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Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening

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Abstract

Larval zebrafish are used extensively for developmental genetic studies due to their salient features, such as small size, external development, optical transparency, and accessibility in large numbers. However, their use for the study of drug and alcohol abuse has not been explored. Here we investigated the response of larval zebrafish to acute treatment of alcohol. Our analyses showed that like adults, the larval zebrafish exhibited a dose-dependent locomotor response to ethanol: intermediate doses led to hyperactivity, whereas high doses have a neurodepressive effect resulting in hypoactivity and sedation. Alcohol also induced morphological changes of melanocytes, providing a visible cellular measure of the biological effects of alcohol in vivo. In addition, alcohol induced thigmotaxis behavior (preference for the edge of a compartment). In the behaviors we analyzed, genetic background influenced the locomotor responses to alcohol. The present study demonstrates that larval zebrafish exert a response to the acute treatment of alcohol, which is genetically modifiable. Therefore, the larval zebrafish represent a tractable vertebrate model system for a large-scale genetic analysis of the biological effects of alcohol.
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Keywords: Alcohol effects; Behavior; Drugs of abuse; Genetics; Larval zebrafish; Locomotor activity

1. Introduction

Ethanol is naturally produced by fruit or cereal fermentation. Since it is soluble in both aqueous and lipid environments, ethanol can easily cross biological membranes and affects virtually all body organs. Behaviorally, acute administration of alcohol in humans leads to disinhibition and euphoria. As doses increase, ethanol causes sedation and even death. It is well established that these effects of ethanol are mediated through many specific proteins in the central nervous system (CNS) (Fadda and Rossetti, 1998). However, the targets of ethanol and their role in generating ethanol-induced behavior in vivo are not well understood.

Although environmental factors influence alcohol consumption and abuse, genes play important roles in alcoholism. Family studies have documented a three- to fivefold increased risk for alcoholism among siblings and first-degree relatives of affected individuals (Cotton, 1979). In addition, twin studies have identified a significant genetic component to alcoholism risk, with estimates of heritability ranging from 50% to 60% (Heath et al., 1997). However, identification of genes involved has been difficult due to the complex nature of the disorder and the lack of sufficient candidate genes.

The use of animal models with similar or related behaviors may provide insights into molecules involved in mediating the biological effects of ethanol. Like humans, rodents exhibit similar acute sensitivity to ethanol. Inbred strains of mice that show different sensitivity to ethanol have been established and used to identify genes by quantitative trait loci analysis (Crabbe et al., 1999). Mice with targeted disruption of the serotonin receptor 5-HT_{1B} display reduced sensitivity to ethanol (Crabbe et al., 1996), whereas mice lacking Fyn-tyrosine kinase are hypersensitive to the hypnotic effects of ethanol (Miyakawa et al., 1997). In addition to rodents, the invertebrate model organism for genetics, *Drosophila*, also exhibits ethanol sensitivity and has been used in a forward genetic study to show that the cAMP signaling pathway is important to mediate the effects of ethanol (Moore et al., 1998).

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The zebrafish, *Danio rerio*, is a small freshwater teleost and a recently established vertebrate model organism for genetics. Like the invertebrate genetic models *Drosophila* and *C. elegans*, zebrafish have the salient features of being small sized, having a large number of progeny, and relatively short life cycle. Being a vertebrate, zebrafish are more structurally homologous to humans, and their genes are about 70–80% identical to human counterparts (Dooley and Zon, 2000), thus making it convenient to identify human orthologues of zebrafish genes. Although zebrafish have been widely used for studies of development, their use in the study of the effects of ethanol has been limited. In addition to several reports documenting the teratogenic effects of ethanol on developing embryos (Baumann and Sander, 1984; Blader and Strahle, 1998; Laale, 1971), two recent reports demonstrate that ethanol modifies multiple behaviors of adult zebrafish in a strain-dependent manner (Dlugos and Rabin, 2003; Gerlai et al., 2000). However, it is cumbersome to use adult zebrafish for genetic screens aimed at identifying molecules mediating the biological effects of ethanol. A significant portion of adult zebrafish are sensitive to handling-induced stress by exhibiting wall-hugging and freezing behavior even after extended habituation (S. Bretaud and S. Guo, unpublished observations), making it difficult to carry out behavioral analysis. In addition, extra breeding and housing is required for behavioral genetic study in adult zebrafish. At present, the neural mechanisms involved in mediating ethanol effects in zebrafish are not known.

In this study, we explored the effects of ethanol on larval zebrafish. Larval zebrafish are postembryonic day 5 to 2–3 weeks of age. They are free living, hunt for food, and escape from predators, thus having a well-established nervous system and possessing many patterns of behavior (Fetcho and Liu, 1998). Yet, the larval zebrafish are much smaller sized than adults (about 2 mm in length), thus they can be easily handled in large quantities. More importantly, they appear to be less sensitive to handling-induced stress than adults (S. Bretaud and S. Guo, unpublished observations), possibly because larval zebrafish are tested in a much larger compartment compared to their size and they can be tested in groups due to the lack of apparent social interaction (S. Bretaud and S. Guo, unpublished observations). Our data reported in this study indicate that similar to adult fish, larval zebrafish exhibit acute sensitivity to ethanol: their locomotor activity was modified in response to different concentrations of ethanol, and such response was genetically modifiable. In addition, upon ethanol exposure, the morphology of melanocytes was altered, and larval zebrafish also exhibited a preference for the edge of a compartment (thigmotaxis). These findings, together with the amenability of larval zebrafish to large-scale genetic screens, permit the identification of molecules involved in mediating the biological effects of ethanol.

2. Methods

2.1. Animal care and maintenance

We kept adult zebrafish in our fish facility at the University of California, San Francisco. Standard fish care and maintenance protocols were carefully followed (Westerfield, 1995). Thus, we kept environmental variance at a minimum for all behavioral experiments. Adult zebrafish were maintained in deionized water containing 200 mg/l Instant Ocean Salt (Aquatic Eco-Systems, Orlando, FL). The water was recirculated after sterilizing by UV light and filtering through mechanical filters and biofilters. Fish were fed twice daily with a mixture of brine shrimp (*Artemia salina*, San Francisco Bay Brand, San Francisco, CA) and flake food (Aquatic Eco-Systems). Two strains that have been extensively bred in the laboratory were used: AB, which originated from Oregon, USA, and WIK, which originated from Germany. Larval zebrafish (also known as fry) were obtained from these strains through natural mating. We raised fry in a 28 °C incubator from birth to 7 days postfertilization (dpf) as previously described (Guo et al., 1999). We used 7-day-old fry in this study. All animal care procedures were approved by the Institutional Review Committee at the University of California, San Francisco.

2.2. Ethanol treatment

To determine the acute sensitivity of larval zebrafish to ethanol, ten 7-day-old fry were transferred to a view chamber ($L \times W \times H$: $8 \times 6 \times 2$ cm) containing blue egg water (0.2 g/l Instant Ocean Salt, 0.12 g/l $CaSO_4$, and 10 µl/l methylene blue). We used 10 fry in a group so that a higher throughput and less variability could be obtained. Fry were allowed to habituate to the new environment for 5 min. A solution of ethanol was gently added to the view chamber to give a defined ethanol concentration: 0% (control), 0.5%, 1%, 1.5%, 2%, 3%, and 4% (v/v) were tested in this study. As a control, we exposed fry to 1.5% methanol to determine whether the hyperactivity behavior that we observed was unique to the effect of ethanol on the CNS or rather a general chemosensory response that resulted from exposure to a noxious compound. Methanol-induced behavior was only assessed for the AB strain fry. The fry behavior was recorded for 20 min using a video camera and the resulting movies were saved for analysis. A 20-min exposure time was chosen to achieve conditions that would be suitable to high throughput in a large-scale forward genetic screen. Consequently, we adjusted internal ethanol levels by varying the concentration of the dose rather than the length of exposure.

2.3. Behavioral analysis

The recorded movies were analyzed using the Dynamic Image Analysis System (DIAS, Solltec, Ohio). DIAS anal-

ysis allowed the quantitative determination of swimming speed and location of the fry at any given time. The mean swim speeds were calculated at 0, 1, 3, 5, 7, 10, 13, 16, and 20 min, and plots were generated using Microsoft Excel. For the analysis of thigmotaxis, the recording chamber was divided into two compartments, one central part and one edge part (see Fig. 4A). The width of the edge was 10% of the length of the view chamber (0.8 cm), thus the edge region made up 41.3% of the total area. An Excel Macro was written to calculate thigmotaxis as the percentage of time fry spent in the edge. Thigmotaxis was measured at the same time intervals as swim speed.

2.4. Measurement of internal ethanol concentration

Ethanol absorption was measured using a protocol already established for *Drosophila* (Moore et al., 1998). To prepare samples for the assay, we exposed groups of sixty 7 dpf fry to 0% (control), 1.5%, and 3% ethanol. To obtain a time-based analysis of ethanol absorption, we prepared samples of AB fry that were exposed to 1.5% ethanol for 1, 10, and 20 min. Absorption at the 3% ethanol exposure was assessed at 20 min. Immediately after exposure, fry were euthanized with 100 mg/l tricaine (3-aminobenzoic acid ethyl ester, Sigma) and rinsed for 30 s in fine mesh with distilled water to remove ethanol from their skin. The fry were frozen on dry ice and homogenized in 500 μl of 50 mM Tris–HCl (pH 7.4). The homogenate was centrifuged at 14,000 rpm for 20 min at 4 °C. Samples were processed using an ethanol assay kit (Diagnostic Chemicals), which uses the enzyme alcohol dehydrogenase to catalyze the conversion of ethanol to acetaldehyde and NADH. Production of NADH corresponds to an increased absorption at 340 nm, which is proportional to the ethanol present in the sample. Moore et al. (1998) estimated the volume of a single adult fruit fly to be ~ 2 μl. By comparison, 7-day old zebrafish fry are smaller than *Drosophila*. By volume displacement, we measured the volume of a single 7-day old fry and found it to be ~ 1 μl. This volume was used to calculate the millimolar ethanol concentration per fry.

2.5. Microscopic analysis of melanocyte morphology

To test the effect of ethanol on melanocyte morphology, 7 dpf fry were exposed to 0% (control), 0.5%, 1.5%, and 3% ethanol for various times (1, 10, and 20 min). After treatment, they were fixed with 4% paraformaldehyde and photographed under a Zeiss compound microscope.

2.6. Statistical analysis

We used SPSS 11.5 for Windows to conduct statistical analyses. In cases of multiple comparisons, we first performed ANOVA and subsequently conducted Dunnett's T3 pairwise comparison post hoc test to determine significant differences between individual treatments. The Dunnett's T3 test was used because it is robust to the analysis of unequal variances. All treatments were compared to the basal level locomotor response (control). Comparisons between AB and WIK strains were performed by assessing the magnitude of the ethanol-induced response compared to each strain's respective basal response. In the case of the WIK strain thigmotaxis data, we used the Student's two-tailed t test because it was a simple comparison of two treatments. Since fry locomotion was relatively uniform across treatments at the beginning of each observation period, we compared different treatments over the time intervals that exhibited the greatest effects of ethanol on behavior. All statistical analyses assumed an alpha level of .05 to determine significance.

The experiments with 1.5% methanol were performed after moving to a different laboratory. Consequently, there was an environmentally induced difference in behavior that affected basal level swim speed. We found this difference to be uniform. To standardize these data with the previous data, we performed control experiments to assess the basal swim speed, and we accounted for the differences by introducing a correction factor into the 1.5% methanol data set. Each datum (1.5% methanol) was multiplied by the ratio of the original basal level response to the new basal level response. There were no significant differences between the speeds of methanol-treated fry and nontreated fry both before ($t = 0.49$, $df = 6$, $P = .64$) and after this correction ($t = -0.81$, $df = 12$, $P = .43$). We did not standardize the thigmotaxis data because we have found no evidence that swim speed affects thigmotaxis (see Sections 3.4 and 4 and Figs. 2 and 4).

3. Results

3.1. Ethanol modified the locomotor activity of larval zebrafish in a dose- and time-dependent manner

Larval zebrafish were much less sensitive to handling-induced stress compared to adults. They also exhibited minimal social interaction, as no clustering was observed. We found significant differences between the ethanol treatments [ANOVA, $F(6,56) = 13.58$, $P < .001$]. Somewhat surprisingly, 0.5% ethanol did not have an effect on the locomotor activity of larval zebrafish during the 20-min observation period (Fig. 1C; Dunnett's T3, $P > .50$), whereas 1.0%, 1.5%, and 2.0% ethanol-induced hyperactivity from 7 min onward to a significant extent (Fig. 1; Dunnett's T3, $P < .05$). 1.5% ethanol led to the highest mean swim speed (Fig. 1A) and the highest increase in locomotor activity, which reached a plateau after 10 min (Fig. 1A). Higher ethanol concentrations than 1.5% did not lead to further increased locomotor activity; rather, larval zebrafish succumbed and became hypoactive and subsequently sedated (Figs. 1A and C). Exposure to 4.0% ethanol caused a hypoactive state that was significantly slower than the basal

level from minute 7 onward (Fig. 1C; Dunnett's T3, $P<.05$). Methanol had no significant effect on swim speed (see Section 2.6; Figs. 1B and C; Dunnett's T3, $P>.50$). Since the activating effect of ethanol at intermediate doses (e.g., 1.5%) took several minutes to occur (Fig. 1A), the behavior observed in larval zebrafish is likely to be mediated by the pharmacological action of ethanol on the CNS. A chemosensory effect of ethanol usually appears much faster; as in the case of *Drosophila*, the chemosensory effect of ethanol occurred within 30 s upon exposure (Scholz et al., 2000). The fact that methanol did not elicit similar effects as ethanol further supported the notion that ethanol acted on the CNS. Taken together, these observations suggest that ethanol enters larval zebrafish and exerts a pharmacological action on the CNS; in addition, there is a particular internal ethanol concentration threshold that leads to hyperactivity. Above this concentration, the effect of ethanol on inducing hyperactivity is compromised or masked by its neurodepressive effect.

To determine the internal ethanol concentrations that led to hyperactivity or hypoactivity, we measured ethanol absorption that corresponded to exposures that caused both the highest hyperactivity (1.5%) and hypoactivity (3.0%) in larval zebrafish. As shown in Table 1, we found a steady increase in ethanol absorption when fry were exposed to 1.5% ethanol, which reached 25 mM, or 0.12% (w/v), per fry at 20 min. At the 3.0% exposure level, the ethanol absorption was much higher (Table 1). WIK fry ethanol absorption was similar to AB (Table 1). These results were consistent with observations of the locomotor activity.

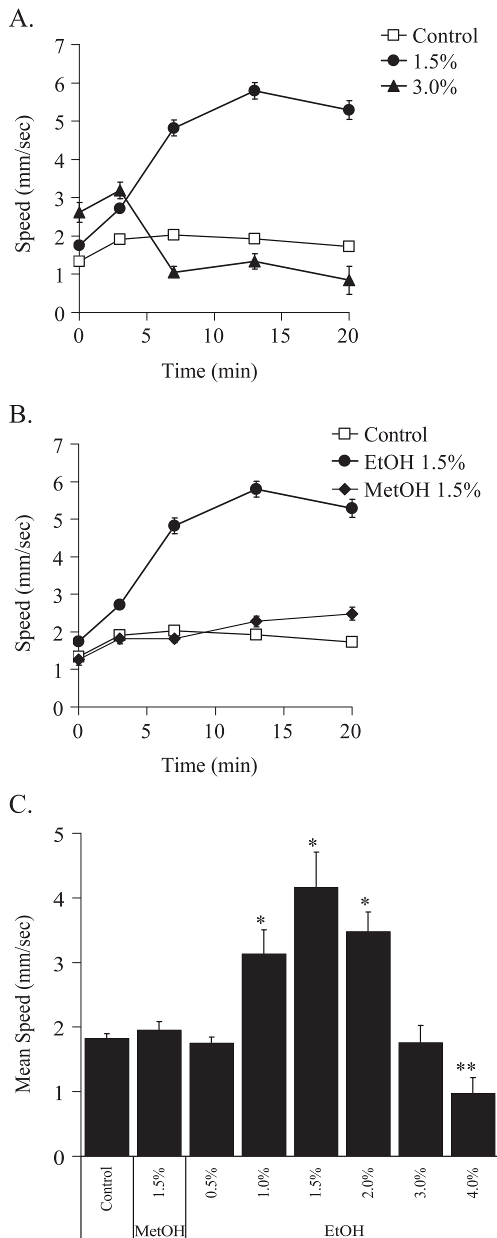

Fig. 1. AB fry locomotion depended on ethanol treatment concentration. (A) The locomotor activity of AB fry was graphed in response to time and ethanol concentration. Percentages indicate concentration of ethanol treatment. The control represents basal swim speed. For simplicity, mean speeds ± S.E.M. were plotted at 0, 3, 7, 13, and 20 min. At certain data points, S.E.M. was too small to report on the figure. From minute 7 onward the differential effects of the three treatments were most pronounced [ANOVA, $F(2,12)=263.7$, $P<.001$]. The greatest hyperactivity was between 10 and 15 min at 1.5% ethanol (Dunnett's T3, $P<.001$) and 3.0% ethanol produced a significant hypoactive response (Dunnett's T3, minutes 7–20, $P<.05$). Sample sizes were in numbers of fry: control, $n=25\times10$; 1.5% ethanol, $n=36\times10$; 3.0% ethanol, $n=6\times10$. (B) The locomotor activity of AB fry was graphed in response to time and both 1.5% methanol and 1.5% ethanol treatments. The control represents basal swim speed. Mean swim speeds ± S.E.M. were plotted at 0, 3, 7, 13, and 20 min. At certain data points, S.E.M. was too small to report on the figure. The 1.5% methanol data were standardized (see Section 2.6). Differences were most significant from minutes 7 to 20 [ANOVA, $F(2,12)=227.4$, $P<.001$]. Unlike ethanol, 1.5% methanol produced no significant effect on swim speed (Dunnett's T3, $P=.17$). Sample sizes were in numbers of fry: control, $n=25\times10$; 1.5% ethanol, $n=36\times10$; 1.5% methanol, $n=8\times10$. (C) Mean speeds ± S.E.M. over the 20-min observation period were reported for each ethanol treatment. * Significant hyperactivity at 1.0%, 1.5%, and 2.0% ethanol compared to the control. ** Significant hypoactivity at 4.0% ethanol compared to the control (minutes 7–20) [ANOVA, $F(7,32)=83.35$, $P<.001$; Dunnett's T3, $P<.05$]. Sample sizes were in numbers of fry: control, $n=25\times10$; 1.5% methanol, $n=8\times10$; 0.5% ethanol, $n=4\times10$; 1.0% ethanol, $n=6\times10$, 1.5% ethanol, $n=36\times10$; 2.0% ethanol, $n=5\times10$; 3.0% ethanol, $n=6\times10$; 4.0% ethanol, $n=2\times10$.

3.2. Genetic background influenced basal and ethanol-induced locomotor activity

To determine if genetic background has an effect on acute sensitivity to ethanol, we tested larval zebrafish derived from the WIK strain, which is known to be highly polymorphic compared to AB (Knapik et al., 1998). Since a correlation has been shown between hyperactivity and the rewarding effects of a number of drugs of abuse including ethanol (Phillips and Shen, 1996), we focused our attention on ethanol-induced hyperactivity for subsequent analysis. As shown in Fig. 2, there were significant differences in both basal and ethanol-induced locomotor activity between AB and WIK [ANOVA, $F(3,32)=8.164$, $P<.001$]. The greatest differences were observed from minute 10 onward

Table 1
Internal ethanol concentration

Treatment	AB		WIK	
	mM	% (w/v)	mM	% (w/v)
Control	0	0	0	0
1.5%, 1 min	8 ± 2	0.04	–	–
1.5%, 10 min	17 ± 2	0.08	–	–
1.5%, 20 min	25 ± 3	0.12	25 ± 2	2
3.0%, 20 min	71 ± 3	0.33	–	–

Internal ethanol concentrations [mM and % (w/v)] were measured as described (see Section 2.4) in 7 dpf larval zebrafish upon acute treatment to 1.5% and 3.0% ethanol in AB and 1.5% ethanol in WIK.

(Fig. 2) [ANOVA, $F(3,12)=288.9$, $P<.001$]. WIK had a significantly higher basal locomotor activity than AB from minute 7 to 20 (Dunnett's T3, $P<.01$). The 1.5% ethanol exposure induced significant hyperactivity in WIK as it did in AB (Fig. 2; Dunnett's T3, minutes 10–20, $P<.01$). However, the effect was much smaller in WIK than in AB (Fig. 2). Since environmental variance has been kept at a minimum for these behavioral experiments, these observations suggest genetic factors that differ between AB and WIK could regulate both basal locomotor activity and ethanol-induced hyperactivity (see also Section 4).

3.3. Effects of ethanol on melanocytes

During the course of studying ethanol's acute effects on locomotor activity, we noticed that ethanol modified the appearance of melanocytes (Fig. 3). The zebrafish melanocytes, also known as melanophores, reside in the dermis layer. Therefore, they are not in direct contact with the external aqueous environment, and are thus exposed to the internal rather than external concentrations of alcohol. Melanocytes in larval zebrafish that were not treated with ethanol had pigmentary organelles, termed *melanosomes*, clustered mainly in the center of the cell (Fig. 3A). Upon treatment with 1.5% ethanol for 1 min, we observed no discernible changes (Fig. 3B). Upon treatment with 1.5% ethanol for 20 min, we observed a noticeable dispersion of melanosomes (Fig. 3C). A similar effect was observed at 3.0% ethanol exposure (picture not shown). We have not observed melanosome dispersion in fry exposed to other compounds, such as fluphenazine, morphine, and amphetamine (data not shown). This observation suggests that the change of melanocyte morphology provides a visible cellular measure of the biological effects of ethanol in vivo. It is not clear at present whether such an effect is mediated

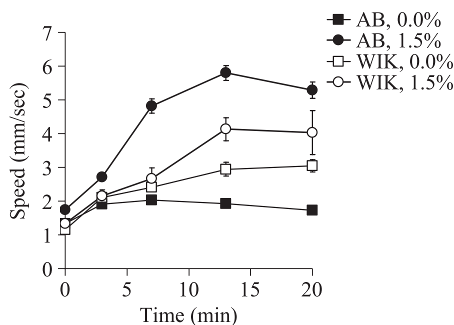

Fig. 2. The effect of ethanol depended on the genetic background of fry. The locomotor activity of fry derived from both the AB and WIK strains was graphed in response to time and ethanol concentration. Percentages indicate concentration of ethanol treatment. Treatments at 0.0% ethanol observed basal locomotor activity. Mean speeds ± S.E.M. were plotted at 0, 3, 7, 13, and 20 min. At certain data points, S.E.M. was too small to report on the figure. WIK strain fry had a higher basal locomotor activity than AB [ANOVA, $F(3,16)=71.58$, $P<.001$; Dunnett's T3, $P<.01$]. 1.5% ethanol treatment had a greater effect on AB than WIK strain fry (minutes 10–20, AB exposed vs. basal mean difference = 3.7 mm/s, WIK exposed vs. basal mean difference = 1 mm/s). Sample sizes were in numbers of fry: AB 0.0% ethanol, $n=25\times10$; AB 1.5% ethanol, $n=36\times10$; WIK 0.0% ethanol, $n=8\times10$; WIK 1.5% ethanol, $n=9\times10$.

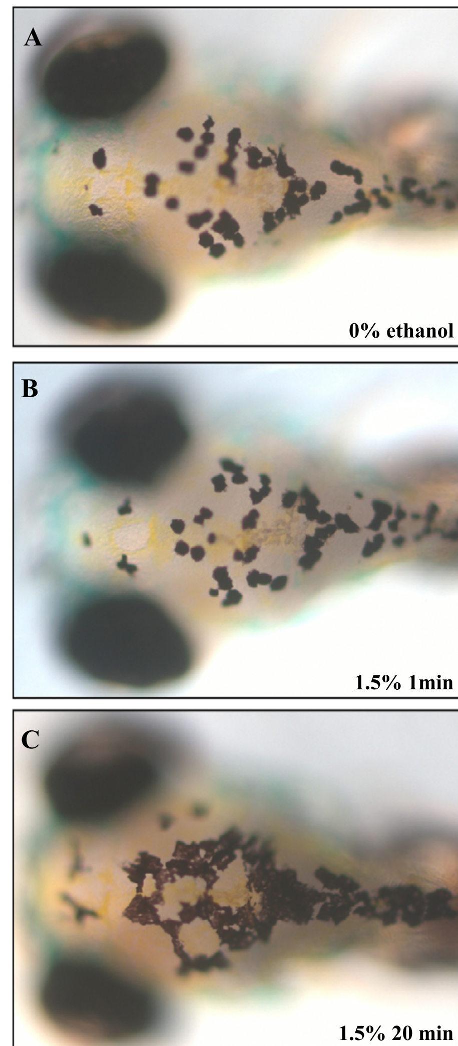

Fig. 3. Ethanol had an effect on the pigment cells of AB fry. The sample size was $n=25$ exhibiting the effect of ethanol on melanocytes. (A) AB fry not treated with ethanol had melanosomes that were clustered in the center of the cell. (B) AB fry treated with 1.5% ethanol for 1 minute showed no change in melanocyte morphology. (C) AB fry treated with 1.5% ethanol for 20 min showed visible dispersion of melanosomes.

through the CNS or is due to direct action of ethanol on melanocytes.

3.4. Ethanol-induced thigmotaxis behavior in larval zebrafish

Thigmotaxis, namely the "wall-seeking" tendency in an open field, is a behavior indicative of anxiety and stress in mice and rats (Treit and Fundytus, 1988). Since very little is known about anxiety-like behavior in zebrafish, we were unable to prove definitively whether thigmotaxis behavior in larval zebrafish was a stress response within the scope of this study. However, thigmotaxis behavior was observed in larval zebrafish upon ethanol exposure. When treated with 1.5% ethanol, AB fry initially preferred the center compartment as compared to untreated control. The differential preference was greatest at minute 3 (Fig. 4B); however, this trend was not significant (Dunnett's T3, P=.07). From minutes 7 to 20, ethanol-treated AB fry had significantly greater thigmotaxis than control (Fig. 4B) [ANOVA, $F(2,12) = 25.06$, $P < .001$; Dunnett's T3, $P < .05$]. Methanol (1.5%) had the opposite result, but the effect was not as great (Fig. 4B and C; Dunnett's T3, $P < .001$; methanol vs. control mean difference = 9.7%, ethanol vs. control mean difference = 17.7%). These data show that the initial contact with ethanol decreased thigmotaxis, but subsequent accumulation of higher internal ethanol concentration increased thigmotaxis. A similar effect was also observed in the WIK strain ($t = -4.978$, $df = 8$, $P < .01$), although the magnitude of thigmotaxis was smaller than that of the AB strain (Fig. 4B and D). We have not observed a significant thigmotaxis response to other compounds, such as amphetamine, morphine, and cocaine (data not shown).

Swim speed did not affect thigmotaxis behavior because increased swim speed did not consistently increase or decrease thigmotaxis. WIK fry had a higher basal level speed (Fig 2; minutes 7–20) [ANOVA, $F(3,16) = 71.58$, $P < .001$; Dunnett's T3, $P < .01$] and a lower basal level thigmotaxis (Fig 4; minutes 10–20 [ANOVA, $F(4,15) = 23.5$, $P < .001$; Dunnett's T3, $P < .01$] than AB control, and AB fry exposed to 1.5% ethanol had the highest speed (Fig. 2; minutes 7–20; Dunnett's T3, $P < .01$) but the highest thigmotaxis (Fig. 4; minutes 10–20; Dunnett's T3, $P < .01$). This analysis suggests that genetic background also influenced the ethanol-induced thigmotaxis behavior.

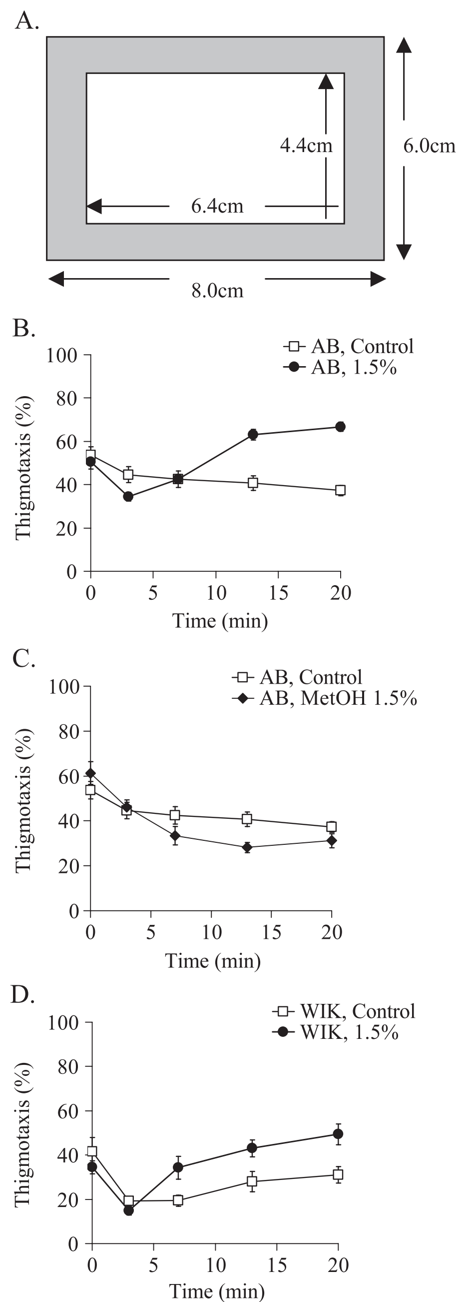

Fig. 4. Ethanol-induced thigmotaxis behavior in AB and WIK strain fry. (A) The view chamber was separated into central and edge parts for the analysis of thigmotaxis behavior. The edge region comprised 41.3% of the total area. Thigmotaxis was the percentage of time fry spent in the edge. A random distribution of fry would correspond to a thigmotaxis of 41.3%. We plotted the thigmotaxis ± S.E.M. at 0, 3, 7, 13, and 20 min in response to control, ethanol, and methanol treatments. At certain data points, S.E.M. was too small to report on the figure. Control measured basal level activity. (B) AB response to 1.5% ethanol. Control fry exhibited 40% thigmotaxis, corresponding to a close to random distribution of the fry. AB fry exposed to 1.5% ethanol initially preferred the center region more than AB control fry at minute 3, but the preference was not significant (Dunnett's T3, P=.07). From minutes 7 to 20, ethanol-treated fry had increased thigmotaxis compared to control [ANOVA, $F(2,12) = 25.06$, $P < .001$; Dunnett's T3, $P < .05$]. Sample sizes were in numbers of fry: AB control, $n = 15 \times 10$; AB 1.5% ethanol, $n = 30 \times 10$. (C) AB response to 1.5% methanol. From minutes 7 to 20, AB fry exposed to 1.5% methanol exhibited less thigmotaxis than control fry [ANOVA, $F(2,12) = 25.06$, $P < .001$; Dunnett's T3, $P < .001$]. Sample sizes were in numbers of fry: AB control, $n = 15 \times 10$; 1.5% methanol, $n = 8 \times 10$. (D) WIK response to 1.5% ethanol. From minute 7 onward, ethanol-exposed WIK fry exhibited greater thigmotaxis than control fry ($t = -4.978$, $df = 8$, $P < .01$), but the difference between ethanol-treated and control was not as great as in the AB fry (AB mean difference = 22.2% of fry, WIK mean difference = 15.3% of fry). Sample sizes were in numbers of fry: WIK control, $n = 8 \times 10$; WIK 1.5% ethanol, $n = 8 \times 10$.

4. Discussion

Alcoholism is known to have a strong genetic basis (Dick and Foroud, 2003). However, at present, we know very little about the identity of these genetic factors. Studies to evaluate the biological effects of ethanol in genetically tractable systems will allow identification of molecules that mediate the biological effects of alcohol, thus providing candidate genes for subsequent human genetic studies of alcoholism.

In this study, we show that larval zebrafish, a genetically tractable system, exhibit acute sensitivity to ethanol in a dose- and time-dependent manner. They initially become hyperactive, and as ethanol accumulates, they become hypoactive and sedated. This is similar to what has been observed in humans and other animal models (Schumann et al., 2003). Furthermore, we show that ethanol-induced hyperactivity is different between two genetic strains, the AB and WIK, which showed no difference in ethanol absorption. It is well known that environment as well as an interaction between the environmental and genetic factors could influence ethanol-induced behavior (Dick and Foroud, 2003), for example, the rearing of the animal as well as the behavioral testing conditions could alter the parameters of the behavior analyzed. In this study, we kept the environmental influences under tight control, thereby we were able to effectively compare different strains and assess the genetic influence on the behavioral effects of ethanol. Therefore, our analyses suggest that ethanol-induced hyperactivity in larval zebrafish is genetically modifiable. Future forward genetic analysis to identify mutations that show altered sensitivity to ethanol shall provide important insights into the genes involved in regulating the behavior.

The interaction of ethanol with multiple brain neurotransmitter systems has been demonstrated in humans and rodents (Phillips and Shen, 1996; Weiss and Porrino, 2002). These include the brain dopamine, GABA, serotonin, and glutamate systems. It is worth mentioning that the brain dopamine, serotonin, and GABA systems have been shown to develop early during embryogenesis and are present in larval zebrafish, providing a physiological basis for their possible involvement (Doldan et al., 1999; Guo et al., 1999; Wullimann and Rink, 2001). It will be interesting to determine in the future whether these neural systems are involved in regulating ethanol-induced behavior in larval zebrafish.

The ethanol concentration used in our study is higher than that used in adult zebrafish studies. In previous studies, adult zebrafish were exposed to ethanol for 1–2 h before behavioral analysis (Dlugos and Rabin, 2003; Gerlai et al., 2000). After this amount of exposure, the internal ethanol concentration is thought to reach equilibrium with that in the tank water. In contrast, we monitored the initial 20-minute response of larval zebrafish upon contact with ethanol to determine their acute sensitivity. Consequently, the internal ethanol concentration was lower than that in the view chamber. Our measurement of internal ethanol concentration indicates that the effect of ethanol on zebrafish might be similar to humans. At the 1.5% level, which induced hyperactivity, the internal ethanol concentration after 20 min of exposure was 0.12% (w/v). This level can affect locomotion similarly in humans, as the legal automobile driving limit is 0.1% in many countries.

In addition to modifying the locomotor sensitivity of larval zebrafish, ethanol exposure changed the morphological appearance of melanocytes. As previously reported, pigment cells in most teleost fish undergo pigment aggregation or dispersion in response to environmental factors including light, physical, and chemical factors (Fujii, 2000). Both neuronal and hormonal mechanisms are thought to regulate this process. In particular, decreases in cAMP levels and/or increases in Ca^{2+} levels within the melanophores can trigger aggregation response, while dispersion responses are induced by opposite changes in cAMP or Ca^{2+} levels (Fujii, 2000). Ethanol is known to modulate cAMP signaling (Diamond and Gordon, 1997). Therefore, it is possible that ethanol directly binds to protein target(s) expressed on melanocytes to elevate intracellular cAMP or Ca^{2+} levels and lead to pigment dispersion. Alternatively, the effect may be mediated through the CNS.

Ethanol also induced thigmotaxis in larval zebrafish. The preference for the edges of an open field has been used as a way of measuring anxiety, fear, and stress in rodents (Simon et al., 1994; Treit and Fundytus, 1988). It is interesting to note that such behavior can also be observed in larval zebrafish, although at present we do not know if such behavior reflects anxiety or fear in larval zebrafish. Nevertheless, initial exposure to ethanol led to a slight reduction of thigmotaxis. Subsequently, as ethanol concentration accumulated, larval zebrafish displayed significantly increased thigmotaxis. It is unlikely that thigmotaxis of larval zebrafish is due to increased locomotor activity, as the WIK strain, which had an increased basal locomotor activity, did not have an increased thigmotaxis.

At present, it is not known what mediates these effects of ethanol in larval zebrafish. The amenability of larval zebrafish to future genetic and pharmacological analysis provides us with an opportunity to reveal the molecular and cellular mechanisms underlying the biological effects of ethanol in vivo.

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