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Gain in gut *Bifidobacteria* improves hippocampal plasticity and cognitive behavior in adult healthy rats

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Author Contributions Statement

T.G, B.F and M.M.C wrote the main manuscript text, prepared figures and performed experiments; L.V., P.C., B.L., Saolini E., and P.R., performed the experiments; Sanna E and B.G designed the experiments and contribute to the final revision of the manuscript.

Abstract

Nowadays, gut microbiota inspires a greatest interest in the field of neuroscience depending on its role in normal physiology and involvement in brain function. This aspect suggests a specific gut-brain pathway, mainly modulated by gut microbiota activity. Among the multiple actions controlled by microbiota at brain level, neuronal plasticity and cognitive function represent two of the most interesting aspects of this cross talk communication. We addressed the possible action of the two months implement of gut *Bifidobacteria* using a mixture of three different strains (B-MIX) on hippocampal plasticity and related cognitive behavior in adult healthy Sprague Dawley rats. B-MIX treatment increases the hippocampal BDNF with a parallel gain in dendritic spines density of hippocampal CA1 pyramidal neurons. Electrophysiological experiments revealed a significant increment of HFS-induced LTP formation on CA1 hippocampal region in B-MIX treated rats. All these effects are accompanied by a better cognitive performance observed in B-MIX treated animals with no impairments in locomotion activity. Therefore, in adult rats, improving the microbiota composition, markedly enhances neuronal plasticity and the function of CNS influencing the cognitive behavior, an effect that may suggest a potential therapeutic treatment in brain diseases associated to cognitive functions.

Introduction

Progress in the field of neuroscience regarding the tight link between the physiology of gut microbiota and its influence on human health, has triggered a huge interest with an increasing clear role of this system in controlling the brain function and behavior in both rodents and humans (Hoban et al., 2016; Mayer et al., 2014; Foster and Neufeld, 2013; Cryan and Dinan, 2012; Collins et al., 2012). In this respect, modifications of gut function and its microbiota composition have been implicated in stress-related disorders such as depression and anxiety, as well as in neurodevelopmental diseases and deficits in cognitive

function (Mayer et al., 2014; Cryan and Dinan, 2012; Rogers et al., 2016; Hsiao et al., 2011; Gareau et al., 2011). To further elucidate the tight relation between gut microbiota and brain function various paradigms, such as germ-free animals, animals with bacterial infections, as well as animals exposed to probiotics have been studied (Cryan and O'Mahony, 2011). Evaluate this aspect may be possible since recent evidences had and are actually proving that the human and rodent microbiota share similar strain of bacteria (Avolio et al 2019; Colica et al., 2017). In addition, the gastrointestinal tract from humans and rodents presents a high grade of similarities including the anatomy and the strains microbiota composition (Nguyen et al., 2015). It has been found that the relative abundances of most of the dominant genera in mouse and human are quite different while Clostridium, Bacteroides and Blautia show a similar relative abundance in both organisms.

Some findings suggested that in rats with impairments in learning and memory, or affected by secondary to diabetes mellitus, the administration of probiotics may restore the functional and molecular alterations in hippocampal LTP (Davari et al., 2013). Other reports in rodents have shown that treatment with a specific strain of *Bifidobacterium* or *Lactobacillus*, may have a positive impact on stress-related changes on gene expression of GABAA receptor subunits, as well as cognitive processes and emotional behavior (Savignac et al., 2015; Bravo et al., 2011).

Neurotrophins such as BDNF may play a pivotal role in regulation of neuronal development and function (Huang and Reichardt, 2001) linked with the activity of gut microbiota, a mechanism that control different forms of long-term plasticity, such as long term potentiation (LTP) and long term depression (LTD), mainly reported in the hippocampal formation (Peters et al., 2018; Panja and Bramham, 2014). In addition, other studies in mice have shown that germ-free animals have a significant decrease in BDNF expression both in the cortex and hippocampus (Gareau et al., 2011; Sudo et al., 2004) and a reduced expression of the NR2B subunit of the N-methyl-D-aspartate (NMDA) receptor (Sudo et al., 2004). Moreover, in the same

areas of rat brain, treatment with probiotics positively regulated the amount of BDNF (Liang et al., 2015; Bercik et al., 2011).

Given that BDNF is also involved in dendritic spine formation and in neuronal growth (Huang and Reichard, 2001), the effect of treatment with a probiotic mixture was investigated on certain forms of synaptic plasticity such as LTP (Distrutti et al., 2014), that is a common marker of brain plasticity that results reduced in aged animals (Rosenzweig and Barnes, 2003). Accordingly, Distrutti and colleagues (Distrutti et al., 2014) have recently suggested that an aged-dependent alteration of gut microbiota is linked with a parallel decrease of BDNF and hippocampal plasticity that could be rescued by probiotics treatment. Interestingly, treatment with different strains of *Bifidobacteria* were also efficacious in counteracting the decreased levels of BDNF observed in patients with schizophrenia and bipolar disorder (Tomasik et al., 2015; Angelucci et al., 2005).

On the basis of these evidences, we evaluated the putative effect on hippocampal plasticity and cognitive behavior elicited by two months chronic treatment with a mixture of three common Bifidobacteria (B. longum, B. breve, B. infants) in the human gastrointestinal tract (B-MIX so called by our convention), since a supportive literature suggests the beneficial effects of these tree strain of bifidobacteria (Toscano et al., 2017; Kobayashi et al., 2017; Wang et al., 2019), that was administered intragastrically to Sprague Dawley adult rats. A Using advanced biomolecular, immunological, electrophysiological and behavioral techniques, we studied the effect of microbiota supplementation on the structural and functional changes of hippocampus, a brain area that plays a crucial role in the control of cognitive behavior both in rodent and humans (Shohamy and Turk-Browne, 2013).

Materials and Methods

Animals

Adult male Sprague Dawley rats (200 gr) (Charles River, Como, Italy) were maintained under an artificial light (12h on-12 h off), constant temperature of 22° ± 2°C, and humidity of 65%. Animal care and handling throughout the experimental procedures were in accordance with the guidelines for care and use of experimental animals of the European Communities Council (2010/63/UE L 276 20/10/2010) and with Italian law (DL: 04.03.2014, N° 26). The experimental protocols were also approved by the Animal Ethics Committee of the University of Cagliari. Furthermore, every effort was made to minimize suffering and reduce the numbers of animals used.

B-MIX treatment

In order to allow an optimal gastric absorption of the B-MIX, rats were subjected to a 4-hour fast before and 1-hour after the treatment. Before and after the treatment rats may consume *ad libitum* normal rodent laboratory chow. Animals were treated with gavage once a day (from 12.00 to 13.00) for 4 weeks. The mix of Bifidobacteria (*B. longum, B. breve, B. infants*) (VALEAS, Milano, IT), was freshly prepared every day from the single bacteria strains (stored at 4°C) and was dissolved in tap water right before the treatment (2 ml/kg) and given at $5x10^9$ CFU/kg of body mass. Proportion of different strains of Bifidobacteria was: *B. breve*, M-16V, $1x10^9$ CFU/kg; *B. longum*, BB536, $3x10^9$ CFU/kg; *B. infants*, M-63, $1x10^9$ CFU/kg. Control animals (CTRL) were treated with a vehicle solution (tap water).

Immunoblot analysis

After sacrifice the brain was rapidly removed from the skull, and the hippocampus was dissected on ice. Total protein was extracted from the tissue with the use of a kit (Membrane and Cytoplasmic Protein Extraction kit, Bio Basic Inc., Markham Ontario, Canada), and the protein concentration of the extract was determined with the use of a DC Protein Assay kit (Bio-Rad, Milan, Italy). For the immunoblot 40 µg of proteins were used in SDS-polyacrylamide gel electrophoresis (NuPAGE Novex 4–12% Bis-Tris Midi Gel, Life Technologies, Monza, Italy). The separated proteins were transferred to a

polyvinylidene difluoride membrane (Immobilon-P; Millipore, Milan, Italy) with the use of a Criterion Blotter (Bio-Rad). The membrane was exposed to 5% nonfat dried milk in Tris-buffered saline containing 1% Tween 20 (TBS-T) before an overnight incubation at 4 °C with rabbit polyclonal antibodies to BDNF (1:400) (Santa Cruz Biotechnology, Heidelberg, Germany) and mouse monoclonal antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000) (Millipore); all antibodies were diluted in TBS-T containing 5% nonfat dried milk. The membrane was then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat secondary antibodies (anti-rabbit, Sigma, Milan, Italy; anti-mouse, Millipore, Milan, Italy) in TBS containing 5% non-fat dried milk and 0.5% Tween 20. An ECL kit (Millipore, Little Chalfont, UK) was used for detection of immune complex. The intensity of immunoreactive bands was quantitated by Geliance 600 Chemi Imaging System (Perkin Elmer, Monza, Italy) and analyzed by the GeneTools software (Perkin Elmer). Values of optical density (OD) for BDNF factors were normalized by the corresponding GAPDH values.

Golgi-Cox staining and dendritic spines morphology

A modified Golgi-Cox protocol was used to process the tissue. Rats (n° = 5 per group) were deeply anaesthetized (4μl x gr of body mass) with a mix of: 1 g sodium pentobarbital, 4.251 g chloral hydrate, 2.125 g $MgSO_4$, 12 ml EtOH, 43.6 ml propylene glycol, adjusted to a total volume of 100 ml with distilled water, and after sacrifice the brains were removed and sectioned by a brain slicer at 5 mm in coronal plane. The slices were subjected to Golgi-Cox staining and post-impregantion of neurons as per the manufacturer's instructions (SuperGolgi kit, Bioenno Tech.). Tissue slices were blocked, super-glued to a metal chuck, and were cut at 100 µm by a vibratome (VT1000S, Leica Microsystems, Wetzlar, Germany) into a water bath. Slices were placed on slides and allowed to dry. The slides were subjected a staining and post-staining as per the manufacturer's instructions. They were next run

through dehydrating series of alcohols and xylenes and coverslipped with Eukitt (Sigma, Milan, Italy).

Image analysis, spine quantification and morphology

The serial sections were chosen and acquired by a microscope motorized x, y and z focus (AxioObserver Z.1, Zeiss, Germany) fitted with a high resolution cooled CCD camera and Axiovision Rec 4.8 software, for high-resolution image acquisition and digital quantitation. In combination with a 100X objective, using a sophisticated and well-established method (Dumitriu et al., 2010), this should represent a 3D quantitative profile of the neurons sampled and prevent a failure to detect less prominent spines. Neurons in the dentate gyrus region which fulfil the following criteria were selected for the analysis: (i) the cells type must be identifiable; (ii) image resolution should be sufficient to visually distinguish dendritic spine formation from variably contrasting background, and (iii) completeness of Golgi impregnation of all dendrites. Neurons with incomplete impregnation or neurons with truncations due to the plane of sectioning were not analyzed.

To ensure standardization of dendritic regions, only branch segments from neurons entering into molecular layer of dentate gyrus region were used in the present analysis. A software package (Axiovision, Zeiss) was used to record the number of distinct spines in the z-stacks (step size 0,5 μm 100X objectives).

Analysis of dendritic spines density and morphology were performed using a semi-automated software NeuronStudio (http://research. mssm.edu/cnic/tools-ns.html), which analyzes in 3D dendritic length, spine density, and morphometric features (i.e., head/neck diameter, length, subtype) for each dendritic spine. Dendritic spine density was calculated as number of spines per 10 μm unit length of dendrite measured Spines were classified as thin or mushroom if the ratio of the head diameter-to-neck diameter was >1.1. If their ratio exceeded this value, spines with a maximum head diameter >0.4 μm were classified as

mushroom or else were classified as thin. Spines with head-to-neck diameter ratios <1.1 were also classified as thin if the ratio of spine length-to-neck diameter was >2.5; otherwise, they were classified as stubby. A fourth category, filopodial spines, was considered to have a long and thin shape with no enlargement at the distal tip, was very seldom observed, and was classified as thin.

All dendritic segments selected for spine density estimations were of similar diameter (~ 1 μm) to minimize the effect of hidden spines above or below the dendrites. Five dendritic branch (15 μm in length) were analyzed in each neuron, and from 5 to 7 neurons for each slice were analized. Spine counts were averaged and spine density was expressed as the number of total spines or spine subtypes per 10 μm of dendrite length.

Preparation of hippocampal brain slices

Coronal brain slices containing the hippocampal region were prepared as previously described (Talani et al., 2016). Briefly, after deep anesthesia with isoflurane, brains were rapidly removed from the skull and transferred to modified artificial cerebrospinal fluid (ACSF) containing (in mM): 220 sucrose, 2 KCl, 0.2 $CaCl_2$, 6 $MgSO_4$, 26 $NaHCO_3$, 1.3 NaH_2PO_4, and 10 D-glucose (pH 7.4, set by aeration with 95% O_2 and 5% CO_2). Coronal brain slices (thickness of 250 or 300 μm) containing were cutted using a vibratome and then immediately transferred to a nylon net submerged in standard ACSF containing (in mM): 126 NaCl, 3 KCl, 2 CaCl2, 1 MgCl2, 26 NaHCO3, 1.25 NaH2PO4, and 10 D-glucose (pH 7.4, set by aeration with 95% O2 and 5% CO2) for at least 40 min at a controlled temperature of 35°C. After subsequent incubation for at least 1 h at room temperature hemislices were transferred to the recording chamber with a constant flow rate of ~2 ml/min of ACSF at controlled temperature of 33°C.

Electrophysiology experiments

Extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) were performed in the stratum radiatum of the CA1 hippocampal region through stimulation of the Schaffer collateral afferents from CA3 as previously described (Sanna et al., 2011; Talani et al., 2011). Responses were triggered digitally every 20 s by application of a constant current pulse of 0.2–0.4 mA with a duration of 60 μs, which yielded a half-maximal response. For I/O an increasing (from 0 to 1 mA, Δ 0.1 mA) stimulating current pulse was given and fEPSP slope was calculated. For induction of LTP, after a 10 min of stable baseline a high frequency stimulation (100 stimuli @ 250 Hz) was applied, and slope of fEPSP was recorded for 1 h and averaged between 50 and 60 min after HFS.

Open field test

Six rats were used for each group. On the test day, rats were brought into the test room under standardized environmental conditions (dim light) and acclimated for 1 h before the beginning of the experiment. PVC cages consisted of a 41×41×30 cm were equipped with two sets of photocells located both at the bottom of both sides and at 20 cm upper the box floor, in order to measure horizontal and vertical movement respectively. Vrious animal activity were acquired with a recorder (Omnitech Electronics, Inc.). Recordings were performed every 5 min over a period of 30 min.

Barnes maze

A set of animals (5 per group) were subjected to Barnes maze in order to test their spatial learning and memory performance as reported previously (Talani et al., 2011; Rosenfeld and Ferguson, 2014). Animals were placed in the middle of a circular platform with 40 equally spaced holes (platform: 122 cm of diameter; holes: 5 cm diameter; 5 cm between each hole). Only one of the holes was connected to a dark chamber called the “target”. Animals were driven to find this box by being exposed to a bright light (200 W, 1400 lumen) fixed 50 cm up of the maze, using 4 different cue panels. The acquisition phase takes

4 days, consisting of a single trial of 3 min. The time needed to identify the target hole (latency) and number of errors to find the target box were thus recorded. The short-term retention on day 5 and long-term retention on day 12 were measured.

Novel Object Recognition (NOR)

One day before the test each animal (5 rats per group) was placed in the PVC arena (50 x 50 x 50 cm) in order to acclimate with the environmental condition. The day of the test, two identical objects (Obj1 and Obj2) were placed in a side into the arena during a 5min sample phase (familiarization). After a 6 h delay each animal was returned to the arena and was exposed to the two object but one of those was exchanged by a new one with different shape and color (familiar and novel) for one single session of 3 min (test) (modified by Bolz et al. 2015). Memory was assessed by comparing the time spent exploring the novel object and the time spent exploring the familiar object. We calculate the recognition index as the percentage time spent to each object compared to the total time spent in objects exploration (Time Obj1 or 2/(Time Obj1+Obj2 x 100) and the Discrimination index (Time Novel / Time familiar + Time novel) that may vary between +1 and -1, indicating more time spent with the novel object (score +1) or more time spent with the familiar one (score -1) or even null preference between objects (score 0).

Statistical analysis

Data are presented as mean ± SEM and compared by one or two-way analysis of variance (ANOVA) and Bonferroni's test or Student's *t* test with the use of Prism software (version 7, Graphpad). A p value of < 0.05 was considered statistically significant.

Results

Change in hippocampal BDNF protein expression in B-MIX treated rats

Since neurotrophins such as BDNF play a pivotal role in neuronal development and function and regulation of different forms of long-term plasticity, such as long term potentiation (LTP) and long term depression (LTD), we firstly evaluated by immunoblot analysis the effect of two months treatment with B-MIX on hippocampal BDNF expression in adult rats. As reported in Fig. 1, B-MIX treatment significantly enhanced BDNF protein expression in the whole hippocampus (unpaired t-Test, $p = 0.04$ vs CTRL, $n = 11\text{-}14$) when compared with levels detected in CTRL animals.

The density of dendritic spines is increased in hippocampal CA1 pyramidal neurons of B-MIX treated rats

Given the crucial involvement of BDNF in the modulating the number and morphology of dendritic spines (von Bohlen Und Halbach and von Bohlen Und Halbach, 2018), we evaluated whether chronic B-MIX administration was also able to modify the dendritic spine density and morphology in the CA1 pyramidal neurons. Using a Golgi-cox staining, we found a significant ($p < 0.001$) increase in total spine density, as calculated in 10 μm sections of both basal and apical dendritic branches of CA1 pyramidal neurons of B-MIX treated rats when compared to vehicle treated animals (Fig. 2A, B, D, E). In a more detailed analysis we divided the visualized spines in three different type based on their different shape: stubby (like a dendritic bulge), thin (long neck and thin head) and mushroom (short neck and big head). As reported in figure 2, treatment with B-MIX leads to a significant ($p < 0.05$) increase of both thin ($p < 0.001$) and mushroom ($p < 0.05$) spines in both apical and basal dendritic branches wile stubby spines resulted unaffected by the treatment (Fig. 2C, F).

B-MIX affects long-term synaptic plasticity in CA1 pyramidal neurons

The increase in both BDNF expression and CA1 dendritic spines density may be predictive of a possible impact of B-MIX treatment on neuronal excitability as well as long-

term plasticity of glutamatergic synapses in the hippocampus. To evaluate this hypothesis, we first recorded dendritic fEPSPs in the CA1 field and applied an input-output paradigm (I–O) to construct a related curve by stimulating the Schaffer's collateral afferents with increasing current intensity (from 0 to 1.0 mA). From the normalized corresponding I–O curves, calculating the stimulation able to evoke the half of the maximal response, we observe no significant difference between B-MIX treated and controls rats (Fig. 3A). Two-way ANOVA confirms a significant effect of stimulation in both experimental groups [$F(10,260) = 247.41$, $p < 0.0001$, $n = 13$] (Fig. 3A) but no difference between I/O curves slope was detected on the stimulation at half response [$F(1,26) = 0.001$, 0.974] (Fig. 3A). To get a more detailed calculation of this parameter, the half of maximal response was calculated in every single recording. Graph on Fig. 3B confirms that no difference was detected between groups (t-Test, $p > 0.05$ vs CTRL). Subsequently, LTP was induced in the CA1 region by HFS that was delivered to the Schaffer's collateral afferents after 10 min of stable baseline. The magnitude of the resulted LTP, calculated by averaging the slope value of fEPSPs recorded during the last 10 min (ie, from 50 to 60 min post HFS), was significantly greater in hippocampal slices obtained from B-MIX treated rats (t-Test, $p < 0.05$ vs CTRL, $n = 13$) (Fig. 3C, D) when compared with that in CTRLs, suggesting a positive effect of B-MIX treatment on the function of hippocampal outputs.

Effect of B-MIX on locomotor behavior in rats.

Given that gut microbiota significantly influences different stress-related behavior (Foster et al., 2017), we used the open field test to evaluate anxiety- and exploratory-related behavior in rats treated with B-MIX. As shown in Fig. 4, chronic administration of B-MIX failed to change all parameters related to locomotion behavior. However, a difference ($p < 0.05$ vs CTRL, $n = 6$) in vertical activity and stereotypical counts has been observed during the last 30 min and the first 5 min in B-MIX treated animals, respectively (Fig. 4 D, E).

Effect of B-MIX treatment on cognitive behavior in rats.

The enhanced extent of LTP observed in the hippocampal CA1 field of B-MIX treated rats prompted us to determine whether this effect could be associated with a better cognitive performance. To do so, we used Barnes maze and NOR to measure spatial learning and memory (Antunes and Biala, 2012; Harrison et al., 2006) in CTRL and B-MIX treated animals, respectively. In the Barnes test rats were trained once a day for four consecutive days and the test was made at day 5 (short-term retention). During both training and test the time needed to find the target hole was significantly decreased in B-MIX animals. Two-way ANOVA revealed a significant effect in each group during days in terms of latency [$F(4,50) = 40.36$, $p = 0.0001$, $n = 6$], and a significant effect of treatment [$F(1,50) = 16$, $p = 0.0002$], with no interaction between groups (Fig. 4F). Furthermore, B-MIX treatment failed to alter the number of errors committed both during training and retention time points (Fig. 4G).

NOR test was performed using different set of animals. During familiarization both experimental groups were exposed to identical objects. As expected, we found no differences in both recognition index (Fig. 5B) and discrimination index (Fig. 5C), that mirrored the interest of the animal for both objects placed in the arena. As the nature of animal behavior, when one of the two objects has been exchanged with a familiar one (test phase), CTRL animals preferred to explore the novel with respect to the familiar as revealed by a higher percentage (+86% vs familiar) of recognition index (Fig. 5D) and a positive (0.28) discrimination index, indicating a remarkable preference for the novel object (Fig. 5E). Interestingly, when B-MIX animal were exposed to NOR test during the test phase they revealed a much greater (180%) recognition index than that observed in CTRL, an effect that was accompanied by a more pronounced discrimination index (0.51) when compared with CTRL animals (t-Test, $p < 0.05$ vs CTRL, $n = 6$).

Discussion

Our data demonstrate that chronic treatment with a mixture of three different strain of *Bifidobacteria* (*longum, breve and infants*) induces a parallel positive changes in brain plasticity and cognitive behavior in male adult rats. In particular, we found that a gain in the amount of specific strains of *Bifidobacteria* in the gut of healthy rats was capable to induce an increase in the content of the neurotrophic factor BDNF with a parallel rise in dendritic spines density detected in CA1 pyramidal neurons of hippocampal formation accompanied by a greater long term synaptic plasticity when compared with that observed in vehicle treated animals. These molecular, structural and functional effects are associated to an increased cognitive behavior in animals exposed to B-MIX without any significant motor impairment.

The significant increase in the expression of BDNF is in line with previous reports suggesting that altering the constitution of gut microbiota may affect the expression of different factors at brain levels, related to neuronal growth and synaptic plasticity in both rodents and humans (Hoban et al., 2016; Liang et al., 2015; Bercik et al., 2011; Tomasik et al., 2015; Allen et al., 2016; Severance et al., 2016; Dickerson et al., 2014). In this respect, aged-related alteration of gut microbiota, lead to profound modifications in expression of genes associated with neural plasticity, such as BDNF (Distrutti et al., 2014), with parallel impairments in long term synaptic function observed at hippocampal level (Davari et al., 2013). More recent data demonstrate that the treatment with a specific *Bifidobacteria* strains, i.e. B. *longum* and *B. infants*, may have a positive impact on stress-related cognitive impairments both in mice (Savignac et al., 2015), and in humans (Allen et al., 2016).

A recent report from Luczynski and colleagues (2016) suggests that the effect of deficiency of gut microbiota might be specific for certain brain areas. In particular, these authors show that germ-free mice exhibit altered stress responses and anxiety-like behavior

that is accompanied by a hypertrophy of dendrites in neurons of basolateral amygdala whereas, the ventral hippocampal pyramidal neurons show dendritic branches with less stubby and mushroom spines, when compared with conventionally colonized animals (Luczynski et al., 2016). Consistent with the above mentioned reports, a very recent finding by Hoban and colleagues (2018), show that microbiota plays a crucial role in the amygdala-dependent memory formation regulating several mechanisms involved in fear recall in mice (Hoban et al., 2018). The evidence that BDNF is involved in the control of dendritic arborization and spine formation (Huang and Reichardt, 2001; von Bhlen Und Halbach and von Bohlen Und Halbach, 2018; Tyler et al., 2002) prompted us to deeply evaluate this aspect in our experimental condition. We show that B-MIX treatment leads to a significant increase in spine density in both basal and apical dendritic branches of CA1 pyramidal neurons, suggesting a boosting in number of connections with glutamatergic presynaptic terminals. A deeply analysis of spine morphology revealed that both thin and mushroom spines but not stubby spines, were affected by the treatment. As recently reported both thin and mushroom spines play a fundamental role in regulating hippocampal plasticity and mushroom spines represent a strong and mature state of the excitatory synapses (Burk et al., 2018; Sala and Segal, 2014). In addition, in line with the latest finding and in agreement with the role of BDNF and dendritic spines in regulating at hippocampal level certain forms of plasticity such as LTP and LTD (Peters et al., 2018; Panja and Bramham, 2014; von Bhlen Und Halbach and von Bohlen Und Halbach, 2018), our data show that chronic treatment of healthy rats with B-MIX may also significantly increase the extent of LTP measured at hippocampal level without influencing the basal excitability of this important brain area. The finding is in agreement with other reports showing that LTP formation is markedly altered in the presence of pathological (Davari et al., 2013) or stress related alteration (Savignac et al., 2015) of gut *bacteria,* conditions restored by a treatment with a specific *Bifidobacteria* strains, may have a positive impact on restoration of such impaired synaptic plasticity.

Neuronal plasticity often correlates with the density of dendritic spines where ligand gated receptors, such as those for AMPA and NMDA, are largely located to the surface of these spines close to the presynaptic element (Megias et al., 2001). Since CA1 pyramidal neurons represent the principal cell types that carry hippocampal output (Graves et al., 2012), our data suggest that B-MIX treatment may induce an increase in the hippocampal output signals to other brain areas functionally linked to this limbic formation in the modulation of learning and memory and emotional state. Since some studies indicate that the increased rearing is related with an enhancement in anxiety levels in mice (Borta and Schwarting, 2005), the decreased effect in vertical activity, observed at the end of the test (Fig. 4D), may mirrored an anxiolytic effect induced by B-MIX treatment. On the other hands the lower amount of stereotyped behavior showed by B-MIX animals at the beginning of the test (Fig. 4E), may be related with lower level of anxiety. Accordingly, other reports suggested that stress may cause an animal to develop abnormal repetitive behavior (Langen et al., 2011). Finally, our molecular and electrophysiological findings are associated to behavioral changes suggesting that the treatment with B-MIX refines the cognitive performances in two different experimental models such as Barnes maze and novel object recognition (NOR), both preferentially used to evaluate the hippocampal function (Antunes and Biala, 2012; Harrison et al., 2006).

In summary, our data demonstrate specific, previously undescribed morphological, neurochemical, and behavioral changes induced by chronic supplementation of intestinal microbiota using a mix of three different strain of nonpathogenic *Bifidobacteria* in heathy rats. Accordingly, we show that this treatment may modulate the glutamatergic transmission in healthy rats and this, in turn, may have beneficial effects on hippocampal plasticity and cognitive behavior. It is worth noting that, the present results, similarly to other reports both in rodents and humans (Savignac et al., 2013; Schmidt et al., 2015), were obtained in healthy animals, thus further information will be obtained by applying such treatment to

animals exposed to different stress insults that may alter the function of the microbiota–gut–brain axis. In fact, as the nature of probiotic bacteria, they actively contribute to the health of the host (Gareau et al., 2010), modulating response to stress with a significant improve the general mood and gut pathologies (Silk et al., 2009; Rao et al., 2009). Although straightforward our data may not illuminate for a precise mechanism as to how such effects are manifested, while strongly strengthen the well-established link between the modulation of gut microbiota and brain plasticity (McVey Neufeld et al., 2016; Leung and Thuret, 2015), suggesting that all these changes may affect the animal cognitive behavior. Different authors suggested the vagus nerve as a key pathway in the gut-brain crosstalk (see Bauer et al., 2019 for review), since it has been shown that vagus nerve stimulation potentiates hippocampal LTP in freely-moving rats (Vazquez et al., 2016; Zuo et al., 2007). The mechanisms of action of probiotics at brain level appear to be still very complex and many efforts have yet to be made to evaluate the properties of different strain of gut bacteria. Our findings suggest the potential utility of these three *Bifidobacteria* strains to ameliorate even in humans emotional and affective diseases as well as the aged-associated cognitive decline.

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Competing interests

The authors declare no competing interests.

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Figure legends

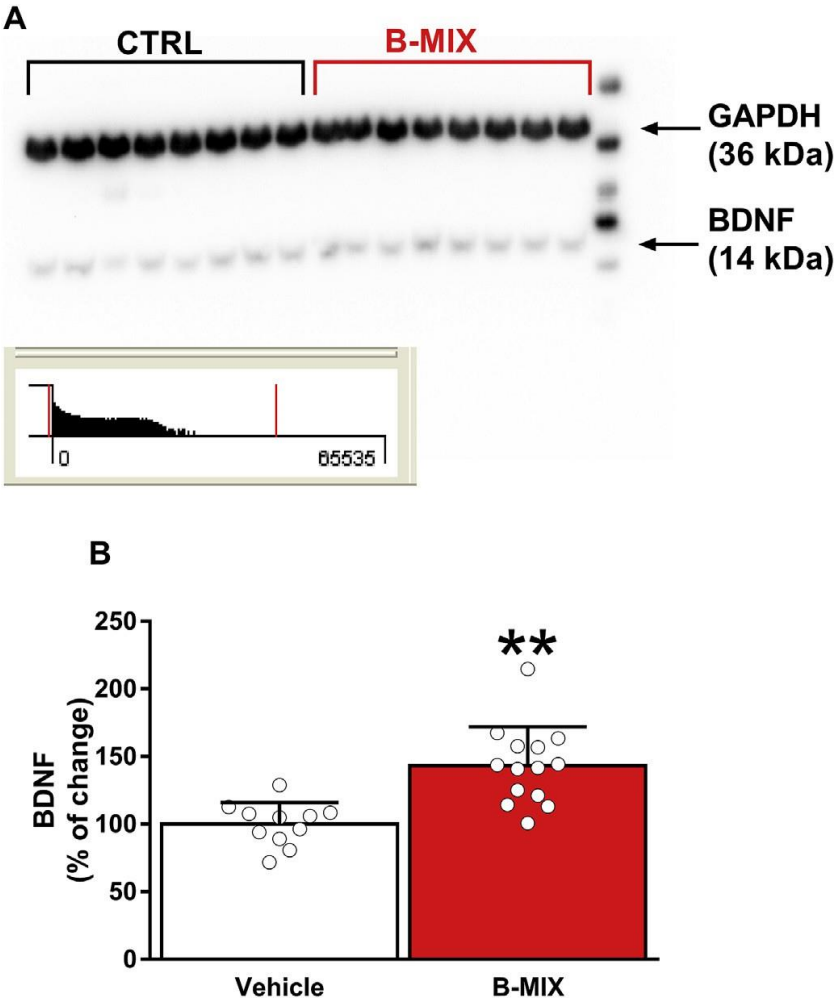

Figure 1. Chronic B-MIX treatment increases BDNF protein levels in the hippocampus. **(A)** Representative blot of a single experiment measuring BDNF protein amounts where both different groups were reported and compared with GAPDH signal, used as internal control. In the inset is reported a screenshot of the software used for protein quantification in which the level of saturation signal (in red) is reported. **(B)** Bargraph representing the percentage of increase in BDNF protein levels in the hippocampus of treated rats compared with controls (CTRL). Data are expressed as means ± SEM of values from 11 (CTRL) to 14 (B-MIX) rats and columns represent the average of two separate experiments. ** $p < 0.001$ vs. CTRL. (unpaired t-Test). The number of sampling (white dots) is different depending on more available animals for the B-MIX group.

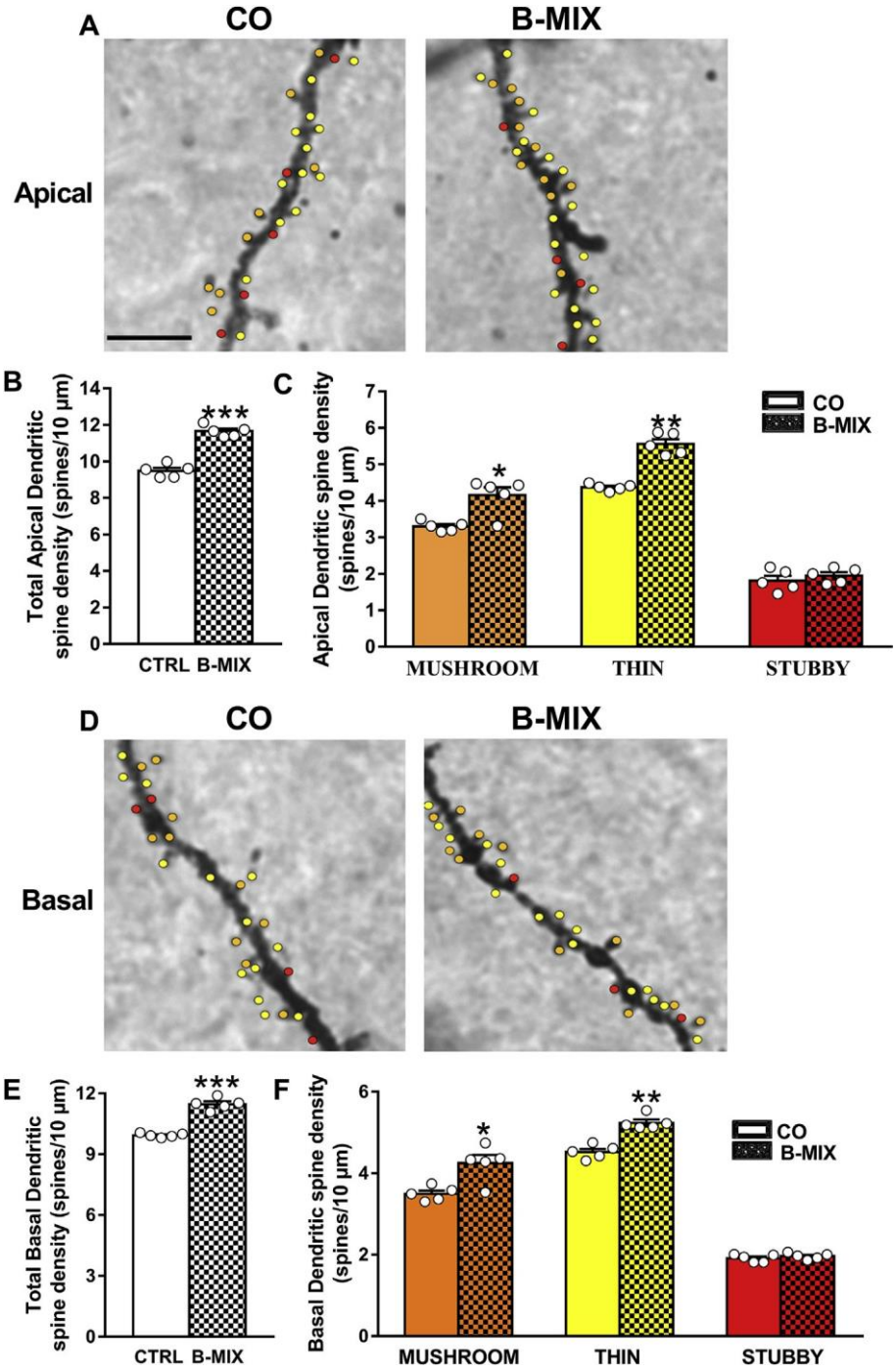

Figure 2. Chronic treatment with B-MIX increases dendritic spine density in the hippocampal CA1 pyramidal neurons. (A, D) Representative images of Golgi impregnated apical (A) and basal (D) dendrites of CA1 pyramidal neurons (scale bar: 5 µm). Color circles are located for the maximal head diameter measured: yellow, thin spine; orange, mushroom spines, red, stubby spines. **(B, E)** Bar graphs summarizing the increased

spine density calculated in a 10-μm dendritic section of the different experimental groups. *** $p < 0.0001$ vs CTRL (unpaired t-Test). **(C, F)** Bar graphs summarizing the change in spine density split by shape and calculated in a 10-μm dendritic section of the different experimental groups. *$p < 0.05$; **$p < 0.001$ vs CTRL (unpaired t-Test). Data are means ± SEM of values from 5 (CTRL) and 5 (B-MIX) rats. White dots on bar graphs represent the average of samples from each animal.

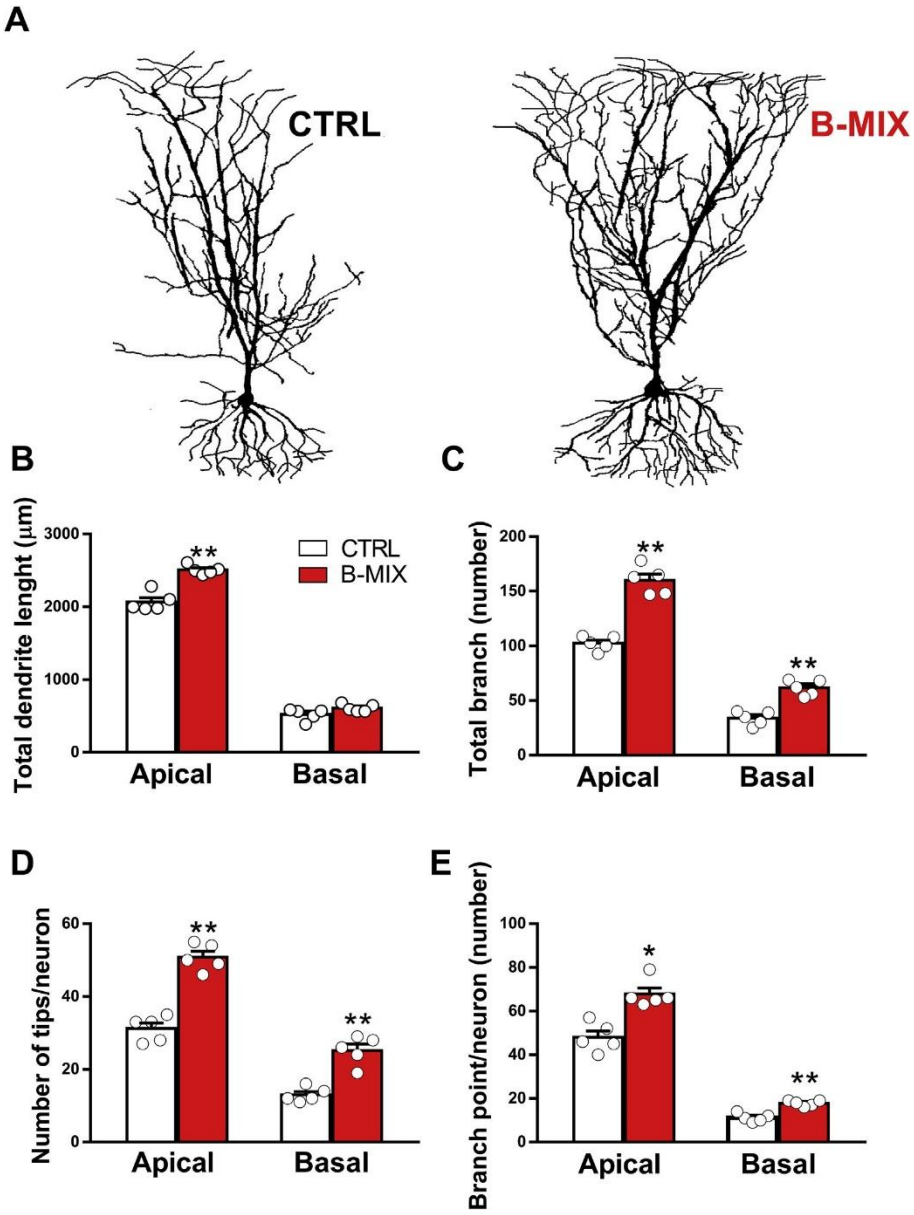

Figure 3. B-MIX treatment increases dendritic arborization on hippocampal CA1 pyramidal neurons. (A) Representative images of Golgi impregnated pyramidal neurons of the hippocampal CA1 region of control and treated rats. **(B–E)** Bar graphs report the averaged total dendrite length (µm) (B), the total branch number (C), the number of tips per neuron (D), and the number of branch points per neuron (E) measured in CA1 apical and basal dendrites areas. Data are reported as means ± SEM and obtained from 5 rats per experimental group. *$p < 0.05$; **$p < 0.001$ vs control. White dots on bar graphs represent the average of samples from each animal.

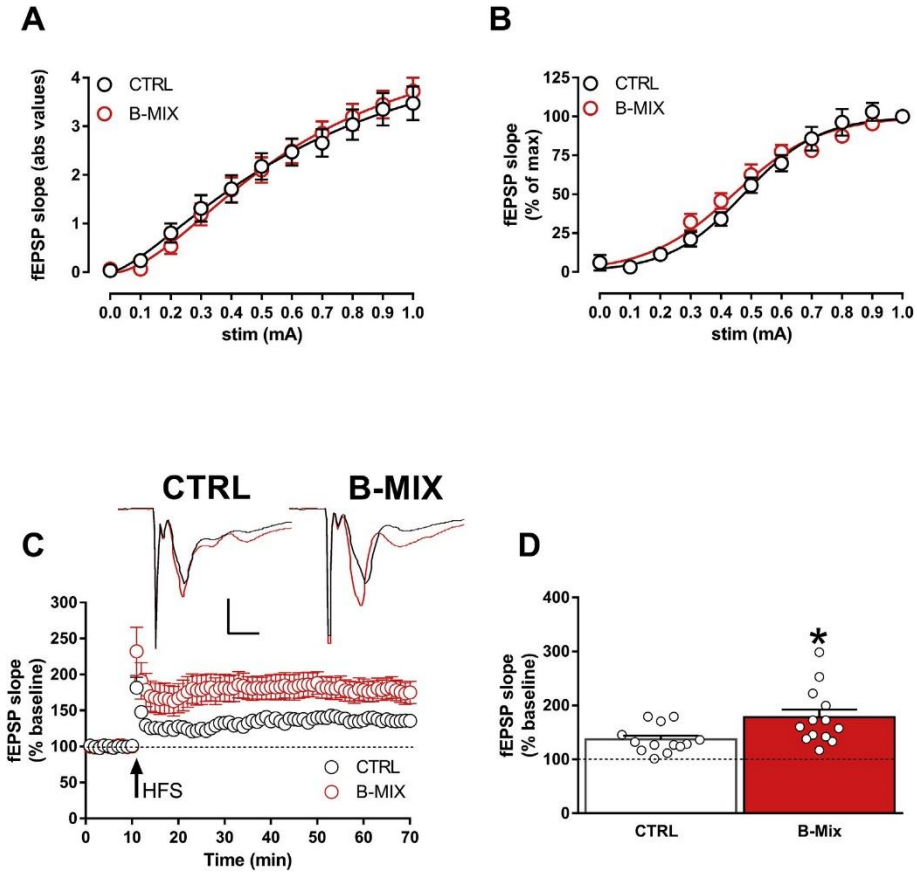

Figure 4. B-MIX improves LTP formation on hippocampal CA1 subfield. (A, B) Input–output (I–O) curves were determined in slices from CTRL and B-MIX treated rats by measuring the fEPSP slope in response to stimulation of Schaffer's collaterals with current steps of increasing intensity (from 0 to 1.0 mA). On A, data are raw data of slopes obtained in every single stimulation, while on B, data are expressed as percentage of the corresponding maximal response for every single recording (n = 13 recordings from 5 animals per group). **(C)** Representative traces of fEPSPs before and 1 h after HFS application (red trace) are shown (scale bars: 1 mV, 5 ms) and scatter plots representing the percentage change in fEPSP slope values induced by HFS (indicated with a black arrow) with respect to baseline in rats of the different experimental groups. **(D)** Bar graph of the averaged fEPSP slope values obtained during the last 10 min of LTP recording, compared with the relative baseline (n = 13 recordings from 5 animals per group). * $p < 0.05$ vs CTRL

(unpaired t-Test). White dots on bar graph represent the effect related to every single recording.

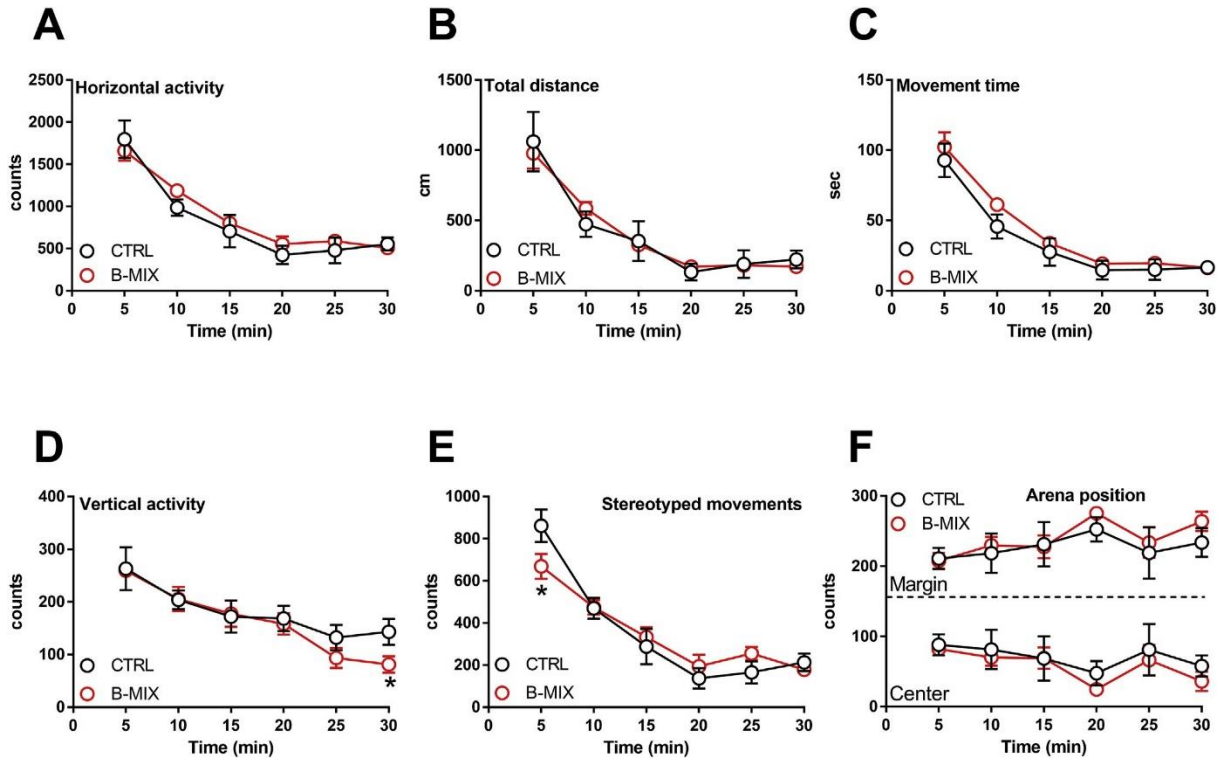

Figure 5. Chronic treatment with B-MIX fails to alter the spontaneous exploratory activity in rats. Exploratory activity was assessed in an open field test and monitored every 5 min for a total 30 min. The different parameters measured are reported as scatter plots in panels **A-F**. Data in graphs are means ± SEM (n = 6 animals per group) . * $p < 0.05$ vs CTRL (unpaired t-Test).

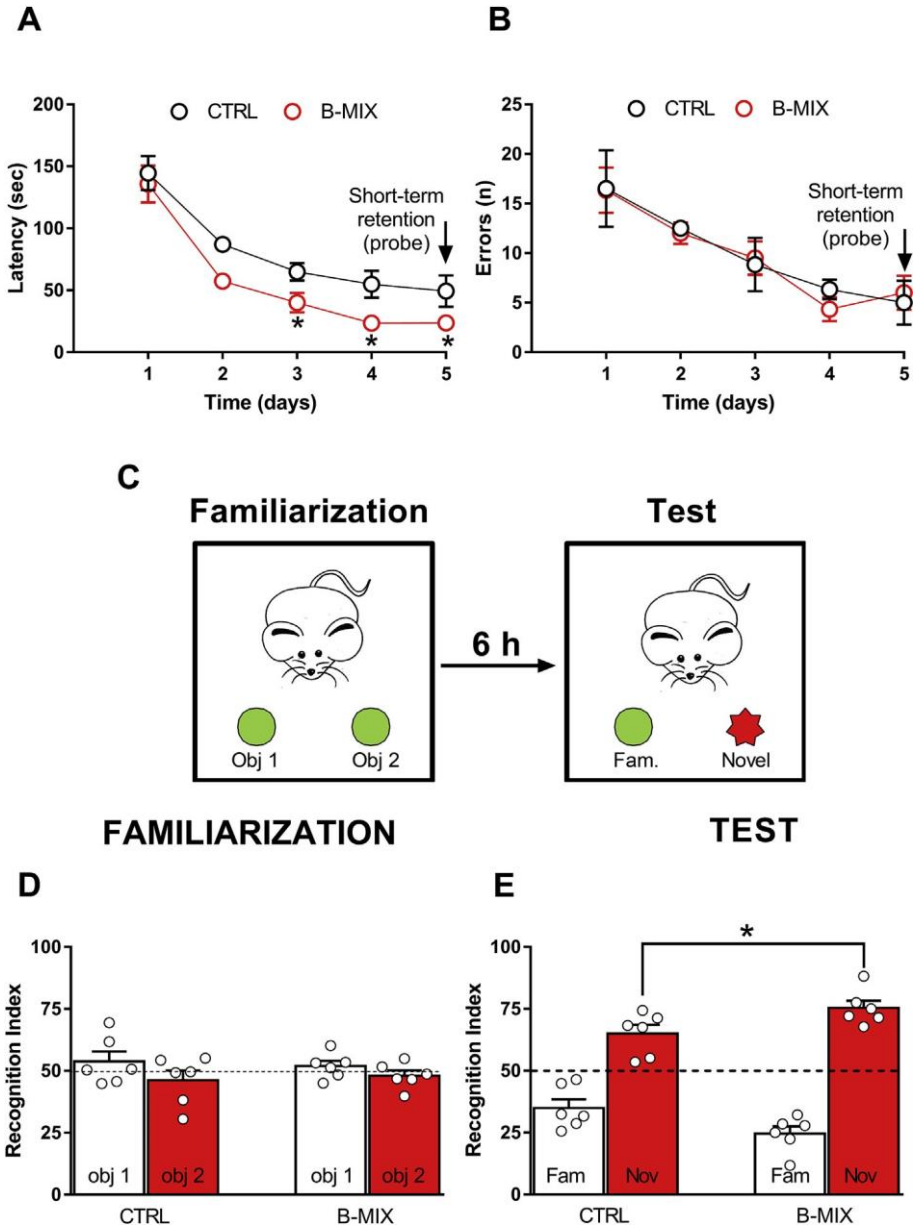

Figure 6. B-MIX enhances spatial learning and recognition memory in rats. **(A, B)** The time needed by each animal to identify the target hole of a Barnes maze (A) and the number of errors (B) are shown for the four consecutive training sessions and during the test on day 5. * $p < 0.0001$ vs CTRL, two-way ANOVA followed by Bonferroni test. (n = 6 animals per group)**.** **(C)** Schematic representation of the spatial object recognition paradigm during familiarization. The test was performed 6h after familiarization. **(D)** Bar graph representing the percentage of recognition index in both CTRL and B-MIX during the familiarization training with identical objects. **(E)** Bar graphs representing the percentage of recognition

index in both CTRL and B-MIX during the test with different objects. * $p < 0.05$ vs CTRL. (unpaired t-Test). n = 6 animals per group. White dots on bar graphs represent the effect related to every single animal.