Amino Acids (2014) 46:921–930
DOI 10.1007/s00726-013-1658-y

ORIGINAL ARTICLE

# Chronic and acute alcohol administration induced neurochemical changes in the brain: comparison of distinct zebrafish populations

**Diptendu Chatterjee · Soaleha Shams · Robert Gerlai**

Received: 24 June 2013 / Accepted: 20 December 2013 / Published online: 1 January 2014


**Abstract** The zebrafish is increasingly utilized in the analysis of the effects of ethanol (alcohol) on brain function and behavior. We have shown significant population-dependent alcohol-induced changes in zebrafish behavior and have started to analyze alterations in dopaminergic and serotoninergic responses. Here, we analyze the effects of alcohol on levels of selected neurochemicals using a 2 × 3 (chronic × acute) between-subject alcohol exposure paradigm randomized for two zebrafish populations, AB and SF. Each fish first received the particular chronic treatment (0 or 0.5 vol/vol % alcohol) and subsequently the acute exposure (0, 0.5 or 1.0 % alcohol). We report changes in levels of dopamine, DOPAC, serotonin, 5HIAA, glutamate, GABA, aspartate, glycine and taurine as quantified from whole brain extracts using HPLC. We also analyze monoamine oxidase and tyrosine hydroxylase enzymatic activity. The results demonstrate that compared to SF, AB is more responsive to both acute alcohol exposure and acute alcohol withdrawal at the level of neurochemistry, a finding that correlates well with prior behavioral observations and one which suggests the involvement of genes in the observed alcohol effects. We discuss correlations between the current results and prior behavioral findings, and stress the importance of characterization of zebrafish strains for future behavior genetic and psychopharmacology studies.

D. Chatterjee · R. Gerlai (✉)
Department of Psychology, University of Toronto Mississauga, 3359 Mississauga Road North, Rm 4023C, Mississauga, ON L5L 1C6, Canada
e-mail: robert_gerlai@yahoo.com

S. Shams · R. Gerlai
Cell and Systems Biology, University of Toronto, Toronto, Canada

**Keywords** Acute and chronic alcohol · Ethanol · Ethyl alcohol · Zebrafish · Neurochemistry

## Introduction

The zebrafish has been increasingly employed in neurobehavioral genetics research. For example, it has been proposed as a tool for the analysis of the effects of alcohol on brain function and behavior (Gerlai et al. 2000).

Both acute and chronic alcohol exposures were found to alter the behavior of adult zebrafish (Gerlai et al. 2000, 2006, 2008, 2009; Dlugos and Rabin 2003; Echevarria et al. 2010; also see Echevarria et al. 2011 for recent review). For example, we employed a chronic × acute between-subject experimental design (Gerlai et al. 2009) and found alcohol-induced impairment of behavioral responses of zebrafish to conspecific images (Gerlai et al. 2009). Furthermore, we demonstrated adaptation to alcohol after chronic exposure and also observed alcohol withdrawal-induced behavioral impairments including reduced or abolished responses to social stimuli (Gerlai et al. 2009). These changes were found robust in one quasi-inbred zebrafish strain, AB, but not in a genetically heterogeneous population called SF. We have also investigated changes in the levels of neurotransmitters dopamine and serotonin and their metabolites DOPAC and 5HIAA, and found significant alcohol effects but with dose response curves idiosyncratic for the alcohol treatment and population used (Gerlai et al. 2009). Although AB fish appeared more responsive to alcohol treatment compared to SF fish both at the level of behavior and neurochemistry, a consistent correlation between these responses could not be established, partly because the neurochemical analysis was not fully randomized. The lack of good correlation also

implied that there may be mechanisms in addition to the studied neurochemicals that may underlie the observed population-dependent alcohol effects.

In the current study, we characterize such possible mechanisms by expanding our analysis to a broader range of neurochemicals. Now, we analyze the level of nine neurochemicals using a fully randomized 2 × 3 (chronic × acute) alcohol exposure between-subject experimental design that resembles the one we used before in the analysis of behavioral responses (Gerlai et al. 2009) but one which has not been conducted for these biological targets. In addition, we also analyze the activity of two enzymes important in aminergic neurotransmission in the brain: tyrosine hydroxylase (TH) and MAO. TH is responsible for catalyzing the conversion of the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), a rate limiting step in the synthesis of dopamine. MAO catalyzes the oxidative deamination of monoamines and as such it is crucial in the metabolic inactivation of aminergic neurotransmitters.

The experimental design employed in the current paper allows us to study, for example, acute alcohol and chronic alcohol exposure-induced adaptation and also possible alcohol withdrawal-induced changes, respectively, now at the level of neurochemistry and enzymatic activity. Furthermore, comparison of the zebrafish populations, AB and SF, also allows us to investigate potential genetic differences in the above traits, a useful first step towards future genetic analyses and also for other approaches including psychopharmacological or drug screening studies.

## Methods

### Animals and housing

Sexually mature 8-month-old zebrafish (*Danio rerio*, 50–50 % males and females) of the AB strain and of the short fin wild type (SF) population were tested (for sample sizes see figure legends). All fish were bred, raised and tested in the vivarium of the University of Toronto Mississauga (Mississauga, Ontario, Canada) under identical conditions, i.e., in the same room, in adjacent tanks with identical fish density, water chemistry, filtration, lighting, heating, feeding and all other environmental parameters, which were described in detail in Gerlai et al. (2009). The rationale for the choice of the zebrafish populations, origin and maintenance is also described in detail elsewhere (Gerlai et al. 2009). Briefly, the AB strain, established over four decades ago, is one of the genetically most well-characterized zebrafish strains which shows homozygosity at more than 80 % of its loci (Lockwood et al. 2004; Guryev et al. 2006). The SF population is also a wild type group but one that was established only about 3 years before the start of this study in our own vivarium (Gerlai et al. 2009). This population was chosen as it may represent the prototypical zebrafish because due to its recently started laboratory breeding and its origin (large commercial breeding facility near the geographic origin of the species) it is expected to have a high heterozygosity ratio and it is thus not expected to exhibit random allele fixation (genetic drift)-induced phenotypical idiosyncrasies. The AB strain and the SF population are referred to as "populations" in this paper.

### Experimental design

A 2 × 3 × 2 (chronic alcohol × acute alcohol × population) fully randomized between-subject experimental design was employed as follows. First, all fish were exposed to a chronic dosing procedure as described in detail elsewhere (Gerlai et al. 2009). Briefly, half of the fish received an escalating alcohol concentration with 0.125 % (vol/vol %) concentration increments once every 4 days (total dose escalation exposure time was 12 days) until reaching the final dose of 0.5 %, at which fish were maintained for an additional 10 days. That is, fish were kept in alcohol solution in their home tank for a total of 22 days as described before (Gerlai et al. 2009). The rationale for the dose escalation has been given elsewhere (Gerlai et al. 2009) but briefly it allowed us to expose the fish to the final dose of 0.5 % alcohol with no mortality or morbidity. As a control, 50 % of our experimental fish received exactly the same maintenance procedure as the chronic alcohol exposed animals but these control fish were not exposed to alcohol (0.0 % chronic dose). Following the chronic dosing procedure fish were randomly assigned to one of three groups and received the acute alcohol treatment, a 1-h long immersion in either 0.0 % alcohol (freshwater), 0.5 % alcohol or 1.0 % alcohol. The rationale for the length of this acute exposure treatment was as follows. We have previously employed the 1-h long immersion in our acute alcohol studies (Gerlai et al. 2000, 2009). Also notably, neurochemical responses to visual stimuli or to immersion in alcohol solution were found to be rapid (Saif et al. 2013). For example, the trajectory of temporal changes in dopamine and serotonin levels induced by acute alcohol treatment closely mimicked the rise of blood alcohol content in the brain of zebrafish (Chatterjee and Gerlai 2009; Dlugos and Rabin 2003) and showed that when alcohol was detectable in the brain (after about 10–15 min) significant neurochemical responses also started to occur. In summary, we expect the 1-h long alcohol immersion to have a robust and measurable effect on neurochemical levels in the zebrafish brain.

Also, importantly, a similar chronic × acute experimental design (but with four instead of the current three

acute doses) was previously used for the analysis of alcohol effects on behavior of zebrafish (Gerlai et al. 2009). However, this fully randomized design has not yet been employed for the quantification of neurochemical responses, the goal of the current study. We chose sample sizes based upon preliminary studies that showed only small variation in the populations and measured $n = 8$ or 7 for neurochemistry and enzymatic activity analysis per cell of the $2 \times 3 \times 2$ experimental design (for more details see below).

### Quantification of neurochemicals

We have selected nine neurochemicals to quantify: dopamine, DOPAC (3,4-dihydroxyphenylacetic acid, dopamine's metabolite), serotonin, 5HIAA (5-Hydroxyindoleacetic acid, serotonin's metabolite), glutamate (glutamic acid), GABA (gamma aminobutyric acid), aspartate, glycine and taurine. The rationale for the choice of these neurochemicals was as follows. The dopaminergic and serotoninergic neurotransmitter systems have been demonstrated to be involved in mediating alcohol's actions in the brain (Crabbe et al. 1996, 2006; Di Chiara and Imperato 1988; Gessa et al. 2004; Lovinger and Zhou 1994; Lovinger 1999; Rodd-Henricks et al. 2000; Sari et al. 2006; Spanagel and Weiss 1999; Thielen et al. 2004) and levels of dopamine, serotonin, DOPAC and 5HIAA have been found to respond to alcohol treatment in the adult as well as in the developing zebrafish (Gerlai et al. 2009; Buske and Gerlai 2011). Acute alcohol exposure inhibits glutamate receptors, e.g., the NMDA-R, while chronic alcohol exposure reduces brain levels of glutamate (Thoma et al. 2011). GABA has been implicated in alcohol-induced functional changes in the brain both in rodents (Feller et al. 1988) and in humans (Maccioni and Colombo 2009). Reduced aspartate levels have been associated with the chronic use of alcohol in humans (Licata and Renshaw 2010). Glycine receptors, which are ligand-gated chloride channels, are the primary targets of alcohol (Vengeliene et al. 2008). Taurine and other related molecules, e.g., the homotaurine-derivative acamprosate (calcium acetylhomotaurinate), can reduce alcohol self-administration and relapse to drinking in both animals and humans (Olive 2002), and taurine has been found to exert protective effects against alcohol in both zebrafish and other vertebrates (Rosemberg et al. 2012).

Methodological details of the quantification of the above neurochemicals in zebrafish have been described elsewhere (Chatterjee and Gerlai 2009; Pan et al. 2012). Briefly, the sampling and analysis procedures were conducted fully randomized across the $2 \times 3 \times 2$ groups. Zebrafish were decapitated and their brains were dissected and sonicated. We did not attempt to obtain brain region-specific samples because the in vivo or ex vivo neuroanatomical landmarks are not well developed for the zebrafish and because the HPLC methods are also not worked out for small tissue samples with this species (Chatterjee and Gerlai 2009). The sonicates (each representing a single zebrafish) were centrifuged and the supernatant was analyzed with high-precision liquid chromatography (HPLC) using a BAS 460 MICROBORE-HPLC system with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, IN) together with a Uniget C-18 reverse phase microbore column as the stationary phase (BASi, Cat no. 8949 for dopamine and serotonin and their metabolites; Cat no. 8912 for amino acids). Standard neurochemicals (Sigma-Aldrich, Oakville, ON, Canada) were used to quantify and identify the peaks on the chromatographs. Results are expressed as nanogram (ng) of neurochemical per milligram (mg) of total brain protein for dopamine and serotonin and their metabolites and pmol/mg protein for amino acids.

### Enzyme essays

Numerous aminergic neurotransmitters and their metabolites were found to respond to alcohol treatment and thus in addition to measuring their levels, the activity of two key enzymes associated with these neurotransmitters, TH and monoamine oxidase (MAO) was also evaluated from a set of fish exposed to chronic and acute alcohol as described above. TH activity was evaluated from whole brain samples according to McGeer et al. (1967). TH catalyzes the conversion of the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and we measured the amount of the latter to quantify the activity of this enzyme. Each brain was homogenized in 100 µl of ice-cold 0.25 M sucrose solution and 30 µl of the homogenate was used for the assay. The product of TH activity, 3,4-dihydroxyphenylalanine, was measured colorimetrically according to Arnow (1937). The results are expressed as nmol of 3,4-dihydroxyphenylalanine formed/µg protein/30 min.

MAO activity was assayed according to Anichtchik et al. (2006). Briefly, each zebrafish brain was homogenized in 100 µl of ice-cold buffer containing 10 mM potassium phosphate, 1 mM EDTA, at pH 7.6 and centrifuged at 5,000 rpm for 5 min. The supernatants were assayed for MAO activities using the color developing reagent containing 1 mM vanillic acid, 500 µM 4-aminoantipyrine and 4U/ml horse radish peroxidase (type II) in 0.2 M potassium phosphate buffer, pH 7.6. After incubation for 1 h, the O.D. at 492 nm was measured and the results were expressed as O.D. at 492/mg protein/h.

### Statistical analysis

Data were analyzed using SPSS (14.0) statistical software. We conducted parametric statistical tests. These tests are

**Table 1** Variance analysis (ANOVA) for the neurochemicals tested revealed several significant effects including that of acute and chronic alcohol treatment and that of population

| ANOVA terms (main factors and interaction terms) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Neurochemica | Population | Acute alcohol | Chronic alcohol | Population × acute | Population × chronic | Acute × chronic | Pop × acute × chronic |
| Dopamine | $F(1,84) = 89.92$, $p < 0.001$ | $F(2,84) = 14.83$, $p < 0.00$ | $F(1,84) = 92.19$, $p < 0.001$ | $F(2,84) = 42.67$, $p < 0.001$ | $F(1,84) = 9.32$, $p < 0.01$ | $F(2,84) = 135.99$, $p < 0.001$ | $F(2,84) = 52.70$, $p < 0.001$ |
| DOPAC | $F(1,84) = 134.9$, $p < 0.001$ | $F(2,84) = 22.19$, $p < 0.001$ | $F(1,84) = 161.6$, $p < 0.001$ | $F(2,84) = 58.47$, $p < 0.001$ | $F(1,84) = 3.11$, $p > 0.05$ | $F(2,84) = 14.08$, $p < 0.001$ | $F(2,84) = 39.90$, $p < 0.001$ |
| Aspartate | $F(1,84) = 314.0$, $p < 0.001$ | $F(2,84) = 334.2$, $p < 0.001$ | $F(1,84) = 380.7$, $p < 0.00$ | $F(2,84) = 39.48$, $p < 0.001$ | $F(1,84) = 3.29$, $p > 0.05$ | $F(2,84) = 6.21$, $p < 0.01$ | $F(2,84) = 0.22$, $p > 0.05$ |
| Serotonin | $F(1,84) = 104.6$, $p < 0.001$ | $F(2,84) = 9.37$, $p < 0.001$ | $F(1,84) = 101.5$, $p < 0.001$ | $F(2,84) = 64.75$, $p < 0.001$ | $F(1,84) = 14.41$, $p < 0.001$ | $F(2,84) = 254.5$, $p < 0.001$ | $F(2,84) = 39.12$, $p < 0.001$ |
| 5HIAA | $F(1,84) = 82.04$, $p < 0.001$ | $F(2,84) = 14.80$, $p < 0.001$ | $F(1,84) = 46.40$, $p < 0.001$ | $F(2,84) = 28.81$, $p < 0.001$ | $F(1,84) = 3.50$, $p > 0.05$ | $F(2,84) = 178.0$, $p < 0.001$ | $F(2,84) = 20.25$, $p < 0.001$ |
| Taurine | $F(1,84) = 478.5$, $p < 0.001$ | $F(2,84) = 0.83$, $p > 0.05$ | $F(1,84) = 4.98$, $p < 0.05$ | $F(2,84) = 93.28$, $p < 0.001$ | $F(1,84) = 170.9$, $p < 0.001$ | $F(2,84) = 6.20$, $p < 0.01$ | $F(2,84) = 25.71$, $p < 0.001$ |
| GABA | $F(1,84) = 1{,}394.0$, $p < 0.001$ | $F(2,84) = 1.10$, $p > 0.05$ | $F(1,84) = 3.41$, $p > 0.05$ | $F(2,84) = 142.4$, $p < 0.001$ | $F(1,84) = 197.2$, $p > 0.05$ | $F(2,84) = 1.91$, $p > 0.05$ | $F(2,84) = 41.22$, $p < 0.001$ |
| Glutamate | $F(1,84) = 124.9$, $p < 0.001$ | $F(2,84) = 115.1$, $p < 0.001$ | $F(1,84) = 153.1$, $p < 0.001$ | $F(2,84) = 17.13$, $p < 0.001$ | $F(1,84) = 13.57$, $p < 0.001$ | $F(2,84) = 4.53$, $p < 0.05$ | $F(2,84) = 2.33$, $p > 0.05$ |
| Glycine | $F(1,84) = 238.7$, $p < 0.001$ | $F(2,84) = 41.37$, $p < 0.001$ | $F(1,84) = 64.10$, $p < 0.001$ | $F(2,84) = 31.36$, $p < 0.001$ | $F(1,84) = 102.1$, $p < 0.001$ | $F(2,84) = 10.70$, $p < 0.001$ | $F(2,84) = 1.31$, $p > 0.05$ |

Several interactions among these factors were also found significant. The neurochemicals tested are listed in the first column. The ANOVA factors (independent variables) are indicated above each column. $F$ values are shown with degrees of freedom indicated in brackets. $p > 0.05$ represents non-significant results

known to be fairly insensitive to the violation of the variance homogeneity and normal distribution criteria especially when sample sizes of the analyzed groups are similar, i.e., differ by a ratio less than two to one (Pagano 1990; May et al. 1989; Glass et al. 1972). Our sample sizes were identical across all treatment groups ($n = 8$ for the neurochemical analyses and $n = 7$ for the enzyme analyses). The normality of distribution cannot be reliably checked when sample sizes are this small and in this case visual inspection of data distribution is recommended. Also notably, the normality of distribution criterion is considered not crucial as long as the distribution appears unimodal and there are no obvious outliers (Pagano 1990; May et al. 1989; Glass et al. 1972). We found group variances to be homogeneous and the data appeared normally distributed (unimodal and apparently symmetrical distribution). Therefore, parametric statistical tests were employed. First, univariate variance analyses (ANOVAs) were performed with 'acute alcohol' (3 levels), 'chronic alcohol' (2 levels) and 'population' (2 levels) as between-subject factors. We report the results of these analyses in Table 1. In case of significant main effects or interaction terms, the data were further analyzed using post hoc univariate one-factorial ANOVA for specific factors followed by Tukey Honestly Significant Difference (HSD) tests, a multiple comparison test method designed to reduce type I error. We report the results of the Tukey HSD test in the text.

## Results

Quantification of neurochemicals demonstrated an apparently robust alcohol treatment effect that was dependent upon the population origin of the fish (Fig. 1). This observation was confirmed by ANOVA (for detailed statistical results see Table 1), which found a significant acute alcohol treatment effect for all neurochemicals measured except for GABA and Taurine. The chronic alcohol treatment effect was found significant for all neurochemicals except for GABA. Significant population differences were revealed in all neurochemicals tested. Acute × chronic treatment interaction was found significant for all neurochemicals, except for GABA. Acute treatment × population interaction was found significant for all neurochemicals tested. Chronic treatment × population interaction was found significant for most neurochemicals, except for DOPAC, 5HIAA, and aspartate. The triple interaction term (acute treatment × chronic treatment × population) was also significant for the majority of neurochemicals measured except for glutamate, glycine and aspartate. These results confirmed our expectations about the chronic alcohol pre-treatment having an effect on how subsequent acute alcohol treatment influences neurochemistry of the zebrafish brain and also demonstrated that the combined alcohol effect depended upon the population of the fish (AB vs. SF).

First, we examine the effect of acute alcohol treatment alone, i.e., the effect of acute alcohol treatment that was preceded by chronic freshwater exposure (Fig. 1, bars corresponding to 0.0 % chronic alcohol dose). Acute alcohol treatment alone significantly increased levels of dopamine, DOPAC, serotonin and 5HIAA (Fig. 1a, b, d and e, first three bars on each panel) in a linear dose-dependent manner in AB fish [Tukey HSD test showed all three dose groups to significantly ($p < 0.05$) differ from each other for each of these neurochemicals]. Importantly, such a linear dose response was not seen in SF population. In these latter fish the acute alcohol-induced dose response curve appears inverted U-shaped for the above neurochemicals (Fig. 1a, b, d and e, 7th to 9th bars) with the intermediate acute dose having the largest value and being significantly different from the other two doses (Tukey HSD, $p < 0.05$). Examination of the effect of acute alcohol treatment (no prior chronic alcohol) on the other neurochemicals showed that these responded differently compared to dopamine, DOPAC, serotonin and 5HIAA. While these latter four neurochemicals showed a linear increase with acute dose, aspartate, taurine, GABA, glutamate and glycine exhibited a significant dose-dependent (linear) decrease in their levels in AB fish in response to acute alcohol treatment (Fig. 1c, f, g, h and i, first three bars on each panel); (Tukey HSD showed each dose to significantly ($p < 0.05$) differ from the another). Notably, the effect of acute alcohol treatment without prior chronic alcohol exposure was similar between AB and SF for aspartate (Fig. 1c) and glutamate (Fig. 1h) in the sense that these neurochemicals showed a significant dose-dependent decrease in both populations. Interestingly, however, such similarity was not observed between AB and SF for taurine or GABA. These two neurochemicals responded the opposite way to acute alcohol alone in these populations, with SF fish showing a significant dose-dependent increase (Tukey HSD, each dose differs from the other significantly, $p < 0.05$) and AB fish showing a decrease. Also, notably, while glycine was found to respond with a significant decrease in their levels to acute alcohol treatment in AB fish, SF fish showed no appreciable change (Tukey HSD found no significant dose differences, $p > 0.05$) in response to acute alcohol treatment alone in this neurochemical.

Next, we examine the effect of chronic alcohol treatment, i.e., how it may have affected the subsequent acute alcohol treatment-induced changes in the two zebrafish populations. After chronic alcohol exposure both AB and SF zebrafish appeared to respond to the acute exposure treatment differently as compared to those that did not

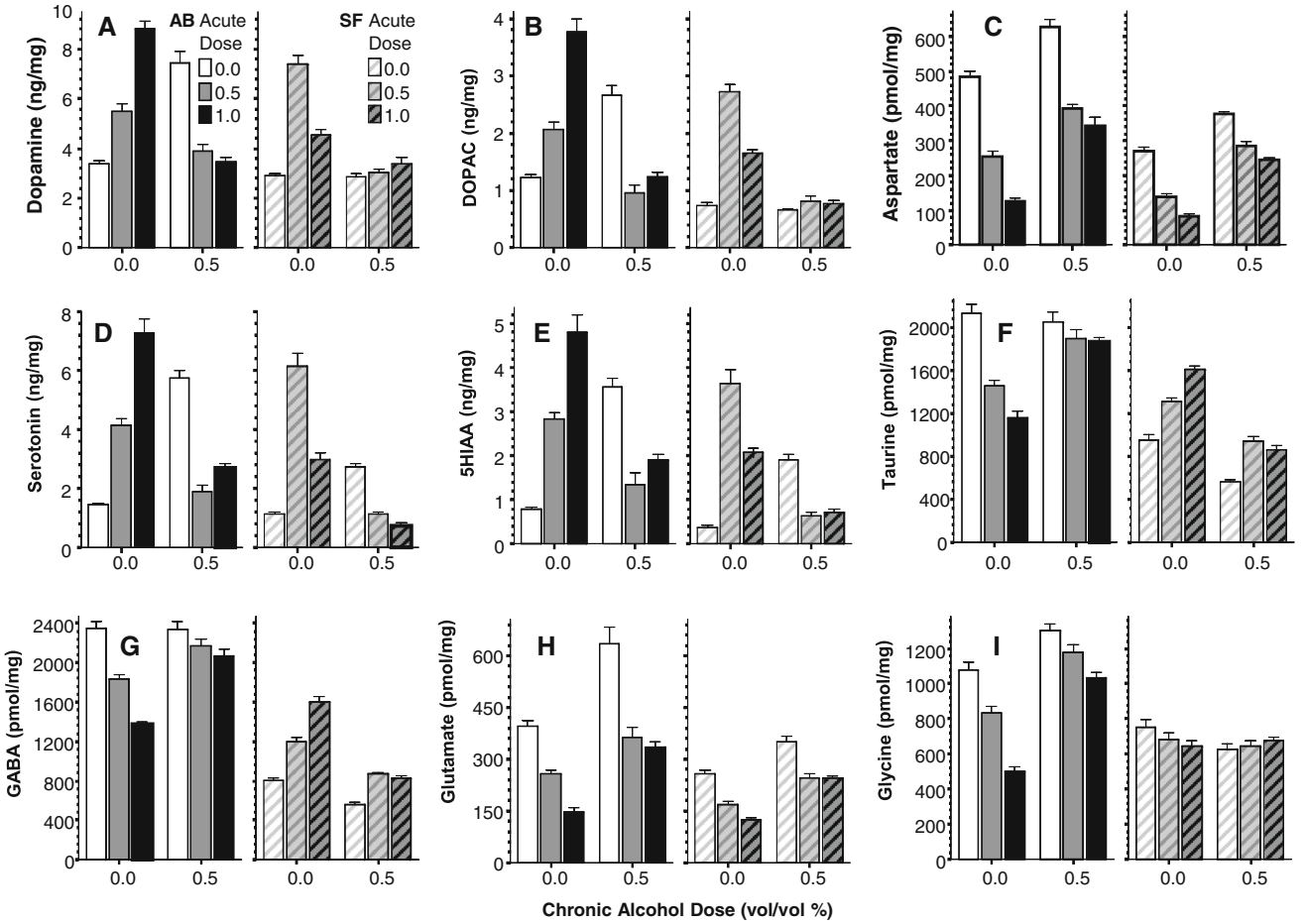


**Fig. 1** Chronic and acute alcohol administration induced changes in neurotransmitters and their metabolites are often population dependent. The quantified weight of neurochemical is expressed relative to total brain protein weight (ng/mg). Mean ± SEM are shown. Sample sizes were $n = 8$ for all groups. Dopamine (**a**), DOPAC (**b**), Aspartate (**c**), Serotonin (**d**), 5HIAA (**e**), Taurine (**f**), GABA (**g**), Glutamate (**h**), Glycine (**i**) are shown. Each panel consists of two sets of graphs. The first set (*solid bars*) shows the results obtained for zebrafish of the AB strain. The second set (*striped bars*) shows the results obtained for zebrafish of the SF population. The concentration of alcohol administered during the chronic pre-treatment is shown on the *X*-axis (with 0.0 representing freshwater control). The concentration of alcohol employed during the 1-h long acute treatment, which was administered immediately after the chronic pre-treatment procedure, is shown: *solid white* (AB) or light grey striped (SF) represents 0.0 % acute alcohol, *solid grey* (AB) or *striped darker* grey (SF) represents 0.5 % acute alcohol, and *solid black* (AB) or *dark grey striped* (SF) represents 1.0 % acute alcohol concentration. For details of methods, statistical analyses and interpretation of results see text

receive the chronic alcohol pre-exposure. But it is also notable that the effect of the chronic pre-treatment differed between the two populations. The pattern of acute alcohol responses after chronic alcohol pre-treatment is similar for dopamine, DOPAC, serotonin and 5HIAA in AB fish. All these neurochemicals showed a robust and significant increase in their levels in response to acute fresh water (i.e., to withdrawal from alcohol after chronic exposure) in AB fish (Tukey HSD, comparison of chronic 0.5 % treated AB fish: the acute 0 % significantly ($p < 0.05$) differs from the other two acute dose groups). However, this robust effect is absent for dopamine and DOPAC in SF fish (Fig. 1a, b, last three bars; Tukey HSD, $p > 0.05$) while still detectable for serotonin and 5HIAA in this population (Fig. 1d, e, last three bars, Tukey HSD, $p < 0.05$). It is also important to point out that AB zebrafish showed signs of adaptation to alcohol after chronic exposure, which is demonstrated by the lack of significant difference between those fish that were freshwater pre-exposed and subsequently acutely freshwater treated and those that received the chronic alcohol treatment followed by the same dose of acute alcohol (0.5 %) treatment. This lack of significant difference is observable in AB fish for dopamine (Fig. 1a, 1st and 5th bars), DOPAC (Fig. 1b 1st and 5th graph), serotonin (Fig. 1d, 1st and 5th graph), 5HIAA (Fig. 1e, 1st and 5th graph) and also for glutamate (Fig. 1h, 1st and 5th bar), for example. Also important to note that fish pre-treated with freshwater and exposed to 0.5 % alcohol showed higher dopamine, DOPAC, serotonin and 5HIAA levels as compared to those that were pre-exposed to chronic alcohol and

placed in the same alcohol dose (0.5 %) acutely. Adaptation to alcohol due to the chronic pre-treatment is also demonstrated by the blunted effect of the highest acute dose (1.0 %) of alcohol in the above neurochemicals. These observations were supported by Tukey HSD tests conducted for the comparison of three zebrafish groups: one, chronic 0.0 % acute 0.0 %; two, chronic 0.5 % acute 0.5 %; and three, chronic 0.5 % acute 1.0 %. These groups were found not to differ significantly ($p > 0.05$) for dopamine, DOPAC, serotonin, 5HIAA and glutamate in AB fish. The other neurochemicals, i.e., aspartate, taurine, GABA and glycine, also show that chronic alcohol pre-treatment blunts the effect of subsequent acute alcohol treatment in AB fish. Importantly, the above observations with regard to adaptation to alcohol held valid for SF fish too. Although occasionally slightly differently, the acute alcohol responses of SF fish were also blunted by prior chronic alcohol treatment, Tukey HSD test found essentially the same results. It showed that the chronic freshwater- and acute freshwater-treated groups did not significantly differ from the chronic alcohol- and acute alcohol-treated groups ($p > 0.05$) for dopamine, DOPAC, serotonin, 5HIAA, aspartate, taurine, GABA and glutamate.

We found significant population and alcohol administration regimen-dependent changes in the levels of dopamine and thus next we examined the activity of TH, a rate limiting enzyme involved in the synthesis of L-DOPA, the precursor of dopamine. Figure 2a shows that TH activity mirrored the changes we observed in dopamine and DOPAC. ANOVA (Table 2) confirmed this observation and revealed a significant acute alcohol treatment effect, a significant chronic alcohol treatment effect, and a significant population effect. Furthermore, all interaction terms were also found significant. Further analyses confirmed that the acute alcohol effects indeed were population and chronic alcohol treatment dependent. For example, fish of the AB strain not pre-treated with chronic alcohol (Fig. 2a, left bar graph, first set of three bars) showed a significant acute alcohol effect ($F(2, 18) = 111.006$, $p < 0.001$) whereby all treatment groups differed from each other (Tukey HSD, $p < 0.001$). SF population zebrafish not pre-treated with chronic alcohol (Fig. 2a, right bar graph, first set of three bars) also exhibited a significant acute alcohol effect (F2, 18) = 75.601, $p < 0.001$) but here, instead of the linear dose response, we found the intermediate dose (0.5 % alcohol) to have the strongest effect (all groups differed significantly ($p < 0.001$) from each other, Tukey HSD). Population × acute treatment interaction is also apparent after chronic alcohol treatment. ANOVA found a significant acute alcohol effect in AB fish ($F(2, 18) = 227.151$, $p < 0.001$; Fig. 2a, left bar graph, second set of three bars) and Tukey HSD showed that the freshwater group (withdrawal from chronic alcohol treatment) significantly

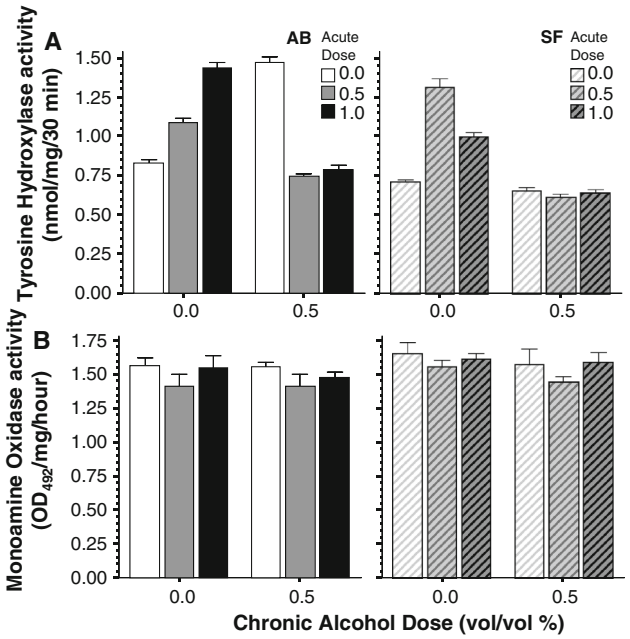


**Fig. 2** The effects of chronic and acute alcohol administration on Tyrosine hydroxylase (**a**) and Monoamine oxidase (**b**) enzyme activity in two zebrafish populations, AB and SF. Tyrosine hydroxylase activity is expressed as nmol of 3,4-dihydroxyphenylalanine per μg of total brain protein per 30 min. Monoamine oxidase activity is expressed as optical density (O.D.) at 492 nm wavelength per mg of brain protein per 1 h. Mean + SEM are shown. Each panel consists of two sets of graphs. The first set (*solid bars*) shows the results obtained for zebrafish of the AB strain. The second set (*striped bars*) shows the results obtained for zebrafish of the SF population. Sample sizes were ($n$) = 7 for each group (cell) of the 2 × 3 × 2 (chronic alcohol × acute alcohol × population) experimental design. Note that the differences among the alcohol treatment groups and populations in Tyrosine hydroxylase activity are very similar to those obtained for dopamine (and DOPAC). Also note that MAO shows no such differences. For details of the quantification and analysis of the enzymatic activity see "Methods". For details of the differences across treatment groups see "Results"

($p < 0.001$) differed from the acute alcohol-treated groups. Importantly, in SF zebrafish, the acute freshwater or alcohol treatment had no significant effect after the chronic alcohol exposure (ANOVA $F(2. 18) = 1.181$, $p > 0.30$; Fig. 2a, right bar graph, second set of three bars).

As shown earlier, significant population-dependent alcohol treatment effects were also found in the levels of metabolites of dopamine and serotonin. MAOs catalyze the oxidative deamination of monoamines, including serotonin and dopamine. Therefore, we examined the activity of MAOs. We observed no robust population or alcohol effects (Fig. 2b). ANOVA (Table 2) partially confirmed this observation and found all effects non-significant except the main effect of acute alcohol treatment. Post hoc Tukey HSD showed that this effect was due to the activity of MAO in the 0.5 % alcohol-treated groups being significantly ($p < 0.05$) less compared to the acute freshwater groups.

**Table 2** Variance analysis (ANOVA) of the data representing tyrosine hydroxylase (TH) enzyme activity revealed significant effects of acute and chronic alcohol treatment and of population

| ANOVA terms (main factors and interaction terms) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Enzyme | Population | Acute alcohol | Chronic alcohol | Population × acute | Population × chronic | Acute × chronic | Pop × acute × chronic |
| TH | $F(1,72) = 209.8$, $p < 0.001$ | $F(2,72) = 3.28$, $p < 0.05$ | $F(1,72) = 216.6$, $p < 0.001$ | $F(2,72) = 83.53$, $p < 0.001$ | $F(1,72) = 59.6$, $p < 0.001$ | $F(2,72) = 266.7$, $p < 0.001$ | $F(2,72) = 78.01$, $p < 0.001$ |
| MAO | $F(1,72) = 3.80$, $p > 0.05$ | $F(2,72) = 3.82$, $p < 0.05$ | $F(1,72) = 1.49$, $p > 0.05$ | $F(2,72) = 0.08$, $p > 0.05$ | $F(1,72) = 0.28$, $p > 0.05$ | $F(2,72) = 0.01$, $p > 0.05$ | $F(2,72) = 0.40$, $p > 0.05$ |

All interaction terms among these factors were also found significant. The effects of these factors and the interactions among them were found non-significant for monoamine oxidase (MAO) activity. The ANOVA factors (independent variables) are indicated above each column. *F* values are shown with degrees of freedom indicated in brackets. $p > 0.05$ represents non-significant results

## Discussion

We uncovered significant interactions between the effects of chronic and acute alcohol treatment that manifested in a population-dependent manner in zebrafish. Behind the numerous significant findings some important patterns may be noted. The first one we discuss concerns the population differences found in response to acute alcohol treatment. Notably, alcohol naïve AB zebrafish responded to acute alcohol treatment with robust changes in all neurochemicals studied and the dose response curves were different from those seen in SF fish. For example, while dopamine, DOPAC, serotonin and 5HIAA showed a linear dose-dependent increase in the AB strain, the SF population exhibited an inverted U-shaped dose response. Also, while alcohol naïve AB zebrafish showed a linear dose-dependent decrease of the levels of aspartate, taurine, GABA, glutamate and glycine in response to acute alcohol treatment, SF fish showed the opposite, a linear dose-dependent increase in two of these neurochemicals (taurine and GABA). In addition to these striking population-dependent acute alcohol treatment-induced differences, we also noted a marked difference between the populations in the effect of withdrawal from alcohol after chronic alcohol exposure. While AB fish showed a robust increase of the levels of dopamine, DOPAC, serotonin, 5HIAA and glutamate, and to a certain degree also of aspartate and glycine in response to alcohol withdrawal, SF fish showed such alcohol withdrawal effects only in the latter three neurochemicals but not in dopamine and DOPAC. Last, but also notably, development of tolerance to alcohol after chronic exposure to this substance appeared similar between the two populations. In summary, the pattern of neurochemical results suggests the two populations differ in their baseline (no-alcohol) state as well as in the way acute alcohol and acute withdrawal from alcohol affects them. Whether these differences are mechanistically related to each other or represent spurious gene associations could be ascertained using a segregating generation of hybrids (for example an F2 generation) between the two populations.

Examination of the activity of two enzymes, TH and MAO, showed that the former was likely to be responsible for some of the alcohol treatment-dependent population differences. The pattern of differences among the groups in activity of TH was practically identical to what we found for dopamine as well as for DOPAC. Thus, we conclude that the group differences in dopamine were likely the result of differences in dopamine synthesis. We also examined whether differential metabolism of dopamine and serotonin may explain the group differences in these neurotransmitters and in their metabolites. However, we found the activity of MAO not to follow the pattern of differences seen among our treatment groups and thus

conclude that this enzyme may not be behind the observed strain-dependent alcohol effects.

We also note a general pattern of findings that is in line with what is known about the phenomenon known as hybrid vigor: finding SF fish less affected by the highest doses of acute alcohol and by acute withdrawal from alcohol after chronic exposure demonstrates phenotypical "buffering" against the effects of alcohol. This buffering effect is likely the result of reduced probability of deleterious recessive alleles being in a homozygous form in this highly heterozygous population. One way such buffering may occur is via compensatory gene expression changes, a hypothesis that may be tested in the future by comparing AB and SF zebrafish using comprehensive DNA microarrays. Using a DNA microarray we previously uncovered almost 2,000 differentially expressed genes, many of which with no known function, responding to chronic alcohol exposure (Pan et al. 2011). Others also found a large number of genes (several hundred) to show differential expression in response to a fluctuating chronic alcohol exposure regimen (Kily et al. 2008 ). These results underscore the conclusion made by others before: alcohol may interact with a large number of biochemical targets (Vengeliene et al. 2008).

Can the observed neurochemical changes induced by alcohol in the two zebrafish populations explain the behavioral effects of alcohol previously published (Gerlai et al. 2009)? Correlations between the previous and our current study are quite apparent. For example, previously we found alcohol naïve zebrafish of the AB strain to respond to acute alcohol treatment with robust impairment of a shoaling response (Gerlai et al. 2009). But the acute effects of the very same alcohol doses were much less robust in SF zebrafish, a finding that mimics what we obtained with neurochemicals here. Also, while the effect of acute withdrawal from alcohol after chronic treatment was highly disruptive, leading to impaired shoaling responses, in AB, this effect was absent in SF. These latter fish continued to shoal normally during acute withdrawal from alcohol, a set of results that again corresponds to what we found with neurochemicals here. Last, significant adaptation, i.e. tolerance to alcohol after chronic exposure, was observed in both populations in their behavior, a finding that parallels our neurochemical results. Although the questions as to which neurochemicals are involved or which ones are most crucial in these behavioral responses to alcohol, and also what biochemical processes may underlie these changes cannot be answered at this point, the current results now allow us to start formulating working hypotheses. For example, the dopaminergic system plays roles in reward and in the development of alcohol addiction in mammals (Setini et al. 2005; Spanagel and Weiss 1999). The dose-dependent increase of dopamine levels in response to acute treatment in AB but not in SF, and the large dopamine level increase in response to acute withdrawal from alcohol after chronic exposure seen in AB but not in SF now warrant detailed follow-up analyses. One may employ a psychopharmacology analysis with dopamine receptor selective drugs to study the involvement of these receptors or initiate a neuroanatomical analysis (immunohistochemistry or in situ hybridization) to identify particular dopaminergic targets and how and in which brain regions they respond to alcohol differently in the two zebrafish populations. One may also study biochemical mechanisms upstream or downstream of dopamine receptors. These studies only represent a subset of possible directions one may take. Similar studies will be conducted to study the involvement of other neurotransmitter systems too. Given the varied effects, one may also suggest a comprehensive transcriptome analysis using DNA microarrays with the $2 \times 3 \times 2$ experimental design.

In closing, we argue that the current study may be viewed as a proof of principle. Zebrafish strains are rarely compared for potential differences in their responses to alcohol. This is the first study in which the effects of chronic and acute alcohol exposure have been tested in a systematic and randomized manner on neurochemistry. Our results demonstrate that characterization of alcohol responses in zebrafish populations can yield positive results. Characterization of zebrafish strains will be useful for two principally different reasons. One, discovery of significant strain differences will allow one to conduct quantitative trait locus analyses to identify loci of genes underlying natural genetic variation in the chosen alcohol related traits. Two, identification of genetically distinct strains that show lack of significant genetic variation in the chosen alcohol related traits will allow one to use these strains as the mutation host and mapping strains of random mutagenesis (forward genetic) studies. In addition to these genetic approaches, well-characterized strains will also facilitate choosing the right genotype for psychopharmacology analyses or drug screens.

**Acknowledgments** Supported by an NIH/NIAAA R01 grant to RG.

**Conflict of interest** The authors declare no conflict of interest of any sort.

## References

Anichtchik O, Sallinen V, Peitsaro N, Panula P (2006) Distinct structure and activity of monoamine oxidase in the brain of zebrafish (*Danio rerio*). J Comp Neurol 498:593–610

Arnow LE (1937) Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixture. Biol Chem 118:531–537

Buske C, Gerlai R (2011) Early embryonic ethanol exposure impairs shoaling and the dopaminergic and serotoninergic systems in adult zebrafish. Neurotoxicol Teratol 33:698–707

Chatterjee D, Gerlai R (2009) High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. Behav Brain Res 200:208–213

Crabbe JC, Phillips TJ, Harris RA, Arends MA, Koob GF (2006) Alcohol-related genes: contributions from studies with genetically engineered mice. Addict Biol 11:195–269

Crabbe JC, Phillips TJ, Feller DJ, Hen R, Wenger CD, Lessov CN, Schafer GL (1996) Elevated alcohol consumption in null mutant mice lacking 5-HT1B serotonin receptors. Nat Genet 14:98–101

Di Chiara G, Imperato A (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci USA 85:5274–5278

Dlugos CA, Rabin RA (2003) Ethanol effects on three strains of zebrafish: model system for genetic investigations. Pharm Biochem Behav 74:471–480

Echevarria DJ, Toms CN, Jouandot DJ (2011) Alcohol-induced behavior change in zebrafish models. Rev Neurosci 22:85–93

Echevarria DJ, Hammack CM, Jouandot DJ, Toms CN (2010) Does acute alcohol exposure modulate aggressive behaviors in the zebrafish (*Danio rerio*), or is the bark worse that the bite? Int J Comp Psychol 23:62–69

Feller DJ, Harris RA, Crabbe JC (1988) Differences in GABA activity between ethanol withdrawal seizure prone and resistant mice. Eur J Pharmacol 157:147–154

Gerlai R, Chatterjee D, Pereira T, Sawashima T, Krishnannair R (2009) Acute and chronic alcohol dose: population differences in behavior and neurochemistry of zebrafish. Genes Brain Behav 8:586–599

Gerlai R, Prajapati S, Ahmad F (2008) Differences in acute alcohol induced behavioral responses among zebrafish populations. Alcohol Clin Exp Res 32:1763–1773

Gerlai R, Lee V, Blaser R (2006) Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (*Danio rerio*). Pharmacol Biochem Behav 85:752–761

Gerlai R, Lahav M, Guo S, Rosenthal A (2000) Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacol Biochem Behav 67:773–782

Gessa GL, Serra S, Vacca G, Carai MA, Colombo G (2004) Suppressing effect of the cannabinoid CB1 receptor antagonist, SR147778, on alcohol intake and motivational properties of alcohol in alcohol-preferring sP rats. Alcohol 40:46–53

Glass GV, Peckham PD, Sanders JR (1972) Consequences of failure to meet the assumptions underlying the use of analysis of variance and covariance. Rev Ed Res 42:237–288

Guryev V, Koudijs MJ, Berezikov E, Johnson SL, Plasterk RH, van Eeden FJ, Cuppen E (2006) Genetic variation in the zebrafish. Genome Res 16:491–497

Kily LJ, Cowe YC, Hussain O, Patel S, McElwaine S, Cotter FE, Brennan CH (2008) Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways. J Exp Biol 211:1623–1634

Licata SC, Renshaw PF (2010) Neurochemistry of drug action: insights from proton magnetic resonance spectroscopic imaging and their relevance to addiction. Ann N Y Acad Sci 1187:148–171

Lockwood B, Bjerke S, Kobayashi K, Guo S (2004) Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening. Pharmacol Biochem Behav 77:647–654

Lovinger DM (1999) 5-HT3 receptors and the neural actions of alcohols: an increasingly exciting topic. Neurochem Int 35:125–130

Lovinger DM, Zhou Q (1994) Alcohols potentiate ion current mediated by recombinant 5-HT3RA receptors expressed in a mammalian cell line. Neuropharmacology 33:1567–1572

Maccioni P, Colombo G (2009) Role of the GABA(B) receptor in alcohol-seeking and drinking behavior. Alcohol 43:555–558

May RB, Masson MEJ, Hunter MA (1989) Application of statistics in behavioral research. Harper & Row Publishers, New York, pp 276–277

McGeer EG, Gibson S, McGeer PL (1967) Some characteristics of brain tyrosine hydroxylase. Can J Biochem 45:1557–1563

Olive MF (2002) Interactions between taurine and ethanol in the central nervous system. Amino Acids 23:345–357

Pagano RR (1990) Understanding statistics, 3rd edn. West Publishing Co, St, Paul. 348

Pan Y, Chaterjee D, Gerlai R (2012) Strain dependent gene expression and neurochemical levels in the brain of zebrafish: focus on a few alcohol related targets. Physiol Behav 107:773–778

Pan Y, Kaiguo M, Razak Z, Westwood JT, Gerlai R (2011) Chronic alcohol exposure induced gene expression changes in the zebrafish brain. Behav Brain Res 216:66–76

Rodd-Henricks ZA, McKinzie DL, Edmundson VE, Dagon CL, Murphy JM, McBride WJ, Lumeng L, Li TK (2000) Effects of 5-HT(3) receptor antagonists on daily alcohol intake under acquisition, maintenance, and relapse conditions in alcohol-preferring (P) rats. Alcohol 21:73–85

Rosemberg DB, Braga MM, Rico EP, Loss CM, Córdova SD, Mussulini BH, Blaser RE, Leite CE, Campos MM, Dias RD, Calcagnotto ME, de Oliveira DL, Souza DO (2012) Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol. Neuropharmacology 63:613–623

Saif M, Chatterjee D, Buske C, Gerlai R (2013) Sight of conspecific images induces changes in neurochemistry in zebrafish. Behav Brain Res 243:294–299

Sari Y, Bell RL, Zhou FC (2006) Effects of chronic alcohol and repeated deprivations on dopamine D1 and D2 receptor levels in the extended amygdala of inbred alcohol-preferring rats. Alcohol Clin Exp Res 30:46–56

Setini A, Pierucci F, Senatori O, Nicotra A (2005) Molecular characterization of monoamine oxidase in zebrafish (Danio rerio). Comp Biochem Physiol B: Biochem Mol Biol 140: 153–161

Spanagel R, Weiss F (1999) The dopamine hypothesis of reward: past and current status. Trends Neurosci 22:521–527

Thielen RJ, Engleman EA, Rodd ZA, Murphy JM, Lumeng L, Li TK, McBride WJ (2004) Ethanol drinking and deprivation alter dopaminergic and serotonergic function in the nucleus accumbens of alcoholpreferring rats. J Pharmacol Exp Ther 309:216–225

Thoma R, Mullins P, Ruhl D, Monnig M, Yeo RA, Caprihan A, Bogenschutz M, Lysne P, Tonigan S, Kalyanam R, Gasparovic C (2011) Perturbation of the glutamate-glutamine system in alcohol d.ependence and remission. Neuropsychopharmacology 36:1359–1365

Vengeliene V, Bilbao A, Molander A, Spanagel R (2008) Neuropharmacology of alcohol addiction. Br J Pharmacol 154: 299–315