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The effect of ethanol on cultures of the fetal rat brain

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Summary

Alcohol is a teratogen that affects the fetus in a negative manner. Because alcohol is an abundant and accepted drug in most societies, children are born by mothers who have been drinking alcohol during pregnancy. The birth defects in these children vary and the most severe form is called fetal alcohol syndrome (FAS). FAS is clinically diagnosed by a number of characteristics: craniofacial anomalies, growth deficiencies and central nervous system dysfunction. The mechanisms behind these alcohol-induced damages are not fully understood. In this study the effect of alcohol on the developing rat cerebellum was investigated. The cerebellum makes up 10% of the human brain volume but contains more neurons than the rest of the brain. If alcohol has neurodegenerative effects, the cerebellum is an ideal place to look. In the second trimester in rodents, the cerebellum is not fully developed and contains many neural stem cells. The neural stem cells give rise to precursor cells which in turn differentiate into neurons and glia cells.

In this study, the short-term and long-term effects of 1‰ alcohol on these cell types in different developmental stages were investigated. My results show that short-term exposure to alcohol has a varying effect on different cell types. Neural stem cells respond to low concentration of alcohol by first increasing their proportion in the cell population, then decreasing. The initial increase may be due to some kind of compensatory mechanism where neural stem cells compensate for some other cell type that decreases. Oligodendrocyte precursor cells decrease while astrocytes increase. The overall cell viability is reduced by low concentration of alcohol (1‰) while high concentration (5‰) kills almost all cells. The reduced viability in 1‰ alcohol treated cultures is delayed and the effect emerges after 8 to 11 days of culture. In this study, I also report that long-term exposure to alcohol has a clear negative effect on neurons and myelinating oligodendrocytes, while astrocytes are moderately affected. These results contribute to the complex image of how alcohol affects the developing brain. Further studies of chronic alcohol exposure to developing brain regions would add knowledge about the effects of this kind of treatment.

Introduction

The mammalian brain is a complex machinery consisting of billions of cells. These cells emerge from a small number of neural stem cells in early embryogenesis. Neural stem cells derive from so called neural precursor cells found in the neural tube (Purves *et al.* 2004). The multiplication, differentiation and maturation of these neural precursor cells into an adult brain are a delicate matter. A wide range of mechanisms, both predestined and random, regulate the formation and arrangement of the brain. Many of these mechanisms act simultaneously and depend upon each other (Purves *et al.* 2004). Because of this intricate multitude of mechanisms and events during brain development, small disturbances can result in immense effects. Such disturbances may be due to exposure to teratogenic substances such as alcohol.

Alcohol is one of the most abundant drugs around the world, and socially accepted in most cultures. For this reason, it is of great importance that the teratogenic effects of alcohol are thoroughly investigated.

Alcohol and the developing brain

Prenatal exposure to alcohol can give various effects on the fetus, from mild forms of growth deficiency to severe physiological and psychological defects. The clinical diagnosis for the severe form is called fetal alcohol syndrome (FAS) and is characterized by craniofacial anomalies, growth deficiencies and central nervous system dysfunction (Goodlett *et al.* 2001). How extensive the negative effects of alcohol on the fetus are depend upon a number of parameters such as dose, time point and duration of exposure (Carloni *et al.* 2004, Goodlett *et al.* 2005). It is also known that the different brain regions have varying susceptibility to alcohol. Autti-Ramo *et al.* (2002) who investigated patients with FAS by neuroimaging, found that different brain regions were diversely affected. One region that was found to have a high susceptibility to alcohol was the cerebellum. This conclusion was based on a significantly reduced size of the brain region. Alcohol has been shown to induce neurodegeneration in the cerebellum as well as other brain regions (Dikranian *et al.* 2005, Olney *et al.* 2000, Rubert *et al.* 2006). The degeneration of cells is due to activated caspases and related proteins which are part of the apoptosis machinery (Dikranian *et al.* 2004, Siler-Marsiglio *et al.* 2005). If the alcohol-induced damages to cerebellum can be understood, the mapping of determining events in the development of FAS and other birth deficiencies due to prenatal alcohol exposure can be identified (Spadoni *et al.* 2006).

The cerebellum

The cerebellum's primary function is to detect and control the position of the body and the limbs. The cerebellum also refines and modulates movements by comparing the information from the cerebral cortex regions that plan and initiate movements and the sensory systems that monitors the actual movement. If there is a difference between these two, the cerebellum modulates the signals so that the "motor error" is reduced. The cerebellum occupies 10% of the total brain volume and contains more neurons than the rest of the brain. The cerebellum, as well as the rest of the brain, is populated by different kinds of brain cells. Each cell type has a specific and vital role in the functional brain.

Nerve cells

There are two major classes of brain cells; neurons and glia cells. Neurons produce and transmit signals in the central and peripheral nervous system. These signals consist of electrical pulses that travel along axons and dendrites between neurons. The brain holds

billions of neurons that are connected in networks by axons and dendrites (Purves *et al.* 2004). Glial cells do not transmit any signals, instead they have an important supporting and modulating function towards neurons. There are three main classes of glia cells in the central nervous system; astrocytes that support and supply neurons with nutrients, oligodendrocytes that myelinate axons and microglia that have an immunodefensive role (Purves *et al.* 2004). The development and migration of glial cells during brain development may be negatively affected by alcohol exposure (Guerri *et al.* 2000, Guizzetti *et al.* 1997). To study and visualize the different nerve cells, cell type specific proteins are used as targets for markers. Neurons and glia cells have different protein expression and these can be used to identify them. Intermediate filament protein α -internexin and cytoskeleton protein β -tubulin are markers for neurons in the central nervous system. Glial fibrillary acidic protein (GFAP) is an intermediate filament protein found in astrocytes. Neural stem cells can be identified by the protein nestin, which is an intermediate filament protein expressed in dividing cells in the early brain development. Immature oligodendrocytes express the proteoglycan NG2 while mature oligodendrocytes express the myelin protein Rip.

During brain development, neurons migrate to their final position with the aid of specialized glia cells called radial glia. These radial glia cells have been found to have a high susceptibility to alcohol, resulting in displaced neurons (Rubert *et al.* 2006). This disturbed migration gives immense effects on the function of the adult brain. It has also been found that children prenatally exposed to ethanol have decreased white matter and/or increased gray matter in their brains (Sowell *et al.* 2002). White matter consists of myelinated axons while neural cell bodies and glial cells make up gray matter.

It is well known that alcohol has a negative effect on the developing brain, but the underlying mechanisms for these negative effects are not clear. For example, the mechanism for cell death resulting from alcohol exposure is not fully understood. There are two types of cell death; necrosis, caused by damage to the cell and apoptosis, In apoptosis, also referred to as programmed cell death, cells commit suicide as a response to specific internal signals (Purves *et al.* 2004). The question has also been raised as to whether alcohol affects the proliferation of new cells rather than the apoptosis machinery (Rubert *et al.* 2006). The smaller brain regions in children with FAS may be due to lowered proliferation during development.

How to study the effect of alcohol in the developing brain

The large number of parameters involved in alcohol exposure makes it difficult to determine exactly which mechanisms are disrupted. Different time points, doses and duration of exposure give different results due to the varying susceptibility to alcohol during brain development (Goodlett *et al.* 2001). To study the effect of alcohol in the fetal brain, researchers often use animal models (i. e. *in vivo* studies) and cell cultures (i. e. *in vitro* studies). Animals used in such studies have similar build-up of the brain as humans, so studying animals gives useful information that can be applied on humans (Goodlett *et al.* 2001). *In vivo* studies give a composite image of the effect of alcohol on the whole brain. The overall response in all cell types is simultaneously studied, as well as the interaction between cells. *In vivo* studies have shown that the susceptibility to alcohol is high during early neonatal life and lower during later postnatal time points (Siler-Marsiglio *et al.* 2005, Light *et al.* 2002). In *in vitro* studies, researchers select cell populations or brain regions to be examined and create artificial environments for them. *In vitro* studies give the researchers opportunity to study individual cells or populations of different cell types. Their molecular properties and response to alcohol can be investigated in great detail. In this study, the

artificial environment contained a constant level of alcohol and this enables me to see the effect of alcohol when it is chronically present without fluctuating.

Apoptosis and necrosis

When cells die by apoptosis, they produce several substances, including caspases. Caspases are enzymes that cleave protein substrates, and their enzymatic activity can be used as an indicator for apoptosis in assays. A colorless substrate molecule is cleaved by caspases producing a colored substance. The intensity of the color is proportional to the amount of active caspases and can be measured spectrophotometrically. When a cell dies because of necrosis, none of these caspases are produced.

Cell viability and proliferation

Cell viability and proliferation rate can be examined by letting viable cells incorporate substances. One such substance is (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), also called MTT. All viable cells have active mitochondria which is the organelle that produces energy for the cell. MTT is taken up by active mitochondria and is converted to insoluble crystals by mitochondrial dehydrogenase. These crystals can then be solubilized and the absorbance measured in a spectrophotometer. The absorbance gives a direct indication of how many cells are viable (Edmondson *et al.* 1988). Another method to visualize the amount of viable cells is to use trypan blue. In viable cells, the cell membrane hinders the dye from entering the cytoplasm and the cells look transparent in the microscope. Dead cells are stained blue because trypan blue is absorbed into the cytoplasm. To study the rate of proliferation in alcohol-exposed cell cultures, a method called BrdU-incorporation can be used. BrdU is an analogue to thymidine and is incorporated into the DNA if cells are exposed to BrdU during replication. The incorporated thymidine analogue can then be detected by immunocytochemistry and the number of proliferating cells thereby visualized.

Immunocytochemistry

Immunocytochemistry enables researchers to target and visualize specific cellular constituents. This method is based on the action of antibodies. A primary antibody binds to its specific antigen, a cellular protein. A secondary antibody conjugated with a fluorescent marker binds to the primary antibody. The secondary antibody carries a fluorescent marker which can be detected in a fluorescence microscope. To visualize the nuclei in cells, the fluorescent stain DAPI that binds strongly to DNA is used.

Aims

The aim of this project was to investigate the effect of ethanol on mixed cultures of embryonic rat cerebellum and to investigate

- a. if alcohol induced cell death and if the cell death was necrosis or apoptosis
- b. how alcohol affected different cell species in short-term cultures
- c. how alcohol affected the different cells in long-term cultures

Results

Cell viability

The primary approach to investigate if alcohol exposure has an effect on cell viability and cell death was to study the overall changes in cell viability in cultures. Cell cultures exposed to a lower dose of alcohol showed a delayed response, where a marked decrease of cell viability was observed at day 11 (Fig. 1). No significant difference between control cultures and 1‰ alcohol treated cultures were found after 5 or 8 days of exposure while there was significantly decreased cell viability for the cultures treated with 5‰ alcohol. After 11 days of exposure, the activity for the 1‰ alcohol treated cultures was significantly decreased compared to control cultures. The cultures treated with 5‰ showed low cell viability already from day 5, significantly less than the 1‰ alcohol treated cultures.

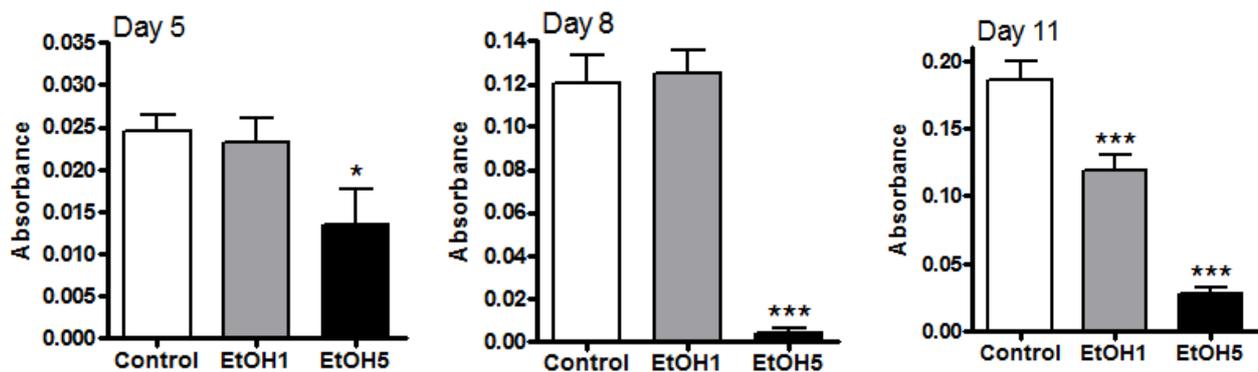


Fig. 1. The viability of cell cultures treated with two different concentrations of alcohol; 1 ‰ (17.1 mM) and 5 ‰ (85.7 mM). Cell cultures from gestation day 17 (E17) rat cerebellum were cultured for 5, 8 or 11 days. A colorimetric assay kit was used to test the viability of the cell cultures. The absorbance is directly proportional to the cell viability. Graphs represent the mean \pm SEM $n=9$, one way ANOVA-test, * $p < 0.05$ *** $p < 0.001$.

Short-term and long-term effect on different cell types

The short-term and long-term effect of alcohol on different cell types was studied with immunostaining. For short-term exposure, the relative proportion of selected cell types were manually counted and the data statistically evaluated by unpaired t-test. The cells were exposed to low concentration of alcohol (1‰) during different time periods. The two different time points; 5 days and 11 days, mirror two different stages in the primary culture. The 5 day culture represents the typical system used for acute exposure by most groups working with cerebellar granule cells. The 11 day culture is unique to this method since cultures normally do not last this long even without treatment. Therefore, 11 day cultures are ideal for studies of proliferation since the stem cells compensate for cell loss, as my results from the cell counting of neural stem cells shows. The results from long-term exposure to alcohol were only evaluated visually. The cell types investigated in short-term exposure were neural stem cells (nestin-positive), oligodendrocytes precursor cells (NG2-positive) and astrocytes (GFAP-positive). In long-term exposure, myelinating oligodendrocytes (Rip-positive), neurons (β -tubulin-positive) and astrocytes (GFAP-positive) cells were studied.

My results showed that the relative proportion of neural stem cells (nestin-positive) significantly increased in 5 day cultures exposed to 1‰ alcohol ($p < 0.001$). After 11 days of culture the proportion had decreased, although not significantly ($p > 0.05$) (Fig. 2A-B). An anti-nestin antibody visualized the population of neural stem cells in the cultures (Fig. 2C-D).

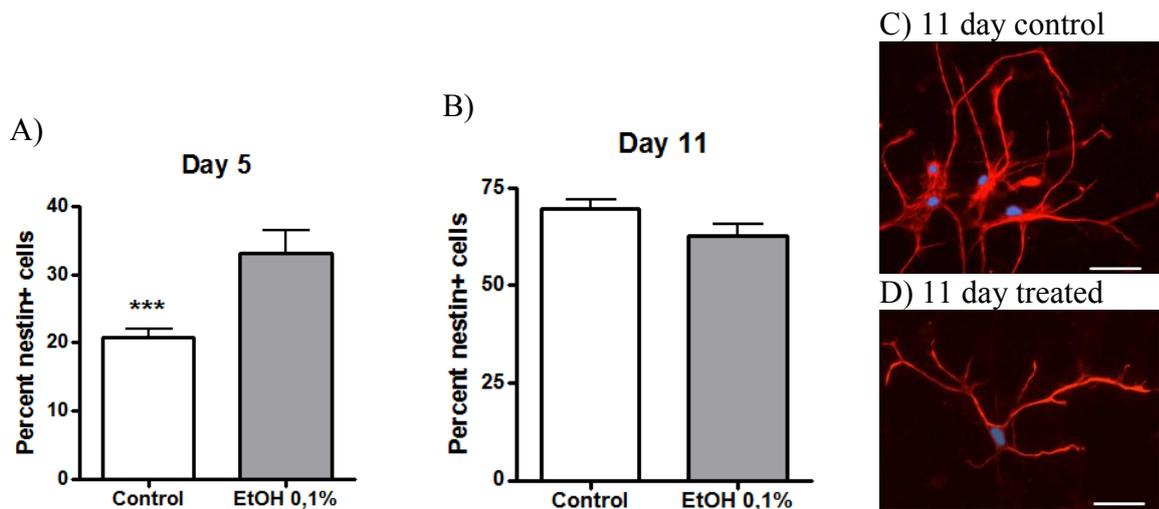


Fig. 2. The effect of alcohol on the fraction of nestin-positive neural stem cells in cell cultures from gestation day 17 rat cerebellum. Treated cultures were exposed to ethanol during the entire culturing. **A,B:** Bars showing mean \pm SEM of 1500-3500 counted cells in random pictures taken with a fluorescence camera after 5 (A) or 11 (B) days. Nestin-positive cells were labeled immunologically, and the percentage of total cells (stained with DAPI) that were nestin+ was calculated $***p < 0.001$, unpaired t-test. **C,D:** Immunofluorescence image showing the population of neural stem cells in control culture (C) and in a culture treated with 1‰ alcohol (D). Nuclei are stained blue (DAPI) while cell bodies and extensions are stained red (nestin). Scale bar = 43 μ m.

The high percentage of neural stem cells in cultures indicated that there was some kind of overlap between the cellular targets for neural stem cells and neurons used in immunocytochemistry based on results from Johannes Molin (2007) who observed similar high proportion of neurons in treated cultures. To investigate if this was true, cell cultures were stained with markers for neural stem cells (nestin) and neurons (β -tubulin). The result (Fig. 3) indicates an overlap between the two markers, thus indicating that the neural stem cells are differentiating into neurons but are still immature and therefore contain both cell markers.

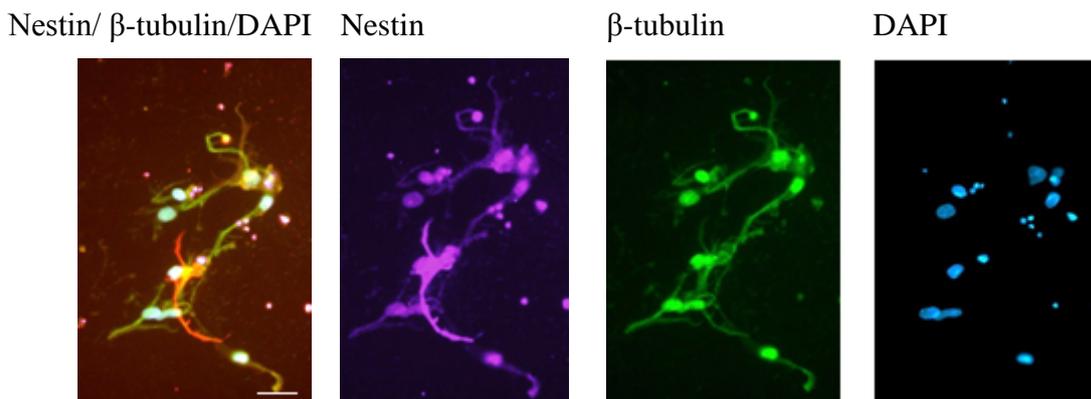
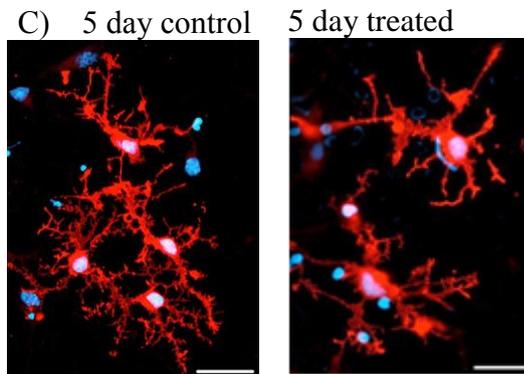
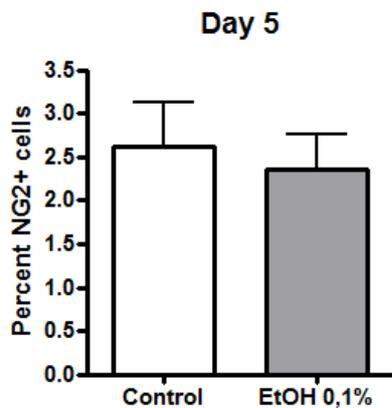


Fig. 3. Immunofluorescence image showing neural stem cells and neurons in cultures from gestation day 17 rat cerebellum. Overlap between protein markers used for identification of neurons (β -tubulin) and neural stem cells (nestin) are indicated by yellowish coloring. Neural stem cells are stained magenta (nestin), neurons green (β -tubulin) and nuclei blue (DAPI). Scale bar = 43 μ m.

For oligodendrocyte precursor cells, the trend was a steadily although not statistically significant reduction in the relative proportion of NG2-positive cells in alcohol treated cultures (Fig. 4).

A)



B)

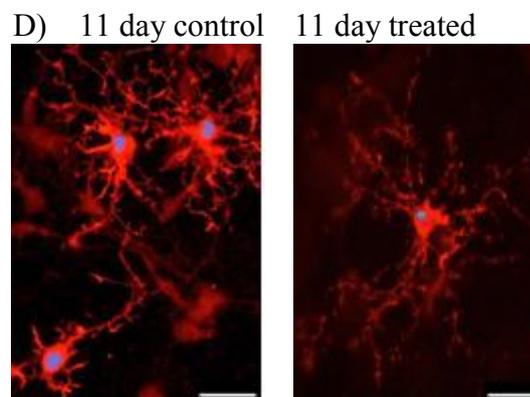
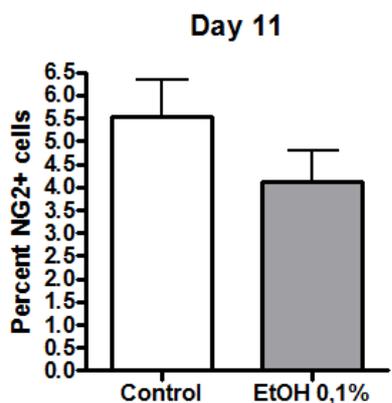
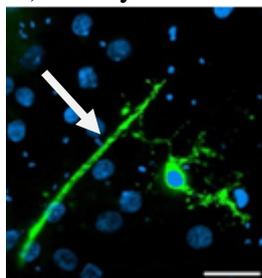


Fig. 4. The effect of alcohol on oligodendrocyte precursor cells in 5 day and 11 day cultures from gestation day 17 rat cerebellum. **A,B:** Bars showing mean \pm SEM of 1500-3500 counted cells in cell cultures, $p > 0.05$, unpaired t-test, for both graphs. NG2-positive cells were labeled immunologically, and the percentage of total cells (stained with DAPI) that were NG2+ was calculated. **C,D:** Representative immunofluorescence showing oligodendrocyte precursor cells stained red (NG2) in control cultures and cultures treated with 1% alcohol. Nuclei are stained blue (DAPI). Scale bar = 43 μ m.

A) 30 day control



B) 30 day treated

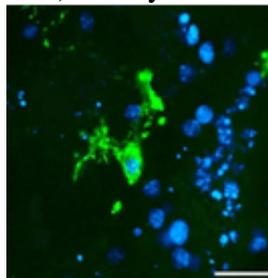


Fig. 5. Long-term effect of alcohol on the development of myelinating oligodendrocytes in cell cultures from gestation day 17 rat cerebellum. **A,B:** Pictures showing myelinating oligodendrocytes (myelin sheath indicated with arrow) in control cultures (A) and 1% alcohol treated cultures (B). Cell bodies and extension are stained green (Rip), nuclei are stained blue (DAPI). Scale bar = 43 μ m.

The long-term effect of alcohol on the development of myelinating oligodendrocytes (Rip-positive) was studied. Control cultures and 1% alcohol treated cultures were examined and compared in a fluorescence microscope. The cultures exposed to alcohol contained no myelinating oligodendrocytes while control cultures contained a few (Fig 5 A-B). Myelinating oligodendrocytes produce myelin sheaths that looks like elongated, thick extensions. In cultures exposed to alcohol there were a few Rip-positive cells with morphology similar to oligodendrocytes, but no myelinating extensions were visible.

My results for astrocytes (GFAP-positive) complement those of a former degree project student's unfinished study of this cell type. Figure 6A shows that the proportion of astrocytes increased in alcohol treated cultures. Though not statistically significant, the trend is supported by results from long-term cultures (Fig. 6B-C). After 35 days of alcohol treatment cultures were overcrowded by astrocytes. Only a small amount of neurons were found. In 35 day control cultures, there were only a few astrocytes, but the culture instead consisted of a massive network of neurons.

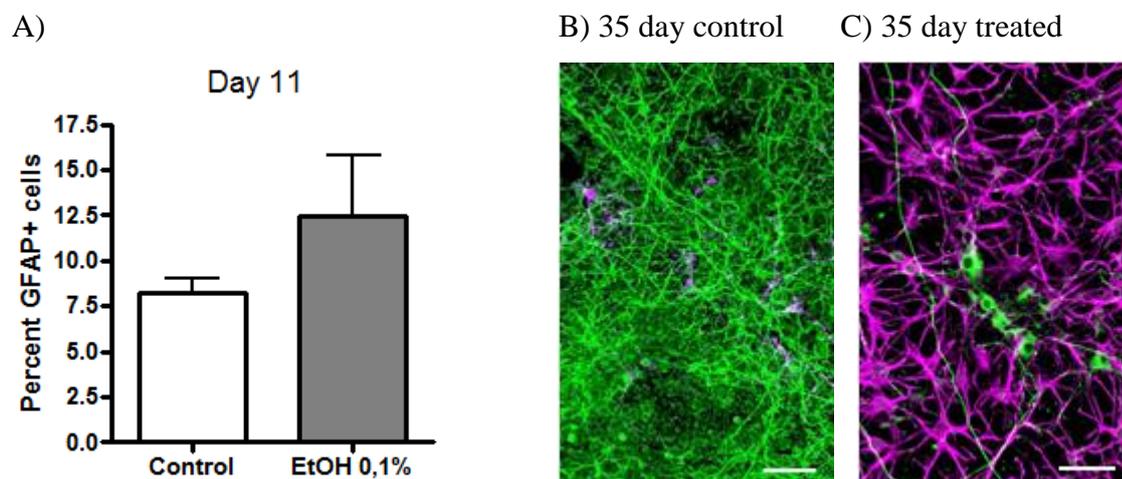


Fig. 6. Short-term and long-term effect of alcohol on the population of astrocytes (GFAP-positive cells) in mixed cultures from fetal rat cerebellum. **A:** The fraction of GFAP-positive cells (astrocytes) in 11 day cell cultures from gestation day 17 rat cerebellum visualized by immunostaining. The alcohol-treated cultures were exposed to alcohol during the entire incubation. The graph represent the mean \pm SEM of 1500-3500 counted cells in randomly chosen fields on cultures, $p > 0.05$, unpaired t-test. The total number of cells (stained with DAPI) was used to calculate the percentage of GFAP-positive cells. **B,C:** Long-term effect (35 days) of alcohol. Immunofluorescence showing the proportion of neurons (α -internexin) stained green and astrocytes (GFAP) stained magenta in control cultures and 1% alcohol-treated cultures. Scale bar = 43 μ m.

Apoptosis assay

When cells undergo apoptosis they initiate and activate a series of events. One of the players in this process is the enzyme caspase-3. To evaluate the extent of apoptosis in alcohol-treated cultures, this enzyme can be used as an indicator. After 5 days of alcohol exposure an increase of active caspase-3 was observed for cultures treated with 5% alcohol ($p < 0.09$). No significant difference between control cultures and alcohol treated cultures was observed after 11 days.

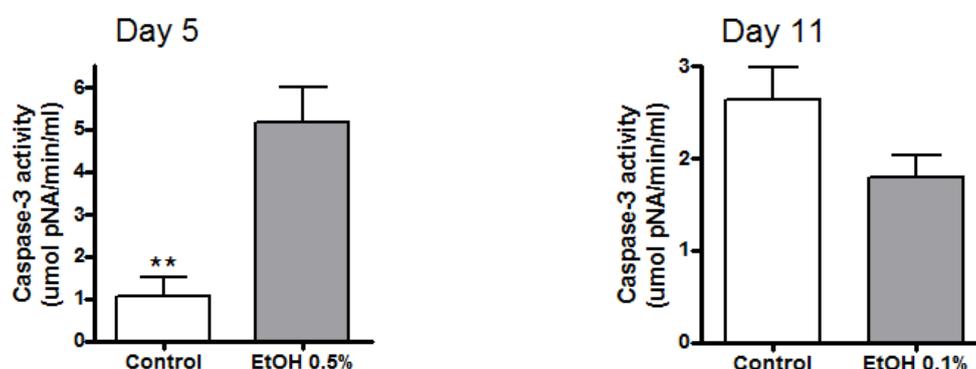


Fig. 7. The apoptotic response in cell cultures treated with alcohol. Primary cultures from E17 rat cerebellum was cultured and exposed to 1% or 5% alcohol for 5 or 11 days. $**p < 0.01$, unpaired t-test, $n=4$.

Proliferation assay

No results were analyzed from the BrdU-incorporation experiment. I did have time to expose the cell cultures to BrdU, fix them and complete the immuno staining. However, counting cells after pictures were taken in the fluorescence microscope will be continued in a subsequent project.

Discussion

How alcohol affects cell viability and apoptosis

The viability test gives information about the overall cell viability in cultures (Cebere *et al.* 1999). The viability in cultures treated with 1‰ alcohol was unchanged for the first 8 days of culture. After 11 days of culture the viability was significantly lower ($p < 0.001$) than control cultures. Cultures treated with 5‰ alcohol maintained a very low viability throughout the tested time period. The delayed decreased viability for the cultures treated with 1‰ alcohol may indicate two things. First, the negative effect of low concentration of alcohol is not acute. Instead the negative effects emerge after a certain time of exposure. Second, the developmental stage at 11 days of culture may represent a window of vulnerability to alcohol.

Although I did not analyze data from the proliferation assay (BrdU-incorporation) some information regarding proliferation and cell viability can be inferred from the immuno staining. Between 5 and 11 days of culture, the percentage of neural stem cells rose from 21% to 70% in control cultures. This is an increase of 49 percentage points. For alcohol treated cultures, the increase was from 33% to 63%, an increase of 30 percentage points. This may indicate that alcohol reduced the proliferation of neural stem cells. The fraction of neural stem cells increased by 235% in control cultures between day 5 and day 11, while alcohol treated cultures increased only by 89%. The proliferation rate for oligodendrocyte precursor cells also decreased in alcohol-treated cultures, control cultures increased by 112% between day 5 and 11 while alcohol treated increased only by 74%. An alternative explanation for the lowered fraction of both neural stem cells and oligodendrocyte precursor cells is that the cells die, either due to necrosis or apoptosis. This was analyzed with an apoptosis kit. Results from day 11 indicated no increase in apoptotic activity. If this is true, the alcohol-induced damage to neural stem cells and oligodendrocyte precursor cells between day 5 and 11 of culture may be due to inhibition of proliferation.

Short-term effect of alcohol

Results from the immunostaining gave detailed information about how different cell types were affected by alcohol exposure. For the shorter time period of 5 days, there was a diverse response to alcohol exposure. The fraction of neural stem cells (nestin-positive) increased significantly ($p < 0.001$) while oligodendrocyte precursor cells (NG2-positive) decreased, although not significantly. Overall cell viability was not different between control cultures and alcohol-treated cultures. These results indicated that the total number of healthy cells remained the same, but their internal organization changed. The increased fraction of neural stem cells may imply that there is some kind of compensatory mechanism in action. The viability of cells exposed to 5‰ alcohol was significantly lower ($p < 0.05$) than control cultures, indicating a neurotoxic effect of alcohol.

At the second time point, 11 day culture, the response to alcohol had changed. There was significantly decreased viability ($p < 0.001$) in cultures exposed to 1‰ alcohol. Cultures exposed to 5‰ alcohol had a very low viability, significantly lower than cultures treated with 1‰ alcohol ($p < 0.001$). This implies that concentrations as high as 5‰ kill almost all cells while lower doses have a more gradual effect. In terms of internal proportions between different cell types, there was a change. The assumed compensatory mechanism from neural stem cells was no longer present. The trend instead was a slight decrease. The same trend was seen for oligodendrocyte precursor cells but the decrease for the two cell types was not significant. The results were different concerning astrocytes (GFAP-positive), which constituted a larger fraction in alcohol-treated cultures than in control cultures.

Long-term effect of alcohol

The long-term effect of alcohol was investigated by culturing cells for 30 to 35 days. Treated cultures were exposed to 1‰ alcohol during the entire time period. Three cell types were studied; neurons (β -tubulin-positive), astrocytes (GFAP-positive) and myelinating oligodendrocytes (Rip-positive). An important finding in this study was the shift of vulnerability in neurons regarding short-term and long-term exposure to a low concentration of alcohol (1‰). According to preliminary results from the Åsa Fex Svenningsen group, short-term exposure does not affect the survival of neurons (Molin, 2007). I studied the long-term effect of alcohol on neurons and found that the initial low sensitivity to alcohol did not persist. After 35 days of exposure almost all neurons were dead. Only a few, large neurons survived the treatment. Control cultures were crowded with small neurons and massive axonal networks. In these cultures, only small astrocytes were present. The negative effect of long-term exposure to low concentration of alcohol was obvious concerning neurons. Mature myelinating oligodendrocytes were found in control cultures. In alcohol treated cultures there were only immature, non-myelinating oligodendrocytes. Negative effect on myelinating oligodendrocytes have been observed in other studies (Hekmatpanah *et al.* 1994). According to previous research, children with FAS have decreased volume of white matter in the cerebellum (Sowell *et al.* 2001). My results support this observation: long-term exposure to a low dose of alcohol has a strong negative effect on the survival of neurons and myelinating oligodendrocytes.

Some of the results in this study need to be corroborated. Additional apoptosis assays for short-term effects of 1‰ and 5‰ alcohol are needed, as well as investigation of the short-term effect of 1‰ alcohol on neurons. The proliferation assay with BrdU-incorporation will show whether the proliferation is lowered by chronically alcohol exposure and if the effect is instant or delayed. Further studies will provide more knowledge about the diverse response seen in this study.

Materials and methods

Animals and Dissection

Pregnant Sprague-Dawley rats from Scanbur BK AB (Sollentuna, Sweden) were used. At gestational day 17 (E17), pregnant rats were euthanized with an overdose of CO₂ and the uterus was removed with scissors and forceps under sterile conditions. The uterus was placed on ice in a 35*10 mm dish (Falcon) with Leibovitz 15 medium (L15) (Gibco Invitrogen, Stockholm, Sweden), and the embryos were detached from the uterus and placenta with scissors and forceps. The embryos were decapitated with a scissor and their heads were then kept on ice-cold L15 on ice until used. A dissection microscope was used for the removal of the cerebellum. The heads were placed under the microscope and the skull was cut open with micro scissors to reveal the lobes and cerebellum. The cerebellums were collected by cutting the anterior end of the cerebellum with the micro scissors and then pulling it in a posterior direction. The isolated cerebellums were kept in L15 on ice. Sterile conditions were maintained throughout the dissection.

Cell Culturing

The cerebellums from a number of rat pups were mechanically dissociated in L15 with a 10-ml pipette. The cell suspension was filtered through a cell strainer into a 15-ml Falcon tube and centrifuged at 160g for five minutes. The supernatant was removed and the pellet was resuspended in 10 ml L15. A 20 µl sample was taken from the cell suspension to determine cell density. Total cell number was counted in a Bürker chamber (0,100 mm, Assistent). The Bürker chamber was misted up and a cover glass was carefully pushed forward onto it. A 20 µl sample from the cell suspension was mixed with an equal amount of Trypan blue (0.4%, Sigma-Aldrich, Stockholm, Sweden). The sample was then carefully pipetted into the chamber. The loaded Bürker chamber was put under a microscope and the average cell number was determined by counting cells from 4-6 grids. The cell suspension was centrifuged a second time, the supernatant removed and the pellet resuspended in Neuro Basal Medium (Gibco Invitrogen) supplemented with serum substitute B27 (2%, Gibco Invitrogen) and glutamine (1.5%, Gibco Invitrogen). The amount of Neurobasal Medium added depended on the required cell density for the different containers the cells were cultured in (table 1).

Table 1.

Type of assay	Cell culturing container	Cell density
Immunocytochemistry	4-well Chamber slides (Nalge Nunc Lab-Tec)	1*10 ⁶ cells/ml
BrdU incorporation	4-well Chamber slides (Nalge Nunc Lab-Tec)	1*10 ⁶ cells/ml
In vitro Toxicology assay kit MTT based	96-well ELISA plate (Cellstar, Greiner Bio-one)	0.6*10 ⁶ cells/ml
Caspase 3 assay Kit	100*10 mm dishes (Falcon)	1*10 ⁶ cells/ml

The cell culturing containers were pre-treated with poly-L-lysine (50 µg/ml, Sigma-Aldrich, Stockholm, Sweden). The containers were filled with sterile filtered poly-L-lysine and incubated at 37°C, 5% CO₂ over night. The following day, the poly-L-lysine was removed and the containers washed twice with sterile water. The procedure was performed under sterile conditions.

Before plating the cell suspension, some cell cultures received ethanol (96%, Solveco, Stockholm Sverige) in concentrations of 1‰ (17.1 mM) or 5‰ (85.7 mM). The preparatory steps before incubation were performed in room temperature in a laminar flow hood. Alcohol-treated cultures were incubated in airtight boxes together with dishes containing ethanol diluted in sterile water corresponding to the ethanol concentration in the medium. Cell cultures were incubated at 37°C, 5% CO₂ and medium and water baths were renewed every third day.

Viability test

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide-test (MTT-test) measures the respiratory activity in cell cultures or cell suspensions. Cells were cultured in ELISA plates (96-well, Cellstar, Greiner Bio-one) pre-coated with poly-L-lysine. After cell counting and centrifugation, the pellet was resuspended in Neuro-Basal Medium. The cell suspension was divided into three 15-ml Falcon tubes, one control and two for ethanol treatment, 1‰ and 5‰ ethanol. The three different cultures were plated on separate ELISA plates, 100 µl/well in columns 2-10. In column 1 and 12, 100 µl of medium without cells were added to avoid evaporation and to serve as blanks in the assay. The plates were incubated at 37°C, 5% CO₂ for 5, 8 or 11 days. Medium was exchanged every third day by gently vacuuming the old medium and replacing it with fresh medium with or without ethanol. After completed incubation, 10 µl MTT (Sigma-Aldrich) was added to 10 randomly chosen wells - including one blank from row 1.

The substance MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is taken up by mitochondria in viable cells. The mitochondrial dehydrogenase cleaves the tetrazolium ring, creating insoluble formazan crystals. After addition of the reagent the plates were incubated for 4 hours in 37° C, 5% CO₂. After this the medium and the MTT solution were carefully vacuum aspirated from the wells. Care was taken to empty the wells thoroughly. To this point all work was performed in a laminar flow hood under sterile conditions. Directly after removal of medium a solubilization solution (Sigma-Aldrich) was added, 100 µl/well. This solubilizes the formazan crystals produced by active mitochondria in viable cells. The ELISA plates were incubated 30 minutes in room temperature on a shaker plate to ensure all MTT was solubilized. Afterwards, the absorbance was measured spectrophotometrically in a Multiscan MS spectrophotometer (Labsystems, Stockholm, Sweden) at a wavelength of 570 nm. The background absorbance was measured at 690 nm and subtracted from the 570 nm measurement. The data was processed in the computer program GraphPad Prism 4 and the statistical significance was evaluated by a one way ANOVA-test. This statistical test was chosen because there were three different groups to be evaluated and compared.

Immunocytochemistry

For immunocytochemistry, cells were grown on 4-well Chamber slides. After incubation (5, 8 or 11 days) alcohol-treated and control cells were fixed in Stefanini fixative (2% formaldehyde, 0.2% picric acid, 10% PBS, pH 7.3) for 20 min. The fixative solution was washed off with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.3) 3 times for approximately 2 minutes each. Primary antibodies were selected (Table 2) and diluted in blocking solution (PBS, 0.25% Triton X-100 Sigma-Aldrich, 0.25% BSA (Bovine serum albumin) Sigma-Aldrich), kept at 4°C. To each well, 150 µl of primary antibody solution was prepared and added. The chamber slides were then incubated at 4°C overnight in a box holding a moist tissue to keep the slides from drying out. The next day, the cells were washed twice with washing solution (PBS, 0.25% Triton X-100) for approximately

15 minutes. Blocking solution was mixed with the appropriate fluorescent secondary antibody and added to the wells, 150 μ l each, and then incubated for 1 hour at room temperature. During incubation, the slides were covered with aluminum foil to avoid bleaching of the fluorescent antibody. After incubation the cells were washed twice with washing solution for 15 minutes. During the second wash, 1 ml DTG mounting medium (1.25 g DABCO (Sigma-Aldrich D-2522), 45 ml glycerol, 5 ml 0.5 Tris pH 8.6 (Sigma-Aldrich) was mixed with 0.33 μ l nuclear marker DAPI (4', 6-diamidino-2-phenylindole 1.25 mg/ml, Sigma-Aldrich). After the second wash, chambers and joints were separated from the slides and superfluous washing solution was removed by tilting the slides against a paper towel. Immediately after removal of washing solution, DTG mounting medium was dripped on the slides to avoid drying. Cover glasses were placed on the slides and pressed down carefully to get rid of air bubbles. The edges were sealed with nail polish and the slides stored at -20°C.

Table 2. Primary and secondary antibodies.

Primary antibody (host, source)	Target	Dilution	Fluorophore (host, source)	Target	Dilution
anti-neslin (mouse, Developmental Studies)	Neural stem cell, intermediate filaments	1:400	Alexa 488 (goat, Molecular Probes)	Mouse-IgG	1:400
anti-NG2 (mouse, Chemicon)	Oligodendrocyte precursors	1:500	Alexa 488 (goat, Molecular probes)	Mouse-IgG	1:400
anti-α-internexin (rabbit, Chemicon)	Axons in central nervous system	1:400	Alexa 488 (goat, Molecular probes)	Rabbit-IgG	1:400
anti-GFAP (rabbit, Sigma)	Astrocytes	1:1000	RRx (goat, GTF /Jackson Probes)	Rabbit-IgG	1:400
anti-β-tubulin (rabbit, BioSite)	Axons in central nervous system	1:500	Alexa 488 (goat, Molecular probes)	Rabbit-IgG	1:400
Anti-Rip (mouse, The developmental studies hybridoma bank, University of Iowa)	Myelinating oligodendrocytes	1:1000	Alexa 488 (goat, Molecular probes)	Mouse-IgG	1:400
Anti-BrdU FITC (rat, BioSite)	Incorporated BrdU in proliferating cells	1:75	No secondary antibody needed	-	-

A fluorescence camera was used (Olympus) to detect the fluorescence from the marked antibodies. 10 pictures were taken from two wells per slide. The pictures were analyzed in the computer program Volocity 4.0 where cells were counted manually. The total number of cells was visualized by DAPI staining while specific cell types were labeled with antibodies. For each cell type, time point and culturing condition approximately 1500-3500 cells were counted. Data was collected and processed and statistical evaluation with unpaired t-test was performed with GraphPad Prism 4.

BrdU incorporation

BrdU (20 μ M, 5-Bromo-2'-deoxyuridine, Sigma-Aldrich) was added to 4-well chamber slides after 5, 8 and 11 days of incubation and left for 24 hours. For each time point, one control and one ethanol treated slide was assayed. The medium was gently removed and the cell cultures fixed with Stefanini fixative for 20 minutes. The wells were then thoroughly rinsed with PBS until all fixative was washed off. The cell cultures were washed with deionized water 3 times 5 minutes and then incubated in hydrochloric acid (2M, Fischer Scientific) for 3 hours at room temperature. After incubation, wells were washed with PBS 4 times 5 minutes and then incubated in blocking solution for 1 hour in room temperature. Rat anti-BrdU FITC antibody (BioSite) was mixed with blocking solution in a 1:75 ratio and added to the wells, 150 μ l to each well. The slides were placed in a box wrapped in foil to avoid bleaching and incubated for 4 hours in room temperature. Cells were then washed 2 times 15 minutes in washing solution and then 15 minutes in PBS. Slides were then mounted and pictures taken and processed in the same way as for immunocytochemistry.

When data had been collected from all time points and alcohol concentrations, statistical analysis was performed in the computer program GraphPad Prism 4. The statistical significance of the variations was evaluated by unpaired t-test.

Apoptosis assay

The Caspase 3 assay kit measures the amount of activated Caspase 3 by adding the substrate acetyl-Asp-Glu-Val-Asp p-nitroaniline. If this substance is hydrolyzed by Caspase 3, it releases p-nitroaniline (p-NA) which produces a color. The assay was performed according to the manual from Sigma (Sigma CASP3C-1KT) except the culturing method. Instead of keeping the cells as a suspension, they were grown on 20*100 mm Falcon dishes pre-coated with poly-L-lysine. For each dish, 10 ml of cell suspension was plated yielding 1×10^6 cells/dish. The dishes were incubated at 37° C, 5% CO₂ for 5 or 11 days. Cells were then carefully scraped off the bottom of the culturing dishes with a cell scraper. The cell suspensions were collected in 2 ml Eppendorf tubes (Eppendorf) and put on ice until used. Briefly, the assay was performed as follows.

The cell suspension was centrifuged and washed before a lysis buffer was added. The cells were kept on ice for 20 minutes and during this time the assay components were prepared. After incubation on ice, the cell suspension was centrifuged and the supernatant collected. The supernatant, reagents and buffers were added in a strict order in a 96-well ELISA plate (Cellstar, Greiner Bio-one) and then incubated at 37°C for 90 minutes. After incubation the absorbance was measured in the same way as for the MTT-test, but this time at 405 nm. To be able to process the data collected from the assay a calibration curve was performed using p-nitroaniline. When data had been collected from all time points and alcohol concentrations, statistical analysis was performed in the computer program GraphPad Prism 4. The statistical significance of the variations was evaluated by unpaired t-test.

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References

- Autti-Ramo I., Autti T., Korkman M., Kettunen S., Salonen O., Valanne L., 2002, *MRI findings in children with school problems who had been exposed prenatally to alcohol*, *Developmental Medical and Child Neurology*, **44**:98-106
- Carloni S., Mazzoni E., Balduini W., 2004, *Caspase-3 and calpain activities after acute and repeated ethanol administration during the rat brain growth spurt*, *Journal of Neurochemistry*, **89**: 197-203.
- Dikranian K., Qin Y-Q., Labruyere J., Nemmers B., Olney J. W., 2005, *Ethanol-induced neuroapoptosis in the developing rodent cerebellum and related brain stem structures*, *Developmental Brain Research*, **155**:1-13
- Edmondson M. J., Armstrong L. S., Martinez A. O., 1988, *A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures*, *Journal of Tissue Culture Methods*, **11**:15-17.
- Gilyarov, A. V., 2008, *Nestin in central nervous system cells*, *Neuroscience and Behavioral Physiology*, **38**:165-169.
- Goodlett C. R., Horn K. H., Zhou F. C., 2005, *Alcohol teratogenesis: mechanisms of damage and strategies for intervention*, *Society for Experimental Biology and Medicine*, **230**:394-406.
- Goodlett C. R., Horn K. H., 2001, *Mechanisms of alcohol-induced damage to the developing nervous system*, *National Institute of Alcohol Abuse and Alcoholism of the National Institute of Health*, **25**:175-184.
- Guerra C., Pascual M., Renau-Piqueras, 2000, *Glia and Fetal Alcohol Syndrome*, *NeuroToxicology*, **22**:593-599.
- Guizzetti M., Catlin M., Costa L. G., 1997, *Effects of ethanol on glial cell proliferation: relevance to the Fetal Alcohol Syndrome*, *Frontiers in Bioscience*, **2**:93-98.
- Hekmatpanah J., Haghigat N., Adams C.R., 1994, *Alcohol consumption by nursing rats and its effect on the cerebellum of the offspring*, *Alcohol Alcohol*, **29**:535-547.
- Cebere A., Cebers G., Liljequist S., *Enhancement of NMDA-induced functional responses without concomitant NMDA receptor changes following chronic ethanol exposure in cerebellar granule cells*, *Naunyn-Schmiedeberg's Arch Pharmacol*, **360**:623-632.
- Light K. E., Brown D. P., Newton B. W., Belcher S. M., Kane C. J.M., 2002, *Ethanol-induced alterations of neurotrophin receptor expression on purkinje cells in the neonatal rat cerebellum*, *Brain Research*, **924**:71-81.
- Molin J., 2007, *The effect of ethanol on cultures of the fetal cerebellum*, Biomedicine program, degree project, Uppsala University
- Nishiyama A., Watanabe M., Yang Z., Bu J., 2002, *Identity, distribution, and development of polydendrocytes: NG2-expressing glial cells*, *Journal of Neurocytology*, **31**:437-455.

Purves D., Augustine G. J., Fitzpatrick D., Hall W. C., LaMantia A-S., McNamara J. O., Williams S. M., 2004, *Neuroscience*, 3:rd ed., Sinauer Associates, Sunderland.

Oberdoerster J., Rabin R. A., 1999, *Enhanced caspase activity during ethanol-induced apoptosis in rat cerebellar granule cells*, *European Journal of Pharmacology*, **385**: 273-282.

Olney J. W., Ishimaru M. J., Bittigau P., Ikonomidou C., 2000, *Ethanol-induced apoptotic neurodegeneration in the developing brain*, *Apoptosis*, **5**: 515-521.

Rubert G., Min~ana R., Pascual M., Guerri C., 2006, *Ethanol exposure during embryogenesis decreases the radial glial progenitor pool and affects the generation of neurons and astrocytes*, *Journal of Neuroscience Research*, **84**:483-496.

Siler-Marsiglio K. I., Paiva M., Madorsky I., Pan Q., Shaw G., Heaton M. B., 2005, *Functional mechanisms of apoptosis-related proteins in neonatal rat cerebellum are differentially influenced by ethanol at postnatal days 4 and 7*, *Journal of Neuroscience Research*, **81**:632-643

Sowell E.R., Thompson P.M., Mattsson S.N., Tessner K.D., Jernigan T.L., Riley E.P., 2002, *Regional brain shape abnormalities persist into adolescence after heavy prenatal alcohol exposure*, *Cerebral Cortex*, **12**:856-865

Spadoni A. D., McGee C. L., Fryer S. L., Riley E. P., 2007, *Neuroimaging and Fetal Alcohol Spectrum Disorders*, *Neuroscience and Biobehavioral Reviews*, **33**:239-245.