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Role of cannabinoid CB1 receptors in modulation of dopamine output in the prefrontal cortex associated with food restriction in rats

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Abstract. An extensive body of literature has documented the importance of endocannabinoid system in the regulation of appetite and feeding behavior in mammals. The cannabinoid CB1 receptor is abundant in the prefrontal cortex (PFC), which plays key roles in working memory and reward. Principal dopaminergic afferents arising from the ventral tegmental area(VTA) are crucial for PFC function and dopamine type 2 (D2) as well as CB1 receptors are co-expressed on ?-aminobutyric acid (GABA)–containing terminals in the PFC. To elucidate the role of CB1 receptors in the regulation of dopamine release in the PFC associated with feeding behavior in rats, we exposed Sprague-Dawley rats to a restricted food regimen, with availability of food limited to a 2-h period daily for 3 weeks. Control rats were given ad libitum access to food. Microdialysis, Western-blot as well as patch clamp experiments were performed. Food-restricted (FR) rats showed a marked increase in the extracellular dopamine concentration in the PFC 80 min before food presentation, with the concentration peaking during food consumption and returning to baseline after food removal. These changes were attenuated by the CB1 antagonist SR141716A but were unaffected by the agonist WIN 55212-2. Patch clamp experiments performed in principal medial prefrontal cortex (mPFC) neurons revealed that during the anticipatory phase before food presentation there is a significant decrease of the inhibitory effect of WIN 55212-2 on the frequency of GABAergic spontaneous inhibitory post-synaptic currents (sIPSCs) in FR rats compared with control animals (one-way ANOVA: $p = 0.017$ vs controls). Moreover, endocannabinoid-dependent depolarization-induced suppression of GABAergic inhibition was abolished in FR rats during this phase (one-way ANOVA: $p = 0.001$ vs controls). The basal sIPSC frequency was also reduced in mPFC neurons of FR rats compared with control animals, suggestive of an altered control of presynaptic GABA release (one-way ANOVA: $p = 0.0001$ vs controls). A complex innervations between mPFC and VTA has been previously described by other authors. Briefly, evidence suggests that endocannabinoids may modulate dopamine release in the PFC indirectly by activating CB1 receptors located on presynaptic GABAergic terminals, thereby inhibiting GABA release and resulting in disinhibition of glutamatergic neurons that project to the VTA. An increase in glutamate release in the VTA would stimulate dopaminergic neurons that project back to the mPFC and thereby increase the release of dopamine in this region. Current-clamp recordings revealed an increased excitability in both mPFC and VTA neurons of FR animals, relative to control animals, an effect that well correlates with the decrease of GABA release observed in mPFC inhibitory synapses (t-Test: $p < 0.05$ vs controls). Finally, CB1 receptor expression in the PFC was reduced in FR rats before food presentation compared with controls accordingly with other previews reports. Together, these data support a role for the endocannabinoid system in regulation of dopamine release in the PFC, and they suggest that the feeding-associated increase in dopamine output in the PFC of FR rats might be due to down-regulation of CB1receptors in this brain region that in turn regulates the function of the whole meso-cortical neuronal circuitry. The authors declare no competing financial interest.

Figure 1. Effects of food restriction and CB1 ligands on extracellular dopamine concentration in the rat mPFC. Rats were trained to consume their daily meal within a 2-h period (11:00 to 13:00 hours) (circle symbols), or fed ad libitum (triangle down symbols), and microdialysis samples were collected from the mPFC before, during, and after food presentation. Animals also received an acute administration of vehicle (open symbols), WIN 55212-2 (5 mg/kg, i.p., closed symbols) (a), or SR141716A (1 mg/kg, i.p., closed symbols)(b) at 40 min before food presentation. Data are expressed as a percentage of basal values and are means ± SEM for at least four rats per group. *P < 0.05, **P < 0.01 versus basal values; #P < 0.01 versus corresponding vehicle value.

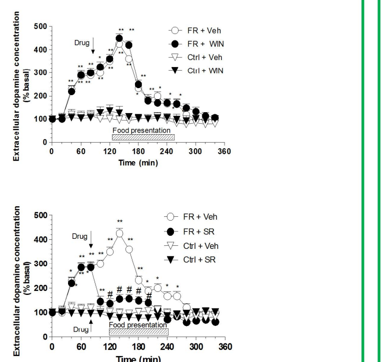


Figure 2. Effects of food restriction on basal sIPSCs and endocannabinoid-dependent DSI in mPFC neurons. (A) Representative traces of sIPSCs recorded from voltage-clamped (–65 mV) mPFC neurons in brain slices from FR rats at various times relative to food presentation. (B, C) Summary of sIPSC amplitude and frequency, respectively, measured for the indicated numbers of cells from control and FR rats as in (a). Data are means ± SEM. *P < 0.05 versus control rats. (D) Depolarization (5 s)–induced reduction in sIPSC frequency (DSI) in mPFC neurons from control rats. (E) Lack of effect of the DSI protocol on sIPSC amplitude in mPFC neurons of control rats. (F) DSI recorded in mPFC neurons from FR rats at 5 min before food presentation. (G) Summary of the percentage change in sIPSC frequency from baseline induced by depolarization in mPFC neurons from control rats or FR rats at different times relative to food presentation. Two-way ANOVA and Bonferroni post-hoc test, *P < 0.05; ***P < 0.0001 versus control.

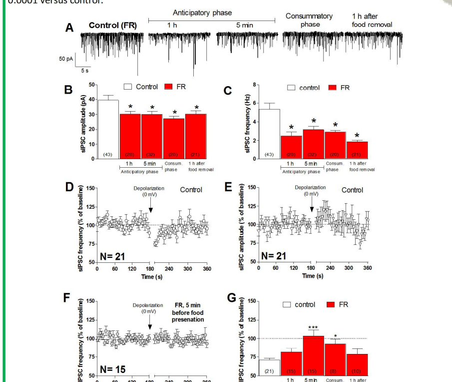


Figure 3. Effects of food restriction on suppression of sIPSC frequency in mPFC neurons by WIN 55212-2, baclofen, or quinpirole. (A) Effect of 5 μM WIN 55212-2 and subsequent coapplication of 1 μM SR141716A on sIPSC frequency in mPFC neurons from control animals. Data are means ± SEM. (B) Effect of 5 μM WIN 55212-2 on sIPSC frequency in mPFC neurons from control rats and FR rats at 5 min before food presentation. Data are means ± SEM. (C) Summary of the percentage change in sIPSC frequency from baseline induced by WIN 55212-2 in mPFC neurons from control and FR rats tested at different times relative to food presentation. Data are means ± SEM for the indicated numbers of neurons. Two-way ANOVA and Bonferroni post-hoc test, **P < 0.01 versus control. (D, E) Effects of 10 μM baclofen and 1 μM quinpirole, respectively, on sIPSC frequency in mPFC neurons from control and FR rats at 5 min before food presentation. Data are means ± SEM. (F) Summary of the percentage change in sIPSC frequency from baseline induced by WIN 55212-2, baclofen, or quinpirole in control and FR rats at 5 min before food presentation. Data are means ± SEM. *P < 0.05 versus baseline, #P < 0.05 versus corresponding control.

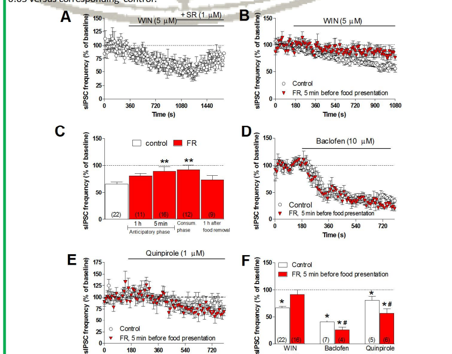


Figure 4. Effect of food restriction on CB1 receptor expression in the mPFC (western blot and immunofluorescence experiments). (A) Protein extracts prepared from the mPFC of control or FR rats at the indicated times relative to food presentation were subjected to immunoblot analysis with antibodies to CB1 and to GAPDH. Representative blot as well as quantitation of the CB1/GAPDH ratio (means ± SEM for 5 to 10 rats) are shown. *P < 0.001 versus control rats. (B) Effect of food restriction on the amount of CB1 receptors in mPFC obtained by confocal images of CB1 receptor (CB1R green, nuclei blu) and relative histogram, *P < 0.001 versus control rats. (C) Confocal images of CB1 receptor (green) localizzazion in CCK positive neurons (red, nuclei blu) and relative histogram, *P < 0.001 versus control rats; (D) Semiquantitative determination of the abundance of the cluster GAD65/CB1 receptor as determined by image analysis of the immunohistochemistry data. Results are expressed as percentage of change in cluster numbers relative to control rats and are mean ± SEM of values of 5 rats for each experimental group. *P < 0.005; **P<0.001 vs control; image scale bar 10 μm.

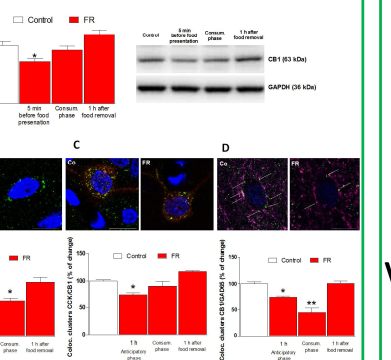


Figure 5. Effect of FR on mPFC and VTA pyramidal neurons excitability. (A) Representative membrane voltage responses to negative (- 80 pA) and positive (140 pA) current pulses applied to single mPFC pyramidal neurons of control and FD rats. (B) Scatter plot representing the quantitative effect of increasing depolarizing current steps on AP frequency in mPFC neurons of control and FR animals. *P < 0.0001, two-way ANOVA. Data are expressed as means ± SEM and were obtained from 8 animals. (C) VTA dopaminergic neurons show a typical Ih current when the membrane is hyperpolarized starting from -100 mV. (D) Representative traces of spontaneous AP firing recorded from single VTA dopaminergic neuron from control and FR rats. (E) Bar graph representing the quantitative effect of FR on spontaneous AP firing recorded in VTA dopaminergic neurons. *P < 0.0001 vs control, unpaired t-Test. Data are calculated from 8 animals, including 16 cells from controls, and 18 cells from FR animals.

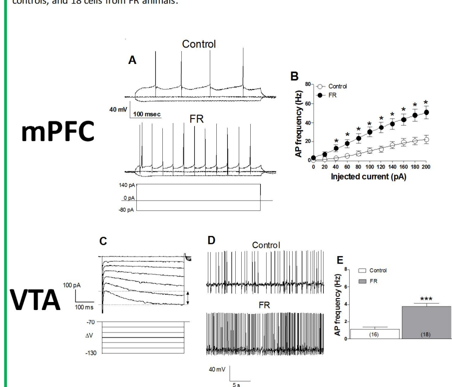
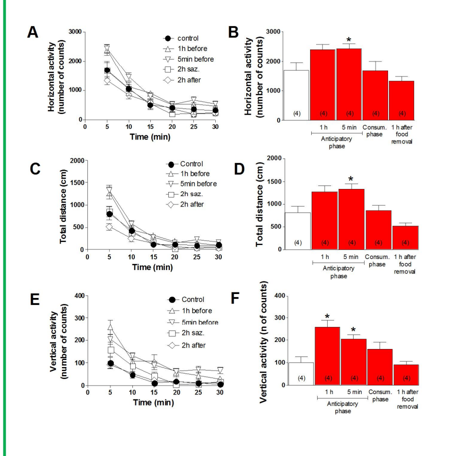


Figure 6. Spontaneous locomotor activity in FR and control animals. Locomotor activity was assessed in a motility meter after 3 weeks of food restriction. The different parameters measured were: horizontal activity (A, B), total distance traveled (C,D), and vertical activity (E, F) that were averaged in bins of 5 min, for 30 min. Data in graphs are means ± SEM (n = 4 per group) of the absolute values of the different measures. *P < 0.05 vs. control animals, one-way ANOVA.



Conclusions

✓The FR paradigm causes a severe increase of DA in rat mPFC relative to food presentation.

✓This effect is strictly correlated to CB1R function on GABAergic terminals.

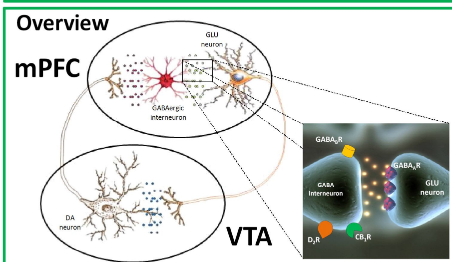
✓The decrease of CB1R is associated with the upregulation of presynaptic receptors such as GABABR and D2R in GABAergic terminals, resulting in a depression of probability of GABA release.

✓The FR-induced decrease of GABA release in mPFC, leads to an increase of the whole mesocortical circuitry with a parallel increase of neuronal excitability on both principal neurons in mPFC and VTA DA neurons.

✓All this changes seem correlated with a general increase of locomotor activity observed in FR animals with respect to controls.

These changes underscore the key role of CB1 receptor signaling in control of the dopamine response to sustained motivated feeding behavior. Our results may provide a basis for the development of strategies that target CB1 receptors for the treatment of obesity and other eating disorders

Overview



Materials and methods.

Animals Male Sprague Dawley CD rats (Charles River, Como, Italy) were bred in our animal facility and maintained under an artificial 12-h-light, 12-h-dark cycle (lights on from 08:00 to 20:00 hours), a constant temperature of 22° ± 2°C, and a relative humidity of 65%. They had free access to water and standard laboratory food at all times until the food restriction regimen was applied. Animal care and handling throughout the experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The experimental protocols were also approved by the Animal Ethics Committee of the University of Cagliari.

Food restriction paradigm Rats with a body mass of 200 to 230 g were assigned either to a control group that received food and water ad libitum or to the FR group. As described previously, FR animals were allowed access to their daily meal (rat food pellets; Standard Diet GLP, Mucedola, Italy) only from 11:00 to 13:00 hours, whereas water was available ad libitum. Body weight and food consumption were measured daily during the 3-week period in which the food restriction regimen was applied. Control animals consumed a constant amount of food (24.6 ± 0.3 g/day) throughout the experimental period. The amount of food consumed by FR animals increased gradually to achieve a constant level (21.7 ± 0.5 g/day) within 10 to 14 days that still significantly (P < 0.05) smaller than that for control rats. Even though rats of both groups initially had a similar body mass, the body mass of FR animals was reduced compared with that of control rats during the initial 4 days as a result of their habituation to food estriction; the rats of both groups subsequently showed similar rates of body mass gain.

Microdialysis and drug treatment Rats were anesthetized by intraperitoneal (i.p.) injection of chloral hydrate (0.4 g/kg), and a concentric dialysis probe was inserted at the level of the mPFC (A +3.2, ML +0.8, V –5.3 relative to the bregma) or nucleus accumbens shell (A +2.2, ML +1, V -7.8) according to the Paxinos atlas47. The active length of the dialysis membrane (Hospal Dasco, Bologna, Italy) was restricted to 4 mm for PFC and 2 mm for NAC. Experiments were performed in freely moving rats ~24 h after probe implantation to allow recovery from surgery procedures. Ringer's solution [3 mM KCl, 125 mM NaCl, 1.3 mM CaCl2, 1 mM MgCl2, 23 mM NaHCO3, 1.5 mM potassium phosphate (pH 7.3)] was pumped through the dialysis probe at a constant rate of 2 μl/min. Samples of dialysate were collected every 20 min from 8:30 to 15:00 hours and immediately analyzed for dopamine by HPLC with electrochemical detection, as previously described48; the detection limit for dopamine was 2 fmol per injection. The average neurotransmitter concentration in the first two samples was taken as 100%, and all subsequent values were expressed as means ± SEM relative to the basal value.

The mean in vitro recovery of the probes was 15 ± 3%. All probes were tested before implantation, and those with a recovery value outside of this range were not used. The absolute concentration of dopamine was not corrected for this value. At the end of each experiment, the placement of the probe was verified histologically. All rats in which the probe was located outside of the target region were excluded from the analysis. Rats were subjected to acute or long-term (twice a day for 21 days) treatment with SR141716A (1mg/kg, i.p.) dissolved in ethanol/Tween 80/saline (1:1:16, v/v/v). WIN 55,212-2 was dissolved in distilled water containing one drop of Tween 80 per milliliter and administered acutely at a dose of 5 mg/kg (i.p.). Both drugs were administered in a volume of 3 ml/kg, and an identical volume of vehicle was administered as a control.

Preparation of PFC and VTA slices and whole-cell patch-clamp recordings. Coronal and horizontal slices containing the mPFC and the VTA, respectively, were prepared from control and FR animals at various time points relative to the 2-h feeding period (60 and 5 min before food presentation; at the end of the consumatory period; and 1 h after food removal) as previously described for the hippocampus.

Whole-cell recordings from mPFC neurons were performed as previously described for the hippocampus. In brief, spontaneous GABA type A (GABAA) receptor–mediated ISPCs (sIPSCs) were recorded in the presence of the ionotropic glutamate receptor antagonist kynurenic acid (1 mM) with the use of an Axopatch 200-B amplifier (Axon Instruments, Sunnyvale, CA, USA), and they were filtered at 2 kHz and digitized at 5 kHz. Off-line analysis of sIPSCs was performed with Minianalysis 6.0 software (Synaptosoft, Decatur, GA, USA). For application of the depolarization-induced suppression of inhibition (DSI) protocol, we recorded sIPSCs under control conditions for 3 min and then imposed a depolarization step from –65 to 0 mV on the postsynaptic neuron for 5 s. DSI was calculated from the sIPSC amplitudes and frequencies measured before (baseline) and after the depolarization step. In a different set of experiments, neuronal excitability was studied in the current-clamp mode by evaluating spontaneous and evoked action potential (AP) firing rate in VTA and mPFC neurons, respectively. DA neurons in the VTA were identified by the presence of an Ih current, as previously reported, which was evoked by incrementally hyperpolarizing (steps of 10 mV) the membrane from an holding potential of - 70 mV.

Immunoblot analysis At the end of the food restriction protocol, rats were killed for immunoblot analysis. The brain was rapidly removed, and the mPFC was dissected on ice, weighed, and stored at –8 80°C until analysis. Total protein was extracted from the tissue with the use of a kit (Membrane and Cytoplasmatic 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). The extract (40 μg of protein in 15 μl) was incubated for 10 min at 70°C and then fractionated by 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), after which the membrane was exposed to 5% nonfat dried milk in Tris-buffered saline containing 1% Tween 20 (TBS-T) before an over-night incubation for at 4°C with rabbit polyclonal antibodies to CB1 (1:500) (Calbiochem, Merk-Millipore, Milan, Italy) and mouse monoclonal antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000) (Millipore) 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% nonfat dried milk and 0.5% Tween 20, after which immune complexes were detected with Luminata Crescendo and Forte substrates (Millipore). The intensity of immunoreactive bands was quantitated with the use of a Geliance 600 Chemi Imaging System (Perkin Elmer, Monza, Italy) and GeneTools software (Perkin Elmer). The intensity of CB1 bands was normalized by that of the corresponding GAPDH band.

Tissue preparation and immunofluorescence

Rats were deeply anesthetized with Equithesin (1 g sodium pentobarbital, 4.251 g choral hydrate, 2.125 g MgSO4, 12 ml EtOH, 43.6 mL propylene glycol, adjusted to a total volume of 100 ml with distilled water) at 0.3 ml/g and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, at 1 hr and 5 min beforefood presentation, during eating phase and 1 h after food removal. Brains were then quickly removed and post-fixed for 4 h with 4% paraformaldehyde. After thorough rinsing, brains were cryoprotected in 20% sucrose overnight at 4°C. The day after sagittal sections of 50 μm thickness were obtained with a vibratome (Leica Microsystems, Milan, Italy) and maintained in antifreeze solution (30% ethylene glycol, 20% glycerol and 50% 0.05 M phosphate buffer v/v) at - 20°C. Indirect immunohistochemistry was performed with free-floating sections as follows: (Method 1, double labeling): the sections were washed with PBS, permeabilized for 1 h with 0.1% Triton X-100 in PBS (PBS-T), incubated for 1 h with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) in PBS-T and incubated overnight with CCK primary antibody. The sections were then rinsed in PBS-T and incubated with CY3-conjugated polyclonal donkey anti-rabbit antibody for 2 h at RT (1:1500 in PBS-T; Millipore). The sections were washed several time in PBS-T, reincubated for 1 h with 10% of goat serum in PBS-T and incubated overnight at 4°C with CB1 receptor primary antibody. The day after the sections were washed several times and incubated for 1 h with CY2-conjugated polyclonal goat antirabbit antibody for 2 h a RT (1:750 in PBS-T; Millipore). After several washing in PBS-T, nuclei were counterstained with 1 μg/ml Hoechst 33342 in PBS for 30 min, rinsed again in PBS and coverslipped with gelatin-glicerol mounting medium (Sigma-Aldrich, Milan, Italy). (Method 2, triple labeling): serial sections were used 10 and processed as described for method 1. After permeabilization with 0.1% Triton X-100 and incubation with normal donkey serum, the sections were incubated with a combinations three primary antibodies (anti CB1 receptor, anti-parvalbumin and anti GAD 65) raised in different species. The sections were then rinsed in PBS-T, incubated with the appropriate secondary antibodies, raise either in goat or donkey, conjugated to one of the following fluorophores cyanine-derived Cy2 (1:750 in PBS-T; Millipore), Cy5 (1:1500 in PBS-T; Millipore) and Dylight 594 (1:1500 in PBS-T; Jackson Immunoreasearch, West Grove, PA), incubated with Hoescht 33342 and coverslipped with gelatin glycerol mounting medium (Sigma-Aldrich, Milan, Italy). Control sections not exposed to primary antibodies did not yield positive staining (results not shown). **Confocal microscopy and data analysis** Acquisitions were carried out using a Leica TCS SP5X Inverted Supercontinuum Confocal Laser Scanning microscope (Leica Microsystems, Heidelberg, Germany) equipped with a diode 405 laser and a white laser and using the multichannel acquisition mode to avoid fluorescence crosstalk. Images (2048 x 2048 pixels) were obtained with a Plan Apo 63X oil immersion objective NA 1.40 at a magnification of 1.4 x 10-2 μm2/pixel and the pinhole set at 1 Airy unit, and then processed with the image-analysis program images NIH Image J software (http://rsb.info.nih.gov/nih-image). Control images were obtained using sections incubated with secondary antibody alone and /or without antibodies. To determine the density of CB1 receptor and their colocalization with CCK or GAD65 proteins, images were first segmented using a threshold that maximized the selection of immunofluorescent puncta over background labeling and then processed with the "colocalization" module, in which a mask is generated from the comparison of two different confocal channels. The number and density of puncta were then calculated with ImageJ (version 1..46a).
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Recording of spontaneous locomotor activity Spontaneous locomotor activity was measured by a motility meter (Omnitech Electronics Inc.) in food restricted (FR) rats at different time points following the three-week period of FR regimen: during the anticipatory phase (1 h and 5 min before food presentation), and after food removal (1 min and 2 h after food removal). A group of rats fed ad libitum were tested as controls. Eight rats were used for each group. On the test day, rats were brought into the test room under standardized environmental conditions and acclimated for 1 h before the beginning of the experiment. They were then individually placed in the test cage. Each cage consisted of a 40X40X30 cm specially designed sound-attenuating shells made of polypropylene and an expanded PVC sheet equipped with two sets of photocells located at right angles to each other, projecting horizontal infrared beams. Animals were isolated from noise of the recorder and printer used to acquire the data by placing these devices in a different room. Horizontal activity (number of photobeam interruptions), total distance traveled (centimeters), locomotion time (seconds), and rest time (seconds) were recorded with Omnitech Electronics, Inc. software. Recordings were performed every 5 min, starting immediately after placing the rat in the cage, over a period of 30 min.

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