Research

G(o) activation is required for both appetitive and aversive memory acquisition in *Drosophila*

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Heterotrimeric G(o) is an abundant brain protein required for negatively reinforced short-term associative olfactory memory in *Drosophila*. G(o) is the only known substrate of the S1 subunit of pertussis toxin (PTX) in fly, and acute expression of PTX within the mushroom body neurons (MB) induces a reversible deficit in associative olfactory memory. We demonstrate here that the induction of PTX within the α/β and γ lobe MB neurons leads to impaired memory acquisition without affecting memory stability. The induction of PTX within these MB neurons also leads to a significant defect in an optimized positively reinforced short-term memory paradigm; however, this PTX-induced learning deficit is noticeably less severe than found with the negatively reinforced paradigm. Both negatively and positively reinforced memory phenotypes are rescued by the constitutive expression of G(o) α transgenes bearing the Cys₃₅Ile mutation. Since this mutation renders the G(o) molecule insensitive to PTX, the results isolate the effect of PTX on both forms of olfactory associative learning to the inhibition of the G(o) activation.

[Supplemental material is available for this article.]

Pavlovian conditioning is a simple form of learning in which neutral stimuli become associated with rewards or punishments. Identifying the molecular mechanism underlying the formation of these associations remains a significant goal in neurobiology. Much progress has been made over the past 30 yr in uncovering the cellular and molecular events required for associative memory formation using negatively and positively reinforced olfactory conditioning assays in *Drosophila melanogaster* (Keene and Waddell 2007; Waddell 2010). In these assays, the flies learn to associate an odor (Conditioned Stimulus paired; CS⁺) with either electric shocks or sugar (Unconditioned Stimulus; US). Both aversive (negatively reinforced) and appetitive (positively reinforced) associative memories require the activity of the mushroom body neurons located in the central brain (Dubnau et al. 2001; Krashes et al. 2007, 2009).

The mushroom bodies (MB) are paired 3rd order olfactory neuropil, each containing ~2000 neurons (Heisenberg 2003; Aso et al. 2009). These neurons have been classified based on their distinct axon projections (Armstrong et al. 1998; Crittenden et al. 1998). The α/β neurons have bifurcated axons that project vertically into the α lobe and horizontally into the β lobe. Similarly, the α'/β' lobes contain the bifurcated axons of the α'/β' neurons, and the γ lobes contain the axons from the γ lobe neurons. These classes of MB neurons have distinct functions during memory formation. Synaptic output from the α'/β' mushroom body neurons and the synaptically connected Dorsal Paired Medial Neurons (DPM) are essential for the acquisition and consolidation of both appetitive and aversive olfactory memories but are not essential for the recall of these memories, leading to the proposal of a reinforcement circuit between these neural structures (Krashes et al. 2007). In contrast, neurotransmission from the α/β lobe neurons is dispensable for acquisition and consolidation but necessary for retrieval, suggesting acquisition of these memories

occurs prior to synaptic release from these neurons (McGuire et al. 2001; Isabel et al. 2004; Krashes et al. 2007). The inhibition of synaptic release from the γ lobe neurons does not significantly impact 3-min memory (McGuire et al. 2001).

The cAMP signaling pathway is required for normal levels of memory formation within these MB neurons. Central to this pathway is the *rutabaga* (*rut*) type I adenylyl cyclase (Levin et al. 1992). The *rut* gene product acts as a coincidence detector, increasing cAMP and cAMP-dependent protein kinase A (PKA) activity synergistically upon dopamine stimulation of G(s)-coupled receptors and acetylcholine-induced Ca²⁺ influx (Tomchik and Davis 2009; Gervasi et al. 2010). Both dopamine and octopamine activate PKA predominantly in the axons of MB neurons in a manner that suggests the presence of distinct subcellular compartments for PKA dynamics and appears to reflect an autonomous property of MB neurons rather than a network effect (Gervasi et al. 2010). While octopamine affects the activation of PKA within the α/β and γ MB lobes, dopamine action is restricted to α lobes by a mechanism requiring *dunce* phosphodiesterase (PDE) activity (Gervasi et al. 2010). Interestingly, *rut* activity is both necessary and sufficient within the γ lobe for acquisition of short-term aversive memories and the activity of this gene within the α/β lobes neurons is not essential, suggesting the *rut*-dependent synergistic activation of PKA in the α lobes by dopamine and acetylcholine are not acting in the formation of aversive short-term memories (Zars et al. 2000; Akalal et al. 2006; Blum et al. 2009). Since the amorphic *rut*¹ allele is capable of supporting significant amounts of both positively and negatively reinforced olfactory memory, additional signaling pathways are also required to form these short-term associative memories.

One such *rut*-independent signaling pathway involves the activation of heterotrimeric G(o) (Ferris et al. 2006). G(o) is abundantly and ubiquitously expressed throughout the adult fly brain, and its α subunit, encoded by the *G(o) α 47A* gene, is the only detected substrate of the S1 subunit of pertussis toxin (PTX) (Thambi et al. 1989; Fremion et al. 1999). Post-developmental PTX expression in MB demonstrated that G(o) activity is required

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in α/β and γ cell populations for associative olfactory memory formation with aversive stimuli (Ferris et al. 2006). The PTX effect appeared to be largely independent of the cAMP pathway, showing additivity with the hypomorphic *rut²⁰⁸⁰* and requirement for activity in different MB neurons.

Herein, we further define the effect of PTX expression on associative memory formation by showing that PTX expression in the α/β and γ lobe neurons affects the kinetics of memory acquisition but does not influence memory stability. However, expression of PTX within the α'/β' neurons did not result in a learning phenotype, indicating the effect is limited to the α/β and γ lobe neurons. We further demonstrate that the PTX expression within these neurons also disrupts short-term appetitive memory. Both aversive and appetitive memory phenotypes were rescued by the expression of a PTX-insensitive G(o) α subunit (PiGo) (Chatterjee et al. 2009), indicating that the effect of PTX on memory was through the ADP-ribosylation of the heterotrimeric G(o) protein.

Results

PTX induction within MB neurons reduces asymptotic learning without affecting memory retention

To dissect the processes of olfactory learning and memory that are dependent on heterotrimeric G(o) signaling within the mushroom body neurons, we examined how PTX affects the kinetics of aversive memory acquisition and retention. PTX was acutely expressed within the adult mushroom body neurons with the P247 Gal4 driver controlled by the *Gal80^{ts}* system (Zars et al. 2000; McGuire et al. 2003; Ferris et al. 2006). Since strong PTX induction protocols result in an almost complete loss of aversive learning, we utilized a more modest induction protocol for these experiments. Two independent *Gal80^{ts}* transgenes were used for a more complete clamping of Gal4 activity during development, and induction was accomplished with a 3-h, 32°C heat treatment followed by a 3-h recovery at 25°C.

The rate of memory acquisition was determined after varying the number of discrete odor (CS⁺) and electric shock (US) pairings using the “short protocol” (Beck et al. 2000). Each CS⁺-US pairing represents one training trial. The induction of PTX within the α/β and γ lobe neurons identified by P247 leads to a significantly reduced Performance Index (PI) at each number of training trials compared to the within-genotype controls ($P < 0.001$, Bonferroni-Dunn) (Fig. 1A). There were no significant differences in performance between the uninduced experimental genotype and the control genotypes in this experiment. Maximal learning was reached after five training trials following PTX induction within the MB neurons, while performance was still ascending after 15 training trials for the uninduced experimental and control genotypes (Fig. 1A). Hence, PTX expression within the MB neurons reduced both the rate of learning and the learning asymptote.

The poor memory acquisition following acute PTX expression within MB neurons does not preclude an effect of this enzyme on memory stability. To address this question, we examined memory decay after equalizing the initial performance levels between the induced experimental and control genotypes (Fig. 1B). The induced PTX genotype was provided five training trials and the control genotype was provided a single training trial. Although memory levels were low in both groups and did not persist beyond 60 min, there were no significant differences in memory found in these flies at the times examined, suggesting that memory stability is not affected by PTX expression within the α/β and γ lobe MB neurons ($P = 0.83$) (Fig. 1B).

We next sought to further identify which mushroom body neurons require G(o) activation for negatively reinforced olfactory memory formation. Previously, we found that PTX expression

within the α/β and γ lobe neurons of the mushroom bodies was capable of disrupting memory formation. We also determined that PTX expression in the α/β core neurons defined by the 17d Gal4 driver, or the DPM neurons defined by the c316 Gal4 driver, did not affect aversive olfactory memory (Ferris et al. 2006). We have further examined whether PTX expression within the α'/β' mushroom body neurons also disrupts aversive memory. The c305a Gal4 line will drive expression preferentially within the α'/β' mushroom body neurons. When PTX expression is driven in conjunction with *Gal80^{ts}* system, we failed to detect a significant defect in negatively reinforced learning (Supplemental Fig. S1). Hence, the effect of PTX expression in the MBs on aversive learning appears limited to the α/β and γ lobe neurons.

PTX expression in MB neurons reduces appetitive memory formation

A lower learning asymptote, as found after PTX induction within MB neurons, may result from a reduction in the salience of the US (Rescorla and Wagner 1972; Tully and Quinn 1985). It is

Figure 1. Acute PTX expression in MB results in impaired memory acquisition, while memory retention is spared. The P247 Gal4 driver in conjunction with the *Gal80^{ts}* system was used to induce PTX within the MB neurons. The genotypes were PTX: *Gal80^{ts20}/+; ry⁵⁰⁶/P247, Gal80^{ts2}*; and the control, *ry: Gal80^{ts20}/+; ry⁵⁰⁶/P247, Gal80^{ts2}*, respectively. (A) Memory acquisition: sub-maximal PTX induction (3 h at 32°C, followed by a 3-h recovery at 25°C) within MB neurons leads to a significantly lower maximal performance level ($F_{(3,201)} = 99.57$; $P < 0.0001$). $n = 8$ for all groups; the values are mean $\pm$ SEM. The learning asymptote is also reached earlier than in control genotypes. (B) Memory decay: the retention levels were determined for the induced PTX and *ry* genotypes after differential training. The induced PTX flies were trained for five training trials and the *ry* flies for one training trial. No significant differences were found between these groups ($F_{(1,140)} = 0.001$; $P = 0.97$) when the level of initial learning is normalized. All values are mean $\pm$ SEM; $n \geq 12$ for all groups.

possible that the reduced processing of US input into the MB neurons could mimic a reduction of salience of the electric shock and lead to a lower asymptote. The MBs are also important centers for appetitive learning (Schwaerzel et al. 2003). If the PTX effect on learning was solely due to an effect on electric shock processing by the mushroom bodies, then PTX expression would not affect appetitive learning. Alternatively, if PTX disrupted positively reinforced memory formation, then the G(o) requirement may also be required for sugar-reinforcement, or G(o) may reside in a shared pathway downstream from the initial acquisition event. To examine the effect of PTX expression on positively reinforced olfactory learning, we utilized a version of the sugar learning paradigm of Krashes et al. (2007), hereafter referred to as the standard appetitive protocol. The expression of PTX was driven by the P247 MB Gal4 line and controlled with two *Gal80^{ts}* insertions as before (Fig. 2). In this experiment, we found a significant effect of both genotype and induction treatment on the performance in the appetitive memory assay. The induction of PTX within the α/β and γ lobe neurons led to a significantly reduced appetitive learning score compared to the control genotypes and the within-genotype uninduced control (Bonferroni-Dunn; $P < 0.0001$ for all comparisons). Interestingly, the induction protocol in the *Gal80^{ts}* PTX flies resulted in a significantly increased performance index (Bonferroni-Dunn; $P = 0.0032$) (Fig. 2). It is possible that the increased temperature used for PTX induction leads to a higher metabolic drive and unequal motivation toward the sugar reward. The induction of PTX driven by P247 does not affect the attraction to the sucrose reward (Supplemental Table S1). Although we did detect some differences in naive odor avoidance between these genotypes and treatments, the effects could not explain the significantly lower appetitive learning scores found after PTX induction. Thus, the PTX expression within the α/β and γ MB lobe neurons disrupts the formation not only of the aversive associative memory, but also of the appetitive memory.

Pertussis-insensitive G(o) α rescues PTX learning phenotypes

To verify that the PTX-induced deficits in both aversive and appetitive learning were due to the specific inhibition of heterotrimeric

Figure 2. PTX expression in MB inhibits appetitive memory. The P247 Gal4 driver and *Gal80^{ts}* system were used to induce PTX within the α/β and γ lobe MB neurons. The induction of PTX leads to significantly lower levels of positively reinforced learning ($F_{(5,72)} = 106.40$; the asterisk designates $P < 0.0001$, significant with Bonferroni-Dunn). Interestingly, the 12-h, 32°C heat induction also resulted in an increase in the performance of the *Gal80^{ts20}/+; ry⁵⁰⁶, UAS-PTX.16/Gal80^{ts2}* control genotype. The values are mean $\pm$ SEM; $n = 13$ for all groups.

G(o) activation, we performed rescue experiments with a PTX-insensitive G(o) α (PiGo) (Chatterjee et al. 2009). Briefly, PTX adds an ADP-ribosyl group onto Cysteine₃₅₁ in the carboxy terminus of G(o) α , inhibiting interactions with the activating G protein-coupled receptor (Katada et al. 1983). In the UAS-PiGo transgene, G(o) α 47A was rendered insensitive to PTX by incorporating the Cysteine₃₅₁ to Isoleucine mutation. The identical mutation in mouse G(o) α retains activity (couples to receptors, effectors, and to the G β/γ subunits), while becoming insensitive to PTX (Jeong and Ikeda 2000). The acute coexpression of PiGo in the adult fly mushroom body neurons using P[MBSwitch]12 did not ameliorate the learning deficit induced by PTX (Supplemental Fig. S2). Similar results were found with three independent UAS-PiGo transgenes (data not shown).

We hypothesized that the rapidity of PTX modification of G(o) ($K_{CAT} = \sim 10 \text{ min}^{-1}$) (Cortina et al. 1991) relative to G(o) activation within mushroom body neurons causes the G β/γ subunits to be irreversibly bound up in ribosylated complexes with wild-type G(o) α . Newly synthesized PiGo subunits would not be able to associate with G β/γ subunits and, hence, could not be activated and would not rescue the PTX phenotype. To test this hypothesis, we sought to constitutively express PiGo, while retaining the ability to induce PTX. This was accomplished using two independent *tet^o-PTX* lines (Chatterjee et al. 2009). In our first experiment, we verified that doxycycline-induced PTX would disrupt aversive learning. In these experiments, the P247 MB Gal4 driver induces the expression of the UAS-*rtTA* tetracycline-responsive transactivator within α/β and γ MB neurons. In the presence of doxycycline, the *rtTA* activates the *tet^o-PTX* transgene (Stebbins et al. 2001; Ford et al. 2007; Chatterjee et al. 2009). Doxycycline was fed to the flies for 24 h, followed by a 24-h recovery period prior to behavioral testing. The induction of *tet^o-PTX* resulted in significantly reduced aversive olfactory learning (Bonferroni-Dunn; $P < 0.0001$), similar to the *Gene-Switch* and *Gal80^{ts}* systems; the induction had no significant effect on the 3-min memory of the control genotypes in this paradigm (Fig. 3).

We next examined whether the constitutive expression of PiGo with the P247 driver would rescue the ability of induced PTX to inhibit associative memory formation (Fig. 4A,B). In these experiments, we examined 3-min memory performance with two independent combinations of *tet^o-PTX*, and UAS-PiGo. For both combinations, the expression of PiGo through development, along with *rtTA*, completely blocked the effect of PTX on aversive memory (Fig. 4A,B). However, the similar coexpression of wild-type G(o) α with PTX failed to effect an increase in aversive memory formation (Fig. 5). Thus, PiGo can functionally rescue the PTX learning phenotype, demonstrating that learning is inhibited by the ribosylation of G(o) α on Cys₃₅₁.

The ability of PiGo to block PTX-associated learning deficits was also examined in the appetitive learning paradigm. In these experiments, we utilized a modified sugar learning paradigm to increase the performance and discriminatory power. Glazing the sucrose-coated filter paper 24 h prior to training and providing a severe starvation period provided higher learning scores (see Materials and Methods). Using this modified appetitive learning protocol, we achieved higher PIs for our control genotype and the uninduced experimental genotype than the previous standard method (Fig. 4C,D). The induction of PTX within the α/β and γ lobe neurons defined by P247 resulted in a modest, but significant reduction of appetitive memory (Fig. 4C,D). Interestingly, PTX expression within the mushroom bodies leads to consistently worse performance in the aversive paradigm than in the appetitive paradigm (Fig. 4; data not shown). The induction of PTX within the mushroom bodies with the *tet^o* system and P247 driver does not affect the naive responsiveness to odors used in this assay, nor the response to electric shock punishment and sugar

Figure 3. PTX expression in MB using *tet^o* system severely reduces aversive learning. The listed genotypes were fed either doxycycline or vehicle for 24 h followed by a 24-h recovery period. The doxycycline induction led to a significant reduction in aversive learning within the *UAS-rtTA, tet^o-PTX.20f/+*; *P247/+* experimental genotype ($F_{(5,71)} = 96.95$; $P < 0.0001$; the asterisk designates $P < 0.0001$, significant with Bonferroni-Dunn), without significant effect on the performance of the control genotypes. All genotypes are in the *w¹¹¹⁸* background. All values are mean $\pm$ SEM; $n \geq 12$ for all groups.

reward (Supplemental Tables S2, S3). Similar to the results from the aversive paradigm, the developmental expression of PiGo blocks the effect of PTX on reward learning, indicating that the effect of PTX expression within the α/β and γ lobe neurons on appetitive learning is also due to the ribosylation of G(o) α on Cys₃₅₁.

G(o) requirement during appetitive learning is distributed within both α/β and γ MB lobes

We next sought to further define which mushroom body neurons require G(o) activation for positively reinforced olfactory memory formation with the *c739* and NP1131 MB Gal4 drivers. The *c739* Gal4 driver has a central α/β lobes expression, while NP1131 is expressed in the γ lobe. For these experiments, we utilized the *tet^o-PTX* expression system. The acute induction of PTX with the *c739* driver within the α/β MB lobes following doxycycline treatment resulted in significant reduction in the appetitive learning (Fig. 6A). The planned within-genotype comparison of *tet^o-PTX.36f/+*; *UAS-rtTA/c739* indicated a significant reduction in PI with the induction of PTX expression ($t = 2.554$; $P = 0.02$). The PI of the induced *tet^o-PTX.36f/+*; *UAS-rtTA/c739* experimental group was also significantly less than the full set of induced genotype controls (Bonferroni-Dunn post-hoc; $P \leq 0.0005$ for all comparisons) (Fig. 6A). PTX expression with the NP1131 Gal4 driver within the γ MB lobes produced a similar outcome. The within-genotype comparison of the *tet^o-PTX.36f/+*; *UAS-rtTA/NP1131* demonstrated a significant reduction in positively reinforced short-term memory ($t = 2.4$; $P = 0.028$) and significant difference from the induced doxycycline-fed control genotypes (Bonferroni-Dunn post-hoc; $P < 0.0001$ for all comparisons) (Fig. 6B). Finally, the PTX induction with *tet^o-PTX.36f*, *UAS-rtTA901*, and the concomitant use of both *c739* and NP1131 Gal4 drivers, has yielded an appetitive STM reduction comparable in magnitude with the individual effect of each of the individual drivers. The within-genotype planned comparison demonstrated that PTX expression in both α/β and γ lobe neurons significantly reduces appetitive STM ($t = 3.21$; $P = 0.006$). The induced experimental genotype also resulted in significantly lower appetitive STM than the induced genotype controls (Bonferroni-Dunn

post-hoc; $P < 0.0001$) (Fig. 6C). Finally, the induction of PTX within the mushroom bodies with doxycycline does not affect the naive responsiveness to odors used in this assay and does not affect the attraction to the sugar reward (Supplemental Tables S4, S5). Together, these data demonstrate that G(o) signaling is required within the α/β and γ lobe neurons for normal appetitive learning.

We have further examined whether G(o) signaling within the α'/β' MB neurons is important for *t* appetitive memory formation. The *tet^o* expression system was used as in the previous experiments (Figs. 4, 6) (*tet^o-PTX.36f* and *UAS-rtTA901*), together with two independent Gal4 drivers—*c305a* and *c320*—to induce acute expression of PTX within α'/β' MB neurons (Krashes et al. 2007). In these experiments, no discernable decreases in appetitive learning were found in the doxycycline-induced PTX expression, the genotypes suggesting that G(o) signaling is not required in the α'/β' neurons for appetitive memory formation (Supplemental Fig. S3A,B).

Discussion

The acute expression of PTX within the α/β and γ lobe neurons defined by the *P247* driver is sufficient to inhibit both aversive and appetitive short-term olfactory memories. We have further shown through transgenic rescue experiments that the PTX inhibition of both aversive and appetitive short-term memories requires the G(o) α_{Cys351} ADP-ribosylation site. PTX will only ribosylate heterotrimers (not individual α subunits), and the consequence of this ribosylation is inhibition of the heterotrimer activation (Katada et al. 1983; Moss et al. 1983). The inhibition of G(o) signaling by PTX is, therefore, extremely specific; since the ADP-ribosylated G(o) heterotrimers cannot be activated, they do not generate ectopic G $\beta\gamma$ subunits, nor do they sequester free G $\beta\gamma$ subunits away from other G α subunits. Hence, the PTX loss-of-learning phenotype and the rescue of this deficit with the expression of G(o) $\alpha_{Cys351Iso}$ demonstrates that G(o) activation is required within the mushroom body neurons for the formation of short-term olfactory associative memories.

Since anatomically distinct regions of the mushroom bodies have distinct roles in associative memory acquisition, stabilization, and recall (Zars et al. 2000; Dubnau and Tully 2001; Krashes et al. 2007, 2009; Blum et al. 2009; Trannoy et al. 2011), the identification of neurons that require G(o) activation provides important insight into the function of this signaling pathway during memory formation. Previously, it was found that PTX would partially affect negatively reinforced learning when expression was limited to either the α/β or γ mushroom body neurons, but when expressed in both subpopulations of neurons, as defined by the *P247* and *c772* Gal4 lines, it would almost completely eliminate memory formation (Ferris et al. 2006). In contrast, the inhibition of G(o) activation within the α/β core neurons or within the DPM neurons had no effect on aversive memories (Ferris et al. 2006). We have further delineated the G(o) requirements during memory formation by excluding α'/β' lobe neurons. The DPM and α'/β' neurons are likely involved in a recurrent circuit that during both appetitive and aversive memory acquisition supports the consolidation of memories within the α/β lobe neurons of the mushroom bodies (Krashes et al. 2007). The activation of G(o) is, therefore, required for aversive memory formation outside of the acquisition and stabilization events that occur within this α'/β' -DPM neuron circuit.

Our data highlight potential functions for this G(o) activation in memory formation. The stability of negatively reinforced olfactory memories appeared unaffected by the inhibition of G(o) activation, which suggests that G(o) activation is required during memory formation but not subsequently. In this last experiment, however, possible effects on memory stability may

Figure 4. The constitutive expression of a pertussis toxin-insensitive G(o) transgene fully rescues the negatively reinforced (A,B) and positively reinforced (C,D) learning deficits resulting from the acute induction of PTX in MB. P247 was used to drive UAS-rtTA and UAS-PiGo transgenes within the α/β and γ lobe MB neurons. (A) Aversive 3-min memory assay with *tet^o-PTX.20f* and UAS-PiGo_{B10E}; PTX induces a significant decrease in learning ($F_{(3,30)} = 47.66$; $P < 0.0001$; Bonferroni-Dunn) that is fully reverted by the expression of PiGo. (B) Aversive 3-min memory assay with females carrying *tet^o-PTX.36f* and UAS-PiGo_{A1H}; PTX induces a significant decrease in learning ($F_{(3,30)} = 40.88$; $P < 0.0001$; Bonferroni-Dunn) that is also fully rescued by the expression of PiGo. (C) Appetitive 3-min memory assay with *tet^o-PTX.20f* and UAS-PiGo_{B10E}; PTX induces a significant decrease in learning ($F_{(3,31)} = 14.68$; $P < 0.0001$; Bonferroni-Dunn), fully reverted by constitutional expression of PiGo. (D) Appetitive 3-min memory assay with females carrying *tet^o-PTX.36f* and UAS-PiGo_{A1H} ($F_{(3,29)} = 13.33$; $P < 0.0001$; Bonferroni-Dunn). The asterisk indicates $P < 0.0001$ with a Bonferroni-Dunn post-hoc test on designated comparisons. All values are mean $\pm$ SEM; $n = 8$ for all groups.

be hidden by the low performance found in flies expressing PTX within their α/β and γ lobe neurons and in the control flies trained with a single CS-US pairing. G(o) activation is also unlikely to be required for the initial encoding of CS strength or identity within the α/β and γ lobe neurons but may be involved in processing the electric shock or in subsequent memory formation processes. This latter conjecture is based partially on the fact that overtraining in the negatively reinforced learning paradigm will not compensate for the learning defect as would be expected if PTX reduced the salience of the odor stimuli (Rescorla and Wagner 1972). Moreover, the inhibition of G(o) activation differentially affected performance in the appetitive and aversive paradigms, which is again inconsistent with a change in odor strength or identity. The lower performance asymptote found in the acquisition curve after G(o) inhibition could, however, reflect a defect in processing the electric shock, in memory formation, or

in reinforcement events acting downstream from this step, as well as in multiple processes. Although transducing the signal for the electric shock within the α/β and γ lobe neurons could account for the severely reduced learning found with PTX expression in both neuron types, it would fail to account for the phenotype found in appetitive memory. G(o) may also be required for a process shared by both appetitive and aversive memory systems that provides more general information about properties of the reinforcement, such as its predictive value, and, as such, could influence memory strength.

Heterotrimeric G(o) signaling

The receptor(s) responsible for activating G(o) during memory formation and the downstream effectors are currently unknown. G(o) signaling pathways in vertebrates are better studied than in *Drosophila* and can offer insight into possible pathways for activation and effectors during *Drosophila* associative memory formation. G(o) is an extremely abundant membrane protein in the vertebrate brain, comprising between 1%–2% of total membrane protein, suggesting a common role in neural signal transduction (Sternweis and Robishaw 1984; Huff and Neer 1986). In vertebrate cells, G(o) is typically activated by GPCRs but can also be activated by non-GPCR receptors such as Amyloid Precursor Protein and GAP-43 (Strittmatter et al. 1991; Nishimoto et al. 1993; Swanson et al. 2005). The GPCRs that activate G(o) are typically of the G(i/o) coupled family and will generally inhibit neural activity; these include opioid receptors, different neuropeptide receptors, subtypes of mGluR receptors, and GABA_B receptors (Hescheler et al. 1987; Kleuss et al. 1991; Taussig et al. 1992; Perroy et al. 2000). However, the specificity of GPCR coupling to specific classes of G proteins is not absolute and may strongly depend on the cellular context of the GPCRs

(Laugwitz et al. 1996; Brydon et al. 1999). The promiscuous coupling of GPCRs may also be regulated through post-transcriptional modification. For example, the palmitoylation of the Endothelin_B receptor results in a shift in coupling from G(i) to G(q) (Okamoto et al. 1997), and the phosphorylation of the β_2 -adrenergic receptor by PKA leads to a G(s) to G(i) shift in coupling (Daaka et al. 1997). Hence, we cannot a priori eliminate any GPCR from consideration based on receptor type and preferred coupling. Given this stipulation, one possible candidate for G(o) activating GPCRs within the α/β and γ lobe neurons is the dDA1 receptor (Gotzes et al. 1994). The dDA1 dopamine receptor is prominently expressed in the α/β and γ lobe neurons of the mushroom bodies where G(o) activation is required (Kim et al. 2003). Moreover, similar to the effects of PTX, dDA1 mutants display severe impairments in aversive memory and less severe impairments in appetitive memory (Ferris et al. 2006; Kim et al. 2007). However, to our knowledge,

Figure 5. The overexpression of wild-type G(o)α in MB fails to rescue the PTX-induced negatively reinforced learning deficit. The *tet^o-PTX.36f, UAS-G(o)α/Y; UAS-rtTA/+; P247/+* induced with doxycycline displayed significantly lower performance as compared to the *UAS-G(o)α/Y; UAS-rtTA/+; P247/+* induced flies ($t = 9.01$; $P < 0.0001$). These induced *tet^o-PTX.36f, UAS-G(o)α/Y; UAS-rtTA/+; P247/+* also performed significantly worse than the within-genotype uninduced control flies ($t = 6.74$; $P < 0.0001$). No significant differences were found between the induced *tet^o-PTX.36f, UAS-G(o)α/Y; UAS-rtTA/+; P247/+* and *tet^o-PTX.36f/Y; UAS-rtTA/+; P247/+* groups. All values are mean $\pm$ SEM; $n \geq 8$ for all groups. Significant differences with the Fischer post hoc test are labeled with a number sign (#), and those with the more stringent Bonferroni-Dunn are labeled with an asterisk (*).

dDA1, which can activate cAMP synthesis, has not been shown capable of coupling to G(o) (Gotzes and Baumann 1996).

Few G(o) effectors have been demonstrated in vertebrates, and in most cases, it is the $\beta\gamma$ subunits that are responsible for actuating signaling. Presynaptic voltage-gated Ca^{2+} channels represent a major effector for G(o) (Strock and Diverse-Pierluissi 2004). G-protein $\beta\gamma$ -subunits inhibit N-type ($\text{Ca}_v2.2$) and P/Q-type ($\text{Ca}_v2.1$) presynaptic Ca^{2+} channels involved in neurotransmitter release (Herlitze et al. 1996; Ikeda 1996), causing a positive shift in their voltage dependence of activation. The inhibition is lifted by high-frequency action potentials (Park and Dunlap 1998). N-type channels also undergo a voltage-independent inhibition that is mediated by the direct binding of G(o)α to the α_{1B} subunit, resulting in an inhibition of Ca^{2+} current even after strong depolarization (Furukawa et al. 1998). These effects of activated G(o) on the presynaptic voltage-gated Ca^{2+} channels were described in cultured sensory neurons from embryonic chick dorsal root ganglion and set in motion by noradrenaline (NA), γ -aminobutyric acid (GABA), serotonin (5-HT), enkephalin, and somatostatin GPCRs (Dunlap and Fischbach 1978, 1981). This voltage-gated Ca^{2+} channels effector pathway for G(o) is also present in central neurons, e.g., in Purkinje cerebellar neurons, elicited through GABA_B receptors (Mintz and Bean 1993), and in sympathetic neurons, e.g., effected through presynaptic α_2 -adrenergic autoreceptors (Lipscombe et al. 1989), adenosine A1 receptors (Zhu and Ikeda 1993), and E2 (PGE2) (Ikeda 1992). The *Drosophila cacophony* voltage-gated Ca^{2+} channel α subunit may be a target for G(o) subunits during memory formation.

Pheromone binding to the VR2 receptors in rodent vomeronasal organs activates G(o), liberating $\text{G}\beta\gamma$, which then activates phospholipase $\text{C}\beta$ to mediate pheromone signal transduction (Krieger et al. 1999; Runnenburger et al. 2002). In *Drosophila*, *plcβ21* is coexpressed with G(o)α in essentially the entire nervous system (Shortridge et al. 1991). Recently, Dahdal et al. found that *Plcβ21* is an effector for G(o) in the *Drosophila* LN_vs neurons (Dahdal et al. 2010). Hence, the loss of *Plcβ21* activation within

Figure 6. The acute expression of the pertussis toxin within the MB using *tet^o* system reduces positively reinforced associative olfactory learning: the G(o) role is distributed between the α/β and γ MB lobes. (A) The c739 Gal4 driver was used to drive PTX expression within the α/β MB lobe neurons. The within-genotype planned comparison is significant (#; $t = 2.554$; $P = 0.02$). The asterisk indicates a significant difference between the induced experimental group and all the induced genotype controls ($P \leq 0.0005$ for all comparisons). All values are mean $\pm$ SEM; $n \geq 8$ for all groups. (B) The NP1131 Gal4 driver was used to express PTX within the γ MB lobe neurons. The induced experimental group was significantly different from its uninduced within-genotype control (#; $t = 2.4$; $P = 0.028$) and from the induced genotype controls (labeled with asterisks; Bonferroni-Dunn; $P \leq 0.0001$ for all comparisons). All values are mean $\pm$ SEM; $n \geq 8$ for all groups. (C) Both c739 and NP1131 Gal4 drivers were used concurrently to drive PTX expression within the α/β and γ MB lobe neurons. The induced experimental group was significantly different from both its uninduced counterpart and from the induced genotype controls by the Bonferroni-Dunn test (labeled with asterisks; $P \leq 0.0001$ for all comparisons). All values are mean $\pm$ SEM; $n \geq 8$ for all groups.

the α/β or γ lobe neurons may account for the loss of short-term memory found after PTX expression.

Lastly, the activation of adenylyl cyclase is an important G-protein signaling pathway involved in associative memory formation (Gervasi et al. 2010; Zars 2010). In vertebrates, G(o) does not appear to signal through adenylyl cyclase (Jiang et al. 1998). In *Drosophila*, G(o) signaling within the mushroom body neurons during memory formation is, at least partially, if not wholly, independent of the *rut* adenylyl cyclase (Ferris et al. 2006). This conclusion was based on several considerations including the significantly stronger phenotype of PTX inhibition as compared to *rut* mutants (Ferris et al. 2006). Additionally, PTX expression within the α/β lobe neurons will inhibit short-term memory, whereas *rut* activity within these neurons is not necessary for negatively reinforced short-term memory (Zars et al. 2000; Ferris et al. 2006; Blum et al. 2009). Moreover, when PTX was lightly induced in *rut*²⁰⁸⁰ homozygotes or heterozygotes, PTX displayed additivity in the short-term memory phenotype (Ferris et al. 2006). When these PTX-*rut*²⁰⁸⁰ “double-mutant” experiments were performed, *rut*²⁰⁸⁰ was reported to be an amorph or, at least, a severe-hypomorph (Han et al. 1992; Mao et al. 2004); more recent data indicates that *rut* mRNA levels are at ~25% wild-type levels in the *rut*²⁰⁸⁰ allele (Pan et al. 2009). Hence, it remains possible that the additivity found between PTX expression and the *rut*²⁰⁸⁰ may be due to residual activity in the *rut*²⁰⁸⁰ allele. Nevertheless, the learning phenotype for *rut*²⁰⁸⁰ is as strong as the reported amorphic *rut*¹ allele, and it even displays haploinsufficiency, arguing that a high level of the enzyme is required to support memory formation (Mao et al. 2004; Ferris et al. 2006; Blum et al. 2009). This, together with the strength of the phenotypic differences and the differences in anatomical requirements for G(o) activation and *rut*, argues against a direct interaction between the cAMP pathway and G(o) activation during negatively reinforced memory formation. In positively reinforced memory, *rut* is required in the α'/β' neurons and the projection neurons of the antennal lobe (Thum et al. 2007). Thus, the role for G(o) activation in positively reinforced memory also maps outside the *rut* domains and, therefore, is also *rut*-independent.

In summary, the activation of G(o) is an essential signaling event for associative memory formation. The G(o) pathway is required for the formation of both appetitive and aversive memories within the α/β and γ lobe neurons. Further elucidation of this pathway within these neurons will likely provide fundamental information on the molecular events underlying memory formation.

Materials and Methods

Fly stocks and husbandry

Flies were raised on cornmeal, sucrose, and yeast agar at 25°C (unless otherwise specified) in 12:12-h light/dark cycles. All tests were conducted on young flies (2–5 d old) during the subjective day.

The *Gal80^{ts20}*, *Gal80^{ts2}*, UAS-PTX.16, UAS-rtTA901, *tet^o-PTX*, and UAS-*PiGo* transgenes were previously described (Stebbins et al. 2001; McGuire et al. 2003; Ferris et al. 2006; Ford et al. 2007; Chatterjee et al. 2009). To avoid position effects of the *tet^o-PTX* and UAS-*PiGo* experiments, two separate pairs of insertion lines were employed: *tet^o-PTX.36f* and UAS-*PiGo_{AIH}* located on the X chromosome and, respectively, *tet^o-PTX.20f* and UAS-*PiGo_{B10E}* on the second chromosome. The *Gal80^{ts}* lines were gifts from S. McGuire (Baylor College of Medicine, Houston), P247 driver was a gift from T. Zars (University of Missouri, Columbia), and c305a and c320 were a gift of Scott Waddell (University of Massachusetts Medical School, Worcester, MA).

Olfactory conditioning

Behavior testing was conducted as previously described (Tully and Quinn 1985; Schwaerzel et al. 2003; Ferris et al. 2006), under dim

red light, at 25°C and 70% humidity. Flies were allowed to acclimate for at least 30 min to the testing environment prior to training. The odor presentations are 1-min-long in the aversive paradigm (shock) and 2-min-long in the appetitive one (sugar), and they are preceded and followed by half-min-long exposures to fresh air. For memory kinetics determination only (Fig. 1), the “short program” was used: 10 sec CS⁺ odor with a 1-sec shock delivered at the 9th second with a 30-sec intertrial interval (Beck et al. 2000). The odorants were dispersed in 10 mL of light mineral oil (0.2% octanol [OCT], 0.1% methylcyclohexanol [MCH], respectively, 0.067% benzaldehyde [BZA]) and delivered through air bubbling at a constant rate of 500 mL/min. OCT/BZA pair was used as conditioned stimuli unless otherwise specified. The performance index (PI) for negatively reinforced learning was calculated as (number of flies choosing CS[−] – number of flies choosing CS⁺)/(number of flies choosing CS[−] + number of flies choosing CS⁺). The PI for positively reinforced learning was calculated as (CS⁺ flies – CS[−] flies)/(CS⁺ flies + CS[−] flies). Approximately 40 flies were used in each trial, and the measurement was typically repeated at least eight times.

For appetitive learning, the stimulus was dried sugar deposited on a filter paper cylinder lining the training chamber. The standard protocol involves a single immersion of the paper in 0.2M sucrose solution, after which it is dried and presented to the flies after a 24-h period of starvation (Krashes et al. 2007). Our optimized protocol used paper glazed with sugar by brief immersion in 2M sucrose and dried under vacuum at 50°C; the entire procedure was repeated three times. The standard starvation time (24 h in a vial containing a Kimwipe wetted with 2.5 mL water) is also extended by an additional prior incubation on 2% sucrose for 24 h under similar conditions. For the appetitive learning experiments in Figure 6 and Supplemental Figure S3, the protocol for positively reinforced associative learning required further adjustment to minimize doxycycline treatment-related mortality: 2 h of recovery on regular fly food after the 24-h treatment with doxycycline/vehicle in 2% sucrose and before the 24-h starvation period. With this adjusted protocol, the experimental variance was reduced (cf. Fig. 4C,D and Fig. 6) but at the expense of lower test sensitivity (smaller reductions in STM found after PTX induction). The higher PIs in the doxycycline-treated flies likely result from the unpalatable taste of the chemical for the flies and consequent additional starving from eating less during the 24 h of the induction treatment. Since the shift in the PIs of the induced flies is in the opposite direction to the PTX-induced phenotype, this does not affect the validity of the result, although it may lead to an underestimation of the phenotype magnitude.

Transgene induction

For the experiments using the *Gene Switch* system, flies were induced with 500 μ M RU486 in 2% sucrose feeding solution for 24 h, delivered 2.5 mL on a kimwipe in a vial with ~40 flies (Roman et al. 2001). After induction, the flies are transferred for 24 h to regular sugar/yeast/cornmeal food for the aversive conditioning. Uninduced flies are treated identically, but the RU486 is not added to the feeding solution. For appetitive conditioning, the induced flies are transferred to a vial with a wetted kimwipe and starved for 24 h prior to training. For the *tet^o* system, the flies were induced on 2% sucrose containing 1 mg/mL doxycycline for 24 h and allowed to recover as before. Occasionally, dead animals that failed to survive the starvation period were removed before testing. For the experiments involving *Gal80^{ts}* system (Fig. 2; Supplemental Fig. S1A), the crosses were raised at 18°C, and induction was accomplished by a 12-h exposure to 32°C, followed by 3 h of recovery at 25°C. Submaximal PTX induction required in the memory kinetics experiments (Fig. 1) was achieved with a 3-h, 32°C induction.

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G(o) activation is required for both appetitive and aversive memory acquisition in *Drosophila*

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