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Effects of a human milk oligosaccharide, 2′-fucosyllactose, on hippocampal long-term potentiation and learning capabilities in rodents☆

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Abstract

Human milk oligosaccharides (HMOs) are unique with regard to their diversity, quantity and complexity, particularly in comparison to bovine milk oligosaccharides. HMOs are associated with functional development during early life, mainly related to immunity and intestinal health. Whether HMOs elicit a positive effect on cognitive capabilities of lactating infants remains an open question. This study evaluated the role of the most abundant HMO, 2′-fucosyllactose (2′-FL), in synaptic plasticity and learning capabilities in rodents. Mice and rats were prepared for the chronic recording of field excitatory postsynaptic potentials evoked at the hippocampal CA3–CA1 synapse. Following chronic oral administration of 2′-FL, both species showed improvements in input/output curves and in long-term potentiation (LTP) evoked experimentally in alert behaving animals. This effect on LTP was related to better performance of animals in various types of learning behavioral tests. Mice were tested for spatial learning, working memory and operant conditioning using the IntelliCage system, while rats were submitted to a fixed-ratio schedule in the Skinner box. In both cases, 2′-FL-treated animals performed significantly better than controls. In addition, chronic administration of 2′-FL increased the expression of different molecules involved in the storage of newly acquired memories, such as the postsynaptic density protein 95, phosphorylated calcium/calmodulin-dependent kinase II and brain-derived neurotrophic factor in cortical and subcortical structures. Taken together, the data show that dietary 2′-FL affects cognitive domains and improves learning and memory in rodents.

Keywords: Human milk oligosaccharides; 2′-Fucosyllactose; Long-term potentiation; IntelliCage; Cognition; Synaptic plasticity

1. Introduction

A growing body of evidence shows that glycans play a pivotal role in the growth and development of several organs, including the brain. Myelinization, formation and function of synapses and, neurotransmitter release are modulated by the glycosylation of structural molecules and receptors of neurons and ancillary cells [1,2]. Glycoconjugates in the human brain increase in the last trimester of gestation until 8 months after birth; a period characterized by the highest synaptic plasticity and rapid development of brain networks [3].

Breastfeeding has been associated with significantly higher scores for cognitive development than formula feeding [4–6]. Human milk is unique regarding the diversity, quantity and complexity of human milk oligosaccharides (HMOs), with more than 150 structures identified so far [7]. In contrast, oligosaccharides in bovine milk, which constitute the base for most infant formulas, are less abundant and complex [8,9].

The most abundant HMO, up to 4.65 g/L [10], is 2′-fucosyllactose (2′-FL). Interestingly, fucosylated glycoproteins were reported in synaptic junctions from rat brain [11], and more recently, a proteomic study identified a number of fucose-bearing proteins that are relevant for synaptic signaling in mouse olfactory bulb [12]. Pioneering studies demonstrated that radioactively labeled fucose ([^{3}H]Fuc) injected intracranially was rapidly incorporated into glycoproteins and transferred to nerve endings [13]. Moreover, several studies showed an increase in the incorporation of [^{3}H]Fuc to hippocampal proteins after different learning paradigms both in rats [14] and chicks [15].

Long-term potentiation (LTP) is an experimentally evoked process whereby synaptic strength is rapidly increased, and it constitutes the key mechanism underlying learning and memory [16]. An enhancement in

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hippocampal-LTP by fucose and 2′-FL was demonstrated *in vitro* [17] and in freely moving rats after an intrahippocampal injection; therefore, a role for both in learning and memory was suggested.

The absorption of 2′-FL from milk has been indirectly shown in breastfed babies, since its presence has been reported in urine but not in plasma [18]. Taking this evidence into account, we hypothesize that milk 2′-FL contributes to improve cognitive abilities in breastfed babies. We show that feeding a diet with 2′-FL increases LTP at the hippocampus, in conscious rats and mice. 2′-FL also enhanced memory consolidation, spatial learning and associative learning. In addition, several molecular brain markers such as postsynaptic density protein 95 (PSD-95), phosphorylated calcium/calmodulin-dependent kinase II (pCaMKII) and brain-derived neurotrophic factor (BDNF) were increased by 2′-FL. In summary, dietary 2′-FL exerts a positive effect on learning and memory in rodents.

2. Materials and methods

2.1. Experimental animals

C57BL/6 male adult mice (2–3.5 months old; 25–30 g) and Sprague–Dawley male adult rats (2.5–4 months old; 250–300 g) were obtained from Charles River Laboratories. Animals were kept on a 12-h light/dark cycle with constant ambient temperature (21.5±1°C) and humidity (55%±8%). Food and water were available *ad libitum*. Upon arrival, mice were housed in groups (n=5 per cage) and rats were kept in pairs. Animals devoted to LTP studies were switched to individual cages after surgery.

Experiments were carried out in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (BOE 252/34367-91, 2005) for the use of laboratory animals in chronic studies. All experimental protocols were also approved by the local Ethics Committee.

2.2. 2´-FL administration

2´FL was always administered orally, in most experiments through the diet. The control diet was a semipurified nutritional regimen that followed AIN-93M guidelines [19] (manufactured and pelletized by Abbott Laboratories). The 2′-FL diet was supplemented with 2′-fucosyllactose (2′-FL, acquired from Inalco Pharmaceuticals) at 0.312% (wt/wt) for mice and 0.625% (wt/wt) for rats. Supplementation with 2′-FL was calculated to provide approximately 350 mg 2′-FL/kg of body weight per day. Since we did not have previous information about feeding duration to affect brain function by this type of compound, we decided to use two feeding regimens, and then the experimental diets were given for 5 weeks in rats (short term feeding) or for 12 weeks in mice (long-term feeding). Feeding times with each diet are indicated with the corresponding experiments. In one experiment, 2´-FL was acutely administered to rats by oral gavage (1g 2´-FL/kg BW) before to run the input/output curves.

2.3. Surgery

Mice were anesthetized with 4% chloral hydrate (0.1 ml/10 g). Once anesthetized, animals were implanted with stimulating and recording electrodes in the hippocampus. Stereotaxic coordinates [20] were followed to implant animals with stimulating electrodes aimed at the Schaffer collateral–commissural pathway of the dorsal hippocampus (2 mm lateral and 1.5 mm posterior to bregma; depth from brain surface, 1.0–1.5 mm). These electrodes were made of 50-μm Teflon-coated tungsten wires (Advent Research Materials Ltd.). In addition, animals were implanted with recording electrodes aimed at the ipsilateral stratum radiatum underneath the CA1 area (1.2 mm lateral and 2.2 mm posterior to bregma; depth from brain surface, 1.0–1.5 mm). Recording electrodes were also made of 50-μm Teflon-coated tungsten wires (Advent Research Materials Ltd.). Electrodes were surgically implanted in the CA1 area using as a guide the field potential depth profile evoked by paired (20–50 ms of interval) pulses presented at the ipsilateral Schaffer collateral pathway. The recording electrodes were fixed at the site where a reliable monosynaptic field excitatory postsynaptic potential (fEPSP) was recorded. A 0.1-mm bare silver wire was affixed to the skull as a ground. The wires were connected to two four-pin sockets (RS-Amidata). The ground wire was also connected to the recording system with a single wire. Sockets were fixed to the skull with the help of two small screws and dental cement (Fig. 1A) [21].

Rats were anesthetized with 4% chloral hydrate at a dose of 1 ml/100 g following a protective injection of atropine sulphate (0.1 mg/100 mg, i.m.). Stereotaxic coordinates [22] were used to implant animals with stimulating electrodes aimed at Schaffer collateral–commissural pathway of the dorsal hippocampus (3.5 mm lateral and 3.2 mm posterior to bregma; depth from brain surface, 1.0–1.5 mm). In addition, animals were implanted with four recording electrodes aimed at the ipsilateral stratum radiatum underneath the CA1 area (2.5 mm lateral and 3.6 mm posterior to bregma; depth from brain surface, 1.0–1.5 mm). Electrodes and the rest of implantation procedures were similar to the above description for mice (Fig. 2A) [21].

2.4. Electrophysiological studies

Electrophysiological studies were started 1 week after surgery. Experimental diets were given for 12 weeks (mice) or 5 weeks (rats) before these experiments. To this end, the experimental animal was placed in a small box located inside a larger Faraday cage. Recordings were carried out using Grass P511 differential amplifiers with a bandwidth of 0.1 Hz–10 kHz (Grass-Telefactor). Synaptic field potentials in the CA1 area were evoked by single, 100 μs, square, biphasic (negative-positive) pulse applied to Schaffer collaterals.

For input/output curves, we used single pulses of increasing intensities (from 0.02 to 0.4 mA in steps of 0.02 mA). Each stimulus was repeated 5 times. Time interval between successive stimulus presentations was >20 s to avoid any after effect of the preceding pair of stimulus (Figs. 1C and 2B). The input/output curves were used in order to determine the intensity of the stimuli to be applied in the LTP protocol.

For the LTP study, the stimulus intensity was set well below the threshold for evoking a population spike, usually 30%–40% of the intensity necessary for evoking a maximum fEPSP response [23]. An additional criterion for selecting stimulus intensity was that a second stimulus, presented 20–50 ms after a conditioning pulse, evoked a larger (>20%) synaptic field potential [24].

For evoking LTP, we used the following high-frequency stimulation (HFS) protocol: each animal was presented with five 200-Hz, 100-ms trains of pulses at a rate of 1/s. These trains were presented 6 times in total, at intervals of 1 min. The 100-μs, square, biphasic pulses used to evoke LTP were applied at the same intensity used for evoking baseline records (see Ref. [21] for further details of this chronic preparation). Baseline records were collected for 15 min with the paired stimuli presented every 20 s. After the HFS protocol, fEPSPs were recorded again for 30 min. Additional recordings were carried out for 15 min during the 1–3 following days (Figs. 1D and 2C, D).

2.5. Histology to test electrode location

At the end of the experiments, animals were deeply reanesthetized (sodium pentobarbital, 50 mg/kg) and perfused transcardially with saline and 4% phosphate-buffered paraformaldehyde. Selected sections (50 μm) including the dorsal hippocampus were mounted on gelatinized glass slides and stained using the Nissl's staining with 0.1% Toluidine blue, to determine the location of stimulating and recording electrodes (Fig. 1B).

2.6. IntelliCage study

The IntelliCage (NewBehavior AG) has been described in detail elsewhere [25–27]. Briefly, it is a computer-based, fully automated testing system used to analyze spontaneous and learning behavior of radiofrequency identification (RFID)-tagged mice in a home–cage environment (Fig. 3A). Each IntelliCage is equipped with four triangular operant learning chambers (corners), RFID readers and several other sensors, allowing for simultaneous monitoring of up to 16 transponder-tagged mice housed in the same cage. Each corner accommodates a single mouse at a time, through an antenna ring detecting the transponder number. In the inner space of the corner, mice encounter a choice between two nosepoke holes, equipped with light-beam nosepoke sensors. The correct nosepoke triggers the opening of a motorized gate, giving access to water bottle nipples. The time and duration of each behavioral event (corner visit, nosepoke and lick), mouse ID and corner ID are automatically recorded through RFID readers, infrared sensors and lickometers.

For the IntelliCage study, mice were divided into two experimental groups (n=28) receiving control or 2′-FL diets for 5 weeks. After 1 week of initial habituation to the testing room, mice were implanted with glass-covered transponders with unique ID codes (Trovan Ltd.). Transponders were injected subcutaneously in the interscapular area under light isoflurane anesthesia. Twenty-four hours after the implantation, mice were placed into the IntelliCages (n=14 per cage) and were maintained there for a total of 5 weeks. Experimental diets were given to mice when they were moved to IntelliCages. The experiments started from an adaptation period with *ad libitum* access to water in all the corners. Different test modules were used in order to obtain information concerning learning behavior (Table 1).

2.6.1. Habituation-free exploration (H-FE)

During this module (3 days) all doors of the IntelliCage were open.

2.6.2. Nosepoke adaptation (NP)

During this module (2 days), all doors were initially closed upon one nosepoke the respective door was opened, once during a visit. Following a successful nosepoke, the water bottle was available for 4 s.

2.6.3. Nosepoking during drinking sessions (NP-DS)

In this module, corner access and water availability were restricted to two drinking sessions per night. The early session lasted between 2315 and 2445, and the late session, between 0615 and 0745.

2.6.4. Place learning during drinking sessions (PL-DS)

In this module (3 days) for each mouse, access to water was restricted to one corner (correct or rewarded corner), and nosepoking in all other corners was ineffective. Drinking corner assignment was randomized within the groups. The ability of each mouse to find the

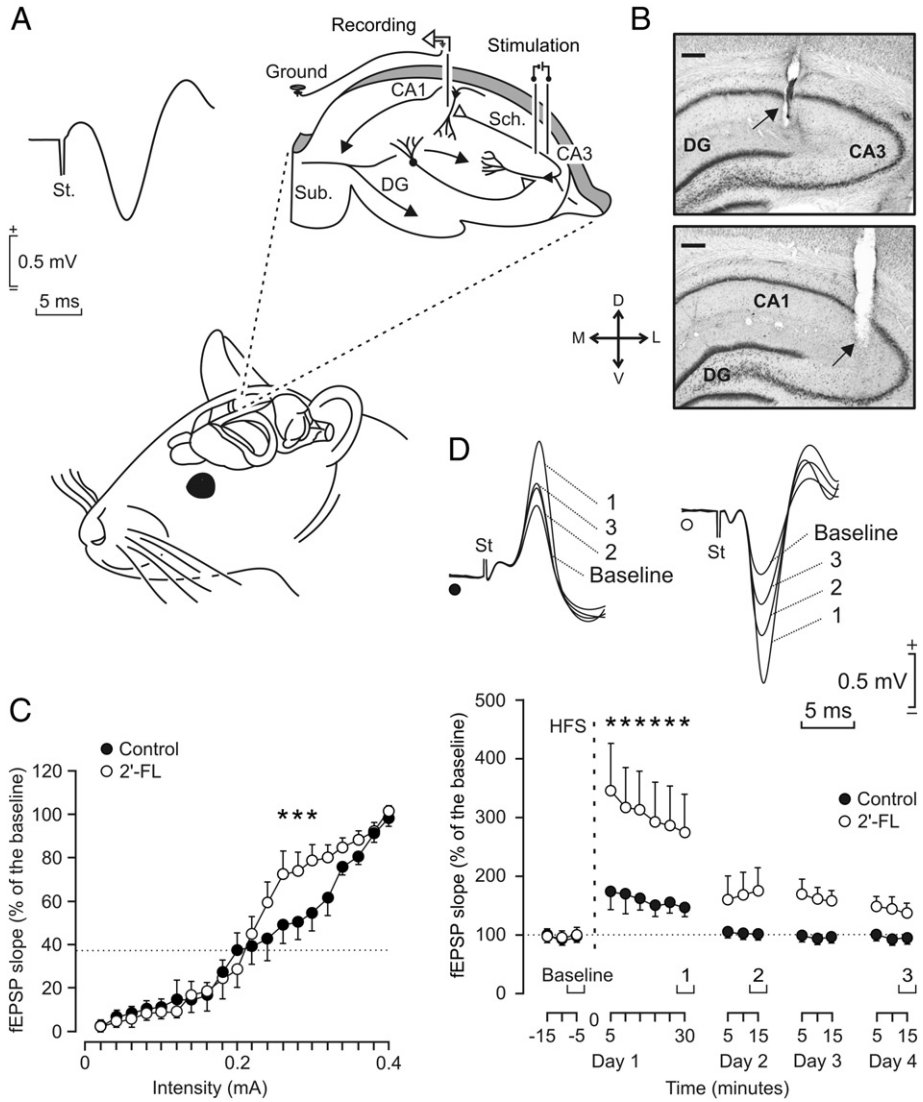

Fig. 1. Administration of 2′-FL potentiates input/output curves and LTP evoked at the hippocampal CA3–CA1 synapse in behaving mice. (A) Mice were chronically implanted with bipolar stimulating electrodes at the Schaffer collaterals and with a recording electrode at the ipsilateral CA1 area. An example of fEPSP (averaged 5 times) evoked at the CA3–CA1 synapse is illustrated at the top left. 2′-FL mice were treated for 12 weeks before the electrophysiological study. (B) Photomicrographs illustrating the location of recording (top) and stimulating (bottom) sites (arrows). Calibration bar is 200 μm. Abbreviations: D, L, M, V, dorsal, lateral, medial, ventral; DG, dentate gyrus; Sub, subiculum. (C) The graphs illustrate performance of the two groups during the input/output test. The slopes of evoked fEPSPs increased steadily with stimulus intensity in both cases; asterisks indicate significant (*P<.05) differences between the 2′-FL (white circles) and control (black circles) groups. (D) LTP study. Animals were stimulated with a HFS protocol after 15 min of baseline recordings. Following HFS, recordings were carried out for up to 4 days. Significant differences (*P<.05) were observed between the 2′-FL and control groups during the 30 min of recordings following the HFS protocol. At the top are illustrated fEPSPs (averaged 5 times) collected at the times indicated in the graph.

correct corner and learn its position provided a measure of place learning. The sessions followed the same schedule as described under (NP-DS).

2.6.5. Extinction of place preference drinking sessions (EP-DS)

In order to extinguish previously learned behaviors, mice were allowed to drink from any corner for 4 days between modules.

2.6.6. Patrolling during drinking sessions (P-DS)

During this module (4 days), access to water was available by a nosepoke in an "active" rewarded corner during drinking sessions. Rewarded corners changed dynamically clockwise: upon a nosepoke in an "active" rewarded corner, the corner became "inactive," and the following clockwise corner became active. Thus, animals had to learn to visit corners clockwise in order to get access to water patrolling different locations (working memory). Drinking corner assignment was randomized within groups.

2.6.7. Extinction of patrolling preference drinking sessions

Mice were allowed to drink from any corner during drinking sessions for 3 days.

2.6.8. Fixed ratio during drinking sessions (FR-DS)

In this module, we evaluated an operant conditioning response. Animals had to nosepoke on a specific schedule of reinforcement in which the number of nosepokes required for getting access to water increased in a fixed rate. Once the task was performed successfully 10 times, the animal was promoted to the next module where more nosepokes were necessary. The first module (fixed ratio) consisted of a single nosepoke, and was assigned to all mice at the beginning of the experiment. Once the mouse had been successful 10 times, the next module was assigned. The number of consecutive nosepokes increased by 4 between modules, i.e., module 1, 1 nosepoke; module 2, 4 nosepokes; and module 3, 8 nosepokes. Drinking corner assignment was randomized within groups. After a 6-day period for extinction of learning, animals were subjected to the same protocol to test a facilitative effect on long term memory recovery.

2.7. Operant conditioning of rats

Animals received experimental diets for 5 weeks. Training and testing took place in basic Skinner box modules (n=5) measuring 29.2×24.1×21 cm (MED Associates). The operant chambers were housed within a sound-attenuating chamber (90×55×60 cm), with constant illumination (19W lamp) and exposed to a 45 dB white noise (Cibertec). Each Skinner box was equipped with a food dispenser from which pellets (MLabRodent

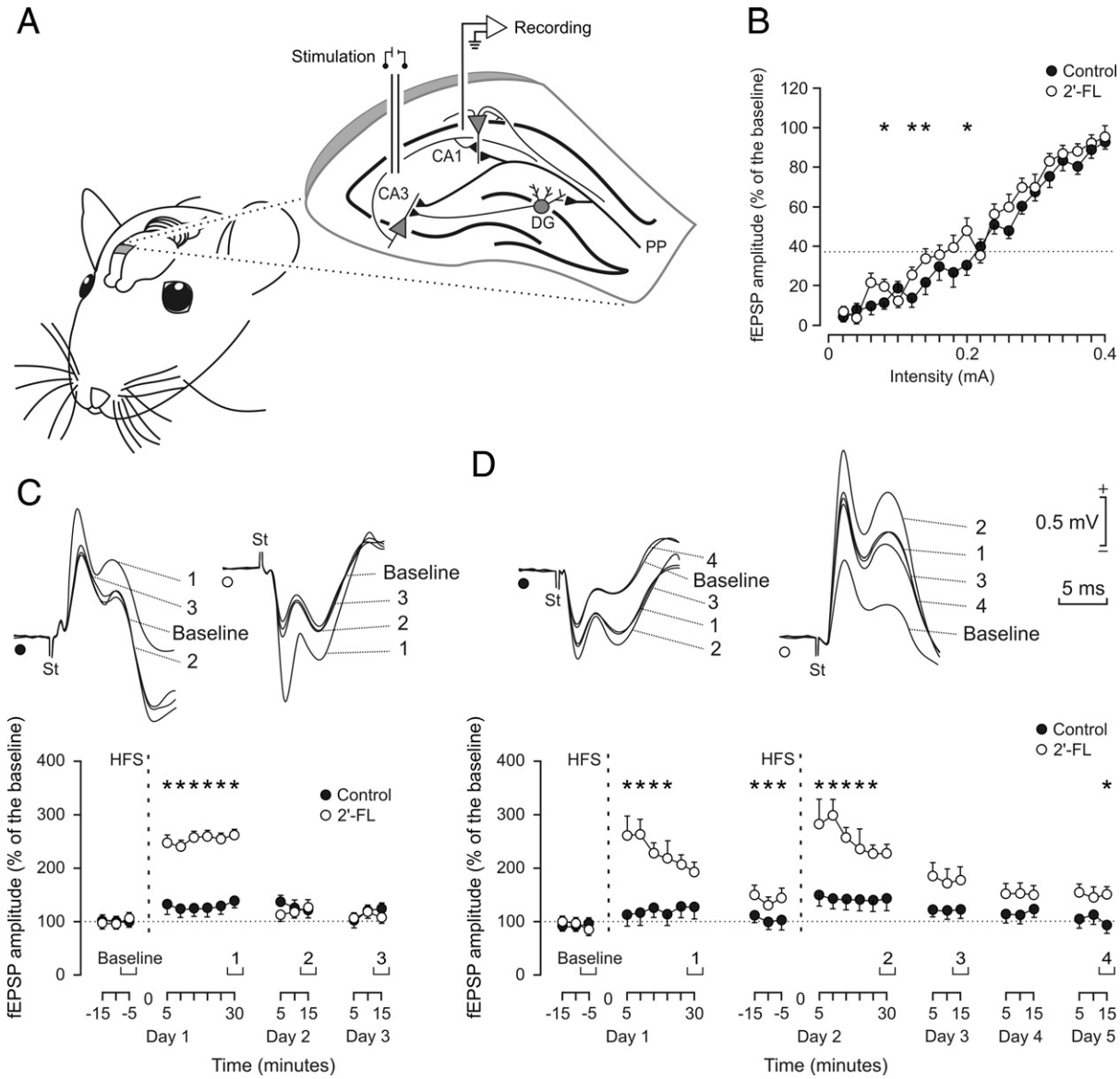

Fig. 2. Administration of 2′-FL potentiates input/output curves and LTP evoked at the hippocampal CA3–CA1 synapse in behaving rats. (A) The diagram illustrated how rats were prepared for the chronic recording of fEPSPs evoked at the hippocampal CA3–CA1 synapse. (B) Input/output curves evoked at the hippocampal CA3–CA1 synapse before (0 min; black circles) and after (180 min; white circles) the administration of 2′-FL by oral gavage. Illustrated data were collected and averaged from 10 recording electrodes (n=4 animals). *P<.05. (C) An LTP test was carried out for two groups of rats (control, black circles; 2′-FL, white circles; n=10 animals per group). 2′-FL rats were treated for 5 weeks before the HFS session. After 15 min of baseline records (Day 1), animals were stimulated with the HFS protocol. Recording was carried out for 30 min after the HFS. Additional recordings were carried out for 15 min for two additional days (Days 2 and 3). The 2′-FL group presented significantly (*P≤.05) larger LTP values than the control group. (D) An additional LTP test was carried out for control (black circles; n=10) and for rats fed for 5 weeks with 2′-FL (white circles; n=10). Animals were stimulated for two successive days with the HFS protocol. Recordings were carried out for 30 min after the two HFS (arrow) protocols (Days 1 and 2). Additional recordings were carried out for 15 min for three additional days (Days 3–5). The 2′-FL group presented significantly larger and longer-lasting LTP values than the control group (*P≤.05). At the top of C and D, selected fEPSPs recorded at the indicated times from control and 2′-FL-treated rats are illustrated.

Tablet, 45mg; Test Diet, Richmond) were delivered by pressing a lever. Before training, rats were handled daily for 7 days and food-deprived to 80%–85% of their free feeding weight. Once the desired weight was reached, animals were placed in the Skinner box for 20 min and allowed to press the lever to receive pellets from the food tray using a fixed-ratio (FR; 1:1) schedule, until reaching criterion. The selected criterion was to press the lever up to 40 times/session and to repeat the same rate during the following session. Animals were allowed a maximum of 10 days to reach the criterion. The start and end of each session were indicated by a tone (2 kHz, 200 ms, 70 dB) provided by a loudspeaker located in the recording chamber (Fig. 4A, B).

Conditioning programs, lever presses and delivered reinforcers were controlled and recorded by a computer, using a MED-PC program (MED Associates).

2.8. Immunohistochemistry protocols

For immunohistochemical procedures, rats were fed control or 2′-FL diets for 5 weeks. Five control and five treated rats were deeply anesthetized (ketamine: 100 mg/kg body weight, i.p.; and xylazine: 5 mg/kg body weight, i.p.), transcardially perfused with 0.01 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Their brains were removed and postfixed 4 h in the same fixative, and cryoprotected with 30% sucrose in 0.1 M phosphate buffer. The brains were embedded in O.C.T. medium (Sakura Finetek) and frozen in 2-methylbutane prechilled in liquid nitrogen. Serial rostrocaudal sections (40 μm) were cut on a cryostat.

Free-floating sections were incubated for 4 h in PBS containing 0.1% Triton X-100, and then in rabbit anti-PSD-95 (1:750; Abcam) antisera diluted in PBS containing 0.2% Triton X-100, overnight at 4°C. After several rinses in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (1:100; Pierce) and processed by the avidin–biotin peroxidase complex procedure (Pierce). The peroxidase activity was demonstrated following the nickel-enhanced diaminobenzidine assay.

Control procedures were carried out on adjacent sections of the same tissues. No immunolabeling was detected when the primary antibody was either omitted or replaced with an equivalent concentration of preimmune serum. Sections were then mounted on slides, dehydrated and covered using DPX (Fluka). Sections were imaged by an Olympus BH-2 microscope and acquired using a Nikon DS-Fi1 camera.

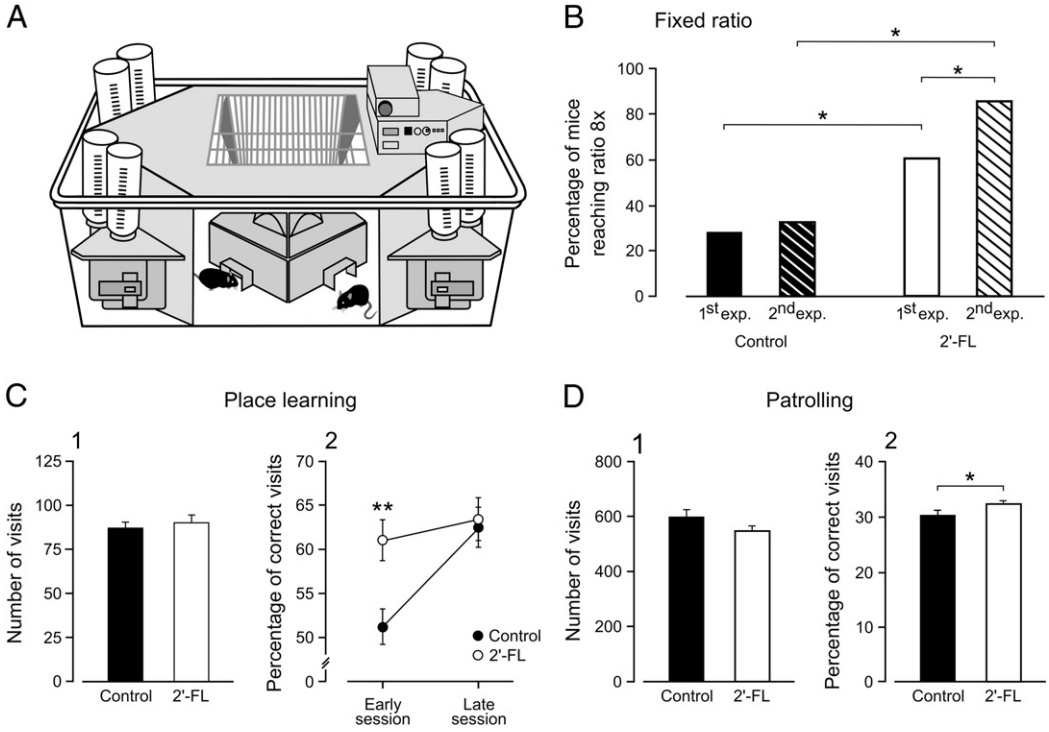

Fig. 3. Chronic administration of 2′-FL to mice potentiates performance in the IntelliCage. (A) Diagram of the IntelliCage, showing the recording chambers, water bottles and mouse shelter. Mice (n=28 per group) were placed in the IntelliCages and their behavioral responses were monitored in an automated manner. Following an adaptation period to the new environment, different protocols specified in the Methods Section 2.6 were carried out. (C1) During the place learning test, no differences were recorded in the total number of visits between the two groups. (C2) The percentage of correct visits in the early session was significantly higher in the 2′-FL group. (D1) During the patrolling test, there were no differences in the total number of visits between the two groups. (D2) The percentage of visits to the active rewarded corner during the 4-day learning sessions was significantly higher in the 2′-FL group. (B) Percentage of mice reaching the fixed ratio 8×. First (plain bars) and second (striped bars) experiments were performed with a 6-day extinction period between both. The 2′-FL group performed significantly better than the control group in both experiments. *Denotes statistical significance between control (black) and 2′-FL diet (white). *P<.05; **P<.01; ***P<.001.

2.9. Western blots procedures and BDNF quantification

For biochemical techniques, animals (10 fed control and 10 fed 2′-FL diet for 5 weeks) were killed by cervical dislocation to avoid interferences of anesthetics with the brain biochemistry; frontal cortex, striatum and hippocampus of rat brains were dissected on ice, flash-frozen with liquid nitrogen and stored at −80°C. The tissue samples were homogenized in 1:3 (wt/vol) homogenization buffer consisting of 30 mM Tris–HCl (pH 7.4) containing 1% NP-40, 0.5 mM phenymethylsulfonyl fluoride, protease and phosphatase inhibitors, and centrifuged for 40 min at 30,000g. All procedures were performed at 4°C. The protein concentration was determined by the Bradford method [28].

Equal amounts of protein extract were loaded and separated on 4% to 12% polyacrylamide gradient gels (Invitrogen). Proteins were transferred to PVDF membranes. Membranes were blocked and incubated overnight at 4°C with diluted policlonal anti PSD-95 (1:1500, Abcam) and anti-p-CaMKIIα (Thr 286) (1:600, Santa Cruz Biotech.) in blocking solution. Bound antibody was revealed by means of an enhanced chemiluminescence kit (ECL plus; Amersham). After immunodetection, membranes were probed with anti-α-tubulin (Sigma) as a loading control. The relative amount of the proteins in each sample was quantified by densitometric scanning.

Total BDNF protein level was determined in the homogenates using the commercial ELISA kit (Abnova) according to the manufacturer's instructions. Results were expressed as pg/mg of protein.

2.10. Statistical analysis

The normality of data distribution and homogeneity of variances was confirmed in each set of data before applying the different statistical tests.

2.10.1. LTP experiments and Skinner box tests

Hippocampal activity and 1-volt rectangular pulses corresponding to stimulus presentations were stored digitally on a computer through an analog/digital converter (CED 1401 Plus), at a sampling frequency of 11–22 kHz and an amplitude resolution of 12 bits. Commercial computer programs (Spike 2 and SIGAVG from CED) were modified to represent extracellular synaptic field potential recordings. Data were analyzed offline for quantification of fEPSP slopes with the help of homemade representation programs [21]. The slope of evoked fEPSPs was collected as the first derivative (i.e., mV/s) of fEPSP records (mV). For this, five successive evoked field synaptic potentials were averaged, and the mean value of the slope was determined for the rise time period (i.e., the period of the slope between the initial 10% and the final 10% of the evoked field potential). Unless otherwise indicated, data are presented as the mean value collected from each experimental group followed by the SEM. Graphic displays were constructed with the help of the SigmaPlot 8.0.2 program (Systat Software, Inc.). Statistical differences between experimental groups and their corresponding control were determined with the help of the SigmaStat 3.0.1 package (Systat Software, Inc.). In particular, possible differences between groups in the Skinner box tests were determined with the help of two-way repeated means of the analysis of variance, with the session (days) or the trial being the repeated means factor. The significance level was established at P=.05 for all tests.

2.10.2. IntelliCage statistical analysis

Data are expressed as the mean value±S.E.M. Differences between the two groups were analyzed by Student's t test. A chi-square test was performed to analyze differences when data were shown as categories. Analyses were conducted using Prism 5 for windows 5.02 (GraphPad Software). Differences were considered significant when P<.05.

Table 1
Summary of IntelliCage modules used for behavioral characterization of mice as described in the Materials and Methods section

Order	Module	Duration (number of days)
1	H-FE	3
2	NP	2
3	NP-DS	2
4	PL-DS	3
5	EP-DS	4
6	P-DS	4
7	Extinction of place preference during drinking sessions	3
8	FR-DS 1st Exp	1
9	EP-DS	6
10	FR-DS 2nd Exp	1

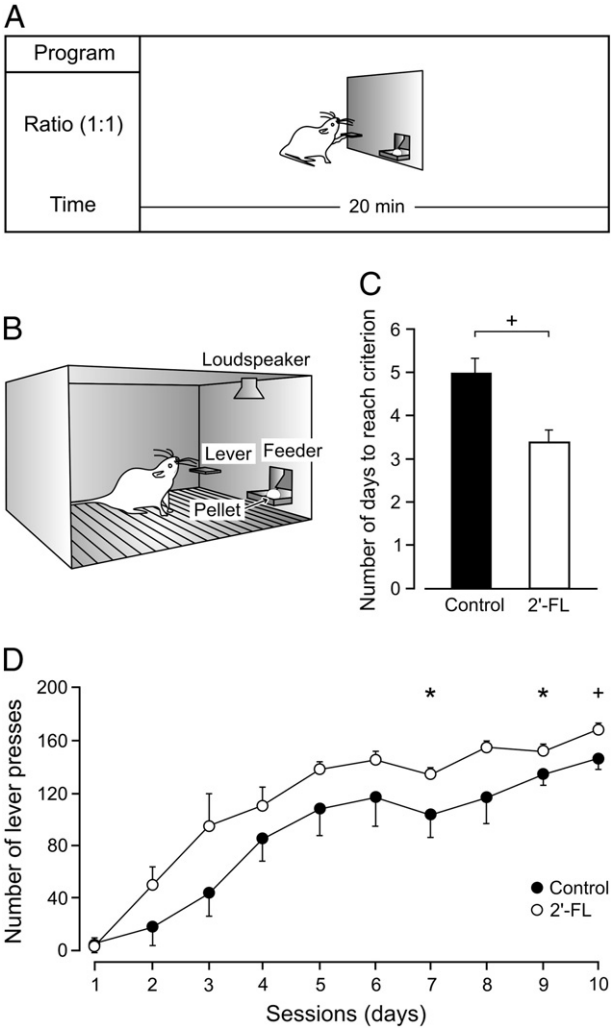

Fig. 4. Chronic administration of 2′-FL potentiates the acquisition of an operant conditioning task in behaving rats. (A, B) Rats (n=10 per group) were trained to press a lever to obtain a food pellet using an FR (1:1) schedule. In this situation, animals press the lever once to obtain a pellet of food. A tone provided by a loudspeaker indicated the beginning and end of the session. Each session lasted for 20 min. (C) 2′-FL-treated (6 weeks) rats reached the criterion in fewer sessions (*P=.053) than the control group. The selected criterion was to press the lever up to 40 times/session and to repeat the same rate during the following session. (D) Training in the Skinner box was maintained for up to 10 days. In this situation, 2′-FL rats performed more lever presses than their littermate controls (*P≤.05).

2.10.3. Histological and biochemical data statistical analysis

For statistical analysis, Student's t test was performed to evaluate the significance of differences between groups, accepting P<.05 as the level of significance.

3. Results

Since the experimental animals were kept in groups during the study, individual food intake measurements could not be done. However, previously we performed a preliminary pilot study in order to check the average intake of animals fed on a 2′-FL diet or control diet and no significant preferences were detected (2.71±0.72 and 2.63±0.79 g of daily intake for the control and 2′-FL supplemented groups, respectively). In addition to that, no differences were found in the body weight of the animals from the 2′-FL group with respect to the control group in any experiment.

3.1. Chronic administration of 2′-FL improves input/output curves and LTP evoked at the hippocampal CA3–CA1 synapse of alert behaving mice

We studied functional properties of the hippocampal CA3–CA1 synapse in control adult mice and in mice fed the 2′-FL diet in 12 weeks. For input/output curves, the two groups of animals (n=8 per group) presented a steady increase in fEPSP slopes in relation to the increased stimulus intensity (range 0.02–0.4 in steps of 0.02 mA; Fig. 1C). As expected, the increase in fEPSP slopes presented sigmoid-like shapes in the two groups of mice [29,30]. Nevertheless, the input/output curves evoked in 2′-FL mice presented a significantly ($F_{(1,7)}$=0.716; P≤.01) steeper increase than that evoked in the control group (Fig. 1C), suggesting greater excitability evoked by 2′-FL administration.

We also examined the evolution of fEPSPs evoked at the CA3–CA1 synapse by single pulses presented before and after an HFS session in the two groups (n=8 animals per group; Fig. 1D). The aim of this experiment was to determine whether this form of in vivo LTP was enhanced by the chronic administration of 2′-FL. Stimulus intensity was set at 35% of the intensity necessary to evoke a maximum fEPSP response for HFS and for both baseline and post-HFS recordings. The HFS session induced a LTP potentiation of fEPSPs evoked by the single pulses presented to the CA3–CA1 synapse in the control group ($F_{(1,7)}$=3.077; P≤.05). LTP lasted less than 24 h in control mice, since the test carried out 24 h after the HFS session did not present any significant difference with baseline records. In contrast, LTP observed in the 2′-FL group reached larger values than that evoked in controls (P≤.05) and lasted for more than 3 days (Fig. 1D). As a whole, these results indicate that chronic administration of 2′-FL causes a larger and longer-lasting LTP response at the hippocampal CA3–CA1 synapse.

3.2. Administration of 2′-FL improves input/output curves and LTP evoked at the hippocampal CA3–CA1 synapse of alert behaving rats

We repeated the set of experiments described above in alert behaving rats to check the effects of 2′-FL administration in a different animal species as well as for shorter periods of time (6 weeks of dietary treatment) or even given acutely by oral gavage.

Even the acute administration of 2′-FL by oral gavage in 3-month-old rats (n=8 per group) evoked an increase in the excitability of hippocampal synapses. As illustrated in Fig. 2B, the use of single pulses of increasing intensities (in steps of 0.02 mA) evoked fEPSP of increasing amplitude at the hippocampal CA3–CA1 synapse. For the sake of simplicity, the maximum mean value collected before 2′-FL administration (black circles, Fig. 2B) was represented as a 100% value. The rest of the collected fEPSP amplitudes were adjusted to this percentage. As illustrated in Fig. 2B, the amplitude of fEPSPs evoked at the CA3–CA1 synapse was significantly larger ($F_{(1,7)}$=3.293; P≤.05) 180 min after 2′-FL administration by gavage, mainly at low stimulus intensities (Fig. 2B).

Accordingly, 2′-FL administration for up to 6 weeks in two additional groups of rats (n=10 per group) evoked significantly ($F_{(1,9)}$=4.868; P≤.05) larger LTP in the CA1 area than in controls following HFS of Schaffer collaterals (Fig. 2C). The larger LTP evoked in treated rats lasted for less than 24 h, because it returned to control values during the test carried out during day 2 (Fig. 2C). In addition to that, a second LTP study was carried out in another two groups of rats (n=10), which received two HFS within a 24-h interval. In this experiment, administration of 2′-FL evoked not only larger but also longer-lasting LTP in the treated group of rats ($F_{(1,9)}$=3.671; P≤.05), as compared to their littermate controls (Fig. 2D). In addition, we included another group of rats (n=10) fed on L-fucose assessed in parallel with the control and 2′-FL groups (data not shown). Data from these L-fucose treated rats showed that the facilitatory effect of

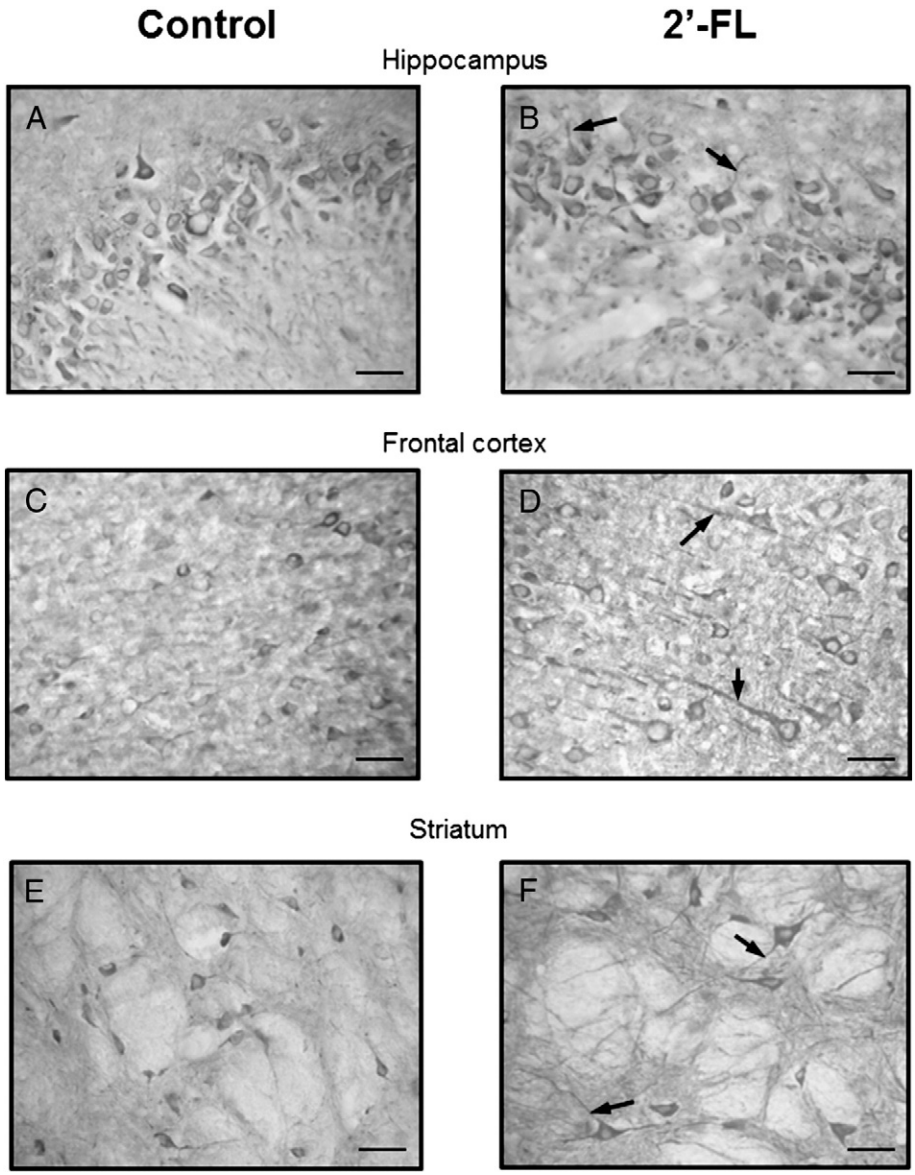

Fig. 5. Chronic administration of 2′-FL increases the expression of PSD-95 in cortical and subcortical structures of treated rats. Following a feeding period of 5 weeks, the expression of the brain marker PSD-95 was examined in immunostained brain sections. (A, B) Hippocampus. (C, D) Frontal cortex. (E–F) Striatum. PSD-95 expression was more intense in hippocampus, cortex and striatum from 2′-FL rats. PSD-95 was detected in soma and dendrites. Pictures were taken at 40×.

dietary 2′-FL on LTP could not be mimicked by L-fucose alone. Thus, the observed changes are specific to intact 2′FL.

3.3. Administration of 2′-FL to mice improves the performance of motor and cognitive tests in the IntelliCage

Mice were adapted to the new environment of the apparatus, and the modules described in Materials and Methods were performed (Table 1).

3.3.1. Place learning

During the early learning session, control and 2′-FL groups did not exhibit differential frequency of corner entries, visiting the corners a similar amount of times (86 in control vs. 87.5 in 2′-FL diet (Fig. 3C1). However, in the same drinking session, the 2′-FL group showed a significant increase (10%) in the percentage of correct visits, compared to the control group (51.2% vs. 61%, P=.0024) (Fig. 3C2). Interestingly, during the late learning session, both groups behaved equally, showing a similar number of both total and correct corner visits (Fig. 3C2).

3.3.2. Patrolling

After 4 days of the patrolling test, the total number of visits was similar in the two experimental groups (Fig. 3D1). However, the percentage of visits to the "correct" rewarded corner was significantly higher in the 2′-FL group compared to controls (30.32% vs. 32.48%, P=.0143) (Fig. 3D2).

3.3.3. Fixed ratio

The module was performed twice with a 6-day extinction period between both tests. During the first test, a significantly higher proportion of the 2′-FL group reached the 8× criterion during the early learning session compared to the control group (60.71% vs. 28.57%, respectively ((χ^2_1, n=56)=5.853, P=.0156). The second time, the facilitating effect of 2′-FL was also found (85.71% vs. 32.14%,

respectively; ((χ^2_1, n=56)=16.60, P<.001) (Fig. 3B). Comparing the behavior of mice during the two fixed ratio experiments, we observed that control mice behaved similarly in both tests. In contrast, more mice from the 2′-FL group reached the 8× criterion the second time the test was performed, with respect to the first test (85.71% vs. 60.71%, (χ^2=4.42 (n=56), P=.0347), respectively (Fig. 3B).

The results of the aforementioned tests clearly indicate that mice fed the 2′-FL-supplemented diet performed better than controls in learning tasks in the IntelliCage system. These data support the positive effects of 2′-FL on learning and memory.

3.4. Administration of 2′-FL improves the performance of rats in a Skinner box task

Rats (n=7 per experimental group) were trained in Skinner boxes to press a lever in order to obtain a small pellet of food (Fig. 4A, B). All of the experimental animals acquired the operant conditioning task with an FR (1:1) schedule (i.e., a food pellet following each lever press). The selected criterion was to press the lever a minimum of 40 times/session for two successive 20-minute sessions. Interestingly, animals treated with 2′-FL (6 weeks) reached the selected criterion in fewer sessions (3.35±0.24 sessions for the 2′-FL group vs. 4.96±0.3 sessions for the control group; P=.053; Mann–Whitney test) than the control group (Fig. 4C). In addition, the 2′-FL group demonstrated a significantly ($F_{(1,7)}$=0.856; P≤.05) better performance than the control group across 10 sessions of the FR (1:1) schedule (Fig. 4D).

3.5. Administration of 2′-FL increases the expression of brain functional markers in rats

In order to analyze the potential effects of 2′-FL intake and its possible positive action on the brain, we studied molecules that may act as markers of synapses and neuronal function. The PSD is a structure composed of both membranous and cytoplasmic proteins localized at the postsynaptic plasma membrane of excitatory synapses, which plays a key role in the synaptic signal transduction [31]. Neurotrophins are proteins closely related to synaptic plasticity, responsible for the activation of protein synthesis locally [32,33]. We selected the neurotrophin BDNF together with two other proteins involved in the LTP process, described as key PSD molecules: the scaffolding protein PSD-95 and the active phosphorylated form of CaMKII [34,35]. CaMKII is a neuronal enzyme that is a well-known effector of calcium- and calmodulin-mediated functions, which is autophosphorylated and rapidly translocated to the PSD or presumed postsynaptic sites after Ca^{2+} influx. Activation of the enzyme is required for LTP since phosphorylation of the AMPA type glutamate receptor, which mediates rapid excitatory synaptic transmission, is correlated with activation and autophosphorylation of the α isoform of CaMKII. Strong evidence identified CaMKII is responsible for molecular memory storage and information processing [36,37].

To investigate whether expression of the selected markers (PSD-95, pCaMKII, BDNF) would be altered following dietary supplementation with 2′-FL, we examined histological and biochemical features in brain samples from rats fed control and 2′-FL diets for 5 weeks. The specific location of the selected biomarkers was analyzed, and we quantified the expression level of the markers in cerebral cortex, striatum and hippocampus.

To determine whether PSD-95 would be differentially expressed in control and 2′-FL-fed rats, we performed immunostaining of brain sections. PSD-95 immunoreactivity was located in neurons distributed within the striatum, hippocampus and, predominantly, cerebral cortex of both experimental groups; however, the signal was enhanced in the 2′-FL diet group. Furthermore, immunolocalization in the 2′-FL group was not only in the neuronal soma but in neurites, as well (Fig. 5B, D, F). Western blot analysis indicated a statistically significant increase of PSD-95 expression in the frontal cortex and hippocampus in the 2′-FL-fed group (Fig. 6A, C, E).

Western blot analysis was also performed to examine expression of pCaMKII. In the hippocampus of rats fed the 2′-FL diet, a statistically significant increase of pCaMKII was observed (Fig. 6B). The frontal cortex or striatum expression showed no differences between the control and 2′-FL groups (Fig. 6D, F).

Finally, we used an ELISA assay to measure the level of BDNF in protein extracts from the cortex, striatum and hippocampus from both experimental groups. The assay detected increased levels of BDNF protein in the striatum and hippocampus of rats fed the 2′-FL-supplemented diet compared to control rats (Fig. 7).

In summary, the 2′-FL diet increased the expression of PSD-95 in the cerebral cortex and hippocampus; it raised protein levels of p-CaMKII in the hippocampus and promoted BDNF expression in the striatum and hippocampus. These results are in agreement with an enhancement of synaptic plasticity in rats fed a 2′-FL-supplemented diet.

4. Discussion

Several studies, including a meta-analysis, have revealed breastfed children exhibit higher intelligence quotients and perform better in intelligence tests than formula-fed children. Differences in the composition of human and bovine milk may be responsible for such differences. HMOs have been postulated as key ingredients for brain development due to their high abundance and variety in maternal milk when compared to bovine milk-based formulas [38]. In particular, 2′-FL is the most abundant HMO in most women's milk [39], while oligosaccharides from cow's milk are mainly sialylated and are present at low levels. Thus, dissecting a role for 2′-FL in cognitive development is of great interest.

The results described in the present study suggest that dietary administration of 2′-FL to rodents has a potentiating effect on hippocampal LTP and also improves performance in various learning tests. As the presence of this oligosaccharide was the only difference in the composition of the diets used, all of the measurable effects can be attributed to 2′-FL. The experiments were done in young adult animals due to the difficulty of measuring some of the variables (particularly behavioral tests and LTP) in pups or weanling rodents. However, this is the first evidence of an active role of 2′-FL on cognitive outcomes, and we considered it significantly enough to be extended to other stages of life.

4.1. 2′-FL effects on LTP evoked at hippocampal synapses

A relatively old report [40] indicated that intrahippocampal administration of L-fucose and 2′-FL had a potentiating effect on the LTP evoked at the perforant pathway-dentate gyrus synapse in freely moving rats. Another study reported that both molecules increased the potentiation of the population spike amplitude and fEPSP after LTP induction in the CA3–CA1 in hippocampal slices from rats [17]. Conversely, L-fucose and 3-FL had no potentiating effects on experimentally evoked LTP [17].

In the present study, we extended these findings to alert behaving mice and rats and showed hippocampal potentiating effects of orally administered 2′-FL. Orally administered L-fucose showed no hippocampal effects.

Among other mechanisms [17,40], injected L-fucose may have an influence on glutamate release at the hippocampal CA3–CA1 synapse [41]. In addition, it has been reported that glycosylation of proteins during a critical time window following HFS of hippocampal slices is necessary for the proper induction and maintenance of the evoked LTP [42]. Similarly, there is evidence supporting a role for fucose and fucosyl derivatives in maintenance of synaptic function. Murrey et al. [43] reported that protein fucosylation regulates synapsin Ia/Ib expression and neuronal morphology in hippocampal neurons and described how other fucosylated sugars,

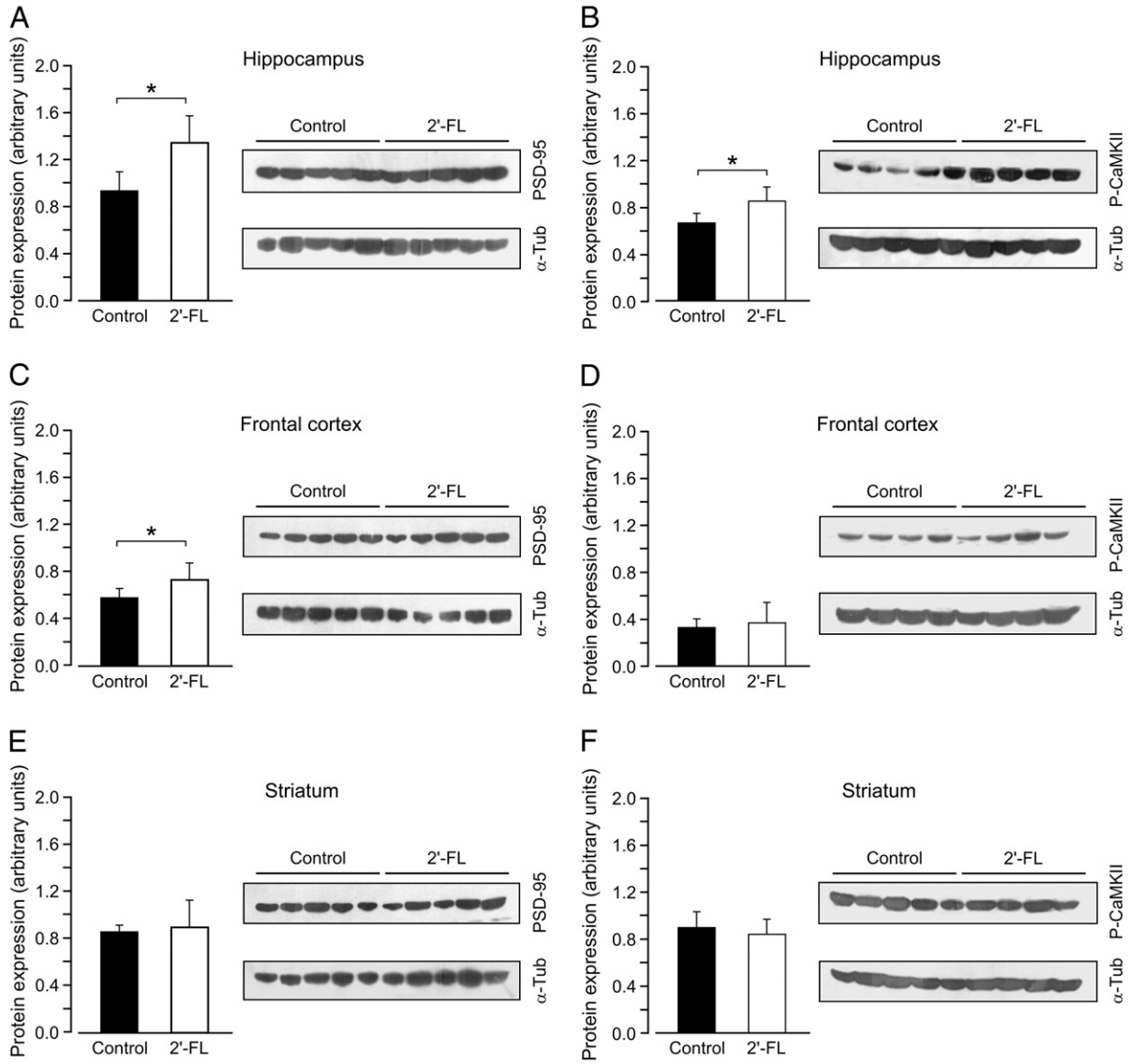

Fig. 6. Chronic administration of 2′-FL increases the expression of PSD-95 and CaMKII in cortical and subcortical structures of treated rats. Following a 5-week feeding period, the expression of brain markers PSD-95 and CaMKII was examined by Western blot. (A, C, E) Expression of PSD-95 in the hippocampus, cortex and striatum. Levels of PSD-95 expression were increased in the hippocampus and cortex of 2′-FL-fed rats. (B, D, F) Expression of CaMKII in the hippocampus, cortex and striatum. CaMKII showed increased levels of expression in the hippocampus of 2′-FL fed rats. Densitometric quantification results, shown at the left-hand side, are the means±S.D. of 10 experimental animals in each group, expressed as a ratio of band intensities of PSD-95 or p-CaMKII relative to α-tubulin. Statistically significant differences from the corresponding control group are shown as * (*P<.05). Representative immunoblots are shown to the right; α-tubulin immunodetection was included as a protein-loading control.

such as fucose-alpha(1-2)-galactose, are involved in neuronal plasticity in mouse olfactory bulbs [12]. However, we have confirmed that ingested L-fucose exerted no effect on LTP, in contrast to previous reports in which it was exogenously added to brain slices or injected into the hippocampus. Interestingly, neither free fucose nor lactose was able to elicit the effect observed for dietary administration of 2′FL. This suggests that molecular integrity of 2′FL is necessary to either directly stimulate elements of the nervous system or indirectly via the microbiota, to induce these effects.

4.2. 2′-FL effects on expression of selected neural function markers

As early as the 1970s, the rapid incorporation of injected L-fucose into neuron glycoproteins and its transport to synapses was demonstrated [11,13,44]. More recently, the epitope Fucα(1–2)Gal has been revealed as a key regulator of neurite outgrowth and synapse formation and maintenance [43,45]. Furthermore, it has been reported that L-fucose increased glutamate release and interfered with competitive glutamate receptor antagonists when applied to hippocampal slices [41].

Considering this former evidence, we decided to test if the ingestion of 2′-FL has any effect on the expression of various key molecules involved in synapse function and neural development. 2′-FL increased the expression of BDNF in the hippocampus and striatum, as well as the amount of cytoplasmic CaMKII in the hippocampus and the expression of PSD95 in the hippocampus and frontal cortex. Regarding this protein, it is worth pointing out that PSD95 was only expressed in the soma from control rats, whereas it was located both in the soma and neuronal prolongations in 2′-FL. The brain area-dependent differences observed in the biomarkers expression could be related to a particular functional effect of 2′-FL in the hippocampus, where LTP was specifically measured and related to some of the tasks such as place.

Although neurological effects of different glycan containing diets have been reported [46], to our knowledge, this is the first time that a

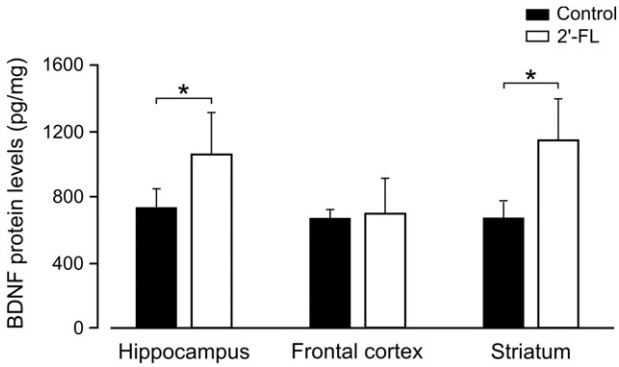

Fig. 7. Chronic administration of 2′-FL increases the protein levels of BDNF in cortical and subcortical structures of treated rats. An ELISA assay was used to quantify the level of BDNF in the cortex, striatum and hippocampus of 2′-FL-fed rats as compared to control (5 weeks of feeding). Both the hippocampus and the striatum from 2′-FL-fed rats showed increased expression of BDNF. Data represent mean±S.D. of 10 animals. Statistically significant differences from the control group are shown as * (*P<.05).

milk oligosaccharide has been shown to modulate the expression of three fundamental molecules in synaptic function in different brain areas related to learning and memory formation. Recently, it has been reported that feeding with non-human prebiotics (FOS, GOS) increased the level of BDNF in rat brains [47]. The authors speculated that prebiotic-induced changes in the intestinal microbiota were responsible for the increased expression of BDNF brain levels. Since microbiota are one of the main components of the brain–gut axis [48], it may be plausible that HMOs modify these brain markers and regulate central nervous system function based on their prebiotic potential.

4.3. 2′-FL effects on learning and memory

Having proven that dietary 2′-FL enhanced LTP and increased the expression of BDNF, CaMKII and PSD95, effects of ingested 2′FL on cognitive skills were assessed. A study in rats previously showed intraperitoneal injection of L-fucose, before training was related to better performance in the shuttle-box avoidance and brightness discrimination task [49]. Sukumar et al. [15] reported an increase in protein fucosylation associated with learning after a passive avoidance task performed in chicks. Later, the same group showed that fucose incorporation was directly associated with engram formation, since no fucose incorporation was observed when the chicks received a shock after training that impaired memory formation, [50].

In the present study, we demonstrated *in vivo* effects of dietary 2′-FL in rats and mice by a classical behavioral test, i.e., Skinner box, and also by the novel Intellicage system. We have shown that 2′-FL intake positively modulated different learning capabilities, accelerated spatial learning after a short feeding (7 days) and increased working memory and associative learning. Although our research on LTP focused on the hippocampus, oral 2′-FL affected not only spatial learning, which is mainly related to hippocampal activity [51], but also working memory and operant conditioning, suggesting that other neuronal circuits outside the hippocampus may be also involved. To our knowledge, this is the first time that an effect of an orally given HMO on learning and memory capabilities has been reported.

4.4. Conclusion and final remarks

In summary, we report here the first evidence for effects of a dietary HMO on brain function and cognition using rodent models. Previous reports regarding the influence of L-fucose and 2′-FL on LTP and learning/memory abilities involved the injection of such molecules, either intraventricularly or intraperitoneally. Conversely, we chose the most natural route of administration and demonstrated clear effects of dietary 2′-FL on LTP, molecular brain function marker expression, and learning and memory; dietary L-fucose showed no measurable effects.

2′-FL is the most abundant oligosaccharide in human milk. HMOs have been ascribed defensive roles in the infant intestine by promoting the growth of beneficial bacteria and as antiadhesive antimicrobials [52,53]. It is well known that HMOs are resistant to hydrolysis in the gastrointestinal tract, but a function exerted outside the intestine had never been shown. Beyond the prebiotic role of HMOs and the influence of microbiota in the brain–gut axis, it has been recently shown that fucosylated HMOs (such as 2′-FL) diminish colon motor contractions in an *ex vivo* model [54], revealing a direct stimulus of enteric nerves by fucosylated HMOs, although the authors also suggest that they may stimulate the brain via the vagus nerve. This result opens a new window of potential effects of HMOs on the nervous system.

Despite the fact that 2′-FL has been found in the urine of breastfed babies [18,55,56], which suggests it is absorbed and transported through the bloodstream without modification, there is no published report confirming the presence of 2′-FL in human plasma. Recently, our group detected 2′-FL in the plasma of rats after oral administration [57], suggesting that a mechanism of export from the intestine into the circulation does exist. However, its presence in the brain has not been reported yet nor a mechanism to cross the blood–brain barrier. Consequently, although the influence of dietary 2′-FL on the enhancement of cognitive abilities has been unambiguously demonstrated in the present work, the mechanism of action has not been elucidated.

Conflict of interest

Authors labeled with [1] and [5] are employees of Abbott Laboratories.

Acknowledgments

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