Dopaminergic Modulation of Short-Term Associative Memory in *Caenorhabditis elegans*

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Abstract

Forgetting, the inability to retrieve previously encoded memories, is an active process involving neurotransmission, second messenger signalling, and cytoskeletal modifications. Forgetting is thought to be essential to remove irrelevant memories and to increase the capacity to encode new memories. Therefore, identifying key regulators of active forgetting is crucial to advance our understanding of neuroplasticity. In this study, we utilised the compact and tractable *Caenorhabditis elegans* model to investigate the role of the neurotransmitter dopamine in forgetting. We conducted butanone associative learning assays based on an established protocol and used mutant strains deficient in dopamine synthesis (tyrosine hydroxylase CAT-2 and dopamine transporter DAT-1) and signalling (G protein-coupled receptors DOP-1, DOP-2, and DOP-3) to assess the impact on learning and memory retention. Learning was measured immediately post-training, and memory retention was evaluated every 0.5 hours up to 2 hours. Our results show that animals lacking dopamine display a modest enhancement in learning relative to wild-type, with the learned association persisting for at least 2 hours after training. We also found that the D2-like receptors DOP-2 and DOP-3 function together to modulate the

23 forgetting process, with D1-like receptor DOP-1 having an antagonistic effect. Furthermore, re-
24 expression of CAT-2 tyrosine hydroxylase in ADE or CEP neurons showed that dopamine
25 synthesis in both neuron types is essential for short-term memory retention, albeit at different
26 time points after learning. These findings highlight the critical role of dopamine in forgetting,
27 consistent with findings in *Drosophila*, and suggest potential relevance for understanding
28 memory retention during healthy ageing and in conditions with dopamine imbalances such as
29 Parkinson's disease.

30

31 **Introduction**

32 The nematode *Caenorhabditis elegans* serves as an excellent model for studying the molecular
33 regulation of memory due to its genetic tractability, well-characterised nervous system, and
34 capacity to learn and remember in ways analogous to higher organisms (Ardiel & Rankin 2010).
35 In *C. elegans* and other animals, memory is categorised into short-term and long-term, based on
36 how it is encoded and its susceptibility to decay (Kandel *et al.* 2014). Conversely, forgetting
37 involves the inability to retrieve previously encoded memories. Research indicates that forgetting
38 is an active process requiring protein synthesis, and its regulation involves processes related to
39 neurotransmission, second messenger signalling, and cytoskeletal modifications (Stein &
40 Murphy 2014; Liu *et al.* 2022). Identifying the key modulators of active forgetting and
41 understanding their mechanisms, distinct from those involved in learning and short- or long-term
42 memory retention, is crucial to advance our knowledge of neuroplasticity.

43 Studies investigating the underlying mechanisms involved in memory regulation and forgetting
44 have reported diverse molecular pathways. Two major regulatory mechanisms have been

identified: 1) the TIR-1 and MAP kinase/JNK pathway, along with associated proteins (membrane protein MACO-1 and receptor tyrosine kinase SCD-2 and its ligand HEN-1) play roles in olfactory adaptation (Inoue *et al.* 2013; Kitazono *et al.* 2017; Arai *et al.* 2022), while 2) the RNA-binding protein MSI-1 regulates forgetting by downregulating the Arp2/3 complex (Hadziselimovic *et al.* 2014) in olfactory conditioning. Other actin regulatory proteins such as α-adducin ADD-1 and Rho family G proteins are implicated in memory loss, albeit with RAC-2 acting independently of actin dynamics (Vukojevic *et al.* 2012; Bai *et al.* 2022). Neurotransmitters such as serotonin and acetylcholine also regulate memory retention and forgetting in *C. elegans* (Liu *et al.* 2022; Niu *et al.* 2022). In addition, dopamine plays a crucial role in modulating short-term associative memory in *C. elegans* and other organisms (McMillen & Chew 2023). Dopamine's role in forgetting is well-documented in studies of aversive olfactory learning in *Drosophila melanogaster*: blocking dopaminergic neurons after learning enhances memory retention, while stimulation decreases or eliminates it (Berry *et al.* 2012; Berry *et al.* 2015; Berry *et al.* 2018). To determine if dopamine's role in forgetting is conserved across species, in this study, we utilised the compact and tractable *C. elegans* to dissect the effect of dopamine on forgetting and to identify the neural circuits involved.

To do this, we conducted butanone associative learning assays according to (Kauffman *et al.* 2011) and tested mutants deficient in dopamine synthesis and signalling. Behavioural tests for forgetting in *C. elegans* involve assessing learned behaviours at various time points post-conditioning to observe when responses revert to baseline levels. Our data indicate that dopamine-deficient animals exhibit a slight improvement in learning compared to wild-type, and the memory of this learned association is retained for at least 2 hours post-training. Additionally, we found that DOP-2 and DOP-3 D2-like receptors work together to modulate the forgetting of

68 this learned association. Furthermore, cell-specific re-expression of CAT-2 tyrosine hydroxylase
69 in dopaminergic neurons suggests that dopamine from both ADE and CEP neurons is required
70 for short-term memory retention, albeit at different times post-training. Taken together, our data
71 showing that dopamine signals are required for forgetting is consistent with findings in other
72 invertebrates (Berry *et al.* 2012). These findings highlight the critical role of dopamine in the
73 process of forgetting and suggest implications for understanding the pathological mechanisms
74 that contribute to cognitive deficits in neurodegenerative diseases such as Parkinson's disease.

75

76 **Results**

77 ***Worms lacking dopamine show extended short-term associative memory.***

78 Dopamine regulates both non-associative and associative learning in *C. elegans* (McMillen &
79 Chew 2023). To determine if dopamine regulates forgetting, we used the olfactory associative
80 memory paradigm (**Figure 1A**) (Kauffman *et al.* 2011) to assess associative memory over time in
81 mutants deficient in dopamine signalling. Briefly, worms are conditioned with the odour
82 butanone (conditioned stimulus) and presence of food (unconditioned stimulus) for 1 hour on
83 solid media, before being placed onto 'hold' plates containing food but no butanone for 0.5 to 2
84 hours. Then, worm populations are assessed for odour preference in a two-choice chemotaxis
85 assay as in (Kauffman *et al.* 2011), where high chemotaxis indices (CIs) indicate strong butanone
86 attraction, and CIs closer to zero represent neutral butanone attraction. 'Naïve' worms do not
87 undergo conditioning and are placed onto chemotaxis plates immediately, while the 'Timepoint
88 0' (t=0) cohort are placed onto chemotaxis plates directly after conditioning. Conditioned worms
89 undergo a 1 hour 'pre-starvation' step to increase the value of the unconditioned stimulus (food)

(Kauffman *et al.* 2011) (**Figure 1Aii)**. To simplify the visualisation of behavioural assay data, we only display comparisons between genotypes at each post-conditioning time point, and between naive and t=0 for wild-type and other genotypes to show if learning has occurred. Other statistical comparisons are described in the text. After conditioning (t=0), wild-type worms show an increased attraction to butanone (shown by a higher chemotaxis index, CI) compared with the naïve cohort, indicating the capacity for associative olfactory learning. This memory decreases over time, returning to baseline (naïve) levels after ~0.5 hours (comparison between CIs of naïve and 0.5 h post-conditioning cohorts: p-value = 0.6754; one-way ANOVA with Dunnett's post-test) (**Figure 1B**).

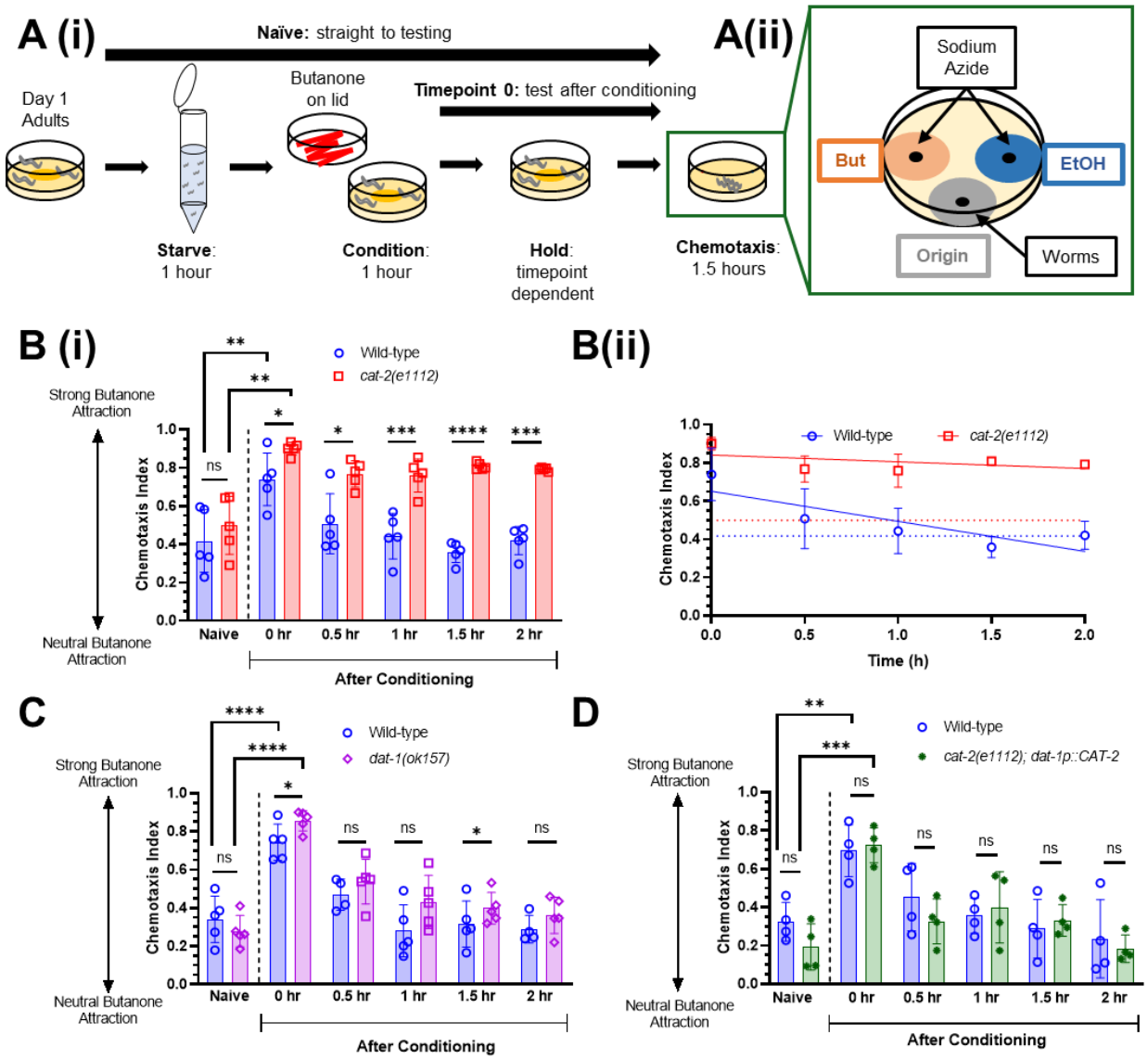
We first tested the *cat-2* tyrosine hydroxylase mutant strain that lacks the enzyme required to synthesise dopamine (Lints & Emmons 1999). These worms have substantially reduced dopamine levels (Sanyal *et al.* 2004). Interestingly, *cat-2* mutant worms show a small but significant improvement in learning, and an increased attraction to butanone for up to 2 hours (comparison between CIs of *cat-2* naïve and 2 h post-conditioning cohorts: p-value <0.0001; one-way ANOVA with Dunnett's post-test), suggesting they retain the memory of the learnt association for a longer period compared with wild-type controls (**Figure 1B**). As a complementary method to compare the rate of memory loss (i.e., the rate of forgetting) between genotypes, we performed linear regression analysis on the chemotaxis indices of conditioned worms at post-conditioning time points (t= 0, 0.5, 1, 1.5, 2). We then compared the slopes of the lines of best fit between the tested genotypes. This is shown for data from wild-type and *cat-2* mutant worms in **Figure 1Bii**. Statistical comparisons from linear regression analysis of short-term associative memory assays in **Figure 1** (and other Figures) are summarised in **Table 1**. We found that the slopes for wild-type (-0.1575) and *cat-2* mutants (-0.0353) are significantly

different, with a p-value of 0.0049 (**Table 1**), consistent with *cat-2* worms showing a slower rate of memory loss.

To determine if a milder reduction in dopamine has the same effect, we next tested *dat-1* mutant worms with a mutation in the DAT-1 sodium-dependent dopamine transporter. These worms are able to synthesise dopamine; however, dopamine reuptake from the synaptic cleft is impaired, reducing the amount of residual dopamine in the nervous system (Formisano *et al.* 2020; Kindt *et al.* 2007). *dat-1* mutant worms showed improved learning compared with wild-type controls immediately after conditioning (t=0) and, unlike wild-type, retained memory of the learnt behaviour at t=0.5 (comparison between naïve and t=0.5 cohorts: p-value <0.01 for *dat-1*, p-value >0.05 for wild-type; one-way ANOVA with Dunnett's post-test). However, *dat-1* mutant worms do not display an extended memory phenotype like *cat-2* mutants (comparisons between naïve and time points after t=0.5: all p-values >0.05; one-way ANOVA with Dunnett's post-test) (**Figure 1C**). In addition, linear regression analysis of the rate of memory loss for wild-type and *dat-1* showed that these slopes were not significantly different (**Table 1**). This suggests that while excess dopamine in the synaptic cleft (as seen in *dat-1* mutants) leads to a memory retention phenotype at 0.5 hours, newly synthesized dopamine is necessary for maintaining memory retention up to 2 hours post-conditioning.

Finally, we tested if re-expression of CAT-2 in dopaminergic neurons (using the *dat-1* promoter) rescues the extended memory phenotype in *cat-2* mutants. We found that transgenic worms re-expressing CAT-2 phenocopied wild-type animals, with no improved learning compared with wild-type at t=0, and memory returned to baseline levels from t=0.5 onwards (comparisons between naïve and t=0.5: p-values >0.05 for both wild-type and CAT-2 rescue worms; one-way ANOVA with Dunnett's post-test) (**Figure 1D**). Linear regression analysis of the rate of memory

136 loss for both strains showed that these slopes were not significantly different (**Table 1**). This
137 indicates that the enzyme required for dopamine synthesis, CAT-2, is required for forgetting and
138 acts in the dopaminergic neurons.



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140 **Figure 1: Worms lacking dopamine show extended short-term associative memory. A) (i)** Schematic
141 of butanone associative learning and short-term memory assay, adapted from (Kauffman *et al.* 2011). Naïve
142 worms are placed directly onto chemotaxis assay plates, while conditioned worms are first starved for 1
143 hour in M9 buffer prior to conditioning on plates with food and butanone (streaked on lid). After
144 conditioning, worms are transferred to 'hold' plates with food (no odour) for 0.5–2 hours before being

placed onto chemotaxis plates. **(ii)** Layout of chemotaxis assay plate indicating where butanone (But), ethanol (EtOH) and sodium azide are spotted. Worms are placed on the 'origin', as indicated. **B) (i)** Wild-type and *cat-2(e1112)* mutant worms tested for butanone associative memory (n = 5 biological replicates). **(ii)** Linear regression of mean CIs for each time point, compared between genotypes. Dashed line indicates CI of 'naïve' cohort for both genotypes. **C)** Wild-type and *dat-1(ok157)* mutant worms tested for butanone associative memory (n = 5 biological replicates). **D)** Wild-type and *cat-2* 'rescue' worms re-expressing CAT-2 in the dopaminergic neurons tested for butanone associative memory (n = 4 biological replicates). For butanone assays (**Bi, C, D**), each data point represents one biological replicate (which includes four technical replicates). Each technical replicate consists of 50-250 worms. Behaviour was quantified using chemotaxis indices (CI) for naïve/untrained worms and conditioned animals at 0, 0.5-, 1-, 1.5- and 2-hours post-conditioning. Statistical analysis: a pairwise t-test was performed to compare genotypes for each time point, and a one-way ANOVA with Tukey's multiple comparisons test was performed to compare post-conditioning t=0 with naïve chemotaxis indices (CIs) between genotypes. Error bars = mean ± SD. p-values are represented by **** ≤ 0.0001; *** ≤ 0.001, ** ≤ 0.01; * ≤ 0.05; ns = non-significant.

Dopamine receptors act synergistically to modulate short-term memory.

In *C. elegans*, dopamine receptors are categorised as ligand-gated ion channels (LGIC) or G protein-coupled receptors (GPCR). Dopaminergic GPCRs are divided into D1-like and D2-like receptors, which stimulate or inhibit adenylyl cyclase, respectively (Chase & Koelle 2007; Girault & Greengard 2004; Mersha *et al.* 2013). DOP-1 is a D1-like receptor, while DOP-2 and DOP-3 are D2-like receptors (Chase & Koelle 2007; Mersha *et al.* 2013) (**Figure 2A**). We first tested *dop-1, dop-2* and *dop-3* single mutants to determine if they showed a memory retention phenotype similar to *cat-2* mutant worms. We found that none of the single mutants showed statistically significant learning or memory differences compared with wild-type controls (**Figure 2B**): all mutants showed intact learning at t=0, and butanone preference had returned to baseline (naïve) levels at t=0.5 (comparisons between naïve and t=0.5: p-values >0.05 for all genotypes; one-way ANOVA with Dunnett's post-test). Linear regression analysis also showed

172 no statistically significant differences between slopes for single mutants compared with wild-
173 type controls for data in **Figure 2Bi, ii** and **iii** (**Table 1**). This indicates that dopamine does not
174 regulate forgetting by acting through DOP-1, DOP-2 or DOP-3 alone.

175 One possibility is that these receptors could be acting in a functionally redundant manner for
176 dopamine signalling to modulate forgetting in *C. elegans*, as in other behaviours such as bacterial
177 avoidance (Chou *et al.* 2022) or the escape response to olfactory cues (Baidya *et al.* 2014).
178 Therefore, we next tested a *dop-1;dop-2;dop-3* triple mutant strain and found that they displayed
179 similar memory compared with wild-type controls. Although their CIs were higher than wild-
180 type at 1,1.5 and 2 h post-conditioning, likely due to their increased preference for butanone in
181 naïve worms, comparisons between naïve and post-conditioning CIs for all time points within the
182 triple mutant strain were not statistically significant ($p>0.05$, one-way ANOVA with Dunnett's
183 post-test) (**Figure 2C**). Moreover, linear regression analysis revealed that the slopes for wild-
184 type and *dop-1;dop-2;dop-3* triple mutants were not significantly different (**Table 1**). We
185 postulated that there could be an antagonistic effect between D1 and D2-like dopamine receptors
186 that could be masking the effects of individual receptors (Chase *et al.* 2004), and so we
187 subsequently tested double mutants for *dop-1;dop-2*, *dop-1;dop-3*, and *dop-2;dop-3* for their
188 capacity to retain memory over time (**Figure 2D**). As in other assays, we found that wild-type
189 controls returned to baseline (naïve) CI values by t=0.5 (or t=1 for **Figure 2Di**). This was also
190 the case for *dop-1;dop-2* (**Figure 2Di**) and *dop-1;dop-3* (**Figure 2Dii**) double mutants, which
191 returned to baseline CI levels by t=1 and t=0.5, respectively. In contrast, the *dop-2;dop-3* double
192 mutants showed elevated CIs post-conditioning that had not returned to baseline by t=2
193 (comparisons between naïve and t>0: p-values <0.01 for all time points; one-way ANOVA with
194 Dunnett's post-test) (**Figure 2Diii**). Linear regression analysis also showed that the slopes for

195 wild-type and *dop-2;dop-3* are close to significantly different (p-value =0.06) (**Table 1**). This
196 suggests that the D2-like receptors DOP-2 and DOP-3 act synergistically to modulate forgetting
197 of an olfactory associative memory, and that the D1-like receptor DOP-1 may antagonise this
198 effect.

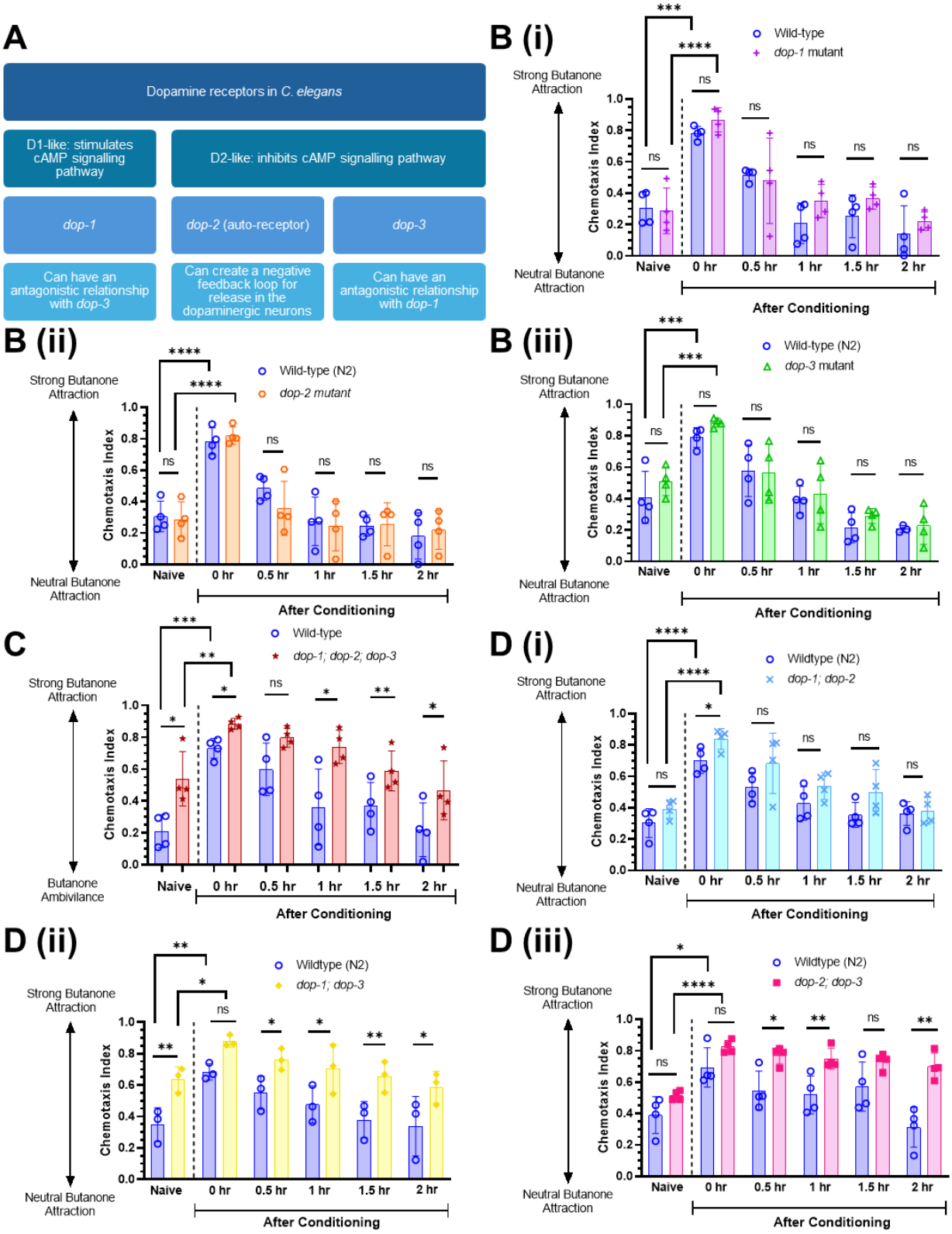


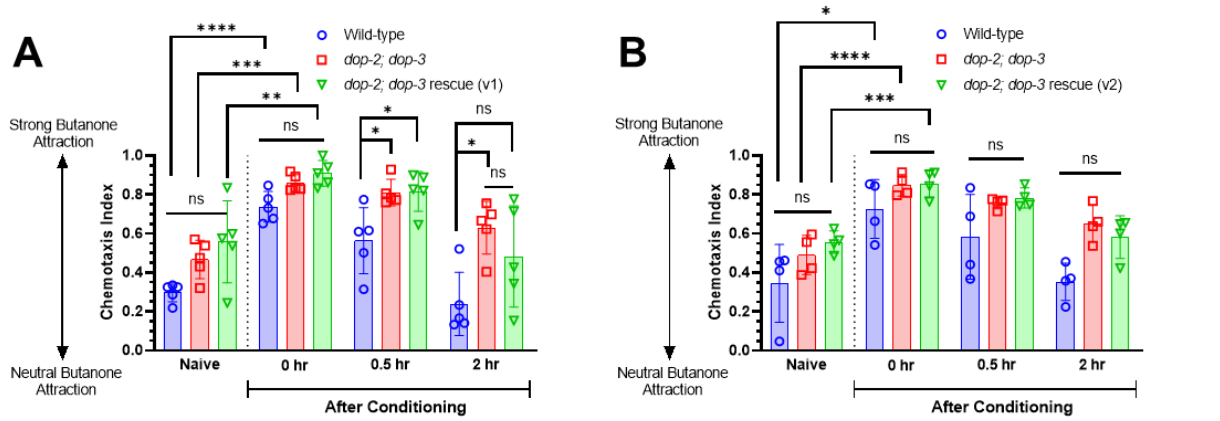
Figure 2: Dopamine receptors act synergistically to modulate short-term memory. **A)** *C. elegans* dopamine receptors DOP-1, DOP-2, and DOP-3 are D1 or D2-like receptors (Chase & Koelle 2007;

Mersha *et al.* 2013). **B)** Butanone associative memory assay for wild-type worms and worms containing single mutations in **(i)** *dop-1(vs100)* (*n* = 4 biological replicates), **(ii)** *dop-2(vs105)* (*n* = 4 biological replicates), and **(iii)** *dop-3(vs106)* (*n* = 4 biological replicates). **C)** Butanone associative memory assay for wild-type and *dop-1;dop-2;dop-3* triple mutant worms (*n* = 4 biological replicates). **D)** Butanone associative memory assay for wild-type and **(i)** *dop-1;dop-2* (*n* = 4 biological replicates), **(ii)** *dop-1;dop-3* (*n* = 3 biological replicates), and **(iii)** *dop-2;dop-3* (*n* = 4 biological replicates) double mutant worms. For butanone assays (**B, C, D**), each data point represents one biological replicate (which includes four technical replicates). Each technical replicate consists of 50-250 worms. Behaviour was quantified using chemotaxis indices (CI) for naïve/untrained worms and conditioned animals at 0, 0.5-, 1-, 1.5- and 2-hours post-conditioning. Statistical analysis: a pairwise t-test was performed to compare genotypes for each time point, and a one-way ANOVA with Tukey's multiple comparisons test was performed to compare post-conditioning t=0 with naïve chemotaxis indices (CIs) between genotypes. Error bars = mean ± SD. p-values are represented by **** ≤ 0.0001; *** ≤ 0.001, ** ≤ 0.01; * ≤ 0.05; ns = non-significant.

Dopamine acts in dop-2/dop-3 expressing neurons to modulate short-term memory

Our data suggests that dopamine acts through D2-like dopamine GPCRs, DOP-2 and DOP-3, to promote forgetting in *C. elegans*. To provide additional evidence for this, we re-expressed DOP-2 and DOP-3 in *dop-2;dop-3* double mutants using a transgene that contains DOP-2 cDNA (corresponding to DOP-2S in (Suo *et al.* 2009)) and DOP-3a cDNA, expressed under the control of 5 kb of promoter sequence for both *dop-2* and *dop-3* (see Experimental Procedures for details). Due to practical limitations on the number of groups that can be handled in a single experiment, for these assays we tested only naïve and post-conditioning t=0, 0.5 and 2 h cohorts for four genotypes: wild-type, *dop-2;dop-3* double mutants, and two versions of the transgenic DOP-2/3 rescue line (**Figure 3A** and **B**). As the DOP-2/3 rescue transgenic lines contain extrachromosomal arrays, there may be minor differences in transgene expression, so we tested two versions to assess the role of DOP-2 and DOP-3 in forgetting. We found for data shown in both **Figure 3A** and **3B** that, as expected, wild-type controls showed intact learning at t=0, as

229 indicated by higher CI compared with naïve worms, and that this had returned to baseline at t=2
230 (comparisons between naïve and t=0, 0.5, and 2 for all genotypes are indicated underneath each
231 graph). Consistent with **Figure 2Diii**, *dop-2;dop-3* mutants also displayed intact learning at t=0,
232 and the memory of this learnt association did not return to baseline levels at t=2 (comparison
233 between CIs for naïve and t=2: p-value <0.05; one-way ANOVA with Dunnett's post-test)
234 (**Figures 3A, 3B**), indicating an extended memory retention phenotype. We found that both
235 DOP-2/3 re-expression lines showed a partial rescue phenotype: transgenic worms showed intact
236 learning at t=0, and had returned to baseline levels at t=2 (comparison between CIs for naïve and
237 t=2: p-value >0.05; one-way ANOVA with Dunnett's post-test) (**Figures 3A, 3B**). Additionally,
238 linear regression analysis showed that slopes of wild-type and DOP-2/3 rescue lines (both
239 versions) are not significantly different (p-values 0.6889 and 0.4740 for **Figure 3A** and **3B**,
240 respectively), while slopes for wild-type vs *dop-2;dop-3* mutants are significantly different for
241 **Figure 3A** (p-value = 0.0188) and trend towards a difference for **Figure 3B** (p-value =0.1442)
242 (**Table 1**). Importantly, the slope for the DOP-2/3 rescue lines falls between wild-type and *dop-*
243 *2;dop-3* mutants for both versions (**Figure 3A:** -0.2414 for wild-type, -0.1161 for *dop-2;dop-3*
244 double mutant, -0.2153 for DOP-2/3 rescue; and **Figure 3B:** -0.1792 for wild-type, -0.09202 for
245 *dop-2;dop-3* double mutant, -0.1363 for DOP-2/3 rescue; **Table 1**). However, the CIs of DOP-
246 2/3 rescue worms were not significantly different from wild-type or *dop-2;dop-3* mutant controls
247 at t=2 (or in naïve worms), suggesting that their forgetting phenotype is intermediate between
248 wild-type and dopamine receptor mutants (**Figures 3A, 3B**). Taken together, these data indicate
249 that re-expression of DOP-2 and DOP-3 in a *dop-2;dop-3* double mutant is able to at least
250 partially rescue the forgetting phenotype observed in the double mutants.



Strain	Naïve vs t=0	Naïve vs t=0.5	Naïve vs t=2
Wild-type	0.0001	0.0116	0.8004
dop-2; dop-3	<0.0001	<0.0001	0.0352
dop-2; dop-3 rescue (v1)	0.0175	0.0882	0.8441

Strain	Naïve vs t=0	Naïve vs t=0.5	Naïve vs t=2
Wild-type	0.0221	0.1710	0.9999
dop-2; dop-3	<0.0001	0.0009	0.0288
dop-2; dop-3 rescue (v2)	0.0003	0.0028	0.9266

Figure 3: Dopamine acts through DOP-2 and DOP-3 to modulate short-term memory. A) Butanone associative memory assay for wild-type worms, *dop-2;dop-3* double mutant worms and *dop-2;dop-3* double mutant worms re-expressing DOP-2 and DOP-3 under the control of their endogenous promoters. DOP-2 in this transgene is a truncated version missing 5 amino acids when compared to the DOP-2S sequence (*n* = 5 biological replicates). **B)** Butanone associative memory assay for wild-type worms, *dop-2;dop-3* double mutant worms and *dop-2;dop-3* double worms re-expressing DOP-2 and DOP-3 under the control of their endogenous promoters. DOP-2 in this transgene exactly matches the published DOP-2S sequence (Suo *et al.* 2009) (*n* = 4 biological replicates). For rescue lines expressing extrachromosomal arrays, only CIs from transgenic worms are displayed here. Each data point represents one biological replicate (which includes four technical replicates). Each technical replicate consists of 50-250 worms. Behaviour was quantified using chemotaxis indices (CI) for naïve/untrained worms and conditioned animals at 0, 0.5-, and 2-hours post-conditioning. Statistical analysis: one-way ANOVA with Dunnett's multiple comparisons test was performed to compare post-conditioning time points with naïve chemotaxis indices (CIs) within a single genotype (shown in tables below each graph) and with Tukey's multiple comparisons test to compare CIs for naïve vs t=0 between genotypes. Error bars = mean ± SD. p-values are represented by **** ≤ 0.0001; *** ≤ 0.001, ** ≤ 0.01; * ≤ 0.05; ns = non-significant.

Dopamine release through ADE and CEP neurons promotes forgetting.

There are eight dopaminergic neurons in hermaphrodite *C. elegans*: two ADE neurons, four CEP neurons, and two PDE neurons (**Figure 4A**), each of which have specific synaptic and

neuromodulatory inputs/outputs to other parts of the nervous system. To investigate whether specific connections are required for memory retention, we examined the role of individual subsets of dopamine-releasing neurons in the process of forgetting. To do this, we assayed transgenic lines in which CAT-2 is specifically re-expressed in the ADE (using an *exp-1* promoter) or CEP neurons (using an *ace-1* promoter), in a *cat-2* mutant background (Chou *et al.* 2022). We first examined the role of ADE neurons in forgetting (**Figure 4B**): wild-type controls displayed the expected phenotype of showing an increased CI (indicating intact learning) at t=0, which had returned to baseline at t=0.5 (comparison between CIs for naïve and t=0.5: p-value >0.05; one-way ANOVA with Dunnett's post-test). We found that ADE::CAT-2 rescue worms showed intact learning at t=0, which had also returned to baseline at t=0.5 (comparison between CIs for naïve and t=0.5: p-value >0.05; one-way ANOVA with Dunnett's post-test), suggesting that expression of CAT-2 from the ADE neurons is sufficient to rescue the forgetting phenotype observed in *cat-2* mutant worms (**Figure 1B**). However, we noticed that the CIs for the ADE::CAT-2 rescue line at t=1.5 and t=2 post-conditioning were significantly higher than wild-type controls, and the comparison between naïve and t=2 for this rescue line showed a trend towards a significant difference (p-value 0.0971) (**Figure 4B**). Indeed, linear regression analysis showed that slopes for wild-type and ADE::CAT-2 rescue worms are significantly different (**Table 1**), suggesting incomplete rescue. Based on this data, we postulate that dopamine release from ADE neurons may have a stronger effect on forgetting at the earlier (t=0.5,1) time points.

We next tested CEP::CAT-2 rescue worms (**Figure 4C**) in the butanone associative memory assay. We found that wild-type worms showed the expected phenotype of intact learning at t=0 and return to baseline (naïve) CI at t=0.5. In contrast, CEP::CAT-2 worms showed significantly higher CIs at t=0 (indicating intact learning), but this did not return to baseline until t=1.5,

suggesting a delayed forgetting phenotype (comparison between naïve and t=0.5 gives p-value of 0.0103; comparison between naïve and t=1 gives p-value of 0.0660 i.e. close to significance threshold; one way ANOVA, Dunnett's post-test). However, the slope of the CEP::CAT-2 rescue line (-0.2469) is similar to, and indeed is not significantly different from, that of wild-type controls (-0.2518) (p-value = 0.9453; **Table 1**). These data suggest that dopamine release from CEP neurons does not facilitate memory returning to naïve levels by t=0.5, but is sufficient to promote a rate of memory loss that is similar to that of wild-type. Taken together with findings from ADE::CAT-2 rescue lines, we propose that the CEPs regulate the rate of memory loss at both early and late time points post-conditioning, but that this requires additional dopamine release from other neurons such as the ADEs, which are critical for forgetting at early time points.

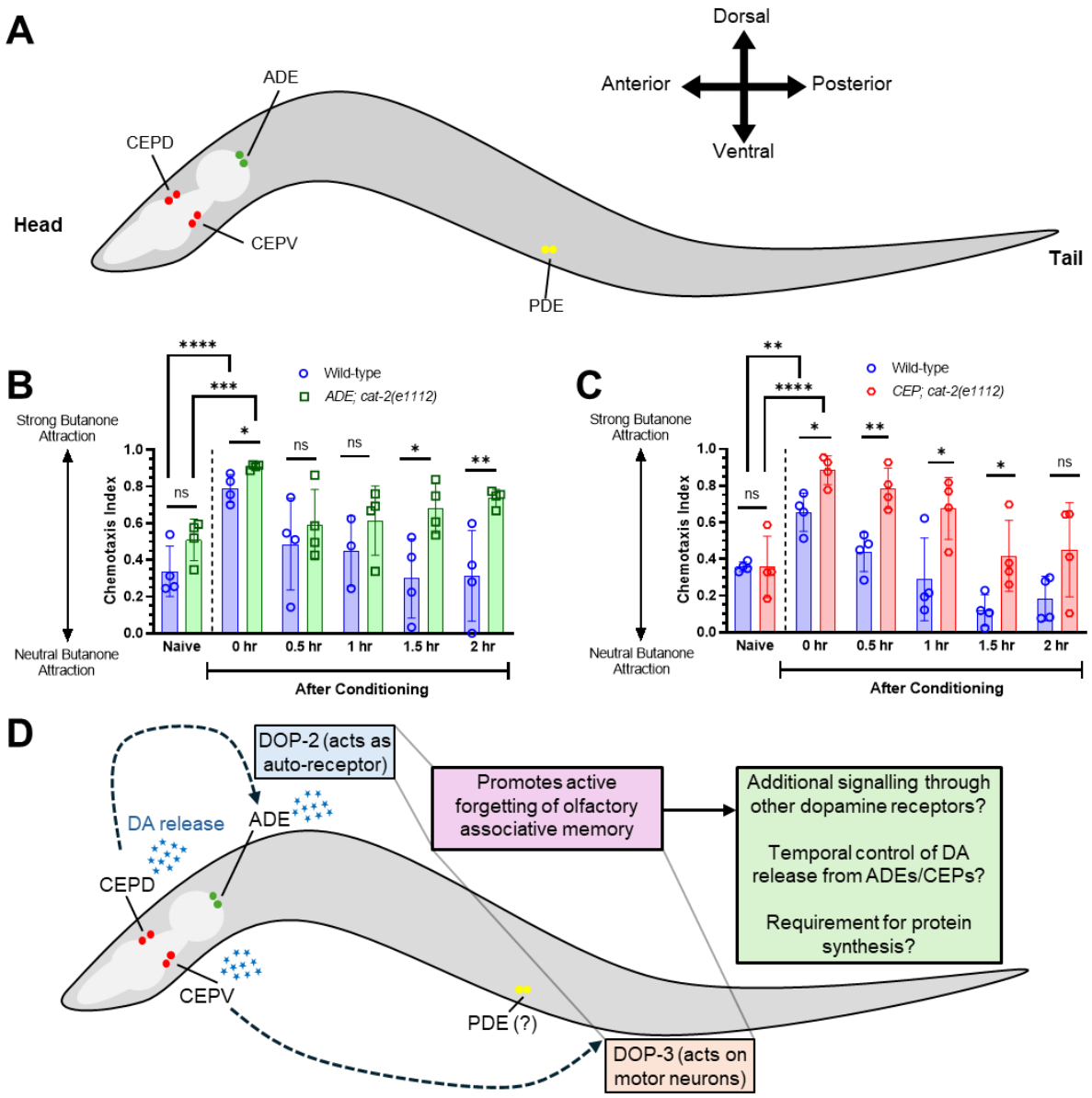


Figure 4: **Dopamine release from ADE and CEP neurons promotes forgetting. A)** Dopaminergic neurons in *C. elegans* are the ADE, CEP (D/V = dorsal/ventral), and PDE neurons. **B)** Butanone associative memory assay for wild-type worms and *cat-2* mutant worms re-expressing CAT-2 in the ADE neurons (n = 4 biological replicates). **C)** Butanone associative memory assay for wild-type worms and *cat-2* mutant worms re-expressing CAT-2 in the CEP neurons (n = 4 biological replicates). Each data point represents one biological replicate (which includes four technical replicates). Each technical replicate consists of 50-250 worms. Behaviour was quantified using chemotaxis indices (CI) for naïve/untrained worms and conditioned animals at 0, 0.5-, 1-, 1.5- and 2-hours post-conditioning. Statistical analysis: a pairwise t-test was performed to compare genotypes for each time point, and a one-way ANOVA with Tukey's multiple

comparisons test was performed to compare post-conditioning t=0 with naïve chemotaxis indices (CIs) between genotypes. Error bars = mean ± SD. p-values are represented by **** ≤ 0.0001; *** ≤ 0.001, ** ≤ 0.01; * ≤ 0.05; ns = non-significant. **D)** Schematic of potential mechanism, summarising data in **Figures 1-4**: our findings show that newly synthesised dopamine from both the ADEs and CEPs is required for forgetting of olfactory associative memory, acting through DOP-2 and DOP-3 receptors.

Table 1: Linear regression analysis for short-term associative memory assays. Slopes from linear regression are presented to 4 decimal places. *n* indicates the number of biological replicates. Statistical comparisons are performed using a two-tailed test (GraphPad Prism). p-values (significance) indicated by ** ≤ 0.01; * ≤ 0.05; ns = non-significant.

Strain 1	**Strain 2**	**Figure**	***n***	**Slope strain 1**	**Slope strain 2**	**p-value**	**Significance**
Wild-type	*cat-2*	1B	5	-0.1575	-0.0353	0.0049	**
Wild-type	*dat-1*	1C	5	-0.2223	-0.2257	0.9512	ns
Wild-type	*cat-2;dat-1p::CAT-2*	1D	4	-0.2167	-0.2148	0.9775	ns
Wild-type	*dop-1*	2Bi	4	-0.3088	-0.2799	0.6808	ns
Wild-type	*dop-2*	2Bii	4	-0.2896	-0.2606	0.6897	ns
Wild-type	*dop-3*	2Biii	4	-0.3141	-0.3168	0.9628	ns
Wild-type	*dop-1, dop-2, dop-3*	2C	4	-0.2500	-0.2084	0.4982	ns
Wild-type	*dop-1, dop-2*	2Di	4	-0.1708	-0.2190	0.3154	ns
Wild-type	*dop-1, dop-3*	2Dii	3	-0.1726	-0.1386	0.5189	ns
Wild-type	*dop-2, dop-3*	2Diii	4	-0.1475	-0.05769	0.0661	ns (close to 0.05)
Wild-type	*dop-2;dop-3* rescue 1	3A	4	-0.2414	-0.2153	0.6889	ns
Wild-type	*dop-2; dop-3*	3A	4	-0.2414	-0.1161	0.0188	*
Wild-type	*dop-2;dop-3* rescue 2	3B	4	-0.1792	-0.1363	0.4740	ns
Wild-type	*dop-2; dop-3*	3B	4	-0.1792	-0.09202	0.1442	ns

Wild-type	*cat-2* ADE rescue	4B	4	-0.2278	-0.05045	0.0406	*
Wild-type	*cat-2* CEP rescue	4C	4	-0.2518	-0.2469	0.9453	ns

Discussion

Our study demonstrates that dopamine is essential for active forgetting in *C. elegans*. Interestingly, the absence of a forgetting phenotype in the dopamine transporter DAT-1 mutant, compared with the strong phenotype observed in CAT-2 tyrosine hydroxylase mutants, suggests that the ability to synthesise and retain high levels of dopamine is necessary for this process. Additionally, our findings indicate that D2-like receptors, specifically DOP-2 and DOP-3, are required for forgetting. Finally, we observed that ADE and CEP dopaminergic neurons both modulate forgetting, with ADE required at early time points, and CEP required at both early and late time points after learning, highlighting the temporal complexity of this process (**Figure 4D**).

The role of dopamine in learning and memory is conserved across species. However, while dopamine synthesis and signalling pathways are highly conserved, dopaminergic circuits between *C. elegans* and other organisms differ. In *C. elegans*, eight dopaminergic neurons act 'extra-synaptically' on over 100 targets, mainly motor neurons. In mammals, dopaminergic neurons are concentrated in the ventral midbrain (SNc and VTA) and project to the basal ganglia, amygdala, and prefrontal cortex. In *Drosophila melanogaster*, small populations of dopaminergic neurons are found throughout the fly brain, with projections innervating almost the entire brain, with dense projections to the mushroom body lobes, which regulate olfactory learning (Riemensperger *et al.* 2005; Marquis & Wilson 2022). Despite these differences, our finding that dopamine is required for forgetting aligns with previous research on the role of dopamine in

346 forgetting in *Drosophila*. In flies, blocking synaptic output from dopaminergic neurons promotes
347 memory retention (i.e. reduces forgetting), whereas activating dopamine neurons decreases
348 memory (Berry *et al.* 2012). Indeed, dopamine is essential for both forgetting and memory
349 acquisition in flies, but this involves different receptors: DAMB for forgetting and dDA1 for
350 acquisition (Berry *et al.* 2012; Himmelreich *et al.* 2017). In our study, we observed a small but
351 robust learning improvement in *cat-2* and *dat-1* mutant worms, as well as in *dop-1;dop-2;dop-3*
352 triple mutants (double mutant combinations of these three receptors also showed a learning
353 improvement, albeit slightly above the alpha value of $p = 0.05$). This indicates that the
354 requirement for dopamine in learning and forgetting is conserved between flies and worms,
355 suggesting it is evolutionary important and likely also retained in higher organisms.

356 Our findings contrast with the work by Raj and Thekkuveettil (Raj & Thekkuveettil 2022), which
357 showed that dopamine deficient *cat-2* mutant *C. elegans* display both a learning defect and a
358 memory defect, i.e. they forget more quickly than wild-type controls. Although both our study
359 and the 2022 study use the butanone associative learning assay, worms in this research were
360 conditioned with agar plates seeded 1-2 days in advance with 100 µL OP50 (and thus already
361 dried on the agar), whereas the Raj and Thekkuveettil study involved conditioning in 400 µL of
362 OP50 pipetted onto unseeded agar plates immediately after worms are added. This means that the
363 worms are being conditioned in liquid, which may influence other locomotion pathways in which
364 dopamine plays an important role, such as in regulating "swimming" in liquid, a distinct form of
365 locomotion separate to crawling on solid media. Dopamine modulates swimming behaviour (Xu
366 *et al.* 2021), swimming-induced paralysis (McDonald *et al.* 2007), and swim-to-crawl transitions
367 when moving from liquid to solid media (Vidal-Gadea *et al.* 2011). As learning is a multifaceted
368 process modulated by multiple internal and external cues, it is not surprising that dopamine could

369 impact memory in different ways depending on the mode of conditioning, for example due to an
370 interaction between the response to the food and odour cues, and the swimming activity. This
371 underscores dopamine's multifaceted role in modulating behaviour and memory in *C. elegans*.

372 Our data show that both DOP-2 and DOP-3 D2-like receptors are required to modulate
373 forgetting. Single mutants of either receptor show memory retention capacity similar to wild-
374 type, while the *dop-2;dop-3* double mutant show enhanced memory retention similar to *cat-2*
375 mutant animals. Interestingly, the rate of memory loss in *dop-1;dop-2;dop-3* triple mutants
376 (indicated by the slope from linear regression) is similar between the triple mutant and wild-type
377 controls. This suggests that while DOP-2 and DOP-3 may promote memory retention (i.e. block
378 forgetting), the D1-like DOP-1 receptor may have an antagonistic effect in the absence of these
379 D2-like receptors. D1- and D2-like receptors are well-known to antagonise each other in signal
380 transduction, and their opposing effects in *C. elegans* have been previously reported in dietary
381 restriction-mediated lifespan extension (Jiang *et al.* 2022), swimming (Xu *et al.* 2021), and basal
382 slowing behaviour in response to a food lawn (Chase *et al.* 2004). We did not test the
383 invertebrate specific DOP-4 receptor or dopamine ligand-gated ion channels such as LGC-53;
384 therefore, a role for these receptors in dopamine-dependent short-term associative memory in *C.*
385 *elegans* remains unknown.

386 Short- and long-term memory differ by the mechanism for encoding and time taken to fully
387 decay. In *C. elegans*, short-term memory is typically considered to be relevant at 1-2 hours post-
388 learning, while long-term memory is evaluated 12-24 hours post-learning (Rahmani & Chew
389 2021; Kauffman *et al.* 2011). In our study, we tested short-term associative memory only up to 2
390 hours post-conditioning. At this time point, dopamine-deficient *cat-2* mutants, as well as *dop-*
391 *2;dop-3* double mutants, still showed robust memory. Identifying when the memory decays to

392 baseline levels may provide further understanding of the role of dopamine in forgetting, such as
393 whether processes associated with long-term memory e.g. *de novo* protein synthesis (Fioriti *et al.*
394 2015), are required. In addition, we found that temporal regulation of dopamine release from
395 ADE neurons and CEP neurons may be required to modulate forgetting of olfactory memories.
396 The mechanistic details of this process, as well as the potential (untested) requirement of PDE
397 neurons, warrant further investigation in future studies.

398 In conclusion, our study leverages the worm's compact nervous system and genetic tractability to
399 provide new insights into the role of dopamine in forgetting. Other studies on olfactory
400 paradigms in worms have identified diverse regulators of forgetting, indicating that multiple
401 pathways and mechanisms are involved in this complex process. Our study adds to this body of
402 knowledge by highlighting the specific role of dopamine and its receptors. In addition, these
403 findings are consistent with research in *Drosophila*, suggesting a mechanism for forgetting that is
404 conserved in even more complex brains. Based on this study, future research could focus on
405 exploring the conserved mechanisms of forgetting across species and investigating potential
406 therapeutic targets for memory-related disorders.

407

408 **Experimental Procedures**

409 **C. elegans *strain maintenance***

410 Young adult (day 1) hermaphrodite *C. elegans* were grown using standard conditions on
411 nematode growth medium (NGM) agar in petri dishes at 22 °C for all experiments (Brenner
412 1974). Worms were cultured at 22 °C for at least two generations prior to all learning assays. For
413 all experiments, animals were fed *Escherichia coli* (*E. coli*) strain OP50.

414 A list of strains and transgenic lines used, including full genotype information, is in **Table 2**.
415 Genotypes of mutant strains were confirmed via PCR, based on information in Wormbase
416 version WS295 (Sternberg *et al.* 2024). Transgenes were generated through standard
417 microinjection procedures as in (Chew *et al.* 2018a; Chew *et al.* 2018b; Gadenne *et al.* 2022).

418

419 **Table 2: Genotype information for strains used in this study.** For transgenic lines generated in this
420 study, numbers in square brackets refer to the concentration injected in ng/µL.

Strain name	**Genotype**	**Source**
Bristol N2	Wild-type	*Caenorhabditis* Genetics Centre (CGC)
CB1112	*cat-2(e1112)*	*Caenorhabditis* Genetics Centre (CGC)
RM2702	*dat-1(ok157)*	*Caenorhabditis* Genetics Centre (CGC)
LX645	*dop-1(vs100)*	*Caenorhabditis* Genetics Centre (CGC)
LX702	*dop-2(vs105)*	*Caenorhabditis* Genetics Centre (CGC)
LX703	*dop-3(vs106)*	*Caenorhabditis* Genetics Centre (CGC)
LX706	*dop-1(vs100); dop-2(vs105)*	*Caenorhabditis* Genetics Centre (CGC)
LX704	*dop-2(vs105); dop-3(vs106)*	*Caenorhabditis* Genetics Centre (CGC)
LX705	*dop-1(vs100); dop-3(vs106)*	*Caenorhabditis* Genetics Centre (CGC)
LX734	*dop-1(vs100); dop-2(vs105); dop-3(vs106)*	*Caenorhabditis* Genetics Centre (CGC)
YLC299	*cat-2(e1112); baIs4 [dat-1p::GFP + dat-1p::CAT-2]*	*cat-2(e1112)* crossed with UA57 from CGC
CLP1318	*cat-2(n4547);twnEx593[Pace-1::cat-2; Pgcy-8::mCherry]*	(Chou *et al.* 2022)
CLP1320	*cat-2(n4547);twnEx599[Pexp-1::cat-2; Pgcy-8::mCherry]*	(Chou *et al.* 2022)
YLC381	*dop-2(vs105)V; dop-3(vs106)X; pepEx040[Pdop-2(5kb)::dop-2S cDNA::let-858 UTR (pYLC102) [50];*	This study

	Pdop-3(5kb)::dop-3a cDNA::let-858 UTR (pYLC104) [50]; ccGFP [50]]	
YLC382	*dop-2(vs105)V; dop-3(vs106)X; pepEx043[Pdop-2(5kb)::dop-2S cDNA::let-858 UTR (pYLC103) [50]; Pdop-3(5kb)::dop-3a cDNA::let-858 UTR (pYLC104) [50]; ccGFP [50]]*	This study

421

422 ***Molecular biology***

423 Transgenes were cloned using the NEB HiFi cloning (NEB #E2621). Promoters for the *dop-2*
424 and *dop-3* gene were both cloned 5 kb before the ATG, starting from
425 CAAGACGGACGCAGTCCG and CTGGAATATTCCAGAACTTTC, respectively. *dop-2* and
426 *dop-3* cDNA were amplified from wild-type N2 RNA, extracted using the using the Monarch
427 Total RNA Kit (NEB #T2010S) and converted to cDNA using the Luna® Universal One-Step
428 RT-qPCR Kit (NEB #E3005S). Two forms of the *dop-2* cDNA sequence were cloned: one
429 aligned to the DOP-2S sequence published in (Suo *et al.* 2009) (pYLC103), the other is identical
430 to this sequence but lacks a AGTACGACGGCAAAT sequence from the 1385th nucleotide
431 position, corresponding to amino acids STTAN (pYLC102). The *dop-3* cDNA sequence aligned
432 with the expected sequence for DOP-3, isoform a (T14E8.3a.1) (Sternberg *et al.* 2024).

433 ***Behavioural assays***

434 The butanone appetitive learning assay (Kauffman *et al.* 2011; Rahmani *et al.* 2024) tests naïve
435 worms, conditioned worms that are tested directly after training (t=0), and conditioned worms
436 that go through a 'hold' period between training and testing (t=0.5–2 h). Briefly, *C. elegans*
437 populations were washed twice in M9 buffer (86 mM NaCl, 42 mM Na_2HPO_4, 22 mM K_3PO_4
438 (pH 6.0), 1 mM M_gSO_4) to constitute the 'naïve' group in butanone chemotaxis assays.
439 Remaining groups were then subjected to a 1-hour starvation period in M9 buffer on a shaker

440 (175 rpm at room temperature). Conditioned groups were then transferred onto NGM plates pre-
441 seeded with 100 µL *E. coli* OP50 and exposed to 10% (v/v) butanone (Sigma-Aldrich, #360473;
442 final concentration, diluted in 95% ethanol), streaked on plate lids, for 1 h. The conditioned
443 groups undergoing the 'hold' period were transferred onto NGM plates pre-seeded with 100 µL
444 *E. coli* OP50 for the assigned time periods. All groups were transferred onto salt-deficient agar in
445 6 cm petri dishes to perform butanone chemotaxis assays as described in Kauffman *et al.* (2010)
446 and Rahmani *et al.* (2024). Butanone chemotaxis assay plates were prepared with two equidistant
447 points both spotted with sodium azide, as well as 95% (v/v) ethanol on one position (control) and
448 10% (v/v) butanone diluted in 95% (v/v) ethanol on the other spot. Butanone chemotaxis
449 behaviour was quantified using the following equation:

450 $$\frac{\#\ worms\ on\ butanone - \#\ worms\ on\ ethanol}{\#\ total\ population - \#\ worms\ on\ origin}$$

451 The chemotaxis index (CI) ranges between 0 (neutral butanone attraction) to 1.0 (complete
452 butanone attraction). Each assay plate constituted one technical replicate; technical replicates
453 were generated from <50 total worms or >250 worms were excluded from the data. On very rare
454 occasions where negative CIs are observed, these data are also excluded as these populations
455 failed to show the standard naïve attraction to butanone. Each biological replicate constituted 3-4
456 technical replicates. At least three biological replicates were performed for each genotype tested,
457 the biological replicates are stated for each experiment in the appropriate figure legend.

458 ***Statistical analysis***

459 Statistical analysis for all experiments was performed using GraphPad Prism version 8.0. In
460 general, where two groups were compared, a paired t-test was used. A one-way ANOVA with
461 Dunnett's multiple comparisons test was performed to compare post-conditioning time points

with naïve chemotaxis indices (CIs) within a single genotype, or with Tukey's multiple comparisons test to compare CIs between genotypes for naïve and t=0. The alpha value for all analyses is set at 0.05. Simple linear regression was performed to calculate the slope of the change in chemotaxis index over time for conditioned worms, and to perform a two-tailed test to determine if the slopes are significantly different between genotypes tested.

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Conflict of interest: The authors declare that they have no conflicts of interest.

Data Availability Section

This study includes no data deposited in external repositories. Reagents including *C. elegans* strains and expression plasmids are available from the corresponding author upon request.

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