

Neuroplasticity in brain reward circuitry following a history of ethanol dependence

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Abstract

Mitogen-activated and extracellular regulated kinase (MEK) and extracellular signal-regulated protein kinase (ERK) pathways may underlie ethanol-induced neuroplasticity. Here, we used the MEK inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (UO126) to probe the role of MEK/ERK signaling for the cellular response to an acute ethanol challenge in rats with or without a history of ethanol dependence. Ethanol (1.5 g/kg, i.p.) induced expression of the marker genes *c-fos* and *egr-1* in brain regions associated with both rewarding and stressful ethanol actions. Under non-dependent conditions, ethanol-induced *c-fos* expression was generally not affected by MEK inhibition, with the exception of the medial amygdala (MeA). In contrast, following a history of dependence, a markedly suppressed *c-fos* response to acute ethanol was found in the medial pre-frontal/orbitofrontal cortex (OFC), nucleus accumbens shell (AcbSh) and paraventricular nucleus (PVN). The suppressed ethanol response in the OFC and AcbSh, key regions involved in ethanol preference and seeking, was restored by pre-treatment with UO126, demonstrating a recruitment of an ERK/MEK-mediated inhibitory regulation in the post-dependent state. Conversely, in brain areas involved in stress responses (MeA and PVN), an MEK/ERK-mediated cellular activation by acute ethanol was lost following a history of dependence. These data reveal region-specific neuroadaptations encompassing the MEK/ERK pathway in ethanol dependence. Recruitment of MEK/ERK-mediated suppression of the ethanol response in the OFC and AcbSh may reflect devaluation of ethanol as a reinforcer, whereas loss of an MEK/ERK-mediated response in the MeA and PVN may reflect tolerance to its aversive actions. These two neuroadaptations could act in concert to facilitate progression into ethanol dependence.

Introduction

Transition to ethanol dependence involves long-term neuroadaptations that lead to excessive voluntary ethanol intake and altered responses to stress (Heilig & Koob, 2007). Long-lasting neural and behavioral plasticity thought to model this process has been observed in laboratory rats following a history of dependence induced by prolonged exposure to ethanol vapor. Repeated cycles of intoxication and withdrawal, which mimic the course of the clinical condition, are the most effective paradigm for inducing these long-term neuroadaptations, together labeled as ‘the post-dependent state’ (Roberts *et al.*, 2000; Rimondini *et al.*, 2002, 2003; O’Dell *et al.*, 2004; Valdez & Koob, 2004; Breese *et al.*, 2005; Sommer *et al.*, 2008).

The post-dependent state is associated with a stable up-regulation in the expression of several genes encoding members of mitogen-activated protein kinase (MAPK) pathways in the pre-frontal cortex (Rimondini *et al.*, 2002). Elevated expression of several MAPKs has also been found in the nucleus accumbens of a genetically selected alcohol-preferring rat line (Arlinde *et al.*, 2004). MAPK pathways, i.e. mitogen-activated and extracellular regulated kinase (MEK) and extracellular signal-regulated protein kinase (ERK), have previously

been implicated in the development of drug dependence. Inhibition of ERK attenuates cocaine-induced hyper-locomotion and antagonizes cocaine-induced expression of the immediate early gene *c-fos* (Valjent *et al.*, 2000), whereas ERK1 null mutants show increased sensitivity to the rewarding properties of morphine (Mazzucchelli *et al.*, 2002). The effects of ethanol on MEK/ERK signaling are more complex. A decrease in phosphorylated ERK in the brain was found during ethanol exposure, whereas ERK phosphorylation increased during withdrawal (Sanna *et al.*, 2002; Roberto *et al.*, 2003; Chandler & Sutton, 2005). However, little is known about the long-term regulation of MEK/ERK signaling following a history of dependence and its possible role in the behavioral phenotype observed in the post-dependent state.

The stimulus-dependent activation of marker genes such as *c-fos* and *erg-1* is mediated in part by MAPK pathways (Bachtell *et al.*, 2002; Schuck *et al.*, 2003). The induction of these immediate early genes is mainly observed in neurons (Chaudhuri *et al.*, 1995; Tsai *et al.*, 2000; Hansson *et al.*, 2003) and is stimulus-specific to a degree that allows classification of psychoactive drugs (Sumner *et al.*, 2004). In rodents, acute ethanol administration at moderate doses is consistently reported to induce *c-fos* expression in both regions associated with aversion, stress responses and sensory information processing [e.g. central amygdala (CeA), hypothalamic paraventricular nucleus (PVN) and Edinger-Westphal nucleus, respectively (Ryabinin *et al.*, 1997)] and regions thought to be involved in positive

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drug reinforcement such as the ventral tegmental area, nucleus accumbens and pre-frontal cortex (Zoeller & Fletcher, 1994; Chang *et al.*, 1995; Hitzemann & Hitzemann, 1997; Ryabinin *et al.*, 1997; Bachtell *et al.*, 2002; McBride, 2002; Crankshaw *et al.*, 2003).

We reasoned that the expression of *c-fos* and *egr-1* after acute ethanol challenge, administered in the presence or absence of the MEK1/2 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (UO126), would delineate structures differentially involved in the initial ethanol response in dependent and non-dependent animals, respectively, and would thus serve as a marker for neuroadaptive processes associated with the development of the dependent state. We focused particularly on forebrain structures known to be involved in the mediation of drug seeking and positive and negative drug reinforcement.

Materials and methods

Animals

The male Wistar rats (Møllegaard, Denmark) weighed 220–250 g at the beginning of the experiment and were housed four per cage under a reversed light/dark cycle with free access to food and water. All experimental procedures using animals were carried out under the National Animal Welfare Act and were approved by the local ethical committees (Stockholm South Animal Ethics Committee, ethics permits S84/98).

Drugs

The MEK1/2 inhibitor, UO126 (Calbiochem, CA, USA), was dissolved in different concentrations (1.25, 2.5 and 5 nmol) in 4% dimethylsulfoxide (DMSO) and 0.9% saline according to Coogan & Piggins (2003). UO126 in 4% DMSO was pre-warmed prior to i.c.v. injection in order to prevent precipitations. UO126 inhibition is non-competitive with respect to MEK substrates, ATP and ERK (Calbiochem).

Animal procedures

Rats were anaesthetized with halothane (1.5% in an air flow of 1.5 L/min) and placed in a Kopf stereotaxic frame, unilaterally implanted with guide cannulae into the right lateral ventricle [coordinates: Bregma posterior, 0.8 mm; lateral, 1.4 mm; ventral, 4.3 mm, according to the atlas of Paxinos & Watson (1998)] and were afterwards handled for 5 min daily for 10 days.

For *in-situ* hybridization, rats were decapitated and brains were quickly removed, snap frozen in liquid isopentane (−40 °C) and stored at −70 °C. Coronal brain sections (10 μm) were cryosectioned at forebrain bregma levels +2.0, 1.8 and −3.0 mm (Paxinos & Watson, 1998) (Fig. 1).

In preliminary experiments we established the effects of UO126 (1, 2.5 and 5 nmol, injected i.c.v.) and vehicle (4% DMSO in 0.9% saline; injection volume, 5 μL; injection time, 5 min) on ethanol-induced *c-fos* expression in ethanol-naive Wistar rats. With the exception of the medial amygdala (MeA) we found no effects of UO126 on ethanol-induced *c-fos* expression (see Fig. 2). Because an apparent lack of effect of UO126 could have been caused by a low MEK response to ethanol, insufficient diffusion of the inhibitor to sites of action, or both, a control experiment was carried out using the robust phospho-ERK response to systemic amphetamine (10 mg/kg) as positive control. UO126 (2.5 nmol in 4% DMSO) administered i.c.v. effectively blocked amphetamine-induced phospho-ERK1/2 immuno-

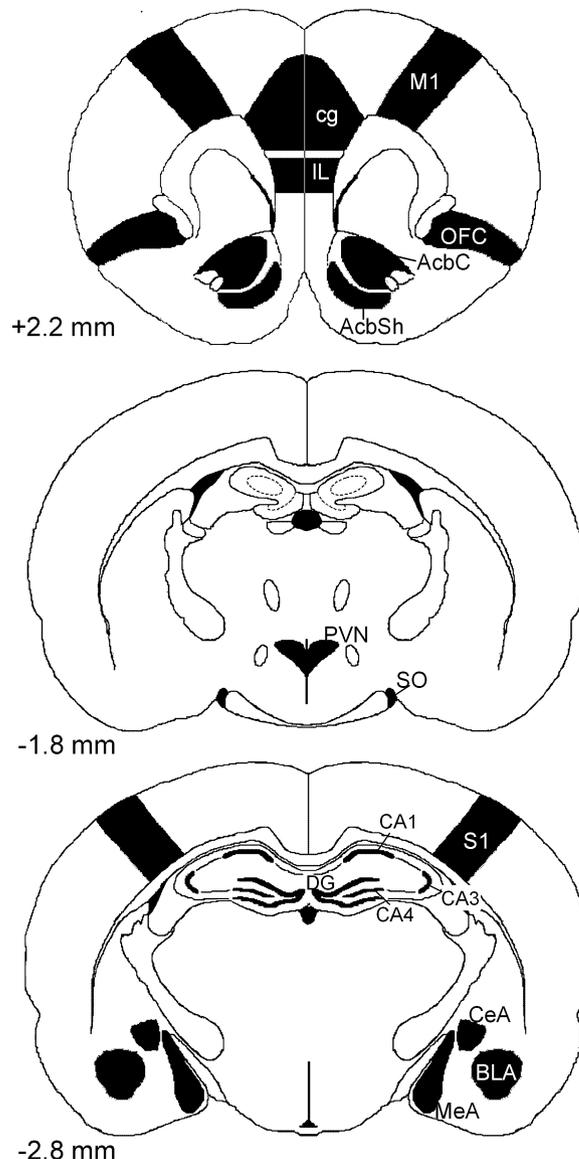


FIG. 1. Schematic representation of the sampled areas for the densitometric evaluation of mRNAs in a coronal section through the rat forebrain at Bregma levels +2 to −3 mm. cg, cingulate cortex; M1, frontal motor cortex; S1, primary sensory cortex; IL, infralimbic cortex; OFC, orbitofrontal cortex; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; CeA, central amygdaloid nucleus; MeA, medial amygdaloid nucleus; BLA, basolateral amygdaloid nucleus; dorsal hippocampal subregions [Cornu Ammon (CA) areas, CA1–CA4]; DG, dentate gyrus; PVN, hypothalamic paraventricular nucleus and SO, supraoptic nucleus.

reactivity in the primary motor cortex at Bregma 2.5 mm (see Supplementary material, Fig. S1).

Experiment I

In order to make Experiments I and II comparable, all rats were injected i.c.v. with vehicle. At 30 min after i.c.v. injections, rats were injected i.p. with either a moderate dose of ethanol (1.5 g/kg, see Ryabinin *et al.*, 1997) or 0.9% saline.

Ethanol vapor exposure

Procedures were carried out as described previously in Rimondini *et al.* (2002). The exposure was as follows: 1 week of habituation to

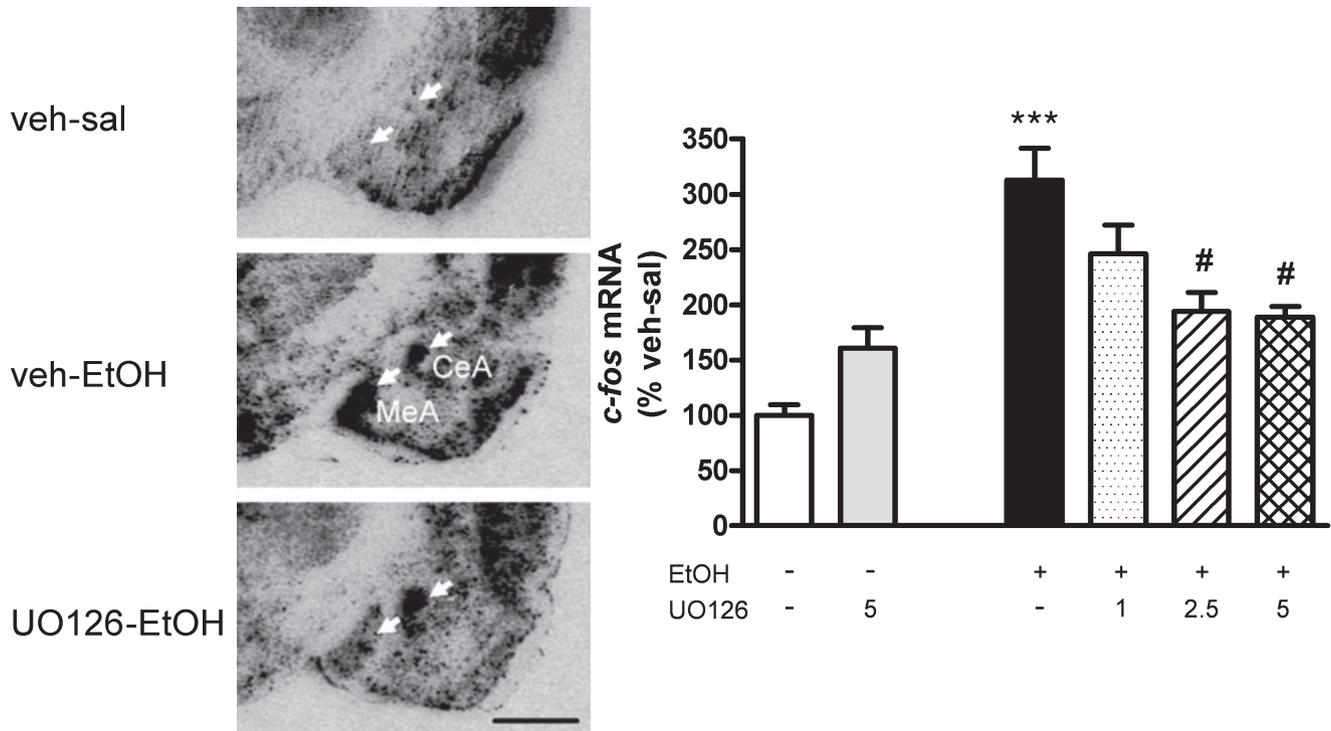


FIG. 2. Right: Bar graph illustrating *c-fos* expression in the MeA after UO126 (1, 2.5 and 5 nmol, i.c.v.) and ethanol (EtOH) (1.5 g/kg, i.p.) treatment in naive Wistar rats. Corrected *P*-values: ****P* < 0.001 vs. vehicle-saline (veh-sal) group, #*P* < 0.05 vs. veh-EtOH control group. Left: Bright-field microphotographs from autoradiograms of *in-situ* hybridization of *c-fos* mRNA in the amygdala region after UO126 (5 nmol, i.c.v.) and EtOH (1.5 g/kg, i.p.) treatment in naive Wistar rats. Arrows indicate *c-fos* mRNA in MeA and CeA. *c-fos* mRNA levels are increased in both MeA and CeA after EtOH challenge and decreased in MeA after UO126 treatment. UO126 treatment shows no effect on EtOH-*c-fos* in CeA. Scale bar, 1 mm. For details on treatment, see Materials and methods.

the chambers (no alcohol), 1 week of continuous exposure to 22 mg/L alcohol to adapt to the novel odor and 7 weeks of exposure to alcohol vapor adjusted to produce blood alcohol concentrations (BACs) of 150–320 mg/dL for 17 h (16:00–09:00 h) each day. Once a week during exposure, rats were weighed and blood was collected from the tail veins. Control rats were housed under the same conditions, except for the addition of ethanol vapor to the air flow. Ethanol vapor exposure was followed by a 3-week period of abstinence in order to eliminate effects of acute withdrawal. After 2 weeks of the abstinence period, a random subgroup of rats from each condition was selected for assessment of voluntary ethanol drinking and placed in single cages for 1 week to habituate to this new environment and reduce potential stress-induced effects on drinking.

Two-bottle free choice

Following completion of the abstinence period, increasing concentrations of ethanol were made available in a 0.2% saccharin solution, as continuous two-bottle free choice between the ethanol/saccharin and saccharin alone solutions. Briefly, the ethanol concentration was increased as follows: days 1–3, 2% ethanol; days 4–7, 4% ethanol; from day 8, 6% ethanol (v/v solutions). The consumption of ethanol/saccharin and saccharin alone was measured on Monday, Wednesday and Friday at the same time. Bottle positions were alternated daily to avoid development of side preference.

Experiment II

Ethanol vapor-exposed and control rats were unilaterally implanted with cannula guides and handled as described above. Based on the

results of the initial experiment, ethanol vapor-exposed and age-matched control rats were i.c.v. injected with either UO126 (2.5 nmol) or vehicle alone using the same injection volume and injection time as described for Experiment I. At 30 min after i.c.v. injections all animal groups received the same dose of ethanol (i.p., 1.5 g/kg) as used in Experiment I.

Rats from both Experiments I and II were decapitated at 45 min after the last injection and trunk blood was collected for blood alcohol and plasma corticosterone measurements.

Radioimmunoassay for corticosterone

Trunk blood was collected in heparine-containing tubes and centrifuged at 2000 g for 20 min at 4 °C. Plasma corticosterone (CORT) levels were determined by radioimmunoassay (Coat-a-count, Diagnostic Products Corporation, Los Angeles, CA, USA). The radioimmunoassay was performed with rat [¹²⁵I]CORT and had a detection limit of ~5.7 ng/mL.

Blood alcohol concentration

Plasma was assayed for ethanol using an Analox system (Analox Instruments Ltd, Lunenburg, MA, USA) according to the manufacturer's instructions.

In-situ hybridization

The rat specific riboprobes for *c-fos* [gene reference sequence in Pub-Med database (<http://www.ncbi.nlm.nih.gov/Entrez/>); NM_022197.1,

position 306–864 bp], *egr-1* [gene reference sequence in PubMed database (<http://www.ncbi.nlm.nih.gov/Entrez/>); NM_012551.1, position 1384–1851 bp] and the *in-situ* hybridization have been recently described (Hansson *et al.*, 2003, 2006). Phosphor imaging plates (Fujifilm for BAS-5000, Fujifilm Corp., Japan) were exposed for 48 h to hybridized sections. Phosphor imager (Fujifilm Bio-Imaging Analyzer Systems, BAS-5000, Fujifilm Corp.) generated digital images were analysed using MCID Image Analysis Software (Imaging Research Inc., UK). Regions of interest were defined by anatomical landmarks as described in the atlas of Paxinos & Watson (1998) and illustrated in Fig. 1. Based on the known radioactivity in the ^{14}C standards, image values were converted to nCi/g. For detailed visualization, slides were subsequently exposed for 1 month to Kodak BioMax MR film (Eastman Kodak Company, UK).

Immunohistochemistry

For immunohistochemical studies another set of normal male Wistar rats were cannulated as described above. At 10 days after surgery rats received i.c.v. either UO126 (2.5 nmol) or vehicle (4% DMSO); 30 min later rats were i.p. injected with either amphetamine (10 mg/kg) or saline. For phospho-ERK immunohistochemistry, rats were killed with a lethal dose of pentobarbital (100 mg/kg, i.p.) 15 min later and perfused intracardially with ice-cold saline and 4% paraformaldehyde. Brains were removed, post-fixed for 1.5 h in the same fixative and transferred to 10% sucrose solution in 0.1 M phosphate buffer for 48 h. Immunohistochemistry was carried out as described in Hansson *et al.* (2003) using the polyclonal rabbit anti-phospho-p44/42 MAPK antibody (1 : 250, Cell Signaling Technology, Inc., Boston, USA). The immunostaining for phospho-ERK is similar to that in Cai *et al.* (2000).

Statistics

Ethanol consumption is expressed as the amount of ethanol ingested per day (g/kg/day). All data are expressed as means \pm SEM. The data met assumptions of normality and homogeneity of variances, and were analysed using standard parametric ANOVA. Region-wise one-way ANOVAs were used to identify ethanol-responsive regions for *c-fos* and *egr-1* expression in Experiment I. Correction was made by Holm's sequential rejective testing procedure with respect to the 16 analysed brain regions (Holm, 1979). Only ethanol-responsive regions as identified in Experiment I were subjected to a two-way ANOVA for ethanol and UO126 effects in Experiment II. In cases where the test suggested UO126 or interaction effects, a planned post-hoc procedure was carried out to test for significant effects between groups using Fisher's PSLD. The number of these tests was added to the initial 16 tests to calculate the respective Holm's correction factor. Raw *P*-values are reported and significance is indicated at levels for $\alpha < 0.05$, $\alpha < 0.01$ or $\alpha < 0.001$.

Results

Experiment I: acute ethanol response in drug-naive animals

At 45 min after acute systemic ethanol administration the BACs in the ethanol-treated groups were between 175 and 228 mg/dL.

Significant induction of *c-fos* by ethanol was found in several cortical regions [anterior cingulate cortex, infralimbic cortex and orbitofrontal cortex (OFC)], the nucleus accumbens (nucleus accumbens shell and nucleus accumbens core), the central and medial nuclei

TABLE 1. Effects of acute ethanol (EtOH) (1.5 g/kg, i.p.) on *c-fos* and *egr-1* gene expression levels in different brain regions of naive Wistar rats

Region	<i>c-fos</i>		<i>egr-1</i>	
	Veh-sal	Veh-EtOH	Veh-sal	Veh-EtOH
cg	22.3 \pm 1.7	31.5 \pm 1.4*	100.7 \pm 5.6	96.0 \pm 2.8
M1	14.4 \pm 1.0	16.2 \pm 1.5	64.3 \pm 5.6	57.2 \pm 3.6
S1	18.8 \pm 1.0	25.1 \pm 0.5	92.0 \pm 3.3	92.4 \pm 3.6
IL	25.8 \pm 1.9	36.5 \pm 1.5**	64.0 \pm 2.6	63.0 \pm 1.6
OFC	20.3 \pm 0.5	27.3 \pm 1.0***	110.7 \pm 5.9	100.9 \pm 4.5
AcbC	4.4 \pm 0.2	11.3 \pm 1.3**	26.6 \pm 1.9	33.6 \pm 1.9
AcbSh	4.2 \pm 0.3	7.4 \pm 0.3***	40.1 \pm 1.1	48.9 \pm 2.0
CeA	4.7 \pm 0.9	18.8 \pm 3.1*	19.5 \pm 1.5	52.6 \pm 9.4**
MeA	5.2 \pm 0.5	16.2 \pm 1.5***	15.6 \pm 0.8	32.7 \pm 1.6***
BLA	9.8 \pm 0.7	10.3 \pm 0.5	35.4 \pm 2.7	42.6 \pm 2.1
CA1	6.6 \pm 0.7	5.7 \pm 0.7	109.9 \pm 3.0	104.2 \pm 2.2
CA3	11.7 \pm 0.8	10.8 \pm 1.2	45.0 \pm 2.4	38.1 \pm 2.5
CA4	14.4 \pm 1.3	12.6 \pm 1.0	41.6 \pm 1.9	43.1 \pm 2.2
DG	5.5 \pm 0.6	4.2 \pm 0.4	25.0 \pm 1.4	26.2 \pm 1.1
SO	13.1 \pm 0.4	42.0 \pm 4.0**	18.5 \pm 1.1	29.7 \pm 2.8*
PVN	37.9 \pm 1.9	254.9 \pm 13***	23.3 \pm 2.2	107.6 \pm 3.9***

Data are expressed as nCi/g (mean \pm SEM), $n = 4\text{--}6/\text{group}$. For details of treatment see Materials and methods. For anatomical abbreviations see Fig. 1; sal, saline; veh, vehicle. Corrected *P*-values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, veh-EtOH vs. veh-sal.

of the amygdala (CeA and MeA), and hypothalamic regions (supraoptic nucleus and hypothalamic PVN). The dorsal hippocampus and basolateral amygdala were unaffected. The results and respective statistics are given in Table 1 and illustrated in Fig. 3.

Acute ethanol challenge had a less pronounced effect on *egr-1* mRNA levels. Induction was found in the CeA and MeA as well as in the supraoptic nucleus and PVN (Table 1, Fig. 3).

Experiment II: effects of UO126 on ethanol-induced marker gene expression in post-dependent animals

Similarly to our previous experiments (Rimondini *et al.*, 2002), daily 17-h exposure to ethanol vapor induced BAC in the range of 150–250 mg/dL, which fell to an undetectable level within 5 h during the ethanol-off period with detectable signs of mild withdrawal such as tail stiffness and piloerection towards the end of the 7-week exposure period. Withdrawal intensity did not reach seizure level.

Effects of intoxication procedure on ethanol consumption and corticosterone levels

After 3 weeks of abstinence, ethanol consumption was assessed in a randomly selected subgroup of rats. We found a greater than twofold increase in voluntary ethanol drinking in the two-bottle free-choice test of exposed rats vs. controls demonstrating long-lasting behavioral plasticity induced by the exposure paradigm [mean daily intake from ethanol bottle (6% v/v), 2.6 \pm 0.16 and 1.2 \pm 0.21 g/kg/day, $F_{1,19} = 55.6$, $P < 0.001$ exposed vs. control].

The remaining rats were implanted with i.c.v. cannula guides and familiarized with the experimental environment during the abstinence period. On the day of the experiment ethanol-dependent rats and controls were given UO126 (2.5 nmol, i.c.v.) or vehicle (4% DMSO, i.c.v.) followed by an acute ethanol challenge (1.5 mg/kg, i.p.). The dose of 2.5 nmol UO126 was chosen based on the results of the initial dose–response experiment (Fig. 2). Rats were killed 45 min after the last injection. At this time point, mean BACs were between 155 and

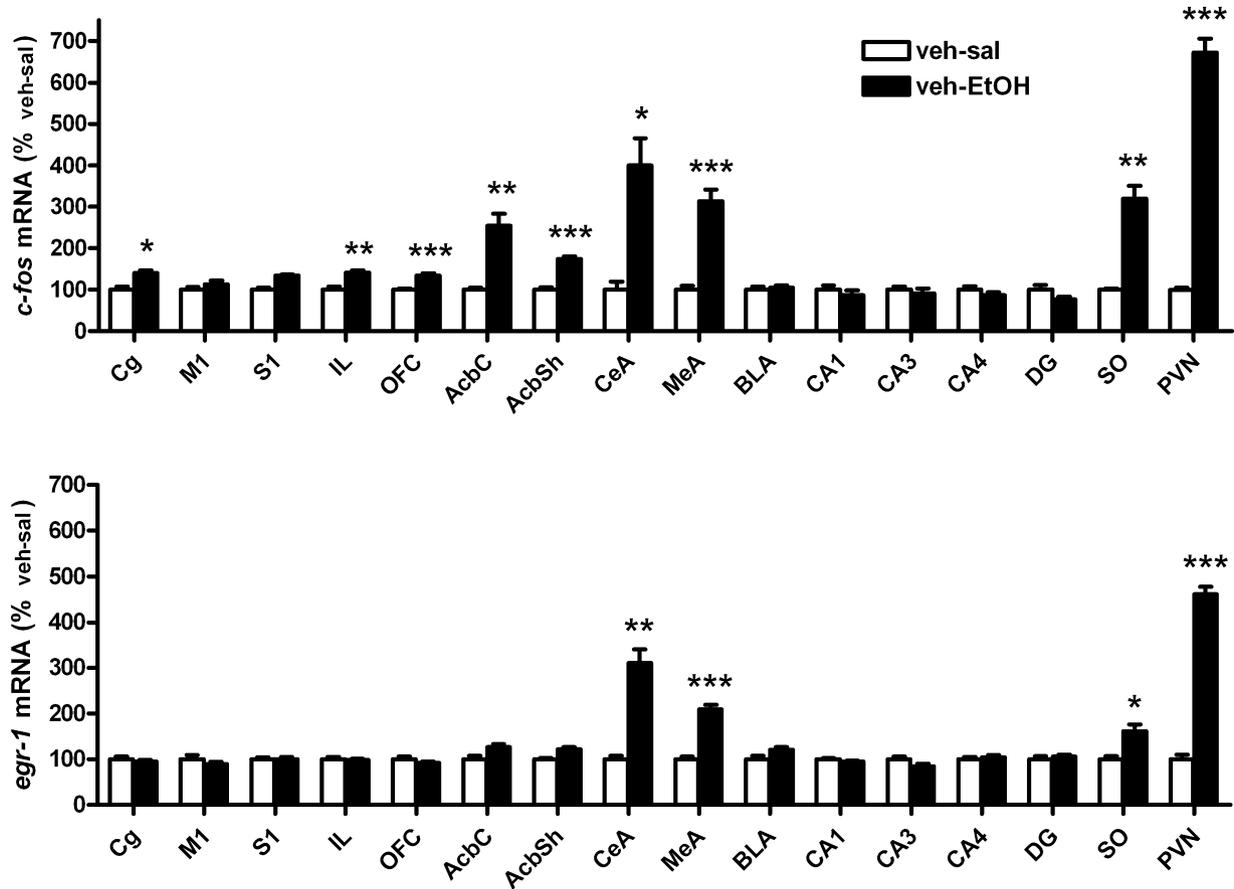


FIG. 3. Ethanol (EtOH)-induced *c-fos* (upper panel) and *egr-1* (lower panel) expression in different forebrain regions of Wistar rats. Bar graphs illustrating *c-fos* and *egr-1* expression at 45 min after EtOH (1.5 g/kg, i.p., black bars) or saline (sal) (i.p., unfilled bars) injection in rats pre-treated with vehicle (veh) (4% DMSO, i.c.v.). Data are expressed as percent of control group (% veh-sal group, mean \pm SEM). Statistical analysis were performed by one-way ANOVA followed by Holm's corrected Bonferroni's post-hoc test ($n = 4-6$ /group, corrected P -values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). For abbreviations see Fig. 1, for details on treatment, see Materials and methods.

170 mg/dL without significant differences between the groups. However, plasma corticosterone levels were significantly lower in exposed vs. control rats (two-way ANOVA; main exposure effect: $F_{1,24} = 9.7$, $P < 0.01$; main UO126 effect: $F_{1,24} = 0.5$, not significant; interaction: $F_{1,24} = 0.1$, not significant).

Ethanol-induced *c-fos* and *egr-1* expression in dependent rats

The transcript levels of *c-fos* were analysed in ethanol-responsive brain regions as identified by Experiment 1 by two-way ANOVA for effects of history of dependence and UO126 treatment. Dependent rats showed a significantly attenuated induction of *c-fos* expression by ethanol in the anterior cingulate cortex, infralimbic cortex and OFC and in the PVN as demonstrated by post-hoc comparison between exposed-vehicle vs. control-vehicle (see Tables 2 and 3, Fig. 4). No such effects were found on *egr-1* expression in any region (Tables 2 and 3).

In post-dependent rats UO126 (2.5 nmol) increased *c-fos* expression in the OFC and nucleus accumbens shell, and decreased *c-fos* in the supraoptic nucleus (post-hoc comparison between exposed-UO126 vs. control-vehicle groups, Table 3 and Fig. 4). Notably, the only UO126-sensitive region in non-dependent rats, the MeA, showed no effect in ethanol vapor-exposed rats (Table 2, Fig. 4). Furthermore, UO126 treatment in dependent rats significantly decreased *egr-1* in the supraoptic nucleus (Table 3).

Discussion

With the exception of the MeA and, to some extent, the PVN, we found that ethanol-induced neuronal activation as probed by *c-fos* and *egr-1* expression is generally not affected by UO126 in non-dependent animals. In contrast, following a history of dependence, ERK pathways are recruited and suppress the cellular response to ethanol in the OFC and nucleus accumbens shell, brain regions known to mediate drug seeking and positive reinforcement. Conversely, the MEK/ERK-mediated cellular response to ethanol originally present in the MeA and PVN, probably related to the behavioral and endocrine stress response to ethanol, is lost (summarized in Fig. 5).

Ethanol effects on marker gene expression in the forebrain of drug-naive animals

We observed *c-fos* responses to acute ethanol in the pre-frontal cortex, nucleus accumbens, centro-medial amygdala and hypothalamic regions. This pattern is likely to reflect simultaneous activation by ethanol of structures that mediate its reinforcing, as well as stress-like actions. Our results are in general agreement with previous studies on ethanol-induced *c-fos* immunoreactivity (Chang *et al.*, 1995; Hitzemann & Hitzemann, 1997; Ryabinin *et al.*, 1997; Ryabinin & Wang, 1998; Knapp *et al.*, 2001; McBride, 2002; Herring *et al.*, 2004) and

TABLE 2. Effects of the MEK1/2 inhibitor UO126 (2.5 nmol, i.c.v.) and acute ethanol (EtOH) (1.5 g/kg, i.p.) on *c-fos* and *egr-1* gene expression levels in different brain regions of 7-week cyclic EtOH-exposed Wistar rats

Region	Control-vehicle	Control-UO126	EtOH-exposed-vehicle	EtOH-exposed-UO126
<i>c-fos</i>				
cg	95.2 ± 5.8	89.4 ± 3.5	75.6 ± 1.9	79.2 ± 3.8
M1	25.8 ± 2.6	30.9 ± 2.2	23.4 ± 1.0	28.4 ± 1.9
S1	68.7 ± 4.6	54.9 ± 4.4	68.0 ± 3.3	58.7 ± 5.7
IL	80.7 ± 4.9	67.4 ± 6.1	58.0 ± 3.3	62.9 ± 6.3
OFC	58.1 ± 2.9	59.7 ± 2.3	42.5 ± 2.0	67.6 ± 2.3
AcbC	24.8 ± 2.1	26.8 ± 1.9	21.8 ± 1.0	27.0 ± 1.7
AcbSh	12.5 ± 1.1	11.9 ± 0.5	10.5 ± 0.4	14.3 ± 1.0
CeA	83.0 ± 2.4	87.2 ± 3.3	92.2 ± 4.7	100.3 ± 9.4
MeA	53.8 ± 1.8	41.1 ± 2.0	45.6 ± 2.2	42.8 ± 3.4
BLA	55.1 ± 3.3	50.7 ± 1.9	60.4 ± 1.4	51.5 ± 2.2
CA1	47.8 ± 3.6	26.3 ± 2.4	53.6 ± 2.6	47.8 ± 3.6
CA3	77.5 ± 5.2	70.6 ± 5.2	80.4 ± 2.2	77.0 ± 5.3
CA4	63.4 ± 2.6	52.0 ± 3.4	68.0 ± 1.8	66.4 ± 4.1
DG	52.5 ± 1.0	31.2 ± 5.7	55.2 ± 3.4	43.3 ± 7.4
SO	268.8 ± 12.7	276.1 ± 23.0	302.6 ± 20.6	172.5 ± 6.3
PVN	380.6 ± 27.2	318.7 ± 10.5	215.8 ± 21.2	244.8 ± 21.3
<i>egr-1</i>				
cg	182.4 ± 7.5	189.6 ± 5.6	176.0 ± 5.1	183.5 ± 6.7
M1	97.5 ± 3.4	105.6 ± 4.1	101.2 ± 5.4	101.5 ± 4.6
S1	191.3 ± 2.2	167.9 ± 6.4	178.0 ± 5.9	176.9 ± 5.4
IL	93.5 ± 3.7	83.7 ± 4.7	76.9 ± 3.8	87.6 ± 3.9
OFC	125.6 ± 3.5	142.3 ± 3.7	132.7 ± 7.3	139.0 ± 5.5
AcbC	48.0 ± 2.0	73.9 ± 2.2	62.4 ± 2.3	64.7 ± 2.1
AcbSh	78.9 ± 1.7	98.2 ± 4.7	90.2 ± 0.8	92.0 ± 2.3
CeA	167.4 ± 15.5	129.9 ± 14.6	141.6 ± 14.7	137.2 ± 12.8
MeA	72.9 ± 4.8	61.6 ± 3.3	59.1 ± 4.8	59.0 ± 3.3
BLA	89.4 ± 2.8	83.3 ± 3.4	84.9 ± 4.3	80.9 ± 2.0
CA1	164.4 ± 6.4	165.3 ± 6.2	180.1 ± 3.1	140.0 ± 4.2
CA3	107.4 ± 7.7	116.9 ± 10.1	108.4 ± 6.9	106.4 ± 5.2
CA4	94.7 ± 4.3	94.4 ± 5.8	90.8 ± 5.8	96.9 ± 4.9
DG	71.0 ± 3.4	68.9 ± 6.2	65.1 ± 4.1	71.5 ± 4.0
SO	125.0 ± 2.8	106.5 ± 8.1	105.8 ± 4.4	77.6 ± 5.3
PVN	143.8 ± 6.1	157.2 ± 11.8	93.9 ± 10.1	102.8 ± 11.7

Data are expressed as nCi/g (means ± SEM), $n = 6-7$ /group. For anatomical abbreviations see Fig. 1 and for details of treatment, see Materials and methods. veh, vehicle.

demonstrate a distinct *c-fos* response profile that is different from other psychoactive drugs.

It has been shown that *c-fos* activation patterns allow classification of drugs according to their neurochemical mechanism of action (Sumner *et al.*, 2004). For instance, psychostimulants seem to consistently activate *c-fos* in pre-frontal and striatal regions, and this effect is likely to be mediated via dopamine. The specific activation pattern caused by ethanol probably reflects this drug acting via a broader range of neurotransmitter systems. Thus, like psychostimulants, ethanol induces *c-fos* in the pre-frontal cortex and ventral striatum but lacks their action in the caudate putamen. Simultaneously, ethanol also induces *c-fos* in regions involved in processing of negative emotions and stress responses, which show consistent *c-fos* activation by antidepressants and some anxiolytics (Sumner *et al.*, 2004). The *c-fos* profile of ethanol is also different from that of acute opioid and endocannabinoid action on this gene (Garcia *et al.*, 1995; Gutstein *et al.*, 1998; Erdtmann-Vourliotis *et al.*, 1999; Valjent *et al.*, 2001; Derkinderen *et al.*, 2003), although these neurotransmitter systems appear to play a key role in mediating the positively reinforcing properties of ethanol. In summary, most drugs of abuse, including ethanol, induce *c-fos* in the nucleus accumbens but show very different activation patterns in other brain regions.

The *egr-1* response to acute ethanol paralleled, in a less pronounced manner, the pattern of *c-fos* expression. In contrast to *c-fos*, *egr-1* has a high basal expression in many brain regions and, because of that, may be less sensitive to induction. The discordance in the regulation of *c-fos* and *egr-1* may also reflect that different neuronal cell populations express these immediate early genes and that these are differentially sensitive to ethanol challenge. For example, *egr-1* was found to be expressed in excitatory but not inhibitory cortical neurons (Ponomarev *et al.*, 2006).

Role of ERK1/2 in ethanol-induced marker gene expression in non-dependent animals

In contrast to *c-fos*, phosphorylation of ERK1/2 shows a more consistent pattern upon acute challenge with addictive drugs, including amphetamine, cocaine, Δ^9 -tetrahydrocannabinol, nicotine and morphine. All of these cause strong phosphorylation of ERK in the pre-frontal cortex, nucleus accumbens, bed nucleus of stria terminalis (BNST) and CeA (for review see Lu *et al.*, 2006; Girault *et al.*, 2007; Zhai *et al.*, 2008). A number of studies have shown that MEK/ERK signaling regulates both *c-fos* and *egr-1* expression (reviewed by Davis, 1995; Seger & Krebs, 1995; Kaufmann *et al.*, 2001) and the ERK activation observed after administration of drugs of abuse other than ethanol is probably a key signal for induction of these genes. Ethanol appears to fundamentally differ from other drugs of abuse in that the MEK/ERK cascade does not seem to mediate ethanol-induced *c-fos* and *egr-1* expression, found to be insensitive to the inhibitor in non-dependent animals. These observations are in agreement with and predicted by studies that show that acute ethanol, rather than activating ERK phosphorylation suppresses it in a wide range of brain regions, including the cerebral cortex, nucleus accumbens and hippocampus (Davis *et al.*, 1999; Kalluri & Ticku, 2002; Chandler & Sutton, 2005; Neznanova *et al.*, 2007).

The one exception to this pattern was found in the MeA, where UO126 significantly blocked ethanol-induced *c-fos* (but not *egr-1*) expression. This MeA-specific effect points to an important functional differentiation between the CeA and MeA in the mediation of ethanol effects. The importance of the CeA in mediating autonomic and behavioral responses to aversive stimuli is well established (Davis, 1992; Möller *et al.*, 1997). Although less is known about the MeA, this structure seems to modulate defensive behavior (Dielenberg *et al.*, 2001; Blanchard *et al.*, 2005). Lesions of the MeA, but not of the CeA, markedly reduce immediate defensive responses, such as freezing and assessment behaviors. Furthermore, the amount of freezing seems to be correlated with *c-fos* expression in the MeA (Chen *et al.*, 2006). Thus, this structure has a highly specialized role in emotional processing and effects of alcohol here may provide a substrate for altered processing of emotional stimuli in alcoholics. Similar to the CeA, the MeA projects to the medial hypothalamus. In fact, it has been pointed out that medial rather than CeA is critical for the activation of the HPA axis in response to emotional stressors (Dayas *et al.*, 1999). Notably, the strongest induction of both marker genes by ethanol in the present study was found in the PVN. Here, MEK/ERK inhibition seemed to have a trend effect on ethanol-induced *c-fos* expression. Together with the MeA finding, these data suggest that MEK/ERK signaling in non-dependent animals is probably involved in the response to ethanol as a stressor (Fig. 5).

Neuroadaptations following ethanol dependence

Following a history of dependence, we found a recruitment of inhibitory MEK/ERK signaling in the OFC and nucleus accumbens

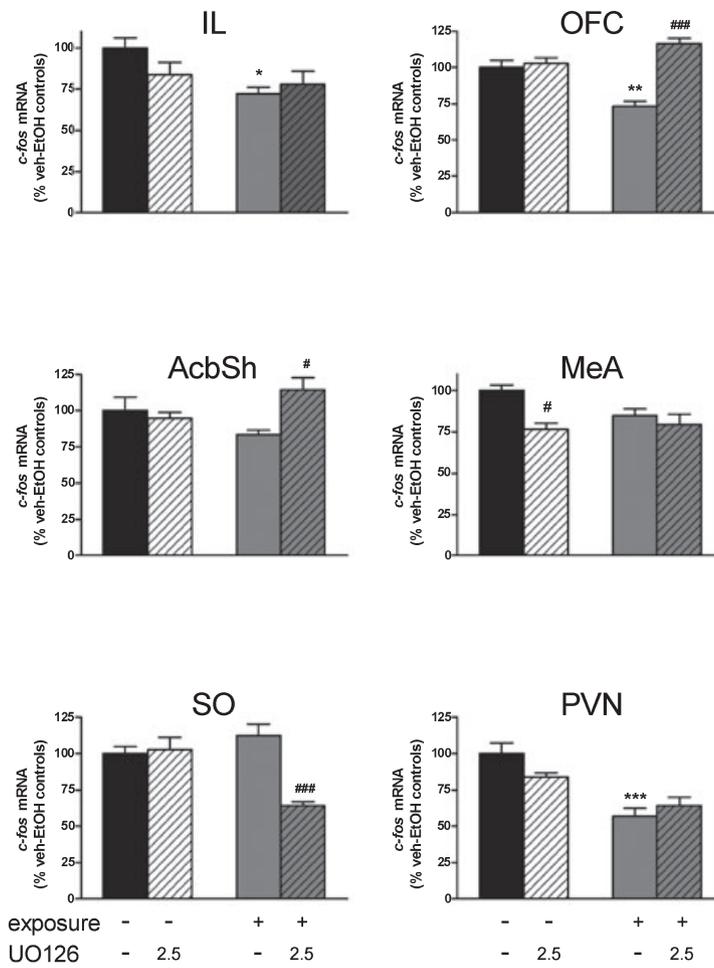
TABLE 3. Regions with statistically significant effects on *c-fos* and *egr-1* expression in Experiment II

Region and contrast	<i>F</i> -value [d.f.]	Planned comparisons			
		<i>P</i> -value	Exp-veh vs. control-veh	Control-UO vs. control-veh	Exp-UO vs. exp-veh
<i>c-fos</i>					
cg					
Exp	15.1 [1,23]	0.000737			
UO	0.1 [1,23]	n.s.			
Exp × UO	1.5 [1,23]	n.s.	0.001740*	n.s.	n.s.
IL					
Exp	6.7 [1,23]	0.016139			
UO	0.6 [1,23]	n.s.			
Exp × UO	3.0 [1,23]	n.s.	0.004659*	n.s.	n.s.
OFC					
Exp	2.4 [1,22]	n.s.			
UO	29.4 [1,22]	0.000019			
Exp × UO	22.6 [1,22]	0.000095	0.000116**	n.s.	0.000001 ^{###}
AcbC					
Exp	0.7 [1,24]	n.s.			
UO	4.2 [1,24]	n.s.			
Exp × UO	0.9 [1,24]	n.s.	n.t.	n.t.	n.t.
AcbSh					
Exp	0.1 [1,23]	n.s.			
UO	4.0 [1,23]	n.s.			
Exp × UO	8.0 [1,23]	0.009697	n.s.	n.s.	0.002002 [#]
CeA					
Exp	3.4 [1,22]	n.s.			
UO	1.0 [1,22]	n.s.			
Exp × UO	0.1 [1,22]	n.s.	n.t.	n.t.	n.t.
MeA					
Exp	1.7 [1,23]	n.s.			
UO	9.8 [1,23]	0.004748			
Exp × UO	4.0 [1,23]	n.s.	0.032436	0.001722 [#]	n.s.
SO					
Exp	4.5 [1,19]	0.048160			
UO	13.8 [1,19]	0.001462			
Exp × UO	17.3 [1,19]	0.000533	n.s.	n.s.	0.000018 ^{###}
PVN					
Exp	37.0 [1,16]	0.000016			
UO	0.7 [1,16]	n.s.			
Exp × UO	5.4 [1,16]	0.034302	0.000036***	0.042449	n.s.
<i>egr-1</i>					
CeA					
Exp	0.4 [1,22]	n.s.			
UO	2.1 [1,22]	n.s.			
Exp × UO	1.3 [1,22]	n.s.	n.t.	n.t.	n.t.
MeA					
Exp	4.0 [1,23]	n.s.			
UO	1.9 [1,23]	n.s.			
Exp × UO	1.9 [1,23]	n.s.	n.t.	n.t.	n.t.
SO					
Exp	16.4 [1,22]	0.000535			
UO	15.4 [1,22]	0.000720			
Exp × UO	0.7 [1,22]	n.s.	0.039708	0.047051	0.001928 [#]
PVN					
Exp	20.9 [1,16]	0.000311			
UO	1.0 [1,16]	n.s.			
Exp × UO	0.04 [1,16]	n.s.	0.012196	n.s.	n.s.

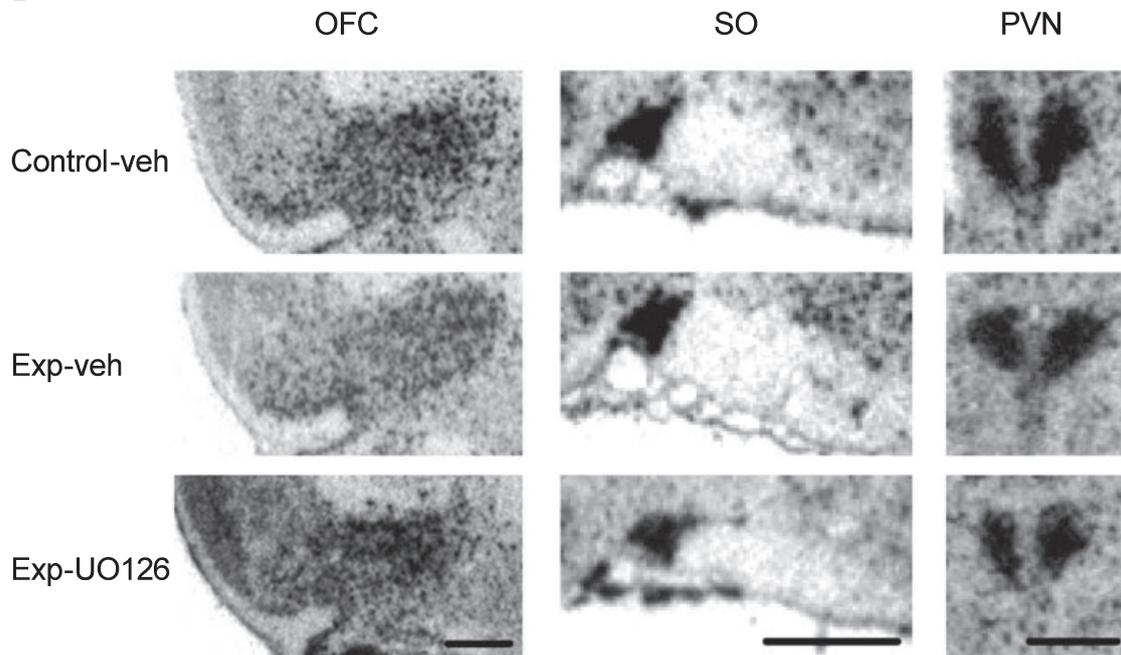
Statistical analysis was performed by two-way ANOVA in brain regions responding to a challenging dose of ethanol (EtOH) as identified in Experiment I to assess EtOH vapor exposure (exp) and the effect of the MEK1/2 inhibitor UO126 (UO) (2.5 nmol, i.c.v.) as well as the interaction of EtOH vapor exposure and MEK1/2 inhibitor in rats injected i.p. with EtOH (1.5 g/kg). In order to correct for multiple tests with a family-wise error rate of 0.05, Holm's corrected Bonferroni's post-hoc test was used for every planned comparison. Corrected *P*-values: *[#]*P* < 0.05, ***P* < 0.01, ***^{###}*P* < 0.001. veh, vehicle; n.s., not significant; n.t., not tested. For anatomical abbreviations see Fig. 1 and for details of treatment, see Materials and methods.

FIG. 4. (A) Bar graphs illustrating the effects of the MEK inhibitor UO126 (2.5 nmol, i.c.v.) or vehicle (veh) (4% DMSO, i.c.v.) on ethanol (EtOH) (1.5 g/kg, i.p.)-induced *c-fos* mRNA in different forebrain regions of rats with a history of EtOH dependence and age-matched controls. Corrected *P*-values: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vehicle-treated EtOH-exposed vs. vehicle-treated control group; [#]*P* < 0.05, ^{###}*P* < 0.001 UO126-treated group vs. corresponding EtOH-exposed or control group. (B) Bright-field microphotographs from autoradiograms of *in-situ* hybridization show the effects of the MEK inhibitor UO126 (2.5 nmol, i.c.v.) on EtOH (1.5 g/kg, i.p.)-induced *c-fos* mRNA levels in the OFC, supraoptic nucleus (SO) and PVN region of rats with a history of EtOH dependence and vehicle-treated age-matched control rats. Scale bar, 1 mm. For abbreviations see Fig. 1, for details on treatment, see Materials and methods. Exp, ethanol vapor exposed.

A



B



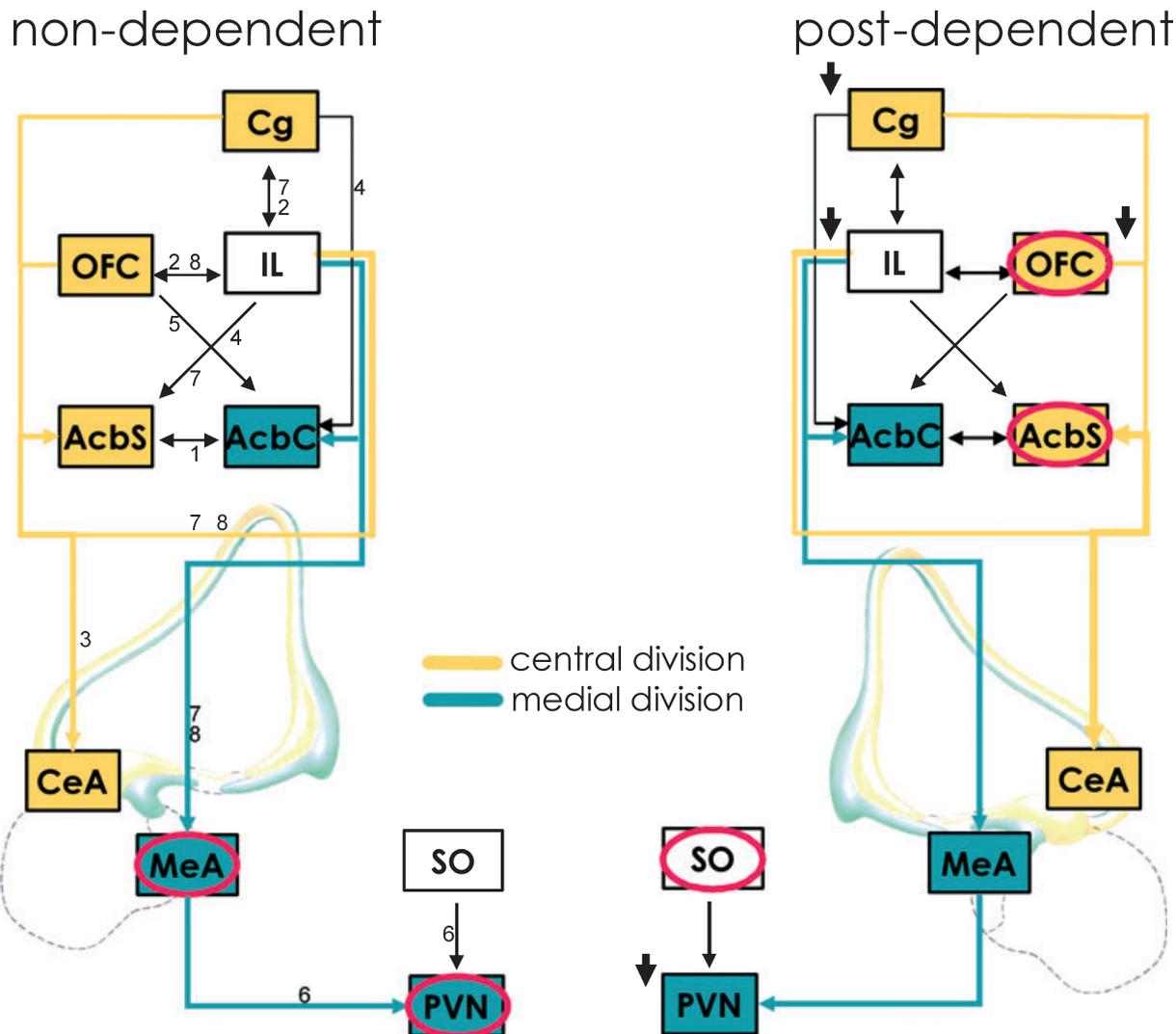


FIG. 5. Schematic representation summarizing the results on ethanol (EtOH)-induced *c-fos* expression and its interaction with the ERK1/2 kinase signaling pathway in brain regions related to the extended amygdala (adapted from Heimer, 2003). Only those regions are shown that react in non-dependent rats (left) with increased *c-fos* to an acute EtOH (1.5 g/kg, i.p.) challenge. Under non-dependent conditions, EtOH-induced *c-fos* expression was generally not affected by MEK inhibition, with the exception of the MeA (red circles) and, to a lesser extent, the PVN. Post-dependent rats (right) show reduced *c-fos* induction in the pre-frontal cortex [anterior cingulate cortex (Cg), infralimbic cortex (IL) and OFC] and nucleus accumbens shell (AcbSh), key components of circuitry mediating positive drug reinforcement, demonstrating a recruitment of an ERK-mediated inhibitory regulation in the post-dependent state. Thus, positive MEK/ERK–EtOH interactions are related to the central division and negative interactions to the medial division of the extended amygdala. In addition to anatomical evidence of a division of the extended amygdala into central and medial parts (Alheid, 2003; Heimer, 2003) our results support the idea of corresponding functional divisions, i.e. devaluation of EtOH as a reinforcer and tolerance to its aversive actions, respectively, which may both take part in the development of EtOH dependence. Brain regions anatomically or functionally related to the central or medial divisions of the extended amygdala are shown in yellow or blue, respectively, and their efferents/afferents are indicated as arrows of the respective color. Black arrows indicate other connections between the regions. For abbreviations see Fig. 1, for details on treatment, see Materials and methods. ¹van Dongen *et al.* (2005), ²Hoover & Vertes (2007), ³McDonald *et al.* (1996), ⁴Reynolds & Zahm (2005), ⁵Schoenbaum & Setlow (2003), ⁶Silverman *et al.* (1981), ⁷Vertes (2004), ⁸Vertes (2006). AcbS, nucleus accumbens shell.

shell, two structures intimately involved in drug seeking and ethanol preference, respectively (Kalivas *et al.*, 2005; Schoenbaum & Shaham, 2008). In the non-dependent state, the *c-fos* response to an acute ethanol challenge in these structures was robust and insensitive to UO126. In contrast, in post-dependent animals, this response was markedly suppressed but was restored by pre-treatment with the MEK inhibitor. This provides evidence for a functional recruitment of MAPK signaling in the post-dependent state and may be a correlate of the up-regulated MAPK expression previously found under these conditions (Rimondini *et al.*, 2002). Recruitment

of inhibitory mechanisms within the OFC–nucleus accumbens circuitry (Homayoun & Moghaddam, 2006) may result in a devaluation of ethanol reward and thus contribute to escalation of drug intake.

The opposite pattern was observed within structures related to stress responses, where ethanol responses found in non-dependent animals were absent or attenuated following a history of dependence. Thus, the ERK-dependent *c-fos* response to acute ethanol found in the MeA of non-dependent rats was eliminated following a history of dependence. A very similar pattern was seen within the hypothalamic PVN.

Together, these data suggest tolerance to ethanol effects within stress-responsive circuitry following a history of dependence. This is in line with neuroendocrine data demonstrating attenuated HPA axis function in post-dependent animals and the suggestion that the HPA axis develops tolerance to the effects of ethanol (Rivier *et al.*, 1990; Zorrilla *et al.*, 2001). Progressive attenuation of ethanol-induced stress responses may remove a brake on excessive ethanol intake and serve as a permissive factor in the development of dependence.

Conclusions

We show that excessive voluntary ethanol intake observed following a history of dependence is accompanied by long-term plasticity of neuronal circuitries mediating acute ethanol effects. ERK pathways within structures that mediate positive and negative drug reinforcement, respectively, are differentially affected by dependence-induced plasticity. Within the former, inhibitory ERK influence is recruited in a manner that may attenuate ethanol reward and lead to compensatory escalation of ethanol intake. Within the latter, tolerance to an acute ethanol challenge evolves, and may be mediated by, a down-regulation of ERK-mediated responses, in particular within the MeA. This may contribute to the development of dependence by removing a brake on excessive ethanol intake.

Supplementary material

The following supplementary material may be found on

<http://www.blackwell-synergy.com>

Fig. S1. UO126 blocked amphetamine-induced phospho-ERK1/2 immunoreactivity in the primary motor cortex.

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Abbreviations

BAC, blood alcohol concentration; CeA, central amygdala; DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; MeA, medial amygdala; MEK, mitogen-activated and extracellular regulated kinase; OFC, orbitofrontal cortex; PVN, paraventricular nucleus; UO126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene.

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