPA in neuronal membranes and can block or slow the deactivation of these receptors (Ahmed and Oswald 2010), suggesting it may act as a cognitive enhancer on a molecular and electrophysiological level.

While the trisynaptic circuit and the olfactory learning circuit are not analogous, they both undergo synaptic plasticity and LTP, which are the physical changes that underly learning and memory processes.

KCs utilize downstream AMPA receptor signaling mechanisms as well as insertion and removal of proteins and receptors in the membrane (Busto, Cervantes-Sandoval, and Davis 2010), which can reportedly be enhanced by properties of piracetam (Ahmed and Oswald 2010; Winblad 2005) Based on the pharmacological properties of piracetam, this drug may improve learning and memory in flies, which could be indicative of piracetam's beneficial effects as a treatment for human memory and learning deterioration. This study tests the hypothesis that piracetam will increase the effectiveness of olfactory associative learning in *Drosophila melanogaster*, possibly through its effects on brain health and promotion of LTP.

Methods

Materials

Flies. Approximately 300 Wild-type *Drosophila melanogaster* were used. They were on a standard fly media diet as in Tully and Quinn's experiment (1985). Fly age, sex, and rearing conditions beyond the standard media diet were not recorded.

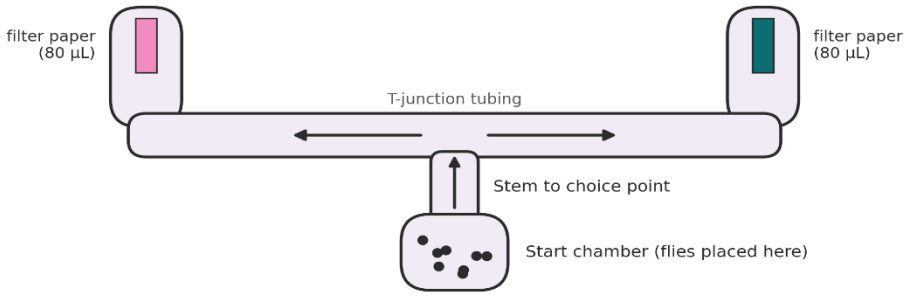


Figure 3. The schematic of the T-maze used in this experiment is a simplified version of the conditioning apparatus used in Tully and Quinn's (1985) experiment.

T-Maze. The apparatus for classical conditioning is derived from Tully and Quinn's (1985) procedure, shown in *Figure 3*. It is composed of 3 test tubes which slip onto the ends of the tubing that the flies pass through to make a choice: two house different odorants on filter paper. Flies enter the smaller chamber and make a choice between odorants.

Learning Index. The Learning Index (LI) is derived from Tully and Quinn's (1985) experiment, where LI = (Number of flies avoiding CS - Number of flies avoiding other odor) / Number of total flies that made a choice. In Group 1 with no conditioning performed, this is referred to as the Preference Index (PI). Because 1-octanol (OC) served as the CS, flies avoiding the CS were those counted in the 4-methylcyclohexanol (MCH) tube; thus, positive index values indicate a preference for MCH while negative values indicate a preference for OC. If all flies chose one odor, the absolute value of the index would be one, and if flies were evenly split between odors or none made a choice, the index would be zero.

Odorants. The odorants in this experiment were 1-octanol (OC) and 4-methyl cyclohexanol (MCH), both of which are aversive to flies (Tully and Quinn 1985). Filter paper was saturated with 80uL

of odorant, 1 in each test tube of the T-maze respectively, and then inserted near the top of the test tube. New odorant filter papers were used with each batch of new flies for consistency and potency.

Procedure

Group 1. The first group consisted of 4 trials in which flies were removed from their tubes with standard media and transferred to the T-maze to test initial preference between odorants. When placed in the T-maze, flies were given 5 minutes to make a choice between odorants. Halfway through the time limit (2.5 minutes), flies were encouraged to make a choice through aversive experimenter action, such as gently tapping the chamber that the flies were placed into or cupping the chamber to produce body heat.

Group 2. The second group consisted of 4 trials in which flies were removed from their tubes with standard media. The flies were transferred to the test tube with OC and given 30 seconds to rest in the tube. Then, they were placed on the vortex machine for 3 seconds and vortexed at speed 8, followed by 30 seconds of rest: this process was then repeated for a total of 5 rounds. Vortexing served as a mechanical aversive stimulus in place of the electric shock used by Tully and Quinn (1985). The flies were then transferred to the test tube containing MCH and rested in the tube for 90 seconds before being transferred to the T-maze. The flies were given 10 minutes to make a choice between odorants. Halfway through the time limit (5 minutes), the same encouragement procedures used in Group 1 were applied.

Group 3. Approximately 48 hours before the drug trials, 79.0uL of liquid piracetam drug media was added to the standard fly diet for a total concentration of 0.5mM ingested before the trials. The third group then followed the same experimental procedure as Group 2.

Results

Statistical analysis was performed using 2-tailed *t*-tests with statistical significance level of 0.05 and $n = 4$ trials per group. Within each group, preference for MCH versus OC was tested with paired t-tests on the trial counts, and indices were compared between groups with Welch's two-sample t-tests. Flies initially preferred OC to MCH, resulting in a negative PI in Group 1 (mean = -0.48), and then were conditioned to prefer MCH, resulting in a positive LI for Groups 2 (mean = 0.43) and 3 (mean = 0.72).

Group 1 flies had a significant initial preference of OC over MCH ($p = .017$). Group 2 significantly preferred MCH to OC after conditioning ($p = .002$), as did Group 3 ($p = .003$). Classical conditioning in Group 2 had a significantly improved average LI compared to Group 1 ($p = .001$). Group 3 had significantly improved average LI compared to Group 2 ($p = .029$) with the addition of piracetam (*Figure 4*). See *Table 1* for raw data.

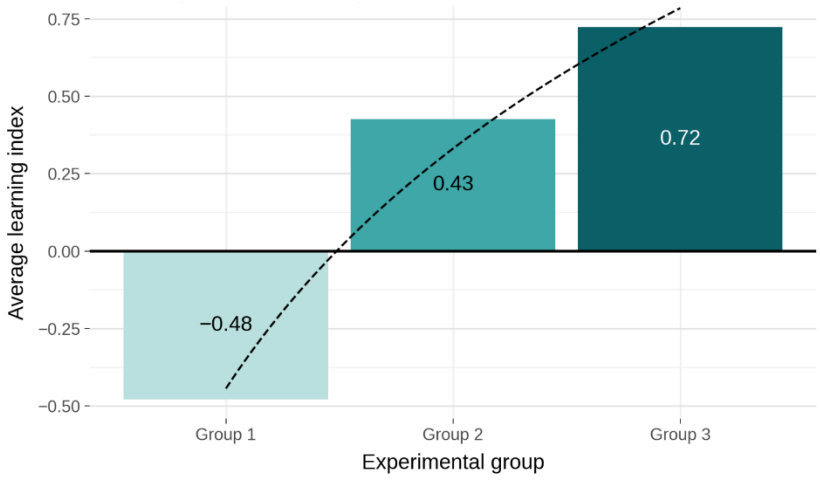


Figure 4. Average index change. This graph demonstrates that the preference index (PI) for Group 1 was negative, and the learning index (LI) for Groups 2 and 3 was positive. Includes logarithmic trendline demonstrating that there is a greater change between Groups 1 and 2 than between Groups 2 and 3.

Table 1

Raw Trial Data, Group Means, and Variability for Experimental Groups

	Group 1			Group 2			Group 3		
Trial	**MCH**	**OC**	**PI**	**MCH**	**OC**	**LI**	**MCH**	**OC**	**LI**
1	3	12	-0.6000	12	5	0.4118	16	1	0.8824
2	4	13	-0.5294	11	4	0.4667	15	4	0.5789
3	4	14	-0.5556	9	4	0.3846	12	3	0.6000
4	5	8	-0.2308	13	5	0.4444	11	1	0.8333
Total	16	47		45	18		54	9	
Mean			-0.4789			0.4269			0.7237
SD			0.1680			0.0361			0.1565
n			4			4			4
SEM			0.0840			0.0180			0.0782

Note. Number of flies choosing each odorant in each of four trials per group, with the resulting index for each trial and group summary statistics. Group 1 was tested without conditioning; Group 2 was conditioned without piracetam; Group 3 was conditioned after 48 hours of exposure to 0.5 mM piracetam. MCH = 4-methylcyclohexanol; OC = 1-octanol; PI = preference index; LI = learning index. Both indices are calculated as (MCH – OC) ÷ (MCH + OC), so negative values indicate a preference for OC and positive values indicate an avoidance of OC, the conditioned stimulus. *SD* = standard deviation; *SEM* = standard error of the mean (*SD* ÷ √*n*); *n* = number of trials; Mean refers to mean index value across trials within each group. Index values are rounded to four decimal places.

Discussion

Due to the significant effect of piracetam on olfactory associative learning in *Drosophila*, this study supports the hypothesis that piracetam improves learning and memory. In Group 1 with no experimental conditioning, *Drosophila* were tested on their initial preference (forming the PI) between two aversive odorants, OC and MCH; however, MCH was more aversive to the flies, resulting in a negative PI. Next, Group 2 underwent Pavlovian conditioning, resulting in a switch to preferring MCH to OC. Finally, Group 3, under the pharmacological influence of piracetam, followed the same conditioning procedures to result in a stronger preference for MCH over OC than in Group 2. Considering that the only alteration in procedure between conditioning groups was the ingestion of piracetam prior to the trials, these results suggest that piracetam enhanced performance in this olfactory conditioning assay, consistent with improved learning and memory.

This beneficial effect of piracetam on learning and memory can possibly be attributed to the molecular properties of piracetam itself, as well as the molecular and electrophysiological properties of LTP and neurons that allow for learning and memory to flourish. Piracetam's role in this improvement may involve the increased cell membrane fluidity in the brain (Winblad 2005), which could allow for increased learning in this case through the olfactory associative learning circuit. Kenyon cells are the key neurons of learning and plasticity in this circuit and therefore can impact the number of receptors available to receive odor-invoked input in the cell membrane (Busto, Cervantes-Sandoval, and Davis 2010): if the membrane is more fluid, it is possible that the synaptic plasticity facilitated by learning will result in an increase or decrease in the number of receptors in the cell membrane. KCs utilize AMPA receptors for downstream signaling mechanisms but mainly are mediated by nAChRs and GABA receptors (Su and O'Dowd 2003): if the membrane is more fluid, more of these receptors can be inserted into the membrane, increasing the control and flexibility of KC signaling.

Piracetam increases the signaling of ionotropic AMPA receptors by enhancing AMPA-induced Ca^{2+} influx and increasing the density of AMPA binding sites in neuronal membranes (Ahmed and Oswald 2010). Ca^{2+} is a significant signaling molecule for neurons, acting as the trigger for NT release after action potentials (Basu and Siegelbaum 2015), and the activation of downstream signaling molecules like CaMKII that play key roles in LTP induction (Basu and Siegelbaum 2015). More NT release as a result of this increased ion flux may lead to increased receptors in this circuit or heightened release of key NTs in olfactory learning like DA, GABA, and ACh (Busto, Cervantes-Sandoval, and Davis 2010), which leads to stronger signaling in cascade pathways and LTP. As piracetam improves neural plasticity (Winblad 2005), LTP also may be enhanced by piracetam through the inherent electrophysiological properties of the induction process as detailed by Basu and Siegelbaum (2015). Through the associative learning circuit, piracetam could act as the trigger for downstream signaling pathways and increased efficacy of the processes that lead to learning and memory in both *Drosophila* and mammals.

While the results of this study are significant, the limitations are also notable. Although flies are relatively easy to obtain with minimal clearances as an animal model, this study was performed over the course of three weeks, resulting in a limited number of trials and experimental techniques available. There also was a high possibility for chance error, in which flies might just not choose an odorant at all and stay

in their original chamber, and experimenter error, given that counting flies manually may be challenging. Due to the limited time frame, bioavailability- the amount of the substance that is free to act on its target- also could be a limitation in the piracetam experimental group. The amount of piracetam able to act on the brain depends on route of delivery (in this experiment, ingestion), dosage, and metabolizing speed and method. Several design limitations also apply. No drug-only control (piracetam without conditioning) was included, so effects of piracetam on odor sensitivity, locomotion, or response to vortexing cannot be separated from effects on learning. Group 1 was given a shorter choice window (5 minutes) than Groups 2 and 3 (10 minutes), which limits direct comparison with baseline. Fly age, sex, and rearing conditions were not recorded, making reproducibility of results a challenge, and only one piracetam dose and four trials per group were tested.

The results of this study contain some critical implications for the field of learning and memory. Firstly, these results emphasize the significance of piracetam's effect on cognition and its possible clinical benefits. Piracetam is not widely understood or accepted: it is prescribed in some European countries to improve brain function but is not yet approved in the United States (National Center for Biotechnology Information 2024). Its site of action and mechanism for improving cognition is not yet known (Tacconi and Wurtman 1986), and there is limited evidence to support its clinical benefit as a cognitive enhancer beyond conditions such as cortical myoclonus in Brown's (1993) study, clearly supporting a need for more research on piracetam's clinical relevance.

This study also raises the possibility that membrane fluidity contributes to learning and memory in both *Drosophila* and mammals, as humans and flies share broadly similar olfactory pathways. Because insect and mammalian olfactory systems share similar organizational features, these results have potential implications on treatments for cognitive impairment, possibly benefitting research in fields including but not limited to neurodegenerative disorders or traumatic brain injuries. These results point to next steps including experiments that uncover the bioavailability of piracetam or studying the effects of piracetam on the learning and memory of mutant flies with disorders that impair cognition. To test if this mechanistic hypothesis of piracetam's improvement of learning and memory is correct, future studies should utilize neurophysiological procedures to examine LTP's impact on the olfactory associative learning circuit on a molecular level.

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