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Tolerance to Anesthesia Depends on Synaptic Proteins

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Abstract

The hypnotic effects of anesthetics are caused by their interactions with neuronal components vital for proper signaling. An understanding of the adaptive mechanisms that lead to the development of anesthetic tolerance can offer insight into the regulation of neuroexcitability and plasticity that alter behavioral output. Here we use genetic and pharmacological manipulation of *Drosophila* to investigate the mechanisms of tolerance to benzyl alcohol. The mutants tested were temperature-sensitive paralytics that interfere with neuronal signaling: two mutations in dynamin that affect vesicle recycling, *shi*^{ts1} and *shi*^{ts2}, and one that affects the voltage-activated Na⁺ channel, *para*^{ts1}. We also used *N*-ethylmaleimide (NEM) to pharmacologically interfere with synaptic function. We found that blocking the generation of action potentials using a temperature-sensitive paralytic mutation does not induce nor prevent the development of functional tolerance to benzyl alcohol, but that disruption of synaptic signaling using mutations in the dynamin gene or by NEM treatment inhibits the induction of tolerance.

Keywords

Tolerance; Shibire; Paralytic; *N*-Ethylmaleimide; Benzyl alcohol; Synapse; Drug abuse; Behavior; *Drosophila*; Anesthetic

Introduction

Drug tolerance is a reduction in an effect of a drug caused by prior drug exposure (Atkinson 2009; Chao and Nestler 2004; Scholz et al. 2000). There are two physiologically distinct origins of tolerance. Pharmacokinetic tolerance, also called metabolic tolerance, arises from an increase in the rate of drug clearance or from a reduction in drug uptake. Pharmacodynamic (or functional) tolerance is an adaptive cellular process that counters the effects of the drug. Functional tolerance that alters a neurally based behavior is of great interest to neurobiologists because such tolerance is assumed to be the product of a change in the activity of the nervous system. Tolerance is an endophenotype of addiction, and thus an understanding of the mechanisms underlying tolerance may provide insight into the mechanics of addiction (Lesch 2005). However, unfortunately for individuals who abuse drugs, tolerance may develop to the pleasurable effects but not to the undesired toxic side effects of the drug. Drug users who have acquired tolerance require a larger dose of the drug to achieve the same hedonic effect. As a result, the increased drug consumption needed to achieve a particular behavioral endpoint leads to an increased potential for organic damage (Kalant 1998).

Volatile organic solvent anesthetics are dirty drugs in that they affect the activity of a wide variety of proteins, including components of the synaptic release machinery, neurotransmitter-gated ion channels, and voltage-gated ion channels (Pittson et al. 2004). Although the relative contributions of these targets toward anesthesia and sedation are not completely understood, it is clear that anesthetics act by reducing excitatory synaptic transmission and enhancing inhibitory synaptic transmission, which leads to a net reduction in neuronal activity (Hemmings 2009; Staiman and Seeman 1977). Tolerance can arise in response to a wide variety of sedating and abused substances, which can include—depending on the behavior measured—inhaled anesthetics (Chalon et al. 1983; Smith et al. 1979). Functional tolerance appears to be a homeostatic response to a reduction in neuronal activity, opposing the sedative effects of the drug by increasing neuronal excitability. This increase may arise from both an augmentation of excitatory inputs and a reduction in inhibitory ones.

Mutant analysis in *Drosophila* has identified genes important for the normal response to anesthetics and alcohol. The identified genes are involved in second-messenger signaling, stress-responses, and memory formation pathways and include proteins such as ion channels, synaptic proteins, neurotransmitter receptors and biosynthetic enzymes (Berger et al. 2008; Cowmeadow et al. 2005; Dzitoyeva et al. 2003; Gamo et al. 1981; Godenschwege et al. 2004; Humphrey et al. 2007; Moore et al. 1998; Scholz et al. 2005; Urizar et al. 2007; Walcourt et al. 2001). Because of the strong conservation of both gene sequence and function between insects and mammals, genes identified in flies typically serve the same role in mammals and vice versa. Thus, mutant analysis in *Drosophila* is a promising approach for identifying genes that are important in drug responses in both flies and mammals.

Here we use the *Drosophila melanogaster* model system to investigate the mechanisms that underlie rapid tolerance to the anesthetic benzyl alcohol. Rapid tolerance arises from a single drug exposure and persists after drug clearance. Previously, we have demonstrated that rapid benzyl alcohol tolerance originates in the nervous system (Ghezzi et al. 2004). Like other anesthetics, benzyl alcohol has been demonstrated to inhibit channel function (Staiman and Seeman 1977; Barrantes 1993; Takadera and Ohyashiki 2008; Haeseler et al. 2000).

Simple behavioral assays can be used to demonstrate the capacity to acquire tolerance. We employ an assay that measures the duration of loss of climbing to compare the period of sedation produced by a single drug sedation to the period produced by a second drug sedation (Wang et al. 2007, 2009; Ghezzi et al. 2004). Animals are said to be capable of acquiring tolerance if they recover more rapidly from the second sedation than the first.

The experiments discussed here aim to address three aspects of tolerance: whether a mere reduction in neuronal signaling can trigger resistance to benzyl alcohol sedation, whether altering synaptic function hampers the development of tolerance; and whether normal electrical signaling is required for the development of the tolerant state.

Materials and methods

Fly stocks

All the flies were raised on standard cornmeal/molasses/agar medium. Flies were kept in a room with a constant temperature (20°C) and a 12:12 h light:dark cycle. Flies that emerged from pupae were collected over a period of 2 days, transferred to bottles containing fresh food, and allowed to age between 3 and 4 days. Then these animals were divided into groups of ten females each under light CO₂ anesthesia and tested the following day. All flies were

between 5 and 7 days of age at the time of testing. The genotypes of mutant stocks used were *para^{ts1}*, *shi^{ts1}*, and *shi^{ts2}*; and the wild-type stock was Canton S.

Coating vials with benzyl alcohol

Benzyl alcohol sedation was induced as previously described (Ghezzi et al. 2004). Briefly, clear glass vials (30 mL) with a rounded bottom were coated with 200 μ L of a 0.4% benzyl alcohol in acetone solution and rotated continuously at room temperature in a Autoblots Micro Hybridization Oven (Bellco Glass, Inc. Vineland, NJ) for 20 min to allow the volatile acetone (vapor pressure at 20°C is 185.6 mm Hg) solvent to evaporate, leaving an even coating of the nonvolatile benzyl alcohol (vapor pressure at 20°C is 0.07 mm Hg) behind. Control vials were produced in the same manner by omitting the benzyl alcohol.

Tolerance behavioral assay

To detect tolerance, we use a climbing reflex assay. Suppression of the climbing reflex is used as a measure of sedation. The period of sedation of animals receiving their first dose of benzyl alcohol is compared to that of animals receiving their second dose. Tolerance is said to have occurred if the animals recover more rapidly from their second sedation than from the first. For the first exposure, five to six groups of 10 age-matched female flies were incubated in benzyl alcohol-coated vials for 15 min, while their control counterparts were placed in clean benzyl alcohol-free vials (control vials). After a sedating 15 min benzyl alcohol exposure the experimental groups were moved to food-filled vials to recover. Twenty-four hours later, both the experimental and control groups were incubated in benzyl alcohol-coated vials for 15 min. Flies were allowed to recover in clean glass vials.

Behavioral recovery was quantified from images taken of the animals recovering. Images were taken at a rate of one frame every 10 s. Flies are normally negatively geotactic, but this behavior ceases while they are sedated. Automated image-processing software was used to detect when the flies recovered from sedation and returned to climbing the walls of their vials (Ramazani et al. 2007). Briefly, the software subtracts images of each vial from the image where all flies are sedated. This resulting subtracted image is devoid of background and contains only white images of flies that have recovered and have resumed climbing. The number of non-black pixels are then counted to generate a quantity that represents the number of flies recovered and returned to negative geotaxis. For each vial, the value at each time point is normalized to a value that represents complete recovery, giving a percent recovery curve. The percent recovery of each vial within a population is then averaged as a function of time and plotted with error bars describing the standard error of the mean (SEM). A leftward shift in the recovery graph indicates resistance to the sedative affects of benzyl alcohol. A statistically significant difference between the two curves is determined using log-rank analysis.

Manipulation of temperature-sensitive mutations

The temperature-sensitive paralytics, *para^{ts1}*, *shi^{ts1}*, and *shi^{ts2}*, were incubated at the nonpermissive temperature of 30°C for 5 h. A cotton plug containing 3 mL of water was inserted into the vial to prevent desiccation during the incubation at elevated temperature. The flies were then placed into vials of food and allowed to recover and recuperate for 24 h, at which time they were given a sedating dose of benzyl alcohol.

To perform benzyl alcohol sedation at the restrictive temperature for *para^{ts1}*, 100 μ L of a 0.2% benzyl alcohol solution dissolved in acetone was pipetted into a clean glass vial and rotated for 15 min at room temperature in an Autoblots Micro Hybridization Oven (Bellco Glass, Inc. Vineland, NJ). Then the vials and *para^{ts1}* flies were separately incubated at 33°C for 5 min (this is sufficient to heat the vials and paralyze the flies). The paralyzed flies were

transferred into the benzyl alcohol-treated vials and exposed to the drug for 15 min at 33°C. After the 15 min treatment, the animals were transferred to humidified, clean, benzyl alcohol-free vials and incubated for 2 h at 33°C in the hybridization oven. The animals were returned to vials of food at room temperature (~22°C) and tested the following day for benzyl alcohol tolerance at room temperature using the standard method.

Electrophysiology

Adult, four-to-seven-day old female flies were positioned such that their heads and 1/4 of their thorax protruded out of a pipette tip. A pulled capillary tube was used to glue the thorax to the pipette tip and the head to the thorax with Loctite® Super Glue (Henkel Corporation, Avon, OH). The pipette tip was then fixed to a magnetic clamp, and the fly was oriented such that the head was horizontal relative to the table. Two 75 µm diameter insulated tungsten wire electrodes (FHC Inc, Bowdoinham, ME) were implanted into the brain and eye of the fly using micro-manipulators (Narishige, Tokyo, Japan). The positive recording electrode was placed in the brain, while the negative and ground electrode were placed in the eye. Signals were amplified with a 10× headstage and with a Microelectrode Amplifier Model 1800 (A-M systems, Inc., Carlsborg, WA), digitized using National Instruments® cRIO 9215, and acquired on a Dell® inspiron 1150 laptop computer using a Labview program written by Dr. Sari Andoni (unpublished). Drug delivery was achieved by the application of a small amount of benzyl alcohol through a pulled capillary onto the abdomen of the fixed animal.

Feeding *N*-ethylmaleimide (NEM)

Clear glass vials containing an NEM (Acros Organics USA, Morris Plains, NJ) or 1% sucrose vehicle-soaked filter paper were prepared by the application of 300 µL of the appropriate solution to the filter paper in a manner that causes the filter paper to adhere to the sides of the vial. Four-to-seven-day-old wild-type adult females were then transferred to the drug- or vehicle-containing glass vials. The animals were allowed to feed for 24 h prior to testing.

Defecation assay

To test if benzyl alcohol sedation alters feeding behavior, we conducted a defecation assay (Edgecomb et al. 1994) to compare the quantity of dyed food consumed before and after benzyl alcohol sedation. This was accomplished by transferring flies to vials containing food mixed with a food coloring tracer. The number of colored defecations is a measure of the quantity of food consumed.

Gas chromatography

Liquid phase gas chromatography was used to assay for the concentration of benzyl alcohol in flies. The samples were prepared for gas chromatography by transferring the flies to a 0.2 mL gas chromatography vial containing 100 µL of acetone. A dilution series of benzyl alcohol in acetone was prepared for the generation of a standard curve. The samples and the benzyl alcohol dilutions were then loaded into the carousel of a SRI 311H 42-vial liquid autosampler, with the vial pressure set to 6 psi and the tray pressure at 60 psi and the injection volume set to 3 µL. The autosampler is connected to an SRI 310 GC® through an on column injection, the samples are injected into a 60 m Restek MXT®-1 column with the hydrogen, carrier (He), and air pressures set to 8, 16, 8 psi respectively and detected through a flame ionized detector during the course of each run. The oven temperature was programed in SRI Peak-Simple software for Windows and was set to hold at 50°C for 1 min and then ramps to 220°C at 20°C/min, and held at 220°C for 5 min. The voltage changes in the detector were acquired and analyzed using SRI PeakSimple software. Benzyl alcohol

concentration was determined by interpolating benzyl alcohol peak area to a standard curve that relates benzyl alcohol peak area to molarity of solution. To determine the molarity of benzyl alcohol in the flies, the molarity of acetone solution was divided by the amount of water contained in the 10 flies assayed (each flies contained ~0.8 μ L water).

Statistical analysis

We use a recovery from sedation assay to determine whether a population of animals has acquired tolerance to the anesthetic benzyl alcohol. In this test, the rate of recovery of a naive population is compared to the recovery rate of a previously sedated population. To evaluate whether the two curves differ significantly, we used log-rank survival statistics. This test is best suited for data in which one is measuring the time to a specific event as in all of the tolerance assays in which we measure the time that it takes for each fly to recover from sedation. The statistic evaluates whether entire recovery curves are statistically different, as opposed to individual data points comprised by the curve. However, in the behavioral assays, we have also included error bars that represent the SEM for each individual data point.

Results

Benzyl alcohol causes a reduction in neural signaling

Benzyl alcohol is an anesthetic that is well tolerated by flies. Like other anesthetics, benzyl alcohol suppresses neural activity in a reversible manner. Previously, Ghezzi et al. (2010) have reported that sedating doses of benzyl alcohol suppress approximately 70% of the evoked responses of the short latency giant fiber escape circuit. Figure 1 shows local field potentials recorded from the fly brain prior to benzyl alcohol application (Fig. 1a) and 10 min after application (Fig. 1b). This benzyl alcohol treatment produced approximately a 20 min reduction in neuronal signaling.

Exposure to this drug induces long-lasting functional tolerance that can be scored behaviorally (Wang et al. 2007; Ghezzi et al. 2004). An example of functional tolerance is presented in Fig. 1c. These curves represent the recovery from benzyl alcohol sedation of two populations of animals. One curve represents a population that is recovering from their first sedation with benzyl alcohol and the other curve represents a population that is recovering from their second sedation. Notice that recovery from the second sedation is more rapid than recovery from the first sedation; the curve is shifted to the left.

A reduction in neural signaling does not phenocopy tolerance

Cross-tolerance to benzyl alcohol is induced by sedative agents, such as chloroform and ethanol, whose chemical structures differ from that of benzyl alcohol (Ghezzi et al. 2004; Cowmeadow et al. 2006). A shared property of all of these agents is that they inhibit neural activity. This led us to ask whether tolerance was merely a response to reduced neural activity. To test whether reduced neural signaling is sufficient to phenocopy tolerance, we suppressed neural activity with temperature-sensitive paralytic mutations and asked whether the suppression induced benzyl alcohol resistance.

We inhibited neuronal signaling at the level of action potential generation and propagation using a mutation in the main *Drosophila* voltage-gated sodium channel gene, *paralytic*. The *paralytic* gene is the major and only confirmed voltage-gated sodium channel gene in the *Drosophila* genome (Lin et al. 2009). Animals carrying the temperature-sensitive *para*^{ts1} allele exhibit near-instantaneous paralysis and recovery when switched between the restrictive temperature and the permissive temperature (Ganetzky 2000). Paralysis is produced by a temperature-induced failure of neuronal action potential propagation. In

para^{ts1} mutants, the CNS neurons do not generate action potentials at the restrictive temperature (Siddiqi and Benzer 1976). The neural blockage of action potential generation in the neurons of the brain and thoracic ganglion by *para*^{ts1} is well documented (Nelson and Wyman 1990)—reported as a loss of the long latency response by Elkins and Ganetzky (1990) and a loss of local field potentials in the brain by Nitz et al. (2002). At the restrictive temperature, the *para*^{ts1} allele is believed to essentially eliminate sodium action potentials in the *Drosophila* CNS. Another voltage-gated sodium channel homolog, NaCP60E, exists in flies. However, its identity as a sodium channel is inferred only from sequence similarity. Mutations in NaCP60E perturb olfactory responses but do not produce paralysis (Kulkarni et al. 2002).

We used *para*^{ts1} to temporarily reduce neuronal signaling and asked whether this reduction induced resistance to benzyl alcohol sedation and thereby phenocopied benzyl alcohol tolerance. Such a result is expected only if tolerance to benzyl alcohol sedation is a response to a gross reduction in neural activity. Age- and sex-matched *para*^{ts1} flies were incubated at 30°C for 5 h and then returned to the permissive temperature and allowed to recover. This prolonged reduction in neuronal signaling represents the maximal duration that does not have deleterious effects on the general health of the animals. Twenty-four hours after the reduction in signaling, benzyl alcohol sensitivity of the heat-treated animals was compared to the benzyl alcohol sensitivity of age- and sex-matched animals that had been maintained at the permissive temperature. Figure 2b shows that a reduction in neuronal signaling did not induce a tolerance-like phenotype in the *para*^{ts1} mutant.

We also tested neuronal blockade induced at the level of the synapse using mutant alleles of the *shibire* gene. The *shibire* gene encodes a *Drosophila* GTPase dynamin that is required for vesicle recycling. Dynamin acts as a “pin-chase” that forms a ring around the neck of clathrin-coated pits and upon hydrolysis of GTP mediates vesicle fission. When viewed by electron microscopy after incubation at the restrictive temperature, the synapses of *shi*^{ts1} mutants exhibit a reduced number of vesicles and clathrin-coated pits that have exaggerated necks (Kosaka and Ikeda 1983). These studies demonstrated that the paralytic phenotype of temperature-sensitive mutations in the *shibire* locus is caused by a reduction in neurotransmitter-containing vesicles due to the inhibition of local vesicle recycling at the synapse.

Figure 2 shows that the transient reduction in neuronal signaling produced by incubating *shi*^{ts1} or *shi*^{ts2} animals at the restrictive temperature did not produce a tolerance-like phenotype. However, whereas, the nearly identical *para*^{ts1} data indicates that reduced neural signaling does not by itself trigger the tolerance phenotype, the *shibire* mutant data must not be interpreted in this manner because we later show that the *shibire* mutation blocks the capacity to acquire tolerance even at the permissive temperature.

***shibire* mutations interfere with the development of tolerance**

We tested the *paralytic* and *shibire* mutants for their ability to acquire tolerance at the permissive temperature. These mutant animals are considered to have nominally normal behavior at the permissive temperature (Kelly and Suzuki 1974; Koenig et al. 1983; Littleton et al. 1998; Siddiqi and Benzer 1976; Tolar and Pallanck 1998; van Swinderen and Greenspan 2003). These mutations are predicted to reduce or eliminate activity at elevated temperatures by affecting unique aspects of neuronal function. We found that they have different effects on the capacity to acquire tolerance to benzyl alcohol sedation at the permissive temperature.

Although mutation-induced paralysis did not phenocopy tolerance, we observed that some of these mutations interfered with the normal acquisition of benzyl alcohol tolerance at the

permissive temperature. While the *para*^{ts1} mutation did not compromise the capacity to acquire tolerance (Fig. 3b), we found that mutations of the dynamin gene, *shi*^{ts1} and *shi*^{ts2}, prevented the acquisition of benzyl alcohol tolerance (Fig. 3c, d) at the permissive temperature. These findings led us to suspect that the cellular machinery involved in proper vesicular endocytosis is required for the development of tolerance.

N-Ethylmaleimide (NEM) alters the sensitivity to benzyl alcohol

The data above suggests that a normally functioning synapse is important for the acquisition of tolerance. Mutant analysis suffers from the caveat that the absence of normal gene function during development may lead to unanticipated developmental changes in the nervous system and these changes may affect the performance of the adult. For this reason, we used the pharmacological agent *N*-ethylmaleimide (NEM) to disrupt synaptic activity. NEM is a widely used reagent that inhibits various synaptic processes including vesicle recycling and fusion (Block et al. 1988; Lledo et al. 1998; Markham et al. 2009). For this set of experiments adult wild-type flies were fed sugar water laced with NEM.

Because our tolerance assay relies on normal climbing behavior, we first identified a dose of NEM that did not affect the negative geotactic response of flies. Three concentrations of NEM-laced food were tested (1.0, 0.1, and 0.01 mM NEM) for their effect on the propensity of flies to climb (Fig. 4). The animals fed 1.0 mM NEM exhibited a marked reduction in geotactic behavior 4 h after their transfer to NEM containing food. These animals continued to show an impaired ability to climb 24 h after the NEM treatment had terminated. While the 0.1 mM NEM dose caused a reduction in climbing during the NEM treatment, normal climbing capacity was observed 24 h following the treatment. Finally, the 0.01 mM NEM dose did not cause any noticeable change in climbing behavior, neither during nor after the NEM treatment.

We then asked whether the two lower concentrations of NEM altered the sensitivity of flies to benzyl alcohol sedation. Flies were allowed to feed on 0.1 or 0.01 mM NEM-laced food for 24 h and then their sensitivity to the drug was measured in a recovery assay. Flies that consumed 0.1 mM NEM food displayed resistance to benzyl alcohol while flies that consumed 0.01 mM NEM displayed no difference in sensitivity to benzyl alcohol compared to control animals (Fig. 5a, b).

NEM interferes with the induction but not the maintenance of tolerance

Because the 0.01 mM NEM dosage did not cause a change in the climbing activity or in the benzyl alcohol sensitivity of flies we chose to use this concentration to test the effects of NEM on the capacity to acquire benzyl alcohol tolerance. Flies were fed 0.01 mM NEM before and after their first benzyl alcohol sedation. These flies failed to develop benzyl alcohol tolerance (Fig. 6a).

The observation that feeding 0.01 mM NEM was able to prevent the acquisition of tolerance suggested that an NEM sensitive component was involved in the process of development of tolerance. Furthermore, the identification of a pharmacological agent with the ability to obstruct the development of tolerance led us to ask if benzyl alcohol tolerance can be resolved into an NEM sensitive phase and an NEM insensitive phase. We observed that flies fed 0.01 mM NEM laced food before but not after their benzyl alcohol sedation did not acquire tolerance to benzyl alcohol (Fig. 6b). However, when flies were fed NEM only after their benzyl alcohol sedation, they retain the capacity to acquire benzyl alcohol tolerance (Fig. 6c). A very seductive explanation for these data is that an NEM-sensitive process is required for the initiation of the tolerance phenotype but not for the maintenance of the tolerance phenotype.

This interpretation is predicated on the assumption that the dose of NEM consumed before and after benzyl alcohol sedation is the same. However, it is possible that benzyl alcohol sedation reduces or interrupts feeding behavior and therefore alters the dosage of NEM. To assay for gross changes in feeding behavior caused by benzyl alcohol sedation, we conducted a defecation assay to determine the amount of food consumed before and after sedation. In this assay, flies consume dye-containing food and the number of colored fecal droplets are counted. Prior benzyl alcohol sedation did not produce a difference in the number of food coloring containing fecal matter droplets deposited overnight on the sides of the vials. Furthermore, the latency to the first colored defecation did not change (data not shown).

Tolerance appears to be a cell-autonomous phenotype

The induction of tolerance may be an emergent property of networks of neurons and NEM, therefore, may block the acquisition of tolerance because it disrupts communication between cells. However, tolerance might also be cell-autonomous. In a cell-autonomous scenario each cell relevant to behavioral tolerance becomes tolerant on its own, without input from neighboring cells. In this latter scenario, NEM would block tolerance because it directly prevents individual cells from appropriately responding to benzyl alcohol. To test the requirement of neuronal signaling during benzyl alcohol exposure for the development of tolerance we employed the *para*^{ts1} mutation. We used the temperature sensitive nature of the *para*^{ts1} allele to produce a reversible block in electrical signaling.

Animals homozygous for *para*^{ts1} show normal development of tolerance to benzyl alcohol (Fig. 3b) and can be used to control action potential production in the central nervous system. We used *para*^{ts1} to produce a temperature-dependent block in action potential propagation during drug exposure.

The *para*^{ts1} homozygotes were exposed to benzyl alcohol vapor while continuously being incubated at 33°C. Control animals were subjected to a mock sedation while at room temperature. Siddiqi and Benzer (1976) have demonstrated that incubating *para*^{ts1} mutants at 32°C blocks spontaneous neural activity and evoked responses even in the high safety-factor giant fiber neurons. However Suzuki et al. (1971) showed that at 34°C five percent of *para*^{ts1} flies had recovered some mobility. Although we cannot be certain that heating *para*^{ts1} mutants has blocked all neural signaling, we did not observe the partial alleviation of the temperature blockade reported by these authors. All animals at the elevated temperature were visually monitored and no movement was observed, suggesting that the temperature-induced blockade of neural signaling was persistent and efficacious. Gas chromatography was used to confirm that the drug was cleared from the animal's body before release from the temperature-induced paralysis. Wild-type Canton S flies were also exposed, at the same time, to the same dose of benzyl alcohol delivered at 33°C in order to confirm that the dose was sufficient to induce sedation. The temperature blockade of neural signaling did not prevent the acquisition of tolerance (Fig. 7).

Discussion

Volatile organic solvent anesthetics induce sedation by altering the excitability of the nervous system. We have shown that *Drosophila* acquire tolerance to the sedating properties of these compounds. Because drugs with very different chemical structures, such as benzyl alcohol and ethanol, induce mutual cross-tolerance (Ghezzi et al. 2004; Cowmeadow et al. 2006), we speculated that tolerance might merely be a homeostatic response to a common effect of these compounds. It seemed probable that the common effect was reduced neural activity. This idea had been previously discounted in Ghezzi et al. (2004) because cold anesthesia failed to phenocopy tolerance. However, this interpretation had the caveat that

cold might also inhibit other enzymatic responses that underlie tolerance. Despite the attractiveness of the hypothesis, here we demonstrate that reduced neural activity does not trigger benzyl alcohol tolerance. In the process of investigating this hypothesis we serendipitously identified *shibire* mutations that interfere with the acquisition of tolerance and demonstrated that tolerance appears to be a cell-autonomous response to drug exposure.

To reduce neural activity in a drug-free manner, we chose to use mutant alleles of the *paralytic* gene (encodes a neuronal voltage-gated Na⁺ channel) and the *shibire* gene (encodes dynamin). Mutant alleles of the *paralytic* and *shibire* genes were first isolated almost 40 years ago and are two of the best characterized *Drosophila* temperature-sensitive paralytic mutations (Grigliatti et al. 1973; Davis 2006). Although both mutations result in a temperature-induced cessation of neural activity, they do so in very different ways. The *para*^{ts1} allele reduces voltage-gated sodium channel activity and prevents action potential propagation while the *shi*^{ts1} and *shi*^{ts2} alleles block synaptic release by interfering with neurotransmitter vesicle recycling. Neither method of reducing neural activity phenocopied tolerance.

There are two possible explanations as to why suppression of neural activity with *para*^{ts1} did not cause resistance to benzyl alcohol: The first possibility is that benzyl alcohol sedation produces a specific pattern of reduced neural activity and that the *para*^{ts1}-induced inhibition of signaling does not follow the appropriate pattern. This seems improbable because flies were able to acquire tolerance in response to drug exposure even while neural signaling was suppressed. The second, more likely, possibility is that tolerance requires a biochemical interaction of the drug with its targets, which in-turn initiates an adaptive mechanism leading to the development of drug tolerance.

We also used the *para*^{ts1} mutation to ask whether the initiation of tolerance required electrical communication between neurons. Interestingly, triggering of the tolerance response does not appear to be dependent on the normal propagation of action potentials to or from the synapse, as indicated by the capacity of *para*^{ts1} animals to acquire tolerance under conditions in which their neural activity is sufficiently compromised to produce uninterrupted physical paralysis for 2 h. If *para*^{ts1} has completely blocked neural signaling then tolerance must be a cell-autonomous response. Unfortunately, we cannot be certain that all central nervous system signaling has been blocked by heat treating *para*^{ts1}.

We were very surprised to find that the *shi*^{ts1} and *shi*^{ts2} stocks failed to acquire tolerance at the permissive temperature. This deficit in tolerance is related to the *shibire* allele itself because a 6-fold backcross against the wild-type Canton S line failed to restore the capacity to acquire tolerance. The absence of tolerance in *shibire* mutants is not caused by an increase in innate resistance to a maximal level beyond which further resistance cannot be induced. If *shibire* mutants have increased innate resistance (pre-tolerance) compared to wild-type animals, then the *shibire* mutants should recover more rapidly than the Canton S stocks. As seen in Fig. 2 this is not the case.

It is not surprising that a mutant allele described as a temperature-sensitive mutation has a phenotype at the permissive temperature. In general, temperature-sensitive mutations in protein-coding genes are thought to result from amino acid sequence changes that cause the mutant protein to be more sensitive than the wild type protein to heat-induced conformational changes that disrupt normal protein activity. Usually, temperature-sensitive mutations respond in a graded fashion with temperature and the permissive temperature is merely one in which the mutant approximates the wild type with respect to a particular phenotype. A graded response has been directly demonstrated for one temperature-sensitive allele of *shibire* (Siddiqi and Benzer 1976). Furthermore, the *shi*^{ts1} and *shi*^{ts2} mutants were

labelled temperature sensitive with regards to their paralytic phenotype. However, this does not mean that these alleles are temperature sensitive for all phenotypes. At the permissive temperature, the *shi^{ts1}* allele has been biochemically shown to cause the production of dynamin complexes that are substantially more stable than the wild-type complex (Chen et al. 2002). Furthermore, at the permissive temperature, the *shi^{ts1}* mutation alters the latency of the giant fiber escape pathway (Hummon and Costello 1989). Our work indicates that with regard to the benzyl alcohol tolerance phenotype that the *shi^{ts1}* and *shi^{ts2}* are not conditional mutants. Related observations have been made for the acquisition of ethanol tolerance (Krishnan, unpublished).

Our mutant and pharmacological analysis of tolerance point to the synapse as an important site for the development of tolerance. This result is congruent with the plethora of studies that identify synaptic processes in adaptive mechanisms such as neuronal homeostasis (Davis 2006). It is important to note that in proteomic analysis of murine models after anesthesia, several synaptic proteins have been shown to be differentially regulated after volatile anesthetic sedation, including dynamin (Futterer et al. 2004; Kalenka et al. 2007). It has been reported that mutations in the *synapsin* gene cause flies to acquire a significantly higher level of ethanol tolerance (Godenschwege et al. 2004). Furthermore, *Syntaxin 1A*, a component of the vesicle-release pathway, has been identified as a target of the commonly used general anesthetic isoflurane (Herring et al. 2009). Recently, dynamin has also been found to associate with the BK-type Ca^{2+} -activated K^{+} channel (Gorini et al. 2010), encoded by the *slowpoke (slo)* gene. We have shown that mutations in *slowpoke* also interfere with the development of tolerance (Ghezzi et al. 2004). Taken together, these observations paint a mechanism of anesthetic tolerance that requires the careful regulation of ion channel density and vesicle fusion at the plasma membrane.

Of particular importance is the observation that a low dose of NEM can block the development of tolerance. Although, our data does not address how the feeding of low dose NEM affects neural signaling, it does demonstrate the presence of an early NEM sensitive window. The NEM sensitive component acts to induce tolerance, but is not necessary for its maintenance. This result indicates that benzyl alcohol sedation activates a trigger for the production of tolerance, which is itself rather stable. Other work has shown that this form of tolerance is dependent on changes in gene expression which occur in the first 6 h after exposure (Wang et al. 2007). Therefore, we surmise that the trigger for tolerance occurs in the synapse and involves a component whose activity is directly or indirectly affected by NEM. This trigger then activates a signaling cascade, that is no longer sensitive to NEM, but that produces the changes in gene expression that cause the animal to become tolerant. The synaptic localization of the trigger for functional tolerance will simplify our search for the molecular mechanism underlying this drug response.

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References

- Atkinson NS. Tolerance in *Drosophila*. *J Neurogenet*. 2009; 23:293–302. [PubMed: 19180359]
- Barrantes FJ. Structural–functional correlates of the nicotinic acetylcholine receptor and its lipid microenvironment. *FASEB J*. 1993; 7:1460–1467. [PubMed: 8262330]
- Berger KH, Kong EC, Dubnau J, Tully T, Moore MS, Heberlein U. Ethanol sensitivity and tolerance in long-term memory mutants of *Drosophila melanogaster*. *Alcohol Clin Exp Res*. 2008; 32:895–908. [PubMed: 18435628]

- Block MR, Glick BS, Wilcox CA, Wieland FT, Rothman JE. Purification of an *N*-ethylmaleimide-sensitive protein catalyzing vesicular transport. *Proc Natl Acad Sci USA*. 1988; 85:7852–7856. [PubMed: 3186695]
- Chalon J, Tang CK, Roberts C, Walpert L, Hoffman C, Ramanathan S, Turndorf H. Murine auto- and cross-tolerance to volatile anaesthetics. *Can Anaesth Soc J*. 1983; 30:230–234. [PubMed: 6336543]
- Chao J, Nestler EJ. Molecular neurobiology of drug addiction. *Annu Rev Med*. 2004; 55:113–132. [PubMed: 14746512]
- Chen ML, Green D, Liu L, Lam YC, Mukai L, Rao S, Ramagiri S, Krishnan KS, Engel JE, Lin JJ, Wu CF. Unique biochemical and behavioral alterations in *Drosophila* shibire(ts1) mutants imply a conformational state affecting dynamin subcellular distribution and synaptic vesicle cycling. *J Neurobiol*. 2002; 53:319–329. [PubMed: 12382260]
- Cowmeadow RB, Krishnan HR, Atkinson NS. The slowpoke gene underlies rapid ethanol tolerance in *Drosophila*. *Alcohol Clin Exp Res*. 2005; 29:1777–1786. [PubMed: 16269907]
- Cowmeadow RB, Krishnan HR, Ghezzi A, Al'hasan Y, Wang YZ, Atkinson NS. Ethanol tolerance caused by slowpoke induction in *Drosophila*. *Alcohol Clin Exp Res*. 2006; 30:745–753. [PubMed: 16634842]
- Davis GW. Homeostatic control of neural activity: from phenomenology to molecular design. *Annu Rev Neurosci*. 2006; 29:307–323. [PubMed: 16776588]
- Dzitoyeva S, Dimitrijevic N, Manev H. Gamma-aminobutyric acid B receptor 1 mediates behavior-impairing actions of alcohol in *Drosophila*: adult RNA interference and pharmacological evidence. *Proc Natl Acad Sci USA*. 2003; 100:5485–5490. [PubMed: 12692303]
- Edgecomb RS, Harth CE, Schneidman AM. Regulation of feeding behavior in adult *Drosophila melanogaster* varies with feeding regime and nutritional state. *J Exp Biol*. 1994; 197:215–235. [PubMed: 7852903]
- Elkins T, Ganetzky B. Conduction in the giant nerve fiber pathway in temperature-sensitive paralytic mutants of *Drosophila*. *J Neurogenet*. 1990; 6:207–219. [PubMed: 2231176]
- Futterer CD, Maurer MH, Schmitt A, Feldmann REJ, Kuschinsky W, Waschke KF. Alterations in rat brain proteins after desflurane anesthesia. *Anesthesiology*. 2004; 100:302–308. [PubMed: 14739804]
- Gamo S, Ogaki M, Nakashima-Tanaka E. Strain differences in minimum anesthetic concentrations in *Drosophila melanogaster*. *Anesthesiology*. 1981; 54:289–293. [PubMed: 6782911]
- Ganetzky B. Genetic analysis of ion channel dysfunction in *Drosophila*. *Kidney Int*. 2000; 57:766–771. [PubMed: 10720927]
- Ghezzi A, Al-Hasan YM, Larios LE, Bohm RA, Atkinson NS. slo K⁺ channel gene regulation mediates rapid drug tolerance. *Proc Natl Acad Sci USA*. 2004; 101:17276–17281. [PubMed: 15569939]
- Ghezzi A, Pohl JB, Wang Y, Atkinson NS. BK channels play a counteradaptive role in drug tolerance and dependence. *Proc Natl Acad Sci USA*. 2010; 107:16360–16365. [PubMed: 20798347]
- Godenschwege TA, Reisch D, Diegelmann S, Eberle K, Funk N, Heisenberg M, Hoppe V, Hoppe J, Klagges BR, Martin JR, Nikitina EA, Putz G, Reifegerste R, Reisch N, Rister J, Schaupp M, Scholz H, Schwarzel M, Werner U, Zars TD, Buchner S, Buchner E. Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. *Eur J Neurosci*. 2004; 20:611–622. [PubMed: 15255973]
- Gorini G, Ponomareva O, Shores KS, Person MD, Harris RA, Mayfield RD. Dynamin-1 co-associates with native mouse brain BKCa channels: proteomics analysis of synaptic protein complexes. *FEBS Lett*. 2010; 584:845–851. [PubMed: 20114047]
- Grigliatti TA, Hall L, Rosenbluth R, Suzuki DT. Temperature-sensitive mutations in *Drosophila melanogaster*. XIV. A selection of immobile adults. *Mol Gen Genet*. 1973; 120:107–114. [PubMed: 4631264]
- Haeseler G, Mamarvar M, Bufler J, Dengler R, Hecker H, Aronson JK, Piepenbrock S, Leuwer M. Voltage-dependent blockade of normal and mutant muscle sodium channels by benzylalcohol. *Br J Pharmacol*. 2000; 130:1321–1330. [PubMed: 10903972]
- Hemmings HCJ. Sodium channels and the synaptic mechanisms of inhaled anaesthetics. *Br J Anaesth*. 2009; 103:61–69. [PubMed: 19508978]

- Herring BE, Xie Z, Marks J, Fox AP. Isoflurane inhibits the neurotransmitter release machinery. *J Neurophysiol.* 2009; 102:1265–1273. [PubMed: 19515956]
- Hummon MR, Costello WJ. Giant fiber activation of flight muscles in *Drosophila*: asynchrony in latency of wing depressor fibers. *J Neurobiol.* 1989; 20:593–602. [PubMed: 2760611]
- Humphrey JA, Hamming KS, Thacker CM, Scott RL, Sedensky MM, Snutch TP, Morgan PG, Nash HA. A putative cation channel and its novel regulator: cross-species conservation of effects on general anesthesia. *Curr Biol.* 2007; 17:624–629. [PubMed: 17350263]
- Kalant H. Research on tolerance: what can we learn from history? *Alcohol Clin Exp Res.* 1998; 22:67–76. [PubMed: 9514287]
- Kalenka A, Hinkelbein J, Feldmann REJ, Kuschinsky W, Waschke KF, Maurer MH. The effects of sevoflurane anesthesia on rat brain proteins: a proteomic time-course analysis. *Anesth Analg.* 2007; 104:1129–1135. [PubMed: 17456663]
- Kelly LE, Suzuki DT. The effects of increased temperature on electroretinograms of temperature-sensitive paralysis mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci USA.* 1974; 71:4906–4909. [PubMed: 4216025]
- Koenig JH, Saito K, Ikeda K. Reversible control of synaptic transmission in a single gene mutant of *Drosophila melanogaster*. *J Cell Biol.* 1983; 96:1517–1522. [PubMed: 6304107]
- Kosaka T, Ikeda K. Possible temperature-dependent blockage of synaptic vesicle recycling induced by a single gene mutation in *Drosophila*. *J Neurobiol.* 1983; 14:207–225. [PubMed: 6304244]
- Kulkarni NH, Yamamoto AH, Robinson KO, Mackay TF, Anholt RR. The DSC1 channel, encoded by the smi60E locus, contributes to odor-guided behavior in *Drosophila melanogaster*. *Genetics.* 2002; 161:1507–1516. [PubMed: 12196396]
- Lesch KP. Alcohol dependence and gene $\times$ environment interaction in emotion regulation: Is serotonin the link? *Eur J Pharmacol.* 2005; 526:113–124. [PubMed: 16288736]
- Lin WH, Wright DE, Muraro NI, Baines RA. Alternative splicing in the voltage-gated sodium channel DmNav regulates activation, inactivation, and persistent current. *J Neurophysiol.* 2009; 102:1994–2006. [PubMed: 19625535]
- Littleton JT, Chapman ER, Kreber R, Garment MB, Carlson SD, Ganetzky B. Temperature-sensitive paralytic mutations demonstrate that synaptic exocytosis requires SNARE complex assembly and disassembly. *Neuron.* 1998; 21:401–413. [PubMed: 9728921]
- Lledo PM, Zhang X, Sudhof TC, Malenka RC, Nicoll RA. Postsynaptic membrane fusion and long-term potentiation. *Science.* 1998; 279:399–403. [PubMed: 9430593]
- Markham MR, McAnelly ML, Stoddard PK, Zakon HH. Circadian and social cues regulate ion channel trafficking. *PLoS Biol.* 2009; 7:e1000203. [PubMed: 19787026]
- Moore MS, DeZazzo J, Luk AY, Tully T, Singh CM, Heberlein U. Ethanol intoxication in *Drosophila*: genetic and pharmacological evidence for regulation by the cAMP signaling pathway. *Cell.* 1998; 93:997–1007. [PubMed: 9635429]
- Nelson JC, Wyman RJ. Examination of paralysis in *Drosophila* temperature-sensitive paralytic mutations affecting sodium channels; a proposed mechanism of paralysis. *J Neurobiol.* 1990; 21:453–469. [PubMed: 2161909]
- Nitz DA, van Swinderen B, Tononi G, Greenspan RJ. Electrophysiological correlates of rest and activity in *Drosophila melanogaster*. *Curr Biol.* 2002; 12:1934–1940. [PubMed: 12445387]
- Pittson S, Himmel AM, MacIver MB. Multiple synaptic and membrane sites of anesthetic action in the CA1 region of rat hippocampal slices. *BMC Neurosci.* 2004; 5:52. [PubMed: 15579203]
- Ramazani RB, Krishnan HR, Bergeson SE, Atkinson NS. Computer automated movement detection for the analysis of behavior. *J Neurosci Methods.* 2007; 162:171–179. [PubMed: 17335906]
- Scholz H, Ramond J, Singh CM, Heberlein U. Functional ethanol tolerance in *Drosophila*. *Neuron.* 2000; 28:261–271. [PubMed: 11086999]
- Scholz H, Franz M, Heberlein U. The hangover gene defines a stress pathway required for ethanol tolerance development. *Nature.* 2005; 436:845–847. [PubMed: 16094367]
- Siddiqi O, Benzer S. Neurophysiological defects in temperature-sensitive paralytic mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci USA.* 1976; 73:3253–3257. [PubMed: 184469]

- Smith RA, Winter PM, Smith M, Eger EI. Tolerance to and dependence on inhalational anesthetics. *Anesthesiology*. 1979; 50:505–509. [PubMed: 572191]
- Staiman A, Seeman P. Conduction-blocking concentrations of anesthetics increase with nerve axon diameter: studies with alcohol, lidocaine and tetrodotoxin on single myelinated fibers. *J Pharmacol Exp Ther*. 1977; 201:340–349. [PubMed: 300798]
- Suzuki DT, Grigliatti T, Williamson R. Temperature sensitive mutations in *Drosophila melanogaster*. VII. A mutations (*para*^{ts1}) causing reversible adult paralysis. *Proc Natl Acad Sci USA*. 1971; 68:890–893. [PubMed: 5280526]
- Takadera T, Ohyashiki T. Benzyl alcohol inhibits *N*-methyl-d-aspartate receptor-mediated neurotoxicity and calcium accumulation in cultured rat cortical neurons. *J Biomed Sci*. 2008; 15:767–770. [PubMed: 18712491]
- Tolar LA, Pallanck L. NSF function in neurotransmitter release involves rearrangement of the SNARE complex downstream of synaptic vesicle docking. *J Neurosci*. 1998; 18:10250–10256. [PubMed: 9852562]
- Urizar NL, Yang Z, Edenberg HJ, Davis RL. *Drosophila* homer is required in a small set of neurons including the ellipsoid body for normal ethanol sensitivity and tolerance. *J Neurosci*. 2007; 27:4541–4551. [PubMed: 17460067]
- van Swinderen B, Greenspan RJ. Salience modulates 20–30 Hz brain activity in *Drosophila*. *Nat Neurosci*. 2003; 6:579–586. [PubMed: 12717438]
- Walcourt A, Scott RL, Nash HA. Blockage of one class of potassium channel alters the effectiveness of halothane in a brain circuit of *Drosophila*. *Anesth Analg*. 2001; 92:535–541. [PubMed: 11159264]
- Wang Y, Krishnan HR, Ghezzi A, Yin JC, Atkinson NS. Drug-induced epigenetic changes produce drug tolerance. *PLoS Biol*. 2007; 5:2342–2353.
- Wang Y, Ghezzi A, Yin JC, Atkinson NS. CREB regulation of BK channel gene expression underlies rapid drug tolerance. *Genes Brain Behav*. 2009; 8:369–376. [PubMed: 19243452]

Fig. 1.

Effects of benzyl alcohol anesthesia. **a** Local field potentials recorded in the fly brain prior to benzyl alcohol application. The scale indicates the dimensions in both panels **a** and **b**. **b** Benzyl alcohol anesthesia depresses the activity of the nervous system. Shown are local field potentials recorded within the fly brain as the animal is sedated with benzyl alcohol. The reduction in bursting activity indicates that benzyl alcohol suppresses neuronal signaling. **c** A tolerance assay showing the behavioral effect of benzyl alcohol sedation. Two populations of animals are sedated with benzyl alcohol and moved to a fresh-air environment at $t = 0$ min. Shown are recovery curves for a population of animals recovering from their first sedation with benzyl alcohol (*gray line*) and a population recovering from their second sedation (*black line*). Plotted are averages of the percentage of flies climbing the walls of the vial. Error bars are shown in *light gray* and represent the standard error of the mean. Flies recovering from their second sedation recover more rapidly than do animals recovering from their first sedation. For these behavioral assays, significant differences between curves was determined by logrank test. $**P < 0.001$

Fig. 2.

A reduction in neural activity does not phenocopy tolerance. Shown are recovery plots of Canton S controls (**a**) and the three temperature-sensitive mutations *para*^{ts1} (**b**), *shi*^{ts1} (**c**), and *shi*^{ts2} (**d**) the day after incubation either at the restrictive temperature (30°C, *black line*), or at room temperature (22°C, *gray line*). The line is the average response ($N = 5$ or 6) and the *light gray* error bars represent the standard error of the mean

Fig. 3.

Mutations in the *Drosophila* dynamin gene disrupt the capacity to acquire tolerance at the permissive temperature. Shown are recovery curves for Canton S controls (a) and the temperature-sensitive mutants *para*^{ts1} (b), *shi*^{ts1} (c), and *shi*^{ts2} (d). The gray line shows the recovery curve of animals recovering from their first benzyl alcohol sedation and the black line represents the recovery from a second benzyl alcohol sedation. Plotted are averages of the percentage of flies climbing the walls of the vial. Error bars are shown in light gray and represent the standard error of the mean. $N = 3$ for (a), 5 for (b) and 6 for (c, d). Permissive temperature is 22°C. ** $P < 0.001$ as determined by log-rank test

Fig. 4. High doses of NEM impair negative geotaxis. Animals were housed in vials containing food laced with three different doses of NEM: 1 mM (*filled circles*), 0.1 mM (*open boxes*) and 0.01 mM (*solid triangles*). The percentage of flies on the walls of the vials is plotted

Fig. 5.

Feeding flies 0.1 mM NEM causes resistance. All plots show the recovery of flies from their first benzyl alcohol sedation. **a** 0.1 mM NEM food causes resistance. Flies that have consumed 0.1 mM NEM (*black line*) recover more rapidly from benzyl alcohol sedation than animals that were not fed NEM (*gray line*). **b** Feeding flies 0.01 mM NEM does not cause a change in sensitivity to benzyl alcohol. Flies that have consumed 0.01 mM NEM (*black line*) recover from benzyl alcohol sedation at the same time as animals not fed NEM (*gray line*). Lines are averages ($N = 6$) and the *light gray* error bars are SEM. $^{**}P < 0.001$ as determined by the log-rank test

Fig. 6.

NEM disrupts the acquisition but not the maintenance of the tolerant state. The graphs are standard rate of recovery from sedation tolerance tests. The *bars* above each graph (labeled 'NEM food') is the treatment paradigm. The black portions denote the periods of time during which the flies consume food containing 0.01 mM NEM. The *arrows* denote the timing of the first benzyl alcohol sedation (or the mock sedation for the control) and the second benzyl alcohol sedation. Immediately after sedation for the second time, the animals are allowed to recover in drug free vials. Animals are said to have recovered once they begin climbing the walls of the vials. **a** Flies that have consumed 0.01 mM before and after the initial benzyl alcohol sedation recover at the same rate from their second sedation (*black*

line) as from their first sedation (*gray line*). Prior sedation does not produce tolerance. **b** Flies that consume 0.01 mM NEM before but not after their first benzyl alcohol sedation also fail to acquire tolerance to benzyl alcohol sedation. **c** Feeding 0.01 mM NEM immediately after but not before the first benzyl alcohol sedation does not prevent tolerance. Following benzyl alcohol sedation, the animals begin consuming the food after approximately 3 h. The rate of accumulation of NEM in the fly is not known. $N = 6$ for **a** and **b** and 4 for **c**. $**P < 0.01$, as determined by the log-rank test

Fig. 7.

Blockade of neuronal action potentials does not prevent the acquisition of benzyl alcohol tolerance. **a** The experimental paradigm used to maintain temperature-induced paralysis before, during, and after the first benzyl alcohol sedation. *para^{ts1}* flies were incubated at 33°C, from 5 min before sedation to 2 h after their first benzyl alcohol sedation. Hemolymph levels of benzyl alcohol have returned to base-line (zero) before the animals are returned to the permissive temperature (data not shown). **b** *para^{ts1}* flies acquire substantial tolerance despite the blockade of neural signaling during drug exposure. The black trace depicts the recovery of flies after their second benzyl alcohol sedation (labeled 2nd benzyl alcohol sedation in part **a**). The gray trace depicts the recovery profile of flies from their first sedation (these flies received a mock sedation at $t = 5$ min). $N = 6$. ** $P < 0.01$ as determined by the log-rank test