Neural correlates of water reward in thirsty Drosophila

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Drinking water is innately rewarding to thirsty animals. In addition, the consumed value can be assigned to behavioral actions and predictive sensory cues by associative learning. Here we show that thirst converts water avoidance into water-seeking in naive Drosophila melanogaster. Thirst also permitted flies to learn olfactory cues paired with water reward. Water learning required water taste and <40 water-responsive dopaminergic neurons that innervate a restricted zone of the mushroom body γ’ lobe. These water learning neurons are different from those that are critical for conveying the reinforcing effects of sugar. Naïve water-seeking behavior in thirsty flies did not require water taste but relied on another subset of water-responsive dopaminergic neurons that target the mushroom body β’ lobe. Furthermore, these naïve water-approach neurons were not required for learned water-seeking. Our results therefore demonstrate that naïve water-seeking, learned water-seeking and water learning use separable neural circuitry in the brain of thirsty flies.

Thirst is a manifestation of an animal’s internal deprivation of water1. Increasing dehydration promotes pursuit of the goal of finding water and drinking. Serving this need requires foraging behavior that is guided by the collection of sensory cues that are present, and the most meaningful, in the environment. Some of these are innately significant and clear, such as water itself, and others are learned as useful signs from knowledge of previous purchase2–4. Therefore, as it forages, an animal needs to integrate the most useful innate and learned cues with its internal state to direct appropriate motivated or goal-directed behavior. How thirst affects the nervous system to control water-seeking behavior is largely unknown.

Dopaminergic neurons are generally considered to signal reward value in the mammalian brain5–7, and recent work in Drosophila has provided inroads to a cellular-resolution analysis of reward value coding8,9. A cluster of approximately 130 dopaminergic neurons that innervate the horizontal lobes of the mushroom body was implicated in conveying positive reinforcement value and found to be required for flies to learn with a sugar reward8,9. Surprisingly, a subset of these rewarding neurons were also demonstrated to be required for flies to evaluate the lesser of two voltages during relative shock-avoidance learning10, suggesting an unforeseen complexity in the function of dopaminergic valuation processes in the fly.

Here we further probed Drosophila reward coding by developing new assays to study water valuation in thirsty flies. We found that valuation of water vapor in naïve flies utilized a different population of rewarding dopaminergic neurons than those that were required for ingested water to provide reinforcement value during reward learning. Furthermore, the water learning neurons were apparently separate from those required for sugar reward learning. The type of rewarding stimulus therefore seems to functionally subdivide the fly dopaminergic system.

RESULTS

Thirsty flies seek water and can learn using water reward

Water-sated flies actively avoid water, preferring to enter a dry tube rather than one that is humid11. As thirsty flies should seek water rather than avoid it, we first determined a time of deprivation after which water becomes attractive to naïve flies. Approach behavior was evident after 6 h of water deprivation, and 90% of flies entered the water tube after 14 h without drinking (Fig. 1a). These data demonstrate that thirsty flies seek water. Thirst therefore changes the flies’ valuation of water from something they consider to be aversive into something they seek, or want.

To determine whether water was rewarding to thirsty flies, we conditioned 16-h water-deprived flies by presenting one of two odors with water in a variant of an established olfactory appetitive learning paradigm12,13. A robust but short-lived olfactory memory was formed when flies were trained at 23 °C (Fig. 1b). When flies were trained at 32 °C, initial memory scores increased and robust performance could be measured at least 24 h later (Fig. 1b). We reasoned that the increased performance might result from the flies being more motivated to drink at higher temperature. Indeed, thirsty flies given 2 min to drink consumed statistically more blue-dyed water at 32 °C than 23 °C (Fig. 1c). Allowing flies to drink to satiety for 1 h before training significantly decreased water memory at both temperatures (Fig. 1b). Therefore thirst and drinking are required for the acquisition of water memory.

We also tested whether thirst is required for behavioral expression of water memory (Fig. 1d and Supplementary Fig. 1a, b). Thirsty flies trained with water displayed significantly greater conditioned odor approach performance than when they were hungry or water sated. (Fig. 1d). A similar specificity of deprivation state dependence was also observed with sugar-reinforced memory. Hungry flies trained with dry sugar expressed significantly greater memory performance when hungry than when thirsty or food sated (Fig. 1e and

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Received 26 June; accepted 1 September; published online 28 September 2014; doi:10.1038/nn.3827
Figure 1 Thirsty flies approach water and can be trained with water reward. (a) Increasing time of water deprivation in naive flies converts avoidance of humidity into attraction (n = 4 for each time point). (b) Water memory performance after training at 23 °C (magenta line) or 32 °C (blue line). Following thirsty flies to drink water for 1 h before training significantly diminished water learning at both temperatures (23 °C, open tan circle; 32 °C, open black circle), n = 20 for the thirsty 23 °C group, n = 15 for the sated 23 °C group, n = 8 for the thirsty 32 °C group, n = 12 for the sated 32 °C group, *P < 0.005 (t-test) for the difference between thirsty and sated groups. (c) Flies consume more blue-dyed water in 2 min at 32 °C than at 23 °C as measured by the absorbance at 625 nm (A625) (*P < 0.0001, n = 4; t-test). (d) Water memory is retrieved more robustly in thirsty than hungry flies (*P < 0.01 as compared to thirsty but not hungry flies, n = 8; n.s., not significant: P = 0.74, n = 8; ANOVA followed by post hoc Tukey’s honest significant difference (HSD) test). (e) Sugar memory is retrieved more robustly in hungry than thirsty flies (*P < 0.001 as compared to hungry but not thirsty flies, n = 12; n.s., P = 0.57, n = 12; ANOVA followed by post hoc Tukey’s HSD test). (f) PPK28 is required for water learning (*P < 0.0001, n = 12; t-test). (g) PPK28 is not required for naive water approach in thirsty flies (P = 0.51, n = 8; t-test). (h) Flies exhibit thirst-dependent attraction to water vapor. All data are mean ± s.e.m. Full descriptions of the fly strains and their corresponding colors used in the bar-graphs can be found in Supplementary Table 1.

Supplementary Fig. 1c,d). Water-reinforced appetitive memory performance therefore exhibits a thirst state dependence that is analogous to that for hunger and carbohydrate memory12,13. In addition, the two types of appetitive memory are independently controlled by the appropriate deprivation states of thirst or hunger.

PPK28 is required for water learning but not naive water-seeking

We next investigated the neural circuitry of water-directed behaviors. Flies can taste water via the osmosensitive ion channel Pickpocket28 (PPK28) that is expressed in gustatory neurons on the proboscis15. Thirsty flies homozygous for ppk28 were defective in water learning (Fig. 1f), despite displaying normal olfactory acuity (Supplementary Fig. 2) and naive water-seeking behavior (Fig. 1g). Finding that performance in our water choice assay did not require water taste lead us to test whether the flies were instead directed by water vapor. We gave water-sated or thirsty flies the choice between entering a tube containing dry air or one with an inaccessible water source at the end. Whereas sated flies preferred the dry tube, thirsty flies approached the water vapor (Fig. 1h). Water seeking therefore utilizes water vapor detection. In contrast, our experiments suggest that water learning requires the flies to taste water in order to stimulate drinking.

Octopamine is not required for water learning

Octopamine has long been considered to signal reward in insects16–19, and recent studies suggest that in Drosophila it exclusively conveys the reinforcing effects of the sweet taste of sugars6. Given the requirement for water taste neurons in learning, we tested whether octopamine was required for water-reinforced learning. Both Tyramine β-hydroxylase (TbhβH18) mutant flies, which lack octopamine20, and flies in which octopaminergic neurons were blocked using Tdc2-GAL4 (ref. 21) to express UAS-shiH13 (ref. 22) displayed water learning that was indistinguishable from that of control flies (Fig. 2a). Therefore octopamine is not critical for water reinforcement. These results suggest that the neural pathways used to learn with sugar and water reward are different.

Water learning utilizes DopR1 in mushroom body γ neurons

Dopamine signaling is essential for reward learning with sugar8,9,23 and conveys both the octopamine-dependent sweet taste signal and that for nutritive value6. We therefore addressed the role of dopamine in water learning. Flies carrying the dumbb or dumb2 mutation in the D1 dopamine DopR1 receptor, which are defective in sugar reward learning23, were significantly impaired in water learning too (Fig. 2b). We were also able to rescue the performance of dumbb mutant flies by re-establishing expression of DopR1 in mushroom body neurons. Expressing UAS-DopR1 in mostly the γ (with NP1131-GAL4 and 201Y-GAL4), but not the αβ (with c379-GAL4) or α′β′ neurons (with c305a-GAL4), produced a significant restoration of memory performance (Fig. 2b). The dumb mutant flies were not defective in water drinking (Supplementary Fig. 3a). Furthermore, despite the dumbb flies having an apparent olfactory acuity defect, restoration of olfaction with the c379-GAL4 and c305a-GAL4 drivers did not correlate with wild-type learning ability (Fig. 2b and Supplementary Fig. 3b). However, as both NP1131-GAL4– and 201Y-GAL4–driven UAS-DopR1 restored olfaction and learning, and the region of clear overlap in expression in these lines is in the γ lobe24, we conclude that key water-reinforcing dopamine signals are likely to be delivered to the γ lobe.

Rewarding dopaminergic neurons reinforce water memory

Previous studies have established that dopaminergic neurons in the protocerebral anterior medial (PAM) cluster that innervate the horizontal lobes of the mushroom body are critical for sugar-reinforced olfactory memory5,7. We therefore used UAS-shiH13 driven by 0273-GAL4 (ref. 9) or R58E02-GAL4 (ref. 8), respectively, to block during training either the entire population of ~130 or a subpopulation of ~90 dopaminergic neurons in PAM. In both cases, water memory formation was significantly impaired at 32 °C (Fig. 2c) but not at the permissive 23 °C (Supplementary Fig. 4a). The olfactory acuities of all strains were not significantly different (3-octanol, P = 0.46; 4-methylcyclohexanol,
P = 0.67; ANOVA; Supplementary Fig. 4b). The 0273-GAL4; UAS-shh1 flies drink significantly less water during the 2-min training cycle (Supplementary Fig. 4c). However, the magnitude of the decreased drinking is unlikely to account for the abolition of memory performance (Fig. 2c) because 0273-GAL4; UAS-shh1 flies still consumed a quantity of water that was comparable to that of wild-type flies at 23 °C (Fig. 1c) and that is sufficient to form robust 3-min water memory (Fig. 1b). Moreover, the R58E02-GAL4; UAS-shh1 flies drank normally during training (Supplementary Fig. 4c). We also tested the role of dopaminergic neurons that have been implicated in aversive reinforncement18,23 by blocking them during training using TH-GAL4 driven UAS-shh1. No defect was observed (Fig. 2d). Lastly, flies in which the PAM neurons were blocked for 30 min after training and during memory testing displayed memory performance that was indistinguishable from that of controls (Fig. 2e). The PAM dopaminergic neurons are therefore required during acquisition but are apparently dispensable for the expression of water memory.

**Water-rewarding dopaminergic neurons innervate γ4**

To identify the water-reinforcing dopaminergic neurons, we visually isolated seven GAL4 lines that express in subsets of the 0273 and R58E02 populations and assayed the consequence of blocking these neurons with UAS-shh1. In this screen, only R48B04; UAS-shh1 flies revealed a significant defect in water learning (Fig. 3a; also see Fig. 3d). Importantly, the water learning defect of R48B04-GAL4; UAS-shh1 was not observed at the permissive 23 °C (Supplementary Fig. 5a), and water consumption (Supplementary Fig. 5b) and olfactory acuity (Supplementary Fig. 5c) were not impaired. Finding a role for R48B04 neurons caught our attention because R48B04 expression is driven by a promoter fragment from the *aoml* octopamine receptor gene26. A previous study showed that *aoml*-dependent signaling in 0104-GAL4 neurons was critical for the short-term memory reinforcing effects of the sweet taste of sugars5. However, octopamine was not required for water learning (Fig. 2a) and 0104-GAL4 blockade did not impair water learning (Fig. 3a), suggesting that water- and sugar-reinforcing neurons might be separable within the neurons labeled by R48B04-GAL4. R48B04 expresses in around 55 dopaminergic neurons in the PAM cluster that can be labeled by immunostaining for the tyrosine-hydroxylase enzyme (Fig. 3b and Supplementary Fig. 5d). We verified that it was the R48B04 dopaminergic neurons that were critical for water learning by removing them from the R48B04 expression pattern using an overlapping R58E02-GAL80 transgene8 (Fig. 3c). This manipulation restored wild-type learning performance (Fig. 3d), suggesting that the R48B04 dopaminergic neurons convey the reinforcing effects of water.

Dopaminergic neurons in R48B04 innervate the γ4, γ5 and β2 zones of the horizontal mushroom body lobes27 (Fig. 3e,f). Although this pattern resembled 0104 innervation (Fig. 3g,h), the discordance of behavioral phenotypes led us to investigate whether R48B04 and 0104 label distinct neurons that innervate similar areas of the mushroom body lobes. When 0104-GAL4 and R48B04-LexA expression was visualized in the same brain, it was evident that 0104 and R48B04 neurons were largely non-overlapping in γ5 and that R48B04 had more neurons in γ4 than 0104 (Supplementary Fig. 5e). In contrast, 0104 and R48B04 labeled the same neurons in β2 (Supplementary Fig. 5f). We therefore generated flies in which 0104 neuron expression was removed from the R48B04 pattern, using 0104-GAL4 to drive a UAS-LexA RNAi transgene in R48B04-LexA flies. Despite the potential caveat of partial interference with RNAi, expressing LexAop-GFP with this combination revealed dopaminergic neurons whose processes were largely confined to the γ4 and γ5 zones of the mushroom body γlobe (Fig. 3i,j), from here on referred to as PAM-γ4/5 neurons.

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![Figure 2](https://example.com/figure2.png) **Figure 2** Water-reinforced learning is independent of octopamine and is supported by dopaminergic signaling to mushroom body γ neurons. (a) Water learning performance is normal for TbhM18 flies, which lack octopamine (*P = 0.0528, n = 24; t-test), and flies in which transmission from Tdc2-GAL4 octopaminergic neurons is blocked by UAS-shhP21 (P = 0.34 as compared to UAS-shhP21 control and *P = 0.41 to Tdc2-GAL4 control, n = 8; ANOVA followed by post hoc Tukey’s HSD test). (b) Flies harboring the dumb1 and dumb2 mutations in the DopR1 dopamine receptor show impaired water learning (*P < 0.0001, n = 27 for wild-type, n = 12 for dumb1 and n = 12 for dumb2; ANOVA followed by post hoc Tukey’s HSD test). Restoring DopR1 expression to mushroom body γ neurons rescues water learning by dumb mutant flies to a level that is indistinguishable from that of wild-type flies (*P = 0.66 as compared to the NP1131 rescue group, n = 11; P = 0.38 to the 201Y rescue group, n = 8; ANOVA followed by post hoc Tukey’s HSD test). (c) Output from 0273- and R58E02-expressing PAM dopaminergic neurons is required for water learning (*P < 0.0001 as compared to both UAS-shhP21 and GAL4-only controls, n = 32, 12, 12, 19 and 20 from left to right; ANOVA followed by post hoc Tukey’s HSD test). (d) Blocking output from TH-GAL4-expressing dopaminergic neurons does not impair water learning (*P = 0.02, n = 11 for UAS-shhP21 and n = 12 for TH-GAL4; UAS-shhP21; n.s., P = 0.12, n = 12; ANOVA followed by post hoc Tukey’s HSD test). (e) Blocking 0273 and R58E02 PAM dopaminergic neurons after training does not disrupt 30-min water memory (*P = 0.76 for R58E02-GAL4; UAS-shhP21, n = 8; n.s., P = 0.31, n = 8; *P = 0.03, n = 8; ANOVA followed by post hoc Tukey’s HSD test). Full descriptions of the fly strains and their corresponding colors used in the bar-graphs can be found in Supplementary Table 1.
Blocking only the PAM-γ4/5 neurons with UAS-shi-ts1 significantly impaired water learning (Fig. 3k). The defect was not observed at the permissive temperature of 23 °C (Supplementary Fig. 5g), and these flies showed normal water consumption (Supplementary Fig. 5h) and odor acuity (Supplementary Fig. 5i) at the restrictive temperature.

To test whether the PAM-γ4/5 neurons can provide instructive reinforcement, we conditioned flies with odor presentation and remote activation of PAM-γ4/5 neurons using LexAop-dTrpA1 (ref. 9). dTrpA1 encodes a transient receptor potential (TRP) channel that conducts calcium and depolarizes neurons when flies are exposed to temperature >25 °C (ref. 28). PAM-γ4/5; LexAop-dTrpA1 flies exhibited appetitive memory that was statistically different from that of all control transgenic flies (Fig. 3l). This learning was not observed without the temperature shift during odor presentation (Supplementary Fig. 5j), and the flies displayed no differences in naive olfactory acuity (Supplementary Fig. 5k). More strikingly, the artificial memory performance was suppressed when the flies were allowed to drink before training, demonstrating that memory implanted by PAM-γ4/5 neurons is water satiable (Fig. 3m). We are at present unable to exclusively manipulate the PAM-γ4 neurons. However, blocking R15A04 neurons did not significantly disrupt water learning (Fig. 3a). Combining R15A04-GAL80 with R48B04-GAL4 revealed that R15A04 expresses in R48B04-labeled dopaminergic neurons that innervate γ5 but not γ4 (Fig. 3n). In addition, removing γ5 expression from R48B04 did not restore wild-type water learning (Fig. 3o). The defect remaining in these flies was not observed at the permissive temperature (Supplementary Fig. 5i), and neither water consumption (Supplementary Fig. 5m) nor olfactory acuity (Supplementary Fig. 5n) was different from that of control...
Figure 4 Naive thirst-dependent water-seeking requires different dopaminergic neurons than those required for water learning. (a) Drinking evokes an increase in intracellular Ca\(^{2+}\) in R48B04 dopamine neurons. Time courses of GCaMP5 responses (percentage change in fluorescence, ∆F/F) to water consumption (solid lines are average traces and shaded areas represent the s.e.m., n = 6 flies). Time of water presentation is indicated by the arrow. Panels show pseudo-colored examples of GCaMP5 fluorescence before (left) and after (right) water consumption. Dashed lines outline the distinct anatomical zones of the mushroom body lobes. Scale bar, 15 μm. (b) Blocking R48B04 dopaminergic neurons converts naive water approach in 16-h water-deprived flies into significant avoidance (*P < 0.0001 from other groups and *P = 0.0009 from zero, n = 8; ANOVA and one sample t-test). (c) The naive water approach defect in thirsty R48B04-GAL4; UAS-shi\(^{ts1}(JFRC100)\) flies (*P < 0.0001 as compared to the other three groups, n = 6, 7, 8 and 8 from left to right; ANOVA followed by post hoc Tukey’s HSD test) is nullified by suppressing expression in dopaminergic neurons using R58E02-GAL80 (P = 0.9 as compared to UAS-shi\(^{ts1}(JFRC100)\) control and P = 0.13 to R48B04-GAL4 control; ANOVA followed by post hoc Tukey’s HSD test). (d) Output from 0104 neurons is required for naive water approach in thirsty flies (*P < 0.0001 as compared to the two controls, n = 7, 8 and 8 from left to right; ANOVA). (e) Blocking R48B04 neurons with R48B04-LexA and LexAop\(^{shi}\) impairs naive water approach in thirsty flies (*P < 0.0005 as compared to the control flies, n = 8; ANOVA followed by post hoc Tukey’s HSD test). The impairment was abrogated by removing expression from 0104 neurons (P = 0.71 as compared to the control flies, n = 8; P = 0.0016 to the unreleased flies, n = 8; ANOVA followed by post hoc Tukey’s HSD test). (f) Blocking 0104 neurons after training does not impair 30-min water memory performance in thirsty flies (P = 0.69 as compared to the two controls, n = 8; ANOVA). All error bars are s.e.m. Full descriptions of the fly strains and their corresponding colors used in the bar graphs can be found in Supplementary Table 1.

transgenic flies at the restrictive temperature. We therefore conclude that the key water-reinforcement signals come from PAM-γ4 neurons.

Drinking water activates rewarding dopaminergic neurons

We also tested whether drinking evoked a response in dopaminergic neurons in thirsty flies by expressing GCaMP5 (ref. 29), a genetically encoded indicator of intracellular calcium, with R48B04-GAL4. Drinking water drove a strong increase in GCaMP5 fluorescence in dopaminergic neuron processes in γ4 and β2, and to a lesser extent in the γ5 zone of the mushroom body (Fig. 4a). These results support the model that water reinforcement is conveyed by PAM-γ4 neurons, and they also suggest a possible role for the β2 and γ5 innervating neurons.

Naive water evaluation requires dopaminergic neurons innervating β2 but is independent of DopR1

We reasoned that water-evoked signals in another zone might represent incentive salience signals that control naïve water-seeking behavior. We therefore investigated a role for these dopaminergic neurons in naive approach to water by thirsty flies. Strikingly, blocking R48B04 neurons converted the behavior of naïve thirsty flies from water approach into water avoidance (Fig. 4b), like that observed in water sated flies (Fig. 1a). This behavioral reversal was not evident at the permissive temperature (Supplementary Fig. 6a). Furthermore, blocking R48B04 neurons had no effect on water avoidance in sated flies (Supplementary Fig. 6b), suggesting that these flies perceive water normally and that output from R48B04 neurons is only required for water approach in thirsty flies. A weaker but significant water approach defect was also observed when we expressed a different UAS-shi\(^{ts1}(JFRC100)\) transgene (JFRC100)\(^{30}\) with R48B04-GAL4 (Fig. 4c). This defect was not observed at the permissive temperature (Supplementary Fig. 6c), and these flies showed normal water avoidance when they were water sated (Supplementary Fig. 6d). Moreover, using R58E02-GAL80 (ref. 8) to suppress expression in the PAM dopamine neurons in this combination removed the behavioral defect caused by blocking R48B04 neurons (Fig. 4c). Unlike with water learning, blocking 0104 neurons also abolished naïve water-seeking behavior in thirsty flies (Fig. 4d and Supplementary Fig. 6a,b). In addition, using the intersection of 0104 and R48B04 to suppress expression in β2 neurons (Fig. 3i,j) restored water-seeking to R48B04; UAS-shi\(^{ts1}\) flies (Fig. 4e and Supplementary Fig. 6e,f).

Taken together, our experiments suggest that the β2 neurons are required for the flies to evaluate water vapor signals in the naïve state, whereas the PAM-γ4 neurons assign water value to odors during learning.

As water learning requires the D1 dopamine receptor (Fig. 2b), we also tested this receptor’s role in naïve water-seeking in thirsty flies (Supplementary Fig. 6g). Surprisingly, the water-seeking behavior of thirsty dumb\(^{1}\) mutant flies was indistinguishable from that of thirsty wild-type flies. We speculate that dopamine signals from the β2 neurons to the β’ tip of the mushroom body are interpreted by a different dopamine receptor or that a cotransmitter of dopamine might regulate naïve water-seeking.

Different neurons control naïve and learned water-seeking

We also tested whether the β2 neurons are required for conditioned odor approach by blocking 0104 neurons immediately after training and during testing of water-reinforced memory. No significant
defect was observed (Fig. 4f), consistent with the results of blocking the entire PAM cluster with either 0273-GAL4 or 58E02-GAL4 (Fig. 2e). Paradoxically, blocking R48B04 neurons after training and during testing of water-reinforced memory significantly enhanced learned odor approach ($P < 0.0001$, ANOVA; Supplementary Fig. 7), a trend that was also apparent when blocking 0273 but not 0104 neurons (Fig. 2e). As blocking 0104 neurons did not have any effect and the R48B04 enhancement is the opposite of the effect observed with naive water-seeking, we conclude that the $\beta^2$ neurons are not required for water-seeking using learned odor cues. However, the data suggest that other R48B04 expressing neurons may limit the efficiency of water-reinforced memory expression.

**DISCUSSION**

Psychologists have split reward into wanting, learning and liking components that can be assessed using drinking and feeding behaviors in animal models. Wanting denotes an animal’s desire to seek the resource, whereas learning assigns the value of the consumed food or water to associated sensory stimuli. An animal is considered to like a substance if it accepts that substance as palatable. Our results clearly demonstrate that separate PAM dopaminergic neurons in the fly are required for naive water-seeking behavior (wanting) and for learning with a water reward. In addition, our data suggest that a different neural mechanism controls learned water-seeking. What about liking? Investigators have used acceptance and facial expression such as tongue protrusion as a sign that mammals like a given tastant. Flies extend their proboscis to palatable substances and retract it when presented with something bitter. Furthermore, proboscis extension is controlled by the motivational state of the fly. None of our neural manipulations that impaired naive water-seeking or water learning disrupted proboscis extension to water in thirsty flies (Supplementary Fig. 8). Hunger-responsive dopaminergic neurons in the subesophageal ganglion have been shown to regulate proboscis extension to sugar. It therefore seems possible that analogous thirst-responsive neurons will control responses to water. Nevertheless, it appears that manifestations of thirst in the fly that resemble wanting, learning and liking are supported by separate neural circuitry, at least some of which involve dopaminergic neurons (Supplementary Fig. 9).

Taken together with previous work, our results here demonstrate an elaborate neural circuitry within the mushroom body that allows independent control of naive and learned appetitive behaviors in the fly. Water reinforcement involves different dopaminergic neurons, and independent mechanisms, from those required for sugar learning. Water reward can be associated with odors through the PAM-$\gamma$4 neurons whereas sugar memory is reinforced by other rewarding dopaminergic neurons in the PAM cluster. Thirst motivates naive water-seeking through the activity of the PAM-$\beta^2$ neurons. Although the behavioral expression of learned approach to water-associated odors is also specifically regulated by thirst, the PAM-$\beta^2$ neurons are not essential for learned approach. Hunger releases the mushroom body MP1 dopaminergic neurons to permit expression of sugar-seeking memory. It will therefore be interesting to determine whether other dopaminergic neurons provide a similar inhibitory control over the expression of water-seeking memory. Our data indicate that some R48B04-labeled neurons may play a role. Having mechanisms to separately learn food and water information and retrieve these memories appropriately permits efficient foraging behavior. In addition, segregating the control of naive water-seeking from water learning and memory expression is likely to permit the fly to seek water using learned distance cues that may predict the presence of water, in addition to the most reliable signal, vapor from the water source itself.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank C.J. Burke for his extensive failings and teachings in the art of water learning. We also thank Y. Huang and R. Brain for technical support and the Bloomington stock center, T. Clandinin (Stanford University), D. Gohl (Stanford University), M. Silles (Stanford University), G. Rubin and the Janelia Farm Project, Y. Ben-Shahar (Washington University), K. Scott (University of California, Berkeley), T. Lee (Janelia Farm Research Campus) and J. Dubnau (Cold Spring Harbor) for fly lines. S.L. was supported by an EMBO Long-Term Fellowship. D.O. was supported by an EMBO Long-Term Fellowship and a Sir Henry Wellcome Postdoctoral Fellowship. V.C. was supported by an Andrew Mason Memorial Scholarship. S.W. is funded by a Wellcome Trust Senior Research Fellowship in the Basic Biomedical Sciences and by funds from the Gatsby Charitable Foundation and Oxford Martin School.

**AUTHOR CONTRIBUTIONS**

S.W. and S.L. conceived this project and designed all experiments. S.L. and V.C. designed and optimized the water-conditioning assay and performed all behavioral experiments. Live imaging was performed by D.O. using custom apparatus and software constructed and programmed by C.T. GAL4 lines were visually screened and selected by W.H. Anatomical data were produced by S.L. and W.H. The manuscript was written by S.W. and S.L.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Fly strains. *Drosophila melanogaster* were raised on standard cornmeal-agar food at 25 °C and 60% humidity under a 12 h/12 h light/dark cycle. In all behavioral studies, 5- to 7-d-old flies of both sexes were used and experiments were performed between 9 a.m. and 6 p.m. The wild-type strain was Canton-S. The *ppk28*, *Tbhl* and *dumbb* mutant strains have been described. The UAS-endsi (X3), UAS-shhi (IFCR100), Tdc2-GAL4, R58E02-GAL4, RS8E02-GAL80, 0273-GAL4 and 0104-GAL4 transgenic strains have been described. R48B04-GAL4 and R15A04-GAL4 flies were raised from the Bloomington stock center. The LexAop-rCD2::GFP, LexAop-shhi and LexAop-TrpA1 strains have been described with two miRNA targeting sites: 5′-CGACAGAATGGCTTATGACG-3′ and 5′-CCTAGAAGACTTACTGTC-3′. The UAS-LexA RNAi flies were raised commercially (BestGene) using routine P-element-directed transformation. R48B04-GAL4 was made by inserting the enhancer fragment of R15A04-GAL80 into the pBPGAL80·Uw-6 vector (Addgene plasmid 26231). The R15A04-GAL80 construct was made by inserting the enhancer fragment of R15A04-GAL80 into the pBPGAL80Uw6 vector (Addgene plasmid 26236). The R48B04 and R15A04 enhancer fragments are from the JFRC FlyLight database. The R48B04-GAL4 fly strains were made commercially (BestGene) by site-specific insertion into the attP40 and attP2 landing sites, respectively. The UAS-LexA RNAi was made as described, with two miRNA targeting sites: 5′-GCAGACACGAGCTCTTACTAGC-3′ and 5′-CCTAGAAGACTTACTGTC-3′. The UAS-LexA RNAi flies were raised commercially (BestGene) using routine P-element-directed transformation.

Water deprivation. Approximately 80 flies per vial were water deprived by housing them for a defined time period with a 2-cm x 6-cm piece of dry sucrose-coated filter paper at 25 °C and 60% humidity. For 6-h quick desiccation, flies were kept in vials containing a 2-cm x 3-cm piece of dry sucrose-coated filter paper above a thick layer of Drierite (Sigma-Aldrich). The flies and sugar paper were separated from the Drierite by a layer of cotton wool. The vials were kept in a sealed box containing a thin layer of Drierite for 6 h.

Naive water choice test. Water-sated or water-deprived flies were given 2 min to choose between a dry filter paper–lined tube and one containing a water-soaked filter paper. The water attraction index was calculated as the number of flies in the wet tube minus the number of flies in the dry tube, divided by the total number of flies in each experiment. For the water vapor choice test, the filter papers were put in an inaccessible compartment at the end of the tube. The flies could therefore detect the vapor but could not touch the water.

Proboscis extension reflex assay. PER was performed as described with a few modifications. Flies deprived of water for 16 h were anesthetized on ice and stuck backside down onto nontoxic adhesive flypaper at 23 °C, 60% humidity. Immobilized flies were then transferred to 32 °C, 60% humidity and left to recover for 30 min. PER was assayed by presenting each fly with a drop of distilled water to either the foreleg or labellum. Water was presented three times per fly. Data represent the percentage of the total water offerings that elicited PER.

Ingestion assay. To measure water consumption, flies were placed in a training tube used in the learning assay and allowed to drink for 2 min. Tubes were lined with a filter paper coated with a thin layer of 1% non-nutritive agar containing distilled water and 0.4% FD&C blue no. 1 food dye (Spectrum). After 2 min, flies were quickly frozen at −20°C to prevent excretion. Twenty flies were homogenized in 500 µl phosphate-buffered saline (PBS) and centrifuged at 18,800 × g for 3 min to clear debris. The supernatant was then mixed with 100 µl PBS and centrifuged again at 18,800 × g for 3 min. The dye in the supernatant was then quantified by measuring the absorbance at 625 nm using a nanodrop. Sugar consumption was measured similarly by replacing water in 1% agar with 3 M sucrose.

Water conditioning. The olfactory water conditioning paradigm was modified from the previously described sugar-reinforced olfactory conditioning paradigm. Odors were 3-octanol (7 µl in 8 ml mineral oil) and 4-methylcyclohexanol (14 µl in 8 ml mineral oil). Flies were exposed to one odor for 2 min in a tube lined with dry filter paper, followed by 30 s of fresh air. The flies were then transferred to another tube lined with water-soaked filter paper and exposed to a second odor for 2 min, followed by 30 s of fresh air. To measure learning (3-min memory), the flies were transferred back into water deprivation vials until the time of memory testing. The performance index was calculated as the number of flies running toward the conditioned odor minus the number of flies running toward the unconditioned odor, divided by the total number of flies in each experiment. A single performance index value is the average score from two experiments in which a different population of the same genotype of flies was trained and tested with each odor paired with reinforcement. To satiate flies with water or food, flies were transferred to vials containing 1% agar or standard molasses-based fly food, respectively. Experiments were performed at 23 °C and 60% humidity, except where noted otherwise. For experiments at 32 °C, the flies were moved to 32 °C 30 min before training and maintained at 32 °C throughout the experiment. For experiments using UAS-shhi, the permissive temperature was 25 °C and the restrictive 32 °C. To block neurotransmission for 3-min memory, flies were shifted from 23 °C to 32 °C for 30 min before training and testing. Alternatively, to block neurotransmission after training, flies were moved to 32 °C for 30 min before testing and maintained at that temperature during testing. Artificial learning pairing odor exposure with lexAop-dTrpA1–mediated neural activation was performed as described. The relevant groups of flies were trained and tested in parallel and the order of groupings randomized. Data collection and analyses were not performed blind to the conditions of the experiments. To accommodate likely differences in genetic background between strains from different sources, each experiment included all relevant control groups, in which transgenic lines were crossed to wild-type flies. Between-group comparisons of independent JFRC FlyLight lines was assumed to be the best control for GAL4 lines from that source that exhibited behavioral phenotypes.

Imaging. Brains were dissected in PBS and fixed in PBS with 4% paraformaldehyde at room temperature for 20 min. They were washed three times, 20 min each, in PBS containing 0.5% Triton-X100 (PBT), followed by 30 min incubation in PBT containing 5% normal goat serum. Anti-GFP (1:1,000; Invitrogen, A11122), anti-TF (1:200; Millipore, AB152) and anti-nc82 (1:50; DSHB) antibodies were added to the solution and brains were incubated overnight at 4 °C. Brains were then washed in PBT three times, 20 min each, at room temperature, followed by incubation in PBT containing Alexa 488–conjugated goat anti-rabbit (1:100; Invitrogen, A11034) and Cy3-conjugated goat anti-mouse (1:200; Jackson ImmunoResearch, 115-165-003) overnight at 4 °C. Brains were then washed in PBT three times, 20 min each, at room temperature, before being mounted on slides with Gold Anti-Fade mounting solution (Invitrogen). Imaging was performed using a Leica TCS SP5X confocal microscope. The resolution of the image stack was 1024 × 1024 with 1 µm step size. Images were processed using Fiji. For each genotype, at least two brains were dissected to confirm that they had the same pattern of expression.

Two-photon in vivo calcium imaging. UAS-GCaMP5;R48B04-GAL4 flies up to 7 d old were water-deprived for 6–8 h, briefly anesthetized on ice and affixed with wax to a custom-built imaging chamber. The head capsule was opened under sugar-free HL3-like saline. Two-photon imaging was performed using a multiphoton imaging system (Scientifica) with a 40× 0.8 NA water-immersion objective controlled by ScanImage 3.8 (ref. 41) software. Fluorescence was excited at 910 nm, 80 MHz repetition rate, ~70 fs, using a Ti:sapphire laser (ChromaCameleon). Images (256 × 256 pixels) were acquired at approximately 6 Hz. Water was delivered to the fly (for ≤10 s) using an automated feeding device while the fly’s drinking behavior was observed using a Stingray CCD camera (Allied Vision Technologies). Two-photon images were analyzed using Fiji/ImageJ. Regions of interest were manually assigned to the anatomically distinct lobe zones. Intensity tables were exported to Microsoft Excel and the ΔF/F was calculated, with an F calculated using the 20th–30th frames. Traces were generated in Prism 6 (GraphPad Software).

Statistical analysis. Data distribution was assumed to be normal. Data were analyzed using Prism 6 (GraphPad Software). We used unpaired two-tailed t-tests with Welch’s correction (not assuming equal s.d.) to compare the difference between two groups. One-way ANOVA with Geisser-Greenhouse
correction (not assuming equal variability of differences) and Tukey's multiple comparisons tests were used to compare more than two groups. Definition of statistical significance was set as \( P < 0.05 \). One-sample t-tests were used to detect the difference between an actual mean and zero. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in many previous publications.

A Supplementary Methods Checklist is available.

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