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Sleep Homeostasis and General Anesthesia: Are Fruit Flies Well-Rested After Emergence From Propofol?

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Abstract

Background—Shared neurophysiologic features between sleep and anesthetic-induced hypnosis indicate a potential overlap in neuronal circuitry underlying both states. Previous studies in rodents indicate that pre-existing sleep debt discharges under propofol anesthesia. We explored the hypothesis that propofol anesthesia also dispels sleep pressure in the fruit fly. To our knowledge, this constitutes the first time propofol has been tested in the genetically tractable model, *Drosophila melanogaster*.

Methods—Daily sleep was measured in *Drosophila* using a standard locomotor activity assay. Propofol was administered by transferring flies onto food containing various doses of propofol or equivalent concentrations of vehicle. High-Performance Liquid Chromatography (HPLC) was used to measure tissue concentrations of ingested propofol. To determine if propofol anesthesia substitutes for natural sleep, we subjected flies to 10 hours (h) sleep deprivation (SD), followed by 6h propofol exposure, and monitored subsequent sleep.

Results—Oral propofol treatment causes anesthesia in flies as indicated by a dose-dependent reduction in locomotor activity (n=11–41 flies from each group) and increased arousal threshold (n=79–137). Recovery sleep in flies fed propofol after SD was delayed until after flies had emerged from anesthesia (n=30–48). SD was also associated with a significant increase in mortality in propofol-fed flies (n=44–46).

Conclusions—Together, these data indicate that fruit flies are effectively anesthetized by ingestion of propofol, and suggest that homologous molecular and neuronal targets of propofol are conserved in *Drosophila*. However, behavioral measurements indicate that propofol anesthesia does not satisfy the homeostatic need for sleep, and may compromise the restorative properties of sleep.

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Introduction

The mechanisms underlying anesthetic-induced loss of consciousness remain nebulous, despite ubiquitous clinical use and the identification of numerous neuronal targets.^{1–3} One emerging hypothesis is that anesthetic drugs reversibly induce unconsciousness by acting on endogenous sleep and arousal circuitry.⁴ For example, sleep duration and sensitivity to anesthesia are controlled by a common pathway in the fly brain.⁵ Though general anesthesia and sleep exhibit obvious clinical differences,⁶ numerous shared neurophysiologic features indicate an overlap in neuronal circuitry underlying both states.⁷ Further evidence supporting this overlap originate from reports that sleep deprivation enhances anesthetic potency.⁸ Fittingly, pharmacological augmentation of arousal (via monoaminergic stimulation) destabilizes the anesthetic state and precipitates emergence.^{9–12}

The convergence between general anesthesia and sleep has led to questions about the extent to which anesthetics may substitute for aspects of natural sleep.¹³ One approach to address this question is to investigate how anesthetics affect various sleep parameters, such as delta power or the homeostatic response to sleep deprivation. Seminal studies conducted in rodents show that rapid eye movement (REM) sleep debt accrues during exposure to volatile anesthesia, as evidenced by decreased REM sleep latency and increased REM sleep time during the active period after emergence.¹⁴ Though the volatile anesthetics fail to fulfill REM sleep requirements, post-anesthesia reductions in delta power indicate that these agents substitute for non-REM (NREM) sleep, excluding halothane, which incurs NREM debt.^{14–16}

In contrast to these findings, the widely-used intravenous agent propofol satisfies the homeostatic drive for both NREM and REM sleep, perhaps reflecting a unique property of this anesthetic. Pre-existing sleep debt is discharged identically under propofol anesthesia as during natural rebound sleep, while new sleep debt does not accumulate.^{17,18} Though these agent-specific effects on sleep homeostasis likely reflect heterogeneity in the molecular and neuronal mechanisms of action of structurally diverse anesthetic ligands, an interesting question of how different hypnotics might fulfill some homeostatic functions of sleep remains unanswered.

The finding in rodents that propofol satisfies sleep requirements is in need of replication under divergent conditions and in other species to better assess its general validity. *Drosophila melanogaster* is an established genetic model for elucidating mechanisms of general anesthesia,¹⁹ and has been used successfully to identify a variety of polymorphisms which confer either hypersensitivity or resistance to anesthetics.²⁰ To our knowledge, propofol has never been tested in *Drosophila* due to practical obstacles imposed by its typically intravenous route of administration. Therefore, we determined whether fruit flies are sensitive to propofol using a behavioral assay^{21,22} and found that flies are effectively anesthetized when propofol is added to the food medium. We first asked whether fruit flies fed propofol are unresponsive to mechanical stimuli as one would expect of truly anesthetized subjects. We next investigated the pharmacokinetic profile of orally ingested propofol in flies to better correlate fly tissue propofol levels with locomotor activity. Finally, we hypothesized that propofol's ability to satisfy the homeostatic requirement for sleep in

rodents should generalize to *Drosophila*, and used a sleep-deprivation paradigm to test this assumption.

Materials and Methods

Fly stocks

Flies were grown on standard dextrose/cornmeal media. Female, Canton-S (wild-type) flies were used exclusively in all experiments. No vertebrate animals were used in these studies.

Behavioral assays

All experiments were performed using flies maintained at 25 °C under 12:12 light: dark (L:D) conditions. Daily sleep was measured by monitoring locomotor activity in flies using the Trikinetics *Drosophila* Activity Monitoring System (DAM2; Trikinetics, Waltham, MA). Flies 1–4 days in age were CO₂ anesthetized on a porous polyethylene/acrylic sorting plate (Tyler Research Co., Edmonton, Alberta Canada) and loaded individually into glass tubes containing 5% sucrose and 2% agar medium. Activity counts correspond to the number of times a fly physically interrupts an infrared light beam perpendicular to the axis of the glass tube. Sleep in this assay is defined as an activity count of zero for a minimum of 5 consecutive minutes.²² Sleep and activity parameters were analyzed using custom software (Insomniac3, RP Metrix, Skillman, NJ). Results of all behavioral assays were restricted to flies that survived for at least 24h following the analysis period. The time of death is determined from the time when all activity counts stop varying from zero for the remainder of the assay.²³

For sleep deprivation (SD) experiments, flies were loaded into two DAM2 activity monitors (32 flies in each monitor) and baseline values were collected for 2–3 days. Flies were subjected to SD during the dark phase, from ZT 14–24 (ZT = zeitgeber time, where ZT 0–12 is lights on and 12–24 is lights off), or as otherwise indicated. SD was performed by attaching one of the activity monitors to a multi-tube vortexer (Corning, Edison NJ). The other monitor was left undisturbed as a control. The vortexer was controlled by software (Trikinetics, Waltham MA) that activated the vortexer for 1 second with inter-stimulus intervals randomly varying between 2–40 seconds. The strength of the vortexer was set to the minimal intensity that was required to maintain wakefulness for at least 10 h (i.e., the duration of the desired sleep deprivation). At the end of the SD period, half of the flies from each monitor were transferred to activity tubes containing propofol mixed with food, while the other half was transferred to tubes with food containing equivalent concentrations of vehicle (see “Preparation and administration of propofol,” below). After 6h, all flies were transferred to activity tubes containing regular food. Results are reported from 2–4 replicate experiments as indicated.

To determine the arousal threshold of flies fed propofol, we again employed the vortexer to stimulate flies at two different intensities, either mild or strong. Mild and strong stimuli were delivered in an alternating pattern, with a 2h interval between successive strong stimuli. Inter-stimulus intervals were a minimum of 30 minutes, such that mild stimulation was applied at 1.5h, 3.5h, and 5.5h during propofol exposure, and strong stimulation was

introduced at 2h, 4h, and 6h after induction of propofol anesthesia. Arousal responses were also monitored for up to 24h after withdrawal, with the strong stimulus applied as indicated. The percentage of flies responding corresponds to the number of sleeping (or anesthetized) flies that were awakened by the vibratory stimuli divided by the total number of flies that were quiescent prior to stimulation. Sleep or consolidated immobility is stipulated as a minimum of zero activity counts for at least 5 minutes prior to the stimulation. Awakening or arousal by the mild stimulus is defined by having any activity counts occurring within the first minute following stimulation. Arousals in response to the strong stimulus are defined by more than 1–2 activity counts within the minute of stimulation to eliminate movement artifact that occurred at this stimulus strength. In each experiment, arousal responses were measured from 16–24 flies for each condition (drug, vehicle, etc). Flies that were already awake at the time of stimulation (i.e., registered >0 activity counts in the five minutes prior to stimulation) were excluded from this analysis. Flies are deemed responsive only if they are also quiescent for a minimum of five minutes prior to stimulation. Results are reported from 4–6 replicate experiments as indicated.

Preparation and administration of propofol

A 100 millimolar (mM) stock solution of propofol was prepared by dissolving 9 microliters (μL) of propofol (2,6-Diisopropylphenol, >97%, Sigma-Aldrich, St. Louis MO) in 491 uL of polyethylene glycol (PEG400). Activity tubes containing various concentrations of propofol were prepared by diluting an appropriate volume of 100 mM propofol stock solution in regular fly food (5% sucrose, 2% agar).

Propofol was administered by individually transferring flies to activity tubes with sucrose food containing various doses of propofol or equivalent amounts of vehicle. Activity data was collected for three baseline days during photoentrainment prior to administering propofol treatment on the fourth day for 6h from ZT 0 to ZT 6 (ZT = zeitgeber time, where ZT 0–12 = lights on, and ZT 12–24 = lights off), or as otherwise indicated. Post-propofol treatment, flies were transferred back to freshly prepared activity tubes containing regular sucrose medium, and behavioral data was collected for an additional 3–7 days after drug exposure.

High-Performance Liquid Chromatography (HPLC) measurement of Propofol concentration

Flies were fed 1.0 mM propofol for 6h from ZT 0 to ZT 6. Flies were collected in Eppendorf tubes over dry ice every 2h during exposure and up to 48h afterwards. Fly heads were separated from bodies by agitating Eppendorf tubes containing frozen whole-flies, and manually dividing the heads from bodies on a piece of weighing paper suspended over dry ice. For evaluating effects of SD on propofol concentrations in flies, flies with and without SD were collected immediately after a 6h treatment with 1.0 mM propofol at ZT 6. Two other groups of flies (sleep deprived and control) were left to recover until ZT 6 the following day. Flies were manually decapitated, and head weights were estimated by weighing whole flies before and after decapitation. Groups of five fly heads and five whole flies were analyzed independently to determine propofol concentrations in tissue. The

experimenter who conducted the HPLC measurements was blind to the condition and time points from which the flies were collected. Results are reported from three replicates.

The tissue from each sample was homogenized in 400 μ L of solution containing a 3:1 volume ratio of acetonitrile and 0.02 molar (M) phosphate buffer. The homogenate was centrifuged (10,000 times gravity at 4 degrees for 20 minutes), and 25 μ L of the supernatant was injected into a Shimadzu HPLC instrument (Shimadzu, Kyoto, Japan).

Calibration curves were run prior to each batch of samples and all had correlation coefficients of 0.99 or better. Calibration curves were prepared as follows: A standard 0.05 mM propofol solution was prepared with methanol, and 2, 5, 10, 15, and 20 μ L of solution were injected into the HPLC used to construct the calibration curve by plotting the propofol peak against the known amount of analytes and fitted by linear regression analysis.

The chromatography system consisted of a Gold system (Beckman Coulter, Jersey City, NJ) separation module and a FP-2020 plus fluorescence detector (Agilent Technologies, Wilmington, DE). Separation was achieved on a Vydac analytical C18 column. The mobile phase was an isocratic mixture of A: acetonitrile, B: 2-propanol, C: 0.02M NaH₂PO₄ buffer. The flow rate was 1.5 mL/minute, and the column temperature was ambient. The fluorescence detector was set at an excitation of 276 nanometers (nm) and emission of 310 nm.

Statistical analyses

Samples of flies were inherently randomized through the loading of glass tubes and activity monitors, as described above (see “Behavioral assays”). Sample sizes were restricted by the size of the DAM2 monitors (32 flies per monitor), by the number of monitors that could be accommodated by the vortexing apparatus, and by the speed in which flies could be manually transferred from regular food to drug in a timely manner, typically 2–4 monitors per experiment.

To analyze recovery sleep following sleep deprivation, we calculated net changes in sleep across 12h periods (day and night) for two days following the SD period using an approach similar to that described by Kuo et al.²⁴ Briefly, baseline values were subtracted from post-deprivation values in individual flies and averaged across handled control (non-deprived) groups. The average control value was then subtracted from values obtained in individual flies in sleep deprived groups. The net changes in sleep are therefore corrected for effects of time in the assay as well as effects of drug. Baseline correction was not applied in the starvation assay due to increased activity in the non-deprived controls. Results were subjected to one-sample t-tests. A Bonferroni correction was applied to p-values for the number of tests performed (n=4; daytime and nighttime points were treated separately). Corrected p-values and all other p-values described of 0.05 or less were considered significant. A two-way analysis of variance (ANOVA) was also performed to evaluate an effect of drug treatment as well as time on recovery sleep.

One-way ANOVA followed by Tukey’s post-hoc comparison was used to evaluate differences in locomotor activity and other sleep parameters among groups exposed to

vehicle or varying doses of propofol to determine whether a significant effect of dosage exists. For arousal threshold experiments, results are reported as percent immobile (sleeping/anesthetized) flies responding to stimuli; Kruskal-Wallis tests were used to evaluate differences among propofol doses with a Bonferroni correction applied to the p-value for each of six time points tested. Survival was analyzed using a Kaplan-Meier estimator followed by a log-rank test. A Cox-proportional hazard regression analysis was performed to evaluate effects of dose and duration of propofol on survival. All statistical analyses were performed using open-access software developed by Hammer et al,²⁵ Paleontological Statistics software (PAST, version 2.17: <http://folk.uio.no/ohammer/past/>).

Results

Propofol dose-dependently reduces locomotor activity and induces an anesthetic-like state in flies

To examine the effect of different doses of propofol on *Drosophila* behavior, we transferred individual flies to activity tubes containing regular sucrose food mixed with varying amounts of propofol. Flies were kept on propofol or an equivalent concentration of vehicle for 6h from zeitgeber time (ZT) 0–6, the first half of the lights-on phase. The highest dose, 3.0 mM propofol, rapidly abolished locomotor activity in all flies (Figure 1A), but killed 66% (21/32) of animals treated with this high dose. Exposing flies to two lower, non-toxic doses of propofol during wakefulness also robustly decreased locomotor activity ($p < 0.0001$ one-way ANOVA, $n=32$ vehicle (VEH) versus $n= 41, 39,$ and 11 flies fed $0.33\text{mM}, 1.0\text{ mM}$ and 3.0 mM propofol, respectively, Figure 1A and 1B). The group of flies fed the lowest dose (0.33 mM propofol) regained baseline activity levels much more rapidly than the groups of flies fed higher doses of propofol (Figure 1A). The emergence period (ZT 7–12) occurring after the flies were returned to fresh, drug-free sucrose activity tubes, most clearly separated the effects of different doses on locomotor activity (Figure 1B). Flies fed the lowest dose of propofol showed activity levels during the emergence period that were significantly higher than that in groups fed 1.0 and 3.0 mM propofol ($p < 0.0002$, Tukey's post-hoc). These differences in activity level during emergence from anesthesia putatively reflect the varying depth of anesthesia and residual drug effects following the 6 hour ingestion of different propofol doses in the food medium.

As consolidated inactivity in flies lasting more than 5 minutes is conventionally defined as sleep, another means of evaluating this data quantifies the epochs during which flies fail to trigger beam breaks. Although these propofol-fed animals could either be in an extended state of propofol-induced immobility or could merely have exited the anesthetic state and fallen asleep as discussed below, Figure 2A demonstrates that inactivity putatively assigned as 'sleep' was increased dose-dependently by propofol exposure. The duration of 'sleep' bouts also reached maximal levels toward the end of the propofol feeding period (Figure 2B). When evaluated across the propofol period as well as during emergence, 'sleep' bout length was strongly influenced by propofol dose (Figure 2C). Specifically, all three doses of propofol significantly increased 'sleep' bout duration during the propofol treatment period ($p < 0.00001$, one-way ANOVA), with the two higher doses (1.0 mM and 3.0 mM) producing significantly longer 'sleep' bouts than the lower dose (0.33 mM ; $p < 0.002$,

Tukey's post-hoc). The 0.33 mM dose also increased bout duration relative to the vehicle control ($p < 0.02$, Tukey's post hoc). During emergence, after all flies were transferred back to regular food (ZT 7–12, Figure 2C), bout duration remained increased in flies that had received 1.0 mM and 3.0 mM relative to lower doses and vehicle controls ($p < 0.001$, Tukey's post-hoc). Together, these data indicate that propofol ingestion by flies dose-dependently reduces locomotor activity with a concomitant increase in consolidated inactivity.

Given that 3.0 mM propofol-fed over a 6h period killed 66% of flies, we attempted to create a deeper anesthetic state by feeding flies high doses of propofol for a shorter duration and wondered whether this might be less toxic. Flies were fed 3.0 mM or 10 mM propofol or equivalent concentrations of vehicle for 1h or 3h, starting at lights on, ZT 0, and then transferred back to regular food. Survival outcome was monitored for 7 days. Similar to the 6h treatment period, high doses of propofol rapidly reduced activity and increased 'sleep' as well as bout duration of inactivity. These high-dose propofol effects persisted for more than 12h irrespective of 1h or 3h treatment periods (Figure 3A,B). For all behavioral parameters measured, activity counts (Fig 3C,D), total 'sleep' time (Fig. 3E,F), and bout length (Fig. 3G,H) per 6h, one way ANOVA showed significant effects of drug ($p < 0.00001$, for 1 hour fly dosing: $n=47$ fed VEH, $n=48$ fed 3.0 mM propofol, and $n=48$ fed VEH and $n=30$ fed 10.0 mM propofol across 3 experimental replicates; for 3 hour fly dosing: $n=47$ fed VEH, $n=31$ fed 3.0 mM propofol, and $n=48$ fed VEH and $n=13$ fed 10.0 mM propofol in total across 3 independent experimental replicates). However, there was no significant effect of dose on behavior within the 1h or 3h feeding times ($p > 0.05$, Tukey's post-hoc), indicating a possible ceiling effect of propofol as delivered in the food medium.

Striking effects of dose and feeding duration of propofol were detected on survival (Figure 3I and 3J). Cox proportional hazards regression analysis showed significant effects of dose (risk ratio, 2.20, $p < 0.0001$) and duration of feeding (risk ratio, 2.08, $p < 0.0002$, $n=191$ flies across all propofol-fed groups). As shown in Fig 3I, most of the flies that succumbed to 1h exposure of the drug did so within the first 48h, while the remaining flies survived for the duration of the assay. Exposing flies to 3h propofol killed roughly 70% of flies receiving 10 mM propofol, and 30% of flies receiving 3 mM propofol (Fig 3J). Few remaining flies succumbed over the next several days. In conclusion, flies show dose-dependent behavioral responses to propofol with a ceiling effect on inactivity at 3.0 mM and increased anesthetic-associated mortality in keeping with high dose propofol exposure in other species.^{26,27}

Propofol-fed flies display increased arousal thresholds consistent with onset of anesthetic state

To further analyze behavioral effects of propofol and to attempt to differentiate between sleeping and anesthetized flies, we assessed the arousal threshold of flies fed non-toxic doses of propofol. We subjected propofol-fed flies to mechanical stimuli using a vortexer and compared their responsiveness to naturally sleeping flies. Flies were deemed responsive only if they were also quiescent for a minimum of five minutes prior to mechanical stimuli. Propofol (0 mM, 0.5 mM, and 1.0 mM) was administered during the second half of the night, from ZT 18 to 0, corresponding to a period during which entrained flies would

normally be sleeping. Propofol-treated flies were significantly less responsive to vibratory stimuli compared with naturally sleeping flies (Figure 4). Propofol-fed flies were nearly unresponsive to the mild stimuli, with a significant effect detected at ZT 19.5 ($p < 0.008$, Kruskal-Wallis; n sleeping flies=79 fed VEH, $n=92$ fed 0.5 mM propofol, and $n=101$ fed 1.0 mM propofol across 6 replicates). Propofol robustly reduced responses to strong stimuli at all time points measured (Figure 4; $p < 0.02$, Kruskal-Wallis, $n = 137$ fed VEH, $n=119$ fed 0.5 mM propofol, and $n=112$ fed 1.0 mM propofol in total across 6 experimental replicates). Although the higher, 1.0 mM dose of propofol almost completely abolished responsiveness and the 0.5 mM dose impaired responsiveness to a lesser degree, these differences were not statistically significant ($p > 0.09$, Mann-Whitney comparison).

Oral propofol is rapidly absorbed in flies but cleared slowly

We next used HPLC to evaluate the pharmacokinetics of ingested propofol in whole flies as well as in isolated fly heads. Groups of five female flies fed 1.0 mM propofol were collected at indicated time points during a 6h propofol treatment and afterward for up to 48h (Figure 5). The concentration of propofol in homogenized fly tissue was then measured at each time point and normalized to the weight of the tissue samples. Propofol accumulated at higher concentrations in heads than throughout the whole fly, with peak concentrations of 1623.0 ± 211.3 nanograms (ng) per milligram (mg) tissue in heads and 243.4 ± 17.3 ng/mg tissue in whole flies (Figure 5) after 6h exposure. Oral ingestion of propofol caused rapid drug accumulation, and in keeping with propofol's high lipophilicity,²⁸ displayed the expected slower clearance (Figure 5). The prolonged clearance of oral 1.0 mM propofol correlates with the sustained reduction in locomotor activity observed in flies during the withdrawal period (Figure 1). These data indicate that the absorption and clearance of orally ingested propofol correlates with fly behavior during exposure.

Propofol anesthesia delays recovery sleep after sleep deprivation

Were propofol anesthesia to substitute for natural sleep, no sleep debt would accrue during propofol exposure and pre-existing sleep debts would be discharged. To determine whether sleep pressure is dissipated under propofol anesthesia, we sleep deprived populations of flies for 10h from ZT 14–24, and individually transferred them to activity tubes containing either 1.0 mM propofol or an equivalent concentration of vehicle for 6h from ZT 0–6. Flies were then returned to activity tubes containing fresh food, and monitored continuously for up to 7 days. Following the 10h SD, vehicle-fed flies showed the expected period of recovery sleep in the morning (ZT 0–6) relative to the non-deprived control (Figure 6A). Net changes in sleep were calculated across 12h periods in the sleep deprived groups and normalized to those in corresponding non-deprived controls (see methods). The increase in sleep in the vehicle group was significant ($p < 0.0001$, one-sample t-test, Bonferroni corrected; $n=48$ flies (SD), and 46 controls across 3 replicates) for the first 12h following SD, while no significant changes were detected at night or for the subsequent day (Figure 6B). Propofol-fed flies showed no significant change in sleep during the first 12h following SD, nor at nighttime ($p > 0.05$, $n=30$ flies (SD), and 45 propofol-fed controls across 3 replicates). However, a significant increase in sleep was detected on the day following propofol treatment (Day 2, Fig 6B; $p < 0.03$, one-sample t-test, Bonferroni corrected). Because sleep deprivation elicited no significant changes in sleep at night in either the vehicle or propofol-fed groups (not

shown), we focused on daytime sleep only and conducted a two-way ANOVA to evaluate effects of drug condition and time on recovery sleep. A significant interaction between drug effect and time was detected ($F_{1,152} = 11.0$, $p < 0.002$), but no effect of drug ($p = 0.41$). These findings indicate that sleep debt does not dissipate during propofol anesthesia, but is instead delayed until after emergence from anesthesia.

Sleep deprivation increases lethality during propofol anesthesia

A criterion for including flies in analyses of sleep and other behavioral parameters is that they survive for at least 24h following the analysis period (see methods). Approximately 95% or greater survival is common for most behavioral studies in flies. However, we noticed that a much larger proportion of propofol-treated flies that were also subjected to SD did not survive and had to be excluded from our analyses described above (see “Behavioral assays” in Materials and Methods). To investigate this issue further, we calculated survival in vehicle- and propofol-fed flies with and without SD (Figure 6C). While 1.0 mM propofol alone had no significant effect on survival ($p = 0.08$, log rank test, $n = 46$ VEH and propofol-fed flies; Fig. 6C, left panel), subjecting flies to 10h SD prior to propofol anesthesia significantly decreased survival outcome ($p < 0.0001$, log rank test, $n = 46$ VEH and 44 propofol-fed flies; Fig. 6C, right panel). This finding suggests that propofol anesthesia may severely impair restorative properties of sleep.

We next assessed propofol concentrations in flies subjected to SD and compared values to those that were not. Groups of flies were subjected to 10h SD from ZT 14–24, and fed 1.0 mM propofol from ZT 0–6. Propofol fed flies that were sleep deprived were collected at ZT 6, along with a non-deprived control. The remaining flies from each group were allowed to recover for 24h and collected the next day at ZT 6, 24h after propofol treatment. Propofol concentration was measured in fly heads using HPLC, as described in Materials and Methods. Results are shown in Figure 6D. All flies fed propofol for 6h had 1438.7 ± 267.1 ng/mg tissue in heads ($N = 3$ replicates, 5 flies each for SD and control groups), most of which was cleared over the ensuing 24h, consistent with the result shown in Figure 5. However, flies subjected to SD had significantly lower concentrations of propofol (by $-45.9 \pm 9\%$) than those in the control group ($F_{1,8} = 11.14$, $p < 0.02$, two-way ANOVA, Figure 6D). This finding indicates that sleep deprived flies may have been more sensitive to propofol such that lower amounts of the drug were sufficient to elicit anesthetic effects.

To test whether propofol anesthesia in sleep deprived flies was equivalent to that in the non-deprived group, we again tested arousal thresholds in flies at different times relative to the propofol or vehicle treatment following sleep deprivation. We subjected additional groups of flies to 10h SD, and applied strong vibratory stimuli (1second pulse; see “Behavioral assays” in Materials and methods) during and after the 6h propofol treatment period. Specifically, stimuli were applied during vehicle or propofol treatment at ZT 4 and 6, during the subsequent nighttime period at ZT 16 and 18, and the following day, at ZT 4 and 6. Responses were determined during drug treatment, 10–12 h after treatment (during the nighttime), and 24 hours later ($N = 4$ experimental replicates). Results are shown in Figure 6E. Kruskal-Wallis tests indicate significant effects during the treatment period ($p < 0.007$, $n = 64$ vehicle and 48 propofol controls; 63 vehicle and 33 propofol fed flies subjected to SD

across 4 experiments), and during the nighttime period ($p < 0.04$). Mann-Whitney comparisons demonstrate onset of the anesthetic state as evidenced by significantly reduced responsiveness of propofol treated flies during the drug exposure period relative to the corresponding vehicle group ($p < 0.04$, Figure 6E, left), but no differences between the propofol-fed sleep deprived and propofol-fed non-deprived groups or in the vehicle-fed sleep deprived and vehicle-fed non-sleep deprived groups, supporting the ability of strong external stimuli to distinguish between sleeping and anesthetized flies. During the night-time recovery period (Fig. 6E, middle), arousal responses of both propofol groups were equivalent to corresponding vehicle groups, but the sleep-deprived vehicle group showed significantly reduced responsiveness as compared to both the non-deprived vehicle and propofol groups ($p < 0.04$). No significant group effects were detected 24h after exposure (Fig. 6E, right). These findings indicate that SD and control groups were anesthetized by propofol to an equivalent extent and recovered in a manner that was also statistically equivalent. These findings also indicate that the net increase in sleep seen in the propofol-fed sleep-deprived group the next morning, on Day 2 (Figure 6B), occurred after flies had emerged from an anesthetized state, supporting the notion that recovery sleep is delayed by propofol anesthesia.

Given that sleep deprived flies ingested lower amounts of propofol, we next tested whether the decrease in survival in propofol-anesthetized flies was attributed to an impaired access to food. Although flies have free access to food, the combination of forced activity by the mechanical stimulus used to keep flies awake for 10h and an anesthetized state (despite oral delivery) that followed for 6h may have reduced the flies' ability to ingest a sufficient amount of food to support survival. To test this possibility, all flies were placed on nutrient-free medium during the propofol treatment period following 10h SD from ZT 14–24. Interestingly, starvation during the 6h period following SD blocked recovery sleep and increased locomotor activity in vehicle-fed flies, but not in propofol-fed flies during the 6h treatment period (Figure 7A and 7B). However, the vehicle-fed group that was subjected to sleep deprivation prior to the starvation period showed a significant increase in sleep that lasted through the second daytime period of recovery (Figure 7C). The propofol-fed group, in contrast, showed recovery sleep on the second day, similar to that observed without starvation (compare Figure 7C to Figure 6B). Two-way ANOVA showed significant effects of propofol ($F_{1,216} = 5.98$, $p < 0.02$), as well as time ($F_{3,216} = 3.05$, $p < 0.03$), with a significant interaction ($F_{3,216} = 3.08$, $p < 0.03$). Thus starving flies both delays as well as prolongs their recovery sleep in response to SD. Propofol anesthetized flies, in contrast, also showed delayed recovery sleep, but in a manner that was similar to non-starved flies, such that the increase in sleep occurred on the second day after SD and after emergence from the anesthesia. If lack of access to the food during propofol anesthesia decreased survival, we would expect that vehicle-treated, starved flies would also show reduced survival after 10h SD. Instead, we found that starving flies for the 6h period after SD had no effect on survival in the vehicle-treated flies, but similarly reduced survival in propofol-treated starved flies (Figure 7D). Thus inadequate access to food does not account for reduced survival in propofol-anesthetized flies following SD.

A shorter SD duration of 6h (from ZT 18–24) also reduced survival in 1.0 mM propofol-treated flies as compared to vehicle controls ($p < 0.00005$ log rank test, $n = 31$, 28 flies for

sleep deprived VEH and PPF groups, respectively across two independent replicates; data not shown). Moreover, 6h SD produced a significant sleep rebound in vehicle-treated flies on the day following SD with a net increase in 93.6 ± 22.0 minutes sleep over the 12h daytime period ($p < 0.001$, one-sample t-test, Bonferroni corrected, $n = 28$ flies across two replicates). An increase in sleep in the propofol-fed flies was also noted on the second day after SD, but this fell short of significance (60.9 ± 34.4 minutes, $p = 0.088$, one-sample t-test, $n = 14$ flies across two replicates). Nonetheless, the reduced survival in the propofol-fed flies support the notion that propofol anesthesia does not satisfy the restorative properties of sleep.

Discussion

The canonical two-process model of sleep holds that sleep is dissociable into circadian and homeostatic components which together orchestrate sleep/wake cycles in addition to numerous other diurnal neurophysiologic outputs.²⁹ Knowledge of the circadian process has advanced with the identification of various clock genes, such as Period (Per) and Timeless (Tim) which comprise a cell-autonomous transcriptional feedback loop, underlying the core molecular clock in *Drosophila*.³⁰ Sleep homeostasis, by contrast, is postulated *a priori* to arise from byproducts of neuronal activity which accumulate during wakefulness and modulate the duration and intensity of sleep (i.e., sleep pressure). It remains controversial, however, whether a unitary account of sleep homeostasis will emerge paralleling the identification of Per and Tim, due to the complexity and redundancy of a number of sleep regulatory substances, such as adenosine,³¹ numerous cytokines^{32,33} as well as *crossveinless-c*³⁴ and *sleepless* in *Drosophila*.³⁵ Tung et al. reported that propofol anesthesia satisfies the homeostatic requirement for both REM and NREM sleep in rodents, such that pre-existing sleep debt dissipates under propofol anesthesia and new debt does not accrue.^{17,18} Though intriguing, this result is in need of replication under disparate conditions and in other species to better assess its general validity and potential relevance to human neurophysiology. Herein, we report that propofol induces a state of general anesthesia in *Drosophila* and provide behavioral evidence that prior sleep debt does not dissipate during propofol anesthesia, which contrasts with previous findings in rodents.¹⁸ Instead, recovery sleep is delayed until after flies have emerged from anesthesia. Moreover, lethality was increased in flies subjected to SD prior to propofol anesthesia, suggesting either that temporal suppression of recovery sleep by propofol compromises resilience to SD or that SD increases sensitivity to the adverse side effects of propofol.

We first demonstrate that flies are effectively anesthetized when propofol is added to the food medium as evidenced by the dose dependent decrease in locomotor activity. The decrease in locomotor activity was limited by the flies' ability to ingest food, as we noted a ceiling effect on behavior at high doses, but not on toxicity. One limitation of the locomotor activity assay we employed is that flies might cease moving yet remain alert, such that the assay is blind to the fly's actual arousal level. Or in other words, how can we be certain that flies are not simply motionless rather than anesthetized? The ceiling effect of high-dose propofol is unlikely due to this limitation. Anecdotally, we found that propofol treatment caused a loss of postural reflexes in *Drosophila*, such that propofol-treated flies were supine and did not attempt to escape during the manual transfer back to fresh activity tubes

containing regular food. Nevertheless, to address the possibility that propofol treatment might specifically impair locomotor activity without affecting arousal at lower, less toxic doses, we subjected anesthetized subjects to mechanical stimuli using a vortexer and compared their responsiveness to naturally sleeping, control flies. Noxious stimuli are sufficient to rouse sleeping flies, but fail to rouse propofol-fed flies. As the immobilizing endpoint requires higher doses of propofol than that required for loss of consciousness,³⁶ our data are congruent with the idea that propofol-induced inactivity likely reflects the true induction of an anesthetic state. Though it remains technically unfeasible to directly measure other clinically significant anesthetic endpoints in flies (e.g., analgesia or amnesia), unresponsiveness to external stimuli and cessation of locomotor activity are *prima facie* tenable indications of the state of general anesthesia.

HPLC measurements of propofol concentration in fly heads following 6h exposure to 1.0 mM propofol were slightly greater than predicted in human brain. This estimate assumes that pericerebral fat body occupies ~20% of the volume in the fly head,³⁷ that it has a partition coefficient similar to octanol (55-fold greater than brain³⁸), and suggests that the actual fly brain propofol levels peak at 97 micrograms (μg)/mL. Given a brain:plasma partition coefficient of 8.2,³⁹ were flies to have plasma, we would predict propofol serum levels corresponding to 11.8 μg /mL. This slightly exceeds the clinical range of 1–10 μg /mL in human plasma.^{40–43} Considering that propofol takes up to 6 hours to reach equilibrium into brain slices,⁴⁴ our HPLC results may cumulatively explain why ingestion of 1mM propofol leads to consolidated inactivity that is most congruent with a state of anesthesia. This lends credence to our findings that 1mM propofol-fed flies are not merely sleeping and should not be roused by vibratory stimuli as these tissue levels are predicted to produce surgical plane of propofol anesthesia. Despite millions of years of evolution separating arthropods from mammals, it is remarkable that such strikingly similar doses of propofol elicit anesthesia in flies. We therefore suggest that homologous molecular and neuronal targets likely mediate the behavioral effects of propofol in flies.

Using rodents to investigate how propofol alters the response to SD is advantageous since propofol can be delivered intraperitoneally or intravenously, allowing more careful titration to ensure that hypnotic doses of anesthetic are maintained. It is well known that anesthetics produce sedation and depress central nervous system function at lower doses prior to wholesale loss of consciousness. However, it might be argued that Tung's result can be rationalized by a transient drop to sub-hypnotic propofol levels, allowing animals to partially emerge from the anesthetic state, intermittently access sleep, and thereby covertly discharge sleep pressure. Inducing anesthesia by adding propofol to the food medium, the method used in the present study, appears even more susceptible to this objection, since oral propofol offers less control over dose than the typical intravenous route.

The pharmacokinetic data directly addresses this difficulty, however. Oral propofol is absorbed rapidly in flies, and levels steadily increase during our 6h exposure. Propofol also slowly clears with detectable amounts remaining in tissue for at least 24h after removal from the drug (Figures 5 and 6D). These findings are consistent with the arousal assay, where anesthetized flies showed significantly reduced responses to mechanical stimuli applied during nighttime (Figure 5) or daytime (Figure 6D) treatment with propofol. Moreover,

ingested propofol preferentially concentrates in neural tissue (on an ng propofol per mg fly tissue basis) rather than in the body of the fly (Figure 5). Intravenously delivered propofol in humans, by contrast, is rapidly cleared from neural tissue and is continually redistributed into other bodily compartments, and hence must be administered as a continuous infusion to compensate for its rapid pharmacokinetic profile.⁴⁵ The authors speculate that propofol largely accumulates in neural tissue in *Drosophila* because 1) ingested propofol passes directly through the proboscis into the head; and 2) propofol taken up into circulating hemolymph is pumped directly into the head.⁴⁶ Together, these findings indicate that it is unlikely that the propofol concentration in the central nervous system would fall to sub-hypnotic concentrations, and may partially account for why results of the current study contrast with the previous work of Tung et al.¹⁸

If propofol substituted for natural sleep, we would expect animals to revert to normal sleep patterns after emergence from anesthesia regardless of prior sleep history. Instead, flies experience a net increase in sleep 24h after treatment. Although traces of drug remained in tissue by this time, flies emerged from an anesthetized state as indicated by their responsiveness to mechanical stimuli that was indistinguishable from vehicle-treated controls. To our surprise, 6h starvation in vehicle-fed flies delayed and extended recovery sleep after a 10h deprivation. Starvation initially suppresses sleep^{47,48} but ultimately causes rebound recovery sleep in adult flies.⁴⁸ The extended recovery sleep in the vehicle-fed group may be explained by the prolonged waking induced by both starvation and sleep deprivation in the current study. In contrast, propofol-fed flies showed the same pattern of recovery sleep whether they were starved during treatment or not. Our explanation for this is that the propofol-fed flies did not experience waking during the starvation period (and were not further sleep deprived); thus the recovery sleep was solely attributed to the prior night-time SD.

While the current findings indicate that propofol does not satisfy the homeostatic need for sleep, whether propofol or other anesthetic drugs produce cognitive or other deficits associated with sleep deprivation will require further study. The current findings show that lethality in anesthetized flies was increased by SD. To our knowledge, SD does not alter the pharmacokinetics of drugs, but pharmacokinetic changes cannot be entirely ruled out. Propofol may instead antagonize the restorative properties of sleep that are necessary for survival.^{49–51} Alternatively, it is likely that as in rodents,⁸ sleep deprivation increased flies' sensitivity to propofol as indicated by HPLC measurements showing reduced ingestion of the drug. Future studies should address these issues by evaluating performance in a learning assay or measuring other physiological parameters associated with sleep such as synaptic scaling.^{52,53}

Taken together, our results indicate that flies are effectively anesthetized by the addition of propofol to the food medium, and importantly, establish *Drosophila* as a suitable genetic model to investigate mechanisms of propofol anesthesia. These data also indicate that sleep debt does not dissipate during propofol anesthesia, nor does propofol substitute for the restorative aspects of natural sleep in *Drosophila*.

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Final Box Summary Statement**What we already know about this topic**

- Though clearly distinct states, sleep and general anesthesia share some clinical features and neurobiological mechanisms
- In contradistinction to the volatile anesthetics, propofol does not accrue sleep debt in rodents.

What this article tells us that is new

- Propofol produced anesthesia in the fruit fly *Drosophila*, but did not dissipate sleep debt or satisfy the homeostatic need for sleep in contrast to rodents.
- Further studies will be required to validate findings in both rodents and flies and reconcile the apparent species-specific differences in the interactions between natural sleep and general anesthesia.

Figure 1. Propofol dose-dependently decreases locomotor activity in *Drosophila*

(A) Mean $\pm$ SEM activity counts per hour is plotted relative to ZT (zeitgeber time).

Horizontal bars (bottom) indicate 12:12 light:dark cycles. Blue horizontal bar indicates 6h

propofol (PPF) or vehicle (VEH) treatment (ZT 0 to ZT 6). (B) Mean $\pm$ SEM total total

activity per 6h is shown for the propofol treatment and withdrawal periods for indicated

doses. Significant differences between the four groups are indicated by asterisks, ** $p <$

0.005, *** $p <$ 0.0005, one-way ANOVA, Tukey's pairwise comparisons. High mortality

was consistently observed in the 3mM group. For this figure and Figure 2, $n = 32$ VEH, and

$n = 41, 39,$ and 11 flies in the 0.33mM, 1.0mM, and 3.0mM groups, respectively across 2–3 experimental replicates.

Figure 2. Propofol dose-dependently increases consolidated immobility

Mean $\pm$ SEM total time 'sleep' (A) and 'sleep' bout length (B) per 15 minutes during the 6h propofol (PPF) or vehicle (VEH) treatment (horizontal bar, zeitgeber time (ZT) 0–6) and the 6h period following treatment (ZT 7–12) is shown for all three doses of propofol and vehicle. Sleep is defined as immobility for a minimum of 5 minutes. (C) Mean $\pm$ SEM total time 'sleep' bout length per 6h is shown for the 6h propofol treatment and withdrawal periods. Gray bars correspond to times during which flies were flipped to and from tubes containing propofol. Significant differences between the four groups are indicated by asterisks, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, one-way ANOVA, Tukey's pairwise comparisons. Data are derived from the same flies depicted in Figure 1.

Figure 3. Effects of duration and dose of propofol treatment on behavior and survival

Results from flies receiving propofol at ZT 0 for one hour (A, C, E, G, and I) are shown in the left panels or three hours (B, D, F, H, and J) are shown in the right panels. Duration of propofol (PPF) (or vehicle, VEH) feeding is depicted by green horizontal bars. (A, B) Representative experiments showing mean $\pm$ SEM activity counts per hour plotted against zeitgeber time (ZT). Flies were fed indicated doses of propofol during the second 24h period shown. $n=15-16$ flies (A), and $7-16$ flies (B). Mean $\pm$ SEM activity counts (C,D), 'sleep' (E,F), and 'sleep' bout length (G,H) per 6h is plotted for the day that flies were treated with propofol. $** = p < 0.00001$, $* = p < 0.005$, one-way ANOVA with Tukey's post-hoc. For one hour treatment, $n=47, 48$ flies fed 3mM VEH and PPF, respectively, and $n=48$ and 30 flies

fed 10mM VEH and PPF, respectively across 3 independent replicates. For three hour treatment, n=47, 31 flies fed 3 mM VEH and PPF, and n=48, 13 flies fed 10mM VEH and PPF, respectively across 3 independent replicates. (I, J) Kaplan-Meier plots of survival is shown for flies treated with 3 mM and 10 mM VEH and PPF as indicated. Survival was measured from ZT 0, at the time when flies were placed on propofol. Both 3mM and 10mM propofol significantly reduced survival when applied for 1h ($p < 0.0002$, log rank test) relative to the corresponding VEH control, and when applied for 3 h ($p < 0.00001$, log rank test) relative to corresponding controls; n=47–48 flies each group across 3 independent replicates.

Figure 4. Propofol-treated flies show increased arousal thresholds

The percentage of flies responding to either mild or strong mechanical stimuli was assessed at indicated zeitgeber times (ZT) for flies fed vehicle (VEH) or two different doses of propofol (PPF) from ZT 18–24. “Mild” and “strong” correspond to the strength of the mechanical stimulus (see methods). The percentage of flies reported are a proportion of sleeping flies from VEH $n=137$, 0.5 mM propofol $n=119$, and 1.0 mM propofol fed $n=112$ total flies tested across six replicate experiments; ** = $p < 0.009$; * = $p < 0.02$ (Bonferroni corrected), Kruskal-Wallis test.

Figure 5. Absorption and Clearance of Ingested Propofol

Mean $\pm$ SEM propofol concentration (ng propofol/mg tissue) is plotted on a logarithmic scale relative to time (in hours) during propofol exposure (1.0 mM, horizontal bar, from ZT 0–6) and afterward for up to 48 hours (N=3 replicates derived from five flies each). A model fit (curve) for each set of data points (heads and whole flies) was produced by a weighted least squares regression fit to a three compartment first-order pharmacokinetic model.

Figure 6. Recovery sleep is delayed in propofol-fed flies after sleep-deprivation

(A) Representative experiment showing mean $\pm$ SEM time sleeping in minutes per hour plotted relative to ZT (zeitgeber time) in flies fed 1.0 mM propofol (PPF, $n=15$) or vehicle (VEH, $n=16$), indicated by the red horizontal bar, during the 6h morning period following a 10h sleep deprivation (SD, gray horizontal bar) in two other groups (VEH+SD, $n=16$, and PPF+SD, $n=7$). (B) Mean $\pm$ SEM net changes in sleep (minutes per 12h) are reported for sleep deprived flies for the day immediately following SD (Day 1), and the next day (Day 2). **= $p<0.0001$, *= $p<0.03$, one-sample t-test, Bonferroni corrected; $n=48$ VEH flies and 30 PPF flies across 3 independent experiments. See text for details. (C) Survival was monitored during the 6h propofol treatment period (from ZT 0–6; see Fig. 4A) and for up to 5–6 days afterward. Survival was plotted using the Kaplan-Meier estimator. Propofol treatment did not significantly alter survival in non-deprived flies (handled control, left panel, $p=0.08$ log rank test, $n=46$ flies each group), but reduced survival in sleep-deprived flies ($p<0.0001$, log rank test, $n=48$, 44 flies for VEH and PPF groups, respectively). (D) Mean $\pm$ SEM propofol concentration (plotted on a logarithmic scale) in fly heads is shown for propofol fed flies (1.0 mM) that were sleep deprived as in (A) and for non-deprived controls (Ctrl); $N=3$ replicates containing 5 flies each. (E) Arousal responses following strong mechanical stimuli are shown for indicated groups during propofol or vehicle treatment (ZT 4–6 refers to stimulus times), 10–12 hours after treatment (Night), and 24h after vehicle or drug treatment (24h recovery). * = $p < 0.04$, Mann-Whitney; percent flies responding are reported as a proportion of sleeping flies from $n=64$ and 48 VEH and propofol controls, respectively, and 63 and 33 VEH and propofol-fed flies, respectively, that were subjected to SD across 4 independent replicates.

Figure 7. Starvation does not affect lethality following sleep deprivation in propofol-anesthetized flies

(A) Representative experiment showing mean $\pm$ SEM time sleeping in minutes per hour plotted relative to ZT (zeitgeber time) as described in Figure 1A. Following 10h sleep deprivation (SD; gray horizontal bar), flies in all groups were placed on starvation medium (2% agar) containing 1.0 mM propofol (PPF) or vehicle (VEH) for 6h (indicated by red-outlined horizontal bar; $n=14-16$ flies for all groups). (B) Mean $\pm$ SEM total activity counts is shown for the 6h period during PPF (right panel) or VEH (left panel) treatment in flies that had been subjected to 10h SD or not. Results are shown for flies that were maintained on regular food (5% sucrose) or starved. Two-way ANOVA showed significant effects of starvation on increasing activity counts ($F_{1,154}=18.77$; $* p < 0.00001$) in VEH fed flies, but not in PPF-treated flies; a significant effect of SD was also detected in VEH flies (left; $F_{1,154}=93.2$; $\# = p < 0.00001$) but not in PPF-fed flies (right). Numbers of flies are indicated

for each group. **(C)** Mean $\pm$ SEM net changes in sleep (minutes per 12h) are shown for sleep deprived flies for indicated 12h periods following 10h SD. ** = $p < 0.002$, * = $p < 0.03$, one-sample t-test, Bonferroni corrected; $n = 32$ and 24 VEH and PPF fed flies, respectively, across two replicates. **(D)** Survival in flies subjected to 10h sleep deprivation followed by 6h propofol or vehicle treatment and starvation is plotted using the Kaplan-Meier estimator. Survival was monitored during the 6h starvation period (from ZT 0–6) and for up to 5–6 days afterward ($p < 0.001$ log rank test, $n = 30$ – 32 flies per group across two independent replicates).