Associative Learning Disrupted by Impaired G_s Signaling in *Drosophila* Mushroom Bodies

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Disruptions in mushroom body (MB) or central complex (CC) brain structures impair *Drosophila* associative olfactory learning. Perturbations in adenosine 3′,5′ monophosphate signaling also disrupt learning. To integrate these observations, expression of a constitutively activated stimulatory heterotrimeric guanosine triphosphate–binding protein α subunit ($G\alpha_s$*) was targeted to these brain structures. The ability to associate odors with electroshock was abolished when $G\alpha_s$* was targeted to MB, but not CC, structures, whereas sensorimotor responses to these stimuli remained normal. Expression of $G\alpha_s$* did not affect gross MB morphology, and wild-type $G\alpha_s$ expression did not affect learning. Thus, olfactory learning depends on regulated G_s signaling in *Drosophila* MBs.

Associative learning can be analyzed on biochemical, neuroanatomical, and behavioral levels. Perturbations in adenosine 3′,5′ monophosphate (cAMP) signaling affect learning in *Aplysia*, *Drosophila*, and mice (*1*–*3*). Gene disruptions of *dunce* (cAMP phosphodiesterase II), *rutabaga* [type I adenylyl cyclase (AC)], and the DC0 catalytic and RI regulatory subunits of cAMP-dependent protein kinase (PKA) impair olfactory learning in flies (*2*, *3*). Neuroanatomically, Dunce, Rutabaga, and DC0 proteins are expressed throughout the brain but are expressed at elevated levels in the MBs (*3*, *4*). Chemical ablation of the MBs, as well as mutations disrupting either MB or CC structures, produce defective olfactory learning (*5*, *6*). No functional data, however, indicate that cAMP signaling within the MBs or CC mediates this type of learning.

To explore this notion, we restricted disruption of the cAMP pathway to the MBs or CC and examined the effects on olfactory learning. We used the P-GAL4 enhancer trap system to target expression of $G\alpha_s$ transgenes within the brain (*7*–*9*). Upon receptor activation, $G\alpha_s$ binds guanosine triphosphate (GTP) and becomes activated, effecting AC stimulation (*10*). The GTPase activity of $G\alpha_s$ hydrolyzes GTP to guanosine diphosphate (GDP), deactivating $G\alpha_s$. The $Gln^{215} \rightarrow Leu^{215}$ (Q215L) mutation in *Drosophila* $G\alpha_s$ impairs this GTPase activity and results in a form of $G\alpha_s$ ($G\alpha_s$*), which constitutively activates AC (*9*, *10*). Transgenic flies capable of expressing either wild-type $G\alpha_s$ ($G\alpha_s$WT) or $G\alpha_s$* under the control of the GAL4-responsive upstream activating sequence (UAS) were generated (*11*). Expression of these transgenes was driven by selected P-GAL4 insertions that demonstrated prominent expression in the MBs or CC (*12*).

To examine associative learning in these flies, a Pavlovian olfactory conditioning assay was used (*13*). In that procedure, flies are trained by exposure to electroshock paired with one odor [octanol (OCT) or methylcyclohexanol (MCH)] and subsequent exposure to a second odor without electroshock. Immediately after training, learning is measured by forcing flies to choose between the two odors used during training. No preference between odors results in a performance index (PI) of zero (no learning), as is the case for naive flies.

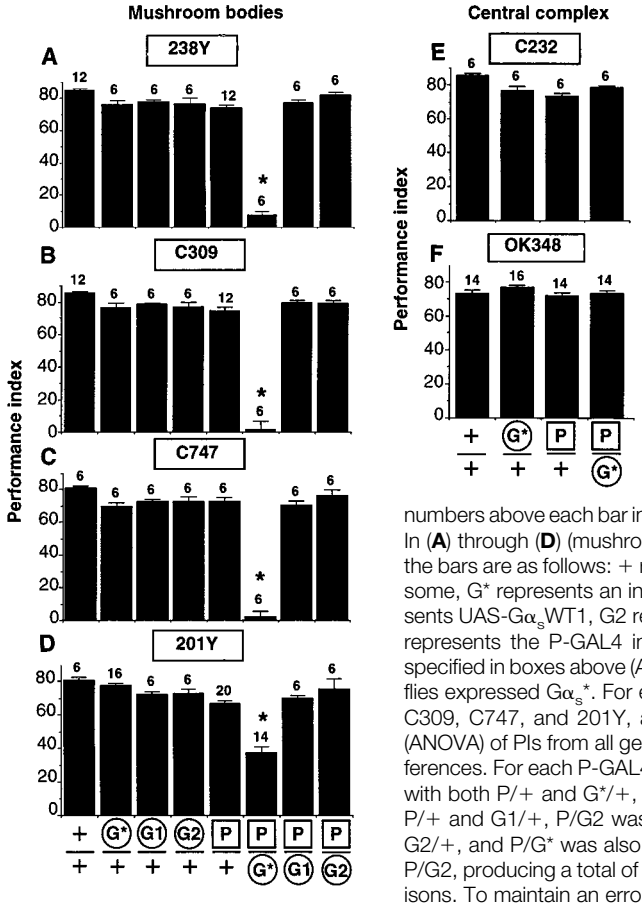

Fig. 1. Associative learning is disrupted by expression of activated but not wild-type $G\alpha_s$. All behavioral experiments were performed blind with respect to genotype. Flies of the Canton-S strain served as a wild-type control for the calibration of teaching machines. Potential genetic background effects dictated that the appropriate controls were those for which flies with either the P-GAL4 or UAS-$G\alpha_s$ insertions were heterozygous. After assays of associative learning, PIs were calculated as described in (*25*). Bars represent mean PIs ± SEMs; numbers above each bar indicate number of PIs per group. In (**A**) through (**D**) (mushroom bodies), the symbols below the bars are as follows: + represents a Canton-S chromosome, G* represents an insertion of UAS-$G\alpha_s$*, G1 represents UAS-$G\alpha_s$WT1, G2 represents UAS-$G\alpha_s$WT2, and P represents the P-GAL4 insertion [insertion numbers are specified in boxes above (A) through (D)]. Hence, only P/G* flies expressed $G\alpha_s$*. For each of the P-GAL4 lines 238Y, C309, C747, and 201Y, a one-way analysis of variance (ANOVA) of PIs from all genotypes revealed significant differences. For each P-GAL4 insertion, P/G* was compared with both P/+ and G*/+, P/G1 was compared with both P/+ and G1/+, P/G2 was compared with both P/+ and G2/+, and P/G* was also compared with both P/G1 and P/G2, producing a total of eight planned pairwise comparisons. To maintain an error rate of $\alpha = 0.05$ in the experiment, the critical *P* value was adjusted to $\alpha = 0.006$ (*26*). In each case, an asterisk above any group indicates significant differences from all other groups (except in 238Y, where P/G2 was significantly different from P/+ but not from G2/+). In (**E**) and (**F**) (central complex), the symbols below the bars are as defined in (A) through (D). For each of the P-GAL4 lines C232 and OK348, PIs from all groups were subjected to a one-way ANOVA, and P/G* was compared with P/+ and G*/+, producing two planned pairwise comparisons. To maintain an error rate of $\alpha = 0.05$ in the experiment, the critical *P* value was adjusted to $\alpha = 0.025$ (*26*). In both cases, no significant differences were detected.

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Avoidance of the odor previously paired with electroshock, however, yields a PI > 0 (with a score of 100 indicating maximal learning).

When $G\alpha_s$* was expressed in MBs by each of three P-GAL4 insertions (238Y, C309, and C747), learning was completely abolished (Fig. 1, A through C). When a fourth MB-expressing P-GAL4 insertion

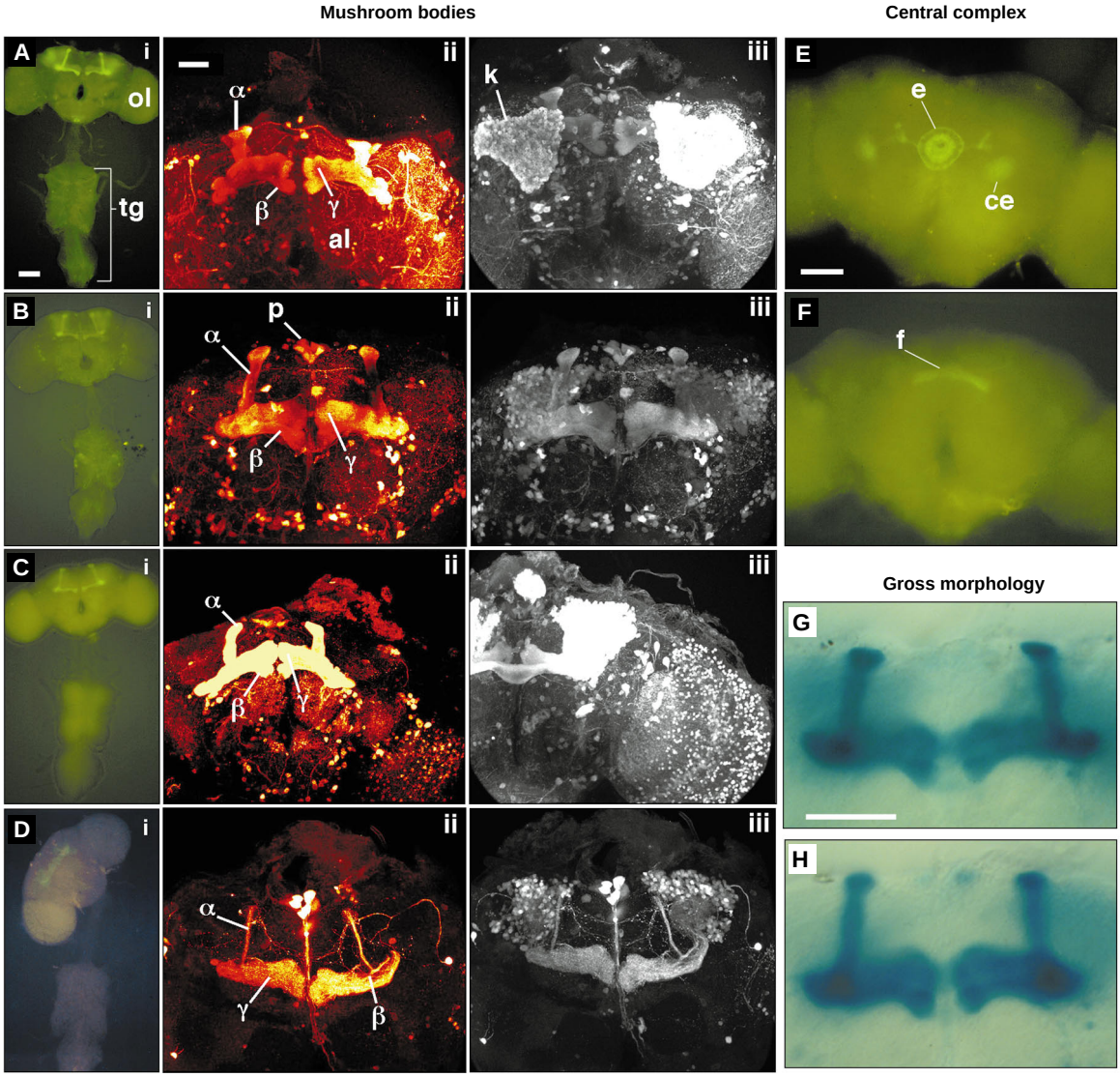

Fig. 2. P-GAL4 expression patterns in adult brains. In all cases, brains were examined as whole mounts. In panel i in (**A**) through (**D**) and in (**E**) and (**F**), brains from P-GAL4 lines crossed to UAS-GFP^{B1} (GFP^{81}, green fluorescent protein, insertion B1) were examined under fluorescence microscopy (*26*). In panels ii and iii in (A) through (D), GAL4-dependent β-Gal expression patterns were visualized immunohistochemically (*17*). In (**G**) and (**H**), brains were stained with X-Gal (5-bromo-4-chloro-3-indol-β-D-galactosidase) (*20*). In panels showing mushroom bodies, (A) shows line 238Y, (B) shows line C309, (C) shows line C747, and (D) shows line 201Y. In panel i of (A), ol indicates optic lobes and tg indicates the thoracic ganglion. The predominant site of expression in lines 238Y, C309, C747, and 201Y was in the MBs. All lines labeled small neuronal subsets in the thoracic ganglion. Panel ii of (A) through (D) shows a three-dimensional confocal reconstruction from a frontal aspect. For clarity, the Kenyon cell body layer of each pattern has been excluded. In lines 238Y, C309, and C747, the α, β, and γ lobes (labeled) of the MBs stained strongly, except that C309 showed less in the core regions of the α and β lobes (*17*). In line 201Y, the MB γ lobe was extensively stained, but only narrow core elements of the α and β lobes showed staining. All lines in (A) through (D) also stained in the pars intercerebralis (p). In line 238Y, a small number of extrinsic neurons arborizing in the γ lobe and neurons spanning the optic lobes, which sent large horizontal tracts to the contralateral lobe and branches to the lateral protocerebrum, were revealed. CC neurons innervating narrow layers of the fan-shaped body and noduli, and a small number of antennal lobe (al) afferents, were weakly stained. In line C309, several classes of optic lobe neurons, a small set of antennal lobe local interneurons, and CC neurons (a subset of the ellipsoid body and fan-shaped body) stained weakly. In line C747, antennal lobe local interneurons were labeled. CC neurons with arbors in the protocerebral bridge, superior arch of the fan-shaped body, ellipsoid body, and noduli stained, which did not appear to overlap with those of 238Y and C309. Large numbers of optic lobe neurons stained, with dense regions of arborization in the medulla. In line 201Y, two lateral neurons resembling MB feedback interneurons were labeled. Panel iii of (A) through (D) shows the MB Kenyon cell body (k) layer. Line 238Y expressed β-Gal in Kenyon cell bodies. Line C747 (*12, 17*) stained more strongly a subset of Kenyon cells similar to those of C309. Line 201Y showed weak expression in a diffuse subset of cell bodies. In panels showing the central complex, (E) shows line C232 (*18*). GFP was detected in cell bodies (ce) and the neuropil (e) of the ellipsoid body. (F) shows line OK348. GFP was detected in cell bodies (not shown) and the neuropil (f) of the fan-shaped body. In panels showing gross morphology (*20*), brains from lines C309/UAS-lacZ (G) and C309/UAS-lacZ/UAS-$G\alpha_s$* (H) are shown. Scale bar in panel i of (A), 50 μm (applies to all i panels); in panel ii of (A), 20 μm (applies to all ii and iii panels); in (E), 50 μm [also applies to (F)]; and in (G), 50 μm [also applies to (H)].

(201Y) was used, expression of $G\alpha_s$* reduced learning by ~50% (Fig. 1D). In contrast, expression of wild-type $G\alpha_s$ in each of these four P-GAL4 lines had no effect on learning (Fig. 1, A through D). Thus, learning deficits resulted from the Q215L mutation constitutively activating $G\alpha_s$* and not simply from misregulation of endogenous signaling by $G\alpha_s$ overexpression (9).

In these four learning-impaired lines, olfactory responses to OCT and MCH were normal. Likewise, responses to electroshock were normal (Table 1). This demonstrates that expression of $G\alpha_s$* did not affect naive sensorimotor responses to electroshock or odors. Thus, all MB P-GAL4 lines expressing $G\alpha_s$*, which showed normal sensorimotor responses, showed learning defects (*14*). When $G\alpha_s$* was expressed in the ellipsoid body or fan-shaped body of the CC (with the use of P-GAL4 C232 or OK348, respectively) (Fig. 2, E and F), learning was unaffected (Fig. 1, E and F). This suggests that such perturbation of G_s signaling in the CC is insufficient to disrupt olfactory learning (*15*).

The expression patterns of MB P-GAL4 lines were examined with confocal microscopy after immunolocalization of expression of the GAL4-driven β-galactosidase (β-Gal) reporter gene (*16*–*18*) (Fig. 2, A through D). In lines 238Y, C309, and C747, where learning was abolished with $G\alpha_s$* expression, extensive expression in all MB lobes was evident. In line 201Y, which yielded a partial learning defect with $G\alpha_s$* expression, the γ lobe stained extensively, whereas only narrow core elements of the α and β lobes were labeled (*17*, *18*). All MB-expressing lines showed GAL4 activity elsewhere in the brain. Optic lobe structural mutants learn normally, which suggests that $G\alpha_s$* expression in the optic lobes did not affect learning (*5*). Within the central brain, the MBs and pars intercerebralis were the only common regions of expression in all learning-impaired lines. Although we cannot exclude a role for the pars intercerebralis in olfactory learning, this structure has not been implicated previously in the process. By contrast, chemical ablation of the MBs is sufficient to abolish olfactory learning (*6*, *15*).

Pan-neural expression of $G\alpha_s$* during development produced neither lethality nor overt behavioral phenotypes, which suggests that perturbation of G_s signaling did not significantly affect basic neuronal function (*19*). Furthermore, gross morphology appeared normal when $G\alpha_s$* and β-Gal were coexpressed in the MBs (*20*) (Fig. 2, G and H). These data suggest that the abolition of learning did not result from maldevelopment of underlying structures. Admittedly, more subtle, undetected changes in MB structure might contribute to learning defects, but chemical ablation experiments show that >96% of MBs must be absent to produce PIs below 20 (*6*). In contrast, PIs of zero were obtained here with no detectable effect on MB morphology.

We have presented in vivo evidence that associative olfactory learning in *Drosophila* requires regulated G_s signaling within MB neurons. MB expression of $G\alpha_s$* can abolish associative learning, whereas null alleles of *dunce* and *rutabaga* exhibit only partial impairments. The *dunce* and *rutabaga* mutations affect only one class of phosphodiesterase or AC, respectively. In flies, three ACs, in addition to Rutabaga, have been identified (*21*). In mammals, $G\alpha_s$ stimulates all ACs to some degree (*22*). Thus, disruption of all AC regulation by $G\alpha_s$* expression could have more drastic effects on signaling than removal of one form of AC (as in *rutabaga*) or cyclic nucleotide phosphodiesterase (as in *dunce*). Alternatively, activation of PKA-dependent phosphorylation through $G\alpha_s$* expression could impede modulatory changes in shared substrates or cellular systems by kinases other than PKA. Another possibility is that $G\alpha_s$ could exert signaling effects other than through the cAMP pathway, such as through direct modulation of channels (*23*). In such a scenario, $G\alpha_s$* expression might also produce learning deficits greater than those observed in *dunce* or *rutabaga* mutants. Further analyses of G_s signaling in *Drosophila* should clarify these issues.

Table 1. Olfactory acuity and shock reactivity are normal in P-GAL4 lines expressing $G\alpha_s$* in the MBs. Symbols in the left-hand column are as defined in the legend of Fig. 1. In each case, $n = 8$, except $n = 6$ for G*/+ in the 238Y MCH 10^{-2} experiment. Olfactory acuity tests with odors at the concentrations used during training and testing (10^0 dilution) and at 10^{-2} dilutions were performed, and PIs were calculated as described in (*25*). For each P-GAL4 line, PIs from four genotypes (+/+, G*/+, P/+, and P/G*) and four odor levels (OCT 10^0, OCT 10^{-2}, MCH 10^0, and MCH 10^{-2}) were subjected to a two-way ANOVA, with genotype and odor level as main effects and genotype × odor level as the interaction term. For each P-GAL4 insertion, P/G* was compared with P/+ and G*/+ heterozygous controls at each of the four odor levels, producing a total of eight pairwise planned comparisons. To maintain an error rate of $\alpha = 0.05$ in the experiment, the critical *P* value was adjusted to $\alpha = 0.006$ (*26*). In no case were significant differences detected. Shock reactivity tests to the training voltage (60 V) and to 20 V were performed as described in (*28*). For each P-GAL4 line, PIs from four genotypes (+/+, G*/+, P/+, and P/G*) and two shock groups (60 and 20 V) were subjected to a two-way ANOVA, with genotype and shock group as main effects and genotype × shock group as the interaction term. For each P-GAL4 insertion, P/G* was compared with P/+ and G*/+ heterozygous controls for both shock groups, producing a total of four pairwise planned comparisons. To maintain an error rate of $\alpha = 0.05$ in the experiment, the critical *P* value was adjusted to $\alpha = 0.013$ (*26*). In no case were significant differences detected.

Lines and genotypes	Olfactory acuity				Shock reactivity	
	OCT dilution		MCH dilution			
	10^0	10^{-2}	10^0	10^{-2}	60 V	20 V
201 Y						
+/+	66 ± 5	44 ± 8	62 ± 5	35 ± 5	93 ± 2	59 ± 6
G*/+	69 ± 4	40 ± 8	70 ± 4	40 ± 7	87 ± 2	48 ± 8
P/+	70 ± 5	37 ± 5	67 ± 3	30 ± 5	89 ± 1	49 ± 9
P/G*	61 ± 6	44 ± 7	70 ± 6	32 ± 5	86 ± 2	46 ± 6
238Y						
+/+	53 ± 4	28 ± 3	63 ± 3	22 ± 2	84 ± 3	25 ± 6
G*/+	58 ± 5	28 ± 3	68 ± 4	28 ± 2	75 ± 2	24 ± 4
P/+	63 ± 4	29 ± 4	71 ± 5	24 ± 3	77 ± 5	31 ± 5
P/G*	64 ± 5	17 ± 8	67 ± 4	21 ± 4	73 ± 3	25 ± 8
C309						
+/+	64 ± 4	32 ± 7	58 ± 4	26 ± 4	84 ± 3	32 ± 7
G*/+	61 ± 3	29 ± 4	63 ± 3	21 ± 3	75 ± 2	26 ± 3
P/+	65 ± 4	32 ± 5	67 ± 4	25 ± 4	72 ± 4	31 ± 5
P/G*	62 ± 3	32 ± 8	66 ± 5	27 ± 5	66 ± 6	25 ± 6
C747						
+/+	53 ± 4	28 ± 3	63 ± 3	22 ± 2	84 ± 3	25 ± 6
G*/+	60 ± 5	27 ± 3	70 ± 3	26 ± 5	73 ± 3	22 ± 4
P/+	59 ± 4	28 ± 5	71 ± 4	30 ± 4	77 ± 3	24 ± 4
P/G*	54 ± 2	35 ± 3	76 ± 3	28 ± 2	68 ± 3	21 ± 4

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11. Complementary DNAs encoding the short forms of $G\alpha_s$WT and $G\alpha_s$* (*9*) were subcloned into pUAST (*7*) and used to transform flies [A. Spradling, in *Drosophila: A Practical Approach*, D. Roberts, Ed. (IRL Press, Oxford, 1986), pp. 75–197]. The UAS-$G\alpha_s$ lines were verified by Msc I digestion of polymerase chain reaction–amplified $G\alpha_s$ transgenes to detect the presence of a site found in $G\alpha_s$WT but lacking as a result of the Q215L mutation.
12. 238Y and 201Y are described in (*17*) and C232 in (*18*). C747 shows identical expression to that of C772 (*17*) (*24*). C309 is described by J. D. Armstrong [thesis, University of Glasgow, Scotland (1995)]. The O'Kane laboratory screened 500 P-GAL4 lines to identify OK66, OK86, OK62, OK107, OK348, and OK415.
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14. In total, we analyzed behaviorally 16 P-GAL4 insertions that expressed to some degree in MBs. Flies with four insertions (OK66, OK86, KL65, and KL107) showed reduced learning in the absence of expression of constitutively activated $G\alpha_s$*, most likely because of genetic background differences. These were not tested further. Flies with eight insertions showed defects in olfactory acuity (30Y, C35, OK62, OK107, C302, OK415, and C532) or shock reactivity (C772), as transheterozygotes with UAS-$G\alpha_s$*. These were eliminated from further behavioral testing. Flies with the remaining four P-GAL4 insertions (201Y, 238Y, C309, and C747) showed disrupted learning but normal olfactory acuity and shock reactivity, as transheterozygotes with at least one UAS-$G\alpha_s$* insertion. We characterized a total of two P-GAL4 insertions (C232 and OK348) that expressed to some degree in the CC.
15. We suggest that olfactory learning results from G_s-dependent convergence of the conditional stimulus (CS) and unconditional stimulus (US) in the MBs. This belief is reinforced by the observation that expression of $G\alpha_s$* in the CC does not affect learning. Formally, however, we cannot exclude the possibility that other sites of convergence of the CS and US contribute to the conditioned behavior, as in honeybees [M. Hammer and R. Menzel, *J. Neurosci.* **15**, 1617 (1995)].
16. Direct immunolocalization of transgenic $G\alpha_s$ forms proved impossible, as endogenous $G\alpha_s$ is expressed throughout the central nervous system (*8*). We have not observed any significant differences in qualitative expression between UAS-regulated insertions [see also (*18*) and D. Lin and C. Goodman, *Neuron* **13**, 507 (1994)]. Thus, P-GAL4–driven reporter gene expression should reflect genuine expression patterns of $G\alpha_s$* under UAS regulation.
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A Mechanism of Drug Action Revealed by Structural Studies of Enoyl Reductase

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Enoyl reductase (ENR), an enzyme involved in fatty acid biosynthesis, is the target for antibacterial diazaborines and the front-line antituberculosis drug isoniazid. Analysis of the structures of complexes of *Escherichia coli* ENR with nicotinamide adenine dinucleotide and either thienodiazaborine or benzodiazaborine revealed the formation of a covalent bond between the 2′ hydroxyl of the nicotinamide ribose and a boron atom in the drugs to generate a tight, noncovalently bound bisubstrate analog. This analysis has implications for the structure-based design of inhibitors of ENR, and similarities to other oxidoreductases suggest that mimicking this molecular linkage may have generic applications in other areas of medicinal chemistry.

ENR catalyzes the final reaction of the fatty acid synthase cycle: the reduction of a carbon-carbon double bond in an enoyl moiety that is covalently linked to an acyl carrier protein. Recent studies have identified ENR as the target for a number of therapeutic agents against *Mycobacterium tuberculosis* (*1*) and *Escherichia coli* (*2*). *Mycobacterium tuberculosis* ENR is the target for a metabolite of isoniazid, a potent drug that is used in the front-line chemotherapeutic treatment of tuberculosis. However, strains of *M. tuberculosis* are emerging that are resistant to isoniazid (*3*), with consequent problems in treatment. *Escherichia coli* ENR is inhibited by a range of diazaborines, heterocyclic boron-containing compounds whose action is thought to lead to the inhibition of cell growth by preventing lipopolysaccharide synthesis (*4*). Biochemical studies on *E. coli* ENR have shown that nicotinamide adenine dinucleotide (NAD^+) is required for diazaborine binding; this finding has led to the suggestion that the drug binds to ENR in association with NAD^+ or that NAD^+ converts the drug to an active form (*5*). To obtain a molecular explanation for the inhibitory activities of this class of antibacterial agents, we determined and analyzed the structure of *E. coli* ENR in complexes with NAD^+ and either thienodiazaborine or benzodiazaborine as well as with NAD^+ alone.

The structure of the ENR-NAD^+ complex was solved to 2.1 Å (*6*); those of the ENR-NAD^+-thienodiazaborine and ENR-NAD^+-benzodiazaborine complexes were solved to 2.2 and 2.5 Å, respectively (*7*) (Table 1 and Fig. 1A). In the final map of the ENR-NAD^+ complex, the electron density is of high quality for most of the protein atoms. However, there is a break in the density for a stretch of 10 amino acid residues; these 10 residues form a loop, between

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