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**Lmo Mutants Reveal a Novel Role for Circadian Pacemaker Neurons in Cocaine-Induced Behaviors**

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**Drosophila** has been developed recently as a model system to investigate the molecular and neural mechanisms underlying responses to drugs of abuse. Genetic screens for mutants with altered drug-induced behaviors thus provide an unbiased approach to define novel molecules involved in the process. We identified mutations in the **Drosophila** LIM-only (LMO) gene, encoding a regulator of LIM-homeodomain proteins, in a genetic screen for mutants with altered cocaine sensitivity. Reduced Lmo function increases behavioral responses to cocaine, while Lmo overexpression causes the opposite effect, reduced cocaine responsiveness. Expression of Lmo in the principal **Drosophila** circadian pacemaker cells, the PDF-expressing ventral lateral neurons (LNvs), is sufficient to confer normal cocaine sensitivity. Consistent with a role for Lmo in LNv function, Lmo mutants also show defects in circadian rhythms of behavior. However, the role for LNvs in modulating cocaine responses is separable from their role as pacemaker neurons: ablation or functional silencing of the LNvs reduces cocaine sensitivity, while loss of the principal circadian neurotransmitter PDF has no effect. Together, these results reveal a novel role for Lmo in modulating acute cocaine sensitivity and circadian locomotor rhythmicity, and add to growing evidence that these behaviors are regulated by shared molecular mechanisms. The finding that the degree of cocaine responsiveness is controlled by the **Drosophila** pacemaker neurons provides a neuroanatomical basis for this overlap. We propose that Lmo controls the responsiveness of LNvs to cocaine, which in turn regulate the flies’ behavioral sensitivity to the drug.

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**Introduction**

Cocaine, a naturally occurring plant alkaloid, is the prototype addictive psychomotor stimulant. It elicits a variety of acute behavioral changes ranging from mood elevation, disinhibition, and motor activation at low doses to compulsive stereotypies and psychosis at higher doses (Gawin 1991). Long-term cocaine use generally results in tolerance to many of its subjective effects, an increased craving towards the drug, and, eventually, drug abuse and addiction.

Cocaine’s primary mechanism of action is to bind and inhibit plasma membrane monoamine transporters, thereby increasing synaptic monoamine neurotransmitter levels and potentiating their actions. Cocaine’s direct role in increasing dopamine (DA) in the nucleus accumbens via inhibition of the DA transporter (DAT) has contributed to the prevalent DA hypothesis of drug addiction, which posits that the shared ability of drugs of abuse to increase DA in the nucleus accumbens underlies their reinforcing properties (Wise and Bozarth 1985; Kuhar et al. 1991). More recent animal studies, however, have shown that cocaine still elicits a robust conditioned place preference in mice with genetic deletions of DAT, suggesting that the rewarding properties of cocaine are not solely mediated by its action on DAT (Sora et al. 1998). Additional studies on mice in which the serotonin transporter or norepinephrine transporter has been deleted, together with studies using selective serotonin transporter or norepinephrine transporter inhibitors, have suggested roles for both serotonin and norepinephrine systems in mediating cocaine’s rewarding properties (Uhl et al. 2002). These data show that the molecular bases for cocaine’s psychostimulant and reinforcing properties are more complicated than once thought, and that a combination of actions at multiple sites may mediate its effects. Indeed, multiple genes and signaling pathways have been implicated in the stimulant and rewarding properties of cocaine in mice (Laakso et al. 2002).

The fruit fly **Drosophila melanogaster** has been advanced as a useful model system for identifying novel genes that regulate behavioral responses to drugs of abuse including cocaine (Wolf and Heberlein 2003). Several of cocaine’s most
characteristic properties have been recapitulated in flies. First, cocaine induces motor behaviors in flies (see below) that are remarkably similar to those observed in mammals (McClung and Hirsch 1998; Bainton et al. 2000). Second, repeated cocaine administration induces behavioral sensitization (McClung and Hirsch 1998), a form of behavioral plasticity believed to underlie certain aspects of addiction (Robinson and Berridge 1993; Schenck and Partridge 1997).

Finally, a key role for dopaminergic systems in mediating cocaine’s effects has been demonstrated through both pharmacologic and genetic methods (Bainton et al. 2000; Li et al. 2000). More importantly, Drosophila studies have identified genes and pathways whose role in cocaine responsiveness had not been anticipated (Hirsh 2001; Rothenfluh and Heberlein 2002). For instance, the Drosophila circadian gene period was identified as a necessary mediator of cocaine sensitization (Andretic et al. 1999). Subsequently, mice carrying various period mutations were found to have altered cocaine sensitization and conditioned place preference (Abarca et al. 2002).

In order to identify novel molecules and pathways involved in behavioral responses to cocaine, we carried out a genetic screen for Drosophila mutants with altered acute responses to cocaine. Here, we report the phenotypic and molecular characterization of mutations in the Drosophila LIM-only gene, Lmo (also called Beadex [Bx]), isolated due to their increased sensitivity to cocaine-induced loss of negative geotaxis. The products of Drosophila and mammalian Lmo genes modulate the function of LIM-homeodomain (LIM-HD) proteins (Milan et al. 1998; Retaux and Bachy 2002), which in turn regulate various aspects of nervous system development, including the specification of neural identity (Thor and Thomas 1997; Hobert and Westphal 2000; Shirasaki and Pfaff 2002; Tsalik et al. 2003). We find that cocaine sensitivity is inversely related to the levels of Lmo function: reduced function causes increased sensitivity, while increased function causes resistance. This bidirectional regulation of cocaine responsiveness is mediated by Lmo function in a small set of neurons, the ventral lateral neurons (LNv), which are the primary circadian pacemaker cells in Drosophila (Helfrich-Förster 1997; Renn et al. 1999). Like other mutants that affect LNv function, Lmo mutants show altered circadian locomotor behaviors. However, the roles of the LNv in regulating cocaine sensitivity and circadian behavioral rhythms are genetically separable, as mutants lacking the neuropeptide PDF—the only known functional output of these neurons—show normal cocaine sensitivity. Thus, Lmo defines a novel role for the circadian pacemaker cells in regulating behavioral responses to cocaine.

Results

Loss- and Gain-of-Function Mutations in the Lmo Locus Show Altered Cocaine Sensitivity

To identify novel molecules involved in cocaine-related behaviors, we carried out a genetic screen for mutants with altered acute responses to volatilized freebase cocaine using the crackometer, a simple assay that measures cocaine-induced loss of negative geotaxis (Bainton et al. 2000). Screening of 400 first chromosome P-element insertions from the EP collection (Röth 1996) led to the identification of five mutants with reduced cocaine sensitivity and seven with increased sensitivity. Two of the mutants conferring increased cocaine sensitivity, EP1306 and EP1383 (Figure 1A), carry a P-element insertion in the promoter region of the Drosophila Lmo locus (Milan et al. 1998; Zeng et al. 1998). Drosophila LMO protein has been shown to inhibit the activity of the LIM-HD transcription factor apterous through its interactions with the LIM-HD activator Chip (Milan and Cohen 1999; Weihe et al. 2001).

Independently, we isolated a P[GAL4] insertion in the Lmo locus, dubbed pipedream (pdrm), that also showed increased cocaine sensitivity (Figure 1A). Finally, heldup (hdp) mutant flies, which carry well-characterized loss-of-function mutations in Lmo (Milan et al. 1998), displayed increased sensitivity to cocaine that is similar in magnitude to that of the EP1306 line (Figure 1A). This increased sensitivity was observed at all cocaine doses tested (Figure 1B and 1C; data not shown). The EP1306 and hdp alleles consistently demonstrated stronger phenotypes than EP1383 and pdrm. All mutants tested performed within the normal range in task-specific baseline behaviors (see Materials and Methods). Finally, we were able to revert the cocaine phenotype of EP1306 by excision of the P element (data not shown). Taken together, these data demonstrate that loss-of-function mutations in Lmo result in increased sensitivity to cocaine.

The dominant Beadex (Bx) mutations were first described in 1925 by Morgan, Bridges, and Sturtevant, and named for their characteristic beaded wing margin (Morgan et al. 1925). More recently, Bx mutants were shown to be overexpression alleles of Lmo (Milan et al. 1998; Shoresh et al. 1998; Zeng et al. 1998). Overexpression results from the insertion of naturally occurring transposable elements into the 3′ UTR of Lmo, which leads to transcript stabilization (Shoresh et al. 1998). The resultant overexpression of Lmo causes decreased apterous activity in the dorsal compartment of the wing and, consequently, disorganization of the wing margin (Morgan and Cohen 1999, 2000).

We tested multiple available Bx alleles, and found that they all caused resistance to the acute effects of cocaine at every dose tested (Figure 2A and 2B). The strength of the cocaine resistance phenotype correlated well with the severity of the beaded wing-margin phenotype, Bx<sup>l</sup> > Bx<sup>g</sup> = Bx<sup>S</sup> = Bx<sup>d</sup> (Figure 2A and 2B; data not shown). Because in Bx mutants Lmo transcripts are stabilized in their normal spatiotemporal pattern (Shoresh et al. 1998), the observed cocaine phenotypes likely result from overexpression, rather than from misexpression of Lmo. Taken together with the loss-of-function effects described above (see Figure 1A and 1B), these data reveal a graded behavioral response to cocaine that is inversely related to Lmo levels.

In order to study Lmo mutant behavior in more detail, we recorded the cocaine-induced patterns of locomotor activity of control and Lmo flies using a movement tracking system (Bainton et al. 2000; Wolf et al. 2002). Upon mock exposure, control flies showed robust locomotor activity, generally walking in straight lines (Figure 1C, top left panel). At relatively low doses of cocaine (75 and 100 µg), flies engaged in stereotyped circling patterns of activity (Figure 1C, top right panels). At higher doses (125–200 µg), flies began to show spasmodic movements (seen as zigzag patterns in movement) and severe hypokinesia (data not shown; Bainton et al. 2000). EP1306 flies, while showing a reduced baseline speed, showed normal patterns of activity upon mock...
exposure (Figure 1C, bottom left panel). Upon exposure to cocaine, these mutant flies showed a shift in the dose-response relationship. At low doses (75 μg), EP1306 flies showed a marked increase in stereotyped circling behavior compared to controls (Figure 1C, bottom middle panel), while at higher doses (100 μg), the mutant flies were more likely to be spasmodic or akinetic (Figure 1C, bottom right panel). Thus, despite the reduced speed observed in the mock exposures, increases in specific cocaine-induced locomotor behaviors demonstrate that EP1306 flies have a shifted dose-response to cocaine: mutant flies exposed to 75 μg of cocaine behaved similarly to control flies exposed to 100 μg of the drug. 

Bx^l flies also showed changes in walking patterns that are consistent with a shift in the cocaine dose-response relationship. After exposure to 100 μg of drug, most control flies showed slow circling behavior and some were akinetic (Figure 2C, top middle panel). Bx^l flies were much less affected, showing increased locomotion (mostly in straight lines), decreased slow circling, and almost no akinesia (Figure 2C, bottom middle panel). At 125 μg, control flies showed very little movement (Figure 2C, top right panel), while most Bx^l flies continued to show circling behaviors seen in control flies at lower doses (Figure 2C, bottom right panel). Bx^l flies, like EP1306 flies, showed reduced activity upon mock exposure (Figure 2C, bottom left panel). Despite this reduced activity, these two mutants showed very different sensitivities to cocaine: EP1306 flies were more affected and Bx^l flies were less affected, suggesting that the reduced activity upon mock exposure is unrelated to the cocaine response. Long-term activity recordings revealed no differences between Bx^l, EP1306, and control lines (data not shown; see below).

In summary, using two different behavioral assays we show that flies carrying loss-of-function mutations in Lmo display increased sensitivity to the effects of cocaine on locomotor behaviors, while gain-of-function mutations show the converse effect, a reduced response to the drug. This inverse relationship between Lmo gene activity and drug responsiveness suggests that Lmo may regulate the expression of genes that might play a direct role in controlling cocaine responses (see Discussion).

**Molecular Characterization of Lmo Mutants**

The Lmo locus produces at least three transcripts by differential promoter use (Figure 3A). The RA and RC transcripts differ only in their 5' UTRs and are predicted to encode identical proteins of 313 amino acids; the RB transcript is predicted to utilize an alternative translational start site. The insertions that produce RA and RC transcripts differ only in their 5' UTRs and are predicted to encode identical proteins of 313 amino acids; the RB transcript is predicted to utilize an alternative translational start site. The insertions that produce RA and RC transcripts differ only in their 5' UTRs and are predicted to encode identical proteins of 313 amino acids; the RB transcript is predicted to utilize an alternative translational start site.
fold more abundant in heads as compared to bodies, suggesting that this transcript is enriched in the nervous system (Figure 3B; see also Figure 4). Importantly, the RA transcript was reduced by greater than 50% in the heads, but not the bodies, of EP1306 flies (Figure 3B); a 40% increase in the RA transcript was observed in the heads of BxJ flies (data not shown). Thus, as predicted by the location of the P-element insertion, the EP1306 mutation causes a reduction in Lmo transcript levels, a finding that is consistent with the observation that the cocaine sensitivity of EP1306 flies is similar to that seen with known loss-of-function alleles of Lmo (the hdp alleles) and opposite to that seen with gain-of-function Bx alleles. In addition, the finding that transcript levels are specifically reduced in the heads of EP1306 flies, and not their bodies, suggests that this mutation affects a nervous-system-enriched (or -specific) Lmo transcript (see below). Finally, none of the P-element insertions in the promoter of the RA transcript leads to a held-up wing phenotype, which is characteristic of loss-of-function hdp alleles (Shoresh et al. 1998; Milan and Cohen 1999). This suggests that the P-element insertions isolated in our genetic screen cause either less severe or more spatially restricted changes in Lmo gene expression.

Restricted Expression of Lmo in the Nervous System Is Sufficient for Wild-Type Cocaine Sensitivity

The pdrm UAS-tauGFP insertion, which acts as a promoter/enhancer detector, is expected to reproduce, at least in part, the expression pattern of the Lmo gene. In adult flies, the pdrm line showed extensive GAL4 expression in the brain as visualized with the UAS–green fluorescent protein (GFP) transgene (Figure 4A). Prominent expression was observed in the antennal lobes (ALs) and the Kenyon cells of the mushroom bodies (MBs), which are major brain centers involved in olfaction and olfactory conditioning, respectively (Stocker 1994; Zars 2000). In addition, GAL4 was expressed in the LNvs, which are the major pacemaker cells regulating circadian locomotor rhythmicity in flies (Renn et al. 1999). Finally, GAL4 expression was seen in neurosecretory cells located in the pars intercerebralis, in glial cells surrounding the optic lobes, and in scattered cells throughout the ventral nerve cord (VNC) (Figure 4A; data not shown). In pdrm larvae and adult flies, GAL4 expression was restricted to the nervous system. GAL4 expression was not detected in larval imaginal discs, such as the wing disc (data not shown), where Lmo expression has been shown to play an important patterning role (Milan and Cohen 1999). Thus, pdrm traps a specific subset of Lmo regulatory elements, possibly those controlling nervous-system-restricted expression of the RA transcript.

We hypothesized that the expression pattern of the pdrm enhancer trap may identify the cells in which Lmo expression is disrupted in the P-element insertion lines, causing increased cocaine sensitivity. If this is the case, pdrm-driven expression of UAS-Lmo transgenes is expected to restore normal cocaine sensitivity to pdrm flies. Indeed, we found that two UAS-Lmo transgenes rescued the cocaine-sensitivity defect of pdrm mutant flies (Figure 4B). This effect was specific to Lmo, as pdrm-driven expression of UAS-tauGFP or UAS-lacZ failed to restore normal behavior. Finally, Lmo mutants that did not contain a GAL4 enhancer trap, such as

Figure 2. Lmo Gain-of-Function Bx Alleles Show Reduced Sensitivity to Cocaine

(A) Cocaine phenotypes of Bx mutants. Male flies hemizygous for Bx alleles Bx2 or Bx3 show significant reductions in sensitivity to cocaine compared to control (Ctl) flies (p < 0.001, n = 12 experiments). Asterisks denote significant differences from control (Student’s paired t-test assuming equal variance).

(B) Dose–response. BxJ flies (filled circles) show reduced sensitivity compared to Ctl flies (open circles) at all doses tested (p < 0.001, n = 16–20 for all doses except for 250 μg, where p = 0.0015, n = 8). Two additional Bx alleles (BxG and Bxk) had similar phenotypes to BxJ (not shown).

(C) BxJ flies show alterations in cocaine-induced locomotor patterns of activity. Flies were exposed to 0, 100, or 125 μg of cocaine, as indicated, for 1 min. Representative traces shown are 30 s of recorded activity of about ten flies starting 30 or 60 s after the end of cocaine exposure (n ≥ 5). Top panels show response of control flies to indicated amounts of cocaine; bottom panels show activity of BxJ flies after cocaine administration. Ctl flies are not shown.

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EP1306, failed to be rescued by the presence of UAS-Lmo transgenes (data not shown). These data demonstrate that the cocaine-sensitivity defect of pdrm flies is due to the loss of Lmo function, and that nervous-system-specific expression of Lmo in cells dictated by the pdrm GAL4 line is sufficient to confer normal cocaine responses.

**Lmo Expression in PDF Neurons Is Sufficient to Confer Normal Cocaine Responses**

In order to refine further the spatial requirements for Lmo function, we attempted to rescue the EP1306 phenotype by expression of Lmo in specific brain regions. EP lines carry a P-element insertion containing multiple UAS sites to which GAL4 can bind to drive expression of adjacent genomic sequences (Rørth 1996). Therefore, mutagenic EP insertions, if oriented appropriately, can be used to drive expression of the disrupted gene. It was demonstrated previously that GAL4-driven Lmo expression can be mediated by the EP1306 and EP1383 insertions (Milan et al. 1998; Zeng et al. 1998). Consistent with this, we were able to rescue the EP1306 phenotype in the presence of the heat-inducible hs-GAL4 driver and UAS-Lmo transgenes. We found that these flies showed a significant decrease in cocaine sensitivity (Figure 5B). The magnitude of the induced resistance was lower than that observed in the Bx\(^+\) strain, but similar to that of weaker Bx alleles. It is possible that overexpression of Lmo with the pdf-GAL4 driver may not be as high as that caused by the Bx\(^+\) mutation, or, alternatively, that overexpression of Lmo in cells other than the LN\(_n\)s mediates the remaining resistance to cocaine observed in Bx\(^+\). Nonetheless, this experiment supports a role for LN\(_n\)s as a site where Lmo regulates sensitivity to cocaine.

In the adult fly, pdf-GAL4 drives expression in small and large LN\(_n\)s (s-LN\(_n\)s and l-LN\(_n\)s) and in a group of neuroendocrine cells located at the very tip of the VNC (J. H. Park et al. 2000; Figure 5C). The LN\(_n\)s express many central clock genes and have been demonstrated, through genetic ablations and electrical silencing studies, to play a central role in maintaining circadian locomotor rhythmicity (Renn et al. 1999; Nitabach et al. 2002; Peng et al. 2003); the function of the pdf-GAL4-expressing cells in the VNC is unknown. The pdrm line drives expression in both the s-LN\(_n\)s and l-LN\(_n\)s, as demonstrated by immunohistochemical analysis of pdrm/UAS-GFP flies with antibodies directed against the PDF precursor PAP (Figure 5C). pdrm does not, however, drive expression in the PDF-expressing cells located in the VNC (data not shown). This overlap in expression of pdrm and pdf-GAL4 in the LN\(_n\)s implicates these few neurons as mediators of the altered cocaine sensitivity observed in Lmo mutants.

Because of the known role of LMOs as regulators of developmentally important transcription factors (Hobert and Westphal 2000), we asked whether the development of PDF-expressing LN\(_n\)s is disrupted in Lmo mutants. Projections of the s-LN\(_n\)s and l-LN\(_n\)s can be visualized with an antibody that recognizes the PDF precursor PAP (Renn et al. 1999). L-LN\(_n\)s make an elaborate network of varicosities on the surface of the optic medulla, and project across the midline to the contralateral LN\(_n\)s, while s-LN\(_n\)s make a very specific projection to the dorsal central brain (Figure 5C). Both sets of LN\(_n\) neurons also make extensive arborizations in the accessory medulla (reviewed in Hellfrich-Förster 2003). In the EP1306 and pdrm mutants, anti-PAP staining revealed that the number and detailed morphology of LN\(_n\) neurons were...
sensitivity. For all genotypes, transgenes (absence of a LMO2 UAS-LMO1/+ transgene alone (white bars) or for non-specific effects of transgene overexpression, of a control GAL4 line (Ctl GAL4, line normal cocaine sensitivity when compared to either UAS-LMO transgenes were also driven by pdrm GAL4. Male flies were hemizygous for pdrm and heterozygous for either of two UAS-LMO transgenes (UAS-LMO1 and UAS-LMO2; hatched black bars), show normal cocaine sensitivity when compared to either UAS-LMO transgene alone (white bars) or UAS-LMO transgenes in the presence of a control GAL4 line (Ctl GAL4, line 8.142 black bars). To control for non-specific effects of transgene overexpression, UAS-GFP and UAS-lacZ transgenes were also driven by pdrm GAL4. Male flies were hemizygous for pdrm (or heterozygous for the control GAL4 insertion) and heterozygous for the specific UAS transgene. One-way ANOVA revealed a significant effect of genotype in the UAS-lacZ group (F = 17.4, p < 0.001), UAS-GFP (F = 19.47, p < 0.001), or no UAS transgene (F = 4.1, p < 0.001) groups, but not in either of the UAS-LMO transgene groups (F = 1.58, p = 0.22 and F = 1.21, p = 0.31 for UAS-LMO1 and UAS-LMO2, respectively); thus, UAS-LMO expression specifically restores normal cocaine sensitivity to pdrm flies. Post hoc pairwise planned comparisons, with the critical p-value adjusted to 0.025, revealed significant differences between the “non-rescued” pdrm/UAS-GFP flies and the appropriate controls (UAS-GFP± or 8.142/UAS-GFP, p < 0.002); similarly, pdrm/UAS-lacZ flies are significantly different from their controls (UAS-lacZ± and 8.142/UAS-lacZ, p < 0.002). Pairwise comparisons revealed no significant differences between “rescued” pdrm/UAS-LMO flies and their “normal” controls (8.142/UAS-LMO1 and UAS-LMO1±, p = 0.99 and p = 0.09, respectively) or pdrm/UAS-LMO flies and their controls (8.142/UAS-LMO2 and UAS-LMO2±, p = 0.09 and p = 0.99, respectively), indicating full rescue of pdrm cocaine sensitivity. For all genotypes, n = 16–20 experiments.

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**Figure 4.** pdrm’s GAL4 Expression Is Sufficient to Drive Lmo-Transgene-Mediated Rescue of Cocaine Sensitivity

(A) pdrm’s GAL4 expression pattern. UAS-GFP reveals GAL4 expression pattern of the pdrm enhancer-trap insertion in MB lobes and calyces, ALs, the large cell bodies of the peptidergic LNvs, and neurons of the pars intercerebralis (PI).

(B) Lmo expression restores wild-type cocaine responses. In the absence of a UAS transgene (“no UAS” columns), hemizygous male pdrm flies (hatched bar) are more sensitive than controls (Ctl GAL4 is line 8.142, solid bar), as shown before in Figure 1A. Male flies hemizygous for pdrm and heterozygous for either of two UAS-LMO transgenes (UAS-LMO1 and UAS-LMO2; hatched black bars), show normal cocaine sensitivity when compared to either UAS-LMO transgene alone (white bars) or UAS-LMO transgenes in the presence of a control GAL4 line (Ctl GAL4, line 8.142 black bars). To control for non-specific effects of transgene overexpression, UAS-GFP and UAS-lacZ transgenes were also driven by pdrm GAL4. Male flies were hemizygous for pdrm (or heterozygous for the control GAL4 insertion) and heterozygous for the specific UAS transgene. One-way ANOVA revealed a significant effect of genotype in the UAS-lacZ group (F = 17.4, p < 0.001), UAS-GFP (F = 19.47, p < 0.001), or no UAS transgene (F = 4.1, p < 0.001) groups, but not in either of the UAS-LMO transgene groups (F = 1.58, p = 0.22 and F = 1.21, p = 0.31 for UAS-LMO1 and UAS-LMO2, respectively); thus, UAS-LMO expression specifically restores normal cocaine sensitivity to pdrm flies. Post hoc pairwise planned comparisons, with the critical p-value adjusted to 0.025, revealed significant differences between the “non-rescued” pdrm/UAS-GFP flies and the appropriate controls (UAS-GFP± or 8.142/UAS-GFP, p < 0.002); similarly, pdrm/UAS-lacZ flies are significantly different from their controls (UAS-lacZ± and 8.142/UAS-lacZ, p < 0.002). Pairwise comparisons revealed no significant differences between “rescued” pdrm/UAS-LMO flies and their “normal” controls (8.142/UAS-LMO1 and UAS-LMO1±, p = 0.99 and p = 0.09, respectively) or pdrm/UAS-LMO flies and their controls (8.142/UAS-LMO2 and UAS-LMO2±, p = 0.09 and p = 0.99, respectively), indicating full rescue of pdrm cocaine sensitivity. For all genotypes, n = 16–20 experiments.

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**Figure 5.** Lmo Expression in PDF Neurons Regulates Cocaine Responses

(A) pdf-GAL4-driven expression of Lmo using the EP1306 element rescues the EP1306 insertional phenotype. Lmo mutants EP1306, hdp, and pdrm, as well as a control EP line (Ctl-1 = EP1413), were tested in the absence (–pdf-GAL4; white bars) and presence (+pdf-GAL4; black bars) of pdf-GAL4. All male flies are hemizygous for the Lmo mutation (or Ctl-1) and heterozygous for pdf-GAL4 (when carrying the transgene). One-way ANOVAs with post hoc planned comparisons (critical p-value adjusted to 0.0125) confirmed that EP1306, pdrm, and hdp flies (in the absence of pdf-GAL4) had significantly increased sensitivity to cocaine compared to control flies (Ctl-1 = EP1413) (p < 0.003, n = 15–26 experiments). One-way ANOVA with post hoc planned comparisons (critical p-value adjusted to 0.01) revealed a significant difference between pdrm/pdf-GAL4 and hdp/pdf-GAL4 flies and their controls (EP1413/pdf-GAL4, pdf-GAL4+, or EP1413+/C0, p < 0.003, n = 24–27 experiments), showing that the presence of pdf-GAL4 does not rescue the cocaine sensitivity of pdrm or hdp flies. In contrast, similar comparisons for “rescued” EP1306/pdf-GAL4 flies revealed no significant differences from their “normal” controls (p ≥ 0.026, n = 27–36). Furthermore, within-group comparisons (+pdf-GAL4) using t-tests indicate a significant difference only in the EP1306 group (p < 0.002). Asterisks denote significant difference between –pdf-GAL4 and +pdf-GAL4 phenotype.

(B) Flies overexpressing Lmo in PDF cells show decreased sensitivity to cocaine. Flies heterozygous for both pdf-GAL4 and either one of two UAS-Lmo transgenes (black bars) were compared to flies carrying UAS-Lmo (white bars) or pdf-GAL4 (gray bar) alone. One-way ANOVA revealed a significant effect of genotype for both UAS-Lmo transgene groups. Post-test planned comparisons, with the critical p-value adjusted to 0.025, showed significant differences between the pdf-GAL4/UAS-Lmo flies and either pdf-GAL4+ (p < 0.02) or UAS-Lmo- controls (p < 0.005). Asterisks denote significant differences, n = 16 experiments.

(C) Confocal images demonstrate overlap between pdrm and PDF expression in the LNvs. In the left panel, UAS-nCD8GFP reveals the pdrm-driven GAL4 expression pattern (green) in the adult brain, and α-PAP staining (magenta) reveals PDF-expressing LNvs. Right panels are close-ups of the cell bodies of the LNvs; white areas correspond to regions of overlap between GFP (green) and PAP (magenta) expression.

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completely normal (data not shown). Thus, Lmo does not appear to play a role in the development of the LN_s, although subtle developmental defects could have been missed.

Lmo Is Required for Robust Circadian Locomotor Rhythms

The increased sensitivity to cocaine of Lmo mutants suggested that these mutations alter LN_s function without grossly affecting LN_v development. In order to determine whether Lmo mutants have a more general dysfunction in the LN_s, we tested Lmo mutants in locomotor rhythm assays. The LN_s are required for the maintenance of circadian locomotor rhythms in constant darkness, and are thus the pacemaker neurons of the fly (Stanewsky 2003). Lmo mutants EP1306, EP1383, hdp_{R26}, and hdp_{rev83} had less robust locomotor rhythms in constant darkness than their control lines (Figure 6A; data not shown), and all alleles had a higher tendency for arrhythmicity (Figure 6B). We used the power of the rhythm as an estimate of the degree of rhythmicity and found that EP1306 flies had weaker behavioral rhythms than EP1383 flies, which, in turn, were less robustly rhythmic than their control line (Ctl-2; Figure 6C); similarly, hdp_{R26} and hdp_{rev83} flies had significantly weaker rhythms than their control line (Ctl-1; Figure 6C). However, there were rhythmic flies in almost all genotypes assayed (Figure 6B), and the period lengths of the rhythms were very similar across the genotypes (see legend to Figure 6 for details). Together, these data demonstrate that disruption of Lmo results in weak circadian rhythms of behavior and suggest a generalized dysfunction of the LN_s in Lmo mutants.

LN_s Regulate Circadian Rhythmicity and Cocaine Sensitivity Independently

The observation that Lmo functions in the PDF neurons to regulate cocaine sensitivity, together with the finding that Lmo mutants show weak circadian locomotor rhythms, suggested that the pathways regulating cocaine sensitivity interact with the circadian clock. Alternatively, Lmo could regulate these two behaviors independently. We addressed these possibilities in two sets of experiments. First, we determined whether cocaine sensitivity was regulated by the circadian clock. For this purpose we entrained flies to light–dark (LD) cycles and then tested them for cocaine sensitivity. During the light and dark phases at 3-h intervals over 24 h (see Materials and Methods). We found that cocaine sensitivity was essentially the same at all times tested (Figure 7A), demonstrating that cocaine responsiveness is not a behavioral output of the circadian clock.

Second, we asked whether PDF, the only known functional output of the LN_s in the context of circadian rhythms, is involved in regulating cocaine sensitivity. pdf mutant flies show a circadian phenotype that has been localized to the LN_s. We found that the pdf^{01} mutant flies, which completely lack PDF (Renn et al. 1999), showed normal cocaine responses compared to the control strain (Figure 7B). To eliminate the possibility that the absence of a phenotype was caused by genetic modifiers in the background of the pdf^{01} strain, we also tested flies carrying the pdf^{01} chromosome over a deficiency for the locus (see Materials and Methods). These pdf^{01} hemizygous flies also displayed normal cocaine sensitivity (Figure 7B). Taken together, these results show that the altered cocaine sensitivity of Lmo flies is not secondary to their abnormal circadian rhythms. Moreover, our data show that cocaine sensitivity and circadian behaviors, although both localized to the LN_s, are genetically separable.

LN_s and Their Synaptic Activity Regulate Acute Cocaine Responsivity

The hypersensitivity of Lmo mutants to cocaine could result from either the disruption of an LN_v output that acts normally to dampen cocaine sensitivity or from an increase in an output of LN_s that normally enhances cocaine sensitivity. In order to differentiate between these two possibilities, we tested flies that lacked LN_s, generated by targeted expression of the cell death gene head involution defective (hid) using pdf-GAL4. This approach was previously used to demonstrate that LN_s are the Drosophila pacemaker neurons responsible for rhythmic locomotor activity (Renn et al. 1999). When tested in the crackometer, flies with LN_v ablation showed reduced sensitivity to cocaine when compared to controls (Figure 8). These results show that LN_s normally act to increase cocaine responsiveness and that this effect is antagonized by Lmo function in these cells (see Discussion).

To confirm that the behavioral resistance observed in LN_v-ablated animals results from loss of neuronal signaling from these cells, rather than from developmental compensations induced by their ablation, we functionally silenced the LN_s either electrically, by targeted expression of the mammalian inward rectifying K⁺ channel (Kir2.1), or synaptically, by expression of tetanus toxin light chain (TeTx) (Sweeney et al. 1995; Baines et al. 2001). These manipulations do not affect LN_v survival or the normal projection patterns of LN_s (Kaneko et al. 2000; Nitabach et al. 2002). Expression of either Kir2.1 or TeTx resulted in reduced cocaine sensitivity similar to that seen with LN_v ablations (Figure 8). Importantly, expression of an inactive TeTx (TeTx^*) did not significantly alter cocaine responses, confirming that the actions of these transgenes were specific to their ability to silence or ablate the LN_s (Figure 8). These data further demonstrate that circadian phenotype does not predict cocaine phenotype, as targeted expression of either hid or Kir2.1 in LN_s causes arrhythmia, while expression of TeTx does not (Renn et al. 1999; Kaneko et al. 2000; Nitabach et al. 2002). In summary, these results confirm a novel role for LN_v activity and synaptic output in increasing behavioral responses to cocaine in a manner independent from its role in regulating circadian locomotor rhythmicity.

Discussion

In a genetic screen for Drosophila mutants with altered acute responses to cocaine, we isolated multiple mutations in the Lmo locus. Behavioral characterization of gain- and loss-of-function alleles of Lmo demonstrates an inverse correlation between Lmo expression levels and cocaine sensitivity. Through targeted expression of Lmo, we show that these altered cocaine responses are caused by differential Lmo expression in the circadian pacemaker neurons, the LN_s. Consistent with a dysfunction of these pacemaker neurons in Lmo mutants is our finding that the mutant flies also show altered circadian locomotor rhythms. However, using a variety of genetic methods to ablate or functionally silence these neurons, we reveal a novel role for the LN_s in...
flies arrhythmic. For example, 13/26 $hdp^{+3}$ flies were arrhythmic, with the other 13 all having weak rhythms. The power of the rhythm was used to estimate the strength of the activity rhythm, with a power of 300 or more classed as a strong rhythm and a power between 300 and 170 classed as a weak rhythm; arrhythmic rhythms were given a power of 170 (for analysis below). Between 24 and 30 flies were assayed for each genotype. There were no major differences in the period length of the rhythmic flies in each genotype (Ctl-1, 23.6 ± 0.3; Ctl-2, 23.5 ± 0.5; EP1306, 23.2 ± 0.4; EP1306, 23.4 ± 0.3; $hdp^{+3}, 24.2 ± 0.5$; and $hdp^{+3}, 23.4 ± 0.3$). (C) Quantitation of the average power of the rhythm with error bars showing standard error of the mean. One-way ANOVA revealed significant differences between genotypes ($p < 0.0001$). Post-hoc t-tests using a Bonferroni correction revealed that the power of the rhythm was significantly different between control flies and the Lmo mutants $hdp^{+3}$, $hdp^{+3}$, and EP1306 ($p < 0.01$). EP1383 flies had a significantly weaker rhythm than Ctl-2 flies ($p < 0.05$). Ctl-1, $w^{1118}$, is the appropriate genetic control for the $hdp$ alleles (black columns); Ctl-2, EP1631, is the appropriate control for EP1306 and EP1383 (gray columns). All flies tested are in the same genetic background, that of the $w^{1118}$ flies. (D) Quantitation of average activity (beam crossings per minute) with error bars showing standard error of the mean. ANOVA did not reveal significant differences between genotypes at the 0.01 level. Ctl-1, $w^{1118}$, is the appropriate genetic control for the $hdp$ alleles (black columns); Ctl-2, EP1631, is the appropriate control for EP1306 and EP1383 (gray columns). All flies tested are in the same genetic background, that of the $w^{1118}$ flies.

Figure 6. Wild-Type Lmo Is Required for Robust Circadian Rhythms of Locomotor Activity

Locomotor activity of control (Ctl-1 and Ctl-2) and Lmo mutant (EP1383, EP1306, $hdp^{+3}$, and $hdp^{+3}$) flies was recorded in constant darkness as previously described (Nita-Bakó et al. 2002). (A) Representative actograms of control and Lmo mutants. Control flies show robust circadian rhythms with clear distinctions between activity during the subjective day and inactivity during the subjective night. The pattern of the EP1306 and $hdp^{+3}$ mutants was more stochastic. (B) Graph showing the proportion of strongly rhythmic (white), weakly rhythmic (gray), and arrhythmic (black) flies for each genotype. Most control flies had strong rhythms (28/30 for Ctl-1 and 27/29 for Ctl-2) while Lmo mutants formed a series with an increasing fraction of the flies arrhythmic. For example, 13/26 $hdp^{+3}$ flies were arrhythmic, with the other 13 all having weak rhythms. The power of the rhythm was used to estimate the strength of the activity rhythm, with a power of 300 or more classed as a strong rhythm and a power between 300 and 170 classed as a weak rhythm; arrhythmic rhythms were given a power of 170 (for analysis below). Between 24 and 30 flies were assayed for each genotype. There were no major differences in the period length of the rhythmic flies in each genotype (Ctl-1, 23.6 ± 0.3; Ctl-2, 23.5 ± 0.5; EP1306, 23.2 ± 0.4; EP1306, 23.4 ± 0.3; $hdp^{+3}, 24.2 ± 0.5$; and $hdp^{+3}, 23.4 ± 0.3$). (C) Quantitation of the average power of the rhythm with error bars showing standard error of the mean. One-way ANOVA revealed significant differences between genotypes ($p < 0.0001$). Post-hoc t-tests using a Bonferroni correction revealed that the power of the rhythm was significantly different between control flies and the Lmo mutants $hdp^{+3}$, $hdp^{+3}$, and EP1306 ($p < 0.01$). EP1383 flies had a significantly weaker rhythm than Ctl-2 flies ($p < 0.05$). Ctl-1, $w^{1118}$, is the appropriate genetic control for the $hdp$ alleles (black columns); Ctl-2, EP1631, is the appropriate control for EP1306 and EP1383 (gray columns). All flies tested are in the same genetic background, that of the $w^{1118}$ flies. (D) Quantitation of average activity (beam crossings per minute) with error bars showing standard error of the mean. ANOVA did not reveal significant differences between genotypes at the 0.01 level. Ctl-1, $w^{1118}$, is the appropriate genetic control for the $hdp$ alleles (black columns); Ctl-2, EP1631, is the appropriate control for EP1306 and EP1383 (gray columns). All flies tested are in the same genetic background, that of the $w^{1118}$ flies.

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modulating cocaine’s acute locomotor responses that is independent of their pacemaker function. These findings add to mounting data in Drosophila and mice supporting a role for circadian genes in cocaine-related behaviors (Andretic et al. 1999; S. K. Park et al. 2000; Abarca et al. 2002; Rothenfluh and Heberlein 2002). Our discovery that cocaine actions are modulated by neurons critical for normal circadian locomotor rhythmicity suggests a basis for this overlap.

**Lmo Functions in PDF Neurons to Regulate Cocaine Sensitivity**

We provide several lines of evidence that levels of Lmo expression in PDF-expressing LNs regulate acute sensitivity to volatilized cocaine in Drosophila. First, loss-of-function mutations in Lmo show increased cocaine sensitivity, a defect that can be reversed by induced expression of Lmo in the LNs. Second, Bx mutants, in which Lmo is overexpressed, show reduced cocaine sensitivity; this resistance can be mimicked by overexpression of Lmo in the LNs. The LNs are a group of 8–10 neurons in each brain hemisphere that express the neuropeptide PDF. These neurons have previously been identified as the circadian pacemaker neurons of adult Drosophila and are involved in modulating rhythmic locomotor behavior (Renn et al. 1999; Blanchard et al. 2001). Interestingly, expression of a mouse homolog of Lmo, Lmo4, is highly enriched in the suprachiasmatic nucleus (SCN) (A. Lasek, D. Kapfhammer, and U. H., unpublished data), the mammalian central pacemaker. Furthermore, microarray analysis revealed that in many tissues, Lmo4 expression varies with circadian time (Panda et al. 2002). These data suggest an evolutionarily conserved role for Lmo in clock neuron function.

LMOs are known to act as regulators of LIM-HD protein activity and stability (Retaux and Bachy 2002). LIM-HD proteins are transcription factors involved in many stages
of nervous system development, from neuronal generation and axon guidance to determination of neuronal subtype identity (Hobert and Westphal 2000). In addition, expression of LIM-HD proteins in postmitotic neurons suggests a role in maintaining the differentiated state of these neurons (Hobert and Westphal 2000). The development and structure of the PDF-expressing LNvs, the neurons to which we localize Lmo action, have been studied in detail (Helfrich-Förster 1997). The LNvs can be divided into two groups of 4–5 neurons based on cell body size, projection pattern, and time of development. The s-LNvs, which arise in early larval development, project to the dorsal central brain, terminating near two sets of dorsal neurons that express clock genes at high levels. The 1-LNvs arise during pupal stages and project onto the surface of the optic medulla, as well as to the contralateral LNvs through fibers running in the posterior optic tract. Both s- and 1-LNvs have dense arborizations in the accessory medulla, a neuropil proposed to be a circadian pacemaker center in cockroaches and crickets (Helfrich-Förster 1998). An assessment of s-LNv and 1-LNv numbers and detailed projection patterns revealed no differences between wild-type flies and Lmo mutants. Furthermore, by both quantitative RT-PCR and immunohistochemistry, we determined that PDF levels are normal in Lmo mutants (data not shown). Expression of PDF, the only known LNv output, is restricted to the LNvs and a few tritocerebral neurons. As PDF expression is a specific marker for the differentiated state of the LNvs, it is unlikely that in Lmo mutants these neurons are grossly abnormal in their terminal differentiation.

The absence of obvious structural abnormalities of LNvs suggests that Lmo may play an active role in regulating cocaine responses. In fact, evidence is mounting that the expression and activity of LMOs are dynamically regulated within the nervous system. For instance, expression of the murine Lmo homologs Lmo1, Lmo2, and Lmo3 is differentially regulated by seizure activity in specific regions of the hippocampus and forebrain of adult mice (Hinks et al. 1997). In addition, gene array experiments have revealed that expression of mammalian Lmo homologs is under circadian regulation in the SCN and is increased in the cerebral cortex during sleep deprivation (Cirelli and Tononi 2000; Panda et al. 2002). Furthermore, Lmo3 was isolated as a transcript upregulated by DA administration in cultured astrocytes (Shi et al. 2001). Lastly, Lmo2 and Lmo4 were recently isolated in a screen for calcium-regulated activators of transcription (Aizawa et al. 2004), suggesting a role for LMOs in regulating gene expression changes induced by neural activity. These data are intriguing in light of the many functional changes that occur in reward pathways in the addicted state. Whether LMO activity and/or expression are regulated by acute cocaine exposure remains to be studied.

Separable Roles of LNvs in Regulating Cocaine and Circadian Behaviors

We provide evidence that the LNvs regulate cocaine-induced behaviors, in addition to their well-known role in controlling circadian locomotor behaviors. Flies with LNv ablations show reduced sensitivity to cocaine, establishing that these cells normally increase behavioral responses to cocaine. Our experiments also indicate that LNvs drive circadian locomotor and cocaine behaviors in distinct ways. First, we found that sensitivity to cocaine is not modulated in a circadian manner, showing that the cocaine response is not simply an output of the central clock. Second, we showed that Lmo mutants, pdf mutants, and flies in which LNvs have been electrically silenced or ablated—all known to display similar locomotor rhythm deficits—show completely uncorrelated cocaine sensitivities: increased, unchanged, and reduced sensitivity, respectively. Lastly, we provide evidence that the LNvs outputs that mediate cocaine and circadian behaviors are divergent. PDF, the only known LNv neurotransmitter, is required for normal locomotor rhythms (Renn et al. 1999). pdf null mutants, however, show normal cocaine responses. Furthermore, while synaptic silencing of PDF neurons does not disrupt circadian locomotor activity (Kaneko et al. 2000), we show here that the same manipulation reduces cocaine responses to the same extent as neuronal ablation. Together, these results imply the existence of an alternate, TeTx-sensitive functional output that mediates LNv modulation of cocaine responses. Interestingly, Blau and colleagues have also hypothesized a PDF-independent LNv output that regulates another rapid behavioral response, larval photophobicity (E. Mazzoni, C. Desplan, and J. Blau, unpublished data).

How might LNvs modulate circadian rhythmicity and cocaine sensitivity independently? It is possible that these cells use distinct output mechanisms, PDF to regulate circadian locomotor rhythms and another unknown signal to regulate cocaine sensitivity. Alternatively, these behaviors could be regulated by distinct subsets of LNvs. For example, several recent findings suggest that 1-LNvs may play a lesser role in regulating circadian rhythmicity (Helfrich-Förster 2003). First, 1-LNvs do not project to the dorsal brain, an area implicated in locomotor rhythmicity (Helfrich-Förster 1997). Second, unlike in s-LNvs, molecular clock cycling is not sustained for long in these cells during free-running

Figure 8. Silencing or Ablating PDF Cells Induces Resistance to Cocaine

Ablation of PDF cells with pdf-GAL4 and UAS-hid reduced sensitivity to cocaine compared to parental lines (pdf-GAL4/+ and UAS-hid/+). Electrical (UAS-Kir2.1β or UAS-Kir2.1α) or synaptic (UAS-TeTx) silencing of PDF cells with pdf-GAL4 phenocopied PDF cell ablations. One-way ANOVAs with post hoc planned comparisons revealed a significant effect of genotype for the UAS-hid (p < 0.003, n = 20), UAS-Kir2.1β (p < 0.008, n = 32), UAS-Kir2.1α (p < 0.002, n = 28), and UAS-TeTx (p < 0.001, n = 28) groups, but not for UAS-TeTxα (p > 0.043, n = 28) (n corresponds to the number of experiments). Critical p-value was adjusted to 0.025. Asterisks denote significant differences in both planned comparisons (pdf-GAL4+ and UAS-transgene− versus pdf-GAL4/UAS-transgene). Variations in phenotype of pdf-GAL4 flies for each set of experiments is caused by day-to-day variability.

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LMO-induced changes in receptor expression are not inconceivable given LMO's interaction with LIM-HD proteins. Changes in LIM-HD protein function have been shown to affect aspects of neuronal subtype identity, including neurotransmitter and receptor expression profiles. For instance, mutations in the Drosophila LIM-HD gene islet cause a loss of DA and serotonin synthesis, while ectopic expression leads to ectopic expression of tyrosine hydroxylase, an enzyme required for DA biosynthesis (Thor and Thomas 1997). In addition, expression of a Drosophila DA receptor in larval neurons requires the function of the LIM-HD gene aporter (Park et al. 2004). The observation that DA receptor expression is also altered in various Caenorhabditis elegans LIM-HD mutants (Tsalk et al. 2003) suggests an evolutionarily conserved role of LIM-HD proteins and possibly LMOs in the regulation of neurotransmitter identity and responsiveness. Consistent with this idea is the finding that Lmo homologs are highly expressed in the mesolimbic DA system of mice (A. Lasek, D. Kapfhammer, and U. H., unpublished observations)—a neural pathway involved in the acute stimulant and rewarding properties of abused drugs. We posit that LMOs are ideally suited to modulate in a subtle manner the neurochemical identity and sensitivity of the nervous system to various stimuli, including drugs of abuse.

How Might LNv's Modulate Cocaine Responses

We have demonstrated that LNv electrical activity and synaptic output contribute to cocaine-induced behavioral responses, raising a number of questions regarding the interaction of cocaine with these neurons and their output. We propose a simple model whereby the activity of some or all LNv's directly increases upon cocaine administration, which in turn results in cocaine-induced changes in locomotion (Figure 9). Interestingly, a recent report showed that cultured LNv neurons can respond to either DA or acetylcholine, but not to glutamate, serotonin, octopamine, or histamine (Wegener et al. 2004). The inferred presence of DA receptors on a subset of LNv's provides a potential mechanism by which LNv activity could be directly increased by cocaine administration, as cocaine's primary mechanism of action is to inhibit the reuptake of DA by DAT. In this model, when the LNv's are ablated or silenced, one site of cocaine action would be eliminated, thus reducing cocaine's effect (Figure 9B).

How would Lmo fit into this model? We propose that in Lmo loss-of-function mutants, LNv output is boosted, possibly because of increased expression of DA receptors (Figure 9C), leading to enhanced cocaine sensitivity. Conversely, increased Lmo expression (in Bx mutants or in flies specifically overexpressing Lmo in PDF cells) would result in reduced receptor content and, consequently, in dampened cocaine sensitivity (Figure 9D). Further studies are needed to identify the putative DA receptor (or other molecules) that functions in LNv's to regulate cocaine-induced behaviors.

Figure 9. A Model for LNv and LMO Regulation of Cocaine Sensitivity
(A) In wild type, LNv's modulate locomotor responses via electrical activity and synaptic transmission. We propose a model in which cocaine acts to directly increase LNv activity. Upon cocaine administration, synaptic DA concentrations are increased (via cocaine's inhibition of the plasma membrane DA transporter). Activation of presumed DA receptors on the LNv (dark arrowheads) stimulates electrical activity and subsequent synaptic output. This activity contributes to the behavioral response of the fly to cocaine. (B) LNv ablation eliminates LNv contribution to the cocaine response, reducing cocaine sensitivity. (C) In our model, Lmo loss-of-function mutants (Lmo<sup>lof</sup>), which have increased cocaine sensitivity, have increased activity/output during the cocaine response. This increased activity may be mediated by increases in receptor content on the LNv, or by recruitment of other LNv's that normally do not participate in the cocaine response. (D) Lmo gain-of-function mutants (Lmo<sup>gof</sup>) mutants have reduced LNv output and reduced cocaine sensitivity. This could also result from a reduction in receptor density.

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Materials and Methods

*Drosophila* culture and strains. All flies were maintained on standard cornmeal molasses agar at 25 °C and 70% humidity under constant weak light. The Reth EP collection was obtained from G. M. Rubin (University of California, Berkeley, California, United States) (Reth 1996). The *p[acman]* allele was originally isolated as a genetic screen of *P(GAL4)* insertions (carrying the GawB element) for altered response to the locomotor-activating effects of ethanol (F. Wolf, unpublished data) and consequently tested for cocaine sensitivity. The location of the insertion was determined by inverse PCR (http://www.fruitfly.org/about/methods/inverse.pcr.html). The *hdp-R26* loss-of-function alleles were provided by S. Cohen (European Molecular Biology Laboratory, Heidelberg, Germany) (Milan et al. 1998). *UAS-Lmo* lines were provided by C. Zeng (University of Wisconsin, Milwaukee, Wisconsin, United States) (Zeng et al. 1998). The *hdp-R28* strain was generated by N. Justice by imprecise excision of the EP1306 line, screening for the *hdp* phenotype (unpublished data). *Rs* alleles and *pdf* deficiencies (*Df(3R)T1-X* and *Df(3R)T1-P*) were obtained from the *Drosophila* Stock Center (Bloomington, Indiana, United States). *w*3, *pdf*71 and *pdf-GAL4* flies were obtained from P. Taghert (Washington University, St. Louis, Missouri, United States) (Rehn et al. 1995). *UAS-Ko2.1* lines were obtained from S. Sweeney (University of California, San Francisco, California, United States) (Baines et al. 2001). *UAS-Trx* lines were generated as previously described (Sweeney et al. 1995; Scholz et al. 2000). *UAS-hid* flies were provided by R. S. Stowers (NASA Ames Research Center, Moffett Field, California, United States) (Zhou et al. 1997). *UAS-tauGFP* and *UAS-dilGFP* lines were obtained from W. Ford (University of California, San Francisco). All lines used for behavioral experiments, unless noted below, were out-crossed for five generations to a *w1118* stock isogenic for Chromosomes II and III. Because *pdf*71 and its background control strain (*w33*) are unmarked, only Chromosomes I and II were replaced via crosses to balancer stocks in the *w1118* genetic background.

**Genetic screen and selection of controls.** The approximately 400 X-linked EP lines were initially screened as hemizygous crossed to a *w; XX/Y* tester strain in the crackermap as described below (*n* = 6–9). These lines distributed normally, and 30 lines with phenotypes greater than 1.5 standard deviation from the mean were out-crossed into our *w1118* background and retested. An additional 70 lines were selected to be out-crossed in order to provide a population distribution from which to select control lines. These 100 out-crossed lines were rescreened for acute responses to cocaine. A group of five control EP lines were chosen based on having a normal acute response (near the mode of the distribution). In all behavioral experiments involving EP lines, at least three of these control lines were tested to ensure that the control shown was representative of the EP population. The specific control EP line shown for each experiment is noted in the figure legends. As a control for the *p[acman]* line we used a *P(GAL4)* insertion (line 8142) that shows a normal response to multidrug-modifying drugs (A. Rothermel-Maia, J. Guarneri, A. Rodan, and F. Wolf, unpublished data). For data shown in Figures 1 and 3, all lines were crossed to a *w; XX/Y* tester strain to reduce possible effects of recessive autosomal modifiers. In addition to the genotypes shown, we tested three P-element lines in the *Lmo* locus that did not produce cocaine phenotypes: *MS1096*, which lies 29 bp downstream of the first RA transcript exon, and two additional EP lines, *EP0443* and *EP1394*, inserted 5′ to *EP1383*; the EP lines have normal wings. *MS1096* has been shown to have a very weak wing venation phenotype, but has otherwise not been phenotypically or molecularly characterized (Milan et al. 1998).

**Behavioral statistics.** For cocaine-sensitivity assays, for all behavioral experiments, 15 male flies were collected under CO2 anesthesia 0–2 d post eclosion (day 12), and tested 2–3 d later. Flies were equilibrated to room temperature for at least 1 h before being exposed to cocaine. Cocaine exposures were performed as described previously (McClung and Hirsh 1998; Bainton et al. 2000), and starlike-induced locomotion was assayed using a genetic background previously (Bainton et al. 2000). A drug effect score was determined as the average (measured every minute over 5 min) number of flies that remained on the bottom of the cylinder, expressed as percent of the total number of flies. Significance was established for each experiment as described in figure legends. Generally, either Student’s paired t-tests assuming equal variance or one-way ANOVAs with post hoc Bonferroni planned comparisons or Tukey-Kramer multiple comparisons were performed in GraphPad Prism 4 (GraphPad, San Diego, California, United States). Error bars in all experiments represent the standard error of the mean. To maintain an experiment-wide error rate of *α* = 0.05, the adjusted error rates were *p* = 0.05/n for the n subsequent planned pairwise comparisons in each experiment. To observe cocaine locomotor activity patterns, ten flies were exposed to volatilized cocaine for 1 min, transferred to a 7.5 cm × 10 cm × 0.5 cm acrylic box, and images were captured on an Apple eMac computer (Apple Computer, Cupertino, California) using Adobe Premiere (Adobe Systems, San Jose, California, United States) at 10 frames/s for 5 min. Flight locomotion was tracked using the dynamic image analysis system software (Solltech, Oakdale, Iowa, United States). All genotypes were tested at each dose multiple times (*n* ≥ 3), and representative data were selected for the figures. Baseline negative geotaxis was measured by mock exposures of flies and subsequent assay in the crackermap as above; all genotypes displayed behavioral scores of less than 10%, with no significant differences between genotypes (*n* > 8).

For circadian experiments, locomotor activity of individual flies was measured using the TriKinetics (Walhesh, Massachusetts, United States) infrared beam-crossing system recording total crossings in 10-min bins. Raw activity histograms were analyzed for circadian rhythms using Actimetrics (Wilmette, Illinois, United States) Clock Lab software. Chi-square periodograms were constructed according to Sokolove and Bushell (1978), and significant circadian rhythmicity was defined as presence of a peak in periodogram power that extends above the 0.01 Chi-prosquare significance line. Since this line is equal to a power of approximately 175 at a period of 24 h, flies with no periodogram peak crossing the significance line were assigned a circadian power of 1.0. This would minimize the circadian power of these flies, and thus is conservative with regard to assessing statistical differences in power between genotypes exhibiting frequent arrhythmicity and those that are predominately rhythmic. For the circadian cocaine-sensitivity experiment, flies were set up and raised in LD, collected under CO2 anesthesia during the light phase 1–2 d after eclosion, and placed back in LD for 2–3 d. At the indicated Zeitgeber times, flies were tested in the crackermap, under lights, within 5 min of being removed from LD conditions.

**Histology.** Expression pattern of the *p[acman]* *P(GAL4)* insertion was examined by crossing to *UAS-GFPT2* and *UAS-mCD8GFP*. All adult and larval preparations were dissected in PBS, fixed in 4% formaldehyde/PBS for 40 min, washed in PBS, and dehydrated in 50% glycerol/PBS for 1 h. Tissue was mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, California, United States) and analyzed with a Bio-Rad confocal microscope with Bio-Rad Lasersharp 2000 software (Bio-Rad, Hercules, California, United States). PAP antibody staining (provided by P. Taghert) was performed on CNS preparations that were dissected, fixed, and washed as above. Specimens were incubated in 1:2,000 dilution of anti-PAP PBT, and with a Texas Red–coupled goat anti–guinea pig secondary antibody, diluted 1:250 (Jackson Laboratory, Bar Harbor, Maine, United States).

**Real-time quantitative RT-PCR.** Flies 2- to 4-d-old were collected and frozen immediately at −80 °C. Heads were removed from bodies using fine tweezers, and separated using a needle from heads and bodies by homogenizing the flies in hot phenol and NTE. Complementary DNA (cDNA) was prepared using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, California, United States) according to the manufacturer’s specifications, with the addition of random sequence hexamers to 2.5 μl of each sample. cDNA was analyzed by quantitative, real-time PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The following probes and primers were designed using ABI PrimerExpress software: *Lmo-RA-Forward*, GAAAGAAGAAAACAGACAGCAACA; *Lmo-RA-Reverse*, ATTTGCAATTTGCGACCTGTGTTAGCT; and *Lmo-RA-Probe*, CICTGCGCCGTGCGTCG. *pdf* probe and primers (CT 4640) were obtained from Applied Biosystems. TaqMan PCR reactions consisted of 50 ng of cDNA, 0.9 μM each diagnostic primer, 0.25 μM diagnostic probe, and 1x final of TaqMan Universal PCR Mastermix (Applied Biosystems) in a reaction volume of 25 μl. The TaqMan PCR cycle computer was used with the following conditions: 50 °C for 2 min to activate the TaqMan enzyme, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample was analyzed in triplicate. As negative controls, we used both no-template and DNase- treated non-reverse-transcribed mRNA samples; no significant amplification was observed in these samples. *pdf* transcript levels were used as an endogenous normalization control for all RNA samples, and relative mRNA abundance was calculated using the comparative delta-delta Ct method. This is noted in figure legends. Quantitative RT-PCR analysis was performed on at least two independent RNA preparations, with similar results.
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Author contributions. LYT and UH conceived and designed the experiments. LYT and JB performed the experiments. LYT and JB analyzed the data. RJB participated in the genetic screen that led to the isolation of EP1306 and EP1383. LYT and UH wrote the paper.

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