Lesions of the Edinger–Westphal nucleus in C57BL/6J mice disrupt ethanol-induced hypothermia and ethanol consumption

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Abstract
The Edinger–Westphal nucleus (EW) is a brain region that has recently been implicated as an important novel neural target for ethanol. Thus, the EW is the only brain region consistently showing elevated c-Fos expression following both voluntary and involuntary ethanol administration. Ethanol-induced c-Fos expression in the EW has been shown to occur in urocortin I-positive neurons. Moreover, previous reports using several genetic models have demonstrated that differences in the EW urocortin I system are correlated with ethanol-mediated behaviours such as ethanol-induced hypothermia and ethanol consumption. The aim of this study was to confirm these relationships using a more direct strategy. Thus, ethanol responses were measured following electrolytic lesions of the EW in male C57BL/6J mice. Both EW-lesioned and sham-operated animals were tested for several ethanol sensitivity measures and ethanol consumption in a two-bottle choice test. The results show that lesions of the EW significantly disrupted ethanol-induced hypothermia, while having no effect on pupillary dilation, locomotor activity or ethanol-induced sedation. In addition, EW-lesioned animals showed significantly lower ethanol preference and total ethanol dose consumed in the two-bottle choice test. EW-lesioned animals also consumed less sucrose than sham-operated animals, but did not have altered preferences for sucrose or quinine in a two-bottle choice test. These data support previously observed genetic correlations between EW urocortin I expression and both ethanol-induced hypothermia and ethanol consumption. Taken together, the findings suggest that the EW may function as a sensor for ethanol, which can influence ethanol consumption and preference.

Introduction
The neurobiological study of alcoholism has primarily focused on neural systems associated with ethanol’s rewarding components such as the mesocorticolimbic dopamine system and the extended amygdala (Koob & Nestler, 1997; Wise, 1998). However, ethanol also targets neural systems outside these conventional systems (Kiiammar et al., 2003). Actions within these systems may represent modes through which ethanol produces behavioural effects contributing to drug consumption. The Edinger–Westphal nucleus (EW) represents a novel neural target that is uniquely sensitive to ethanol. Chang et al. (1995) first observed that an acute injection of ethanol resulted in elevated c-Fos expression in the EW. Similar findings have been repeatedly shown using a variety of voluntary ethanol consumption models (Topple et al., 1998; Bachtell et al., 1999; Weitemier et al., 2001; Ryabinin et al., 2003).

The EW is a midbrain nucleus often recognized for its role in the oculomotor response where cholinergic neurons relay information to the ciliary ganglion (Burd & Williams, 1989; Trimarchi, 1992; Klooster et al., 1993). However, EW projections to areas such as the lateral septum (LS), dorsal raphe (DR), spinal cord, parabrachial nucleus, periaqueductal grey, reticular formation and cerebellum implicate its position in more complex neural circuits (Roste & Dietrichs, 1988; Spence & Saint-Cyr, 1988; Klooster et al., 1993; Bittencourt et al., 1999). Similarly, areas projecting to the EW include not only the olivary pretectal nucleus thought to play a role in the oculomotor response (Klooster et al., 1995; Klooster et al., 2000), but also hypothalamic nuclei (Saper et al., 1976; Koss, 1986; Zheng et al., 1995) and the locus coeruleus (Loewy et al., 1973; Breen et al., 1983). It was recently discovered that the 40-amino acid peptide, urocortin-I (Ucn I), is preferentially expressed in the EW (Vaughan et al., 1995; Yamamoto et al., 1998; Bittencourt et al., 1999; Weninger et al., 2000). Ucn I is structurally and functionally similar to corticotropin-releasing factor (CRF) and it binds to CRF receptors with equal or greater affinity than CRF itself (Vaughan et al., 1995; Reyes et al., 2001). In addition to the prominent Ucn I projections to the spinal cord, EW Ucn I-containing neurons project to the LS and DR where a high density of CRH-R2 receptors are located (Bittencourt et al., 1999).

Recent work demonstrates that ethanol-induced c-Fos expression occurs within Ucn I cells of the EW (Bachtell et al., 2002; Ryabinin et al., 2003). Ucn I expression in the EW correlates with several ethanol-mediated responses. A positive relationship exists between Ucn I expression in the EW and ethanol-induced hypothermia and ethanol consumption (Bachtell et al., 2002, 2003b). Taken together, these results suggest that Ucn I-containing neurons in the EW are influenced by ethanol intoxication and may regulate ethanol intake. Based on these relationships, it is hypothesized that electrolytic lesions of the EW will diminish the hypothermic response to ethanol and decrease ethanol preference. The aim of the present studies was to test...
these hypotheses in order to confirm a role for the EW with ethanol-induced hypothermia and ethanol consumption.

Materials and methods

Animals

Male C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME) were housed four animals per cage upon arrival. One week following arrival, animals underwent surgery as detailed below. All animals were 7–8 weeks of age at the beginning of the surgical procedure. C57BL/6J mice were selected for these analyses because of their well-characterized behavioural responses (e.g. ethanol-induced hypothermia and ethanol drinking). Animals were maintained on a 12-h light : 12-h dark cycle with lights on at 06:00 h. Water and food were available ad libitum throughout the experiment in the home cage. All animal procedures were in accordance with National Institutes of Health guidelines.

Surgical procedures

Mice were anaesthetized with 4 mL/kg body weight of a cocktail containing ketamine (30 mg/mL), xylazine (3.0 mg/mL), and acepromazine (0.6 mg/mL) for the surgical procedure. Heart rate (30–50 beats/s) and breathing rates (12–18 breaths/s) were monitored throughout the session to assess appropriate anaesthesia level. Mice are known to show extreme hypothermia under anaesthesia. Therefore, rectal temperatures were monitored throughout surgery and a heating pad was used to maintain the animal’s normal temperature (37°C). While anaesthetized, mice were positioned in a stereotaxic apparatus (Model EW40M, Cartesian Research, Inc., Sandy, OR). This apparatus allows for angular insertion of electrodes. Insulated (except for the 500 μm tip) stainless steel electrodes (SNE-300, Rhodes Medical Instruments, Inc, Woodland Hills, CA) were stereotaxically guided into the EW nucleus (A/P, −3.2 mm; M/L, 0.0 mm; D/V, −3.75 mm from Bregma) at a 2° angle through a small hole drilled in the skull (Franklin & Paxinos, 1997). The angled placement was necessary to avoid damaging the midsagittal sinus. The electrode was connected to the positive terminal of a lesion making current device (Model 3500, Ugo Basile, Comerio, Italy). The negative terminal was used to ground the animal via a connection to the animal’s ear. Preliminary experiments have revealed that 5 s of 400 μA current is sufficient to produce a lesion approximately 200 μm in diameter. Following application of electrical current, electrodes were removed, the skin sutured and animals were removed from the apparatus. The electrode was lowered into the EW of sham-operated animals, however, no current was applied. During the immediate postoperative period, animals were kept warm with a heating pad and monitored for unobstructed respiration and appropriate body temperature until the effects of general anaesthesia had dissipated (generally 3 h). Food and fluid intake were monitored daily during the subsequent two days of recovery. Each subject was also monitored for normal signs of alertness and motor activity during the subsequent two days of the postoperative recovery period. Animals were given 1 week to fully recover from the surgery and were then introduced to the first ethanol test (hypothermia).

Ethanol sensitivity measurements

Mice from both sham and lesion groups were tested for various ethanol-related responses including hypothermia, locomotor activation, and sedation. The experiments were designed to minimize the number of used animals and to avoid the potential concern of ethanol tolerance. Thus, we performed the experiments in the reverse order to our previous genetic studies showing EW Ucn contribution to ethanol’s regulation of temperature, but not locomotor activity or sedation (Bachtell et al., 2002). In these previous experiments, locomotor activity and duration of sedation was assessed prior to temperature assessments. Here, temperature studies were performed prior to locomotor activity and sedation studies. Should our lesion studies agree with our previous genetic studies, the concern for tolerance would be eliminated. Finally, the various ethanol sensitivity tests were also spaced with at least 1 week between assessments. To our knowledge, there are no studies reporting tolerance in C57BL/6J mice 1 week following a single ethanol injection. For the sensitivity experiments, two cohorts of 30 male C57BL/6J mice were used. Animals from both cohorts were tested for the hypothermic effects of ethanol. To evaluate these effects, animals were first assessed for baseline rectal temperatures, which was immediately followed by an injection of 3.0 g/kg ethanol (i.p.). Rectal temperatures were subsequently measured at 15 and 30 min postethanol injection.

One week following the hypothermia assessments, pupil measurements were made in all mice. These assessments were performed in a dark room. A fibre optic light, which was positioned 50 mm from the animals nose provided indirect lighting. In preliminary assessments, it was observed that this indirect source of lighting provided optimal visualization of the pupil. An infrared camera, capable of video recording in low light environments, was positioned 50 mm from the animal’s left eye. While the animal was gently restrained by the scruff at the nape of the neck, the pupillary response to the light was recorded for 2–3 s. Pupil diameters were later measured by an observer blind to the animal’s group assignment using the ImageJ software (National Institute of Health, Bethesda, MD, USA). Total area in pixels was used as the dependent variable.

One week after pupil assessments, mice from cohort 1 were then tested for the locomotor effects of ethanol using a two-day procedure. Animals were first habituated to a saline injection and the activity chambers during the first day. On the next day, all animals were injected with 1.8 g/kg ethanol (i.p.) dissolved in saline (20% v/v). On both days, animals were placed into dimly lit activity chambers enclosed in sound attenuating boxes two minutes following the injection. Activity was assessed by an automated photobeam system that included a 10 × 12 array of photocells, situated 1 cm off the floor, surrounding a 21 × 25 × 18 cm Plexiglas arena (San Diego Instruments, San Diego, CA). Activity was assessed in five-minute intervals for a total of 15 min. The distance travelled during each interval was used as a measure of activity.

Two weeks following pupil assessments, animals from cohort two were tested for the loss of righting reflex using a 4.0 g/kg dose of ethanol. Animals were first injected and the time until the onset of loss of the righting reflex (LORR) was measured. The time to recover the righting reflex (two rightings within 30 s) was then recorded. Upon recovery of righting reflex, mice were killed by cervical dislocation and trunk blood was collected for blood ethanol concentration (BEC) measurements. Brains were removed for verification of lesion placement using the thionin staining protocol below.

Several dependent variables resulted from these behavioural assessments. Responses following the 3.0 g/kg dose of ethanol included baseline rectal temperature (°C) and ethanol-induced hypothermia (°C) at the 15 and 30 min time points. The 15 and 30 min time points following the 3.0 g/kg dose were based on the well-characterized hypothermic response in C57BL/6J mice (Crabbe et al., 1994). Locomotor activity is reported graphically in its raw form.
following a saline and 1.8 g/kg ethanol injection. However, statistical analysis of the locomotor activity measure was performed separately on locomotor activity on Day 1 following saline injections and using a difference score calculated by subtracting Day 1 activity (saline vehicle) from Day 2 activity (1.8 g/kg ethanol). The following three measures were evaluated following the 4.0 g/kg dose of ethanol: (i) time until LORR onset; (ii) duration of LORR and (iii) blood ethanol concentration from trunk blood at recovery. Preliminary findings in unoperated mice showed that the 4.0 g/kg dose produced a more reliable onset and duration of LORR than the 3.0 g/kg dose.

**Ethanol drinking procedures**

An independent set of 60, ethanol-naïve C57BL/6J mice were used for assessing the effects of EW lesions on ethanol drinking. Following surgical procedures and postoperative recovery (5–8 days), the experiment was initiated by individually housing the animals in metal hanging racks. The animals were allowed 5 days to habituate to the new housing conditions. During this time, animals were given continuous access to one bottle containing tap water. On the first through the fourth day of the experiment, animals were given continuous access (24 h) to two 50 mL cylinders (one containing tap water and one containing 3% ethanol in tap water). On the fifth through eighth and ninth through twelfth day of the experiment, the ethanol concentration was 6% and 10%, respectively. On days 13 and 14, the ethanol was increased to 20%. Body weights and fluid consumption (g) from both cylinders were recorded daily at 09:00 h (two hours after light onset). Bottle positions were alternated daily to avoid development of a position preference. Both preference measures (g ethanol consumed/g total fluid consumed) and consumption measures (g/kg/day) were calculated and used as dependent variables.

**Sucrose and quinine consumption**

In an effort to rule out differences in taste sensitivity that would affect drinking an ethanol-containing solution, consumption tests of sucrose and quinine were performed (Thiele et al., 1998). Immediately following the two-bottle choice ethanol drinking procedure, consumption and preference for sucrose and quinine solutions were compared in both the EW-lesioned and sham-operated group. On days 15 and 16, animals were presented with one bottle of 1.7% sucrose and one bottle of tap water. On days 17 and 18, the sucrose concentration was increased to 4.3% sucrose. On day 19 and 20, the animals were presented with one bottle of 0.03 mM quinine and one bottle of tap water. On days 21 and 22, the quinine concentration was increased to 0.1 mM quinine. Body weights and fluid consumption (g) from both cylinders were recorded daily at 09:00 h (two hours after light onset). Bottle positions were alternated daily to avoid development of a position preference. Preference measures for sucrose (g sucrose solution consumed/g total fluid consumed) and quinine (g quinine solution consumed/g total fluid consumed) were calculated at each concentration and consumption measures (g/kg) were calculated and used as dependent variables.

**Histology**

All animals were killed by CO2 asphyxiation and brains removed. Dissected brains were postfixed overnight in 2% paraformaldehyde in 10 mM (pH 7.4) phosphate-buffered saline (PBS), cryoprotected with 30% sucrose in PBS and sectioned coronally on a cryostat. Floating 40 µm sections were collected and stored in PBS. Four to six sections containing the EW were mounted on gelatin-coated glass slides and thionin staining was performed to detect accurate placement of lesions.

**Ucn I immunohistochemistry**

The floating sections remaining (~3–4 per animal) following histological confirmation of lesions were processed for Ucn I immunohistochemistry according to previously published protocols (Bachtell et al., 2002). Briefly, endogenous peroxidase activity was quenched by 0.3% peroxide in PBS. Blocking was performed with 4% horse serum. A goat polyclonal antibody against an epitope at the C terminus of rat Ucn I (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:10 000. Biotinylated goat secondary antibody was used to detect the primary antibody (Vector Laboratories, Burlingame, CA, USA). The immunoreaction was detected with the Vectastain ABC kit (Vector Laboratories), and enzymatic development was accomplished with the Metal Enhanced DAB kit (Pierce, Rockford, IL, USA). Ucn I-positive cells were topographically identified as EW according to the brain atlas (Franklin & Paxinos, 1997). However, peptidergic neurons in the mouse EW proper are not necessarily identical to the EW functionally defined as cholinergic neurons projecting to the ciliary ganglion (Erichsen & May, 2002; Vasconcelos et al., 2003).

Analysis of Ucn I processes in the lateral septum (LS) and dorsal raphe (DR) was performed on a subset of floating sections containing these brain regions using a modified immunohistochemistry protocol as reported previously (Bachtell et al., 2003b). Endogenous peroxidase activity was quenched by 0.3% peroxide in PBS. Sections were incubated for 5 h in a blocking solution containing 2% BSA and 1 mg/mL heparin. After blocking, sections were incubated overnight in rabbit Ucn antibody corresponding to amino acids 105–120 of human pro-Ucn (Sigma, St. Louis, MO). Biotinylated rabbit secondary antibody was used to detect the primary antibody (Vector Laboratories). The immunoreaction was detected using the Vectastain ABC kit, and enzymatic development was accomplished with the Metal Enhanced DAB kit. A previous report demonstrated the specificity of the positive staining achieved in the LS for Ucn I, compared with Ucn II, Ucn III, and CRF (Bachtell et al., 2003b).

Ucn I-immunoreactive projections in the LS appeared as dark-brown beaded varicosities and were manually counted due to the sparse distribution in the LS region. One varicosity, regardless of length, was counted as one Ucn I fibre using 20× objective magnification. Varicosities across ten LS-containing sections per animal spanning +1.2 to +0.3 mm from Bregma were summed up to a single data point per animal. As we are using an inbred mouse strain, and EW lesions are unlikely to change the actual size of LS, the resulting numbers reflect the number of fibers in the similar areas of LS in lesioned and control animals. Ucn I immunoreactivity in the DR appeared as numerous beaded varicosities and isolated beads distributed throughout the DR. Ucn I-immunoreactive projections in two sections per subject containing the dorsal portion of the DR (−4.48 to −4.60 mm from Bregma) were quantified automatically by ImageJ software using 40× objective magnification. First, digitized images of each lateral half of the DR (Fig. 2A and B) were converted to 8-bit grey images. A threshold was then applied so that background was eliminated and only the dark Ucn I-immunoreactive beads remained. The beads in the resulting image were then quantified automatically, summed for each single section (two images per section), and averaged over the two sections per subject to create a single data point for each subject. The area of DR used in Ucn I quantification was equal across all subjects and corresponded to the entire visual field of...
the camera, which at this magnification corresponds to 22.5 μm × 16.67 μm. Quantification of Ucn I-immunoreactive fibers in all brain regions was performed by an observer blind to the experimental group of the animal.

**Blood ethanol measurement**

Trunk blood was collected in microcentrifuge tubes upon killing and placed on ice. All samples were immediately centrifuged at ~ 20 000 g for 20 min. Serum was collected and stored overnight at ~ 20 °C. Blood samples (3 μL) from individual animals were used to assess NAD-ADH reactivity with the spectrophotometric NAD-ADH Detection System (Sigma Diagnostics, St. Louis, MO, USA). A set of ten standards ranging from 10 to 3000 mg/dL was performed to assess the accuracy of the NAD-ADH Detection system.

**Statistical analysis**

Data were analysed by one-way or mixed-design ANOVA where appropriate with P < 0.05 considered statistically significant. In cases where multifactorial ANOVA identified significant interactions between factors, the Fisher PLSD was used as a posthoc test to follow-up. Due to a substantial number of measurements performed on a total of 120 mice used in this study prior to identification of lesion location, a few animals had to be excluded from some tests due to recording errors. The exact number of animals used in each statistical analysis with verified lesion location is shown in Table 1A, B. The results of posthoc tests are shown in the figures.

**Results**

**Histology**

Coronal brain sections encompassing the EW were examined under a microscope for accuracy of the lesion and the degree of tissue damage. Accuracy of the lesions were judged based on the extent of midline destruction of the large cell bodies known to be contained within the EW. Tissue damage produced by the lesion was limited to a 200 μm diameter region encompassed within the medial longitudinal fasciculus, which was sufficient to destroy a large portion of the EW with little damage to the surrounding areas (Fig. 1). Accurate lesions were observed in approximately one-third of the animals that underwent surgery for EW lesions (32 out of 90). Accurate placement of electrodes in the EW of sham-operated animals was not assessed directly because there was no obvious damage to the EW region in sham-operated animals several weeks following the surgery (Fig. 1). Some animals (n = 19) recovered from surgery with a significant list and possessed varying degrees of circling locomotor behaviour. This behaviour ceased within a few days following the surgery. Of the animals that developed this behaviour postoperatively, most (n = 17) possessed lesions missing the EW laterally. Some animals (n = 10) with electrical current delivery did not fully recover from surgery and died postoperatively. Importantly, at the time of experimental assessments, animals with accurate lesions displayed no overt behavioural side-effects that impeded our ability to record any of the measures outlined below. In particular, we observed that EW-lesioned animals continued to locomote, displayed exploratory activity, and ate normally compared with sham-operated control animals. This is agreement with lack of reports of effects of EW lesions in larger animals on such behaviours (Pierson & Carpenter, 1974; Pickard et al., 2002; Smeraski et al., 2004). Preliminary analyses revealed that animals with misplaced lesions performed similar to the sham control animals, however, because of unknown effects of the electric current application and disparate locations of the missed lesions, these animals were eliminated from the final analyses.

**Assessment of Ucn I immunoreactivity**

Immunohistochemistry was performed to identify the damage to the Ucn I cell bodies in the EW. As can be seen in Fig. 1B and D),...
accurate lesions of the EW completely ablated the Ucn I-positive cells in the EW. In order to assess the effects of ablating a large portion of the Ucn I cell bodies in the EW, we performed immunohistochemistry for Ucn I in the LS and DR, two regions containing Ucn I immunoreactive projections in C57BL/6J mice (Bachtell et al., 2003b; A. Z. Weitemier and A. E. Ryabinin, unpublished observations). The results indicate a significant reduction in the number of Ucn I-positive processes in the LS of EW-lesioned compared with sham-operated animals ($F_{1,15} = 10.36, P < 0.006$, Fig. 2). In addition, a reduction in the number of Ucn I-positive processes in the DR was also observed in the EW-lesioned mice compared with the sham-operated control animals ($F_{1,8} = 23.79, P < 0.002$ in the sensitivity experiment, Fig. 2; $F_{1,10} = 22.26, P < 0.001$ in the preference experiment).

**Effect of Edinger–Westphal nucleus lesions on pupillary responses**

The EW has been implicated in oculomotor functions where disinhibition of the nucleus produces a mydriatic response to light (Westphal, 1887; Warwick, 1954; Roste & Dietrichs, 1988; Burde & Williams, 1989; Trimarchi, 1992; Klooster et al., 1993). It is therefore of interest to test the influence of EW lesions on pupillary responses. In comparison with sham-operated control mice, there was no significant difference in the basal pupil diameter of the EW-lesioned mice ($F_{1,32} = 1.617, P = 0.21$). It is believed that the EW provides tonic inhibition, which when removed, results in pupillary dilation. Removal of this tonic inhibition through an EW lesion would thus be expected to produce a persistently large pupil. This was not observed and, in fact, a trend toward a smaller pupil was observed in the EW-lesioned animals (336.28 ± 21.58) vs. the sham-operated (378.56 ± 25.59). Visualization of the pupillary constriction response was made in response to a brief flash of light. Although quantification of this response was not performed, there were no detectable differences between the pupil constriction response of sham-operated and EW-lesioned mice.

**Effect of Edinger–Westphal nucleus lesions on ethanol sensitivity measures**

Animals with EW lesions were compared to sham-operated controls on a number of ethanol sensitivity measures including ethanol-induced hypothermia, sedation, and locomotor activation. In our previous study, we observed a positive correlation between hypothermic responses to ethanol and Ucn I levels in the EW (Bachtell et al., 2002). Based on these findings, one would predict EW-lesioned animals, possessing blunted Ucn I levels, would have a diminished hypothermic response to ethanol. Indeed, thermoregulatory responses appeared different in the EW-lesioned animals vs. sham-operated controls (Fig. 3). Although not statistically significant, animals with EW lesions tended to have lower baseline body temperatures ($F_{1,34} = 3.73, P = 0.062$). This was observed only in the first cohort of the analysis, but not in the second, and is likely a result of high baseline temperatures recorded in the first cohort suggesting that the statistical trend may be influenced by environmental factors. Ethanol-induced hypothermia was significantly blunted at both 15 min and 30 min postinjection in EW-lesioned animals compared with sham-operated animals (effect of lesion $F_{1,34} = 9.64, P < 0.005$). In addition, there was a significant effect of time on ethanol-induced hypothermia ($F_{1,34} = 20.11, P < 0.0001$) reflecting lower body temperatures in both groups at 30 min postinjection, but no significant interaction of lesion and time ($F_{1,34} < 1$, not significant (NS)). The effect of lesion on hypothermia was apparent in both cohorts and did not appear to be significantly influenced by the high baseline temperatures or baseline differences observed in cohort 1. In agreement with this, inclusion of the cohort as a factor into a mixed design ANOVA (between, group × cohort; within, time) did not confirm any significant interactions of cohort with other factors (group by cohort, $F_{1,32} < 1$, NS; time by cohort, $F_{1,32} < 1$, NS; group by time by cohort, $F_{1,32} = 3.89, P = 0.057$ reflecting a weaker
hypothemic effect in the second cohort at the 30 min time point). Furthermore, the differences in ethanol-induced hypothermia were not a result of altered metabolism of ethanol because blood ethanol concentration measurements were equivalent between the lesioned mice and sham controls ($F_{1,16} = 1.81, P = 0.2$).

Our previous findings in several genetic models revealed no significant relationships between other ethanol sensitivity measures and Ucn I levels in the EW. Therefore, we would not expect to find differences between the sham-operated and EW-lesioned animals in these same behaviours. Thus, a mixed design ANOVA (group x time) revealed no significant differences between EW-lesioned and sham-operated control animals in locomotor activity following saline injections ($F_{1,15} < 1, NS$, Fig. 4A). There was a significant effect of time on locomotor activity ($F_{2,30} = 48.39, P < 0.0001$), but no significant interaction of time by lesion ($F_{2,30} < 1, NS$) reflecting no effect of lesion on locomotor habituation. The lesion also did not affect the difference in the locomotor activity following injection of 1.8 g/kg of ethanol ($F_{1,15} < 1$, NS). There was no effect of time on this measure ($F_{2,30} < 1$, NS) and no time by lesion interaction ($F_{2,30} = 1.54, P = 0.22$). Similarly, there was no effect of lesion on ethanol-induced sedation ($F_{1,16} < 1$, NS, Fig. 4B). It should be noted that none of the mice displayed increases in locomotor activity in response to 1.8 g/kg ethanol. This is characteristic of the C57BL/6J mouse strain and may prohibit the detection of differences in this response (Crabbe et al., 1982). Regardless, these tests of ethanol sensitivity not only support relationships between Ucn I and ethanol sensitivity measures obtained in our previous genetic studies (Bachtell et al., 2002), but also reveal specificity of the EW in thermoregulatory responses over other sensitivity measures.

**Effect of Edinger–Westphal nucleus lesions on ethanol consumption**

Previous reports suggest that Ucn I in the EW may be important for ethanol consumption. This relationship was demonstrated in the C57BL/6J and DBA/2J inbred mouse strains, the B6D2 F2 mice, as well as the high alcohol preferring and low alcohol preferring selected mouse lines (Bachtell et al., 2002, 2003b). The relationship in all cases was positive, indicating that more Ucn I in the EW predicted high ethanol consumption. Thus, it could be hypothesized that animals with high levels of Ucn I in the EW (sham-operated control) would drink more than those animals with low levels of Ucn I in the EW (EW-lesioned animals).

Comparison of EW-lesioned and sham-operated animals on the two-bottle choice ethanol consumption test confirmed this hypothesis. Hence, EW-lesioned mice consumed significantly less ethanol at the 3%, 6%, and 10% concentrations of ethanol (Fig. 5A). A mixed design analysis of variance revealed that there was an overall effect of lesion ($F_{1,20} = 18.67, P < 0.0005$). The daily dose of ethanol significantly increased as the concentration of ethanol was increased ($F_{3,60} = 98.93, P < 0.0001$). In addition, significant interactive effects between group and concentration were detected and shown in Fig. 5 ($F_{3,60} = 4.67, P < 0.01$), which reflect a lack of difference between EW-lesioned mice and sham controls at the 20% solution.

Based on these results and our previous reports, we would expect a similar decrease in the preference for the ethanol-containing bottle to the water-containing bottle. Accordingly, analysis of the preference ratios at 3%, 6%, and 10% ethanol concentrations demonstrated that EW-lesioned mice had significantly diminished preference for ethanol compared with sham-operated animals (Fig. 5). A mixed design
effect of Edinger-Westphal nucleus lesions on taste sensitivity

Because the differences in ethanol consumption and preference can be driven by altered reactivity to taste, we compared EW-lesioned and sham-operated controls on consumption and preference of a sweet and bitter solution. The results of these tests suggest that differences in ethanol consumption and preference observed between the EW-lesion and sham-operated groups are likely not due to altered taste sensitivity. Animals from both groups strongly preferred both the 1.7% and 4.3% sucrose solution (Fig. 6A). Animals from both groups showed significantly higher preference for the 4.3% sucrose over water vs. the preference for the 1.7% sucrose over water (F1,20 = 108.46, P < 0.0001). There was no main effect of group on sucrose preference (F1,20 < 1, NS) and no significant interactive effects between the group and concentration on preference (F1,20 < 1, NS). Consumption of the sucrose solution was significantly higher in the sham-operated animals compared with the EW-lesioned animals (n = 10) (**P < 0.0001, posthoc comparison). Consumption of 20% ethanol was not significantly different. (B) EW-lesioned animals demonstrate a decreased preference for ethanol compared with sham-operated animals (*P < 0.005, posthoc comparison). EW-lesioned and sham-operated animals preferred ethanol similarly at the 20% concentration.

Fig. 5. Lesions of the EW diminished ethanol consumption. (A) EW-lesioned animals (n = 12) consumed less daily ethanol across the 3%, 6% and 10% ethanol concentrations compared with sham-operated animals (n = 10) (**P < 0.0001, posthoc comparison). Consumption of 20% ethanol was not significantly different. (B) EW-lesioned animals demonstrate a decreased preference for ethanol compared with sham-operated animals (*P < 0.005, posthoc comparison). EW-lesioned and sham-operated animals preferred ethanol similarly at the 20% concentration.

Analysis of variance for the preference ratios detected significant group differences (F1,20 = 12.78, P < 0.005). The preference ratios across the ethanol concentrations significantly increased as the concentration of ethanol was increased until the 10% concentration, but decreased in the sham-operated animals at the 20% concentration (F3,60 = 6.80, P = 0.0005). In addition, the lack of difference in the preference measure at the 20% concentration was reflected in significant interactive effects between group and concentration (F3,60 = 7.34, P < 0.0005).

Fig. 6. EW-lesioned (n = 12) and sham-operated (n = 10) animals performed similarly on the taste sensitivity tests. (A) Both EW-lesioned and sham-operated animals strongly preferred sucrose at the 1.7% and 4.3% concentrations. (B) Consumption of the sucrose solution was significantly different between EW-lesioned and sham-operated animals. Sham-operated animals consumed more sucrose than EW-lesioned animals. (C) Both EW-lesioned and sham-operated animals avoided quinine at the 0.03 and 0.1 mM concentrations. (D) Consumption of quinine at both concentrations was equivalent between the two groups.

Differences between the EW-lesioned animals and sham-operated animals were observed in total fluid consumption and water consumption throughout the two-bottle procedure (Fig. 7). There was a significant effect of group on total fluid consumption (F1,20 = 19.54, P < 0.005), significant change in total fluid consumption during the two-bottle procedure across days (F1,140 = 60.37, P < 0.0001) and significant interactive effects between group and day on total fluid consumption (Fig. 7A, F1,140 = 3.73, P = 0.001). In addition, analyses performed on water consumption during the two-bottle choice test revealed no significant effect of group (F1,20 < 1, NS), significant effect of days (F1,140 = 54.38, P < 0.0001), and a significant group–days interaction (F1,140 = 8.00, P < 0.0001). These effects reflected that EW-lesioned animals consumed more water when 3%, 6% or 10% ethanol was presented in the alternative bottle, less total fluid when sucrose or quinine was presented in the alternative bottle, and less water when highly aversive quinine solution was presented as the alternative fluid (Fig. 7B).

Discussion

This series of experiments assessed the effects of electrolytic lesions of the EW on several ethanol-mediated responses in male C57BL/6J mice. The observations made here parallel those observed in previous reports using genetic models and provide substantial evidence of the EW’s involvement in several ethanol-mediated behaviours. Thus, it was observed that EW lesions selectively disrupted ethanol-induced hypothermia, while other measures of ethanol sensitivity such as locomotor activity and ethanol-induced sedation were unchanged compared with sham-operated control animals. In addition, mice with EW lesions displayed decreased preference for ethanol-containing...
solutions and decreased ethanol consumption using the two-bottle choice test. In agreement with the involvement of Ucn I in regulation of food consumption (Spina et al., 1996), we also observed decreased consumption of sucrose in EW-lesioned mice. However, because these lesions decreased preference for ethanol but not sucrose in the two-bottle test, a preferential involvement of the EW in ethanol-related behaviours may be inferred.

Novel functions of the Edinger–Westphal nucleus

The results of this report provide support for recent speculation that the EW may have functioning beyond that of supporting ocular accommodation (Roste & Dietrichs, 1988; Burde & Williams, 1989; Trimarchi, 1992; Klooster et al., 1993). Perhaps contributing to the controversy is the less than clear definition and precise identity of the EW. Some investigators define the EW as the medial visceral cell column and the lateral visceral cell column plus the anterior median nucleus (Warwick, 1954; Piersson & Carpenter, 1974; Benevento et al., 1977). In other cases, the EW is simply defined as the cluster of cells supplying preganglionic parasympathetic input to the ciliary ganglion (Akert et al., 1980). However, this latter distinction is not clear because retrograde tracers injected into the mammalian ciliary ganglion produce labelling in several midbrain nuclei of the oculomotor complex, but often do not label the morphologically identified EW (Ishikawa et al., 1990; Erichsen & May, 2002). The observations made in this report in rodents with EW lesions lend support to a more diverse function in that EW lesions had no effect on the pupil diameter and the pupillary response to light.

Raising additional concern are species differences in the content and composition of EW cells. For example, the rodent EW is a primary source of the neuropeptide Ucn I in the brain (Vaughan et al., 1995; Bittencourt et al., 1999), while the avian EW appears to be absent of Ucn I (Cavani et al., 2003). Thus, the peptidergic neurons in the mouse EW proper are not necessarily identical to the EW functionally defined as cholinergic neurons projecting to the ciliary ganglion (Erichsen & May, 2002; Vasconcelos et al., 2003). Therefore, it appears that the rodent EW, as traditionally identified, is more functionally diverse than originally thought. For example, the presence of Ucn I in the EW suggests that it may mediate anxiety-related behaviours. Ucn I mRNA expression in the EW was shown to up-regulate following 3-h restraint stress (Bittencourt & Sawchenko, 2000). In addition, induction of c-Fos was observed in EW after acute lipopolysaccharide and pain stress, but not after all types of stress (Kozicz et al., 2001; Kozicz, 2003).

The Edinger–Westphal nucleus’ role in ethanol-induced hypothermia

Our findings suggest that the EW is an important mediator of thermoregulatory systems following ethanol administration. Other recent studies also support the EW’s role in temperature regulation. Thus, transneuronal labelling was observed in EW neurons following injection of a pseudorabies virus injected into the wall of the ventral tail artery of rats, which is a primary mediator of increased sympathetic blood flow for heat loss (Smith et al., 1998). In addition, animals acutely exposed to both warm and cold ambient temperatures show elevated c-Fos expression in the EW compared with control animals (Bachtell et al., 2003a). Therefore, the EW Ucn I system may represent a novel midbrain component of a thermoregulation system.

In agreement with this idea, it has been shown that members of the CRF/Ucn family such as CRF, sauvagine, and urotensin, produce thermogenic actions when administered centrally (Le Feuvre et al., 1989). Thus, central and peripheral administration of CRF and sauvagine induce a dose-dependent hypothermia in rats. Ucn I administration is known to produce potent and long-lasting hypotension and vasodilation (Vaughan et al., 1995; Lubomirov et al., 2001). These alterations may contribute to thermoregulatory processes. However the role of the CRF/Ucn family is still poorly understood and demands further investigation.

The Edinger–Westphal nucleus’ role in ethanol consumption

Our results support previous findings suggesting that genetic predispositions to high and low alcohol consumption involve differences in Ucn I expression in the EW (Bachtell et al., 2003b). Thus, by ablating a significant portion of the EW Ucn I cells we observed significant disruption of the preference for ethanol observed in sham-operated animals. The CRF/Ucn system has been implicated in ethanol consumption previously. For example, George et al. (1990) demonstrated that high alcohol preferring Wistar rats possessed elevated CRF-like immunoreactivity in the hypothalamus. Comparisons in CRF levels and electroencephalographic activity between the alcohol preferring and nonpreferring selected lines of rats show that preferring rats have depressed CRF levels in the hypothalamus, amygdala, and cortex while also having enhanced electroencephalographic responses to exogenous CRF (Ehlers et al., 1992). It is therefore conceivable that differences in other components important for regulating ethanol consumption could exist in the CRF/Ucn system. Based on the results of this report and our previous findings, the EW Ucn I system appears important for determining ethanol preferences.
An important caveat with this conclusion, however, is that we failed to observe a difference in ethanol preference between EW-lesioned and sham-operated animals at the 20% ethanol concentration. The lack of group differences in ethanol preference is due to the substantial decrease in preference observed in the sham-operated animals, which displayed strong preferences for the 6% and 10% ethanol concentrations. The lack of preference for the 20% ethanol concentration may actually indicate ethanol aversion in the sham-operated animals as a slight aversion to the high concentration of ethanol was observed. This is fairly typical for such a high concentration of ethanol (McMillen & Williams, 1998; Savelieva et al., 2002; Hall et al., 2003). If this observation is an indication of ethanol aversion, EW-lesioned animals displayed values equal to those of sham-operated animals, suggesting that the EW has no influence on ethanol aversion. However, this result remains unclear as strong ethanol aversions were not observed in the sham-operated animals. One can conclude however, that none of the ethanol concentrations tested produced ethanol preference in the EW-lesioned animals, while strong preferences were detected in the sham-operated animals. This finding suggests that the EW is an important mediator in the willingness to ingest ethanol. Further studies should focus on the role of the EW in ethanol aversion for a more complete understanding.

**Mechanisms of the Edinger-Westphal nucleus influence on ethanol-induced hypothermia and preference**

Ucn I immunoreactive projections in two brain areas outside the region of the EW were quantified in EW-lesioned and sham animals to confirm that lesion-induced ablation of EW cell bodies affected Ucn I output to the LS and DR (Bittencourt et al., 1999). The EW lesion-induced reduction in Ucn I fibers confirms a functional connection between the EW and both the LS and the DR in mice. It is possible therefore, that the EW regulates ethanol-induced hypothermia and ethanol consumption through projections to these brain areas.

The DR seems a likely target for thermoregulatory functions of EW given its close proximity to EW, Ucn I-immunoreactive fibers, and CRF R2 receptors. Involvement of the DR in thermoregulation has been shown in several studies. Thus, injection of a number of pharmacological agents into DR leads to decreased body temperature (Higgins et al., 1988; Hillegaart, 1991; Ginefra-Gavet & Garet, 1993), and lesions of the DR attenuate hypothermia produced by systemic apomorphine and kainic acid injections (Przewlocki, 1977; Turski & Kleinrok, 1980). Moreover, the DR has been implicated in ethanol-induced hypothermia by genetic studies of selectively bred long-sleep and short-sleep mice (French & Weiner, 1991).

One cannot exclude that the regulation of alcohol consumption by the EW is also mediated through the DR, for example through anxiolytic effects of DR-derived serotonin. However, it seems more likely that this regulation involves the LS, which has long been proposed to be involved in regulation of drinking and thirst. It is not surprising that arginine vasopressin and angiotensin II, two other peptides known to regulate fluid consumption have prominent innervation of this brain area (Biegon et al., 1984; Brinton et al., 1984; Ivovino & Stardo, 1985; Vasudev et al., 1986; Ostrowski et al., 1992). Thus, fluid consumption leads to activation of neurons in the LS (Mink et al., 1983; Gonzalez-Lima et al., 1993), while electric stimulation of neurons in the LS markedly decreases osmotic thirst threshold (Szczechanska-Sadowska et al., 1981). Electrolyte and chemical lesions of the LS lead to increased fluid consumption (Taghzouti et al., 1985; Ivovino & Stardo, 1985).

Importantly, this increase is accompanied by an increase in preference for consumption of other fluids vs. water (Vasudev et al., 1985). The latter study is in agreement with effects of EW lesions on consumption of water or total fluid in the two-bottle choice procedure observed in our study, and deserving separate investigation. We hypothesize that the EW, being highly sensitive to ethanol, provides a signal to the LS through its Ucn I-containing projections, which act on CRF-R2 receptors prominently expressed in the LS (Van Pett et al., 2000).

The actions of Ucn in the LS are most likely inhibitory because, in contrast to other brain areas, CRF was shown to have inhibitory action on LS neurons (Siggins et al., 1985). In agreement with this hypothesis, we have observed that voluntary alcohol consumption in mice leads to a dose-dependent suppression of c-Fos expression in the LS (Ryabinin et al., 2003). Future studies should address the roles of Ucn I innervation of DR and LS in ethanol-induced hypothermia and alcohol consumption by investigating effects of injections of Ucn-I into these brain areas.

**Conclusions**

The experiments presented here extend previous genetic studies demonstrating that the EW Ucn I system is highly sensitive to ethanol and may regulate ethanol-induced hypothermia and ethanol consumption. These results provide direct support for these findings using an electrolytic strategy. Thereby, animals with EW-lesions demonstrate decreased ethanol-induced hypothermia and ethanol consumption contradicting the traditional functions attributed to the EW and suggests a novel role for the EW to regulate temperature and consumption.

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**Abbreviations**

CRF, corticotropin releasing factor; DR, dorsal raphé; EW, Edinger–Westphal nucleus; LORR, loss of righting reflex; LS, lateral septum; NS, not significant; PBS, phosphate-buffered saline; Ucn I, urocortin I.

**References**


