

## Increased Volatile Anesthetic Requirement in Short-sleeping *Drosophila* Mutants

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**Background:** Anesthesia and sleep share physiologic and behavioral similarities. The anesthetic requirement of the recently identified *Drosophila* mutant *minisleeper* and other *Drosophila* mutants was investigated.

**Methods:** Sleep and wakefulness were determined by measuring activity of individual wild-type and mutant flies. Based on the response of the flies at different concentrations of the volatile anesthetics isoflurane and sevoflurane, concentration-response curves were generated and EC<sub>50</sub> values were calculated.

**Results:** The average amount of daily sleep in wild-type *Drosophila* (n = 64) was 965 ± 15 min, and 1,022 ± 29 in *Na[bar<sup>38</sup>]* (P > 0.05; n = 32) (mean ± SEM, all P compared to wild-type and other shaker alleles). *Sb<sup>mins</sup>* flies slept 584 ± 13 min (n = 64, P < 0.01), *Sb<sup>102</sup>* flies 412 ± 22 min (n = 32, P < 0.01), and *Sb<sup>120</sup>* flies 782 ± 25 min (n = 32, P < 0.01). The EC<sub>50</sub> values for isoflurane were 0.706 (95% CI 0.649 to 0.764, n = 661) and for sevoflurane 1.298 (1.180 to 1.416, n = 522) in wild-type *Drosophila*; 1.599 (1.527 to 1.671, n = 308) and 2.329 (2.177 to 2.482, n = 282) in *Sb<sup>102</sup>*, 1.306 (1.212 to 1.400, n = 393) and 2.013 (1.868 to 2.158, n = 550) in *Sb<sup>mins</sup>*, 0.957 (0.860 to 1.054, n = 297) and 1.619 (1.508 to 1.731, n = 386) in *Sb<sup>120</sup>*, and 0.6154 (0.581 to 0.649, n = 360; P < 0.05) and 0.9339 (0.823 to 1.041, n = 274) in *Na[bar<sup>38</sup>]*, respectively (all P < 0.01).

**Conclusions:** A single-gene mutation in *Drosophila* that causes an extreme reduction in daily sleep is responsible for a significant increase in the requirement of volatile anesthetics. This suggests that a single gene mutation affects both sleep behavior and anesthesia and sedation.

DESPITE the widespread use of volatile anesthetics in medical practice, the specific mechanisms of action of inhalational agents remain largely unknown. This hampers efforts to make general anesthesia more individually tailored, more effective, and more convenient for the patient. Also, insuffi-

cient knowledge of underlying mechanisms of anesthesia is associated with lack of predictive values regarding anesthesia-related complications; *i.e.*, incidence of awareness, side effects of anesthetics, and hemodynamic compromise. More importantly, the individual anesthetic requirement markedly differs between patients and is largely unpredictable. At present, dosing recommendations are based on expert knowledge that in turn depends on patient characteristics such as age and weight, whereas genetic factors that undoubtedly play a major role in the existing differences in anesthetic requirement remain mostly elusive.

*Drosophila melanogaster* represents a powerful model for studying many aspects pertaining to the interaction between neural function and genetic properties.<sup>1</sup> The fruit fly has a complex nervous system consisting of tens of thousands of neurons organized into circuits that control complex behavior, uses many of the same neurotransmitters as vertebrates, and possesses homologous neurotransmitter receptors and ion channels. Moreover, like mammals, fruit flies exposed to volatile anesthetics proceed through an excitable state, followed by an uncoordinated state, and then an unresponsive and immobile state.<sup>2,3</sup>

Recently, some of us identified *minisleep* (*Sb<sup>mins</sup>*), a *Drosophila* strain that sleeps significantly less than its wild-type counterpart, thereby linking a genetic mutation to a difference in a complex process like sleep behavior.<sup>4</sup> Although anesthesia and sleep are not identical, both share remarkable physiologic and behavioral similarities that may rely partly on identical mechanisms and common molecular targets. Therefore, we were interested in testing whether this short-sleeping *Drosophila* line comparably shows a differing sensitivity to volatile anesthetics.

### Materials and Methods

#### Animals

*Drosophila melanogaster* were bred in the laboratory at 21°C, 68% humidity, on yeast, dark corn syrup, and agar food. For determination of sleep and wakefulness, male and female fruit flies were used in equal numbers. To exclude age-associated effects, only young flies (≤ 2 weeks) were tested for all experiments.<sup>5</sup> *Drosophila* stocks used were *Sb<sup>mins</sup>*, *Sb<sup>102</sup>*, *Sb<sup>120</sup>* *Na[bar<sup>38</sup>]*, and wild-type Canton-S. To remove modifiers, stocks were consequently outcrossed for at least five rounds to Canton-S background as described before.<sup>4</sup> Canton-S is not known to be resistant to volatile anesthetics. *Sb<sup>mins</sup>*,

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*Sh*<sup>102</sup>), and *Sh*<sup>120</sup>) are different mutant alleles of the *Shaker* locus, encoding the alpha subunit of a tetrameric voltage-dependent potassium channel.<sup>6</sup> The *Na* gene encodes a sodium leak channel,<sup>7</sup> which exerts opposite effects on excitability to the *Shaker* gene; e.g., *Na[bar<sup>38</sup>]* is known to be hypersensitive to volatile anesthetics.<sup>8</sup>

#### Determination of Locomotor Activity

Sleep and wakefulness were determined from individual fruit flies placed in a *Drosophila* activity monitoring system (Trikinetics, Waltham, MA) at constant environmental conditions. Activity measurement was recorded for consecutive 1-min periods for 1 week after 1 day of adaptation, and analyzed with custom-designed software developed in our laboratory. As described before, sleep was defined as any period of uninterrupted behavioral immobility (0 counts per minute) lasting > 5 min.<sup>4,9,10</sup> The total duration of sleep episodes was then calculated exactly to the minute.

#### Measurement of Anesthetic Sensitivity

Anesthetic sensitivity was tested in a custom-made *Drosophila* anesthesia chamber (V = 200 ml) connected to isoflurane or sevoflurane vaporizers, respectively, with a constant flow of 1.6 l/min. For each experiment, at least 10 young ( $\leq$  2 weeks) wild-type or mutant strain fruit flies were placed inside the chamber and exposed to distinct anesthetic concentrations. After a 10-min exposure the chamber was rotated and shaken for 2 s under the control of a motor, which caused the flies to fall from their current position to the bottom of the chamber. With this accepted method of sleep deprivation,<sup>10</sup> we were able to distinguish between sleep and anesthesia. The numbers of mobile and immobile flies were counted by a blinded observer, but a convulsion was not considered movement. The results were recorded for subsequent statistical analysis. All experiments were carried out at constant environmental temperature of 21°C, and concentrations of the volatile anesthetics were continuously monitored at the chamber outflow with a Datex-Ohmeda Capnomac Ultima (GE Healthcare, Chalfont St. Giles, England).

#### Statistical Analysis

A Student *t* test was used to assess statistically significant differences for periods of sleep and wakefulness between *Drosophila* strains. Based on the response of the flies at different concentrations of isoflurane and sevoflurane, concentration-response curves were generated according to the method of Waud for quantal biologic responses.<sup>11</sup> The half-maximum effective concentration (EC<sub>50</sub>) values and 95% CIs were calculated and compared for statistically significant differences using GraphPad Prism version 4.03 for Windows (GraphPad Software, La Jolla, CA).

## Results

For determination of locomotor activity, male and female *Drosophila* were used in equal numbers. As described before, the duration of sleep and wakefulness was different in wild-type *Drosophila* and *Shaker* mutants.<sup>4</sup> The average amount of daily sleep in wild-type *Drosophila* (n = 64) was 965 ± 15 min (mean ± SEM), as compared with 584 ± 13 min for *Sh<sup>mns</sup>* flies (n = 64, *P* < 0.01), and as compared with wild-type *Sh<sup>120</sup>* and *Sh<sup>102</sup>*; 412 ± 22 min for *Sh<sup>102</sup>* flies (n = 32, all *P* < 0.01) and 782 ± 25 min for *Sh<sup>120</sup>* (n = 32, all *P* < 0.01). Thus, the short-sleeping phenotype was most pronounced in *Sh<sup>102</sup>*, moderately less expressed in *Sh<sup>mns</sup>* and weakest in *Sh<sup>120</sup>*. *Na[bar<sup>38</sup>]* showed a sleeping phenotype comparable to wild-type (1,022 ± 29 min, n = 32, *P* > 0.05).

Response of different *Drosophila* strains to the volatile anesthetics isoflurane and sevoflurane measured at various concentrations ranging from 0.13 to 5% for isoflurane and from 0.21 to 4% sevoflurane, respectively, yielded specific concentration-response curves. The EC<sub>50</sub> values for both volatile anesthetics, isoflurane and sevoflurane, were significantly increased statistically in fruit flies expressing the short-sleeping phenotype, and decreased in *Na[bar<sup>38</sup>]*, as compared to wild-type *Drosophila*. Moreover, EC<sub>50</sub> values for isoflurane and sevoflurane were associated with the severity of the short-sleeping phenotype. The differences in the anes-

**Table 1. Isoflurane EC<sub>50</sub> of Short-sleeping *Na[bar<sup>38</sup>]* and Wild-type *Drosophila* Calculated from Dose-response Curves**

Strain	Isoflurane EC <sub>50</sub> Value (95% CI)	<i>P</i> Values
Wild-type (n = 661)	0.706 (0.649 to 0.764)	
<i>Sh<sup>120</sup></i> (n = 297)	0.957 (0.860 to 1.054)	< 0.0001 as compared with wild-type < 0.0001 as compared with <i>Sh<sup>mns</sup></i> < 0.0001 as compared with <i>Sh<sup>102</sup></i>
<i>Sh<sup>mns</sup></i> (n = 393)	1.306 (1.212 to 1.400)	< 0.0001 as compared with wild-type < 0.0001 as compared with <i>Sh<sup>102</sup></i>
<i>Sh<sup>102</sup></i> (n = 308)	1.599 (1.527 to 1.671)	< 0.0001 as compared with wild-type
<i>Na[bar<sup>38</sup>]</i> (n = 360)	0.6154 (0.581 to 0.649)	< 0.0001 as compared with wild-type

Isoflurane EC<sub>50</sub> values are presented as means with 95% CI, and were calculated from dose-response curves generated in multiple experiments at different concentrations.

**Table 2. Sevoflurane EC<sub>50</sub> of Short-sleeping *Na[bar<sup>38</sup>]* and Wild-type *Drosophila* Calculated from Dose-response Curves**

Strain	Sevoflurane EC <sub>50</sub> (95 % CI)	P Values
Wild-type (n = 522)	1.298 (1.180 to 1.416)	
<i>Sh<sup>120</sup></i> (n = 386)	1.619 (1.508 to 1.731)	< 0.0005 as compared with wild-type < 0.0001 as compared with <i>Sh<sup>mns</sup></i> < 0.0001 as compared with <i>Sh<sup>102</sup></i>
<i>Sh<sup>mns</sup></i> (n = 550)	2.013 (1.868 to 2.158)	< 0.0001 as compared with wild-type < 0.005 as compared with <i>Sh<sup>102</sup></i>
<i>Sh<sup>102</sup></i> (n = 282)	2.329 (2.177 to 2.482)	< 0.0001 as compared with wild-type
<i>Na[bar<sup>38</sup>]</i> (n = 274)	0.934 (0.823 to 1.041)	< 0.0001 as compared with wild-type

Sevoflurane EC<sub>50</sub> values are presented as means with 95% CI, and were calculated from dose-response curves generated in multiple experiments at different concentrations.

thetic requirement of *Sb<sup>mns</sup>*, *Sb<sup>102</sup>*, *Sb<sup>120</sup>*, and *Na[bar<sup>38</sup>]* were also found to be statistically significant. The results for isoflurane and sevoflurane are summarized in tables 1 and 2, respectively. Typical concentration-response curves are shown in figure 1.

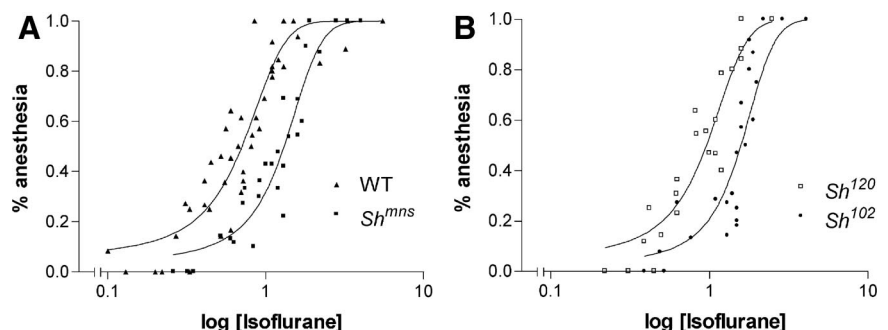
## Discussion

The main findings of our study are that mutations that cause a short-sleeping phenotype in *Drosophila* are also responsible for a significant difference in anesthetic requirement, and that the quantity of volatile anesthetic required to anesthetize *Drosophila* is associated with the severity of the short-sleeping phenotype.

Although sleep and general anesthesia are not identical, there has been increasing consensus that both states are neurophysiologically related. It has been shown that anesthetics may act partly by duplicating activities of brain regions important in initiating or maintaining sleep, and the effects on regional neuronal activity suggest activation of endogenous sleep-promoting pathways.<sup>12-14</sup> Sleep deprivation potentiates anesthetic-induced loss of the righting reflex, and anesthetic agents increase sleep when administered into brain regions known to regulate sleep.<sup>15,16</sup> In addition, the neurotransmitter adenosine increases the sleep requirement, enhances anesthetic potency, and delays recovery from halothane anesthesia.<sup>17-19</sup> Animal experiments suggest that anesthetic agents induce loss of consciousness, at least in part, *via* activation of endogenous, nonrapid eye movement, sleep-promoting hypothalamic pathways.<sup>20,21</sup>

Differences in the anesthetic sensitivity to volatile anesthetics have been reported for several mutations in genes affecting ion channels, and neurotransmitters and their receptors in *Caenorhabditis elegans* and *Drosophila*.<sup>22-25</sup> However, until now there have been no reports of common mechanisms of naturally occurring sleep and anesthesia on a molecular level.

*Drosophila* is an ideal model for investigating mechanisms involved in anesthesia in humans, as these flies have a complex nervous system and possess many of the same ion channels, neurotransmitters, and neurotransmitter receptors as vertebrates. Recently, some of us identified *Sb<sup>mns</sup>*, a *Drosophila* strain exhibiting an extreme reduction in sleep requirement, as compared with wild-type flies. We also found that other severe loss-of-function mutations of *Shaker*, including *Sb<sup>102</sup>*, were short sleepers, while weak hypomorph alleles such as *Sb<sup>120</sup>* show only little variance.<sup>4</sup> Previous electrophysiological and molecular studies found that the *Shaker* current and a normal-sized protein product were completely absent in short-sleeping mutants such as *Sb<sup>102</sup>*, whereas in *Sb<sup>120</sup>* mutants the *Shaker* current is present, although reduced.<sup>26,27</sup> With the present study, we demonstrate that a single-gene mutation affecting sleep regulation in *Drosophila* is also associated with an increased anesthetic requirement in these fruit flies. Moreover, the severity of the short-sleeping phenotype among different alleles was consistent, with an increased anesthetic requirement in *Drosophila*. The fact that the hypersensitive strain *Na[bar<sup>38</sup>]* does not show a significant long-sleeping phenotype underscores the relation-



**Fig. 1. Isoflurane dose-response curves. (A), dose-response curve for wild-type (WT) *Drosophila* and *Shaker* mutant *Sb<sup>mns</sup>*. (B), dose-response curves for *Shaker* mutants *Sb<sup>120</sup>* and *Sb<sup>102</sup>*.**



ship between the *Shaker* gene, sleep, and anesthetic requirement. Moreover, it should be mentioned that other authors have identified *Na[bar<sup>38</sup>]* as a long-sleeper.<sup>28</sup> This might be as a result of differences in the presence of genetic modifiers.

In contrast to intravenous anesthetics and opioids that have been shown to exert their anesthetic and analgesic properties mainly because of specific receptor-ligand interactions, the mechanism of action of volatile anesthetics remains largely elusive. In this study we showed that a mutation in a voltage-gated potassium channel powerfully affects the anesthetic requirement of *Drosophila*. *Shaker* controls membrane repolarization after action potentials and presynaptic transmitter release.<sup>6</sup> Neurotransmitters and their receptors have been well conserved during evolution, and homologous ion channels in vertebrates have similar properties.<sup>29</sup> Also, our study and previous quantitative comparisons of the EC<sub>50</sub> values of volatile anesthetics reveal an impressive correlation between *Drosophila* and humans,<sup>3</sup> although the EC<sub>50</sub> calculated after a 10-min exposure may reflect a complex mixture of pharmacokinetic and pharmacodynamic effects of the mutation. Furthermore, it is important to know that looking at different anesthetic endpoints in *Drosophila* may lead to completely different results.<sup>2,30</sup>

Our findings may have implications for at least two reasons: They demonstrate a link between sleep and anesthesia on a molecular level, and they show that a single-gene mutation can have a drastic effect on the susceptibility to volatile anesthetics.

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