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ORIGINAL INVESTIGATION

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High alcohol/sucrose consumption during dark circadian phase in C57BL/6J mice: involvement of hippocampus, lateral septum and urocortin-positive cells of the Edinger-Westphal nucleus

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Abstract *Rationale:* Identification of the neuroanatomical substrates regulating alcohol consumption is important for the understanding of alcoholism. Previous studies mapping changes in brain activity used rodent models of alcohol drinking with relatively low alcohol intakes. *Objectives:* This study was aimed to identify brain regions changing activity after high voluntary intake of alcohol-containing solutions. *Methods:* Adult male C57BL/6J mice were trained to drink a 10% ethanol/10% sucrose solution in daily 30-min limited-access sessions during the dark phase of the circadian cycle. Control groups of animals consumed 10% sucrose or water. Analysis of c-Fos immunohistochemistry (as a marker for neuronal activity) was performed at 90 min after the last alcohol drinking session. *Results:* The limited access procedure led to high intakes (2.9±0.3 g/kg) and blood alcohol concentrations of 251±46 mg%. Expression of c-Fos was significantly higher in the alcohol/sucrose group than both the water and sucrose groups in the Edinger-Westphal nucleus, and significantly lower in the alcohol/sucrose group than two control groups in hippocampal subregions, posterior hypothalamus and dorsal lateral septum. Double immunohistochemistry showed that alcohol-induced c-Fos-positive cells in the Edinger-Westphal nucleus co-localized with the neuropeptide urocortin. In addition, intake and/or blood alcohol concentrations correlated with c-Fos expression in specific subregions of the hippocampus, hypothalamus, prefrontal cortex, lateral septum and midbrain. *Conclusions:* The dark phase voluntary limited-access procedure in mice leads to intakes of alcohol-containing solutions that are considered highly intoxicating. Brain regions showing alcohol-specific changes in c-Fos expression after this procedure can be connected into a novel neurocircuit, including lateral septum, hippocampus, hypothalamus, and the Edinger-Westphal nucleus.

Keywords Ethanol · Self-administration · c-Fos · Inducible transcription factor · Mice

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Introduction

Alcoholics frequently consume large quantities of alcohol-containing beverages. Understanding the neuronal substrates and pathophysiological consequences of this behavior is therefore of great importance. Recent studies show that voluntary self-administration of alcohol-containing solutions in rats or mice changes activity in brain regions different from those affected by experimenter-administered alcohol (Porrino et al. 1998a, 1998b; Bachtell et al. 1999; Ryabinin 2000). Analysis of models of voluntary alcohol self-administration therefore becomes especially important. However, an obstacle in many alcohol self-administration studies is low voluntary consumption of alcohol-containing solutions. Thus, voluntary alcohol self-administration in unselected non-fluid-deprived rodents does not typically reach intoxicating doses and does not lead to signs of alcohol dependence (Cunningham et al. 2000).

Brain regions changing activity after drinking of alcohol-containing solutions have been mapped by analyzing expression of inducible transcription factors (ITFs) (Topple et al. 1998; Bachtell et al. 1999; Ryabinin et al. 1999, 2001; Weitemier et al. 2001). This approach is based on the notion that basal expression of ITFs (c-Fos, c-Jun, JunB, Zif268 and several others) in neurons is very low but, upon stimulation of the neurons (including actions of agents leading to neuronal depolarization), a rapid transient induction of these proteins occurs (Morgan et al. 1987; Sagar et al. 1988).

Recent studies show that voluntary self-administration of an alcohol-containing solution produces a distinct pattern of c-Fos expression from involuntary alcohol administration (Topple et al. 1998; Bachtell et al. 1999;

Ryabinin et al. 1999, 2001; Weitemier et al. 2001). One effect most frequently observed in such studies is induction of c-Fos in the Edinger-Westphal nucleus (EW). In addition, alcohol also tends to suppress c-Fos expression in hippocampal areas, an effect more easily observed when basal levels of c-Fos have been elevated by environmental factors, e.g., exposure to environmental stress (Bachtell et al. 1999; Ryabinin et al. 1999). In a majority of these studies, intake of self-administered alcohol was 2 g/kg or lower (Topple et al. 1998; Ryabinin et al. 2001; Weitemier et al. 2001). However, in one study, C57BL/6J (C57) mice self-administered approximately 2 g/kg during most of the procedure, but on the last day (day of c-Fos analysis) registered a large fluctuation from their average intake and self-administered 2.6 g/kg (Bachtell et al. 1999). In this study, increased c-Fos expression was observed in a group drinking alcohol-containing solution not only in the EW, but also in the core of nucleus accumbens (AcbC) and medial portion of the central nucleus of amygdala (CeM). It was unclear whether induction of c-Fos in AcbC and CeM in one study occurred because of specific actions of alcohol or due to stress/novelty after an unpredicted alcohol intoxication. An important experiment to evaluate these two possibilities is the investigation of c-Fos expression after high but stable intakes of alcohol.

In order to achieve higher intakes of alcohol in a mouse model of voluntary consumption of alcohol-containing beverages, we took advantage of diurnal variations in rodent activity. Previous studies reported that voluntary alcohol intakes are higher during the dark phase of the circadian cycle where the peak fluid consumption occurred between 2 h and 4 h after the beginning of the dark period (Freund 1970; Goldstein and Kakihana 1977; Kurokawa et al. 2000). We hypothesized, therefore, that higher intakes of ethanol/sucrose could be achieved by moving the 30-min drinking session into the dark phase of the circadian cycle, allowing us to investigate brain regions showing ethanol-specific changes in c-Fos expression.

In the present study, we show that C57 mice voluntarily consume a 10% ethanol/10% sucrose solution leading to intake of about 3 g/kg ethanol during the 30-min drinking session at 2.5 h into the dark circadian phase. These high voluntary intakes lead to ethanol-specific changes in c-Fos expression in several subregions of the prefrontal cortex, hippocampus, EW, septum, hypothalamus and midbrain. These findings are synthesized into a hypothesized neurocircuitry that may be involved in response to ethanol intoxication and in regulation of alcohol self-administration.

Materials and methods

Animal procedures

All animal procedures followed "Principles of laboratory care" (NIH publication no. 85–23, revised 1996). Male C57 mice (n=30), 7 weeks old, were purchased from Jackson Laboratories and housed four per cage on a 12-h/12-h light/dark cycle (lights off at 1700 hours). There was ad libitum access to food (LabDiet 5001) at all times. At 1 week post-arrival, animals were housed individually in metal hanging racks. A limited-access alcohol/sucrose drinking procedure was used (Grant and Samson 1985; Bachtell et al. 1999; Ryabinin et al. 1999). After 1 week of habituation to these housing conditions, animals were water-deprived for 22 h, and then were given access to a 20% sucrose solution for 2 h. Fluids were presented in 25-ml graduated glass cylinders with stainless-steel drinking spouts inserted through the front of the cage. This day was designated as day 0. During the next 9 days, access to sucrose was gradually decreased from 2 h to 30 min, and access to water was increased from 0 h to 20 h. On day 12, the sucrose solution was changed to 10%. Beginning on this day, the schedule of access to drinking solutions and procedure was kept constant: access to water was from 2100 hours to 1700 hours the next day; at 1900 hours, body-weight measurement; 1930–2000 hours, access to test solutions. During days 12–29, ten subjects (the sucrose group) continued to receive the 10% sucrose solution. In another ten animals (the alcohol/sucrose group), ethanol was gradually added to the 10% sucrose solution at increasing concentrations from 0% (on day 12), 2% (v/v, during days 13–14), 5% (days 15–16), 7.5% (days 17–18), to 10% (days 19–29). A third group of ten animals ingested only water (the water group). On day 29, to achieve a similar time of sacrifice after alcohol drinking, a 1-min interval was implemented between putting the bottles into individual cages. At 1.5 h after the drinking session, animals were individually placed into a CO_2 chamber in the dark housing room for euthanasia. The chamber was then immediately transported to the adjacent lighted procedure room to allow for brain isolation. This time point of sacrifice was selected because, at this interval, expression of c-Fos expression peaks after most types of neuronal stimulation (Morgan and Curran 1991). The interval between euthanasia of each mouse was 1–2 min. All animals were sacrificed on the same day. Trunk blood was collected at time of sacrifice for blood alcohol concentration (BAC) analysis.

Immunohistochemical procedures

Immunohistochemistry was performed according to previous protocols (Ryabinin and Wang 1998; Bachtell et al. 1999). Briefly, dissected brains were postfixed overnight in 2% paraformaldehyde in isotonic sodium phosphate buffered saline (PBS) and cryoprotected with 30% sucrose in PBS. Forty-micrometer frozen coronal sections were collected in PBS from Bregma level 1.70 mm to –4.24 mm according to the Mouse Brain Atlas (Franklin and Paxinos 1997). Immunohistochemical analysis for c-Fos was performed on every fifth section from approximate Bregma levels of 1.1 mm to –3.9 mm (25 sections per animal). Endogenous peroxidase activity was inhibited by pretreatment with 0.3% hydrogen peroxide. Blocking was performed with 6% goat serum. Rabbit polyclonal antibodies against amino acids 210–335 of human c-Fos protein that are not cross-reactive with FosB, Fra-1 and Fra-2 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) were used in a dilution of 1:10,000. The immunoreaction was detected with Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA). Enzymatic development was done with the Metal Enhanced diaminobenzidine (DAB) kit (Pierce, Rockford, Ill., USA).

Double immunohistochemistry

In all cases, double immunohistochemistry was initiated as described above for c-Fos immunohistochemistry with the exception of 4% horse serum being used for blocking. Following the first reaction (c-Fos) and DAB staining, unbound avidin and biotin resulting from initial reactions were blocked using the avidin/biotin blocking kit (Vector Laboratories). Sections were then incubated for 4 h in 4% horse serum (Vector Laboratories), followed by

incubation with the goat anti-Ucn primary antibody (Research Diagnostics, Inc., Flanders, N.J.) at a dilution of 1:10,000. The immunoreaction was detected with Vectastain ABC kit as above, while enzymatic development was performed with Vector VIP staining kit (Vector Laboratories). To confirm specificity of the staining, parallel sections were taken through the identical procedure with omission of either the c-Fos- or the Ucn-specific antibodies.

Blood alcohol concentrations

After collecting trunk blood from sacrificed animals, BACs were determined using the nicotinamide adenine dinucleotide (NAD)–alcohol dehydrogenase (ADH) detection system (Sigma Diagnostics, St. Louis, Mo., USA).

Data analysis

Drinking and body weight data were analyzed using a mixed-design analysis of variance (ANOVA). Consumption and body weight values on the final 11 days were used as the within variable, while group served as the between variable (10% sucrose, 10% sucrose/10% ethanol, or water). Quantitative image analysis was performed using a system consisting of an Olympus microscope BX40 and Sony CCD IRIS/RGB video camera connected to a Power PC. A single section from each animal was used to represent each of the analyzed brain regions. While data analysis from a single section may bias the overall results with regard to anterior–posterior distributions within a given region, it is believed that careful selection of sections and matching between animals maximizes confidence in comparisons across subjects (Saper 1996). Each digitalized video image was analyzed using NIH image 1.62 software. An image was adjusted through background subtraction such that neighboring areas with no immunostaining would contain no positive signals. Remaining grains in a size range from 7 to 50 pixels were automatically counted. All counting was performed by the same individual blind to the group of the analyzed animal. The value for a single matched region was summed for each bilateral area and used as a single data point for statistical analysis using a one-way ANOVA. For analysis of some brain regions, we were not able to match sections from all 30 animals to the same location. In such situations, counts in this brain region for this animal were not performed. Therefore, the actual number of animals per group analyzed for c-Fos expression is 8–10. Differences between individual groups were evaluated post-hoc using the Fisher test. Colocalization was assessed visually by counting c-Fos positive nuclei, Ucn-containing cells and the occurrence of c-Fos positive nuclei localized within Ucn-containing cells.

Comparison between groups of animals is the conventional approach to map changes in c-Fos expression. However, group comparison can miss brain regions in which effects of drugs are dose dependent. Therefore, the alcohol/sucrose group was also analyzed for correlations between c-Fos expression, intake and BAC on the last day of the behavioral experiment. To confirm alcohol specificity of identified correlations, brain regions with significant correlations between c-Fos expression and alcohol intake values were further analyzed for correlations between c-Fos, sucrose and water intake. Effects with $P<0.05$ were considered statistically significant. Although possibility of type-I error is high in mapping studies due to analysis of multiple brain regions, such alpha levels are typically used in order not to miss brain regions with weak effects (Porrino et al. 1998b; Smith et al. 2001). Confirmation of brain regions found by mapping studies should be done by other neuroanatomical and pharmacological methods.

Results

Alcohol consumption data

As expected, mice showed reliable drinking behavior during the 30-min limited-access procedure (Fig. 1). Consumption of 10% ethanol/10% sucrose on the last day of the experiment led to intake of 2.9±0.3 g/kg ethanol. BACs measured in the trunk blood of animals sacrificed for immunohistochemical analysis averaged 250.74±46.33 mg/dl. Despite the high doses of ethanol reached in the alcohol/sucrose group, the groups never differed in their body weight, indicating that the alcohol drinking sessions were too short to have a nutritional impact on the observed effects (Fig. 1). Behavioral assessments of mice in this study were not performed as they could influence results of c-Fos immunohistochemistry.

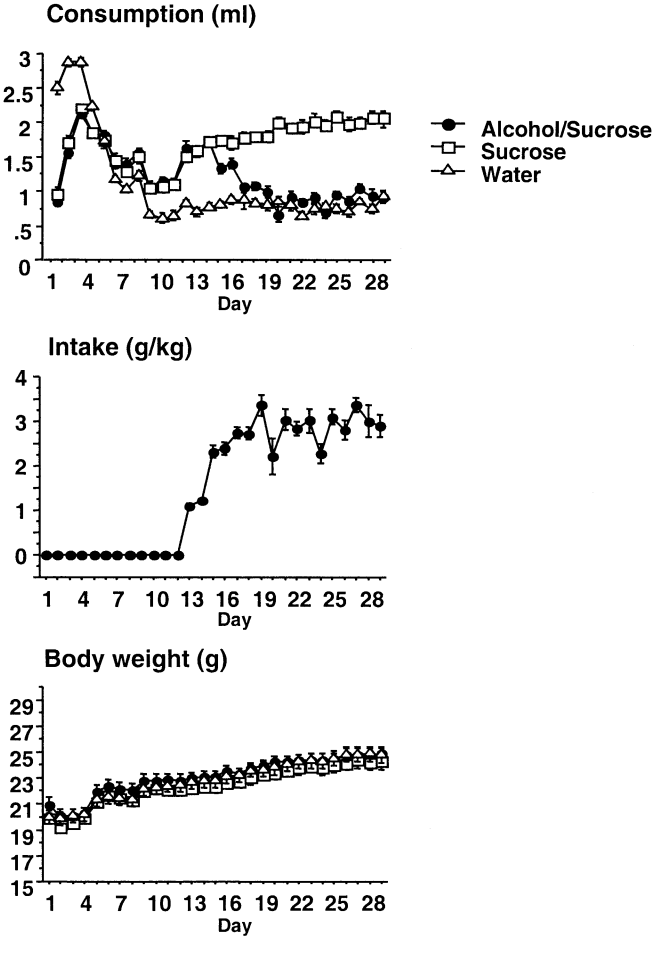

Fig. 1 Consumption, intake and body weight during the 30-min limited-access drinking procedure. Data are shown as mean±SEM. Consumption in sucrose is significantly higher than in the alcohol/sucrose and water groups ($P<0.05$). There was no significant difference in body weights between groups

Table 1 Number of c-Fos-positive cells after voluntary alcohol consumption during the dark phase of the circadian cycle in mice. Data shown as mean number of Fos-positive cells/slice±SEM. *NS* one way ANOVA not significant, *AcbC* core of nucleus accumbens (+1.1 mm), *AcbS* shell of nucleus accumbens (+1.1 mm), *AHP* posterior portion of anterior hypothalamus (–1.22 mm), *ArcLP* lateroposterior arcuate nucleus (–2.5 mm), *BLA* basolateral amygdala (–1.2 mm), *BST* bed nucleus of stria terminalis (+0.5 mm), *CA1* (–1.5 mm), *CA2* (–1.5 mm), *CA3* (–1.5 mm), *CeC*, *CeL*, and *CeM* capsular, lateral, and medial divisions of central nucleus of amygdala (–1.2 mm), *Cg* cingulate cortex (+0.7 mm), *DG* dentate gyrus (–1.5 mm), *DpG* deep gray layer of superior colliculus (–3.1 mm), *EW* Edinger-Westphal nucleus (–3.8 mm), *InG* intermediate gray layer of superior colliculus (–3.1 mm), *LH* lateral hypothalamus (–0.9 mm), *LHb* lateral habenula (–1.2 mm), *LSD*, *LSI* and *LSV*, lateral septum dorsal, intermediate and ventrale (+0.7 mm), *M* motor cortex (–1.2 mm), *MPA* medial preoptic area (+0.3 mm), *MS* medial septum (+0.7 mm), *OPT* olivary pretectal nucleus (–2.5 mm), *Pa* paraventricular nucleus of hypothalamus (–0.9 mm), *PH* posterior hypothalamus (–2.5 mm), *Pir* piriform cortex (–1.2 mm), *PMV* ventral premammillary nucleus, *PrC* precommissural nucleus (–2.5 mm), *PV* paraventricular nucleus of thalamus (–0.9 mm), *RI* rostral interstitial nucleus (–2.5 mm), *RSA* retrosplenial agranular cortex (–1.2 mm), *S* somatosensory cortex (–1.2 mm), *SO* supraoptic nucleus (–0.8 mm), *SuMM* medial supramammillary nucleus (–2.5 mm), *VLG* ventrolateral geniculate nucleus (–2.5 mm), *VMH* ventromedial hypothalamus (–1.2 mm), *VTA* ventral tegmental area (anterior portion: –3.1 mm), *Xi* xiphoid thalamic nucleus (–1.2 mm), *ZI* zona inserta (–1.2 mm). Brain sections were subjectively matched at Bregma levels shown in parentheses according to the Mouse Brain Atlas (Franklin and Paxinos 1997)

	Water	Sucrose	Alcohol/sucrose	ANOVA
Amygdala				
BLA	23.8±2.5	23.5±1.4	18.8±2.9	NS
CeL/C	6.6±1.6	10.7±2.1	9.4±1.9	NS
CeM	15.7±2.0	14.2±1.8	11.1±1.5	NS
Hippocampus				
CA1	5.8±1.3	7.3±1.8	1.7±0.8*,#	$F_{2,27}$=4.5, P=0.021
CA2	5.4±1.2	3.5±0.5	1.0±0.4*,#	$F_{2,27}$=7.4, P=0.003
CA3	5.8±0.9	8.1±1.3	3.6±1.4#	$F_{2,27}$=3.5, P=0.045
DG	7.0±1.4	7.3±1.4	4.0±1.1	NS
Hypothalamus				
AHP	27.0±4.0	27.4±3.0	27.7±5.9	NS
ArcLP	14.9±1.9	16.6±3.7	13.2±2.4	NS
LH	17.0±2.2	10.2±2.1*	8.5±1.4*	$F_{2,27}$=5.4, P=0.011
MPA	10.0±0.8	10.9±0.8	10.9±0.8	NS
Pa	3.2±0.5	4.9±0.4*	5.4±0.3*	$F_{2,27}$=7.3, P=0.003
PH	59.1±6.8	65.0±8.5	32.7±3.5*,#	$F_{2,26}$=6.3, P=0.006
PMV	1.3±0.8	0.9±0.6	3.4±1.6	NS
SO	4.6±2.2	2.4±0.4*	2.7±0.4*	$F_{2,27}$=5.1, P=0.013
VMH	2.2±0.4	3.4±0.5	2.6±0.5	NS
Neocortex				
Cg 1	69.3±17.7	59.8±10.1	58.5±13.3	NS
Cg 2	87.9±20.8	85.4±18.9	68.9±9.3	NS
M	3.4±1.0	4.9±1.1	3.4±0.7	NS
Pir	8.7±1.4	8.6±1.4	8.3±1.9	NS
RSA	2.7±0.7	4.7±0.6	3.4±0.7	NS
S	10.8±2.1	19.3±3.7	10.6±3.3	NS
Septum				
LSD	15.0±1.4	16.8±2.0	9.6±1.6*,#	$F_{2,26}$=4.8, P=0.017
LSI	12.2±1.5	13.0±1.2	9.8±1.7	NS
LSV	26.6±3.9	27.3±3.7	20.8±3.7	NS
MS	6.2±1.0	5.5±1.1	3.8±1.1	NS
Striatum				
AcbC	3.7±0.9	3.7±0.6	4.0±0.9	NS
AcbS	8.8±1.2	9.8±1.0	11.7±1.6	NS
BST	12.6±1.6	15.9±1.7	12.3±1.1	NS
Thalamus and midbrain				
DpG	14.9±1.3	17.2±2.2	15.4±1.8	NS
EW	7.0±0.8	11.8±1.1*	26.6±1.1*,#	$F_{2,27}$=107, P<0.0001
InG	7.2±1.1	7.1±1.0	4.4±1.0	NS
LHb	7.8±2.0	6.2±1.3	7.1±1.7	NS
OPT	10.3±1.8	10.6±2.7	5.7±1.7	NS
PrC	131±10.7	140.7±13.6	123±13.2	NS
PV	12.3±3.9	16.8±1.9	15.0±1.8	NS
RI	28.0±9.4	44.9±7.9	39.1±11.5	NS
SuMM	34.9±5.8	38.0±6.8	34.7±3.7	NS
VLG	41.6±3.1	49.2±5.8	31.7±4.8#	$F_{2,25}$=3.6, P=0.043
VTA	15.4±2.5	33.8±5.8*	15.7±4.6#	$F_{2,26}$=5.5, P=0.01
Xi	2.5±0.5	3.4±0.9	5.7±1.6	NS
Zi	22.2±3.8	24.4±3.9	20.1±3.3	NS

P>0.05, post-hoc * Significantly different from water # Significantly different from sucrose

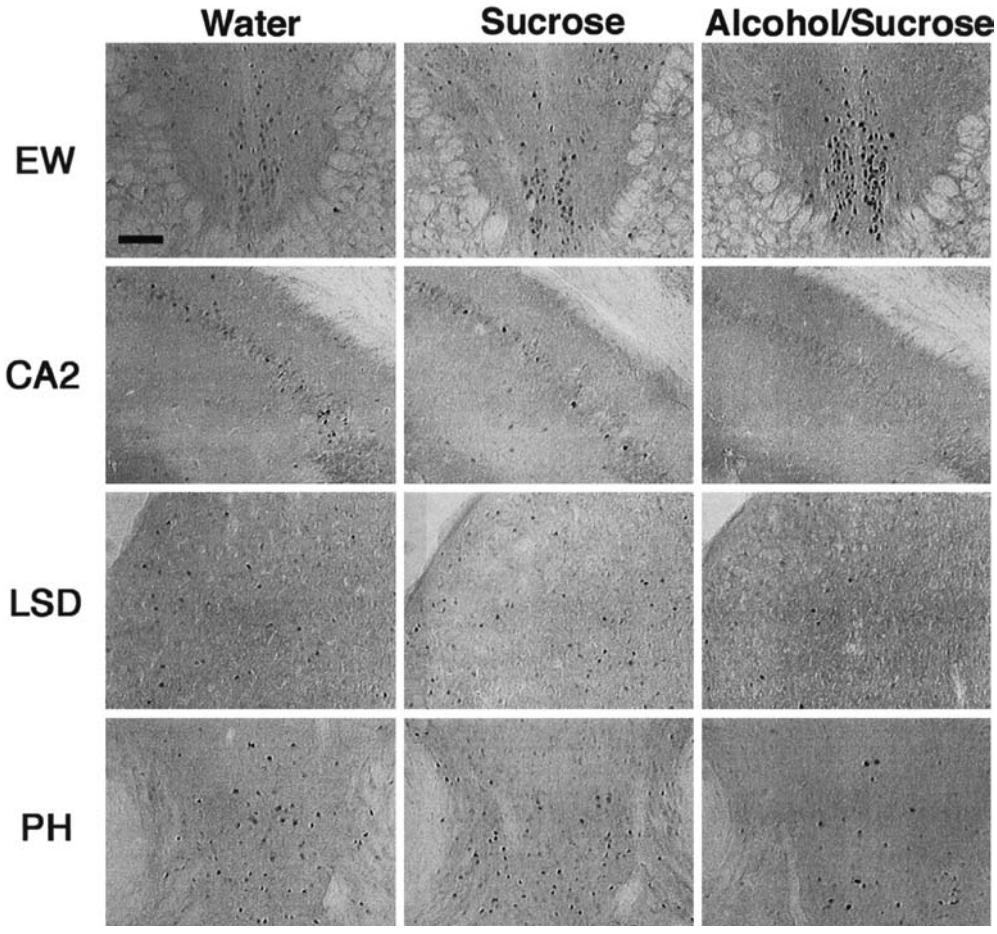

Fig. 2 Expression of c-Fos in the Edinger-Westphal nucleus (EW), CA2 region of hippocampus, dorsal lateral septum (LSD) and posterior hypothalamus (PH) after the 30-min limited-access drinking procedure. Scale bar corresponds to 100 μm. Note higher density of c-Fos-positive cells in EW, and lower density of c-Fos-positive cells in CA2, LSD and PH of animal in alcohol/sucrose group than in other groups

Immunohistochemical analysis

Expression of c-Fos was analyzed in 42 brain areas selected on the basis of previous studies showing regulation of c-Fos expression or deoxyglucose utilization following alcohol administration, or on the basis of high number of c-Fos-positive cells observed in the present study. As expected from studies analyzing circadian rhythms of c-Fos expression (Kononen et al. 1990; Grassi-Zucconi et al. 1993), the basal number of c-Fos-positive cells in many brain regions was substantially higher than in previous experiments performed in the light part of the circadian cycle. Particularly high basal numbers of c-Fos-positive cells were found in the PH, Cg1, Cg2, PrC and VLG (abbreviations of brain region names and their coordinates can be found in Table 1).

A one-way ANOVA performed on the number of c-Fos-positive cells identified significant differences between groups in 12 brain regions. Alcohol-specific effects defined by statistically significant post-hoc differences between the alcohol/sucrose and both control groups were found in CA1, CA2, PH, LSD, and EW (Fig. 2, Table 1). The only region showing ethanol-specific increase in c-Fos expression was the EW. Higher levels of c-Fos expression in EW of the sucrose group versus the water group were also observed.

CA1, CA2, PH and LSD showed ethanol-specific decreases in c-Fos expression (there was no difference between the water and the sucrose groups, but a significant difference between the alcohol/sucrose group and the two control groups). In addition, CA3 and VLG showed significantly lower levels of c-Fos expression in the alcohol/sucrose group versus the sucrose but not the water groups, a finding that could reflect a weaker effect of ethanol in these structures.

Differences in c-Fos expression between groups suggesting regulation that is not specific to ethanol were also found in VTA and three hypothalamic areas. In the VTA, both the alcohol/sucrose group and the water group showed significantly lower c-Fos expression than the sucrose group. Expression of c-Fos in LH and SO was significantly lower in the water group than the sucrose and the alcohol/sucrose groups, while c-Fos expression in the Pa was significantly higher in the water group than the sucrose and alcohol/sucrose groups.

Double immunohistochemistry with antibodies recognizing c-Fos and urocortin showed that 91.6% of alcohol-induced c-Fos-positive neurons in EW contained urocortin (Fig. 3). Interestingly, although animals in the water and sucrose groups had significantly lower numbers of c-Fos-positive cells, 79.3% and 81.7% of the expressed c-Fos colocalized with urocortin in these groups, respec-

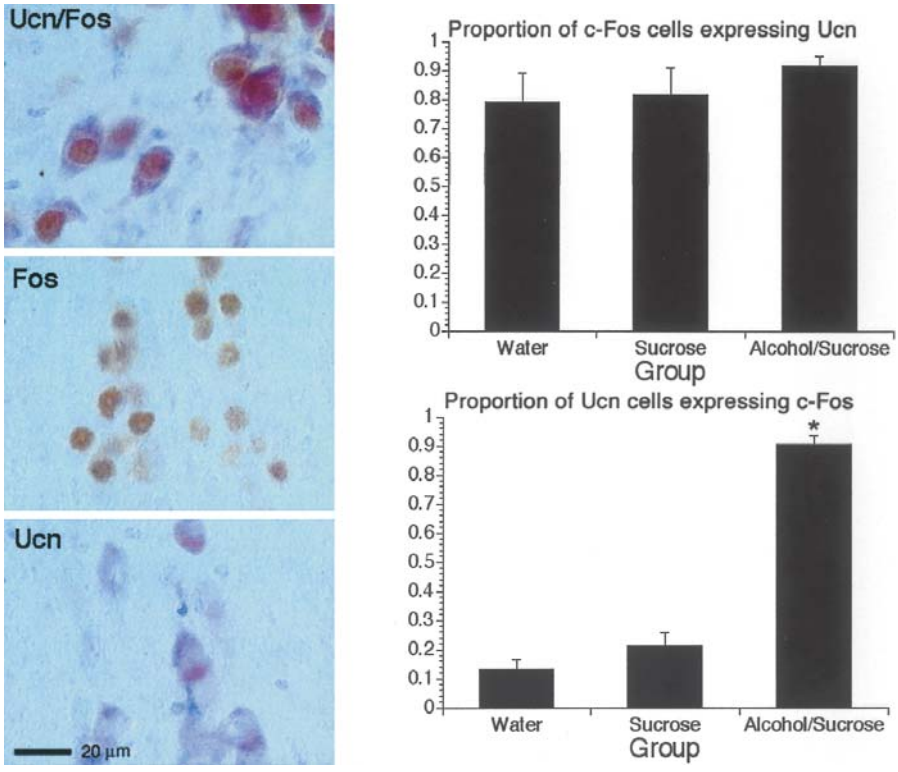

Fig. 3 Co-expression of c-Fos and urocortin in EW after the 30-min limited-access drinking procedure. *Left panel* Double-immunohistochemical staining in animals of the alcohol/sucrose group using antibodies against urocortin and c-Fos (*top*), only against c-Fos (*middle*) and only against urocortin (*bottom*). *Right panel* Proportion of c-Fos-positive cells expressing urocortin (*top*) and proportion of urocortin-positive cells expressing c-Fos (*bottom*). Data are shown as mean ratio±SEM. Note that the majority of c-Fos expression occurs in urocortin-positive cells in the alcohol/sucrose group, and that the majority of urocortin-expressing cells show induction of c-Fos in the alcohol/sucrose group. *Significantly different from other groups ($P<0.05$)

Table 2 Correlation of alcohol intake and c-Fos expression. Only brain regions with statistically significant correlations are shown. NS indicates not statistically significant ($P>0.05$). For abbreviations of brain regions see legend to Table 1

	Correlation coefficient between the number of c-Fos-positive cells and	
	Ethanol intake	BAC
Hippocampus		
CA1	–0.80 (P=0.0033)	–0.86 (P=0.0006)
CA2	–0.79 (P=0.0042)	–0.85 (P=0.001)
CA3	–0.67 (P=0.03)	–0.84 (P=0.0014)
DG	NS	–0.75 (P=0.011)
Hypothalamus		
VMH	NS	0.69 (P=0.024)
Neocortex		
Cg 1	–0.63 (P=0.0498)	–0.65 (P=0.039)
Septum		
LSD	–0.73 (P=0.025)	NS
LSI	–0.68 (P=0.041)	NS
Thalamus and midbrain		
OPT	NS	–0.75 (P=0.011)

tively. Of even more importance, it was found that 90.7% of urocortin cells in the EW expressed alcohol-induced c-Fos, while 13.2% and 21.5% of the urocortin cells expressed c-Fos in the water and sucrose groups, respectively.

To confirm the relationship between c-Fos expression and alcohol consumption, and to identify additional ethanol-responsive brain regions not identified by ANOVA, we also calculated correlations between number of c-Fos-positive cells, alcohol intake and BACs (Table 2). Negative correlations between alcohol intake and number of c-Fos-positive cells were found in LSD, CA1, CA2 and CA3, confirming ethanol specificity of the effects observed in these areas with the between-group ANOVAs. This specificity was further confirmed by negative correlations between BACs and c-Fos expression in CA1, CA2 and CA3 (Table 2).

In addition, negative correlations were found in several other areas that did not show significant differences between groups according to ANOVA. Significant negative correlations between c-Fos expression and ethanol-related measures and c-Fos expression were found in DG, Cg1, LSI and OPT. A positive correlation between c-Fos expression and BACS was found in the ventromedial

hypothalamus (VMH). To confirm ethanol specificity of these correlations, areas showing significant correlations but not group differences were further analyzed for correlations between c-Fos expression and consumption of water or sucrose in the corresponding groups. These correlations were not statistically significant ($P>0.05$).

Discussion

Analysis of alcohol consumption

The outcome of the present study was consistent with previous reports showing higher fluid consumption during the dark part of the circadian cycle in rodents (Freund 1970; Goldstein and Kakihana 1977). Thus, during an average limited-access session in the present study, mice consumed 2 ml of 10% sucrose, 0.9 ml of the 10% sucrose/10%ethanol solution or 0.8 ml water. This is in comparison with 1.6 ml of 10% sucrose, 0.6 ml of 10% sucrose/10% ethanol and 0 ml of water consumed by C57 mice in previous experiments (Bachtell et al. 1999; Ryabinin et al. 2001). Higher consumption of solution resulted in higher intakes of ethanol (2.9± 0.3 g/kg in comparison with an average of 1.9 g/kg in previous studies). The higher volumes of the 10% ethanol/10% sucrose solution consumed during a 30-min session resulted in BACs (250 mg%) that are considered highly intoxicating. Although such high BACs may seem surprising, they are in agreement with time courses of BACs measured in mice after intraperitoneal injections of similar doses of ethanol using gas chromatography (Grisel et al. 2002). To our knowledge, such intakes and BACs are the highest achieved in alcohol self-administration studies in non-deprived rodents. The main goal of the present study was to identify brain regions showing ethanol-specific responses after alcohol/sucrose self-administration. We did not evaluate the state of intoxication in alcohol-consuming mice behaviorally; however, a recent study demonstrated high levels of intoxication following similar alcohol intakes in water-deprived C57 mice (Finn et al. 2001). Future studies should take advantage of this procedure to address the levels of intoxication and alcohol dependency of animals voluntarily consuming such high doses of alcohol.

Ethanol and induction of c-Fos expression

In agreement with all previous studies using the voluntary alcohol self-administration procedure, consumption of ethanol/sucrose strongly induced c-Fos expression in EW (Topple et al. 1998; Bachtell et al. 1999; Ryabinin et al. 2001; Weitemier et al. 2001). Induction of c-Fos in EW has also been consistently observed after involuntary modes of alcohol administration (Chang et al. 1995; Ryabinin et al. 1997; Bachtell and Ryabinin 2001; Knapp et al. 2001), suggesting that c-Fos expression in this area is due to pharmacological actions of ethanol. EW has received little attention in alcohol research because it has been primarily considered in oculomotor adaptation functions. However, evidence is accumulating that EW is involved in other functions, including temperature regulation, nociception and anxiety (Loewy et al. 1978; Innis and Aghajanian 1986; Smith et al. 1998; Weninger et al. 1999). Moreover, EW is the main brain source of the neuropeptide urocortin, the alternative and strongest ligand of CRF receptors (Vaughan et al. 1995; Bittencourt et al. 1999). Our recent studies using inbred mouse strains suggest that urocortin-containing neurons of EW are involved in regulation of ethanol-induced hypothermia and alcohol consumption in mice (Bachtell et al. 2002). In the present study, we show through double immunohistochemistry that induction of c-Fos after alcohol consumption occurs in urocortin-containing neurons of EW. The induction of c-Fos in EW, however, is not correlated with intake of alcohol or BACs. Our previous studies with injections of ethanol showed that ethanol-induced c-Fos expression in EW reaches a plateau at 2.4 g/kg (Bachtell et al. 2002). Eight of ten mice in the alcohol/sucrose group exceed this dose, which could contribute to lack of correlation between c-Fos expression and alcohol-related measures. A weak, but statistically significant increase in c-Fos expression was also observed in EW of sucrose-consuming animals versus the water controls. A tendency toward such an increase was consistently observed in our previous studies (Bachtell et al. 1999; Ryabinin et al. 2001), although the difference between the water and sucrose groups reached statistical significance only in the present study. Since the differences in c-Fos expression in EW cannot be attributed to differences in volumes of consumed fluids (sucrose > alcohol/sucrose = water), we hypothesize that the large volumes of consumed sucrose in this study produced effects that were similar in some pharmacological or behavioral aspects to the effects to alcohol.

The finding that EW is the only brain region showing higher expression in the alcohol/sucrose versus sucrose and water groups is in agreement with three previous studies (Topple et al. 1998; Ryabinin et al. 2001; Weitemier et al. 2001). In addition, this finding suggests that induction of c-Fos in AcbC and CeM found in one of our previous studies (Bachtell et al. 1999) is not ethanol specific. In the latter study, mice consumed an average of 2 g/kg per day, but on the last day of the study unexpectedly increased their consumption to 2.6 g/kg. This is in contrast with the other three studies where alcohol-induced c-Fos in EW occurred in animals consistently consuming 2 g/kg of ethanol or lower. Since exposure to environmental novelty is capable of inducing c-Fos expression in the nucleus accumbens and central nucleus of amygdala (Honkaniemi et al. 1992; Ericsson et al. 1994; Beck and Fibiger 1995; Ryabinin et al. 1999), we believe that induction of c-Fos in CeM and AcbC observed in one study is not due to specific actions of ethanol. Instead, it is thought that these effects are due to stress of unexpected intoxication after a single session with a high dose of ethanol. Therefore, it is extremely

important that caution be taken so that animals do not experience one-time fluctuations on the day of immunohistochemical analysis. The present study is not hampered by this unexpected novelty in that animals in the alcohol/sucrose group consistently consumed nearly 3 g/kg ethanol throughout the procedure.

A positive correlation was noted between expression of c-Fos in VMH with BACs but not with intake of sucrose or water, suggesting involvement of VMH in the effects of alcohol. However, neither differences between groups nor correlations with ethanol intake were found significant. Therefore, significance of ethanol's effects in VMH is difficult to interpret.

Ethanol and suppression of c-Fos expression

In addition to ethanol-specific induction of c-Fos, we have also observed specific suppressive effects of ethanol. These include subregions of hippocampus, lateral septum, OPT, PH, prefrontal cortex (Cg1), and VLG. In general, suppressive effects of ethanol are in agreement with 2-deoxyglucose mapping showing suppression of metabolic activity in many areas after voluntary alcohol self-administration in rats (Williams-Hemby et al. 1996; Smith et al. 2001). Suppressive effects of ethanol in hippocampal subregions have been identified by us previously. These effects have been observed after both injections and self-administration of alcohol (Ryabinin et al. 1995, 1997, 1999; Bachtell et al. 1999). It has been suggested that alcohol exerts direct effects on hippocampal neurons (Browning et al. 1992; Ryabinin 1998). This idea is supported by the strong negative correlation of BACs and c-Fos expression in hippocampus in this study.

Suppressive effects of alcohol self-administration in Cg1, LSD, LSI, OPT, PH, and VLG, however, are being shown here for the first time. These effects may not have been observed previously because of two reasons: (1) these effects could require higher doses of ethanol not achieved in previous studies and (2) suppressive effects of ethanol can only be observed when basal levels of c-Fos expression are increased, e.g., due to higher activity in the dark phase of the circadian cycle. Whatever the reason, the effects in many of these regions agree with the connections of the EW nucleus and have allowed the development of a hypothetical neurocircuit. Thus, in rodents EW has been shown to send urocortin projections to the LSD/LSI border region (Bittencourt et al. 1999). Interestingly, this region is also known to have high levels of CRF-R2 mRNA expression (Van Pett et al. 2000). Activation of CRF receptors in this region has been shown to inhibit extracellular recording potentials upon i.c.v. administration of corticotropin releasing hormone, which supports the suppression of c-Fos expression noted here by alcohol (Siggins et al. 1985).

The lateral septum also receives prominent projections from several subregions of the hippocampus (Risold and Swanson 1997). It is possible that downregulation of c-Fos expression in LSD/LSI is due to innervation from both the EW and hippocampus. In addition to LSD/LSI, EW also has reciprocal projections to OPT, which in turn also has reciprocal connections to VLG (Paxinos 1985). Functional significance of these brain regions for alcohol's effects is unclear, but effects in these areas confirm that projection areas of EW are affected following ethanol's effects on EW.

Connections between VTA and EW have also been shown (Hogg 1966; Breen et al. 1983). However, the c-Fos response in VTA is different from other brain regions. Ethanol is also known to have direct effects on VTA (Wise and Rompre 1989; Di Chiara 1995; Koob and Moal 1997). Interestingly, in this study only c-Fos expression in VTA parallels volumes of consumed fluid between groups. However, c-Fos expression in VTA did not correlate with the amount of consumed fluid within any of the groups suggesting that activity of VTA is not simply related to the amount of consumed fluid but could be regulated by additional factors. Surprisingly, regulation of VTA in our study did not result in regulation of c-Fos expression in the nucleus accumbens, one of its prominent target regions. However, the prefrontal cortex (Cg1) is regulated by alcohol/sucrose consumption in this study. It is possible that this regulation occurs through innervation from a subpopulation of VTA neurons, or from the CA1 region of the hippocampus (Berger et al. 1976; Swanson 1981; Paxinos 1985).

Finally PH, showing lower expression of c-Fos in the alcohol/sucrose group versus the sucrose and water groups, receives projections from many of the areas identified above, including lateral septum, hippocampus and cingulate cortex (Cavdar et al. 2001).

Conclusions

Increased sensitivity of c-Fos expression mapping because of high ethanol intakes and the ability to detect suppressive effects of ethanol allowed us to identify several brain regions changing their expression during ethanol/sucrose self-administration. Interestingly, this pattern of brain regions is substantially different from those observed previously after forced ("involuntary") modes of alcohol administration (Chang et al. 1995; Hitzemann and Hitzemann 1997; Ryabinin et al. 1997; Thiele et al. 1997).

Importantly, many identified brain regions are connected to each other, allowing us to propose an hypothetical neurocircuit. We propose that direct action of ethanol affects activity of urocortin-positive neurons of EW. Ethanol also directly affects activity of hippocampus and VTA. However, ethanol's effects in these two regions depend on additional factors (e.g., environmental stress, etc.). Hippocampus and urocortin-positive cells of EW send projections to LSD/LSI, which project to hypothalamic subregions (such as PH and VMH) and could regulate consumption of alcohol/sucrose. Hypothalamic subregions also receive projections from the prefrontal

cortex (Cg1), which in turn receive projections from VTA and hippocampus.

Taken together, a neurocircuit containing EW, LSD/LSI, hippocampus, prefrontal cortex, VTA and subregions of hypothalamus could be important for the consumption of highly intoxicating doses of alcohol, and should be investigated by direct manipulation of the identified brain regions.

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