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Dopaminergic Modulation of Arousal in *Drosophila*

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Summary

Background: Arousal levels in the brain set thresholds for behavior, from simple to complex. The mechanistic underpinnings of the various phenomena comprising arousal, however, are still poorly understood. *Drosophila* behaviors have been studied that span different levels of arousal, from sleep to visual perception to psychostimulant responses.
Results: We have investigated neurobiological mechanisms of arousal in the *Drosophila* brain by a combined behavioral, genetic, pharmacological, and electrophysiological approach. Administration of methamphetamine (METH) suppresses sleep and promotes active wakefulness, whereas an inhibitor of dopamine synthesis promotes sleep. METH affects courtship behavior by increasing sexual arousal while decreasing successful sexual performance. Electrophysiological recordings from the medial protocerebrum of wild-type flies showed that METH ingestion has rapid and detrimental effects on a brain response associated with perception of visual stimuli. Recordings in genetically manipulated animals show that dopaminergic transmission is required for these responses and that visual-processing deficits caused by attenuated dopaminergic transmission can be rescued by METH.
Conclusions: We show that changes in dopamine levels differentially affect arousal for behaviors of varying complexity. Complex behaviors, such as visual perception, degenerate when dopamine levels are either too high or too low, in accordance with the inverted-U hypothesis of dopamine action in the mammalian brain. Simpler behaviors, such as sleep and locomotion, show graded responses that follow changes in dopamine level.

Introduction

Behavioral performance is determined to a large degree by an animal's level of arousal. An optimal arousal level is required for proper cognitive and motor performance, and it is the result of an interaction between mechanisms controlling endogenous states and stimuli from the environment. An understanding of neural mechanisms determining the arousal level underlying behaviors is essential for understanding both normal and aberrant states.

The extensive literature on the effects of psychostimulants such as cocaine, amphetamine, and methamphetamine on brain function and behavior universally point to the arousing properties of these drugs. The multiple behavioral consequences of psychostimulant administration have all been associated with changes in the extracellular concentration of the neurotransmitters dopamine, serotonin, and noradrenaline. Psychostimulants either block transporters for these neurotransmitters, thereby preventing their clearance from the synaptic cleft (cocaine), or in addition promote their release from the presynaptic neuron (amphetamines) [1, 2]. The arousing impact of psychostimulants depends on the dose given and spans a range of cognitive and motor effects, from those that are beneficial at low doses to those that are detrimental for cognitive and behavioral functioning at higher doses. Low doses in humans improve selective attention, reaction time, and accuracy [3–5]. In contrast, high doses induce hyperactive and stereotypical locomotor activity in rodents and lead to impulsive and distractive behavior in humans and rodents [6, 7]. Psychostimulants are also widely used in treatments for narcolepsy; their arousing effects suppress sleep and consolidate periods of wakefulness [8, 9]. Furthermore, psychostimulants counteract the negative effects of sleep deprivation by improving cognitive and motor performance in humans during periods of extended wakefulness [10, 11]. Whereas hyperactivity and the reinforcing effects of psychostimulants leading to addiction have been studied extensively, much less is known about the arousal-inducing effects at low doses.

Attempts to understand the consequences for sleep and arousal of low psychostimulant doses have focused on the role of dopamine. Wake-promoting effects of METH in rodents have most often been associated with the enhancement of dopaminergic transmission, decreased activity of dopamine transporters, and stimulation of D1 and D2 receptors [12, 13]. Studies in rodents, in which the wake-promoting effects of amphetamine and/or methylphenidate were compared to those of the stimulant caffeine, indicated that psychostimulant effects depend on the enhancement of dopaminergic transmission whereas caffeine effects do not [9]. Electrophysiological and microdialysis studies from mammalian brains argue for the activity of noradrenergic neurons from the locus coeruleus in maintaining wakefulness [14, 15]. However, there seems to be agreement that activation of dopaminergic transmission predominates as a mechanism through which psychostimulants maintain wakefulness [12].

As in mammals, *Drosophila* exhibits behavioral states spanning the full continuum of arousal, from general anesthesia and sleep to visual discrimination [16]. Inactive states that predominate during the night, and which are associated with increased arousal thresholds and decreased brain activity, are analogous to sleep in mammals [17–20]. On the other extreme of this continuum, volatilized cocaine induces hyperactive and stereotypical behaviors, and intermittent exposure to the same drug concentration will lead to behavioral sensitization [21]. Recent advances in recording of brain activity from flies responding to sensory stimuli have made

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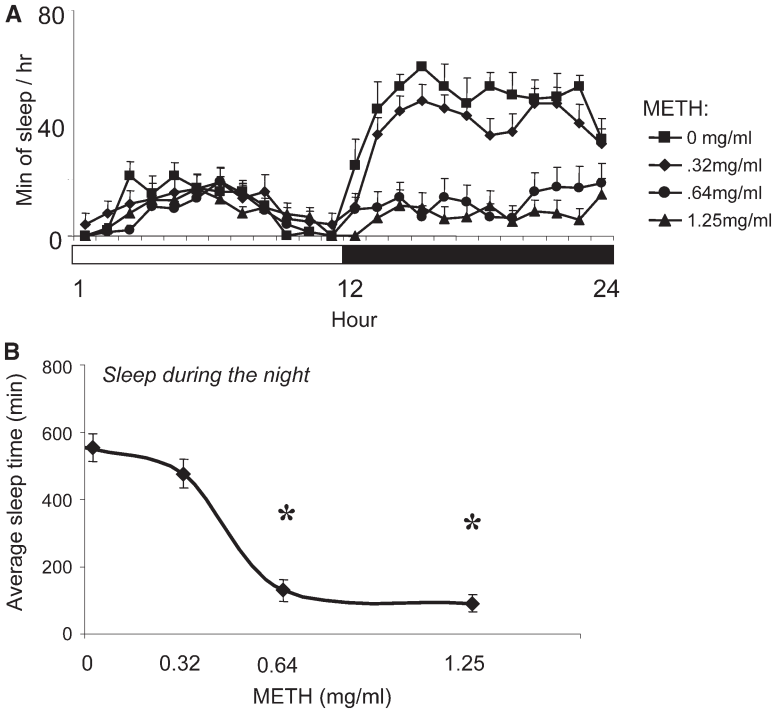

Figure 1. Oral Administration of METH Prevents Sleep During the Night

(A) Minutes of sleep per hour were calculated on the basis of activity recorded in a 5 min time window during 24 hr, in 12 hr of light and 12 hr of dark (hour 1 = lights on, hour 12 = lights off). METH was mixed in regular food, and wild-type female flies of the *Canton-S* variety were exposed to it for 12 hr from the time of lights off, with the following numbers of flies in each category: 0 mg/ml, n = 7; 0.32 mg/ml, n = 13; 0.64 mg/ml, n = 13; 1.25 mg/ml, n = 16.

(B) Average amount of sleep during 12 hr of METH exposure on the basis of the experiment shown in (A). There is a significant decrease in sleep amount during the night at 0.64 mg/ml METH (Student's t test, * $p < 0.01$) and 1.25 mg/ml METH (* $p < 0.01$). Panels show average values with corresponding standard error.

it possible to correlate behavioral performance with changes in local field potentials (LFPs) in the animal's brain [22]. These electrophysiological studies in *Drosophila* showed not only that distinct arousal states in the fly can be determined by looking at locomotor output (the only method available in the past) but also that they can be inferred from analyzing changes in brain activity [19, 20, 22].

In this study, we combine several approaches to investigate how changes in dopaminergic transmission affect different measures of arousal. We studied arousal changes induced by feeding flies methamphetamine (METH) and asked how it affects behaviors of different complexity, from simple behaviors, such as sleep and locomotion, to complex behaviors, such as visual perception and courtship behavior.

Results

Wake-Promoting Effects of Methamphetamine in *Drosophila*

We measured average sleep time in METH-exposed and sham-treated wild-type *Canton-S* female flies, as described [17, 23]. METH was administered orally, mixed in the regular fly food, during 12 hr of darkness when sleep predominates in *Drosophila*. Figures 1A and 1B show a significant effect of METH on average sleep time, an effect evident as a decrease in minutes of sleep per hour at 0.64 mg/ml or higher doses (0 mg/ml METH = 556 ± 66 min versus 0.64 mg/ml METH = 128.9 ± 32 min, $p < 0.01$; 1.25 mg/ml METH = 90.4 ± 27 min, $p < 0.01$). Sleep time decreases immediately upon transfer to METH-containing food, and the effect persists for the entire 12 hr exposure, suggesting an absence of adaptation to the drug during this time (Figure 1A).

Further analysis revealed that METH affected several sleep parameters. The decrease in average sleep time during 0.64 and 1.25 mg/ml METH administration was due to changes in both sleep- and wake-bout number and duration (Figures 2A–2D). METH significantly increased wake-bout duration (0 mg/ml METH = 21 ± 4 min versus 0.64 mg/ml METH = 269 ± 63 min, $p < 0.01$; 1.25 mg/ml METH = 222 ± 72 min, $p < 0.01$) and decreased the number of bouts (0 mg/ml METH = 10.6 ± 0.8 versus 0.64 mg/ml METH = 5.1 ± 1, $p < 0.01$; 1.25 mg/ml METH = 6.1 ± 1, $p < 0.01$), thus leading to consolidation of wakefulness. This was accompanied by a significant shortening of sleep-bout duration—i.e., when METH-treated flies fell asleep, they woke up sooner than controls (0 mg/ml METH = 100 ± 25 min versus 1.25 mg/ml METH = 17 ± 8 min, $p < 0.01$)—and a tendency for sleep-bout number to decrease (flies initiated sleep less often, i.e., they were less sleepy). Taken together, these observations suggest that METH consolidates wakefulness in flies by interfering with sleep maintenance.

Accordingly, the drug increased the latency to sleep, as shown by a delay of onset for the first sleep episode in METH-fed flies (Figure 2E). At the time of lights off, sham-treated controls take an average of 25.7 min to start their first sleep bout. If, at the time of lights off, flies are exposed to food containing METH, the latency to sleep significantly increases (0.32 mg/ml METH = 72.3 ± 11 min, $p < 0.01$). Interestingly, sleep latency was the most sensitive sleep-related measure; no significant difference was observed in other sleep parameters at 0.32 mg/ml METH. This observation further supports the conclusion that METH interferes with the sleep pro-

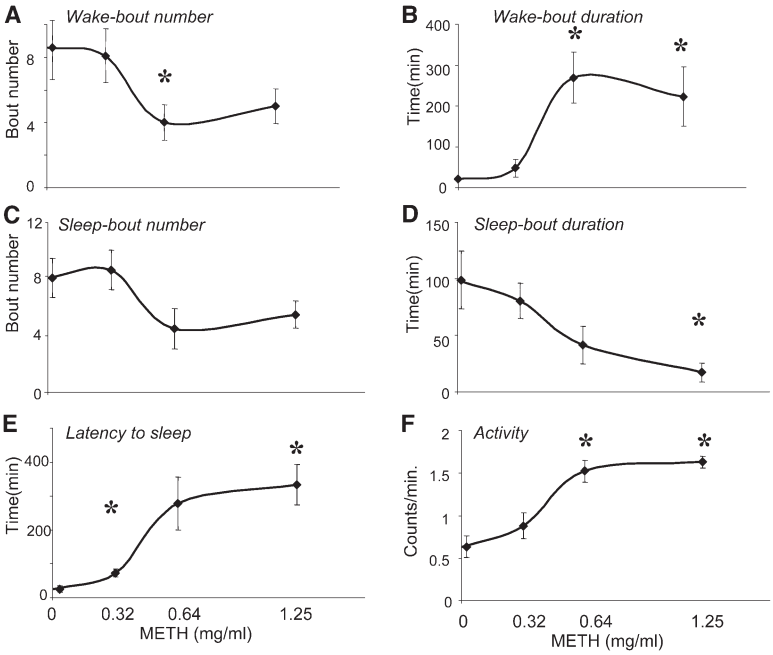

Figure 2. METH Administration Consolidates Wakefulness, Delays Sleep Initiation, and Increases Waking Activity

(A–D) Average wake-bout number decreases (A) (0.64 mg/ml METH, * $p < 0.016$) and duration increases (B) with increasing METH dose (0.64 mg/ml, $p < 0.01$; 1.25 mg/ml, * $p = 0.01$). (C) Nonsignificant decrease in sleep-bout number upon METH administration and (D) significant decrease in sleep-bout duration at 1.25 mg/ml METH (* $p < 0.01$).

(E) Dose-dependent increase in latency for the first sleep-bout onset after METH administration: 0.32 mg/ml, $p < 0.01$; 1.25 mg/ml, * $p < 0.01$.

(F) Average counts during waking increase (0.64 mg/ml, * $p < 0.01$; 1.25 mg/ml, * $p < 0.01$). All parameters calculated on the basis of recordings from the experiment described in Figure 1. Panels show average values with corresponding standard error. Asterisks denote significant differences based on Student's t test, where the significance level was adjusted with a Bonferroni correction for multiple comparisons.

cess by preventing initiation, i.e., by suppressing sleep drive, as has been described in humans [24].

Because activity levels can vary greatly during waking episodes, we calculated activity as counts per waking minute (counts/min). Using this measure, we observed that METH treatment at a concentration of 0.64 mg/ml or higher not only increases wakefulness but also significantly increases activity during waking episodes (0 mg/ml METH = 0.63 ± 0.1 counts/min versus 0.64 mg/ml METH = 1.52 ± 0.1 counts/min, $p < 0.01$; 1.25 mg/ml METH = 1.63 ± 0.1 counts/min, $p < 0.01$) (Figure 2F). An increase in locomotion is frequently used as a criterion of increased general arousal in other experimental organisms [25]; hence, by this measure, METH seems to increase general arousal in flies as well.

Although we did observe a trend for dose-dependent changes in many measures, the lack of a strong dose-response regression for all measures (except for sleep latency) could be attributed to the nature of our METH administration. Variation originating from individual differences in activity, food intake, metabolism, and responsiveness to METH could obscure a clear dose-dependent effect.

In order to establish to what extent the sleep and activity changes following METH administration are due to changes in dopaminergic transmission, we exposed flies to an inhibitor of dopamine synthesis, 3-iodo-tyrosine (3IY). This drug inhibits activity of the rate-limiting enzyme—tyrosine hydroxylase—in dopamine synthesis and significantly decreases steady-state amounts of dopamine after 2 days of feeding [26]. The drug was mixed into the food, and flies were exposed to it while their sleep and activity were monitored. 3IY resulted in a significant increase in sleep during the day (flies already sleep approximately 50 min of every hour during the night, so it was unlikely to get a significant effect during the night) (Figure 3). An effect was already evident on the first day of treatment, became maximal by day 2, and stabilized at that level. Significantly, this increase in sleep was not accompanied by a change in the level of activity during active periods; average counts/min during the day remained unchanged between the baseline day without drug and all the subsequent days of treatment. This finding is important because it shows that some aspects of neural control of

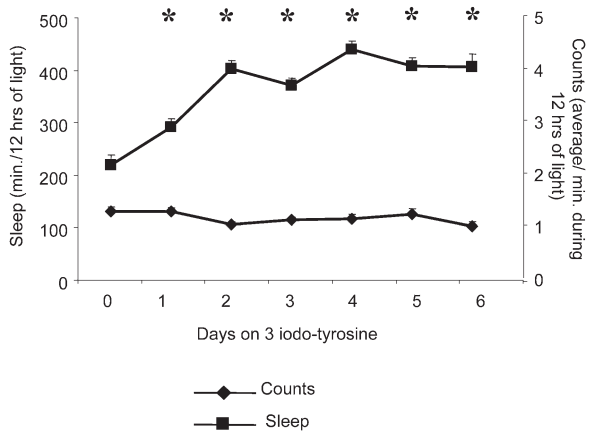

Figure 3. Preventing Dopamine Synthesis Increases Average Sleep Time

Effects of a dopamine-synthesis inhibitor, 3IY, on average sleep time during 12 hr of lights on (left Y-axis) and corresponding average counts during waking (right Y-axis) in wild-type females ($n = 28$) with corresponding standard error. Flies were recorded during the 24 hr baseline day. On day 2, at the time of lights-on, flies were transferred onto food containing 5 mg/ml 3IY and maintained on it for 5 more days. An effect of 5 mg/ml 3IY during 5 days is compared to the baseline day (day 0). Statistical significance was determined by Student's t test and adjusted for multiple comparisons with Bonferroni correction (* $p < 0.01$).

sleep are distinct from those controlling general activity; thus, it is possible to change one without affecting the other. Moreover, it shows that decreasing dopamine production has the opposite effect on sleep from that of exposing flies to a psychostimulant drug known to increase dopaminergic function. This suggests that the average amount of sleep in flies is significantly influenced, at least in part, by changes in dopaminergic transmission. (We were unable to use targeted, conditional gene expression [see below and Experimental Procedures] to determine whether suppression of dopamine release leads to a sleep phenotype similar to that which pharmacological suppression of dopamine synthesis with 3IY leads to because the high temperature required for conditional suppression with UAS-*shi*^{ts1} [see below and Experimental Procedures] is incompatible with long-term sleep recording, which is normally conducted at 25°C.)

As a test of the accuracy of the 5 min data-collection bins used in the foregoing studies, we also performed METH and 3IY treatments with 1 min collection bins for increased resolution while maintaining the 5 min definition of sleep we have routinely used. No differences were found between the two modes of analysis for METH-treated flies in either sleep amount or activity (Figures S1A and S1B in the Supplemental Data available with this article online) or for 3IY-treated flies for sleep amount (Figure S1C). Activity (counts/waking min) in 3IY-treated flies differed slightly between 1 min and 5 min collection bins, indicating that activity actually increased modestly while sleep was increasing (Figure S1D).

Wake-Promoting Effects of Methamphetamine in Flies with Increased Sleep Need

In addition to promoting active wakefulness, METH also counteracts the effects of sleep loss in flies. We tested the efficacy of the wake-promoting effects of METH in flies with elevated sleep need. Flies were first sleep deprived, via mechanical deprivation for 9 hr from ZT15–ZT24, and were then placed on METH-containing food (1.25 mg/ml) for 12 hr (ZT1–ZT12) (ZT1 = lights on; ZT12 = lights off). Figure 4 histograms show the average amount of daytime sleep (ZT1-ZT12) for the baseline day (day 0) and Recovery + METH day (day 2). Average sleep on day 1 (when sleep deprivation occurs during the night) and day 3 (when flies are left undisturbed) for all of the groups is similar to their day 0 baseline levels (data not shown). In the group subjected to sleep deprivation only (SD), a homeostatic increase in the average sleep amount occurs after mechanical deprivation (SD, day 0 = 176 ± 27 min; SD, day 2 = 305 ± 42 min; $p = 0.01$). METH potently prevented sleep in flies that were not sleep deprived (METH), demonstrating that METH is as efficient during the day as it is during the night. In the daytime, it significantly decreased sleep during the flies' characteristic noontime siesta (METH, day 0 = 218 ± 32 min; METH, day 2 = 50 ± 18 min; $p < 0.01$). Most importantly, after METH administration, the amount of sleep during the recovery period after deprivation (SD + METH) is indistinguishable from that on the pretreatment day (SD + METH, day 0 = 213 ± 27 min; SD + METH, day 2 = 160 ± 38 min; $p > 0.05$). Cumulative plotting of the data shows that the SD + METH group began diverging from the SD group shortly after the start of METH treatment, with significant differences observed from ZT8–ZT16 (hour 32–hour 40) (Figure 4, inset). Interestingly, METH decreased the amount of sleep in the SD + METH group only to the baseline day 0 level but not to the level observed in the non-sleep-deprived METH group, indicating that the efficacy of a given dose of METH is influenced by the level of sleep drive. The SD + METH group has a greater sleep drive than the METH group; thus, the same METH concentration led to higher sleep suppression in the latter condition.

Increased Sexual Arousal in Methamphetamine-Fed *Drosophila* Males

One of the well-documented effects of METH in experimental animals is a decrease in arousal threshold. Behavioral responsiveness has been measured previously in *Drosophila* via vibrational or visual stimuli to characterize differences between sleep and wakefulness [17–20]. A different kind of test is needed to determine whether responsiveness is relevant for brain functions associated with more complex behaviors, such as courtship. For this purpose, we measured several temporal parameters that constitute the behavioral-courtship sequence. A single male was presented with a virgin female (either intact or decapitated, see Experimental Procedures) and then observed for the following: (1) latency to courtship (time at which the male fly starts displaying any of the defined courtship steps); (2) courtship index (time spent courting the female; the time is normalized for total time leading to copulation); and (3) latency to copulation.

When tested with intact virgin females, METH-fed (0.85 mg/ml) males showed a tendency toward decreased courtship latency, although the difference was not statistically significant (data not shown). We reasoned that by adapting the assay so as to lengthen the latency to courtship, we would increase the resolution. We therefore tested males in a modified courtship assay where the object of courtship was a decapitated female; decapitated females produce increased latencies to courtship because they lack the ability to move [27]. We observed that METH-treated males started courtship toward decapitated females significantly faster than did the control males (METH = 40.2 ± 7 s versus control = 70.2 ± 12 s, $p < 0.05$), suggesting that METH-fed males were more aroused and had a lower threshold to initiate courtship than control males (Figure 5).

Further indication that METH increased sexual arousal comes from the observation that the METH-treated group showed a significantly greater courtship index (more time spent courting) with both intact and decapitated females (intact, METH = 0.83 ± 0.1 versus control = 0.63 ± 0.1, $p = 0.04$; decapitated, METH = 0.44 ± 0.1 versus control = 0.2 ± 0.1, $p = 0.03$) (Figure 5B). This suggests that, in addition to being more highly aroused, METH-fed males were unable to adapt their behavior properly to signals (or lack thereof) given by the females or that they were unable to exit from a stereotyped behavioral loop. This observation of a potentially

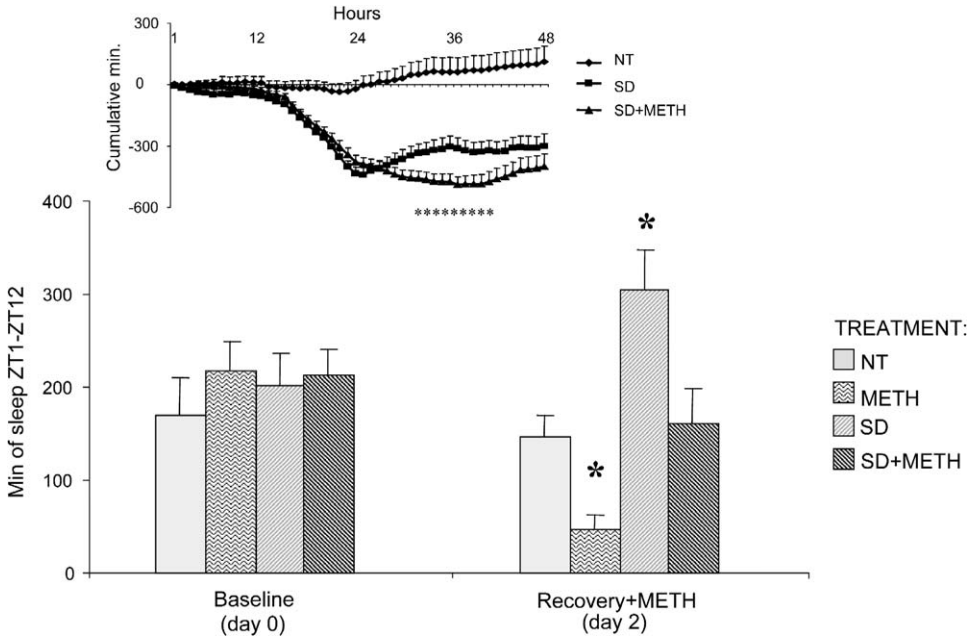

Figure 4. METH Prevents Recovery Sleep in Sleep-Deprived Flies

Average sleep amount during 12 hr of light (ZT1–ZT12) with corresponding standard error for the following: baseline day (day 0) and Recovery + METH (day 2). The No Treatment (NT) group, n = 16, received no treatment during the same time period. The methamphetamine group (METH), n = 13, received 1.25 mg/ml METH on day 2 from ZT1 to ZT12. The SD group, n = 9, was sleep deprived on day 1 (data not shown) and was left to recover undisturbed on day 2. The SD + METH group, n = 19, was sleep deprived on day 1 and exposed to 1.25 mg/ml METH on day 2. (* denotes significant difference from baseline, $p < 0.05$ by Student's t test.)

Inset: Cumulative plot of difference between day 1 and day 2 versus corresponding day 0 with corresponding standard error for NT, SD + METH, and SD groups. During the first 12 hr of the SD treatment, sleep is similar to the baseline day. Both groups start losing sleep at hour 15 when sleep deprivation begins, and by the time deprivation ends (hour 24), both groups lose equivalent amounts of sleep (SD + METH = –378 ± 34 min, SD = –435 ± 32 min). During METH treatment (hour 25–36), SD gains sleep back whereas SD + METH shows no sleep gain. The difference is statistically significant between hour 32 and hour 40 at $p < 0.05$ or less by Student's t test (corresponding to ZT8–ZT16).

nonfunctional increase in arousal prompted us to examine the latency to copulation. We found that METH feeding substantially decreased the number of males that copulated in the given time period. Compared to 82.3% of control males that started copulating within 30 min, only 57.5% of METH males did so (Figure 5C). Copulation kinetics indicate that when METH-fed males did copulate, the average latency was significantly increased (control = 448.1 s, METH = 705.8 s, $p < 0.05$). (Figure 5D).

These findings show that METH increases sexual arousal by shortening the latency to initiate courtship; however, this increase was ultimately maladaptive because the latency to copulation increased and, consequently, copulation success decreased. One explanation for this result might be that general arousal in METH-fed flies increased to a level where it interferes with performance of the complex behavior required to complete courtship and mating. Another less-global possibility is that physiological inhibition of dopamine release in specific circuits might be required for copulation in flies.

Brain Electrophysiological Signatures of Acute Methamphetamine Exposure

We investigated the effect of METH on a different form of arousal in *Drosophila* by measuring brain activity in response to visual stimuli. Previous work has shown that the perception of salient visual stimuli in this organism is associated with changes in 20–30 Hz local field potentials (LFP) in the fly's medial protocerebrum (mpc) [22]. We reasoned that this approach might provide insight into METH-induced performance deficits because responding to salient stimuli is essential in various stages of courtship.

We recorded baseline activity from three sites on a vertical axis in the protocerebrum in response to visual stimuli (a moving black bar, see [22] and the Supplemental Experimental Procedures) and then tested the effect of METH (1.25 mg/ml) on these responses by administering the drug to the tethered fly in a small piece of food, on which it would feed for up to 1 min. This led to significant brain-activity changes that were observable in the first 800 s window and that persisted for at least 2400 s: a consistent decrease in the 20–30 Hz response to a visual stimulus in all three channels (Figure 6A). The kinetics of this effect differed somewhat for each recording position: The medial channel was more rapidly affected than the ventral channel (in the medial channel, a significant decrease compared to baseline was seen by 1600 s, compared to by 2400 s for the ventral channel, $p < 0.05$). The dorsal channel showed a similar decrease at all three times, but it was only significant for the entire epoch (0–2400 s) collapsed together ($p < 0.05$). Given the time course of METH effects, these region-specific differences in ki-

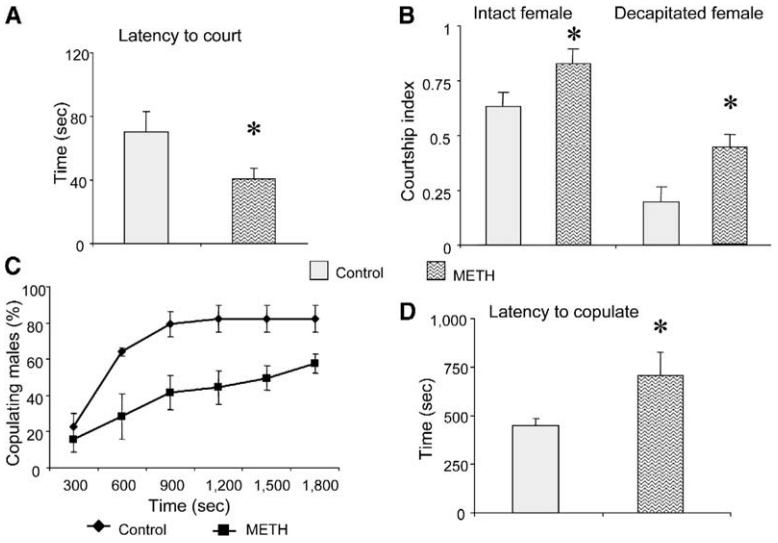

Figure 5. Males on METH Show Shorter Latency to Courtship but Longer Latency to Copulation and Longer Duration of Courtship

(A) METH (0.85 mg/ml) decreases latency to courtship toward decapitated females. METH = 40.7 ± 12.6 s, n = 23; control = 70.2 ± 12.6 s, n = 18 (* p = 0.03).

(B) METH increases courtship duration (expressed as the courtship index) toward intact and decapitated females. Toward intact females, the following courtship durations were observed: control = 0.63 ± 0.07, n = 18; METH = 0.83 ± 0.07, n = 19 (* p = 0.04). Toward decapitated females, the following courtship indices were observed: control = 0.20 ± 0.07, n = 7; METH = 0.44 ± 0.06, n = 17 (* p = 0.03). Behavior was monitored for 10 min and performed as in (A).

(C) METH slows down copulation kinetics. Cumulative frequency based on 4 independent experiments (METH, n = 39; control, n = 40) showing the percentage of flies that start copulating within a given time window. Observations lasted 30 min (* p < 0.01).

(D) METH increases latency to copulation. Data are based on the experiment shown in (C) and calculated on the basis of flies that started copulating within 30 min (80% controls and 56% METH): control = 448.1 ± 39.5 s, METH = 705.8 ± 119.9 s (* p = 0.02). All panels show average values and corresponding standard error. Statistical significance was determined with Student's t test.

netics are more likely to reflect intrinsic physiological interactions in the fly brain rather than differential access to the drug.

Another major effect of METH administration on brain activity was a decrease in the correlation between the average brain LFP activity at all frequencies (10–100 Hz in the medial channel) and the fly's own movement (Figure 6B), significant 1600 s after METH ingestion. This is reminiscent of previous findings in which a similar uncoupling characterizes periods when a normal fly is responding to a visual stimulus in its environment [20]. Thus, METH concentrations that produce increased behavioral activity in our locomotor and sleep assays interfere with proper function in courtship, visual response, and LFP-movement coupling.

Visual Perception Is Dependent on Transmission from Dopaminergic Neurons

In order to identify the neurotransmitter system through which METH affects these physiological correlates of arousal in the fly, we obtained recordings from transgenic animals in which synaptic release from dopaminergic and serotonergic neurons is under conditional control. We focused on these systems because converging evidence from higher vertebrates and *Drosophila* indicates that monoaminergic neurons are a target of psychostimulants such as cocaine and amphetamine [28, 29]. Furthermore, in humans, monkeys, and rodents, dopamine signaling is involved in the processing of both nonrewarding salient stimuli and rewarding stimuli [30, 31]. Finally, in mammals, cognitive and behavioral effects of METH administration have been associated with changes in dopaminergic transmission [32, 33].

To reversibly attenuate synaptic release in both dopaminergic and serotonergic neurons, we drove expression of a temperature-sensitive *shibire* mutation with *Dopa decarboxylase* controlling sequences (*Ddc*-GAL4/UAS-*shi^{ts1}*, [34]). In addition, we further restricted expression exclusively to dopaminergic neurons by using *Tyrosine hydroxylase* controlling sequences (*Th*-GAL4/UAS-*shi^{ts1}*, [35]). Both strains were tested for their 20–30 Hz brain response to the same visual object rotating in open loop, as described previously [22]. In comparison to baseline recordings at room temperature (22°C), heating flies to 38°C in both transgenic strains showed a significant decrease in the 20–30 Hz response, which recovered when returned to room temperature, indicating that dopamine release is required for the brain's response to the visual stimulus (Table 1). In contrast, *Th-GAL4*/+ and UAS-*shi^{ts1}*/+ control animals did not show a decreased 20–30 Hz response at 38°C (Figure S2). The fact that 20–30 Hz brain activity is attenuated with both GAL4 drivers suggests that dopamine plays the dominant role in these measures of visual perception, or else it suggests that modulating serotonergic transmission simultaneously has no major effect.

Transgenic animals of the same genotypes were also tested for visual fixation, a behavioral measure of visual perception, in a closed-loop flight arena (see [22] and Experimental Procedures). In this assay, perception is inferred from the animal's tracking response in which it holds the virtual object in one part of its visual field, usually in front. Both *Ddc*-GAL4/UAS-*shi^{ts1}* and *Th*-GAL4/UAS *shi^{ts1}* genotypes performed this visual task at the permissive temperature (22°C) before and after heat treatment but displayed a significant decrement in fixation behavior (while still "flying") at 38°C, the restrictive temperature (Table 1), indicating a requirement for dopamine. It was not possible to test the effect of METH on tracking behavior because METH-fed flies would not fly in the flight arena. (A previous study of the dependence of flight fixation behavior on dopamine reported that only chronic blockade, with tetanus toxin or *shi^{ts1}*, affected the behavior, whereas transient blockade with *shi^{ts1}* did not [36]. We attribute the difference in the present study to our standard use of a

Table 1. Dopamine Is Required for Behavioral Tracking and for the 20–30 Hz Response to a Visual Stimulus

	20–30 Hz Response				Behavioral Tracking			
	n	*PRE*	*HEAT*	*POST*	*n*	*PRE*	*HEAT*	*POST*
Ddc-GAL4/UAS-*shi*^{ts1}	4	1.0	0.45 ± 0.04*	0.99 ± 0.15	4	2.4 ± 0.13	0.61 ± 0.13*	3.1 ± 0.57
Th-GAL4/UAS-*shi*^{ts1}	5	1.0	0.60 ± 0.08*	1.20 ± 0.22	5	2.4 ± 0.49	0.83 ± 0.03*	2.6 ± 0.47
Th-GAL4/+	4	1.0	1.14 ± 0.16	1.11 ± 0.24	2	2.6 ± 0.26	2.4 ± 0.59	2.1 ± 0.46

The effects of the restrictive temperature (HEAT, 38°C) on the 20–30 Hz brain response and on behavioral tracking were contrasted to baseline responses and behavior at room temperature (PRE, 22°C). Recovery data (POST, 22°C) immediately followed the heated sessions. During the 100 s of behavioral tracking in a closed-loop flight arena, visual perception is inferred from an animal's ability to hold the virtual object in one part of its visual field. Different flies were used for either behavioral or brain-recording paradigms (* denotes significant differenence from baseline, $p < 0.05$). For UAS-*shi*^{ts1}/+ control data, see Supplemental Experimental Procedures and [22].

higher temperature [38°C versus 30°C], which is more effective in rapidly inactivating gene product in *shi*^{ts} heterozygotes [37].)

On the basis of preceding observations showing that a correlate of visual perception is impaired in situations where dopaminergic transmission is decreased (Table 1), and knowing METH's ability to induce dopamine release [1], we hypothesized that we should be able to rescue the compromised visual response in these transgenic flies at the restrictive temperature by exposing them to METH. In the following experiments, we again used *Ddc*-GAL4/UAS-*shi*^{ts1} and *Th*-GAL4/UAS *shi*^{ts1} to attenuate dopaminergic transmission and then followed the same regimen of temperature shifts with a brief feeding on METH-containing food, as described earlier. We thus performed three recording sessions: a baseline without METH and two after METH feeding, the first at 300 s and the second at 1500 s after drug administration. We found that METH administration rescued the 20–30 Hz response at the restrictive temperature (38°C) in flies with either GAL4 driver, such that the response at the high temperature was either the same as or greater than the baseline response within each treatment condition (Figures 7A and 7B; sessions 1 and 2). Control genotypes (*Th*-GAL4/+ and UAS-*shi*^{ts1}/+) show no heat-induced decrease in the 20–30 Hz response and thus no rescue of heat-induced effects with METH (see Figure S2).

This rapid restorative effect of METH on dopamine function in transgenic flies is in contrast to the negative effect, presented earlier, that METH had in wild-type flies, where it attenuated the 20–30 Hz response to a visual stimulus. Thus, METH can either increase or decrease the response depending on the baseline level of dopamine present in the nervous system. METH restores the 20–30 Hz responses in transgenic flies, where synaptic release from dopaminergic neurons was attenuated, and degenerates the response in wild-type flies with normal levels of dopamine. These observations have a double importance. First, they argue that

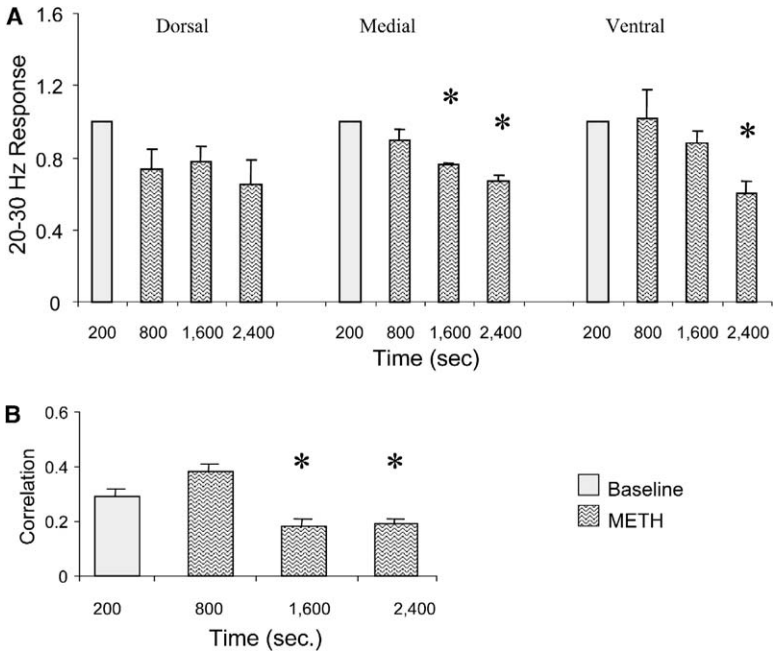

Figure 6. METH Degrades Brain Response to a Visual Stimulus and Uncouples Brain Activity from Movement

(A) A 20–30 Hz response to a visual stimulus, from dorsal, medial, and ventral protocerebral LFP recordings averaged for a 200 s window before METH administration (baseline) and for three consecutive 800 s windows after METH administration. METH was administered at a concentration of 1.25 mg/ml mixed in regular fly food and was ingested by the fly for < 1 min while it was in the recording arena (* $p < 0.05$, t test, n = 4 flies). The decrease in 20–30 Hz response occurred without any overall decrease in average 20–30 Hz activity (see Supplemental Experimental Procedures).

(B) Correlation between movement and power of brain LFP activity in the 10–100 Hz frequency range averaged for a 200 s window before METH administration and three consecutive 800 s windows after METH administration. Movement was monitored with a recording electrode in the thorax, as described [20] (* $p < 0.05$, t test). The observed METH-induced decorrelation was not simply a function of changes in average activity; average brain activity and average movement were not significantly changed, compared to baseline, in our tethered preparation (data not shown). All panels show average values with corresponding standard error.

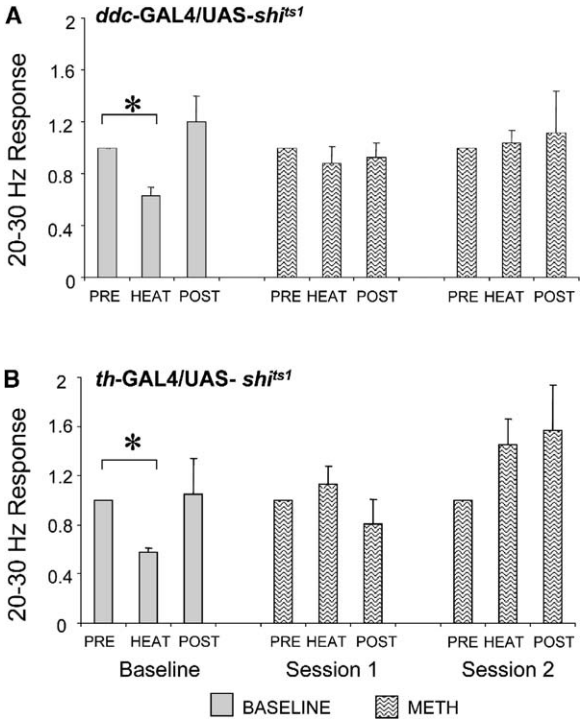

Figure 7. METH Antagonizes Effects of Decreased Dopaminergic Release on Visual Perception

The 20–30 Hz visual response (see Experimental Procedures) was measured before (PRE), during (HEAT), and after (POST) the temperature was raised to 38°C, the restrictive temperature for *shi*^{ts1}. HEAT and POST response values within each group (Baseline, Session 1, and Session 2) are normalized to the PRE value. Brief feeding on food containing 1.25 mg/ml METH rescues the 20–30 Hz visual response at the restrictive temperature in two recording sessions at 300 s (Session 1) and 1500 s (Session 2) after the feeding. (A) The 20–30 Hz visual response in *Ddc*-GAL4/UAS-*shi*^{ts1} (n = 4 flies) and in (B) *Th*-GAL4/UAS-*shi*^{ts1} (n = 4 flies) is shown. Statistical significance (*) was determined by the Tukey-Kramer multiple-comparison method ($p < 0.05$). All panels show average values with corresponding standard error.

METH ingestion in flies leads to changes in dopaminergic transmission, and second, they suggest that there exists an optimal level of dopamine release for optimal behavioral performance. Increasing dopamine release, as in METH-fed wild-type flies, or decreasing it, as in transgenic flies, is detrimental for visual perception and behavioral performance.

Discussion

Our results show that changes in dopaminergic transmission modulate levels of arousal in *Drosophila* for behaviors of varying complexity. Sleep and locomotion show graded responses that follow changes in dopamine level, and drug concentrations that promote wakefulness were detrimental to courtship success. Neural correlates of visual perception, on the other hand, degenerate when dopamine levels are either too high or too low.

Methamphetamine Effects on Sleep and Activity

The most obvious effect of feeding METH to *Drosophila* is a general arousal increase manifested as a decrease in average sleep time (even in flies that have significantly increased sleep need) and an increase in average activity when awake. The following similar behavioral effects have been reported in mammals: a decrease in sleep amount, consolidation of periods of wakefulness, and improved vigilance during extended sleep deprivation [24, 25]. Our findings complement previously published work that studied the behavioral effects of volatilized-cocaine exposure in *Drosophila* [21, 28, 38] and addressed issues of acute behavioral sensitization, whereas our feeding protocol investigates chronic changes in arousal. It is important to note that METH feeding to flies has in no case induced the same kind of stereotypical, hyperkinetic, or uncoordinated behaviors seen with volatilized cocaine.

The opposing effects on average sleep time of METH versus 3IY, drugs that have been shown to have opposing effects on the concentration of dopamine, agree well with those in mammalian studies in which administration of low concentrations of D1 and D2 dopamine-receptor agonists promote active wakefulness and in which blockade of those receptors leads to sedation [13]. We observed that decreasing dopamine concentration with 3IY has a selective effect on sleep, whereas increasing it with METH affects both average sleep and activity, suggesting that sleep time is more sensitive than locomotor activity to perturbations in the neurotransmitter concentration. Similarly, in rodents, the METH-induced decrease in sleep is inseparable from its motor-activating effects, whereas another wake-promoting substance, modafinil, whose activity appears to be mediated by dopamine, does not lead to increased locomotor activity [25, 39]. Thus, drugs, such as 3IY or modafinil, that selectively influence the dopaminergic system produce a more selective effect on sleep. In *Drosophila*, as in mammals, locomotor-activating effects of METH at low doses are likely to be mediated by the combined action of the drug on multiple transmitter systems.

We (B.v.S. and R.J.G.) have previously proposed that arousal levels in the fly are a function of the degree of coupling among various parts of the nervous system [20]. This was seen physiologically during sleep in the uncoupling of peripheral responses to visual stimuli from the CNS [22] and in the uncoupling of movement from brain LFPs during a putative intermediate stage of sleep [20]. At the high end of the arousal scale, it is seen in the increased coherence between central brain sites during a visual-discrimination task [22].

In light of these previous findings, it may seem paradoxical that METH reduces the correlation between brain LFPs and movement while at the same time producing an increase in wakefulness and locomotor activity. This apparent paradox may be explained, however, by reference to another previous finding: Presentation of a visual stimulus to a fly also reduces the correlation between brain LFPs and movement [20]. Both of these results suggest that the LFP-movement correlation decreases when the fly is "distracted" by something: the visual stimulus in one case and METH in the other. For the visual stimulus, it is likely that the LFP-movement

coupling is being replaced by a specific coupling, such as the coherence increase seen during visual discrimination, among other brain regions [22]. METH, in contrast, is likely to be inducing nonspecific brain activity, uncoupled from the fly's sensory input.

A further possible consequence of a nonspecific, METH-induced uncoupling relates to the restorative functions of sleep. If one considers that brain LFPs are generally uncoupled from movement and from sensory input in the intermediate state preceding quiescent sleep [20], then perhaps some of the restorative functions of sleep are being carried out during that time. If so, then the dramatic reduction in quiescent sleep in METH-fed flies and the suppression of a homeostatic response to sleep deprivation in these flies may result from the partial fulfillment of some sleep functions during their prolonged periods in this state of LFP uncoupling from sensory stimuli and movement.

Methamphetamine Effects on Visual Perception and Courtship Behavior

Our finding that central visual perception is impaired by manipulation of dopamine, whether by increasing its action (METH) or by suppressing its release (shi^{ts1}), agrees well with the hypothesis of an inverted-U functional-response curve corresponding to increasing dopamine signaling in prefrontal cortex [40, 41]. When human subjects are given low doses of amphetamine, their cognitive performance will depend on the level of dopaminergic signaling in the prefrontal cortex. The same concentration of amphetamine enhanced performance for subjects with low prefrontal dopamine and caused deterioration in subjects with high prefrontal dopamine.

The effects of METH on courtship may resemble those on visual perception with respect to the requirement for an optimal setting of arousal level. The METH-induced increase in sexual arousal is defined by the latency to initiate courtship; however, this high level of arousal appears to be detrimental for the completion of the entire complex behavioral sequence. Males may persist in particular courtship steps longer because of their inability adequately to interpret and respond to female behavior, consistent with our finding that central visual processing is impaired after METH administration. Dopaminergic effects on courtship have been shown previously, where inhibition of dopamine synthesis during development in males increased the latency to initiate courtship and to copulate [42]. The possibility that the effects we report on visual perception and courtship might be due merely to primary visual defects is unlikely for several reasons. First, the dopamine-depleted flies in the cited study [42] were normal for phototaxis. Second, although vision is not essential for courtship, the lack of it produces an increase in courtship latency but no impairment to copulation [43]. Thus, the effects we observe are likely to be central rather than peripheral and more involved in the modulation of overall arousal than in the primary sensory response.

The Complexity of Behavioral Arousal

Arousal has been defined operationally as a state in which "an animal is more responsive to a wide variety of external stimuli spanning sensory modalities and is more motorically active" [44]. Our results suggest that the situation is more complex and nuanced [20]. Not all behaviors show a graded arousal change correlating with changes in dopaminergic activity. METH concentrations that lead to a gradual increase in locomotor activity (without hyperactivity or loss of coordination) and a decrease in average sleep time produce maladaptive arousal in the context of more complex behaviors. Performance of complex behaviors degenerates when dopamine levels are either too high or too low, as seen also in mammalian brain [40, 41]. Although we favor the idea that the observed effects of dopamine in *Drosophila* are acting primarily through its effect on arousal, we recognize the possibility of alternative explanations involving more restricted actions, yet to be identified, of central dopaminergic circuits on particular aspects of behavior.

Our findings suggest that courtship and visual perception in *Drosophila* display a complex response to changes in dopaminergic activity, whereas sleep and locomotor activity give a more linear response. Similar observations have been reported on the actions of drugs, such as volatile general anesthetics, that decrease general arousal, where complex behaviors are more susceptible to the sedating effects of these agents [45, 46]. This commonality suggests that neural mechanisms governing behaviors of varying degrees of complexity have evolved corresponding degrees of sensitivity to changes in the neuromodulatory milieu of an organism, with more primitive or basic behaviors showing greater robustness. On a more practical note, this finding indicates that locomotion alone is too crude an indicator of changes in the arousal of a fly, especially for more complex behaviors [16].

These explanations fit well with the role of dopamine as a key component of neuromodulatory "value" systems in the brain. Such systems have been shown to play an important role in conferring salience on particular stimuli, either intrinsically as part of the animal's heredity or adaptively when paired with specific sensory inputs [47–49]. In vertebrates, these functions have been attributed to diffusely ascending systems, employing biogenic amines as neurotransmitters. In the fly brain, the dopaminergic and octopaminergic systems have been shown to play such a role in aversive and appetitive conditioning, respectively [50]. These systems are generally nonspecific, both anatomically, in the sense that their projections are diffuse, and physiologically, in the sense that they provide general reinforcement (positive or negative) to more restrictively stimulated sensory or motor systems. The interaction between relatively specific sensory and motor systems, on the one hand, and relatively nonspecific value systems, on the other, thus underlies much of the brain's combinatorial versatility.

In this formulation, too much dopaminergic transmission would be as dysfunctional as too little, disrupting the balance between specific input and value-system modulation. Thus, nonspecific arousal producing sleep loss, increased activity, and overly stereotypical, unsuccessful courtship would have a common etiology

with the failure of the visual response: a failure of regulation of the animal's value system.

Experimental Procedures

Animals

Flies were grown in 40 ml vials on standard agar and yeast-based food [17] and housed in humidified incubators at 25°C, 60% humidity on a 12 hr light-dark cycle. We used wild-type *Canton-S* strain and progeny from the following transgenic strains: *Ddc*-GAL4 (kindly provided by J. Hirsh), *Th*-GAL4 (kindly provided by S. Birman), UAS-*shi*^{ts1} (kindly provided by T. Kitamoto). All measurements were performed on flies that were 4–6 days old.

Behavioral Measurements

Sleep

Sleep was measured in *Canton-S* females as described previously [17]. Mechanical sleep deprivation was performed as described [23]. In order to minimize environmental variance, we collected data for the dose-response experiments within same session, and we carried out all treatments within the dose-response or the sleep-deprivation experiments during the same night. Statistical significance was determined by Bonferroni corrected t test ($p < 0.02$). Methamphetamine hydrochloride and 3-iodo-L-tyrosine were obtained from Sigma-Aldrich Corp (St. Louis, MO).

Courtship Assay

Courtship of *Canton-S* males and virgins was tested as described in [51] and [52]. On the test day, experimental males were transferred onto food with 0.85 mg/ml METH from ZT2–ZT6, and control males were transferred to a new vial of regular food. All females were decapitated [51] except for those used for the measurement of copulation latency.

Electrophysiology

Recordings were made in *Canton-S* female flies from the protocerebrum referenced to an optic lobe with either single-channel glass electrodes or multichannel silicon electrodes, as described previously [19, 22]. All data were normalized to baseline recordings before heating (or METH feeding) for tests of significant transgenic (or METH-induced) effects ($p < 0.05$, by t test).

Visual-Fixation Behavior

Closed-loop experiments in the flight arena were performed as described previously [22], each fly was tested in triplicate, data were averaged, and significant differences ($p < 0.05$) from baseline were assessed by t test, as was significant fixation (compared to zero, or no fixation).

Supplemental Data

Supplemental Data include two figures and detailed Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/13/1165/DC1/.

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