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Strain dependent gene expression and neurochemical levels in the brain of zebrafish: Focus on a few alcohol related targets

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Abstract

The zebrafish is becoming increasingly popular in behavior genetics because it may allow one to conduct large scale mutation and drug screens facilitating the discovery of mechanisms of complex traits. Strain differences in adult zebrafish behavior have already been reported, which may have important implications in neurobehavioral genetics. For example, we have found the AB and SF strains to differ in their behavioral responses to both acute and chronic alcohol exposure. In the current study, we further characterize these strains using semi-quantitative RT-PCR to measure the expression of ten selected genes and HPLC to measure the levels of nine neurochemicals. We chose the target genes and neurochemicals based upon their potential involvement in alcohol and other drugs of abuse related mechanisms. We quantified the expression of the genes encoding D1-R, D2a-R, D4a-R dopamine receptors, $GABA_A$-R, $GABA_B$-R1, GAD1, MAO, NMDA-R (NR2D subunit), 5HT-R1bd and SLC6 a4a. We found the gene encoding D1 dopamine receptor over-expressed and the genes encoding $GABA_{B1}$ receptor and solute family carrier protein 6 (SLC6) 4a under-expressed in SF compared to AB. We also found the level of all (dopamine, DOPAC, Serotonin, GABA, Glutamate, Glycine, Aspartate, Taurine) but one (5HIAA) neurochemicals tested decreased in SF as compared to AB. These results, combined with previously identified behavioral differences between the AB and SF strains, demonstrate the importance of strain characterization in zebrafish. They now also allow formulation of working hypotheses about possible mechanisms underlying the differential effects of acute and chronic alcohol treatment on these two zebrafish strains.

Keywords

gene expression; neurotransmitters; RT-PCR; HPLC; strain differences; zebrafish

1. INTRODUCTION

The zebrafish has been argued to strike an optimal compromise between system complexity and practical simplicity [15]. While it shares numerous features, from neuroanatomy [46] to nucleotide sequence of genes [40], with those of higher order vertebrates including mammals, it is small (4 cm long) and easy to keep and breed in large numbers in the laboratory. Also importantly, the zebrafish has become one of the preferred model

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organisms of geneticists, and several forward and reverse genetic techniques have been developed for this species [15]. Due to these genetics tools, the zebrafish is becoming increasingly utilized in numerous fields of biology, including brain and behavior research.

In behavioral brain research studies, the genetic background of the individuals is important to consider [12]. In the rodent literature, one can enjoy a wealth of knowledge about features of different inbred and outbred strains. This knowledge is virtually nonexistent for zebrafish as the few strains and populations available for research have not been characterized with regard to their brain function or behavior. In this study, two populations of zebrafish, AB and short fin wild type (SF), were chosen for analysis. We refer to these populations as "strains" although it must be noted that the SF strain has not been genetically characterized and is expected to be genetically heterogeneous. SF originates from a breeding facility in Singapore near the natural geographic origin of zebrafish and in this facility the effective population size has been large. As such, the SF population has been argued to represent the prototypical zebrafish, a quasi-natural gene pool [13, 14]. On the other hand, the AB strain has been bred using closed sib-mating for four decades, has been shown to be homozygous at over 80% of its loci [19, 34] and has been genetically well characterized and often used in experimental studies [17]. Briefly, these two strains are expected to be genetically different at many of their loci and indeed, they have been found to perform differently in response to both acute [13] and chronic alcohol treatment [14]. Significant differences among other zebrafish strains have also been identified. For example, behavioral responses to drugs of abuse, including alcohol, have been reported [8, 13, 14, 18]. In this paper we continue the characterization of the two zebrafish strains, AB and SF, in which we discovered robust alcohol induced differences.

In our previous behavioral studies, using a social preference (shoaling response) paradigm, we discovered that fish of the AB strain are significantly affected by acute alcohol treatment [14]. These fish reduced their shoaling behavior in response to intermediate doses of alcohol (0.25-0.50%, vol/vol percentage), concentrations that had no sedative or motor function impairing effects. On the other hand, fish of the SF strain remained much less affected by these acute alcohol doses. Also importantly, the AB fish showed significant adaptation to alcohol after prolonged (3 week long) exposure to this substance. The development of tolerance was demonstrated by the absence of acute alcohol effects subsequent to the chronic exposure [14]. That is, acute alcohol could not diminish shoaling responses if administered after chronic treatment in AB fish. Notably, the AB fish also responded to withdrawal from chronic alcohol exposure with cessation of shoaling responses [14]. Interestingly, development of tolerance and the effect of withdrawal from alcohol after chronic treatment was either absent or was significantly blunted in SF fish [14]. The mechanisms underlying these differences are not known but given that the fish of these two strains were bred, raised, maintained and tested under identical conditions and at the same time and in a randomized manner, the strain specific alcohol effects were concluded to be due to the genetic differences between these strains [14].

In the current study, we decided to follow up on these promising behavioral findings by further characterizing the AB and SF strains. We chose ten genes whose expression levels we tested using semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and nine neurochemicals whose levels we tested using High Precision Liquid Chromatography (HPLC). The choice of these targets (genes and neurochemicals) was based upon their involvement in alcohol mediated effects in mammalian species as well as in zebrafish [4, 38], but it is notable that they represent only a very small fraction of possible molecular players involved [38].

Briefly, we chose a number of genes encoding different dopamine receptor subtypes because dopamine receptors in particular and the dopaminergic system in general have been shown to play roles in reward and these mechanisms are known to be engaged by drugs of abuse, including alcohol [30]. For the same reason, we measured the level of dopamine and its metabolite DOPAC. We tested the expression of two GABA-receptor genes and also measured the level of the neurotransmitter GABA because the GABA-ergic system has been implicated in alcohol's actions in rodents as well as in humans, for example, in the context of alcohol seeking, consumption and withdrawal to mention but a few alcohol related processes [10, 28, 31]. In addition, we also measured the expression level of a gene encoding GAD, or glutamic acid decarboxylase, an enzyme involved in the synthesis of GABA [7]. Monoamine oxidase (MAO) is a mitochondrial enzyme involved in the metabolism of biogenic amines and thus it is responsible for the degradation of a number of neurotransmitters [43]. It has been implicated in human alcoholism but also importantly, similarly to the above listed genes, the gene encoding this enzyme has been found to be differentially expressed in response to chronic alcohol treatment in zebrafish [38]. The N-methyl D aspartate receptor, or NMDA-R, is a ligand (glutamate) and voltage gated cation (calcium) channel that has been shown to mediate numerous alcohol induced changes including the amnesic effects of alcohol in mammals. For example, its NR2B subunit has been proposed as a target for treatment of alcohol dependence [33]. Here we analyze the level of expression of the gene encoding this subunit and also quantify the amount of glutamate, the ligand of this receptor and of several other glutamatergic neurotransmiter receptors implicated in alcoholism [33]. The serotoninergic system has also been implicated in alcoholism and in fact serotonin's metabolites have been suggested as biomarkers of alcohol intake and abuse [6]. Here we quantify the expression of a zebrafish gene encoding a serotonin receptor and also measure the level of serotonin and its metabolite 5HIAA. The last gene on our list of targets is one that encodes a solute carrier family protein (SLC6). These proteins have been known to play roles in the transport of small molecules including neurotransmitters across vesicular and plasma membranes and as such they have been suspected to mediate some effects of alcohol. Recently their involvement in chronic alcohol induced changes has been unequivocally demonstrated in zebrafish [38]. Last, it is notable, that practically every neurotransmitter system has been implicated in alcohol's actions in the brain either as a result of alcohol having a direct effect on the respective neurotansmitter receptors or via other mechanisms including the intricate interactions among the neurotransmitter systems [3]. Thus, here we decided to monitor nine neurochemicals including the above mentioned ones and also aspartate, glycine, and taurine.

We must emphasize that the above list of targets is far from exhaustive when one considers the potential molecular players implicated in alcohol's actions in the brain. Furthermore, and also importantly, we note that the current study was not designed to address the question of causality, i.e. gene expression or neurochemical level differences between the studied strains do not necessarily explain how and why alcohol induced behavioral responses differ between these strains. Nevertheless, we hope that the current study will allow us to formulate some working hypotheses as to the potential mechanisms underlying such differences and, on the long run, will contribute to the understanding of the neurobiology and genetics of alcohol abuse.

2. MATERIALS AND METHODS

2.1 Animals and Housing

Adult (6-month old) zebrafish of both strains were bred, raised in our vivarium (University of Toronto Mississauga, Mississauga, ON, Canada). The progenitors of AB fish were obtained from the Zebrafish International Research Centre (ZIRC; Eugene, Oregon). The progenitors of SF fish were obtained from a local pet store (Big Al's Aquarium Warehouse,

Mississauga, ON, Canada) and the second filial generation of these fishbred in-house was used in our experiments. In each experimental group, ten fish were housed in 6 l transparent acrylic tanks, with approximately 50–50% males and females. All fish were kept on a constant 14h: 10h light: dark cycle at 27°C and housed in a standard manner as described previously [14]. All experiments involving the treatment and handling of fish were conducted according to local, provincial and Federal guidelines and were approved by the Local Animal Care Committee of the University of Toronto.

2.2 Semi-quantitative RT-PCR

In order to investigate potential gene expression differences between AB and SF zebrafish, we employed a sensitive semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis. We obtained RNA samples from 20 adult zebrafish of each strain selected randomly from their aquaria. First the body weight and length of fish were measured and found statistically indistinguishable between the two strains (not shown). Subsequently, the fish were sacrificed by decapitation and the entire brain of the fish (without the eyes) was quickly removed from the skull, immediately frozen in liquid nitrogen and subsequently stored at −80°C until RNA extraction.

Total RNA was extracted from the brain with the TRIzol reagent according to the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA). In order to minimize between-animal variability and to obtain sufficient amount of RNA, individual samples were randomly pooled. Each pool consisted of a balanced contribution of RNA from four different randomly selected fish of the same strain. We used a minimum of four pools of samples for each zebrafish strain (each pool containing brain tissue from 2 females and 2 males) for statistical comparison. The total RNA was purified using the RNeasy Mini Kit (QIAGEN Inc. Mississauga, CA). The RNA quantity and quality were determined using a Spectrophotometer NanoDrop ND-1000 (NanoDrop Technologies, Inc. Wilmington, DE, USA). For most genes RNA quality was determined to be excellent but for the minority of genes we had to exclude certain pools (hence the variation between 4 and 5 pools per strain). For remaining pools, total RNA was used for semi-quantitative RT-PCR.

Ten genes were selected as targets for investigation of potential gene expression level differences between AB and SF zebrafish. The sequences corresponded to the D1-R (Danio rerio dopamine receptor D1), D2a-R (Danio rerio dopamine receptor D2a), D4a-R (Danio rerio dopamine receptor D4a), GABA-Ra (Danio rerio gamma-aminobutyric acid (GABA) A receptor), $GABA_B$-R1 (Danio rerio gamma-aminobutyric acid (GABA) B receptor, 1), GAD1 (Danio rerio glutamate decarboxylase 1), MAO (mono-amine oxydase), NMDA-R2D (Danio rerio similar to Glutamate [NMDA] receptor subunit NR2D), 5HT-R1bd (Danio rerio 5-hydroxytryptamine (serotonin) receptor 1bd), SLC6 a4a (Danio rerio solute carrier family 6 member 4A) proteins respectively. These genes were chosen because of their known involvement in alcohol induced functional changes in the brain in mammals as well as in zebrafish [38]. In addition, β-actin was regarded as a house keeping gene and was used as control. The assumption that β-actin expression levels do not differ between SF and AB was confirmed previously [38] and in the current study as well (data not shown). The intensity of bands obtained for the target genes was normalized to the β-actin 2 (Danio rerio bactin2).

PCR primer pairs were designed for each gene using the Primer3 software application (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/), and sequence data were acquired from GenBank (Table 1). In general, primers with 40-60% GC and temperature of 55-60°C were preferred. The primers were finalized when both primer sequences showed homology to the same gene and all pairs produced a single amplicon. All primer sequences used for

Semi-quantitative RT-PCR are reported in Table 1. The primers were synthesised by Sigma-Aldrich (Oakville, Canada).

A 0.5 μg aliquot of total RNA from each pooled sample was used as a template and ProtoScript M-MULV Taq RT-PCR Kit (New England Biolabs Ltd. Pickering, CA) was chosen for reverse transcription (RT). The reaction mixture (20 μl) contained the following components: 20X RT buffer, 40 mM dNTP, 100 μM oligo-dT_{23} primer, 20 units of RNase inhibitor and 10 units of Moloney murine leukemia virus reverse transcriptase (MMLV) enzyme. The reaction was carried out at 70°C for 5 min, 42°C for 60 min, followed by enzyme inactivation at 80°C for 5 min as specified by the protocol (New England Bio labs Ltd). The cDNA solution was brought to 50 μl volume with water and stored at -20°C. A negative RT control reaction was also performed.

Target genes were amplified from 5μl aliquots of the first-strand cDNA. PCR was carried out in a total reaction volume of 50 μl, containing 10 μM of 3' and 5' primers specific for the given target gene, and the Taq 2X Master Mix of 25 μl (New England Bio labs Ltd). The PCR reaction was started with 5 min incubation at 94°C, followed by 30 sec at 94°C, 1 minute of annealing at the temperature, 1 min at 72°C, and an additional 7 min at 72°C. Linear amplification range for each gene was first determined by performing PCR reactions at 25, 30, 35, and 40 cycles. The positive loading control GADPH and negative control reaction (no reverse transcriptase) were amplified following the protocol (New England Bio labs Ltd) to examine the quality of kit components, and to ensure there is no DNA contamination.

2.3 Quantification of gene expression results

10 μl of each PCR product was electrophorised on 1.5% agarose gel and stained with ethidium bromide. The intensities of the bands were evaluated with the Quantity One (version 4.6) software application (Bio-Red Laboratories, Inc. Hercules CA), and plotted to establish the linear phase of amplification. PCR reactions were then completed for the two populations of zebrafish within the linear phase of the amplification. β-actin was used as a house keeping gene. The RT-PCR values obtained for the target genes were normalized to β-actin (by dividing their values with that of β-actin). These normalized values were used to calculate SF/AB ratios. The rationale for calculating the SF/AB ratio was that variations between gels due to technical issues would inflate the error variance of signal intensity obtained across gels. Given that each gel contained not only a β-actin loading control but also equal number of samples from both strains, calculating the ratio between the strain values minimized the technical error related variation and maximized our ability to obtain sufficient statistical power. A value above 1 represents relative overexpression of the given gene in SF fish as compared to AB and a value below 1 means underexpression. Statistical analysis of these values was conducted using SPSS. Two tailed one-sample t-tests were conducted to compare each ratio to 1. The null hypothesis (equal expression level of the gene in SF and AB, i.e. SF/AB = 1) was rejected when its probability was found to be smaller than 0.05.

2.4 Neurochemical analysis using HPLC

In addition to measuring gene expression we also quantified the level of 9 neurochemicals: dopamine, its metabolite DOPAC (3,4-Dihydroxyphenylacetic acid), serotonin (5-HT or 5-hydroxytryptamine), its metabolite 5HIAA (5-Hydroxyindoleacetic acid), GABA (Gamma Aminobutyric Acid), Glutamate (or Glutamic acid), Glycine, Aspartate and Taurine. The rationale for conducting this analysis was that post-transcriptional gene regulation remains undetectable to gene expression analyses and previously alcohol induced genotype dependent neurochemical changes were detected in mammals as well as in zebrafish [14].

The fish used for neurochemical analysis were identical in age, size, and strain origin to those utilized in the gene expression analysis. The methods of analysis of neurochemicals have been specifically adopted and modified to zebrafish as described before [4]. Fish were decapitated rapidly and their brains were dissected on ice under a dissecting microscope. The brains were frozen in a microcentrifuge tubes (1 brain per tube) at -80 °C. For HPLC sample preparation, the tubes were taken out of the freezer and the brains were suspended in artificial cerebrospinal fluid (ACSF, Harvard, 10 μl/ brains). The brains were sonicated 4 times (2 sec pulse each time) on ice. 2 μl of the sonicate from each sample was analyzed for protein content by BioRad protein assay reagent (BioRad, Hercules, CA, United States). To each tube, 1 μl of stabilizer (0.2N perchloric acid and 1.0 M ascorbic acid) was added, and the sonicates were centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was collected carefully and stored at -80 °C until use. HPLC analysis for the amino acid neurotransmitters was carried out using a BAS 460 MICROBORE HPLC system with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, IN, USA) together with a Uniget C-18 reverse phase microbore column (BASi, Cat no. 8912; analytical - 1 × 150 mm, 5 μm ODS) as the stationary phase. The mobile phase consisted of 0.15 M sodium acetate buffer, 1 mM EDTA, pH 5.4 and 50% acetonitrile. The flow rate was 0.8 ml/min.

For *o*-phthalaldehyde (OPA) derivatization, 5 μl of homo-serine (125 pmol in ACSF) was added as the internal standard to 5 μl of sample. Then 5 μl of OPA reagent was added to the sample and mixed well by vortex. The mixture was incubated at room temperature for 3 min. Immediately after incubation, 5 μl of the reaction mixture was injected onto the column for the analysis. The working electrode (Uniget 3 mm glassy carbon, BAS P/N MF-1003) was set at 750 mV vs. Ag/Ag/Cl reference electrode. Detection gain was 1.0 nA, filter was 0.2 Hz and detection limit was set at 100 nA. Standard neurochemicals (all from Sigma) were used to quantify and identify the peaks on the chromatographs. For this purpose, standard solutions of 1 mg per ml (in HPLC grade water) were made with pure dopamine, DOPAC, serotonin, 5HIAA, glutamate, aspartate, glycine, taurine and GABA and diluted with artificial cerebrospinal fluid (ACSF, Harvard) accordingly to get the desired concentrations of the stock solutions for running in HPLC. The sensitivity was selected at the concentration at which we achieved a signal to noise ratio exceeding 3:1.

Preparation of OPA reagent: The OPA reagent was prepared according to Peinado et al. [39]. After 27 mg of OPA were dissolved in 0.5 ml of methanol, 10 μl of 2-methyl-2-propanethiol (tert-butylthiol) was added to the solution which was then slowly diluted with 4.5 ml of 0.1M borate buffer, pH 9.5. The solution was filtered through a 0.22 μm syringe filter. The solution was kept at room temperature in a brown bottle and was used for up to 1 month with 5 μl of tert-butylthiol added weekly.

To make the analysis comparable to the gene expression analysis, all neurochemical analyses were conducted in a systematically ordered manner, that is a pair of AB and SF samples were run at the same time. First, neurochemical levels were normalized to the standards and total brain protein. Subsequently, the ratio of neurochemical levels between SF and AB samples was calculated for each neurochemical analyzed. This ratio was analyzed with SPSS (version 14 for the PC) using one sample t-test. A ratio significantly exceeding 1 indicated higher neurochemical level in SF vs. AB and vica versa.

3. RESULTS

The results of gene expression analyses are shown in Figure 1. t-tests indicated that the gene for the D1 receptor was significantly over expressed ($t = 6.833$, $df = 3$, $p < 0.05$) in SF as compared to AB (that is the SF/AB ratio was significantly above 1), and the gene for GABA1 ($t = -4.776$, $df = 3$, $p < 0.05$) and for SLC-6 ($t = -6.422$, $df = 3$, $p < 0.01$) was

significantly underexpressed in SF as compared to AB. The expression levels of the other genes were not significantly different between these two strains, i.e. the SF/AB expression ratio was not statistically distinguishable from 1 for these genes.

The quantification of neurochemicals revealed a fairly consistent picture (Figure 2): all but one appeared underexpressed in SF compared to AB, i.e. the SF/AB ratio appeared to be less than 1. t-tests showed that the ratio of SF/AB was significantly below 1 for all neurochemicals ($t > I\text{-}7.59I$, $df = 11$, $p < 0.0001$), except for 5HIAA ($t = -1.075$, $df = 11$, $p > 0.30$).

4. DISCUSSION

In the current study we identified significant gene expression and neurochemical level differences between two zebrafish strains, AB and SF. Finding strain differences between genetically distinct populations is not surprising and studies reporting such differences abound in the behavior genetics literature [5, 11, 35]. In case of the AB and SF strains, distinct genotype specific features are also expected because these strains have highly different breeding histories (AB has been bred in the laboratory for four decades and 80% of its loci are homozygous [19, 34], whereas the SF strain is a genetically heterogeneous stock originating from a commercial breeder in Singapore). Briefly, random genetic drift and accumulation of spontaneous mutations have likely made these strains different. Although expected, strain differences in zebrafish have rarely been studied. Specifically, our knowledge with regard to strain differences in drug abuse related phenotypes in zebrafish is rather limited, a hiatus that has started to be filled only recently [8, 14, 25, 36]. We have conducted one of the first detailed analyses of zebrafish strains in the context of alcohol effects on brain function and found intriguing behavioral differences between AB and SF zebrafish [14]. The former turned out to be highly responsive to both the acute and chronic effects of alcohol while the latter exhibited only blunted or no appreciable responses, at least as measured at the level of behavior.

At this point, we do not know what neurobiological and molecular mechanisms may underlie the alcohol dependent strain differences but it must be noted that heterozygous individuals or genetically heterogeneous populations have long been known to often show better buffering against changes in the environment including external insults [21]. Alcohol exposure may be one such insult and the higher frequency of homozygous loci in the AB fish genome thus may have made these fish more responsive (vulnerable) to alcohol. One of the most well studied neurotransmitter systems implicated in mediating the actions of drugs of abuse such as alcohol, is the dopaminergic system.

Dopamine, a neurotransmitter regulating a variety of functions, e.g. motor function, learning and reward [1, 16, 27, 30, 41], plays a fundamental role in alcoholism in humans and in mediating alcohol's actions in other vertebrates too [3, 4, 14, 44, 47]. The expression levels of four dopamine receptor genes (D1, D2, D3, and D4) previously identified in zebrafish [1, 23, 27, 42] were tested in the current study. The results revealed over-expression of the gene encoding the D1-receptor in SF as compared to AB zebrafish. This is noteworthy as D1-R is one of the most abundantly expressed postsynaptic dopamine receptors in the zebrafish brain [23, 42]. Accompanying the overexpression of D1-R in SF relative to AB, we detected significantly reduced dopamine and DOPAC levels in the brain of SF fish as compared to AB fish. This is again notable as alcohol exposure has been shown to alter the amount of dopamine in zebrafish and in other vertebrates as well [4, 14]. It is thus possible that the dopaminergic system is hypo-reactive in SF (or hyper-reactive in AB), which may explain the blunted alcohol responses in SF fish compared to AB fish found before [14].

The under-expression of the gene encoding the GABA-B1 receptor in SF relative to AB is also noteworthy. GABA is a major inhibitory neurotransmitter in the CNS of vertebrates [7, 9]. Its actions are mediated by ionotropic ($GABA_A$) and metabotropic ($GABA_B$) type receptors, which are widely distributed throughout the central nervous system [2, 29, 31]. $GABA_B$ receptors play roles in the regulation of neuronal excitability and synaptic transmission [26] and GABA has been implicated in alcohol responses both in rodents [10] and in humans [28]. The amount of neurotransmitter GABA was found significantly reduced in SF fish compared to AB in the current study. Given the known involvement of GABA-mediated mechanisms associated with alcohol's actions [2, 3, 10], one could speculate that the currently discovered baseline differences in GABA levels may have contributed to the previously observed behavioral differences between these strains in their responses to alcohol [14]. Notably, the GABA-ergic and the dopaminergic neurotransmitter systems have been found to be highly conserved across vertebrates, including the zebrafish and mammalian species [30, 32, 42], thus further examination of these strain differences may lead to translationally relevant findings.

The under-expression of the SLC6 gene in SF relative to AB is also worth discussing. SLC6 is a member of a large protein family, the solute carrier family of proteins involved in trafficking molecules across the membrane of neurons. SLC6 is known to regulate synaptic transmission by mediating neurotransmitter recycling [20] and it has been found associated with chronic alcohol exposure in zebrafish [38]. Given that SLC6 expression is reduced in SF relative to AB, it is plausible that the blunted responses to chronic alcohol treatment seen in SF may be partly due to under-expression of this gene.

Last, we consider the reduced levels of Aspartate, Glutamate, Glycine, Serotonin and Taurine found in the brain of SF fish relative to AB fish. Reductions in Aspartate levels have been found associated with neuronal damage, reduction in synaptic density, and dysregulated neurotransmission [24]. Interestingly, reduced Aspartate levels have been associated with the chronic use of alcohol in humans [24]. Thus, differences in aspartate levels between Sf and AB zebrafish may have also contributed to the alcohol induced behavioral differences observed before [14]

Glutamate is a major excitatory neurotransmitter responsible for mediating fast synaptic neurotransmission with important roles in synaptic plasticity and drug abuse related adaptive changes in the brain. Acute ethanol exposure inhibits Glutamate receptors, e.g. NMDA-R, while chronic alcohol exposure significantly reduces brain levels of Glutamate [45]. One may speculate, therefore, that reduced baseline Glutamate levels found in SF zebrafish may make these fish less sensitive to the effects of chronic alcohol treatment due to a floor effect.

Glycine receptors, a ligand-gated chloride channel, are known as one of the primary targets of alcohol [48]. Glycine receptors are known to mediate numerous functions including that of the mesolimbic dopaminergic system involved in reward as well as drug abuse [48]. Interestingly, the glycine reuptake inhibitor Org25935, acting on the glycine transporter 1 (GlyT1), was found to decrease alcohol intake and preference in rats and to increase extracellular Glycine levels [48]. Thus it is plausible that the difference in Glycine levels we report here for alcohol naïve SF vs. AB zebrafish may contribute to the alcohol induced behavioral differences between these strains found before [14].

Serotonin receptors are also known to directly bind alcohol: alcohol potentiates a number of these receptors [48]. Serotonin reuptake inhibitors were found to reduce alcohol consumption in rodents [48] and serotonin transporters are believed to play key roles in human alcoholism as well as depression [22]. A central serotonin deficit is thought to be involved in the pathogenesis of alcohol dependence [22]. Briefly, the serotoninergic system

is known to play intricate roles in alcohol abuse and alcoholism. Differences in overall brain serotonin levels between the SF and AB zebrafish strains in alcohol naïve fish may thus translate to strain-dependent behavioral responses to alcohol.

Taurine is an abundant amino acid in the CNS and plays roles in osmoregulation, neuroprotection and neuromodulation [40]. Interestingly, both taurine and alcohol exert positive allosteric modulatory effects on ligand-gated chloride channels, i.e. $GABA_A$ and glycine receptors, as well as inhibitory effects on calcium channels including NMDA-R [37]. Also importantly, taurine and several related molecules including the homotaurine derivative acamprosate (calcium acetylhomotaurinate) can reduce alcohol self-administration and relapse to drinking in both animals and humans [37]. Thus differences in baseline taurine levels between SF and AB zebrafish may have significant consequences in the way these strains respond to alcohol treatment.

It is important to stress again that our current findings on the differences between the alcohol naïve fish of the two strains only represent possible associations with the alcohol induced strain differences. Although these associations do not contradict what we know about the psychopharmacology and the neurobiological mechanisms of alcohol, they do not represent causal discoveries for three main reasons. One, differences between strains and correlations among such differences may be due to spurious genetic associations. Two, the number of targets we studied is certainly well below of what actually may be engaged by alcohol [38]. Three, all the analyses (including gene expression and HPLC) were based upon samples from whole brain tissue extracts and thus we could not identify brain region specific differences between the strains. The question of causality, i.e. the role of the molecular players we speculate about with regard to their involvement in alcohol induced differences between SF and AB fish, will thus have to be ascertained in the future. Nevertheless, our current findings, together with the previously identified behavioral differences between the studied two zebrafish strains [14], suggest that zebrafish will be a useful tool with which one may identify the molecular and neurobiological bases of alcohol's actions in the vertebrate brain.

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Highlights

- We analyzed mRNA expression of ten genes and amount of nine neurochemicals in the zebrafish brain.
- Two strains of zebrafish, SF and AB, were compared.
- mRNA expression level differences between the strains were found in 3 genes.
- All but one neurochemical tested was decreased in SF compared to AB.
- The results demonstrate significant strain differences that extend our previous knowledge of alcohol induced genotype dependent behavioral characteristics of these strains.

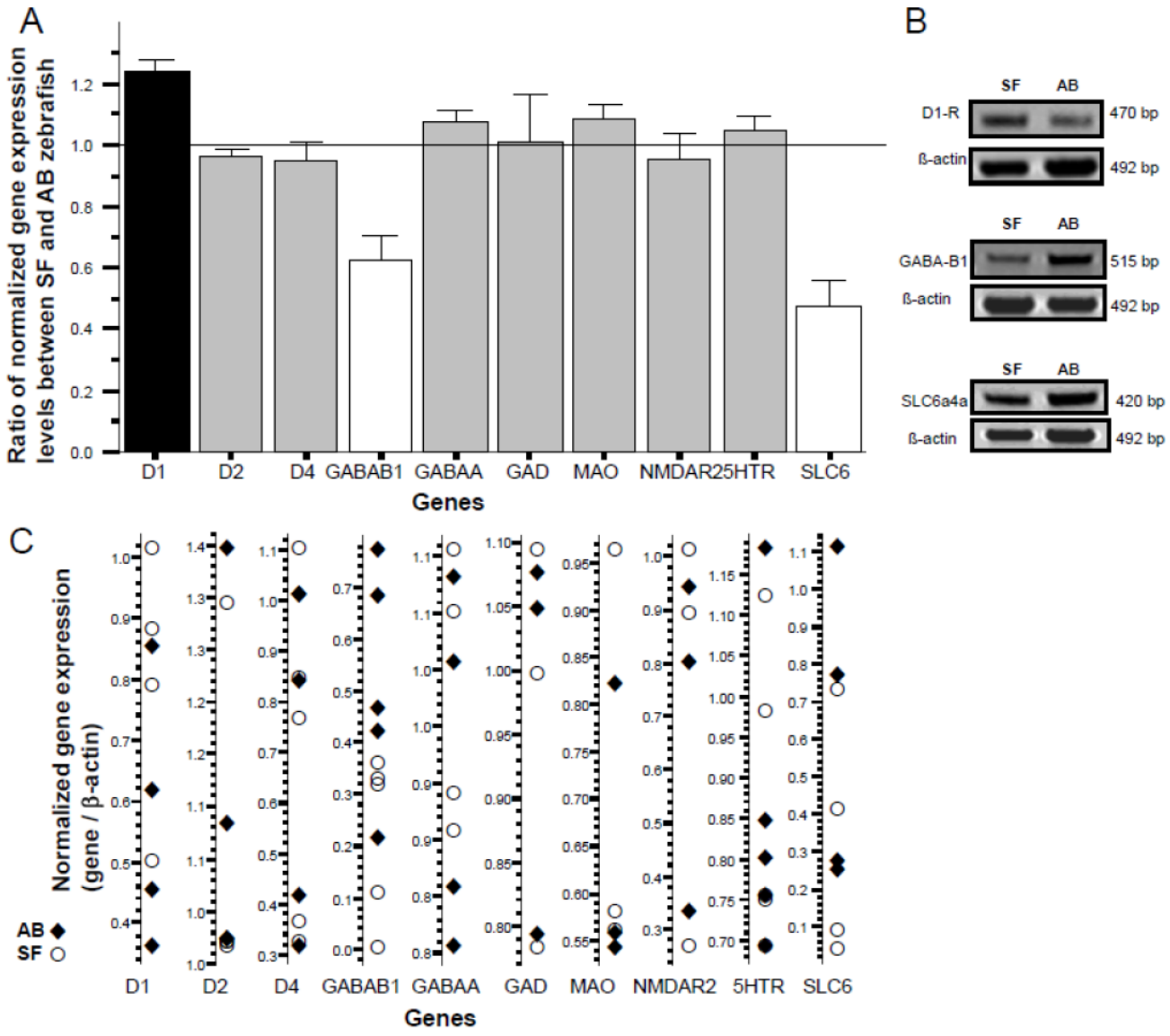

Figure 1.

Panel A: Ratio (SF/AB) of normalized mRNA expression levels quantified for 10 select genes. The gene encoding the Dopamine 1 receptor (D1) is overexpressed while the genes encoding the $GABA_B$ 1 receptor (GABA1) and Solute Carrier Family member 6 protein (SLC6) are underexpressed in fish of the SF strain as compared to fish of the AB strain. Mean ± S.E.M. are shown. The results are shown as the ratio of gene expression (normalized to β-actin) between SF and AB samples. Note that in case of equal gene expression levels in SF and AB fish, the ratio is 1, indicated by the horizontal line. Significant overexpression (SF/AB > 1) is indicated by black, significant underexpression (SF/AB < 1) is indicated by white filling of the bars. Panel B: Representative examples of signal intensities found on gels for the three genes in which significant mRNA expression differences were detected between SF and AB zebrafish. The genes are indicated on the left side of the gel image, while the size of the corresponding DNA fragment is shown on the right side. The strain origin of the sample is shown above the gel images. Panel C: Scaterplots corresponding to each gene tested. Each black diamond represents a pool of four AB individuals and each

open circle a pool of four SF individuals. Note that the scale, indicated to the left of each of the scatterplots, is different for each gene. For details of methods, see Methods section. For statistical analysis and its results, see Results section.

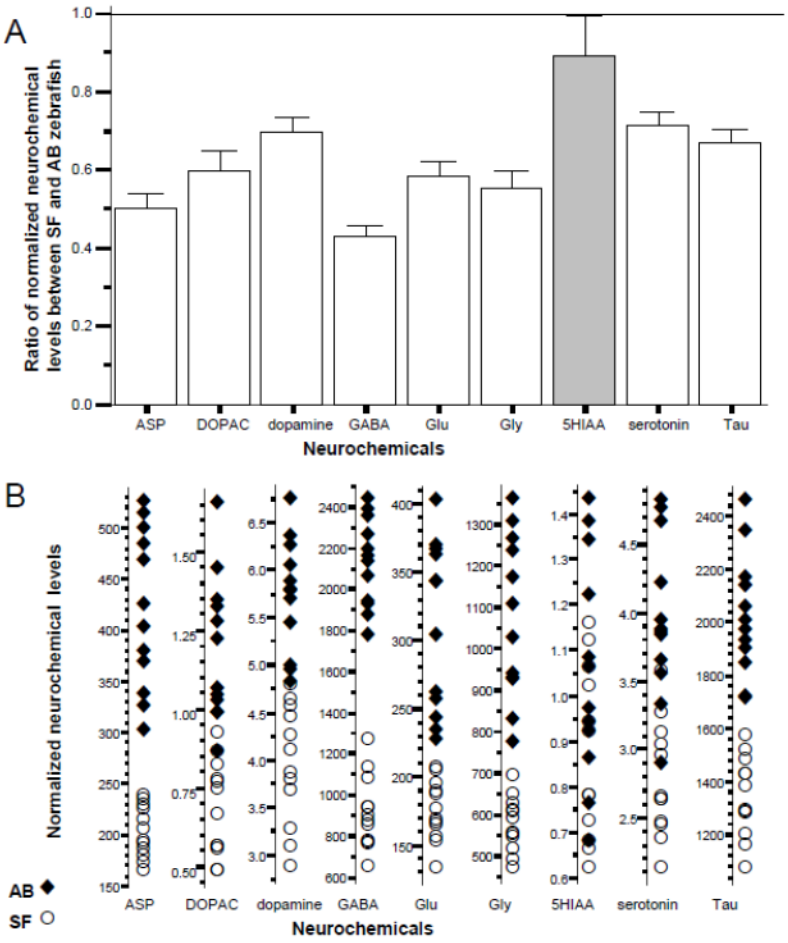

Figure 2.

Panel A: Ratio (SF/AB) of normalized (ng of neurochemical / mg of brain protein for dopamine, serotonin and their metabolites and pmol / mg brain protein for amino acid neurotransmitters) neurochemical levels from whole brain tissue samples of zebrafish. All but one (5HIAA) neurochemicals showed significant reduction in SF relative to AB strain of zebrafish. Mean ± S.E.M. are shown. Data are expressed as ratio of the amount of neurochemicals found in the brain of SF fish vs. AB fish normalized to total brain protein. A ratio of 1, indicated by the solid horizontal line, represents equal neurochemical level between SF and AB fish. Neurochemicals for which the SF/AB ratio was found significantly lower than 1 are indicated by the white bars. Panel B: Scatterplots showing the distribution of data for the nine neurochemicals. Each black diamond represents a single AB individual and each open circle a single SF individual. For details of methods see Methods section. For statistical analysis and its results, see Results section.

Table 1

Primer sequences for semi-quantitative PCR.

Genes Description	Primers for semi-quantitative RT-PCR	Amplicon size (bp)	URL
Danio rerio dopamine receptor D1 (drd1)	5’-3’ ACTGCATGGTTCCTTTTTGC 3’-5’ GGATTTGTGCTGTCCGTTTT	470	http://www.ncbi.nlm.nih.gov/gene/568126
Danio rerio dopamine receptor D2a (drd2a)	5’-3’ CCCGATGATGTGAAACTGTG 3’-5’ CAGGAGGGACTTGGCAGTAA	493	http://www.ncbi.nlm.nih.gov/gene/282557
Danio rerio dopamine receptor D3 (drd3)	5’-3’ CCTTGGGCAGTTTATTTGGA 3’-5’ TTCTCTTTGTGTGCGTCGTC	500	http://www.ncbi.nlm.nih.gov/gene/282554
Danio rerio dopamine receptor D4a (drd4a)	5’-3’ GATGGAGTTTGGTCCCTCAA 3’-5’ CCAACATGGACACAGGATCA	571	http://www.ncbi.nlm.nih.gov/gene/503564
Danio rerio monoamine oxidase (mao)	5’-3’ ACCAACTCAAAACCGCATTC 3’-5’ CTCATTCACCGTCCTGACCT	571	http://www.ncbi.nlm.nih.gov/gene/404730
Danio rerio 5-hydroxytryptamine (serotonin) receptor 1bd (htr1bd)	5’-3’ CAGCTCCGATGAGTTTTTCC 3’-5’ CATGGTTCTCCGTTTCGAGT	430	http://www.ncbi.nlm.nih.gov/gene/556429
Danio rerio solute carrier family 6 member 4A (slc6a4a)	5’-3’ ACTGCACCCACTACCTGTCC 3’-5’ ATGCCAGGAGAACACCAAAG	420	http://www.ncbi.nlm.nih.gov/gene/664719
Danio rerio gamma-aminobutyric acid (GABA) A receptor (gaba1)	5’-3’ AAGAGCCAAAACCCAAACCT 3’-5’ CCTTTACAGACACGGCCATT	566	http://www.ncbi.nlm.nih.gov/gene/768183
Danio rerio gamma-aminobutyric acid (GABA) B receptor, 1 (gabab1)	5’-3’ CATCGTCTGCCTCTCATTCA 3’-5’ AGCCATAAACCACACCAAGC	515	http://www.ncbi.nlm.nih.gov/gene/558708
Danio rerio glutamate decarboxylase 1 (gad1),	5’-3’ GCTGAAATACGGGGTCAGAA 3’-5’ GTGGCATTCACAAACAGTGG	507	http://www.ncbi.nlm.nih.gov/gene/378441
Danio rerio similar to Glutamate [NMDA] receptor subunit (NMDAR2D)	5’-3’ GGGTGTTGCGTCTGAGGTAT 3’-5’ AGAAAAGCCGAGGGAGAGAC	508	http://www.ncbi.nlm.nih.gov/gene/614087
Danio rerio β-actin2 (β-actin 2)	5’-3’ AAGGCCAACAGGGAAAAGAT 3’-5’ CTCGTGGATACCGCAAGATT	492	http://www.ncbi.nlm.nih.gov/gene/57935