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Chronic ad libitum ethanol exposure impairs corticolimbic and cerebellar structural neuroplasticity in rats

Claudia Rebeca Mendoza [1,2], Leonardo Aguilar-Hernández [1], David J. Apam-Castillejos [1], Andrea Judith Vázquez-Hernández [1], Alfonso Díaz [1], Hiram Tendilla-Beltrán [3], Gonzalo Flores [1]* and Fidel de la Cruz-López [2]*

[1] Laboratorio de Neuropsiquiatría, Instituto de Fisiología, Benemérita Universidad Autónoma de Puebla (BUAP), Puebla, Mexico.
[2] Escuela Nacional de Ciencias Biológicas (ENCB), Instituto Politécnico Nacional (IPN), Mexico City, Mexico.
[3] Centro de Investigación en Reproducción Animal, Cinvestav-Universidad Autónoma de Tlaxcala, Tlaxcala, Mexico.
* Correspondence: gonzaloflores56@gmail.com; Tel.: 2222295500 ext 7322
flacruz90@gmail.com; Tel.: 5557296000 ext 52333

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Abstract: Consequences of chronic ethanol exposure on cognitive and motor functions are widely studied due to the neurodegeneration that ethanol produces in the cerebellum and other brain areas, including some corticolimbic regions. However, there is scarce information about the structural neuroplasticity effects of chronic ethanol exposure that ultimately lead to characteristic neurodegenerative consequences. For this purpose, we evaluated the effects of chronic ethanol exposure in adult male rats on exploratory behavior (locomotor activity induced by a novel environment) and structural neuroplasticity in corticolimbic and cerebellar neurons. After 90 days of *ad libitum* ethanol (10%) exposure, the locomotor behavior of the animals did not differ from that of the control group (exposed to water). Structural neuroplasticity was assessed using the Golgi-Cox technique in neurons from corticolimbic areas and the cerebellum. The findings revealed that ethanol exposure induced basilar dendritic atrophy without modifying the dendritic spine density in pyramidal cells in prefrontal cortex layers 3 and 5, the CA1 region of the dorsal hippocampus, and the basolateral amygdala. In contrast, ethanol exposure hypotrophied the dendritic arbor of Purkinje cells and reduced the density of dendritic spines in these cells. These data contribute to the knowledge of the neuroplasticity-related mechanisms underlying the neurodegenerative consequences of chronic ethanol exposure and its cognitive implications.

Keywords: Dendritic spines; Pyramidal neurons; Purkinje cells; Corticolimbic; Alcohol use disorder

1.0 INTRODUCTION

Ethanol is an ancient psychostimulant widely used in societies worldwide. When abused, it can lead to a chronic consumption disorder described as alcohol use disorder (AUD). According to the World Health Organization (WHO), AUD is a causal factor for numerous diseases, including mental issues and non-communicable diseases like liver cirrhosis and certain cancers (Roerecke & Rehm, 2014). One of the most well-documented organs damaged in AUD is the brain, as ethanol easily crosses the blood-brain barrier (BBB) and impacts various neurotransmission systems (glutamate, GABA, dopamine, serotonin, acetylcholine, etc.), leading to cognitive and motor dysfunctions (Rao & Topiwala, 2020). It is worth mentioning that alcohol has a biphasic effect: at low doses (blood alcohol concentration, BAC = <100 mg/dL), it has a psychostimulant effect, and there are even reports of benefits from its consumption. However, at high doses (toxic doses, BAC = >300 mg/dL), it is considered a depressant (Tizabi et al., 2018).

The main mechanism of action of ethanol in the brain is due to its GABAergic agonism. When it acts on $GABA_A$ receptors in the cerebellum and amygdala, it results in motor incoordination, anxiolytic effects, and changes in mood (Roberto et al., 2012; Rossi & Richardson, 2018). $GABA_A$ receptor activity produces Cl^- ion influx, hyperpolarizing nerve cells, and reducing excitability, explaining the anxiolytic effect (Davies, 2003). These effects are evident in GABAergic structures such as the cerebellum, in which impaired function and atrophy are common in AUD patients, and underlie the neurobiology of motor dysfunction in the disorder (Mitoma et al., 2021). But beyond the cerebellum, other brain structures can be structurally and functionally damaged in AUD, as the GABAergic ethanol effects can impair excitatory neurotransmission, mainly through the alteration of inhibitory interneuron activity in cortical areas (Hughes et al., 2020; Joffe et al., 2021).

The mechanism of action of ethanol is complex, as it alters different proteins and receptors. Ethanol inhibits the function of the NMDA glutamatergic receptor at both low and high doses. On the other hand, ethanol is an agonist of glycine receptors, acetylcholine receptors (nAChR), and serotonin 5-HT-3 receptors. The latter, although known as an excitatory receptor, is usually expressed in GABAergic interneurons. Therefore, its activation by ethanol contributes to the well-known inhibitory actions of alcohol at high doses (Vengeliene et al., 2008).

Preclinical (Dahchour & De Witte, 2003) and clinical studies (Tsai et al., 1998) have demonstrated that the excitatory/inhibitory imbalance in corticolimbic regions contributes to dependence, tolerance, and withdrawal symptoms due to ethanol exposure. Moreover, both in AUD patients and rodents, ethanol exposure increases NMDA receptor expression in multiple regions, including the hippocampus and cerebellum (den Hartog et al., 2017; Hoffman & Tabakoff, 1994; Mira et al., 2019), which also contributes to the excitatory/inhibitory imbalance beyond its GABAergic mechanisms. Also, glutamatergic activity leads to excitotoxicity, causing neuronal death, and ultimately to atrophy or volume reduction in multiple brain areas, which has been reported in AUD patients (Daviet et al., 2022; Fama et al., 2021; Mechtcheriakov et al., 2007; Sullivan & Pfefferbaum, 2023).

One study revealed that, after chronic intermittent ethanol exposure, the neurons of layer 5 prefrontal cortex (PFC) exhibit impaired excitability without a modification in dendritic spine density (Kroener et al., 2012). This is interesting because dendritic spines represent the main site of postsynaptic glutamatergic neurotransmission, with more than 90% of this phenomenon occurring in these structures. Moreover, the PFC has reciprocal glutamatergic connections with the hippocampus and the basolateral amygdala (BLA), and this circuit plays a role in limbic-related functions such as decision-making, learning, memory, and stimulus/reward-directed behavior (Reyes-Lizaola et al., 2024). All of the aforementioned behaviors are impaired in people with AUD (Bailey et al., 2018; Logge et al., 2023), making the study of ethanol effects on the structural neuroplasticity of corticolimbic regions relevant.

In this study, the effects of chronic *ad libitum* ethanol exposure on behavior, specifically locomotor activity, as well as on the structural neuroplasticity of pyramidal neurons in the corticolimbic system, including the PFC, the hippocampus, the BLA, and Purkinje cells in the cerebellum, were evaluated.

2.0 MATERIALS AND METHODS

2.1 Experimental design

Male Sprague-Dawley rats, 60 days of age (considering birth as day 0) and weighing between 250–300 g at the beginning of the experiment, were obtained from the Claude Bernard Animal Facility at Benemérita Universidad Autónoma de Puebla. They were housed in a controlled environment at 18–23°C and 50–60% humidity, with 12-hour light/dark cycles (lights on at

8:00 am), and were kept in acrylic cages with free access to Lab Diet MR 5008 food. All protocols described in this study were approved by the Institutional Animal Care Committee (100235377-UALVIEP-24/1) and adhered to the technical specifications for the Production, Care, and Use of Laboratory Animals in Mexico (NOM-062-ZOO-1999) and the ARRIVE (Animal Research: Reporting of *in vivo* Experiments) guidelines.

Two experimental groups of 10 animals were formed and categorized as the control group (with *ad libitum* access to water) and the ethanol group (with *ad libitum* access to 10% ethanol in water, diluted from 96% ethanol) for 90 days. The dose and duration of ethanol exposure were selected based on several considerations.

First, multiple studies have reported behavioral disturbances, including cognitive impairment, following chronic exposure (more than six weeks in adults) to 10% ethanol in rodents (Eisenhardt et al., 2015; Martinez et al., 2016; Mormede, 2004). Second, a 10% ethanol solution is considered a low dose due to its biochemical effects on hepatic and mitochondrial function, as well as on the mesolimbic dopaminergic pathway (Aguiar et al., 2009; Jin et al., 2023; Lograno et al., 1993; Puzziferri et al., 2000). Third, this concentration is capable of producing a BAC in the range of 80–100 mg/dL in voluntary consumption protocols (Fadda et al., 1999). Finally, rats chronically exposed to 10% ethanol as a sole source of fluid (Aguiar et al., 2009) exhibit structural changes in different brain areas that may be associated with deficits in various cognitive and behavioral functions (Han et al., 2020). Furthermore, animals were weighed before the ethanol exposure period (control group: 244 ± 13.5 g; chronic ethanol exposure group: 267 ± 15.8 g) and at the end of the 90-day intake period (control group: 355 ± 19 g; chronic ethanol exposure group: 383 ± 17 g). Following the exposure period, behavioral assessments were conducted, and brain samples were collected (**Figure 1A**).

2.2 Behavioral test

Twenty-four hours after the 90-day exposure to either ethanol or water, the animals' locomotor activity in response to a novel environment was evaluated. For this purpose, individual cages (20 x 40 x 30 cm) equipped with 8 pairs of photo-beam detectors on the lateral walls and connected to a computer counter (Tecnología Digital, Mexico) were used. Each rat was placed in an activity cage for 60 minutes, and the number of counts (photo-beam interruptions) was recorded (Morales-Medina et al., 2008; Tendilla-Beltrán et al., 2016). Immediately after the end of the behavioral test, the rats were euthanized with a sodium pentobarbital overdose (75 mg/kg, i.p.), underwent intracardiac perfusion with a saline solution, and the brains were extracted.

2.3 Golgi-Cox stain

The extracted brains were placed in Golgi-Cox solution and stored in darkness for 30 days. After this period, the Golgi-Cox solution was replaced with a 30% sucrose solution for seven days before brain sectioning. Coronal brain tissue sections and sagittal cerebellar sections (200 µm thickness) were made using a motorized manual vibratome (Campden Instrument, MA752, Leicester, UK). The sections were mounted on slides that had been coated with 2% gelatin. Subsequently, the tissues were hydrated and revealed according to the following procedure: the slides were dipped in ammonium hydroxide (50% in water) for 30 minutes, rinsed with distilled water for 1 minute, dipped in Kodak Rapid Fixer (1:3 in water) for 30 minutes, rinsed with distilled water for 1 minute, dehydrated with increasing concentrations of ethanol (70% for 1 minute, 95% for 1 minute, 100% for 5 minutes twice), and then cleared in xylene for 15 minutes. Finally, the slides were mounted with synthetic resin for microscopy (Coatl-Cuaya et al., 2022; Gibb & Kolb, 1998; Silva-Gómez et al., 2003).

2.4 Neuronal and dendrite reconstructions

Neuronal and dendrite reconstructions were conducted based on the references from "The Rat Brain in Stereotaxic Coordinates" atlas (Paxinos and Watson, 1998). Pyramidal neurons from the medial prefrontal cortex (mPFC) layers 3 and 5, the basolateral amygdala (BLA), and the CA1 region of the dorsal hippocampus, as well as Purkinje cells from the cerebellar region, were identified in the animals. Ten isolated and well-impregnated neurons from each region (5 from each hemisphere) were traced for each brain using an optical microscope attached to a camera lucida (40X). Additionally, a portion of the distal dendrite (with a minimum length of 30 µm) was traced from the same neurons, with a magnification of 100X for dendritic spine quantification (Alcantara-Gonzalez et al., 2010; Silva-Gómez et al., 2003; Tendilla-Beltrán et al., 2016).

2.5 Sholl analysis and dendritic spine quantification

For each neuronal reconstruction, each dendrite's order was differentiated by color, labeled as first (emerging from the soma), second (bifurcated from the first), third, and up to the 'n' order. Differentiated neuronal reconstructions were analyzed using the Sholl method

(Sholl, 1953), employing a template of concentric circles spaced 10 µm apart. The intersections between dendrites and circles were then counted, and 3 morphometric parameters were estimated: the complexity of the dendritic arbor from soma (dendritic arbor length by its distance to the soma), the length per dendritic order, and the total dendritic length (Silva-Gómez et al., 2003; Tendilla-Beltrán et al., 2016). Additionally, to determine the density of dendritic spines, in the reproduced dendritic segments, the average number of dendritic spines within three segments of 10 µm each was calculated (Flores et al., 2005; Tendilla-Beltrán et al., 2019).

2.6 Statistical analysis

The data are expressed as mean ± SEM, and individual values are shown in the figures. The data on total locomotor activity, total dendritic length, and dendritic spine density were analyzed with Student t-test. Data on locomotor activity for every 10 minutes were analyzed using two-way repeated-measures ANOVA, with periods and ethanol exposure as independent variables. Data on arborization and length per dendritic order were analyzed using two-way ANOVA, with distance to soma and dendritic order, respectively, as independent factors, depending on the case. In some cases, ethanol exposure was considered the other independent factor. If a significant interaction was detected, a Bonferroni test for post hoc analysis was performed. All results were considered significant at a p-value of <0.05. These analyses were conducted using the GraphPad PRISM 9.0 software.

3.0 RESULTS

3.1 Chronic ethanol exposure did not modify exploratory activity in the open field test

After 90 days of ethanol exposure, locomotor activity in response to a novel environment was evaluated in the animals. Animals chronically exposed to ethanol did not exhibit differences in their locomotion behavior at any of the 10-minute intervals analyzed compared with the control group (time x ethanol: $F(5,90) = 1.133$, $p=0.3489$; **Figure 1B**). Additionally, when total locomotor activity over 60 minutes was analyzed, there was no difference between groups ($t(18) = 0.7917$, $p=0.4388$; **Figure 1C**).

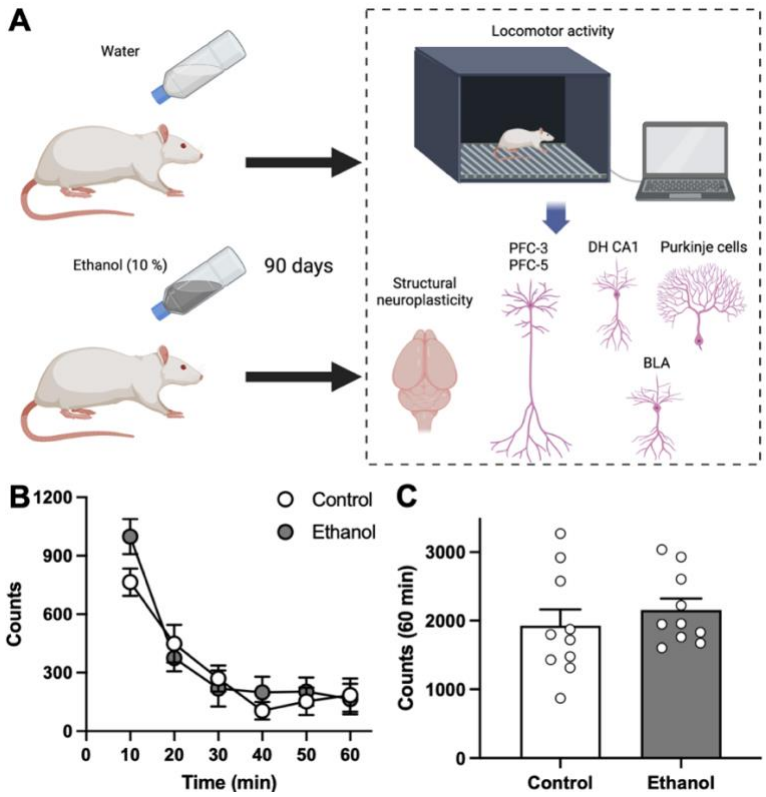

Figure 1: Experimental design (A) and behavioral tests (B-C). **(B)** Temporal profile: during each of the 6 periods of 10 minutes, the number of counts did not change between control and ethanol-exposed rats. **(C)** Accumulated activity: the total number of movements during the 60 minutes of the test did not change between groups. *BLA=basolateral amygdala; DH CA1=CA1 region of the dorsal hippocampus; PFC-3=prefrontal cortex layer 3; PFC-5=prefrontal cortex layer 5. Panel A of the figure was created with BioRender.com.*

3.2 Chronic ethanol exposure leads to atrophy of the basilar dendritic arbor in mPFC pyramidal neurons without affecting the dendritic spine density

Some samples were excluded because the staining quality did not allow for a clear observation of the neural structures of interest, especially the dendritic spines. Only the brains in which 10 neurons were found from each of the regions of interest with the aforementioned characteristics, were included in the study. Because of this, the sample size for structural neuroplasticity assessments is n = 5–6 animals per group. Chronic ethanol exposure produced similar effects in the pyramidal neurons of PFC layers 3 and 5, reducing the length of the basilar dendritic arbor without affecting the dendritic spine density.

Regarding mPFC neurons, in layer 3 (**Figure 2A-B**), ethanol reduced the arborization of the neurons in distal segments: from 90 to 160 µm from the soma (ethanol x distance to soma: $F(29,270) = 2.492$, $p<0.0001$; post hoc: $p<0.05$; **Figure 2C**). Furthermore, ethanol reduced the length of dendritic orders as a main effect (ethanol x dendritic order: $F(7,72) = 1.204$, $p=0.3120$; ethanol: $F(1,72) = 15.08$, $p=0.0002$; **Figure 2D**). This was confirmed by the reduction of the total dendritic length, as animals chronically exposed to ethanol showed a decrease in this parameter compared to control rats ($t(9) = 2.809$, $p=0.0204$; **Figure 2E**). However, ethanol consumption did not modify the dendritic spine density in distal segments of these neurons ($t(9) = 0.1898$, $p=0.8537$; **Figure 2F**). In mPFC layer 5 pyramidal neurons (**Figure 2G-H**), the chronic ethanol exposure reduced the basilar arborization in both proximal and distal segments: from 50 to 180 µm from the soma (ethanol x distance to soma: $F(29,270) = 10.23$, $p<0.0001$, post hoc: $p<0.05$; **Figure 2I**). Moreover, ethanol reduced the length of the third dendritic order in these neurons (ethanol x dendritic order: $F(7,72) = 6.594$, $p<0.0001$, post hoc: $p<0.001$; **Figure 2J**). Also, ethanol reduced the total dendritic length of the basilar arbor of these cells ($t(9) = 5.208$, $p=0.0006$; **Figure 2K**), without affecting the dendritic spine density ($t(9) = 0.4147$, $p=0.6881$; **Figure 2L**).

3.3 Chronic ethanol exposure causes basilar dendritic arbor atrophy in dorsal hippocampus pyramidal neurons without affecting dendritic spine density

In the CA1 region of the dorsal hippocampus (**Figure 3A-B**), chronic ethanol exposure drastically reduced the arborization of the pyramidal cells almost throughout the entire basilar portion: from 30 to 190 µm from the soma (ethanol x distance to soma: $F(29,270) = 11.90$, $p<0.0001$, post hoc: $p<0.05$; ethanol: $F(1,270) = 516.6$, $p<0.0001$; **Figure 3C**). Additionally, ethanol reduced the length of the fourth and fifth dendritic orders in these cells (ethanol x dendritic order: $F(8,81) = 4.600$, $p=0.0001$, post hoc: $p<0.0001$; ethanol: $F(1,81) = 36.71$, $p<0.0001$; **Figure 3D**). The reduction in arborization and dendritic order lengths impacted the total dendritic length (basilar portion) of these neurons by reducing it in comparison with the control group ($t(9) = 15.70$, $p<0.0001$; **Figure 3E**). Interestingly, dendritic spine density remains unchanged due to ethanol exposure in these cells ($t(9) = 0.8408$, $p=0.4223$; **Figure 3F**), consistent with findings in the PFC pyramidal neurons.

3.4 Chronic ethanol exposure leads to atrophy of the basilar dendritic arbor in BLA pyramidal cells without affecting dendritic spine density

In BLA pyramidal cells (**Figure 3G-H**), chronic ethanol exposure induced a general reduction of the basilar dendritic arbor of these neurons from 30 to 170 µm from the soma (ethanol x distance to soma: $F(29,240) = 5.694$, $p<0.0001$, post hoc: $p<0.05$; ethanol: $F(1,240) = 258.9$, $p<0.0001$; **Figure 3I**). Additionally, ethanol reduced the length of the third dendritic order (ethanol x dendritic order: $F(8,72) = 2.942$, $p=0.0067$, post hoc: $p<0.0001$; ethanol: $F(1,72) = 18.92$, $p<0.0001$; **Figure 3J**). When the total dendritic length of the basilar arbor of these neurons was analyzed, ethanol was found to reduce it ($t(8) = 3.566$, $p=0.0073$; **Figure 3K**). Finally, as found in the PFC and hippocampus, chronic ethanol exposure did not modify the dendritic spine density in the distal basilar dendrites of the BLA pyramidal neurons ($t(8) = 0.7932$, $p=0.4506$; **Figure 3L**).

3.5 Chronic ethanol exposure leads to dendritic arbor atrophy and a reduction in the dendritic spine density in the cerebellar Purkinje cells

In cerebellar Purkinje cells (**Figure 4A-B**), chronic ethanol exposure led to a reduction in arborization in specific segments of the neurons (ethanol x distance to soma: $F(34,280) = 2.553$, $p<0.0001$; ethanol: $F(1,280) = 166.7$, $p<0.0001$; **Figure 4C**), including segments from 70 to 90 µm and from 130 to 170 µm from the soma (post hoc: $p<0.05$). The analysis per dendritic order revealed a reduction in the length of distal orders from the thirteenth to the twentieth (ethanol x dendritic order: $F(29,240) = 4.443$, $p<0.0001$, post hoc: $p<0.05$; ethanol: $F(1,240) = 82.75$, $p<0.0001$; **Figure 4D**). Moreover, ethanol reduced the total dendritic length of these neurons in comparison with the control group ($t(8) = 3.796$, $p=0.0053$; **Figure 4E**). Notably, in these GABAergic cells, chronic ethanol exposure induced a reduction in the dendritic spine density in the distal dendrites ($t(8) = 5.562$, $p=0.0005$; **Figure 4F**).

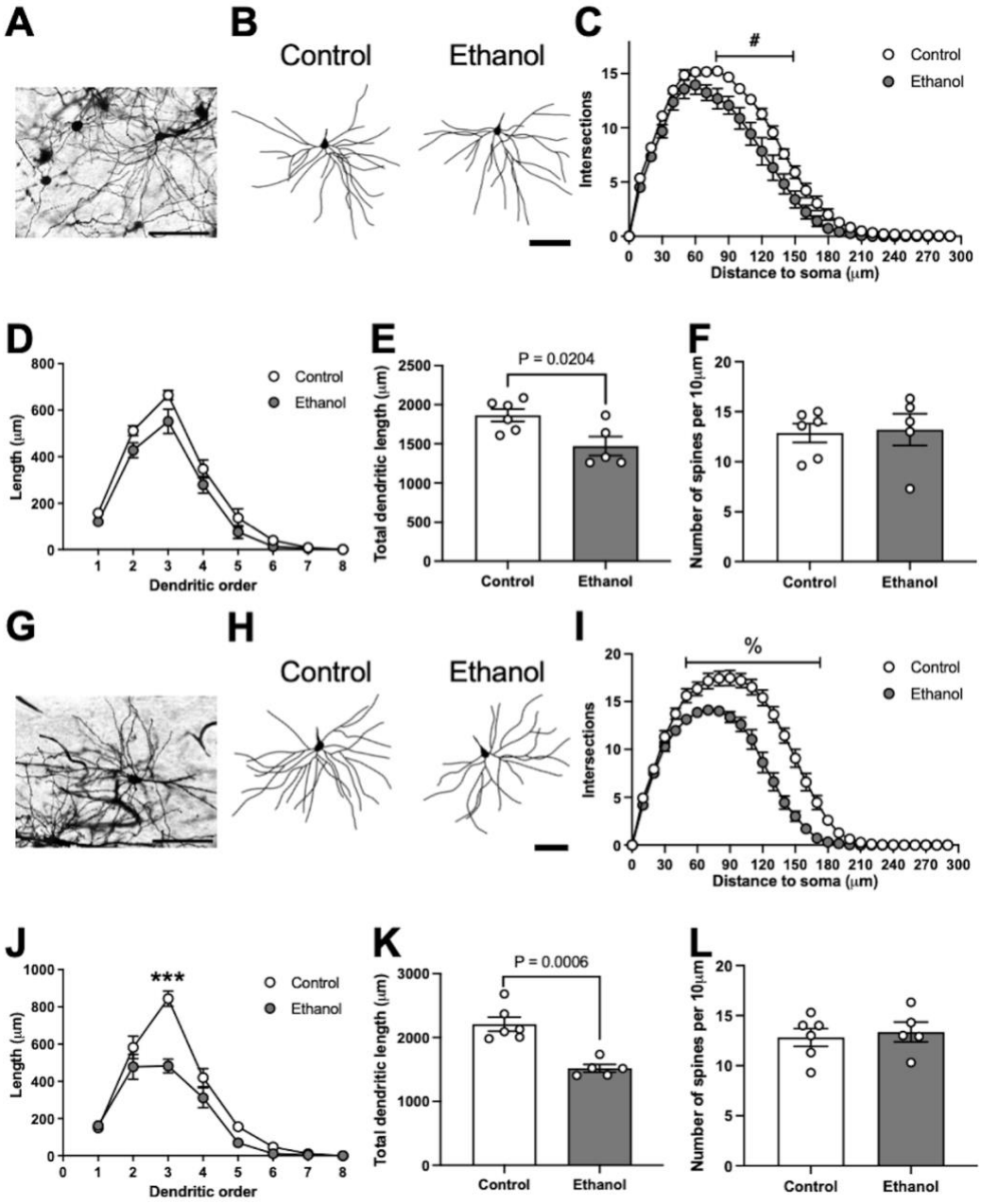

Figure 2: Structural neuroplasticity of the mPFC pyramidal neurons in layer 3 (A-F) and layer 5 (G-L). (A) Photomicrograph of mPFC layer 3 pyramidal neuron. **(B)** Representative neuronal reconstructions of mPFC layer 3 pyramidal neurons of control and ethanol groups (scale bar = 100 µm). Temporal profile. **(C)** Arborization. Ethanol reduced the arborization of these neurons in distal segments, from 90 to 160 µm from the soma [ethanol x distance to soma: $F(29,270) = 2.492$, $p<0.0001$; post hoc: #$p<0.05$ for 90 and 160 µm, $p<0.01$ for 90, 100, and 150 µm, $p<0.001$ for 120 and 140 µm, $p<0.0001$ for 130 µm]. **(D)** Length per dendritic order. Ethanol reduced the length of dendritic orders as a main effect [ethanol $F(1,72) = 15.08$, $p=0.0002$]. **(E)** Total dendritic length. In animals chronically exposed to ethanol, the total dendritic length of these neurons was reduced compared to the control rats [$t(9) = 2.809$, $p=0.0204$]. **(F)** Dendritic spine density. Ethanol consumption did not modify the number of dendritic spines in distal segments of these neurons. **(G)** Photomicrograph of mPFC layer 5 pyramidal neuron**. (H)** Representative neuronal reconstructions of mPFC layer 5 pyramidal neurons of control and ethanol groups (scale bar = 100 µm). Temporal profile. **(I)** Arborization. The chronic ethanol exposure reduced the basilar arborization in both proximal and distal segments, from 50 to 180 µm from the soma [ethanol x distance to soma: $F(29,270) = 10.23$, $p<0.0001$; post hoc: %$p<0.05$ for 50 and 60 µm, $p<0.01$ for 70 µm, $p<0.001$ for 80 µm, $p<0.0001$ for 90-180 µm]. **(J)** Length per dendritic order. Ethanol reduced the length of the third dendritic order in these neurons [ethanol x dendritic order: $F(7,72) = 6.594$, $p<0.0001$, post hoc: ***$p<0.001$]. **(K)** Total dendritic length. Ethanol reduced the total dendritic length of the basilar arbor of these cells [$t(9) = 5.208$, $p=0.0006$]. **(L)** Dendritic spine density. Ethanol consumption did not modify the number of dendritic spines in distal segments of these neurons.

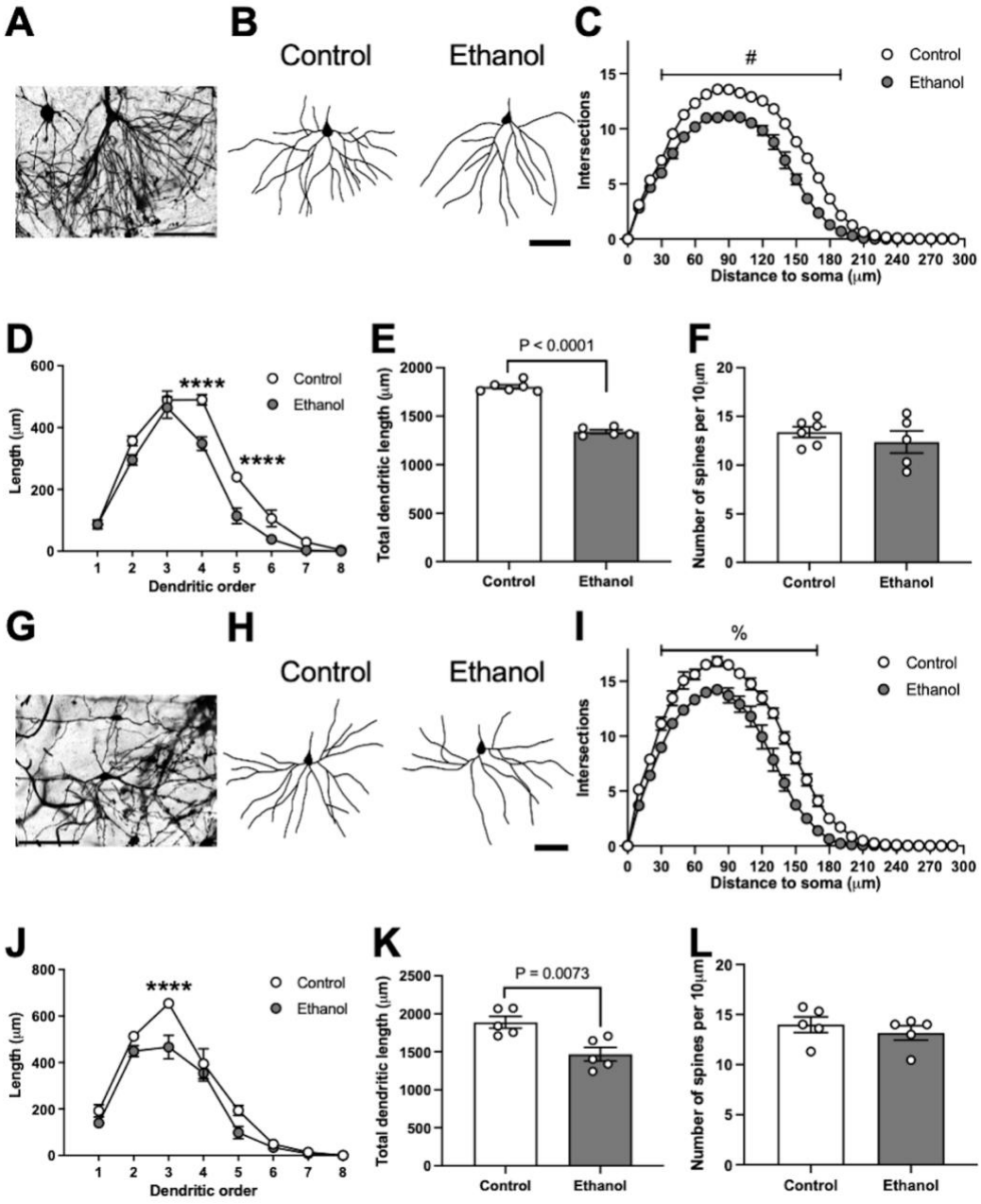

Figure 3: Structural neuroplasticity of the CA1 dorsal hippocampus (A-F) and BLA pyramidal neurons (G-L). (A) Photomicrograph of CA1 dorsal hippocampus pyramidal neuron. **(B)** Representative neuronal reconstructions of CA1 dorsal hippocampus pyramidal neurons of control and ethanol groups (scale bar = 100 μm). Temporal profile. **(C)** Arborization. Chronic ethanol exposure reduced the arborization of the pyramidal cells almost throughout the entire basilar portion: from 30 to 190 μm from the soma [ethanol x distance to soma: $F(29,270) = 11.90$, $p<0.0001$; #$p<0.05$ for 30 μm, $p<0.01$ for 190 μm, $p<0.0001$ for 40-180 μm]. **(D)** Length per dendritic order. Ethanol reduced the length of the fourth and fifth dendritic orders in these cells [ethanol x dendritic order: $F(8,81) = 4.600$, $p=0.0001$, post hoc: ****$p<0.0001$]. **(E)** Total dendritic length. In the ethanol-exposed rats, the total dendritic length (basilar portion) of these neurons was reduced compared to the control group [$t(9) = 15.70$, $p<0.0001$]. **(F)** Dendritic spine density. Ethanol consumption did not modify the number of dendritic spines in distal segments of these neurons. **(G)** Photomicrograph of BLA pyramidal neuron. **(H)** Representative neuronal reconstructions of BLA pyramidal neurons of control and ethanol groups (scale bar = 100 μm). Temporal profile. **(I)** Arborization. Chronic ethanol exposure induced a general reduction of the basilar dendritic arbor of these neurons, from 30 to 170 μm from the soma: [ethanol x distance to soma: $F(29,240) = 5.694$, $p<0.0001$; post hoc: %$p<0.05$ for 30 μm, $p<0.01$ for 40, 60, 70 μm, $p<0.001$ for 50, 80-100, 170 μm, $p<0.0001$ for 100-160 μm]. **(J)** Length per dendritic order. Ethanol reduced the length of the third dendritic order [ethanol x dendritic order: $F(8,72) = 2.942$, $p=0.0067$; post hoc: ****$p<0.0001$]. **(K)** Total dendritic length. Ethanol reduced he total dendritic length of the basilar arbor of these neurons [$t(8) = 3.566$, $p=0.0073$]. **(L)** Dendritic spine density. Ethanol consumption did not modify the number of dendritic spines in distal segments of these neurons.

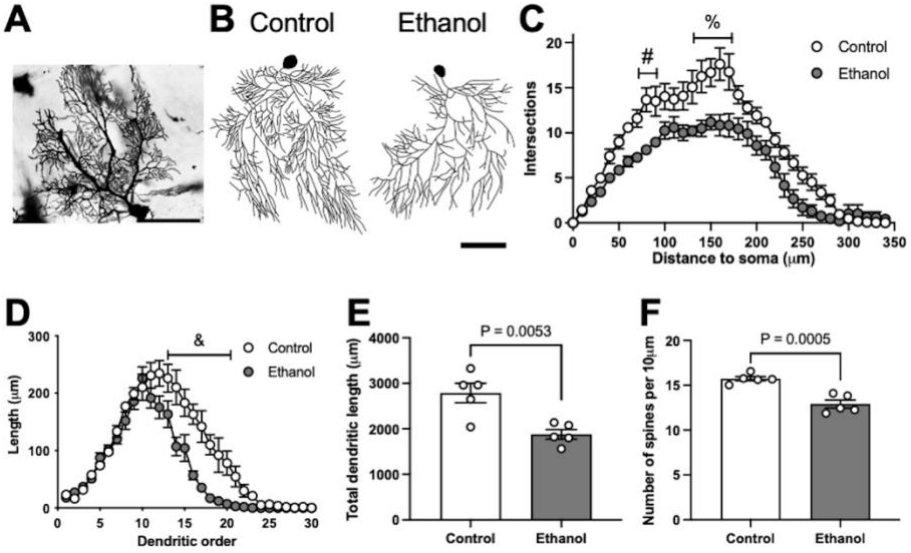

Figure 4: Structural neuroplasticity of cerebellar Purkinje cells. (A) Photomicrograph of cerebellar Purkinje cells. **(B)** Representative neuronal reconstructions of cerebellar Purkinje cells of control and ethanol groups (scale bar = 100 μm). Temporal profile. **(C)** Arborization. Chronic ethanol exposure led to a reduction in arborization in specific segments of these neurons [ethanol x distance to soma: $F(34,280) = 2.553$, $p<0.0001$; post hoc: #$p<0.05$ for 70 and 90 μm, $p<0.01$ for 80 μm; %$p<0.01$ for 130, 150 μm, $p<0.001$ for 140 and 170 μm, $p<0.0001$ for 160 μm]. **(D)** Length per dendritic order. Ethanol reduced the length of distal orders from the thirteenth to the twentieth [ethanol x dendritic order: $F(8,81) = 4.600$, $p=0.0001$; post hoc: &$p<0.05$ for 13th order, $p<0.01$ for 15th and 20th orders, $p<0.001$ for 19th order, $p<0.001$ for 14th, 16th-18th orders]. **(E)** Total dendritic length. Ethanol reduced the total dendritic length of these neurons compared to the control group [$t(8) = 3.796$, $p=0.0053$]. **(F)** Dendritic spine density. Chronic ethanol exposure induced a reduction in the number of dendritic spines in the distal dendrites [$t(8) = 5.562$, $p=0.0005$].

4.0 DISCUSSION

In this research, it was demonstrated that 90 days of *ad libitum* ethanol exposure did not impair exploratory behavior in male adult rats. However, chronic ethanol exposure led to impairments in structural neuroplasticity, including reductions in the dendritic arbor complexity and length of the basilar arbor in pyramidal neurons of the PFC, dorsal hippocampus, and BLA. Interestingly, the dendritic spine density in these neurons remained unaffected by ethanol. Another studied region was the cerebellum, where ethanol reduced both the dendritic arbor length and the dendritic spine density in Purkinje cells.

Exploring a novel environment is an innate behavior in rodents. A common measure to assess exploratory behavior is locomotor activity in response to novelty, as it reflects aspects of cognitive function (McGregor et al., 2020; Pisula & Siegel, 2005; Whishaw et al., 2006). In the present study, we found that chronic ethanol exposure did not alter the number of movements at any 10-minute interval during the evaluation period, nor did it affect the total number of movements over the 60-minute test. In chronic 10% ethanol exposure protocols involving voluntary consumption for 40 days (Mendoza et al., 2024) and 84 days (Peng et al., 2024), animals did not show changes in locomotor activity, which is consistent with our findings. Similarly, in ethanol-preferring animals, long-term consumption of 10% ethanol for 7 months (210 days) did not alter the distance traveled in open-field tests, a measure comparable to locomotor activity (Xu et al., 2021). Notably, although locomotor activity remained unchanged in all these protocols, ethanol exposure did affect other behavioral domains, such as pain perception, motor coordination, recognition memory (Xu et al., 2021), and anxiety-like behaviors (Mendoza et al., 2024; Peng et al., 2024), in alignment with our results. However, the absence of more detailed analyses, such as grooming behavior and motor coordination testing, as well as memory evaluation, is a limitation of the present study.

Regarding structural neuroplasticity in mPFC neurons, animals exposed to ethanol exhibited a reduction in the complexity and length of the basilar dendritic arbor. This finding is consistent with previous reports (Díaz et al., 2016; Kroener et al., 2012; Lawson et al., 2022). Interestingly, chronic ethanol exposure did not alter the density of basilar dendritic spines in these neurons,

which aligns with a previous study showing that five days of ethanol exposure (20% v/v in water) reduced the dendritic complexity in the basilar, but not apical, arbor of layer 3 mPFC neurons in mice subjected to fear conditioning and extinction (Lawson et al., 2022). In that same study, however, ethanol reduced dendritic spine density in mPFC neurons, an effect not observed in our data. This discrepancy may be due to differences in the timing or context of ethanol administration. Due to its mechanism of action as a $GABA_A$ receptor agonist, ethanol can affect multiple brain regions, including those of the corticolimbic system. Notably, increased activity in the prelimbic cortex following ethanol exposure may lead to reduced activity in the ventral tegmental area and, consequently, a decrease in the dopaminergic signaling through the mesocortical pathway (Ferreira et al., 2021). This could favor glutamatergic activity and help preserve spine density. Nonetheless, further studies are required to confirm this hypothesis.

Memory impairments are common in patients with chronic AUD and are often attributed to the dysfunction of multiple brain areas, including the hippocampus (Sullivan & Pfefferbaum, 2023). The lack of a memory performance evaluation in the present study is a limitation, rendering this aspect of the discussion plausible but ultimately speculative. In our analysis, the hippocampal CA1 pyramidal neurons of ethanol-exposed rats exhibited reduced basilar arbor complexity and length compared to the control group. These alterations may be linked to the cellular effects of ethanol on glutamatergic neurotransmission, which inhibits NMDA receptors and impairs long-term potentiation (LTP) (Ramachandran et al., 2015; Tizabi et al., 2018). NMDA receptor activity influences neuronal morphology via brain-derived neurotrophic factor (BDNF), a key regulator of synaptic plasticity (Afonso et al., 2019). BDNF exerts its effects through the receptor tyrosine kinase type B (TrkB), activating signaling pathways that promote neurotrophic functions such as survival, growth, and differentiation through transcription factor activation (Pradhan et al., 2019). In the hippocampus, BDNF facilitates the induction and maintenance of LTP, suggesting a crucial role in ethanol-induced neuroplastic changes. Regarding the density of dendritic spines, no changes were observed between groups. However, previous studies have shown that chronic ethanol exposure can transiently reduce dendritic spine activity and density in CA1 pyramidal neurons (Gass & Olive, 2012).

We also analyzed the BLA, a region crucial for reward-based decision-making, spatial memory, and the formation of reward-related memories through its connections with the PFC and the hippocampus (Yang & Wang, 2017; Yizhar & Klavir, 2018). Evidence suggests that chronic ethanol consumption disrupts glutamatergic projections from the PFC. Nevertheless, pyramidal neurons in the BLA appear to maintain their basic synaptic firing and membrane properties (Crofton et al., 2022). Consistent with these findings, we observed a reduction in the complexity and length of BLA pyramidal neurons, but no change in dendritic spine density. These results align with studies in pubertal and adult mice exposed to chronic intermittent ethanol, which reported no change in the number of dendritic spines but observed alterations in the proportion of spine types (Jury et al., 2017). This may reflect a compensatory mechanism for reduced dendritic length, but further research is necessary to explore this possibility.

Ethanol also enhances GABAergic activity at both pre- and postsynaptic levels in BLA neurons, through a mechanism involving cannabinoid type 1 (CB1) receptors (Varodayan et al., 2017). *Ex vivo* studies in rats show that ethanol increases the activation of GABAergic interneurons in the BLA, likely through a rapid, local neuroinflammatory response (Munshi et al., 2023). In line with this, studies in mice have shown that ethanol consumption increases the number of hyperreactive astrocytes in the BLA (Brewton et al., 2023). Neuroinflammation, in turn, has been associated with reductions in dendritic length (LaFever et al., 2022; Tamakoshi et al., 2020; Tendilla-Beltrán et al., 2019). Thus, the dendritic shortening observed in our study may result from both inflammatory processes and increased inhibitory transmission induced by ethanol.

Finally, we examined the impact of chronic ethanol exposure on the structural neuroplasticity of cerebellar Purkinje cells. The cerebellum is a key target in alcohol research due to its role in motor control and its susceptibility to alcohol-induced neurodegeneration (García-Dolores et al., 2025; Luo, 2015). We found that ethanol exposure caused significant atrophy in Purkinje cells, both in dendritic structure and at the spine level. Given that Purkinje cells are GABAergic (Llinás & Sugimori, 1992), this may explain their heightened vulnerability to ethanol's effects. As previously reported, cerebellar damage leads to motor impairments (Luo, 2015). In our study, we did not observe changes in overall locomotor activity between ethanol-exposed and control rats. However, this test

primarily reflects exploratory behavior and may mask motor deficits, especially since novelty and ethanol-induced craving can increase locomotion (Acevedo et al., 2013; Donaire et al., 2018). This addresses the study's limitation related to the lack of motor coordination assessment in the animals. Since the 1970s, ethanol has been shown to impair the electrophysiological properties of Purkinje cells (George & Chu, 1984), and cause reductions in rough endoplasmic reticulum, dendrites, and soma volume (Lewandowska et al., 1994), neuronal loss (Northup, 1976), and dendritic arbor atrophy (Tavares et al., 1983). The latter is consistent with our results. These effects may stem from ethanol's GABAergic agonism, resulting in sustained hyperpolarization, as well as from ethanol-induced neuroinflammation. Specifically, ethanol activates NF-kB and TLR4 pathways, contributing to structural damage (Rossetto et al., 2021). Interestingly, caffeine appears to mitigate ethanol-induced inflammation in the cerebellum via adenosine receptor-related mechanisms (Rossetto et al., 2021). Further studies should investigate the role of these receptors in ethanol-induced neuroplasticity across brain regions.

It is important to acknowledge certain limitations of this study. Chief among them is the exclusive use of male rats, as numerous reports have demonstrated sex-related differences in behavioral and neurochemical responses to ethanol (McElroy et al., 2023; Pirino et al., 2022; Vetter-O'Hagen et al., 2009). Additionally, we were unable to measure ethanol consumption or BAC, nor behaviors such as rearing frequency, grooming, memory performance, or motor coordination. These limitations restrict our ability to fully characterize the impact of ethanol exposure on cognitive and behavioral function.

5.0 CONCLUSIONS

In conclusion, chronic ethanol exposure induced basilar dendritic atrophy without affecting spine density in pyramidal neurons of layers 3 and 5 of the mPFC, the CA1 region of the dorsal hippocampus, and the BLA. In contrast, Purkinje cells exhibited marked dendritic hypotrophy accompanied by a reduction in distal dendritic spine density. These findings provide important insights into neuroplasticity-related mechanisms underlying the neurodegenerative effects of chronic ethanol exposure and their potential cognitive implications.

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