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Olfactory learning skews mushroom body output pathways to steer behavioral choice in *Drosophila*

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Learning permits animals to attach meaning and context to sensory stimuli. How this information is coded in neural networks in the brain, and appropriately retrieved and utilized to guide behavior, is poorly understood. In the fruit fly olfactory memories of particular value are represented within sparse populations of odor-activated Kenyon cells (KCs) in the mushroom body ensemble. During learning reinforcing dopaminergic neurons skew the mushroom body network by driving zonally restricted plasticity at synaptic junctions between the KCs and subsets of the overall small collection of mushroom body output neurons. Reactivation of this skewed KC-output neuron network retrieves memory of odor valence and guides appropriate approach or avoidance behavior.

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Current Opinion in Neurobiology 2015, **35**:178–184

This review comes from a themed issue on **Circuit plasticity and memory**

Edited by **Thomas Mrsic-Flogel** and **Alessandro Treves**

For a complete overview see the Issue and the Editorial

http://dx.doi.org/10.1016/j.conb.2015.10.002

Introduction

Progress in understanding the neural mechanisms of learning and memory has come from studies in several organisms. Some of the animal models were initially chosen because of a unique experimental strength, such as large accessible cells that facilitate cellular and physiological analyses; or complex circuits that more obviously resemble those present in humans. Olfactory learning and memory has been studied in the fruit fly *Drosophila* for >40 years [1,2]. Flies can be taught to associate odors with punishing shock, heat or bitter-taste, or rewarding sugars or water [1–5,6••]. After training they either avoid an odor predicting unpleasantness or approach an odor expecting reward. The *Drosophila* brain has approximately 100,000 neurons and recent progress suggests that the fly mushroom body (MB), an ensemble of around 2200 intrinsic neurons, might be part of a circuit upon which the traditional cellular, systems and behavioral neuroscience boundaries can be bridged. Furthermore, it is now appreciated that the fan-out fan-in neural architecture of the MB shares structural features and perhaps a coding logic with that of the cephalopod vertical lobe and the mammalian olfactory, cerebellar and hippocampal structures [7,8,9•]. Studying the reduced complexity of the MB should therefore be generally informative.

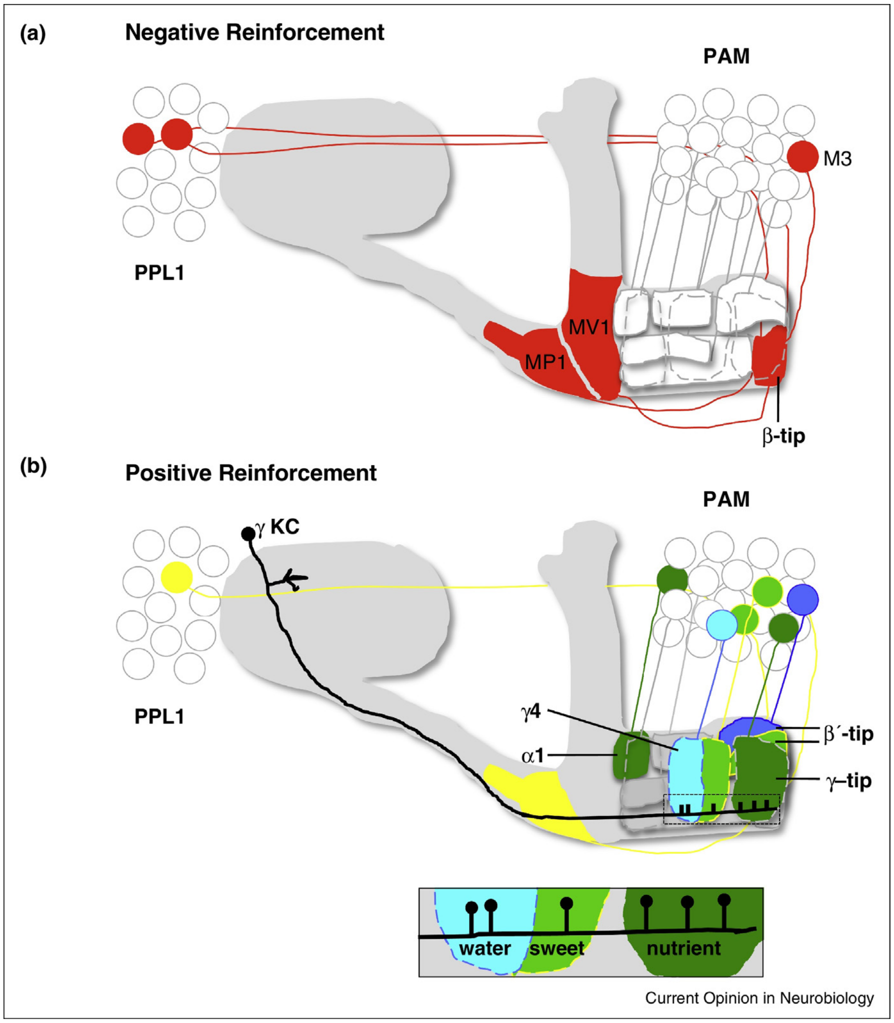
Odors are sparsely represented in MB Kenyon cells

Flies detect odors in the environment using peripheral olfactory sensory neurons on their antennae and maxillary palps. These neurons send this information to glomeruli in the fly antennal lobes where it is processed and transferred to a subpopulation of 150 projection neurons (PNs). PNs project from the antennal lobes to the calyx of the MB and the lateral horn (LH). Classically the MBs have been considered to be the pathway for learning, while the LH guides innate odor-responses [10–12], although this is now recognized to be an oversimplification [13••,14,15]. Each of the 2200 MB KCs receives input from ∼6 randomly chosen PNs [16,17,18•] providing a large fan-out expansion in the coding space for odors. Recordings from KC somata suggest that the αβ, α′β′ and γ subclasses fire relatively few times per odor exposure [17,19,20] and strong input to at least half of the KC's dendritic claws correlates with the cell reaching threshold to fire [21••]. In addition to the PN-KC connectivity, KCs drive local GABAergic inhibition in the calyx which isolates the strongly odor-activated KCs from the rest of the population [22,23]. As a result only 5–20% of the overall KC ensemble responds to a given odor [19]. Interestingly, activity in ∼5% of the total KC population of randomly chosen αβ and γ KCs was optimal to substitute for an odor stimulus during aversive learning and retrieval [24•]. Therefore randomly distributed sparse combinations of cells in the KC population provide an association matrix in which to store odor-specific memory. This is important because it illustrates that a vast number of stimuli can be encoded in the KC ensemble, if they reach a significant combinatorial representation.

Learning assigns values to specific zones on odor-activated KCs

Different fly dopaminergic neurons (DANs) provide positive and negative value signals [25]. Most of the DANs that innervate the MB reside in two discrete clusters called PPL1 and PAM (Figure 1). Each PPL1 neuron that innervates the MB projects presynaptic terminals to a unique zone on the vertical α or α′ lobes, or heel and

Figure 1



Schematics of reinforcing dopaminergic neurons that innervate the MB. **(a)** The MB-MP1 [PPL1-γ1pedc] and MB-MV1 [PPL1-γ2α′1] DANs in the protocerebral posterior lateral (PPL) 1 cluster provide negative reinforcement signals. The MB-MV1 neuron projects to the lower stalk and junction region and the MB-MP1 neuron innervates the heel and distal peduncle. In addition, the aversive MB-M3 (PAM-β2β′2a) neuron from the protocerebral anterior medial (PAM) cluster ramifies on the tip of the β lobe. All neurons shown have an identical paired neuron that primarily innervates the contralateral MB lobes. **(b)** DANs in the PAM cluster mostly provide positive reinforcement signals. PAM DANs representing sugar sweetness (green with yellow outline), nutritious value of sugar (darker green) and water (blue) project to discrete zones of the horizontal β, β′ and γ MB lobes (marked with dotted outlines for γ); sweet taste to β′2am and γ4, sugar nutrient to γ5b (tip) and α1 (and possibly β1 β2, not illustrated), and water to a subregion of γ4 that appears distinct from the sweet-taste DANs. In addition, naïve evaluation of water vapor in thirsty flies requires a DAN that innervates β′2p. Reward type is therefore differentially represented in the DAN population and along the MB lobes. Several of the PAM DANs also have a projection to the contralateral MB. A single γ KC is shown with inset illustrating a model where adjoining segments of the KC arbor contain KC presynaptic terminals that are reinforced by DANs for water, sweet taste, or sugar nutrient value. These presynapses are assumed to wire to MBONs with a very similar zonally restricted anatomy to that of the DANs. Cell body position is not stereotyped and diagrams are not intended to be anatomically accurate. These illustrations are edited from [25].

surface of the peduncle. Several of them can convey negative reinforcement value during learning (Figure 1a) [26–28].

Aversive stimuli such as electric shock, high temperature and bitter substances/insect repellent appear to bottleneck onto the same negatively reinforcing DANs (MP1 [PPL1-γ1pedc] and MV1 [PPL1-γ2α′1]) suggesting that reinforcing DANs coding aversion may lack information of the quality of the stimulus and simply represent stimulus magnitude [3,4]. A different DAN (aSP13 [PAM-γ5]) innervating the tip of the MB γ lobe has been implicated in courtship conditioning [29].

Positive reinforcement signals are provided by subsets of the approximately 100 neurons in the PAM cluster [30,31] and they predominantly innervate adjacent zones on the horizontal β, β′ and γ lobes (Figure 1b). Perhaps surprisingly, discrete PAM DANs convey the reinforcing effects of the sweet taste and nutrient value of sugar [32••,33••] and of water reward [6••]. In addition, identified sugar and water responsive DANs project to unique zones on the MB lobes suggesting that reward identity, and therefore the respective learning-related plasticity, is represented in different places along the axon of an individual KC (Figure 1b).

It appears that reinforcement is not uniform across all the KCs in a DAN-marked zone. Some aversively reinforcing DANs do not innervate the $\alpha\beta_c$ neurons [34], which suggests that certain KC representations of odors may already be skewed for valence. Interestingly, the αβ core neurons are crucial for the retrieval of approach memories [34] and for time-consuming odor choices [35].

DAN zones have corresponding MB output neurons

Outputs from the 2200 KCs fan-in onto 34 MBONs of 21 types [36,37••]. Strikingly the dendrites of each of these MBONs are largely confined to a single, or a few, DAN zones. For instance, the axons from sugar rewarding dopaminergic neurons overlap with the dendrites of the M4/6 (or MBON-β2β′2a, MBON-β′2mp and MBON-γ5β′2a) MBONs on the tips of the MB horizontal lobes [13••]. Similarly, another MBON is dendritic in a zone of the γ lobe that receives water-reinforcing DAN input [6••,37••]. Since each type of DAN contacts a defined stretch of an individual KCs axonal arbor (Figure 1b), they are likely to only modify *en passant* KC output synapses onto MBONs in their respective zone. Such an organization predicts that water memory implements unique KC-MBON connections to those used for sugar memories. Since other sugar and water-independent DANs provide positive reinforcement when they are artificially activated [32••,34], we speculate that other KC-MBON zones might represent different rewarding events, such as additional components of food, sex and sleep.

Learning skews the odor-drive to collections of KC-MBON junctions

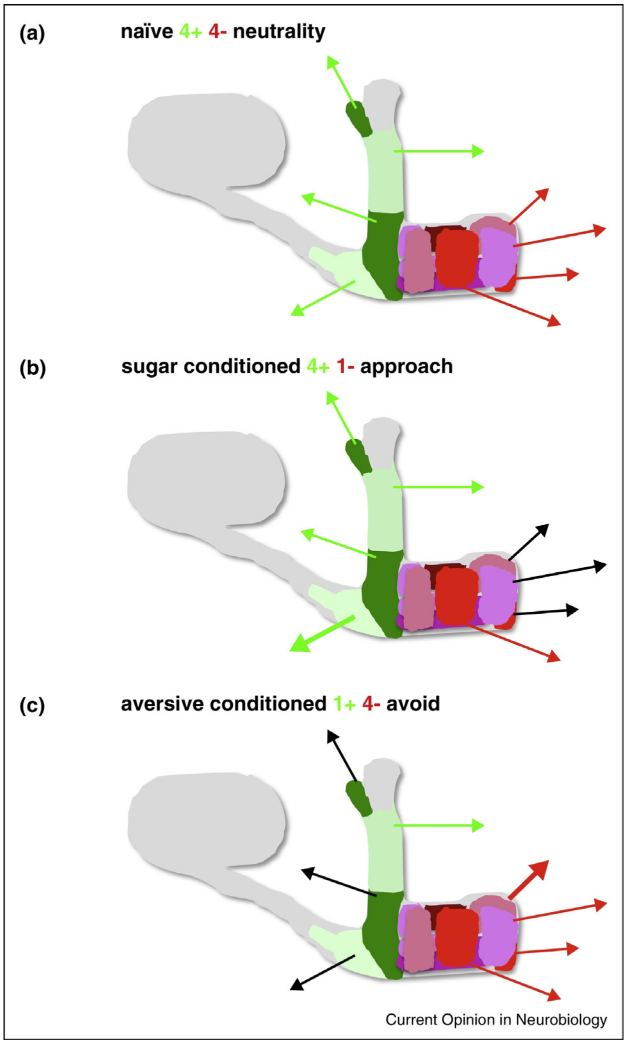
Evidence suggests that dopamine drives learning via the presynaptically expressed dDA1 receptor in KCs [38,39] and several studies have demonstrated dopamine-driven plasticity of KC responses [40•,41]. If learning modifies the output of odor-activated KCs, this should be evident in the activity of the MBONs. Indeed, aversive learning has been reported to depress the odor-drive to the MB vertical lobe outputs MB-V2α [MBON-α2sc] and MB-V2α′ [MBON-α′3] [42] whereas reward learning potentiates drive to MB-V3 [MBON-α3] [43], although others reported potentiation of MB-V3 [MBON-α3] responses after aversive training [44]. In addition, the relative odor-drive to dendrites of β′2 outputs on the horizontal lobe tips was shown to be bi-directionally altered by learning [13••]. Aversive training potentiated responses [13••,45••] whereas appetitive training depressed them [13••]. Taken together these studies demonstrate that learning changes the relative odor drive to identified MBONs that are required for memory expression. However, how does a change of drive to a particular MBON translate to a change in odor-driven behavior? A clear answer to this question was provided by experiments that manipulated the activity of the M4 [MBON-β2β′2a, MBON-β′2mp] and M6 [MBON-γ5β′2a] outputs [13••]. Blocking these neurons to mimic the reward learning induced depression of the KC-M4/6 connection, converted odor avoidance into odor approach in naïve flies. Furthermore, optogenetic activation of the M4 [MBON-β2β′2a, MBON-β′2mp] and M6 [MBON-γ5β′2a] neurons drove avoidance behavior. A parallel extensive study activated individual pairs of almost all of the MBONs [46••]. Many of the glutamatergic MBONs on the horizontal lobe, including M4/6 neurons, triggered avoidance whereas some cholinergic MBONs on the vertical lobes and the GABA-ergic MVP2 [MBON-γ1pedc > α/β] output on the heel and peduncle directed approach [46••]. Co-activating opposing MBON pathways neutralized behavior [46••].

The observed learning-related changes of odor-drive to MBONs, and intrinsic valence of particular MBONs support a model wherein learning skews collections of KC-MBON pathways that are ordinarily balanced in naïve flies (Figure 2a). Appetitive learning promotes odor approach by depressing odor-drive to avoidance MBON pathways and perhaps strengthening approach pathways (Figure 2b). In contrast aversive learning promotes odor avoidance by depressing odor-drive to MBON pathways that direct approach while strengthening those for avoidance (Figure 2c). During memory testing, reactivation of these skewed KC-MBON networks by the trained odor retrieves the memory valence and either leads to odor-approach or avoidance behavior.

The requirement for MBON output has been shown to shift with phases of aversive memory [45••], reminiscent of the previously established temporally evolving requirement for output from the different, α′β′ and αβ classes of KCs for memory processing and the expression of particular memory phases [47–49]. It will be important to understand how appropriate behavioral instruction is maintained as the anatomical substrate changes.

Although we have focused on olfactory memory, recent studies have shown that the *Drosophila* MB also plays a crucial role in visual [50] and taste memories [51,52]. If parallel sets of KCs represent olfactory, gustatory and visual stimuli, the same reinforcing DAN systems could

Figure 2



A piano-playing model for learned valence in the KC-MBON network. **(a)** The canonical view on higher order processing in the fly brain places the LH (not shown) as instrumental for the expression of innate odor-driven behaviors. Experiments blocking all synaptic output from KCs either by ablation or acute silencing suggested the MBs were dispensable for innate odor-driven behavior, but essential for learned responses. However recent findings demonstrate that blocking the MBONs from the tips of the horizontal MB lobes radically alters naïve and learned odor-driven behaviors [13••,15•]. In addition, the activity of particular MBONs is now known to favor either avoidance (red arrows) or approach (green arrows) [13••,46••]. It therefore seems logical that the contribution of these MBONs is integrated and balanced in the naïve fly, leading to an apparent lack of contribution from the MB and neutrality in naïve odor-driven tasks. For simplicity we illustrated the balance as equal numbers of outputs (4 plus and 4 minus = zero, neutrality), but it need only be balanced by the relative weights. **(b)** Reward learning with sugar depresses the odor-specific KC connections to avoidance MBONs (black arrows). In addition it modulates/enhances approach connections (thicker green arrow). This skewed balance (4 plus and 1 minus) now favors odor-driven approach. **(c)** Aversive conditioning depresses the odor-specific KC connections to approach MBONs (black arrows). In addition it enhances avoidance connections (thicker red arrow). This skewed balance (1 plus and 4 minus) now favors odor-driven avoidance. Avoidance neurons are glutamatergic whereas approach neurons are cholinergic or GABAergic [13••,46••].

intersect all these information streams and thereby simultaneously assign value through learning to odors, visual features and tastes. These memories would then be stored using a similar mechanism to that illustrated for odors (Figure 2), where the MBON drive from KCs that are activated by a specific taste or visual feature would be skewed either towards approach or avoidance. It will be important to determine the extent to which these modalities and memories are integrated within the MB network.

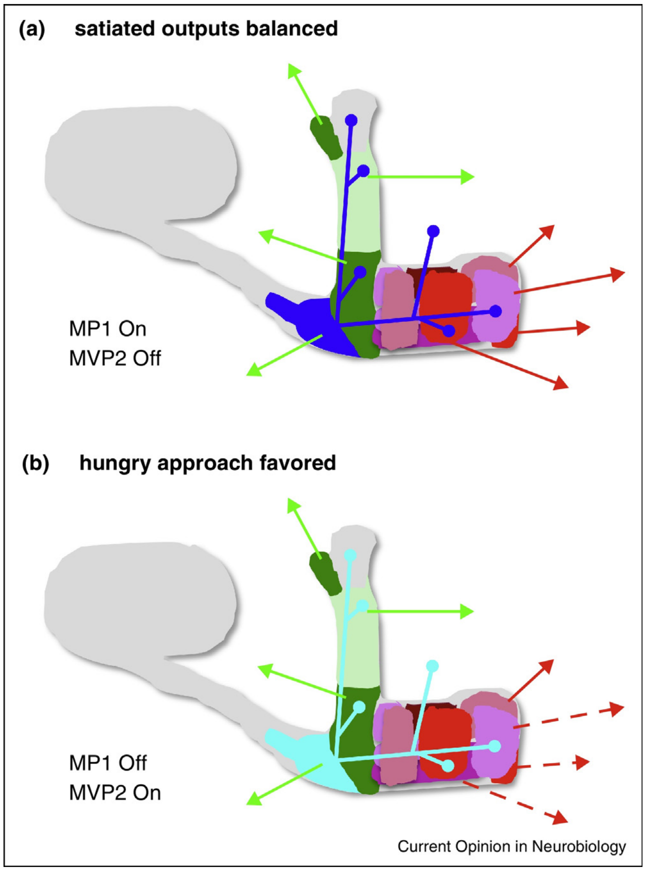
State-dependence — an additional level of dopaminergic control

Sugar memory is most robustly expressed in hungry flies [53] whereas thirst promotes the expression of water memory [6••]. It seems possible that forming these memories in different zones on the MB provides a simple organizational scaffold onto which additional control can be differentially exerted. The MB-MP1 [PPL1-γ1pedc] DANs provide the inhibitory constraint of satiety on the expression of sugar memory [53]. The MB-MP1 [PPL1-γ1pedc] neurons can also convey short term aversive memory reinforcement [28] suggesting that negative reinforcement and motivational processes are tightly interlinked in the MB. MP1 [PPL1-γ1pedc] neurons have also been implicated in the transition between different memory phases [54,55] and forgetting [56]. It is interesting to note that the MB-MP1 [PPL1-γ1pedc] neurons occupy the same zones in the heel and peduncle as the GABAergic MVP2 [MBON-γ1pedc > α/β], whose activation drives approach behavior [46••]. The anatomy of MVP2 [MBON-γ1pedc > α/β] suggests that they are feed-forward local MB inhibitory interneurons (Figure 3). It therefore seems plausible that the internal state of hunger also skews the balance of MBON pathways so that those favoring approach are preferentially activated by relevant trained odors. In addition, such a function would indicate that the first layer of MBON integration is within the MB itself. It will be important to determine the role of other neurons that connect MBON zones [32••,36,37••] and whether the thirst-dependence of water memory expression [6••] involves a similar DAN control mechanism to that of sugar memory. DANs that innervate the tip of the β′ lobe control the thirst dependence of water vapor seeking in naïve flies [6••]. In addition, DANs and MBONs have been implicated in hunger-dependent modulation of naïve responses to carbon dioxide [15•], temperature preference [57,58], and the regulation of sleep [46]. Therefore, DAN-driven modulation of the MB does not exclusively gate learned behaviors and might more broadly control the expression of state-dependent goal-directed behaviors.

Where do the MBONs go?

Some cholinergic MBONs project presynaptic terminals into the LH [42,46••] suggesting that part of the MB-routed learned odor information is reunited and integrated with the more direct PN-driven activity in the LH.

Figure 3



Model how local feed-forward inhibitory interneurons in the MB could mediate the motivational control of sugar memory retrieval. **(a)** The MB-MP1 [PPL1-γ1pedc] DANs that innervate the heel and peduncle of the MB provide the inhibitory constraint of satiety on the expression of sugar reward memory [53]. The MB-MP1 presynaptic terminals overlap with the dendrites of the GABAergic MVP2 [MBON-γ1pedc > α/β] (dark blue) [46••] suggesting that MB-MP1 DANs drive plasticity between KC synapses in these regions and the MVP2 MBONs. In the satiated fly the MB-MP1 DANs are tonically active/on and therefore inhibit odor-drive to MVP2, reducing feed-forward inhibition to MBON junctions, such as M4 [MBON-β2 β′2a, MBON-β′2mp] and M6 [MBON-γ5β′2a] outputs on the horizontal lobe tips that drive avoidance. This situation inhibits the expression of reward memories. **(b)** In hungry flies the MB-MP1 neurons are inhibited/turned off by the action of Neuropeptide F [53]. This results in increased odor-drive to MVP2 and therefore more feed-forward inhibition (MVP2 neuron now light blue) to MBON avoidance pathways (dashed red arrows). This situation favors expression of conditioned odor approach behavior. Interestingly, only nutrient-dependent sugar memory expression requires the flies to be hungry [32••] and MVP2 innervates the relevant α1 zone of the MB. Furthermore, water-reinforced memory expression is promoted by thirst and not hunger and the MVP2 neuron does not seem to have an arbor in the γ4 water-reinforcement zone. A similar mechanism could provide state-dependence to visual and tastant memories.

Other cholinergic and glutamatergic MBONs project to a region of neuropil called the superior medial protocerebrum, or SMP, and additional surrounding zones [46••]. Interestingly, these zones also contain the dendritic arbors of many classes of DANs [13••,37••]. Detailed anatomical studies suggest that the dendrites of the DANs innervating a particular MB zone closely overlap with the presynaptic boutons of their corresponding MBONs [37••]. This arrangement suggests that recurrent connections exist between KCs, MBONs and DANs [13••,15•,37••]. These microcircuit motifs could serve stimulus re-evaluation functions integrating MB output and reinforcing stimulus-specific information; for example, the reliability of shock punishment, sugar or water reward, or relative shock value [34]. A full understanding of how avoidance and approach behaviors are generated will require knowledge of multimodal processes in the MB, the complex MBON interconnections in the LH and SMP, and ultimately how the downstream circuits controlling locomotion are instructed.

Conflict of interest statement

We confirm that there are no known conflicts of interest associated with this manuscript and there has been no significant financial support for this work that could have influenced its outcome. Both authors have read and approve of this manuscript.

Acknowledgements

We thank Suewei Lin for comments on the manuscript. DO was supported by an EMBO Long-Term Fellowship and a Sir Henry Wellcome Postdoctoral Fellowship. SW is funded by a Wellcome Trust Senior Research Fellowship in the Basic Biomedical Sciences and by funds from the Gatsby Charitable Foundation, the Oxford Martin School and the Bettencourt-Schueller Foundation.

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