

**An optogenetic analogue of second-order reinforcement in *Drosophila***

**König et al**

**Biology Letters**

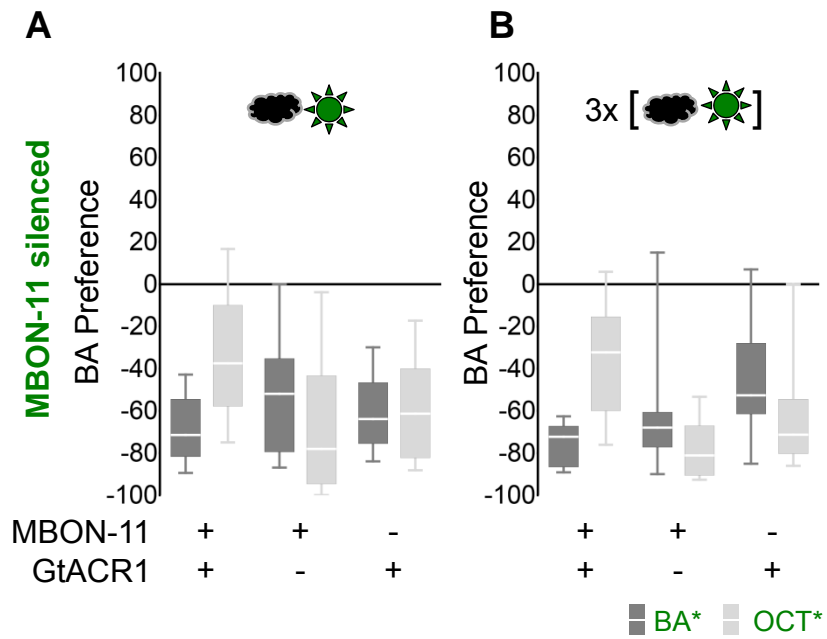
**Supplemental Material**

**Figure S1**

**Figure S2**

**Figure S3**

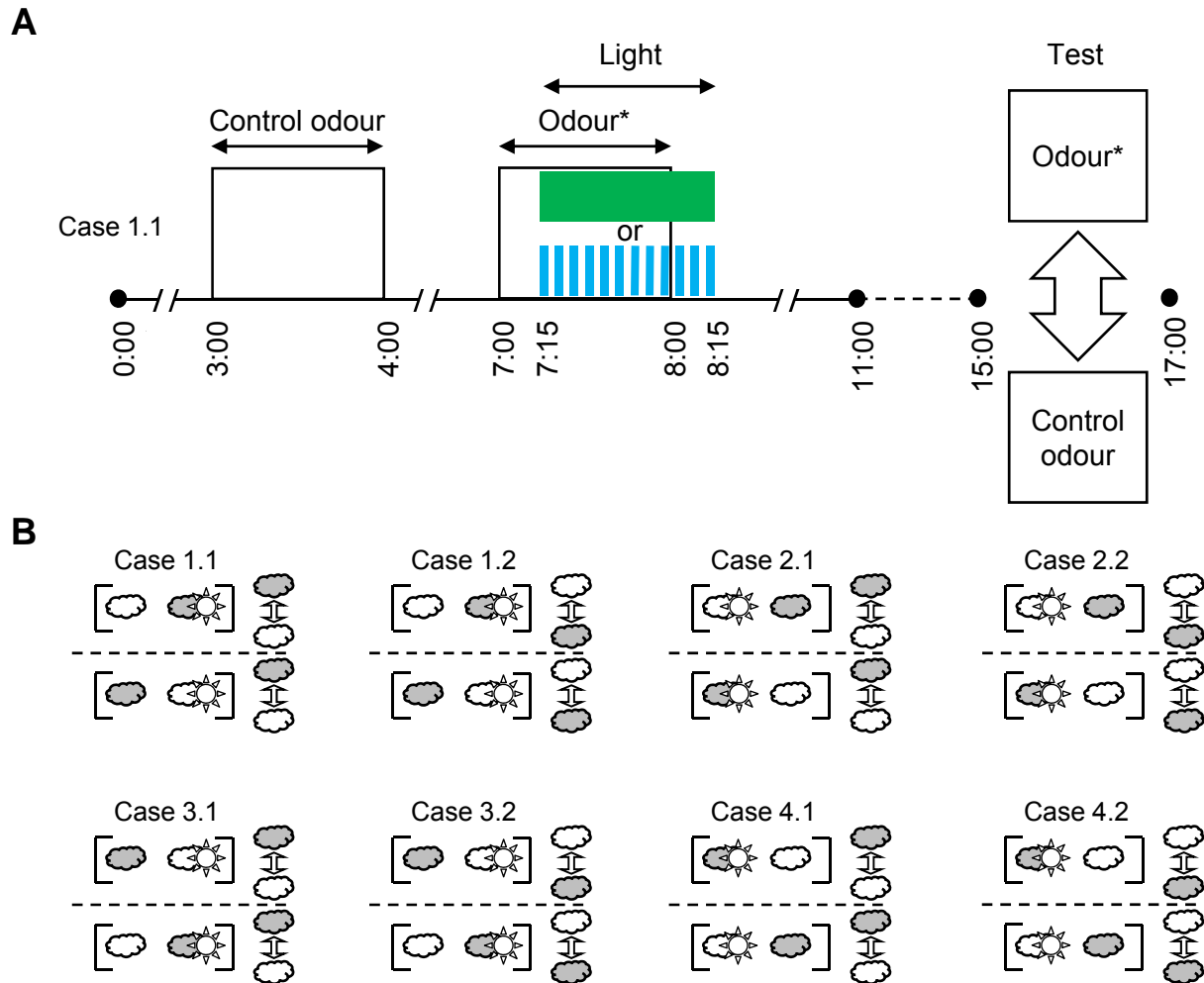
**Figure S4**



**Figure S1**

**(A)** BA Preference scores underlying the associative memory scores shown in Figure 1B. Dark and light grey boxes present the relative preference for BA when BA or OCT, respectively, was paired with green light presentation. Sample sizes can be found in Supplementary Material, Table S1. Other details as in the legend of Figure 1.

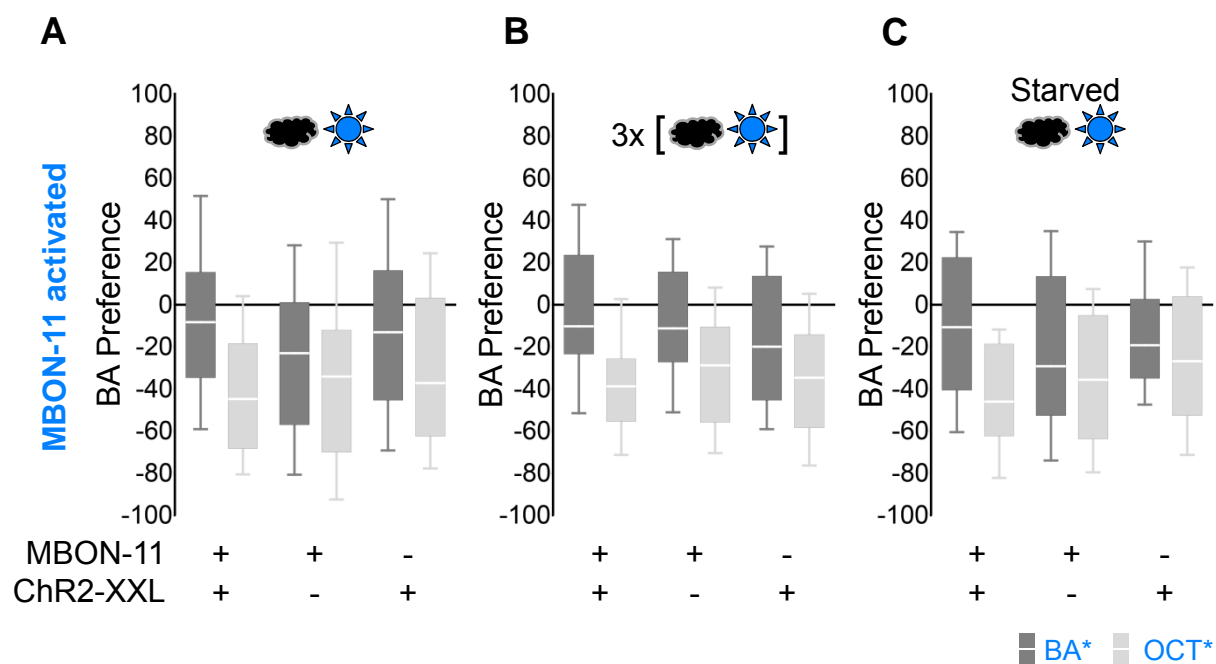
**(B)** BA Preference scores underlying the associative memory scores shown in Figure 1C. Other details as in (A).



**Figure S2**

**(A)** Sketch of the temporal relationships of events during training and testing, for Case 1.1 as an example. At time 0:00 min, cohorts of ~ 100 flies were gently loaded into the experimental setup. From 3:00 min on, the Control odour was presented for 1 min. At time 7:00 another odour was presented for 1 min followed by light presentation at 7:15 min; this odour is designated Odour\*. To apply blue (activation) or green (silencing) light, we used 2.5 cm-diameter and 4.5 cm-length hollow tubes, with 24 LEDs of 465 nm (blue) or 520 nm (green) peak wavelength mounted on the inner surface. These tubes fitted around transparent training tubes harbouring the flies. Blue light was applied as 12 pulses, each 1.2 s-long and followed by the next pulse with a 5 s onset-to-onset interval; green light was presented continuously. The absolute irradiance in the middle of the training tube during light was  $200 \mu\text{W}/\text{cm}^2$  (blue) and  $140 \mu\text{W}/\text{cm}^2$  (green) as measured with an STS-VIS Spectrometer (Ocean Optics, Ostfildern, Germany). For the test, which took place from 15:00 min on, the flies were transferred to a choice point of a T maze with Control odour and the Odour\* presented from either side. After 2 min, the arms of the maze were closed and the flies were counted. Training was performed in dim red light, the test in darkness.

**(B)** Sketches of all combinations of temporal events and spatio-temporal permutations of performing the experiment. Open clouds indicate OCT, clouds with grey fill indicate BA; the star indicates light presentation, which could be either green or blue. For each Case, top and bottom rows depict reciprocal training conditions. Cases 1.1 and 1.2 differ from one another in terms of Control odour and Odour\* position within the setup (back or front) during the test; the same for 2.1/2.2 etc. Additionally, in Cases 1 and 3 the Control odour was presented before the Odour\*, differing from Cases 2 and 4, where the Odour\* was presented before the Control odour. Furthermore, Cases 1 and 2 started with OCT as the first odour whereas Cases 3 and 4 started with BA.

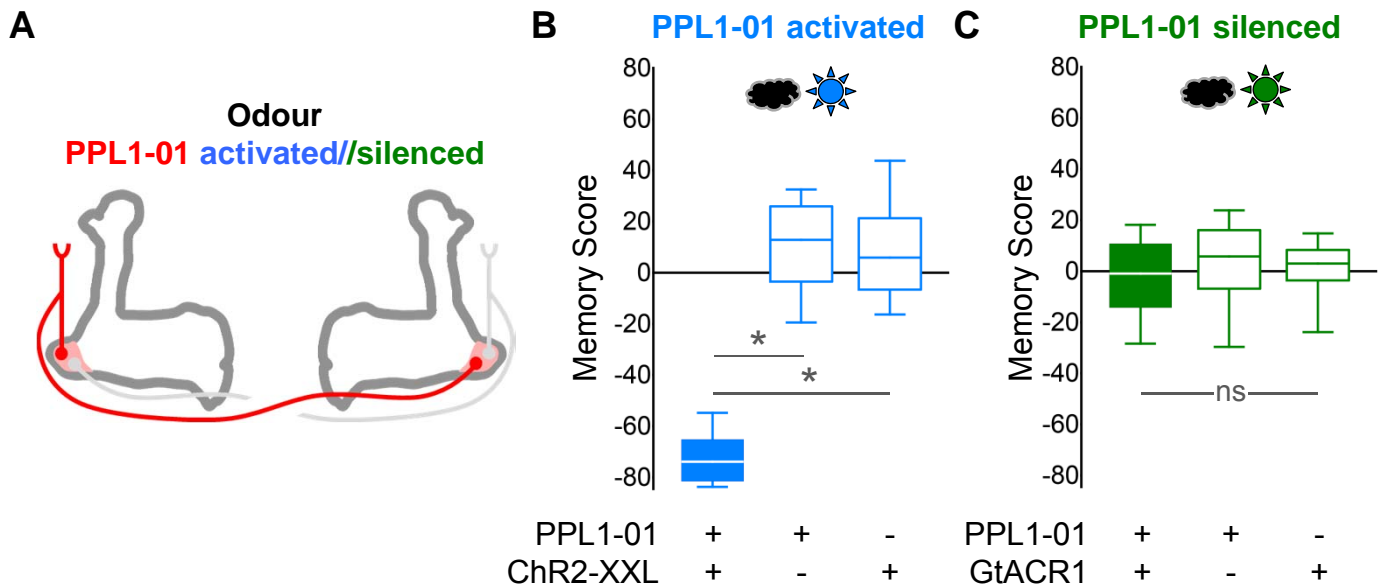


### Figure S3

**(A)** BA Preference scores underlying the associative memory scores shown in Figure 2A. Dark and light grey boxes present relative preference for BA when BA or OCT, respectively, was paired with green light presentation. Sample sizes can be found in Supplementary Material, Table S1. Other details as in the legend of Figure 1.

**(B)** BA Preference scores underlying the associative memory scores shown in Figure 2B. Other details as in (A).

**(C)** BA Preference scores underlying the associative memory scores shown in Figure 2C. Other details as in (A).



### Figure S4

**(A)** Sketch of how the dopaminergic mushroom body input neuron PPL1-01 innervates the mushroom body (after 17).

**(B)** Presenting an odour (black cloud) with blue light (blue star) leads to aversive associative memory in flies expressing the blue-light-gated cation channel ChR2-XXL in PPL1-01, but not in genetic controls.

**(C)** Presenting an odour (black cloud) with green light (green star) to silence PPL1-01 using the green-light-gated anion channel GtACR1 does not lead to associative memory. Sample sizes and statistical results can be found in Table S1. Other details as in the legend of Figure 1.