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Reduced density of dendritic spines in pyramidal neurons of rats exposed to alcohol during early postnatal life

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ABSTRACT

Dendritic spines are the main postsynaptic sites of excitatory connections of neocortical pyramidal neurons. Alterations of spine shape, number, and density can be observed in different mental diseases, including those caused by developmental alcohol exposure. Pyramidal neurons of layer 2/3 are the most abundant cells of the neocortex and represent the main source of associative cortico-cortical connections. These neurons are essential for higher functions mediated by the cortex such as feature selection and perceptual grouping. Furthermore, their connections have been shown to be altered in experimental models of fetal alcohol spectrum disorders.

Here, we used a Golgi-like tracing method to study the spine density of layer 2/3 associative pyramidal neurons in the somatosensory cortex of adult rats exposed to alcohol during the first postnatal week.

The main result of the present study is represented by the decreased spine density in the apical dendrite of alcohol-treated rats, as compared to controls. As to the basal dendritic tree, there were no significant differences between the experimental and the control group.

A decreased density of dendritic spines in the apical dendrite may impair the excitatory input onto pyramidal neurons, thus resulting in a widespread alteration of the cortical information flow.

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1. Introduction

It has long been recognized that the effects of exposure to ethanol during brain development, usually referred to as fetal alcohol spectrum disorders (FASD), represent one of the leading causes of intellectual disability in humans (Abel and Sokol, 1986). Ethanol-induced damage involves the whole developing brain, resulting in morphological and functional alterations of corpus callosum (Bookstein et al., 2001), hippocampus (Barnes and Walker, 1981), neocortex (Wass et al., 2001; Granato et al., 2003, 2012; Granato, 2006; Granato and De Giorgio, 2014), basal ganglia (De Giorgio et al., 2012), cerebellum (Lee et al., 2008; Kumar et al., 2013), and amygdala (Diaz et al., 2014). These impairments can lead to negative effects on motor control, behavior, cognition, decision making throughout the lifespan (Kodituwakku, 2007).

Neocortical pyramidal neurons of layer 2/3 (L2/3 PNs) play a key role for cortical operations (LaBerge, 2006) and their dendritic spines, which are the main postsynaptic sites of excitatory connections, are fundamental to perform PNs functions (Spruston, 2008). Dendritic spines are distributed through the whole dendritic tree and anomalies of their morphology, distribution, or density may underlie several functional alterations (DeFelipe and Farinas, 1992; Matsuzaki et al., 2004), thus accounting for the neurocognitive disability observed in FASD (Cui et al., 2010).

In the present study, we investigate the density and distribution of dendritic spines in L2/3 PNs of rats exposed to alcohol during the first postnatal week, a developmental period corresponding to the brain growth spurt and to the third trimester of gestation in humans (Dobbing and Sands, 1979; Valenzuela et al., 2012).

2. Materials and methods

All the experiments were conducted in accordance with the Society for Neuroscience Policies on the use of animals and humans in neuroscience research.

Newborn Wistar rats were given ethanol by inhalation from the second through the sixth postnatal day (P2-P6; P0 is the birth date).

Abbreviations: ANCOVA, analysis of covariance; bAP, backpropagating action potential; BDA, biotinylated dextran amine; C, control group; Ca^{2+}, calcium; Et, ethanol group; FASD, fetal alcohol spectrum disorders; L2/3, cortical layers 2 and 3; L5, cortical layer 5; NMDA, *N*-methyl-D-aspartic acid; NND, nearest neighbor distance; P0, birthday; P2, second postnatal day; P6, sixth postnatal day; PNs, neocortical pyramidal neurons; VGCC, voltage gated calcium channels.

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In previous experimental studies (Granato and Van Pelt, 2003; Granato et al., 2003), we have demonstrated that pyramidal neurons are affected by alcohol exposure during this developmental window and that the detrimental outcome on their dendritic trees is permanent and well recognizable during adulthood (i.e., at P90). Two litters (ten pups each) underwent the alcohol exposure procedure (Et group). An air pump (air flow = 3 l/min) was connected to a vaporization chamber kept at the constant temperature of 38 °C. Ethanol (95% v/v) was injected into the vaporization chamber at a rate of 2.5 ml/min. The ethanol vapors were conveyed to a plexiglass cage in which the pups were placed, after separation from the mothers, for 3 h a day. A small hole (5 mm diameter) on one side of the cage and a fan allowed the air circulation inside the cage, where the temperature was kept at 22–24 °C. Two additional litters (ten pups each) served as control (C) group. These animals were separated from the mothers and placed in the plexiglass cage for 3 h a day, but the exposure to alcohol vapors was omitted. Although in principle there might be a synergy between the effects of alcohol and those of maternal separation, we decided not to perform experiments on additional groups (i.e., animals not separated from the mothers). In fact, in previous experiments dealing with the electrophysiological properties of pyramidal neurons we observed no significant interaction between the two factors (alcohol treatment and maternal separation; Granato et al., 2012).

At P90, four male rats from the Et group (two from each litter) and four male rats from the C group (two from each litter) received cortical injections of a solution containing 10% biotinylated dextran amine (BDA) and 10 mM *N*-methyl-D-aspartic acid (NMDA) in 0.01 M phosphate buffer, pH 7.4. Animals were deeply anaesthetized (ketamine 90 mg/kg, xylazine 10 mg/kg i.p.) and placed on a stereotaxic frame. Injections of BDA–NMDA were made using glass micropipettes coupled to the a pneumatic picopump and were aimed at the sensorimotor cortex of the right side (1.20–0.70 mm anterior to the bregma; 2–4 mm lateral to the midline). Delivered volumes ranged from 0.1 to 0.2 μl, divided into two or three penetrations.

After a survival time of 96 h, animals were deeply anaesthetized and transcardially perfused with phosphate buffered saline followed by 4% buffered paraformaldehyde. Brains were removed, cryoprotected in 30% buffered sucrose, and cut on a freezing microtome into 50 μm thick coronal sections. Associative cortico-cortical neurons retrogradely labeled with BDA were evidenced by incubating sections in avidin–biotin complex (1:100), using the diaminobenzidine as chromogen and a nickel intensification.

The sections were observed under a Nikon E600 light microscope and PNs were chosen for analysis when most of their dendritic tree was visible (basal dendrites, apical dendrite including the tuft, and oblique collaterals; see Fig. 1). The observer (ADG) was blind respect to the treatment group. One animal of the Et group was discarded, due to poor retrograde labeling. The chosen neurons were reconstructed with Neurolucida (MBF Bioscience, Williston, VT) and the dendritic spines were marked as 1, 2, . . ., *n*, according to the order of the dendritc branch they were emerging from. Altogether, 15 neurons from the C group (4 neurons from 3 animals and 3 from the fourth animal) and 18 neurons from the Et group (6 neurons from each animal) were reconstructed. For the quantitative analysis, the spine density was calculated separately for basal, apical, and oblique dendrites. Data were pooled for the same branch order within the same neurons (for instance, the spine density of second-order basal dendrites of each neuron was pooled, regardless of whether they belong to different primary basal dendrites). Among the 33 reconstructed neurons, only those displaying labeling of 4, 3, and 2 dendritic branch orders were used for the quantitative analysis of basal, apical, and oblique dendrites, respectively.

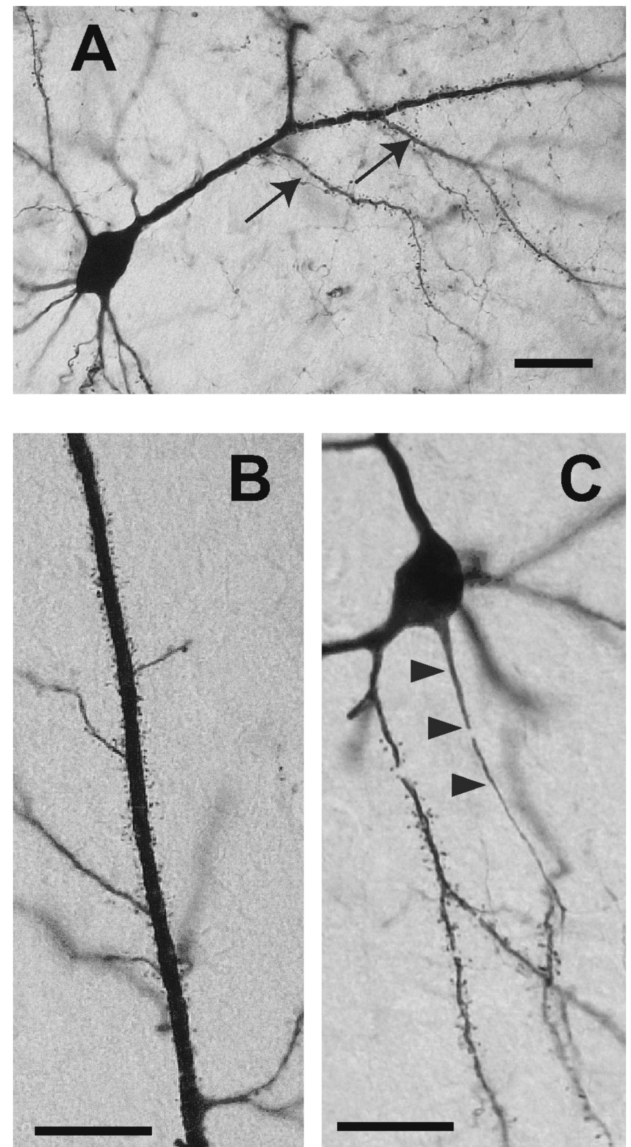

Fig. 1. Dendritic trees and spines of layer 2/3 pyramidal neurons labeled by retrograde transport of BDA–NMDA. A. Soma, basal, and apical dendritic arbor of a L2/3 PN from a control case. Arrows indicate the oblique collateral branches emerging from the main apical trunk. B. Principal trunk of the apical dendrite. C. Soma and basal dendrites. Spines on 3rd and 4th order branches are visible. Arrowheads indicate the initial segment of the axon. Scale bars = 25 μm.

The statistical analysis was performed using the ANOVA for mixed design, with the dendritic order as within-subjects factor and the treatment (alcohol vs. control) as between-subjects factor.

3. Results

We have used a tracing strategy based on the simultaneous cortical injection of the retrograde tracer BDA and of the glutamate receptor agonist NMDA (Jiang et al., 1993). Due to the activity-dependent enhancement of tracer uptake, retrogradely labeled cells are filled in a Golgi-like fashion. Therefore, the dendritic morphology of neurons with known projections and their spines can be studied in detail (Minciacchi et al., 2010).

In all the cortico-cortical neurons considered for the quantitative analysis, the retrograde filling of dendritic shafts and spines was nearly complete in the branches of all orders (Figs. 1 and 2).

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