

Astrocyte-to-neuron H_2O_2 signalling supports long-term memory formation in *Drosophila* and is impaired in an Alzheimer's disease model

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Yasmine Rabah¹, Jean-Paul Berwick^{1,2}, Nisrine Sagar^{1,2}, Laure Pasquer¹, Pierre-Yves Plaçais¹ & Thomas Preat¹✉

Astrocytes help protect neurons from potential damage caused by reactive oxygen species (ROS). While ROS can also exert beneficial effects, it remains unknown how neuronal ROS signalling is activated during memory formation, and whether astrocytes play a role in this process. Here we discover an astrocyte-to-neuron H_2O_2 signalling cascade in *Drosophila* that is essential for long-term memory formation. Stimulation of astrocytes by acetylcholine induces an increase in intracellular calcium ions, which triggers the generation of extracellular superoxide ($\text{O}_2^{\bullet-}$) by astrocytic NADPH oxidase. Astrocyte-secreted superoxide dismutase 3 (Sod3) converts $\text{O}_2^{\bullet-}$ to hydrogen peroxide (H_2O_2), which is imported into neurons of the olfactory memory centre, the mushroom body, as revealed by in vivo H_2O_2 imaging. Notably, Sod3 activity requires copper ions, which are supplied by neuronal amyloid precursor protein. We also find that human amyloid- β peptide, implicated in Alzheimer's disease, inhibits the nAChR $\alpha 7$ astrocytic cholinergic receptor and impairs memory formation by preventing H_2O_2 synthesis. These findings may have important implications for understanding the aetiology of Alzheimer's disease.

Reactive oxygen species (ROS) such as superoxide ($\text{O}_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are distinctive among metabolites because of their physiological ambivalence¹. On the one hand, ROS are toxic byproducts that can irreversibly oxidize macromolecules, and powerful scavenging enzymes are active to protect cells from ROS damage. The production of ROS is closely linked to the activity of the mitochondria in the production of ATP, and neurons are particularly susceptible to ROS damage due to their high energy requirements and high content of unsaturated fatty acids that are prone to peroxidation². On the other hand, ROS play a positive role by activating signalling pathways involved in physiological functions such as cell survival, growth or response to stress^{1,2}. ROS signalling occurs mainly through reversible oxidation of redox-sensitive

cysteines, which requires a sharply regulated spatiotemporal increase in ROS concentration³. In vitro studies of long-term potentiation in mice have shown that neuronal ROS signalling plays a positive role in synaptic plasticity^{4,5}. Recent in vivo studies in *Drosophila* have shown that ROS signalling is required during development for activity-dependent plasticity in motoneurons^{6,7}. While pioneering studies have reported that mutant mice with altered ROS metabolism display learning or memory defects^{2,8}, it remains unknown how ROS signalling is recruited in vivo for memory formation. Astrocytes are known to regulate neuronal redox homeostasis and cognition in mice^{9,10}. In particular, it was shown that under physiological conditions mitochondrial ROS in astrocytes stimulate nuclear factor erythroid-2-related factor 2 (NRF2) transcription

¹Energy & Memory, Brain Plasticity Unit, CNRS, ESPCI Paris, PSL Research University, Paris, France. ²These authors contributed equally: Jean-Paul Berwick, Nisrine Sagar. ✉e-mail: thomas.preat@espci.fr

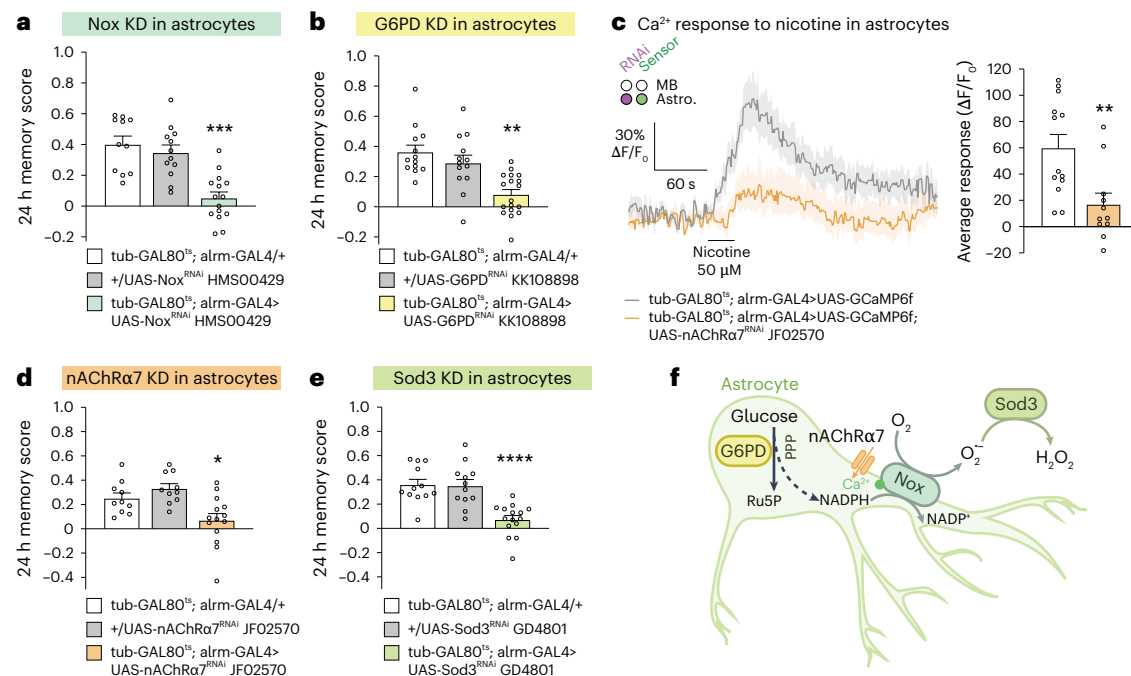


Fig. 1 | Astrocyte-derived H₂O₂ is required for LTM formation. **a**, Nox knockdown (KD) in adult astrocytes impaired memory after spaced conditioning ($n = 11$ (first group), $n = 12$ (second group), $n = 15$ (third group); $F_{2,35} = 17.43$, $P = 0.000006$). **b**, G6PD KD in adult astrocytes impaired memory after spaced conditioning ($n = 13$ (first and second groups), $n = 18$ (third group); $F_{2,41} = 12.98$, $P = 0.00004$). **c**, The fluorescent calcium sensor GCaMP6f was expressed in adult astrocytes. Nicotine stimulation (50 μ M) elicited calcium increase in astrocytes, which was significantly decreased in nAChR7 KD ($n = 13$ (first group), $n = 11$ (second group), $t_{22} = 3.15$, $P = 0.005$). **d**, nAChR7 KD in adult astrocytes impaired memory after spaced conditioning ($n = 10$ (first and second groups), $n = 14$ (third group); $F_{2,31} = 7.81$, $P = 0.002$). **e**, Sod3 KD in adult astrocytes

impaired memory after spaced conditioning ($n = 12$ (first and second groups), $n = 15$ (third group); $F_{2,36} = 16.02$, $P = 0.00001$). **f**, Scheme of the molecular actors involved in astrocytes for LTM. Nox, activated by calcium entry through nAChR7, produces superoxide O₂^{•-} from O₂ using the NADPH co-factor derived from the PPP. Extracellular O₂^{•-} is converted to H₂O₂ by extracellular Sod3 secreted from the astrocytes. Ru5P, ribulose-5-phosphate. Data are presented as mean $\pm$ s.e.m. Genotype sample sizes are listed in the legend in order of bar appearance. Significance level of a two-sided t -test or the least significant Tukey pairwise comparisons following one-way analysis of variance (ANOVA). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

factor expression, which promotes glutathione biosynthesis by astrocytes and antioxidant protection of neighbouring neurons¹⁰; however, it is not known whether neurons can trigger beneficial ROS production by astrocytes, and thereby if astrocytes are involved in the initiation of neuronal ROS signalling for synaptic plasticity.

Building a mechanistic view of ROS signalling during memory formation is essential not only from a brain physiology perspective, but it could also improve our understanding of the onset of Alzheimer's disease (AD), a neurodegenerative disease characterized in particular by memory loss and defects in redox homeostasis^{11,12}. At the neuropathological level, AD is characterized by the progressive formation in the brain of amyloid plaques that correspond to the extracellular accumulation of amyloid β (A β) peptide, generated by cleavage of the transmembrane amyloid precursor protein (APP), and by the formation of intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein¹³. Early synaptic dysfunction has been associated with AD^{12,14}, which correlates with cognitive decline¹⁵; however, the cascade of events leading to this fatal condition remains unclear.

Here, we have addressed these key brain physiology and pathology issues in *Drosophila* by combining brain H₂O₂ imaging using recently developed roGFP2-Tsa2ΔC_R ultrasensitive sensors¹⁶ with functional characterization of ROS-related pathways during memory formation. We report that long-term memory (LTM) formation requires the establishment of a local H₂O₂ gradient in neurons of the olfactory memory centre, which follows the activation of ROS-producing enzymes in astrocytes. This signalling cascade is impaired by AD-related A β 42. Our results provide a new framework to understand neuroglia interactions during memory formation in normal conditions and in AD.

Results

Astrocytes generate extracellular ROS for long-term memory
AD has been extensively linked to defects in redox homeostasis^{11,12}. The objective of this study was to exploit the strengths of *Drosophila* genetics and in vivo imaging capabilities to further elucidate the relationships between A β , ROS and cognition; however, rather than artificially increasing ROS production and assessing its impact on memory formation, our approach was to investigate the potential beneficial effects of ROS on memory formation, with the understanding that this physiological pathway might be affected by A β in an AD-related model. Given the important role of neuron–glia metabolic coupling in *Drosophila* memory formation^{17–19}, we sought to address these questions by investigating the potential role of astrocytes in neuronal ROS signalling.

We observed that inhibiting NADPH oxidase (Nox) expression in adult astrocytes impairs olfactory LTM in *Drosophila* (Fig. 1a). The only function of the transmembrane enzyme Nox is to produce extracellular O₂^{•-} from O₂ and intracellular NADPH³, and therefore our observation was potentially important from a ROS signalling perspective. For this experiment, we used a well-established paradigm of associative aversive olfactory conditioning^{20,21} named spaced conditioning (Methods), along with inducible and spatially controlled expression of a Nox-targeting RNAi. A single Nox enzyme exists in *Drosophila*, which shows strong sequence similarity with the human Nox5 (ref. 22). Similarly to Nox5 (ref. 3), *Drosophila* Nox is activable by Ca²⁺ thanks to intracellular EF-hands domains that bind Ca²⁺ (ref. 23).

LTM formation requiring Nox activity in astrocytes depends on de novo protein synthesis²¹ in neurons of the olfactory memory centre, the mushroom body (MB)^{24,25}. Conversely, anaesthesia-resistant

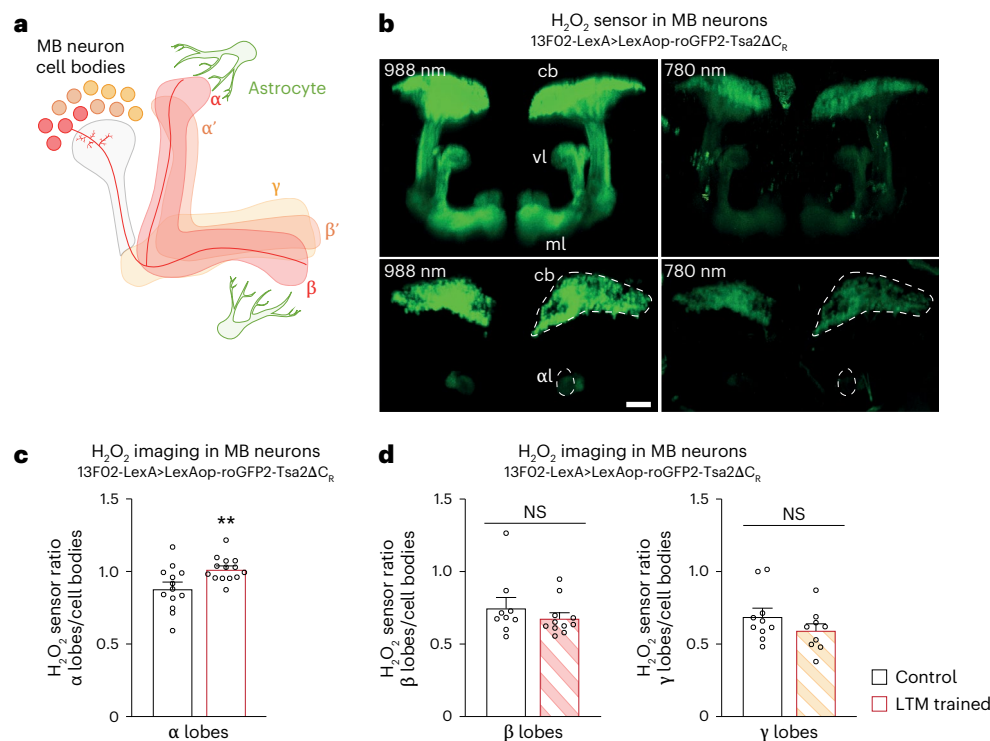


Fig. 2 | An H₂O₂ gradient forms in MB α lobes upon LTM formation.

a, Illustration of MB structure. Axons of MB neurons follow a branching pattern that forms vertical lobes (α and α') and medial lobes (β , β' and γ). **b**, Images of the roGFP2-Tsa2ΔC_R H₂O₂ sensor expressed in MB neurons. 3D reconstructions of image stacks (128- μ m-thick stack, 1 μ m Z-step) of the H₂O₂ sensor in MB neurons at the two excitation wavelengths (988 and 780 nm) (top). Cb, cell bodies; vl, vertical lobes (α and α'); ml, medial lobes (β , β' and γ). Horizontal plane with regions of interest, cell bodies and α vertical lobes delimited with dashed lines (bottom). Scale bar, 30 μ m. **c**, The H₂O₂ level in α lobes normalized

to the cell bodies value is increased upon spaced training, in comparison to the non-associative unpaired control ($n = 13$ (first group), $n = 14$ (second group); $t_{25} = 2.82$, $P = 0.009$). **d**, The H₂O₂ level in β or γ lobes normalized to the H₂O₂ level in cell bodies after spaced training was similar to the unpaired control (β , $n = 9$ (first group), $n = 11$ (second group), $t_{18} = 0.94$, $P = 0.36$; γ , $n = 10$ (first group), $n = 9$ (second group), $t_{17} = 1.30$, $P = 0.21$). Data are presented as mean $\pm$ s.e.m. Genotype sample sizes are listed in the legend in order of bar appearance. Significance level of a two-sided t -test. ** $P < 0.01$; NS, not significant, $P > 0.05$.

memory (ARM), which forms after massed conditioning (Methods) and does not depend on de novo protein synthesis²¹, was not affected by *Nox* knockdown in adult astrocytes (Extended Data Fig. 1a). In addition, *Nox* knockdown did not affect perception of the stimuli used for conditioning (Supplementary Table 1), and LTM was normal when *Nox* RNAi expression was not induced (Extended Data Fig. 1a). To further ensure the specificity of *Nox* implication in LTM, we measured the immediate memory capacity after spaced conditioning of flies expressing *Nox* RNAi in adult astrocytes. Our results demonstrated that *Nox* knockdown did not affect immediate memory, confirming that superoxide production by astrocytes is required specifically for LTM, as measured at 24 h after spaced conditioning (Extended Data Fig. 1b). Last, we confirmed the involvement of *Nox* in adult astrocytes for LTM with a second nonoverlapping *Nox* RNAi (Extended Data Fig. 1c and Supplementary Table 1). These controls were performed in a systematic manner throughout the course of this study. With regard to text simplification, knockdown flies exhibiting the aforementioned phenotypes (impaired LTM as measured at 24 h after conditioning, assessed with two RNAis; normal ARM as measured at 24 h after massed conditioning; normal immediate memory after spaced conditioning; normal stimuli perception; normal LTM in the absence of RNAi induction) will be simply reported as displaying a specific LTM defect.

The *Nox* substrate NADPH is mainly produced by the pentose phosphate pathway (PPP)²⁶. To study this pathway we inhibited in adult astrocytes the expression of *G6PD*, the enzyme catalysing the first step of the PPP²⁶, and we observed a specific LTM defect (Fig. 1b, Extended Data Fig. 1d,e and Supplementary Table 1). Given that the PPP generates many intermediates involved in various biosynthetic processes,

including fatty acids and nucleotides²⁶, an additional experiment was conducted to ensure that inhibiting the PPP in astrocytes did not affect different forms of olfactory memory in an unspecific manner. Thus, in addition to the assessment of ARM following massed conditioning and immediate memory following spaced conditioning, the middle-term memory capacity following a single conditioning cycle was evaluated after *G6PD* knockdown in adult astrocytes. *G6PD* knockdown flies displayed normal middle-term memory (Extended Data Fig. 1d). The essential role of astrocytic PPP in LTM formation was confirmed by inhibiting the expression in astrocytes of *Pgd* and *Pgls*²⁶, two other enzymes in this pathway (Extended Data Fig. 1e–g and Supplementary Table 1). Altogether, these results support the notion that astrocytic *Nox* generates O₂^{•−} required for LTM formation.

As stated above, similar to the human *Nox5*, *Drosophila* *Nox* is activated by Ca²⁺. Acetylcholine (ACh) is the major excitatory neurotransmitter of *Drosophila* brain neurons, and in particular MB neurons are cholinergic²⁷. Among the ionotropic cholinergic receptors, the channel formed of nAChR α 7 subunits is the most permeable to Ca²⁺ (ref. 28) and is expressed in human astrocytes²⁹. To examine whether nAChR α 7 cholinergic stimulation induces a Ca²⁺ flux in *Drosophila* astrocytes, we expressed the GCaMP6f Ca²⁺ fluorescent reporter in astrocytes and stimulated the brains of live flies with nicotine, an nAChR α 7 agonist. Nicotine stimulation induced an increased astrocytic Ca²⁺ concentration in control flies (Fig. 1c). This effect was dampened when nAChR α 7 expression was inhibited in astrocytes (Fig. 1c). We next addressed the role of astrocytic nAChR α 7 in memory and showed that nAChR α 7 knockdown in adult astrocytes specifically impaired LTM (Fig. 1d, Extended Data Figs. 1b and 2a,b and Supplementary Table 1). These

results suggest that during spaced conditioning ACh release can activate Ca^{2+} signalling in astrocytes through nAChR $\alpha 7$ and is required for LTM. Consistent with our findings, astrocytic nAChR $\alpha 7$ was implicated in the persistence of fear memory in mice³⁰.

While $\text{O}_2^{\cdot -}$ is a highly reactive and therefore toxic ROS with a short half-life, H_2O_2 , which is less reactive and has a longer half-life, is more suitable for ROS signalling^{1,31}. Astrocytic Nox activity at the plasma membrane produces extracellular $\text{O}_2^{\cdot -}$, whose conversion into H_2O_2 is catalysed by the only secreted superoxide dismutase (Sod), Sod3 (ref. 32). In the *Drosophila* brain, Sod3 is more strongly expressed in astrocytes than in neurons, as shown by single-cell transcriptomics data³³ (Extended Data Fig. 2c). Sod3 knockdown in adult astrocytes specifically impaired LTM (Fig. 1e and Extended Data Figs. 1b and 2d,e and Supplementary Table 1). Altogether, these results suggest that a nAChR $\alpha 7$ –Nox–Sod3 signalling cascade takes place upon LTM formation, which is triggered by ACh stimulation of astrocytes, resulting in extracellular H_2O_2 formation (Fig. 1f).

Like the tripartite synapse in the mammalian brain³⁴, astrocytic processes overlap with synapses in the *Drosophila* brain³⁵. We therefore wondered whether astrocytes might be a source of beneficial ROS imported by neurons during LTM formation. In particular, is H_2O_2 signalling involved during LTM formation in the MB?

An H_2O_2 gradient in MB α lobe supports LTM formation

The MB is a bilateral structure consisting of about 2,000 cholinergic neurons²⁷ in each brain hemisphere. Axons of the MB neurons form bundles and branch out, giving shape to anatomical structures called lobes that synapse with the MB output neurons. MB neurons are classified into three different subtypes: the α/β neurons, whose axons branch to form an α vertical projection and a medial β projection (Fig. 2a); the α'/β' neurons; and the γ neurons, which form a single medial γ lobe. The axonal projection of the α lobe is specifically involved in LTM formation^{20,36}. We aimed to image H_2O_2 in MB neurons upon LTM formation. Recently, the ability to observe physiological H_2O_2 levels has become possible with the development of an ultrasensitive H_2O_2 fluorescent sensor, roGFP2-Tsa2AC_R¹⁶, which we imported and characterized in *Drosophila* (Fig. 2b and Extended Data Fig. 3a).

To observe change in H_2O_2 dynamics, flies expressing the H_2O_2 sensor in the MB were imaged between 0.5 h and 2 h after spaced conditioning, a time window when early LTM-encoding events occur^{17,37}. Control flies were submitted to non-associative unpaired protocol that does not allow memory formation. Notably, in flies trained for LTM, we observed an increased H_2O_2 level in the MB α lobe (Fig. 2c), and no increase in the MB β and γ medial lobes (Fig. 2d). An additional point must be considered to interpret the H_2O_2 imaging data. As we previously demonstrated, *Drosophila* LTM formation involves activation of the PPP in MB neurons, fuelled by Glut1-mediated glucose import¹⁷. As PPP activity generates the reducing agent NADPH, its activation in the MB is expected to result in a global decrease in ROS concentration (compare with scheme in Extended Data Fig. 3b). Indeed, detailed examination of imaging data revealed a decreased H_2O_2 level in MB cell bodies in flies trained for LTM (Extended Data Fig. 3c). Notably, when glucose import was impaired in adult MB, the decreased H_2O_2 response in MB neuron cell bodies after spaced conditioning was abolished, but the H_2O_2 increase was observed in the α lobe (Extended Data Fig. 3d). Altogether, these data reveal the existence in LTM-forming flies of an H_2O_2 gradient within the MB neurons, generated by H_2O_2 influx within the α vertical lobe. These results are in agreement with the fact that the α lobe is specifically involved in LTM formation^{20,36}.

To test whether astrocyte activity modulates H_2O_2 signalling in the MB LTM centre, we imaged H_2O_2 in MB neurons while interfering with the H_2O_2 -producing cascade in astrocytes. The increased level of H_2O_2 in the MB α lobe was lost by inhibiting the expression of either Nox, nAChR $\alpha 7$, G6PD or Sod3 in adult astrocytes (Fig. 3a). These results indicate that the H_2O_2 gradient formed in MB neurons after

spaced conditioning originates from astrocytic Nox and Sod3 activity in response to ACh stimulation. To substantiate this hypothesis, we intended to deplete the extracellular H_2O_2 generated by astrocytic Sod3 during LTM formation by expressing a secreted form of the human catalase in astrocytes⁷. Catalase is responsible for catalysing the breakdown of H_2O_2 into H_2O and O_2 . Notably, the typical increase in H_2O_2 observed in the α lobe following spaced conditioning was absent in the presence of the secreted catalase (Fig. 3b). In accordance with the imaging data, the LTM of flies that expressed the secreted catalase was found to be specifically impaired (Fig. 3c, Extended Data Fig. 3e and Supplementary Data Table 2). These findings substantiate the hypothesis that the H_2O_2 gradient observed in the MB during LTM formation is due to extracellular SOD3 activity.

A redox relay cascade is required in MB for LTM formation

How does H_2O_2 produced extracellularly by astrocytic enzymes enter MB neurons upon LTM formation? Although H_2O_2 can diffuse across the plasma membrane, H_2O_2 import is strongly facilitated by channels of the aquaporin protein family³⁸. We tested the potential involvement of the main *Drosophila* aquaporin, AQP, in LTM (Fig. 4a). AQP knockdown in adult MB α/β neurons specifically impaired LTM (Fig. 4b, Extended Data Fig. 4a,b and Supplementary Table 3). The LTM defect following AQP knockdown was linked to a loss of the H_2O_2 increase in the α lobe after spaced conditioning (Fig. 4c). Three additional aquaporin orthologues have been identified in *Drosophila*: drip, bib and prip⁷. The inhibition of their expression in adult α/β MB neurons did not affect LTM (Extended Data Fig. 4c–h), indicating that AQP plays a specific role in H_2O_2 import following SOD3 activity.

Our results support the idea that H_2O_2 signalling in α/β neurons occurs for LTM formation. H_2O_2 signalling is primarily characterized by the reversible oxidation of target cysteines on signalling proteins, which subsequently modifies their activity. This is achieved either by direct action of H_2O_2 following an increase in local concentration, or through a cascade of redox-sensitive relay enzymes, starting with the Cys-based peroxidase peroxiredoxin (Prx), an H_2O_2 scavenger (Fig. 4a)^{39,40}. We identified Prx2 as the major Prx involved in LTM formation (Fig. 4d, Extended Data Fig. 5a–c and Supplementary Table 4). Oxidized Prx are reduced by thioredoxin (Trx)⁴⁰, and we showed that *Drosophila* Trx2 is specifically involved in LTM (Fig. 4e, Extended Data Fig. 5b,d,e and Supplementary Table 4). Last, Trx is reduced by a Trx reductase (Trxr) that oxidizes NADPH into NADP^+ in the process. We identified Trxr1 as a major actor of LTM formation in α/β neurons (Fig. 4f, Extended Data Figs. 5b and 6a,b and Supplementary Table 4). Prx H_2O_2 scavenging enzymes are involved in the general maintenance of redox homeostasis^{39,40}. Consequently, the inhibition of Prx2 expression may potentially impact α/β MB neurons in an unspecific manner. To eliminate this possibility, we conducted an additional experiment, measuring middle-term memory after a single conditioning cycle of Prx2, Trx2 or Trxr1 knockdowns. This control is of particular significance given that middle-term memory is encoded by α/β neurons, as is LTM⁴¹. Knocking down Prx2, Trx2 or Trxr1 in α/β neurons did not affect middle-term memory, demonstrating the specificity of the effect on LTM (Extended Data Figs. 5a,d and 6a).

Altogether, these results reveal a cascade of redox-sensitive enzymes required for LTM formation in α/β MB neurons. This cascade could be involved in the regulation of gene expression required for LTM formation^{1,21}.

Appl delivers extracellular copper for SOD3 activity

We have shown that H_2O_2 is generated extracellularly by Sod3, which requires extracellular Cu^{2+} for its catalytic activity³². Because of its potential toxicity, free Cu^{2+} is maintained at a very low concentration in cells and tissues by copper-binding proteins⁴². This raises the question of how sufficient Cu^{2+} levels can be accumulated at the MB synapses to sustain during LTM formation the activity of Sod3 secreted

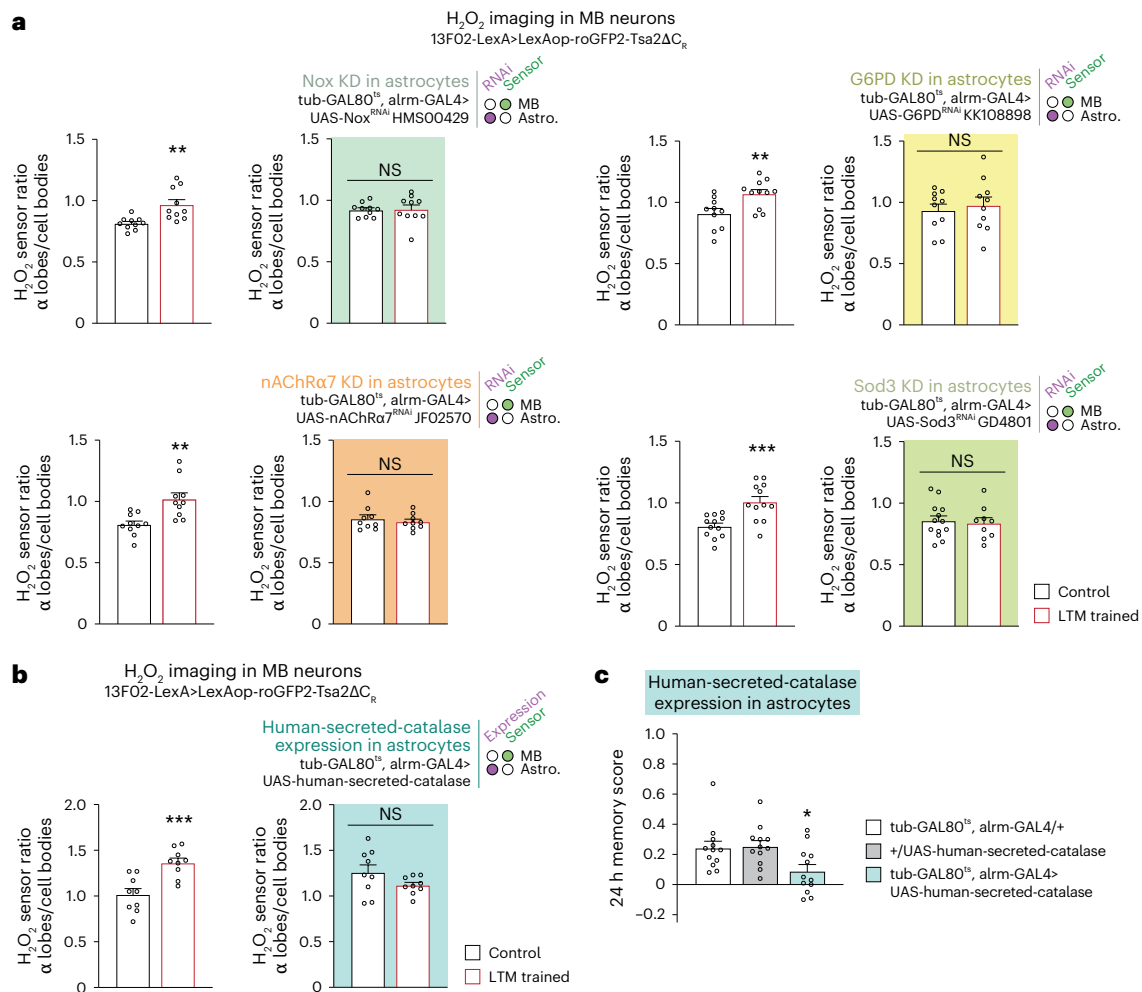


Fig. 3 | Astrocytes are a source of H_2O_2 imported into MB α lobes.

a, Measurements of H_2O_2 levels in MB neurons after spaced training in the context of astrocytic KDs. The increase in the α lobes/cell bodies H_2O_2 level elicited by spaced training was impaired in KD of *Nox* ($n = 10$, $t_{18} = 0.11$, $P = 0.91$; wild-type control: $n = 10$, $t_{18} = 3.47$, $P = 0.003$), *G6PD* ($n = 10$ (first and second groups), $t_{18} = 0.49$, $P = 0.63$; wild-type control: $n = 10$ (first group), $n = 11$ (second group), $t_{19} = 3.01$, $P = 0.007$), *nAChRα7* ($n = 9$, $t_{16} = 0.63$, $P = 0.54$; wild-type control: $n = 10$, $t_{18} = 3.57$, $P = 0.002$) and *Sod3* ($n = 13$ (first group), $n = 9$ (second group), $t_{20} = 0.33$, $P = 0.75$; wild-type control: $n = 12$, $t_{22} = 3.93$, $P = 0.0007$). Data

are presented as mean $\pm$ s.e.m. **b**, Expression of human-secreted-catalase in astrocytes abolished the increase in the α lobes/cell bodies H_2O_2 level elicited by spaced training ($n = 9$, $t_{16} = 1.596$, $P = 0.13$; wild-type control: $n = 9$, $t_{16} = 4.136$, $P = 0.0008$). **c**, Expression of human-secreted-catalase in astrocytes impaired 24 h memory after spaced conditioning ($n = 12$, $F_{(2,33)} = 4.603$, $P = 0.017$). Data are presented as mean $\pm$ s.e.m. Genotype sample sizes are listed in the legend in order of bar appearance. Significance level of two-sided *t*-test or the least significant Tukey pairwise comparison following one-way ANOVA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant, $P > 0.05$.

by astrocytes. We hypothesized that Appl, the single fly orthologue of APP transmembrane protein, might provide Cu^{2+} for astrocyte-derived Sod3 activity based on the following observations: (1) the APP extracellular domain has several Cu^{2+} binding sites^{43,44} whose function remains poorly understood⁴⁵, and *Drosophila* Appl carries in its conserved E2 ectodomain the four histidines that are characteristic of the M1 high-affinity Cu^{2+} binding site⁴⁶; (2) APP enables intracellular copper to be transported out of neurons, as APP overexpression decreases Cu^{2+} content in neurons in vitro, whereas APP loss of function leads to copper accumulation⁴⁷; (3) Appl knockdown in MB neurons induces an LTM defect⁴⁸; and (4) Appl exhibits neuronal expression and is enriched in α/β MB neurons⁴⁹, which are involved in LTM^{20,36}. We confirmed this strong α/β neuron expression with an HA-tagged Appl line generated by CRISPR (Fig. 5a and Extended Data Fig. 7a). Furthermore, we showed that knocking down Appl specifically in α/β neurons with RNAi expression induces an LTM defect (Fig. 5b, Extended Data Fig. 7b,c and Supplementary Table 5).

If Appl does provide copper to Sod3, the H_2O_2 gradient that forms after spaced conditioning should require Appl. Indeed, we observed

that the H_2O_2 gradient after spaced conditioning was abolished when Appl expression was inhibited in adult MB (Fig. 5c).

To further support the idea that Appl and Sod3 are involved in the same pathway, we searched for a synergistic interaction between Appl and Sod3 using mild inhibition of their expression. The objective was to demonstrate that the concomitant reduction in Appl and Sod3 expression could result in a more pronounced LTM impairment than that observed in single knockdowns. The flexibility of the GAL4/GAL80^{ts} system, which is induced by elevated temperature, was used to express Appl RNAi for a brief period of 1 day, instead of the usual 3 days of RNAi expression. At the behavioural level, the induction of Appl or Sod3 RNAi for a single day in MB neurons and astrocytes did not result in an LTM impairment (Fig. 5d). Next, we asked whether the brief simultaneous induction of Appl and Sod3 RNAi would affect LTM, thus revealing a synergistic interaction. Strikingly, LTM was specifically abolished when both Appl and Sod3 expressions were inhibited in MB neurons and astrocytes for one day (Fig. 5d, Extended Data Fig. 7d and Supplementary Table 5). Of note, the simultaneous mild inhibition of Appl and Sod3 expressions in MB alone or astrocytes alone did not

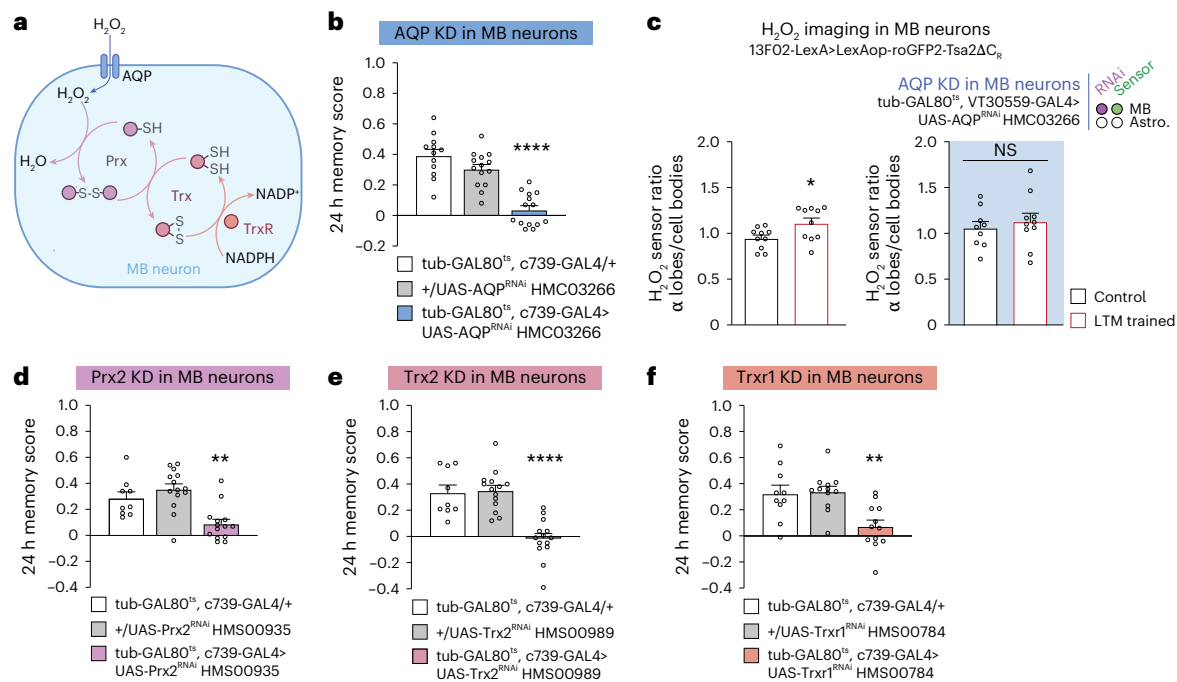


Fig. 4 | A redox-sensitive cascade is recruited upon H_2O_2 entry into MB neurons for LTM formation. **a**, Putative redox relay cascade downstream of H_2O_2 import in MB neurons. **b**, AQP KD in adult α/β MB neurons impaired memory after spaced conditioning ($n = 12$ (first group), $n = 14$ (second and third groups), $F_{2,37} = 31.24$, $P = 0.00000001$). **c**, The increase in H_2O_2 level in α lobes/cell bodies elicited by spaced training ($n = 10$, $t_{18} = 2.42$, $P = 0.0261$) was impaired in AQP KD in adult MB neurons ($n = 9$ (first group), $n = 10$ (second group), $t_{17} = 0.59$, $P = 0.56$). **d**, Prx2 KD in adult α/β MB neurons impaired memory after spaced conditioning ($n = 9$ (first group), $n = 14$ (second and third groups), $F_{2,34} = 12.08$, $P = 0.0001$).

e, Trx2 KD in adult α/β MB neurons impaired memory after spaced conditioning ($n = 9$ (first group), $n = 14$ (second and third groups), $F_{2,37} = 14.43$, $P = 0.0000007$). **f**, Trxr1 KD in adult α/β MB neurons impaired memory after spaced conditioning ($n = 10$ (first group), $n = 12$ (second and third groups), $F_{2,27} = 20.30$, $P = 0.0012$). Data are presented as mean $\pm$ s.e.m. Genotype sample sizes are listed in the legend in order of bar appearance. Significance level of a two-sided t -test or the least significant Tukey pairwise comparison following one-way ANOVA. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$; NS, not significant, $P > 0.05$.

impact LTM (Extended Data Fig. 7e,f). Altogether, we can conclude that the inability to form LTM in double-knockdown flies results from the simultaneous mild decrease of *Appl* expression in the MB, and of *Sod3* expression in astrocytes. To further substantiate this finding, we conducted imaging of H_2O_2 dynamics in the double knockdown following brief RNAi expression. Formation of the H_2O_2 gradient after spaced conditioning was abolished in flies expressing both *Appl* and *Sod3* RNAi, whereas it remained unaltered in flies expressing a single RNAi, aligning with the behavioural data (Fig. 5e). These results outline the existence of a strong functional interaction between *Appl* and *Sod3*, and are in agreement with the hypothesis that *Appl* supplies copper for *Sod3* activity during LTM formation.

To clarify the involvement of the *Appl*–*Sod3* interaction in the early phase of LTM formation rather than in memory recall, which occurs 24 h after conditioning in our experiments, we examined the performance of flies in which the brief *Appl* and *Sod3* RNAi expression was initiated immediately following spaced training, as opposed to before training. The memory performance of flies expressing both *Appl* and *Sod3* RNAi for 1 day after training was not affected (Extended Data Fig. 7g), whereas control flies expressing both RNAi for 1 day before training exhibited an LTM defect (Extended Data Fig. 7g). These results lend support to the hypothesis that *Appl*–*Sod3* interaction is involved in an early step of LTM formation, rather than in LTM recall.

APP physiological function has been linked to many pathways^{50,51}. To further demonstrate that the LTM defect of the *Appl* knockdown is indeed due to a lack of copper, we aimed to rescue the LTM defect of the *Appl* mutant by feeding flies with copper-complemented food. Notably, the memory performance of the *Appl* knockdown was fully rescued by 1 mM copper feeding before conditioning (Fig. 6a). In agreement with this, the H_2O_2 gradient was restored after spaced conditioning

in the *Appl* knockdown supplemented with copper (Fig. 6b). Thus, copper-supplemented food can compensate for *Appl* knockdown at both the behavioural and H_2O_2 signalling levels. Altogether, these results strongly support the idea that the main function of *Appl* during LTM formation in MB α lobe^{20,36} is to deliver extracellular copper for *Sod3*.

This leads to the question of why the increase in H_2O_2 occurs only in the α lobe of the MB, rather than in both the α and β lobes, which are two axonal projections of the same MB neurons. We postulated that this may be due to the activity of *Appl* itself, given that its copper-delivering property is permissive for H_2O_2 production by *Sod3*. The expression of *Appl* was monitored in the α and β lobes following spaced conditioning using the *Appl*-HA line and anti-HA immunohistochemistry. The level of *Appl*-HA expression was normalized to that of green fluorescent protein (GFP), which was expressed homogeneously in MB α/β neurons using the GAL4/UAS system. Notably, *Appl*-HA expression was specifically elevated in the α lobe following spaced conditioning, whereas no increase was observed in the β lobe (Fig. 6c). Furthermore, no increase in *Appl*-HA was observed in the α lobe following massed conditioning, a condition that does not engage H_2O_2 signalling in α/β neurons (Extended Data Fig. 8a). These findings support the notion that an elevation in *Appl*-HA levels within the α lobe results in an augmentation of copper release in the surrounding area, thereby facilitating the localized activation of *Sod3* secreted by astrocytes.

To further illustrate the pivotal function of the E2 copper-binding domain of *Appl* in LTM, we generated via CRISPR a constitutive *Appl* mutant for histidines of the evolutionarily conserved E2 copper-binding domain. E2 histidines at positions 535 and 539 were replaced by arginines (Methods). It should be noted that these mutations were introduced into the *Appl* gene at its typical chromosomal location,

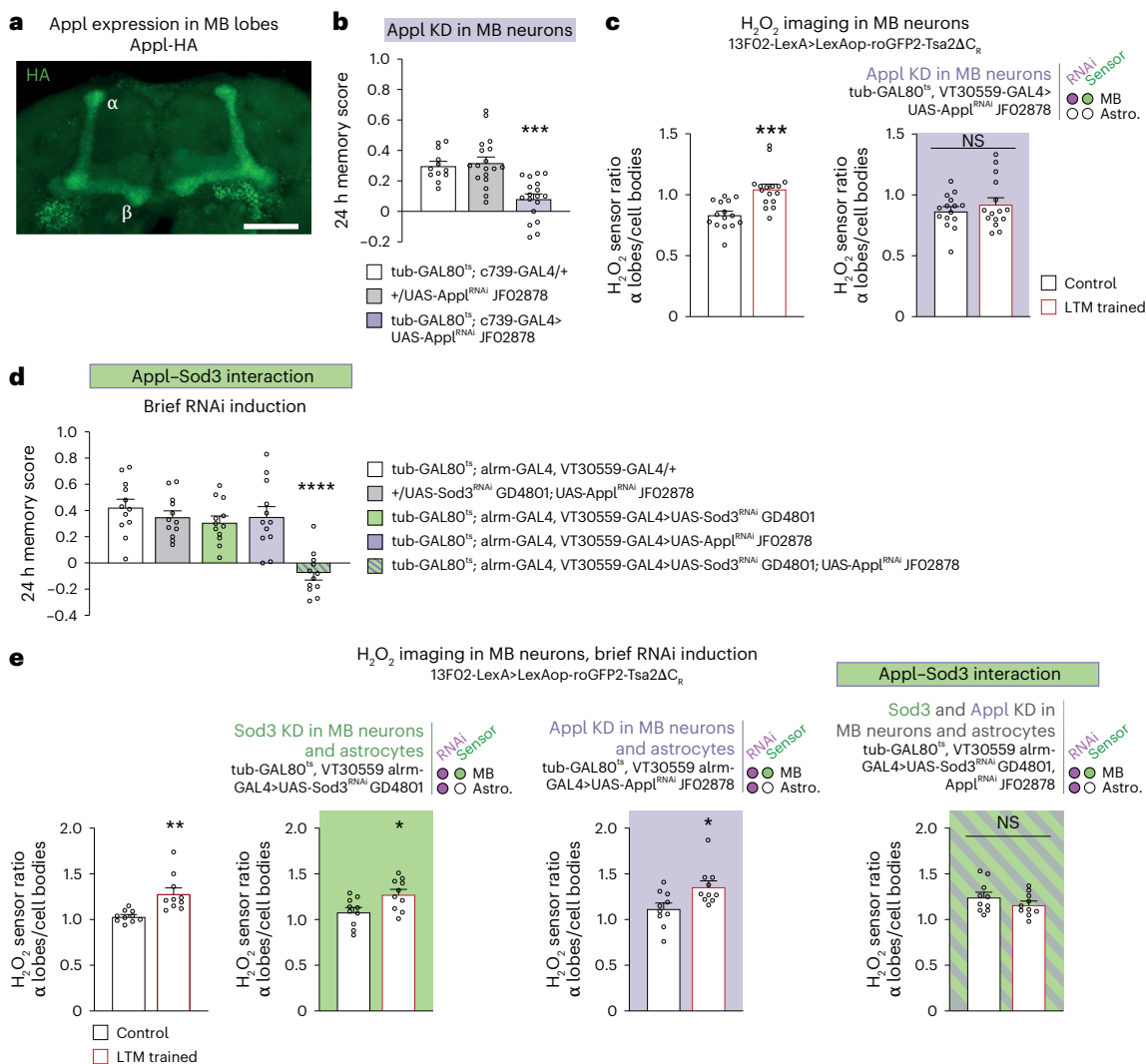


Fig. 5 | *Appl* interacts with *Sod3* during LTM formation. a, Image (maximum intensity projection of 22-μm-thick Z-stack acquisition, Z-step 1 μm) of C-terminal HA-tagged Appl immunostaining showing the high expression of HA-tagged Appl in α/β MB neurons (green). Scale bar, 40 μm. **b**, Appl KD in adult α/β neurons impaired memory after spaced conditioning ($n = 12$ (first group), 18 (second and third groups), $F_{2,45} = 15.58$, $P = 0.000007$). **c**, The increase in H₂O₂ level in α lobes/cell bodies elicited by spaced training ($n = 15$ (first group), $n = 16$ (second group), $t_{29} = 4.30$, $P = 0.0002$) was impaired in Appl KD in adult MB neurons ($n = 15$, $t_{28} = 0.88$, $P = 0.39$). **d**, Mild decreases in both Appl and Sod3 expression in MB neurons and astrocytes impaired memory after

spaced conditioning ($n = 12$, $F_{4,55} = 13.22$, $P = 0.0000001$). This mild decrease was obtained by activating RNAi expression for 1 day instead of 3 days. **e**, Mild decrease in both Appl and Sod3 expression in MB neurons and astrocytes prevents increase in H₂O₂ level elicited by spaced training (wild-type control: $n = 10$, $t_{18} = 3.741$, $P = 0.0015$; Sod3 KD: $n = 10$, $t_{18} = 2.651$, $P = 0.0163$; Appl KD: $n = 10$, $t_{18} = 2.655$, $P = 0.0161$; Sod3 and Appl KD: $n = 10$, $t_{18} = 1.262$, $P = 0.2229$). Data are presented as mean $\pm$ s.e.m. Genotype sample sizes are listed in the legend in order of bar appearance. Significance level of a two-sided t -test or the least significant pairwise Tukey comparison following one-way ANOVA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; NS, not significant, $P > 0.05$.

without affecting regulatory sequences. Appl^{H(535)R,H(539)R} mutant with modified copper-binding histidines exhibited an impairment in LTM (Fig. 6d). ARM and middle-term memory remained unaltered in Appl^{H(535)R,H(539)R} flies (Extended Data Fig. 8b), whereas middle-term memory is affected in Appl knockdown⁵². The olfactory acuity and shock reactivity of the Appl^{H(535)R,H(539)R} mutant were normal (Supplementary Table 6). Notably, the memory defect of Appl^{H(535)R,H(539)R} mutant was rescued by 24 h 2 mM copper feeding (Fig. 6e). These findings further substantiate the pivotal and specific role of the copper-binding property of Appl in LTM formation.

Aβ42 inhibits astrocytic nAChRα7 and prevents H₂O₂ signalling

Following the observation that Appl is involved in the activation of H₂O₂ signalling, we wondered whether, conversely, the toxic derivative of APP, amyloid β (Aβ), can inhibit this signalling pathway. Notably,

human Aβ42 is a strong ligand for the nAChRα7 cholinergic receptor⁵³. At very low concentrations (<100 pM), which we cannot generate in *Drosophila* using available genetic expression systems, oligomeric Aβ42 was previously shown in vitro to activate nAChRα7 (ref. 54); however, in the 10–100 nM range, Aβ42 becomes a strong inhibitor of nAChRα7, preventing activation of the receptor by its natural ligand, ACh⁵⁵. As we showed here that nAChRα7 activation in astrocytes initiates an H₂O₂ signalling pathway, we postulated that secreted Aβ might impede this process. Because Appl shows no clear sequence similarity with APP at the level of the Aβ sequence⁵⁶, we expressed a secreted form of the human Aβ42 peptide, as frequently performed in *Drosophila* to study the consequences of brain amyloid expression^{57,58}. The human Aβ42 peptide was expressed with a signal sequence that ensures its secretion into the extracellular space⁵⁶. After several days of constitutive human Aβ42 expression in the *Drosophila* brain, diffuse aggregates are observed, along with neurodegenerative defects⁵⁷. These defects are

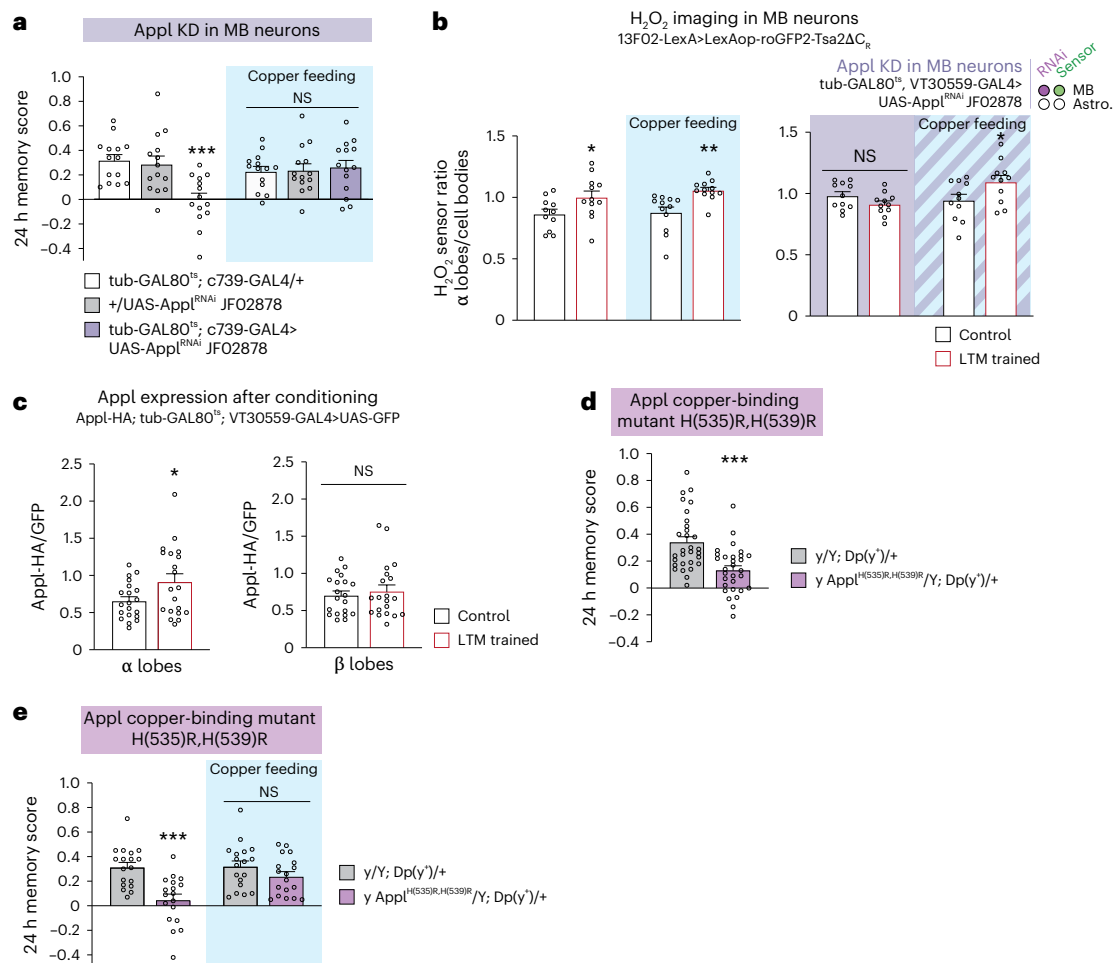


Fig. 6 | Appl provides copper to sustain Sod3 activity during LTM formation.

a, Copper feeding (1 mM) 24 h before spaced training restored normal memory capacity in flies with an *Appl* KD in adult α/β MB neurons (two-way ANOVA, $n = 14$, copper treatment: $F_{1,78} = 0.94$, $P = 0.33$; genotype: $F_{2,78} = 4.48$, $P = 0.01$; interaction: $F_{2,78} = 6.70$, $P = 0.002$). **b**, Copper feeding (1 mM) 24 h before training restored the H₂O₂ level in α lobes/cell bodies in MB neuron presynaptic terminals of an *Appl* KD in adult MB neurons (right) (two-way ANOVA, $n = 11$, copper treatment: $F_{1,40} = 3.07$, $P = 0.087$; training: $F_{1,40} = 0.92$, $P = 0.34$; interaction: $F_{1,40} = 7.01$, $P = 0.012$). In wild-type controls (left), the increase in H₂O₂ level in the α lobes/cell bodies elicited by spaced training was not affected by copper feeding (two-way ANOVA, $n = 11$ (first, third and fourth groups), $n = 12$ (second group), copper treatment: $F_{1,43} = 0.71$, $P = 0.40$; training: $F_{1,43} = 15.32$, $P = 0.0003$; interaction: $F_{1,43} = 0.30$, $P = 0.59$). **c**, *Appl* expression is increased in MB α lobes after spaced training. Control flies

received odorant and shock stimuli temporally dissociated, a condition that does not induce LTM (α lobes: $n = 20$, $t_{38} = 2.181$, $P = 0.0355$; β lobes: $n = 20$, $t_{38} = 0.5444$, $P = 0.5894$). **d**, Mutations of copper-binding histidines H(535) and H(539) affect memory after spaced training ($n = 30$, $t_{38} = 4.169$, $P = 0.0001$). **e**, Copper feeding (2 mM) 24 h before spaced training restored normal memory capacity in *Appl^{H(535)R,H(539)R}* flies (two-way ANOVA, $n = 18$, copper treatment: $F_{1,68} = 5.603$, $P = 0.0208$; genotype: $F_{1,68} = 17.22$, $P = 0.00009$; interaction: $F_{1,68} = 4.932$, $P = 0.0297$). Data are presented as mean $\pm$ s.e.m. Genotype sample sizes are listed in the legend in order of bar appearance. Significance level of a two-sided *t*-test or the least significant Tukey pairwise comparison following one-way ANOVA or Šidák pairwise comparison following two-way ANOVA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant, $P > 0.05$.

not observed after 3 days of Aβ42 induction⁵⁷. As we aimed to evaluate the potential acute effect of Aβ42 on astrocytic nAChRα7, expression was activated in MB α/β neurons for a short 24-h period. We observed that the calcium response to nicotine was inhibited in the presence of Aβ42 (Fig. 7a), suggesting that human Aβ42 secreted by MB neurons inhibits astrocytic nAChRα7. We then showed that expressing Aβ42 in MB for 24 h affected LTM specifically (Fig. 7b, Extended Data Fig. 9a and Supplementary Table 7). Last, we showed that Aβ42 inhibits formation of the H₂O₂ gradient in MB neurons upon spaced conditioning (Fig. 7c). These results demonstrate that an acute expression of toxic Aβ42 in young flies prevents H₂O₂ formation by astrocytes after spaced conditioning, and therefore LTM formation.

Discussion

ROS are byproducts produced mainly by mitochondria, and they are particularly toxic for neurons². It has been suggested that ROS play a

negative role in normal cognitive aging⁵⁹, and that a defect in redox homeostasis is involved in the memory impairment that is characteristic of AD^{59,60}. Glia help neurons fighting oxidative stress in various ways, including the production of reductive glutathione^{10,61,62} or accumulation of peroxidized lipids transferred from neurons⁶³; however, ROS also play physiological roles¹, and modulating the level of enzymes involved in ROS metabolism affects synaptic plasticity² and learning and memory^{8,10}. Nevertheless, the precise mechanisms controlling ROS signalling during memory formation have remained poorly known, in particular because imaging physiological ROS variations during memory formation was out of reach until the development of a genetically encoded highly sensitive sensor¹⁶. Moreover, it was not known if glia could transfer beneficial ROS to neurons for neuronal plasticity and memory formation. Our data now provide a mechanistic understanding of the role of H₂O₂ signalling in vivo during memory formation. We have deciphered a pathway by which astrocytes deliver beneficial

H₂O₂ to neurons for LTM formation, a phenomenon we have named astrocyte-to-neuron H₂O₂ signalling (ANHOS) (Fig. 7d). The production of ROS by astrocytic Nox and Sod3 enzymes, which follows ACh neurotransmitter-induced Ca²⁺ increase, ensures a local H₂O₂ accumulation in the MB α lobe, the specific axonal projections encoding LTM^{20,36}. Following H₂O₂ import, a signalling cascade is initiated by redox-sensitive enzymes⁶⁴ comprising Prx2, Trx2 and Trx1 (Fig. 7d). We put forth the proposition that this cascade serves as a trigger for LTM formation through the reversible oxidation of specific cysteines in LTM-relevant signalling proteins, in conjunction with the potential direct action of H₂O₂ itself. Although the future investigation of these molecular changes will be important, assessing transient changes of oxidation states is technically challenging, and this will require targeting the small subpopulation of α/β neurons that respond to the odorant used during LTM conditioning⁶⁵. What is the potential connection between ANHOS and cAMP-response element binding protein (CREB), a transcription factor that has been demonstrated to play a pivotal role in LTM formation in *Drosophila* and mice^{24,25}? Mitogen-activated protein kinases (MAPKs) have been demonstrated to activate CREB by phosphorylation, thereby facilitating memory consolidation^{66,67}. Moreover, kinases of the MAPK family, such as p38a and ERK, can be activated by H₂O₂ signalling^{68,69}. It is therefore proposed that the link between H₂O₂ signalling in the α lobe and the regulation of transcription by CREB may imply the activation of the MAPK pathway and retrograde signalling from the axonal projection to the nucleus.

Sod3 secreted by astrocytes plays a central role in ANHOS, as it generates the H₂O₂ molecules that will be imported by neurons. Sod3 requires a source of free copper for its catalytic activity. Although the free copper concentration remains extremely low in the brain because of its toxicity, copper concentrations around 100 μ M have been reported at the synaptic cleft in mammals⁷⁰. Our results in *Drosophila* suggest that an essential source of copper for the activity of Sod3 secreted by astrocytes comes from neuronal Appl, which exhibits an evolutionarily conserved copper-binding site on its extracellular domain. Indeed, *Appl* and *Sod3* knockdowns display a strong synergistic interaction (Fig. 5d,e) and feeding copper to *Appl* knockdown flies rescued the LTM defect (Fig. 6a) and reestablished the H₂O₂ gradient in the olfactory memory centre (Fig. 6b). In addition, mutation of two of four evolutionarily conserved histidines within the E2 copper-binding domain of Appl resulted in impaired LTM (Fig. 6d), whereas middle-term

memory remained unaltered (Extended Data Fig. 8b). Conversely, *Appl* knockdown affects both LTM and middle-term memory⁵², which further delineates the specific role of the copper-binding domain in

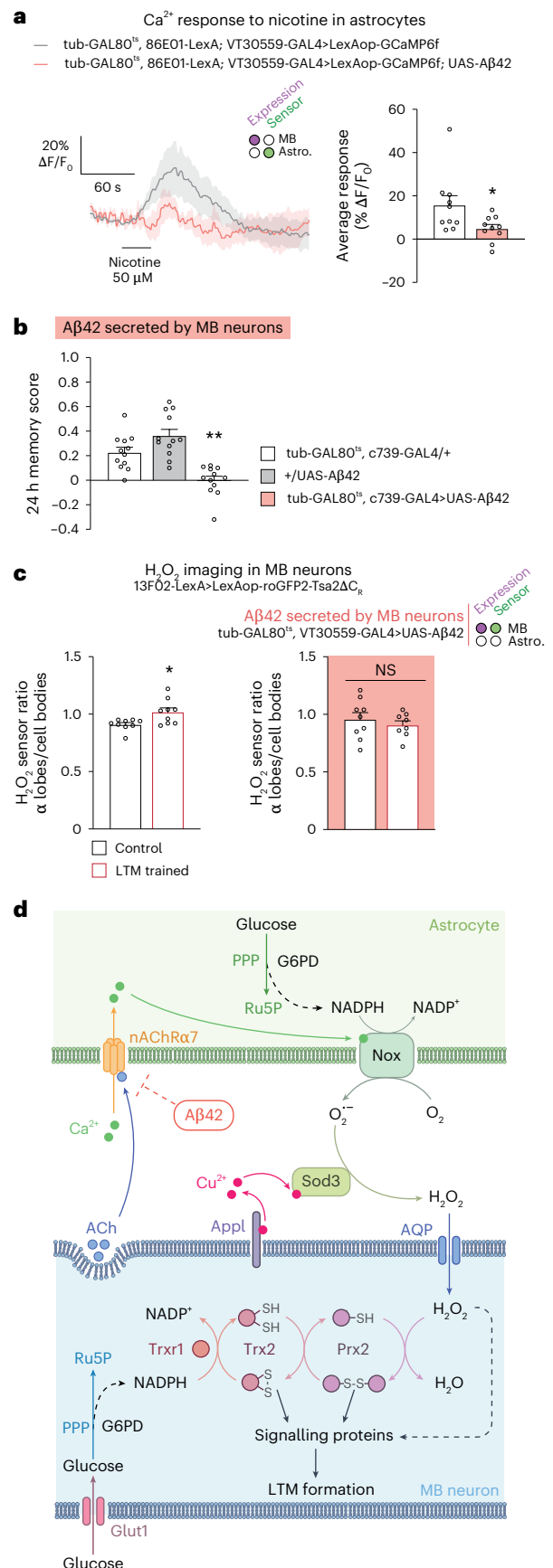


Fig. 7 | Secreted human A β 42 inhibits astrocyte-neuron H₂O₂ signalling.

a, Nicotine stimulation (50 μ M) elicited increased calcium in astrocytes, which was significantly decreased when secreted human A β 42 was expressed by MB neurons ($n = 10$, $t_{18} = 2.29$, $P = 0.03$). **b**, Human A β 42 secretion by adult α/β neurons impaired memory after spaced conditioning ($n = 12$, $F_{2,33} = 18.51$, $P = 0.000004$). **c**, Spaced training elicited an increase in the H₂O₂ level of the α lobes/cell bodies with the unpaired control ($n = 9$, $t_{16} = 2.75$, $P = 0.014$), which was impaired by human A β 42 secretion by adult MB neurons ($n = 9$ (first group), $n = 8$ (second group), $t_{15} = 0.72$, $P = 0.48$). **d**, Model of ANHOS for LTM formation. ACh release from MB neurons²⁷ upon spaced training activates astrocytic nicotinic receptor nAChR α 7, which induces a calcium elevation in astrocytes, resulting in Nox activation. Nox produces extracellular O₂⁻ in the presence of NADPH co-factor derived from astrocytic PPP. In the presence of Cu²⁺ delivered by MB neuronal Appl, extracellular astrocytic Sod3 converts O₂⁻ to H₂O₂. H₂O₂ enters MB neuron lobes through the AQP channel and fuels the redox-sensitive cascade composed of Prx2, Trx2 and Trx1 enzymes. Regeneration of reduced forms of these enzymes is provided by PPP-derived NADPH produced during LTM formation¹⁷. Oxidized Prx2 and Trx2 can activate signalling cascades (to be identified), which allows LTM formation. In a parallel pathway, H₂O₂ can directly oxidize signalling proteins (dotted line). A β 42 impedes ANHOS by inhibiting nAChR α 7. Data are presented as mean $\pm$ s.e.m. Genotype sample sizes are listed in the legend in order of bar appearance. Significance level of a two-sided t -test or the least significant Tukey pairwise comparison following one-way ANOVA. * $P < 0.05$; ** $P < 0.01$; NS, not significant, $P > 0.05$.

LTM. The study of the *Drosophila* histidine E2 mutant is particularly important because, to the best of our knowledge, no mouse mutants for E2 copper-binding histidines have been reported so far. Feeding copper to *Appl* E2 mutant rescued the LTM defect (Fig. 6e), outlining the essential role of the copper-binding property of *Appl* in LTM formation.

Altogether, our results suggest that the copper-binding property of *Appl* and its functional interaction with *Sod3* are the major roles of *Appl* in LTM formation⁴⁸. Of note, expression of extracellular *Sod3* in the human brain is much stronger in astrocytes than in neurons⁷¹, as in *Drosophila*³³ (Extended Data Fig. 2c). In mammals, APP binds copper^{43,44}; it is expressed at the presynaptic active zone of neurons, where neurotransmitters are released^{72,73}, and it is involved in synaptic plasticity and memory^{51,74}. We propose that our model developed in *Drosophila* (Fig. 7d) can be generalized to these mammalian data, leading us to conclude that APP activates astrocytic *Sod3* for memory formation in mammals. In the mammalian brain, glutamate is the predominant excitatory neurotransmitter. The ANHOS cascade may be activated not only at the cholinergic synapse, but also at the glutamatergic synapse, following calcium signalling in astrocytes that results from the activity of glutamatergic neurons⁷⁵.

From a pathological standpoint, our findings demonstrate that human A β 42 exerts an inhibitory effect on the ANHOS cascade. This is evidenced by its capacity to impede the Ca²⁺ influx observed in astrocytes following nAChR α 7 stimulation, as well as the H₂O₂ increase in the MB LTM centre subsequent to spaced conditioning. It is likely that the effect of A β 42 involves the inhibition of nAChR α 7 in astrocytes, which in turn prevents the formation of ROS by astrocytic Nox and *Sod3*. We propose that this inhibitory process is at play during AD initiation. Indeed, several observations are in agreement with the pathophysiological hypothesis that impairment of astrocytes by diffusible A β 42 oligomers at cholinergic synapses may occur precociously in the AD brain: (1) soluble synaptic A β 42 correlates better with the pattern of cognitive decline in AD than amyloid plaques¹⁵; (2) nAChR α 7 is expressed by human brain astrocytes, in particular in the hippocampus²⁹, a neural structure that plays a major role in episodic and spatial memory; (3) A β 42 inhibits nAChR α 7 in vitro at a nM concentration⁷⁶; and (4) in humans, pathology of the basal forebrain cholinergic neurons precedes cortical defects but is predictive of future memory deficits^{77,78}. In addition, as revealed in a global data-mining survey, *Sod3* has been associated with AD in several ‘omics’ approaches, although its implication remains to be understood⁷⁹. Our ANHOS model integrates observations of early AD and cholinergic neurons, and proposes a key role for *Sod3* in AD. In normal conditions, cholinergic neurons of the basal forebrain, which send diffuse projections to many cortical areas as well as the hippocampus, are involved in memory formation^{77,80}. The transition from a physiological condition to an AD condition might therefore involve a switch from an ANHOS activation by ACh to ANHOS inhibition via increased A β 42 production.

Methods

Experimental model

Flies (*Drosophila melanogaster*) were raised on standard medium (inactivated yeast 6% w/v; corn flour 6.7% w/v; agar 0.9% w/v; methyl-4-hydroxybenzoate 22 mM), at 18 or 23 °C (depending on the experiments; see respective details below) and 60% humidity in a 12-h light–dark cycle. The study was performed on 1–3-day-old adult flies. For behavioural experiments, both male and female flies were used. For imaging experiments, female flies were used because of their larger size. Before imaging or behavioural experiments, we chose flies informally in a random manner from a much larger group raised together for all studies; there was no formal randomization procedure for selecting flies. Data collection and analysis were not performed blind to the conditions of the experiments. Transgenic flies were outcrossed for five generations to a reference strain carrying the *w¹¹¹⁸* mutation in an otherwise Canton-Special genetic background. Because TRIP RNAi

transgenes are labelled by a *y⁺* marker, these lines were outcrossed to a *y⁺ w^{67c23}* strain in an otherwise Canton-Special background. All strains used in this study are described in Supplementary Table 8.

Behavioural experiments

For behavioural experiments, flies were raised on standard medium at 18 °C and 60% humidity in a 12-h light–dark cycle. We used the GAL4/GAL80^{TS} TARGET system⁸¹ to inducibly express RNAi constructs exclusively in the MB or astrocytes of adult flies, and not during development. To achieve the induction of RNAi expression, adult flies were kept at 30.5 °C for 3 days before conditioning, unless otherwise specified. To induce A β 42 carrying the pre-proenkephalin signal peptide for secretion⁵⁸, adult flies were kept at 30.5 °C for 24 h before conditioning. To test the interaction of *Appl* and *Sod3*, adult flies were kept at 30.5 °C for 24 h to achieve mild expression of RNAis before conditioning (Fig. 5d and Extended Data Fig. 7g) or after conditioning (Extended Data Fig. 7g). Otherwise, for non-induced experiments, experimental flies were transferred before conditioning to fresh bottles at 18 °C.

All behavioural experiments, including the sample sizes, were conducted similarly to other studies from our laboratory^{17,20,37}. Groups of 20–50 flies were subjected to one of the following olfactory conditioning protocols at 25 °C: a single training cycle (1 $\times$), five consecutive associative training cycles (5 $\times$ massed training), or five associative cycles spaced by 15-min inter-trial intervals (5 $\times$ spaced training). A non-associative control protocol (unpaired protocol) was also employed for imaging experiments and *Appl*-HA immunocytochemistry experiments, during which the odour and shock stimuli were delivered separately in time, with shocks occurring 3 min before the first odourant. Conditioning was performed using previously described barrel-type machines that allow the parallel training of up to six groups^{17,20,36}. Throughout the conditioning protocol, each barrel was plugged into a constant air flow at 2 l min⁻¹. For a single cycle of associative training, flies were first exposed to an odourant (the CS⁺) for 1 min, while 12 pulses of 5-s long 60-V electric shocks were delivered; flies were then exposed 45 s later to a second odourant without shocks (the CS⁻) for 1 min. The odourants 3-octanol (Fluka 74878, Sigma-Aldrich) and 4-methylcyclohexanol (Fluka 66360, Sigma-Aldrich), diluted in paraffin oil to a final concentration of 2.79 $\times$ 10⁻¹ g l⁻¹, were alternately used as conditioned stimuli. During unpaired conditionings, the odour and shock stimuli were delivered separately in time, with shocks occurring 3 min before the first odourant.

Flies were kept at 18 °C on standard medium between conditioning and the memory test, except in experiments in which immediate memory was tested after spaced conditioning. The memory test was performed in a T-maze apparatus⁸², 24 h after massed or spaced training, at 25 °C. The memory test was performed at 2 h after 1 $\times$ training. Each arm of the T-maze was connected to a bottle containing 3-octanol and 4-methylcyclohexanol, diluted in paraffin oil to a final concentration identical to the one used for conditioning. Flies were given 1 min to choose between either arm of the T-maze. A performance score was calculated as the number of flies avoiding the conditioned odour minus the number of flies preferring the conditioned odour, divided by the total number of flies. A single performance index value is the average of two scores obtained from two groups of genotypically identical flies conditioned in two reciprocal experiments, using either odourant (3-octanol or 4-methylcyclohexanol) as the CS⁺. The indicated ‘*n*’ is the number of independent performance index values for each genotype. To avoid giving disproportionate statistical weight to a small number of flies, rare behavioural experiments involving a group of fewer than six flies were excluded.

The shock response tests were performed at 25 °C by placing flies in two connected compartments; electric shocks were provided in only one of the compartments. Flies were given 1 min to move freely in these compartments, after which they were trapped, collected and counted. The compartment where the electric shocks were delivered

was alternated between two consecutive groups. Shock avoidance was calculated as for the memory test.

Because the delivery of electric shocks can modify olfactory acuity, our olfactory avoidance tests were performed on flies that had first been presented another odour paired with electric shocks. Innate odour avoidance was measured in a T-maze similar to those used for memory tests, in which one arm of the T-maze was connected to a bottle with odour diluted in paraffin oil and the other arm was connected to a bottle with paraffin oil only. Naive flies were given the choice between the two arms during 1 min. The odour-interlaced side was alternated for successively tested groups. Odour concentrations used in this assay were the same as for the memory assays. At these concentrations, both odorants are innately repulsive.

In vivo calcium imaging

Calcium imaging experiments were performed on flies expressing the GCaMP6f calcium sensor in astrocytes via the *alrm-GAL4* driver, in combination with *UAS-GCaMP6f*. Transgenes were expressed in astrocytes using the inducible *tub-GAL80^{ts}*; *alrm-GAL4* driver. As carried out previously in our laboratory for imaging experiments^{17,37}, flies were raised at 23 °C to increase the expression level of genetically encoded sensors without allowing RNAi expression. Adult flies were kept at 30.5 °C for 3 days before conditioning to achieve the induction of RNAi expression.

As in all previous imaging work from our laboratory, all in vivo imaging was performed on female flies, which are preferred as their larger size facilitates surgery. A single fly was picked and prepared for imaging as previously described^{17,37}. In brief, the head capsule was opened and the brain was exposed by gently removing the superior tracheae. The head capsule was bathed in artificial haemolymph solution for the duration of the preparation. The composition of this solution was: NaCl 130 mM (Sigma, S9625), KCl 5 mM (Sigma, P3911), MgCl₂ 2 mM (Sigma, M9272), CaCl₂ 2 mM (Sigma, C3881), D-trehalose 5 mM (Sigma, 9531), sucrose 30 mM (Sigma, S9378) and HEPES hemisodium salt 5 mM (Sigma, H7637). At the end of surgery, any remaining solution was absorbed and a fresh 100-μl droplet of this solution was applied on top of the brain. Two-photon imaging was performed using a Leica TCS-SP5 upright microscope equipped with a ×25, 0.95 NA water-immersion objective. Two-photon excitation was achieved using a Mai Tai Deep-See laser tuned to 910 nm. The frame rate was one image per second. Recordings were acquired in the astrocytic region in the anterior part of the brain (where MB vertical lobes are located), at approximately 30 μm depth from the top of the brain. Calcium imaging experiments with nicotine stimulation were performed as previously described¹⁷. Nicotine was freshly diluted from a commercial liquid (Sigma, N3876) into the saline used for imaging on each experimental day. A perfusion setup at a flux of 2.5 ml min⁻¹ enabled the time-restricted application of 50 μM nicotine on top of the brain¹⁷. Baseline recording was performed during 1 min, after which the saline supply was switched to drug supply. The solution reached the in vivo preparation within 30 s. The stimulation was maintained for 30 s, before switching back to the saline perfusion for an additional 5 min. The GCaMP6f intensity was measured in the anterior part of the brain where MB vertical lobes are located. GCaMP6f signal was calculated over time after background subtraction and normalized by a baseline value calculated over the 30 s preceding drug injection using MATLAB software (MathWorks).

In vivo H₂O₂ imaging

H₂O₂ imaging experiments were performed on flies expressing the excitation ratiometric H₂O₂ sensor roGFP2-Tsa2ΔC_R¹⁶ in MB neurons via the 13F02-LexA driver, in combination with LexAop-roGFP2-Tsa2ΔC_R (generated in this study). Transgenes were expressed in astrocytes using the inducible *tub-GAL80^{ts}*; *alrm-GAL4* driver, or in MB neurons using the inducible *tub-GAL80^{ts}*; VT30559-GAL4 driver. For imaging experiments, flies were raised at 23 °C, except for the imaging experiment after brief RNAi expression for which flies were raised at 18 °C (Fig. 5e). Adult

flies were kept at 30.5 °C for 3 days before conditioning to achieve the induction of RNAi expression. For experiments on conditioned flies, data were collected indiscriminately from 30 min to 2 h after training.

Surgery was performed as for calcium imaging. At the end of surgery, any remaining solution was absorbed and a fresh 100-μl droplet of saline solution was applied on top of the brain. Two-photon imaging was performed using a pulsed infra-red laser (Insight X3, Spectra Physics), coupled to a Leica SP8 Dive upright microscope equipped with a ×20, 1.0 NA water-immersion objective and non-descanned spectral hybrid detectors. Emission was collected from 500 to 570 nm.

To characterize the roGFP2-Tsa2ΔC_R probe for two-photon excitation microscopy, excitation spectra were measured from 780 to 1,060 nm in steps of 8 nm. Oxidation of the probe was obtained by applying 2 mM H₂O₂ (final concentration) on top of the brain. Spectra in the basal and oxidized states were measured before and 15 min after H₂O₂ treatment, respectively. Excitation wavelengths of 780 nm and 988 nm were selected for further experiments, as they maximized the sensor emission ratio (ratio 780 nm to 988 nm) between the basal and oxidized states. Experiments after conditioning were performed as follows: on a given brain area, two z-stacks (one at each acquisition wavelength) were consecutively acquired with a step of 1 μm and a line averaging of 3. The detector gain was strictly similar between the two stacks and was set to the 988 nm excitation wavelength at the highest value to avoid any pixel saturation; however, because of differential signal strength within different cellular compartments of MB neurons, two sets of stacks were acquired for each brain: one encompassing the MB lobes, and one covering the soma and calyx area, with different detector gains. For data analysis, the average intensity of three consecutive planes of the stack was calculated for each region of interest at the two excitation wavelengths. Oxidation of the sensor by H₂O₂ was measured by the ratio of the signal collected upon 780 nm excitation over the signal collected upon 988 nm excitation (referred to as 'H₂O₂ sensor ratio'), using Leica Microsystems software LAS X Small (offline version).

Dietary copper supplementation

Copper (II) sulfate pentahydrate (Thermo Fisher Scientific, 197730010) was added to melted standard food medium to achieve a final concentration of 1 mM (Fig. 6a,b), a concentration that does not affect survival rate under chronic exposure⁸³. Flies were kept on regular food medium for 48 h at 30.5 °C to achieve RNAi induction and then transferred to copper-enriched medium for 24 h at 30.5 °C for both behavioural and H₂O₂ imaging experiments. No lethality was observed with this copper feeding diet. Control flies were transferred on regular medium for 24 h at 30.5 °C.

For the copper rescue of *App^{1H(S35),H(S39)}* flies (Fig. 6e), copper (II) sulfate pentahydrate was added to melted standard food medium to achieve a final concentration of 2 mM. Flies were kept on copper-enriched medium for 24 h at 18 °C before spaced conditioning.

Immunohistochemistry

Before dissection, whole adult female flies (2–4 days old) were fixed in 4% paraformaldehyde in PBST (phosphate-buffered saline (PBS) containing 1% Triton X-100) at 4 °C overnight. Brains were dissected in PBS solution and rinsed three times for 20 min in PBST, blocked with 2% bovine serum albumin (Sigma-Aldrich, A9085) in PBST for 2 h at room temperature, and then incubated with primary antibodies at 1:200 dilution (rat anti-HA, Roche, ROAHAHA 11867423001, clone 3F10) in the blocking buffer (2% BSA in PBST) at 4 °C overnight. The following day, brains were rinsed three times for 20 min in PBST, and incubated with secondary antibodies at 1:400 dilution (goat anti-rat Alexa 488, Invitrogen, A11006) in blocking buffer for 3 h at room temperature. Brains were rinsed for a further three times for 20 min in PBST and were mounted in ProLong Mounting Medium (Lifetechnology) for imaging. Images were acquired with a Nikon A1R confocal microscope with a ×20 objective.

In experiments where Appl-HA expression was quantified in MB neurons following spaced conditioning, artificial GFP expression was used as an expression control. *Appl-HA/+; tub-GAL80^{ts/+}; VT30559-GAL4>UAS-mCD8GFP* females were raised at 18 °C and transferred for 1.5 days at 30.5 °C to induce GFP expression in MB neurons. The flies were trained using the various protocols. Two hours following conditioning, adult females were fixed in 4% paraformaldehyde in PBST (PBS containing 1% Triton X-100) at 4 °C overnight. For immunohistochemical analysis, brains were dissected in PBS solution and rinsed three times for 20 min in PBST. They were then blocked with 2% bovine serum albumin (Sigma-Aldrich, A9085) in PBST for 2 h at room temperature. Subsequently, the samples were incubated with primary antibodies at a dilution of 1:200 dilution for rat anti-HA (Roche, ROA-HAHA 11867423001, clone 3F10) and 1:400 dilution for mouse anti-GFP (Developmental Studies Hybridoma Bank, DSHB-GFP-12A6, clone 12A6) in blocking buffer (2% BSA in PBST) at 4 °C overnight. On the subsequent day, the brains were rinsed three times for 20 min in PBST and incubated with secondary antibodies at a dilution of 1:400 (goat anti-rat Alexa 594, Invitrogen, A11007 and goat anti-mouse Alexa 488, Invitrogen, A11029) in blocking buffer for 3 h at room temperature, and subsequently placed at 4 °C overnight. Subsequently, the brains were rinsed three times for 20 min in PBST and mounted in ProLong Mounting Medium (Life Technologies) for imaging. Images were obtained using a Nikon A1R confocal microscope with a ×20 objective.

Western blot

Proteins were extracted from adult female heads after liquid nitrogen snap freezing and mechanical grinding in lysis buffer containing sucrose 100 mM, KH₂PO₄/HPO₄[−] 40 mM, EDTA 30 mM, KCl 50 mM, 0.25% Triton X-100, dithiothreitol 10 mM, PMSF 0.5 mM and 1× protease inhibitors (Roche, 11836153001). Protein extracts containing loading buffer NOVEX Tricine SDS sample buffer (Thermo Fisher Scientific, LC1676) were run in NOVEX 10–20% Tricine gels (Thermo Fisher, EC66252BOX) and then transferred on nitrocellulose membranes. Membranes were saturated with 15% non-fat milk, PBS–Tween 0.2% for 1 h. Membranes were incubated with primary anti-HA antibodies diluted in PBS–Tween 0.2% blocking medium (mouse anti-HA, 1:5,000 dilution, BioLegend, 901513, clone 16B12) overnight at 4 °C under agitation. Membranes were rinsed five times for 8 min in PBS–Tween 0.2% and incubated with secondary antibodies for 2 h at room temperature under agitation (HRP anti-mouse, 1:10,000 dilution, Promega, W4021). Membranes were further rinsed five times for 8 min in PBS–Tween 0.2%. Revelation was conducted using NOVEX ECL substrate (Thermo Fisher, WP20005) and chemiluminescence was detected with ImageQuant LAS4000. The same procedure was then applied to the same membranes with anti-tubulin antibodies (mouse anti-tubulin, 1:40,000 dilution, Sigma-Aldrich, T16199, clone DM1A). The molecular weight ladder corresponds to Invitrogen SeeBlue Plus2 (LC5925).

Quantitative PCR

The efficiency of the knockdowns (KDs) used in this study was validated by quantitative PCR with reverse transcription (RT–qPCR) to measure the messenger RNA of the target gene (Supplementary Table 9). Female flies carrying the elav-Gal4 pan-neuronal driver or the repo-Gal4 pan-glial driver were crossed either with males carrying the specified UAS-RNAi or with CS males. Fly progeny were reared at 25 °C throughout their development. Then, 0–1-day-old flies were transferred to fresh food for 1 d before RNA extraction. RNA extraction and complementary DNA synthesis were performed as described elsewhere^{68,69} using the same reagents: the RNeasy Plant Mini kit (QIAGEN), RNA MinElute Cleanup kit (QIAGEN), oligo(dT)20 primers and the SuperScript III First-Strand kit (Life Technologies). The level of complementary DNA for each gene of interest was compared against the level of the α -Tub84B (Tub, CG1913) reference cDNA. Amplification was performed using a LightCycler 480 (Roche) and the SYBR Green I Master mix

(Roche). Reactions were carried out in triplicate. The specificity and size of amplification products were assessed by melting curve analyses. Expression relative to the reference was expressed as a ratio ($2^{-\Delta C_p}$, where C_p is the crossing point). The primers used in this study and results of RT–qPCR data are presented in Supplementary Table 9. For KDs that did not yield memory impairments (negative results), both RNAis were checked by qPCR. For KDs that were induced with two distinct RNAis, at least one of the two RNAi lines was validated by qPCR. Exceptions were for those RNAi constructs that were already validated by RT–qPCR in previous studies, in which case the corresponding reference is indicated (Supplementary Table 10).

Generation of transgenic flies

For the generation of the *LexAop-roGFP2-Tsa2ΔC_R* *Drosophila* line, the p415 TEF roGFP2-Tsa2ΔC_R plasmid (Addgene, #83238)¹⁶ was digested by NotI and XbaI. The resulting 1,416-bp fragment was purified by electrophoresis and cloned into a pJFRC19 plasmid (13XLexAop2-IVS-myr::GFP, Addgene, #26224)⁸⁴. The resulting construct was verified by sequencing (the molecular cloning was outsourced to RD-Biotech, France). Transgenic fly strains were obtained by site-specific embryonic injection of the resulting vector in the attP18 landing site (X chromosome), which was outsourced to Rainbow Transgenic Flies.

The *Appl-HA* line was generated using the CRISPR approach (outsourced to inDroso). The 3×HA sequence (GCCGCCGTGTACCCCTACGACGTGCCCGACTACGCCGGCTACCCCTACGCCGTGCCCGACTACGCCGGCTCCTACCCCTACGACGTGCCCGACTACGCCCGCCGCC) preceded by a linker (sequence: GGCGTGGGC) was inserted between the 11th exon and the 3' UTR of the *Appl* gene using a guide RNA targeting the sequence AAGTGAAG | GAGTAAGCGAGA. The genomic edition was strictly restricted to the 3×HA tag and linker, preventing any alterations due to the presence of a selection marker (scarless).

Generation of *Appl* copper-binding mutant by CRISPR

The *Appl*^{H(535)R,H(539)R} lines were generated using the CRISPR approach, which was outsourced to Rainbow Transgenic Flies. To generate *Appl*^{H(535)R,H(539)R}, two guide RNAs targeting CCCACGCCTTGGCCCACTAC|CGG and GCGCGCCCTGCACAAGGACCG|GGG were employed. The original wild-type genomic sequence GCCCTGCACAAGGACCGGGCCACACGCCTTGGCCCACTACCGGCACCTATTGAACTCTGG, which corresponds to the amino-acids sequence ALHKDRAH(535)ALAH(539)YRHLNS, was altered to GCCCTGCACAAGGACCGGGCCCGCGCCTTGGCCCGCTACCGGCACCTATTGAACTCTGG, which corresponds to the amino-acids sequence ALHKDRAR(535)ALAR(539)YRHLNS. The CRISPR strategy was designed to introduce only the specified H/R modifications, and in particular, no marker was associated with the CRISPR-induced mutation. The presence of mutations in *Appl* was confirmed by Rainbow Transgenic Flies through genomic sequencing.

Appl mutations were generated on a X chromosome carrying *y w* mutations in an uncontrolled genetic background. To introduce the Canton-Special background appropriate for behavioural experiments, we crossed and *y Appl*^{H(535)R,H(539)R} *w* females to wild-type Canton-Special males. At the next F1 generation *y Appl*^{H(535)R,H(539)R} *w*/+ females were crossed to Canton-Special males. Given the close proximity of the *y* gene to *Appl* and the very low rate of recombination between these two loci (less than 0.03%, as estimated from Flybase data⁸⁵), the presence of *y* mutation was employed to recover *y Appl* mutations in a Canton-Special background. In the F2 generation, recombination between the *y Appl* and *w* loci was screened for, and *y Appl*^{H(535)R,H(539)R} *w*⁺/Y males in a Canton-Special background were recovered. Homozygous *y Appl*^{H(535)R,H(539)R} lines were generated. Concurrently, the parental *y w* line, which was utilized to induce the CRISPR mutations, was outcrossed with the identical protocol to generate a control *y* line in a Canton-Special background.

In light of the evidence indicating that *y* mutations impact LTM (based on our own unpublished observations), to conduct behavioural

experiments we crossed *yApp^{1H(535)R,H(539)R}* females to males carrying the *y⁺* duplication *PBac[y⁺]-attP-3B/VK00033* (ref. 86) in a Canton-Special background, designated *Dp(y⁺)*. The offspring were evaluated in behavioural assays to determine the phenotypes of hemizygous *yApp^{1H(535)R,H(539)R}/Y;Dp(y⁺)/+* males compared with *y/Y;Dp(y⁺)/+* males.

Sod3 transcript levels

Relative expression of *Sod3* in astrocytes and α/β neurons was obtained from published single-cell transcriptomic data³³, according to clustering performed in the original study where astrocytes are found in cluster 10 and α/β in cluster 22.

Quantification and statistical analysis

All data are presented as mean $\pm$ s.e.m. For behaviour experiments, two groups of about 30 flies were reciprocally conditioned, using respectively octanol or methylcyclohexanol as the CS⁺. The memory score was calculated from the performance of two groups as described above, which represents a single experimental replicate. For imaging experiments, one replicate corresponds to one fly brain. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications^{17,19,37}. Data distribution was assumed to be normal but this was not formally tested. Comparisons of the data series between two conditions were achieved by a two-tailed unpaired *t*-test. Comparisons between more than two distinct groups were made using a one-way analysis of variance (ANOVA) test, followed by Tukey pairwise comparisons between the experimental groups and their controls. ANOVA results are presented as the value of the Fisher distribution $F_{x,y}$ obtained from the data, where *x* is the number of degrees of freedom between groups and *y* is the total number of degrees of freedom for the distribution. For copper-rescue experiments (Fig. 6a,b), two-way ANOVA tests were performed, followed by Šidák's multiple comparisons test. Statistical tests were performed using the GraphPad Prism 8 software. In the figures, asterisks denote the significance level of the *t*-test, or of the least significant pairwise comparison following ANOVA, with **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS, not significant, *P* > 0.05. Figures were designed using Adobe Illustrator.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

No datasets that require mandatory deposition into a public database were generated during the current study. Source data that are reported as graphs on figures and extended data figures are available as Supplementary Information alongside the paper. Additional raw data will be shared with no restriction by the corresponding authors upon request. This study made use of the SCOPE database of single-cell transcriptomics in the fly brain to compare *Sod3* expression in neurons and glia (Extended Data Fig. 2c) at https://scope.aertslab.org/#/Davie_et_al_Cell_2018/Davie_et_al_Cell_2018%2FAerts_Fly_AdultBrain_Filtered_57k_loom/gene. Source data are provided with the paper.

Code availability

This paper does not report original code. Any additional information required to reanalyse the data reported in this paper is available from the corresponding authors upon request.

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Author contributions

T.P. conceived and supervised the work; N.S. performed the behavioural analyses, with the support of Y.R., J.-P.B. and L.P.; P.-Y.P. developed the assay for in vivo H₂O₂ imaging using the roGFP2-Tsa2 Δ C₈ sensor; Y.R. and J.-P.B. performed H₂O₂ imaging; Y.R. performed calcium imaging; L.P. characterized the *Appl-HA* line; N.S. performed the copper-rescue experiments, immunohistochemistry experiments and RT-qPCR experiments; T.P. wrote the main text of the manuscript; and Y.R., J.-P.B. and P.-Y.P. contributed to writing the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Thomas Preat.

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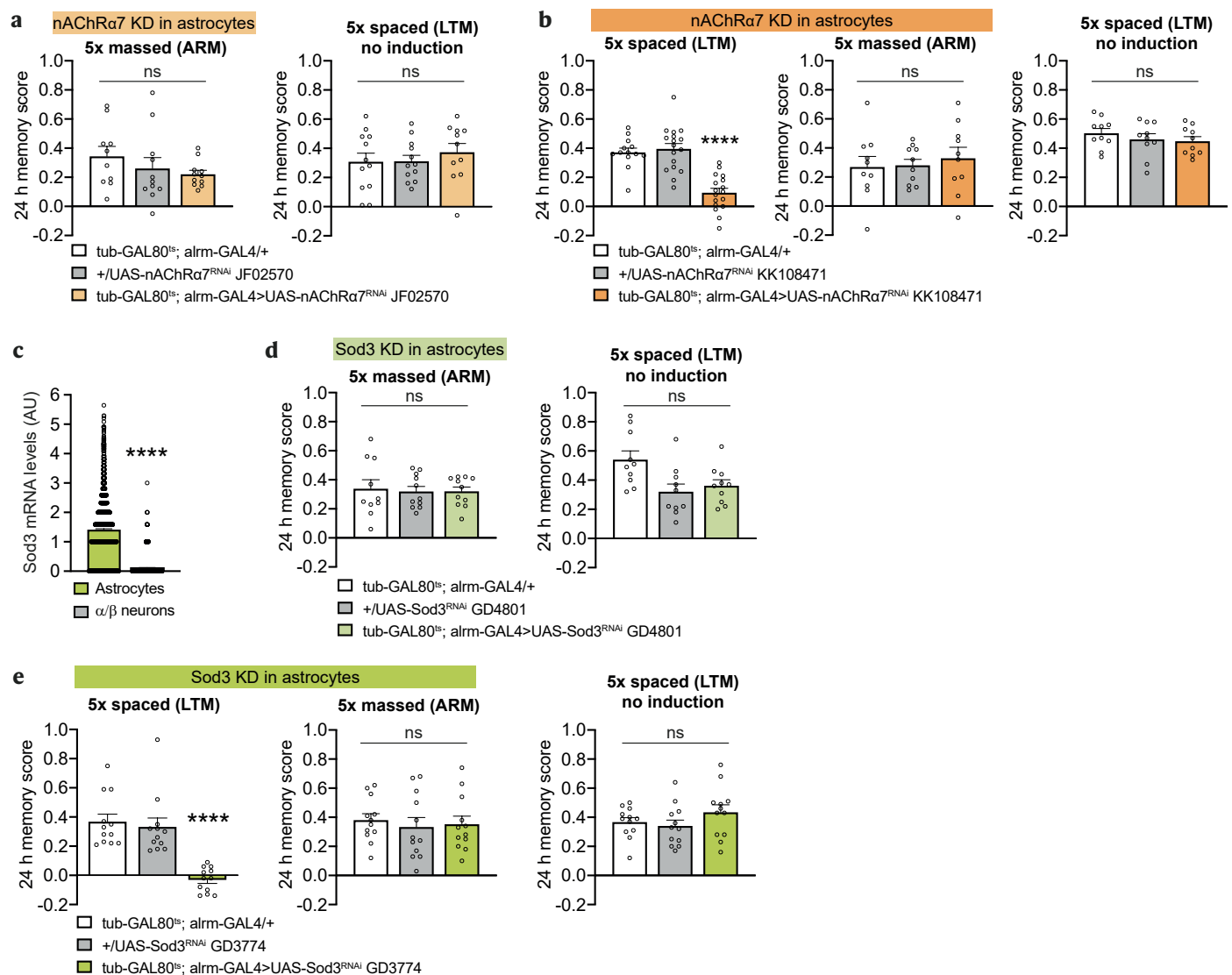
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Extended Data Fig. 1 | Controls for behavioural experiments, related to

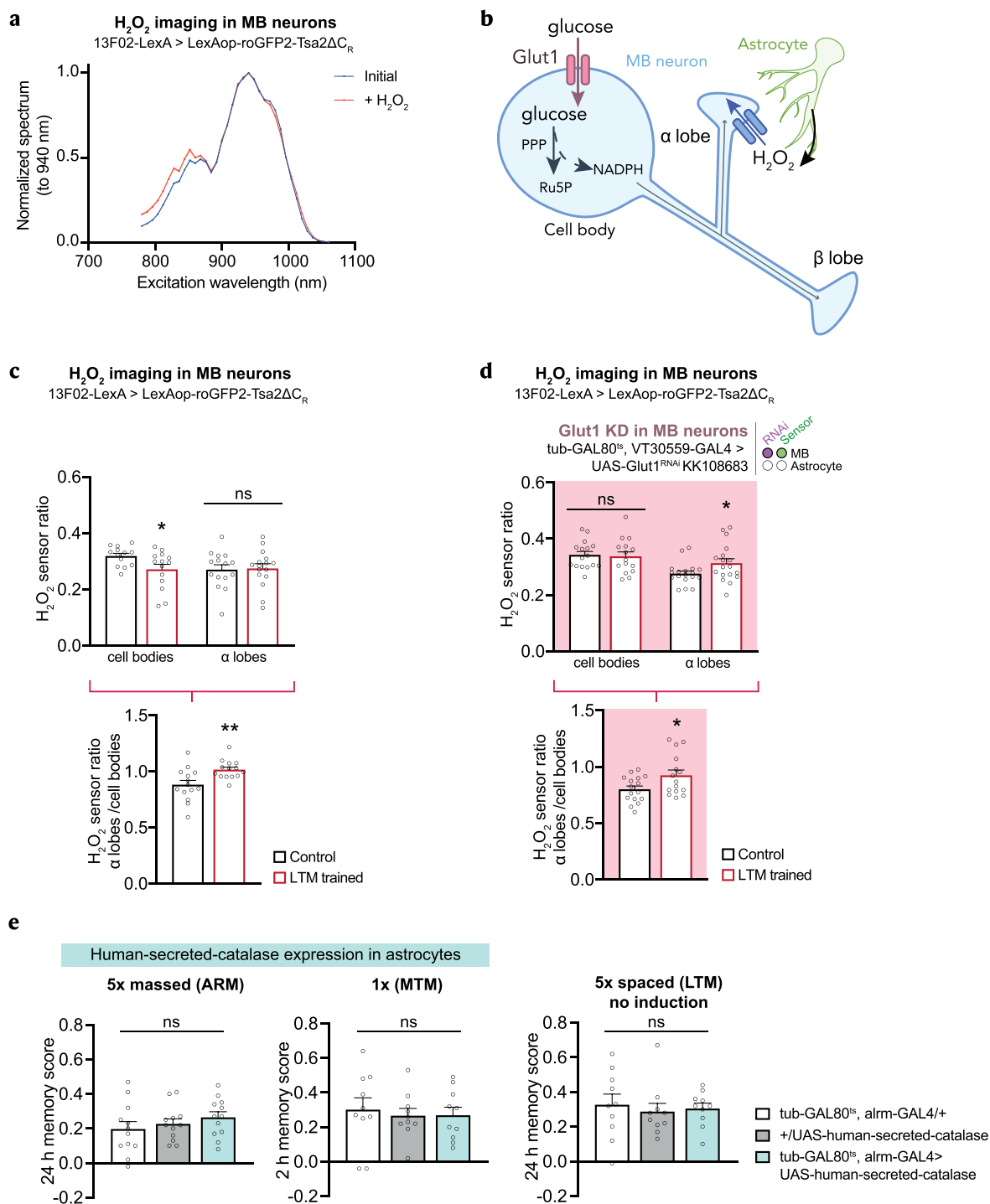
Fig. 1. (a) Memory after massed conditioning was not affected by *Nox* knockdown (KD) in adult astrocytes ($n = 10$ (first group), $n = 11$ (second and third groups), $F_{2,29} = 1.17$, $P = 0.32$). Without *Nox* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 0.67$, $P = 0.52$). (b) Immediate memory after spaced conditioning was not affected by *Nox*, *nAChR α 7*, or *Sod3* KD ($n = 12$, $F_{6,77} = 1.233$, $P = 0.2989$). (c) Memories at 24 h were assessed with a second RNAi against *Nox*. *Nox* KD impaired memory after spaced training ($n = 12$ (first group), $n = 17$ (second and third groups), $F_{2,43} = 24.03$, $P = 0.00000009$), but did not affect memory after massed training ($n = 12$, $F_{2,33} = 0.018$, $P = 0.98$). Without *Nox* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 0.38$, $P = 0.68$). (d) Memory after massed conditioning was not affected by *G6PD* KD ($n = 11$, $F_{2,30} = 2.93$, $P = 0.07$). Memory at 2 h after 1x conditioning was not affected by *G6PD* KD ($n = 15$, $F_{2,42} = 1.528$, $P = 0.2287$). Without *G6PD* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 0.49$, $P = 0.62$). (e) Immediate

memory after spaced conditioning was not affected by *G6PD*, *Pgd* or *Pgls* KD ($n = 12$, $F_{6,77} = 0.4174$, $P = 0.8653$). (f) *Pgd* KD impaired memory after spaced training ($n = 12$, $F_{2,33} = 4.92$, $P = 0.01$), but did not affect memory after massed training ($n = 10$, $F_{2,27} = 0.31$, $P = 0.73$). Memory at 2 h after 1x conditioning was not affected by *Pgd* KD ($n = 15$, $F_{2,42} = 3.582$, $P = 0.0366$). Without *Pgd* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 0.61$, $P = 0.55$). (g) *Pgls* KD impaired memory after spaced training ($n = 12$, $F_{2,33} = 14.75$, $P = 0.00003$), but did not affect memory after massed training ($n = 12$, $F_{2,33} = 0.24$, $P = 0.79$). Memory at 2 h after 1x conditioning was not affected by *Pgls* KD ($n = 15$, $F_{2,42} = 2.078$, $P = 0.1379$). Without *Pgls* RNAi induction, memory after spaced training was normal ($n = 12$, $F_{2,33} = 3.11$, $P = 0.06$). Data are presented as mean $\pm$ SEM. Sample sizes for each genotype are listed in the legend in the order of bar appearance. Significance level of the least significant Tukey pairwise comparison following one-way analysis of variance (ANOVA): * $P < 0.05$; **** $P < 0.0001$; ns: not significant, $P > 0.05$.



Extended Data Fig. 2 | Controls for behavioural experiments, related to Fig. 1. (a) Memory after massed conditioning was not affected by *nAChRα7* KD in adult astrocytes ($n = 10$ (first group), $n = 11$ (second and third groups), $F_{2,29} = 1.07$, $P = 0.36$). Without *nAChRα7* RNAi induction, memory after spaced training was normal ($n = 12$ (first and second groups), $n = 11$ (third group), $F_{2,32} = 0.47$, $P = 0.63$). (b) Memories at 24 h were assessed with a second RNAi against *nAChRα7*. *nAChRα7* KD in adult astrocytes impaired memory after spaced training ($n = 13$ (first group), $n = 17$ (second and third groups), $F_{2,44} = 26.95$, $P = 0.00000002$), but did not affect memory after massed training ($n = 10$, $F_{2,27} = 0.24$, $P = 0.79$). Without *nAChRα7* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 0.71$, $P = 0.50$). (c) Relative expression of *Sod3* in astrocytes and α/β MB neurons according to published single-cell transcriptomics³³. Levels of *Sod3* mRNA are higher in astrocytes as compared to α/β MB neurons ($n = 1407$

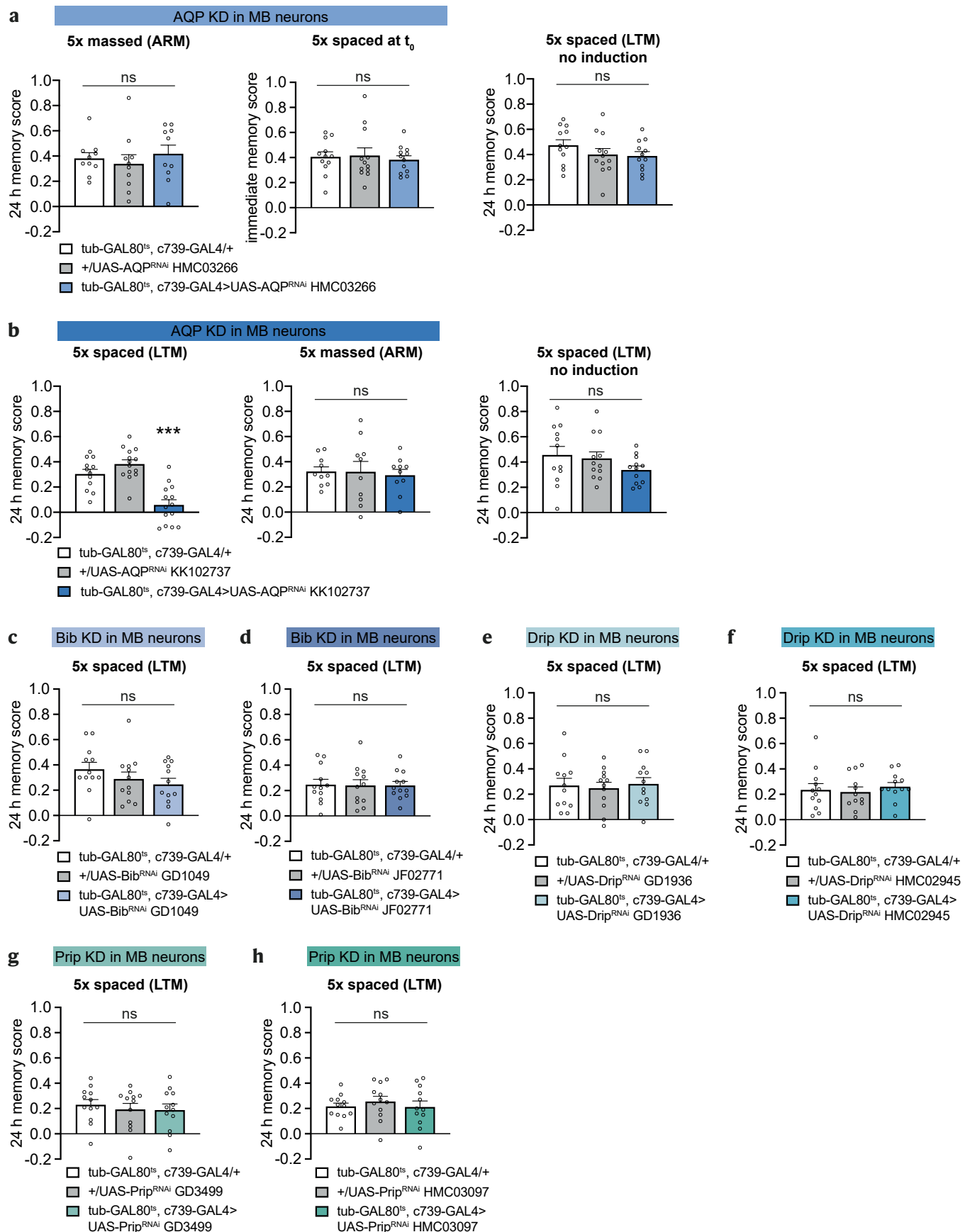
(first group), $n = 672$ (second group), $t_{2077} = 28.04$, $P < 0.000000000000001$). (d) Memory after massed conditioning was not affected by *Sod3* KD in adult astrocytes ($n = 10$ (first group), $n = 11$ (second and third groups), $F_{2,29} = 0.06$, $P = 0.94$). Without *Sod3* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 5.15$, $P = 0.01$). (e) Memories at 24 h were assessed with a second RNAi against *Sod3*. *Sod3* KD in adult astrocytes impaired memory after spaced training ($n = 12$, $F_{2,33} = 20.88$, $P = 0.000001$), but did not affect memory after massed training ($n = 12$, $F_{2,33} = 0.19$, $P = 0.83$). Without *Sod3* RNAi induction, memory after spaced training was normal ($n = 12$, $F_{2,33} = 1.35$, $P = 0.27$). Data are presented as mean $\pm$ SEM. Sample sizes for each genotype are listed in the legend in the order of bar appearance. Significance level of a two-sided t-test or the least significant Tukey pairwise comparison following one-way analysis of variance (ANOVA): **** $P < 0.0001$; ns: not significant, $P > 0.05$.



Extended Data Fig. 3 | See next page for caption.

Extended Data Fig. 3 | roGFP2-Tsa2ΔC_R imaging in MB neurons, related to Figs. 2 and 3. (a) Fluorescence excitation spectra of roGFP2-Tsa2ΔC_R upon 2-photon excitation in basal and oxidized states. H₂O₂-induced oxidation of the probe led to an increase in the excitation spectrum at 780 nm and a slight decrease at 988 nm, allowing ratiometric measurement of the oxidation level of the probe (ratio 780 nm/988 nm). (b) Intracellular pathways recruited during LTM formation in MB neurons. Upon spaced training, glucose intake in MB neuron cell bodies via the Glut1 transporter is increased to fuel the PPP, which produces reductive power in the form of NADPH²⁶. (c) Measurements of H₂O₂ levels in cell bodies and α lobes using the roGFP2-Tsa2ΔC_R H₂O₂ sensor expressed in MB neurons. H₂O₂ level (measured as 780/988 nm ratio) decreased in cell bodies upon spaced training (n = 13 (first group), n = 14 (second group), $t_{25} = 2.27$, $P = 0.03$), while it did not vary in α lobes (n = 15 (first group), n = 16 (second group), $t_{29} = 0.18$, $P = 0.86$). The H₂O₂ level in α lobes normalized to the cell bodies value is increased upon spaced training in adult MB neurons (values, bottom panel, same value as in Fig. 2c). (d) *Glut1* KD in MB neurons abolished the

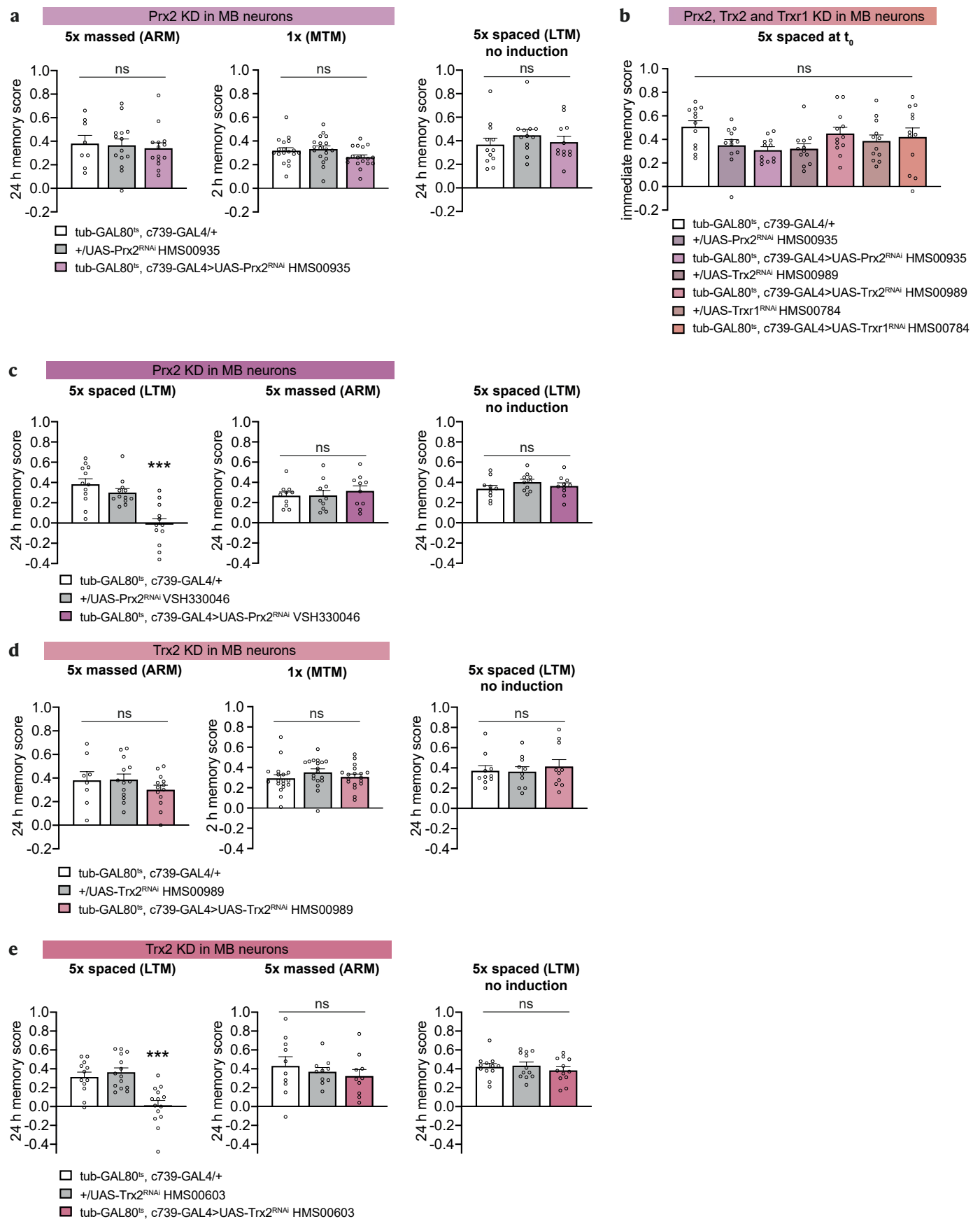
decrease in H₂O₂ level in cell bodies (n = 16 (first group), n = 15 (second group), $t_{29} = 0.25$, $P = 0.81$, top panel), revealing an increase in H₂O₂ level in α lobes after spaced training (n = 18, $t_{34} = 2.06$, $P = 0.047$, top panel). The H₂O₂ level in α lobes normalized to the cell bodies value is increased upon spaced training when *Glut1* was knocked down in adult MB neurons (n = 16 (first group), n = 15 (second group), $t_{29} = 2.66$, $P = 0.013$, bottom panel). (e) Memory after massed conditioning was not affected by human-secreted-catalase expression in astrocytes (n = 12, $F_{2,33} = 1.026$, $P = 0.9116$). Memory at 2 h after 1x conditioning was not affected by human-secreted-catalase expression in adult astrocytes (n = 10, $F_{2,27} = 1.171$, $P = 0.1304$). Without human-secreted-catalase induction, memory after spaced training was normal (n = 10, $F_{2,27} = 1.539$, $P = 0.8436$). Data are presented as mean ± SEM. Sample sizes for each genotype are listed in the legend in the order of bar appearance. Significance level of a two-sided t-test or the least significant Tukey pairwise comparison following one-way analysis of variance (ANOVA): * $P < 0.05$; ** $P < 0.01$; ns: not significant, $P > 0.05$.



Extended Data Fig. 4 | See next page for caption.

Extended Data Fig. 4 | Controls for behavioural experiments, and analysis of alternative *Drosophila* aquaporins, related to Fig. 4. (a) Memory after massed conditioning was not affected by *AQP* KD in adult α/β MB neurons ($n = 10$, $F_{2,27} = 0.40$, $P = 0.68$). Immediate memory after spaced conditioning was not affected by *AQP* KD in adult MB neurons ($n = 12$, $F_{2,33} = 0.1461$, $P = 0.8647$). Without *AQP* RNAi induction, memory after spaced training was normal ($n = 12$, $F_{2,33} = 1.20$, $P = 0.32$). (b) Memories at 24 h were assessed with a second RNAi against *AQP*. *AQP* KD in adult α/β MB neurons impaired memory after spaced training ($n = 12$ (first group), $n = 14$ (second and third groups), $F_{2,37} = 21.55$, $P = 0.0000006$), but did not affect memory after massed training ($n = 10$, $F_{2,27} = 0.08$, $P = 0.92$). Without *AQP* RNAi induction, memory after spaced training was normal

($n = 12$, $F_{2,33} = 1.40$, $P = 0.26$). (c) and (d) *Bib* KD in adult α/β MB neurons did not impair memory after spaced training (c: $n = 12$, $F_{2,33} = 1.327$, $P = 0.2791$; d: $n = 12$, $F_{2,33} = 0.0081$, $P = 0.99$). (e) and (f) *Drip* KD in adult α/β MB neurons did not impair memory after spaced training (e: $n = 12$, $F_{2,33} = 0.1146$, $P = 0.8921$; f: $n = 12$, $F_{2,33} = 0.2708$, $P = 0.7644$). (g) and (h) *Prip* KD in adult α/β MB neurons did not impair memory after spaced training (g: $n = 12$, $F_{2,33} = 0.2466$, $P = 0.7829$; h: $n = 12$, $F_{2,33} = 0.3601$, $P = 0.7003$). Data are presented as mean $\pm$ SEM. Sample sizes for each genotype are listed in the legend in the order of bar appearance. Significance level of the least significant Tukey pairwise comparison following one-way analysis of variance (ANOVA): **** $P < 0.0001$; ns: not significant, $P > 0.05$.

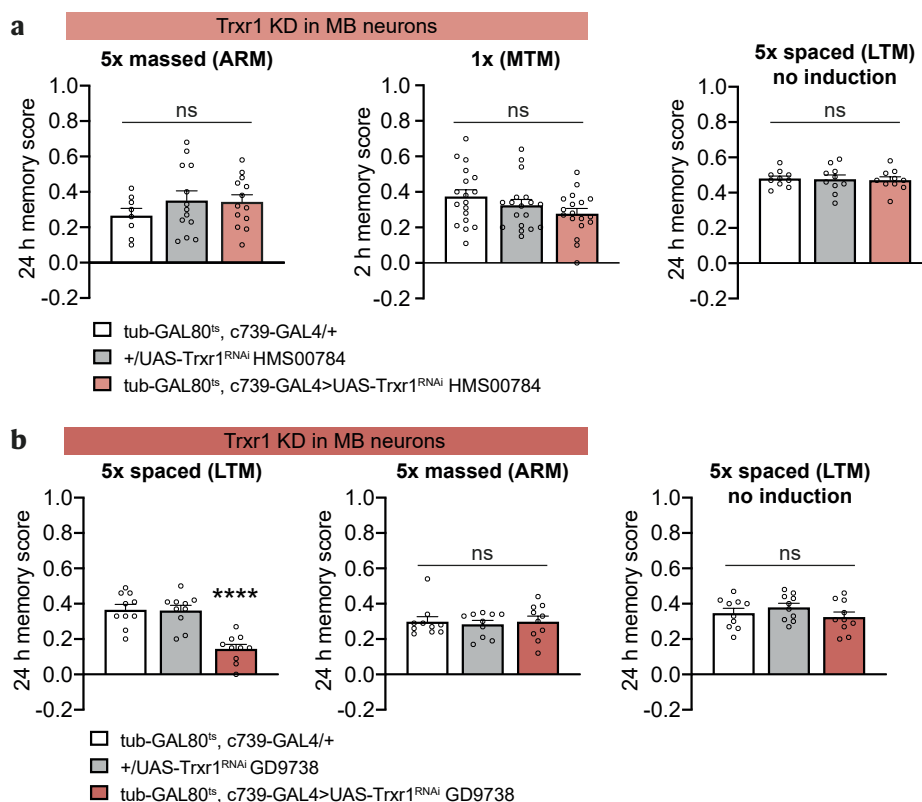


Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | Controls for behavioural experiments, related to Fig. 4.

(a) Memory after massed conditioning was not affected by *Prx2* KD in adult α/β MB neurons ($n = 8$ (first group), $n = 14$ (second and third groups), $F_{2,33} = 0.13$, $P = 0.88$). Memory at 2 h after 1x conditioning was not affected by *Prx2* KD in adult α/β MB ($n = 12$, $F_{2,51} = 2.288$, $P = 0.1118$). Without *Prx2* RNAi induction, memory after spaced training was normal ($n = 12$, $F_{2,33} = 0.64$, $P = 0.53$). (b) Immediate memory after spaced conditioning was not affected by *Prx2*, *Trx2* or *Trxr1* KD in adult α/β MB ($n = 12$, $F_{6,77} = 1.958$, $P = 0.0821$). (c) Memories at 24 h were assessed with a second RNAi against *Prx2*. *Prx2* KD in adult α/β MB neurons impaired memory after spaced training ($n = 12$, $F_{2,33} = 16.48$, $P = 0.00001$), but did not affect memory after massed training ($n = 10$, $F_{2,27} = 0.33$, $P = 0.73$). Without *Prx2* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 1.09$, $P = 0.35$). (d) Memory after massed conditioning was not affected by *Trx2* KD in adult α/β

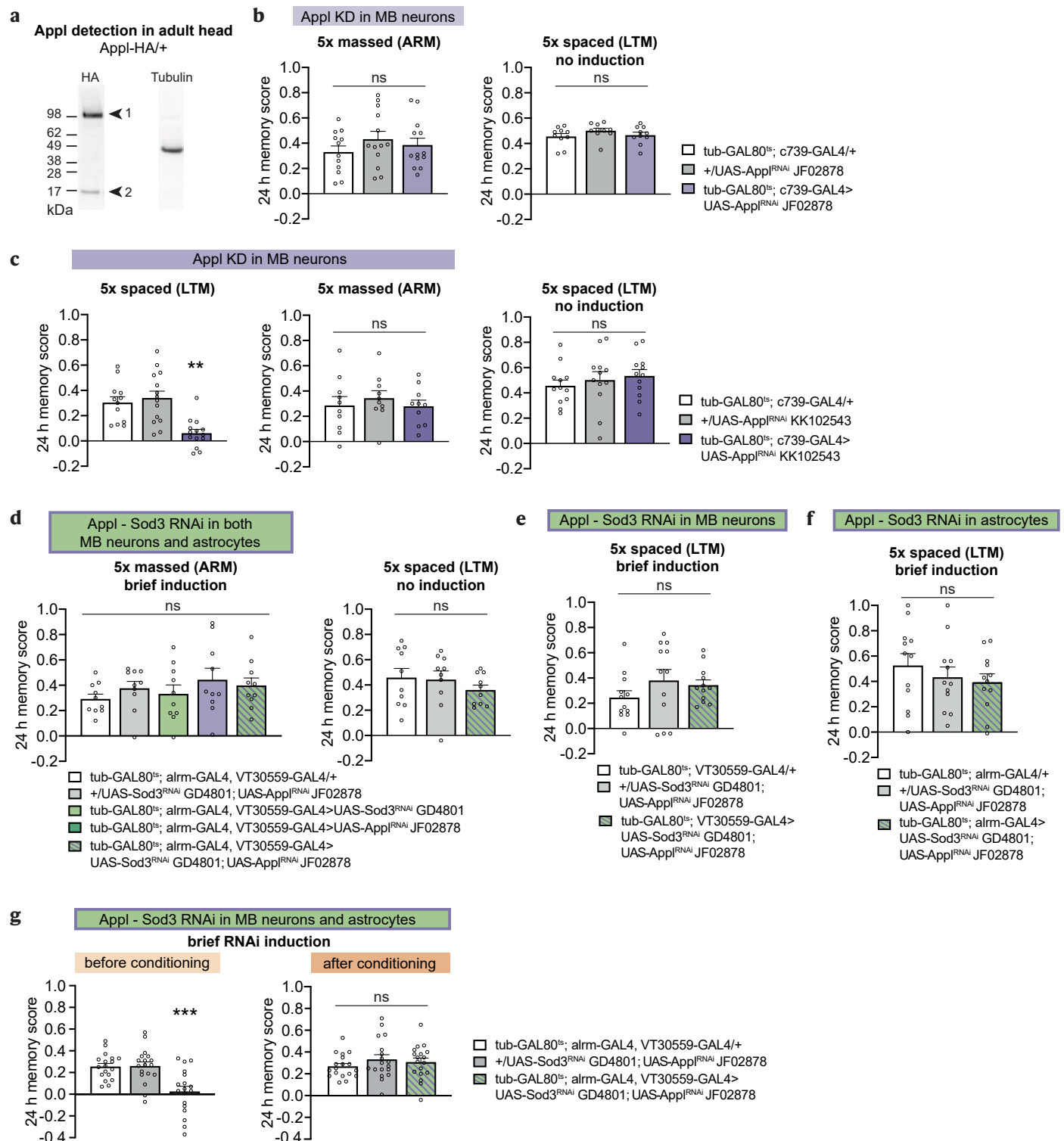
MB neurons ($n = 8$ (first group), $n = 13$ (second and third groups), $F_{2,31} = 0.99$, $P = 0.38$). Memory at 2 h after 1x conditioning was not affected by *Trx2* KD in adult α/β MB ($n = 18$, $F_{2,51} = 0.9106$, $P = 0.4087$). Without *Trx2* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 0.23$, $P = 0.80$). (e) Memories at 24 h were assessed with a second RNAi against *Trx2*. *Trx2* KD in adult α/β MB neurons impaired memory after spaced training ($n = 12$ (first group), $n = 14$ (second and third groups), $F_{2,37} = 14.43$, $P = 0.00002$), but did not affect memory after massed training ($n = 10$, $F_{2,27} = 0.54$, $P = 0.59$). Without *Trx2* RNAi induction, memory after spaced training was normal ($n = 12$, $F_{2,33} = 0.47$, $P = 0.63$). Data are presented as mean $\pm$ SEM. Sample sizes for each genotype are listed in the legend in the order of bar appearance. Significance level of the least significant Tukey pairwise comparison following one-way analysis of variance (ANOVA): *** $P < 0.001$; ns: not significant, $P > 0.05$.



Extended Data Fig. 6 | Complementary controls for behavioural experiments, related to Fig. 4.

(a) Memory after massed conditioning was not affected by *Trxr1* KD in adult α/β MB neurons ($n = 8$ (first group), $n = 13$ (second and third groups), $F_{2,31} = 0.81$, $P = 0.45$). Memory at 2 h after 1x conditioning was not affected by *Trxr1* KD in adult α/β MB ($n = 18$, $F_{2,51} = 2.075$, $P = 0.1361$). Without *Trxr1* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 0.05$, $P = 0.95$). **(b)** Memories at 24 h were assessed with a second RNAi against *Trxr1*. *Trxr1* KD in adult α/β MB neurons impaired memory after spaced

training ($n = 10$, $F_{2,27} = 20.30$, $P = 0.000004$), but did not affect memory after massed training ($n = 10$, $F_{2,27} = 0.09$, $P = 0.92$). Without *Trxr1* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 1.07$, $P = 0.36$). Data are presented as mean $\pm$ SEM. Sample sizes for each genotype are listed in the legend in the order of bar appearance. Significance level of the least significant Tukey pairwise comparison following one-way analysis of variance (ANOVA): **** $P < 0.0001$; ns: not significant, $P > 0.05$.

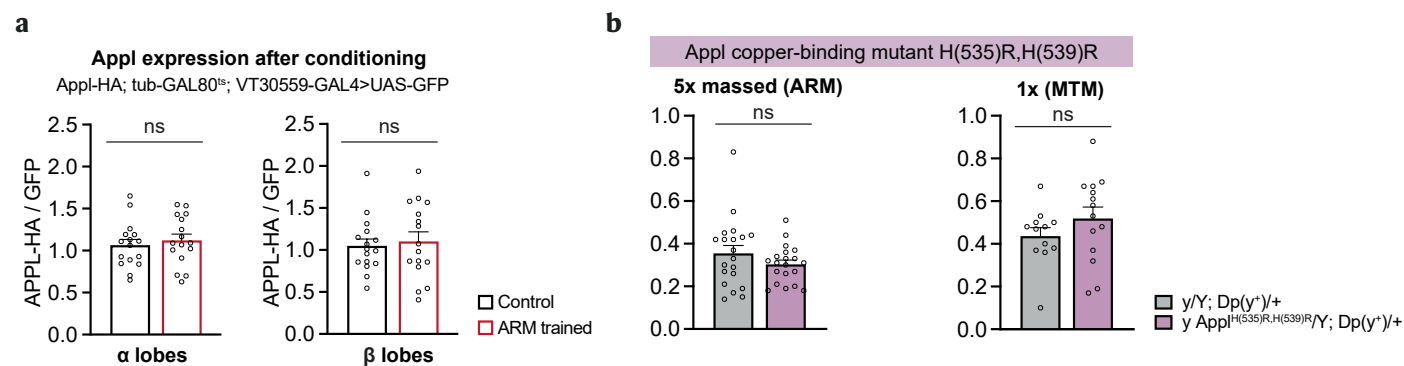


Extended Data Fig. 7 | See next page for caption.

Extended Data Fig. 7 | APPL-HA western analysis and additional behavioural experiments, related to Fig. 5.

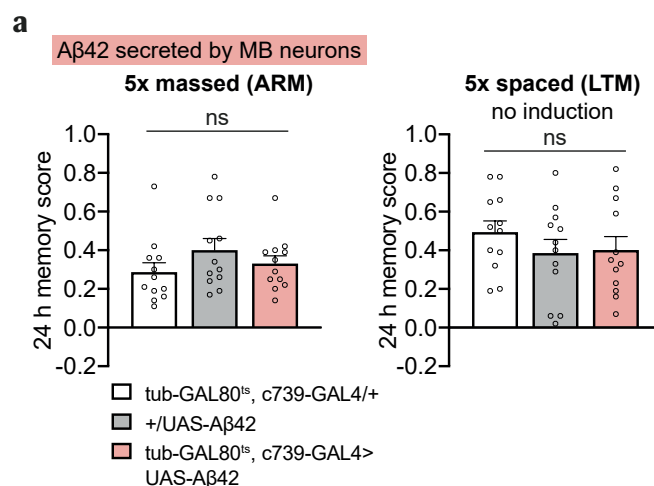
(a) Western blot against HA-tagged Appl. The expected size bands are observed at 99 kDa (label 1) and 15 kDa (label 2), corresponding to full-length APPL and the CTF- α fragment, respectively. CTF- α is generated by α -secretase cleavage of full-length Appl. Tubulin was used as a loading control. Twenty replicates of this Western analysis were performed. **(b)** Memory after massed conditioning was not affected by *Appl* KD in adult α/β MB neurons ($n = 12$ (first group), $n = 13$ (second and third groups), $F_{2,35} = 0.84$, $P = 0.44$). Without *Appl* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 1.19$, $P = 0.32$). **(c)** Memories at 24 h were assessed with a second RNAi against *Appl*. *Appl* KD in adult α/β MB neurons impaired memory after spaced training ($n = 12$ (first group), $n = 14$ (second and third groups), $F_{2,37} = 11.84$, $P = 0.0001$), but did not affect memory after massed training ($n = 10$, $F_{2,27} = 0.36$, $P = 0.70$). Without *Appl* RNAi induction, memory after spaced training was normal ($n = 12$, $F_{2,33} = 0.52$, $P = 0.60$). **(d)** Mild decreases in both *Appl* and

Sod3 expression in adult MB neurons and astrocytes did not affect memory after massed training ($n = 10$, $F_{4,45} = 0.83$, $P = 0.515$). Without *Appl* and *Sod3* RNAi induction, memory after spaced training was normal ($n = 10$, $F_{2,27} = 0.72$, $P = 0.50$). **(e)** Mild decreases in both *Appl* and *Sod3* expression in adult MB neurons alone did not affect memory after spaced training ($n = 12$, $F_{2,33} = 1.18$, $P = 0.32$). **(f)** Mild decreases in both *Appl* and *Sod3* expression in adult astrocytes alone did not affect memory after spaced training ($n = 12$, $F_{2,33} = 0.70$, $P = 0.50$). **(g)** Brief 1 day induction of both *Appl* and *Sod3* RNAi in astrocytes and MB neurons before spaced conditioning impaired 24 h memory ($n = 18$, $F_{2,51} = 11.88$, $P = 0.00006$), whereas brief 1 day induction of both *Appl* and *Sod3* RNAi after spaced conditioning did not impair 24 h memory ($n = 18$, $F_{2,51} = 0.8017$, $P = 0.4541$). Data are presented as mean $\pm$ SEM. Sample sizes for each genotype are listed in the legend in the order of bar appearance. Significance level of the least significant Tukey pairwise comparison following one-way analysis of variance (ANOVA): ** $P < 0.01$; *** $P < 0.001$; ns: not significant, $P > 0.05$.



Extended Data Fig. 8 | Controls for immunochemical and behavioural experiments, related to Fig. 6. (a) No change of Appl expression in MB α and β lobes after massed training (α lobes: $n = 16$, $t_{30} = 0.5812$, $P = 0.5655$; β lobes: $n = 16$, $t_{30} = 0.3759$, $P = 0.7096$). (b) Memory after massed conditioning was not affected in *Appl*^{H(535)R,H(539)R} mutant ($n = 20$, $t_{38} = 1.244$, $P = 0.2209$). Memory after

1x conditioning was not affected in *Appl*^{H(535)R,H(539)R} mutant ($n = 12$ (first group), $n = 14$ (second group), $t_{24} = 1.191$, $P = 0.2452$). Data are presented as mean $\pm$ SEM. Sample sizes for each genotype are listed in the legend in the order of bar appearance. Significance level of a two-sided t-test: ns: not significant, $P > 0.05$.



Extended Data Fig. 9 | Controls for behavioural experiments, related to

Fig. 7. (a) Memory after massed conditioning was not affected by secreted human A β 42 secretion by adult MB neurons ($n = 12$, $F_{2,33} = 1.31$, $P = 0.28$). Without A β 42 induction, memory after spaced training was normal ($n = 12$, $F_{2,33} = 0.77$,

$P = 0.47$). Data are presented as mean $\pm$ SEM. Sample sizes for each genotype are listed in the legend in the order of bar appearance. Significance level of the least significant Tukey pairwise comparison following one-way analysis of variance (ANOVA): ns: not significant, $P > 0.05$.

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Software and code

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Data collection	In vivo imaging data were obtained on two distinct commercial Leica microscopes and acquired through the Leica proprietary softwares.
Data analysis	- Statistical analysis was done using GraphPad Prism 8 (GraphPad Software). - H2O2 and calcium in vivo imaging data were analyzed manually (ROI tracing and intensity calculation) using LAS-X (3.5.7.23225) and MATLAB (9.4.0.813654) softwares. - Figures were designed on Adobe illustrator 2022.

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No datasets that require mandatory deposition into a public database were generated during the current study. Source data that are reported as graphs on figures

and extended data figures are available as Supplementary Information alongside the paper. Additional raw data will be shared with no restriction by the corresponding authors upon request.

This study made use of the SCOPE database of single-cell transcriptomics in the fly brain to compare Sod3 expression in neurons and glia (Extended Data Fig. 2c) (https://scope.aertslab.org/#/Davie_et_al_Cell_2018/Davie_et_al_Cell_2018%2FAerts_Fly_AdultBrain_Filtered_57k.loom/gene).

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Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

Primary antibodies:

- rat anti-HA, Roche, # ROAHAHA 11867423001, clone 3F10.
- mouse anti-HA, Biolegend # 901513, clone 16B12.
- mouse anti-tubulin, Sigma Aldrich # T16199, clone DM1A.
- mouse anti-GFP Developmental Studies Hybridoma Bank, # DSHB-GFP-12A6, clone 12A6

Secondary antibodies:

- goat anti-rat Alexa 488 Invitrogen # A11029
- HRP anti-mouse Promega # W4021
- goat anti-rat Alexa 594 Invitrogen # A1107

Validation

- rat anti-HA: doi:10.1242/bio.060335
- mouse anti-HA: doi.org/10.1038/s41467-021-20898-x
- mouse anti-alpha tubulin: doi: 10.1111/accel.13137
- mouse anti GFP: doi: 10.3390/biology10080729

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals

Drosophila melanogaster flies were raised on standard food medium containing yeast, cornmeal and agar, on a 12h:12h light-dark cycle at 18°C with 60% humidity. The Canton-Special (CS) strain was used as the wild-type strain. All lines were out-crossed for at least three generations to flies carrying a CS wild-type background. The study was performed on 0-3-day-old adult flies. For behavior experiments, both male and female flies were used. For imaging experiments, female flies were used.

List of Drosophila strains:

tub-GAL80ts; alrm-GAL4 (de Tredern et al. 2021)
 tub-GAL80ts; c739-GAL4 (Turrel et al. 2018)
 tub-GAL80ts, 13F02-LexA; VT30559-GAL4 (de Tredern et al. 2021)
 tub-GAL80ts, 13F02-LexA; alrm-GAL4 (This study)
 tub-GAL80ts; alrm-GAL4, VT30559-GAL4 (This study)
 86E01-LexA (Bloomington Drosophila Stock Center (BDSC) 54287)
 tub-GAL80ts, 86E01-LexA; VT30559-GAL4 (This study)
 UAS-NoxRNAi HMS00429 (BDSC 32433)
 UAS-NoxRNAi KK111991 (Vienna Drosophila Resource Center (VDRC) 102559)
 UAS-G6PDRNAi KK108898 (VDRC 101507)
 UAS-PgdRNAi HMC05959 (BDSC 65078)
 UAS-PglRNAi HMS02626 (BDSC 42933)
 UAS-nAChRa7RNAi JF02570 (BDSC 27251)
 UAS-nAChRa7RNAi KK108471 (VDRC 100756)
 UAS-Sod3RNAi GD4801 (VDRC 37793)
 UAS-Sod3RNAi GD3774 (VDRC 8760)
 UAS-Glut1RNAi KK108683 (VDRC 101365)
 UAS-AQPRNAi HMC03266 (BDSC 51504)
 UAS-AQPRNAi KK102737 (VDRC 109314)
 UAS-Prx2RNAi HMS00935 (BDSC 34971)
 UAS-Prx2RNAi VSH330046 (VDRC 330046)
 UAS-Trx2RNAi HMS00989 (BDSC 34019)
 UAS-Trx2RNAi HMS00603 (BDSC 33721)
 UAS-Trxr1RNAi HMS00784 (BDSC 32984)
 UAS-Trxr1RNAi GD9738 (VDRC 47308)
 UAS-AppRNAi JF02878 (BDSC 28043)
 UAS-AppRNAi KK102543 (VDRC 108312)
 UAS-Aß42 (Finelli et al. 2004)
 UAS-GCaMP6f (BDSC 42747)
 LexAop-GCaMP6f (BDSC 44277)
 LexAop-GCaMP6f; UAS-Aß42 (This study)

UAS-GCaMP6f; UAS-nAChRa7RNAi JF02570 (This study)
 LexAop-roGFP-Tsa2DCR (This study)
 LexAop-roGFP-Tsa2DCR; UAS-Glut1RNAi KK108683 (This study)
 LexAop-roGFP-Tsa2DCR; UAS-NoxRNAi HMS00429 (This study)
 LexAop-roGFP-Tsa2DCR; UAS-G6PDRNAi KK108898 (This study)
 LexAop-roGFP-Tsa2DCR; UAS-nAChRa7RNAi JF02570 (This study)
 LexAop-roGFP-Tsa2DCR; UAS-Sod3RNAi GD4801 (This study)
 LexAop-roGFP-Tsa2DCR; UAS-AQPRNAi HMC03266 (This study)
 LexAop-roGFP-Tsa2DCR; UAS-ApplRNAi JF02878 (This study)
 LexAop-roGFP-Tsa2DCR; UAS-A842 (This study)
 APPL-HA (This study)
 APPL-HA; tub-GAL80ts; VT30559-GAL4 (this study)
 y ApplH(535)R,H(539)R (this study)
 PBac{y[+]-attP-3B}VK00033 (BDSC 9750)
 UAS-human-secreted-catalase (Dhawan et al. 2021)
 UAS-BibRNAi GD1049 (VDRC 8893)
 UAS-BibRNAi JF02771 (BDSC 27691)
 UAS-DripRNAi GD1936 (VDRC 51936)
 UAS-DripRNAi HMC0945 (BDSC 44661)
 UAS-PripRNAi GD3499 (VDRC 8124)
 UAS-PripRNAi HMC03097 (BDSC 50695)

Wild animals

The study did not involve wild animal

Reporting on sex

For behavior experiments, mixed populations of males and females were used. For imaging experiments, females were used.

Field-collected samples

The study did not involve samples collected from the field

Ethics oversight

No ethical approval or guidance was required since in this study we used *Drosophila melanogaster*.

Note that full information on the approval of the study protocol must also be provided in the manuscript.