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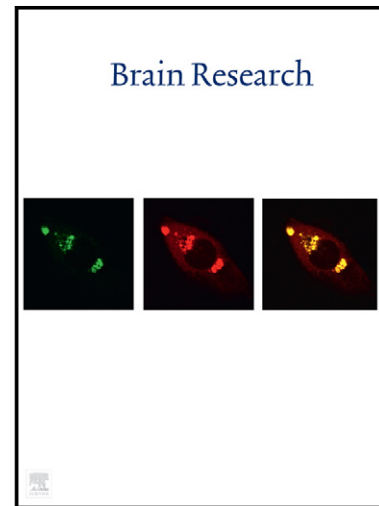
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# Author's Accepted Manuscript

Developmental exposure to ethanol increases the neuronal vulnerability to oxygen-glucose deprivation in cerebellar granule cell cultures

Diana Le Duc, Ana Spataru, Mihai Ceanga, Leon Zagrean, Torsten Schöneberg, Emil C. Toescu, Ana-Maria Zagrean



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1                   **Developmental exposure to ethanol increases the neuronal**  
 2                   **vulnerability to oxygen-glucose deprivation in cerebellar granule cell**  
 3                   **cultures**

4  
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22 **Abstract**

23 Prenatal alcohol exposure is associated with microencephaly, cognitive and  
24 behavioural deficits, and growth retardation. Some of the mechanisms of ethanol-  
25 induced injury, such as high level oxidative stress and overexpression of pro-  
26 apoptotic genes, can increase the sensitivity of fetal neurons towards  
27 hypoxic/ischemic stress associated with normal labour. Thus, alcohol-induced  
28 sequelae may be the cumulative result of direct ethanol toxicity and increased  
29 neuronal vulnerability towards metabolic stressors, including hypoxia. We examined  
30 the effects of ethanol exposure on the fetal cerebellar granular neurons' susceptibility  
31 to hypoxic/hypoglycemic damage. A chronic ethanol exposure covered the entire  
32 prenatal period and 5 days postpartum through breastfeeding, a time interval partially  
33 extending into the third-trimester equivalent in humans. After a binge-like alcohol  
34 exposure at postnatal day 5, glutamatergic cerebellar granule neurons were cultured  
35 and grown for 7 days *in vitro*, then exposed to a 3-hour oxygen-glucose deprivation to  
36 mimic a hypoxic/ischemic condition. Cellular viability was monitored by dynamic  
37 recording of propidium iodide fluorescence over 20 hours reoxygenation. We  
38 explored differentially expressed genes on microarray data from a mouse embryonic  
39 ethanol-exposure model and validated these by real-time PCR on the present model.  
40 In the ethanol-treated cerebellar granule neurons we find an increased expression of  
41 genes related to apoptosis (*Mapk8* and *Bax*), but also of genes previously described as  
42 neuroprotective (*Dhcr24* and *Bdnf*), which might suggest an actively maintained  
43 viability. Our data suggest that neurons exposed to ethanol during development are  
44 more vulnerable to *in vitro* hypoxia/hypoglycemia and have higher intrinsic death  
45 susceptibility than unexposed neurons.

46

47 **Highlights:**

48 - Fetal ethanol exposure increases neurons' vulnerability towards metabolic stressors

49 - Maternal alcoholism decreases offsprings' neuronal tolerance to hypoxia/ischemia

50 - Ethanol alters expression of genes associated with apoptosis and neuroprotection

51

52 **Keywords:** *prenatal ethanol exposure; oxygen-glucose deprivation; cerebellum;*

53 *neuronal vulnerability; differential gene expression.*

54

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## 55 1 Introduction

56 Prenatal alcohol exposure secondary to maternal ethanol consumption causes  
57 deleterious effects on fetal brain development (Falk, 2008). Even brief exposure to  
58 ethanol can result in growth retardation, craniofacial (Ismail et al., 2010) and systemic  
59 congenital abnormalities (Stratton, 1996). The damaging effects of ethanol on the  
60 developing nervous system have been thoroughly described since fetal alcohol  
61 syndrome was first investigated (Jones and Smith, 1975). The extent of neuronal  
62 impairment can lead accordingly to intellectual deficits, seizures, and even paraplegia  
63 (Streissguth and O'Malley, 2000). Fetal ethanol exposure determines an attenuation of  
64 hypoxic vasodilation that limits fetal oxygen delivery to the brain during hypoxic  
65 episodes (Mayock et al., 2007) and leads to altered glucose transport and metabolism  
66 (Fattoretti et al., 2003), promoting cellular damage during physiological peripartum  
67 hypoxia (Bakker and van Geijn, 2008). Moreover, chronic binge alcohol consumption  
68 has been shown to alter maternal uterine vascular function, leading to lowered blood  
69 supply to the placenta and consequently to the fetus (Subramanian et al., 2014).  
70 The cerebellum is highly sensitive to both, ethanol (Goodlett and Eilers, 1997) and  
71 hypoxia/ischemia (Cervos-Navarro et al., 1991; Goodlett and Eilers, 1997). Ethanol  
72 exposure during development causes an increased apoptotic cell death rate of both,  
73 cerebellar Purkinje and granule cells (Bhave and Hoffman, 1997), responsible for the  
74 severe motor impairments commonly associated with cerebellar dysfunction

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### Abbreviations

BAL, blood alcohol level; CGCs, cerebellar granule cells; C<sub>T</sub>, cycle threshold; Ctr, control; EM+/-G, experimental medium with/without glucose; EtOH, ethanol; GO, gene ontology; OGD, oxygen-glucose deprivation; PI, propidium iodide; qPCR, quantitative polymerase chain reaction.

75 (Goodlett and Eilers, 1997). Neonatal cerebellum in rats shows a clear temporal  
76 window of vulnerability during the first 10 days after birth, a period corresponding to  
77 Purkinje cell dendritic outgrowth and synaptogenesis and to the third pregnancy  
78 trimester in humans (Dobbing and Sands, 1979; Goodlett et al., 1990). Within this  
79 period, cerebellar granule cells (CGCs) display a higher susceptibility to ethanol on  
80 postnatal days 4-6 (Goodlett et al., 1998), when proliferation of CGCs precursors  
81 towards postmitotic cells still occurs (Luo, 2012) and even short ethanol exposures  
82 can lead to significant loss of cerebellar neurons by intricate mechanisms such as  
83 inhibition of neurotrophic action, decrease in the pro-survival CREB binding protein  
84 expression (Guo et al., 2011), increased oxidative stress (Kotch et al., 1995), and  
85 activation of pro-apoptotic pathways (Light et al., 2002).

86 We investigated whether and how ethanol exposure influences the neuronal response  
87 to hypoxia/ischemia. Thus, CGCs cultures obtained from pups exposed to ethanol  
88 during fetal life and until postnatal day 5 were used in an *in vitro* oxygen-glucose  
89 deprivation (OGD) model. The model allows the evaluation of neurons' *in vitro*  
90 maturation after an *in vivo* ethanol exposure, which intercepts the maximum  
91 cerebellar vulnerability to ethanol, before the completion of neurite formation. Acute  
92 and delayed neuronal vulnerability secondary to OGD was assessed by a 20 hours-  
93 dynamic measurement of cellular death using propidium iodide (PI) fluorometry. Our  
94 data suggest that neurons exposed to ethanol during development are more vulnerable  
95 to *in vitro* hypoxia/hypoglycemia. To check whether our results are confirmed on a  
96 molecular level and to support a possible *in vivo* extrapolation of the results, we  
97 further analyzed gene expression profiles from publicly available microarray data in a  
98 mouse model of embryonic ethanol exposure. This revealed several biological  
99 pathways that showed differential regulation after ethanol exposure, including

100 embryonic organ and neuronal morphogenesis. Additionally, we validated genes by  
101 real-time PCR on the *in vitro* CGC model and identified some candidate genes which  
102 could be responsible for the low adaptive response to hypoxia/hypoglycemia of  
103 cerebellar granule cells derived from pups exposed to ethanol during the brain growth  
104 spurt period.

## 105 **2 Results**

### 106 *2.1 Morphological assessment*

107 Morphology and cell maturation of cultured CGCs were assessed in phase-contrast  
108 microscopy. There were no obvious morphological differences between cultures  
109 originating from ethanol-treated and non-treated animals (Fig. 1A). The  
110 morphological assessment at the end of the reoxygenation protocol revealed the  
111 nature and the extent of damage induced by OGD and/or ethanol pretreatment (Fig.  
112 1B). Phase-contrast microscopy showed an increased level of cell injury in ethanol-  
113 exposed neurons, ranging from cytoplasmic swelling, loss of phase-bright contours,  
114 and intercellular connections in ethanol control group cultures, to loss of membrane  
115 integrity and the presence of multiple cell ghosts in the ethanol-treated cultures  
116 exposed to OGD (Fig. 1B). Cell death was confirmed on the same microscopic fields  
117 by PI fluorescence (Fig. 1B).

### 118 *2.2 The response of ethanol-treated neurons to oxygen-glucose deprivation*

119 We first assessed the effects of ethanol pre-exposure on the neuronal response to an  
120 OGD protocol. Neuronal viability at the end of a 3-h OGD exposure before the  
121 initiation of reoxygenation was measured by PI fluorescence. Figure 2A indicates that  
122 ethanol-treated neurons showed a significantly higher susceptibility to OGD exposure  
123 ( $7.0 \pm 2.0\%$  cell death,  $n = 5$ ) comparing to non-treated neurons exposed to OGD



124 (0.65 ± 0.10% cell death, n = 4), p-value = 0.03. In the groups maintained in control  
125 normoxic/normoglycemic conditions, there was also a small but significant difference  
126 in survival between the non-treated control neurons and ethanol control neurons (0.26  
127 ± 0.05% cell death, n = 4 and 0.62 ± 0.09% cell death, n = 6, respectively, p-  
128 value = 0.01).

129 Reoxygenation at the end of the OGD period resulted in additional neuronal death,  
130 with an increased death in the non-treated population at 20 h of reoxygenation (Fig.  
131 2B). Figure 2B also shows that the increased neuronal vulnerability, as an effect of  
132 ethanol pre-exposure, is maintained over the 20-h period of reoxygenation following  
133 OGD (33.4 ± 2.3% cell death, n = 5 for the ethanol-treated neurons, vs. 22.4 ± 1.1%  
134 cell death, n = 4 for the non-treated neurons, p-value = 0.004).

### 135 2.3 *Dynamic recording of neuronal death during reoxygenation*

136 Neuronal delayed death was dynamically recorded for 20 h by PI signal to gain  
137 further insight into how ethanol pre-exposure affects neuronal susceptibility to  
138 different levels of metabolic deficits. Figure 3A provides typical examples of such  
139 recordings for each of the conditions used in this study. Maintenance of the primary  
140 neuronal cultures for 20 h in the low-nutrient PI-containing experimental medium  
141 could result in metabolic stress, which induced a low, but constant rate of neuronal  
142 cell death in control cultures. For the first few hours of PI-signal recording, there was  
143 no significant difference between the ethanol-treated and the non-treated neurons. The  
144 sensitising effect of ethanol pretreatment became manifest only after 10-11 h in  
145 control cultures. In case of the more demanding metabolic stress induced by OGD, the  
146 higher death vulnerability of the ethanol-treated neurons was clearly manifest from  
147 the beginning of reoxygenation period (Fig. 3A). These results suggest that neurons  
148 from ethanol-exposed pups have an additional susceptibility to hypoxic-ischemic

149 challenge that is separate from the toxic effect of ethanol alone. Figures 3B and 3C  
150 illustrate these differences across the experimental set using an hourly rate of  
151 neuronal cell death as the measured parameter. Figure 3B shows the sensitising effect  
152 of ethanol pretreatment becoming manifest only in the latter part of the experimental  
153 protocol in control cultures (p-value < 0.001, n = 6 ethanol-treated control group and  
154 n = 4 non-treated control group).

155 After OGD exposure, ethanol-treated cultures showed a significantly different  
156 vulnerability in comparison to control cultures (p-value < 0.001, n = 4 for non-treated  
157 group and n = 5 for ethanol-treated group). OGD induced extensive damage in the  
158 ethanol group during early reoxygenation, as shown by hourly cell death rate  
159  $2.9 \pm 0.4\%/hour$  compared to ethanol non-treated cultures  $0.73 \pm 0.17\%/hour$  (Fig.  
160 3C). Given the significant death of the ethanol-treated neurons during the first part of  
161 the dynamic recording, hourly cell death rate in late reoxygenation was higher in the  
162 control group (Fig. 3C), but overall OGD-induced cell death was still higher in the  
163 ethanol group at the end of reoxygenation ( $66.5 \pm 2.9\%$  vs.  $41.5 \pm 2.1\%$ ) (Fig. 3A).

#### 164 2.4 *Microarray gene expression in control and ethanol-exposed mouse embryos*

165 In the attempt to understand the molecular mechanism which renders ethanol-treated  
166 neurons more vulnerable to metabolic stresses, we analyzed gene expression data  
167 available on a public-domain microarray dataset (GSE9545 from Gene Expression  
168 Omnibus Database), which provides the nearest model example to our experimental  
169 model. In this experiment whole embryo mouse cultures were performed on four  
170 control embryos and four ethanol-treated ones, the latter characterized by a phenotype  
171 of open neural tubes (Wang et al., 2008). Transcriptomic studies suggest that  
172 teratogenic effects observed in whole embryo cultures are relevant to previously

173 identified mechanisms of toxicity *in vivo* (Genschow et al., 2002; Luijten et al., 2010;  
174 Zhou et al., 2011).

175 Given our *in vitro* observations suggesting a metabolic deficiency induced by ethanol  
176 exposure of the offspring, we assessed the effect of ethanol exposure on whole  
177 embryo changes in gene expression in various sets of relevant genes. Thus, informed  
178 by our *in vitro* data we used an additional model as a probe for the potential changes.  
179 Validation of the observed vulnerability on a molecular level in the whole embryo  
180 cultures model would provide an important link to the *in vivo* developmental toxicity  
181 (Robinson et al., 2012a).

182 A widely used systems biology technique to highlight biological processes is gene  
183 category over-representation analysis (Park et al., 2014). To perform this analysis  
184 genes are grouped into categories by a common biological property and then tested to  
185 find categories that are over-represented. To this end gene ontology (GO) pathways  
186 (Ashburner et al., 2000) that are affected by exposure to ethanol were investigated.  
187 After removing genes which were not reliably detected, we ranked and clustered  
188 16,117 genes according to the expression fold change between the ethanol-exposed  
189 and control embryos. Genes clustered in 161 GO categories after correcting for  
190 multiple testing by the stringent method of family-wise error rate (FWER < 0.01)  
191 (Krzywinski and Altman, 2014). Categories that could be relevant to the higher  
192 susceptibility towards metabolic stressors secondary to ethanol exposure of the  
193 embryo were further analyzed (Table 1).

194 To identify genes that might be responsible for the observed neuronal susceptibility  
195 towards metabolic stressors of ethanol-exposed embryos we first screened the GO  
196 category “oxidation-reduction process” (GO:0055114, p-value = 8.80e-08,

197 FWER < 1.0e-04). This category had an enrichment in genes that were low ranked  
198 according to the fold change between the ethanol-exposed group and control, which is  
199 translated in a higher number of down-regulated genes than expected by chance.  
200 *Dhcr24* (gene coding 24-dehydrocholesterol reductase, p-value = 0.006, fold  
201 change = 1.13) and *Cp* (gene coding ceruloplasmin, p-value < 0.001, fold  
202 change = 0.71) were the most significantly up and down-regulated, respectively  
203 (Table 2).

204 The category “mitochondrial respiratory chain” (GO:0005746, p-value = 6.27e-06,  
205 FWER = 0.003) was enriched in genes that were low ranked, but among the 45 genes  
206 in the node none fulfilled the fold change criterion of up- / down-regulation by at least  
207 10%.

208 Genes that might be responsible for the developmental effects of ethanol on embryos  
209 were revealed by focusing on two GO biological processes: “*in utero* embryonic  
210 development” (p-value = 2.04e-06, FWER = 0.003) and “nervous system  
211 development (p-value = 5.5e-08, FWER = 0.0001)”. This resulted in 4 significantly  
212 up-regulated genes involved in embryonic development (GO:0001701) (Table 2), of  
213 which *Nrk* (gene coding Nik-related protein kinase, p-value = 0.02, fold  
214 change = 1.18) was the most significant. Additionally, 7 genes clustering in this  
215 category (Table 2) were significantly down-regulated, with *Slit2* (gene coding Slit  
216 homolog 2 protein, p-value = 0.002, fold change = 0.89) most significant.  
217 Significantly differentially expressed genes, which clustered in the nervous system  
218 development category (GO:0007399) are presented in Table 2, with 19 up-regulated  
219 and 21 down-regulated representatives. Of these the most significant up- / down-  
220 regulated ones were respectively: *Pitx1* (gene coding paired-like homeodomain

221 transcription factor 1, p-value = 0.003, fold change = 1.13) and *Slit2*, which clustered  
222 in both the previous and this category.

223 2.5 *Gene expression quantification on cerebellar granule neurons cultures from*  
224 *ethanol-exposed and control rat offsprings*

225 To verify which genes could be responsible for the higher vulnerability of ethanol-  
226 treated neurons to metabolic stresses, we quantified gene expression levels for  
227 candidates obtained from the microarray analysis. The overlap of differentially  
228 expressed genes on the CGCs model and whole embryo cultures lends further support  
229 to the validity of the identified candidate genes.

230 We validated five genes as possible candidates: *Dhcr24*, *Bdnf*, *Mapk8*, *Bax*, and  
231 *Slc2a4* (Fig. 4). It is common that given the different techniques, qPCR and  
232 microarray measurements show different fold changes of the genes, even when the  
233 same samples are measured (Lussier et al., 2015). In the case of *Dhcr24* the fold  
234 change between the ethanol group and the control is 23 ( $\Delta\Delta C_T = 4.55$ ), while the  
235 microarray data showed an increase of only 1.13 fold. *Bdnf* shows a marked up-  
236 regulation of 32 fold ( $\Delta\Delta C_T = 5.04$ ), while in the microarray data the gene is found to  
237 be down-regulated in the ethanol group (fold change = 0.81, Table 2).

238 Although the last three genes did not show differential expression in the whole mouse  
239 embryo culture model, the rationelle for their measurement was the involvement in  
240 the GO categories which showed disruption and may be relevant for the ethanol-  
241 induced increased susceptibility towards metabolic stressors (Table 1). While  
242 MAPK8 interacting proteins 1 and 2 showed an up regulation after ethanol exposure  
243 (Table 2, GO:0032872), *Mapk8* showed no significant regulation (fold  
244 change = 1.04). Our measurements revealed a 7.1 fold up-regulation of *Mapk8* in the

245 ethanol group ( $\Delta\Delta C_T = 2.82$ ). *Bax* and *Slc2a4* were also up-regulated in the ethanol  
246 group samples to 7.95 fold and 41 fold, respectively ( $\Delta\Delta C_{T_{Bax}} = 2.99$  and  
247  $\Delta\Delta C_{T_{Slc2a4}} = 5.39$ ).

248 Genes involved in the oxidative stress response (*Cp*, *Snca*, *Gss*) failed to reach  
249 significance in the qPCR measurements.

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### 251 3 Discussion

252 Our study indicates that ethanol exposure during nervous system development results  
253 in a significantly higher neuronal vulnerability to metabolically demanding  
254 conditions, either under chronic conditions, such as maintenance in a low nutrient  
255 medium, or in more acute conditions, such as OGD, which is the *in vitro* equivalent of  
256 hypoxia/ischemia.

257 In their review, Bosco and Diaz hypothesised that ethanol-induced fetal growth  
258 retardation occurs most likely as a result of hypoxia and increased oxidative/nitrative  
259 stress, which interfere with cellular processes that require oxygen in order to function  
260 adequately, such as placental transport (Bosco and Diaz, 2012). Although tested only  
261 on one type of cells, the *in vitro* CGCs results support this hypothesis since ethanol  
262 exposure during brain growth spurt aggravates the cellular damage induced by a  
263 hypoxic/ischemic injury (Figs. 2 and 3).

264 To validate the ethanol-induced vulnerability in an additional model, but also to  
265 understand the mechanisms that underlie the ethanol-sensitizing effect to both chronic  
266 and acute metabolic challenges, we performed gene expression data analysis on a  
267 publicly available microarray dataset from whole embryo cell cultures. Whole embryo  
268 cell cultures show gene expression patterns very similar, over time, to *in utero*  
269 embryos; and, in the case of rat, cultures are matching to a great extent the gene  
270 expression profile in human embryos undergoing neurulation and early  
271 embryogenesis (Robinson et al., 2012b). Therefore, given the high *in vivo* translation  
272 potential of whole embryo cell cultures (Genschow et al., 2002), the results can  
273 benefit of better support for a possible *in vivo* extrapolation. However, whole embryos  
274 display tissue heterogeneity, which may mask some changes in specific tissues or  
275 cells (Zhou et al., 2011). Hence, we validated some of the candidate genes on the *in*

276 *in vitro* model of CGCs cultures originating from offspring exposed to ethanol during  
277 cerebellar development.

278 We assessed expression patterns of genes involved in pathways correlated with a  
279 higher metabolic vulnerability. Mitochondrial dysfunction plays a key role in hypoxic  
280 neuronal injury and could therefore be a determining factor in the CGCs' damage  
281 caused by ethanol preexposure. Upon inquiry of the "mitochondrial respiratory chain"  
282 GO category, there was a significant enrichment with down-regulated genes, but  
283 given the strict cutoffs set for candidate genes, none of the genes in this cluster  
284 reached the threshold for significance. Since a strict cut-off to limit false-positive  
285 results leads to an increase of the number of false negative results (Kowalchuk and  
286 Keselman, 2001), and also because oxidative stress has been recently described as an  
287 important factor in the pathophysiology of alcohol-induced impairment (Joya et al.,  
288 2014), we further investigated other related pathways.

289 A close inspection of the GO category "oxidation-reduction process" revealed an  
290 ethanol-induced up-regulation of *Dhcr24* shown to exert a neuroprotective effect  
291 against reactive oxygen species resulted from endoplasmic reticulum stress (Lu et al.,  
292 2014). The down-regulation of *Cp*, an important antioxidant molecule, is in  
293 accordance to previous studies on hippocampi after chronic ethanol exposure (Saito et  
294 al., 2002). Another down-regulated gene of the pathway is *Snca*, a synaptic molecule  
295 involved in neurodegenerative disorders and an oxidative stress protector of neuronal  
296 cells (Hashimoto et al., 2002). While *Dhcr24* was verified by qPCR on the CGCs  
297 model, *Cp* and *Snca* failed to reach significance. Still, we cannot exclude that on the  
298 whole embryo level oxidative stress plays an important role in the ethanol-induced  
299 pathology. However, the proapoptotic mitochondrial-membrane associated *Bax*  
300 showed an elevated expression in the ethanol group on CGCs. It has recently been



301 shown that ethanol exposure of rats on postnatal day 4 leads to an increased  
302 activation of BAX (Heaton et al., 2015), which together with high expression levels  
303 of the gene, could lead to apoptosis. Nonetheless, under basal conditions we could not  
304 detect morphologically higher apoptotic levels in the ethanol group (Figures 1 and  
305 3A). Overexpression of *Dhcr24* was shown to inhibit apoptotic cell signaling, hence it  
306 seems reasonable to assume that the observed upregulation of *Dhcr24* is a  
307 counterbalance that prevents the commitment on the activated apoptotic pathway in  
308 CGCs.

309 Additionally, it is notable that *Bdnf*, a gene that appears down-regulated following  
310 ethanol exposure of embryos, can have protective effects on cerebellar granule  
311 neurons under low glucose conditions (Vakili Zahir et al., 2012). *Bdnf* was shown to  
312 prevent JNK and p38 activation in stress conditions and thus increase cell viability.  
313 The CGCs *in vitro* expression data confirm the overexpression of *Mapk8 (Jnk1)*,  
314 which is known to promote apoptosis (Dhanasekaran and Reddy, 2008). Conversely,  
315 *Bdnf* is up-regulated after ethanol exposure in the CGCs model, unlike on whole  
316 embryo cultures. It seems likely that up-regulation of both genes maintains a balance  
317 in the basal viability and we hence observe a higher vulnerability only under  
318 challenging conditions.

319 We show that *Slc2a4*, the transcript coding for the insulin-sensitive glucose  
320 transporter GLUT4, is up-regulated after ethanol exposure in CGCs. GLUT4 was  
321 described to be present in significant amounts in CGCs and was shown to be up-  
322 regulated in the CGCs of a diabetic, hyperinsulinemic mouse model (Vannucci et al.,  
323 1998). CGCs showed an opposite expression profile in the diabetic mice when  
324 compared to periferic tissues in which *GLUT4* transcript was decreased. Additionally,  
325 prenatal alcohol exposure induces impaired glucose tolerance (Chen et al., 1996).

326 Thus, *Slc2a4* might be a key candidate to explain the impaired cerebellar glucose  
327 metabolism and high susceptibility to *in vitro* hypoxia and hypoglycemia in cultured  
328 CGCs from prenatally ethanol-exposed rats.

329 *Adm*, a shared down-regulated gene between “embryonic and nervous system  
330 development” GO categories, was shown to lead to lower resistance to hypobaric  
331 hypoxia in mice that were *Adm* conditional knockouts in the central nervous system  
332 (Fernandez et al., 2008). Moreover evidence points towards an involvement of this  
333 gene in placentation and regulation of fetal perfusion (Wilson et al., 2004), which  
334 together with the known ethanol cytotoxic effects on trophoblast cells (Clave et al.,  
335 2014) may support an altered placental function in ethanol drinking dams. Although  
336 not significantly disrupted in CGCs, the expression of the *Adm* gene seems to be more  
337 relevant on an entire organism level, such that mild impairment of the placental  
338 function could result in decreased neuronal viability.

339 Taken together ethanol might confer a higher risk towards hypoxic/ischemic events,  
340 which can lead to growth retardation as suggested by Bosco and Diaz (Bosco and  
341 Diaz, 2012) given the confirmed predisposition towards hypoxia and glucose  
342 deprivation in the neurons. Furthermore, in CGCs, survival was reduced, with a  
343 certain lag time, even under less severe metabolic conditions, such as the *in vitro*  
344 maintenance of neurons in an artificial environment, with a relative reduction in the  
345 supply of nutrients (Fig. 3). We show that in our experimental model genes involved  
346 in apoptosis have a higher expression level after ethanol treatment. However,  
347 compensatory mechanisms involving neuroprotective genes, which we show to be up-  
348 regulated, are probably responsible for the apparent similar viability between the  
349 ethanol-exposed and control neurons.

350 In sum, the present results suggest an ethanol-induced reduction of the cellular  
351 adaptation to stress, which was verified in both, the *in vitro* CGCs cultures and on a  
352 molecular level in whole embryo cultures. The gene profiles and the observed pattern  
353 of ethanol-treated neurons impairment should both together contribute to the  
354 generation of *in vivo* studies concerning secondary prevention of brain damage, such  
355 as avoiding additional mild hypoxic events or metabolic stressful circumstances in the  
356 newborn.  
357

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## 358 **4 Experimental Procedure**

### 359 *4.1 Animals*

360 Sixteen pregnant Wistar rats and thirty-one of their litters from the breeding colony at  
361 Carol Davila University of Medicine and Pharmacy were used in this study. All  
362 animal procedures were carried out with the approval of the local ethics committee for  
363 animal research of Carol Davila University of Medicine and Pharmacy (Bucharest,  
364 Romania), and in accordance with the European Communities Council Directive  
365 86/609/EEC on the protection of animals used for scientific purposes. All efforts were  
366 made to minimize the number of animals used and their suffering.

### 367 *4.2 Ethanol treatment*

368 Pregnant rats were randomly assigned on the day of confirming pregnancy, to either  
369 an ethanol group or a non-treated group and housed separately thereafter. Both groups  
370 had free access to food and liquids, and the ethanol group dams were given ethanol  
371 20% v/v in the drinking water to induce ethanol exposure damage to the litter (Snyder  
372 et al., 1992). To validate the level of ethanol exposure, blood alcohol levels (BAL)  
373 were randomly measured during the entire setup time frame using an enzymatic, UV  
374 method (Dialab, Austria). This type of exposure resulted in maternal BAL of  
375  $112.7 \pm 19.3$  mg/dL ( $n = 6$ ) (data represent mean value  $\pm$  SEM). Nutrition and  
376 drinking patterns were daily monitored for both groups, and no nutritional deficits  
377 were observed. Mean block-food ingestion did not significantly differ between  
378 ethanol treated and non-treated group, with an average calorie intake of  
379  $269.1 \pm 6.8$  kcal/kg body weight/day (data represent mean value  $\pm$  SEM). Liquids  
380 consumption in the non-treated group was  $129.2 \pm 5.3$  ml/kg body weight/day. The  
381 fluid ingestion in the ethanol treated group was  $139.6 \pm 9.5$  ml/kg body weight/day, of

382 which an average of  $22.0 \pm 1.6$  g ethanol/kg body weight/day. The body weight did  
383 not differ significantly between the two groups at different pregnancy stages.

384 To mimic ethanol exposure during the third trimester of pregnancy in humans, full-  
385 term litters, known to correspond developmentally to the end of the second trimester  
386 in humans, were further exposed to ethanol through lactation until postnatal day 5, as  
387 the dams continued to receive ethanol 20% v/v in the drinking water (Olney et al.,  
388 2000). BAL was monitored during this period, by random serum measurements, and  
389 reached values of  $109.7 \pm 43.9$  mg/dl in pups ( $n = 4$ ). As ethanol exposure through  
390 breastfeeding might not be sufficient to induce a similar to human third-trimester  
391 exposure causing cerebellar dysfunction (Cebolla et al., 2009), postnatal day 5 pups  
392 were exposed to a “binge-like” pattern of ethanol consumption, common in women  
393 who drink during pregnancy (Maier et al., 1997). This ethanol acute exposure was  
394 mimicked by a 3-hour vapor inhalation (Heaton et al., 2000b), resulting in a BAL of  
395  $461.5 \pm 165.8$  mg/dl ( $n = 4$ ) in pups, a concentration comparable to the high levels  
396 seen in previous human and animal studies (Tran et al., 2005). The chronic and acute  
397 combinatorial model of ethanol exposure corresponds to the behavioral pattern of  
398 steady drinkers (Epstein et al., 1995). Cerebella did not significantly differ in either  
399 morphology or weight compared to unexposed pups.

#### 400 4.3 *Cerebellar granule neurone cultures and oxygen-glucose deprivation exposure*

401 The exposure model was further transferred into an *in vitro* setup allowing a better  
402 assessment of the deleterious effects which ethanol exerts on cerebellar granule  
403 neurons. CGCs cultures are widely used for the *in vitro* study of neuronal ischemia  
404 (Kalda et al., 1998) or ethanol neurotoxicity (Heaton et al., 2000a; Luo, 2012) since  
405 they provide a homogenous population of glutamatergic neurons in which  
406 experimental conditions can be precisely controlled (Contestabile, 2002). Primary

407 cultures of cerebellar granule neurons were obtained as previously described (Toescu,  
408 1999) immediately after the acute ethanol exposure or a sham air exposure, for  
409 ethanol and non-treated pups, respectively. Briefly, the dissociated cell suspensions  
410 from cerebella of 5-day-old pups were plated at a density of 200,000 cells/well into  
411 96-well plates (Nunc) coated with poly-D-lysine (Sigma-Aldrich) in complete BME  
412 culture medium (Sigma-Aldrich) containing 2 mM L-glutamine (HyClone), 32 mM  
413 glucose, 10% heat-inactivated horse serum (Sigma-Aldrich), antibiotic-antimycotic  
414 (GIBCO/Invitrogen) and 25 mM KCl, to promote CGCs viability in culture (Gallo et  
415 al., 1987). Cytosine-arabioside (Sigma-Aldrich, 10  $\mu$ M) was added after 24 h to stop  
416 the glial cells proliferation. There were no apparent differences between the cultures  
417 generated from ethanol-treated group and the cultures from the non-treated grup, in  
418 regard to morphology or number of viable neurons. Cultures were grown in a  
419 humidified incubator at 37°C and 5% CO<sub>2</sub> for 7 days until they reached a maturation  
420 level that allowed evaluating different experimental conditions. The percentage of  
421 glial cells was no more than 1% as assessed by glial fibrillary acidic protein and  $\beta$ -  
422 tubulin immunostaining. The cultures were grown in ethanol-free solutions to  
423 promote similar adaptation to *in vitro* state for both groups. Control condition and  
424 OGD which mimics ischemia *in vitro* (Goldberg et al., 1986), were carried out as  
425 previously described (Ceanga et al., 2010), by shifting cultures in a serum-free  
426 experimental medium (EM) with or without glucose (EM+G, EM-G, containing in  
427 mM: 120 NaCl, 25 KCl, 0.62 MgSO<sub>4</sub>, 1.8 CaCl<sub>2</sub>, 10 HEPES,  $\pm$  11.1 glucose, pH 7.4).  
428 Briefly, OGD was initiated by washing cultures with EM-G to remove glucose before  
429 adding deoxygenated EM-G. Then, cultures were immediately placed into a  
430 humidified hypoxia chamber (Billups-Rothenberg Inc., Del Mar, CA) which was  
431 flushed with 100% N<sub>2</sub> for 10 min, sealed, and placed into an incubator at 37°C for 3 h,

432 an OGD exposure time that we previously found to induce neuronal damage (Zagrean  
433 A.M., 2007-Personal Communication). Reoxygenation was induced by replacing EM-  
434 G with normoxic EM+G. Control cultures went through identical steps except they  
435 were kept in normoxic EM+G in a 5% CO<sub>2</sub> incubator at 37°C (control condition).

#### 436 4.4 *Experimental groups*

437 Four experimental groups were defined to evaluate the neuronal vulnerability to a  
438 metabolic stress, considering both the *in vivo* ethanol treatment and the *in vitro*  
439 exposure of CGCs cultures to OGD or control conditions: two ethanol-treated groups,  
440 an ethanol OGD group and an ethanol control group, and two non-treated groups, a  
441 non-treated OGD group and a non-treated control group.

#### 442 4.5 *Assessment of cellular viability*

##### 443 4.5.1. *Microscopic examination*

444 Morphology and cell maturation were assessed in phase-contrast microscopy with a  
445 Zeiss Axiovert25 inverted microscope at 400× magnification during the cultures'  
446 growth period and no morphological difference between ethanol-exposed neurons and  
447 neurons from non-ethanol treated pups were observed. Cellular morphology was also  
448 assessed immediately after OGD or control conditions. The wells containing disrupted  
449 cultures were discarded. The cellular damage induced by ethanol pretreatment and  
450 OGD was further morphologically assessed in phase-contrast microscopy at the end of  
451 reoxygenation period.

##### 452 4.5.2. *PI fluorometry*

453 The rate of delayed cell death was monitored for the next 20 hours after control and  
454 OGD conditions by use of a vital dye, PI (Sattler et al., 1997), which becomes  
455 fluorescent upon binding the DNA of membrane-compromised cells (Vornov et al.,

1995). Cells were washed once with 200  $\mu$ l EM+G immediately after OGD or control conditions, then 100  $\mu$ l PI solution (50  $\mu$ g/ml in EM+G) was added in each well. The initial level of PI fluorescence in a sequence of five readings at 60 seconds interval was read in a multimode detector (DTX880, Beckman Coulter) set in fluorescent mode with an excitation wavelength of 535 nm and an emission wavelength of 625 nm. Dynamic PI measurements were initiated at every 15 minutes for 20 hours of reoxygenation at 37°C. At the end of reoxygenation time, the PI solution was exchanged with 25  $\mu$ l ethanol (100%)/well to induce maximal death. After ethanol evaporation the PI solution was added back and the maximal signal (corresponding to the total number of cells) was measured in a sequence of 5 readings at 60 second intervals.

#### 4.6 *Microarray gene expression analysis*

Microarray expression data was obtained from the public database Gene Expression Omnibus under the accession number [GSE9545](#). We analyzed the dataset that measured the global gene expression profiles in whole mouse embryo cultures, comparing control with alcohol treated embryos that had a clear phenotype which consisted in open neural tubes. A full description of the experimental ethanol model is available in the study of Wang et. al (Wang et al., 2008). Microarray expression was calculated using the *affy* (Gautier et al., 2004) and *gcrma* (Wu et al., 2004) of the Bioconductor (Gentleman et al., 2004) extensions to the R statistical programming environment (<http://www.R-project.org>). Genes that were not reliably detected in at least one of the two conditions were removed from further analysis. To identify metabolic pathways influenced by ethanol exposure genes were clustered in gene ontology categories (Ashburner et al., 2000) using *FUNC* package (Prufer et al., 2007) with a Wilcoxon ranking test according to the fold change of gene expression.



481 Family-wise error rate ( $< 0.01$ ) was used to correct for multiple testing hypothesis  
482 (Krzywinski and Altman, 2014). On the gene expression level a two sample *t*-test was  
483 used for statistical analysis. The MicroArray Quality Control (MAQC) project has  
484 shown that results of typical statistical differential expression tests with p-value as  
485 significance threshold need to be filtered and sorted by effect strength (fold-change)  
486 to attain robust comparisons across platforms and sites (Consortium et al., 2006).  
487 Hence, genes were defined as candidates if they clustered in a significant GO  
488 category, had a p-value  $< 0.05$  and a fold change between the ethanol-treated and  
489 control group either lower than 0.9, or higher than 1.1. Significance level of genes in  
490 the analyzed GO categories was established according to p-value and fold change  
491 between the ethanol group and control.

#### 492 4.7 Cerebellar granule neurons cDNA preparation and quantification by qPCR

493 CGCs originating from rat pups exposed to ethanol, or under control conditions were  
494 obtained as described above. Offsprings originated from two dams for each group,  
495 cerebella were collected, three and four cell cultures were made for the control and  
496 ethanol-group, respectively. Total RNA was isolated using 500  $\mu$ l TRI REAGENT™  
497 (Sigma Aldrich) according to the manufacturer's instructions. 550 ng of RNA were  
498 reverse transcribed (Superscript, Invitrogen™) with oligo(dT) primer in a total  
499 reaction volume of 20  $\mu$ l. 1  $\mu$ l of cDNA was further subjected to real-time PCR using  
500 Platinum-SYBR Green qPCR Supermix (Invitrogen), forward and reverse primers  
501 (0.9  $\mu$ M), and ROX (100 nM, 5-carboxy-X-rhodamine, passive reference dye).  
502 Primers were designed with the Primer3 software for the following genes: *Cp*  
503 (forward primer: 5'-CATGTGGATGCTCCAAAAGA-3', reverse primer: 5'-  
504 GGTTCGAGCAGAAGGTTTT-3'), *Dhcr24* (forward primer: 5'-  
505 GCTCTCCCTCATCTTCGACA-3', reverse primer: 5'-

506 TGAGACAGTGAGCCATCCAG-3', *Adm* (forward primer: 5'-  
507 CTCGACACTTCCTCGCAGT-3', reverse primer: 5'-  
508 AGACGTGCTCTGCTTGTCT-3'), *Bdnf* (forward primer: 5'-  
509 AGGAGCGTGACAACAATGTG-3', *Snca* (forward primer: 5'-  
510 AGAAAACCAAGCAGGGTGTG-3', reverse primer: 5'-  
511 CCCTCCACTGTCTTCTGAGC-3'), reverse primer: 5'-  
512 CGTGGACGTTTGCTTCTTTC-3'), *Mapk8* (forward primer: 5'-  
513 CGGAGATTCTACATTCACAGTCC-3', reverse primer: 5'-  
514 CGCTTAGCATGGGTCTGATT-3'), *Bax* (forward primer: 5'-  
515 GCTGGACACTGGACTTCCTC-3', reverse primer: 5'-  
516 CTCAGCCCATCTTCTTCCAG-3'), *Slc2a4* (forward primer: 5'-  
517 GGCCGGGACACTATAACCCTA-3', reverse primer: 5'-  
518 GCCAAGCACAGCTGAGAATA-3'), *Gss* (forward primer: 5'-  
519 AGGGGGTGTGCTGAGGT-3', reverse primer: 5'-  
520 CTGGCTGACAGCATCTACCA-3'), and *B2m* (forward primer: 5'-  
521 ACATCCTGGCTCACACTGAA-3', reverse primer: 5'-  
522 CCGGATCTGGAGTTAAACTGG-3'). PCR was performed in an MX 3000P  
523 instrument (Stratagene, La Jolla, CA) using the following protocol: 5 min 50°C, 2 min  
524 95°C, and 40 cycles of 15 s 95°C, 30 s 60°C. A product melting curve was recorded  
525 to confirm the presence of a single amplicon. The correct amplicon size was  
526 confirmed by agarose gel electrophoresis. Threshold ( $C_T$ ) values were set within the  
527 exponential phase of the PCR. *B2m* shows constant level of expression in both  
528 ethanol-treated and control cell cultures ( $C_T$  values of  $17.7 \pm 0.5$  and  $17.9 \pm 0.2$ ,  
529 respectively), which supports its use as housekeeping gene (Butte et al., 2001). The  
530 housekeeping gene *B2m* ( $\beta$ 2-microglobulin) was used for normalization

531  $\Delta C_T (C_{T(\text{gene})} - C_{T(B2m)})$  (Livak and Schmittgen, 2001). The relative expression levels  
 532 are given as the difference between the  $\Delta C_T$  corresponding to the non-treated and  
 533 ethanol-treated groups, respectively, ( $\Delta\Delta C_{T(\text{gene})} = \Delta C_{T(\text{gene Non-treated Group})} -$   
 534  $\Delta C_{T(\text{gene Ethanol Group})}$ ). Gene regulation ratios between the ethanol and control groups  
 535 are given as  $2^{\Delta\Delta C_T}$  values (Livak and Schmittgen, 2001). Gene regulation was  
 536 statistically evaluated by subjecting the  $\Delta C_T$  values to a two-sided, unpaired Student's  
 537 *t*-test.

#### 538 4.8 Statistical analysis for CGCs cultures viability experiments

539 For each group, the experiments were performed at least 4 times, on independent  
 540 CGCs cultures from litters of different dams, with multiple wells for each condition.  
 541 Statistical analysis was performed considering the different litters as biological  
 542 replicates, while corresponding number of culture wells were considered technical  
 543 replicates. To account for both technical and biological variance we used a standard  
 544 equation for error propagation, as described by Taylor:

545  $SD = \sqrt{SD_{\text{biological replicates}}^2 + SD_{\text{technical replicates}}^2}$  (Taylor, 1997). Numerical results are given  
 546 as mean  $\pm$  SEM.

547 To analyze whether cell death was influenced by time or assessment conditions, a  
 548 multiple regression model was run. No interaction between general predictors was  
 549 taken into account, as theory suggests there should be none. Into this we included  
 550 conditions and time (covariate) as the two predictors. Prior to running the model we  
 551 checked covariate and cell death for having approximately simetrical distributions  
 552 (which was the case), and z-transformed covariate to a mean of zero and a standard  
 553 deviation of one to achieve easier interpretable coefficients (Forstmeier and  
 554 Schielzeth, 2011). We checked model diagnostics like distribution of residuals,

555 Cook's distance and  $dfbetas$  and none of these indicated outliers, or obvious  
556 deviations from the assumptions of normality and homogeneity of residuals. The  
557 model and most diagnostics were run using functions available in R (Team, 2011).  
558 Generalized Variance Inflation Factors were derived using the function `vif` of the R  
559 package `car` (Weisberg, 2011). Overall the two parameters used for analysis:  
560 "assessment conditions" and "time", both influenced cell death at a significant level  
561 (comparison of full to the null model results in  $F_{3,319} = 1163$ ,  $p < 0.001$ ). The cell  
562 death increased in time (estimate = 9.9613, SE = 0.2425,  $t_{319} = 41.079$ ). Cell death  
563 was also influenced by the assessment conditions as shown by comparing the full  
564 model with a reduced one not comprising exposure conditions revealed and resulting  
565 in an  $F_{3,319} = 987.67$ , and  $p < 0.001$ ). This validated the data without running pair wise  
566 comparisons and allowed for pair wise post-hoc comparisons to analyze the impact on  
567 cell survival between different conditions. A value of  $p < 0.05$  was considered  
568 statistically significant.  
569

570 **Conflict of interest statement**

571 The authors declare that they have no competing interests.

572 **Authors contributions**

573 DLD: Participated in the design of the research, experimental procedures, data  
574 analysis, microarray gene expression analysis, and manuscript writing.

575 AS: Participated in the design of the research, experimental procedures, and data  
576 analysis.

577 MC: Participated in data analysis.

578 LZ: Participated in manuscript writing.

579 TS: Participated in manuscript writing and qPCR experiments design.

580 ECT: Participated in data analysis and manuscript writing.

581 AMZ: Participated in the design of the research, experimental procedures, data  
582 analysis, and manuscript writing.

583 All authors have approved the final article manuscript.

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592

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772 **Figure legends:**

773 **Figure 1. Microscopy images of CGCs cultures originating from ethanol-treated**  
774 **or non-treated rat pups. A.** Phase-contrast microscopy of CGCs cultures on *in vitro*  
775 day 7 before exposure to OGD and/or normoxic low nutrient medium used for control  
776 shows similar morphology in the ethanol-treated and non-treated groups. **B.** Phase-  
777 contrast microscopy (upper pannel) and fluorescent (bottom pannel) images taken on  
778 the same field from non-treated and ethanol treated CGCs stained with propidium  
779 iodide, after 20 hours of reoxygenation following OGD or control conditions.  
780 Microphotographs were taken with a Zeiss Axiovert25 inverted microscope at 400x  
781 magnification. Scale bar represents 10  $\mu$ m.

782 **Figure 2. Cell death of ethanol-treated and non-treated cerebellar granule**  
783 **neurons exposed to OGD. A.** At the beginning of reoxygenation cell death was  
784 found significantly increased in the ethanol-treated group exposed to OGD. Cell death  
785 was assessed by propidium iodide fluorescence. **B.** The effect of OGD on ethanol-  
786 treated and non-treated cultures after 20 hours of reoxygenation is expressed as % cell  
787 death difference between OGD and control cultures. At this time point, OGD-induced  
788 cell death remained significantly increased in the ethanol group, comparing with the  
789 non-treated group. Error bars represent SEM,  $n \geq 4$  cell cultures from unrelated pups.

790 **Figure 3. Cell death over 20 hours of reoxygenation in ethanol-exposed and non-**  
791 **treated cerebellar granule neurons subjected to OGD. A.** OGD-induced higher  
792 cell death rates in ethanol-exposed neurons in comparison to non-treated ones. Under  
793 control conditions the difference between ethanol-exposed cells and non-treated ones  
794 became manifest after 10 hours of reoxygenation. Cell death was assessed every  
795 15 minutes for 20 hours by propidium iodide fluorescence. **B.** In control cultures,  
796 hourly cell death rate calculated during early (2-7 h) and late (12-17 h) reoxygenation,

797 showed that the sensitising effect of ethanol pretreatment becomes manifest only  
798 during the late reoxygenation. **C.** OGD effect on hourly cell death rate, calculated as  
799 difference between OGD and control cultures, was significantly higher during early  
800 reoxygenation in ethanol-exposed neurons compared to non-treated ones. Due to the  
801 extensive damage during early reoxygenation in ethanol group, the hourly cell death  
802 rate during the late reoxygenation period became higher in non-treated cultures. Error  
803 bars represent SEM,  $n \geq 4$  CGCs cell cultures from unrelated pups.

804 **Figure 4. qPCR measurement of genes hypothesized to be influenced by prenatal**  
805 **alcohol exposure.** Five genes are significantly up-regulated in the ethanol-exposed  
806 neurons compared to the non-treated ones. The measured genes are involved in the  
807 GO categories shown to be disrupted on a microarray dataset from whole embryo cell  
808 cultures after ethanol exposure (Tables 1 and 2).  $\beta$ 2-microglobulin (*B2m*) expression  
809 levels are similar between the ethanol-exposed ( $17.7 \pm 0.5$ ) and non-treated CGCs  
810 cultures ( $17.9 \pm 0.2$ ).  $C_T$  values for *B2m* were used for normalization  $\Delta C_T (C_{T(\text{gene})} -$   
811  $C_{T(B2m)})$ . Error bars represent SEM,  $n = 3$  and  $n = 4$  for CGCs cultures of non-treated  
812 and ethanol-treated groups, respectively.

813

814 **Table 1.** Gene Ontology categories relevant for an increased susceptibility towards  
 815 metabolic stressors observed in ethanol-exposed embryos. GO enrichment was tested  
 816 using a Wilcoxon rank test from FUNC package, according to expression fold change  
 817 between ethanol-exposed and control embryos. FWER = family wise error rate. First  
 818 two nodes are enriched in low ranked genes and the others in high ranked ones.

Root node name	Node name	Node ID	No. of genes in node	raw p-value	FWER
biological process	oxidation-reduction process	GO:0055114	587	8.80E-08	< 1.0E-04
cellular component	mitochondrial respiratory chain	GO:0005746	45	6.27E-06	0.003
biological process	regulation of stress-activated MAPK cascade	GO:0032872	133	3.28E-06	0.005
biological process	in utero embryonic development	GO:0001701	346	2.04E-06	0.003
biological process	nervous system development	GO:0007399	1392	5.50E-08	< 1.0E-04

819

820 **Table 2.** Differentially expressed genes which clustered in “oxidation-reduction  
 821 process” GO:0055114, “regulation of stress-activated MAPK cascade” GO:0032872,  
 822 “*in utero* embryonic development” GO:0001701, and “nervous system development”  
 823 GO:0007399 categories. p-values were calculated gene-wise using a two sample *t*-test  
 824 and fold EtOH/Ctr represents the fraction between the mean expression value in the  
 825 ethanol-exposed versus control embryos.

826 GO:0055114

Gene name	Gene description	p-value	Fold EtOH/Ctr
<i>Dhcr24</i>	24-dehydrocholesterol reductase	0.006	1.13
<i>Fads2</i>	fatty acid desaturase 2	0.018	1.13
<i>Adh1</i>	alcohol dehydrogenase 1 (class I)	0.026	1.12
<i>Gpd1l</i>	glycerol-3-phosphate dehydrogenase 1-like	0.037	1.15
<i>Cp</i>	ceruloplasmin	< 0.001	0.71
<i>Blvrb</i>	biliverdin reductase B (flavin reductase (NADPH))	< 0.001	0.80



<i>Pnpo</i>	pyridoxine 5'-phosphate oxidase	< 0.001	0.89
<i>Snca</i>	synuclein, alpha	0.004	0.85
<i>Bdnf</i>	brain derived neurotrophic factor	0.011	0.81
<i>Ppp1r3c</i>	protein phosphatase 1, regulatory (inhibitor) subunit 3C	0.017	0.87
<i>Aifm2</i>	apoptosis-inducing factor, mitochondrion-associated 2	0.028	0.87
<i>Ppox</i>	protoporphyrinogen oxidase	0.029	0.87

827 GO:0032872

Gene name	Gene description	p-value	Fold EtOH/Ctr
<i>Mapk8ip1</i>	mitogen-activated protein kinase 8 interacting protein 1	0.014	1.18
<i>Nrk</i>	Nik related kinase	0.021	1.18
<i>Ephb1</i>	Eph receptor B1	0.026	1.13
<i>Mapk8ip2</i>	mitogen-activated protein kinase 8 interacting protein 2	0.029	1.12
<i>Ctgf</i>	connective tissue growth factor	0.013	0.86
<i>Ncor1</i>	nuclear receptor co-repressor 1	0.016	0.77
<i>Gadd45a</i>	growth arrest and DNA-damage-inducible 45 alpha	0.030	0.89
<i>Eda2r</i>	ectodysplasin A2 receptor	0.044	0.85

828 GO:0001701

Gene name	Gene description	p-value	Fold EtOH/Ctr
<i>Nrk</i>	Nik related kinase	0.020	1.18
<i>Epb4.115</i>	erythrocyte protein band 4.1-like 5	0.020	1.11
<i>Eif4e2</i>	eukaryotic translation initiation factor 4E member 2	0.020	1.10
<i>Wnt2</i>	wingless-related MMTV integration site 2	0.042	1.11
<i>Slit2</i>	slit homolog 2	0.002	0.89
<i>Cebpb</i>	CCAAT/enhancer binding protein (C/EBP), beta	0.011	0.86
<i>Maff</i>	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	0.011	0.87
<i>Adm</i>	adrenomedullin	0.021	0.86
<i>Cyr61</i>	cysteine rich protein 61	0.028	0.89
<i>Klf1</i>	Kruppel-like factor 1 (erythroid)	0.028	0.79
<i>Pdgfb</i>	platelet derived growth factor, B polypeptide	0.037	0.89

829 GO:0007399

Gene name	Gene description	p-value	Fold EtOH/Ctr
<i>Pitx1</i>	paired-like homeodomain transcription factor 1	0.003	1.13
<i>Clmn</i>	calmin	0.005	1.14
<i>Lrp8</i>	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	0.008	1.16
<i>Fzd3</i>	frizzled homolog 3 (Drosophila)	0.011	1.14
<i>Astn1</i>	astrotactin 1	0.023	1.17
<i>Ephb1</i>	Eph receptor B1	0.026	1.13
<i>En2</i>	engrailed 2	0.027	1.28
<i>Apcdd1</i>	adenomatosis polyposis coli down-regulated 1	0.027	1.13

<i>Ncdn</i>	neurochondrin	0.027	1.11
<i>Lpar3</i>	lysophosphatidic acid receptor 3	0.027	1.11
<i>Sall1</i>	sal-like 1 (Drosophila)	0.028	1.12
<i>Mapk8ip2</i>	mitogen-activated protein kinase 8 interacting protein 2	0.029	1.12
<i>Sphk2</i>	sphingosine kinase 2	0.034	1.12
<i>Pax7</i>	paired box gene 7	0.038	1.11
<i>Sox1</i>	SRY-box containing gene 1	0.041	1.16
<i>Pou3f1</i>	POU domain, class 3, transcription factor 1	0.041	1.37
<i>Wnt2</i>	wingless-related MMTV integration site 2	0.042	1.11
<i>Pcsk9</i>	proprotein convertase subtilisin/kexin type 9	0.046	1.12
<i>Prdm6</i>	PR domain containing 6	0.047	1.13
<i>Slit2</i>	slit homolog 2	0.002	0.89
<i>Tenm2</i>	teneurin transmembrane protein 2	0.005	0.83
<i>Limk1</i>	LIM-domain containing, protein kinase	0.006	0.90
<i>Arhgef28</i>	Rho guanine nucleotide exchange factor (GEF) 28	0.008	0.86
<i>Ntf3</i>	neurotrophin 3	0.009	0.88
<i>Cebpb</i>	CCAAT/enhancer binding protein (C/EBP), beta	0.011	0.86
<i>Gfra1</i>	glial cell line derived neurotrophic factor family receptor alpha 1	0.011	0.79
<i>Bdnf</i>	brain derived neurotrophic factor	0.011	0.81
<i>Nrxn1</i>	neurexin I	0.013	0.86
<i>Ncor1</i>	nuclear receptor co-repressor 1	0.016	0.77
<i>Ndr1</i>	N-myc downstream regulated gene 1	0.018	0.77
<i>Trp63</i>	transformation related protein 63	0.019	0.90
<i>Adm</i>	adrenomedullin	0.021	0.86
<i>Dlc1</i>	deleted in liver cancer 1	0.022	0.76
<i>Ndr2</i>	N-myc downstream regulated gene 2	0.025	0.87
<i>Itga1</i>	integrin alpha 1	0.028	0.82
<i>Foxd1</i>	forkhead box D1	0.030	0.88
<i>Etv1</i>	ets variant gene 1	0.030	0.89
<i>Sema3d</i>	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	0.031	0.85
<i>Ankrd1</i>	ankyrin repeat domain 1 (cardiac muscle)	0.038	0.89
<i>Lrrk2</i>	leucine-rich repeat kinase 2	0.040	0.88

830

831

832 **Highlights:**

833 - Fetal ethanol exposure increases neurons' vulnerability towards metabolic stressors

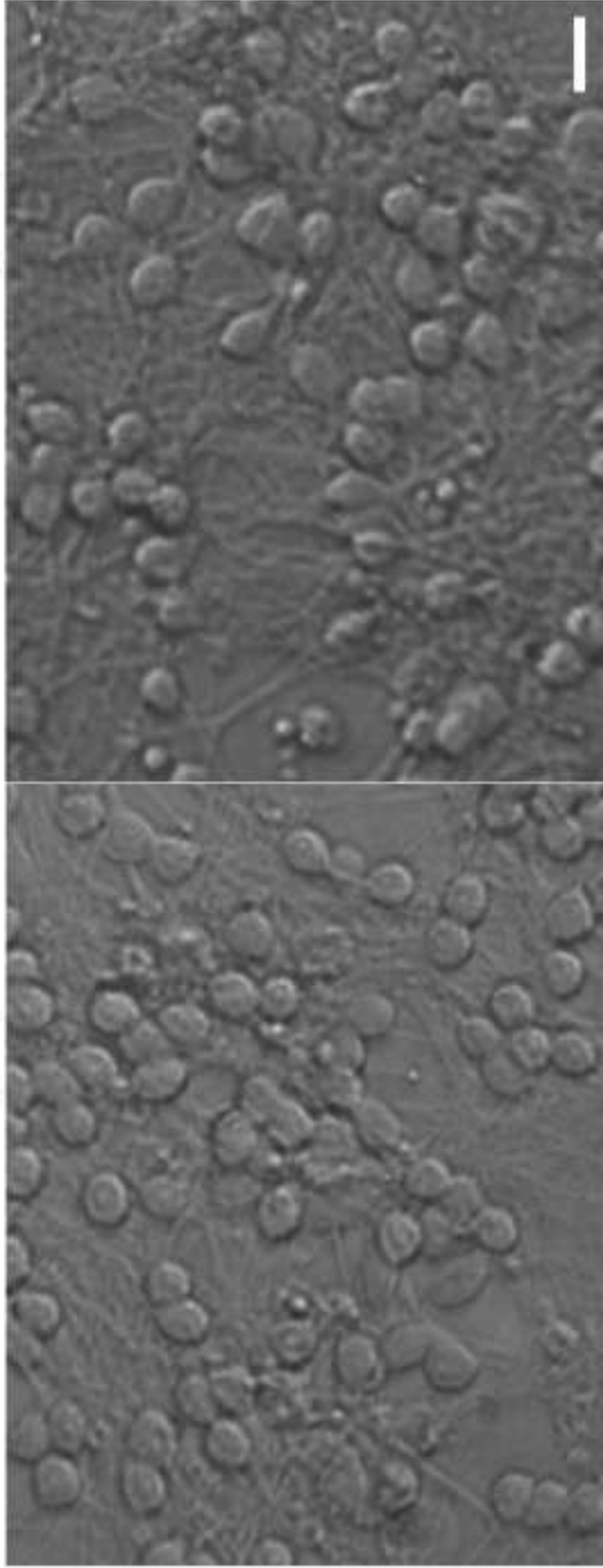
834 - Maternal alcoholism decreases offsprings' neuronal tolerance to hypoxia/ischemia

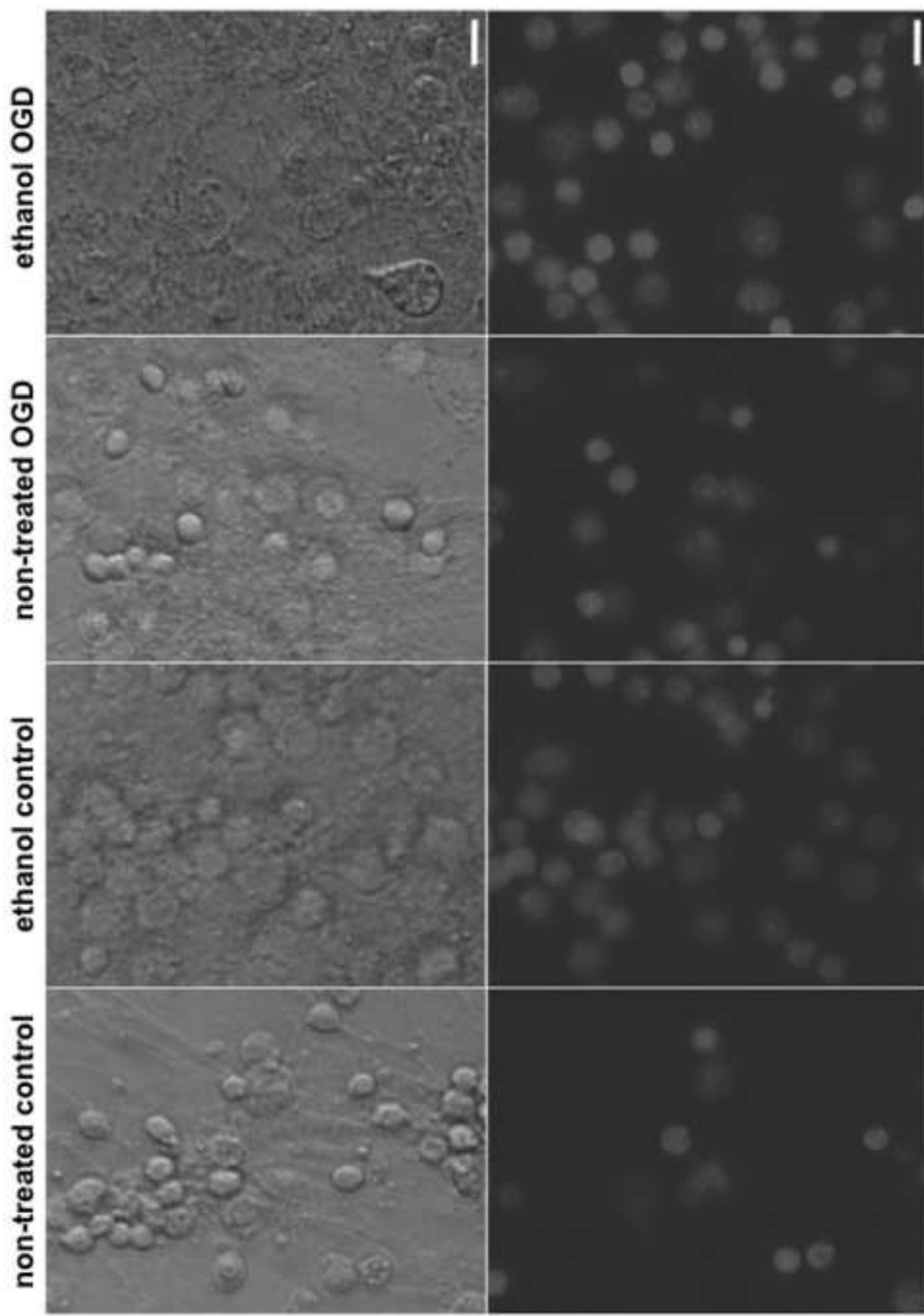
835 - Ethanol alters expression of genes associated with apoptosis and neuroprotection

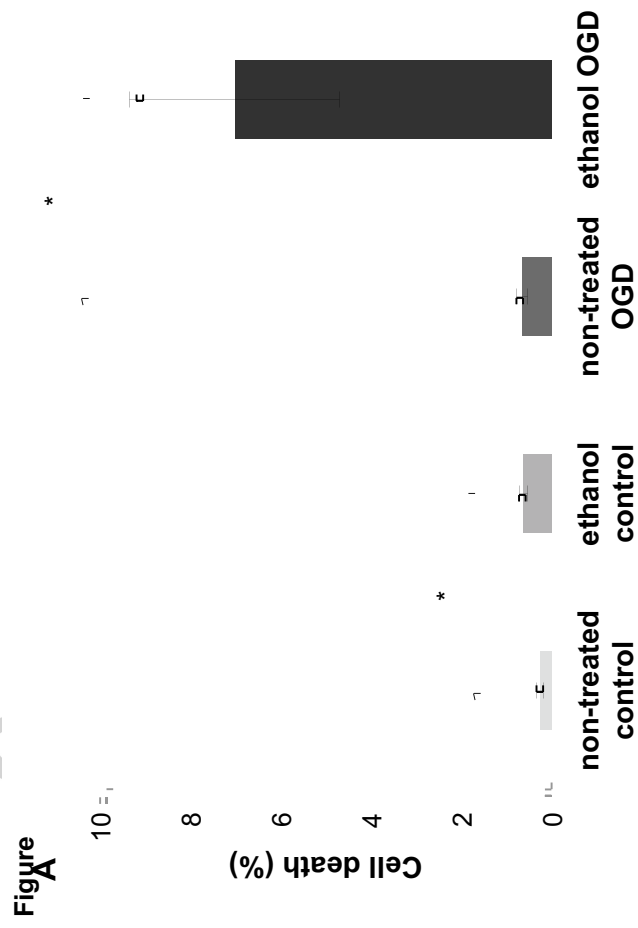
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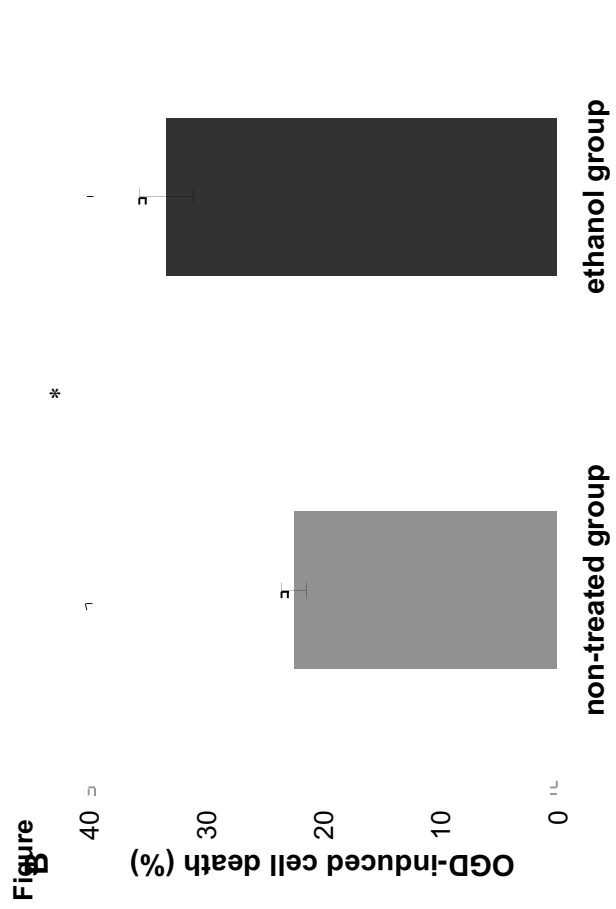
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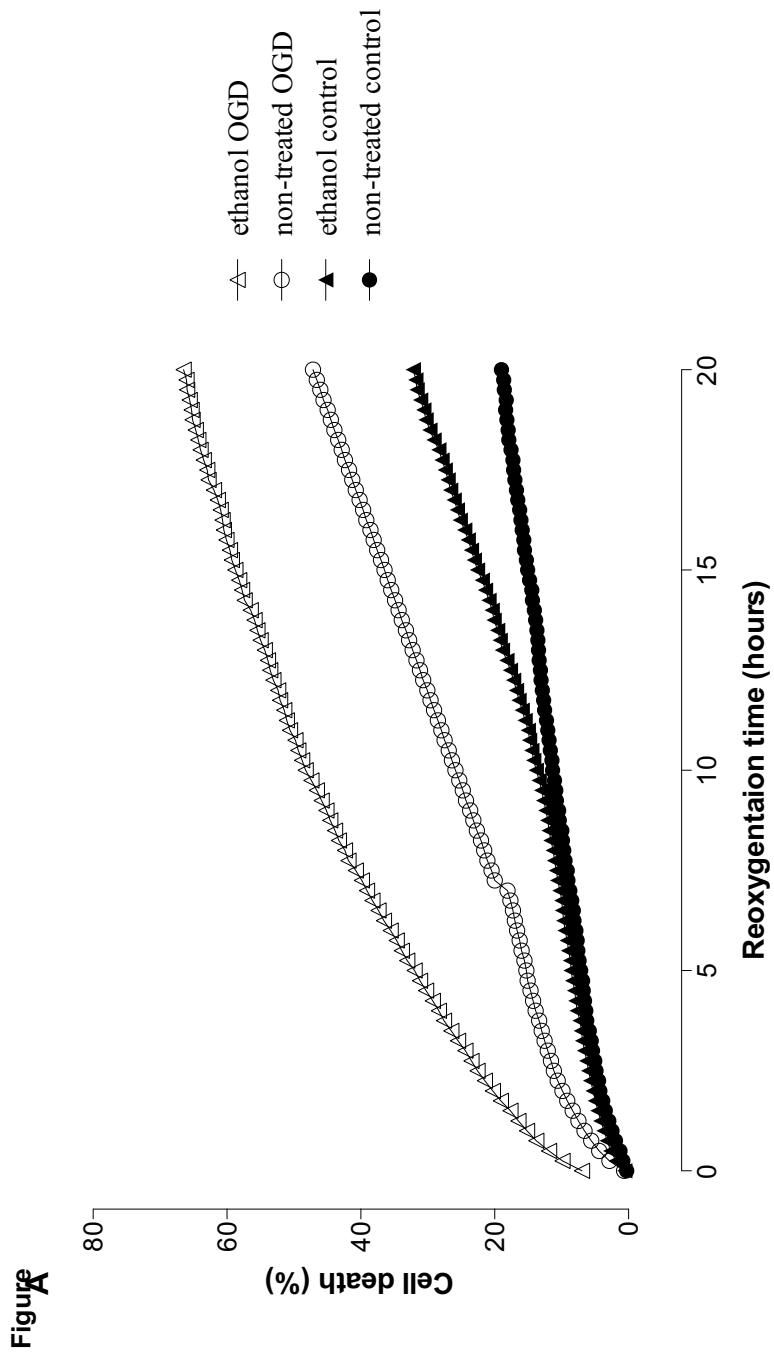
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**A****non-treated group****ethanol-treated group**

**B**

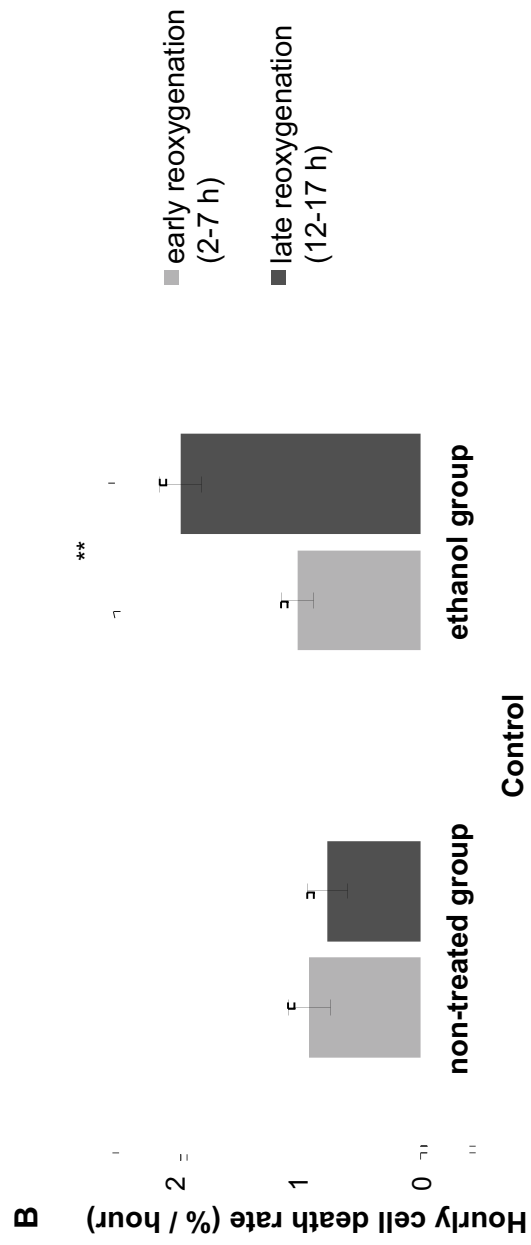








A



Figure

27

