● **PERSPECTIVE**

Fetal alcohol spectrum disorder: molecular insights into neural damage reduction

Fetal alcohol spectrum disorders (FASD) is a group of entirely preventable, lifelong conditions, which occur upon maternal alcohol use during pregnancy. This can result in severe consequences for the newborn and ultimately the family. It is usually characterized by delays in development and motor function, craniofacial abnormalities, and difficulties with learning, memory, speech, and academic achievement. According to the German guidelines for fetal alcohol syndrome (FAS) diagnosis, the prevalence of FASD ranges between 0.02–0.8% of all annual births and often the disorder is not recognized (Landgraf et al., 2013). The U.S. National Institutes of Health regard FAS as the most common nonhereditary cause of mental retardation. Thus, preventing programs, like the one undertaken by the Australian Government, which appointed a National FASD Technical Network (Elliott, 2015), may seem a very reasonable strategy. However, preventing programs for FASD focus mainly either on primary prevention, by increasing pregnant women's awareness of ethanol consumption risks, or on tertiary prevention which supposes early recognition of the condition and social support in the form of an improved developmental framework of the affected individual. Secondary prevention of the disorder, which includes early detection by screening pregnant women for ethanol consumption and control of the progression from a preclinical pathological condition to a severe form of the disease, is considered to be most challenging (Elliott, 2015). Although the most important form of prevention is abstinence from alcohol during pregnancy, offsprings from women with known gestational alcohol misuse could still benefit from a secondary form of FASD prevention, which might hinder the progression to FAS. However, therapy approaches are precluded by our limited knowledge of molecular mechanisms responsible for neuronal damage.

Bosco and Diaz (2012) hypothesized that hypoxia and increased oxidative/nitrative stress are responsible for the decreased fetal growth, characteristic to FASD.

Under normal circumstances, during the transition from intra- to extrauterine life, newborns exhibit more or less pronounced levels of hypoxia, for which they are equipped with adaptive mechanisms that allow a striking high tolerance (Singer, 1999). We thus inquired whether alcohol exposure during the cerebellar growth spurt could lead to alterations of adaptive mechanisms to metabolic stressors (Le Duc et al., 2015).

To this end we exposed rats to 20% v/v ethanol in drinking water during the entire pregnancy period to induce ethanol exposure damage to the litter (**Figure 1**). For the rat neonatal cerebellum, the first 10 days after birth correspond to the third pregnancy trimester in humans, when Purkinje cells grow dendrites and form synapses (Dobbing and Sands, 1979). Thus, in our study dams continued to receive ethanol 20% v/v in the drinking water and consequently offsprings were exposed to ethanol through lactation until postnatal day 5, followed by an acute 3-hour ethanolvapor inhalation on postnatal day 5. Steady drinkers often show a behavioral pattern of chronic and acute binge ethanol consumption (Epstein et al., 1995), that was mimicked by our ethanol exposure model (**Figure 1**).

On postnatal day 5 cerebella were collected from ethanol exposed and control offsprings and the granule neurons (CGNs) were cultured for 7 days *in vitro* (**Figure 1**). The first line of investigation focused on the morphology and *in vitro* adaptability of the neurons. No significant differences were observed between the ethanol exposed and control CGNs for the 7 day period (Le Duc et al., 2015). This suggested that despite the long term, heavy ethanol exposure, CGNs retained similar adaptation capacities to an *in vitro*, nutrition rich environment as unexposed neurons. However, a direct ethanol exposure of CGN cultures causes a concentration-dependent depletion of the neurons (Pantazis et al., 1993) and *in vivo* ethanol exposure of the pups on postnatal day 7 leads to alteration in Rho GTPases signaling and increased apoptosis (Joshi et al., 2006). Taken together, the present lines of evidence could imply that while under ethanol exposure CGNs suffer extensive damage, but bringing previously exposed CGNs to normal conditions allows for their normal maturation.

Further, we investigated the impact of metabolic stressors on CGNs, which, despite the ethanol exposure during development, showed normal morphology in cell culture. Thus, after 7 days *in vitro* we exposed CGN cultures from ethanol-treated and control animals to either a serum free medium or to 3-hour oxygen glucose deprivation (OGD) (**Figure 1**). To assess the damage extent we performed dynamic propidium iodide measurements for 20 hours at 37°C in an exposure medium which contained glucose, but was serum free, mimicking nutritional metabolic stress.

Cellular death evaluation at the end of the 3-hour OGD revealed a significantly higher damage in ethanol-treated neurons compared to control (7% *vs.* 0.65%). Maintenance of CGNs in normoxic/normoglycemic conditions resulted in a small but significant difference in survival between the non-treated and the ethanol exposed groups (0.26% cell death *vs.* 0.62%). Thus, exposure of CGNs to ethanol during development causes a higher death susceptibility to an acute metabolic stress (Le Duc et al., 2015).

We then inquired the neuronal vulnerability after the 20 hour reoxygenation time. The effect of ethanol pre-exposure on CGNs' susceptibility to OGD induced death was maintained over the 20 hour period of reoxygenation (33.4% cell death *vs.* 22.4% for the non-treated neurons).

To gain further insight into how ethanol pre exposure affects neuronal susceptibility to different levels of metabolic deficits we next focused on the dynamic recordings of neuronal delayed death over a 20 hour period (**Figure 1**). The low-nutrient propidium iodide containing experimental medium represented a metabolic stress, which induced a low, but constant rate of neuronal cell death in all CGN cultures. For the first few hours of propidium iodide signal recording we observed no significant difference between the ethanol pre-exposed neurons and the control ones. The sensitising effect of ethanol exposure became manifest between the cultures kept in normoxic/normoglycemic conditions only after 10–11 hours. The more damaging metabolic stress induced by OGD, resulted in a higher death

vulnerability of the ethanol treated neurons from the beginning of reoxygenation period (Le Duc et al., 2015). These results suggest that ethanol exposure of CGNs during development alters adaptive mechanisms to metabolic stressors, but the effect becomes apparent only after a highly damaging or prolonged metabolic insult. Such observations could be relevant for secondary prevention strategies of FASD, which aim to address the pre clinical pathological condition in order to hinder the progression to FAS. In our setup, despite the higher vulnerability of ethanol exposed neurons, increased cellular death happened only after specific triggers.

With a view to better understand the molecular mechanisms underlying FASD and given the observed effects of the different stressors on the CGNs exposed to ethanol during development, we were further prompted to examine dysregulations in gene expression. Making use of a publicly available microarray dataset, in which whole embryo cultures were treated with ethanol to mimic FASD (Wang et al., 2008), we inquired Gene Ontology (GO) categories, which were affected by ethanol exposure. We focused specifically on five categories which were significantly affected in the ethanol exposed embryo cultures: oxidation-reduction process, mitochondrial respiratory chain, regulation of stress-activated MAPK cascade, *in utero* embryonic development, and nervous system development. The first two showed an enrichment in genes which were down-regulated as a result of ethanol treatment, while the last three were enriched in up-regulated genes. Using this approach we were able to define candidate genes, which were further tested in our experimental model. The GO category "oxidation-reduction process" revealed an ethanol-induced up-regulation of *Dhcr24* and a down-regulation of *Cp* and *Snca*. *Dhcr24* was verified by qPCR on the CGNs model, but *Cp* and *Snca* failed to reach significance. Additionally, the proapoptotic mitochondrial-membrane associated *Bax* was highly expressed in the ethanol group on CGNs, which could lead to apoptosis (Le Duc et al., 2015). However, under basal conditions we observed no change in the morphology of CGNs that could be indicative for increased apoptosis in the ethanol group. Overexpression of *Dhcr24* was shown to inhibit apoptotic cell signalling (Lu et al., 2014), which could suggest an adaptive gene regulation to prevent the commitment on the activated apoptotic pathway in CGNs.

Bdnf was a gene that clustered in both the "oxidation-reduction process" and "nervous system development" Gene Ontology categories and appeared down-regulated following ethanol exposure of embryos. *Bdnf* acts protective on cerebellar granule neurons under low glucose conditions by preventing JNK and p38 activation (Vakili Zahir et al., 2012). On our *in vitro* model we measured an over expression of *Mapk8 (Jnk1)*, which is known to promote apoptosis. Unlike on the whole embryo cultures *Bdnf* was up-regulated after ethanol exposure in our setup (Le Duc et al., 2015). It seems likely that up-regulation of *Bdnf* occurs as a counterbalance mechanism to maintain the viability of CGNs and we hence observe a higher vulnerability only under challenging conditions.

Based on our experimental data we propose that neurons exposed to ethanol during development show impaired adaptation to metabolic demanding circumstances. However, kept under normal conditions, there are compensatory mechanisms

that allow for an apparent similar viability as that of normal developing neurons. This could imply that secondary prevention in FASD may be highly beneficial by reducing the stress to which newborns from alcoholic mothers are exposed. Future research should address, preferably in *in vivo* studies whether reducing the mild hypoxia, which accompanies normal labour leads to a reduction in the brain damage of offsprings with FASD and consequently a better long term prognostic.

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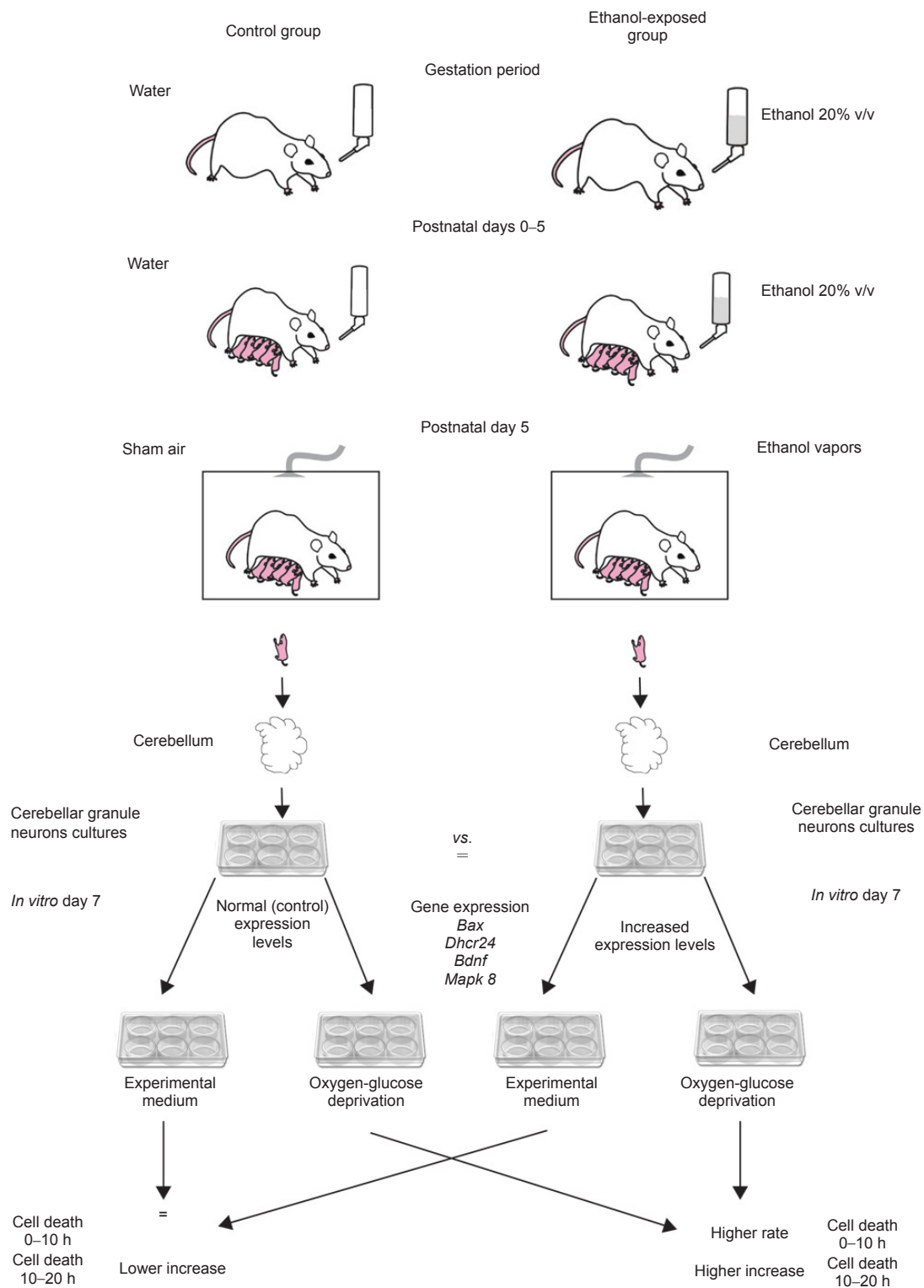
Figure 1 The impact of fetal ethanol exposure on metabolic stressors susceptibility.
After pregnancy confirmation rats were randomly assigned to the control or ethanol-exposed group. Ethanol was administered in 20% v/v in the drinking tab water throughout the entire pregnancy period and continued for 5 days postpartum. On postnatal day 5 offsprings together with the dam were exposed either to a 3-hour ethanol vapor inhalation or sham air. Cerebella were collected and granule neurons were cultured *in vitro*. On *in vitro* day 7 gene expression levels were measured for the cerebellar granule neurons cultures. To assess the impact of metabolic stressors cultures were exposed either to experimental medium without fetal bovine serum or oxygen-glucose deprivation. Cerebellar granule neurons from rats exposed to ethanol during development showed similar viability to the control for the first 10 hours of experimental medium exposure. Between 10–20 hours of dynamic measurement control cultures exposed to experimental medium had a lower increase in cell death compared to ethanol-exposed neurons. Conversely, oxygen-glucose deprivation exposure produced higher damage in cerebellar granule neurons cultures from the beginning of cell death assessment. This suggests, that for mild stressors cerebellar granule neurons possess adaptation mechanisms despite ethanol exposure during development, but longer periods or more demanding metabolic insults result in higher vulnerability of the cells.