Cellular/Molecular

Small-Molecule Screen in Adult *Drosophila* Identifies VMAT as a Regulator of Sleep

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Sleep is an important physiological state, but its function and regulation remain elusive. In *Drosophila melanogaster*, a useful model organism for studying sleep, forward genetic screens have identified important sleep-modulating genes and pathways; however, the results of such screens may be limited by developmental abnormalities or lethality associated with mutation of certain genes. To circumvent these limitations, we used a small-molecule screen to identify sleep-modulating genes and pathways. We administered 1280 pharmacologically active small molecules to adult flies and monitored their sleep. We found that administration of reserpine, a small-molecule inhibitor of the vesicular monoamine transporter (VMAT) that repackages monoamines into presynaptic vesicles, resulted in an increase in sleep. Supporting the idea that VMAT is the sleep-relevant target of reserpine, we found that VMAT-null mutants have an increased sleep phenotype, as well as an increased arousal threshold and resistance to the effects of reserpine. However, although the VMAT mutants are consistently resistant to reserpine, other aspects of their sleep phenotype are dependent on genetic background. These findings indicate that small-molecule screens can be used effectively to identify sleep-modulating genes whose phenotypes may be suppressed in traditional genetic screens. Mutations affecting single monoamine pathways did not affect reserpine sensitivity, suggesting that effects of VMAT/reserpine on sleep are mediated by multiple monoamines. Overall, we identify VMAT as an important regulator of sleep in *Drosophila* and demonstrate that small-molecule screens provide an effective approach to identify genes and pathways that impact adult *Drosophila* behavior.

Introduction

Sleep is an important physiological state, as evidenced by the fact that we spend a third of our lives in this state. Additionally, sleep deprivation causes cognitive and health deficits, indicating that it plays an essential role in physiological homeostasis. Sleep is not just required in humans; all well-studied animals exhibit sleep-like states. Among these animals are common model organisms, including mice, zebrafish, flies, and nematode worms (Allada and Siegel, 2008; Mackiewicz et al., 2008; Raizen et al., 2008; Bushey and Cirelli, 2011). These models and others are being used to investigate outstanding questions regarding the purpose of sleep and its regulation.

The rest state in the fly shares many commonalities with human sleep behavior (Shaw et al., 2000, Hendricks et al., 2000). For example, sleeping flies stop moving and assume a stereotyped posture. They also exhibit an increased arousal threshold, meaning that they require a stronger stimulus to reinitiate activity. Importantly, flies display a homeostatic need for sleep, such that they compensate for periods of sleep deprivation with subsequent rebound. *Drosophila melanogaster* follow a diurnal pattern, resting mostly during the night and taking a mid-afternoon "siesta."

The simplicity of behavioral assays using *Drosophila*, combined with the ease of genetic screens, has led many researchers to turn to this model to elucidate the genetic and molecular mechanisms underlying sleep regulation. Genetic screens have uncovered a few low-sleeping mutants, but these studies have not yet led to a cohesive account of sleep regulation. Given that sleep appears to be an essential process (Rechtschaffen et al., 1983; Shaw et al., 2009), it is likely that many sleep-regulating genes are also essential. In other words, loss of these genes may cause lethality or gross developmental problems, precluding their detection in traditional genetic screens. To complement previous genetic screens and to find novel sleep-regulatory molecules and pathways, we conducted a small-molecule screen for sleep phenotypes in adult *Drosophila*. Here, we report the findings from this screen, which indicate a strong effect of monoaminergic neurotransmission in regulating sleep quantity. Using both pharmacological and genetic approaches, we investigated the role of one of our hits in regulating sleep behavior.

Materials and Methods

Flies. Wild-type iso31 flies (Ryder et al., 2004) were used for drug screen and subsequent experiments. $VMAT^{p1}$ mutants were a kind gift of the Krantz laboratory (University of California–Los Angeles, Los Angeles, CA). TrH^{co1440} (BSC10531), $Hdc^{MB07212}$ (BSC25260) mutants were or-

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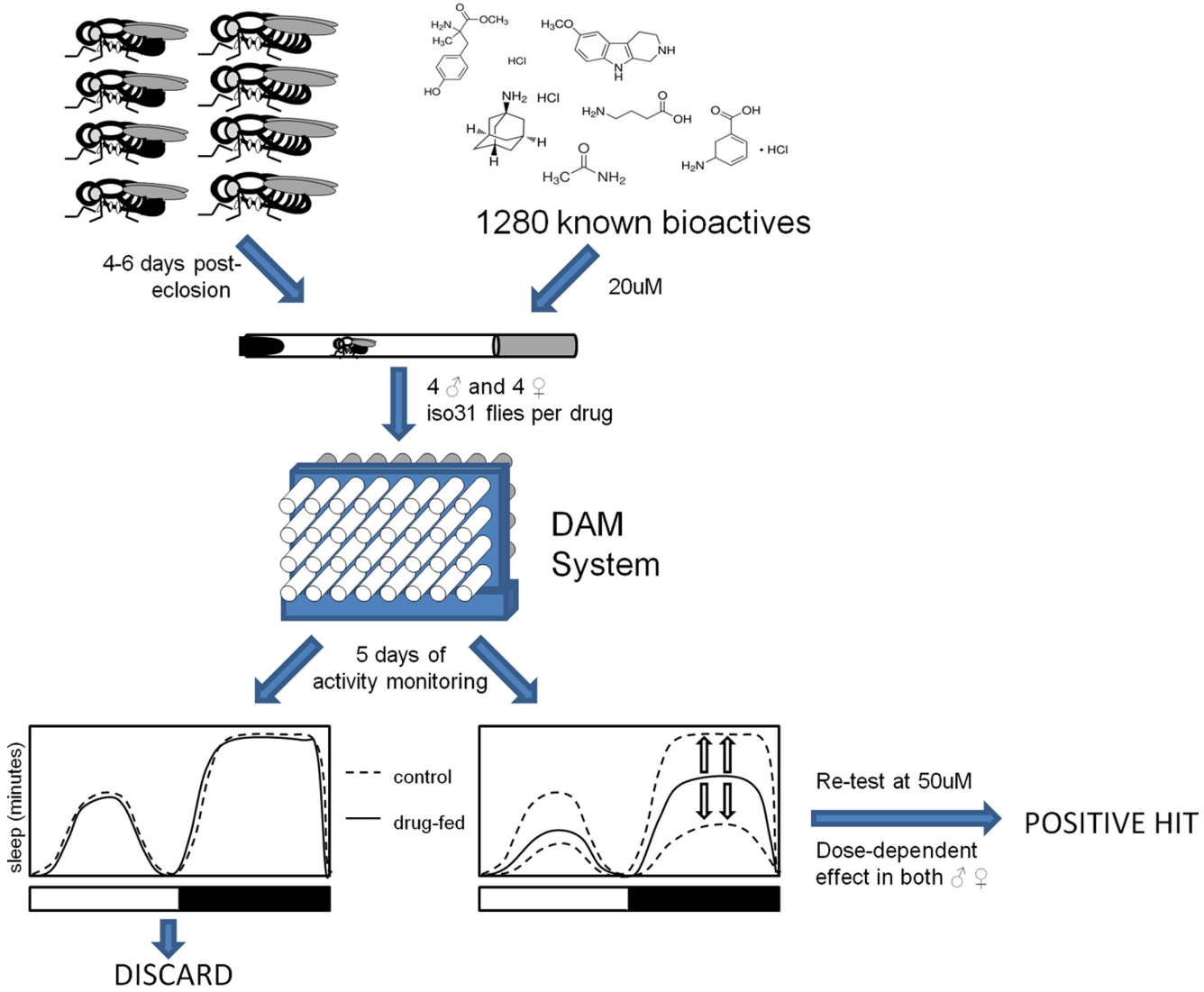

Figure 1. Schematic of the small-molecule screen. Four male and 4 female flies were screened for each of 1280 known bioactive drugs. At 4 – 6 d after eclosion, adult flies were put in tubes with food containing 20 μM of drug. These tubes were placed in monitors, and locomotor activity was measured for 5 d using the Drosophila Activity Monitoring (DAM) System. Using pySolo, sleep profiles were generated for males and females for each drug and compared with flies fed 0.01% DMSO (control). If a drug altered sleep in both males and females, it was retested at 50 μM. If the drug had a dose-dependent effect, it was considered a positive hit.

dered from the Bloomington Stock Center (Bloomington, IN), and *Gad1*^{f00602} was ordered from the Exelixis collection at Harvard Medical School. The temperature-sensitive tyrosine hydroxylase mutant *ple*^{ts} (Pendleton et al., 2002) was a kind gift from Dr. Ralph Hillman (New York University, NY). The octopamine synthesis mutant *TbH*^{nm18} was previously published (Crocker and Sehgal, 2008).

Drug feeding. We used the LOPAC 1280 drug library (Sigma-Aldrich), which is made up of bioactive molecules with known molecular targets, of which approximately half are involved in neurotransmission. Four- to 6-day-old adult isogenic (iso31) flies were given access to drugs at 20 μM, mixed into their 2% agar and 5% sucrose food, *ad libitum* for 1 week. Flies were kept in incubators at 25°C on a 12 h light/dark schedule. During this time, locomotor activity of flies was monitored using the Drosophila Activity Monitoring System (Trikinetics). Sleep behavior was calculated and averaged for four male flies and four female flies per drug treatment. Sleep graphs and calculations of sleep quantity for all experiments were generated using PySolo (Gilestro and Cirelli, 2009). For drugs that produced qualitative changes in sleep profile or quantitative changes in total minutes of sleep per day, four male and four female flies were tested again at 50 μM drug. Drugs that showed a reproducible, dose-dependent effect on sleep quantity were considered screen hits. For reserpine (Sigma-Aldrich), the stock solution was made at 10 mM in DMSO. This stock solution was diluted in 2% agar/5% sucrose food to final concentrations of 20 and 50 μM for the original screen and 10 μM for all subsequent experiments. A total of 0.2% DMSO vehicle controls were used as a comparison for 20 μM drug feeding during the screen, and 0.1% DMSO vehicle controls were used as a comparison for 10 μM reserpine feeding in subsequent experiments. For the *ple*^{ts} mutants, flies were kept at a restrictive temperature of 29°C for 24 h before placing on DMSO and reserpine and were kept at this temperature for the duration of behavioral monitoring.

Arousal threshold. Arousal threshold assay was conducted as previously published (Wu et al., 2008). Mechanical stimuli were applied manually by tapping a dowel on the behavior tubes containing the flies. Weak (one light tap), medium (one strong tap), and strong (six strong taps) stimuli were applied to behavior tubes at ZT16, ZT18, and ZT20, respectively. The percentage of spontaneously sleeping flies awoken was calculated for each genotype and stimulus.

Sleep deprivation. Flies were deprived during the final 6 h of the night (ZT18-ZT24) using a vortex to shake flies for 2 s of every 20 s, at random intervals (Huber et al., 2004). Amount of sleep lost was calculated by subtracting minutes of sleep during deprivation from the minutes of

sleep during the same interval on the previous night. Sleep regained the following morning was calculated by subtracting the minutes of sleep during the first 3, 6, or 12 h the morning before deprivation from the same interval after deprivation.

PCR. The wild-type vesicular monoamine transporter (VMAT) allele was genotyped using VMATp1-F (5′-ATC GGG GGA TGC TTG ATA TT-3′) and VMATp1-R (5′-ATC CGA ATC GGG AAC AGA T-3′) primers, and the mutant $VMAT^{p1}$ allele was genotyped using the Plac1 (5′-CAC CCA AGG CTC TGC TCC CAC AA-3′) primer and VMATp1-R primers. PCR was conducted with GoTaq Flexi (Promega), with the following cycling conditions: 95°C for 2 min, then 30 cycles of 95°C for 30 s, 52°C for 1 min, 72°C for 1 min, and final extension at 72°C for 5 min.

Sleep latency. Latency to sleep was calculated by counting the number of minutes between lights off and the first stretch of 5 consecutive minutes with zero beam crosses, as recorded by the Drosophila Activity Monitoring System.

Results

A small-molecule screen identifies sleep-modulating compounds

We screened 1280 small molecules for their effect on sleep:wake rhythms in the adult fly (Fig. 1). Observation of daily locomotor behavior allowed for quantitative comparisons of total sleep time, daytime and nighttime sleep, and qualitative assessment of sleep patterns, rhythm strength, and anticipation of light/dark transitions. Each drug was assayed in a limited number of flies to enhance throughput; therefore, only drugs with strong effects on sleep were identified as having an effect above individual variation. Additionally, all compounds were fed to flies at a relatively low dose that caused minimal lethality. Many of these drugs showed an effect in only one sex and were discarded from further testing. Even with these constraints, we were able to identify 38 compounds that affected sleep at the initial concentration. Only those drugs found to have a dose-dependent effect on sleep in both sexes when tested at a higher concentration were considered hits. With these stringent criteria, we initially found six compounds that qualified: five that decreased sleep and one that increased sleep. One of the sleep-reducing compounds, the cholinergic agonist carbachol, did not continue to have an effect in subsequent studies (data not shown). The four remaining sleep-reducing drugs caused a significant reproducible decrease in nighttime sleep at a 20 μM concentration and a further reduction in sleep levels at 50 μM. These sleep-promoting drugs are pergolide methanesulfonate (Fig. 2*a*), R(−)-2,10,11-trihydroxyaporphine hydrobromide (Fig. 2*b*), paliperidone (Fig. 2*c*), and 1,3-dipropyl-7-methylxanthine (Fig. 2*d*). The screen revealed a single sleep-promoting drug, reserpine (Fig. 2*e*). Reserpine caused a significant increase in sleep during both the day and night, especially at light/dark transitions when flies are most active ($p = 0.000161$ by one-way ANOVA with Tukey *post hoc* comparison for both the 20 μM and 50 μM reserpine-fed flies compared with DMSO controls). In female flies, sleep increased by 400 min at 20 μM reserpine and by 470 min at 50 μM reserpine (Fig. 3*a*). Male flies showed a similar significant behavioral response to drug treatment (data not shown).

Genetic ablation of VMAT alters sleep behavior

Reserpine is an inhibitor of the VMAT. To determine whether the effects of reserpine on sleep were mediated by its inhibition of VMAT, we compared the sleep phenotype of reserpine-treated flies with that of a VMAT-null mutant, $VMAT^{p1}$ (Simon et al., 2009). The $VMAT^{p1}$ homozygous mutant has the same significantly increased sleep quantity as flies fed 50 μM reserpine ($p = 0.000144$ for $VMAT^{p1}/VMAT^{p1}$ compared with iso31 as shown by one-way ANOVA with Tukey *post hoc* comparison) (Fig. 3*a*).

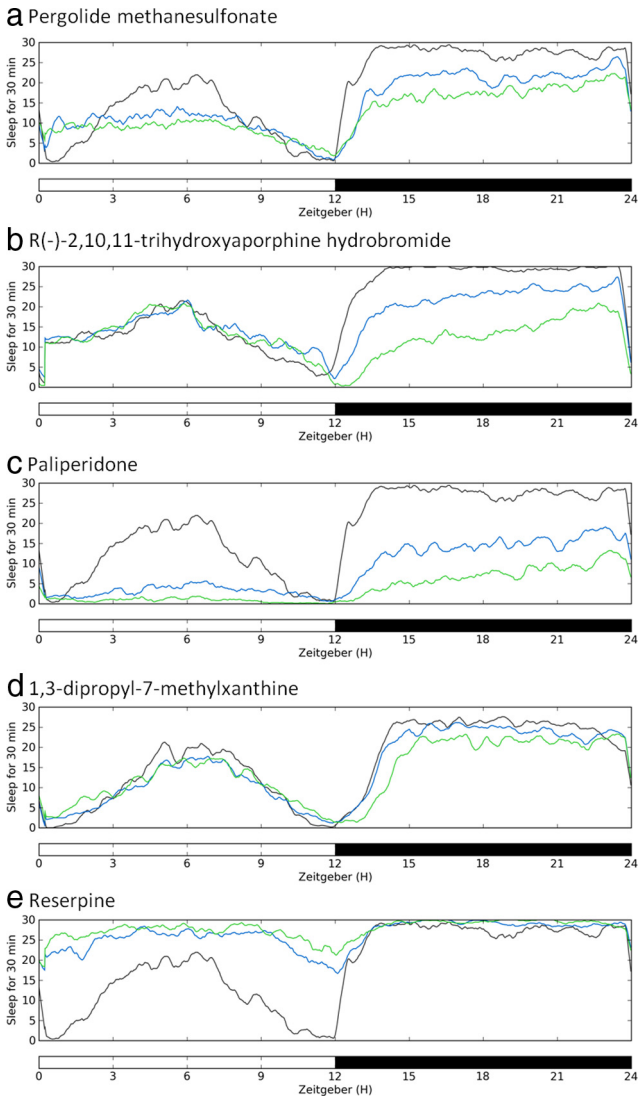

Figure 2. Small-molecule screen identifies sleep-modulating compounds. Sleep profiles for female flies fed 0.1% DMSO (black line), 20 μM drug (blue line), and 50 μM drug (green line) for each of the positive hits from the screen: pergolide methanesulfonate (***a***), R(−)-2,10,11-trihydroxyaporphine hydrobromide (***b***), paliperidone (***c***), 1,3-dipropyl-7-methylxanthine (***d***), and reserpine (***e***). Sleep is plotted as minutes of sleep per sliding 30 min window across a 24 h period (12 h light, white bar; and 12 h dark, black bar) with averaged data from 4 female flies over 5 d of recording.

Importantly, inhibition of VMAT by drug treatment or genetic mutation does not render flies hypoactive, as measured by activity index (activity per waking minute) (Fig. 3*b*), indicating that the effect is specific for sleep.

We next asked whether inhibition of VMAT causes increased sleep depth in addition to increased sleep quantity. To assess sleep depth, we measured arousal threshold, in other words, the ability of the animal to wake up with sensory stimulation. We delivered weak, medium, and strong stimuli to flies at different times of the night and counted the number aroused in different fly lines. We found that a smaller percentage of the drug-treated iso31 and the $VMAT^{p1}$ homozygous mutants awaken in response to these mechanical stimuli, compared with untreated iso31 flies (Fig. 3*c*). The contribution of genotype to arousability at all stimulus intensities was significant ($p < 0.0001$ by two-way ANOVA).

We also measured the effect of VMAT inhibition on the homeostatic rebound that follows a period of sleep deprivation. We

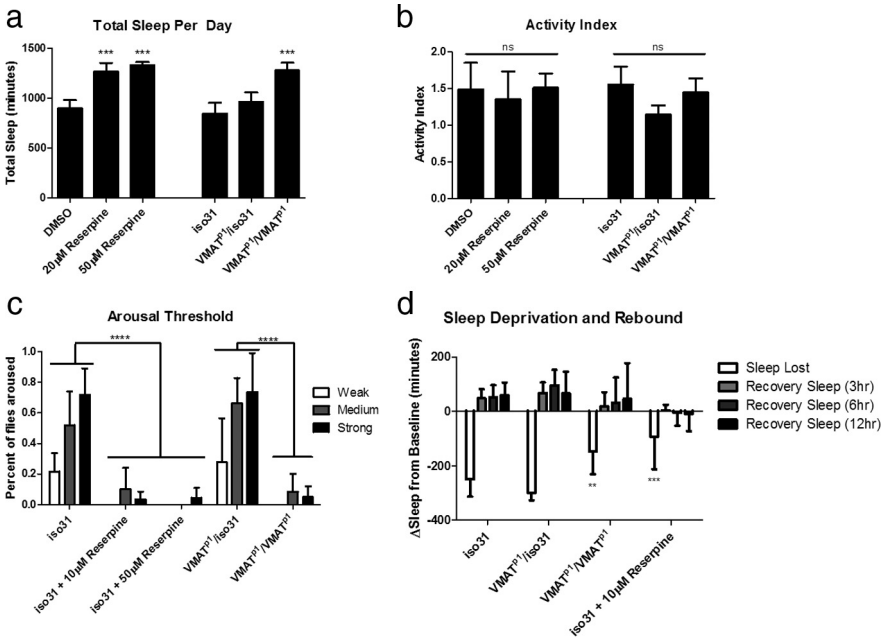

Figure 3. Genetic ablation of *VMAT* alters sleep behavior. ***a***, Total minutes of sleep per 24 h day plotted for wild-type (iso31) flies fed 0.1% DMSO (vehicle control), 20 μM reserpine, and 50 μM reserpine, as well as for iso31, $VMAT^{p1}$/iso31, and $VMAT^{p1}/VMAT^{p1}$ fed sucrose/agar food. $n = 8$ for each genotype/treatment. ***$p < 0.001$. ***b***, Activity index (infrared beam crosses per waking minute) for these reserpine-treated and $VMAT^{p1}$ mutant flies shows these flies are not hypoactive. ***c***, The percentage of sleeping flies that were aroused from sleep by a weak (white bars), medium (gray bars), or strong (black bars) stimulus at ZT16, ZT18, or ZT20, respectively. Data are averaged from three separate experiments. ****$p < 0.0001$, the effect of treatment/genotype on arousability. ns, Not significant. ***d***, Flies were deprived of sleep during the second half of the night (ZT18-ZT24). Minutes of sleep lost during this period (white bar) are plotted as a negative number, and rebound sleep was measured during the first 3 (light gray bar), 6 (gray bar), and 12 h (black bar) the following morning. **$p < 0.01$, the amount of sleep lost compared with iso31 controls. ***$p < 0.001$, the amount of sleep lost compared with iso31 controls. $n = 16$ for each genotype/treatment.

deprived flies of sleep during the second half of the night and assayed rebound sleep the following morning. Drug-treated and homozygous mutant flies were less effectively deprived than wild-type and heterozygous flies ($p = 0.00322$ and $p = 0.000185$ for iso31 vs $VMAT^{p1}/VMAT^{p1}$ and reserpine-treated flies, respectively, by one-way ANOVA with Tukey's *post hoc*), further supporting the idea that sleep depth is increased by inhibition of VMAT. Mutant and drug-treated flies experienced slightly less rebound sleep as well, although this difference did not reach significance because of large interindividual variance (Fig. 3*d*). The apparent reduction in sleep rebound by these flies is likely the result of the relatively ineffective deprivation and already elevated baseline sleep levels.

Effects of reserpine on sleep map to the VMAT gene

In $VMAT^{p1}$ flies, the VMAT gene is disrupted by insertion of a PLacW transposon in the fifth exon of the *VMAT* gene (Bellen et al., 2011). To confirm the presence of this transposon, we genotyped $VMAT^{p1}$ flies by PCR. The wild-type VMAT allele, detected using primers specific for the VMAT genomic sequence, was amplified from iso31 flies and from flies heterozygous for the mutation, but not from homozygous mutants. Heterozygous and homozygous mutants were positive for the mutant *VMAT* allele, which was detected using one *VMAT* primer and one transposon-specific primer (Fig. 4*a*,*b*).

If effects of reserpine on sleep are mediated through inhibition of VMAT, then *VMAT* mutants should be resistant to reserpine. Indeed, the $VMAT^{p1}$ mutant does not show a further sleep increase after reserpine administration (Fig. 4*c*). Additionally, reserpine resistance of the $VMAT^{p1}$ mutant is not complemented by the deficiency Df(2R)BSC306, which spans the *VMAT* locus (Fig. 4*c*). Together, these data indicate that *VMAT* mutants are resistant to reserpine, and the effect maps to the *VMAT* locus.

The sleep phenotype of VMAT mutants is background dependent

To determine whether the sleep phenotype of the $VMAT^{p1}$ mutation is independent of genetic background, we outcrossed the mutation for five generations into an iso31 background. The presence of the transposon was confirmed by PCR (Fig. 4*b*). Surprisingly, outcrossed *VMAT* mutant flies ($VMAT^{p1}(5x)$ flies) slept for approximately the same number of minutes per day as the iso31 control flies (846 min/d for iso31 females vs 881 min/d for $VMAT^{p1}(5x)$/$VMAT^{p1}(5x)$; Fig. 5*a*,*b*). Despite the largely normal baseline sleep behavior, however, the $VMAT^{p1}(5x)$ flies consistently exhibited decreased sleep latency, which means that they fell asleep more quickly than wild-type or heterozygous flies after lights out ($p = 0.035$ by unpaired *t* test with Welch's correction for unequal variances; Fig. 5*c*). Additionally, these mutant flies retained an increased arousal threshold after outcrossing (Fig. 5*d*), similar to the original mutant strain ($p = 0.0100$ by two-way ANOVA for genotype contribution to arousability). Also, like the original mutant, the $VMAT^{p1}(5x)$ mutants were less efficiently sleep-deprived ($p = 0.0003$ by one-way ANOVA) but showed a rebound the following morning proportional to the sleep lost during deprivation (Fig. 5*e*).

Importantly, $VMAT^{p1}(5x)$ mutants were still resistant to the sleep-promoting effects of reserpine (Fig. 5*b*). Because these mutants have normal baseline sleep, the lack of a response to reserpine cannot be the result of a "ceiling" effect. These data establish that VMAT is required for effects of reserpine on sleep.

Effects of reserpine on mutants of different monoaminergic systems

VMAT is a transporter protein that packages all monoaminergic neurotransmitters into presynaptic vesicles. To determine which of the monoamines is responsible for the sleep phenotype produced by reserpine, we fed reserpine to mutants deficient for the various monoamines. Mutants deficient in the synthesis of dopamine (ple^{ts}), serotonin (TrH^{c01440}), octopamine (TbH^{nm18}), histamine ($Hdc^{MB07212}$), and the amino acid-derived neurotransmitter GABA ($Gad1^{f00602}$) all responded to reserpine (Fig. 6). Because dopamine synthesis is required during development, the only viable mutant (*ple*) is temperature-sensitive, and so the drug treatment and sleep behavior for this mutant were measured at the restrictive temperature of 29°C. Reserpine increased sleep significantly for all of the neurotransmitter mutants (comparing total sleep of DMSO controls with 10 μM reserpine-treated flies of the following genotypes, $p = 0.000139$ for iso31, $p = 0.00355$ ple^{ts} at 29°C, $p = 0.000144$ for TrH^{co1440}, $p = 0.000137$ for TbH^{nm18}, $p = 0.000137$ for $Hdc^{MB07212}$, and $p = 0.000151$ for $Gad1^{f00602}$ by two-way ANOVA

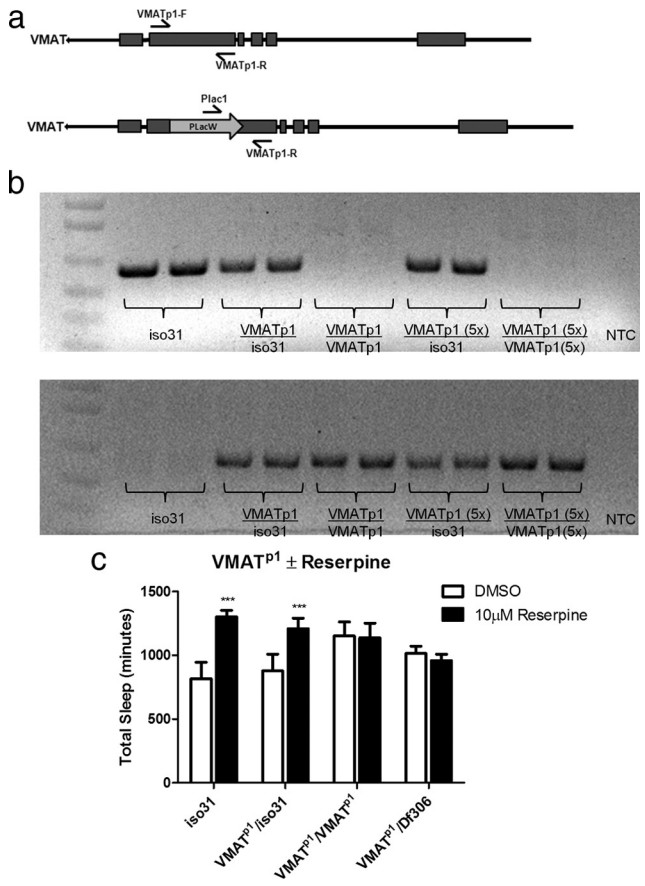

Figure 4. Effects of reserpine on sleep map to the *VMAT* gene. ***a***, The VMAT gene, with dark gray boxes representing exons, showing locations of PCR primers used to genotype the wild-type *VMAT* (top) and P-element-containing mutant $VMAT^{p1}$ (bottom) alleles. ***b***, PCR amplifies the wild-type *VMAT* allele (top) in iso31 and $VMAT^{p1}$/iso31 heterozygous flies and the $VMAT^{p1}$ mutant allele (bottom) in heterozygous and homozygous $VMAT^{p1}$ mutant flies. ***c***, Total minutes of sleep per 24 h day for wild-type, heterozygous mutant, homozygous mutant, and mutant/deficiency *trans*-heterozygotes fed 0.1% DMSO (white bars) or 10 μM reserpine (black bars). $^{***}p < 0.001$. $n = 16$ for each genotype/treatment.

with Bonferroni multiple comparisons), indicating that no single neurotransmitter system is required for sleep-promoting effects of reserpine. VMAT inhibition likely increases sleep by interfering with the signaling from more than one neurotransmitter system simultaneously.

Discussion

Genetic screens for sleep phenotypes have led to the isolation of a few mutants, including *Shaker* (Cirelli et al., 2005), *sleepless* (Koh et al., 2008), *insomniac* (Stavropoulos and Young, 2011), and cyclinA1 (Rogulja and Young, 2012). Other mutations that cause reduced sleep were identified by chance, including *fumin* (Kume et al., 2005) and several mutations in the calcineurin signaling pathway (Nakai et al., 2011), or through assays of candidate genes (Yuan et al., 2006; Crocker and Sehgal, 2008, 2010; Sehgal and Mignot, 2011). Although these studies give valuable insight into molecular underpinnings of sleep behavior, they do not paint a complete picture of the molecular machinery of sleep regulation.

We note that traditional genetic screens may be limited in their ability to uncover molecules that regulate behavior because of factors, such as redundancy, lethality, developmental compensation, and developmental defects, which may mask or conflate adult phenotypes. The study of sleep is particularly susceptible to these limitations, as long-term sleep deprivation leads to death (Rechtschaffen et al., 1983; Shaw et al., 2009). In addition, sleep-regulating genes tend to also be required for other functions. One way to bypass the limitations intrinsic to traditional genetic screens is to use adult-specific manipulations. We asked whether we could use a small-molecule screen to discover new sleep-modulating proteins.

Small-molecule screens in whole animals are rare, especially when measuring a behavioral output. A small-molecule screen for aberrant sleep behavior in zebrafish assayed larvae, through automated methods, for effects of almost 4000 drugs (Rihel et al., 2010). Conducting a drug screen in *Drosophila*, although more labor-intensive, was important to find new sleep-modulating molecular targets in a well-established sleep model. Additionally, an unparalleled genetic toolkit is available in *Drosophila* for confirming and elaborating on drug screen findings.

In the screen reported here, we searched for drugs that dose-dependently and reproducibly affected sleep behavior in both male and female adult flies. Two of the compounds that met these strict criteria are dopamine receptor agonists. Other drugs that increase dopamine signaling, such as methamphetamine and cocaine, are known to increase arousal in humans and model organisms, including *Drosophila*. Inhibition of dopamine biosynthesis biochemically (Andretic et al., 2005) and genetically (Riemensperger et al., 2011) has the opposite effect, increasing sleep amount in the fly. Also, the dopamine type 1 receptor, DopR, promotes arousal at appropriate times in the circadian sleep/wake cycle (Lebestky et al., 2009). The identification of small molecules targeting dopaminergic signaling validates the power of the drug screen to identify sleep-regulatory pathways.

Another sleep-inhibiting molecule identified in the screen was methylxanthine, which is a caffeine analog. Caffeine is well known as a robust wake-promoting stimulant. Although the target of its action in *Drosophila* is still unclear, its effects on behavior are similar to those in mammals/humans (Wu et al., 2008). As in the case of the molecules that affect dopaminergic signaling, the identification of a caffeine analog speaks to the efficacy of the small-molecule screen reported here. This screen also identified an atypical antipsychotic, paliperidone, as a sleep-inhibiting molecule. The target of this antipsychotic is not known, but its effect on sleep supports reports of links between sleep and affective disorders (Wulff et al., 2010).

Surprisingly, only one drug from the screen was found to increase sleep: reserpine. Although reserpine, which is typically used to treat hypertension and is also indicated as an antipsychotic, was shown many years ago to have a tranquilizing effect, it has not been mechanistically linked to sleep (Monroe et al., 1955; Steiner et al., 1963). We now have the genetic tools to understand the nature of this effect in a controlled and systematic manner and its implications for the normal regulation of sleep and wake. Reserpine inhibits the function of the VMAT, a transmembrane protein that transports monoaminergic neurotransmitters into presynaptic vesicles to prepare them for release. Vertebrates have two VMAT genes, VMAT1 and VMAT2, whereas flies have only one.

One common caveat in pharmacological studies is the possibility of off-target effects. We show that a $VMAT^{p1\text{-}null}$ mutant (Simon et al., 2009) has increased sleep. More importantly, this mutant is resistant to the effects of reserpine, indicating that the long-sleeping phenotype is not the result of off-target effects. An apparent increase in sleep can sometimes result from sickness or physical impairment. However, despite sleeping significantly more, the reserpine-fed and $VMAT^{p1}$ mutant flies do not have a decreased activity index. In addition, outcrossed *VMAT* mutants, which have normal levels of baseline sleep, are unresponsive to

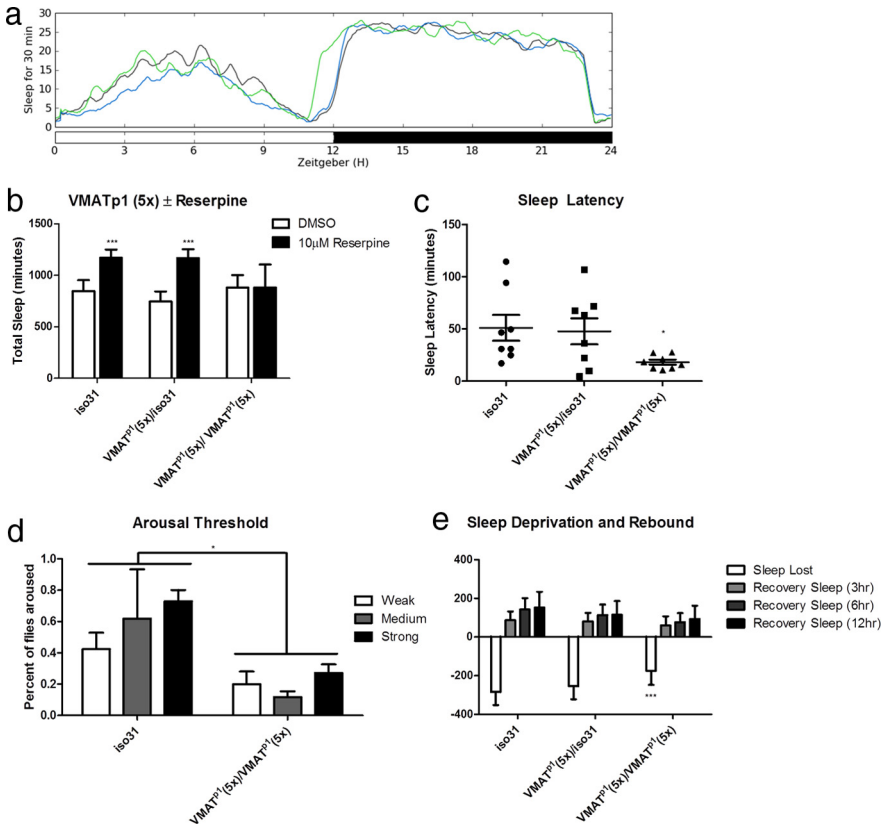

Figure 5. The sleep phenotype of VMAT mutants is background dependent. ***a***, Sleep profile for iso31 (black line), $VMAT^{p1}(5x)$/iso31 (blue line), and $VMAT^{p1}(5x)/VMAT^{p1}(5x)$ (green line) outcrossed mutant flies. Sleep is plotted as minutes of sleep per sliding 30 min window across a 24 h period (12 h light, white bar; and 12 h dark, black bar) with averaged data from 16 female flies over 5 d of recording. ***b***, Total sleep per 24 h period quantified for these flies fed 0.1% DMSO (white bars) or 10 μM reserpine (black bars). $^{***}p < 0.001$. ***c***, Scatter plot of latency to sleep, or minutes between lights off and the first sleep bout. Horizontal line corresponds to group mean. $^{*}p < 0.05$. ***d***, The percentage of sleeping flies that were aroused from sleep by a weak (white bars), medium (gray bars), or strong (black bars) stimulus at ZT16, ZT18, or ZT20, respectively. Data are averaged from three independent experiments. $^{*}p < 0.05$, the effect of genotype on arousability. ***e***, Flies were deprived of sleep during the second half of the night (ZT18-ZT24). Minutes of sleep lost during this period (white bar) are plotted as negative numbers, and rebound sleep was measured during the first 3 (light gray bar), 6 (gray bar), and 12 h (black bar) the following morning. $^{***}p < 0.001$, the amount of sleep lost compared with iso31 controls. $n = 16$ for each genotype.

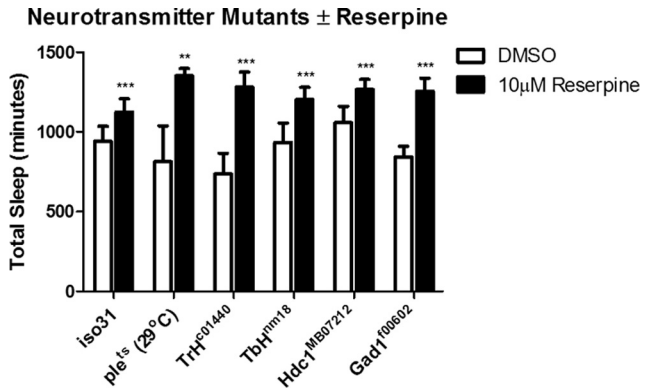

Figure 6. Effects of reserpine on mutants of different monoaminergic systems. Total sleep per 24 h period plotted for mutants defective in the synthesis of the neurotransmitters dopamine (ple^{ts} at 29°C), serotonin (TrH^{co1440}), octopamine (TbH^{nm18}), histamine ($Hdc^{MB07212}$), and GABA ($Gad1^{f00602}$), fed 0.1% DMSO (white bars) or 10 μM reserpine (black bars). $^{**}p < 0.01$. $^{***}p < 0.001$.

reserpine. Together, these data show that reserpine specifically increases sleep by inhibiting VMAT.

Alterations in sleep duration are often accompanied by changes in sleep depth, as measured by arousal threshold. Previous studies have shown that short-sleeping mutants tend to have decreased arousal thresholds during normal sleep (Koh et al., 2008). Interestingly, the long-sleeping $VMAT^{p1}$ mutant has an increased arousal threshold, suggesting that they sleep more deeply (Andretic and Shaw, 2005). In general, loss of VMAT appears to increase sleep drive or decrease the ability to maintain wakefulness, as demonstrated also by the increased latency to sleep in flies carrying a five-generation outcrossed $VMAT^{p1}$ allele. These outcrossed flies no longer have increased daily sleep, but they also display an increased arousal threshold. On the other hand, $VMAT^{p1}$ flies have a normal rebound after deprivation, supporting the idea that the response to sleep deprivation is less tightly correlated with other measures of sleep.

The less severe phenotype of the outcrossed allele is consistent with other studies that have noted the importance of genetic background in animal behaviors, including sleep (Zimmerman et al., 2012). In an iso31 background, inhibition of VMAT throughout development with the $VMAT^{p1}$ mutation does not alter daily sleep, but sleep is increased sleep when VMAT is inhibited acutely in adults. Thus, developmental compensation mechanisms may account for the discrepancy between the sleep phenotype of the drug-fed and mutant flies in the iso31 genetic background. The original mutant background likely confers less developmental compensation, as these flies have the same long-sleeping phenotype as the drug-fed flies.

VMAT plays a presynaptic role in signaling by many different neurotransmitters, including the monoamine neurotransmitters dopamine, serotonin, histamine, and octopamine. Additionally, recent evidence suggests that VMAT transports the amino acid neurotransmitter GABA (Tritsch, 2012). Many of these neurotransmitters, including dopamine (Andretic et al., 2005; Kume et al., 2005), octopamine (Crocker and Sehgal, 2008), serotonin (Yuan et al., 2006), and GABA (Agosto et al., 2008), have been independently implicated in regulating sleep behavior. We found that mutants deficient for each of these neurotransmitters displayed increased sleep after reserpine feeding, suggesting that no single neurotransmitter system accounts for the impact of VMAT inhibition on sleep. Similarly, Chen et al. (2013) found that circadian rhythms are perturbed in $VMAT^{p1}$ mutants, and rescue of this phenotype requires VMAT in multiple neuronal populations. In mammals as well, Coulter et al. (1971) demonstrated that the effect of reserpine on sleep cannot be attributed to reductions in serotonin or norepinephrine. Our findings contribute to a picture of sleep regulation driven by a robust network of neurotransmission that requires VMAT in multiple neuronal populations.

Altered VMAT function has previously been studied in the context of many neuropsychiatric and neurological diseases, including depression, bipolar disorder, schizophrenia, and Parkinson's disease

(Wimalasena, 2011). These diseases are accompanied by an increased prevalence of sleep perturbations, although these have not yet been linked to VMAT. Understanding the role of VMAT in sleep may elucidate the pathophysiology of sleep perturbations in the disorders noted here, as well as the natural regulation of sleep in healthy flies and humans.

The potential for screens in *Drosophila* to identify drugs for human use is high. Although numerous side effects make reserpine suboptimal as a treatment, more specific inhibitors of VMAT-2 may be tolerated better by patients and improve their use as a sleep aid. The screen reported here identified a single sleep-promoting drug, but expanded screens could identify many more potential pharmacotherapies. We used a drug library with known biological targets, which may have biased the findings toward well-studied pathways. Additionally, a low concentration of drug was used to reduce lethality, meaning that only drugs with the strongest impacts on sleep were found. Now that the utility of these screens has been proven, larger and more aggressive screens can be used to identify other novel modulators of behavior.

In conclusion, we used a small-molecule screen to discover regulators of sleep phenotype. Using a genetic approach to confirm one of these drugs, we found that VMAT is required to establish normal sleep duration and arousal state, presumably by regulating transmission of several neurotransmitters. The role of genetic background in the expressivity of the VMAT phenotype highlights the strong effect of developmental compensation on behaviors, such as sleep, and the importance of targeting pathways acutely in adults to look at adult behavior. Small-molecule screens in live animals provide a powerful tool for dissecting molecular mechanisms of adult behavior.

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