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Halothane Presynaptically Depresses Synaptic Transmission in Wild-type Drosophila *Larvae But Not in Halothane-resistant (*har*) Mutants*

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Background: **General anesthetics produce important changes in neural function, but the relation between the many individual changes produced by anesthetics in neural components and the responsiveness of the whole organism is uncertain. An analysis of genetically altered animals that have modified responses to volatile anesthetics may help to allay this uncertainty.**

Methods: **The authors evaluated the effect of halothane on synaptic transmission at the larval neuromuscular junction in wild-type (Ore-R) and halothane-resistant (*har*) mutants of *Drosophila melanogaster*. The body wall muscles, which are innervated by glutamatergic nerves, were voltage clamped at −60 mV using the patch-clamp technique in the whole cell configuration. Nerve-evoked excitatory junctional currents and miniature excitatory junctional currents were recorded. The effects of halothane on the amplitude of these currents were compared in Ore-R and two *har* mutants derived from the Ore-R strain. The time course and frequency of miniature excitatory junctional currents also were analyzed in the presence of halothane.**

Results: **In Ore-R, halothane (1.8%; 1.01 mM) significantly reduced the amplitude of nerve-evoked excitatory junctional currents (61.9 ± 17% of control, mean ± SD; n = 7), but not that of miniature excitatory junctional currents. Conversely, in two *har* mutants, halothane had no effect on the amplitude of either nerve-evoked excitatory junctional currents or miniature excitatory junctional currents. In Ore-R, the frequency of miniature excitatory junctional currents was decreased significantly in the presence of halothane (0.9–2.7%; 0.52–1.46 mM), whereas halothane did not change the frequency in two *har* mutants. The miniature excitatory junctional current decay time constant, thought to reflect the kinetic properties of junctional glutamate receptor channels, was not changed by halothane in either the Ore-R strain or the *har* mutants.**

Conclusions: **Halothane depresses synaptic transmission at the wild-type *Drosophila* neuromuscular junction, most likely by affecting presynaptic properties. The absence of an effect by halothane in the *har* mutants provides evidence that the depression of presynaptic function at the glutamate-mediated synapses is an important contributor to the way halothane alters the responsiveness of the whole animal. (Key words: *Drosophila melanogaster*; excitatory junctional currents; glutamate; neuromuscular junction; patch clamp; volatile anesthetics.)**

ALTHOUGH chemical synapses in the central nervous system are thought to be particularly important candidates for the main sites of anesthetic action,[1,2] little is known about the relation between the many individual changes produced by anesthetics in neural components and the responsiveness of the whole organism. An analysis of genetically altered animals that are resistant or sensitive to inhalation anesthetics ultimately may allow us to understand the molecular and cellular mechanisms by which general anesthetics alter behavioral states. Recently, *Drosophila melanogaster* mutants that showed an abnormal response to the inhalation anesthetic halothane were isolated.[3] Some of these mutants show unusual motor behavior in the absence of halothane, suggesting that some components of the nervous system of *har* mutants have morphologic or physiologic (or both) alterations that cause the altered response to anesthetics. However, despite the findings of previous genetic, behavioral, and pharmacologic studies,[4,5] it remains unknown whether synaptic transmission in these mutants is affected differentially by inhalation anesthetics such as halothane.

In this study, we evaluated the effect of halothane on glutamate-mediated synaptic transmission at the neuromuscular junction (NMJ) of wild-type *Drosophila* larvae and those of *har* mutants. We found that halothane inhibits synaptic transmission by affecting presynaptic

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properties and that the *har* mutants are resistant to this effect of halothane.

Methods

Fly Stocks

The Oregon-R (Ore-R) strain of *D. melanogaster* was used as a wild type throughout this investigation. Stocks of two strains of mutant flies (*har* 38, *har* 85), derived from Ore-R, were the gifts of Dr. Howard A. Nash.[3] These mutants have similarly altered patterns of sensitivity to different anesthetics, show similarly abnormal behaviors in the absence of halothane, map to the same genetic locus, and fail to complement one another fully.[4] Thus, they are likely to be independent alleles of a gene that contributes to the structure or function (or both) of the fly's nervous system.

Preparation

First instar larvae were collected shortly after hatching and dissected using a pair of sharp needles under [Ca^{2+}]-free *Drosophila* saline (composed of 140 mM NaCl, 2 mM KCl, 6 mM $MgCl_2$, and 5 mM HEPES–NaOH, adjusted to pH 7.1 with NaOH) as described previously.[6,7] Briefly, the body wall of the first instar larvae was cut along the dorsal midline to keep the central nervous system intact. After the removal of the gut, gonads, fat bodies, and trachea, the internal surface of the body wall was exposed. To hold the specimen flat on a rectangular cover glass surface (60 × 20 mm), two dental floss fibers that were approximately 10 mm were used. These fibers were placed in parallel approximately 1 mm apart and were taped onto a cover glass. The dissected animal was inserted under the dental floss fibers with the internal side facing upward and spread open. A Plexiglas frame, (Dow Corning, Midland, MI 2 mm thick, was pasted on the cover glass with vacuum grease) to form a 1-ml chamber that was filled with recording solutions (to be described).

The specimen was treated with collagenase (Sigma Chemical Co., St. Louis, MO; 1 mg/ml in low [Ca^{2+}] saline) for 3–5 min at room temperature to facilitate gigaseal formation. The ionic content of low [Ca^{2+}] saline was 149 mM NaCl, 2 mM KCl, 5 mM HEPES–NaOH, and 0.1 mM $CaCl_2$, pH 7.1. After the enzyme treatment, the preparation was washed several times with [Ca^{2+}]-free *Drosophila* saline and the chamber was filled again with recording solution.

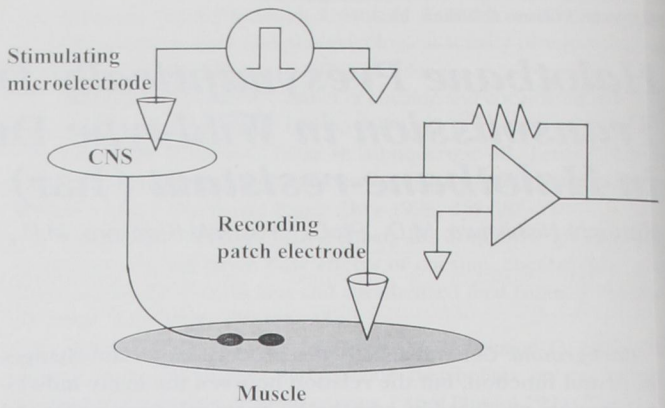

Fig. 1. The experimental setup to record nerve-evoked excitatory junctional currents. A recording chamber (1 ml) containing the preparation was mounted on the stage of an upright microscope (Diaphot; Nikon, Tokyo, Japan) and the specimen was viewed with a ×40 objective lens. A stimulating microelectrode and recording patch electrode were placed with visual control. Abdominal longitudinal muscles 6 or 7 of the first instar larvae were voltage clamped at −60 mV using the patch-clamp technique in the whole cell configuration.

Electrophysiologic Experiments

The properties of glutamate-mediated synaptic transmission of *Drosophila* larvae have been characterized extensively electrophysiologically[6–9] and morphologically.[10] Experiments were performed at identified NMJs on muscles 6 or 7 in abdominal segments (A2–A4) using the standard whole cell patch-clamp technique.[11] Muscle cells were voltage clamped at −60 mV by using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) in the whole cell configuration. Nerve-evoked excitatory junctional currents (EJCs) and miniature excitatory junctional currents (MEJCs) were recorded as follows (fig. 1). To record nerve-evoked EJCs, the central nervous system was stimulated electrically. A stimulating microelectrode was placed near the midline of the central nervous system, and positive pulses (2 μA, 2 ms in duration, 0.1 Hz), which were adjusted in the amplitude to evoke maximal stable EJCs, were delivered every 10 s. After filling with 4 M K-acetate, the microelectrode had a resistance of approximately 10 MΩ. With this arrangement, as verified by the short latency with which synaptic currents appeared after the onset of stimulus, the motoneurons innervating the clamped muscle cell were excited directly by the stimulating pulse. Recording patch electrodes were made from soft, glass capillaries (Drummond Scientific Co., Broomall, PA), which had a resistance of approximately 5 MΩ after being filled with the internal solution (composed 158 mM KCl, 5 mM EGTA, 10 mM HEPES–NaOH, and 2 mM adenosine triphosphate, pH, 7.1). Stocks of adenosine triphosphate

stored at −20°C were dissolved in the internal solution shortly before use.

In all experiments, *Drosophila* saline containing 0.5 mM [Ca^{2+}] was used as the recording solution. This [Ca^{2+}] concentration was relatively less than those of previous reports[8,9] to compensate for the early developmental stage of the muscle examined. As we described previously,[6] increasing [Ca^{2+}] concentrations (> 1 mM) frequently caused contraction of the muscles and their detachment from the body wall in first instar larval muscles, but 0.5 mM [Ca^{2+}] prevented this and still allowed evoked EJCs and MEJCs to be recorded. In MEJCs recordings, 1 μM tetrodotoxin was included in the recording solution to prevent motor nerve firing. All experiments were performed at room temperature (18–24°C).

Application and Measurement of Halothane

Halothane (Takeda Chemical Industry, Osaka, Japan) was applied by the perfusion system (approximately 2 ml/min) with *Drosophila* saline containing 0.5 mM [Ca^{2+}]. To make a solution of a desired concentration of the volatile anesthetic, halothane delivered from a calibrated commercial vaporizer (Fluotec 3; Fraser Harlake, Orchard Park, NY) was bubbled into the perfusate for at least 15 min before switching from control recording solutions. Halothane-containing solutions were perfused through a 1.5-mm Teflon (Cole-Parmer Instrument Company, Chicago, IL) tube using a gravity-feed and vacuum system. Solutions in the recording chamber were sampled, and anesthetic concentrations of halothane were determined at a later date using gas liquid chromatography. Standard halothane concentrations of 0.9, 1.8, and 2.7% used in our experiments corresponded to concentrations of 0.52 ± 0.08 mM, 1.01 ± 0.12 mM, and 1.46 ± 0.15 mM, respectively (mean ± SD, from three measurements). In many cases, successful recordings from one muscle cell were completed within 1 h; therefore, we considered that the loss of halothane was negligible. This reagent contained 0.01% thymol as a preservative. We evaluated the effect of relevant concentrations of thymol (0.15 μM) on synaptic currents at the *Drosophila* NMJ, but the thymol itself at the concentrations used in our study had no significant effect on these currents.

Data Analysis

All currents recorded were filtered at 5 kHz, digitized at sampling rates of 10 kHz, and analyzed using pCLAMP 6.02 software (Axon Instruments), as described previously.[7] At the same time, real-time current recordings were made using a paper recorder (Nihon-Kohden, Tokyo, Japan). Kinetic analysis of MEJCs was performed on averaged traces (usually 10–20 traces). The rise time of synaptic currents was measured at 10–90% of the peak amplitude, and the decay phase was well-fitted with a single exponential curve[6]: $y = Ae^{-t/\tau}$ for single exponential decay, where A is the peak amplitude of MEJCs and τ is the time constant of decay. Measurements are given as the mean ± SD, with an n value that is equal to the number of cells studied. Differences among multiple groups were tested by one-way analysis of variance, as indicated. *P* values less than 0.05 were considered significant.

Results

The Effect of Halothane on Nerve-evoked Excitatory Junctional Currents

The results are based on recordings from muscles 6 or 7 at abdominal segments of the first instar larvae; a representative example is shown in figure 2A. Halothane produced a marked and consistent depression of nerve-evoked EJC amplitude at a concentration of 1.8% in wild-type (Ore-R) larvae (61.9 ± 17% of control, mean ± SD; n = 7, $P < 0.01$; fig. 2B). The time course of the effects of halothane on EJCs amplitude was relatively slow: 5–10 min were necessary to reach steady state levels of depression, and recovery required approximately 5 min. This effect of halothane on nerve-evoked EJCs in Ore-R was observed in a dose-dependent manner (fig. 2C). However, we could not measure the amplitude of evoked EJCs at higher concentrations of halothane (> 2.7%) because muscles contracted frequently and detached from the body wall. Therefore, we could not determine the concentration for 50% depression of nerve-evoked EJCs amplitudes in Ore-R. Conversely, halothane (0.9–2.7%) had no significant effect on the amplitude of EJCs in *bar* 38 and 85 mutants.

The Effect of Halothane on Miniature Excitatory Junctional Currents

Amplitude. Infrequently occurring MEJCs, caused by quantal release of glutamate, were recorded in 0.5 mM [Ca^{2+}] saline containing 1 μM tetrodotoxin (fig. 3A). As shown in table 1, the amplitude of MEJCs in the Ore-R strain is unaffected by halothane (1.8%). Furthermore, the *bar* mutations have no significant effect on the amplitude of MEJCs in the absence or presence of anesthetic.

Rise Time and Decay Time Constants

Miniature EJCs have a fast rising phase and a slower decay phase, shown in figure 3B. The rise time in control

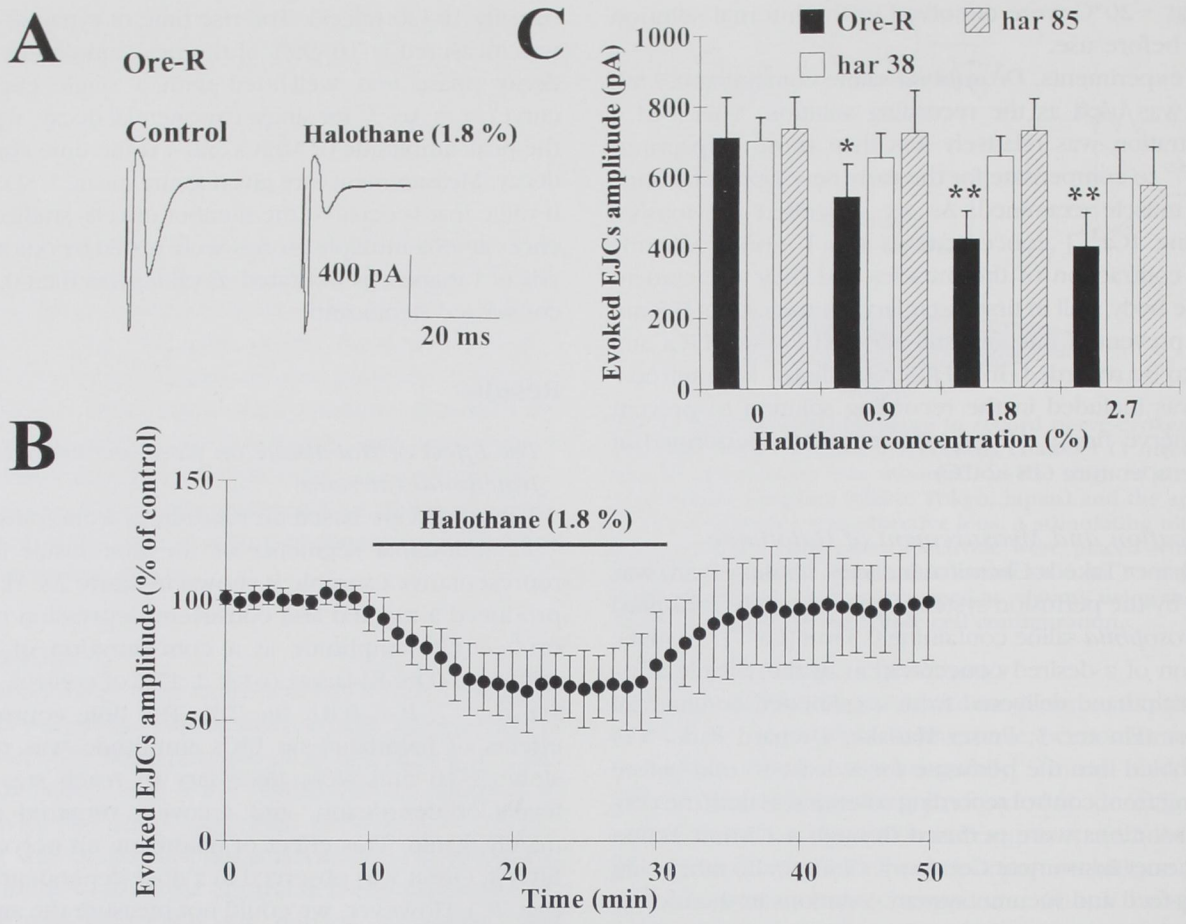

Fig. 2. Halothane decreases the mean amplitude of nerve-evoked excitatory junctional currents (EJCs) in Ore-R. Abdominal muscles 6 or 7 (A2–A4 segment) of early stages of the first instar larvae were voltage clamped at −60 mV in the whole cell configuration. Electric stimuli (2 μA, 2 ms, 0.1 Hz) were given through a microelectrode (10 MΩ) placed near the midline of the central nervous system. Recording solution included 0.5 mM [Ca^{2+}]. (*A*) Sample traces of nerve-evoked EJCs recorded in control saline (left) and in halothane-containing saline (right), respectively. (*B*) A time-*versus*-amplitude graph of nerve-evoked EJC amplitudes in Ore-R shows a marked reduction during a 1.8% halothane application (20 min). Points represent the mean ± SD for seven determinations from separate preparations. The mean of 10 control data was used as 100%. (*C*) Halothane significantly decreased the mean amplitude of nerve-evoked EJCs in Ore-R (n = 7), but not in two *bar* mutants (n = 6, each). *$P < 0.05$, **$P < 0.01$ *versus* 0 mM halothane.

saline was 0.55 ± 0.15 ms (n = 15) in Ore-R, 0.59 ± 0.14 (n = 16) in *bar* 38, and 0.52 ± 0.16 (n = 14) in *bar* 85. There was no significant difference among these values. The rise time was not affected in the presence of halothane (1.8%). We also evaluated the effect of halothane on the decay phase of MEJCs. Figure 3C shows averaged traces of MEJCs recorded from both Ore-R and *bar* 38. The results are summarized in table 1. At a concentration of 1.8%, halothane did not significantly affect the decay time constant of either Ore-R or mutant larvae.

Frequency

Ten minutes after bath application of halothane, the frequency of MEJCs was decreased reversibly in Ore-R (fig. 4A). As shown in figure 4B, the addition of halothane (0.9–2.7%) in the bath solution significantly decreased the frequency of MEJCs in Ore-R (10.2 ± 4 events/min in control, 2.6 ± 2.1 events/min in 2.7% of halothane; n = 8, $P < 0.01$ *vs.* control). Conversely, halothane did not change the frequency of MEJCs in two *bar* mutants.

Discussion

The primary finding of this study is that, in wild-type (Ore-R) larvae of *D. melanogaster*, halothane significantly reduces the amplitude of nerve-evoked EJCs but not that of MEJCs. These data strongly suggest that the primary anes-

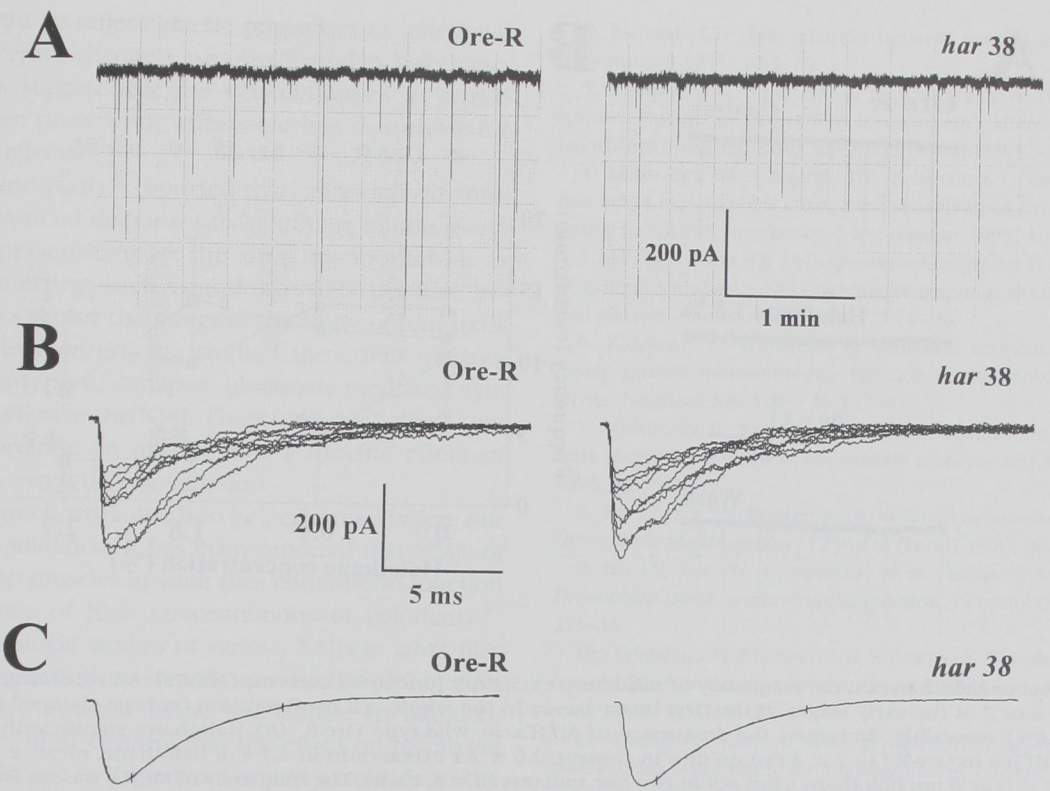

Fig. 3. Sample traces of miniature excitatory junctional currents (MEJCs). (*A*) Single identified muscles 6 or 7 at early stages of the first instar larvae (A2–A4 segment) were voltage clamped at −60 mV in the whole cell configuration in 0.5 mM [Ca^{2+}] saline containing 1 μM tetrodotoxin (left, Ore-R; right, *har* 38). (*B*) Ten sample traces were superimposed to measure the time course of MEJCs (left, Ore-R; right, *har* 38), as described previously.[6] (*C*) A single exponential curve was fitted to the decay phase of averaged MEJCs between two vertical bars, as described in Materials and Methods.

thetic action is on presynaptic neurotransmitter release mechanisms. This conclusion is supported by our findings that the frequency of MEJCs of wild-type larvae is decreased by the presence of halothane, but the rise-time and decay-time constant of MEJCs are unaffected by the drug. To our knowledge, this is the first demonstration in this model organism of a specific effect of a general anesthetic on synaptic transmission.

Although clinically relevant concentrations of anesthetics alter the performance of so many cellular systems,[1] it has been nearly impossible to determine whether a particular anesthetic action makes an important contribution to the clinical state. However, we have found that two mutant strains of *D. melanogaster* are insensitive to the presynaptic effects of halothane on neuromuscular transmission. Specifically, the two effects of halothane seen in Ore-R, reduction of the amplitude of nerve-evoked EJCs and reduction of the frequency of MEJCs, are not evident in mutant larvae, even at concentrations well above those that produce effects in Ore-R. Adults of these mutant strains have altered behavioral responses to halothane,[3] which is why they were selected. At least we can conclude that the altered sensitivity of presynaptic glutamate release to halothane contributes to the altered effects of halothane on behavior. If so, our results provide evidence of the biologic significance of presynaptic neurotransmitter release as an anesthetic mechanism.

The mechanism by which halothane presynaptically depresses the amplitude of nerve-evoked EJCs in Ore-R

Table 1. Timecourse of MEJCs

	Mean Amplitude (pA)		Decay Time Constant (ms)	
	Control Saline	Halothane (1.8%)	Control Saline	Halothane (1.8%)
Ore-R	238 ± 36	225 ± 19	5.3 ± 0.6	5.9 ± 0.8
har 38	188 ± 25	210 ± 22	5.9 ± 0.9	6.3 ± 0.9
har 85	217 ± 48	220 ± 28	5.7 ± 1.0	5.7 ± 0.7

Values are mean ± SD (n = 15, each).

Statistical significance was not evident using analysis of variance.

MEJC = miniature excitatory junctional current.

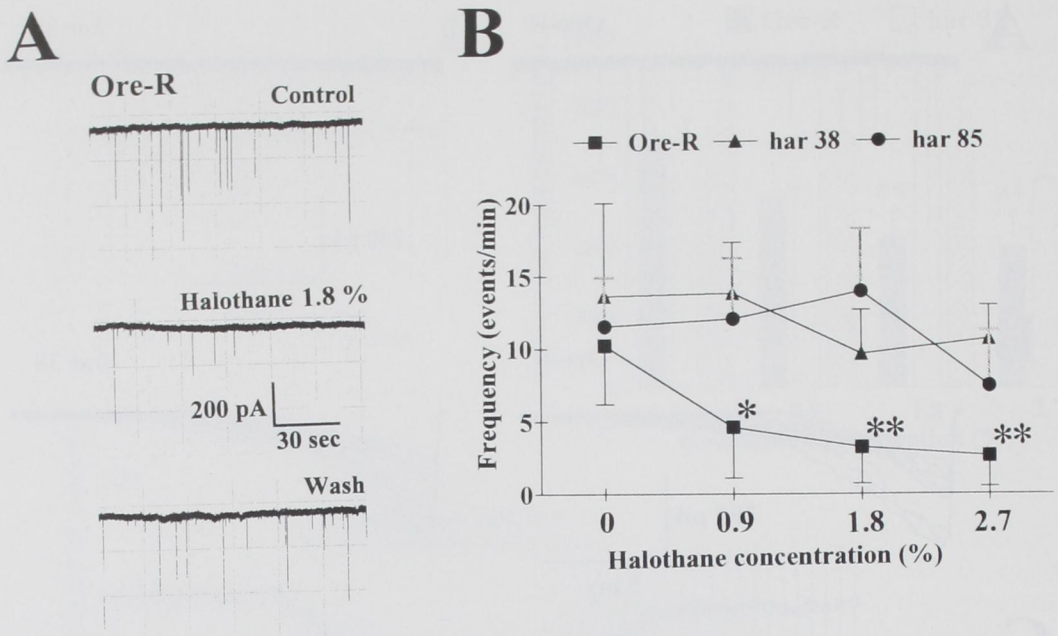

Fig. 4. The effect of halothane on the frequency of miniature excitatory junctional currents (MEJCs). All recordings were obtained from muscles 6 or 7 at the early stages of the first instar larvae in the whole cell configuration (voltage clamped at −60 mV). (*A*) Halothane (1.8%) reversibly decreased the frequency of MEJCs in wild-type Ore-R. (*B*) Halothane significantly decreased the frequency of MEJCs in Ore-R (10.2 ± 4 events/min in control, 2.6 ± 2.1 events/min in 2.7% of halothane, mean ± SD; n = 8, *P < 0.05, **P <0.01 *versus* 0 mM halothane), but not in two *bar* mutants (n = 5, each). The frequency of MEJCs was measured before and 10 min after the bath application of halothane.

remains to be elucidated. In general, multiple steps are involved in neurotransmitter release after action potentials arrive in the presynaptic terminal.[12] The plasma membrane depolarizes as a result of activation of voltage-gated Na^+ channels, which elicit [Ca^{2+}] entry through voltage-gated [Ca^{2+}]. This [Ca^{2+}] entry is coupled to the exocytosis of synaptic vesicles. Each of these steps can be modulated by cellular regulatory mechanisms, such as phosphorylation, and by specific drugs. One possible mechanism by which halothane reduces nerve-evoked glutamate release is the inhibition of presynaptic Na^+ or [Ca^{2+}] channels, or both. Unfortunately, we could not determine the effect of the [[Ca^{2+}]] concentration on evoked EJCs in the presence of halothane, because muscles easily detached and contracted from the body wall in higher [Ca^{2+}] concentrations.

Miniature EJCs recorded in our experiments most resulted from a spontaneous quantal release of neurotransmitter from synaptic vesicles, rather than from spontaneous firing of motor nerves. In all MEJCs recordings, 1 μM tetrodotoxin, which has been shown to block Na^+ inward currents in *Drosophila* neurons,[13] was included in the recording solution. Although many invertebrate muscles receive glutamate-mediated and γ-aminobutyric acid (GABA)–mediated dual innervation, there is no indication of GABA-mediated inhibitory innervation at the *Drosophila* NMJ. First, argiotoxin (12 μM), a spider toxin that blocks *Drosophila* glutamate receptor channels, completely blocked MEJCs and evoked EJCs, as described previously.[6] Second, EJCs could be evoked by puff-applied L-glutamate using a pressure injection system. Third, our preliminary experiments have shown that the bath application of picrotoxin (for 20 min, 50 μM), a GABA–chloride channel blocker, had no significant effect on nerve-evoked EJCs recordings of Ore-R in the presence of halothane. Therefore, we concluded that the effects of halothane on GABA-mediated inhibitory innervation were negligible in our experiments.

There is no reason to believe that the presynaptic depression of neurotransmitter release is the only important action of general anesthetics. Many electrophysiologic and biochemical studies have shown anesthetic actions at postsynaptic sites, especially ligand-gated ion channels such as glutamate,[14,15] $GABA_A$,[16,17] and glycine receptors.[18] These results clearly show that anesthetics can exert a specific action on these ion channel properties. However, our results showed that halothane had no significant effect on the time course of MEJCs; the decay time constant of

MEJCs, thought to reflect kinetic properties of junctional glutamate receptor channels, was unaffected by halothane. These results suggest that the concentrations in which halothane have presynaptic effects are less than those for postsynaptic effects.

Campbell and Nash[19] reported that, although *bar* mutants show reduced responses to halothane in one assay, they are hypersensitive to the drug in another. It is difficult to interpret such complex results because behavioral assays report the integrated activity of hundreds of neurons. In contrast, we studied the effect of halothane on one type of synapse: glutamate-mediated synaptic transmission at the NMJ. Therefore, we believe that our results provide an insight into a specific effect of halothane on synaptic transmission.

Of course, much work needs to be performed before our conclusion is solidified. It has been reported that NMJs of flight and jump muscles in adult flies continue to function in the presence of high concentrations of halothane.[20] Detailed physiologic studies of various NMJs in adult flies need to be evaluated to identify quantitative differences and similarities between larvae and adults. In addition, genetic studies must confirm that the effect on halothane sensitivity in larvae of the mutant strains results from the same mutation that induces an altered anesthetic response in the adult. In this context, it would also be of value to determine whether the *bar* 38 and *bar* 85 mutations alter behavioral responses of *Drosophila* larvae to halothane. As has been noted,[21] gene knockout technology should produce mutant mice that lack various putative anesthetic targets. It will then be of interest to compare the relative influence of postsynaptic effects on the behavioral response to anesthetics with the presynaptic effects highlighted by our study in *Drosophila*.

In conclusion, halothane inhibits synaptic transmission at the larval *Drosophila* NMJ, most likely by affecting presynaptic properties, and *bar* mutants resist this effect of halothane. The absence of the effect of halothane in the *bar* mutations provides evidence that depression of presynaptic function at glutamate-mediated synapses is an important contributor to the way halothane alters the responsiveness of the whole animal.

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