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Research Report

Expression of c-Fos in the mouse Edinger–Westphal nucleus following ethanol administration is not secondary to hypothermia or stress

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Abstract

Restraint stress, lipopolysaccharide (LPS), and ethanol (EtOH) administration have all been found to induce c-Fos in the brain, and to cause hypothermia. The present study was designed to assess whether the c-Fos expression that occurs in the Edinger–Westphal nucleus (EW) after EtOH administration is independent of the hypothermia or any stress effects that occur. To test this, we used restraint stress and LPS in addition to EtOH, and also examined two control areas, the dorsal raphe nucleus (DRN) and the periaqueductal gray (PAG), in addition to EW. Male C57BL6/J mice were used. Groups of mice received intraperitoneal (IP) injections of EtOH (2 g/kg), LPS (600 μg/kg or 50 μg/kg), or saline. A separate group of mice received no injection, but were placed in plastic restrainers for the entirety of the experiment. For all groups, core temperatures were monitored rectally every 30 min for 3 h postinjection, after which, the animals were sacrificed. Then, the number of Fos-positive cells in the brain regions of the EW, DRN, and PAG was quantified. Both EtOH and restraint stress induced a transient hypothermia, where core temperature (T_c) declined immediately and then rose again. Both doses of LPS induced a slower developing, longer lasting hypothermia, while saline had no effect on T_c. Only EtOH induced a significant amount of c-Fos in EW, while both doses of LPS and restraint stress induced c-Fos in DRN, and only restraint stress caused induction in PAG. These data demonstrate that activation of EW after EtOH is unrelated to hypothermia or stress.

Theme: Neural basis of behavior
Topic: Stress

Keywords: Lipopolysaccharide; Restraint; Temperature; Dorsal raphe; Periaqueductal gray

1. Introduction

Alcoholism and alcohol abuse are widespread problems, and the intoxicating and addictive effects of alcohol involve many neural pathways, which remain, as of yet, incompletely defined. The identification and characterization of brain regions that mediate alcohol's effects on the body are important for the understanding of alcohol abuse and the development of treatments for it.

One brain area that is uniquely sensitive to ethanol (EtOH) is the Edinger–Westphal nucleus (EW). The EW has long been thought to be a cholinergic nucleus associated with oculomotor function [24,44]. However, recent evidence suggests that the area broadly defined as the EW can be subdivided into two populations of neurons: one cholinergic that projects to the ciliary ganglion, and one named non-preganglionic EW expressing the peptide urocortin 1 (Ucn1) that projects to several brain regions including the lateral septum, dorsal raphe (DRN), and spinal cord, and may be involved in functions such as temperature regulation, EtOH consumption, and stress [6,7,43]. The nucleus morphologically defined as EW in the mouse consists of exclusively non-preganglionic EW neurons [46]. This nucleus is activated, in mice and rats, as measured by increased c-Fos expression, following both involuntary administration and voluntary consumption of EtOH

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[1,8,33,39,45]. Studies also indicate that the EW may be involved in EtOH-induced hypothermia, as mice selectively bred for differences in EtOH-induced hypothermia differ in the content of Ucn1 in EW [3], and electrolytic lesions of the EW significantly attenuate EtOH-induced hypothermia in C57BL6/J mice, as well as leading to a decrease in the number of Ucn1 terminal fibers in DRN [4], an area known to be involved in thermoregulation [20].

The EW has also been found to be activated in response to stress, administration of the immune stimulatory compound, lipopolysaccharide (LPS), and body temperature changes induced by various means. For example, Kozicz and colleagues [16,25] have found that restraint stress and administration of LPS induced c-Fos in the EW of rats. LPS is often used to produce fever and/or hypothermia in experimental models of inflammation; however, it is also used as a stressor. Varying the ambient temperature around animals also induces Fos in the EW [3]. Moreover, drugs that affect temperature, in addition to EtOH and LPS, also affect Fos expression in this region; for example: morphine [2,35], MDMA [36], and methylphenidate (a dopamine stimulant) [40].

It is often difficult to distinguish temperature effects from stress effects due to the fact that so many stressors also cause temperature changes. Even restraint stress, which is considered a psychological stressor, is known to cause hypothermia [37]. Therefore, since EtOH and LPS administration, and restraint stress are all capable of causing hypothermia, we wanted to examine whether activation of EW following EtOH administration is secondary, due to changes in body temperature or stress. We also wanted to examine c-Fos induction in both the DRN and the periaqueductal gray (PAG) as control brain regions known to respond to changes in body temperature and stress.

2. Materials and methods

2.1. Animals

Male C57BL6/J mice (N = 35) were obtained from Jackson Laboratories (Bar Harbor, ME), and weighed 25–30 g at the time of experimentation. The mice were housed in groups of 5 in standard plastic cages, with mouse chow and water available ad libitum. The vivarium temperature was kept at 19–22 °C, and the light:dark cycle was 12:12 (lights on at 0700 h PST). All tests were conducted during the light phase. All animal procedures were in accordance with National Institutes of Health guidelines for the care and use of laboratory animals.

2.2. Drugs

Lipopolysaccharide (*Escherichia coli* serotype 0111:B4, Sigma Chemical Co., St. Louis, MO) was suspended in pyrogen-free saline at a concentration of either 600 μg/ml or 50 μg/ml. Ethanol used was in a 20% (v/v) solution in pyrogen-free saline.

2.3. Procedure

All testing was conducted in the animals' home cages, and the animals had been accustomed to handling, rectal temperature measurements, and the IP injection procedure prior to testing; however, they had not been accustomed to the restrainers. At 9:00 am, each animal had a baseline temperature taken, and then received an IP injection of LPS (600 μg/kg), LPS (50 μg/kg), EtOH (2 g/kg), or saline (1 ml/kg) (N = 7/group). Another group of mice received no IP injection, but were put in individual restrainers for the duration of testing (N = 7/group). The core temperature (T_c) of each animal was then measured every 30 min for the next 3 h. At the end of the test session, the animals were killed by cervical dislocation, and the brains were removed for immunohistochemical processing. This time point was chosen because c-Fos protein induction is known to reach maximum at about 1 h after neuronal stimulation and last for approximately 2 h [21], and we expected that our treatments would result in hypothermia starting 30 min following treatment.

2.4. Immunohistochemistry

Immunohistochemistry was performed according to previously published protocols [3]. Briefly, brains were post-fixed overnight in 2% formaldehyde in isotonic sodium phosphate buffered saline (PBS) and cryoprotected with successive overnight incubations in 20 and 30% sucrose in PBS with 0.1% NaN_3. Forty micrometer frozen coronal sections were cut on a cryostat (from Bregma level −2.9 to −4.0 mm for EW and PAG; from −4.3 to −4.9 for DRN), according to the Mouse Brain Atlas of Paxinos and Watson and collected in PBS with 0.1% NaN_3. Endogenous peroxidase activity was inhibited by pretreatment with 0.3% hydrogen peroxide. Blocking was performed with 3% goat serum. Anti-rabbit c-Fos-specific primary antibody was used in dilution 1:10,000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreaction was detected with Vectastain ABC kit (Vector Laboratory Inc., Burlingame, CA, USA). Enzymatic development was performed with the Metal Enhanced DAB kit (Pierce, Rockford, IL, USA).

2.5. Data analysis

To analyze the core temperature data, a one-way analysis of variance was performed on mean group core temperatures (area under the curve calculated by the trapezoidal method) in each experiment to determine the main effect of treatment. For c-Fos quantification, 3 brain sections were counted per animal, those that corresponded to plates 59–61, respectively, in the atlas of Franklin and Paxinos [15]; and the mean number of Fos-positive cells among these was