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Functional Ethanol Tolerance in *Drosophila*

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Summary

In humans, repeated alcohol consumption leads to the development of tolerance, manifested as a reduced physiological and behavioral response to a particular dose of alcohol. Here we show that adult *Drosophila* develop tolerance to the sedating and motor-impairing effects of ethanol with kinetics of acquisition and dissipation that mimic those seen in mammals. Importantly, this tolerance is not caused by changes in ethanol absorption or metabolism. Rather, the development of tolerance requires the functional and structural integrity of specific central brain regions. Mutants unable to synthesize the catecholamine octopamine are also impaired in their ability to develop tolerance. Taken together, these data show that *Drosophila* is a suitable model system in which to study the molecular and neuroanatomical bases of ethanol tolerance.

Introduction

Low doses of ethanol in naïve individuals lead to intoxication, with pleasurable effects such as euphoria and loss of social inhibitions, as well as aversive effects such as loss of motor coordination, dehydration, and sedation (reviewed in Fadda and Rossetti, 1998). However, chronic exposure to high ethanol doses is toxic, leading to severe and irreversible damage to the brain, liver, and kidneys. Alcohol abuse is facilitated by the development of tolerance, most simply defined as an acquired resistance to the effects of the drug. In humans, tolerance is thought to develop rapidly to the aversive effects of ethanol, and to a lesser extent to its pleasurable properties. This initial imbalance between aversive and rewarding effects has been suggested to encourage increased intake, which over time leads to the development of physical dependence and possibly addiction (reviewed in Tabakoff et al., 1986).

Rodents have proven to be good animal models in which to study alcohol tolerance and dependence (reviewed in Crabbe et al., 1994). As in humans, ethanol in rodents induces locomotor abnormalities, hypothermia, hypnosis, and eventually death (Lê and Mayer, 1996). Different forms of tolerance can be induced with protocols that vary the dose and/or frequency of ethanol administration. Tolerance that develops after a single short-term exposure is referred to as rapid tolerance, whereas tolerance measured after repeated or sustained exposures is referred to as chronic tolerance. The physiological bases for tolerance include changes in the disposition of ethanol, such as absorption, excretion, or metabolism (metabolic or pharmacokinetic tolerance), and the development of resistance to ethanol at the cellular level (functional or pharmacodynamic tolerance) (Kalant et al., 1971; Tabakoff et al., 1986). Functional tolerance is at least in part achieved by adaptive changes in the central nervous system (CNS) that may bear similarities to the mechanisms underlying learning and memory (reviewed in Cunningham et al., 1984; Fadda and Rossetti, 1998). Consistent with this hypothesis, administration of neuropeptides and neurotrophins that affect neuronal plasticity can accelerate the acquisition and slow down the dissipation of tolerance in mice (Crabbe et al., 1980; Lê et al., 1982; Szabó et al., 1988; Hoffman, 1994; Szabó and Hoffman, 1995). Selective breeding experiments in mice have shown that tolerance to some of the effects of ethanol can be attributed to genetic factors (Erwin and Deitrich, 1996; Gallaher et al., 1996). However, to our knowledge, no single gene has yet been associated with reduced or increased tolerance to ethanol.

Our laboratory uses *Drosophila melanogaster*, with its accessibility to genetic and molecular analysis, as a model organism in which to dissect the molecular mechanisms underlying various responses to ethanol. We reported previously that exposure to ethanol induces behaviors in flies that are quite similar to those observed in mammalian models and in humans. Furthermore, we have shown that responses to acute ethanol exposure are amenable to pharmacological and genetic manipulations (Moore et al., 1998; Bainton et al., 2000; Singh and Heberlein, 2000). Here we extend these studies to the analysis of acquired resistance, or tolerance. We show that *Drosophila* develop tolerance to the effects of ethanol on motor coordination and sedation after single and multiple exposures to ethanol. This tolerance is functional, as it cannot be accounted for by changes in ethanol absorption and/or metabolism, but relies on the functional and structural integrity of specific central brain regions, and it requires the catecholamine octopamine. These studies provide a foundation for the identification of genes mediating tolerance development.

Results

Drosophila Develop Tolerance after a Single Ethanol Exposure

Upon exposure to ethanol vapor, adult *Drosophila* become hyperactive, uncoordinated, and eventually sedated (Bainton et al., 2000; Singh and Heberlein, 2000). The sensitivity of a population of flies to ethanol can be measured using the inebriometer, an apparatus that quantifies ethanol-induced loss of postural control (Cohan and Hoffman, 1986; Weber, 1988). Briefly, flies are introduced into the top of a 4-foot glass column through

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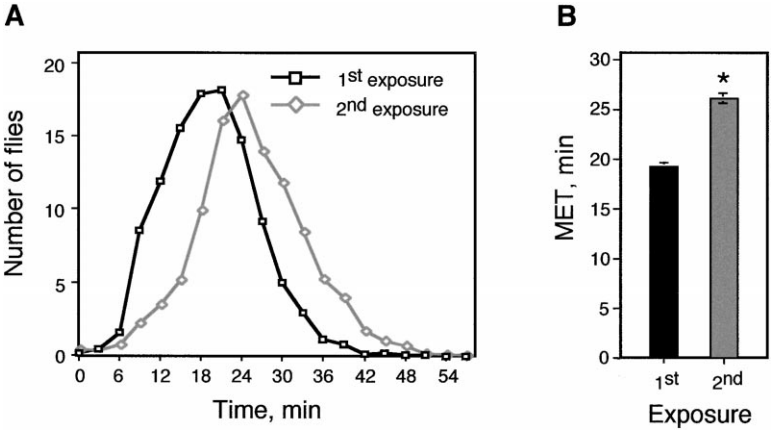

Figure 1. Ethanol Tolerance Measured in the Inebriometer

The sensitivity to ethanol vapor of a population of ~100 flies was measured in the inebriometer, a column that separates flies based on their ability to maintain postural control. At our standard ethanol concentration, wild-type flies "eluted" with a normal distribution (A) and a mean elution time (MET) of 19.5 ± 0.4 min (B). When these flies were reintroduced into the inebriometer 4 hr after the first exposure, their elution profile was also normal but shifted to the right (A): their new MET was 26.2 ± 0.6 min, which corresponds to a 34.2% ± 3.3% increase in resistance. A typical experiment is shown in (A). Error bars represent the standard error of the mean (SEM). n = 19, *p < 0.0001. In all figures, n corresponds to the number of experiments, not the number of flies.

which ethanol vapor of controlled concentration circulates. As they become intoxicated, flies progressively lose postural control and tumble downwards; their fall is retarded by their ability to cling to oblique mesh baffles distributed along the length of the column. At our standard ethanol concentration, wild-type flies reproducibly eluted from the inebriometer with a mean elution time (MET) of ~20 min (Figures 1A and 1B; Moore et al., 1998; Singh and Heberlein, 2000).

To determine if flies develop tolerance to the motor-impairing effects of ethanol, they were reintroduced into the inebriometer 4 hr after the first exposure. The 4 hr interval was chosen to allow flies to recover from the first exposure (see below). Flies were more resistant to the second exposure, displaying a MET of 26.2 min ± 0.6, an increase of nearly 7 min from the MET of their first exposure (19.5 min ± 0.4; Figure 1B). We define tolerance as the relative increase in MET between the first and the second exposure, which for wild-type flies corresponded to ~35% under our standard experimental conditions.

To obtain a more detailed account of ethanol-induced behaviors, we documented the locomotion patterns of naïve and tolerant flies using a locomotor tracking system. Groups of 20 flies were videotaped while exposed to ethanol vapor in a viewing chamber. Traces representing the locomotion of individual flies during a 10 s interval were generated using the Dynamic Image Analysis System (see Experimental Procedures). The effect of ethanol on the velocity of locomotion is shown in Figure 2A. Naïve flies showed an immediate and transient increase in walking velocity, which was maximal 30 s after the start of ethanol exposure and subsided by about 2 min. This transient hyperactivity response, which occurs before an increase in brain ethanol levels was detectable, is mediated by chemosensory systems (Fred Wolf and U.H., unpublished data). A more sustained phase of increased walking activity was observed between 4 and 6 min; after that, flies became gradually less active, reaching complete immobility after 10–12 min of ethanol exposure. The locomotor behavior of the same flies when reexposed to ethanol 4 hr later showed several significant differences from the first exposure (Figure 2A). Whereas the initial and transient hyperactivity was unchanged, walking speed was increased and sedation was delayed. The latter pattern of locomotor behavior is very similar to that displayed by naïve flies exposed to a lower ethanol dose (data not shown). The locomotor traces of naïve and tolerant flies at various times prior to and during ethanol exposure are shown in Figure 2B. The most obvious differences were seen after 7.5 and 12.5 min of ethanol exposure: at these times, most naïve flies were completely sedated, whereas the majority of tolerant flies were still quite mobile. These locomotor tracking experiments strongly suggest that ethanol tolerance, defined by an increased MET in the inebriometer, is caused primarily by a delay in sedation.

Development of Tolerance Is Dose Dependent

To determine the relationship between ethanol dose and tolerance, we exposed flies for defined periods of time in exposure tubes (see Experimental Procedures) and assayed them in the inebriometer 4 hr later (Figure 3A). The amount of ethanol that flies absorbed in these experiments was estimated by measuring the ethanol concentration in fly extracts prepared after each exposure (Figure 3B). The degree of tolerance increased as a function of exposure time, reaching a maximum of nearly 70% at 40 min (Figure 3A), a time when the ethanol absorbed reached 40 mM (Figure 3B). With exposures of 40 min and beyond, however, a subset of flies began eluting in less than 6 min (Figure 3A, white bars), while the remainder showed a typical elution profile with normal distribution and a MET of ~32 min (Figure 3A, gray bars). The fast-eluting hypersensitive flies appeared not to have recovered from their first exposure, although they contained undetectable ethanol levels by the time they were tested in the inebriometer (data not shown). Dose-dependent development of tolerance was also achieved by exposing flies for a fixed time to ethanol vapor of different concentrations (see Experimental Procedures), confirming that the increase in ethanol tolerance described above (Figure 3A) is not a consequence of the length of time spend in the exposure tubes.

After a 20 min exposure, which corresponds to the MET of a typical first inebriometer "run," alcohol concentration in the flies was ~30 mM (Figure 3B). Ethanol at

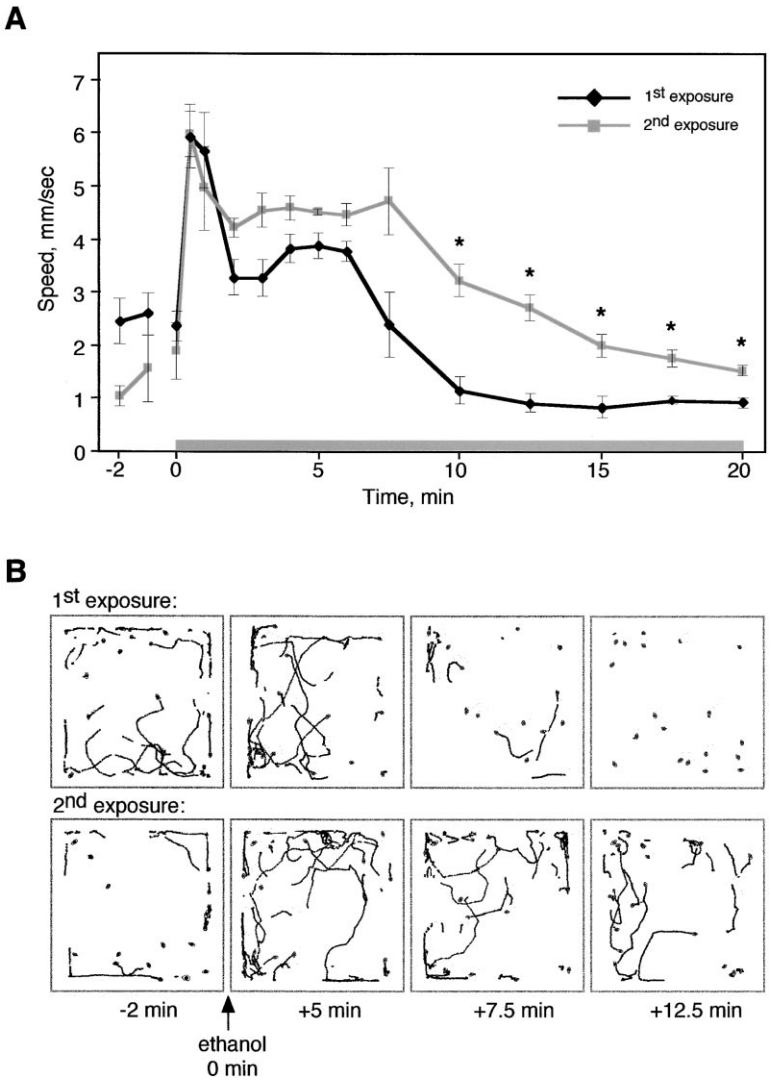

Figure 2. Ethanol Tolerance Measured in a Locomotor-Tracking System

(A) The average locomotor velocity of a population of 20 flies is shown as a function time; ethanol exposure is initiated at time 0. Compared to naïve flies (black trace), tolerant flies (gray trace) showed an increase in locomotion and a delay in sedation. The second exposure was initiated 4 hr after the start of the first. Asterisks correspond to time points with significant differences (*$p \leq 0.02$, $n = 3$).

(B) The locomotor traces of 20 flies corresponding to 10 s periods at the indicated times are shown. While all flies were completely sedated after 12.5 min of first exposure, most flies were still walking during the same time interval when tested 4 hr later.

Error bars in (A) are SEM. Gray horizontal bar in (A) indicates ethanol exposure period.

a similar concentration in the blood of naïve humans would cause severe intoxication (30 mM = 140 mg/dl or 0.14%). No lethality was observed at this dose, and flies recovered from sedation in about 10 min. The ethanol concentration that caused 50% lethality (LD50) was nearly five times higher (~140 mM = 650 mg/dl or 0.65%) and was achieved after an 85 min exposure (Figure 3B); again, this ethanol concentration is similar to that causing lethality in nonalcoholic humans (500 mg/dl). We conclude that standard inebriometer exposures (on average 20 min) lie within the linear range of the dose–response curve and should therefore allow the detection of increases or decreases in tolerance induced by genetic, behavioral, or pharmacological manipulations. Rapid tolerance has been defined in rodents as an increase in resistance to ethanol induced by a single, moderate-to-high ethanol dose and is measured after the ethanol from the initial exposure has been completely eliminated (Crabbe et al., 1979). Rodents develop rapid functional tolerance to the hypothermic, motor-impairing, and hypnotic effects of ethanol (reviewed in Lê and Mayer, 1996). Our results show that flies similarly develop rapid tolerance to the sedative and/or motor-impairing effects of ethanol.

Kinetics of Tolerance Development

To determine the kinetics of tolerance development and decay, we exposed flies in the inebriometer twice at various time intervals (Figure 4). Maximal tolerance, a 63% increase in MET, was achieved with a 2 hr interval. Tolerance decreased quickly, reaching ~40% after 4 hr. It then decayed more slowly and was still detectable 24 hr after the first exposure, but had disappeared by 36 hr (data not shown). The biphasic nature of the kinetics of tolerance dissipation suggests the involvement of two mechanistically different processes. Our attempts to demonstrate a role for de novo protein synthesis have so far been inconclusive; administration of inhibitors led to an increase in ethanol resistance, making an assessment of tolerance development problematic. For our standard tolerance experiments, the 4 hr interval was chosen over the 2 hr interval that induces maximal tolerance for the following reasons. First, we wanted to ensure that flies had completely eliminated all ethanol ab-

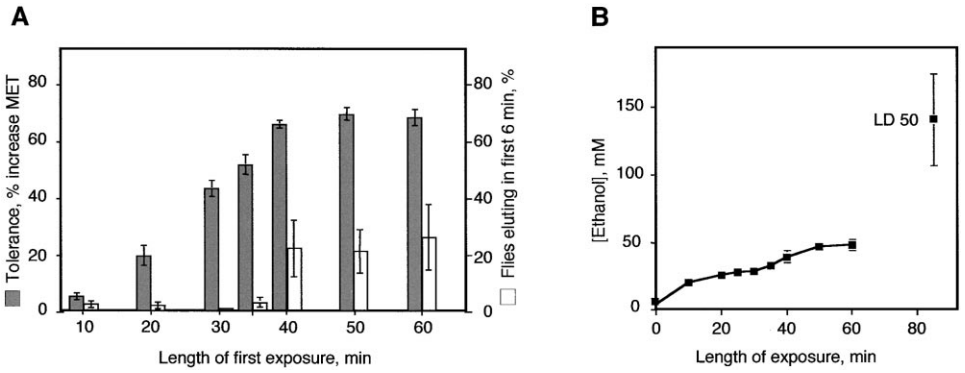

Figure 3. Dose–Response Curve

(A) Groups of ~100 flies were preexposed to ethanol vapor in perforated tubes for the indicated lengths of time and tested in the inebriometer 4 hr later. Tolerance was calculated with reference to the mean elution time (MET) of naïve flies tested on the same day. Tolerance (gray bars) gradually increased with longer preexposures, reaching a plateau by 40 min. With preexposure times of 40 min and higher, a substantial portion of the flies tested displayed hypersensitivity and eluted from the inebriometer in less than 6 min (open bars). They were not included in the MET calculation. These flies were not dead and eventually recovered. The basis of their hypersensitivity is unknown. n = 4–7.

(B) The concentration of absorbed ethanol was measured in whole fly extracts after exposure to ethanol vapor in perforated tubes for defined periods of time. An exposure of 20 min, corresponding to the MET of a typical first inebriometer exposure, led to the accumulation of 30 mM (± 5 mM) ethanol in the flies. The LD50 was reached with an 85 min exposure, which led to the accumulation of 140 ± 33 mM ethanol. n = 9, except for 50 and 60 min time points, where n = 6; error bars represent SEM.

sorbed and had had an opportunity to rehydrate and feed. Second, the intermediate level of tolerance observed after a 4 hr interval is dose dependent and more amenable to genetic analysis.

Tolerance Is Not Based on Pharmacokinetic Alterations

A possible mechanism for the development of tolerance is metabolic adaptation. As in mammals, the first step of ethanol metabolism in flies involves its oxidation to acetaldehyde by the enzyme alcohol dehydrogenase (Adh). Adh expression or activity could be increased as a result of the first exposure, leading to slower accumulation of ethanol, and the need for a longer second exposure to reach the same intoxicating concentration. Alternatively, the rate of ethanol absorption could have decreased between first and second exposure. If such pharmacokinetic alterations were solely responsible for the tolerance we observe, the fly alcohol content leading to loss of postural control should be the same in the first and second exposure. To address this issue, we compared the ethanol content of naïve and tolerant flies under conditions that induce loss of postural control.

Flies were exposed to ethanol vapor in tubes for 20 min, the typical MET of a first inebriometer exposure, and reexposed 4 hr later for 27 min, the typical MET of a second inebriometer exposure (see Figure 1). Extracts were prepared at various times during this procedure to estimate alcohol content. Naïve flies lost postural control after the first 20 min of exposure, at which time they had absorbed alcohol to a final concentration of 32 mM (Figure 5). All ethanol was eliminated by the end of a typical "recovery" period (3 hr, 40 min). After 20 min of second exposure, the alcohol content was comparable (31 mM); however, flies had not yet lost postural control. The latter occurred after 27 min of exposure; by this time, ethanol concentration in the flies had reached 42 mM (Figure 5). It therefore took 32% more ethanol to elicit the same behavioral response (here loss of postural control) in tolerant versus naïve flies. Overall, the rate of ethanol absorption and/or metabolism was not noticeably altered between the first and second exposure. We conclude that other physiological or behavioral adaptations have occurred allowing the organism to cope with higher ethanol concentrations. Therefore, by definition, we are measuring functional tolerance.

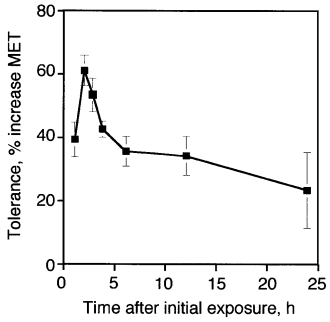

Figure 4. Kinetics of Tolerance Development and Dissipation

Flies were exposed to ethanol once in the inebriometer and then tested in the inebriometer after various time intervals. Tolerance was calculated for each set of flies as the percent increase in mean elution time (MET) between the second and first exposures. n = 39 (0 hr and 4 hr); n = 4–19 (all other time points); error bars represent SEM.

Structural Integrity of the Central Brain Is Required for Normal Tolerance

Functional tolerance is thought to rely on neuronal adaptations. Consistent with this, pharmacological and surgical lesion experiments in rodents have shown that specific brain regions are required for the development of functional tolerance to ethanol (Tabakoff and Ritzmann, 1977; Lê et al., 1981). To demonstrate a role for the fly brain in tolerance development, we tested a collection of

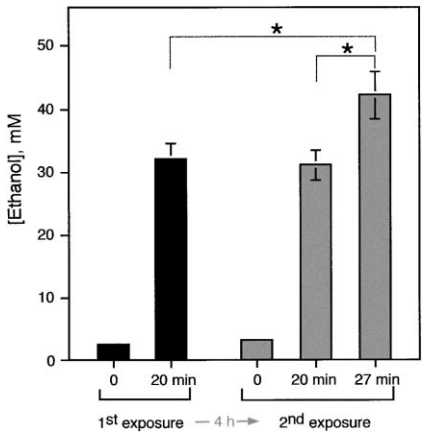

Figure 5. Functional Ethanol Tolerance

Ethanol concentration was measured in fly extracts after various lengths of exposure in tubes. It reached 32.0 ± 2.6 mM after a 20 min exposure. This time corresponds to the mean elution time (MET) of a first inebriometer exposure, and 32 mM therefore represents the average dose required to induce loss of postural control in naïve flies. Upon second exposure 4 hr later, ethanol concentration in extracts remained 31.0 ± 2.3 mM after 20 min, but reached 42.2 ± 3.7 mM by 27 min, the MET of a typical second inebriometer exposure. $n = 15$, $*p \leq 0.0001$; error bars represent the SEM.

Drosophila mutants with structural brain abnormalities (Heisenberg and Bohl, 1979; Strauss and Heisenberg, 1993; de Belle and Heisenberg, 1996). The lesions are located primarily in two central brain regions, the mushroom bodies (MBs), known to mediate classical olfactory conditioning (Heisenberg et al., 1985; de Belle and Heisenberg, 1994; Zars et al., 2000), and the central complex (CC), involved in the higher control of locomotion and flight (Strauss and Heisenberg, 1993; Ilius et al., 1994; Martin et al., 1999).

We tested 13 mutant strains, corresponding to ten loci, in the inebriometer (see Experimental Procedures for details). Those strains that displayed normal ethanol sensitivity—central-brain-deranged (*cbd*^{892}), minibrain (*mnb*^{1}), ellipsoid-body-open (*ebo*^{1}, *ebo*^{3}, and *ebo*^{4}), central-complex (*cex*^{1}), central-complex-broad (*ccb*^{2}), vacuolar-pedunculi (*vap*^{1}), and small-optic-lobes (*sol*^{1})—were tested for ethanol tolerance. Whereas some mutants (*ebo*^{1}, *ebo*^{3}, *ebo*^{4}, and *sol*^{1}) appeared normal, others (*cex*^{1}, *ccb*^{2}, and *vap*^{1}) showed significantly reduced ability to develop tolerance (Figure 6A and data not shown). Ethanol absorption and metabolism were normal in these mutants (data not shown).

From this analysis, we cannot ascertain exactly which brain regions are responsible for tolerance development, because the mutations affect more that one brain structure. For example, *ccb*^{2} has severe disruption of the entire CC (Strauss and Heisenberg, 1993) and *vap*^{1} shows vacuoles in both the MBs and the CC (de Belle and Heisenberg, 1996); *cex*^{1} displays more subtle CC defects (Strauss and Heisenberg, 1993). Moreover, all alleles of *ebo*, which disrupt the CC to varying degrees (Strauss and Heisenberg, 1993), show normal tolerance. Taken together, our data suggest that specific neuronal structures or networks, involving the CC and possibly the MBs, are involved in the development of rapid tolerance to ethanol in flies. However, because traditional mosaic analysis (Hotta and Benzer, 1972) cannot be carried out for population-based behavioral assays such as ours, a defect outside of the nervous system cannot be completely ruled out.

Functional Integrity of the Central Brain Is Required for Normal Tolerance

To confirm a role for the central brain in ethanol tolerance, we used the binary UAS/GAL4 system (Brand and Perrimon, 1993) to target the expression of tetanus toxin (TeTx) in specific brain regions. TeTx cleaves neuronal synaptobrevin, and expression of a TeTx light chain transgene (UAS-TeTxLC) in *Drosophila* neurons eliminates evoked, but not spontaneous, synaptic vesicle release at the neuromuscular junction (Sweeney et al., 1995). We chose a group of P[GAL4] lines with expression patterns (available at http://www.molgen.gla.ac.uk/flytrap/html/) encompassing the CC and MBs, the two brain structures primarily affected in the mutants described above (Figure 6A). Male flies carrying one copy of a particular P[GAL4] insertion in the presence or absence of one copy of the UAS-TeTxLC transgene were tested for ethanol sensitivity and tolerance in the inebriometer (Figure 6B) (see Experimental Procedures).

Unfortunately, most P[GAL4] lines with expression in the MBs were not viable (10 of 11 tested) or showed obvious locomotor alterations (1 of 11 tested) in the presence of the UAS-TeTxLC transgene and could therefore not be tested for ethanol-induced behaviors. This lethality is probably caused by TeTxLC expression in sites other than the MBs, since complete chemical ablation of these structures does not affect viability (de Belle and Heisenberg, 1994). Several P[GAL4] lines with expression in the CC—c561, 007Y, 078Y, and 064Y—were viable and displayed normal ethanol sensitivity in the presence of the UAS-TeTxLC transgene (see Experimental Procedures). Among these, c561 developed normal tolerance, while 007Y and 078Y showed reduced tolerance. Interestingly, line 064Y, which is X linked, displayed reduced tolerance that is TeTxLC independent in hemizygous males; this P[GAL4] insertion may thus inactivate a gene required for normal tolerance (see Experimental Procedures for details).

While the main purpose of the P[GAL4]/UAS-TeTxLC experiments was to confirm that central brain integrity is needed for normal tolerance to develop, we were able to draw a few more specific structure–function relationships. Lines 007Y and 078Y, both of which showed a TeTxLC-dependent reduction in tolerance (Figure 6B), are expressed in a subset of CC neurons, the small field neurons that connect the EB with other CC structures (Hanesch et al., 1989; Martin et al., 1999; Renn et al., 1999; Figure 6C). Moreover, line 064Y, in which the P[GAL4] insertion appears to disrupt a gene needed for tolerance development (Figure 6B), is also expressed, albeit weekly, in a few EB neurons of undefined subtype (Renn et al., 1999). However, TeTxLC expression driven by line c561, which targets the R1 subtype of EB neurons (Renn et al., 1999; Figure 6C), did not affect ethanol tolerance.

The combined analysis of structural brain mutants (Figure 6A) and flies with targeted inactivation of central

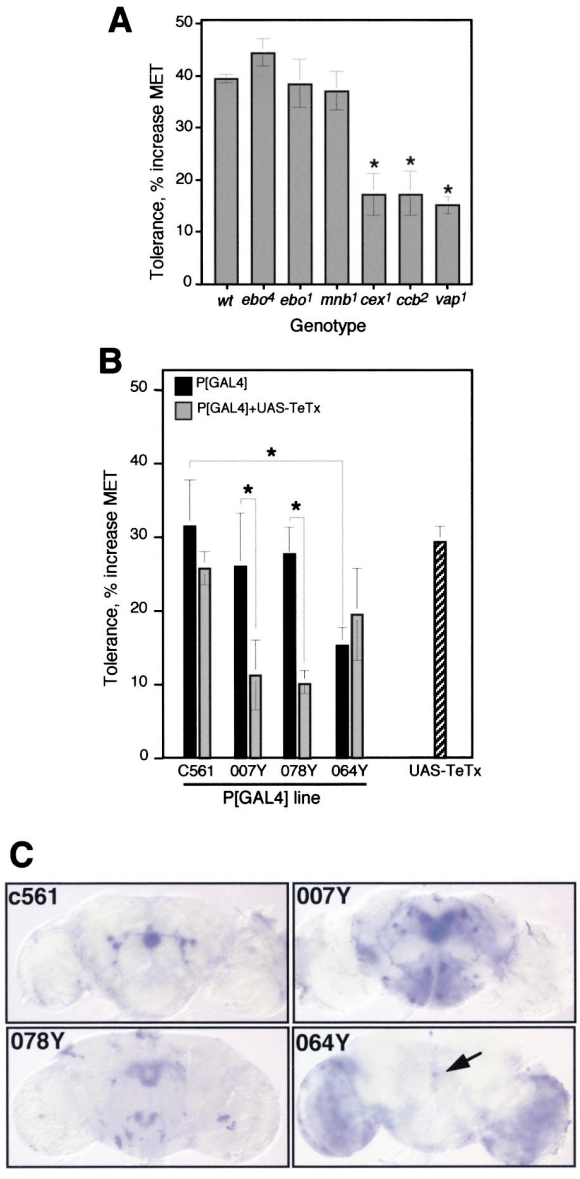

Figure 6. Involvement of the Brain in Tolerance Development

(A) Mutants with various structural abnormalities in the central brain that showed normal sensitivity in the inebriometer were tested for ethanol tolerance 4 hr after the initial exposure. Significant differences from wild type were observed with *cex*^{1}, *ccb*^{2}, and *vap*^{1}. n = 5–7, *p ≤ 0.006.

(B) Male flies carrying a UAS-TeTxLC transgene and the indicated P[GAL4] insertion were tested for ethanol tolerance in the inebriometer. All P[GAL4] lines showed normal tolerance in the presence of an inactive TeTxLC transgene control (Sweeney et al., 1995). Asterisks denote significant differences (*p < 0.01, n = 4–6).

(C) The brains of flies carrying the particular P[GAL4] insertions and a UAS-lacZ reporter were stained for β-galactosidase activity, which is detected in both the neuronal cell bodies and processes. Line c561 shows specific expression in the R1 neurons of the EB; line 007Y is expressed in small field neurons of the ellipsoid body (EB) as well as scattered neurons throughout the central brain; line 078Y is also expressed in the small field neurons of the EB and a few additional cells; line 064Y is expressed weekly in an undefined subtype of EB neurons (arrow) and quite broadly in the optic lobes (OL). The OL expression in line 064Yis probably not responsible for the tolerance phenotype as flies with severe OL abnormalities show normal ethanol tolerance (data not shown). (For a more detailed description of the expression patterns, see Martin et al., 1999; Renn et al., 1999). Lines 064Y and c561 are X linked, and the others are autosomal.

brain regions (Figure 6B) allows us to conclude that specific central brain regions are needed for the expression of ethanol tolerance. While we could not confirm or exclude a role for the MBs, a group of neurons that, based on their structure, appears to connect various CC components, seems to play a role in tolerance development. These so-called small field neurons have been shown before to control the patterns of spontaneous locomotor activity (Martin et al., 1999).

Flies Lacking Octopamine Show Impaired Tolerance

In mice, partial destruction of brain noradrenergic systems completely blocks the development of functional ethanol tolerance (Ritzmann and Tabakoff, 1976; Tabakoff and Ritzmann, 1977). In invertebrates, octopamine acts as a neurohormone, neuromodulator, and neurotransmitter in processes that are analogous to those involving noradrenaline in vertebrates (reviewed in Evans, 1980; Roeder, 1999). Octopamine is synthesized from tyramine by tyramine β-hydroxylase (TβH); *Drosophila* TβH mutants lack octopamine, and the mutant females have a defect in egg laying (Monastirioti et al., 1996).

To ascertain a possible role for octopaminergic systems in ethanol tolerance, we tested flies carrying a loss-of-function *TβH* allele, *TβH*^{nM18}, in the inebriometer. Whereas ethanol sensitivity was normal, the ability of *TβH*^{nM18} flies to develop tolerance, measured 4 hr after the initial ethanol exposure, was diminished by 50%–60% (Figure 7A). A similar defect in tolerance was observed when *TβH*^{nM18} flies were exposed to ethanol using a protocol that produces maximal tolerance; specifically, flies were run in the inebriometer four times at 2 hr intervals (Figure 7B). The latter experiment shows that flies lacking octopamine have a marked defect in tolerance that cannot be explained by an alteration in the kinetics of its acquisition. *TβH*^{nM18} mutant flies have increased levels of the octopamine precursor tyramine (Monastirioti et al., 1996). To confirm that the tolerance defect was caused by lack of octopamine rather than an increase in tyramine, we fed flies tyramine (20 mg/ml) for 2 days and then tested them for tolerance in the inebriometer. This feeding protocol, which was used successfully to restore the defect in cocaine sensitization displayed by mutant flies lacking tyramine (McClung and Hirsh, 1999), did not affect ethanol tolerance (data not shown).

In summary, these data show that octopamine is needed for tolerance development in *Drosophila*. Since brain noradrenergic systems are involved in this process in rodents (reviewed in Tabakoff et al., 1986), these results uncover further similarities between the underlying mechanisms in mammals and flies.

Discussion

Contrary to earlier notions that tolerance simply reflects a physiological adaptation to the effects of ethanol,

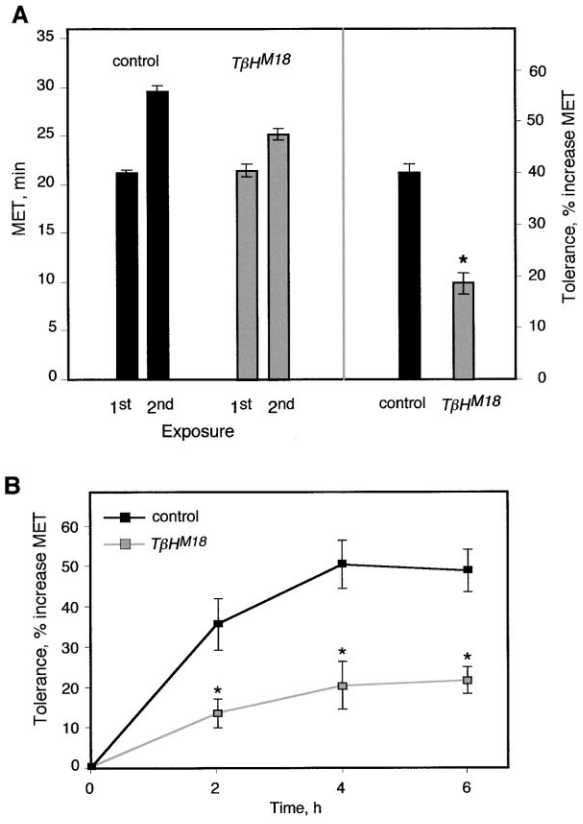

Figure 7. Role of Octopamine in Ethanol Tolerance

Flies carrying a loss-of-function mutation in the gene encoding tyramine β-hydroxylase (TβH), and who are thus unable to synthesize octopamine, were tested for tolerance development in the inebriometer.

(A) *TβH* flies are indistinguishable from the wild-type control in the first inebriometer exposure, but showed significantly reduced tolerance when tested 4 hr later. n = 18, *p < 0.0001.

(B) *TβH* mutant flies were also defective in a chronic tolerance paradigm, in which flies are exposed to ethanol in the inebriometer at 2 hr intervals. n = 7, *p < 0.001.

studies with rodents have revealed a complex phenomenon subject to genetic and environmental influences (reviewed in Lê and Mayer, 1996; Fadda and Rossetti, 1998). However, the molecular mechanisms underlying tolerance have remained elusive. We have developed a paradigm for ethanol tolerance in *Drosophila*, an ideal system for such analysis.

Adult *Drosophila* are sensitive to relatively low ethanol doses that induce a series of reversible behavioral alterations, including hyperactivity, loss of motor coordination, and sedation (Moore et al., 1998; Bainton et al., 2000; Singh and Heberlein, 2000). Here we describe their behavioral responses to repeated ethanol exposures. We find that flies develop rapid tolerance, as a single intoxicating dose of ethanol makes them subsequently more resistant to the uncoordinating and sedating effects of ethanol. This tolerance cannot be accounted for by changes in ethanol absorption or metabolism and is therefore functional tolerance, likely reflecting an adaptation of the nervous system. Consistent with the latter is our observation that genetic manipulations that disrupt the structural and functional integrity of specific regions of the central brain reduce the development of tolerance. Moreover, we show that octopaminergic systems are involved in tolerance acquisition. Interestingly, the quantitative aspects of ethanol tolerance in flies, such as the extent of maximal tolerance and the kinetics of its dissipation, are similar to those previously described for rodent models (Crabbe et al., 1979; Buck et al., 1991; Khanna et al., 1991). Taken together, these data demonstrate that *Drosophila* is a suitable model system in which to study the molecular mechansims that regulate various aspects of ethanol tolerance.

It is important to contrast our approach with previous studies in *Drosophila* that have used the term "ethanol tolerance" to describe the flies' ability to withstand the toxic effects of ethanol (reviewed in Geer et al., 1993). Such assays usually involve growing flies continuously on culture medium containing relatively high concentrations (up to 10%) of ethanol and measuring the rate of survival to the adult stage. This form of tolerance is thought to rely primarily on enzymatic or cellular functions that contribute to metabolism and/or elimination of ethanol, or protection against it (Geer et al., 1988). It is therefore equivalent to what has been described in rodents as dispositional tolerance (Khanna and Israel, 1980). Our tolerance paradigm differs in two important ways. First, we measure intoxication, manifested as impaired locomotor behavior, in response to discrete doses of ethanol well below the toxicity threshold. Second, we measure the flies' acquired resistance, or tolerance, to the effects of ethanol on postural control and locomotion rather than survival. This paradigm closely resembles those used in mammalian models for functional tolerance. We expect that the mechanisms underlying these two different forms of tolerance (dispositional versus functional) are for the most part nonoverlapping. For instance, alteration in ethanol metabolism caused by induction of Adh is a crucial component of dispositional tolerance in *Drosophila* larvae (reviewed in Geer et al., 1993). In contrast, we did not observe noticeable changes in the rate of ethanol disposition during our exposure regimens. This is consistent with observations that Adh gene expression in adult flies appears insensitive to induction by ethanol (Kerver and Van Delden, 1985).

Surgically induced brain lesions in rodents have identified a role for specific neural pathways (such as the pathway from the median raphe nucleus to the dorsal hippocampus) in the development of ethanol tolerance (Lê et al., 1981). We found that *Drosophila* mutants with structural abnormalities in certain central brain regions, including the CC and the MBs, had a reduced ability to develop tolerance. This deficiency is likely due to defects in specific brain regions, since not all brain mutants affect tolerance in the same fashion. However, the extent of the brain deformities (for a detailed description, see Strauss and Heisenberg, 1993) was not obviously correlated with the degree of tolerance impairment. For example, a *cex* mutant that has relatively subtle CC malformations showed strongly reduced tolerance; on the contrary, *ebo* mutants, which show severe abnormalities in most CC structures, developed tolerance normally. Interestingly, the effect was particular to tolerance, since these mutants displayed normal sensitivity

to the effects of acute ethanol exposure on postural control. This was surprising considering the severity of the brain lesions caused by some of these mutations. Similar conclusions were reached from experiments with TeTxLC-mediated inactivation of specific neuronal populations. Unfortunately, a role for the MBs could not be ascertained, as most P[GAL4] lines with MB expression caused TeTxLC-dependent lethality. However, we were able to identify a population of CC neurons—the small field neurons that connect various CC structures (Hanesch et al., 1989; Martin et al., 1999; Renn et al., 1999)—that appear to play a role in the development of ethanol tolerance. Interestingly, these same neurons have been shown to regulate the pattern in which flies initiate spontaneous locomotion (Martin et al., 1999). Further studies and more specific tools are needed to define the exact neural circuits that modulate the effects of ethanol on postural control and the adaptations that ensue and to ascertain if the TeTx-induced behavioral defect are secondary to developmental defects.

In invertebrates, octopamine modulates many physiological processes, such as olfaction, vision, and taste, as well as more complex phenomena, such as learning and memory, motivation, and rhythmic behaviors (reviewed in Roeder, 1999). In *Drosophila*, octopamine has been implicated in egg-laying, conditioned courtship, and olfactory learning (Dudai et al., 1987; O'Dell, 1993; Monastirioti et al., 1996). Octopaminergic neurons, ~70 in adult *Drosophila*, are distributed throughout the nervous system (Monastirioti, 1999), and a *Drosophila* octopamine receptor is preferentially expressed in the mushroom bodies and the ellipsoid body of the central complex (Han et al., 1998). Octopamine is believed to regulate in invertebrates many of the functions that noradrenaline carries out in vertebrates; noradrenaline is not found in invertebrates, including *Drosophila* (Restifo and White, 1990). For example, both catecholamines act as peripheral stress hormones that prepare the organism for a fight or flight response and they alter metabolism in anticipation of energy-demanding situations (reviewed in Roeder, 1999). In the central nervous system, octopamine and noradrenaline modulate the initiation of behaviors and motivational state, respectively. Here we show that octopamine plays a role in tolerance development in *Drosophila*; specifically, *TβH* mutant flies, which are unable to synthesize octopamine, have strongly reduced tolerance. However, tolerance development is not completely abolished in *TβH* flies, implicating additional mechanisms. While we have not excluded a role for additional neurochemical systems, we have found that flies with pharmacologically induced dopamine depletions develop tolerance normally (data not shown), although they show reduced ethanol-induced locomotor stimulation (Bainton et al., 2000). These findings are intriguing, as rodent noradrenergic systems have been implicated in ethanol tolerance (reviewed in Tabakoff et al., 1986), and they provide further evidence for similarities between the neural mechanisms of ethanol tolerance in flies and mammals. Whether octopamine acts as a neurotransmitter/neuromodulator or as a neurohormone in this process remains to be studied.

There is good evidence that in mammals tolerance is in part a learned response similar to Pavlovian conditioning (reviewed in Cunningham et al., 1984; Tabakoff et al., 1986; Lê and Mayer, 1996; Fadda and Rossetti, 1998). For example, environmental cues associated with repeated drug administration can serve as conditioned stimuli that elicit a conditioned response upon administration of placebo. Conversely, conditioned tolerance is strongly reduced when the drug is administered in a novel environment. This type of conditioned tolerance has been demonstrated for many of the effects of ethanol, such as hypothermia, narcosis, analgesia, and lethality (Lê et al., 1979; Mansfield and Cunningham, 1980; Wenger et al., 1981). It is unclear whether, in our current tolerance assay, learning or conditioning plays an important role. However, preliminary experiments reveal that a mutation in the *Drosophila* cAMP-specific phosphodiesterase *dunce* (Qui et al., 1991), originally isolated due to its learning and memory defects in an olfactory conditioning paradigm (Dudai et al., 1976), shows strongly reduced tolerance (C. M. S. and U. H., unpublished data). An involvement of *Drosophila* octopamine systems in learning (Dudai et al., 1987) and in tolerance development is also suggestive of a learned component to tolerance. Moreover, in the honeybee, octopamine has been shown to substitute for sucrose in a classical conditioning paradigm (Hammer and Menzel, 1998), implying a for a role for octopamine, not only in the behavioral plasticity underlying learning and memory, but also in reward systems.

To gain access to the molecular mechanisms underlying ethanol tolerance, we have begun a genetic screen in *Drosophila* for mutations that alter tolerance to the effects of ethanol on postural control. We have evidence that mutations in single genes can indeed increase or decrease ethanol tolerance. We expect that the characterization of these loci will provide interesting insights into the molecular mechanisms controlling ethanol tolerance, the relevant neuronal circuits involved, and potentially the involvement of learning in tolerance formation.

Experimental Procedures

Behavioral Tests

Exposures in the inebriometer were carried out as previously described (Moore et al., 1998). The ethanol concentration, which is controlled by adjusting the relative flow of ethanol vapor and humidified air, corresponded to 50/45 ethanol/air. Between exposures, flies were allowed to recover at 25°C in food vials closed with humidified cotton plugs. The second inebriometer exposure was normally initiated exactly 4 hr after the start of the first exposure. Differences in MET were analyzed with the Students t test (two-tailed test assuming equal variance of the mean).

For exposures of controlled length, such as those used in the dose–response curve shown in Figure 3A, flies were placed in perforated 50 ml Falcon tubes and exposed to ethanol vapor of the same concentration as in the inebriometer (50/45). Flies were then introduced into the inebriometer 4 hr after the start of the first exposure. The effect of ethanol dose on tolerance was also determined after exposure of flies for 20 min to ethanol vapor of fixed concentrations, and the MET was measured 4 hr later. Tolerance development (percent tolerance) was calculated relative to the MET of flies in the standard inebriometer exposure. The following data (percent tolerance) were obtained with ethanol vapor of increasing concentrations (ethanol vapor/air ratio): 25/50, 6%; 50/50, 21%; 50/25, 29%; 50/0, 62%.

Locomotor tracking system: 20 male flies were acclimated for 7 min to a 60 × 60 × 15 mm acrylic box in the presence of a regulated flow of humidified air. They are then exposed to a mixture of ethanol

vapor and humidified air (relative flow 40/25 ethanol/air) delivered at the same flow rate as humidified air alone. The flies were filmed with a digital video camera (Sony DCR-TRV900) beginning 2 min prior to ethanol exposure. Specific 10 s time intervals were captured on an Apple G4 PPC using Adobe Premiere (Adobe Systems) as QuickTime movies (Apple Computer) at 30 frames per second. Simultaneous measurement of the movements of 20 individual flies is accomplished by the DIAS Dynamic Image Analysis System (Solltech). The average speed is calculated, in Microsoft Excel, from the total distance traveled by the population over a 10 s period divided by the total number of flies.

Drosophila Stocks and Genetics

Our wild-type control strain, PZ control, contains a PZ[*rosy*$^+$] insertion (Mlodzik and Hiromi, 1992) at a silent location on the X chromosome, in an otherwise isogenic *ry*^{506} background. This stock has normal acute and chronic responses to ethanol and is used as the wild-type control for mutants generated in our laboratory using the PZ[*rosy*$^+$] element. The insertion is maintained as a homozygous stock, and experimental males are obtained from crossing PZ control males to attached-X,*w*^{1118}/Y females. Flies are grown on standard media without yeast, at 25°C in 70% relative humidity. Approximately 100 2-day-old males are collected from these crosses and placed for 2 additional days in vials at 25°C to recover from CO_2 treatment before all behavioral experiments. Thus, all behavioral experiments are carried out with 3- to 4-day-old flies.

EMS-induced and/or spontaneous brain mutants, in a wild-type Berlin genetic background (de Belle and Heisenberg, 1996), were kindly provided, along with wild-type-Berlin control stocks, by Martin Heisenberg. They include: agnostic (allele *agn*^{1}; Savvateeva et al., 1991), central-complex-broad (allele *ccb*^{1}; Strauss and Heisenberg, 1993), central-brain-deranged (allele *ceb*^{892}; Strauss and Heisenberg, 1993), central complex (allele *cex*^{1}; Strauss and Heisenberg, 1993), ellipsoid-body-open (alleles *ebo*^{1}, *ebo*^{3}, and *ebo*^{4}; Strauss and Heisenberg, 1993), mushroom-body-deranged (allele *mbd*^{1}; Heisenberg et al., 1985), minibrain (allele *mnb*^{1}; Fischbach and Heisenberg, 1984), no-bridge (allele *nob*^{1}; Strauss et al., 1992), small-optic-lobes (allele *sol*^{1}; Fischbach and Heisenberg, 1981), and vacuolar-pedunculi (allele *vap*^{1}; de Belle and Heisenberg, 1996). The mean elution times (METs) of these mutants were compared to the MET of wild-type Berlin control, which is slightly more resistant than PZ control flies and elute with a MET of 21.5 ± 0.4 min (n = 15). The initial MET for the mutants tested for ethanol tolerance was as follows: *ebo*^{4} (23.1 ± 0.7, n = 9), *ebo*^{1} (23.6 ± 0.6, n = 6), *mnb*^{1} (24.1 ± 1.0, n = 5), *cex*^{1} (23.8 ± 0.5, n = 8), *ccb*^{2} (20.5 ± 0.6, n = 5), *vap*^{1} (23.5 ± 0.5, n = 7). TβH mutant flies (allele *TβH*^{M18} were kindly provided by Maria Monastirioti (Monastirioti et al., 1996). The wild-type control flies used for the TβH experiments (Figure 7) was a *w*^{1118} strain; this strain was used because the *TβH*^{M18} mutation is in a *w*$^-$ background.

The P[GAL4]/UAS-TeTxLC flies were generated by crossing homozygous females carrying the various P[GAL4] insertions to homozygous males carrying an autosomal UAS-TeTxLC transgene; male progeny was tested for ethanol tolerance. The UAS-TeTxLC stock was generated by mobilization of one of the existing transgenes (Sweeney et al., 1995). This was done because all six original UAS-TeTxLC carrying fly stocks obtained from S. Sweeney and C. O'Kane showed increased ethanol sensitivity in the absence of P[GAL4] drivers, probably due to leaky TeTx expression. The MET of our new UAS-TeTxLC line (PIN7-2) was 18.8 ± 0.4, n = 9. All P[GAL4] lines, kindly provided by K. Kaiser and C. O'Kane were out-crossed for five generations to our laboratory isogenic *w*^{1118} stock. After outcrossing, they all remained somewhat more resistant than our wild-type laboratory strains, with METs centered around 23 min. The initial MET of these P[GAL4] lines was as follows: c561 (25.3 ± 1.0), 007Y (24.0 ± 0.9), 078Y (23.1 ± 1.3), 064Y (23.0 ± 0.8). In the presence of an inactive UAS-TeTxLC transgene (Sweeney et al., 1995), the percent tolerance of these P[GAL]4 lines was normal.

We found that P[GAL4] line 064Y, originally described as an autosomal insertion (http://www.molgen.gla.ac.uk/flytrap/html/), carries at least two P elements, one of which is X linked. We have separated the P elements and found that the X-linked element is responsible for the tolerance defect and was used in our experiments.

Measurement of Ethanol Concentration

Ethanol concentration in whole fly extracts was measured with a spectrophotometric assay as described previously (Moore et al., 1998). For PZ control flies, all measurements were carried out at least twice and in duplicate. Brain mutants and TβH flies were exposed to ethanol for 30 min, and ethanol content was measured in extracts prepared at 0, 30, 60, and 210 min. Measurements carried out in quadruplicate showed no significant deviation from controls.

Histology

To determine the GAL4 expression patterns, flies from our outcrossed P[GAL4] stocks were crossed to a line carrying the β-galactosidase reporter gene under the control of a UAS enhancer element (UAS-LacZ 4.2.1; BL#1776). β-galactosidase activity staining was performed on 5- to 10-day-old adults as described previously (Renn et al., 1999). Staining was allowed to develop at 37°C for 16 hr (078Y, 007Y, c561) or 38 hr (064Y). Samples were washed and mounted in 80% glycerol in PBS. Nomarsky images were obtained on a Zeiss Axioskop II microscope. In some cases, our staining pattern differs from those previously published, which may be attributed to the elimination of additional P-[GAL4] insertions over the course of outcrossing.

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