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High-Resolution Analysis of Ethanol-Induced Locomotor Stimulation in *Drosophila*

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Understanding how ethanol influences behavior is key to deciphering the mechanisms of ethanol action and alcoholism. In mammals, low doses of ethanol stimulate locomotion, whereas high doses depress it. The acute stimulant effect of ethanol has been proposed to be a manifestation of its rewarding effects. In *Drosophila*, ethanol exposure transiently potentiates locomotor activity in a biphasic dose- and time-dependent manner. An initial short-lived peak of activity corresponds to an olfactory response to ethanol. A second, longer-lasting period of increased activity coincides with rising internal ethanol concentrations; these closely parallel concentrations that stimulate locomotion in mammals. High-resolution analysis of the walking pattern of individual flies revealed that locomotion consists of bouts of activity; bout structure can be quantified by bout frequency, bout length, and the time spent walking at high speeds. Ethanol exposure induces both dramatic and dynamic changes in bout structure. Mutants with increased ethanol sensitivity show distinct changes in ethanol-induced locomotor behavior, as well as genotype-specific changes in activity bout structure. Thus, the overall effect of ethanol on locomotor behavior in *Drosophila* is caused by changes in discrete quantifiable parameters of walking pattern. The effects of ethanol on locomotion are comparable in flies and mammals, suggesting that *Drosophila* is a suitable model system to study the underlying mechanisms.

Key words: Drosophila*; behavior; ethanol; locomotion;* amnesiac*;* rutabaga

Ethanol is one of the most widely abused drugs in the world, yet our understanding of the mechanisms by which it regulates brain function and behavior is incomplete. Ethanol does not appear to have a singular molecular target, and historically its effects in the nervous system had been attributed primarily to nonspecific changes in the properties of neuronal membranes. Recent evidence shows, however, that the functions of a number of specific brain proteins, including several ligand- and voltage-gated ion channels (for review, see Peoples et al., 1996; Harris, 1999), are modified by ethanol. How ethanol acts on specific proteins and how these effects relate to ethanol-induced behaviors is poorly understood and the subject of intensive study.

In animal models, a common response to acute exposure to drugs of abuse is a change in locomotor behavior. Rodents show a time- and dose-dependent locomotor response to acute ethanol administration: low doses stimulate and high doses depress locomotion (for review, see Phillips and Shen, 1996). Although still controversial, the locomotor-activating effects of drugs of abuse, including alcohol, have been proposed to be a manifestation of their positive reinforcing or rewarding properties (Wise and Bozarth, 1987). Consistent with this notion are observations that some of the neural circuits and neurochemical systems that are central to the reinforcing effects of ethanol, such as the mesolimbic dopamine pathway, also regulate the acute stimulant effects of the drug (Phillips and Shen, 1996; Phillips et al., 1998; Cunningham et al., 2000; Risinger et al., 2000).

Identifying the mechanisms by which ethanol stimulates locomotion is an important step toward understanding the more complex behaviors that accompany addiction. We chose to study the effects of ethanol on locomotion in the relatively simple, genetically accessible fruit fly *Drosophila melanogaster*. Acute ethanol exposure increases *Drosophila* walking speed and turning, and prolonged exposure (or higher doses) leads to loss of postural control and sedation (Moore et al., 1998; Singh and Heberlein, 2000; Parr et al., 2001). In addition, flies develop functional tolerance when exposed to ethanol more than once (Scholz et al., 2000). Here we describe the development of an automated high-resolution locomotor tracking system that continuously monitors the simultaneous movement of >150 flies. We find that, during continuous exposure to ethanol vapors, flies increase locomotor activity in two discrete phases in a time- and dose-dependent manner. The levels of locomotor activity are modulated by sensory inputs, internal ethanol accumulation, ethanol metabolism, and ethanol-induced sedation. *Drosophila* locomotion occurs in short bouts of activity that are separated by pauses or rests. During ethanol exposure, the length of activity bouts and the time walking at high speeds are dynamically altered, whereas bout frequency shows an immediate increase but then remains primarily unchanged. The ethanol-sensitive mutants *amnesiac* (*amn*) and *rutabaga* (*rut*) show different and specific defects in ethanol-induced locomotor activity and activity bout structure. Thus, acute ethanol stimulation of locomotion in *Drosophila* resembles

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the behavior of vertebrates, and these behaviors can be dissected genetically.

MATERIALS AND METHODS

Fly strains, conditions, and genetics. All flies were maintained at 25°C and 70% humidity and were grown in constant light. Strains are as reported previously (Moore et al., 1998). The X-linked mutants amn^{chpd} and rut^{769} and the control line PZ-control each contain a PZ[ry^+] transposable element in a ry^{506} genetic background. Although these mutants were outcrossed for five generations to the parental ry^{506} strain, we cannot rule out a contribution of closely linked modifiers to their locomotor phenotypes. To reduce the effects of recessive autosomal modifier loci, 20 mutant males were crossed to 20 attached-X (XX/Y) females. Twenty patriclinous male progeny that are hemizygous for the parental male X chromosome and heterozygous for parental autosomes were collected 1–3 d after eclosion (day 12 after egg laying). To eliminate any effects of CO_2 anesthesia, flies were kept an additional 2 d before testing (day 14). Flies behaved identically from day 13 to day 15 (data not shown). Because outcrossing *Adh* mutant stocks [mutants lacking functional alcohol dehydrogenase (Adh)] to a standard background is difficult because of the lack of closely linked markers, we instead tested transallelic *Adh* flies that were homozygous mutant for *Adh* and heterozygous for potential autosomal modifier loci. Flies homozygous for one *Adh* mutant allele were crossed to flies homozygous for a second allele, and the transallelic progeny were tested. The transallelic combinations were confirmed to be mutant for *Adh* by demonstrating a lack of recovery from ethanol-induced sedation. Flies heterozygous at the *Adh* locus recovered normally and also developed a normal locomotor tracking profile (data not shown). The *Adh* strains used were Adh^{n1}, Adh^{fn23} pr^1 cn^1, and Adh^{fn6} cn^1;ry^{506} and were obtained from the *Drosophila* Stock Center (Bloomington, IN).

Locomotor tracking assay. All locomotor tracking experiments were done with an exposure chamber placed horizontally on top of a light box and were filmed from above. The exposure chamber was maintained at 20°C. In all ethanol exposure experiments, flies were acclimated to the chamber for 9 min before the start of ethanol exposure. At this time, locomotion was variable. However, ethanol-induced behavior was independent of the levels of locomotion before the start of the ethanol exposure (see Fig. 2*B*; data not shown). Baseline locomotion data were obtained after acclimating flies for longer time periods (as indicated in the figure legends). Flies were placed in a 60 × 60 × 15 mm clear acrylic chamber (referred to as square chamber) that contained inlet and outlet ports for vapor delivery. A second device, the "booz-o-mat," was developed by us to increase assay throughput. Flies were placed into 16 × 125 mm cylindrical tubes with perforations clustered at the rounded base. Eight tubes were fitted into a horizontal rack that visually isolates flies in different tubes. Ethanol and water vapor were produced as described previously (Moore et al., 1998), controlled by three 150 mm correlated flowmeters (Cole Parmer, Vernon Hills, IL), and delivered simultaneously to the eight tubes. Ethanol delivery was equivalent to all tubes because ethanol-induced behavior was indistinguishable between tubes (data not shown). The motion of flies in all eight tubes was recorded simultaneously. Total flow rates for the square chamber were 65 U and for the booz-o-mat were 150 U. These flow rates were empirically determined for each device to reduce nonstimulated locomotor activity to baseline within 30 min. Mixtures of air and ethanol are noted as ratios (for example, a mixture of 50 U ethanol and 100 U of humidified air is written as E/A 50:100). All tracking assays were repeated on at least 3 different days to incorporate into the results the sometimes large day-to-day variations in behavior.

For all locomotor assays, fly positions were recorded with a digital video camera (TRV-900; Sony, Tokyo, Japan) connected via IEEE-1394 interface to an Apple (Cupertino, CA) computer with a Motorola G4 processor and captured with either Adobe Premiere (Adobe Systems, San Jose, CA) or VideoScript (VideoScript, Corrales, NM). Films were recorded at 10 frames per second (fps), a sufficient rate for capture of the full range of locomotor speeds. Films were analyzed with a modified version of DIAS 3.2 (Solltech, Oakdale, IA) that was controlled by the OneClick 2.0 scripting language (Westcode Software, San Diego, CA). DIAS analysis identified individual flies as dark objects on a light background, traced the paths of individual objects between frames, and calculated the position and speed of each object. The resulting data files were summarized with programs written in Perl. Incomplete traces of paths taken for individual flies occurred when one or more flies came within close proximity of one another, often resulting in a larger number of identified objects than flies and in periods in which individual flies were not traced. For computing the population average locomotor velocity, we summed the total path length of all objects over an observation period and divided by the number of objects and total time. Thus, the average locomotor velocity measures fly velocity only when each fly is being tracked.

Locomotor velocity patterns. The position of 20 flies in the square exposure chamber was measured at 100 msec intervals (10 fps) over consecutive 1 min periods, resulting in a potential 600 measurements of locomotor velocity per fly per minute. The frame-to-frame data for each object was smoothed with a weighted moving average window of three frames to reduce noise introduced by variation in object outlines between frames. Periods of activity and inactivity were defined as follows. Activity was defined as periods when objects were moving faster than 1 mm/sec. Periods of inactivity were identified as three or more consecutive measurements (>300 msec) of an object moving <1 mm/sec. Periods of inactivity or activity of <5 sec occurring at either the start or end of the detection period of an object were discarded to reduce data analysis method artifacts. Activity bout length was measured as time between activity bout onset and offset. The frequency of activity bouts was calculated as the number of bout onsets per minute and included bouts that initiated when an object was first detected if that bout continued for at least 5 sec. Both bout length and bout frequency were averaged across all objects for a given period. Time spent moving faster than 20 mm/sec was determined by counting the number of measurements above 20 mm/sec divided by the total number of measurements.

Ethanol concentration measurements. Whole flies (20) were frozen on dry ice and homogenized in 200 μl of ice-cold 50 mM Tris, pH 7.5, and centrifuged for 20 min at 4°C to remove particulates. Homogenate (10 μl) was then added to 500 μl of reagent from an ethanol detection kit (Sigma, St. Louis, MO), and concentration was determined according to the instructions of the manufacturer as described previously (Moore et al., 1998). Values are reported as ethanol levels in treated minus untreated flies.

Surgery. Male flies were anesthetized either on ice or with CO_2. Antennal segment 3 was removed with fine forceps. Aristae and maxillary palps were cut with ultrafine microdissecting scissors (Fine Science Tools, Foster City, CA). Flies were allowed to recover for 1–2 d before testing. Recovery was not necessary; flies tested 3 hr after surgery responded identically.

Statistics. Error bars in the figures are SEM. One-way ANOVA with fixed effects was used to test for significance, unless otherwise noted. When more than two conditions were compared, Newman–Keuls *post hoc* analysis was done with a critical p value adjusted to maintain an experiment-wide error rate of $\alpha = 0.05$.

RESULTS

Ethanol-induced locomotor activation

To study *Drosophila* locomotor behavior, we developed an automated motion tracking system (Fig. 1*A*) (see Materials and Methods). In this assay, 20 or more flies of the same genotype are placed into a translucent exposure chamber and allowed to acclimate in a stream of humidified air. Flies are then continuously exposed to ethanol vapor. Fly movement is recorded via digital video at 10 fps. The motion of individual flies is then determined via a combination of computational methods (see Materials and Methods). Traces of the paths of 20 flies taken from critical time points during exposure to a moderate dose of ethanol are shown in Figure 1*B* and described in more detail below. Two exposure chambers were developed: a square chamber was used for detailed analysis of fly movement patterns, and, to increase throughput, we developed the booz-o-mat, a system that allows the simultaneous analysis of eight groups of flies (see Materials and Methods).

The average walking speed of the population of flies exposed to a moderate ethanol dose is shown in Figure 2*A*. Immediately after switching from air to ethanol vapor (time 0), flies rapidly increased locomotion to peak average speeds of ~10 mm/sec; this initial activity subsided within 1 min. Locomotor velocity then increased more gradually to peak levels of 10 mm/sec between 5

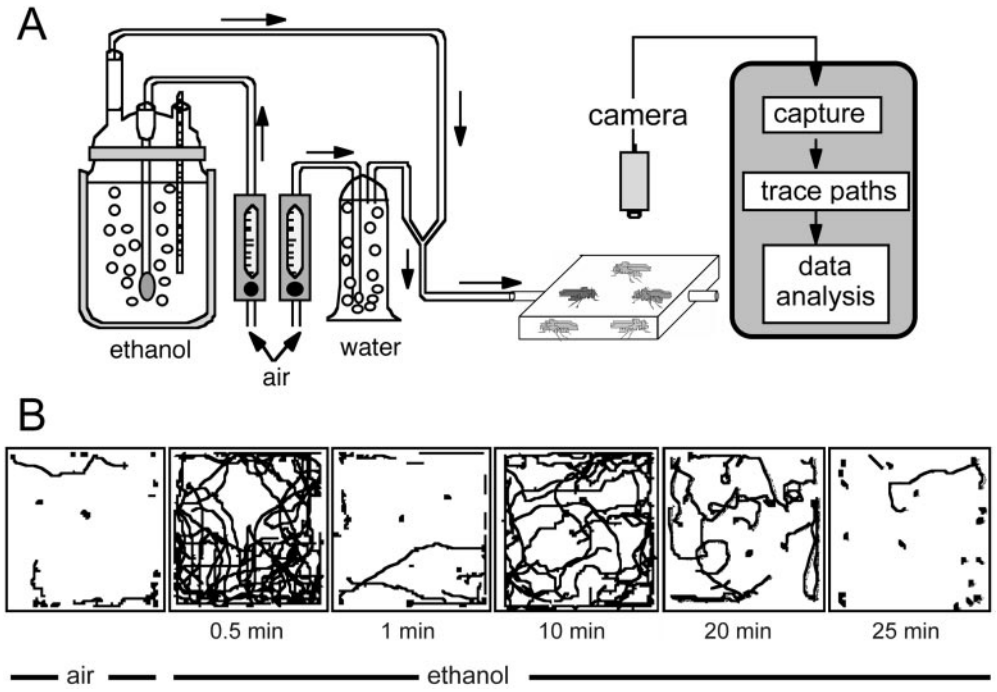

Figure 1. Effect of ethanol on locomotion. *A*, Apparatus for tracking *Drosophila* locomotion. Air at controlled flow rates is bubbled through 95% ethanol maintained at 20°C and separately through water. Humidified air and ethanol vapor are mixed and delivered to an exposure chamber made of clear plastic. Flies are filmed with a digital video camera, and video is captured directly onto a computer. Fly motion is detected by frame-to-frame changes in position by the program DIAS. Locomotor velocity and patterns are calculated from the raw data by programs written in Perl. *B*, Example traces show the path taken by 20 wild-type flies over a 10 sec period at 10 fps at E/A 40:25 in the square chamber. This dose is slightly higher that that used for most experiments and is to reveal the whole range of behaviors seen and recorded by the tracking system. The *first panel* (*air*) depicts typical behavior of flies in a stream of humidified air; little locomotion is observed. During switching from air to ethanol vapor, flies show an immediate and transient peak of activity (*0.5 min*) that subsides by 1 min of exposure. A second more prolonged hyperactive period peaks at ~10 min of exposure. Locomotor activity is reduced by 20 min of exposure and is almost absent after 25 min. In contrast to the immobile flies seen during air exposure, which are standing and often grooming, immobile flies observed after a 25 min ethanol exposure have lost postural control, are lying on their sides or backs, and are resistant to mechanical stimulation.

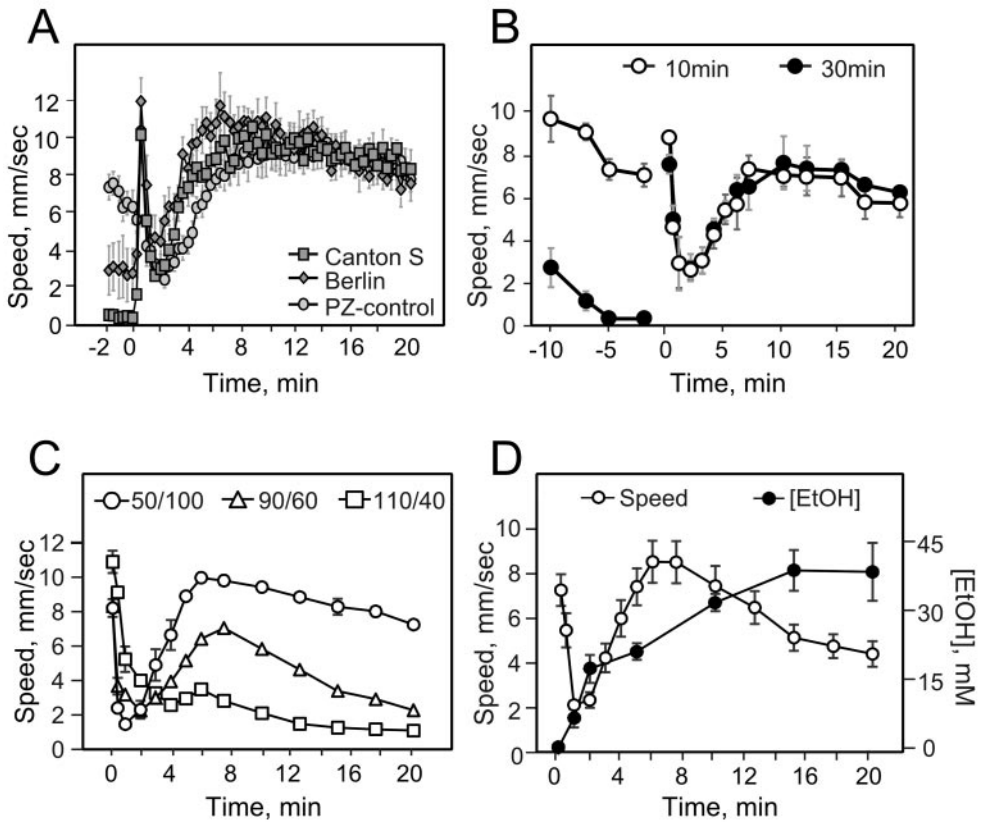

Figure 2. The wild-type hyperactive response is dose sensitive and correlates with ethanol accumulation. *A*, The wild-type locomotor response to ethanol. Population average walking speed for two wild-type strains (Canton S and Berlin) and a control strain (PZ-control) was calculated for 10 sec periods every 30 sec from 2 min before ethanol exposure onward in the square chamber. Ethanol exposure begins at 0 min in this and all subsequent figures and is continuous for 20 min. Ethanol vapor concentration was E/A 30:35 ($n = 3$ for each genotype). *B*, Effect of chamber acclimation on ethanol-induced locomotion. Control flies were allowed to acclimate to the square exposure chamber for 10 or 30 min, and the velocity 10 min before ethanol exposure was calculated. The level of activity before ethanol exposure did not affect ethanol-induced locomotor activity. Ethanol vapor concentration was E/A 30:35 ($n = 3$). *C*, Dose dependence of the hyperactive response. Groups of 20 control flies were exposed to the indicated doses of ethanol in the booze-o-mat. Low (E/A 50:100; $n = 14$), moderate (E/A 90:60; $n = 17$), and high (E/A 110:40; $n = 3$) doses are shown. *D*, Ethanol accumulation. Ethanol concentrations in whole fly extracts were measured while simultaneously determining locomotor velocity in the booze-o-mat at E/A 70:80 ($n = 3$). Error bars in this and all subsequent figures indicate the SEM.

and 10 min of ethanol exposure. During continued exposure to a moderate dose, flies reduced their locomotor speed (Fig. 2*A*,*C*). Eventually, flies lost postural control and became immobile (Fig. 1*B*); these flies recovered when a stream of humidified air replaced ethanol vapor, demonstrating that immobile flies were sedated and not dead (data not shown). The two periods of increased locomotor activity were termed the startle response, because it was found to be a response to the smell of ethanol (see below), and the hyperactive phase, likely attributable to internal accumulation of ethanol affecting nervous system function.

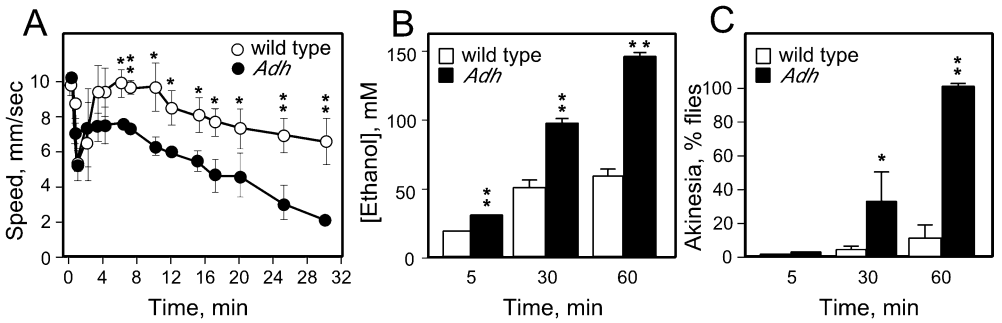

Figure 3. Ethanol metabolism affects ethanol-induced hyperactivity. *A*, Population average locomotor velocities for the wild-type strain Canton S (*white circles*; $n = 3$) and *Adh* mutant flies (*black circles*) exposed to moderate ethanol doses (E/A 70:80 in the booz-o-mat). *Adh* is combined data from three transallelic combinations of the *Adh* mutants Adh^{fn6}, Adh^{fn23}, and Adh^{n1} ($n = 3$ for each transallelic pair). All allele combinations produced similar results (control vs *Adh*; $^{*}p < 0.05$; $^{**}p < 0.01$). *B*, *Adh* flies show increased concentrations of ethanol, even at 5 min of exposure to moderate ethanol doses (E/A 70:80 in the booz-o-mat) ($n = 6$; $^{**}p < 0.01$; two-tailed *t* test). *C*, *Adh* flies became akinetic more quickly than control flies (E/A 70:80 in the booz-o-mat) ($n = 9$; $^{*}p < 0.05$; $^{**}p < 0.001$; two-tailed *t* test). Akinesis is defined as the number of immobile flies lying on their backs for at least 10 sec at a given time point.

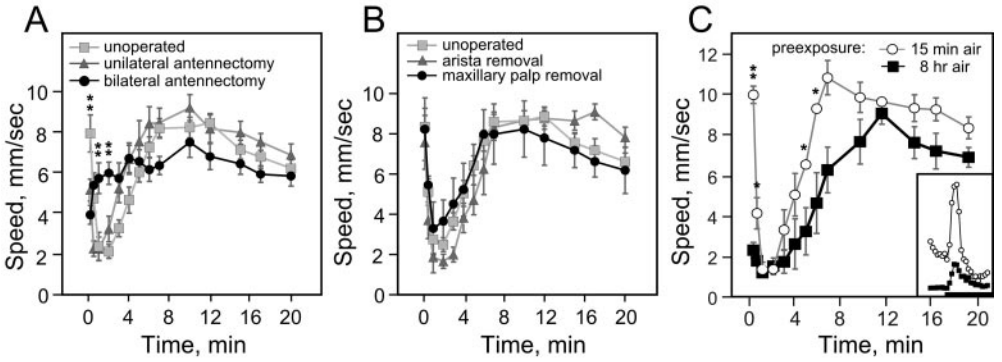

Figure 4. Sensory input regulates hyperactivity. *A*, Surgical removal of the third antennal segment affects startle and quiescence, but hyperactivity is relatively normal. The third segment of the bilaterally paired antennae (containing 1200 olfactory neurons) was removed either unilaterally (*triangles*; $n = 6$) or bilaterally (*circles*; $n = 7$) from control flies (unoperated, *squares*; $n = 11$). Unilateral antennectomy reduced startle ($p < 0.05$) without affecting hyperactivity. Bilateral antennectomy ablated startle ($p < 0.01$) and increased locomotor activity during quiescence (1 and 2 min; $p < 0.01$) but only weakly affected hyperactivity at one time point (7 min; $p = 0.03$). All assays were done in the square chamber at E/A 30:35. *B*, Other sensory organs are dispensable for ethanol-induced locomotor activation. The aristae project from the third antennal segment and were removed in the third antennal segment surgery. Maxillary palps are a second olfactory organ with 120 olfactory receptor neurons ($n = 4$). *C*, Long acclimation periods abolish startle but maintain hyperactivity. Control flies were preexposed to humidified air for 15 min (*circles*; $n = 3$) or 8 hr (*squares*; $n = 4$) and then exposed to ethanol for 20 min (E/A 30:35). Locomotor activity was strongly different between conditions after 10 sec (9.4 vs 1.9 mm/sec; $p = 0.0003$) and weakly different at 30 sec, 6 min, and 7 min ($p < 0.05$). *Inset*, Startle response at high temporal resolution. Speed was sampled every 5 sec and averaged over 5 sec.

The standard laboratory wild-type strains Canton S and Berlin had nearly identical locomotor activity profiles in response to a moderate ethanol vapor concentration (Fig. 2*A*). PZ-control, a laboratory strain selected previously as a control for its normal behavioral responses (see Materials and Methods), and a wild *Drosophila* strain (collected in Mendocino County, CA) also had comparable locomotor activity profiles (Fig. 2*A*; data not shown). We conclude that the population average locomotor activity profiles of Canton S, Berlin, and PZ-control define the wild-type locomotor response to ethanol.

During introduction into the filming chamber, and before ethanol exposure, flies exhibited variable levels of locomotion, which decayed to a low basal level within 1 hr (Fig. 2*B*). To test whether the levels of locomotor activity observed before ethanol exposure affected the responsiveness to ethanol, we allowed flies to acclimate to their environment for 10, 30, or 60 min, at which times they showed high, moderate, and low locomotor activity, respectively. Regardless of the time of acclimation, flies developed identical locomotor activity profiles during exposure to ethanol (Fig. 2*B*; data not shown). Thus, the effects of ethanol on locomotion are independent of the flies' walking speed before exposure. We suspect that ethanol is a strong stimulus that can override environmental or internal cues that modulate spontaneous locomotion in flies. In subsequent experiments, flies were exposed to ethanol vapor after a 9 min acclimation period.

Dose sensitivity and ethanol accumulation

In rodents, low doses of ethanol stimulate locomotion, whereas high doses are sedating (for review, see Phillips and Shen, 1996). To ascertain the effect of ethanol dose on fly behavior, we exposed flies to a range of ethanol vapor concentrations regulated by adjusting the relative flow of ethanol vapor and humidified air, the ethanol/air (E/A) ratio. The olfactory startle response showed a saturable dose–response curve; high ethanol concentrations induced high maximal startle velocities (W. Cho, F. W. Wolf, and U. Heberlein, unpublished observations). The second hyperactive phase showed a more complex dose dependency. Maximal locomotor stimulation was observed with a relatively low ethanol concentration (E/A 50:100) (Fig. 2*C*). With increasing ethanol concentrations (E/A 90:60 and 110:40), the extent and duration of the hyperactive phase diminished in a dose-dependent manner (Fig. 2*C*). Higher doses resulted in delayed onset of hyperactivity and an accelerated rate of sedation. However, very low ethanol doses (E/A 15:135 and 25:125) were also less stimulating than E/A 50:100 (data not shown). Thus, locomotor activation responded in a nonlinear manner to changes in ethanol vapor dose and in a manner that is consistent with findings in rodents.

To determine the relationship between ethanol levels and locomotor behavior, we measured ethanol concentrations absorbed by the flies throughout the exposure period. Low but measurable

levels of ethanol accumulated after 2 min of exposure to a moderate ethanol dose (Fig. 2*D*). At the peak of the hyperactive phase, ethanol levels reached ~20 mM. Ethanol levels continued to rise as flies gradually became sedated, reaching ~40 mM after 20 min, a time when ~30% of flies were immobilized (see below). Thus, the transition from locomotor stimulation to sedation is correlated with increasing internal ethanol levels. This suggests that the reduction in locomotor activity during the sedation phase is not caused solely by adaptation to the stimulant effects of ethanol during the assay. Consistent with this is the observation that the number of flies that fall and fail to regain upright posture increased with time of exposure at moderate doses of ethanol (see Fig. 5*A*, *inset*). These sedated flies are distinguished from flies that are simply not walking by the fact that they have lost postural control, lying on their sides or backs (this is monitored visually).

Role of ethanol metabolism

The first step of ethanol metabolism involves ethanol oxidation to acetaldehyde catalyzed by alcohol dehydrogenase (Adh). To determine whether ethanol metabolism influences locomotor activity in the time frame of our assay, we tested mutants lacking functional Adh. *Adh* mutants had a normal startle response and then, after a brief quiescence, entered into a period of hyperactivity (Fig. 3*A*). The hyperactive phase was, however, lower in magnitude and subsided more rapidly in *Adh* mutants than in controls. To determine whether accelerated ethanol accumulation in *Adh* mutants might explain the altered behavior, we measured ethanol levels in flies at various times after ethanol exposure. The amount of ethanol accumulated was higher in *Adh* mutants, even at the earliest time assayed, 5 min after the start of the exposure (Fig. 3*B*). This suggests that *Adh* mutant flies transition to the sedative phase more quickly than controls, which is reflected as a reduction in the hyperactive phase. Consistent with an early onset of sedation is the finding that the proportion of akinetic flies was substantially higher in *Adh* mutants than in controls (Fig. 3*C*). Currently, we are unable to determine whether *Adh* mutants also decrease hyperactivity through a sedation-independent mechanism, because the early phases of sedation (such as the onset of uncoordinated behavior) are not well defined in our assay. Thus, ethanol pharmacokinetics regulate the degree of hyperactivity.

Sensory inputs regulating ethanol-induced locomotion

The immediate and transient hyperactive phase, or startle, occurred before detectable ethanol accumulation in the flies (Fig. 2*D*). *Drosophila* sense volatile odors via bilaterally paired antennae and maxillary palps, and sense humidity and sound via aristae located on the third antennal segment (Sayeed and Benzer, 1996; de Bruyne et al., 2001). Removal of the third segment of one antenna reduced ethanol-induced startle (unilateral antennectomy; this also removes arista), and removal of both antennal third segments completely ablated startle (bilateral antennectomy) (Fig. 4*A*) (W.C., F.W.W., and U.H., unpublished observations). Neither removal of both aristae nor both maxillary palps had an effect (Fig. 4*B*). Thus, the startle response appears to be a behavioral response to the smell of ethanol, which is sensed by the antennae. Importantly, all groups of operated flies developed a relatively normal hyperactive response to ethanol (Fig. 4*A*,*B*), indicating that this phase of hyperactivity does not rely solely on sensory input from the antennae, aristae, or maxillary palps. Interestingly, bilateral antennectomy caused a precocious onset of the hyperactive phase (Fig. 4*A*). This suggests that a process of attenuation of the olfactory-mediated startle response that precedes the hyperactive phase negatively regulates locomotion and thus the start of this phase of hyperactivity. Competition between the attenuation-mediated brake on locomotion and the direct activating effects of ethanol in the CNS would then shape the kinetics of hyperactivity onset. Alternatively, olfactory-mediated startle and locomotor activity suppression could be independent processes, both requiring intact antennae.

The experiments described above suggested that ethanol-induced hyperactivity could be achieved in the absence of an olfactory startle response; the kinetics of hyperactivity onset were, however, abnormal. We therefore used additional manipulations to determine whether startle and hyperactivity could be dissociated. First, we took advantage of the finding that startle magnitude diminishes over time if flies are left undisturbed (and without food) in the humidified exposure chamber. Decay of startle magnitude is detectable after 60 min in a stream of humidified air, and this decay progresses relatively linearly toward a baseline response within a few hours (W.C., F.W.W., and U.H., unpublished observations). When flies that were acclimated to the exposure chamber for 8 hr were exposed to ethanol vapor, they developed a negligible startle (Fig. 4*C*). However, these acclimated flies reacted with a robust hyperactive phase that was qualitatively similar to nonacclimated flies of the same genotype. Second, the startle response can be rapidly habituated by exposing flies to four short bursts of ethanol separated by rests in humidified air (W.C., F.W.W., and U.H., unpublished observations). Startle-habituated flies also developed a normal hyperactive response (data not shown). Curiously, the chamber-acclimated flies (Fig. 4*C*) did not show the precocious onset of hyperactivity seen with antennectomized flies, suggesting that these manipulations, although able to dissociate the two phases of locomotor activity, do so by different mechanisms. In summary, we show that startle and hyperactivity are separate locomotor responses that likely define two input pathways to the motor output circuitry. The magnitude of the startle response, then, is a measure of the ability of flies to process external stimuli and to mount an appropriate locomotor response. On the other hand, the hyperactive phase is likely a locomotor response to the direct action of ethanol in the fly's nervous system. We note, however, that both methods used to eliminate the olfactory startle also reduced, although not substantially, the maximal locomotor activity achieved during the hyperactive phase, suggesting a modulation of this phase by olfactory inputs.

Together, the data discussed thus far show that ethanol vapor evokes two phases of increased locomotor activity in wild-type *Drosophila*: an olfactory-mediated startle response is followed by a more prolonged hyperactive phase. Olfactory input and ethanol dose influence the kinetics of onset of hyperactivity, and ethanol dose, ethanol metabolism, and onset of sedation influence the decrease in locomotion after peak hyperactivity.

Locomotion in ethanol-sensitivity mutants

Mutants with altered sensitivity to ethanol have been isolated previously (Moore et al., 1998; Singh and Heberlein, 2000). Of these, *amnesiac* (*amn*) and *rutabaga (rut*) show similarly increased sensitivity to ethanol in the inebriometer, an apparatus that quantifies the effects of ethanol on postural control (Weber and Diggins, 1990). The *amn* gene encodes a putative neuropeptide with some similarity to vertebrate PACAP (pituitary adenylate cyclase-activating peptide) (Feany and Quinn, 1995) and the *rut*

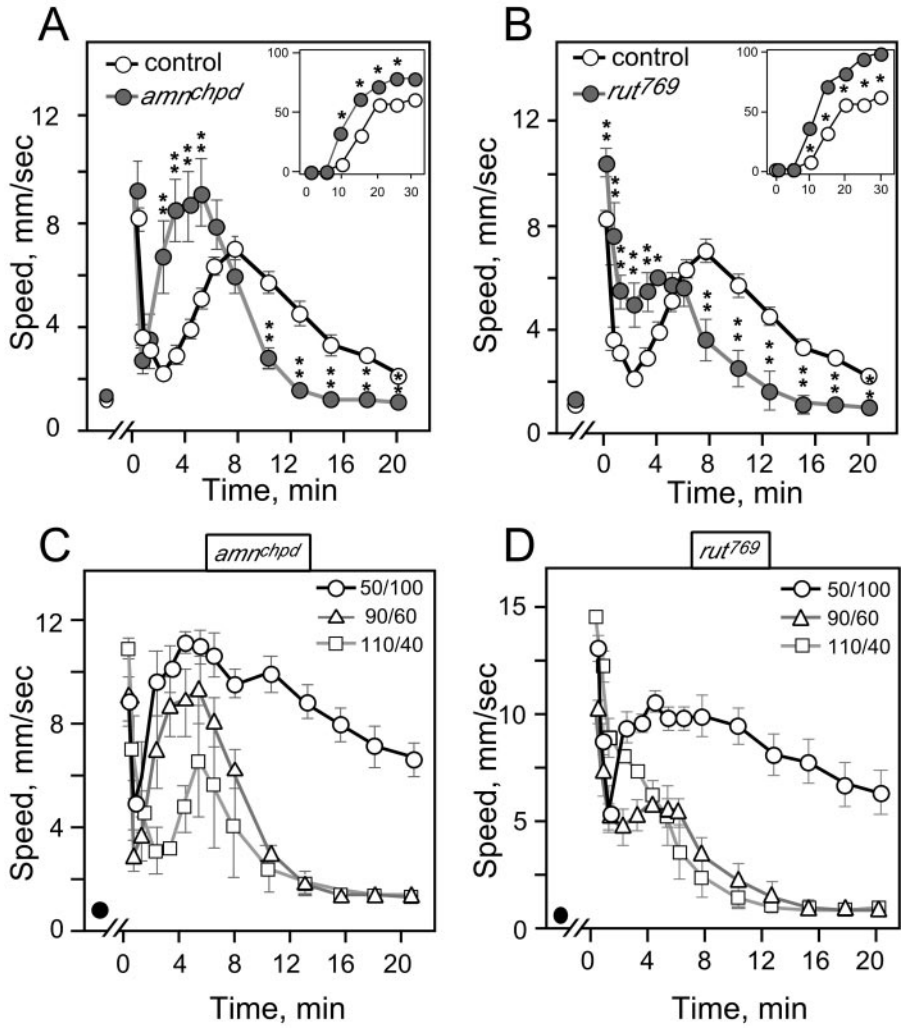

Figure 5. Ethanol sensitivity mutants have genotype-specific defects in ethanol-induced hyperactivity. *A*, *B*, Comparison of mutant ($n = 5$) with control ($n = 17$; data same as in Fig. 2*C*) flies at a moderate ethanol dose (E/A 90:60) in the booz-o-mat. *Insets* show the time course for akinesia in the same experiments: the number of immobile flies lying on their backs for at least 10 sec (expressed as percentage of total flies; *y*-axis) was counted at the given times (in minutes; *x*-axis). $^{*}p < 0.05$; $^{**}p < 0.01$. *C*, *D*, Ethanol sensitivity mutant dose responses. Low ethanol dose corresponds to E/A 50:100 (*circles*; $n = 7$), moderate dose to E/A 90:60 (*triangles*; $n = 5$), and high dose to E/A 110:40 (*squares*; $n = 3$). As for wild-type flies (Fig. 2*C*), lower ethanol doses stimulate locomotion, whereas higher doses are sedating. Baseline locomotor activity for each genotype (*circles* in the *bottom left corner* of each *panel*) was obtained after a 30 min acclimation period, a time when locomotor activity had stabilized.

gene a calcium/calmodulin-sensitive adenylyl cyclase (Livingstone et al., 1984; Levin et al., 1992). Because activation of PACAP receptors increases cAMP levels (for review, see Vaudry et al., 2000), *amn* and *rut* mutants are both expected to impair cAMP signaling. To ask how the effect of ethanol on postural control relates to its effect on locomotion, we analyzed the mutants in the locomotor tracking system. amn^{chpd} contains a P-element insertion in the *amn* coding region (Moore et al., 1998), and rut^{769} contains a P-element in the regulatory region of the *rut* gene (Levin et al., 1992).

Both ethanol-sensitive mutants tested displayed altered ethanol-induced locomotor behaviors (Fig. 5). amn^{chpd} flies developed a normal startle response, suggesting that these flies can react normally to the smell of ethanol (Fig. 5*A*). Like control flies, amn^{chpd} slowed briefly before entering into a sustained period of hyperactivity. These flies, however, reached maximal hyperactivity sooner and were somewhat more hyperactive (maximal speed of 9.0 ± 0.4 mm/sec achieved by amn^{chpd} at 5 min vs 6.8 ± 0.4 mm/sec at 7.5 min for control) and sedated more quickly than controls. rut^{769} flies showed a significantly stronger startle response to ethanol vapor than control flies at all doses tested (13.2 ± 0.6 mm/sec for rut^{769} vs 8.0 ± 0.5 mm/sec for control at E/A 50:100; $p < 0.0001$) (Fig. 5*B*,*D*). Whether this is caused by increased olfactory acuity or a more easily activated motor response is not known. The locomotor activity profile of *rut* flies was similar to that of *amn* flies (Fig. 5*B*); both mutants showed precocious hyperactivity and sedation. The maximal locomotor speeds achieved by *rut* flies were, however, generally lower than those seen with *amn* (5.5 ± 0.6 mm/sec for rut^{769} vs 9.0 ± 1.4 mm/sec for amn^{chpd} at 5 min exposure; $p = 0.004$). At least part of the decline in locomotor activity of *amn* and *rut* flies is attributable to sedation, because both genotypes showed increased akinesia relative to controls from 10 min onward at E/A 90:60 (Fig. 5*A*,*B*, *insets*). At 25–30 min, however, rut^{769} flies were significantly more akinetic than amn^{chpd} flies (97.3 ± 1.7% akinetic for rut^{769} vs 69.0 ± 8.2% for amn^{chpd} at 30 min; $p = 0.008$).

At low ethanol doses (E/A 50:100), both amn^{chpd} and rut^{769} had locomotor tracking profiles that resembled controls (compare Figs. 2*C*, 5*C*,*D*). At higher doses, each strain showed distinct profiles (Fig. 5*A*,*B*), suggesting that the mutants were not simply shifted in their ability to respond to a particular ethanol dose. Because ethanol absorption and metabolism are normal in *amn* and *rut* flies (Moore et al., 1998), we conclude that these mutants have increased sensitivity to both the stimulant and sedative effects of ethanol.

Locomotor activity bout structure

We next turned our attention to a more detailed analysis of *Drosophila* locomotor behavior. It has been reported previously that mice alternate between short periods of activity and inactiv-

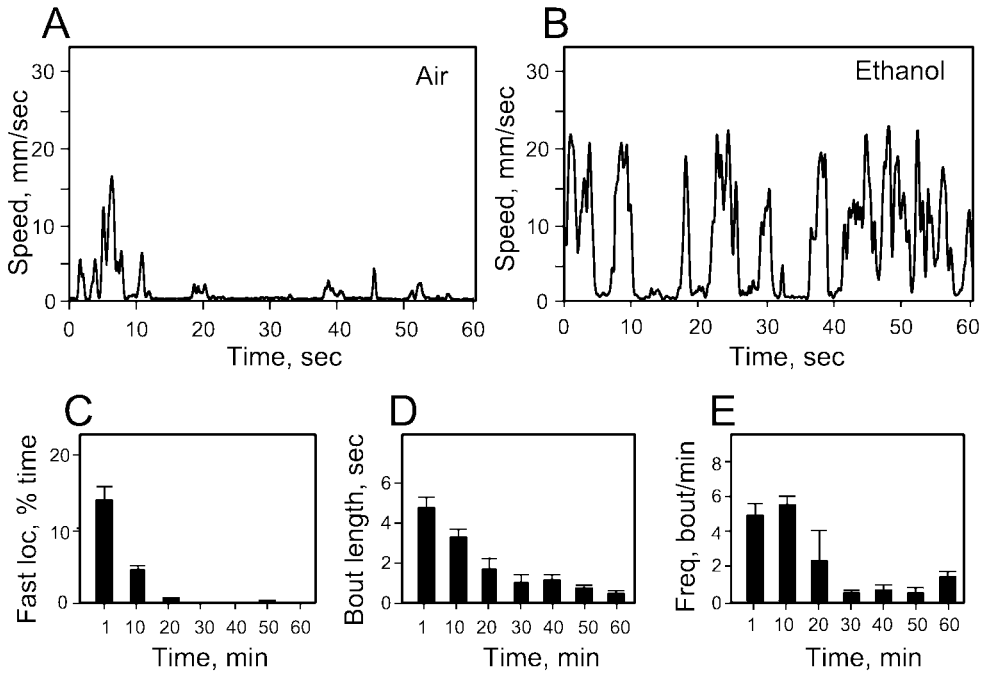

Figure 6. Patterns of locomotor activity. Representative traces of the speed of a single fly over 1 min exposure to humidified air (*A*) and ethanol (*B*) (E/A 30:35 in the square chamber). Fly speed was sampled 10 times per second over the course of 1 min. *C–E*, Measures of activity bout structure as flies acclimate to their environment in a stream of humidified air. Percentage of time spent moving at speeds >20 mm/sec (*C*), activity bout length (*D*), and activity bout frequency (*E*). For each measure, data sampled at 10 fps was averaged across a 1 min time interval for 20 flies ($n = 3$; E/A 0:65 in the square chamber).

ity and that activity is increased in response to ethanol (Smoothy and Berry, 1985). To determine whether flies behave similarly, we analyzed the locomotor speed of individual flies at 100 msec intervals instead of analyzing the average speed of the population. As shown in Figure 6*A*, flies moved in bouts; short periods of activity were separated by periods of inactivity. Fly locomotor behavior could therefore be described by specific parameters, such as bout frequency, bout length, and the time spent walking at high speed, three parameters that we cumulatively term activity bout structure. We then developed algorithms to extract these parameters from frame-to-frame positional data (see Materials and Methods). Based on measures of basal activity, we categorized flies as active if they moved faster than 1 mm/sec for at least 300 msec. The frequency and length of activity periods were then derived from this definition of activity. Because of the dramatic effect of ethanol on average locomotor speed (see above) we defined a parameter of "fast locomotion," which corresponds to the fraction of time that flies spent moving faster than 20 mm/sec. We chose this threshold because nonstimulated flies rarely move faster than 20 mm/sec (Fig. 7), although they can achieve peak locomotor speeds of up to 35 mm/sec (Strauss and Heisenberg, 1993) (our unpublished data). Bout frequency is dependent on activity bout length and the amount of time between activity bouts.

We first determined activity bout structure as flies acclimated to the exposure chamber (Fig. 6*C–E*). Spontaneous locomotor activity during acclimation to a novel environment has been documented previously in *Drosophila*. For example, when individual flies are placed in narrow rectangular chambers, they maintain a moderate level of activity that decays to a low and stable level in ~2 hr (Martin et al., 1999). In our assay, 1 min after being placed in the exposure chamber, the average bout length was 4.7 ± 0.5 sec, bout frequency was 5.0 ± 0.5 bouts/min, and flies moved faster than 20 mm/sec 13.9 ± 1.7% of the time. By 30 min of acclimation in a constant stream of humidified air, flies were substantially calmer; bout length was reduced to 0.9 ± 0.4 sec, frequency was reduced to 0.6 ± 0.2 bouts/min, and flies only exhibited fast locomotion 0.04 ± 0.04% of the time. Thus, as flies gradually adapted to their new environment, or recovered from the stimulation caused by their introduction into the chamber, they moved more slowly, less frequently, and for shorter periods of time. After reaching this state, flies showed very little spontaneous locomotion; this may be attributable, in part, to the low flow of humidified air circulating through the chamber under baseline conditions, which we found to have a calming effect on flies (data not shown).

Effect of ethanol on activity bout structure

We next determined how activity bout structure was altered by ethanol exposure. As shown in Figure 7*A–C*, flies showed an immediate increase in all three parameters of bout structure during ethanol exposure. After the olfactory startle (minute 0, corresponding to the first minute of exposure), flies transiently decreased fast locomotion and activity bout length but maintained activity bout frequency. As hyperactivity developed, fast locomotion increased from 1.0 ± 0.4% at 2 min to a peak of 5.3 ± 1.1% after 7 min of exposure to a moderate ethanol dose. Concurrently, flies increased the average length of activity bouts from 1.8 ± 0.2 sec at 2 min to a peak of 5.4 ± 0.6 sec at 12 min of exposure. Bout frequency did not change appreciably throughout the ethanol exposure. Thus, the development of ethanol-induced hyperactivity as measured by population average velocity was the combined result of more time spent moving fast and longer periods of movement. Interestingly, increases in locomotor velocity appeared to contribute more to hyperactivity early, and increases in bout length continued to contribute at later exposure times. As flies began to sedate, they reduced fast locomotion. Surprisingly, little if any change in bout length or bout frequency was observed during this phase. Thus, in these early stages of sedation, reduced population average velocity was primarily attributable to a reduction in fast locomotion.

Activity bout structure in ethanol sensitivity mutants

Although startle (first minute of exposure) was normal, the activity bout structure of *amn* flies was dramatically different from

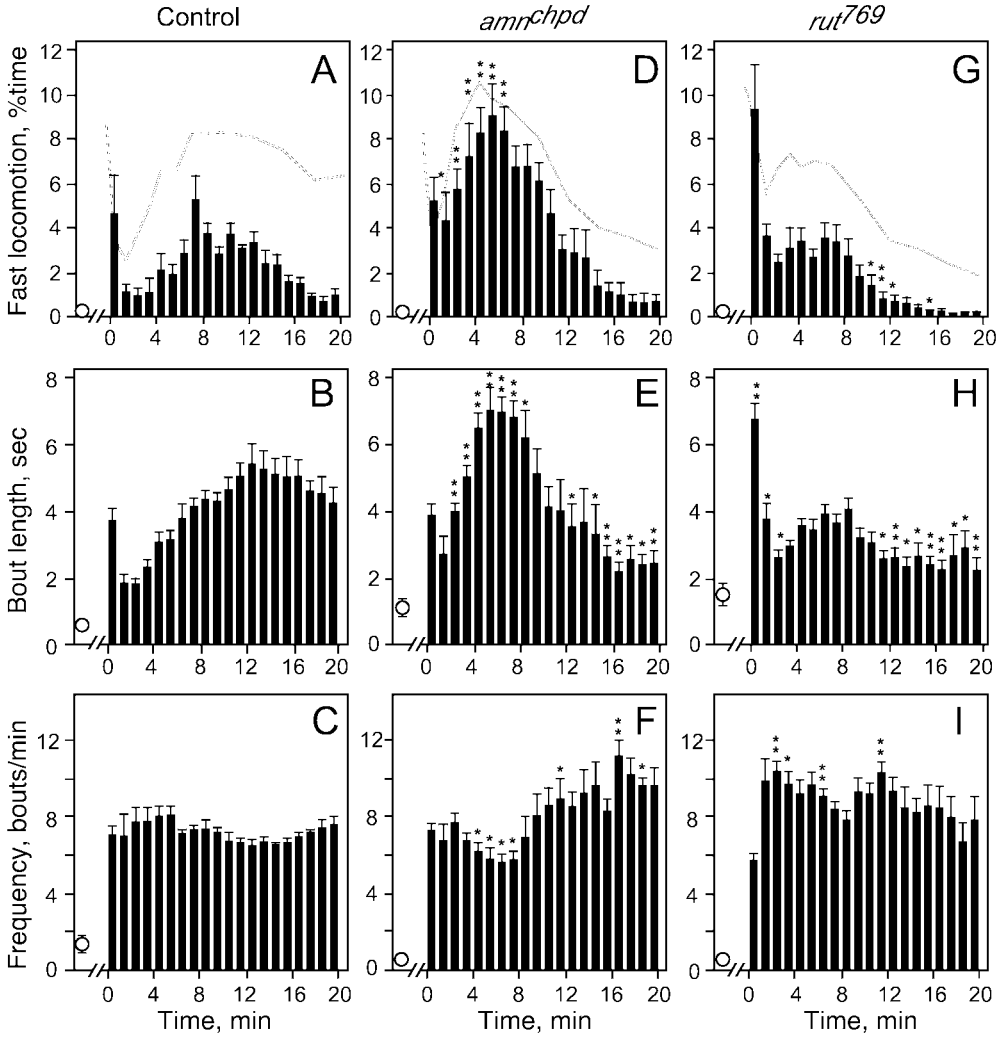

Figure 7. Locomotor activity patterns of control flies and of ethanol-sensitive mutants during ethanol exposure. Time spent moving at speeds >20 mm/sec (fast locomotion; *top row*), activity bout length (*middle row*), and activity bout frequency (*bottom row*). For each measure of locomotor pattern, data sampled at 10 fps was averaged across 1 min time intervals for populations of 20 flies (E/A 30:35; $n = 8$). Superimposed on the fast locomotion panels are the population average locomotor velocities for each genotype derived from the same dataset. The activity bout structure for flies acclimated to the exposure chamber in a stream of humidified air for 50 min is indicated by an *open circle* to the *left* of the ethanol exposure data ($n = 3$). *A–C*, Control strain PZ-control. *D–F*, amn^{chpd}. *G–I*, rut^{769}. For control flies, exposure to ethanol resulted in significant increases over baseline for all three measures ($p < 0.0001$ compared with the first minute of ethanol exposure for each measure). During continuous ethanol exposure, fast locomotion and activity bout length changed significantly over time ($p < 0.0001$), whereas bout frequency did not ($p = 0.46$). *Asterisks* in *D–F* correspond to comparisons of control versus amn^{chpd}, and *asterisks* in *G–I* correspond to comparisons of control versus rut^{769} ($^{*}p < 0.05$; $^{**}p < 0.01$).

controls at later exposure times (Fig. 7*D–F*). The strong, early hyperactivity of amn^{chpd} mutants was temporally coincident with a marked increase in both fast locomotion (8.4 ± 1.1% for amn^{chpd} vs 2.9 ± 0.7% for control at 6 min; $p < 0.001$) and activity bout length (6.9 ± 0.5 sec for amn^{chpd} vs 3.8 ± 0.5 sec for control at 6 min; $p < 0.001$). During this period of hyperactivity, the frequency of activity bouts of amn^{chpd} flies decreased slightly (5.6 ± 0.5 bouts/min for amn^{chpd} vs 7.2 ± 0.2 bouts/min for control flies; $p = 0.01$). Thus, amn^{chpd} flies initiated activity bouts less often than controls but stayed active longer and spent more time moving fast. Interestingly, as amn^{chpd} flies began to sedate, bout frequency increased, from 5.6 ± 0.5 to 10.2 ± 0.9 bouts/min ($p < 0.01$). Additionally, as amn^{chpd} flies sedated, their bout length was shorter than that of controls (2.4 ± 0.4 sec for amn^{chpd} vs 4.3 ± 0.5 sec for controls; $p < 0.01$). Thus, in addition to reacting strongly to the stimulant effects of ethanol, amn^{chpd} flies may also have an altered ability to terminate periods of locomotor activity.

Compared with controls, the increased startle of rut^{769} flies was composed of increased fast locomotion (9.3 ± 2.0% for rut^{769} vs 4.7 ± 1.8% for control during the first min; $p < 0.0001$) and activity bout length (6.7 ± 0.5 sec for rut^{769} vs 3.7 ± 0.4 sec for control for the first minute; $p < 0.0001$) (Fig. 7*G*,*H*); bout frequency was, however, only weakly increased (Fig. 7*I*). The precocious onset of activity and sedation in rut^{769} correlated well with the degree of fast locomotion (Fig. 7*G*); activity bout length and bout frequency of *rut* flies remained relatively constant throughout the exposure (Fig. 7*H*,*I*). Sedation was composed of reduced fast locomotion (0.2 ± 0.1% for rut^{769} vs 1.6 ± 0.3% for control at 15 min; $p = 0.02$) and reduced activity bout length (2.4 ± 0.3 sec for rut^{769} vs 5.0 ± 0.7 sec for control at 15 min; $p = 0.02$) but normal bout frequency.

Interestingly, these two ethanol-sensitive mutants had both differences and similarities in ethanol-induced bout structure changes. The most striking differences between amn^{chpd} and rut^{769} were seen in fast locomotion and activity bout length from 2 to 8 min of exposure. During this period, amn^{chpd} flies moved at >20 mm/sec more often (9.0 ± 1.5% for amn^{chpd} vs 2.7 ± 0.4% for rut^{769} at 5 min; $p < 0.001$) and had a greatly increased bout length (7.0 ± 0.7 sec for amn^{chpd} vs 3.4 ± 0.3 sec for rut^{769} at 5 min; $p < 0.001$). During this same period, rut^{769} flies had an increased bout frequency relative to amn^{chpd} (9.7 ± 0.7 bouts/min for rut^{769} vs 5.8 ± 0.6 bouts/min for amn^{chpd} at 5 min; $p < 0.001$). Thus, by all three measures, rut^{769} differed from amn^{chpd} in response to ethanol during the hyperactive phase. In contrast, as hyperactivity subsided, amn^{chpd} and rut^{769} had a similar reduction in activity bout length relative to control flies (2.4 ± 0.3 sec rut^{769} vs 2.6 ± 0.3 sec amn^{chpd} at 15 min; $p = 0.69$). These data suggest that, although qualitatively similar, *amn* and *rut* have fundamentally different locomotor responses to ethanol.

DISCUSSION

We describe a high-resolution analysis of *Drosophila* locomotor behavior in the absence and presence of ethanol. Using a population-based assay that quantifies locomotion in an automated manner, we show that ethanol has complex effects on locomotion: an initial olfactory startle response is followed by a more sustained period of hyperactivity that in turn gives way to sedation. Additionally, we discovered that flies normally walk in short bouts of locomotor activity. Analysis of the structure of these activity bouts revealed that ethanol exerts its effects by dynamically altering several parameters of walking behavior during the course of exposure. Ethanol stimulation of locomotion coincided with an increase in fast locomotion and an increase in the length of activity periods. The sedative effects of ethanol seemed to be mediated, at least in the early phases, by a reduction in locomotor speed without changes in activity bout length or frequency. Importantly, we show that both overall locomotor activity levels and the underlying bout structure of locomotion are affected by two previously identified mutations with altered sensitivity to the effect of ethanol on postural control.

Assays for measuring the effect of ethanol on locomotion

Ethanol-induced locomotor stimulation in *Drosophila* has been reported previously. In a single fly line-crossing assay, a time course of hyperactivity similar to that described here was observed (Bainton et al., 2000; Singh and Heberlein, 2000). Comparable results were also obtained in the inebri-actometer, a device that measures single fly activity (Parr et al., 2001). In this apparatus, ethanol vapor is delivered to an array of small tubes, each containing one fly. The number of times each fly crosses an infrared beam located at the center of the tube is measured. The assay described here differs in several ways. First, we measured the simultaneous activity of 20 or more genetically identical flies in a single chamber. Whereas the average behavior of multiple individual flies does not differ from that of a population of flies, the locomotor behavior of any one single fly is significantly more variable than that of a group (data not shown). Second, video tracking allowed for direct and continuous measurement of fly position and thus walking speed; this is not possible in either line-crossing or beam-breaking assays. Finally, computer automation of the assay and the development of a multiple-chamber exposure device (see Materials and Methods) allowed for greatly increased throughput. These improved methods led us to discover previously unreported aspects of *Drosophila* behavior. Increased temporal and spatial resolution allowed the detection of the olfactory startle response and also allowed the detection and quantification of activity bout structure of freely moving flies (see below). Analysis of other parameters of locomotor behavior could be developed within the framework of our assay, including measurements of turning and orientation, fly position with respect to one another, and positional preference in the environment. It will be particularly interesting to determine how ethanol influences these behaviors.

Phases of ethanol-induced locomotor stimulation

Flies show a biphasic locomotor response to ethanol. An initial peak of hyperactivity is induced by the smell of ethanol, whereas a delayed hyperactive phase is caused primarily by rising internal ethanol levels, likely acting directly on the flies' nervous system. The timing and magnitude of the delayed hyperactive phase are influenced by the olfactory-mediated startle and by the onset of sedation. Two manipulations that ablate the startle, surgical removal of the major olfactory organs and environmental acclimation, do not block ethanol-induced hyperactivity. However, flies with bilateral antennectomy show a precocious onset of hyperactivity. We suspect that the acute olfactory stimulus of the startle leads to an adaptation or desensitization of olfactory input that is accompanied by a depression of locomotor-activating circuits. Alternatively, olfactory stimulus may inhibit locomotion.

After reaching its peak, locomotor activity declines gradually as ethanol levels rise, and its sedative effects begin to take effect. If ethanol metabolism is impaired as a result of mutations in *Adh*, then flies hyperactivate less and sedate sooner. This suggests that ethanol pharmacokinetics regulate the extent and duration of ethanol-induced hyperactivity.

Role of the cAMP pathway in locomotor stimulation by ethanol

The locomotor response to ethanol is altered by two mutations that affect cAMP signaling, amn^{chpd} and rut^{769}. Both mutants showed precocious ethanol-induced hyperactivity and premature sedation, suggesting that the products of these two genes normally temper the effects of ethanol during both the onset of hyperactivity and sedation. *amn* and *rut* mutants are known to have defects in behavioral plasticity: both were originally isolated as mutants with olfactory learning and memory defects (Quinn et al., 1979; Aceves et al., 1983) and subsequently shown to have defects in some forms of habituation (Duerr and Quinn, 1982; Engel and Wu, 1996). It is therefore possible that the early onset of hyperactivity in these mutants is attributable to a defect in adaptation to the olfactory stimulus. Similarly, during the course of continuous ethanol exposure, amn^{chpd} and rut^{769} may fail to adapt to the disruptive effects of ethanol on locomotor circuit function and therefore sedate sooner.

In addition to these similarities, *amn* and *rut* mutants also displayed differences in their locomotor responses to ethanol. rut^{769} startled more strongly than amn^{chpd}. However, the magnitude of hyperactivity, measured as population average velocity, as well as bout length and fast locomotion, were strikingly reduced in rut^{769} compared with amn^{chpd}. Given the central role of cAMP signaling, it is likely that these genes have some nonoverlapping roles in locomotor hyperactivity. Interestingly, although both mutants displayed precocious hyperactivity, rut^{769} flies showed an increased frequency and length of activity bouts, whereas amn^{chpd} flies increased fast locomotion and the length of activity bouts. Thus, these two mutations may also impinge on locomotor output differently during hyperactivity onset.

Several genes involved in cAMP signaling have been shown to regulate locomotion in flies. Mutations in the protein kinase A catalytic subunit (*pka-C1*) or its type II regulatory subunit (*pka-RII*) disrupt circadian changes in spontaneous locomotion (Majercak et al., 1997; Park et al., 2000). *rut*, *amn*, and *pka-C1* mutants, as well as flies expressing ubiquitously a PKA inhibitory transgene, are sensitive to ethanol-induced loss of postural control (Moore et al., 1998; Rodan et al., 2002), whereas *pka-RII* mutants are resistant to ethanol-induced sedation (Park et al., 2000). Normal function of the cAMP pathway is also involved in the control of locomotion and its regulation by ethanol in mice. For example, mice lacking the PKA-RIIβ subunit have defects in spontaneous motor activity and in responses to ethanol (Brandon et al., 1998; Thiele et al., 2000). In addition, mice lacking one copy of the gene encoding $G\alpha_s$, or expressing a PKA inhibitory transgene in the forebrain, display increased sensitivity to the sedative

effects of ethanol (Wand et al., 2001). The consequences of these genetic manipulations on the stimulant effects of ethanol have, to our knowledge, not been reported.

Bout structure of locomotion

What motivates flies to start and stop moving in our assay is not clear. It is possible that visual perception of objects in the environment, including other flies and the walls of the exposure chamber, could contribute to the fine grain periodicity of fly locomotion. Olfactory or tactile cues may also stimulate or suppress locomotion. Alternatively, locomotor periodicity may be an innate behavioral rhythm that is independent of sensory input, perhaps serving to increase exploration of the environment (Smoothy and Berry, 1985). We have not yet attempted to separate these potential influences on activity bout structure. Episodic locomotor behavior has been documented in many organisms, including nematodes and rodents (Pierce-Shimomura et al., 1999; Waggoner et al., 2000). Rats or mice placed in a novel environment alternate between forward locomotion and observational behavior (Drai et al., 2000). Interestingly, mice given an injection of a low dose of ethanol and placed in an open arena have been reported to increase locomotion in bouts of activity (Smoothy and Berry, 1985). Similar to data reported here, ethanol-stimulated locomotion was attributable to increases in the length and magnitude of activity bouts. At the same time, the mice showed increased periods of immobility, a behavior not observed in flies (data not shown). Thus, mice and *Drosophila* show changes in locomotor behavior during ethanol exposure that are similar even when assayed at this high level of resolution. Short periods of locomotor inactivity might provide opportunity for animals to assess their local environment during exploratory behavior, such as during the search for food, mates, or shelter.

Including the analysis described here, there are now three known layers of temporal organization for locomotor behavior in *Drosophila*. First, locomotor activity levels are under circadian regulation, with peaks of activity, each lasting several hours, observed at dawn and dusk (for review, see Hall, 1990). Second, flies placed into a new environment show moderate levels of spontaneous locomotor activity, which was found to occur in bouts with an average length of just under 3 min (Martin et al., 1999). Finally, we show here that fly locomotion consists of even shorter activity bouts, lasting just a few seconds. Although it is reasonable to assume that the activity bouts described here are components of higher-order locomotor behaviors, how these different layers of regulation of locomotion relate to each other remains to be determined.

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