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Lasting changes induced by mild alcohol exposure during embryonic development in BDNF, NCAM and synaptophysin positive neurons quantified in adult zebrafish

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Abstract

Fetal Alcohol Spectrum Disorder (FASD) is one of the leading causes of mental health issues worldwide. Analysis of zebrafish exposed to alcohol during embryonic development confirmed that even low concentrations of alcohol for a short period of time may have lasting behavioral consequences at the adult or old age. The mechanism of this alteration has not been studied. Here, we immersed zebrafish embryos into 1% alcohol solution (vol/vol%) at 24 hours post-fertilization (hpf) for 2 hours, and analyze potential changes using immunohistochemistry. We measured the number of BDNF (brain derived neurotrophic factor) and NCAM (neuronal cell adhesion molecule) positive neurons and the intensity of synaptophysin staining in eight brain regions: lateral zone of the dorsal telencephalic area, medial zone of the dorsal telencephalic area, dorsal nucleus of the ventral telencephalic area, ventral nucleus of the ventral telencephalic area, parvocellular preoptic nucleus, ventral habenular nucleus, corpus cerebella and inferior reticular formation. We found embryonic alcohol exposure to significantly reduce the number of BDNF and NCAM positive cells in all brain areas studied as compared to control. We also found alcohol to significantly reduce the intensity of synaptophysin staining in all brain areas except the cerebellum and preoptic area. These neuroanatomical changes correlated with previously demonstrated reduction of social behavior in embryonic alcohol exposed zebrafish, raising the possibility of a causal link. Given the evolutionary conservation across fish and mammals, we emphasize the

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Competing Interests

No conflicts of interests.

Authors Contributions

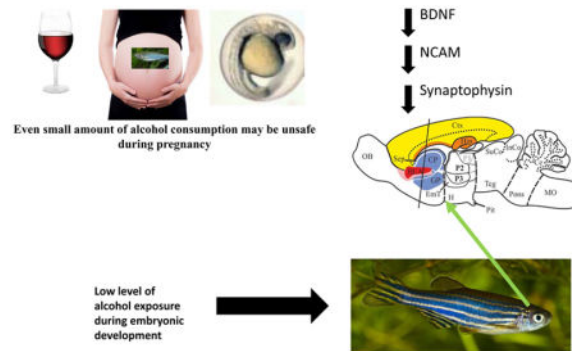
All authors contributed to the conception and design of this study. Acquisition of the data was conducted by Samantha Mahabir, Diptendu Chatterjee, Dipashree Chatterjee and Keith Misquitta. Analysis and interpretation of data was carried out by Robert Gerlai and Samantha Mahabir. Drafting of the manuscript was done by Samantha Mahabir, Diptendu Chatterjee and Dipashree Chatterjee. Critical revisions were conducted by Robert Gerlai.

Data Accessibility

All data including original blots, unedited picture files, and counts are maintained at the University of Toronto Mississauga Vivarium, Mississauga, ON. All data can be accessed by contacting Samantha Mahabir at samantha.mahabir@mail.utoronto.ca.

implication of our current study for human health: even small amount of alcohol consumption may be unsafe during pregnancy.

Graphical Abstract



Keywords

Fetal alcohol spectrum disorder (FASD); prenatal; teratogen; neuronal protein; brain plasticity

Introduction

Brain development involves a variety of processes that support cellular proliferation, apoptotic cell death, cellular differentiation, cell migration and the formation of synapses. Exposure to alcohol at different stages of pregnancy may disrupt several of these processes. Fetal alcohol spectrum disorder (FASD) represents a cluster of diseases with differing symptom sets and severity, all resulting from the developing fetus being exposed to alcohol in utero (see review by Riley *et al.*, 2011). Although its specific mechanisms and mode of action are unclear, the particular effects of alcohol are known to depend upon the developmental stage at which alcohol reached the embryo, the concentration and duration of exposure to this substance, as well as the genotype and health status of the mother and the fetus (Riley & McGee, 2005; Ramsay, 2010). FASD may include both physical and behavioral abnormalities, such as anatomical malformations, problems with learning and memory, difficulties with comprehension or with following directions, reduced ability to control emotions, impaired communication, abnormalities with a variety of social behaviors, and issues even with daily life skills, for example, eating and bathing (see review by O'Connor & Paley, 2009). FASD is the leading cause of preventable developmental disability in the world. That is, the global prevalence of FASD among children and youth is approximately 1% of the general population (Lange *et al.*, 2017; Popova *et al.*, 2016). Efficient treatment for FASD does not exist, despite that embryonic alcohol exposure exerts life-long suffering for the patient and his/her family. Thus, FASD represents a clear and urgent unmet medical need.

Animal models have been developed in the hope that they will speed up discovery of the mechanisms of FASD and in turn will lead to the development of treatment for FASD patients (Meyer *et al.*, 1990; Becker *et al.*, 1996; Fernandes & Gerlai, 2009; Kelly *et al.*,

2009; McClure *et al.*, 2011; Church *et al.*, 2012). The zebrafish is one of these animal models proposed for the analysis of FASD (Fernandes & Gerlai, 2009) as it offers some advantages. For example, zebrafish eggs are externally fertilized and develop outside of the mother, which allows one to precisely control the timing and dose of alcohol exposure during embryonic development (Tanguay & Reimers, 2008). Furthermore, ontogenesis is fast (it completes in 5 days), and the development of the zebrafish brain has been well mapped in terms of anatomical changes, patterns of expression of numerous genes and important markers aiding structural and functional characterization of developmental processes (Wullimann *et al.*, 1996).

Previously, exposure to low concentrations of alcohol (up to 1%, vol/vol bath) employed for a short period of time (2 hours) during embryonic development was shown not to induce observable, gross, physical abnormalities, but to lead to significant reduction in the response of the exposed fish to social stimuli (Fernandes & Gerlai, 2009; Fernandes *et al.*, 2015a, Buske & Gerlai, 2011). This impaired shoaling response was found to be accompanied by reduced levels of neurotransmitters dopamine, serotonin and their metabolites in the adult fish brain (Buske & Gerlai, 2011; Mahabir *et al.*, 2014; Fernandes *et al.*, 2015b). In addition, short duration exposure to low levels of alcohol during embryonic development was also found to lead to impaired learning in zebrafish (Fernandes *et al.*, 2014). Other mechanisms potentially underlying the effects of embryonic alcohol exposure on shoaling in zebrafish are not known.

In the present study, we examine whether exposure of zebrafish embryos to a low concentration of alcohol (1% vol/vol bath immersion) for a short period of time (2 hours) results in alteration in the expression of three neuronal proteins important for zebrafish brain development and plasticity: brain derived neurotrophic factor (BDNF), neuronal cell adhesion molecule (NCAM) and synaptophysin, a synaptic protein. The developmental stage at which alcohol is administered is identical to what was employed before, and the concentration we chose corresponds to the highest dose that was previously found to induce robust behavioral impairment of social behavioral responses, and abolishment of dopaminergic responses to social cues in zebrafish without gross anatomical alterations (Fernandes & Gerlai, 2009; Buske & Gerlai, 2011; Fernandes *et al.*, 2015b). Coincidentally, this external bath concentration and exposure regimen was found to lead to an alcohol dose inside the immersed zebrafish egg that corresponds to blood alcohol levels slightly under the considered legal limit for driving in North America (Mahabir *et al.*, 2014).

We chose BDNF as one of the molecules of focus in this study because it plays a vital role during brain development as well as in the adult brain. It is a nerve growth factor that is also involved in the survival of a variety of neurons in mammals such as the dorsal root ganglion cells, hippocampal and cortical neurons (Liu *et al.*, 2016; Lipsky & Marini, 2007). BDNF is known to modulate the function of both excitatory (glutamatergic) (Martin & Finsterwald, 2011) as well as inhibitory (GABAergic) synapses (Wardle & Poo, 2003) and is also known to play roles in the establishment of hippocampal long-term potentiation (LTP), i.e. in synaptic plasticity (Bekinschtein *et al.*, 2008; Xu *et al.*, 2000). BDNF also plays a significant role in neurogenesis (Bath *et al.*, 2012; Egeland *et al.*, 2015), another process which it mediates via activity-dependent synaptic plasticity learning and memory (Lu, 2003; Yamada

et al., 2002). We also chose to measure the expression of NCAM, a neuronal cell surface glycoprotein, because in mammals it is known to mediate cell signaling, guidance, cellular differentiation, cell adhesion, neurite outgrowth, axon guidance, neural cell migration, myelination and synapse formation, crucial processes during brain development (Hildebrandt *et al.*, 2007). Furthermore, in the adult mammalian brain, NCAM mediates neuronal plasticity as well as nerve regeneration (Rønn *et al.*, 1998; Rønn *et al.*, 2000a) via intracellular signaling through Src non-receptor protein tyrosine kinases (Maness *et al.*, 1996). Last, we chose synaptophysin, a synaptic vesicle membrane protein which constitutes about 7–10% of the total synaptic vesicle proteins, because it is involved in fundamental processes of neurotransmitter packaging and neurotransmitter vesicle functions (Leube, 1995; Leube *et al.*, 1989; Kwon & Chapman, 2011; Faludi & Mirnics, 2011; Eastwood *et al.*, 2000), and its manipulation has been shown to alter complex learning and memory processes in mice (Schmitt *et al.*, 2009).

Using immunohistochemistry, we examined the expression of these neuronal proteins in eight brain areas of the adult zebrafish: lateral zone of the dorsal telencephalic area (DI), medial zone of the dorsal telencephalic area (Dm), dorsal nucleus of the ventral telencephalic area (Vd), ventral nucleus of the ventral telencephalic area (Vv), parvocellular preoptic nucleus (PPa), ventral habenular nucleus (Hav), corpus cerebelli (CCe) and inferior reticular formation (IRF).

In rats, the ventral tegmental area (VTA) and nucleus accumbens (nAC) are involved in dopamine-signaling dependent functions associated with reward, pleasure, cognition, drug addiction and in general, motivation. In the zebrafish brain, evolutionarily, functionally and anatomically homologous areas to these mammalian regions are the diencephalic posterior tuberculum and ventral telencephalic area (Vd and Vv respectively) (Rink & Wullimann, 2001; 2002). The ventral telencephalic (Vv) area represents the subpallium (Wullimann & Rink, 2002). The dorsal nucleus of the ventral telencephalic area (Vd) is considered to be part of the striatum and contains dopaminergic cells and fibers (Rink & Wulliman, 2001), while the ventral nucleus of the ventral telencephalic area (Vv) is considered to be part of the septum (Wullimann & Rink, 2002). In mammals, the hippocampus is primarily responsible for relational learning, the cerebellum is involved in motor function and simple associative learning, and the amygdala is involved in fear and other emotion related behavioral phenomena (Eichenbaum *et al.*, 1994). The corresponding homologous brain areas in zebrafish are the lateral zone of the dorsal telencephalic area (DI, equivalent to the hippocampus), medial zone of the dorsal telencephalic area (Dm, equivalent to the amygdala) and cerebellum (Mueller *et al.*, 2011; Braford, 1995; Castro *et al.*, 2006; Northcutt, 2006; Portavella *et al.*, 2002; 2003; Rodriguez *et al.*, 2005; Ganz *et al.*, 2015). The dorsal telencephalic area in the zebrafish brain represents the pallium (Ganz *et al.*, 2015). The parvocellular preoptic nucleus (PPa) is involved in zebrafish agonistic behavior (Larson *et al.*, 2006) and is suggested to be a homolog of the supraoptic nucleus in mammals (Herget *et al.*, 2014). Furthermore, serotonin has been shown to be the neurotransmitter linked to aggression, and in the mammalian brain, the serotonergic center is the lateral habenula, which is comparable to ventral habenula in zebrafish (Popova, 2008; Amo *et al.*, 2010). Serotonergic cell populations have also been shown to emerge in the reticular

formation area of the hindbrain (Panula *et al.*, 2010) as well as it contains dopaminergic and noradrenergic neuron populations (Ma, 1997).

Thus, analysis of the three chosen neuronal markers in these eight zebrafish brain areas should provide us with a reasonable overview of potential neuroanatomical and functional changes that embryonic alcohol exposure may have induced. Although detailed analysis of the relevance of these brain areas, and molecular targets, for particular aspects of zebrafish behavior is often lacking, we hope that our pioneering study will allow the establishment of working hypotheses as to how exposure to alcohol during embryonic development leads to lasting functional changes in the vertebrate brain. We hypothesize that a single, short, low concentration alcohol exposure during development will result in a change in expressions of BDNF, NCAM, and synaptophysin in adult zebrafish brains. A change in these neuronal proteins could offer a plausible explanation for the altered brain functions (learning deficit, and impaired social behavioral responses) in adult life that has been reported in zebrafish as well as in the milder forms of FASD in humans. Changes in these molecular targets could explain the previously reported reduction of levels of dopamine, serotonin and their metabolites in the zebrafish brain. Furthermore, analysis of these molecular targets may lead to identification of biomarkers that could be used as diagnostic tools for FASD.

Materials and Methods

Housing and alcohol treatment

Adult zebrafish (*Danio rerio*) of the AB strain were bred in-house (University of Toronto Mississauga Vivarium, Mississauga, ON, Canada) to obtain fertilized eggs. All experiments described below were approved by the University of Toronto Animal Care Committee, and were conducted in accordance with the guidelines of the Canadian Council for Animal Care (CCAC). The eggs were kept in system water, reverse osmosis filtered and sterilized water supplemented with 60 mg/l Instant Ocean Sea Salt (Big Al's Pet Store, Mississauga, ON, Canada). At 24 hours post-fertilization (hpf) each group of eggs was immersed in either 0% or 1% (vol/vol) alcohol solution for 2 hrs. Subsequently, the eggs were washed with system water three times. The concentration, and the timing of exposure, employed in the current study was based upon prior studies that showed administration of 1% alcohol during this stage of development to result in robust and significant behavioral changes in adult zebrafish without any observable deleterious morphological effects or negative influence on growth rate and mortality (Fernandes & Gerlai, 2009; Buske & Gerlai, 2011). It is also notable that immersion in the 1% alcohol solution was shown to result in alcohol concentration inside the egg that was about 1/14th of the bath concentration, i.e. 0.07% (Mahabir *et al.*, 2014). The legal limit for driving in most parts of North America is between 0.05 and 0.08%, thus the 0.07% alcohol concentration inside the zebrafish egg is comparable to what one may expect to reach the human fetus when a pregnant woman consumes moderate to low amounts of alcohol.

Eggs hatched at approximately 3 days post-fertilization (dpf). At 5 dpf (free swimming stage) the fish were placed in nursery racks where they were initially fed Larval Artificial Plankton 100 (dried fish formula with particle size below 100 μm , ZeiglerBros, Inc., Gardners, PA, USA) and subsequently freshly hatched brine shrimp nauplii (*Artemia salina*).

At 3 weeks post-fertilization the developing fish were fed a 1:1 mixture of flake food (Tetramin Tropical fish flake food, Tetra Co, Melle, Germany) and powdered spirulina algae (Jehmco Inc., Lambertville, NJ, USA), which continued to adulthood.

Tissue fixation cryoprotection, and tissue processing

Adult zebrafish from control and alcohol exposed groups were quickly decapitated and the heads immediately placed in 4% paraformaldehyde (in PBS) for 24 h at 4°C. Heads were subsequently sequentially placed in 10% (2 h), 20% (4 h), and 30% (24 h) sucrose at 4°C in order to cryoprotect the tissue before sectioning.

The entire zebrafish head was sectioned at 30 microns using a Microm HM 520 Cryostat after mounting with HistoPrep frozen tissue embedding media (Cat. No. SH75-125D; Fisher Scientific) at -25°C. Tissue sections were transferred onto Fisherbrand Superfrost Plus Microscope slides and were dried for an additional hour. Slides were subsequently stored at -80°C until immunohistochemistry was performed. Slides were dried at 45°C on a slide warmer for 1h before staining. Background autofluorescence was reduced by incubating with 1 mg/ml sodium borohydride in ice-cold sPBS for 30 min, and rinsed with sPBS three times. Following sodium borohydride treatment, sections were incubated in 5% normal goat serum in sPBS containing 0.3% Triton X-100 for 20 min to block non-specific binding. Sections were then incubated in the primary antibodies: anti-BDNF (rabbit polyclonal, Chemicon, Temecula, CA, Cat# AB1779SP); anti-NCAM (Sigma Chemicals, St. Louis, MO) (Cat# ABIN335373, AB_10766836); anti-synaptophysin (Sigma Chemicals, St. Louis, MO) (Cat# ABIN335305, AB_10768391) (1:200 dilution using 5% normal goat serum in sPBS) overnight at 4°C. The slides were then washed with sPBS four times (5 min each), and incubated with the secondary anti-rabbit antibody coupled to FITC at 1:200 dilution using 5% goat serum in sPBS for BDNF, or anti-mouse antibody coupled to TRITC at 1:200 dilution using 5% goat serum in sPBS for NCAM and synaptophysin for 2 h at room temperature in the dark. The sections were then washed four times (5 min each) with sPBS and counterstained with 0.5 µg/mL DAPI in sPBS for 10 min in the dark and subsequently washed with sPBS four times. Slides were then mounted using Shandon PermaFluor Mountant with Fisherbrand coverslips. Sections were dried overnight in the dark and subsequently stored at 4°C. For our negative control experiment, we examined non-specific binding by the secondary antibody by substituting the primary antibody with 5% normal goat serum in sPBS.

Image analysis

Immunoreactive cells were imaged using an immunofluorescence microscope (Olympus BX60), and quantified using Image Pro Plus (Media Cybernetics, Inc.) as described before for rat brain (Chatterjee et al, 2007). Appropriate excitation filters for FITC, TRITC and DAPI were used to obtain images at 400x magnification. Different brain regions were identified on the basis of neuroanatomical landmarks, and pictures were taken from identical sites. Schematic diagrams of the sites where photomicrographs were taken are shown in figures 2, 4 and 6. For analysis, pictures were taken of a particular brain area from 4 consecutive sections. Pictures were taken at 400x magnification for counting using a fixed exposure period for each type of stain (100 ms for DAPI and 6 s for BDNF, NCAM and

synaptophysin). The exposure time chosen was based upon the most optimal signal to noise ratio. Number of immunoreactive cells (for BDNF and NCAM positive cells) were quantified using Image-Pro Plus (Media Cybernetics, Inc.) using an automated count with a minimum cut-off area size of 200 pixels. For synaptophysin immunoreactivity, we have counted the pixels (as a measure of synaptophysin immunoreactivity) using Image-Pro Plus (Media Cybernetics, Inc.) using an automated pixel count. Background parameters for all pictures were set to identical settings using the digital control of the Image-Pro Plus software (Media Cybernetics, Inc.). In addition, the size of the area within which quantification of positively stained structures was performed was standardized for all three neuronal markers analyzed, in order to allow us direct comparison of quantified results across the selected brain regions. The sample size (n) of control and treated fish was 6, and the number sections analyzed per area of brain was 4.

Western blot analysis

Zebrafish brains were dissected and stored at -80°C until use. For NCAM and synaptophysin western blots, brains were sonicated in $30\mu\text{L}$ of Tris-buffered saline (TBS). Protein concentration was determined using Bio-Rad protein assay dye reagent (Bio-Rad). 4X Laemmli buffer was added to each sample (3 volume sample lysis + 1 volume sample buffer) and boiled for 2 min. For BDNF western blot, the tissue was prepared different to facilitate better dissolution of BDNF from tissue. For BDNF western blot, dissected tissues were homogenized in 10 volumes of 100mM phosphate buffer containing 1mM EDTA, 2M guanidine hydrochloride (pH7.2), and three protease inhibitors (10mM N-ethylmaleimide, 0.36mM pepstatin, and 1mM PMSF). Homogenates were subsequently sonicated and processed in Laemmli buffer as described above for NCAM and synaptophysin. Twenty micrograms of protein from each sample were loaded onto each lane of a 7.5% polyacrylamide gel (Bio-Rad) and separated by electrophoresis. Separated proteins were transferred to a nitrocellulose membrane using a Bio-Rad mini transblot apparatus. Membranes were incubated with 5% non-fat dry milk in TBS containing 0.1% Tween-20 for 2 h at room temperature to block nonspecific binding. The membrane was then incubated in the primary antibodies: anti-BDNF (rabbit polyclonal, Chemicon, Temecula, CA, Cat# AB1779SP); anti-NCAM (mouse monoclonal, Sigma Chemicals, St. Louis, MO, Cat# C-9672); anti-synaptophysin (mouse monoclonal, Sigma Chemicals, St. Louis, MO, Cat# S-5768) respectively (all primary antibodies were 1:200 dilution using 5% normal goat serum in sPBS) overnight at 4°C . The membranes were subsequently washed with TBS containing 0.1% Tween-20 four times (5 min each). The membranes were then incubated in a secondary anti-rabbit antibody coupled to HRP (1:10000 dilution for BDNF antibody) or in anti-mouse antibody coupled to HRP (1:10000 dilution for NCAM and synaptophysin antibody) for 1 h at room temperature and subsequently washed with TBS containing 0.1% Tween-20. Immunoreactivity was visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Bio-Sciences Corp.), exposed to X-Ray film for 30 sec and developed in a Kodak automated developer. Side by side we have blotted solubilized rat brain homogenates with the same anti-NCAM, anti-synaptophysin and anti- BDNF antibody to confirm the staining of specific proteins in the zebrafish brain.

Statistical analysis

SPSS (version 14.1) was used for data analysis. For each neuronal marker, a repeated measures Univariate Variance Analysis (ANOVA) was performed with two factors: Brain Area (the repeated measures within subject factor with 8 levels) and Embryonic Alcohol Treatment (the between subject treatment factor with 2 levels). Subsequently, as appropriate, a post hoc t-test with Holm-Bonferroni correction (to reduce type 1 error without inflating type 2 error) was performed for each brain area to compare the two treatment groups. The null hypothesis was rejected when its probability (p) was less than 0.05.

Results

Specificity of the BDNF, NCAM and synaptophysin antibody

The antibodies employed in this study were developed using mammalian species. Although high nucleotide sequence homologies between zebrafish and human genes have been found and although similarly high amino-acid sequence homologies between zebrafish and human protein homologs have been well established, antibodies developed for particular mammalian molecular targets may not always work in zebrafish. To investigate whether our chosen antibodies have indeed specificity of binding for their intended targets we conducted western blot analyses. These analyses revealed that the anti-BDNF, anti-NCAM and anti-synaptophysin antibody used in this study detected identical bands in both rat and zebrafish brain homogenates. Anti-synaptophysin antibody recognized a band at 34 kDa and BDNF recognized a band at 18 kDa in both rat and zebrafish brain homogenates. The anti-NCAM antibody, as expected from product insert of the antibody (<https://www.sigmaaldrich.com/catalog/product/sigma/c9672?lang=en®ion=CA>), recognized two proteins, which corresponded to the molecular weight of 180 kDa and 140 kDa subunits of NCAM protein, both in rats and zebrafish brains. The results shown in Fig. 1 thus suggest that the antibodies used in this study specifically recognized the BDNF, NCAM and synaptophysin protein in the zebrafish brain, and were not binding other brain proteins.

Quantification of BDNF, NCAM and synaptophysin

Representative immunostained photomicrographs of different brain areas of zebrafish labeled with anti-BDNF, anti-NCAM, and anti-synaptophysin antibodies are shown in Fig. 2, 4, and 6 respectively. The schematic diagrams of the areas analyzed are also shown in these images. Panel “A” shows the representative immunostaining of control fish brain (brain of fish that received no alcohol during embryonic development). Panel “B” displays DAPI stains of the same area. Panel “C” displays the superimposed image of Panels “A” and “B”. Panel “D” showing the representative immunostaining in brains of embryonic alcohol treated fish (fish that received 1% alcohol during embryonic development). Panel “E” displays DAPI stains of the same area. Panel “F” displays the superimposed image of Panels “D” and “E”. It is important to note that the pattern of DAPI stained nuclei were denser than immunostained cells, as DAPI stained all cells in any area, but immunostaining only stained a target-positive population, i.e. a sub-population, of neurons in that area. Furthermore, we found no significant differences in the total number of DAPI stained nuclei in each area studied between control and 1% alcohol treated fish (data not shown). Therefore, any difference observed in the immunostained cells between control and alcohol treated fish is

likely not due to alcohol treated animals having a reduction in overall number of cells, but rather to changes specific to the immunostained target neuron population.

The number of BDNF positive cells quantified in different areas of the brains of control and 1% alcohol exposed zebrafish (expressed as number of positive cells/mm²area) is shown in Fig. 3. Repeated measures ANOVA detected a significant brain area effect ($F(7, 70) = 819.72, p < 0.001$) demonstrating that BDNF positive cells are not distributed evenly across the eight brain structures studied. It also found the effect of embryonic alcohol treatment to be significant ($F(1, 10) = 667.81, p < 0.001$), confirming the lasting effect of early embryonic alcohol exposure on the brain in adult zebrafish. Last, it also detected a significant brain area x alcohol interaction ($F(7, 70) = 96.43, p < 0.001$) suggesting that the lasting effect of embryonic alcohol exposure was not equal in magnitude for all brain areas studied.

Although the magnitude of alcohol effect varied across brain areas, post hoc two-sample t-tests revealed a significant reduction in the number of BDNF positive cells in fish exposed to alcohol compared to fish that received no alcohol in the lateral zone of the dorsal telencephalic area (D1) ($t(10) = 22.23, p < 0.001$), medial zone of the dorsal telencephalic area (Dm) ($t(10) = 11.00, p < 0.001$), dorsal nucleus of the ventral telencephalic area (Vd) ($t(10) = 7.10, p < 0.001$), ventral nucleus of the ventral telencephalic area (Vv) ($t(10) = 9.39, p < 0.001$), corpus cerebelli (CCe) ($t(10) = 11.39, p < 0.001$), parvocellular preoptic nucleus (PPa) ($t(10) = 6.74, p < 0.001$), ventral habenular nucleus (Hav) ($t(10) = 10.67, p < 0.001$) and inferior reticular formation (IRF) ($t(10) = 13.38, p < 0.001$).

Figure 5 shows the number of NCAM positive neurons (expressed as number of positive cells/mm²area) quantified from 8 brain areas of adult zebrafish that were exposed either to embryonic alcohol or to fresh water (control). The figure demonstrates a robust reduction of the number of NCAM positive cells in all brain areas studied. It also suggests an unequal distribution of NCAM positive cells across the brain areas studied. Repeated measures ANOVA confirmed these observations and showed a significant brain area effect ($F(7, 70) = 1004.98, p < 0.001$) as well as a significant alcohol effect ($F(1, 10) = 350.32, p < 0.001$). In addition, the brain area x alcohol interaction term was also found to be highly significant ($F(7, 70) = 78.55, p < 0.001$) demonstrating that although alcohol significantly reduced the number of NCAM positive neurons in the brain of adult fish, this reduction was brain area dependent. Post hoc two sample t-test comparisons of embryonic alcohol treated and control fish showed that the alcohol treatment significantly reduced the number of NCAM positive neurons in the lateral zone of the dorsal telencephalic area (D1) ($t(10) = 15.35, p < 0.001$), medial zone of the dorsal telencephalic area (Dm) ($t(10) = 9.36, p < 0.001$), dorsal nucleus of the ventral telencephalic area (Vd) ($t(10) = 5.62, p < 0.001$), ventral nucleus of the ventral telencephalic area (Vv) ($t(10) = 7.10, p < 0.001$), corpus cerebelli (CCe) ($t(10) = 9.46, p < 0.001$), parvocellular preoptic nucleus (PPa) ($t(10) = 6.49, p < 0.001$), ventral habenular nucleus (Hav) ($t(10) = 10.19, p < 0.001$) and inferior reticular formation (IRF) ($t(10) = 7.08, p < 0.001$).

Figure 7 shows the intensity of synaptophysin immunostaining (expressed as number of pixels x 1000/mm² area) in 8 brain areas of adult fish that were exposed either to alcohol or to freshwater during embryonic development. Similarly to BDNF and NCAM, embryonic alcohol exposure appears to have reduced the signal compared to control. Repeated

measures ANOVA found the effects of brain area ($F(7, 70) = 167.92, p < 0.001$) and of alcohol treatment ($F(1, 10) = 467.25, p < 0.001$) significant. The interaction between these two factors was also significant ($F(7, 70) = 38.82, p < 0.001$), suggesting that although alcohol in general reduced the intensity of synaptophysin immunostaining, this effect was dependent upon the brain area in which it was quantified. Post hoc two-sample t-tests comparing alcohol and freshwater treated fish found a significant reduction in the intensity of synaptophysin in fish exposed to alcohol compared to fish that received no alcohol in the lateral zone of the dorsal telencephalic area (D1) ($t(10) = 15.73, p < 0.001$), medial zone of the dorsal telencephalic area (Dm) ($t(10) = 5.95, p < 0.001$), dorsal nucleus of the ventral telencephalic area (Vd) ($t(10) = 4.12, p = 0.0122$), ventral nucleus of the ventral telencephalic area (Vv) ($t(10) = 8.73, p < 0.001$), ventral habenular nucleus (Hav) ($t(10) = 17.10, p < 0.001$) and inferior reticular formation (IRF) ($t(10) = 11.34, p < 0.001$). However, the alcohol effect was found non-significant for the corpus cerebelli (CCe) ($t(10) = 2.19, p = 0.1075$) and parvocellular preoptic nucleus (PPa) ($t(10) = -1.33, p = 0.2253$),

Discussion

Previously, low doses of ethanol administered for a short period of time during the embryonic development of zebrafish were shown to induce lasting changes in the behavior. These alterations included reduction or abolishment of response to computer generated social stimuli (Fernandes & Gerlai, 2009; Fernandes *et al.*, 2015a; 2015b), and impaired shoaling with live conspecifics (Buske & Gerlai, 2011), as well as impaired associative learning (Fernandes *et al.*, 2014). The mechanisms underlying these embryonic alcohol exposure-induced behavioral changes are largely unknown, although some neurotransmitter systems (particularly the dopaminergic system) have already been implicated (Buske & Gerlai, 2011; Fernandes *et al.*, 2015b; Mahabir *et al.*, 2013). Social cognition as well as learning and memory are supported by a complex array of molecular mechanisms (Bicks *et al.*, 2015; Mayford *et al.*, 2012), and alcohol is known to interact with many of them (Wozniak *et al.*, 2004). Due to the fact that alcohol interacts directly and indirectly with a large number of molecular targets and thus influences numerous biochemical pathways, its effects on embryonic development are also expected to be rather complex, developmental-stage and alcohol-concentration dependent, analysis of which necessitates comprehensive systematic time-course studies. However, comprehensive analysis of embryonic alcohol induced mechanistic changes would be a substantial undertaking. Instead of such a comprehensive analysis, we decided to focus on a particular embryonic stage, the 24th hpf time point of development that has been shown to be particularly sensitive to alcohol effect in the context of social behavior; a single dose (1% external bath concentration) of alcohol that has been shown to abolish responses to social stimuli; and only three neuronal markers that are fundamentally important in many processes underlying cognitive and including social behavioral functions. The three chosen targets, BDNF, NCAM and synaptophysin, are also known to play crucial roles during brain development, and are likely to be engaged by embryonic alcohol exposure.

Using an immunohistochemical approach, we investigated potential effects of embryonic alcohol exposure on the expression of these neuronal markers quantified in the fully developed adult zebrafish. We found a robust reduction in the number of BDNF and NCAM

positive cells and also the strength of staining for synaptophysin in the brains of adult zebrafish that received embryonic exposure to alcohol. This is notable, because the changes were observed in fully mature adult zebrafish, thus demonstrating the long-lasting consequences of early developmental exposure to alcohol. It is also important to note that, although we found the alcohol effect significant in most brain regions, the magnitude of this effect depended upon the particular brain region studied. In other words, the impact of embryonic alcohol treatment varied according to brain region. How these immunohistochemical/molecular changes may contribute to alteration of behavior is not known at this point.

The BDNF gene is well conserved across species, from fish to mammals (Heinrich & Pagtakhan, 2004; Aid *et al.*, 2007; Pruunsild *et al.*, 2007), suggesting that this protein has a strong functional significance as well as translational relevance. Many neurodevelopmental diseases characterized by deficits with synaptic plasticity have been associated with abnormalities in the expression of BDNF (Alberch *et al.*, 2004; 2006; Arancio & Chao, 2007). BDNF plays a vital role during the development of the brain (Cohen-Cory *et al.*, 2010). For example, BDNF is involved in dendritic development, synaptic connectivity and growth and branching of axon terminals (Zweifel *et al.*, 2005; Ming *et al.*, 2001;). In adulthood, BDNF plays a pivotal role in the molecular mechanisms of synaptic plasticity, and many studies demonstrate the involvement of BDNF in learning and memory, neurogenesis, synaptic morphological rearrangements and plasticity (Hall *et al.*, 2000; Mu *et al.*, 1999; Cunha *et al.*, 2010; Waterhouse *et al.*, 2012; Bath *et al.*, 2012 and Vilar & Mira, 2016; An *et al.*, 2008; Tongiorgi *et al.*, 2006). These studies indicate the importance of BDNF both in the adult and in the developing brain, and how alteration of this protein can impact the neuronal circuit.

BDNF is also known to modulate monoaminergic synaptic transmission, for example, antidepressants primarily acting on the serotonergic and noradrenergic systems increase BDNF mRNA and protein expression (Lee & Kim, 2010; Binder & Scharfman, 2004). Importantly, BDNF expression has been demonstrated in numerous parts of the zebrafish brain including those studied here (Cacialli *et al.*, 2016). We speculate that the reduction of BDNF expressions we observed in brain areas where dopaminergic neurons reside (Vd, D1, Dm) and in areas where serotonergic neurons are found (Hav and IRF) may have contributed to the impaired shoaling and learning performance reported previously for zebrafish exposed to alcohol during embryonic development (Fernandes & Gerlai, 2009, 2014). It is possible that abnormal functioning of the dopaminergic system may have diminished the motivation to join a shoal, and alteration of the serotonergic system may have modified fear or aggression related phenotypes, which could have contributed to modification of cognitive processes involved in shoaling and learning.

NCAM is closely linked to cellular processes involving neural development and plasticity (see Hinsby *et al.*, 2004 for review) and learning and memory (Bailey *et al.*, 1992; Mayford *et al.*, 1992; Schuster *et al.*, 1996). Polysialylated NCAM (PSA-NCAM), or embryonic NCAM (eNCAM), plays a role in nervous system development and plasticity (Rutishauser & Landmesser, 1996;; Durbec & Cremer, 2001; Kleene & Schachner, 2004). During development, PSA-NCAM is expressed with axon outgrowth, branching and contact

formation in the corticospinal and the retinotectal systems as well as in the formation of the neuromuscular junction (Daston *et al.*, 1996; Fraser *et al.*, 1984; Yin *et al.*, 1995; Williams *et al.*, 1996; Landmesser *et al.*, 1990). In the adult brain, the degree of polysialylation of NCAM (PSA-NCAM) is low and most NCAM molecules play a role in stabilizing structures. In vitro studies indicate the role of NCAM in long term potentiation (LTP) (Luthi *et al.*, 1994; Rønn *et al.*, 2000a; Muller *et al.*, 1996) as well as neurite extension (Hildebrandt *et al.*, 2007). The mechanisms underlying NCAM's role in synaptic plasticity, regeneration, or adult learning and memory is still poorly understood.

The expression of NCAM was also affected in all of the brain regions we studied. NCAM has been found to play a role in the serotonergic system and in the development and survival of dopaminergic neurons (Stork *et al.*, 2000; Kohl *et al.*, 2013; Ghitza *et al.*, 2010; Xiao *et al.*, 2009). Based on these studies, the reduced NCAM expression in the brain of embryonic alcohol exposed zebrafish may explain the lower serotonin and dopamine levels as well as the reduced levels of metabolites of these neurotransmitters previously reported as resulting from embryonic alcohol exposure in zebrafish (Buske & Gerlai, 2011). Thus, both BDNF and NCAM may underlie the previously observed behavioral abnormalities of embryonic alcohol exposed fish, including impaired social behavior and learning performance.

Synaptophysin is exclusively localized to synaptic vesicles and is widely used as a marker for pre-synaptic terminals. It is one of the most frequently used protein markers of synapse formation and synaptic plasticity in the brain (Counts *et al.*, 2006; Reddy *et al.*, 2005; Knaus *et al.*, 1986). Synaptophysin also plays a vital role in learning and memory (Sze *et al.*, 1997; Frick & Fernandez, 2003). A study by Sun *et al.* (2007) indicated that synaptophysin may be involved in plasticity-related changes after injury of hippocampal neurons and in age-related cognitive impairment (Smith *et al.*, 2000). Recent studies also revealed increased synaptophysin in hippocampal neurons in aged brains, indicating the possibility of compensation for the cognitive decline associated with aging (Grilloa *et al.*, 2013). Though synaptophysin is one of the major synaptic vesicle proteins, its involvement in the regulation of neurotransmission is unknown (McMahon *et al.*, 1996).

In our study, the expression of synaptophysin was significantly reduced by embryonic alcohol treatment in the D1, Dm, Vd, Hav, Vv and IRF regions of zebrafish exposed to embryonic alcohol treatment as compared to fish that received no alcohol. Notably, we found no such alcohol effect on the cerebellum and preoptic area. These results suggest a decrease in synaptic connections, which likely include dopaminergic and serotonergic synapses, in D1, Dm, Vd, Vv, Hav and IRF, but no synaptic level alterations in the cerebellum and preoptic regions. This finding is intriguing as embryonic alcohol treatment has been found to decrease serotonin and dopamine levels in a dose dependent manner (Mahabir *et al.*, 2014) as well as to impair behaviors that may be mediated or influenced by these neurotransmitter systems (reward, learning, social interactions) in adult zebrafish (Fernandes & Gerlai, 2009, 2014).

Very few studies reported on the effects of prenatal ethanol on BDNF (Caldwell *et al.*, 2008; Feng *et al.*, 2005; Maier *et al.*, 1999), NCAM and synaptophysin (Medina, 2011; Klintsova *et al.*, 2013; Kumar *et al.*, 2013) in the vertebrate brain, and in all of these studies and others

mentioned in this paper, the concentration of alcohol employed during development was high and the duration of exposure was long, thus these studies may only have translational relevance for the most extreme cases of FASD. Our current study is the first to show decreased expressions of BDNF, NCAM, and synaptophysin in the brain of adult zebrafish due to a single, short and low dose alcohol exposure during development. Prior published results (e.g. Fernandes *et al.*, 2015b; Mahabir *et al.*, 2013; Buske & Gerlai, 2011) and our own unpublished findings suggest that the alcohol exposure regimen and timing employed in the current study particularly affects the dopaminergic and serotonergic neurotransmitter systems leaving other neurotransmitter systems, e.g. the GABAergic and glutamatergic, relatively intact at least in the context of neurochemical levels analyzed using HPLC. Thus, our current study together with the previous findings offer working hypotheses about the mechanisms underlying the altered brain functions, e.g. learning deficit and impaired social behavioral responses, detected previously in fully mature or old zebrafish, and, as such, opens new research lines into the analysis of the effects of embryonic alcohol exposure in vertebrates.

Our results also open the possibility of identifying potential biomarkers that may be used for diagnosing FASD, a major limitation in the human clinic. In summary, given the translational relevance of the zebrafish (Gerlai, 2010), mechanistic understanding of the actions of alcohol in this species may have important clinical implications for FASD in humans, the ultimate goal of our study.

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Abbreviations

BDNF	Brain derived neurotrophic factor
CCe	Corpus cerebelli
DAPI	4',6-diamidino-2-phenylindole
Dl	Lateral zone of the dorsal telencephalic area
Dm	Medial zone of the dorsal telencephalic area
dpf	days post-fertilization
EDTA	Ethylenediaminetetraacetic acid
eNCAM	embryonic Neuronal Cell Adhesion Molecule
FASD	Fetal alcohol spectrum disorder
FITC	Fluorescein isothiocyanate
GABA	Gamma Amino Butyric Acid
Hav	Ventral habenular nucleus

hpf	hours post-fertilization
HRP	Horse Radish Peroxidase
HPLC	High Performance Liquid Chromatography
IRF	Inferior reticular formation
LTP	Long-term potentiation
nAC	nucleus accumbens
NCAM	Neuronal cell adhesion molecule
PBS	Phosphate Buffered Saline
PMSF	phenylmethylsulfonyl fluoride
PPa	Parvocellular preoptic nucleus
PSA-NCAM	Polysialylated Neuronal Cell Adhesion Molecule
TBS	Tris Buffered Saline
TRITC	Tetramethylrhodamine-isothiocyanate
Vd	Dorsal nucleus of the ventral telencephalic area
VTA	ventral tegmental area
Vv	Ventral nucleus of the ventral telencephalic area

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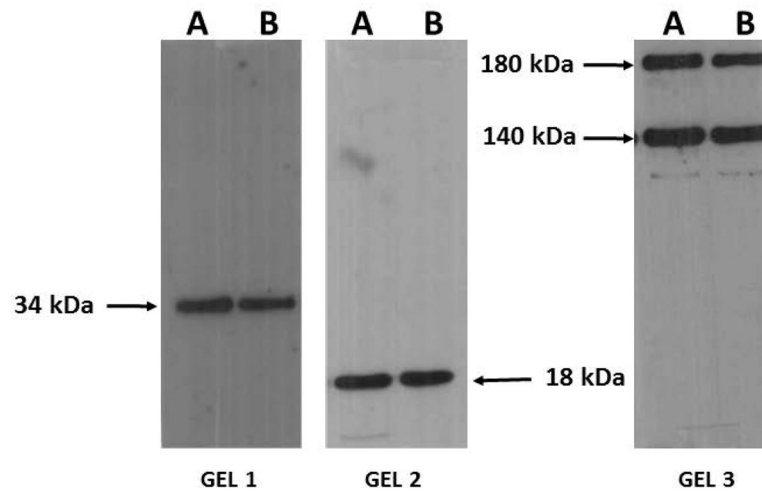


Figure 1. Western blot analysis of rat (A) and zebrafish (B) brain homogenates with anti-synaptophysin (gel 1), anti-BDNF(gel 2) and anti-NCAM(gel 3) antibodies used in the present study. Molecular weights of the proteins recognized by each antibody are indicated by arrows. Note the close correspondence of signals between rat and zebrafish brains.

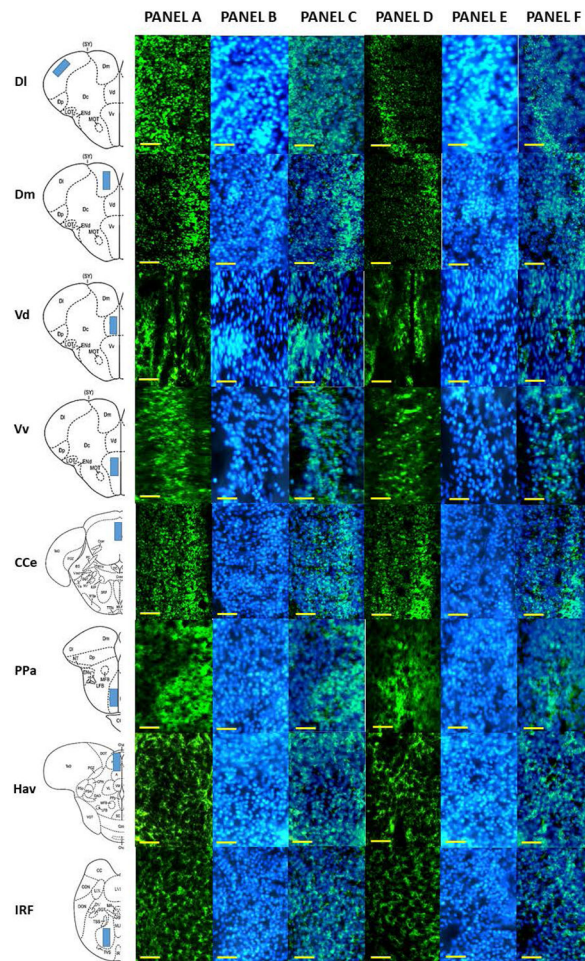


Figure 2.

Representative photomicrograph of different areas of adult zebrafish brain stained with anti-BDNF antibody. The first column of the figure displays a schematic diagram of the areas analyzed. Panel A depicts the anti-BDNF stained brain section of control fish (0% alcohol exposed) and Panel B depicts a DAPI stained brain section of the corresponding area. Panel C shows Panel A and B superimposed. Panel D depicts the anti-BDNF stained brain section of 1% alcohol exposed zebrafish, and Panel E depicts a DAPI stained brain section of the corresponding area. Panel F displays Panel D and E superimposed. The scale bar represents 10 μ m.

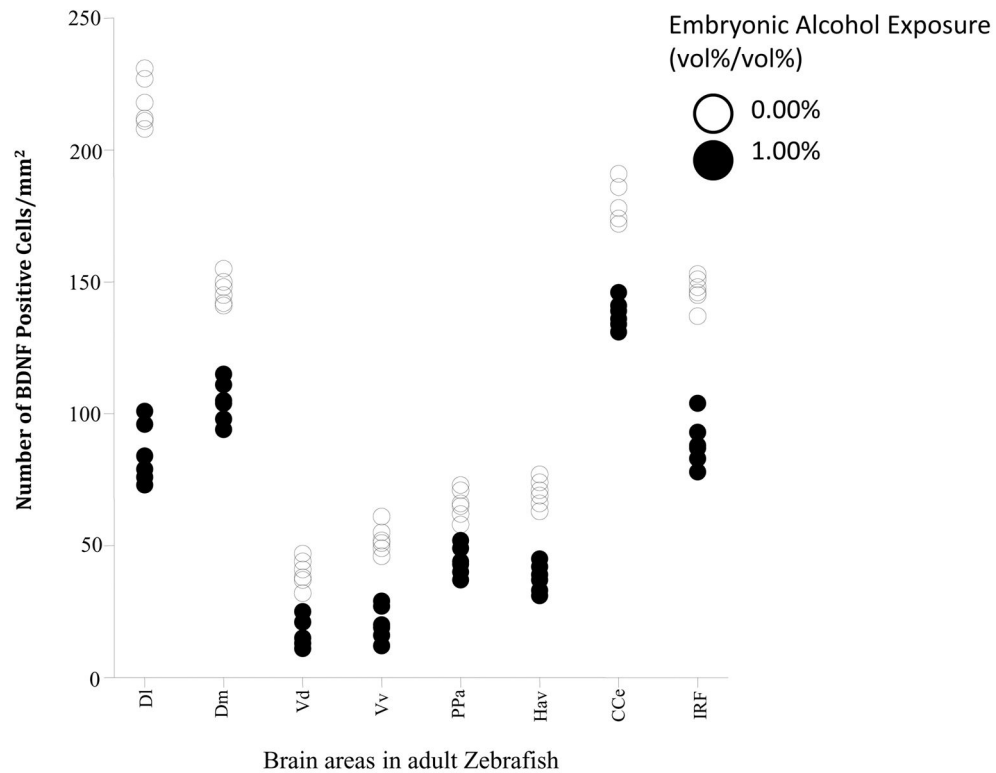


Figure 3.

Scatter-plot showing number of BDNF positive cells in different areas of the adult zebrafish brain. Note that alcohol (0%, control, white; or 1% alcohol black) was delivered during embryonic development and immunostaining to detect BDNF positive cells was conducted in adult zebrafish. Each point represents results from a single subject. The different brain areas analyzed were: lateral zone of the dorsal telencephalic area (DI), medial zone of the dorsal telencephalic area (Dm), dorsal nucleus of the ventral telencephalic area (Vd), ventral nucleus of the ventral telencephalic area (Vv), parvocellular preoptic nucleus (PPa), ventral habenular nucleus (Hav), corpus cerebelli (CCe) and inferior reticular formation (IRF). n=6, and 4 sections were used per animal.

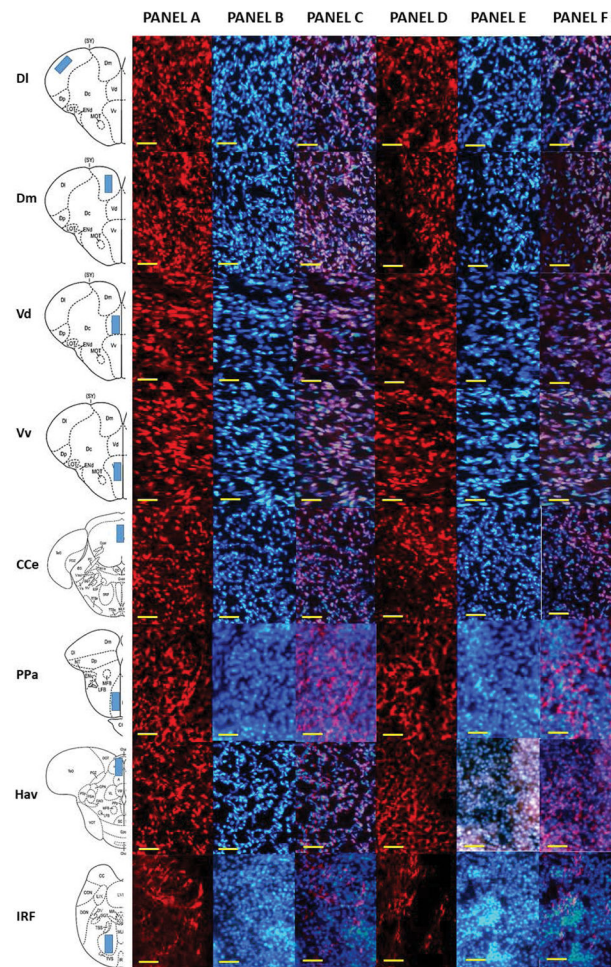


Figure 4.

Representative photomicrograph of different areas of adult zebrafish brain stained with anti-NCAM antibody. The first column of the figure displays a schematic diagram of the areas analyzed. Panel A depicts the anti-NCAM stained brain section of control fish (0% alcohol exposed), and Panel B depicts a DAPI stained brain section of the corresponding area. Panel C shows Panel A and B superimposed. Panel D depicts the anti-NCAM stained brain section of 1% alcohol exposed zebrafish, and Panel E depicts a DAPI stained brain section of the corresponding area. Panel F displays Panel D and E superimposed. The scale bar represents 10 μ m.

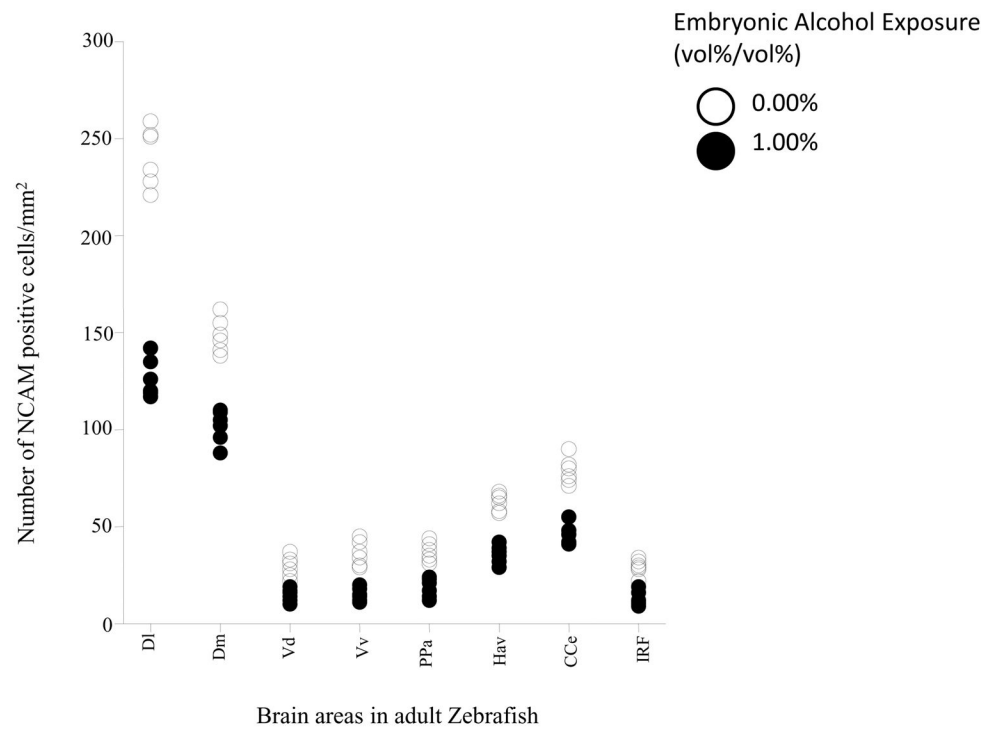


Figure 5.

Scatter-plot showing number of NCAM positive cells in different areas of the adult zebrafish brain. Note that alcohol (0%, control, white; or 1% alcohol black) was delivered during embryonic development and immunostaining to detect NCAM positive cells was conducted in adult zebrafish. Each point represents result from a single subject. The different brain areas analyzed were: lateral zone of the dorsal telencephalic area (DI), medial zone of the dorsal telencephalic area (Dm), dorsal nucleus of the ventral telencephalic area (Vd), ventral nucleus of the ventral telencephalic area (Vv), parvocellular preoptic nucleus (PPa), ventral habenular nucleus (Hav), corpus cerebelli (CCe) and inferior reticular formation (IRF). n=6, and 4 sections were used per animal.

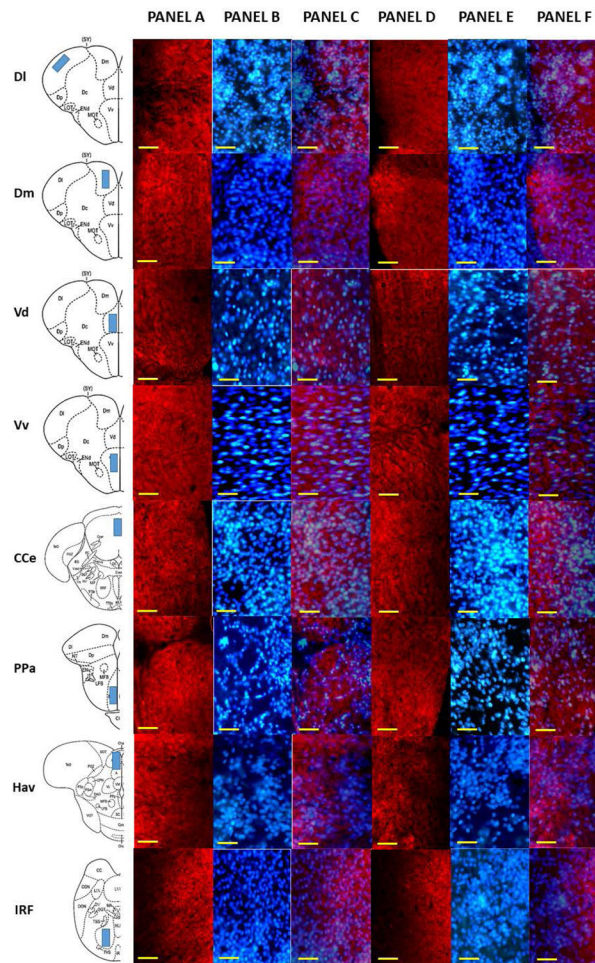


Figure 6.

Representative photomicrograph of different areas of adult zebrafish brain stained with anti-synaptophysin antibody. The first column of the figure displays a schematic diagram of the areas analyzed. Panel A depicts the anti-synaptophysin stained brain section of control fish (0% alcohol exposed), and Panel B depicts a DAPI stained brain section of the corresponding area. Panel C shows Panel A and B superimposed. Panel D depicts the anti-synaptophysin stained brain section of 1% alcohol exposed zebrafish, and Panel E depicts a DAPI stained brain section of the corresponding area. Panel F displays Panel D and E superimposed. The scale bar represents 10 μ m.

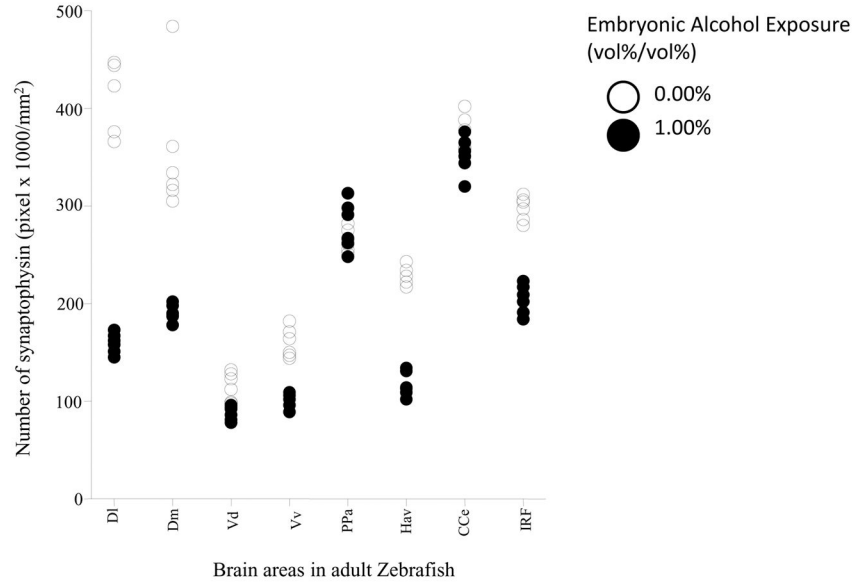


Figure 7. Scatter-plot showing intensity (as measured by pixel count) of synaptophysin positive staining in different areas of the adult zebrafish brain. Note that alcohol (0%, control, white; or 1% alcohol black) was delivered during embryonic development, and immunostaining to detect synaptophysin expression was conducted in adult zebrafish. Each point represents result from a single subject. The different brain areas analyzed were: lateral zone of the dorsal telencephalic area (DI), medial zone of the dorsal telencephalic area (Dm), dorsal nucleus of the ventral telencephalic area (Vd), ventral nucleus of the ventral telencephalic area (Vv), parvocellular preoptic nucleus (PPa), ventral habenular nucleus (Hav), corpus cerebelli (CCe) and inferior reticular formation (IRF). n=6, and 4 sections were used per animal.