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Research paper

Chronic benzodiazepine treatment decreases spine density in cortical pyramidal neurons

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HIGHLIGHTS

- Remodeling of excitatory neurons in parallel to change in GABAergic neurotransmission.
- Not known whether benzodiazepines modify structure of pyramidal neurons.
- Chronic treatment (21 days) with diazepam.
- Treated mice showed decrease in spine density in cingulate cortex pyramidal neurons

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ABSTRACT

The adult brain retains a substantial capacity for synaptic reorganization, which includes a wide range of modifications from molecular to structural plasticity. Previous reports have demonstrated that the structural remodeling of excitatory neurons seems to occur in parallel to changes in GABAergic neurotransmission. The function of neuronal inhibitory networks can be modified through GABAA receptors, which have a binding site for benzodiazepines (BZ). Although BZs are among the most prescribed drugs, is not known whether they modify the structure and connectivity of pyramidal neurons. In the present study we wish to elucidate the impact of a chronic treatment of 21 days with diazepam (2 mg/kg, ip), a BZ that acts as an agonist of GABAA receptors, on the structural plasticity of pyramidal neurons in the prefrontal cortex of adult mice. We have examined the density of dendritic spines and the density of axonal *en passant* boutons in the cingulate cortex. Although no significant changes were observed in their anxiety levels, animals treated with diazepam showed a decrease in the density of spines in the apical dendrites of pyramidal neurons. Most GFP-expressing *en passant* boutons in the upper layers of the cingulate cortex had an extracortical origin and no changes in their density were detected after diazepam treatment. These results indicate that the chronic potentiation of GABAergic synapses can induce the structural remodeling of postsynaptic elements in pyramidal neurons.

1. Introduction

Different regions of the adult CNS, retain a remarkable capacity for synaptic reorganization, which is dependent on experiences [3] and includes a wide range of modifications: from those affecting the molecular machinery to those involving changes in the structure of neurons and their contacts [31]. The structural plasticity of dendritic branches and axonal boutons contributes to the remodeling of specific functional circuits. Different cortical regions undergo plasticity during adulthood, specially in response to aversive experiences such as chronic stress [17,24] or after antidepressant treatment [8]. These structural changes have been extensively described in pyramidal cells, involving the extension/retraction of dendrites and changes in the density of dendritic spines [27,8]. The dynamics and density of dendritic spines is also modulated by local neuronal activity [16]. Similar structural reorganizations can be observed at the presynaptic level, specially in *en passant* boutons, protuberances along an axon, which frequently constitute synaptic contacts. Changes in the density of these axonal boutons are also connected to the level of activity of pyramidal cells [32].

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Alterations in cortical inhibitory networks have a strong impact on the physiology of principal neurons and recent evidence suggests that they may also have an important effect on the structure of these excitatory neurons. However, a direct evidence of this effect is still lacking. Recent evidence from our laboratory suggests that changes in the expression of the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), a molecule related to neuronal structural plasticity, which expression is restricted to mature neocortical interneurons [29,7] have a strong impact on the structure and neurotransmission of cortical inhibitory networks [21]. Interestingly, treatments that increase PSA-NCAM expression, such as chronic fluoxetine administration, also increase the spine density in the apical dendrites of cortical pyramidal neurons [28,8], suggesting that alterations in inhibitory networks may in turn affect the morphology of pyramidal neurons. Moreover, previous studies have indicated that changes in inhibition seem to occur prior to changes in excitatory connections, suggesting a possible role for interneurons in the induction of plasticity in excitatory circuits. In studies focused on sensory deprivation, after a focal retinal lesion, the density of inhibitory neuron spines in the visual cortex decreases, presumably causing a loss of glutamatergic input to these cells. This may result in a reduced level of inhibition, which in turn may trigger a cascade of plastic changes that eventually lead to structural plasticity of excitatory cells [12].

A tempting strategy to increase our knowledge on this matter is to act directly on the GABAA receptors. The benzodiazepines (BZs) are allosteric GABAA receptor agonists which have potent anxiolytic properties [18,19]. Although BZs are among the most prescribed drugs due to their anxiolytic, sedative-hypnotic, anticonvulsant and muscle relaxant effects, it is not known whether they have an effect on the structure and connectivity of cortical pyramidal neurons. In order to evaluate directly the impact of an increased inhibition on the structure of pyramidal neurons, we have subjected mice to a chronic treatment with diazepam (2 mg/kg, ip for 21 days). This treatment **is** directed to determine structural changes, independently of whether it induces anxiolytic effects. The density of dendritic spines in the apical dendrites of pyramidal neurons and the density of axonal *en passant* boutons were examined in the cingulate cortex by confocal microscopy, using a strain of transgenic mice with fluorescent pyramidal neurons, which allows detailed analysis of their morphology.

2. Material and methods

2.1. Animals

Fourteen B6.Cg-Tg (Thy1-YFPH) adult male mice (3 months old, 26.8 ± 5 g) purchased from Jackson laboratories and bred in our animal facility, were used to carry out the chronic benzodiazepine treatment. Additionally, 4 control mice were used for the study of the phenotype of *en passant* boutons. These mice express spectral variants of GFP (yellow-YFP) at high levels in motor and sensory neurons, as well as in subsets of central pyramidal neurons [22]. Animals were maintained under standard conditions of temperature and humidity, with food and water *ad libitum* and normal light/dark cycle (lights on: 8:00–20:00). All animal experimentation was conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee on Bioethics of the Universitat de València (2014/040/UVEG/004). Every effort was made to minimize the number of animals used and their suffering.

2.2. Animal housing

Animals were separated into the experimental groups ($n = 7$ diazepam; $n = 7$ saline) and housed in standard cages of three or four animals per cage. Animals used for the phenotype study of *en passant* boutons were housed in standard cages ($n = 4$). Mice were allowed to habituate to the new cages and conspecifics at least one week prior to the start of the experiments.

2.3. Chronic drug treatment with diazepam

A light-dark test [2] was conducted during the light phase in the day before the start of the drug treatment, in order to study the initial anxiety level of mice and to homogeneously assign them in the different experimental groups. Behavior measurements were the time spent (in seconds) in the light compartment relative to that spent in the dark compartment [26]. All procedures were studied using a video tracking system designed to automate testing in behavioral experiments (Stoelting Any-Maze, USA).

Mice were injected intraperitoneally (i.p) daily with a non sedative dose of diazepam, 2 mg/kg (Sigma–Aldrich, St. Louis, MO) or with vehicle (saline) for 21 days at 9:30–10:00 a.m. Compounds were prepared per day as solutions in physiological saline containing a drop of Tween 80 (0.1%) and sonicated to enhance dissolution. Mice were weighed before each injection to receive the appropriate dose (0.2 mg/ml).

2.4. Open field test

The open field test was developed for the study of emotionality [9] and consists in subjecting an animal to an unknown environment from which escape is prevented by surrounding walls [30]. The test was performed during the light phase, 24 h after the last injection of diazepam. Each mouse was placed gently into the center of the enclosure for 10 min of free exploration. This test was used to study the anxiety level after chronic diazepam treatment. To assess the spatial organization of locomotor activity, the following parameters were recorded: total distance, mean speed, total mobile time, total immobile time, line crossings, immobility latency, freezing episodes, entries to periphery or center zone, latency to first entry, periphery time mobile, periphery time immobile, periphery time freezing, center time mobile, center time immobile and center time freezing. Locomotor activity and anxiety level of mice was recorded and analyzed using a video tracking system and software (Stoelting Any-Maze, USA).

2.5. Perfusion and microtomy techniques

After the last animal was tested in the open field, mice were perfused transcardially (in the same order as in the behavior test) under deep sodium pentobarbital anesthesia (150 mg/kg) with 0.9% saline for three minutes and then for twenty-five minutes with 4% paraformaldehyde in phosphate buffer (PB) 0.1 M, ph 7.4. Control animals destined for the phenotyping of *en passant* boutons were processed identically, without being subjected to behavioral tests. Mice were decapitated and heads were stored for thirty minutes at 4 °C. Then, brains were extracted and the two hemispheres separated. To study the density of *en passant* boutons and of dendritic spines, the right hemisphere of each animal was cut in coronal 100-μm thick sections using a vibratome (Leica VT 1000E). Slices were collected in three subseries and stored at 4 °C in PB 0.1 M with sodium azide (0.05%). To study the phenotype of *en passant* boutons the brains were frozen and cryoprotected in 30% sucrose in PB 0.1 M. Then, coronal sections (50 μm) were obtained with a freezing-sliding microtome (Leica SM2000R), collected in 10 sub-