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ORIGINAL ARTICLE

Differential Hippocampal Response to Chronic Alcohol Consumption of Young Adult and Mature Adult Rats

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Abstract — Aims: Early ethanol consumption could be a risk factor for young brain integrity and its maturation, and also for the development of addictive behaviors in adulthood. Neuronal nitric oxide synthase (nNOS) expressing neurons are specifically located in the subgranular layer (SGL) of dentate gyrus and may be relevant for hippocampal neurogenesis. The focus of this work is aimed to determine local changes in the nNOS-like immunoreactive (nNOS-LIR) cell populations of the SGL after chronic ethanol exposure in young adult and mature adult rats. **Methods:** We used the nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase (NADPH-d) reaction as a qualitative marker of nNOS enzyme activity. We also analyzed the nNOS-LIR cell density by the nNOS immunocytochemistry in order to compare these two methods of labeling. Dorsal striatum (CPu) was also analyzed in order to compare two neural areas with high nNOS-LIR cell density. **Results:** The young adult group showed less hippocampal NADPH-d^+ cell density than the mature adult group. Interestingly, the NADPH-d^+ cell density was increased in the SGL of the young adult ethanol-treated group, whereas it decreased in the mature adult ethanol-treated group, when compared with their respective controls. No change was observed in any of the groups for the hippocampal nNOS-LIR cell density and no differences could be established in CPu for nNOS-LIR and NADPH-d^+ cell densities in any of the groups studied. **Conclusion:** The NADPH-d expression is affected by chronic ethanol exposure in opposite ways between both age groups studied. Further studies are needed to evaluate the relative importance of these findings, especially when considering human subjects.

INTRODUCTION

Although the legal drinking age in Spain, as in other countries, is 18 years, the average age of the first alcohol consumption in Spain is 13.8 years, and there has been no modification of this age in the last decade. More concerning, at ages between 16 and 17 years old, the rate of ethanol consumption is not different from the one observed at the legal drinking age of 18 (Plan Nacional Sobre Drogas, 2007). The latter age period has been considered as the 'adult age', but it is still far from the complete maturation of the human brain (Gogtay *et al.*, 2004). For this reason, we consider of interest this age period from 16 to 20 years as a special scenario to study the effects of drugs.

In the last decade, several studies have focused on the effects of ethanol on the nitrergic system. Nitric oxide (NO) plays a role in the development of tolerance to ethanol or other drugs, and this role may be similar to the one of NO in learning and memory processes, involving facilitation of transmission in certain *N*-methyl-*D*-aspartate synapses (Khanna *et al.*, 1993; Kolesnikov *et al.*, 1993, 1997).

Many and diverse data have been published about the effects of ethanol on the NO system, and these differences may be related to several factors such as the duration of treatment and age (Jang *et al.*, 2002). Spanagel *et al.* (2002) reported that the mice lacking the neuronal type of NO synthase (nNOS) gene showed high ethanol consumption compared with the wild type and are more resistant to the sedative effects of ethanol. This group also analyzed that non-selective nNOS inhibitors surprisingly decreased the ethanol consumption in both the nNOS knock-out mice and the wild type, leading to the conclusion that non-selective nNOS inhibition exerts its role independently of the nNOS gene. These data reinforce the relevance of the nNOS enzyme on central nervous system ethanol effects.

Nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase (NADPH-d) is considered as a good nNOS-rel marker, being the only activity preserved in nervous paraformaldehyde-fixed tissue (Bredt *et al.*, 1991; Hope *et al.*, 1991; Vincent and Kimura, 1992). NADPH-d reaction assesses the ability of the reductase domain to convert NADPH into $NADP + H^+$ and therefore is able to reduce nitro blue tetrazolium salts to a blue water-insoluble formazan complex (Bredt *et al.*, 1991; Dawson *et al.*, 1991; Hope *et al.*, 1991). The significance of this reaction is relevant since the oxygenase domain needs one electron to convert L-arginine to L-citrulline and this electron is released from the reductase domain (Schmidt *et al.*, 1991; Stuher and Ikeda-Saito, 1992).

Hippocampus has been pointed out as a high sensitive area to the effects of ethanol (Ryabinin, 1998; Herrera *et al.*, 2003; Johnsen-Soriano *et al.*, 2007), and newborn cells produced in the subgranular layer (SGL) are quite sensible to the effects of ethanol exposure. More accurately, ethanol-induced oxidative stress represents a key factor in this deleterious process since it can be reversed by antioxidants (Herrera *et al.*, 2003; Johnsen-Soriano *et al.*, 2007). NO forms nitrite (NO_2^-) and nitrate (NO_3^-) and easily reacts with molecules such as oxygen, superoxide, thiyl, lipid peroxyl and other free radicals (Wink and Mitchell, 1998; Chiueh and Rauhala, 1999), but NO can also form *S*-nitrosothiols, as *S*-nitrosoglutathione, with effective antioxidant properties (Do *et al.*, 1996; Chiueh and Rauhala, 1999; Foster and Stamler, 2004). Hippocampus has a relevant neuronal population expressing nNOS in the whole hippocampal area, but specifically shows nNOS-LIR cells in the

SGL. This SGL nNOS-LIR cell population has been correlated with the production of new cells in the SGL (Islam *et al.*, 2003).

Considering the different roles of NO in nervous tissue as a transmitter, neuroprotectant or even as pro-oxidant, and the anatomical distribution of nNOS-LIR cells in the SGL, close to the neural stem cells, the aim of the study is addressed to analyze the effects of a mild-chronic ethanol exposure in SGL NADPH-d$^+$ cells (as a reporter of nNOS activity) in two close, but different, neural maturation steps.

MATERIALS AND METHODS

Experimental design

Twenty-four male Sprague–Dawley (S-D) rats were divided into two groups according to their weight as a measure of age. The young adult group consisted on 12 male S-D rats weighing 161.58 ± 3.20 g, and the mature adult group consisted of 12 male S-D rats weighing 306.58 ± 2.08 g (Table 1). Animals were housed in individual cages with a 7 a.m. to 7 p.m. dark-light cycle, controlled temperature and humidity. All animal manipulations were done according to international regulations of European Parliament and of Council (2003/65/CE) and were approved by the Animal Care Committee of the University Cardenal Herrera—CEU.

Ethanol treatment-liquid diet

The young adult group and the mature adult group were randomly subdivided into two subgroups ($n = 6$ each), receiving either control or alcohol liquid diet (Lieber *et al.*, 1965), and then paired according to weight as previously reported (Herrera *et al.*, 2003; Johnsen-Soriano *et al.*, 2007). Liquid ethanol and control diets were purchased from BioServ (Frenchtown, NJ, USA), and prepared in liquid form with either ethanol plus water (resulting a 6.4% v/v ethanol concentration), or water alone. These diets have been developed to supply isocaloric intake in both alcoholic and non-alcoholic conditions, by supplementing the latter with dextrinated maltose accordingly (ethanol-derived calories at 6.4% ethanol concentration provides 350 kcal/l).

Ethanol liquid diet was administered *ad libitum* for 6 weeks, and control animals received the volume of the corresponding paired on the following day (pair-fed control). Body weight was initially and finally recorded.

Blood ethanol levels

Ethanol blood level was randomly measured along the last 3 weeks of treatment using a standardized colorimetric assay kit (BIOLABO, Maizy, France). Blood samples were obtained from the tail.

Table 1. Weight and daily liquid diet consumption (mean ± SEM) of the experimental groups

	Young adult control	Young adult ethanol	Mature adult control	Mature adult ethanol
Initial weight (gr)	161.0 ± 4.0	163.0 ± 6.0	304.0 ± 3.0	310.0 ± 3.0
Final weight (gr)	306.6 ± 7.0	292.0 ± 9.0	414.0 ± 6.0	407.0 ± 8.0
Diet (ml/day)	69.3 ± 3.0	65.1 ± 4.0[a]	83.5 ± 5.0	83.0 ± 4.0[b]

[a]Corresponds with 4.4 ml ETOH/day.
[b]Corresponds with 5.3 ml ETOH/day.

nNOS immunocytochemistry

For histological processing, rats were anesthetized with sodium pentobarbital and perfused with saline (250 ml 0.9% NaCl) followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Brains were post-fixed in this paraformaldehyde solution for 24 h and then placed in a 30% sucrose solution for 24 h. Forty micrometer thick sections were obtained using a cryostat. Sections were rinsed with 0.01 M PBS, pH 7.0 and blocked with 30% H_2O_2 for 20 min followed by incubation overnight with a primary rabbit anti-nNOS (Santa Cruz, Santa Cruz, CA, USA) (dilution 1:500 in PBS with 0.3% Triton X-100 and 5% normal goat serum). Sections were rinsed in PBS and incubated at room temperature, with shaking for 1 h in 0.4% biotinylated anti-rabbit immunoglobulin G. Finally, sections were rinsed and re-incubated with avidin-biotin complex (Vector Burlingame, CA, USA), and reaction was developed with diaminobenzidine.

NADPH-diaphorase histochemistry

Sections were rinsed with 0.01 M PB and then incubated at 37°C in darkness for 2 h in 0.1 M PB, pH 7.4, containing 0.5 mg/ml β-NADPH (Roche), 0.5 mg/ml Nitroblue Tetrazolium (Sigma Química, Alcobendas SP) and 0.8% of Triton X-100 (Sigma Química, Alcobendas SP). The reaction was stopped by addition of 0.01 M PB.

Quantification and statistical analysis

NADPH-d positive (NADPH-d$^+$) and nNOS-LIR cells in the SGL were assessed by counting the number of cells with visible somata of a limited area in two sections, between −3.40 and −4.00 Bregma, from five or six animals per group. The region of interest was the space corresponding to the granule cell layer (GCL) (200 µm width), included along each blade of the full length of the dentate gyrus. The cells included within this area or in contact with the GCL limit were included, and those in contact with the opposite limit (close to the hilus) were excluded.

The NADPH-d$^+$ and nNOS-LIR cells in CPu were obtained from four randomized microphotographs (×20 magnification) of adjacent sections, between +1.50 and +0.12 Bregma, from five or six animals per group. A limited surface of 572 square pixels was defined and cells included within or in contact with the superior right side were included.

Pixel squared surfaces were converted in all cases to square millimeters, and data were expressed as cell number/mm^2.

Repeated measures from the same animal and group were analyzed by a mixed and nested analysis of variance (ANOVA) design to compare the cell densities between groups followed by a *post hoc* Tukeýs honestly significant difference comparison analysis to identify the differences between groups. One factor ANOVA analysis was performed to compare the different methods of labeling. *P*-level of significance was set at 0.05.

RESULTS

Blood alcohol concentration analysis

The mean serum alcohol concentrations were 23.8 ± 3.1 mM in the young adult ethanol-treated group, 27.3 ± 3.5 mM in the mature adult ethanol-treated group and 0 or negligible in both the young adult and mature adult control groups.

Table 2. SGL and CPu NADPH-d$^+$ and nNOS$^+$ cell densities, expressed as the mean ± SEM, of the different experimental groups ($n = 6$)

	SGL		CPu	
Groups	NADPH-d	nNOS	NADPH-d	nNOS
Young adult control	91.8 ± 10.6†	219.6 ± 19.2	410.4 ± 34.0	333.5 ± 39.2
Young adult ethanol	166.8 ± 22.6*	218.1 ± 18.5	410.4 ± 46.4	461.7 ± 35.2
Mature adult control	137.9 ± 12.0	197.4 ± 14.0	311.5 ± 44.0	401.9 ± 38.8
Mature adult ethanol	95.2 ± 9.9**	190.8 ± 19.6	339.2 ± 35.0	441.8 ± 26.0

$^\dagger P < 0.05$ vs mature adult control.
*$P < 0.05$ vs young adult control.
**$P < 0.05$ vs mature adult control group.

NADPH-diaphorase histochemistry

The NADPH-d reaction is considered as a marker of the nNOS activity in aldehyde-fixed sections of neuronal and other tissues (Dawson *et al.*, 1991; Hope *et al.*, 1991; Vincent and Kimura, 1992). The NADPH-d$^+$ cell density values are summarized in Table 2 for SGL and CPu. The SGL NADPH-d$^+$ cell density was firstly analyzed in order to detect group differences F (3, 17) = 177.301, $P < 0.001$, and *post hoc* comparisons showed statistically significant differences. The young adult control group was significantly lower than the mature adult control group ($P = 0.041$) (Fig. 1). Chronic ethanol treatment increased the SGL NADPH-d$^+$ cell density in the young adult group, whereas it decreased in the mature adult group ($P = 0.001$) (Fig. 1).

No NADPH-d$^+$ cell density differences could be found in CPu for none of the groups studied (Fig. 3C).

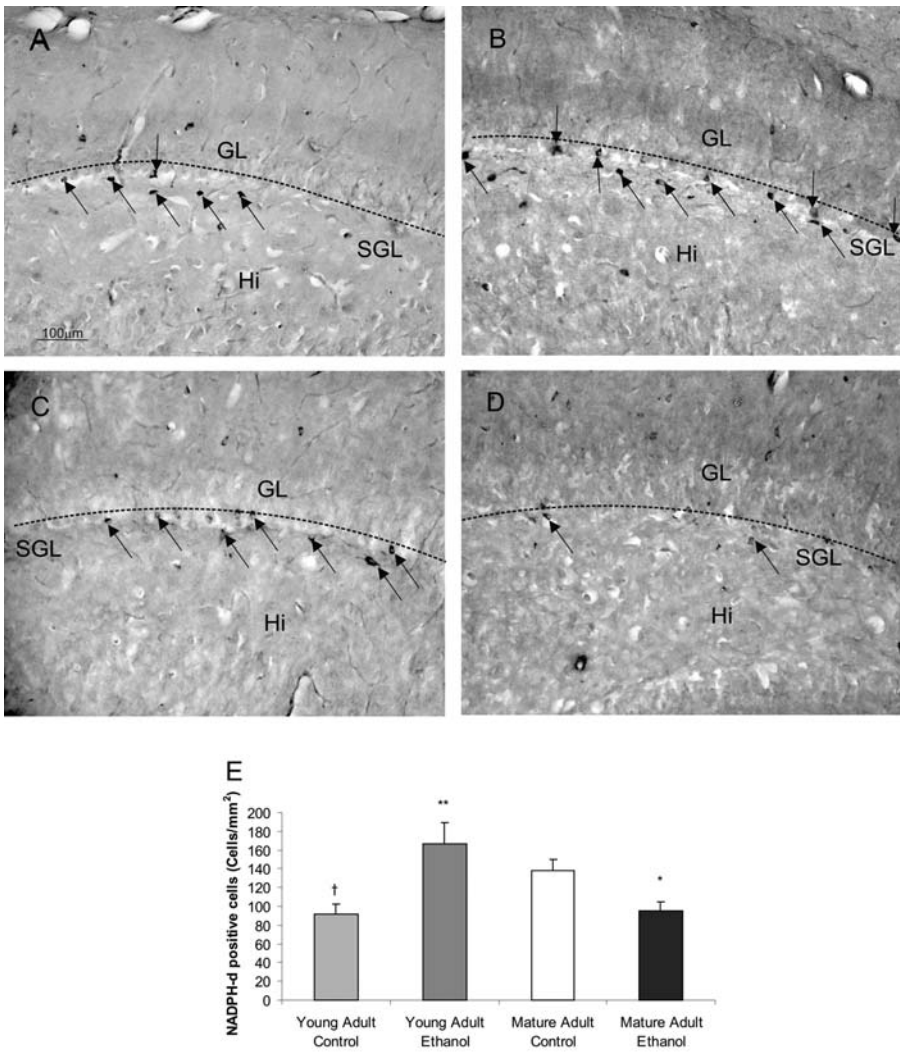

Fig. 1. NADPH-d histochemistry in hippocampus of: (**A**) young adult control group; (**B**) young adult ethanol-treated group; (**C**) mature adult control group and (**D**) mature adult ethanol-treated group. SGL, GL and Hi. The arrows show positive NADPH-d labeling. (**E**) Graphic representation of NADPH-d positive cell density in the SGL of hippocampus. *$P < 0.05$ vs adolescent control group; $^\dagger P < 0.05$ vs control adult group; **$P < 0.05$ vs control adult group.

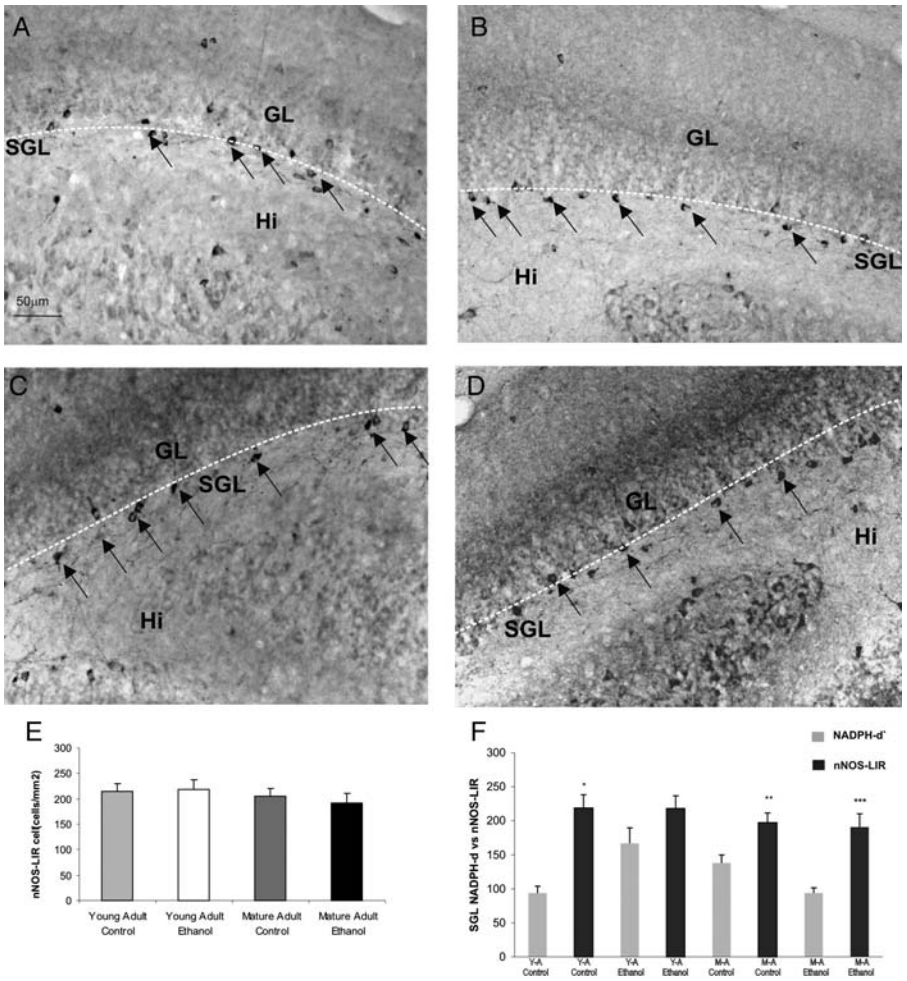

Fig. 2. nNOS-LIR cells in hippocampus of: (**A**) young adult control group; (**B**) young adult ethanol-treated group; (**C**) mature adult control group and (**D**) mature adult ethanol-treated group. SGL; GL is the granular layer and (Hi) is the Hilus of hippocampus. The arrows show positive nNOS-LIR labeling. (**E**) Graphic representation of the nNOS-LIR cell density in the SGL of hippocampus for the experimental groups. (**F**) The graphic comparison in the SGL for both labeling methods (NADPH-d vs nNOS-LIR) within ages and treatments, $*P < 0.05$; $**P < 0.05$; $***P < 0.05$.

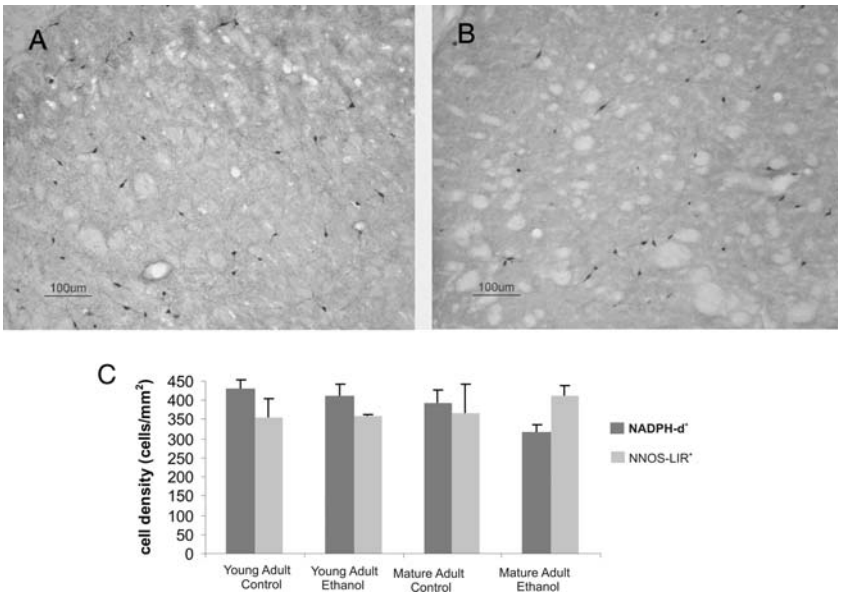

Fig. 3. (**A**) CPu NADPH-d histochemistry and, (**B**) CPu nNOS-LIR cells for every experimental cases; (**C**) Graphic comparison within groups and ages of CPu NADPH-d$^+$ and nNOS-LIR cell densities.

nNOS-LIR

The nNOS-LIR cell densities in both, the SGL or CPu, were unaltered, independently of the age or treatment F (3, 16) = 0.574 (P = 0.641) (Table 2; Figs. 2 and 3).

nNOS-LIR vs NADPH-d

Since NADPH-d$^+$ neurons are nNOS neurons (Bredt *et al.*, 1991; Dawson *et al.*, 1991; Hope *et al.*, 1991; Morris *et al.*, 1997) we compared these two markers in the SGL of hippocampus and CPu within the same group of treatment and age.

SGL nNOS-LIR cell densities were significantly higher than the NADPH-d$^+$ cell densities in all groups studied: Y-A (control) F (1, 8) = 58.837; P < 0.001; M-A (control) F (1, 8) = 14.38; P = 0.005; M-A ethanol (ETOH) F (1, 8) = 53.357; P < 0.001, except in the young adult ethanol-treated group: Y-A (ETOH) F (1, 8) = 2.299; P = 1.68.

No differences were found in CPu when both types of labeling were compared within ages and treatments (Fig. 3C).

DISCUSSION

One of the most important findings herein is the observed difference between the NADPH-d$^+$ cell densities in young adult and mature adult hippocampus. Indeed, the NADPH-d$^+$ cell density in the SGL differs significantly between the two studied ages, being higher in the mature adult group. Interestingly, there are no differences in striatal NADPH-d$^+$ cell densities between any of the groups studied. This result indicates a different hippocampal 'starting point' between both ages. Other interesting finding is that 6 weeks of daily ethanol exposure produced opposite responses in the SGL NADPH-d$^+$ cell populations: while the young adult group increased the NADPH-d$^+$ cell density, the mature adult reduced it, while CPu NADPH-d$^+$ cell density remained unaltered in all cases. These findings may apparently disagree with previous reports; chronic ethanol exposure decreases nNOS-mRNA levels in hippocampus while increases those in striatum. Chronic ethanol exposure also increased striatal nNOS kinetic parameters (*K*m and *V*max) (Naassila *et al.*, 2003). Interestingly, the cerebellar nNOS activity is resistant to ethanol exposure *in vitro* and *in vivo* (Ikeda *et al.*, 1999). Our results must be analyzed by considering the significance of the NADPH-d reaction. This reaction involves the NOS reductase domain and we have counted the number of cells labeled with the formazan blue color independently of the intensity of color. In other words, it could be interpreted as an on/off switching mechanism of the reductase domain that would apparently make these cells appear/disappear; the nNOS immunocytochemistry was performed in order to asses the actual state of the nNOS-LIR cell population. In fact, when SGL nNOS-LIR and NADPH-d$^+$ cell densities were compared, the nNOS-LIR cell density was significantly higher than the NADPH-d$^+$ cell density, except for the young adult ethanol-treated group comparison, where NADPH-d$^+$ cell density increases after ethanol consumption and the difference disappears (Fig. 3). A possible explanation would certainly fit with the proposed hypothesis that the reductase domain of nNOS could be switched on/off. In fact, this is what would occur in striatum when nNOS-LIR and NADPH-d$^+$ cell densities are compared: no differences could be established in any case (Fig 1), allowing the proposal that chronic ethanol consumption may differentially affect the nNOS reductase domain depending on the neural area and age. It has been shown that acute ethanol administration reduces the NADPH-d$^+$ cell density in rat SGL in a dose and duration-dependent manner (Jang *et al.*, 2002). This result agrees with those reported herein, but further co-labeling studies should be addressed to confirm whether all these NADPH-d$^+$ cells are actually nitrergic neurons or not.

The differential response of the SGL NADPH-d$^+$ cell populations to chronic ethanol exposure can be explained by considering the basal differences found already between ages. However, about the meaning of this differential response, a redox-related possible adaptation mechanism could be hypothesized, since the hippocampus is sensitive enough (in terms of oxidative stress) to the effects of ethanol, as has been repeatedly described (Herrera *et al.*, 2003; Johnsen-Soriano *et al.*, 2007) . It may also fit in the former report of NO acting both as neuroprotector or neurotoxic (Mohanakumar *et al.*, 2002).

The unresolved question is the significance of the basal SGL NADPH-d expression differences found between ages as explanation of the opposite response to ethanol exposure in terms of NADPH-d reactivity. In our opinion, the young adult period could be a particularly interesting scenario, slightly different from the mature adult age, in order to analyze the effects of ethanol in this very decisive period of life.

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