A temporally restricted function of the Dopamine receptor Dop1R2 during memory formation

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SUMMARY

Dopamine is a crucial neuromodulator, which is involved in many brain processes, including learning and the formation of memories. Dopamine acts through multiple receptors and controls an intricate signaling network to regulate different tasks. While the diverse functions of dopamine are intensely studied, the interplay and role of the distinct dopamine receptors to regulate different processes is less well understood. An interesting candidate is the dopamine receptor Dop1R2 (also known as Damb), as it could connect to different downstream pathways. Dop1R2 is reported to be involved in forgetting and memory maintenance, however, the circuits requiring the receptors are unknown. To study Dop1R2 and its role in specific spatial and temporal contexts, we generated a conditional knock-out line using the CRISPR-Cas9 technique. Two FRT sites were inserted, allowing flippase-mediated excision of the dopamine receptor in neurons of interest. To study the function of Dop1R2, we knocked it out conditionally in the Mushroom body of *Drosophila melanogaster*, a well-studied brain region for memory formation. We show that Dop1R2 is required for later memory forms but not for short-term memories for both aversive and appetitive memories. Moreover, Dop1R2 is specifically required in the the α/β-lobe and the α'/β'-lobe but not in the γ-lobe of the Mushroom body. Our findings show a spatially and temporally restricted role of Dop1R2 in the process of memory formation highlighting the differential requirement of receptors during distinct phases of learning.

Keywords:
Dop1R2, conditional knock-out, Long-term memory, Mushroom body lobes

INTRODUCTION

The neuromodulator dopamine is involved in a plethora of brain functions. Among them are learning and memory, reinforcement signaling, reward, arousal, and motor functions (Klein et al., 2019; Missale et al., 1998; Tritsch & Sabatini, 2012). Perturbations in the dopaminergic system are associated with diseases like Parkinson's disease, addiction, Depression, Schizophrenia, and many more (Klein et al., 2019; Missale et al., 1998; Tritsch & Sabatini, 2012). Therefore, it is important to understand dopamine signaling in greater detail allowing to develop more effective therapeutics or preventive measures.

Dopamine is synthesized from tyrosine in dopaminergic neurons and binds to G-protein coupled receptors (GPCRs) (Klein et al., 2019; Missale et al., 1998; Yamamoto & Seto, 2014). These dopamine receptors have seven transmembrane domains, with an intracellular C-terminus and an extracellular N-terminus. The receptors interact with different G-Proteins, which are heterotrimeric protein complexes consisting of an α-, β-, and γ-subunit. The G-protein complex binds to the third intracellular loop and the C-terminus of the receptors. Humans and other mammals express five different dopamine receptors, separated into two types. Type 1 dopamine receptors elevate protein kinase A (PKA) signaling and cyclic adenosine monophosphate (cAMP) levels via the alpha subunit $G\alpha_s$, whereas type 2 receptors inhibit PKA activity via $G\alpha_{i/o}$ thus reducing cAMP levels (Klein et al., 2019; Missale et al., 1998; Tritsch & Sabatini, 2012). Downstream of cAMP and PKA, transcriptional activation mediated by c-AMP response element-binding protein leads to the expression of immediate early genes as a response to dopamine signaling (Carlezon et al., 2005; Neves et al., 2002). This pathway is a key regulator of long-term memory (LTM) formation (Alberini & Kandel, 2014; Kaldun & Sprecher, 2019; Kandel, 2012). Moreover, Dopamine receptors are able to modulate internal Ca^{2+} levels by engaging the Phospholipase C signaling pathway. Furthermore, the G-Protein β and γ subunits can directly modulate voltage-gated and ligand-gated ion channels (Klein et al., 2019; Ledonne & Mercuri, 2017; Missale et al., 1998; Nishi et al., 2011; Tritsch & Sabatini, 2012). However, the coordination of the receptors and the different dopamine mediated processes in specific circuits is still a mystery. Therefore, an accessible and well-characterized model is required, like the olfactory circuit of *Drosophila melanogaster*. The

connectome of this circuit is known, and it can be manipulated with the sophisticated genetic tools of the fly [Takemura, 2017 #383; Li, 2020 #1015][Owald, 2015 #200]. Similar to mammals, the fly uses dopamine in learning, memory, forgetting, negative and positive reinforcement, locomotion, and sleep and arousal regulation (Berry et al., 2012; Burke et al., 2012; Karam et al., 2020; Sabandal et al., 2021; Sabandal et al., 2020; Siju et al., 2021; Siju et al., 2020; Sitaraman et al., 2015; Tomita et al., 2017; Waddell, 2013; Yamamoto & Seto, 2014). Additionally, L-Dopa, the precursor for dopamine and a powerful receptor agonist, as well as other pharmaceutics have been shown to function in the fly (Yamamoto & Seto, 2014). Thus, the dopaminergic system can be studied in *Drosophila*, taking advantage of the available genetic tools.

The fly uses four different dopamine receptors. Dop1R1 (Dumb) (Kim et al., 2003; Sugamori et al., 1995) and Dop1R2 (Damb) (Feng et al., 1996; Han et al., 1996) are type 1 receptors. Dop2R is a type 2 receptor (Hearn et al., 2002), and dopEcR is a type 1 dopamine receptor that also uses ecdysone as a ligand (Srivastava et al., 2005). All four receptors were shown to be involved in learning, memory and forgetting (Berry et al., 2012; Karam et al., 2020; Kim et al., 2007; Lark et al., 2017; Qi & Lee, 2014; Qin et al., 2012; Scholz-Kornehl & Schwarzel, 2016; Sun et al., 2020; Zhou et al., 2019). While it is well established that Dop1R1 is crucial for learning and short-term memory (Kim et al., 2007; Qin et al., 2012), the role of the other receptors is less clear. Dop1R2 is an interesting candidate to study, due to its ability to couple to two different G-proteins, $G\alpha_s$, which engages the cAMP pathway and $G\alpha_q$, which couples to Ca^{2+}-signaling (Han et al., 1996; Himmelreich et al., 2017; Sun et al., 2020). This could allow Dop1R2 to modulate learning and memory in a complex fashion. The receptor is mainly expressed in the Mushroom body (MB) (Crocker et al., 2016; Croset et al., 2018; Han et al., 1996; Kim et al., 2007; Lark et al., 2017), an important brain region for olfactory associative learning (Aso, Hattori, et al., 2014; Aso, Sitaraman, et al., 2014; Cognigni et al., 2018). Work on a mutant line suggests that Dop1R2 is involved in forgetting (Berry et al., 2012). However, a study using an RNAi-line suggests that the receptor plays a role in memory maintenance (Sun et al., 2020). As these studies used different learning assays – aversive and appetitive respectively as well as different methods, it is unclear if Dop1R2 has different functions for the different reinforcement stimulus. To resolve this problem, we generated a transgenic line to conditionally knock down Dop1R2, in a spatial and temporal specific manner. Using CRISPR-Cas9 and homology-directed repair (HDR), we included FRT sites in the

endogenous locus of *Dop1R2* for flippase-mediated excision. In addition, we inserted an HA-Tag, to monitor the spatial localization of the receptor. This also allows us to visualize the efficiency of the flip-out. We used this line to study the role of the receptor in learning and memory in the MB for both aversive and appetitive conditioning. Upon flip out of the receptor in the MB, 2h memory and long-term memory (LTM) are impaired. Similar results are obtained when we flip out Dop1R2 specifically in the α/β-lobes and α'/β'-lobes of the MB, which are involved in those memory phases. Therefore, Dop1R2 is required in the α/β-lobes and α'/β'-lobes for later memory forms.

116 # RESULTS

117

118 ***Generation of the Dop1R2 conditional knock-out line***

119 To be able to study both the requirement of Dop1R2 in specific neurons as well as the
120 localization of the receptor we generated a transgenic line that allows defined spatial
121 and temporal knock-out of the receptor. In short, we inserted two FRT sites for
122 flippase-mediated excision (Golic & Lindquist, 1989; Gratz et al., 2014) as well as a
123 3xHA-tag to study the localization of the receptor. The Ha-Tag was chosen to minimize
124 interference with the receptor structure. A representation of the receptor is depicted in
125 Figure 1A. The 3rd intracellular loop between transmembrane domains (TMDs) five
126 and six as well as the C-terminal tail are required for G-protein binding (Missale et al.,
127 1998). The position of the FRT sites and the HA-tag is indicated. Figure 1B gives an
128 overview of the strategy. The endogenous locus is cut twice by CRISPR-Cas9 to
129 replace it with the Dop1R2 sequence carrying the FRT sites and the HA-Tag, using a
130 donor plasmid as template for homolog-directed repair. The donor plasmid also
131 contains the region directly upstream and downstream of the exchange site as
132 homology arms to align the plasmid. The plasmid carrying the sequence for the two
133 gRNAs as well as the donor plasmid were injected into embryos with maternal Cas9
134 expression (*nos>Cas9*). Using the Gal4-UAS system to express flippase in neurons
135 of interest the receptor can be flipped out in those neurons while keeping it functional
136 in the rest of the brain. The first FRT site was placed in the intron before the first coding
137 exon, which contains all seven TMDs (Figure 1C). Since Dop1R2 has three transcript
138 isoforms with differences in the C-terminus the second FRT site was placed at the end
139 of the last common exon. Thus, FLP-mediated recombination will lead to deletion of
140 the two common coding exons including all TMDs. Successful insertion was verified
141 by sequencing.

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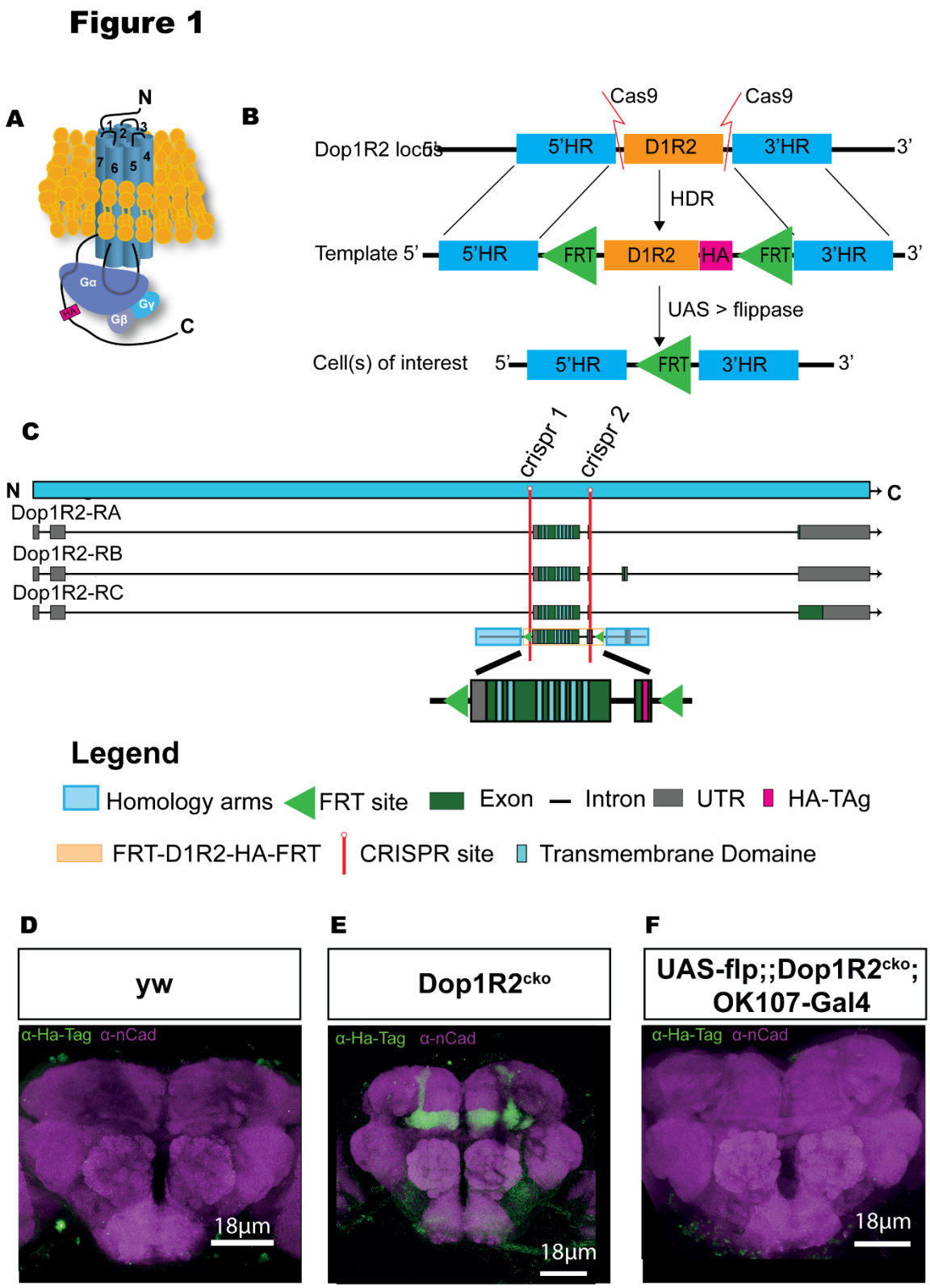

Fig 1. Generation of a conditional knockout allele for Dop1R2.

A) Schematic representation of the receptor structure and interaction with the G-Protein. The FRT sites and HA-Tag are indicated B) Schematic representation of the conditional knock-out system. The endogenous *Dop1R2* was replaced using CRISPR-Cas9 mediated homology-directed repair (HDR) from a donor plasmid. The plasmid contained the common coding exons of *Dop1R2* with an HA-Tag in

149 the C-Terminus and two FRT sites flanking this sequence. In the resulting *Dop1R2*^{cko} allele the inserted
150 HA-Tag and *Dop1R2* sequence can be removed by flippase (FLP) recombinase in cells of interest. C)
151 Schematic representation of the *Dop1R2* gene locus with 3 different transcript isoforms. The location
152 of the two used CRISPR sites are highlighted in red. The positions of the transmembrane domains in
153 the isoforms and in the donor plasmid, are indicated.D-F) Dop1R2::HA expression in a frontal brain
154 confocal section of D yw, E Dop1R2^{cko} or F) UAS-flp;; dop1R2^{cko}, OK107-Gal4 flies aged one week.
155 The HA-Tag was visualized using an anti-HA-Tag antibody (green). Brain structures were labeled with
156 anti-N-cadherin (nCad, magenta) antibodies. Scale bar: 18 µm.
157
158 ***Dop1R2^{cko} is in the Mushroom body***
159 To monitor the localization of the tagged dopamine receptors we first stained brains
160 with an antibody against the HA-Tag in one-week-old flies. In the control line (Figure
161 1D) the HA-tag does not show any specific staining. In the dop1R2^{cko} line, we see a
162 clear signal in the entire MB (Figure 1E). This matches previous reports as well as
163 single-cell RNAseq data showing that *Dop1R2* is expressed in the MB (Croset et al.,
164 2018; Han et al., 1996).
165 After flipping the receptor out in the MB using the MB-specific OK107-Gal4 driver in
166 combination with UAS-flp abolishes the signal, demonstrating the efficiency of the FRT
167 sites (Figure 1F). Therefore, the flip-out system seems to work as planned.
168
169 ***Short-term memory is not affected by the loss of Dop1R2***
170 We wanted to see if flipping out Dop1R2 in the MB affects memory acquisition and
171 STM by using classical olfactory conditioning. In short, a group of flies is presented
172 with an odor coupled to an electric shock (aversive) or sugar (appetitive) followed by
173 a second odor without stimulus. For assessing memory flies can freely choose
174 between the odors either directly after training (STM) or at a later timepoint.
175 To ensure that the introduced genetic changes to the Dop1R2 locus do not interfere
176 with behavior we first checked the sensory responses of that line (Sup A-D). The
177 Dop1R2^{cko} line shows comparable odor responses (Sup A+B) as well as sugar and
178 shock response (Sup C+D) to the control line. Aversive STM (Sup E), as well as
179 aversive 2h memory (Sup F), are not significantly different from the control line.
180 Moreover, appetitive STM is also comparable to the control (Sup G). Thus, the
181 introduced changes to dop1R2 do not interfere with normal receptor function.
182 To test the requirement of dop1R2 for short-term memory we assessed memory
183 performance directly after training for flies with flipped-out dop1R2 in the whole MB or

184 in individual lobes along with the parental controls. The flies were aged for a week
185 before undergoing classical olfactory conditioning. Both aversive STM and appetitive
186 STM were tested. But first, we ensured that the whole MB flip-out line responds
187 normally to the used odors and stimuli. Flp/+;; Dop1R2^{cko}; OK107-Gal4/+ flies show
188 comparable responses as the parental controls to both odors (Sup H+I), shock (Sup
189 J) and sugar (Sup K).
190 For aversive STM flipping out Dop1R2 in the whole MB does not change STM
191 compared to parental controls (Figure 2A). Next, we flipped Dop1R2 in the γ-lobe
192 using 5HTR1B-Gal4. This MB lobe is involved in STM (Blum et al., 2009; Trannoy et
193 al., 2011; Zars et al., 2000). Matching the results for the whole MB flip out, we do not
194 see a change in the performance score (Figure 2B). Similar results are obtained when
195 we flip the receptor in the α/β-lobes using c739-Gal4 (Figure 2C) or α'/β'-lobes using
196 c305a-Gal4 (Figure 2D).
197 Reward STM is also not changed when Dop1R2 is flipped out in the whole MB (Figure
198 2E) in the γ-lobe (Figure 2F), the α/β-lobes (Figure 2G) or the α'/β'-lobes (Figure 2H).
199 Taken together the results indicate that Dop1R2 is not required for STM in the MB
200 lobes.

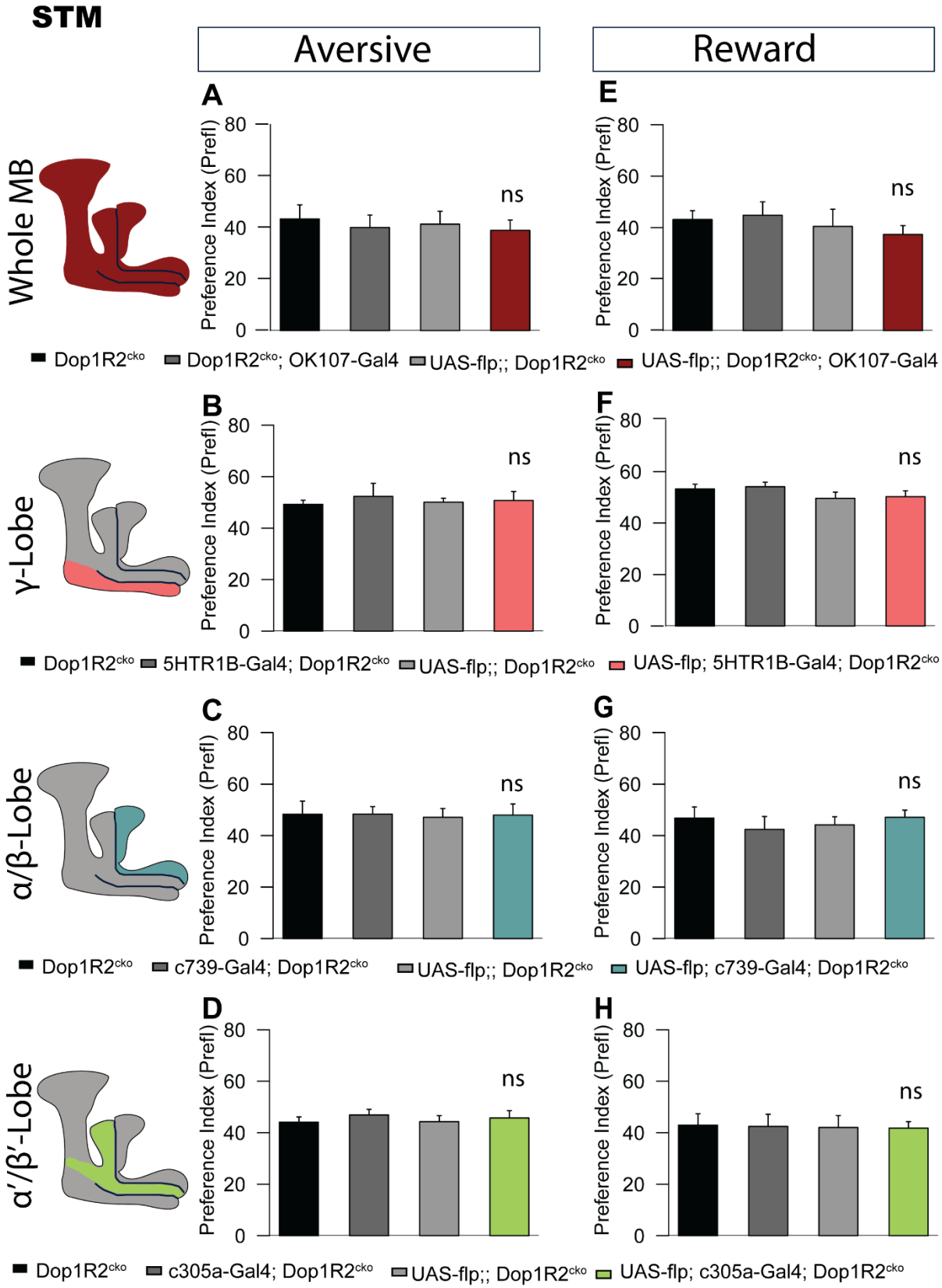

Fig 2. Short-term memory of flies with knock-out of Dop1R2 in the mushroom Body. A-D) Aversive training, E-H) reward training. A) and E) Whole MB flip-out using OK107-Gal4 and parental controls. B) and F) γ-lobe flip-out using 5HTR1B-Gal4 and parental controls. C) and G) α/β-lobe flip-out using c739-Gal4 and parental controls. D) and H) α'/β'-lobe flip-out using c305a-Gal4 and parental

controls. No performance impairment was observed in any of the tested conditions. See S2 for sensory controls and S1 Table for the data. Bar graphs represent the mean, and error bars represent the standard error of the mean. For each shown graph the N = 12. Asterisks denote significant differences between groups (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ns: $p>0.05$) determined by ANOVA Tukey HSD.

2h memory is impaired by loss of Dop1R2

As Dop1R2 was previously described to be involved in forgetting and/or memory maintenance we wanted to assess later time points after training.

First, we looked at two hours after aversive training. Flip-out in the whole Mushroom body leads to a reduced performance score (Figure 3A). Next, we wondered which MB lobe might cause this reduction. Therefore, we flipped out Dop1R2 in γ-lobe using 5HTR1B-Gal4, in the α/β-lobes using c739-Gal4 or α'/β'-lobes using c305a-Gal4. Loss of Dop1R2 in the γ-lobe does not reduce the memory performance (Figure 3B).

However, loss of Dop1R2 in both the α/β-lobes (Figure 3C) or the α'/β'-lobes (Figure 3D) impaired 2h memory after aversive training.

We tested the same for reward memory using sugar as reinforcement. Flip out of Dop1R2 in the whole MB (Figure 3E) results in a reduced performance score. Loss of Dop1R2 in the γ-lobe does not affect memory performance (Figure 3F). As for aversive training, flip out of Dop1R2 in the α/β-lobes (Figure 3G) or the α'/β'-lobes (Figure 3H) impaired 2h memory after reward training. However, the reduction is not as severe as for aversive training. Taken together the results indicate that Dop1R2 is required for aversive 2h memory as well as reward 2h memory in the α/β-lobes and the α'/β'-lobes but is dispensable in the γ-lobe.

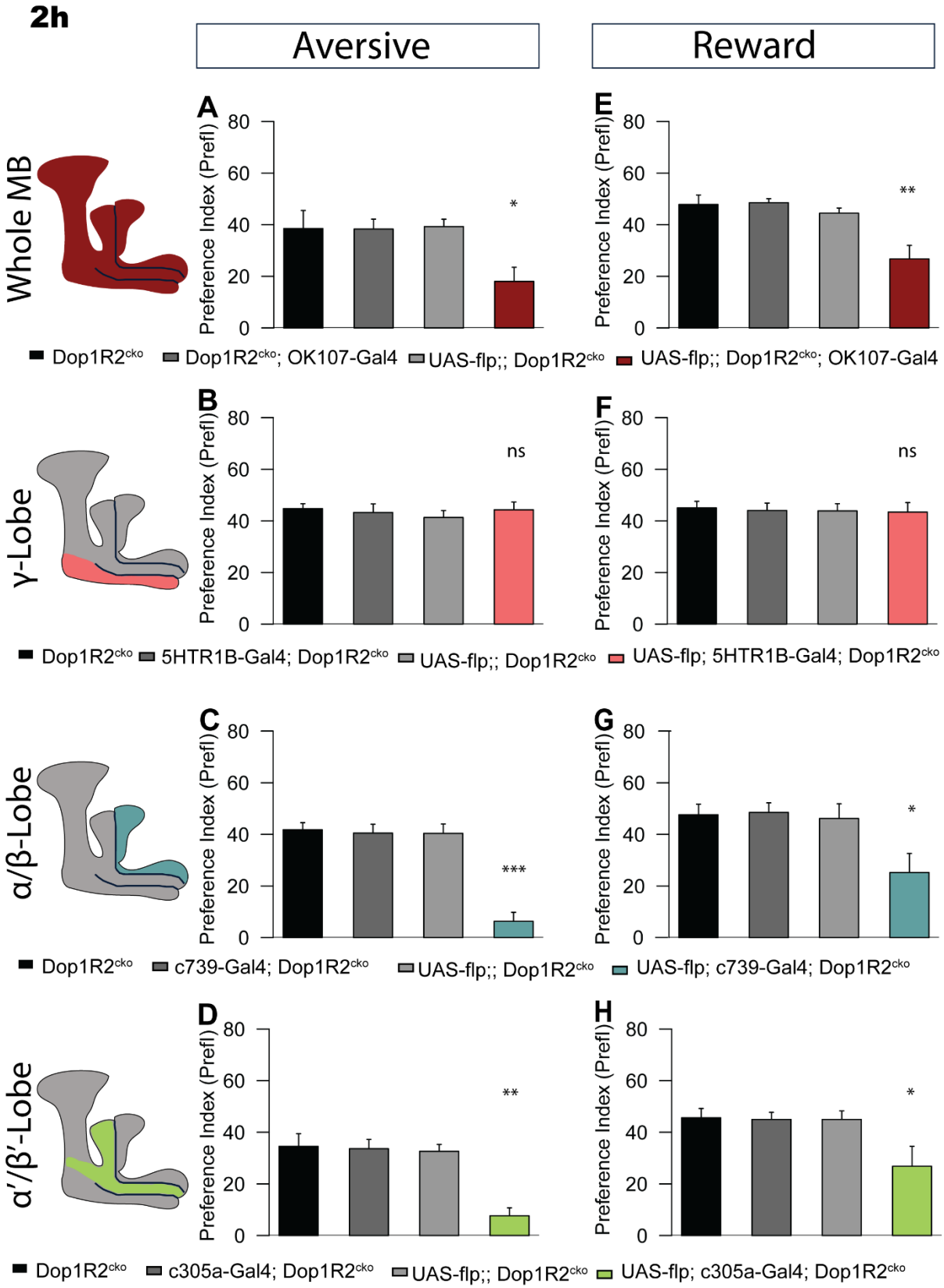

Fig 3. 2h memory of flies with knock-out of Dop1R2 in the mushroom Body. A-D) Aversive training, E-H) reward training. A) and E) Whole MB flip-out using OK107-Gal4 and parental controls. B) and F) γ-lobe flip-out using 5HTR1B-Gal4 and parental controls. C) and G) α/β-lobe flip-out using c739-Gal4 and parental controls. D) and H) α'/β'-lobe flip-out using c305a-Gal4 and parental controls. For whole

MB flip-out, α/β-lobes and α'/β'-lobes both aversive and appetitive 2h memory performance is impaired. Loss of Dop1R2 in the γ-lobe does not affect 2h memory. See S2 for sensory controls and S1 Table for the data. Bar graphs represent the mean, and error bars represent the standard error of the mean. For each shown graph the N = 12. Asterisks denote significant differences between groups (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ns: $p>0.05$) determined by ANOVA Tukey HSD.

24h memory is impaired by loss of Dop1R2

Next, we wanted to see if later memory forms are also affected. One cycle of reward training is sufficient to create LTM (Krashes & Waddell, 2008), so we looked at 24h appetitive memory.

Flipping out *dop1R2* in the whole MB causes a reduced 24h memory performance (Figure 4A). No phenotype was observed when dop1R2 was flipped out in the γ-lobe (Figure 4B). However, similar to 2h memory, loss of dop1R2 in the α/β-lobes (Figure 4C) or the α'/β'-lobes (Figure 4D) causes a further reduction in memory performance. Thus, dop1R2 seems to be involved in appetitive LTM in the α/β-lobes and the α'/β'-lobes.

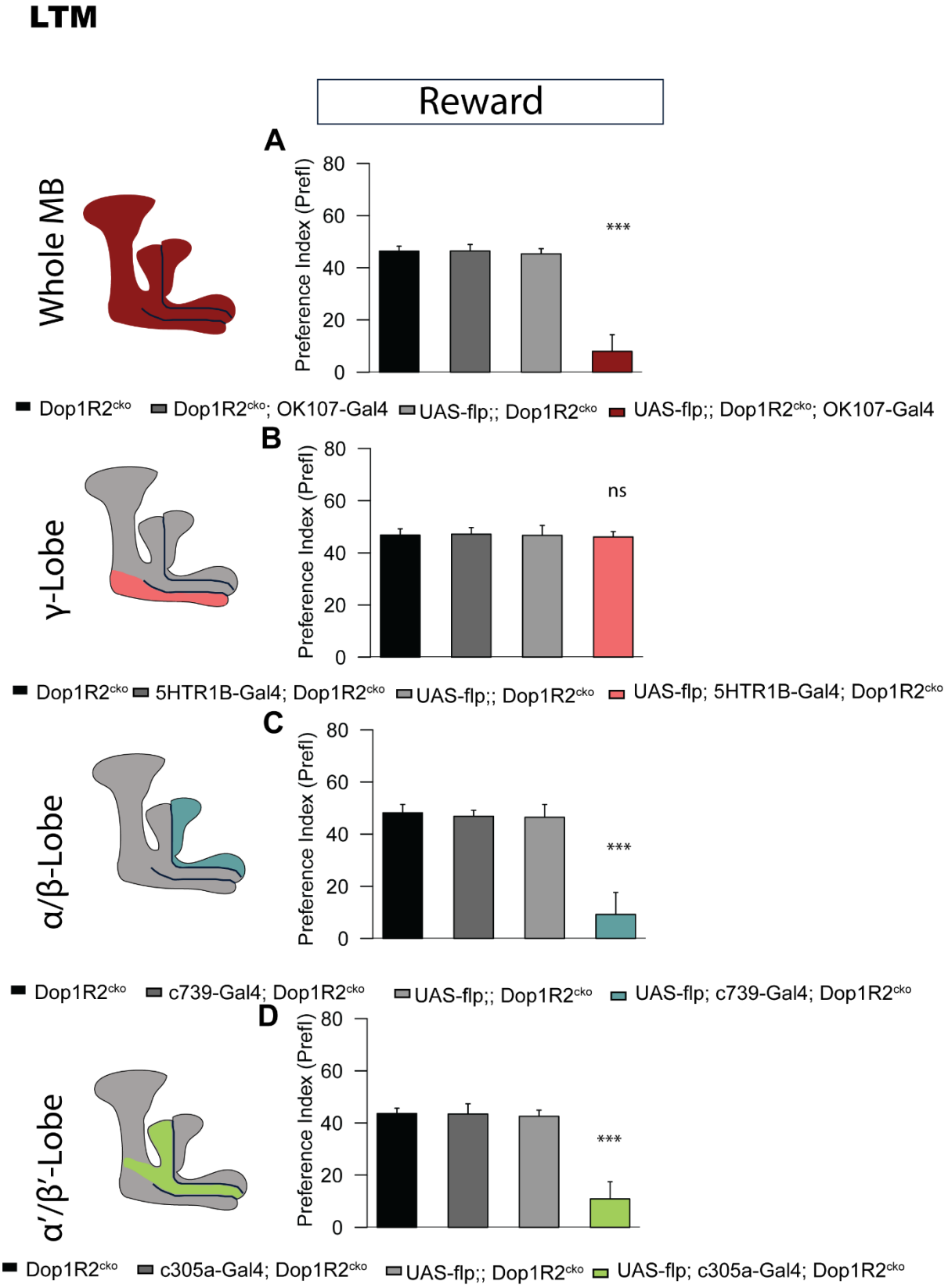

Fig 4. 24h reward memory of flies with knock-out of Dop1R2 in the mushroom Body. A) Whole MB flip-out using OK107-Gal4 and parental controls. B) γ-lobe flip-out using 5HTR1B-Gal4 and parental controls. C) α/β-lobe flip-out using c739-Gal4 and parental controls. D) α'/β'-lobe flip-out using c305a-Gal4 and parental controls. For whole MB flip-out, α/β-lobes and α'/β'-lobes appetitive 24h memory

performance is impaired. Loss of Dop1R2 in the γ-lobe does not affect 24h memory. See S2 for sensory controls and S1 Table for the data. Bar graphs represent the mean, and error bars represent the standard error of the mean. For each shown graph the N = 12. Asterisks denote significant differences between groups (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ns: $p>0.05$) determined by ANOVA Tukey HSD.

Discussion

We have generated a conditional knock-out line for the dopamine receptor Dop1R2 following a similar approach as in Widmer et al. 2018 (Widmer et al., 2018). To achieve this, FRT sites were inserted in front of the start codon and in the C-Terminus of Dop1R2 using CRISPR-Cas9 mediated homology-directed repair. In addition, an HA-Tag was inserted to be able to visualize the dopamine receptor expression as well as verify successful flip out. Using an anti-HA-Tag antibody we were able to visualize Dop1R2 in the MB of the generated line. This matches previous reports for the expression of *Dop1R2* (Crocker et al., 2016; Croset et al., 2018; Han et al., 1996; Kim et al., 2007; Sun et al., 2020). Moreover, upon knocking out Dop1R2 with an MB-specific driver, the HA-tag labeling disappears indicating that the conditional knock-out system works. The HA-Tag could also be useful to study the localization of Dop1R2 within the MB lobes, for example if it is close to synapses of dopaminergic neurons.

To get a better overview of Dop1R2's role in the Mushroom body we analyzed appetitive memory at different timepoints after training in the individual MB lobes. Loss of *Dop1R*2 in the whole MB as well as the α/β-lobe or the α'/β'-lobe impairs 24h reward memory. This observation matches previous studies. Using *Dop1R2-RNAi* in the MB, Sun et al. (Sun et al., 2020) showed, that STM is intact, while LTM is impaired. When knocking down the Raf/MAPK pathway they get a similar phenotype. Moreover, expression of a constitutive active *Raf* allele rescues the Dop1R2 dependent LTM deficit and Dop1R2 seems to be required for the phosphorylation of the MAPK. Therefore, Dop1R2 dependent activation of the Raf/MAPK pathway is required for stabilization of reward LTM memory. Another study shows that reward LTM requires food with a high energetic value (Musso et al., 2015). This signal seems to be relayed by the dopaminergic neuron (DAN) MB-MP1, that showed temporally restricted oscillating activity early post-training(Musso et al., 2015; Pavlowsky et al., 2018). Furthermore, knock-down of Dop1R2 using RNAi impaired reward LTM while leaving

289 STM intact. Therefore, dopaminergic signaling through MB-MP-1 and Dop1R2 could
290 indicate the energetic value of the reward and decide if LTM should be formed or not.
291 Interestingly, the MB-MP1 DAN arborizes in the spur of the γ-lobe as well as the inner
292 core of the pedunculus, which consists of the axons of the α/β Kenyon Cells (KCs)
293 (Tanaka et al., 2008), which according to our results require Dop1R2 for reward LTM.
294 Loss of Dop1R2 in the Mushroom Body Output neuron (neuron) MVP2 also impaired
295 reward LTM while leaving STM intact(Pavlowsky et al., 2018). This GABAergic MVP2
296 MBON forms a feedback circuit with the MB-MP1 DAN. After training, the oscillatory
297 activity of MB-MP1 is enhanced, while MVP2 is inhibited. After 30min, MVP2 gets
298 activated and MB-MP1 is inhibited. Moreover, Dop1R2 seems to be required for
299 modulating this feedback loop.
300 The MBONs are an important contributor to memory. Moreover, MBONs receive
301 dopaminergic input and seem to express dopamine receptors (Crocker et al., 2016)
302 We showed that Dop1R2 is required in the α/β-lobe and the α'/β'-lobe for reward LTM
303 formation. Interestingly, MBONs which are involved in reward LTM(Ichinose et al.,
304 2015; Owald et al., 2015; Plaçais et al., 2013), have arborization in the α/β-lobe and
305 the α'/β'-lobe. Thus, Dop1R2 could modulate these connections as well.
306 All in all, Dop1R2 is required for reward LTM formation and loss of the receptor impairs
307 LTM. Dop1R2 seems to influence reward LTM in different ways. Firstly, by acting on
308 the Raf-MAPK pathway to stabilize memory, secondly by relaying the energetic
309 content of the reward and third, by modulating the MB-MP1-MVP2 loop.
310 For aversive conditioning, we observe that loss of Dop1R2 in the MB leads to impaired
311 2h memory, whereas STM is intact. Moreover, Dop1R2 seems to be required in the
312 α/β-lobe and the α'/β'-lobe. A previous study using a mutant for Dop1R2, that also
313 affects the neighboring gene GC1907, observed higher memory retention(Berry et al.,
314 2012). Further, lack of Dop1R2 impairs reversal learning. It is proposed that Dop1R2
315 acts on the RAC-forgetting pathway (Cervantes-Sandoval et al., 2016; Shuai et al.,
316 2010). Interestingly, Dop1R2 can act on two different downstream second messenger
317 systems by using two different G-Proteins(Himmelreich et al., 2017). By coupling to
318 $G_{\alpha s}$ the cAMP pathway is activated. By coupling to $G_{\alpha q}$ the Ca^{2+} messenger system is
319 activated. Furthermore, knocking down $G_{\alpha q}$ pan-neuronally or in the MB leads to
320 memory enhancement 3h after training, but not 6h after training (Himmelreich et al.,
321 2017). Thus, Dop1R2 could regulate the RAC forgetting pathway through $G_{\alpha q}$.
322 However, also other receptors could use $G_{\alpha q}$ and mediate forgetting.

Berry et al. also observed that blocking output of DANs inhibits forgetting, while activating DANs accelerates forgetting (Berry et al., 2012). This modulation seems to require ongoing activity of the MP1 DAN together with further DANs. Interestingly, using a spaced training protocol and a Dop1R2 line another study showed impaired LTM(Placais et al., 2017). Furthermore, after spaced training, flies have a higher energy uptake and the energy metabolism is upregulated in the MB. This increase in energy consumption is mediated by dopaminergic signaling from the MB-MP1 DAN. Loss of Dop1R2 in the MB abolishes the increase in energy consumption. Therefore, Dop1R2 seems to be important for aversive LTM formation by regulating energy consumption. Thus, like for reward LTM formation, aversive LTM seems to require sufficient energy. Starved flies reduce the formation of aversive LTM(Plaçais & Preat, 2013; Plaçais et al., 2012). This information seems to be relayed through ongoing oscillation of MB-MP1 and Dop1R2 after training. In addition, the MVP2 MBON might also be involved(Ueoka et al., 2017). Therefore, the gating mechanism in both aversive and reward LTM formation seems to require Dop1R2(Pavlowsky et al., 2018). In addition, the MAPK signaling pathway might also be required in aversive LTM formation by activating transcription factors like CREB and c-fos(Miyashita et al., 2018).

Both Dop1R2 and the ongoing activity of MB-MP1 seem to have multiple roles directly after training in a short time window (Berry et al., 2012; Placais et al., 2017; Plaçais et al., 2012). The circuit acts like a gating mechanism for LTM to ensure, that there is enough energy for continuing LTM formation. The MBON MVP2 acts as feedback loop to regulate the activity of the DAN. Moreover, both the RAC and the Raf/MAPK signaling pathway seem to be engaged to either forget or stabilize the memory. In aversive memory formation, loss of Dop1R2 could lead to enhanced or impaired memory, depending on the activated signaling pathways and the internal state of the animal. For reward memory formation, loss of Dop1R2 seems to impair memory. Moreover, Rac does not have an effect on reward memories(Yang et al., 2023).

However, it still remains unclear, how all of these aspects are integrated and if there is a hierarchical order.

DANs can produce aversive and appetitive associations depending on the temporal presentation of odor cue and reinforcement stimulus(Handler et al., 2019). Thus, dopaminergic signaling is able to modify the KC-MBON synapses bi-directionally. The Dop1R1-$G_{\alpha s}$-cAMP pathway seems to detect the temporal coincidence of the stimuli

357 whereas the Dop1R2-$G_{\alpha q}$-Ca^{2+} pathway detects the temporal ordering(Handler et al.,
358 2019). Dop1R2 mutant flies seem to be able to form an odor association but are not
359 able to update it. Further, Dop1R2 is required for the potentiation of the KC-MBON
360 synapse following backward pairing.
361 Taken together, Dop1R2 has multiple roles during memory formation and integrates
362 different signals, including the detection of the order of stimuli, the internal state, like
363 energy levels and forgetting and maintenance signals.
364 The receptor does not seem to be required for STM but for later timepoints. Previous
365 studies looking at the temporal requirement of the lobes (Guven-Ozkan & Davis, 2014;
366 Perisse et al., 2013) defined the γ-lobe to be responsible for memory acquisition (Blum
367 et al., 2009; Trannoy et al., 2011; Zars et al., 2000). The α/β-lobe and its output is
368 involved in LTM (Akalal et al., 2011; Blum et al., 2009; Cervantes-Sandoval et al.,
369 2013; Huang et al., 2012; Ichinose et al., 2015; Krashes & Waddell, 2008; Trannoy et
370 al., 2011). The function of the α'/β'-lobe seems to be LTM consolidation (Cervantes-
371 Sandoval et al., 2013; Krashes & Waddell, 2008). However, both lobes also seem to
372 have a role in middle-term memory (Bouzaiane et al., 2015; Scheunemann et al.,
373 2012; Shyu et al., 2019; Turrel et al., 2022). As loss of Dop1R2 in the γ-lobe or the
374 whole MB does not impair STM, we conclude that the receptor is not required for this
375 memory type. However, Dop1R2 is expressed in the γ-lobe (Crocker et al., 2016;
376 Croset et al., 2018), so it might be required for other types of behaviors.
377 The impairment of reward LTM upon knock-out of Dop1R2 in the α/β-lobe and the
378 α'/β'-lobe matches the described role of these neurons. So, both the α/β-lobe and the
379 α'/β'-lobe require Dop1R2 for LTM. Interestingly, both lobes also seem to require
380 Dop1R2 for two-hour memories. As the MB-MP1 DAN is active in this time window as
381 well, this would suggest that Dop1R2 function at this time point is important for correct
382 LTM formation.
383 This would indicate, that Dop1R1 is the main contributor to STM while Dop1R2 is
384 responsible for later memory stages. It would be interesting to know how the switch
385 from Dop1R1 dependency to Dop1R2 occurs.
386 As Dop1R2 is required in the MBON MVP2 for reward LTM, it would be exciting to see
387 if it is also required in other MBONs or neurons outside the MB. Besides learning and
388 memory, the MB also uses dopamine to regulate sleep. This tool offers the opportunity
389 to study both of these aspects in neurons of interest.

The genetic tool we generated here to study the role of the Dop1R2 dopamine receptor in cells of interest, provides versatile possibilities as it can be used in combination with the powerful genetic tools of *Drosophila*. Using this line, we could show, that Dop1R2 is specifically required for later memory stages of both aversive and appetitive memory in the α/β-lobe and the α'/β'-lobe.

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Author Contribution

Conceptualization: SGS, Data curation: JCK, Formal Analysis: JCK, Funding acquisition: SGS, Investigation: JCK, NK, CF, Methodology: JCK, CF, Project administration: SGS, Resources: SGS, Supervision: SGS, Validation, SGS, CF, Visualization: JCK, NK, Writing – original draft: JCK

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Declaration of Interests

The authors declare no competing interests.

FIGURE LEGENDS

Fig 1. Generation of a conditional knockout allele for Dop1R2.
A) Schematic representation of the receptor structure and interaction with the G-Protein. The FRT sites and HA-Tag are indicated B) Schematic representation of the conditional knock-out system. The endogenous *Dop1R2* was replaced using CRISPR-Cas9 mediated homology-directed repair (HDR) from a donor plasmid. The plasmid contained the common coding exons of *Dop1R2* with an HA-Tag in the C-Terminus and two FRT sites flanking this sequence. In the resulting *Dop1R2*^{cko} allele the inserted HA-Tag and *Dop1R2* sequence can be removed by flippase (FLP) recombinase in cells of interest. C) Schematic representation of the *Dop1R2* gene locus with 3 different transcript isoforms. The location of the two used CRISPR sites are highlighted in red. The positions of the transmembrane domains in the isoforms and in the donor plasmid, are indicated.D-F) Dop1R2::HA expression in a frontal brain confocal section of D yw, E Dop1R2^{cko} or F) UAS-flp;; dop1R2^{cko}, OK107-Gal4 flies aged one week. The HA-Tag was visualized using an anti-HA-Tag antibody (green). Brain structures were labeled with anti-N-cadherin (nCad, magenta) antibodies. Scale bar: 18 µm.

Fig 2. Short-term memory of flies with knock-out of Dop1R2 in the mushroom Body. A-D) Aversive training, E-H) reward training. A) and E) Whole MB flip-out using OK107-Gal4 and parental controls. B) and F) γ-lobe flip-out using 5HTR1B-Gal4 and parental controls. C) and G) α/β-lobe flip-out using c739-Gal4 and parental controls. D) and H) α'/β'-lobe flip-out using c305a-Gal4 and parental controls. No performance impairment was observed in any of the tested conditions. See S2 for sensory controls and S1 Table for the data. Bar graphs represent the mean, and error bars represent the standard error of the mean. For each shown graph the N = 12. Asterisks denote significant differences between groups (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ns: $p>0.05$) determined by ANOVA Tukey HSD.

Fig 3. 2h memory of flies with knock-out of Dop1R2 in the mushroom Body. A-D) Aversive training, E-H) reward training. A) and E) Whole MB flip-out using OK107-Gal4 and parental controls. B) and F) γ-lobe flip-out using 5HTR1B-Gal4 and parental controls. C) and G) α/β-lobe flip-out using c739-Gal4 and parental controls. D) and H)

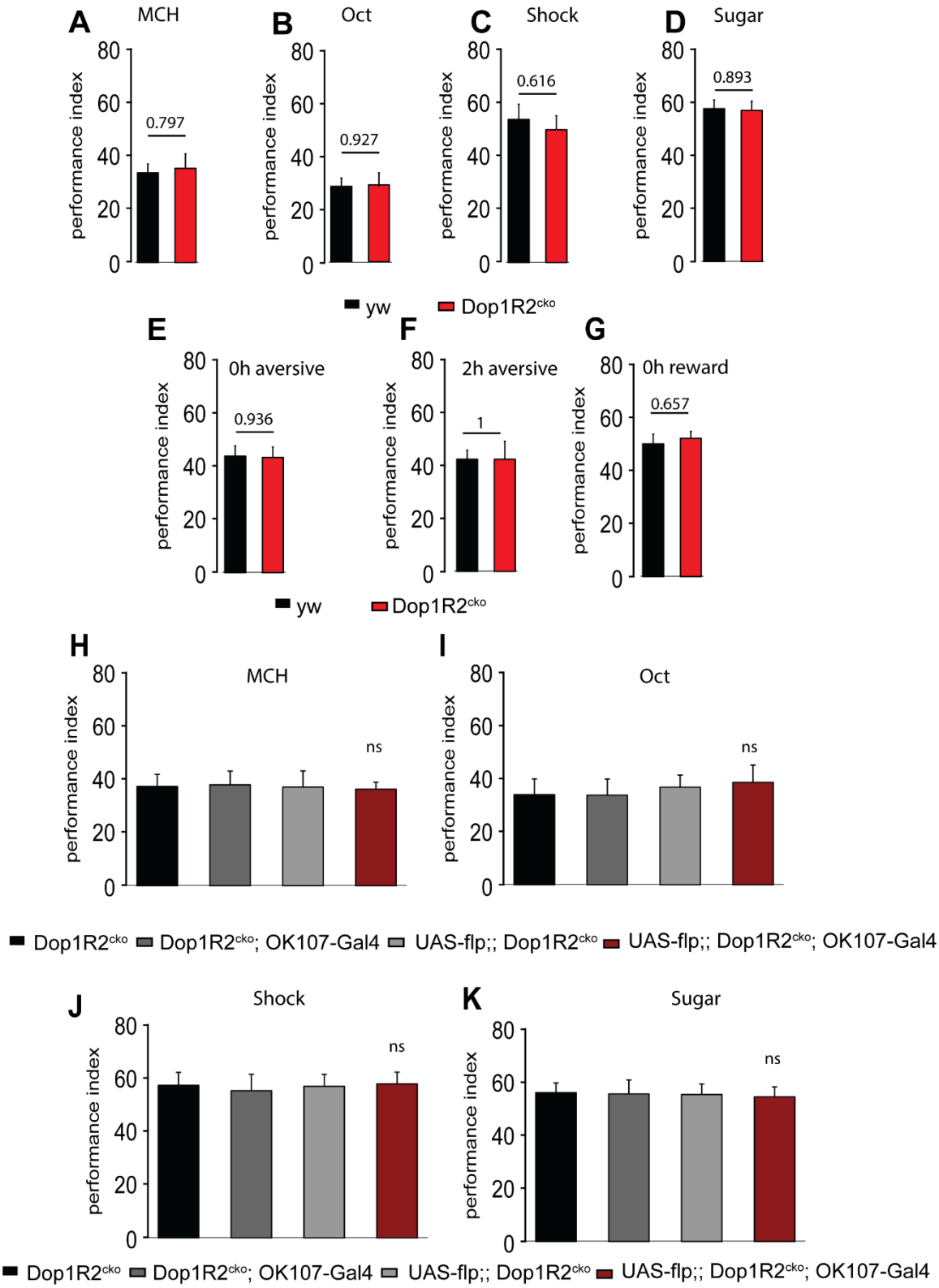
α'/β'-lobe flip-out using c305a-Gal4 and parental controls. For whole MB flip-out, α/β-lobes and α'/β'-lobes both aversive and appetitive 2h memory performance is impaired. Loss of Dop1R2 in the γ-lobe does not affect 2h memory. See S2 for sensory controls and S1 Table for the data. Bar graphs represent the mean, and error bars represent the standard error of the mean. For each shown graph the N = 12. Asterisks denote significant differences between groups (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ns: $p>0.05$) determined by ANOVA Tukey HSD.

Fig 4. 24h reward memory of flies with knock-out of Dop1R2 in the mushroom Body. A) Whole MB flip-out using OK107-Gal4 and parental controls. B) γ-lobe flip-out using 5HTR1B-Gal4 and parental controls. C) α/β-lobe flip-out using c739-Gal4 and parental controls. D) α'/β'-lobe flip-out using c305a-Gal4 and parental controls. For whole MB flip-out, α/β-lobes and α'/β'-lobes appetitive 24h memory performance is impaired. Loss of Dop1R2 in the γ-lobe does not affect 24h memory. See S2 for sensory controls and S1 Table for the data. Bar graphs represent the mean, and error bars represent the standard error of the mean. For each shown graph the N = 12. Asterisks denote significant differences between groups (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ns: $p>0.05$) determined by ANOVA Tukey HSD.

Supplementary FIGURE LEGENDS

S1. Sequences for cloning Dop1R2^{cko}. A) Sequence of *Dop1R2* with crispr sites. B) Sequence of the Donor plasmid with all three fragments of Dop1R2, the FRT sites and the HA-Tag

Supp Learning



S2. Sensory tests of the dop1R2 conditional knock-out line. A) Response of yw and Dop1R2^{cko} to MCH B) Response of y,w and Dop1R2^{cko} to Oct A) Response of yw and Dop1R2^{cko} to Shock A) Response of y,w and Dop1R2^{cko} to Sugar. E) 0h aversive memory of yw and Dop1R2^{cko} F) 0h aversive memory of y,w and Dop1R2^{cko} G) 0h aversive memory of yw and Dop1R2^{cko}. In all tested conditions the

Dop1R2^{cko} shows no significant difference to the control line. H-K) Sensory responses of flies with Dop1R2 knock-out in the whole MB alongside the parental control lines. H) Response to MCH, I) Response to Oct, J) Response to Shock, H) Response to Sugar. Loss of Dop1R2 in the whole MB does not affect sensory responses. See S1 Table for the data. Bar graphs represent the mean, and error bars represent the standard error of the mean. For each shown graph the N = 12. Asterisks denote significant differences between groups (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ns: $p>0.05$).

Tab1. Performance score of all the learning assays.

METHODS

Lead Contact and Data Availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Simon Sprecher (simon.sprecher@unifr.ch). All used data is in this manuscript.

Table 1: Key Resource Table

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
Genetic Reagent *Drosophila melanogaster* both male and female	y[1] w[*] P{y[+t7.7]=nos-phiC31\int.NLS}X; P{y[+t7.7]=CaryIP}su(Hw)attP6	Bloomington Stock Center	RRID:BDSC_32232	
Genetic Reagent *Drosophila melanogaster* both male and female	w;; Dr e/TM3	Bloomington Stock Center	RRID:BDSC_36305	
Genetic Reagent *Drosophila melanogaster* both male and female	w[1118], 20XUAS-FLPG5.PEST	Bloomington Stock Center	RRID:BDSC_55807	
Genetic Reagent *Drosophila*	OK107-Gal4	Kyoto Stock Center	DGGR_106098	

melanogaster both male and female				
Genetic Reagent *Drosophila melanogaster* both male and female	5HTR1B-Gal4	Bloomington Stock Center	RRID:BDSC_27636	
Genetic Reagent *Drosophila melanogaster* both male and female	c739-Gal4	Hiromu Tanimoto (Tohoku UniversityJapan)	RRID:BDSC_7362	
Genetic Reagent *Drosophila melanogaster* both male and female	c305a-Gal4	Bloomington Stock Center	RRID:BDSC_30829	
Genetic Reagent *Drosophila melanogaster* both male and female	yw	Bloomington Stock Center	RRID:BDSC_1495	
Genetic Reagent *Drosophila melanogaster* both male and female	FRT-Dop1R2-HA-FRT Dop1R2^{cko}	This paper		See Materials and Methods section: Creation of Dop1R2^{cko}

antibody	Mouse monoclonal α-HA clone 12CA5	Roche	RRID :AB _514505 11583816001	1:200
antibody	Rat momoclonal α-Droso-N-cadherin (Ncad)	Developme ntal Studies Hybridoma Bank	RRID :AB_52812 1 DN-Ex #8	1:30
Plasmid	pCFD4-U6_1_6_3tandemgRNAs plasmid	Simon Bullock	RRID:Addgene_ 49411	

Fly Husbandry

Drosophila melanogaster flies were reared in plastic vials on standard cornmeal food (12g Agar, 40g Sugar, 40g Yeast, 80g Cornmeal per Liter) and transferred to fresh food vials every 2-3 days. Flies were generally kept at 25°C, 60-65% humidity, and exposed to 12 h light and 12 h darkness with light onset at 8 am. The following fly lines were used: y[1] w[*] P{y[+t7.7]=nos-phiC31\int.NLS}X; P{y[+t7.7]=CaryIP}su(Hw)attP6 (abbreviated *nos>Cas9* in this paper; BL 32232) for microinjection and as PCR template, w;; Dr e/TM3 (BL 36305) was used as 3rd chromosomal balancer line. w[1118], 20XUAS-FLPG5.PEST (BL 55807) was used as UAS-flp. OK107-Gal4 (106098) was obtained from the Kyoto stock center. The 5HTR1B-Gal4 line (BL 27636) and the c305a-Gal4 (BL 30829) line are from Bloomington stock center. The c739-Gal4 line was gifted to us by H. Tanimoto (Tohoku University Japan). The yw (BL 1495) line was used as the control line.

Generation of dop1R2^{cko}

For generating the conditional knock-out line we needed three regions: 1) The 5' flanking sequence upstream of the first FRT site as homologous region, 2) The 3' flanking sequence downstream of the second FRT site as homologous region, 3) the sequence in between the two FRT sites hereafter named Dop1R2 coding fragment. To obtain the dopamine receptor fragments, genomic DNA from *nos >Cas9* flies was used as the template for the PCRs. The primers used for the different PCR fragments are shown in Table 2. The 3' and 5' fragments of Dop1R2 were sub-cloned into pBluescript II SK(+) vector (pBS) with adequate restriction enzymes – SpeI and SmaI for the 5' fragment and XhoI and Acc65I for the 3' fragment. The Dop1R2 coding fragment was

cloned with the Invitrogen TOPO kit. After sequence confirmation the fragments were cloned subsequently into pBS-FRT-3xHA-FRT. The vector is modified from the vector used by Widmer et al. (Widmer et al., 2018). The GFP was replaced by 3xHA using 3xHA Nco fw cacatggtTACCCATACGATGTTCCTGACTATGCGGGCTATCCCTATGACGTCCCG GACTATGCAGGATCCTATCCATATGACGTTCCAGATTACGCTgca and 3xHA H3 re agcttgcAGCGTAATCTGGAACGTCATATGGATAGGATCCTGCATAGTCCGGGACG TCATAGGGATAGCCCGCATAGTCAGGAACATCGTATGGGTAc as oligos whereas the FRT site and the pBS backbone were kept. The GPBS fragment was cloned in-frame in front of the 3xHA tag using AgeI and BstEII-HF as restriction enzymes.

The two guide RNAs were chosen using cripsr optimal target finder (http://targetfinder.flycrispr.neuro.brown.edu/). The used sequences (Table 1) were integrated into PCR primers to amplify a fragment of the pCFD4-U6_1_6_3tandemgRNAs plasmid (a gift from Simon Bullock, addgene plasmid # 49411 (Port et al., 2014) that was cloned into BbsI digested pCFD4-U6_1_6_3tandemgRNAs vector using Gibbson assembly (NEB). Insertion of the gRNAs was confirmed by sequencing. A mix containing 0.4 mg/ml template and 0.2 mg/ml of the guide RNAs plasmid was injected into *nos>Cas9* embryos. The injected flies were crossed with a 3rd chromosomal balancer line. The F1 generation was crossed again with the 3rd chromosomal balancer flies. As soon as eggs or larvae were visible the adults were sacrificed to check for the *Dop1R2* construct via PCR. Positive hits were then sequenced, and a stock was established.

Table 2: Primers and gRNAs for generating Dop1R2 conditional knock-out lines.

	Primer Name	Sequence
Dop1R2		
Cloning Primers	Dop1R2_5'_fw_SpeI	GCTGCAG**ACTAGT**CAGCCACCACA
	Dop1R2_5'_re_SmaI	CCTGAA**CCCGGGG**ATAAACTTTACCATAATGC
	Dop1R2_fragment_fw_AgeI	GTTCG**ACCGGT**GATTGCATTGTGTTCACCAG
	Dop1R2_ fragment _re_BstEII	GGGCTT**GGTAACC**ACGACGAATCTTGCGTGGACAG
	Dop1R2_3'_fw_XhoI	ATT**CTCGAG**AAGTATCAACCCACGATGCGTTC
	Dop1R2_3'_re_Acc65I	GGCAAC**GGTACC**AGATGCAGATACCG
gRNAs	Dop1R2_gRNA_5'	ACGAACTTAAGATAAAGTGTCGG
	Dop1R2_gRNA_3'	GCATCGTGGGCTGGTACTTCCGG
Screening & Sequencing	Dop1R2_Screening_for	TATCCCTATGACGTCCCGGAC
	Dop1R2_Screening_re	GACAGGTTGAGTGATGCGCC
	Dop1R2_5'FRT_for	GGCTACACATCATTTTATGCCAG
	Dop1R2_5'FRT_re	GTTCCTGTGCCTGATTCTGTTC

	Dop1R2_3'FRT_for	TCCTAACTGGCTTCTCTTCC
	Dop1R2_3'FRT_re	AGCGCTTAATTCACGAAAGC

Immunostainings

Brains from 5–8-day old flies were dissected in PBS (Bio-Froxx 1346LT050) and fixed in 3.7% formaldehyde for 25 min at RT. The brains were washed with 1X PBS containing 0.5% Triton X-100 (Carl Roth 3051.3) (0.5% PBST) before incubating the primary antibodies o/n at 4°C. The primary antibodies were mouse α-HA clone 12CA5 (Roche) 1:200, α-Droso-N-cadherin (Ncad) rat (Iowa H.B: DN-EX8) 1:30. After the brains were washed in 0.5% PBST again, they were incubated o/n at 4°C with the secondary antibodies. The following secondary antibodies were used: Goat α-rat Alexa 647 (Molecular probes A-21247) and Goat α-Mouse Alexa 488 (Molecular probes A11029) 1:200. The brains were washed again and mounted in self-made mounting media (90% Glycerol (Fischer Scientific Catalog No. BP229-1), 0.5% N-propyl gallate (Sigma P3130), 20 mM Tris (Fischer Scientific, Catalog No. BP152-5), pH 8.0) (Adapted from NIC Harvard Medical School). The brains were imaged using a confocal microscope (Leica STELLARIS 8 FALCON) at 40X magnification with the Plan APO 40x/1.10 water immersion objective at 1,024 × 1,024 pixels resolution and 600Hz scan rate. Images were processed with Fiji ImageJ and Adobe Illustrator.

Learning Apparatus

For behavior experiments, we used a memory apparatus that is based on Tully and Quinn's design and modified to allow conducting four memory experiments in parallel (CON-Elektronik, Greussenheim, Germany). Experiments were performed at 23-25°C and 65-75% relative humidity. The training was performed in dim red light and memory tests were done in complete darkness. The two odors used were 3-octanol (3-Oct) (Sigma-Aldrich 218405) and 4-methyl-cyclohexanol (MCH) (Sigma-Aldrich 66360) diluted in paraffin oil (Sigma-Aldrich 18512) 1:100 respectively. 260µl of the diluted odors were presented in a plastic cup of 14 mm in diameter. A vacuum membrane pump ensured odor delivery at a flow rate of 7 l/min.

Aversive olfactory conditioning

For aversive conditioning, groups of 50-100 flies of mixed sex were loaded in tubes lined with an electrifiable copper grid. The position in the machine and the sequence

in which the genotypes were tested were randomized. Experiments in which more than half of the flies died, the flies did not move or there were technical problems with the machine, as well as human errors were excluded. The training was conducted in the morning. After an accommodation period of 90 s, the first odor was presented for 60 s. In parallel, 12 pulses of 100 V for 1.5 s were delivered with an interval of 3.5 s. After 30 s of flushing with fresh air, the second odor was presented for 60 s. For the subsequent group of flies, the order of the two odors was reversed. For measuring 0 h performance flies were tested about 3 minutes after the end of the conditioning. To determine 2h memory performance, flies were transferred to food vials after conditioning and kept at 25°C until the test. For each genotype and condition the biological replicate is N=12.

Appetitive olfactory conditioning

Before appetitive conditioning, groups of 50 to 100 flies with mixed sex were starved for 19 to 21 h in plastic vials containing damp cotton at the bottom. Experiments in which more than half of the flies died, the flies did not move or there were technical problems with the machine, as well as human errors were excluded. The position in the machine and the sequence in which the genotypes were tested were randomized. The training was conducted in the morning. The conditioning protocol consists of a 90-s accommodation period, 120 s of the first odor, 60 s of fresh air followed by 120 s of the second odor. During the first odor, flies are in a conditioning tube lined with filter paper that was soaked in water the day before the experiment and left to dry overnight. For the second odor, flies are transferred to a conditioning tube lined with a filter paper that was soaked with a 1.5 M sucrose (Sigma-Aldrich, Cat# 84100-1KG; CAS Number 57-50-1) solution on the day before and left to dry at RT. After conditioning, flies were either directly tested for STM or put back in starvation vials until the memory test 2 h later. For 24-h memory, flies were fed for 3 h after training before starving them again. One experiment consisted of 2 reciprocal conditionings, in which the odor paired with sucrose was reversed. For each genotype and condition the biological replicate is N=12.

Memory tests

Flies were loaded into a sliding compartment and transferred to a two-arm choice point. Animals were allowed to choose between 3-octanol and 4-methyl-cyclohexanol. After

60 s, flies trapped in both arms were collected separately and counted. Based on these numbers, a preference index was calculated as follows:
PREF = $((N_{arm1} - N_{arm2})\ 100) / N_{total}$ the two preference indices were calculated from the two reciprocal experiments. The average of these two PREFs gives a memory performance index (PI). PI = $(PREF_1 + PREF_2) / 2$

Sensory Accuracy tests

Flies were tested for their ability to sense the two used odors 3-octanol and 4-methyl-cyclohexanol as well as electric shock. Therefore, the flies were loaded into a sliding compartment and brought to a two-arm choice point. The flies were allowed to freely choose between an arm containing the stimulus and a neutral arm. All experiments were carried out in the dark. Afterward, the flies in each arm were counted and a preference index was calculated.
For testing the odor response, the flies could choose between one of the odors in the same concentration as used for the behavior experiment and the same amount of paraffin oil for 120s.
Preference index PI = $((N_{air}-N_{odor})100)/N_{total}$.
For shock response, the flies could freely choose for 60s between a cooper-grid lined tube getting pulses of 100V or a cooper-grid lined tube getting no electric shock. Preference index PI = $((N_{No\ shock}-N_{shock})100)/N_{total}$.
For testing sugar sensitivity, a group of flies was starved for 1 to 21 h in a tube with damp cotton on the bottom. They could choose for 120 s between a tube lined with filter paper that was soaked in 1.5 M sucrose solution the day before or a tube lined with filter paper that was soaked in distilled water the day before. Preference index PI = $((N_{sucrose} - N_{water})100) / N_{total}$

Statistical analysis

To compare performance indices between different groups we used One-way ANOVA (ANalysis Of VAriance) with posthoc Tukey HSD (Honestly Significant Difference) Test Calculator for comparing multiple treatments in R with the package multcomp. In the case of two groups, we performed a *t*-test for comparison.

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