BK channels play a counter-adaptive role in drug tolerance and dependence

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Disturbance of neural activity by sedative drugs has been proposed to trigger a homeostatic response that resists unfavorable changes in net cellular excitability, leading to tolerance and dependence. The Drosophila slo gene encodes a BK-type Ca$^{2+}$-activated K$^+$ channel implicated in functional tolerance to alcohol and volatile anesthetics. We hypothesized that increased expression of BK channels induced by these drugs constitutes the homeostatic adaptation conferring resistance to sedative drugs. In contrast to the dogmatic view that BK channels act as neural depressants, we show that drug-induced slo expression enhances excitability by reducing the neuronal refractory period. Although this neuroadaptation directly counters some effects of anesthetics, it also causes long-lasting enhancement of seizure susceptibility, a common symptom of drug withdrawal. These data provide a possible mechanism for the long-standing counter-adaptive theory for drug tolerance in which homeostatic adaptations triggered by drug exposure to produce drug tolerance become counter-adaptive after drug clearance and result in symptoms of dependence.

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Conservation of the balance between excitation and inhibition of neural activity is critically important for the proper function of the nervous system. Many alcohols, anesthetics, and abused volatile solvents alter this balance, resulting in behavioral manifestations that range from hyperexcitability to sedation. In 1968, W. R. Martin [1] proposed a counter-adaptive theory for drug tolerance. This theory states that drug exposure triggers adaptive homeostatic changes that oppose drug effects to produce drug tolerance, but that upon drug clearance these same changes become counter-adaptive and produce symptoms of dependence [2, 3]. These ideas have been extended to account for the changes in motivation that underlie drug-seeking behavior as proposed by the opponent-process model [3, 4].

In Drosophila, rapid tolerance to the sedative effects of alcohols and anesthetics is mediated by an increase in the expression of the slo gene. A single exposure to a sedating dose of ethanol or the anesthetic benzyl alcohol induces transcription of slo. Consequently, flies display a reduced response to a subsequent sedation with the same drug dose just 24 h after their first exposure. In these experiments, previously treated flies recover from sedation and resume normal behavior significantly earlier than naive flies. Transcription from the slo gene was shown to be both sufficient and necessary for the development of tolerance to the behavioral effects of benzyl alcohol, as a mutation that eliminates slo expression prevents tolerance, and expression from an inducible slo transgene mimics tolerance in naive animals [5, 6].

In flies, slo encodes the pore-forming subunit of the BK-type Ca$^{2+}$-activated K$^+$ channel [7]. This channel responds both to increases in free Ca$^{2+}$ and to changes in membrane potential and is thus believed to play a major role in shaping neuronal excitability and regulating synaptic transmission [8]. Because of its central role as a regulator of excitability of nerve terminals, we have proposed that this channel is a likely contributor to the homeostatic mechanism that resists unfavorable changes in net cellular excitability and mediates tolerance to sedation [5]. Here, we electrophysiologically test whether drug-induced slo expression acts as a neural excitant that could directly counter the depressant effects of the drug and if, after drug clearance, the change in slo expression generates a symptom of drug withdrawal.

Results
We used the giant fiber (GF) pathway of Drosophila to test if drug-induced BK channel expression enhances neural excitability and thereby helps the nervous system to resist subsequent sedation. In this pathway, the GF neurons conduct action potentials from the brain to the thoracic ganglion. Within the thoracic ganglion, the GF forms an electrical synapse with an interneuron that connects to a motor neuron innervating the dorsal-longitudinal flight muscles (DLM) (Fig. 1A) [9, 10]. In the GF preparation, one stimulates the GF in the brain and records its response in the DLM. Each GF action potential gives rise to a single action potential in the DLM. We quantify the capacity of the GF pathway to fire repetitively as a biologically meaningful measure of the excitability of the pathway [11, 12].

To determine the following-frequency profile of the GF pathway, three trains of 10 stimuli were delivered and the number of responses was counted. The frequency of the stimulus trains was gradually increased, and as the refractory period of the neuron was exceeded, the incidence of failures increased (Fig. 1B and C). A failure is the absence of an action potential in the muscle in response to an evoked stimulation. In order for this method to accurately report the following frequency of the GF pathway neurons, the following frequency of the DLM must substantially exceed that of the neurons. At stimulation frequencies higher than 80 Hz, the GF circuit becomes refractory and fewer responses are detected. However, using direct muscle simulation we show that the muscle will fire successfully at stimulus frequencies that exceed 300 Hz (Fig. 1C). These results indicate that the failures recorded from the GF pathway are neuronal rather than muscular in nature.

If increased slo expression enhances neural excitability by reducing the refractory period, then we should observe a higher following frequency in animals in which expression of the slo gene was induced. We first determined if slo induction caused by prior benzyl alcohol sedation enhances the following frequency of the GF pathway and whether this change could be attributed exclusively to increased slo expression. A single benzyl alcohol sedation, 24 h prior, which substantially increases slo expression [5], increases the following frequency of the GF pathway. This increase is shown by a significant rightward shift in the frequency-response curve and by an increase in the FF50 (following frequency with a 50% response rate) in animals previously treated with benzyl alcohol (Fig. 2A).

Because a loss of slo expression prevents acquisition of sedation-induced tolerance [5], we used mutant analysis to test whether slo...
expression is also required for the electrophysiological adaptation to drug sedation. Because elimination of slo expression in muscles causes broadening in DLM action potentials and interferes with this assay, we used the ash2\(^{18}\)/slo\(^{4}\) transheterozygous double-mutant to eliminate slo expression in neurons but not in muscles. The intrinsic electrophysiological properties of DLM in ash2\(^{18}\)/slo\(^{4}\) transheterozygotes appear normal (13). Interestingly, in these transheterozygous mutants, prior sedation fails to produce an increase in following frequency (Fig. 2B), indicating that slo expression in the nervous system is required for the sedation-induced reduction in the neural refractory period.

To determine if an increase in slo expression would phenocopy the electrophysiological effect of sedation, we used a transgenic fly carrying a heat-inducible copy of a slo cDNA in a slo\(^{4}\) background (B52H). We do not observe wide muscle-action potentials in the thoracic ganglion. Note that the capacity of the muscle to follow high-frequency stimulations far exceeds that of the GF pathway. Error bars are SEM, n = 5 each.

In mammals, withdrawal of sedative drugs has been linked to increased neural excitability, and symptoms can include the presence of seizures (15). Because slo\(^{4}\) mutants had previously been shown to decrease overall seizure susceptibility in flies (16), we hypothesized that the increased excitability caused by induction of slo might increase the probability of seizures. For this reason, we tested the effects of prior benzyl alcohol sedation and slo induction on susceptibility to seizures. In flies, seizures can be evoked by stimulation with high-frequency electroconvulsive shock (ECS). In this assay, a stimulation train in the fly brain triggers a stereotypical seizure repertoire that can be effectively recorded at the DLM. The seizure consists of a short, high-frequency, spontaneous initial discharge followed by a period of response failure and a subsequent secondary delayed discharge just before recovery of low-frequency evoked responses (Fig. 3A) (17, 18). Because seizure occurrence is an all-or-nothing event and is highly dependent on
expression was induced, we observed an increased seizure susceptibility. The protocol used to induce the B52H transgene did not cause a significant change in seizure susceptibility of nontransgenic animals (Fig. 3D). These results indicate that increased slo expression is sufficient to enhance seizure susceptibility in flies.

In the preceding portions of this article, we have not directly assayed tolerance but have inferred that it has developed based on previously published observations (5) and based on the appearance of increased neural excitability after drug exposure. Here, we assay tolerance in individual animals by using behavioral and electrophysiological methods (Fig. 4). Flies were sedated in a vapor chamber for 20 min. Some of the animals were immediately tested for behavioral recovery and others were assayed for seizure susceptibility and the capacity to follow high-frequency stimulation. Tolerance can be detected with all three measures by comparing responses of the animals as they recover from their first and second sedations.

We first measured functional behavioral tolerance by measuring the time required for animals to regain postural control after drug sedation (Fig. 4A and B). Following the first drug exposure,
flies required an average of $25 \pm 4$ min to recover from sedation. However, recovery from the second bout of drug sedation was substantially accelerated with an average recovery time of $13 \pm 3$ min.

Because we wished to directly correlate behavioral and electrophysiological measures of tolerance, we could not determine the following frequency and seizure threshold in the standard manner (stimulation at a variety of test frequencies or potentials interspersed with rest periods) because the duration of the standard protocol far exceeds the period required for recovery from sedation. Instead, we measured the capacity of the GF to follow a single high-frequency stimulus train immediately after the 20-min period of sedation in the vapor chamber. Similarly, to measure susceptibility for seizures, we used a single stimulation potential: the minimal stimulation potential that produced seizures in 100% of the wild-type control animals. In this manner, the capacity to follow a high-frequency stimulation and the seizure potential could be recorded in individual animals during the sedation recovery period. This approach permits direct comparisons of the behavioral and electrophysiological attributes.

The capacity of benzyl alcohol to inhibit the activity of the GF pathway also showed functional tolerance. Fig. 4C shows that the capacity of the GF to follow a 200-Hz stimulation was reduced by sedation. We counted the number of responses to 30 stimuli delivered at 200 Hz, 2 min after the benzyl alcohol exposure period. Awake, drug-naive, wild-type animals responded to an averaged of 17.9 ± 1.7 of 30 stimulations, whereas flies recovering from their first sedation respond to only 5.3 ± 1.6 of 30 stimulations. This finding demonstrates that benzyl alcohol substantially enhances the refractory period of the GF pathway. In response to the second benzyl alcohol sedation, the GF pathway shows a markedly improved capacity to follow the stimulation train and responds to 11.3 ± 2 of 30 stimulations. Here, therefore, tolerance is manifest as a reduction in the capacity of the drug to inhibit the response of the GF. As shown in Fig. 4D, the tolerance observed in this unique assay is not the product of differential drug loading during the first and second rounds of drug exposure.

In the same way, seizure activity was probed in sedated flies immediately after removal from the vapor chamber. A 50-V ECS was used to evoke seizures every 10 min after the 20-min sedation period. Although this voltage is sufficient to elicit seizures in 100% of awake flies (naive or previously sedated) (Fig. 3B), seizures in flies under the effects of anesthesia were substantially inhibited (Fig. 4E). Notice that the capacity of the anesthetic to inhibit seizure is substantially reduced in animals recovering from their second round of sedation. Two minutes after the sedation protocol, none of the flies experiencing their first exposure exhibited seizures, but 33% of flies recovering from their second sedation produced evoked seizures. As the effects of the anesthetic wear off, evoked-seizure activity is regained in an average of $39 \pm 3.8$ min after the first exposure and $10 \pm 2.4$ min after the second exposure (Fig. 4E and F).

Discussion

The increase in seizure susceptibility after benzyl alcohol sedation represents a remarkable parallel to drug-withdrawal symptoms observed in humans and in other mammalian models of this condition (15). Interestingly, as with drug tolerance, increased seizure susceptibility can be provoked merely by induction of the slo gene. Fig. 5 is a schematic model of how drug-induced modulation of a single gene can produce an adaptation that underlies both tolerance and withdrawal-like symptoms. Previously, Lin and Nash (11) demonstrated that volatile-solvent anesthetics produce a dose-dependent reduction in the following frequency of this neural pathway in concordance with our own data. These data suggest that anesthetic act, at least in part, by increasing the neuronal refractory period, which in turn results in neuronal failure and ultimately sedation (Fig. 5A and B). Accordingly, here we show that prior sedation of flies results in a significant increase in the following frequency of the GF pathway, indicative of an adaptive, opposing response to the acute effect of volatile-solvent anesthesia (Fig. 5C and D). This adaptation is slo-dependent in that mutations in slo block it. Most important, this adaptation can also be produced by the induction of slo expression from a transgene. These results unambiguously indicate that an increase in slo neural expression is sufficient to reduce the refractory period of the pathway.

This model is consistent with the idea that increased neural expression of slo can act as a neural excitant. The role of neural excitant is an unusual one to postulate for a K+ channel, as these channels are commonly associated with cell repolarization and suppression of neural activity. Certainly, in some preparations, increased BK channel activity reduces neural excitability (19, 20). However, BK channel activity has long been positively correlated with neural excitability (21–23).

Recently, increased BK channel activity has also been linked to epilepsy. Mice lacking the β4-BK channel auxiliary subunit show increased BK channel activity that augments high-frequency firing and leads to temporal lobe seizures (24). In humans, a gain-of-function mutation in human slo (KCNMA1) that increases BK channel open probability has been shown to be a cause of coexistent generalized epilepsy and paroxysmal dyskinesia (25). In this instance, treatment with paxilline—a specific blocker of BK channels—is associated with reduced neuronal activity. Interestingly, some individuals carrying a gain-of-function mutation are sensitive to alcohol-induced dyskinesias (25).

It has been proposed that although an increase in BK channel activity can limit the instantaneous response of the neuron, the increase enhances the capacity for repetitive neural activity by reducing the neural refractory period (21, 22). There is strong evidence that increased BK channel activity can reduce the refractory period by repolarizing the synapse before the activation of other classes of K+ channels can occur (26). Activation of these K+ channels would lead to a long-lasting hyperpolarization that would slow the firing rate. Additionally, in posterior pituitary nerve terminals, BK channel-induced after hyperpolarization has been implicated in enhancing neuronal firing frequency by speeding the recovery of Na+ channels from inactivation (27).

Neural pharmacodynamic tolerance to any drug is likely to involve many components (28–31). However, the slo gene is uniquely positioned to be a key part of such a homeostatic response. Because the encoded channel has the highest conductance of any neural ion channel, small changes in its density can have a large influence on membrane excitability. This influence allows for a fast, low-cost adaptation that can influence a myriad of neural components, including calcium channel kinetics, action potential propagation, and neurotransmitter release. In fact, modulation in slo expression may be a generalized homeostatic strategy in response to sudden changes in neural activity. Increased slo expression is triggered by a myriad of sedative compounds, whereas treatments predicted to increase neural excitability reduce slo expression (5).

Intriguingly, the BK channel has been implicated in the production of ethanol tolerance in mammals (32). Recent studies to investigate the physiological mechanism of ethanol tolerance in the rat hypothalamic neurolymphophyseal explant model show evidence of reduced BK channel expression following ethanol exposure. In these studies, the explant is chronically exposed to ethanol, which causes a reduction in BK channel density through the internalization of membrane channels and the down-regulation of ethanol-sensitive BK isoforms caused by microRNA degradation of selected mRNA splice variants (33, 34). These results contrast with the alcohol/anesthetic-induced increase in BK channel expression that we observe in Drosophila (5, 6). There are many potential sources that could account for this difference and the meaning of the difference will not be resolved here. One possible source of the discrepancy is that our data reflects the net change in the entire brain and it is possible that this change is the product of a decrease in expression in one neural structure (as in the mammalian model)
that is masked by increases in other regions of the *Drosophila* brain. Alternatively, the root of the difference could be accounted for by differences in the treatment protocol (continuous 24 h ethanol exposure vs. a single 15- to 20-min exposure in flies), which may generate different molecular outcomes. Our treatment paradigm was developed to reliably produce tolerance that could be assayed in a simple behavioral assay. The benzyl alcohol and ethanol treatments consisted of sedating doses leading to whole-animal paralysis, whereas in the mammalian studies, ethanol treatments consisted of doses that would not induce rapid sedation in whole animals. Rather than inducing sedation, the lower dose used in the mammalian studies may be excitatory for some neural circuits (35–37) and in humans are not immediately sedating and can cause euphoria and behavioral disinhibition (38). Interestingly, in flies, non-sedating doses of benzyl alcohol that induce behavioral hyperexcitability also evoke a reduction in slo gene expression and produce sensitization to subsequent sedation. The response of the fly to a reduced dose of benzyl alcohol has only been studied cursory (5).

These results support the counter-adaptive theory for drug tolerance, in which withdrawal-like symptoms are a by-product of a homeostatic neuroadaptive process, and they provide a well-founded mechanism for the phenomenon of dependence. In the current study, slo induction represents a feed-forward neuroadaptive process that underlies drug tolerance. In the absence of the drug, the increased expression of slo leads to an allstatic state that causes a symptom of withdrawal—an increase in seizure susceptibility—and demonstrates a common origin for tolerance and withdrawal hyperexcitability in *Drosophila*.

### Materials and Methods

**Fly Stocks.** *Drosophila* stocks used were Canton S (wild type), B52H (w1118; B52H; slo<sup>−</sup>), w<sup>1118</sup>, and the ash2<sup>1</sup>/slo<sup>4</sup> transheterozygote. Stocks were raised on standard cornmeal agar medium in a 12/12 h light/dark cycle. Newly eclosed flies were collected over a 1- to 2-d interval and studied 3 to 4 d after eclosion. The B52H transgene (5) expresses a slo cDNA from an hsp70 promoter. B52H was induced at 37 °C for 30 min in a humidified incubator. To account for the nonspecific effects of temperature, a mock induction was performed using *w<sup>1118</sup>* flies. The ash2<sup>18</sup> mutation is a chromosomal deletion that removes the neural promoters from the slo gene and extends into the neighboring ash2 gene. The ash2<sup>18</sup> allele is recessive lethal. Previously, we have shown that ash2<sup>1</sup>/slo<sup>4</sup> transheterozygotes do not show neural expression but have normal muscle expression of the slo gene. Furthermore, flight muscles in ash2<sup>1</sup>/slo<sup>4</sup> transheterozygotes have normal electrical properties (13).

**Drug Treatment.** Preexposure to benzyl alcohol was performed using 30-mL glass vials coated with benzyl alcohol as described in detail in *SI Materials and Methods* and in Ghezzi et al. (5). One group of 10- to 15 age-matched female flies was placed in a benzyl alcohol vial and exposed until sedation (10–15 min, approximately). A second group was placed in a benzyl alcohol-free control vial for the same time and did not sedate. Both groups were returned to food vials and allowed to recover for 24 h before testing. For the tolerance assays, single flies were exposed to benzyl alcohol using a custom-built benzyl alcohol vapor chamber described in *SI Materials and Methods* and Fig. 51. Single age-matched female flies were placed in the chamber and allowed to sedate during a 20-min benzyl alcohol exposure. After this period, flies were immediately removed from the chamber and tested for behavioral recovery, seizure susceptibility, and the ability to follow a high frequency stimulus.

**Electrophysiological Assay.** Individual adult female flies were set up for electrophysiological recordings as described in detail in *SI Materials and Methods*. Using micromanipulators (Narishige), a 200-μm diameter uninsulated tungsten-wire electrode (FHC Inc.) electrolytically sharpened to approximately 5 μm was placed on each of the compound eyes. A 75-μm diameter recording electrode electrolytically sharpened to approximately 5 μm was inserted through the dorsal cuticle into the right-uppermost DLM that lies just beneath the cuticle. Finally, a 200-μm diameter reference electrode, electrolytically sharpened to approximately 5 μm, was inserted into the abdomen (11). Stimulating potentials were generated using a 548 square pulse stimulator, isolated with a SIUS Stimulus Isolation Unit (Grass-Telefactor). Responses from the DLM muscles were amplified with a Microelectrode Amplifier Model 1800 (A-M Systems, Inc.), digitized by a DigiData 1200 (Axon Instruments) and recorded for analysis on a PC using FETCHEX, pCLAMP 6 software (Axon Instruments). To determine the threshold potential to efficiently stimulate the GF pathway, single 0.1-ms pulses of increasing voltages were delivered to the eyes at 5-s intervals. Once the threshold for a muscle response was reached, the latency to the response after each stimulus was measured from the beginning of the stimulus artifact to the beginning of the evoked muscle response. The amplitude of the stimulus was increased until a response with a constant short-latency of ±1.4 ms was detected (typically around 20–30 V).

**Determination of Neuronal Following Frequency.** The following frequency of the GF pathway was determined using a stimulus protocol that consisted of sets of three trains of 10 suprathreshold stimuli (5 V over the short-latency threshold) at increasing frequencies. The stimulus frequency was increased by 20 Hz after each set, starting at 40 Hz up to 220 Hz. The animals were allowed to rest for 5 s between each stimulus train and 15 s between each three-train set.
Determination of Seizure Susceptibility. To determine seizure susceptibility, the same electrophysiological paradigm was used with minor modifications (see SI Materials and Methods for details). Seizure threshold was determined using a 1.5-s-long, high-frequency ECS consisting of 0.1-ms pulses delivered at 200 Hz. Single ECS were applied at increasing voltages until a seizure was detected by the recording electrode in the DLM. A seizure consists of a high-frequency initial discharge, followed by a prolonged period of evoked response failures (Failure), followed by a delayed discharge, and ended with a return of normal capacity to respond to evoked stimuli. The stimulation potential was increased from 10 to 50 V in increments of 5 V, with an 8-min rest period between each ECS. The minimum voltage that induced a complete seizure repertoire was recorded as the voltage threshold for that fly. For each fly, low-frequency stimulation (0.5 Hz, 30 V, 0.1-ms pulses) was applied before and after each ECS to assess the health of the GF pathway, and to determine the duration of the failure period induced by each seizure episode (17, 18).

Behavioral and Electrophysiological Tolerance Assay. For behavioral recovery, individually sedated flies were transferred from the vapor chamber to small plastic Petri dishes and allowed to recover. For each fly, recovery time was the period between removal from the sedation chamber and the return of the righting reflex as indicated by a return to a natural standing position. For the seizure susceptibility and following frequency assays, benzyl alcohol-sedated flies were immediately set up for electrophysiology. Seizure susceptibility was probed every 10 min using a 50-V, 1.5-s, electroconvulsive shock of 200 Hz, 0.1-ms pulses, for 60 min, starting at 2 min after removal from the vapor chamber (because of a 2-min mounting delay). In the same flies, following frequency was determined by counting the successful DLM responses to the first 30 stimuli of the first ECS (delivered 2 min after removal of benzyl alcohol).

Measurement of Benzyl Alcohol Concentrations. Benzyl alcohol concentration was measured in the sedated flies immediately after removal from the vapor chamber using liquid-phase gas chromatography, as described in detail in SI Materials and Methods.

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