Concerted Actions of Octopamine and Dopamine Receptors Drive Olfactory Learning

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Aminergic signaling modulates associative learning and memory. Substantial advance has been made in *Drosophila* on the dopamine receptors and circuits mediating olfactory learning; however, our knowledge of other aminergic modulation lags behind. To address this knowledge gap, we investigated the role of octopamine in olfactory conditioning. Here, we report that octopamine activity through the β -adrenergic-like receptor Oct β 1R drives aversive and appetitive learning: Oct β 1R in the mushroom body $\alpha\beta$ neurons processes aversive learning, whereas Oct β 1R in the projection neurons mediates appetitive learning. Our genetic interaction and imaging studies pinpoint cAMP signaling as a key downstream effector for Oct β 1R function. The *rutabaga*-adenylyl cyclase synthesizes cAMP in a Ca^{2+} /calmodulin-dependent manner, serving as a coincidence detector for associative learning and likely representing a downstream target for Oct β 1R. Supporting this notion, the double heterozygous *rutabaga/+;oct β 1r/+* flies perform poorly in both aversive and appetitive conditioning, while individual heterozygous *rutabaga/+* and *oct β 1r/+* flies behave like the wild-type control. Consistently, the mushroom body and projection neurons in the *oct β 1r* brain exhibit blunted responses to octopamine when cAMP levels are monitored through the cAMP sensor. We previously demonstrated the pivotal functions of the D₁ receptor dDA1 in aversive and appetitive learning, and the α 1 adrenergic-like receptor OAMB in appetitive learning. As expected, *oct β 1r* genetically interacts with *dumb* (dDA1 mutant) in aversive and appetitive learning, but it interacts with *oamb* only in appetitive learning. This study uncovers the indispensable contributions of dopamine and octopamine signaling to aversive and appetitive learning. All experiments were performed on mixed sex unless otherwise noted.

Key words: cAMP; dopamine; learning; memory; mushroom body; octopamine

Significance Statement

Animals make flexible behavioral choices that are constantly shaped by experience. This plasticity is vital for animals to appropriately respond to the cues predicting benefit or harm. In *Drosophila*, dopamine is known to mediate both reward-based and punishment-based learning while octopamine function is important only for reward. Here, we demonstrate that the octopamine–Oct β 1R–cAMP pathway processes both aversive and appetitive learning in distinct neural sites of the olfactory circuit. Furthermore, we show that the octopamine–Oct β 1R and dopamine–dDA1 signals together drive both aversive and appetitive learning, whereas the octopamine–Oct β 1R and octopamine–OAMB pathways jointly facilitate appetitive, but not aversive, learning. This study identifies the cognate actions of octopamine and dopamine signaling as a key neural mechanism for associative learning.

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Introduction

Experiential learning facilitates animals to promptly respond to pleasant or noxious cues for approach or avoidance, respectively, for fitness and survival. In *Drosophila melanogaster*, olfactory classical conditioning is widely used to assess the capacity of the organism to learn and remember the odor associated with electric shock punishment (aversive) or sugar reward (appetitive) for delineating the neural, cellular, and molecular mechanisms. Olfactory learning and memory require modulatory actions of

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multiple aminergic systems including dopamine (DA), octopamine (OA; an invertebrate counterpart of norepinephrine), and serotonin (5-HT; Schwaerzel et al., 2003; Kim et al., 2007a,b; Johnson et al., 2011; Sitaraman et al., 2012; Aso et al., 2014a,b; Iliadi et al., 2017). The fundamental question of how diverse aminergic signals converge within the learning and memory pathways remains incompletely resolved. The actions of modulatory neurotransmitters like DA and OA are initiated on their binding to one or more of their respective receptors in overlapping or parallel neural circuits, conveying additive, synergistic, or antagonistic interactions via intracellular effectors to acquire, store, or retrieve information. OA and 5-HT, for example in aversive conditioning, exert additive effects on intermediate memory through their actions on distinct mushroom body (MB) substructures (Wu et al., 2013), whereas DA via DAMB in the MB has an antagonistic effect by promoting forgetting (Berry et al., 2012). All three monoamines are important for olfactory learning as well; however, the nature of interactions and circuits that DA, OA, and 5-HT shape the ability of the fly to acquire aversive and appetitive memories are unknown.

The best characterized of all three monoamines in olfactory conditioning is DA (Güven-Ozkan and Davis, 2014; Aso et al., 2014a,b; Cognigni et al., 2018). Ectopic activation of discrete PPL1 or PAM DA neurons projecting to the MB substitutes the aversive or appetitive unconditioned stimulus (US), respectively, when paired with the conditioned stimulus (CS; Claridge-Chang et al., 2009; Liu et al., 2012). The DA signals for aversive and appetitive learning require the D₁ receptor dDA1 in the MB, while ongoing DA activity after learning through the D₅ receptor DAMB regulates forgetting (Kim et al., 2007a; Berry et al., 2012; Qin et al., 2012). The dual functions of DA for memory acquisition and forgetting are executed through the following divergent intracellular effectors: dDA1 engages Gas/cAMP signaling, whereas DAMB recruits Gαq/Ca²⁺ signaling (Himmelreich et al., 2017). These findings posit DA as a major contributor to aversive as well as appetitive learning and memory.

OA is a major neuromodulator for appetitive learning. The *tβh* mutant lacking tyramine β hydroxylase (TβH), a rate-limiting enzyme for OA biosynthesis, is defective in the acquisition of appetitive memory in olfactory conditioning (Schwaerzel et al., 2003; Kim et al., 2007b). The function of OA in appetitive learning engages the α1-like OA receptor OAMB in the MB αβγ neurons (Han et al., 1998; Kim et al., 2013). The role of OA in aversive olfactory learning, however, is yet unsettled. Two studies report contrasting findings on the *tβh* mutant: Schwaerzel et al. (2003) demonstrate normal performance, whereas Iliadi et al. (2017) report poor performance in aversive conditioning. It is worth noting that the discrepancy is not due to the nature of mutation in *tβh*, the mutant genetic background, or conditioning parameters. Intermediate-term aversive memory, nonetheless, is shown to require the OA signal through the β-like OA receptor Octβ2R in the MB α'β' neurons (Wu et al., 2013). In operant courtship conditioning, a different type of aversive conditioning, OAMB in the MB αβ neurons, mediates the OA signal for short-term memory of courtship suppression (Zhou et al., 2012). While these reports illustrate the functions of OA in aversive memory, the key issue that is yet unresolved but important regarding mechanism is whether the OA signal is involved only in appetitive learning or both appetitive and aversive learning. In this report, we address this issue and further delineate the

neural sites and intracellular effectors critical for olfactory conditioning.

Materials and Methods

Drosophila strains and culture. The wild-type strain used in this study is *Canton-S*, and the mutant defective in Octβ1R is the piggyBac transgenic line *octβ1r^{f02819}* generated by the Gene Disruption Project (Thibault et al., 2004). The *octβ1r^{f02819}* in the *w¹¹¹⁸* genetic background was obtained from the Bloomington Drosophila Stock Center (BDSC; stock #18589) and placed in the *Canton-S* genetic background by backcrossing with Cantonized *w¹¹¹⁸* for six generations and then replacing the X chromosome containing *w¹¹¹⁸* with the *Canton-S* X chromosome with the normal *w* gene (hereafter called *octβ1r*). We previously described *oamb*, *octβ2r*, *dumb*, and *elav-GAL4*, *GAL80^{ts}* (Lee et al., 2003; Kim et al., 2007a; Lim et al., 2014). The Deficiency (*Df*) lines *Df(3R)Exel6191* (stock #7670) and *Df(3R)BSC685* (stock #26537), *tdc2-GAL4* (stock #9313), *elav-GAL4* (stock #8765), *OK107-GAL4* (stock #854), *c739-GAL4* (stock #7362), *c305a-GAL4* (stock #30829), and *UAS-Epac1-camps* (stock #25407 and #25408) were obtained from the BDSC; *Tβh RNAi* (v51667) were obtained from the Vienna Drosophila Resource Center; *pBDP-GAL4* was provided by David Anderson (California Institute of Technology, Pasadena, CA); *NP1131-GAL4* and *APL-GAL4* (VT43924-GAL4 recombined with UAS-GAL4 on the third chromosome) were provided by Josh Dubnau (Stony Brook University School of Medicine, Stony Brook, NY) and Ann-Shyn Chiang (National Tsing Hua University, Hsinchu City, Taiwan); and *NP225-GAL4* was provided by Andreas Thum (University of Konstanz, Konstanz, Germany).

The open reading frame of Octβ1R (Maqueira et al., 2005) was cloned under UAS in the gateway vector pTW (Akbari et al., 2009), and transgenic UAS-Octβ1R lines were generated by germ line transformation in *w¹¹¹⁸* embryos as previously described (Lim et al., 2014). Germ-line-transformed lines were outcrossed with Cantonized *w¹¹¹⁸* for at least six generations, and the transgenes were placed in the *octβ1r* genetic background for rescue experiments. All fly lines were maintained on standard cornmeal/agar/yeast medium. Flies were collected under CO₂ within 2 d after eclosion and 65–70 flies representing a group were housed together in a food vial at 25°C with ~50% relative humidity under the 12 h light/dark cycle before testing. Unless otherwise stated, the 4- to 7-d-old flies of mixed sex were used for all experiments. For the temporal control of GAL4 activity, the control and experimental flies carrying *elav-GAL4*, *GAL80^{ts}* were reared at 30°C for the induction of GAL4 activity or at 20°C for no induction. For developmental induction, flies were reared at 30°C until a mid-pupal stage and then maintained at 20°C before conditioning. For adulthood induction, flies were reared at 20°C throughout development and for 1 d after eclosion, and then maintained at 30°C for 3 d before conditioning. For the genetic interaction experiments, the virgin females of *octβ1r* or *Canton-S* were crossed with *rutabaga* (*rut*), *dumb*, or *oamb* males. In the case of *rut*, which is on the X chromosome, only the female progeny of *octβ1r* or *Canton-S* crossed with *rut* were used for conditioning.

RNA analysis. The transcript levels of *Canton-S* and *octβ1r* flies were analyzed as previously described (Lim et al., 2014). Three independent sets of 50 heads per genotype were homogenized in 10 μl of RLT lysis buffer (Qiagen) with the KONTES Micro Tissue Grinder (Thermo Fisher Scientific), which was then applied to the QIAshredder Spin Column (Qiagen). Total RNA was extracted using the RNeasy Protect Mini Kit (Qiagen), and then cDNA was generated using the Invitrogen SuperScript III First-Strand Synthesis System (for RT-PCR). Quantitative PCR was conducted using the iQ SYBR Green Supermix Kit (Bio-Rad) and the StepOnePlus Real-Time PCR Detection System (Applied Biosystems) per the manufacturer instructions. The 100, 200, and 400 ng of cDNA samples were run in triplicate, and the reactions were performed with the primer sets for *octβ1r*, the experimental gene, and *rp49*, a ribosomal protein gene as a control, to quantify relative expression levels. The primer sets were designed to span at least one intron and checked for specificity using the BLAST (FlyBase Consortium, 2003) against the *Drosophila* genome. The PCR primers

used in this study are as follows: for *octβ1r*, forward—TGT GCA GCC ACT GGA CTA TC, reverse—TAT GGC GTA TGC CTT GTT CA; for *rp49*, forward—TAC ACGG CCC AAG ATC GTG AA, reverse—GTT CGA TCC GTA ACC GAT GT.

Behavioral tests. All experiments were performed blindly (i.e., an experimenter does not know the genotype of the fly lines when conducting all experiments). The protocol described by Kim et al. (2007a,b) were used for aversive and appetitive conditioning with slight modifications. Training and tests were conducted under dim red light and 50–60% relative humidity. Odorants used for conditioning were 2% ethyl acetate (EA; catalog #319902–500, Sigma-Aldrich) and 2.2% isoamyl acetate (IAA; catalog #112674–500, Sigma-Aldrich). For appetitive conditioning, flies were starved for 18–20 h in the vials containing double-layered, water-soaked Kimwipes before training. A group of 65–70 flies were exposed to the first odor (CS⁺) paired with 12 pulses of 90 V electric shock (US) for aversive conditioning or 2 M sucrose (US) for appetitive conditioning for 1 min followed by 30 s of rest (interstimulus interval). After exposure to the second odor (CS[−]) without electric shock or sucrose for 1 min, flies were tested immediately (3 min), 1 h, or 3 h after training. To measure the capacity of flies to learn and remember, the flies were gently transferred to a T-maze and tested for 2 min to assess their avoidance or preference of the CS⁺ odor. A second group of flies was simultaneously trained with the odors presented in a reversed order to account for any possible odor bias in conditioning. For the short program single-training trial (1T) in aversive conditioning (Beck et al., 2000), flies were exposed to CS⁺ odors for 10 s with a single pulse of 90 V electric shock followed by 30 s rest and then CS[−] odors for 10 s before tests. For the two-training trial (2T), flies received another 1T training program with 15 min intertraining interval (ITI). The performance index (PI) was calculated by subtracting the percentage of flies that made an incorrect choice from the percentage of flies that made a correct choice. The average PI of the two groups of flies conditioned with counterbalanced odors was used as one data point. For 1 and 3 h memory tests, trained flies were gently transferred back into their respective vials (food-containing vials for electric shock-conditioned flies or vials with water-soaked Kimwipes for sugar-conditioned flies) and maintained at room temperature before tests.

Control behaviors were examined as previously reported (Kim et al., 2007a, 2013) and include olfactory acuity, shock avoidance, and sugar preference without conditioning. Briefly, flies were placed in a T-maze and allowed to choose (odor vs air for olfactory acuity; 90 or 30 V vs no shock for shock avoidance; 2 or 0.2 M sucrose vs water for sugar preference). An average PI of the counterbalanced set as noted above was calculated and reported as one data point.

cAMP imaging. The 4- to 7-d-old flies expressing the cAMP sensor Epac1-camps in the MB or projection neurons (PNs) in the wild-type or *octβ1r* genetic background were used for imaging. A fly brain was dissected within 3 min in the ice-cold hemolymph-like saline HL3 (70 mM NaCl, 5 mM KCl, CaCl₂ 1.5 mM, 20 mM MgCl₂, 10 mM NaHCO₃, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES, pH 7.1) and placed on the microscope glass containing either HL3 (baseline or vehicle control) or HL3 with 100 μM OA (treated), which was then covered with a coverslip. To create space between a microscope glass and a coverslip so as not to compress the brain, two layers of the scotch tape (3M) were placed on a microscope glass. The cAMP sensor Epac1-camps was excited at 440 nm, and emission was detected at 480 nm for CFP and 540 nm for YFP (yellow fluorescent protein). Images were collected using the 20× objective in the LSM700 confocal microscope (Zeiss) at every micrometer with the 512 × 512 pixel resolution. Each brain was used only once for imaging, thus different brains were used for imaging baseline and OA treatments. For quantification, images in grayscale were used to measure fluorescence intensity per pixel in each optical section using ImageJ software (NIH). Fluorescence intensity per pixel was calculated by [Integrated density/total area] per region of interest (ROI). We defined ROI in the major brain structures, including MB and PN. The non-ROI was defined in the anterior inferior medial protocerebrum (AIMPR) where the Epac1-camps was not induced. The fluorescence intensity for each CFP or YFP was calculated by [Integrated density/area]_{ROI} − [Integrated density/area]_{non-ROI}. The cAMP response was

determined by calculating the inverse FRET ratio, which is $|\Delta R/R_0|$, where $R = \text{CFP/YFP}$ and $\Delta R = R_{\text{treated}} - R_{\text{nontreated}}$ was used for data presentation (Shafer et al., 2008; Tomchik and Davis, 2009). To determine the timeline of cAMP responses, the changes in Epac1-camps FRET were measured at several time points (i.e., 1, 3, 5, 10, or 30 min) after OA or HL3 (control) treatment. For representative images of CFP and YFP emission in a fire scale, see Figures 4, A and B, and 5C, which exemplify relative cAMP levels.

Experimental design and statistical analyses. The 4- to 7-d-old flies of mixed sex were used for all experiments, with the exception of the genetic interaction experiment involving *rut*. As noted above, *rut* is on the X chromosome; thus, only female *rut* heterozygotes and female *rut* and *octβ1r* double heterozygotes were used. All behavioral experiments were performed blindly to the experimenter, and the control and experimental groups were tested in the same session in a randomized order. Multiple independent sets of flies obtained from different crosses and cultures were used for behavioral analyses. Statistical analyses were performed using the JMP (SAS) and Minitab 16 (Minitab). The raw data were analyzed using the Anderson–Darling goodness-of-fit or Shapiro–Wilk normality test for distribution and the Levene’s test for equality of variances, and are reported as the mean ± SEM. The normally distributed data were analyzed by a two-tailed Student’s *t* test for two groups or ANOVA followed by *post hoc* Tukey’s multiple-comparison or Dunnett’s tests for three or more groups. The non-normally distributed data were analyzed by Kruskal–Wallis and *post hoc* Mann–Whitney tests. Significant difference among the groups under comparison was determined using an α level of 0.05 in all analyses. All raw data files are available on request.

Results

Octβ1R is essential for aversive learning

To clarify whether OA is involved in aversive learning, we used the RNA interference (RNAi) approach to reduce expression of the OA biosynthetic enzyme *TβH*. The *TβH* knock-down flies were then examined in olfactory conditioning to assess their avoidance of the odor associated with electric shock. The *GAL4* drivers expressed in a majority of OA neurons (*tdc2-GAL4*) and a pair of the *tdc2-GAL4*-negative anterior paired lateral (APL; *APL-GAL4*) OA neurons (Busch et al., 2009; Wu et al., 2013) were used for *TβH* RNAi. Compared with the control *pBDP-GAL4* (promoterless *GAL4*)/*UAS-tβh-RNAi* flies, both *tdc2-GAL4/UAS-tβh-RNAi* and *APL-GAL4/UAS-tβh-RNAi* flies performed poorly when tested right after training (ANOVA: $F_{(2,27)} = 31.4$, $p < 0.0001$; Fig. 1A). To clarify whether developmental or adulthood *TβH* knockdown (KD) is responsible for poor performance, we used *elav-GAL4, GAL80^{ts}* (Kim et al., 2007a) to induce *TβH* knockdown only during development, only during adulthood, or throughout development and adulthood. The flies with developmental *TβH* knockdown showed normal performance in olfactory conditioning (*t* test: $t = -0.12$, $p = 0.9074$; Fig. 1B, developmental KD) similar to the flies without knockdown (*t* test: $t = -0.11$, $p = 0.9168$; Fig. 1B, no KD). The flies with adulthood *TβH* knockdown, however, displayed poor performance (*t* test: $t = -5.55$, $p < 0.0001$; Fig. 1B, adulthood KD) similar to the flies with constant knockdown (*t* test: $t = 5.7$, $p < 0.0001$; Fig. 1B, development and adulthood KD). These results together indicate that OA is important for aversive olfactory conditioning and that multiple OA neurons are involved in the process.

Next, we investigated which OA receptor is important. The $\alpha 1$ -like OAMB receptor is not crucial for aversive learning (Kim et al., 2013); thus, we focused on the β -like OA receptors Octβ1R and Octβ2R. The *octβ1r* allele contains a piggyBac transposon in the second intron of the *octβ1r* gene with a splicing acceptor in the transposon in the right orientation to

Figure 1. OA and its receptor Oct β 1R are required for aversive olfactory learning. **A**, T β H knockdown (*UAS-t β h RNAi*) in either OA/TA (*tdc2-GAL4*) or APL (*APL-GAL4*) neurons led to significantly impaired learning compared with the control (*pBDP-GAL4*) in aversive olfactory conditioning ($***p < 0.0001$, $n = 10$). **B**, Uninduced (no KD) or developmental T β H knockdown had no effect on olfactory learning; however, adulthood or constant (development and adulthood KD) T β H knockdown led to impaired learning (KD; $***p < 0.0001$, $n = 12$). **C**, Top, A scheme depicting the approximate location of the piggyBac insertion (a gray broken line with the filled triangle; SA, splice acceptor) in the second intron of the *oct β 1r* gene along with the Oct β 1R translation start site (gray arrow), coding regions (orange boxes), noncoding regions (gray boxes), and introns (gray lines; not scaled). Bottom left, The RT-PCR analysis on the RNA isolated from the heads of *Canton-S* and *oct β 1r* flies showed clear reduction in Oct β 1R mRNA in the *oct β 1r* flies. Rp-49 was used as an internal control. Bottom right, Quantitative PCR analysis revealed $\sim 75\%$ reduction of Oct β 1R mRNA in the *oct β 1r* flies compared with the control *Canton-S* ($**p < 0.01$, $n = 3$). **D**, *oct β 1r* flies exhibited defective performance immediately (3 min), 1 h, and 3 h after training in aversive conditioning ($**p < 0.001$, $***p < 0.0001$, $n = 6$), but *oct β 2r* and *Canton-S* flies showed comparable performance at all time points examined. **E**, The homozygous *oct β 1r* mutant or the flies with the transheterozygous *oct β 1r* and *Df* alleles [*Df-E*, *Df(3R)Exel6191*; *Df-B*, *Df(3R)BSC685*] displayed significantly reduced learning scores compared with either *w* control or the heterozygotes *oct β 1r/+*, *Df-E/+*, and *Df-B/+* ($***p < 0.0001$, $n = 6$). **F**, The learning performance of both *Canton-S* and the homozygous *oct β 1r* mutant flies was significantly improved after the second submaximal training (2T) with 15 min ITI in the short program of olfactory conditioning ($***p < 0.0001$, $n = 12$).

0.0011; Fig. 1C), indicating that *oct β 1r* is a hypomorphic allele. We have previously shown that the *oct β 2r* mutant is a null allele for Oct β 2R (Lim et al., 2014). When tested immediately (3 min), 1 or 3 h after electric-shock olfactory conditioning, the *oct β 2r* flies showed comparable performance and memory decay to the wild-type *Canton-S*, whereas the *oct β 1r* flies displayed substantially poor performance right after training and subsequent time points (two-way ANOVA: $F_{(8,45)} = 50.64$, $p < 0.0001$; genotype effect: $F = 118.4$, $p < 0.0001$; time effect: $F = 64.5$, $p < 0.0001$; interaction effect: $F = 9.8$, $p = 0.0001$; Fig. 1D). These data suggest that aversive olfactory learning requires Oct β 1R but not Oct β 2R.

To ascertain the phenotype of *oct β 1r* to the *oct β 1r* locus, we tested the *oct β 1r* transheterozygotes over the *Df* lines *Df(3R)Exel6191* and *Df(3R)BSC685* containing complete deletion of the *oct β 1r* gene. The heterozygotes *oct β 1r/+*, *Df(3R)Exel6191/+*, and *Df(3R)BSC685/+* had learning scores comparable to that of *Canton-S*; however, the transheterozygotes *oct β 1r/Df(3R)Exel6191* and *oct β 1r/Df(3R)BSC685* performed poorly like the *oct β 1r* homozygotes (ANOVA: $F_{(6,35)} = 33.39$, $p < 0.0001$; Fig. 1E). This substantiates that the loss of *oct β 1r* function rather than background genetic loci accounts for the learning phenotype. Two submaximal training trials (2T) with 15 min rest (ITI) in the short program of olfactory conditioning are known to improve the performance of the learning mutant *rut* (Beck et al., 2000). As in the regular electric shock conditioning, the *oct β 1r* flies displayed impaired performance compared with *Canton-S* in the 1T short program (Fig. 1F; two-tailed t test: $t_{(22)} = -11.69$, $p < 0.0001$). The 2T training with 15 min ITI, however, improved performance in the *oct β 1r* flies (Fig. 1F; two-tailed t test on 1T vs 2T: $t_{(22)} = 6.47$, $p < 0.0001$) similar to *rut*, further supporting the role of Oct β 1R in aversive olfactory learning. We next asked whether the learning phenotype could be due to abnormal sensory modalities. When tested with two different odor concentrations or shock intensities, *Canton-S* and *oct β 1r* flies showed comparable olfactory acuity and shock avoidance (Table 1). Together, the

results support that the function of Oct β 1R is needed to acquire aversive memory of olfactory information associated with electric shock.

Table 1. Sensory modality tests including olfactory acuity, shock avoidance and sugar preference

		<i>Canton-S</i>	<i>octβ1r</i>	<i>p</i> Value
Olfactory acuity	2% EA	63.69 ± 2.54	66.93 ± 3.18	0.45
	0.2% EA	11.26 ± 2.88	13.89 ± 4.42	0.63
	2.2% IAA	62.87 ± 2.31	67.29 ± 3.73	0.34
	0.22% IAA	11.83 ± 3.67	12.6 ± 2.92	0.87
Shock avoidance	90 V	65.04 ± 5.23	61.92 ± 4.69	0.67
	30 V	36.48 ± 3.89	31.67 ± 3.43	0.38
Sugar preference	2 M sucrose	63.00 ± 1.91	56.15 ± 3.96	0.16
	0.2 M sucrose	43.07 ± 2.96	37.81 ± 4.60	0.36

Canton-S and *octβ1r* flies exhibited similar avoidance of the CS odors or the US electric shock as well as comparable preference to the US sugar, all of which were tested at two different doses ($n = 6$). Data are the mean ± SEM, unless otherwise indicated.

The site of the function of Octβ1R for aversive memory formation is the MB αβ neurons

The genomic enhancer-driven reporter expression and single-cell RNA-sequencing studies report Octβ1R expression in many neural structures including the PN and the MB (Pfeiffer et al., 2008; Li et al., 2017; Davie et al., 2018). To identify the functional site of Octβ1R, we performed rescue experiments using the GAL4/UAS system (Brand and Perrimon, 1993). We targeted the transgenic Octβ1R expression in the *octβ1r* mutant background pan-neuronally or in the second-order neurons (PN carrying olfactory information from the antennal lobe to MB) or third-order neurons (MB) for the olfactory pathway, both of which are key neural structures for olfactory learning and memory (Güven-Ozkan and Davis, 2014). Pan-neuronal Octβ1R expression (*elav-GAL4*) fully reinstated the learning performance of *octβ1r* (ANOVA: $F_{(8,45)} = 42.21$, $p < 0.0001$; Fig. 2A). When targeted in a subset of neural structures, Octβ1R in all MB neurons (*OK107-GAL4*), but not in the PN neurons (*NP225-GAL4*), led to full rescue of the *octβ1r* learning phenotype (Fig. 2A). The MB has three distinct substructures, namely αβ, α'β', and γ neurons (Crittenden et al., 1998; Aso et al., 2014a), and they are engaged in distinct functions (Güven-Ozkan and Davis, 2014; Cognigni et al., 2018). The cell type-level transcriptome analysis indicates that Octβ1R is abundant in all MB substructures (Shih et al., 2019). To narrow down the functional site within the MB, we used the substructure-specific GAL4 drivers along with the *MB247-GAL4* expressed in αβ and γ for rescue experiments (Fig. 2A). The *octβ1r* flies with reinstated Octβ1R expression in the αβ neurons (*MB247-GAL4* and *c739-GAL4*) exhibited *Canton-S*-like performance (Fig. 2A), indicating full rescue. The Octβ1R re-expression in other MB substructures, namely α'β' (*c305a-GAL4*) or γ (*NP1131-GAL4*), had no effect (Fig. 2A). These results pinpoint the MB αβ neurons as the major functional substrate where Octβ1R processes aversive learning.

Multiple lines of evidence show persistent Octβ1R expression from late embryonic to adult stages (Chintapalli et al., 2007; Graveley et al., 2011; Brown et al., 2014). To assess whether the phenotype of the *octβ1r* is attributable to developmental or physiological anomaly, we restored Octβ1R expression in the *octβ1r* mutant either during development or adulthood using the TARGET system (McGuire et al., 2003; Kim et al., 2007a, 2013). For induction of Octβ1R expression, the *octβ1r* flies carrying *elavGal80^{ts}/UAS-Octβ1R* were reared at 30°C either during development or at the adulthood while the uninduced controls were kept at 20°C throughout development and adulthood (Fig. 2B). Octβ1R induced at the adult stage rescued the impaired learning phenotype of *octβ1r* in aversive conditioning (ANOVA: $F_{(3,48)} = 50.64$, $p < 0.0001$; Fig. 2B), whereas induction only during

development failed to do so (ANOVA: $F_{(3,44)} = 43.64$, $p < 0.0001$). These data indicate that the function of Octβ1R in aversive learning is physiological as opposed to developmental.

OA–DA interplay and intracellular signaling crucial for aversive learning

Our earlier studies (Kim et al., 2007a, 2013) demonstrate that aversive learning requires the MB DA receptor dDA1 but not the MB OA receptor OAMB. Since the MB is a functional site for Octβ1R as well, we asked whether Octβ1R and dDA1 act in collaboration for aversive learning by using a genetic interaction approach. When subjected to electric shock conditioning, all heterozygous receptor mutants showed normal learning, indicating the recessive nature of *octβ1r* and *dumb* in this process. The *octβ1r/dumb* transheterozygotes, on the contrary, displayed significantly impaired performance (ANOVA: $F_{(5,36)} = 13.62$, $p < 0.0001$; Fig. 3A). This was not due to a simple combination of two heterozygous mutations since the *octβ1r/oamb* transheterozygotes showed normal learning. These data suggest that dDA1 and Octβ1R have parallel functions to process aversive learning.

Like mammalian β-adrenergic receptors, Octβ1R stimulates an increase in the intracellular cAMP level when studied in heterologous cells *in vitro* (Maqueira et al., 2005). The cAMP signaling pathway is an integral cellular mechanism for olfactory learning in flies (Güven-Ozkan and Davis, 2014). We conducted *ex vivo* imaging to visualize and quantify the changes in cAMP levels in the MB lobes of *Canton-S* and *octβ1r*. For the task, we used the pan-MB *OK107-GAL4* to drive the cAMP sensor Epac1-camps, where an increase in cAMP causes a decrease in FRET between CFP and YFP (Nikolaev et al., 2004). We first determined the timeline of the cAMP response in the wild-type brain expressing Epac1-camps in the MB on 100 μM OA or HL3 (control) treatment. The maximal increase in cAMP levels was evident within 1 min after OA treatment and sustained at least for 5 min (Fig. 4A); thus, we measured the cAMP response at 3 min after OA treatment in subsequent experiments. Compared with the control, the OA-induced cAMP response was substantially dampened across all MB subregions examined (α3/α'3, α2/α'2, α1/α'1/γ2, β1/2, and γ3–5), except for β'1/2 and γ1/heel (Fig. 4B,C). To clarify whether cAMP serves as a major downstream effector of Octβ1R for olfactory learning, we examined the genetic interaction of *octβ1r* and *rut* defective in the adenylyl cyclase (AC) that converts ATP to cAMP. Similar to *octβ1r/+*, the *rut* heterozygotes (*rut/+*) showed normal performance but the *rut/+;octβ1r/+* double heterozygotes exhibited poor learning (Fig. 3B). These observations corroborate that OA signaling through Octβ1R recruits cAMP to promote aversive learning.

Octβ1R in the PN plays a major role in appetitive learning

OA is a major transmitter for appetitive conditioning (Schwaerzel et al., 2003; Kim et al., 2007b) and its activity through α1-like OAMB in the MB is indispensable for appetitive memory formation (Kim et al., 2013). We asked whether β-like OA receptors are also important for appetitive learning. The *Canton-S*, *octβ1r*, and *octβ2r* flies were conditioned with 2 M sucrose as US and the same odorants were used for aversive conditioning as CS. *Canton-S* and *octβ2r* showed comparable performance when tested immediately, 1 h, and 3 h after training; however, *octβ1r* displayed poor performance at all time points examined (two-way ANOVA: $F_{(11,60)} = 22.29$, $p < 0.0001$; genotype effect: $F = 25.26$, $p < 0.0001$; time effect: $F = 58.82$, $p < 0.0001$; interaction effect: $F = 3.03$, $p = 0.0118$; Fig.

Figure 2. *Octβ 1R* in the MB $\alpha\beta$ neurons promotes acquisition of aversive memory. **A**, Restored *Octβ 1R* (*UAS-Octβ 1R*) in all (*elav-GAL4*), pan-MB (*OK107-GAL4*), MB $\alpha\beta\gamma$ (*MB247-GAL4*), or MB $\alpha\beta$ (*c739-GAL4*) neurons rescued the learning defect of *octβ 1R* ($p < 0.0001$, $n = 6$). In contrast, the flies with *Octβ 1R* re-expression in other MB neurons (MB γ , *NP1131-GAL4*; or MB $\alpha'\beta'$, *c305a-GAL4*) or the PN (*NP225-GAL4*) performed poorly similar to *octβ 1R* mutants ($p > 0.05$, $n = 6$). **B**, Normal aversive learning was reinstated in *octβ 1R* mutants when *Octβ 1R* was restored during adulthood (induced, temperature shift to 30°C; uninduced, no temperature shift; $p < 0.0001$, $n = 13$), but not during development ($p > 0.05$, $n = 12$). In this and other figures, letters on the bars denote either a significant difference among groups (different letters, i.e., “a” and “b”) or no difference (same letters).

Figure 3. Interaction between *Octβ 1R* and dDA1 concertedly drives aversive learning via cAMP. **A**, The flies with the transheterozygous mutations in *octβ 1R* and *dumb* (a mutant allele for dDA1) showed impaired learning (*octβ 1R/dumb*; $p < 0.0001$, $n = 7$), but the *octβ 1R* and *oamb* transheterozygotes (*octβ 1R/oamb*) were comparable to either *Canton-S* control or the heterozygotes *octβ 1R/+*, *dumb/+*, and *oamb/+* ($p > 0.05$, $n = 7$). **B**, The *rut/+; octβ 1R/+* double heterozygotes displayed impaired acquisition similar to *octβ 1R* mutants ($p > 0.05$, $n = 8–9$).

5A). The impaired performance was not due to anomalous sugar perception (Table 1) or genetic background since the transheterozygotes *octβ 1R/Df(3R)Exel6191* and *octβ 1R/Df(3R)BSC685* also performed poorly like the *octβ 1R* mutant (data not shown). These results illustrate the crucial role of *Octβ 1R* for acquisition of appetitive memory as well. The impaired appetitive learning phenotype of *octβ 1R* was fully rescued when *Octβ 1R* was reinstated in all neurons (*elav-GAL4*) or the PN (*NP225-GAL4*) in the *octβ 1R* background (ANOVA: $F_{(6,77)} = 31.01$, $p < 0.0001$; Fig. 5B). Interestingly, the learning defect of *octβ 1R* was slightly improved when *Octβ 1R* was restored in the MB $\alpha\beta$, $\alpha'\beta'$, or γ neurons (Fig. 5B). These data suggest that *Octβ 1R* has redundant functions in multiple neural sites for appetitive learning with quantitatively different contributions, but its major functional site is the PN. Consistently, the OA-induced cAMP levels were significantly dampened in the PN dendrites at the AL and axons at the MB calyx area (Fig. 5C,D; dendrites: $t_{(22)} = -8.52$, $p < 0.0001$; axons: $t_{(22)} = -13.64$, $p < 0.0001$, two-tailed t test).

To elucidate the relative contributions of *Octβ 1R* and OAMB to appetitive learning, we examined genetic

interaction. Unlike the individual *octβ 1R/+* and *oamb/+* heterozygotes that showed normal appetitive learning, the *octβ 1R/oamb* transheterozygote showed drastically diminished learning, which is comparable to that of the homozygous *octβ 1R* flies (ANOVA: $F_{(8,56)} = 68.91$, $p < 0.0001$; Fig. 5E). The DA receptor dDA1 plays a significant role in appetitive memory acquisition (Kim et al., 2007a); thus, we were curious whether *Octβ 1R* and dDA1 work together for appetitive learning. Similar to the *octβ 1R* and *oamb* genetic interaction, the *octβ 1R/dumb* transheterozygote exhibited poor learning (Fig. 5E). These findings indicate that the concerted actions of *Octβ 1R*, OAMB, and dDA1 mediate appetitive learning.

Discussion

Historically, OA is known to mediate reward-based but not punishment-based learning in *Drosophila* and other invertebrates including honeybees, crickets, and crabs (Farooqui et al., 2003; Schwaerzel et al., 2003; Kaczer and Maldonado, 2009; Mizunami and Matsumoto, 2017). This selective role of OA was first demonstrated by Schwaerzel et al. (2003) based on the observation that *tβh* mutants are defective in appetitive learning, but not aversive learning. Recently, Iliadi et al. (2017) challenged this notion and showed that *tβh* mutants are impaired in aversive learning. Both studies examined the same mutant allele and used the same conditioning parameters including CS odorants and US electric shock intensities; thus, the discrepancy in their findings remains puzzling. In this report, by adopting an

RNAi approach, we provide the independent evidence that substantiates the finding of Iliadi et al. (2017) and further demonstrate that more than one type of OA neuron processes aversive learning through *Octβ 1R* in the MB $\alpha\beta$ neurons.

OA neurons have widespread projections in the brain and are classified into 28 types based on their projection patterns, as follows: 27 types positive for *tdc2-GAL4* and 1 type [i.e., anterior paired lateral (APL; *APL-GAL4*) neurons] negative for *tdc2-GAL4* (Busch et al., 2009; Wu et al., 2013). Our data indicate that both *tdc2-GAL4* and APL OA neurons are important for aversive learning. Notably, the MB is the only brain structure innervated by both *tdc2-GAL4* and APL neurons. Four types of *tdc2-GAL4* OA neurons (namely, VPM-3, VPM-4, VPM-5, and VUMa2) project to the calyx where the MB receives olfactory input and the γ lobes comprising MB axons (Busch et al., 2009), whereas the APL neurons innervate all MB structures where they are both presynaptic and postsynaptic (Liu and Davis, 2009; Wu et al., 2013). The APL neurons use both OA and GABA as neurotransmitters and engage in a feedback loop wherein the MB

activates the APL and the APL inhibits the MB, thereby controlling odor discrimination (Liu and Davis, 2009; Wu et al., 2013; Lin et al., 2014). This feedback loop is modulated by DA, which suppresses the APL through dD2R for CS processing (Zhou et al., 2019). It is tempting to speculate that the APL uses OA output for CS processing through Oct β 1R in the $\alpha\beta$ neurons. The tdc2-GAL4-positive OA neurons mediating olfactory conditioning have not been identified in *Drosophila*; however, in the honeybee, the OA-VUMmx1 neuron projecting to the calyx and the antennal lobe processes appetitive US (Hammer, 1993). The fly homolog of VUMmx1 is the OA-VUMa2 neuron that is positive for tdc2-GAL4 (Busch et al., 2009), serving as a likely candidate to process appetitive US. There is no information on the OA-VPM neurons or their homologs in other insects; nonetheless, their projection onto the MB calyx and lobes makes them potential candidates to process aversive US. This predicts that discrete OA neurons take part in the CS and US pathways for aversive learning, and this notion is consistent with the finding by Boto et al. (2019) that two distinct types of DA neurons independently regulate the CS and US pathways.

The modes of the OA and GABA transmissions of the APL (i.e., whether they are released together or independently and whether they act on the same or different postsynaptic sites) are unknown. It is thus difficult to assign the effects of synaptic activity manipulations (e.g., blockade via Shⁱs and activation via TrpA1) to OA or GABA actions. However, the Gad and Rdl RNAi studies indicate that selective suppression of the APL GABA output or the GABA input to the MB enhances aversive learning (Liu et al., 2009; Liu and Davis, 2009), which is contrary to our finding that selective suppression of the APL OA output or the OA input to the MB through Oct β 1R impairs aversive learning. The opposite actions of OA and GABA released from the APL onto the MB implicate that their release dynamics or functional sites may be distinct. In addition to aversive learning, the APL OA signal modulates amnesia-resistant memory (ARM) consolidation and expression through Oct β 2R in the MB $\alpha'\beta'$ neurons (Wu et al., 2013; Yang et al., 2016). We found normal 3 h memory of *oct β 2r* but impaired 3 h memory of *oct β 1r* mutants, which are different from the findings (normal 3 h ARM of *oct β 1r* and impaired 3 h ARM of *oct β 2r*

Figure 4. The *octβ1r* mutant has the dampened cAMP response in the MB on OA treatment. **A**, Left, Representative confocal images of CFP and YFP emission in the MB lobes of the control (*UAS-Epac1-camps/+;OK107/+*) brains at 3 min after HL3 (vehicle; baseline) or OA treatment. The MB lobes are outlined by broken white lines. Right, The cAMP response was measured at various time points after vehicle (baseline) or OA treatment in the MB lobes of the control (*UAS-Epac1-camps/+;OK107/+*) brains (*** $p < 0.0001$, $n = 4-6$). The letter codes ("a," "b," and "c") denote significant differences ($p < 0.0001$) among the OA-induced cAMP responses at different time points. Scale bar, 25 μ m. **B**, Representative confocal images depicting the cAMP level (represented by CFP emission shown in a fire scale) between baseline and OA treatment in the brains of the control (*UAS-Epac1-camps/+;OK107/+*) or *octβ1r* mutant (*UAS-Epac1-camps/+;octβ1r;OK107/+*). OA treatment drastically increased cAMP levels (elevated CFP signal) in the control but not in the *octβ1r* MB. Shown are subregions ($\alpha3/\alpha'3$; $\alpha2/\alpha'2$; $\beta1/2$ and $\beta'1/2$; $\gamma1/heel$; $\gamma2/\alpha1/\alpha'1$ and $\gamma3-5$) of the MB lobes that are delineated by broken white lines. The images and quantifications were performed on the brains at 3 min after vehicle or OA treatments. Scale bar, 25 μ m. **C**, Quantification of the peak cAMP response revealed that all subregions except for the $\beta'1/2$ and $\gamma1/heel$ in the *octβ1r* MB had significantly dampened responses to OA (* $p < 0.05$, ** $p < 0.01$, $n = 10$).

Figure 5. The action of Oct β 1R in the PN fully supports appetitive learning. **A**, *octβ1r* mutants showed defective performance immediately (3 min), 1 h, and 3 h, but not 6 h, after training in appetitive olfactory conditioning (* $p < 0.05$, ** $p < 0.001$, $n = 6$). **B**, Restored Oct β 1R (via *UAS-Octβ1R*) expression in either all neurons (*elav-GAL4*) or the PN (*NP225-GAL4*) fully rescued the learning phenotype of *octβ1r* (the letter code “a”; $p > 0.05$, $n = 12$). However, the *octβ1r* flies with Oct β 1R re-expression in individual MB subsets (γ , *NP1131-GAL4*; $\alpha\beta$, *c739-GAL4*; $\alpha'\beta'$, *c305a-GAL4*) showed marginal yet significant improvement in olfactory learning (the letter codes “c” and “b”; $p < 0.05$, $n = 12$). **C**, Representative confocal images showing the cAMP levels in the PN dendrites and axons of the baseline versus OA-treated control (*NP225-GAL4/+;UAS-Epac1-camps/+*) and *octβ1r* mutant (*NP225-GAL4/UAS-Epac1-camps;octβ1r*). OA treatment increased cAMP levels (elevated CFP signal) in both PN dendrites and axons to a lesser extent in the *octβ1r* mutant compared with the control. Scale bar, 25 μ m. **D**, Quantified peak cAMP responses. The PN dendrites and axons of *octβ1r* had significantly dampened responses to OA (** $p < 0.0001$; $n = 12$). **E**, The flies with the transheterozygous mutations in *octβ1r* and *dumb* (*octβ1r/dumb*) or *octβ1r* and *oamb* (*octβ1r/oamb*), or the double heterozygous mutations in *octβ1r* and *rut* (*rut/+;octβ1r/+*) showed impaired appetitive learning compared with *Canton-S* or the heterozygotes *octβ1r/+*, *dumb/+*, and *oamb/+* (** $p < 0.0001$, $n = 7–9$).

mutants) of the Wu et al. (2013) study. This could be due to several differences that include CS odorants, the nature and genetic backgrounds of *octβ1r* and *octβ2r* mutants (RNAi-mediated knockdown causing 40–60% reduction in mRNA levels in the study by Wu et al., 2013), and the methods to measure 3 h memory (ARM measured after cold shock). The follow-up studies

incorporating these factors would clarify the differences in 3 h memory. Together, the APL OA signals promote aversive olfactory learning and ARM memory through separate β -like receptors located in the distinct MB subsets, namely Oct β 1R in $\alpha\beta$ and Oct β 2R in $\alpha'\beta'$, respectively.

OA increases cAMP in all MB and PN dendritic and axonal structures (Tomchik and Davis, 2009), which we confirmed in this study. We further demonstrate that the OA-induced increase in cAMP is mediated by Oct β 1R in all MB and PN, which is the first to clarify Oct β 1R as an upstream signal for the cAMP increase and the first to substantiate the *in vitro* analysis on Oct β 1R (Maqueira et al., 2005) *ex vivo*. Similar to OA, DA activates the increase in cAMP in all MB structures (Tomchik and Davis, 2009). Notably, both Oct β 1R and dDA1 are present in all MB neurons (Kim et al., 2003; Shih et al., 2019), yet their activities only in the $\alpha\beta$ and γ neurons, respectively, are required for aversive learning (this study; Kim et al., 2007a; Qin et al., 2012). This could be due to selective OA/DA input that exclusively activates $\alpha\beta$ -Oct β 1R/ γ -dDA1 or selective activity of the MB output neurons (MBONs) in $\alpha\beta/\gamma$ (Aso et al., 2014a) that convey the Oct β 1R/dDA1 information. Together, our study indicates that aversive learning is processed by two parallel circuits consisting of the well characterized DA (PPL1 γ 1pedc)-dDA1 (MB- γ)-MBON (MBON- γ 1pedc > α/β , MBON- γ 5 β' 2a β' 2mp; Aso et al., 2014b; Oswald et al., 2015; Yamazaki et al., 2018), and the OA-Oct β 1R (MB- $\alpha\beta$)-MBON (this study) pathways (Fig. 6). The MBONs with dendrites in $\alpha\beta$ (7 MBON types of a total of 22; Aso et al., 2014b) are good candidates to process the OA-Oct β 1R information for aversive learning. Supporting this notion, the MBON- β 2 β' 2a activity modulates olfactory conditioning (Oswald et al., 2015). Alternatively, the MBONs with dendrites in $\alpha\beta$ as well as γ may convey both Oct β 1R and dDA1 information for aversive learning. For example, MBON- γ 1pedc > α/β has dendrites in γ 1pedc and $\alpha\beta$ s and axons in $\alpha\beta$ lobes, crepine, and superior intermediate protocerebrum, and its activity modulates aversive learning (Perisse et al., 2016), thus serving as a good candidate conveying both Oct β 1R and dDA1 signals for learned avoidance behavior. An anomaly in either Oct β 1R or dDA1 signaling to MBON- γ 1pedc > α/β would lead to poor aversive learning.

Figure 6. Working model for the pathways that OA and DA process aversive and appetitive olfactory learning. In aversive conditioning, the OA and DA neurons deliver the aversive US information to Oct β 1R in the MB $\alpha\beta$ and dDA1 in the MB γ , respectively, which, in concert with the CS input, activate the coincidence detector Rut-AC. The parallel cAMP signaling in the $\alpha\beta$ (1, red box) and γ (2, red box) modulates the MB outputs to MBONs promoting avoidance behavior. Abnormal signaling in either OA-Oct β 1R in $\alpha\beta$ or DA-dDA1 in γ leads to defective aversive learning. In appetitive conditioning, the collective activation of OA-Oct β 1R in the PN (3, blue box), which projects to the MB, as well as OA-OAMB and DA-dDA1 in the MB $\alpha\beta\gamma$ (4, blue box) recruit both Rut-AC and phospholipase C (PLC) to stimulate the cAMP and Ca^{2+} signaling, which in turn modulate the MB outputs to the MBONs promoting approach behavior. Thus, the OA and DA systems act together for aversive and appetitive learning through distinct neural sites and intracellular signaling.

Rut-AC is a coincidence detector for associative learning and memory (Güven-Ozkan and Davis, 2014). We show that *oct β 1r* and *rut* are genetically interacting for aversive learning. The previous findings on the functional site of Rut-AC are somewhat confusing. Blum et al. (2009) identify γ neurons as the site that Rut-AC mediates aversive learning, whereas Zars et al. (2000) and Akalal et al. (2006) demonstrate that Rut-AC in both γ and $\alpha\beta$ neurons is important. The major difference is the *rut* allele: Blum et al. (2009) used *rut¹* but Akalal et al. (2006) and our study used *rut²⁰⁸⁰*, which displays a more severe learning deficit than *rut¹*. Thus, Rut-AC likely serves as a key downstream effector for both Oct β 1R and dDA1 in $\alpha\beta$ and γ , respectively. Regarding the downstream effectors of cAMP, Oct β 1R and dDA1 seems to involve distinct signaling pathways. Gervasi et al. (2010) demonstrate that OA activates protein kinase A (PKA) in $\alpha\beta$ in a Rut-AC-dependent manner but that DA does not induce PKA activation in γ . *In vivo* functional imaging, on the contrary, reveals that an elevated cAMP level leads to enhanced calcium responses to CS odors in γ but not $\alpha\beta$ (Boto et al., 2014). It is tempting to speculate that the γ dDA1–cAMP pathway directly activates cyclic-nucleotide gated ion channels while the $\alpha\beta$

Oct β 1R–cAMP pathway recruits PKA to modify ion channel activity or other downstream effectors for aversive learning.

OA is a major neuromodulator for appetitive learning, and α 1-like OAMB in $\alpha\beta\gamma$ is a key receptor conveying the OA signal (Kim et al., 2013). Here, we show that Oct β 1R is also important for appetitive learning, and this dual function is similar to dDA1 (Kim et al., 2007a). However, there is a major difference: dDA1 processes both aversive and appetitive learning in MB γ , but the functional sites of Oct β 1R are distinct (i.e., MB $\alpha\beta$ for aversive learning and PN for appetitive learning). Thum et al. (2007) demonstrate that Rut-AC restored in either MB or PN fully rescues the phenotype of the *rut* mutant in appetitive conditioning, suggesting multiple yet redundant neural sites for Rut-AC-dependent learning. It is yet unclear whether dDA1 recruits Rut-AC for appetitive memory; however, our study clarifies that the PN Rut-AC is activated by Oct β 1R for the acquisition of appetitive memory. Together, OA mediates positively reinforced learning through the following two parallel circuits: the OA-Oct β 1R–Rut-AC pathway in PN and the OA-OAMB–intracellular calcium pathway in MB $\alpha\beta\gamma$.

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