J. Bacteriol. **170**, 3350 (1988)]. To distinguish residues in the adhesin from residues in the chaperone, FimH residues will be denoted by an H, and FimC residues by a C, after the residue number.
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Requirement of Circadian Genes for Cocaine Sensitization in *Drosophila*

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The circadian clock consists of a feedback loop in which clock genes are rhythmically expressed, giving rise to cycling levels of RNA and proteins. Four of the five circadian genes identified to date influence responsiveness to free-base cocaine in the fruit fly, *Drosophila melanogaster*. Sensitization to repeated cocaine exposures, a phenomenon also seen in humans and animal models and associated with enhanced drug craving, is eliminated in flies mutant for *period*, *clock*, *cycle*, and *doubletime*, but not in flies lacking the gene *timeless*. Flies that do not sensitize owing to lack of these genes do not show the induction of tyrosine decarboxylase normally seen after cocaine exposure. These findings indicate unexpected roles for these genes in regulating cocaine sensitization and indicate that they function as regulators of tyrosine decarboxylase.

In response to exposure to volatilized free-base cocaine, *Drosophila* perform a set of reflexive behaviors similar to those observed in vertebrate animals, including grooming, proboscis extension, and unusual circling locomotor behaviors (*1*–*3*). Additionally, flies can show sensitization after even a single exposure to cocaine provided that the doses are separated by an interval of 6 to 24 hours (*1*). Sensitization, a process in which repeated exposure to low doses of a drug leads to increased severity of responses, has been linked to the addictive process in humans (*4*–*6*) and is potentially involved in the enhanced craving and psychoses that occur after repeated psychostimulant administration.

We have shown circadian variation in the agonist responsiveness of *Drosophila* nerve cord dopamine receptors functionally coupled to locomotor output (*7*). This variation is dependent on the normal functioning of the *Drosophila period* (*per*) gene, the founding member of the circadian gene family (*8*, *9*). Because changes in postsynaptic dopamine receptor responsiveness are also seen during cocaine sensitization in vertebrates (*10*–*12*), we examined flies mutant in circadian functions for alterations in responsiveness to cocaine.

Wild-type (WT) flies or flies containing a *per* null mutation, per^o, were exposed to 75 μg of cocaine four times over 2 days, and the fraction of flies showing severe responses was quantified after each exposure (Fig. 1A). Whereas WT flies showed sensitization after the initial cocaine exposure, per^o flies showed no sensitization either to a normal or increased dose even after repeated exposures. As with WT flies, per^o flies showed a dose-dependent increase in the severity of responses, and the normal cocaine-induced types of behaviors were observed (*13*).

per alleles that either shorten or lengthen the circadian periods show distinct patterns of cocaine responsiveness. The short-period mutants per^S and per^T (*14*, *15*) both showed increased responsiveness to the initial cocaine exposure and weak sensitization to a second 75-μg exposure (Fig. 2A), with only the sensitization of per^S showing statistical significance. Sensitization is not observed in these lines when tested with other cocaine doses (*16*). The long-period mutant per^{L1} (*17*) showed a normal initial cocaine response but no sensitization to a subsequent exposure.

Similarly, other circadian genes showed effects on cocaine sensitization: Both *clock* and *cycle* mutants failed to sensitize when given two doses of cocaine (Fig. 2B). Because these mutants showed an increased sensitivity to the

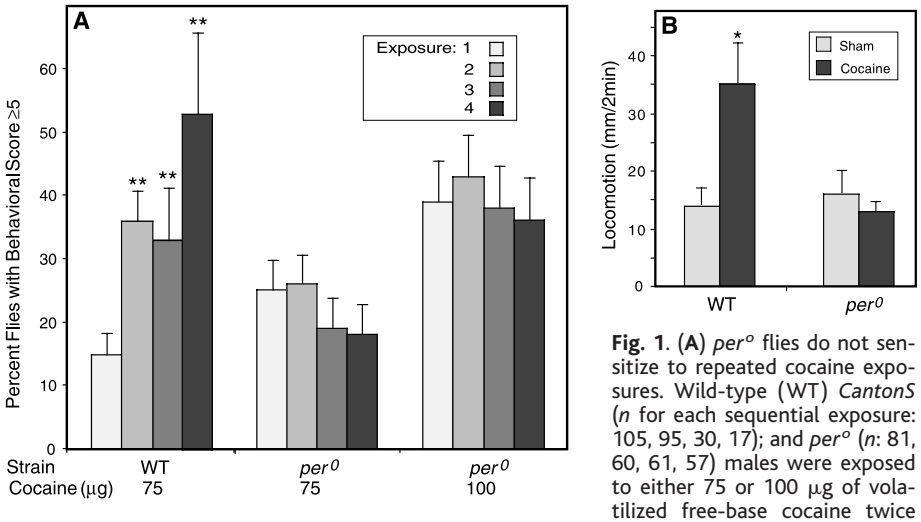

Fig. 1. (**A**) per^o flies do not sensitize to repeated cocaine exposures. Wild-type (WT) *CantonS* (*n* for each sequential exposure: 105, 95, 30, 17); and per^o (*n*: 81, 60, 61, 57) males were exposed to either 75 or 100 μg of volatilized free-base cocaine twice per day at 6-hour intervals for 2 days, and the behavioral responses were scored during the 5 min after exposure with a behavioral scale (*1*). Behavioral scores range from 0 (normal behavior) to 7 (death). Behavioral scores of ≥5 indicate rapid twirling, erratic jumping, or paralysis. Significant differences in responses to the first versus subsequent exposures (χ^2 test): $^{**}P \leq 0.01$. Error bars are standard deviations calculated for binomial distributions. All behavioral analyses were performed blindly; strains were given drugs in random order by placing a numbered tag in the video field during videotaping. The evaluator assayed behaviors blindly, and was unblinded only after all flies had been scored. (**B**) per^o flies do not modulate quinpirole responsiveness after cocaine exposures. WT $Oregon^R$ and per^o were exposed to 75 μg of volatilized cocaine three times over 2 days and decapitated 4 hours after the last exposure. Flies were decapitated and assayed for locomotion with 2 mM quinpirole as described (*24*), with modifications (*7*, *31*). Average locomotion ± SEM is shown (*n* = 30 to 50 flies). Significant differences between sham- and cocaine-treated flies are indicated ($^*P \leq 0.05$, Student's *t* test).

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first exposure (*16*), cocaine doses were decreased to 50 μg. The inability of *clock* and *cycle* to sensitize is markedly similar to the behavior of *per^o* mutants. The gene product of *timeless* (*tim*), TIM, is required for nuclear translocation of PER and its stability in the cytoplasm; in *tim^o* mutants, cytoplasmic PER is degraded and *per* mRNA levels are constant (*18*–*20*). Cocaine responses in *tim^o* mutant flies were normal (Fig. 2B), both in initial responsiveness and in showing a robust sensitized response to the second exposure.

Recently, a *doubletime* (*dbt*) protein with homology to human casein kinase Iε was identified and shown to be required for phosphorylation of PER (*21*). We tested cocaine responses in two viable *dbt* mutants, *dbt^S* and *dbt^L*, which shorten and lengthen the circadian locomotor period, respectively (*22*). *dbt* mutants required a substantially higher cocaine dose to show behaviors normally observed at 75 μg (Fig. 2B), but even at these higher doses *dbt* flies did not show significant sensitization. If the role of *dbt* in cocaine responsiveness is analogous to its role in circadian behavior, then PER phosphorylation status may be important in regulating both initial cocaine responsiveness and sensitization.

Modulation of dopamine receptor responsiveness is important in both the sensitization to cocaine in vertebrate animals and in the circadian modulation of locomotion in *Drosophila* (*7*, *23*). We tested whether cocaine-sensitized flies would show an increase in the responsiveness of the nerve cord dopamine D2-like receptors by using a preparation of behaviorally active decapitated flies that allows direct addition of drugs to the nerve cord (*24*). After decapitation, cocaine-sensitized WT flies locomoted significantly more than sham-treated controls in response to the dopamine D2-like agonist quinpirole (Fig. 1B). However, there was no increase in quinpirole responsiveness of *per^o* flies that did not sensitize to repeated cocaine exposures. Thus, similar to the inability of *per^o* mutant to modulate receptor responsiveness as a function of the time of day (*7*), *per^o* is unable to modulate dopamine receptor responsiveness after cocaine exposure. The observation that cocaine sensitization is associated with increased responsiveness of postsynaptic dopamine receptors shows additional similarities between this system and that in higher vertebrates, where a similar relation holds (*12*, *23*).

In *Drosophila*, sensitization requires the trace amine tyramine because the mutant *inactive*, which is defective in sensitization, shows both reduced tyramine and reduced levels of the enzyme involved in tyramine synthesis, tyrosine decarboxylase (TDC) (*25*). An active role for tyramine in sensitization is indicated because TDC enzyme activity is induced after a single cocaine exposure, with a time course consistent with that for the development of sensitization (*25*). To test if the correlation between induction of TDC activity and behavioral sensitization holds for the circadian mutants, we measured TDC activity in the circadian mutant flies after a single exposure of cocaine (Fig. 3). In contrast to WT flies, in which TDC activity was induced after cocaine exposure, the *per^o*, *cycle*, and *clock* lines that are defective in sensitization showed no such induction; only *tim^o*, which showed normal sensitization, induced TDC activity. It thus seems likely that the transcriptional regulator PER, presumably in conjunction with CLOCK and CYCLE, is a direct or indirect regulator of TDC after exposure to cocaine.

The sensitization defects in *inactive* and *per* mutant flies can be distinguished by differences in the ability of tyramine feeding to restore sensitization. The locomotor and cocaine sensitization defects in *inactive* mutant flies can be rescued by feeding tyramine to adults (*25*), but the sensitization defect in *per^o* flies is distinct, because feeding tyramine to *per^o* adults did not rescue sensitization (Fig. 4). We presume that tyramine from the food can enter tyramine nerve terminals in *inactive* flies, where it is still subject to a cocaine-stimulated release mechanism that mediates sensitization. The failure of tyramine feeding to rescue sensitization in *per^o* flies is most readily understood if the *per* gene

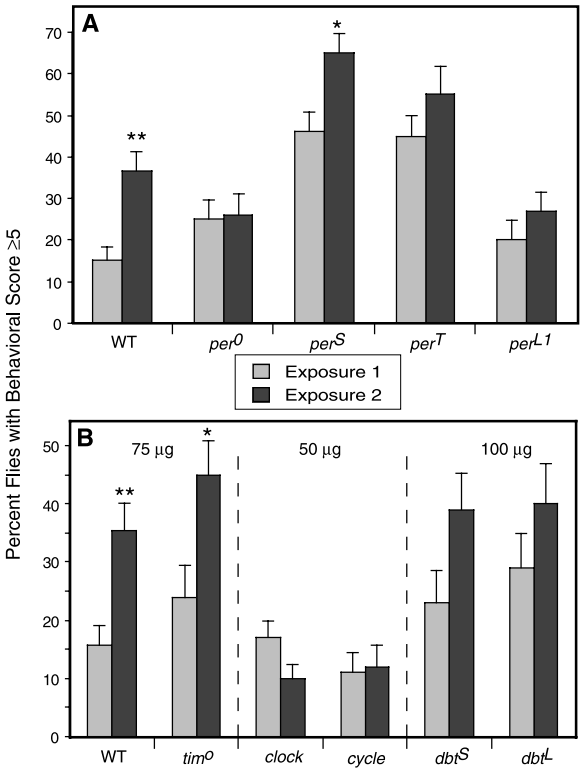

Fig. 2. Circadian mutants show altered cocaine responses. (**A**) *per* mutants. Flies carrying *per* mutations, as indicated, were exposed twice to 75 μg of volatilized cocaine 6 hours apart. The number of flies assayed, for first and second exposures, is as follows: WT *CantonS*, n = 105, 95; *per^o*, n = 81, 60; *per^S*, n = 114, 112; *per^T*, n = 88, 52; *per^{L1}*, n = 86, 83. (**B**) Other circadian mutants. As in (A), except that cocaine doses were adjusted to compensate for differences in cocaine responsiveness to the initial dose: WT *CantonS* exposed to 75 μg of cocaine, n = 105, 95; *tim^o*, n = 66, 63. Circadian mutants exposed to 50 μg of cocaine: *clock*, n = 187, 182; and *cycle*, n = 79, 79. *dbt* mutants were exposed to 100 μg of cocaine: *dbt^S*, n = 59, 55; *dbt^L*, n = 52, 51. In both (A) and B), significant differences in responses to the first versus second exposures are indicated ($^*P \leq 0.05$, $^{**}P \leq 0.01$; χ^2 test).

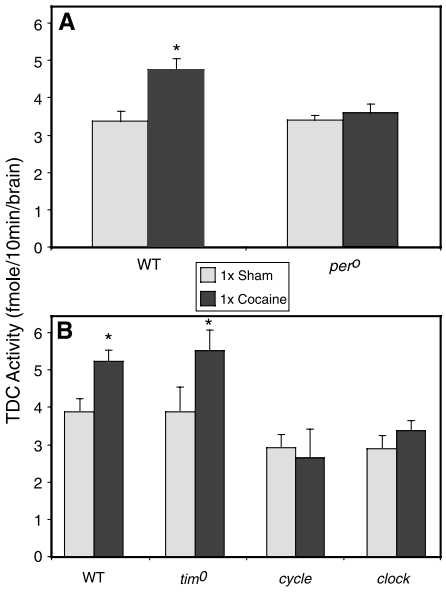

Fig. 3. (**A**) *per^o* flies and (**B**) *cycle* and *clock* flies do not induce TDC activity after cocaine exposure. Assays within each panel were carried out with the same batch of TDC assay mix because different batches can show small variations in activity. TDC assays on dissected adult brains were performed as described (*25*, *32*). Wild-type *CantonS*, *per^o*, and *tim^o* flies were exposed to 75 μg of volatilized cocaine or a sham treatment, and brains were dissected 6 hours later. *clock* and *cycle* flies were exposed to 50 μg of cocaine to compensate for the increased cocaine responsiveness of these lines. The hypomorphic *dbt^L* mutant shows marginal induction of TDC that does not reach statistical significance, consistent with the weak effects of this allele on cocaine sensitization (*16*). The number of independent samples assayed for each strain is as follows: (A) WT: n = 9; *per^o*: n = 9. (B) WT: n = 3; *tim^o*: n = 5; *cycle*: n = 3; *clock*: n = 3. Pairwise comparisons were performed between sham and cocaine values by Student's t test ($^*P \leq 0.05$).

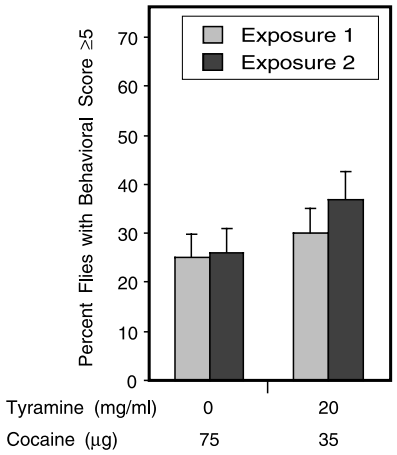

Fig. 4. Tyramine feeding increases initial cocaine responsiveness of *per^o* flies but does not restore sensitization. *per^o* flies were fed on instant food (Carolina Biologicals, Burlington, NC) with or without tyramine (20 mg/ml) for 2 days (0 mg/ml tyramine, $n = 81, 73$; 20 mg/ml tyramine, $n = 73, 57$). Flies were exposed to the indicated amounts of volatilized cocaine and assayed as in Fig. 3.

product is required for this regulated release.

Similar to *inactive* (*25*), tyramine increases initial cocaine responsiveness in *per^o* flies. Exposure of tyramine-fed *per^o* flies to 35 μg of cocaine induced behaviors normally seen in control flies exposed to 75 μg (Fig. 4). Thus, although long-term increase of tyramine levels can affect initial cocaine responsiveness, it is not sufficient for sensitization in flies lacking normal *per* function.

A unifying feature of most genes that regulate circadian rhythmicity in *Drosophila* and vertebrates is the PAS dimerization domain, common to a subset of basic helix-loop-helix transcription factors (*26*, *27*). Within the circadian cycle, CLOCK/CYCLE heterodimers activate *per* transcription, whereas PER/TIM heterodimers inhibit the activity of CLOCK/CYCLE (*28–30*). We find that mutations in *per*, *clock*, and *cycle* share the same cocaine phenotype: a deficiency in the ability to sensitize after one or more drug exposures. This similarity leads us to suspect that as in circadian behaviors, these genes are functioning in a common pathway.

In contrast to the above mentioned genes, the *tim^o* mutant showed normal cocaine responses. The implication of this finding is twofold. First, there must be an as yet unidentified PER binding partner that is specifically involved in regulation of drug responsiveness. Second, drug responsiveness is likely regulated by *per* expression in a set of cells distinct from those involved in circadian function. In *tim^o* mutants, PER levels are constitutively low (*19*, *20*); if the same TIM-containing cells were involved in circadian and cocaine responses, *tim^o* flies should not sensitize.

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13. A failure to sensitize, as is seen with the circadian mutants, is a rare phenotype among the mutants that have been screened in this laboratory, having been seen previously only for the mutant *inactive* (*25*). A number of behavioral mutants have been screened, including *dunce*, *rutabaga*, *shibire*, and several alleles of *amnesiac*, and mutants in cell signaling pathways including *Gprk2*, *trp*, and *Nf1*. None of these lines shows significant aberrations in cocaine responsiveness or sensitization (C. McClung, J. Walman, J. Hirsh, unpublished data). A number of WT strains, including the background strains from which the circadian mutants were isolated, similarly show only minor variations in initial responsiveness to cocaine, with no variation in the ability to sensitize. In addition, forward-based screens show a phenotype of failure to sensitize at extremely low frequency.
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31. Flies were exposed to 75 μg of volatilized cocaine three times over the course of 2 days and decapitated 4 hours after the last exposure as described (*24*). A 2 mM solution of the dopamine D2-like agonist quinpirole was applied to the cut end of the nerve cord, made up in 3% green food coloring to assess diffusion into the nerve cord. Locomotion after drug application was video-recorded for 2 min.
32. Hand-dissected *Drosophila* adult brains (four brains in 12 μl) were homogenized in 50 mM tris (pH 7.5), 1 mM phenylthiourea. Forty-eight microliters of assay mix [0.1 M sodium phosphate buffer (pH 6.8), 0.1 mM pyridoxal phosphate, 0.1 mM EDTA, and [^{3}H]tyrosine (20 μCi/ml)] was added to 12 μl of brain homogenate and incubated for 10 min at 31°C. Conversion to tyramine was linear over the 10-min incubation time. Reactions were stopped by addition of 150 μl of chloroform containing 0.1 M diethylhexylphosphoric acid, followed by addition of 400 μl of 0.05 M sodium phosphate buffer (pH 6.8). After brief vortexing, tubes were centrifuged to separate phases, and the aqueous phase was discarded. Aqueous washing of the organic phase was repeated two times, after which the organic phase was pipetted into scintillation vials, dried, and counted.
33. We thank B. Condron, M. Sankovic, and C. Green for helpful comments on the manuscript. Cocaine was supplied by the National Institute of Drug Abuse Drug Supply Program. We thank J. Hall and M. Young for providing circadian mutants. Supported by NIH predoctoral fellowship DA05942 (R.A.) and grant GM/DA 27318 (J.H.).

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Dynamical Role of Predators in Population Cycles of a Forest Insect: An Experimental Test

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Population cycles occur frequently in forest insects. Time-series analysis of fluctuations in one such insect, the southern pine beetle (*Dendroctonus frontalis*), suggests that beetle dynamics are dominated by an ecological process acting in a delayed density-dependent manner. The hypothesis that delayed density dependence in this insect results from its interaction with predators was tested with a long-term predator-exclusion experiment. Predator-imposed mortality was negligible during the increase phase, grew during the year of peak population, and reached a maximum during the period of population decline. The delayed nature of the impact of predation suggests that predation is an important process that contributes significantly to southern pine beetle oscillations.

Ecologists have been trying to solve the puzzle of population cycles for at least three-quarters of a century (*1*). One class of ecological system that seems particularly prone to population oscillations is insects attacking forest trees (*2, 3*). Because these insects cause

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