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The influence of orexins on ethanol-induced behavioral sensitization in male mice

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HIGHLIGHTS

- Ethanol-induced behavioral sensitization activated orexin neurons in the LH.
- Blockade of orexin 1 receptors prevented expression of behavioral sensitization.
- Orexin system participates in ethanol-induced behavioral sensitization in mice.

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ABSTRACT

Recent evidence indicates the involvement of orexin in reward circuitry and drug addiction. In the present study we evaluated the role of orexin in ethanol-induced behavioral sensitization. In the first experiment, Swiss male mice received seven administrations of saline or ethanol (2.2 g/kg, i.p., chronic), every other day. On the last day of treatment, half of saline-treated mice received a saline injection (saline) whereas the other half received 2.2 g/kg of ethanol (i.p., acute). Behavioral sensitization was assessed by locomotor activity tests and after the last one, immunoreactivity for orexin and Fos (ORX + Fos-ir) was assessed in the lateral hypothalamic area. Chronic ethanol treatment produced behavioral sensitization and a trend for greater ORX + Fos-ir. In the second experiment, mice were treated as in Experiment 1 and type 1 orexin receptor antagonist, SB334867 (20 mg/kg), was administered before the ethanol challenge successfully blocking the expression of sensitization in mice chronically treated with EtOH. These results indicate that orexin plays a role in ethanol-induced behavioral sensitization.

1. Introduction

Orexins are two neuropeptides, Orexin-A and B, derived from the same precursor molecule (pre-pro-orexin) and produced by a few thousand neurons located in the perifornical, lateral (LH) and dorsal hypothalamus [7,32]. These neurons project widely throughout the brain [25] and regulate numerous endocrine functions and motivated behaviors, such as food intake and sleep/wake cycle [10,31]. Interestingly, the LH is related to drug addiction, whereas the perifornical area and dorsomedial hypothalamus regulate arousal and stress response [11]. These neuropeptides bind to two receptors coupled to G protein, with different affinities and distribution in the brain, being orexin 1 receptors (Ox1r) expressed in prelimbic, infralimbic, and insular cortices, bed nucleus of the stria terminalis, ventral tegmental area (VTA) and locus coeruleus [23,39].

A role for these neuropeptides in drug addiction was proposed, based on the initial demonstration of activation of orexinergic neurons in morphine-, cocaine-and food-induced conditioned place preference [11]. Morphine withdrawal increases Fos expression in the LH, which is blocked by SB334867, an Ox1r antagonist [18]. SB334867 also blocks cue-, stress- or orexin-A-induced reinstatement of cocaine- [3,37,41] and nicotine-seeking behavior [27]. Orexins are also involved in EtOH drinking and seeking behaviors [16,19]; intra-VTA infusion of orexin-A increases EtOH intake [34], which is blocked by SB334867 [20].

Behavioral sensitization is widely used to study neural plasticity induced by drugs of abuse [14,33] and is characterized by a progressive increase in locomotor response after repeated administration of the drug [26], resulting in neural plasticity that leads to long-term changes in dopamine-containing neurons that project from the VTA to NAcc and prefrontal cortex [30]. This circuitry

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becomes sensitized to drugs and drug-related stimuli, leading to drug-seeking behaviors [29]. Orexin is also involved in behavioral sensitization to psychostimulants [28,42], since repeated systemic administration or intra-VTA infusion of SB334867 prevents this phenomenon from occurring [2,28,35].

We hypothesized that orexin would be recruited during behavioral sensitization to EtOH. To test this hypothesis we evaluated the number of Fos-positive orexin neurons in the LH in mice submitted to chronic treatment with a stimulant dose of EtOH and the effects of Ox1r blockade on the expression of behavioral sensitization induced by EtOH, by treating mice with the antagonist SB334867 before a challenging administration of drug.

2. Methods

2.1. Animals

Male Swiss albino mice, 3 month-old, weighing 30–50 g (obtained from the Center for Development of Experimental Models in Medicine and Biology – Universidade Federal de São Paulo), housed under standard laboratory conditions of 12 h light/dark cycle (lights on from 7:00 h to 19:00 h), 22–26 °C and 40–70% humidity, were used in all experiments. Food and water were provided *ad libitum*. All studies were conducted in accordance with the strictest ethical principles of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised in 1996. All efforts were made to minimize animals' suffering and to keep the number of animals to a minimum. This project was approved by the Ethics Committee in Research from Universidade Federal de Sao Paulo (CEP 1013/09).

2.2. Drugs

EtOH (Synth®, Diadema, Brazil) solution was prepared with saline (15%, w/v in 0.9% NaCl p/v); SB334867 (Tocris Bioscience®, cat # 1960) was prepared in 10% (v/v) hidroxipropil-beta-cyclodextrin (HPBCD) and 2% (v/v) dimethyl sulfoxide (DMSO).

2.3. Apparatus

All locomotor activity tests were carried out in Opto-Varimex activity cages [model Opto-M3, with acrylic boxes (47.5 cm × 25.7 cm × 20.5 cm); Columbus Instruments, Columbus, OH], which detect locomotion by the interruption of 16 pairs of photoelectrical beams connected to a digital counter.

2.4. Experiment 1: orexin + Fos-ir following induction of behavioral sensitization to EtOH

2.4.1. Experimental procedure

Each mouse was placed in the activity cage (Opto-Varimex) for 15 min, to assess baseline activity. Forty eight hours later, 20 animals received saline 0.9% (SAL, i.p., 0.1 ml/10 g of body weight) and 12 animals received 2.2 g/kg of EtOH solution (15% w/v, i.p.) every other day, for 13 days (chronic group). These mice were tested, every 96 h, in the activity cages, for 15 min, immediately after each administration on days 1 (Test – T1), 5 (T2), 9 (T3) and 13 (T4). On the last day of treatment (T4), nine of the 20 SAL pre-treated mice received a SAL injection (saline group), whereas the remaining 11 mice received 2.2 g/kg of EtOH (acute group). Ninety min after T4, all mice were perfused.

This dose of EtOH was selected from previous studies carried out in our laboratory [1,15].

2.4.2. Tissue preparation and double labeling immunohistochemistry

Mice were deeply anesthetized with a solution containing ketamine (80 mg/Kg, i.p) and xylazine (30 mg/Kg, i.p) and perfused intracardially with 0.9% saline solution followed by 4% formaldehyde in phosphate buffer (PB, Sigma–Aldrich, pH 7.4). They were decapitated and the whole head remained in the same fixative solution for 18 to 24 h at room temperature for fixation. The brains were removed from the skull and kept in 1% formaldehyde at 4 °C until processing of the material. The brains were sectioned on a vibratome at 40 μm thick coronal sections in 1-to-6 serial sections along the rostro-caudal axis.

Enzymatic double immunohistochemistry was performed on free-floating sections, which were blocked for endogenous peroxidase, alkaline phosphatase and biotin, followed by protein blocking. Primary rabbit anti-Fos (1:2500, Santa Cruz biotechnology, Inc, cat # sc-52) was incubated overnight at 4 °C in 0.1% BSA/0.1 M Tris buffer solution. After washing, sections were incubated for 90 min at room temperature with donkey anti-rabbit biotinylated secondary antibody (1:250, Santa Cruz biotechnology, Inc., cat # sc-2089). Sections were then rinsed in Tris buffer and incubated with HRP-streptavidin according to the manufacturer's instructions (DAKO Denmark, A/S, cat # 0397). The antigen-antibody complex (Fos immunolabel) was detected with 3,3′diaminobenzidine (DAB) with metal enhancer rendering a blue reaction product (DAKO, Denmark, cat # S196131). Subsequently sections were incubated with goat anti-orexin-A in 0.1% BSA/0.1 M Tris buffer solution (1:1.000, Santa Cruz biotechnology, Inc., cat # sc-8070) for 2 h at room temperature. After washing, sections were incubated for 90 min at room temperature with donkey anti-goat (1:300 Santa Cruz biotechnology, Inc., cat # sc-3854), followed by incubation with Alkaline Phosphatase according to the manufacturer's instructions (Vector Laboratories Inc., cat # AK-5000). Orexin-A visualization was achieved by incubation with Red Alkaline Phosphatase substrate (Vector Laboratories Inc., cat # AK-5100). After washing, sections were mounted on DAKO glass slides, dehydrated in alcohol and coverslipped. Anatomical regions were defined according to Franklin and Paxinos' mouse brain atlas [9].

Immunoreactivity was quantified by visual cell counting at 40× magnification by an experimenter blind to the group coding. Double labeled neurons were easily identified as dark blue nuclei and brown neuronal bodies (Fig. 2D). All labeled cells in the LH of both hemispheres of each section were counted and averaged for total immunoreactive cells, in a total of six sections (along the rostrocauldal axis) per rat. Values obtained were expressed as number of positively stained neurons.

Digital photomicrographs were taken with a Nikon Eclipse E600 upright microscope equipped with Plan Apo objectives and connected to a Dell workstation computer by the SPOT RT digital camera. Color photomicrographs were transformed into TIFF images with 300 pixels resolution with the aid of Adobe Photoshop CS software, which was also used for figure composition.

2.5. Experiment 2: blockade of expression of EtOH behavioral sensitization with Ox1r antagonist

All mice ($n = 71$) were placed in the activity cages, for 15 min, for assessment of baseline activity. Induction of behavioral sensitization followed the same protocol described previously (SAL group, 0.1 ml/10 g of body weight, i.p.: $n = 36$ and EtOH group, 2.2 g/kg, i.p., 0.1 ml/10 g: $n = 35$). The treatments were withdrawn for a five-days period, after which all animals were submitted to two challenges. In the first one, all animals received SAL (SAL challenge) and were immediately placed in the activity cages for 15 min, to test for hyperactivity conditioned to the environment. Forty-eight hour later, mice received vehicle or SB334867 (10 or 20 mg/kg,