

# Ethanol-Sensitive Brain Regions in Rat and Mouse: A Cartographic Review, Using Immediate Early Gene Expression

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**Background:** Ethanol addiction has been conceptualized as a progression from occasional, impulsive use to compulsive behavior. Ethanol-dependence is a chronic pathology with repeated cycles of withdrawal, craving, and relapse. Specific molecular and cellular mechanisms underlie these transition stages.

**Methods:** This review aimed at elucidating whether there are also adaptations in the pattern of brain regions responding to ethanol. This paper reviews the evidence in rodents for activation of specific brain regions, assessed by induction of IEG expression, following acute and chronic ethanol exposure.

**Results:** The review sheds light on the specific patterns of response in regions of the brain to different types of ethanol exposure and shows that activation of specific brain regions may occur in particular phases of the development of ethanol addiction. Some brain regions respond consistently following acute or chronic treatments or withdrawal: the prefrontal cortex; nucleus accumbens; lateral septum; hippocampus; periolocomotor urocortin-containing cells population (pIIIu), also known as Edinger-Westphal nucleus; central nucleus of the amygdala; and the paraventricular nucleus of hypothalamus. The two last brain areas are particularly activated by relapse-inducing stressors. It is of interest that the amygdala, hippocampus, and prefrontal cortex, which belong to the reward system, are activated by cue-induced relapse to ethanol self-administration in rodents and humans, while activation of these regions is reversed with anticraving compounds. Following chronic exposure, IEG induction desensitizes while withdrawal reactivates these regions.

**Discussion:** Some responding regions are implicated in reward related processes (VTA, extended amygdala, hypothalamus, hippocampus, prelimbic cortex, ventral part of lateral septum) and some others in aversive-related processes (area postrema, nucleus of solitary tract).

**Conclusion:** A better understanding of the neural circuits affected by ethanol and their adaptations during the development of ethanol addiction will provide new opportunities for developing appropriate therapies.

**Key Words:** Mesolimbic Dopamine Circuitry, Immediate Early Gene, c-Fos, Egr-1, Extended Amygdala.

**A**DDICTION IS DEFINED as a “chronically relapsing disorder characterized by compulsive drug-taking, an inability to limit the intake of drugs and the emergence of a withdrawal syndrome during cessation of drug taking (dependence)” (Koob et al., 1998). The state of addiction develops gradually, and there are 3 main stages of transition. The first

is the occasional/intermittent acute drug administration phase, followed by chronic, controlled drug use, in turn followed by the compulsive, out-of-control drug use. Abstinence may be marked by intense and prolonged drug-seeking behaviour, that may lead to relapse (Kalivas, 2005; Nestler, 2004; Torres and Horowitz, 1996). Specific molecular mechanisms underlie these different stages of transition and are responsible for progression from tolerance to dependence, withdrawal, craving, and relapse (Koob et al., 2004; Nestler et al., 2001; Ron and Jurd, 2005). Addiction is thought to depend on molecular and cellular adaptations that lead to persistent changes in transcription, translation, and synaptic morphology that are extremely long-lived (Kalivas, 2005; Koob et al., 1998, 2004; Nestler, 2004; Nestler et al., 1999). The stability of some of these adaptations led to the hypothesis that long-term plasticity (i.e., the regulation of gene expression) is one important underlying mechanism involved in these persistent modifications (Nestler et al., 1999; Ron and Jurd, 2005).

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The immediate-early genes (IEGs) belong to a class comprising genes that are rapidly activated, usually in a transient fashion, in response to intracellular signalling cascades (Sng et al., 2004). Some IEGs encode transcription factors and their induction is responsible for the regulation of transcription of some particular genes. Thus, the IEG encoded transcription factors are implicated in orchestrating changes in gene expression that underlie long term neuronal plasticity (Nestler et al., 1999). Transient stimulus-induced expression of IEGs is believed to be involved in the transduction of extracellular stimuli into prolonged modifications of cellular function (Sabban and Kvetnanský, 2001). While some of these cellular and molecular mechanisms are not addiction-related, some of them are thought to mediate the transitional events leading to addiction.

In the last 15 years, the expression of IEGs has become an important focus in the field of addiction because it has been shown that acute or chronic intake of drugs of abuse changes IEG expression (Kalivas, 2005; Nestler, 2004; Ryabinin, 2000). These changes have attracted much experimental attention as these genes may be part of the neurobiological substrates underlying addiction (Torres and Horowitz, 1996). Thus, the mapping of the drug-induced IEG expression in the brain has been used to identify brain regions that may be involved in the development of tolerance, sensitization, craving, withdrawal, relapse, and addiction.

This paper will review the evidence in rodents for activation of specific brain regions, assessed by induction of IEG expression, following acute and chronic ethanol exposure. We focus on establishing which particular regions of the rodent brain showed IEG expression after acute and chronic ethanol exposures and also during withdrawal. Our aim is to shed light on the regional changes that could underlie the transition from occasional drug use to abuse and addiction (Koob et al., 1998).

This review focuses on 2 particular IEGs, c-Fos and Egr-1, as in neuroscience the mapping of these IEGs has become an established methodology (Nagahara and Handa, 1995; Ryabinin, 2000). The IEGs of the Fos family encode nuclear proteins that are components of the AP-1 transcription factor complex. There are 4 Fos-related genes, referred to as c-Fos, fra1, fra2, and fosB (Yen et al., 1991). These genes encode c-Fos and the isoforms of c-Fos called FRAs (Fos-related antigens), Fra-1, Fra-2, FosB, and the truncated forms of FosB, i.e., delta FosB and the chronic FRAs (for a review see Hope, 1998; Nestler et al., 1999). These Fos family proteins heterodimerize with Jun family proteins (c-Jun, JunB, or JunD) in order to bind to the AP-1 site that is present in the promoter of numerous genes. The AP-1 binding results in either activation or repression of transcriptional activity. These Fos family proteins are rapidly induced in specific brain areas: this induction peaks at 1 to 3 hours, after which expression typically decreases back to basal levels within 16 hours (Chang et al., 1995; Morgan and Curran, 1989). C-Fos expression increases as a result of neuronal activation, and c-Fos is widely used for monitoring the brain areas activated as a result of different manipulations: administration of drugs

of abuse, of antidepressants treatments or following kainate lesions (for a review see Herdegen and Leah, 1998; Nestler et al., 1999; Borlikova et al., 2006). Egr-1 (Early Growth Response 1) is a member of the zinc finger family of IEGs (for a review see O'Donovan et al., 1999). It has been suggested that Egr-1 may be a marker of synaptic plasticity rather than neuronal activity (Jones et al., 2001; Knapska and Kaczmarek, 2004; Yano and Steiner, 2005).

The majority of data presented in this review are from immunocytochemistry (ICC), while other data are from in situ hybridization (ISH). The difference between these 2 techniques is that they analyze protein versus RNA levels, and also that they use different approaches: ICC studies primarily count positive cells, while ISH studies analyze density of signal or count grains/cell. These technical issues reflect different mechanisms. ICC measures a change in protein-expressive cells, whatever the amplitude of this expression. ISH gives the density of RNA expression, whatever the number of cells that contains the studied RNA. Therefore, these technical approaches could lead to different results concerning the expression of the protein or the RNA.

To determine the neurobiological substrates of ethanol addiction, it is crucial to identify the neuronal networks that are activated by ethanol (Bachtell et al., 1999; Koob et al., 1998). Induction of c-Fos or Egr-1 IEGs has been used to identify neural structures that may change their activity in response to drug challenges. It is important to analyze several IEGs in mapping studies, as the level of IEG induction can vary in strength depending on the IEG, and the species and the strains on which the tests are conducted.

Beside their relevance as mapping tools, c-Fos and Egr1 could be specifically involved in ethanol effects. The AP-1 transcription factor complex results from the heterodimerization of one protein of the Fos family with one protein of the Jun family. AP-1 controls both basal and inducible transcription of several genes. Interestingly, AP-1 is a regulator of the *N*-methyl-D-aspartate R2B (NR2B) gene transcription (Qiang and Ticku, 2005), the gene that encodes the protein subunit NMDAR2B of the NMDA receptor. This subunit has been shown to be of particular interest with regard to ethanol addiction. Long-term administration of a beverage of 10% ethanol for 12 weeks in rats up-regulated NR2B protein density in the hippocampus (Bhupanapadu Sunkesula et al., 2008). The treatment of cultured cortical neurons with ethanol for 5 days increased NR2B mRNA density (Sheela Rani and Ticku, 2006), increased the AP-1 binding activity and promoter activity, and increased FosB protein (Qiang and Ticku, 2005). Moreover, drug-induced alterations in the extracellular signal regulated kinase (ERK) signalling pathway in the mesocorticolimbic dopamine system contribute to the drug's rewarding effects (Valjent et al., 2006; Zhai et al., 2007). The expression of c-Fos and Egr1 are both under the control of NMDA and D1 receptors via the ERK pathway (Lu et al., 2006; Valjent et al., 2004). Consequently, ethanol-induced regulation of c-Fos and Egr-1 expression could play a role in the long-term cellular adaptations induced by ethanol abuse. Considering

these elements, we have made an effort in this review to map IEG expression changes at different stages of ethanol exposure, such as acute and chronic exposure and withdrawal.

## IEG EXPRESSION AFTER ACUTE ETHANOL USE

### *c-Fos and Fos-Family Proteins Expression After Acute Ethanol Treatment*

There have been numerous studies of brain regions sensitive to acute ethanol administration in rats and mice. All the major anatomical parts of the brain show changes in c-Fos protein and/or mRNA following acute ethanol administration, as listed below (see also Table 1): *cerebral cortex*: infralimbic, orbital, insular, cingulate, piriform and motor cortices, parietal association and temporal association cortices; subcortical *telencephalon*: nucleus accumbens (both core and shell parts), lateral septum, bed nucleus of the stria terminalis (BNST), striatum (or caudate putamen), globus pallidus, ventral pallidum, and entopeduncular nucleus (also referred to as medial globus pallidus); several nuclei of the amygdala (central nucleus, lateral, basolateral, and medial amygdala); the *diencephalon*: paraventricular and rhomboid nuclei of the thalamus, paraventricular nucleus of the hypothalamus (especially its anterior parvocellular part), anterior and dorsal hypothalamus; supraoptic and suprachiasmatic nuclei; subthalamic nucleus and lateral geniculate nucleus, the *midbrain*: substantia nigra (SN) *pars compacta* (SNc) and *pars reticulata* (SNr), ventral tegmentum area (VTA) and in urocortin containing periculomotor neurons (pIIIu), previously known as the preganglionic Edinger-Westphal nucleus (Horn et al., 2008; May et al., 2008), the *hindbrain*: locus coeruleus, area postrema, parabrachial nucleus, and nucleus of the solitary tract (Bachtell and Ryabinin, 2001; Bachtell et al., 2002; Canales, 2004; Chang et al., 1995; Crankshaw et al., 2003; Criado and Morales, 2000; Hansson et al., 2008; Hitzemann and Hitzemann, 1997; Knapp et al., 2001; Kolodziejska-Akiyama et al., 2005; Ogilvie et al., 1998; Ryabinin and Wang, 1998; Ryabinin et al., 1997, 2000; Thiele et al., 1997, 2000; Turek and Ryabinin, 2005).

Ethanol-induced expression of c-Fos is dose-dependent and increases between 0.5 to 3 g/kg of ethanol (Chang et al., 1995). Induction of protein expression is observed from 1 to 4 hours after ethanol administration (Chang et al., 1995), and the maximum level of induction is most often recorded after 2 hours. As can be expected, mRNA is expressed more rapidly than Fos protein—within 1 hour—and, depending on the method of administration, may be found as early as 10 minutes after intragastric (i.g.) ethanol administration (Ogilvie et al., 1998). As c-Fos expression is particularly sensitive to stress stimuli, animals are often habituated to handling and injections before experiments in order to minimize c-Fos basal levels (Bachtell et al., 2002; Ryabinin et al., 1997). There is almost no c-Fos expression in most brain regions of rats receiving intraperitoneal (i.p.) injections of saline, if animals are habituated to these injections (Ryabinin et al., 1995).

The evaluation of ethanol-induced c-Fos or other IEG expression is hampered by the different experimental paradigms used for ethanol administration and the delay that has been chosen to observe IEG expression following ethanol administration. For example, 3 g/kg i.g. or i.p. ethanol injections in rats produce different temporal patterns of *c-Fos* mRNA and Fos protein inductions in the parvocellular paraventricular hypothalamic nucleus. Following i.p. ethanol injection, levels of paraventricular nucleus c-Fos mRNA was already significantly elevated within 10 minutes, peaked 60 minutes after injection, and remained elevated for 4 hours. In contrast, the paraventricular nucleus of rats injected intragastrically with ethanol showed 2 smaller rises in c-Fos mRNA levels at 30 minutes and at 4 hours after injection (Ogilvie et al., 1998). The difference in time courses could be related to differences in blood ethanol concentrations (BECs) achieved by the 2 different routes of administration. I.p. or i.g. 3 g/kg ethanol both evoked a peak of BEC within 15 minutes of administration, that remained elevated for at least 3 hours. However, at this 15-min peak, the i.p. injections doubled the BEC relative to the i.g. administrations (Ogilvie et al., 1998). While the time courses of the response differ, it is clear that i.p. and i.g. administrations induced c-Fos expression increase in the paraventricular nucleus of the hypothalamus (Knapp et al., 2001). The central nucleus of amygdala (CeA), one of the major ethanol-responding regions, shows c-Fos expression after either i.p. injection and ethanol inhalation (Ryabinin et al., 1997) or i.g. administration (Knapp et al., 2001), but does not show c-Fos induction after intracerebroventricular (i.c.v.) administration (Crankshaw et al., 2003). Again, this difference could be due to different BECs achieved in the different experiments. These examples show that the parameters of route, dose, and BECs may influence both the c-Fos induction time-course and the regions responding (for a review see Ryabinin and Weitemier, 2006).

Besides differences in ethanol-responding regions attributed to different methods of ethanol administration or to different BECs achieved, discrepancies between strains must be considered as well. For example, there was an ethanol-induced expression of c-Fos protein in the CeA, paraventricular nucleus of the hypothalamus, entopeduncular nucleus, SNr, dorsomedial caudate-putamen and core and shell of the nucleus accumbens in mice following an acute i.p. administration of ethanol 0.5, 1, 2, and 4 g/kg. This increase of c-Fos was dose dependent and was greater after the 4 g/kg ethanol dose than following the 0.5 g/kg dose. This induction was strain dependent: in the CeA for example, the induction was lower in C57BL6/J mice as compared to DBA/2/J mice at all the doses tested (Hitzemann and Hitzemann, 1997).

### *Egr1 Protein Expression After Acute Ethanol Treatment*

Acute ethanol administration induces expression of Egr-1 mRNA or protein in the frontal and piriform cortices of rats

**Table 1.** Regions Showing Acute Alcohol Induced c-Fos, Fos B, and Egr-1 Expression Changes (mRNA and Protein)

Ethanol treatment	mRNA or protein	Type of regulation	Regions	Delay after ethanol administration	Species	References
1 and 3 g/kg, i.p.	c-Fos ICC	Increase	Nucleus accumbens core Nucleus accumbens shell CeA Paraventricular nucleus of hypothalamus (parvocellular part) Locus coeruleus Parabrachial nucleus Nucleus of the solitary tract Area postrema Infralimbic cortex Orbital cortex Insular cortex Parietal association cortex Temporal association cortex Nucleus accumbens core Nucleus accumbens shell BNST Caudate-Putamen CeA Paraventricular nucleus of thalamus Paraventricular nucleus of hypothalamus pilu	2 hours	Rat (Long-Evans)	Thiele et al., 1997
1.5 g/kg, i.p.	c-Fos ICC	Increase		2 hours	Rat (Sprague-Dawley)	Ryabinin et al., 1997
1.5 g/kg, i.p. 1.5 g/kg, i.p.	c-Fos ICC c-Fos ISH	Reduction Increase	Locus coeruleus Nucleus of the solitary tract CA1, CA3 layers of the hippocampus Cingulate cortex Infralimbic cortex Orbital cortex Nucleus accumbens core Nucleus accumbens shell CeA Medial amygdala Paraventricular nucleus of hypothalamus (parvocellular part)	2 hours 45 minutes	Rat (Sprague-Dawley) Rat (Wistar)	Ryabinin et al., 1997 Hansson et al., 2008
1.5 and 3.5 g/kg, i.p.	c-Fos ICC	Increase	Supraoptic nucleus Nucleus of the solitary tract Locus coeruleus Ventrolateral medulla pilu CeA CeA Medial amygdala Paraventricular nucleus of hypothalamus (parvocellular part)	2 hours	Rat (Long-Evans)	Thiele et al., 2000
2 g/kg, i.p. 2 g/kg, i.p. 2 g/kg, i.p.	c-Fos ICC c-Fos ICC c-Fos ICC	Increase Increase Increase	Nucleus accumbens core Nucleus accumbens shell CeA Medial amygdala Paraventricular nucleus of hypothalamus (parvocellular part)	3 hours 2 hours after the injection 2 hours after the injection	Mouse Rat (Sprague-Dawley) Rat (Sprague-Dawley)	Turek and Ryabinin, 2005 Criado and Morales, 2000 Ryabinin et al., 2000
2.4 g/kg, i.p	Egr-1 ICC	Increase	Paraventricular nucleus of hypothalamus (parvocellular part) Piriform cortex Lateral amygdala CeA BNST	100 mn	Mouse C57BL/6J	Bachtell and Ryabinin, 2001
2.4 g/kg, i.p.	c-Fos ICC	Increase	Cingulate cortex Piriform cortex Motor cortex Nucleus accumbens core Nucleus accumbens shell BNST Lateral amygdala Basolateral amygdala CeA pilu Paraventricular nucleus of hypothalamus (parvocellular part) Paraventricular nucleus of thalamus Medial terminalis Intermediate layer of the superior colliculus	100 mn	Mouse C57BL/6J	Bachtell and Ryabinin, 2001

Table 1. (Continued)

Ethanol treatment	mRNA or protein	Type of regulation	Regions	Delay after ethanol administration	Species	References
2.5 and 4 g/kg, i.p. 2.5 and 4 g/kg, i.p.	Egr-1 ICC c-Fos ICC	Increase Increase	CeA Nucleus accumbens Nucleus of the solitary tract Parabrachial nucleus Paraventricular nucleus of thalamus	2 hours 2 hours	Rat (Sprague-Dawley) Rat (Sprague-Dawley)	Canales, 2004 Canales, 2004
2.5 g/kg, i.p.	c-Fos ICC	Increase	CeA BNST, dorsolateral Paraventricular nucleus of hypothalamus	2 hours 15 minutes	Rats (Sprague-Dawley)	Knapp et al., 2001
3 g/kg, i.p.	c-Fos ICC	Increase	CeA BNST Paraventricular nucleus of hypothalamus	2 hours	Rats (Sprague-Dawley)	Chang et al., 1995
3 g/kg, i.p.	c-Fos ICC	Increase	Locus coeruleus Parabrachial nucleus Paraventricular nucleus of hypothalamus	2 hours	Rat (Sprague-Dawley)	Ogilvie et al., 19981
3 g/kg, i.p.	c-Fos ISH	Increase	Paraventricular nucleus of hypothalamus (parvocellular part)	1 hour	Rat (Sprague-Dawley)	Zoeller and Fletcher, 1994
3 g/kg, i.p.	c-Fos ISH	Increase	Paraventricular nucleus of hypothalamus (parvocellular part)	1 hour	Rat (Sprague-Dawley)	Ogilvie et al., 1998
4 g/kg, i.p.	c-Fos ICC	Increase	Paraventricular nucleus of hypothalamus (parvocellular part) Nucleus accumbens, core Nucleus accumbens, shell Globus pallidus Ventral pallidum Caudate-Putamen (dorso-medial) Entopeduncular nucleus (or medial globus pallidus)	1 hour	Mouse (DBA/2J)	Hitzemann and Hitzemann, 1997
4 g/kg, i.p.	c-Fos ICC	Increase	CeA Paraventricular thalamus Subthalamic nucleus SNr SNr VTA Motor cortex Lateral septum, ventral BNST Anterior hypothalamus, CeA	2 hours	Mouse (DBA/2J)	Ryabinin and Wang, 1998
4 g/kg, i.p.	c-Fos ICC	Increase	Paraventricular nucleus of thalamus Paraventricular nucleus of hypothalamus Lateral septum, ventral Ventral pallidum Globus pallidus Entopeduncular nucleus (or medial globus pallidus) Paraventricular nucleus of thalamus Supraoptic nucleus Suprachiasmatic nucleus Rhomboid nucleus of thalamus Paraventricular nucleus of hypothalamus Dorsal hypothalamic area Dorsomedial hypothalamic area Lateral geniculate nucleus Subthalamic nucleus SNr SNr pilu Lateral parabrachial nucleus Locus coeruleus	2 hours	Mouse (C57BL/6J)	Kolodziejaska-Akiyama et al., 2005

Table 1. (Continued)

Ethanol treatment	mRNA or protein	Type of regulation	Regions	Delay after ethanol administration	Species	References
4 g/kg, i.p.	c-Fos ICC	Reduction	Intergeniculate leaf SNpc	2 hours	Mouse (C57BL/6J)	Kolodziejaska-Akiyama et al., 2005
4 g/kg, i.p.	Fos B ICC	Increase	CeA	2 hours	Mouse (DBA/2J)	Ryabinin and Wang, 1998
5 g/kg, i.p.	Egr-1 ISH	Increase	Frontal cortex Lateral part of the caudate putamen Motor cortex	45 minutes	Rat (Wistar)	Thiriet et al., 2000
5 g/kg, i.p.	c-Fos ISH	Increase	Caudate putamen Insular cortex	45 minutes	Rat (Wistar)	Thiriet et al., 2000
4 hours ethanol vapour-inhalation	c-Fos ICC	Increase	Parietal Association cortex Nucleus accumbens core and shell BNST Caudate Putamen CeA	1 hour	Rat (Sprague-Dawley)	Ryabinin et al., 1997
			Paraventricular nucleus of thalamus Paraventricular nucleus of hypothalamus pilu VTA			
			Locus coeruleus Nucleus of the solitary tract CA1, CA3 layers of the hippocampus			
4 hours ethanol vapour-inhalation	c-Fos ICC	Reduction	Paraventricular nucleus of hypothalamus (parvocellular part) Paraventricular nucleus of hypothalamus (parvocellular part) Paraventricular nucleus of hypothalamus (parvocellular part)	2 hours	Rat (Sprague-Dawley)	Ryabinin et al., 1997
3 g/kg, i.g.	c-Fos ICC	Increase	BNST, dorsolateral	1 hour	Rat (Sprague-Dawley)	Ogilvie et al., 1998
4 g/kg, i.g.	Egr-1 ISH	Increase	Paraventricular nucleus of hypothalamus	30 mn	Rats (Wistar)	Ueyama et al., 1999
3 g/kg, i.g.	c-Fos ISH	Increase	BNST, dorsolateral	10 mn and 4 hours	Rat (Sprague-Dawley)	Ogilvie et al., 1998
4 g/kg, i.g.	c-Fos ICC	Increase	Paraventricular nucleus of hypothalamus CeA	135 minutes	Rats (Sprague-Dawley)	Knapp et al., 2001
			pilu			
i.c.v. (1 $\mu$ l EtOH + 5 $\mu$ l aCSF)	c-Fos ICC	Increase	Nucleus accumbens, shell Lateral septum, ventral BNST Paraventricular nucleus of hypothalamus	1.5 hours	Rat (Sprague-Dawley)	Crankshaw et al., 2003

All studies were performed on male animals.

ICC, immunocytochemistry; ISH, in situ hybridization; aCSF, artificial cerebral spinal fluid; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of amygdala; pilu, perilocomotor urocortin-containing cells population, also known as Edinger-Westphal nucleus; SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmentum area. Articles are cited in the order of i.p., ethanol vapours, i.g. and i.c.v. administrations (in the order of increasing doses when necessary).

and mice (Bachtell and Ryabinin, 2001; Thiriet et al., 2000) and in subcortical telencephalon: striatum (Thiriet et al., 2000), BNST (Bachtell and Ryabinin, 2001), and central and lateral nuclei of amygdala (Bachtell and Ryabinin, 2001; Canales, 2004). In the diencephalon, a particularly strong expression was shown in the parvocellular paraventricular nucleus of hypothalamus (Ueyama et al., 1999) (see Table 1). In contrast with c-Fos mRNA or protein levels that show light or no basal level in the brain of rats or mice (Bachtell and Ryabinin, 2001; Ogilvie et al., 1998; Thiriet et al., 2000), there is a basal level of expression of Egr1 mRNA in some regions. A basal level of Egr1 mRNA was found in all layers of the neocortex, pyramidal cell layers in the hippocampus, especially in the CA1 region, dentate gyrus but not in the parvocellular paraventricular hypothalamic nucleus (Depaz et al., 2000; Ueyama et al., 1999). This basal level was found in control untreated rats, whether habituated or not for 1 week to the stress of handling. In control mice progressively habituated to handling and i.p. injections, for a period of 2 weeks before the acute final i.p. saline injection, Egr-1 immunoreactivity was found in cortex, striatum, lateral septum ventrale, basolateral nucleus of amygdala, BNST, CA3 region of hippocampus, dentate gyrus, parvocellular paraventricular hypothalamic nucleus and intermediate layer of the superior colliculus (Bachtell and Ryabinin, 2001). Thus, basal levels of Egr-1 mRNA or protein were detected in control animals (Bachtell and Ryabinin, 2001; Depaz et al., 2000; Ueyama et al., 1999).

In the CA1 region of hippocampus and in neocortex, the basal levels of Egr1 mRNA are unchanged by oral ethanol administration (Ueyama et al., 1999), and surprisingly, there is no Egr-1 protein or mRNA expression in the nucleus accumbens after ethanol administration in rats (Canales, 2004; Thiriet et al., 2000). Another mouse study also found that ethanol did not induce Egr-1 immunoreactivity in nucleus accumbens, hippocampus, amygdala, or cortex (Bachtell and Ryabinin, 2001). All together these studies indicate that analysis of different IEGs is needed in order to map ethanol-responding regions.

Moreover, levels of Egr-1 protein or mRNA do not accurately reflect the functional activity of Egr-1-mediated-transcription per se. There are indeed some proteins that suppress Egr-1-mediated transcription: these proteins are named NAB1 and NAB2 (after NGFI-A binding proteins, as Egr-1 is also named NGFI-A). NAB1 and NAB2 proteins bind to Egr-1 and exert a repression of its transcriptional function (O'Donovan et al., 1999). The induction of Egr-1 protein occurs at the same time and in the same brain regions as its transcriptional repressor NAB2 mRNA following acute or chronic cocaine administrations, and this induction is believed to contribute to a down-regulation mechanism (Jouvert et al., 2002). Thus, mapping of ethanol -induced Egr-1 activation enables identification of the brain structures implicated in ethanol responses, but does not allow us to establish conclusively the functional role of this activation.

## IEG EXPRESSION AFTER EXPOSURE TO CHRONIC ETHANOL TREATMENT

### *c-Fos and Fos-Family Proteins Expression in Response to Chronic Ethanol Intake*

Various paradigms of chronic exposure to ethanol (for at least 14 days) have been shown to induce c-Fos expression in both rats and mice. In Table 2 we listed results that show c-Fos and Egr-1 induction or reduction after subchronic treatments (2 to 13 days) as well as after chronic treatments (14 days and more). Figure 2 presents regions showing c-Fos or Egr1 induction or reduction after chronic treatments only.

The responses observed following chronic treatment occurred in a restricted set of brain regions compared to those responding to acute treatment. Regions strongly reactive to acute i.p. ethanol injection, including parietal cortex, temporal cortex, nucleus accumbens, and lateral septum, showed no c-Fos induction following chronic ethanol i.p. injections, as compared to saline-injected animals (Ryabinin et al., 1997). In the orbital and insular cortices, BNST, CeA, caudate putamen, and pIIIu, c-Fos induction was observed following i.p. injections of ethanol 1.5 g/kg for 14 days in rats, as compared to 14 day-saline injected animals, but this induction was of smaller amplitude than after acute treatment (Ryabinin et al., 1997). In the pIIIu, this observation was confirmed by Chang and colleagues (1995). Their results showed that the increased c-Fos response observed was of milder amplitude after chronic i.p. injections of ethanol for 17 or 24 days than after acute injection. In the paraventricular hypothalamic nucleus and CeA of rats, acute ethanol induced c-Fos expression disappeared at the end of a chronic treatment with ethanol 3 g/kg i.p. injected twice a day for 17 or 24 days, as compared to saline-injected rats (Chang et al., 1995). These observations led to the conclusion that chronic ethanol exposure, at least following i.p. injections, results in a desensitization of the c-Fos immunoreactivity response seen with acute ethanol treatment in the nuclei involved in the acute response. This finding suggests a general tolerance to ethanol with regard to c-Fos induction (Chang et al., 1995; Ryabinin et al., 1997). This desensitization has been observed in the CeA, anterior hypothalamus, paraventricular nucleus of hypothalamus, and in the ventral part of lateral septum after treatments as short as 4 days (4 g/kg/d i.p. in mice) (Ryabinin and Wang, 1998). This desensitization could account for the absence of c-Fos induction sometimes observed after chronic ethanol administration. Indeed, chronic ethanol ingestion for 6 weeks in adult male and female rats resulted in no effect on c-Fos mRNA expression, visualized by RT-PCR performed in midbrain, cortex, brainstem and cerebellum (Nakahara et al., 2002). In addition, chronic exposure to ethanol vapour did not increase c-Fos expression in the rat brain regions examined (Wilce et al., 1993).

However, the milder responses to chronic ethanol are not always observed for a given region. The paraventricular nucleus of hypothalamus is variously recorded as showing desensitization after chronic i.p. injections of ethanol in mice

**Table 2.** Regions Showing Chronic-Alcohol-Treatment Induced c-Fos, Fos B, Chronic FRAs, and Egr-1 Expression Changes (Protein)

Ethanol treatment	Duration of chronic treatment	mRNA or protein	Type of regulation	Regions	Delay after ethanol administration	Species	References
4 g/kg, i.p., once a day	4 days	c-Fos ICC	Increase	Paraventricular nucleus of thalamus	2 hours	Mouse (DBA/2J)	Ryabinin and Wang, 1998
4 g/kg, i.p., once a day	4 days	Fos B ICC	Increase	Motor cortex Retrosplenial cortex BNST Lateral septum, ventral Anterior cortical amygdaloid nucleus CeA Lateral amygdala Lateral hypothalamus Paraventricular thalamic nucleus	2 hours	Mouse (DBA/2J)	Ryabinin and Wang, 1998
4 g/kg, i.p., once a day	4 days	FRAs ICC	Increase	Motor cortex Somatosensory cortex Anterior cortical amygdaloid nucleus CeA Paraventricular nucleus of hypothalamus Paraventricular nucleus of thalamus	2 hours	Mouse (DBA/2J)	Ryabinin and Wang, 1998
Self administration of 10% ethanol in drinking, 30-mn limited daily access, resulting in 2 g/kg/d (free choice)	9 days	c-Fos ICC	Increase	plllu	2 hours	Mouse (C57BL/6J)	Sharpe et al., 2005
Self administration of 10% ethanol in drinking, 30-mn limited daily access, resulting in 2 g/kg/d (free choice)	9 days	c-Fos ICC	Decrease	Cingulate cortex Lateral septum, dorsal Medial septum CA1 region of the hippocampus Basolateral amygdala VTA	2 hours	Mouse (C57BL/6J)	Sharpe et al., 2005
1.5 g/kg, i.p.	14 days	c-Fos ICC	Increase, of a milder amplitude than after acute treatment	Orbital cortex Insular cortex CeA plllu	2 hours	Rat (Sprague-Dawley)	Ryabinin et al., 1997
1.5 g/kg, i.p.	14 days	c-Fos ICC	Increase, of a stronger amplitude than after acute treatment	Caudate-Putamen Paraventricular nucleus of hypothalamus Medial Preoptic area of hypothalamus Lateral Hypothalamus Nucleus of the solitary tract	2 hours	Rat (Sprague-Dawley)	Ryabinin et al., 1997
1.5 g/kg, i.p.	14 days	c-Fos ICC	Decrease, of a stronger amplitude than after acute treatment	CA1, CA3 layers of the hippocampus Deniate gyrus	2 hours	Rat (Sprague-Dawley)	Ryabinin et al., 1997
Self administration of 5% ethanol solution, 30-mn limited daily access (free choice)	14 days	c-Fos ICC	Increase	plllu	90 mn	Mouse C57/BL/6J	Ryabinin et al., 2003
Self administration of 5% ethanol solution, 30-mn limited daily access (free-choice)	14 days	c-Fos ICC	Decrease	Lateral septum, dorsal CA1 and CA2 layers of the hippocampus Posterior hypothalamus area	90 mn	Mouse (C57/BL/6J)	Ryabinin et al., 2003

Table 2. (Continued)

Ethanol treatment	Duration of chronic treatment	mRNA or protein	Type of regulation	Regions	Delay after ethanol administration	Species	References
Self administration of 5% ethanol solution, 30-min limited daily access (free-choice)	14 days	c-Fos ICC	Increase	plllu	90 mn	Mouse (C57/BL/6J)	Bachtell et al., 2003
Self administration of 5% ethanol solution, 30-min limited daily access (free choice)	14 days	c-Fos ICC	Decrease	Lateral septum, dorsal	90 mn	Mouse (C57/BL/6J)	Bachtell et al., 2003
Ethanol vapour chambers, maintaining a blood alcohol level $\geq 200$ mg/dl	14 days	Egr-1 ICC	Increase	CA1 layer of the hippocampus Dentate gyrus	1 hour	Rat (Wistar)	Depaz et al., 2000
Ethanol vapour chambers, maintaining a blood alcohol level $\geq 200$ mg/dl	14 days	Egr-1 ICC	Decrease	CA2 layer of the hippocampus CA3 layer of the hippocampus	1 hour	Rat (Wistar)	Depaz et al., 2000
3 g/kg i.p. twice daily (16% in saline)	17 or 24 days	c-Fos ICC	Increase, of a milder amplitude than after acute treatment	plllu	2 hours	Rat (Sprague-Dawley)	Chang et al., 1995
Self administration of sucrose-10% ethanol solution (free choice)	18 days	c-Fos ICC	Increase	Nucleus accumbens, core CeA plllu	90 mn	Mouse (C57/BL/6J)	Bachtell et al., 1999
Self administration of 10% ethanol solution (sole fluid source)	30 days	c-Fos ICC	Increase	Nucleus accumbens		Rat (Wistar)	Yoshimoto et al., 2000
Forced oral consumption of beer for 14 days, followed by self administration of 4% ethanol beer, 30-min limited daily access	36 days	c-Fos ICC	Increase	plllu	90 mn	Rat (Wistar)	Topple et al., 1998
Self administration of 10% ethanol solution, 30-min limited daily access (free choice)	40 days	c-Fos ICC	Increase	plllu	90 mn	Alko Alcohol Rat (F81)	Weitemier et al., 2001

All studies were performed on male animals. In the case of ethanol administered as drinking solution, the percentage of ethanol is progressively increased in the first few days to reach the final concentration delivered to animals for the following days of treatment (Moy et al., 2000).  
 ICC, immunocytochemistry; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; plllu, periolomotor urocortin-containing cells population, also known as Edinger-Westphal nucleus; SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmentum area. Articles are cited in the order of increasing duration of chronic treatments

(Ryabinin and Wang, 1998) and rats (Chang et al., 1995), and showing a stronger response following chronic i.p. ethanol injections for 14 days than following acute i.p. treatment (Ryabinin et al., 1997). This stronger response was not observed when rats were placed in a new environment after each ethanol i.p. injection; in this study, the paraventricular hypothalamic nucleus response was desensitized. Thus, it seems that exposure to a novel environment regulates the paraventricular hypothalamic nucleus response to ethanol. In CeA, c-Fos induction was recorded following ethanol i.p. injections for 14 or 24 days in rats, as compared to saline-injected animals, and this response was desensitized compared to acute treatment (Chang et al., 1995; Ryabinin et al., 1997). C-Fos induction was also observed in CeA following forced oral consumption of a 10% ethanol solution containing sucrose in mice, as compared to water-drinking and sucrose-drinking control mice (Bachtell et al., 1999).

Some other regions present a stronger response to chronic than to acute ethanol. In the nucleus of the solitary tract, medial preoptic area, and lateral hypothalamus, c-Fos induction was observed following i.p. 1.5 g/kg ethanol injections for 14 days in rats, as compared to 14 day-saline injected animals, with larger responses than after acute treatment (Ryabinin et al., 1997).

In the nucleus accumbens there are inconsistencies concerning c-Fos expression following chronic ethanol that could be explained by the different paradigms of ethanol administration. C-Fos induction was recorded in the core of the nucleus accumbens following self administration of unsweetened 10% ethanol solution, given as the sole source of fluid for 30 days in rats, as compared to water-drinking rats (Yoshimoto et al., 2000); as well as following a limited access ethanol/sucrose procedure, during which mice self-administered sucrose-10% ethanol solution 30 minutes a day for 18 days, as compared to water- or sucrose-drinking mice (Bachtell et al., 1999). No induction was recorded following i.p. injections of ethanol for 14 days, as compared to saline injected rats, neither in the core nor in the shell (Ryabinin et al., 1997). According to these results, the *per os* route of chronic ethanol administration seems to be crucial and responsible for the c-Fos induction in nucleus accumbens.

In pIIIu, as reviewed in Ryabinin and Weitemier (2006), the c-Fos expression was induced following numerous different types of chronic ethanol administration. It was observed following i.p. injections of ethanol 3 g/kg for 17 or 24 days or 1.5 g/kg for 14 days, compared to saline-injected rats (Chang et al., 1995; Knapp et al., 2001; Ryabinin et al., 1997). It was also observed following a limited-access ethanol/sucrose drinking procedure for 9 days (subchronic treatment) and for 14 to 18 days (chronic treatment) in mice (Bachtell et al., 1999, 2003; Ryabinin et al., 2003; Sharpe et al., 2005).

As summarized in Table 2 and Fig. 2, regions responding with c-Fos induction to chronic exposure to ethanol for at least 14 days and following different kinds of ethanol administration, in both rats and mice brains are the orbital and insular cortices, nucleus accumbens, caudate putamen, CeA,

paraventricular nucleus of the hypothalamus, medial preoptic area and lateral hypothalamus, pIIIu and nucleus of the solitary tract (Bachtell et al., 1999, 2003; Chang et al., 1995; Ryabinin et al., 1997, 2003; Sharpe et al., 2005; Yoshimoto et al., 2000). Table 2 also indicate regions that have shown decreases in c-Fos expression following subchronic or chronic limited-access to sucrose-ethanol solution as compared to water-drinking mice and/or to sucrose-drinking mice. Subchronic ethanol self-administration for 9 days induced a decrease in c-Fos expression in cingulate cortex, lateral and medial septum, basolateral amygdala, the CA1 layer of the hippocampus, and VTA (Sharpe et al., 2005). Following 14 days of chronic treatment, the dorsal part of the lateral septum, the CA1 and CA2 layers of the hippocampus and posterior hypothalamus also showed a decrease in c-Fos immunoreactivity (Bachtell et al., 2003; Ryabinin et al., 2003).

Another member of the Fos family proteins has attracted attention from researchers: the Fos B protein and its isoform delta Fos B. Thus, while acute administration of ethanol only induced Fos B immunoreactive cells in 1 brain region in mice—the CeA—it has been observed that a subchronic ethanol treatment for 4 days (4 g/kg, i.p., once a day) induced Fos B immunoreactive cells in the whole amygdaloid complex (anterior cortical amygdaloid nucleus, CeA, lateral amygdala, BNST), in the lateral hypothalamus, motor and retrosplenial cortices, shell part of the nucleus accumbens, lateral septum, and paraventricular nucleus of thalamus (Ryabinin and Wang, 1998). Further, ethanol given to mice in a sucrose solution for 18 days induced FosB immunoreactive cells in pIIIu and CeA (Bachtell et al., 1999).

These observations are in keeping with the hypothesis that there could exist a shift from strong c-Fos induction after acute treatments towards Fos B, deltaFos B, and chronic FRAs induction after chronic treatments (Nestler et al., 1999; Ryabinin and Wang, 1998). The FosB gene encodes the Fos B protein and its truncated forms delta FosB and the chronic FRAs (for a review see Hope, 1998). The native deltaFosB is a relatively unstable protein of 33 kD with a half-life of 10 to 12 hours. Its 2 isoforms are both very stable proteins of 35 kD and 37 kD respectively, originally termed “chronic FRAs” and today named deltaFosB<sub>35–37</sub>. They are a particular case among the IEG family. While all other members of the family are produced rapidly and transiently in response to a stimulus, returning to basal levels within hours, the 2 deltaFosB isoforms show in vivo half-lives estimated at weeks (for a review see Nestler et al., 1999; Kelz and Nestler, 2000).

These 2 stable isoforms deltaFosB<sub>35–37</sub> are induced in the brain after repeated exposure to drugs of abuse as well as to other stimuli such as stress or antidepressant treatments (for review see Kelz and Nestler, 2000). The initial stimulus induces the native deltaFosB whereas the repeated ones induce an accumulation of deltaFosB<sub>35–37</sub>. DeltaFosB<sub>35–37</sub> isoforms have thus been proposed to serve as a marker of chronic neuronal perturbation, in the same way that c-Fos serves as a marker for acute stimulation (for a review see Ron and Jurd, 2005). By using an inducible expression system, it

has been shown that expression of deltaFosB<sub>35-37</sub> in the nucleus accumbens of mice induced an increase in sensitivity to the rewarding effects of cocaine (Kelz and Nestler, 2000). Moreover, different studies have shown that chronic exposure to cocaine, amphetamine, morphine or nicotine causes the persistent expression of highly stable isoforms of deltaFosB in the striatum (Hope, 1998; Kelz et al., 1999; McDaid et al., 2006; Nestler et al., 2001; Wang et al., 2005; Zachariou et al., 2006). These observations led to the hypothesis that deltaFosB works as a molecular switch which gradually converts acute responses into relatively stable adaptations to repeated stimuli, and therefore could constitute a neuronal basis of the long-lasting adaptations that take place in addiction (Hope, 1998; Nestler, 2004; Nestler et al., 1999). To date we have no reports as to whether deltaFosB accumulates in the course of long-term ethanol treatments, and we have to keep in mind that ethanol may or may not resemble cocaine or others drug of abuse, regardless of chronicity. Further studies are still needed to test this hypothesis.

#### *Egr1 Protein Expression in Response to Chronic Ethanol Exposure*

Egr-1 is essential for the transition from short- to long-term synaptic plasticity, for the expression of long-term memories (Jones et al., 2001) and for the reconsolidation of hippocampus-dependent fear-memory (Lee et al., 2004a). Thus, Egr-1 may participate in mediating long-term adaptations produced in the brain by ethanol. One study showed Egr-1 induction after a long-term ethanol treatment (see Table 2). Rats maintained for 14 days in ethanol vapour chambers developed an increased Egr-1 immunoreactivity in the CA1 layer and in the dentate gyrus of the hippocampus, and a reduced Egr-1 immunoreactivity in the CA2 and CA3 layers (Depaz et al., 2000). This observation conflicts with the lack of change in Egr-1 immunoreactivity in the hippocampus or other regions after exposure to 10% ethanol solutions for 18 days in mice (Bachtell et al., 1999).

### IEG RESPONSE EXPRESSION DURING WITHDRAWAL

As for acute or chronic treatments, studies concerning withdrawal from ethanol exposure differ in the duration of exposure (see Table 3), the method of ethanol administration (i.p., i.g., ethanol liquid diet as the sole source of fluid or ethanol vapour) or in the time interval chosen after last exposure to test withdrawal effects (always chosen as the peak of behavioural signs of ethanol withdrawal). Moreover, the IEG inductions recorded might not be specific to a withdrawal effect per se. Animals are usually experiencing withdrawal for the first time, and this could be sufficient to produce an anxiety/novelty-related IEG response.

Despite these discrepancies and issues, we tried to list withdrawal responding regions with the different paradigms, as detailed in Table 3. Withdrawal induced in rats an increase in

c-Fos immunoreactivity in all major part of the brain: In the *cerebral cortex*: medial prefrontal, infralimbic cingulate, piriform, and temporal cortices, in the subcortical *telencephalon*: lateral and medial septum, core and shell of nucleus accumbens, claustrum, globus pallidus, ventral pallidum, BNST, dorsomedial caudate putamen, anterior olfactory nucleus, olfactory tubercle, several nuclei of amygdala, dentate gyrus, and CA1, CA2, and CA3 regions of hippocampus; in the *diencephalon*: habenula, lateral geniculate, anterior and paraventricular nuclei of thalamus, anterior and posterior areas of hypothalamus, dorsomedial and paraventricular hypothalamic nuclei; in the *midbrain*: SN, VTA, pIIIu, and central grey; in the *hindbrain*: locus coeruleus, para- and presubiculum (Knapp et al., 1998; Moy et al., 2000; Olive et al., 2001; Putzke et al., 1996) (see Table 3 and Fig. 3).

In rats, there was an increase in c-Fos immunoreactive cells in central and basolateral nuclei of amygdala, CA3 layer of hippocampus, nucleus accumbens and periaqueductal grey at the end of repeated withdrawal experience. This repeated withdrawal paradigm consisted in a 24-day period of ethanol-enriched diet interspersed by 2 withdrawal intervals each lasting for 3 days (Borlikova et al., 2006). It is noteworthy that, in these same regions, there was no significant increase in c-Fos immunoreactive cells during the first withdrawal following a 24-day period of continuous ethanol-enriched diet. These data suggest an increased ability of repeated withdrawal to activate neuronal circuits. As the regimen of repeated withdrawals sensitize rats to pentylenetetrazole-induced seizures (Stephens et al., 2001), the increase in c-Fos immunoreactivity following repeated withdrawals may reflect the increase in seizure sensitivity.

In the paraventricular hypothalamic nucleus of rats, there was no increase in c-Fos immunoreactivity after withdrawal from 14 or 24 days of ethanol-containing beverage (Borlikova et al., 2006; Knapp et al., 1998; Moy et al., 2000). In mice with withdrawal-induced seizures after chronic ethanol exposure, there was a c-Fos protein expression in numerous brain regions, including the paraventricular hypothalamic nucleus, the striatum, olfactory tubercle, amygdala, hypothalamus, SN, and VTA (Olive et al., 2001) (see Table 3). In this regard, it is noteworthy that seizures could be a possible confounding factor, since seizures induce IEG expression. Thus, differential patterns of IEG induction following ethanol withdrawal could result directly from the presence of seizures in the tested animals and from the severity of the ethanol withdrawal syndrome.

In rats chronically intoxicated by ethanol vapour for 35 days, the DNA binding activities of AP-1 were evaluated 16 hours after the withdrawal, by electrophoretic mobility shift assays. These experiments showed that AP-1 binding activity was increased in the cortex, hippocampus, and cerebellum. The composition of the AP-1 DNA-binding complex varied, depending on the brain region and the time after the withdrawal. In the cortex, c-Fos was present at the time of withdrawal and increased 16 hours later. In the hippocampus and cerebellum, the AP-1 DNA-binding complex was devoid

**Table 3.** Regions Showing Ethanol Withdrawal-Induced c-Fos Expression (Protein or mRNA)

Ethanol treatment	Duration of chronic treatment before withdrawal	c-Fos mRNA or protein	Type of regulation	Regions	Delay after last ethanol administration	Species	References
4 g/kg, i.p.	Single injection	ICC	Increase	Cingulate cortex Entorhinal cortex Prelimbic cortex Nucleus accumbens, shell Nucleus accumbens, core Lateral septum Dorsolateral caudate putamen BNST CA3 layer of the hippocampus Lateral amygdaloid nucleus CeA Medial amygdaloid nucleus Basomedial amygdaloid nucleus SNr SNC Lateral globus pallidus Medial globus pallidus Ventral pallidum Subthalamic nucleus VTA pIlu	7 hours	Mouse DBA2J (D2) and C57BL/6J (B6)	Kozell et al., 2005
Intragastric 15% ethanol based diet	4 days	ICC	Increase	Medial prefrontal cortex Orbital cortex Cingulate cortex Piriform cortex Frontal cortex Retrosplenial cortex Clausstrum Tenia tecta Nucleus Accumbens shell Nucleus Accumbens core Dorsal striatum Anterior lateral septum Medial septum CA3 layer of the hippocampus Paraventricular nucleus of the hypothalamus Paraventricular nucleus of the thalamus CeA	12–14 hours	Rat (Sprague–Dawley)	Knapp et al., 1998
7% ethanol liquid diet as the sole source of food	14 days	ICC	Increase	Basolateral nucleus of the amygdala Cortical nucleus of the amygdala Medial amygdala Medial lateral habenula Central grey Inferior colliculus Locus coeruleus Medial prefrontal cortex Piriform cortex Cingulate cortex Clausstrum Nucleus accumbens shell Lateral septum Paraventricular nucleus of the thalamus Central grey	8–10 hours	Rat (Sprague–Dawley)	Knapp et al., 1998

Table 3. (Continued)

Ethanol treatment	Duration of chronic treatment before withdrawal	c-Fos mRNA or protein	Type of regulation	Regions	Delay after last ethanol administration	Species	References
5% ethanol liquid diet as the sole source of food	14 days	ICC	Increase	Piriform cortex Infralimbic cortex Cingulate cortex Nucleus accumbens, shell Nucleus accumbens, core Olfactory tubercle Lateral septum Medial septum BNST Dentate gyrus CA1, CA2 and CA3 layers of the hippocampus CeA Medial amygdaloid nucleus Basolateral amygdaloid nucleus Anterior olfactory nucleus Dorsomedial caudate putamen Anterior area of the hypothalamus Dorsomedial hypothalamus area Paraventricular nucleus of hypothalamus Posterior area of the hypothalamus SN Lateral geniculate nucleus of the thalamus Ventral pallidum VTA	8 hours (peak of behavioural signs of withdrawal)	Mouse BA2J	Olive et al., 2001
20% ethanol solution as sole source of fluid	14 days	ISH	Increase	Cingulate cortex Globus pallidus Medial habenular nucleus Anterior thalamic nucleus Temporal Association cortex Hippocampus Cerebellum Locus coeruleus Para- and presubiculum Medial prefrontal cortex Lateral septal nucleus Nucleus accumbens, shell CeA Nucleus accumbens, core CA3 layer of the hippocampus CeA Basolateral nucleus of the amygdala Periaqueductal grey	24 hours (peak of behavioural signs of withdrawal)	Rat (Wistar)	Putzke et al., 1996
7% ethanol liquid diet as the sole source of food	14 days	ICC	Increase		8 hours	Rat (Sprague-Dawley)	Moy et al., 2000
7% ethanol liquid diet as the sole source of food	30 days, interrupted 2 times for 2 days (repeated withdrawal)	ICC	Increase		8 hours	Rat (Lister hooded)	Borlikova et al., 2006

All cited studies were performed on male animals.

ICC, immunocytochemistry; ISH, in situ hybridization; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of amygdala; pIlu, perifornic urocortin-containing cells population, also known as Edinger-Westphal nucleus; SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmentum area. Articles are cited in the order of increasing duration of treatment that precedes withdrawal.

of c-Fos at the time of the withdrawal, but contained Fos B, C-Jun, JunB, and JunD; while 16 hours later, the AP-1 DNA-binding complex contained c-Fos. This demonstrates a withdrawal-induced c-Fos expression in the hippocampus and cerebellum (Beckmann et al., 1997). After a 14-day chronic ethanol treatment, given as a 20% solution as the sole beverage, there was a strong withdrawal-induced hippocampal c-Fos mRNA density. Interestingly, the anti-craving drug acamprosate, which reduces the incidence of relapse in weaned alcoholics, diminished this withdrawal-induced c-Fos expression (Putzke et al., 1996). This last result therefore indicates that neuronal activity in this brain structure may have a critical role in ethanol relapse.

At the level of synaptic transmission, it is thought that up-regulation of NMDA receptors and down-regulation of GABA<sub>A</sub> receptors after chronic ethanol exposure may contribute to the ethanol-withdrawal syndrome (Beckmann et al., 1997). The regulation of c-Fos and Egr1 IEGs after withdrawal could reflect the change in chronic ethanol-induced NMDA receptors expression, as the cellular events elicited by the stimulation of NMDA receptors lead to the expression of these 2 IEGs (Lu et al., 2006). In addition, GABA<sub>A</sub> and NMDA receptor neuroadaptations observed during ethanol-withdrawal may explain, at least in part, the decrease in withdrawal-induced c-Fos expression by acamprosate (Putzke et al., 1996). In this regard, acamprosate has been shown to modulate NMDA and GABA<sub>A</sub> receptor function (Naassila et al., 1998; Pierrefiche et al., 2004) and a major mechanism of action is the reduction of cerebral hyper-excitability by interaction with glutamate receptors.

One study evaluated c-Fos expression induced by ethanol withdrawal following a single ethanol i.p. injection (Kozell et al., 2005). C-Fos expression was studied in 2 strains of mice (Kozell et al., 2005), that differ in the severity of handling-induced convulsions following ethanol withdrawal (Crabbe et al., 1983). The DBA/2J (D2) strain is characterized by a severe ethanol withdrawal while the C57BL/6J (B6) strain shows a mild ethanol withdrawal. C-Fos immunocytochemistry was performed 7 hours after a single 4 g/kg i.p. ethanol administration, a time that corresponded to a peak in acute ethanol withdrawal severity. As c-Fos induction after acute ethanol treatment disappears within 3 hours, the c-Fos induction recorded 7 hours after the acute injection is attributable to the “withdrawal” effects from acute treatment. The withdrawal-vulnerable D2 strain of mice, that had withdrawal-induced convulsions, showed a stronger neural activation, observed through c-Fos expression, than the B6 strain that did not have withdrawal-induced convulsions. However, there is an induction of c-Fos even in mice that do not display convulsions following withdrawal. The D2 and B6 strains both presented an increase in c-Fos expression in numerous brain regions, including the extended amygdala, the striatum and nucleus accumbens, the SN and the VTA, the pIIIu, the globus pallidus and the ventral pallidum, the septum as well as the prelimbic and limbic cortices (see Table 3). The c-Fos activation in these regions could therefore

result from the ethanol withdrawal itself, and not from withdrawal-induced seizures. In this context, it is important to keep in mind that c-Fos activation is observed during withdrawal and that the level of activation can be exacerbated by the severity of seizures. This effect may explain the differences between species and strains of animals. The hippocampus showed poor activation after acute ethanol withdrawal. This observation is surprising, as the hippocampus projects to and modulates the functions of the extended amygdala, which in turn exhibits c-Fos induction in acute ethanol withdrawal (Kozell et al., 2005).

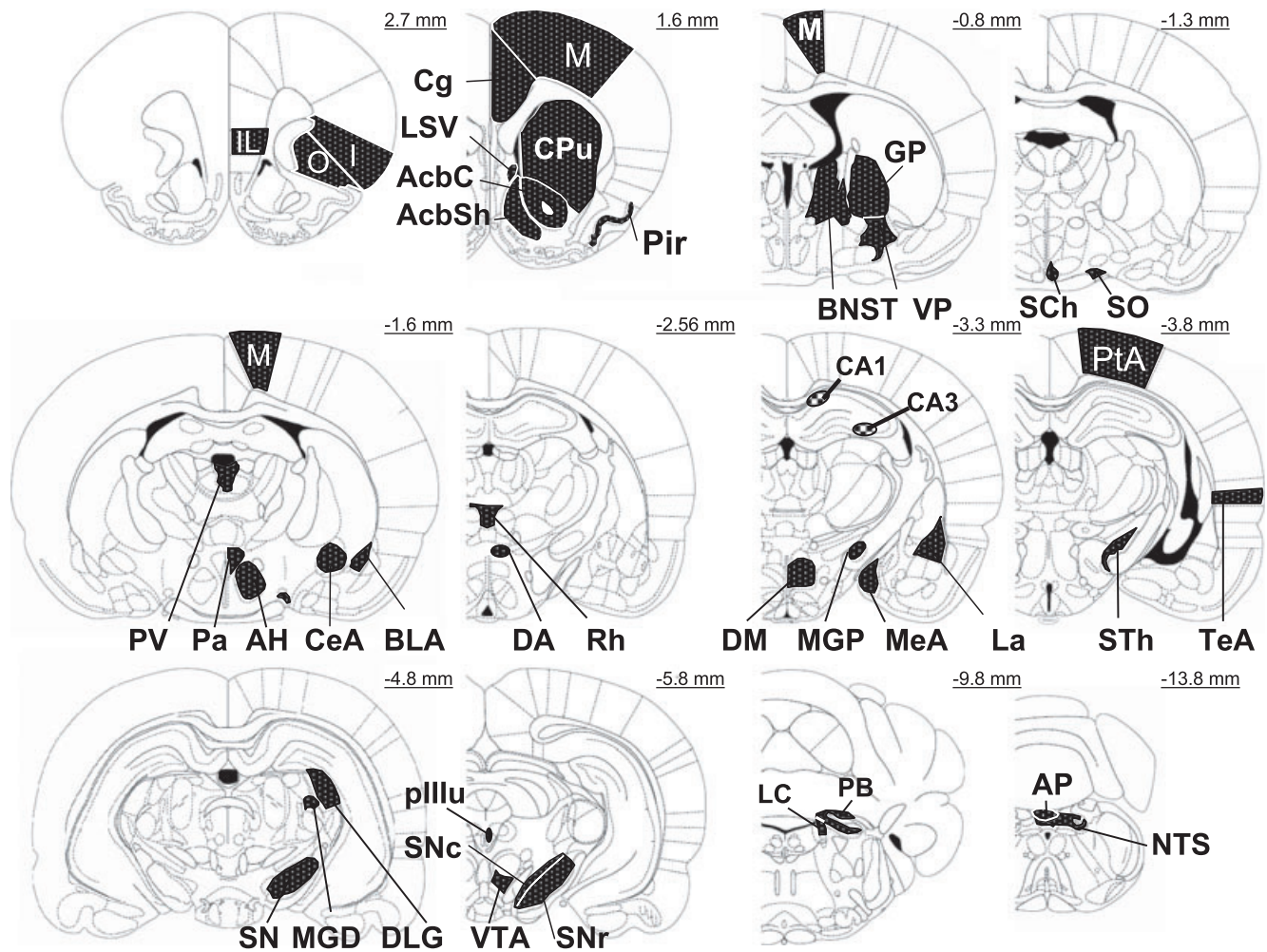
Another study evaluated the effect on c-Fos expression of withdrawal from a subchronic 4-day treatment with i.g. administration of ethanol-containing diet in rats. This paradigm induced c-Fos immunoreactivity in a large set of regions, including the medial prefrontal cortex (i.e., prelimbic and cingulate cortices), the amygdala, the septum, the nucleus accumbens, and the paraventricular nucleus of the hypothalamus (see Table 3) (Knapp et al., 1998).

#### ANALYSIS OF REGIONS SENSITIVE TO ETHANOL ADMINISTRATION

We have sought to identify the differences in ethanol-responsive regions in the brain of rats and mice following acute and chronic ethanol use and withdrawal. Acute ethanol administration induces either neuronal activity or synaptic plasticity in numerous brain regions, including different cortical areas (prefrontal, limbic, motor, and parietal) the basal ganglia, several nuclei of the amygdala, thalamus and hypothalamus, the mesencephalic SN, VTA, and pIIIu, together with brainstem regions such as locus coeruleus, parabrachial nucleus, area postrema, and nucleus of the solitary tract (see Fig. 1).

There is a global desensitization in the response to ethanol after chronic treatment with regard to c-Fos and Egr-1 induction, and most regions that responded to acute ethanol intoxication did not show c-Fos or Egr1 induction after chronic ethanol treatment. Following chronic treatment, the responding regions are restricted to orbitofrontal and insular cortices, core and shell of nucleus accumbens, lateral septum, CeA, paraventricular, lateral and posterior nuclei of the hypothalamus, pIIIu, and the nucleus of the solitary tract (see Fig. 2). Withdrawal induces in turn activation in a large set of brain regions, in a pattern quite similar to the pattern of regions activated following acute ethanol treatment (see Fig. 3). The hippocampus appears to be a special case, in most cases showing a decrease in c-Fos and/or Egr1 immunoreactivity after acute and chronic treatments, and an increase in neuronal activity—considering c-Fos immunoreactivity—following withdrawal from chronic ethanol.

Most of the regions activated by acute ethanol treatments, chronic ethanol treatments or withdrawals are part of the mesolimbic dopamine circuitry, which is responsible at least in part for reward processes in the brain. This circuitry involves the VTA and nucleus accumbens, the extended

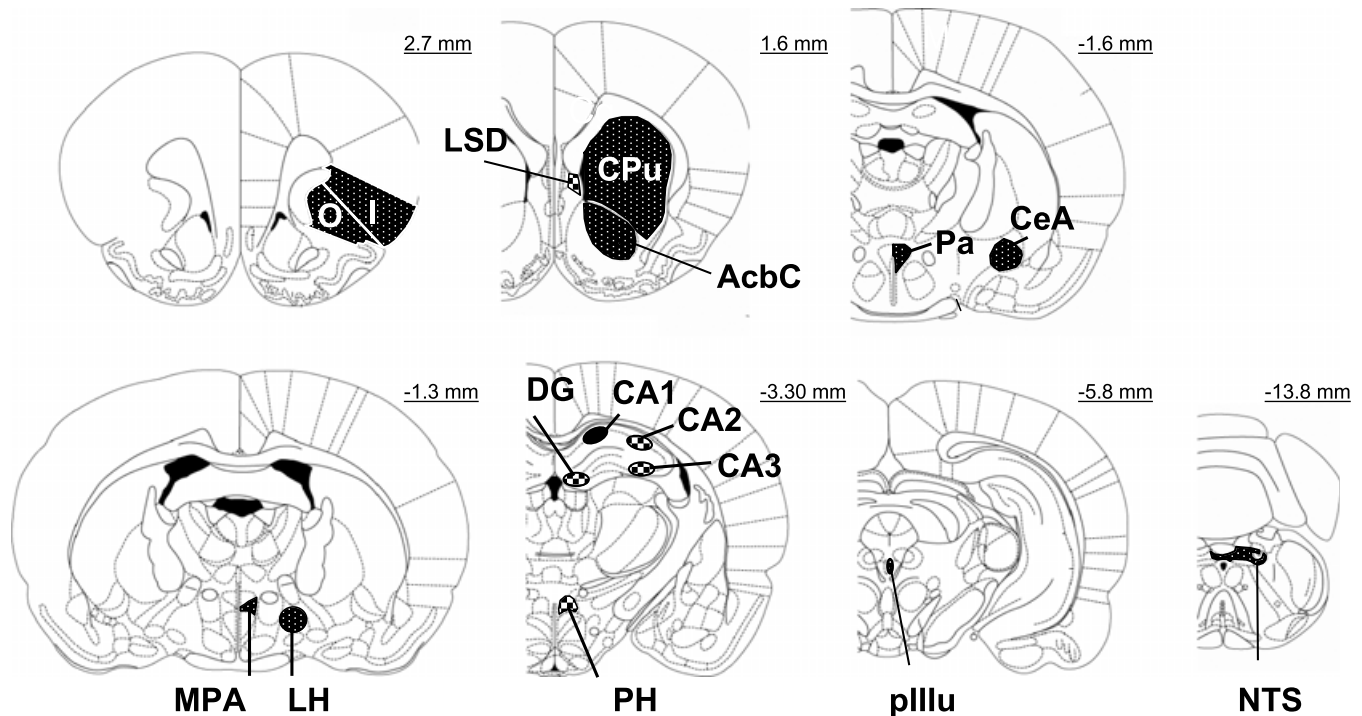


**Fig. 1.** C-Fos or Egr1 mRNA or protein increase ■ and decrease ▣ in the brain of rats and mice after acute ethanol administration, as detailed in Table 1. Brain regions were identified by reference to the atlas of Paxinos and Watson (1998; reprinted with permission from Elsevier). The Bregma levels are indicated next to each section. Abbreviations: IL, infralimbic cortex; O, orbital cortex; I, insular cortex; M, motor cortex; Pir, piriform cortex; Cg, cingulate cortex; CPu, caudate putamen (i.e., striatum); AcbC, core of the nucleus accumbens; AcbSh, shell of the nucleus accumbens; LSV, lateral septum – ventral; BNST, bed nucleus of the stria terminalis; GP, globus pallidus; VP, ventral pallidum; Sch, suprachiasmatic nucleus; SO, supraoptic nucleus; AH, anterior hypothalamus; PV, paraventricular thalamic nucleus; Pa, Paraventricular nucleus of hypothalamus; CeA, central nucleus of the amygdala; BLA, basolateral amygdala; DA, dorsal hypothalamic area; DM, dorsomedial hypothalamic area; Rh, rhomboid thalamic nucleus; MeA, medial amygdala; La, lateral amygdala; MGP, medial globus pallidus (i.e., entopeduncular nucleus); CA1 and CA3 layers of the hippocampus; DM, dorsomedial hypothalamic area; STh, subthalamic nucleus; PtA, parietal association cortex; TeA, temporal association cortex; DLG, dorsal lateral geniculate nucleus; MGD, dorsal medial geniculate nucleus; SNc, substantia nigra *pars compacta*; SNr, substantia nigra *pars reticulata*; VTA, ventral tegmentum area; pIllu, periculomotor urocortin-containing cells population (also known as Edinger-Westphal nucleus); LC, locus coeruleus; PB, parabrachial nucleus; NTS, nucleus of the solitary tract; AP, area postrema. A few structures that presented changes in the mouse brain that were not observed in the rat brain are not reported in this figure: the intermediate layer of the superior colliculus, the medial terminalis (Bachtell and Ryabinin, 2001), and the intergeniculate leaf (Kolodziejska-Akiyama et al., 2005).

amygdala (formed by BNST, CeA, and a transition area in the shell of nucleus accumbens), the lateral hypothalamus, pallidum, striatum, hippocampus, and prefrontal cortex (Kauer and Malenka, 2007; Koob, 2008). It is thought that “the reinforcing effects of drugs of abuse can hijack these circuits to produce the pathological behaviour that defines addiction” (Kauer and Malenka, 2007). This hijacking may be progressive, as the ethanol intake is repeated. In fact, the pattern of regions responsive to ethanol shows transitions from a large set of regions activated by acute ethanol administration toward a restricted set of regions activated by chronic ethanol. In turn, the withdrawal seems to reactivate regions that had become desensitized, such as the infralimbic, piri-

form and cingulate cortices, striatum, paraventricular nucleus of the thalamus, BNST, pallidum, basolateral nucleus of the amygdala, anterior hypothalamus, dorsomedial hypothalamic area, the SN, the VTA, and the locus coeruleus. This reactivation, that occurs when ethanol is withdrawn, suggests that these regions may be implicated in craving and relapse.

In the nucleus accumbens, acute ethanol administration induced no change in the Egr-1 mRNA level in rats (Thiriet et al., 2000) with low or even no c-Fos expression in rats and mice (Crankshaw et al., 2003; Hitzemann and Hitzemann, 1997; Ryabinin and Wang, 1998; Ryabinin et al., 1997, 2000). This absence of c-Fos and Egr-1 expression is at variance with cocaine effects, as acute administration of cocaine induces a

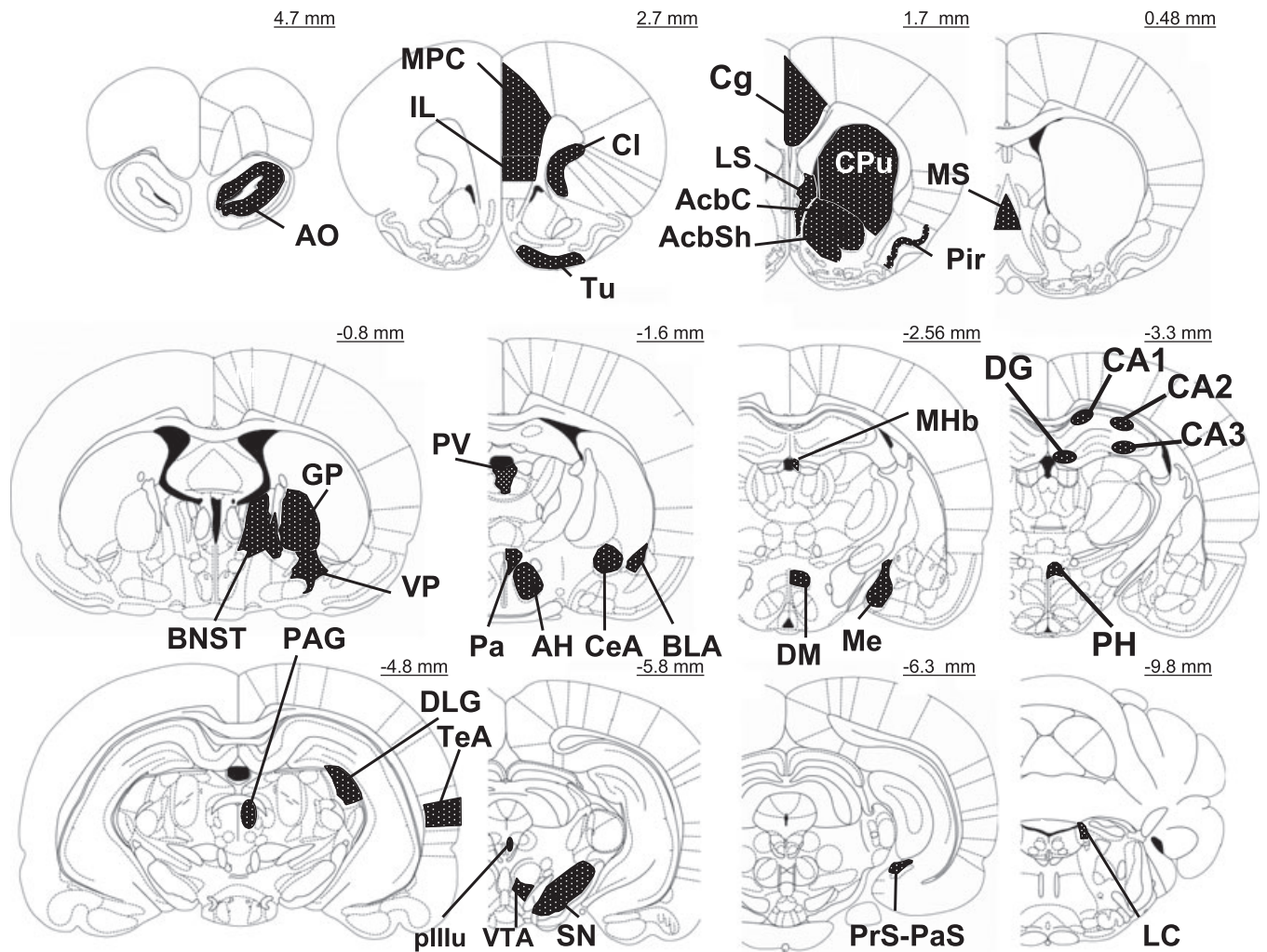


**Fig. 2.** C-Fos or Egr1 mRNA or protein increase ■ and decrease □ in the brain of rats and mice after chronic ethanol administration (14 days and more), as detailed in Table 2. Brain regions were identified by reference to the atlas of Paxinos and Watson (1998; reprinted with permission from Elsevier). The Bregma levels are indicated next to each section. Abbreviations: O, orbital cortex; I, insular cortex; AcbC, core of the nucleus accumbens; LSD, lateral septum dorsal; Pa, paraventricular nucleus of hypothalamus; CeA, central nucleus of the amygdala; LH, lateral hypothalamus; MPA, medial preoptic area of the hypothalamus; CA1, CA2, and CA3 layers of the hippocampus; DG, dentate gyrus; PH, posterior hypothalamus area; pIllu, periculomotor urocortin-containing cells population; NTS, nucleus of the solitary tract.

strong c-Fos and Egr-1 expression in regions involved in the positive-reinforcing effects of this drug, i.e. striatum and nucleus accumbens (Ryabinin et al., 2000; Thiriet et al., 2000). In contrast, contingent 30 minutes a day limited-access to ethanol for 18 days or more induce c-Fos expression in the nucleus accumbens in mice (Bachtell et al., 1999) and rats (Yoshimoto et al., 2000). Interestingly, this induction seems in relation with contingent administration, as there is no c-Fos response in nucleus accumbens in rats following chronic i.p. treatments (Ryabinin et al., 1997). The nucleus accumbens has been suggested to be involved in the maintenance of alcohol ethanol consumption (Bachtell et al., 1999). Furthermore, the nucleus accumbens shows an increase in c-Fos immunoreactivity following ethanol withdrawal in rats and mice (Knapp et al., 1998; Moy et al., 2000; Olive et al., 2001). The shell of the nucleus accumbens presents c-Fos mRNA induction in response to pharmacological and environmental stressors, respectively yohimbine i.p. injection or footshock, that are effective in inducing reinstatement of ethanol seeking in rats. These results may point to the shell of the nucleus accumbens being involved in the processes of stress-induced relapse (Funk et al., 2006). As chronic treatment is required to induce a strong neuronal activation in the nucleus accumbens—with regard to IEG induction—adaptations of the neuronal response in this region may be part of the changes responsible for the transition from occasional ethanol use to chronic abuse. In fact, the nucleus accumbens has a key role

in reward-dependent learning, and adaptations in the excitatory synaptic inputs onto medium spiny neurons of the nucleus accumbens seem to be particularly important for mediating addiction-related changes in behaviour (for a review see Kauer and Malenka, 2007).

The VTA showed IEG induction in response to acute and subchronic ethanol treatments, but not following chronic treatments (see Tables 1 and 2). It also showed a response after withdrawal from chronic ethanol treatment in mice, although the c-Fos induction could be due to the withdrawal-induced seizures (Olive et al., 2001) (see Table 3). The VTA is a key region of brain circuitry related to reward. It is the dopaminergic cell body region from which the reward-implicated mesolimbic dopamine projection originates, to connect to several forebrain regions, including a prominent terminal area, the nucleus accumbens. One major and common effect of most drugs of abuse, including ethanol, is an enhancement of activity in the mesolimbic dopamine system, which leads to an increase in extracellular dopamine concentrations in target areas (Imperato and Di Chiara, 1986). This effect is significant because the mesolimbic dopamine system is thought to be a critical substrate for the control of motivated and goal-directed behaviour (Horvitz, 2000). The neurobiological processes by which ethanol seeking and consumption are established and maintained are thought to involve areas of the brain that mediate motivated behaviour, such as the mesolimbic dopamine system. The exact molecular and cellu-



**Fig. 3.** C-Fos mRNA or protein increase in the brain of rats and mice after withdrawal following chronic ethanol administration (14 days and more), as detailed in Table 3. Brain regions were identified by reference to the atlas of Paxinos and Watson (1998; reprinted with permission from Elsevier). The Bregma levels are indicated next to each section. Abbreviations: AO, anterior olfactory nucleus; MPC, medial prefrontal cortex; CI, claustrum; IL, infralimbic cortex; Tu, olfactory tubercle; Pir, cortex piriform; AcbC, core of the nucleus accumbens; AcbSh, shell of the nucleus accumbens; CPu, caudate putamen (i.e., striatum); LS, lateral septum; Cg, cingulate cortex; MS, medial septum; BNST, bed nucleus of the stria terminalis; GP, globus pallidus; VP, ventral pallidum; AH, anterior hypothalamus; CeA, central nucleus of the amygdala; PV, paraventricular thalamic nucleus; Pa, paraventricular nucleus of hypothalamus; DM, dorsomedial hypothalamic area; Me, medial amygdala; MHb, medial habenula; CA1, CA2, and CA3 layers of the hippocampus; DG, dentate gyrus; PH, posterior hypothalamic area; DLG, dorsal lateral geniculate nucleus of the thalamus; TeA, temporal association cortex; PAG, periaqueductal gray; SN, substantia nigra; VTA, ventral tegmentum area; pIIIu, periculomotor urocortin-containing cells population; PrS-PaS, para- and presubiculum; LC, locus coeruleus.

lar mechanisms through which ethanol alters the mesolimbic dopamine system are still not entirely clear. Previous work suggests that ethanol promotes mechanisms that are known to alter synaptic communication between neurons in the mesolimbic dopamine system, and these mechanisms may contribute to the long lasting changes in ethanol-seeking behaviour that occur in alcoholism (Maldve et al., 2002). Importantly, rats have been shown to self-administer ethanol directly into the posterior VTA and it has been suggested that activation of VTA dopaminergic neurons is involved in this process (Rodd et al., 2004). In rats, acute and subchronic exposure to ethanol causes an increase in the firing rate of dopaminergic neurons in VTA both in vivo and in vitro (Brodie et al., 1990; Diana et al., 1992a, 1993b; Gessa et al., 1985). This increased firing rate could be linked to the c-Fos

induction recorded in the VTA following acute and subchronic ethanol administration.

Conversely, the number of spontaneously active DA neurons in the VTA (Shen and Chiodo, 1993) and their firing rates (Diana et al., 1992b, 1993a, 1996) are reduced during ethanol withdrawal in rats. This suggests that the depression of activity of the mesolimbic dopaminergic system in ethanol withdrawal may be relevant to the dysphoria experienced in human ethanol withdrawal syndrome. It is noteworthy that decreased VTA firing rates were observed in rats during withdrawal, but to our knowledge c-Fos expression in the VTA has not been investigated during withdrawal in rats. C-Fos induction in the VTA during withdrawal was recorded in mice undergoing withdrawal-induced seizures (Olive et al., 2001). So, the c-Fos induction observed could be related to

the seizures rather than the ethanol withdrawal itself. This hypothesis is reinforced by the study of Dave and colleagues (1990). These authors showed that c-Fos mRNA levels, measured by Northern blot analysis, were greatly increased in the brains of mice undergoing ethanol withdrawal seizures, while in mice that did not have withdrawal seizures, c-Fos mRNA levels did not increase significantly. mRNA levels were measured in the cerebral cortex, cerebellum, and hippocampus but not in the VTA. Taken all together, these data indicate that seizures may influence the induction of c-Fos.

The amygdala, and particularly the CeA, is extremely responsive to acute and chronic administration of ethanol (Chang et al., 1995; Morales et al., 1998; Ryabinin et al., 2000). Induction in this region takes place principally in GABAergic neurons co-expressing pro-enkephalin peptide (Bachtell and Ryabinin, 2001; Criado and Morales, 2000), an observation that may underlie the profound implication of the GABAergic neuronal system in the effects of ethanol intoxication. Activation of the central and medial nuclei of the amygdala may be linked to ethanol-seeking behaviour, as these 2 regions have been shown to be involved in drug-seeking behaviour. ERK, that controls both c-Fos and Egr-1 expression (Lu et al., 2005), is expressed in the mouse central and basal amygdala in response to addictive drugs such as cocaine, morphine or nicotine (Valjent et al., 2004). Moreover, the expression of ERK has been shown to be activated in the CeA after cue-induced relapse to cocaine-seeking, while the inhibition of ERK in this nucleus decreased cocaine-seeking after withdrawal (Lu et al., 2005). Another response of the central and medial nuclei of the amygdala has been specifically associated with increased ethanol intake in rats: The brain-derived neurotrophic factor (BDNF) plays an important role in synaptic plasticity. BDNF antisense oligodeoxynucleotide infusions directly into the central and medial nuclei of the amygdala, but not in the basolateral amygdala, increased ethanol intake in rats in a 2-bottle free choice paradigm (Pandey et al., 2006). These results show that the central and medial nuclei of the amygdala play a crucial role in regulating ethanol-drinking.

Understanding the mechanisms involved in ethanol addiction relapse is an important challenge. In this regard, identification of brain structures activated by ethanol-paired cues, in a drug-free state, is of great interest. Experiments which re-expose rats to ethanol-paired cues, in the absence of ethanol itself, provide information about the brain circuitry involved in ethanol-seeking. In rats previously trained to self-administer ethanol, ethanol-paired cues, and ethanol-paired environments (i.e., contexts) induced relapse to self-administration, even after a protracted abstinence (30 days). This re-exposure to ethanol-paired cues and context induces c-Fos expression in the amygdala, specifically in the basolateral, lateral, and central nuclei (Radwanska et al., 2008). Furthermore, in rats previously trained to lever press for an ethanol solution, the re-exposure to the ethanol-associated context increased their operant behaviour on the previously active lever, while ethanol-seeking reinstatement was associated with increased c-Fos

mRNA expression in the lateral and basolateral amygdala (Marinelli et al., 2007). The basolateral and central amygdaloid nuclei also present c-Fos mRNA induction in response to stressors that induce reinstatement of ethanol seeking (Funk et al., 2006). Thus, the central, lateral, and basolateral amygdala are involved in cue-induced relapse in drug-seeking, and in the control of the ability of environmental stimuli to form Pavlovian associations with reinforcing stimuli (for a review see Radwanska et al., 2008). The amygdala is also involved in the withdrawal-induced anxiety through amygdaloid GABA<sub>A</sub> receptors: intra-amygdala infusion of flumazenil—an antagonist of the benzodiazepine receptor—inhibits the development of anxiety that is sensitized by repeated ethanol withdrawal (Knapp et al., 2007).

The BNST is also strongly responsive to ethanol. This region is part of the extended amygdala and thought to play a critical role in the reward system (Kauer and Malenka, 2007). The BNST participates in the stress-related and reward-related limbic circuit, as it receives glutamatergic projections from the prefrontal cortex, and noradrenergic projections from the locus coeruleus, the nucleus of the solitary tract and the area postrema (Nieuwenhuys, 1985).

The lateral septum is activated in rats and mice following acute ethanol treatments (Crankshaw et al., 2003; Kolodziejaska-Akiyama et al., 2005; Ryabinin and Wang, 1998). However, following subchronic and chronic treatments (for 9 days and more), c-Fos immunoreactivity in the lateral septum is decreased (Bachtell et al., 2003; Ryabinin et al., 2003; Sharpe et al., 2005). This decrease has been linked to the involvement of the Ucn1 innervation from the pIIIu to the lateral septum in the regulation of ethanol consumption (Ryabinin and Weitemier, 2006). Withdrawal from acute ethanol injection and from chronic treatments produced an increase in c-Fos immunoreactivity in the lateral septum (Knapp et al., 1998; Kozell et al., 2005; Moy et al., 2000; Olive et al., 2001). This activation in the lateral septum may be related to the anxiety state that can occur in ethanol withdrawal (Knapp et al., 1998).

The different regions activated by ethanol may be activated variously, by the ethanol's rewarding effects or by its aversive properties. In mice trained in a procedure known to produce a conditioned place preference (CPP) for ethanol, the VTA, BNST, SNc, dorsomedial hypothalamus, lateral and central nuclei of the amygdala, CA1 and CA3 layers of the hippocampus, and dentate gyrus all showed c-Fos expression in response to the conditioned stimulus. These results indicate that these brain regions could be implicated in drug-reward and drug-seeking (Hill et al., 2007).

When consumption of a novel taste is followed by exposure to a toxin, animals will avoid consumption of that taste in the future, a learned response known as the "conditioned taste aversion" (CTA) (Thiele et al., 1996). Ethanol is characterized in part by high aversive properties. Studies on brain regions activated by ethanol-induced CTA learning are scarce. However, there are reports that ethanol-induced CTA is associated with activation of several brainstem areas hypothesized to be central for the aversive circuitry such as area postrema,

nucleus of the solitary tract and lateral parabrachial nucleus (Thiele et al., 1996). Interestingly, acute ethanol administration induces neuronal activity in these 3 regions (see Table 1). This activation in these regions may be related to the aversive properties of ethanol (Grancha et al., 2002; Houpt et al., 1996).

The propensity to consume ethanol may relate to individual's sensitivity to the motivational effects of ethanol which are related to its rewarding and/or aversive properties. Pre-clinical studies have used drug learning paradigms, such as CPP and CTA learning, to examine the neurobiological substrates involved in the motivational properties of ethanol. Ethanol-induced CTA and CPP may be mediated by similar neural substrates and a role for the GABA<sub>A</sub> receptor has been suggested in modulating the motivational effects of ethanol (Chester and Cunningham, 1996). Previous reports demonstrated that rats that expected beer but did not receive it showed substantial c-Fos induction in specific regions including the prelimbic cortex, lateral septum (ventral part), caudate-putamen, lateral hypothalamus, basolateral amygdala, CA3 region of the hippocampus, dorsolateral periaqueductal gray, and the pIIIu (Toppo et al., 1998). Crankshaw and colleagues (2003) showed that a single i.c.v. ethanol injection, which induced a conditioned taste preference for saccharin, significantly increased c-Fos immunoreactivity in the BNST, the shell part of the nucleus accumbens, the hypothalamic paraventricular nucleus and the lateral septum (ventral part), an observation that demonstrates a role in reward for these structures.

The paraventricular nucleus of the hypothalamus is highly ethanol-reactive in mice and rats, and this reactivity persists after chronic treatment (Ryabinin et al., 1997). In rats there was no increase in c-Fos immunoreactivity in the paraventricular hypothalamic nucleus after withdrawal from 14 days of ethanol-containing beverage (Knapp et al., 1998; Moy et al., 2000). In mice, withdrawal following chronic exposure induced c-Fos protein expression in the paraventricular nucleus of hypothalamus (Olive et al., 2001). This responsiveness is believed to be due to a hypothalamic-pituitary-adrenal axis activation by ethanol (Lee et al., 2004b; Ogilvie et al., 1998). A specific expression of c-Fos in the hypothalamic paraventricular nucleus is indeed observed following acute and chronic stress (Romeo et al., 2007). Moreover, in some cases, the observed activation of the paraventricular nucleus of the hypothalamus may be due to the withdrawal-induced seizures experienced by these mice (Olive et al., 2001).

All parts of the neocortex show ethanol-responsiveness: the prefrontal cortex (orbital, infralimbic, prelimbic, insular), the limbic cortex (cingulate, piriform), and the motor, parietal, and temporal cortices (see Tables 1–3). In the prefrontal cortex, the neuronal activity could be particularly related to ethanol-seeking, as c-Fos expression in the prefrontal cortex induced by a re-exposure to ethanol-paired cues was significantly higher after 30 days of abstinence as compared to 24-hour withdrawal (Wedzony et al., 2003). The ventro-lateral orbital region of the prefrontal cortex is an area of the brain

important in maintaining representations of reward value and for guiding behaviour thereby (Pickens et al., 2005).

In the hippocampus, although some studies failed to show any basal level of c-Fos, for example in home cage water-drinking mice (Ryabinin et al., 2001), most studies reported a basal level of c-Fos positive cells in the CA1 and CA3 regions and in the dentate gyrus of unstimulated rodents. Those unstimulated animals were either i.p. saline-injected rats (Ryabinin et al., 1995, 1997) saline-injected mice after a habituation period of handling and saline injection for 10 days (Ryabinin and Wang, 1998) or home cage water-drinking mice (Bachtell et al., 1999, 2003; Ryabinin et al., 2003; Sharpe et al., 2005). Acute ethanol administration reduces the expression of c-Fos protein in the hippocampus, an effect even more noticeable when c-Fos expression in this structure has been increased through a previous stimulus such as restraint stress or novel environment (Ryabinin, 1998; Ryabinin et al., 1995, 1997). This particular responsiveness (i.e., loss of c-Fos expression) of the hippocampus following acute ethanol administration may be connected to the role of this region in learning and memory (Bachtell and Ryabinin, 2001; Davis et al., 2003; Jones et al., 2001; Ryabinin, 1998). In rats, ethanol seeking was associated with increased c-Fos mRNA expression in hippocampus CA3 layer (Marinelli et al., 2007). These findings imply that the dorsal hippocampus is involved in the learning of ethanol-associated cues and may have a critical role in cue-induced reinstatement of ethanol-seeking, a process dependent on the mu and delta opioid receptors (Marinelli et al., 2007).

Acute ethanol-induced c-Fos activation occurs in the locus coeruleus (A6 group), 1 major noradrenergic nucleus in the brain. The locus coeruleus sends noradrenergic projections to numerous ethanol-responsive regions: the neocortex, the dentate gyrus and CA1 and CA3 layers of the hippocampus, the paraventricular nucleus of the hypothalamus, the septum, the BNST, the central and basal nuclei of the amygdala, the dorsal thalamus, the medial and lateral geniculate nucleus, the periaqueductal grey, and the superior colliculus (Nieuwenhuys, 1985). Furthermore, stimulation of the locus coeruleus neurons is thought to play an important role in drug dependence and reward (Bell and Grant, 1998; Rasmussen et al., 1990).

In the studies discussed in this review, the dorsal raphe has never been shown to present any IEG induction or reduction after ethanol treatment. This is surprising, as the dorsal raphe sends serotonergic projections to all parts of the neocortex, and in particular to the cingulate cortex, the striatum, the nucleus accumbens, the globus pallidus, the septum, the anterior and basal nucleus of amygdala, the dentate gyrus and the CA1 and CA3 layers of the hippocampus, the paraventricular thalamus, the hypothalamus, the suprachiasmatic nucleus, the SNc, the VTA, the locus coeruleus (Nieuwenhuys, 1985), and the pIIIu (Ryabinin and Weitemier, 2006), all these regions being ethanol-responsive. However, as reviewed by Ryabinin and Weitemier (2006), the Ucn1 projection from

the pIIIu to the dorsal raphe is apparently not involved in the regulation of ethanol intake.

In the pIIIu, the c-Fos induction observed after acute treatment persists after chronic treatment, although in chronic treatments the induction is weaker (Bachtell et al., 1999; Chang et al., 1995; Ryabinin et al., 1997; Sharpe et al., 2005). The induction of c-Fos in the pIIIu by ethanol is not secondary to the stress of ethanol administration or to ethanol-induced hypothermia (Turek and Ryabinin, 2005). Withdrawal after a single injection of ethanol (Kozell et al., 2005) increased c-Fos immuno-reactivity in the pIIIu. So far as we know the effects of withdrawal from chronic ethanol have not been investigated in this brain region.

A very important point to consider when comparing IEG expression patterns is the means of administration, i.e., non-contingent administration (i.p. injections or vapour inhalation) or contingent administration as for example the most frequently used 30 minutes a day limited-access session. As a matter of fact, there is a different set of regions activated following these 2 types of administration after chronic treatment (see Table 2). Lateral septum and nucleus accumbens seem specifically targeted after chronic daily 30 minute-limited-access session of ethanol in mice (Bachtell et al., 1999, 2003; Ryabinin et al., 2003; Sharpe et al., 2005) and rats (Topple et al., 1998; Weitemier et al., 2001; Yoshimoto et al., 2000). There is no reported response in the lateral septum and nucleus accumbens in rats following chronic i.p. treatments (Ryabinin et al., 1997) or exposure to ethanol vapour (Depaz et al., 2000). In contrast, the CeA, pIIIu, and hippocampus are targeted as well after noncontingent i.p. administration (Ryabinin et al., 1997) or ethanol vapour inhalation (Depaz et al., 2000) rather than after free-choice self-administration of ethanol during limited-access session (Bachtell et al., 1999, 2003; Ryabinin et al., 2003; Sharpe et al., 2005; Topple et al., 1998; Weitemier et al., 2001).

A differential pattern of brain region activation may arise from different conditions of ethanol administration. In this regard expectation and conditioning have been shown to modulate the reinforcing effects of drugs of abuse. For example, the ability of drugs to increase dopamine in nucleus accumbens, an effect associated with their reinforcing value (Di Chiara and Imperato, 1988) is larger when animals are given cocaine in an environment where they had previously received it and therefore expected it (Duvauchelle et al., 2000), or when animals self-administer cocaine than with involuntary administration (Hemby et al., 1997). Similarly, cocaine-induced changes in regional brain metabolism, which is an indicator of brain function (Sokoloff et al., 1977), differ when animals self-administer cocaine from when administration is involuntary (Graham and Porrino, 1995) and when cocaine is given in a conditioned environment versus their home cage (Knapp et al., 2002). It is also important to keep in mind that voluntary ethanol intake is a necessary precondition for developing addictive behaviour (for a review see Spanagel, 2003).

In the pIIIu, the c-Fos induction is present whatever the means of administration, i.e., voluntary intake or

experimenter-administered (for a review, see Ryabinin and Weitemier, 2006). In the pIIIu, ethanol-induced expression of c-Fos is dependent upon GABA<sub>A</sub> receptor activation and occurs in neurons expressing Ucn1, an observation that reveals the involvement of this neuropeptide in ethanol-related behaviours. In fact, the pIIIu appears to be a major target of ethanol-induced activation. The entire Ucn1 neuro-circuitry seems to be regulated by long-term ethanol exposure and in turn regulates ethanol consumption. Furthermore, this regulation may occur through projections from the pIIIu to the lateral septum (for a review, see Ryabinin and Weitemier, 2006).

## CONCLUSION

This review has aimed to establish which ethanol-induced IEGs-regulations are present in acute and chronic ethanol intoxication, in withdrawal and in cue-induced relapse to ethanol-seeking. Addiction develops as a transition from initial to habitual to relapsing drug use (Kalivas, 2005). Elucidating the link between drug-induced neuroplasticity in brain regions and the stages of transition in drug intake will improve our understanding of the neurobiology of addiction.

Our analysis of studies on ethanol-responsive brain regions has shown that the map of the activated regions of the brain is not the same after acute or after chronic ethanol intake, or after chronic ethanol withdrawal. Ethanol administration induces intra-neuronal molecular adaptations, as revealed by c-Fos and Egr1 regulations. The neuronal adaptations are not restricted to the intra-neuronal compartment; there are also adaptations in the neuronal activity of inter-connected brain regions. Thus, the different stages of addiction may engage different or additional brain circuitries. These regional changes may be one of the neuronal supports of the transition from occasional ethanol intake toward compulsive use. Very interestingly, it was recently shown that the acute-ethanol-induced c-Fos expression in cingulate, infralimbic and orbitofrontal cortices and in paraventricular nucleus of the hypothalamus was significantly attenuated in rats with a history of dependence. The state of ethanol addiction determines the amplitude of the c-Fos response in these brain regions (Hansson et al., 2008).

There is a "desensitization" of acute ethanol-activated brain regions after chronic intake, and this effect may result in a restricted set of chronic ethanol-activated regions. These regional changes reveal that there is a "footprint" in the brain following repeated ethanol intoxication that may account for the altered reactivity of the brain after chronic exposure. The restricted set of regions includes the extended amygdala, nucleus accumbens, paraventricular nucleus of the hypothalamus, nucleus of the solitary tract, and pIIIu. These regions belong either to the mesolimbic dopamine system and its associated limbic structures implicated in addiction (Kauer and Malenka, 2007) or to the noradrenergic ascendant system (whose somas are located in locus coeruleus and nucleus of the solitary tract) or to the urocortin system (whose somas

are located in the pIIIu). This restricted set of regions focuses on the crucial circuitries involved in reward processing.

A major shortcoming of IEG induction studies (see Borlikova et al., 2006) is that they are done at a particular time after stimulus and the complete kinetics of the induction is rarely undertaken. New technological approaches will allow neuro-anatomists to go beyond this limitation. For example, hybridization of new DNA probes complementary to c-Fos has been tracked by Magnetic Resonance Imagery in the brain of rats previously administered with amphetamine, and showed robust c-Fos mRNA induction in the nucleus accumbens, the striatum and the medial prefrontal area in real time (Liu et al., 2007).

This review focused on c-Fos and Egr1, the IEGs that have so far been investigated for ethanol responsiveness. These are the preferred IEGs for mapping regions of the brain responsive to different types of stimulus, such as drugs of abuse, antidepressants treatments or kainate lesions (for a review see Nestler et al., 1999). In the future, some other targets which seem to participate in the cellular responses responsible for addiction will be of great interest to study. Among these are the Homer proteins family (Chandler et al., 2006; Szumlinski et al., 2008), and particularly Homer 2 (McBride et al., 2005); ERK, which controls c-Fos and Egr-1 expression (Girault et al., 2007; Hansson et al., 2008; Lu et al., 2006), CREB (Pandey et al., 2006), DARP-32 (Ron and Jurd, 2005), cocaine- and amphetamine-regulated transcript (CART) peptides (Hubert et al., 2008) or Arc (Schochet et al., 2005). So far, the study of ethanol effects on these potential targets has received very little or no attention.

Despite some limitations, the brain regions mapped using IEG expression in rats and mice do correlate with regions that clinical studies have shown to be part of ethanol addiction circuitry. Most clinical studies investigated the activated brain regions after re-exposure to ethanol-associated cues which act as secondary reinforcers and can induce relapse in detoxified alcoholics.

Human imaging studies show an overall reduction in prefrontal cortical measures of cellular metabolism and blood flow in individuals addicted to ethanol (Goldstein and Volkow, 2002). This hypofrontality, including regions such as anterior cingulate and ventral orbital cortex, has been characterized as a strong indicator of reduced ability to regulate drug-seeking (Baler and Volkow, 2006). Several studies have observed that ethanol-associated pictures elicit increased activation of the prefrontal cortex, anterior cingulate cortex, striatum, and visual association centres in ethanol-dependent subjects compared with healthy controls (Braus et al., 2001; George et al., 2001; Wrase et al., 2002, 2007). Grüsser and colleagues (2004) showed in ethanol-dependent patients that increased activation of the cingulate and adjacent medial prefrontal cortex and striatum predicted relapse. Furthermore, ethanol-associated cues elicited increased activation in the amygdala/hippocampal area, an area of particular importance for drug reward, stress/anxious responding, learning and emotion, in ethanol-dependent subjects compared with

healthy controls (Schneider et al., 2001). Imaging studies also indicated that other brain regions are involved in ethanol addiction, an observation that may suggest the recruitment of far more complex networks. In this regard, cerebellar activation has been associated with cue-induced ethanol craving (Olbrich et al., 2006) and another study has shown that the precuneus, a brain region activated by visual imagery and conscious recollection, displayed an increased activation in detoxified alcoholic subjects (Heinz et al., 2007). Preclinical (Marinelli et al., 2007; Radwanska et al., 2008; Wedzony et al., 2003) and clinical studies identified similar brain regions recruited by exposure to ethanol-associated cues. The recruited regions are the amygdala, the hippocampus and the prefrontal cortex.

Another important challenge is to study the effects of anticraving compounds on brain regions activated by ethanol-associated cues. Such studies may reveal which areas of the brain have a possibly critical role in mediating cue-induced relapse in ethanol consumption. A recent preclinical study indicated that the hippocampus, amygdala and paraventricular nucleus of the hypothalamus are potential substrates for the antirelapse effects of naltrexone (Dayas et al., 2007). A recent clinical report has also shown a reduction in right thalamic activation elicited by ethanol versus control cues in ethanol-dependent subjects after acute intake of the atypical dopamine D2/3 blocker amisulpride (Hermann et al., 2006).

Preclinical and clinical studies have indicated the major neuronal substrates which underlie ethanol addiction and they comprise, at least, 4 interdependent and overlapping circuits: (i) reward, regulated by nucleus accumbens, ventral pallidum, and hypothalamus; (ii) motivation and/or drive, influenced by the orbito-frontal cortex, (iii) memory and learning, regulated by the amygdala and hippocampus; and (iv) cognitive control, influenced by the prefrontal cortex and the cingulate cortex (Kalivas and Volkow, 2005; Volkow and Li, 2005).

Identification of rodent brain regions activated following ethanol or ethanol-associated cues exposure and human neuroimaging studies allow the determination of specific neurobiological substrates involved in the different steps of development of ethanol dependence and in cue-induced ethanol craving in ethanol-abstinent patients. The successful long-term treatment of ethanol dependence and maintenance of abstinence may require agents that act on different neuronal networks (reward systems, memory network, prefrontal cortex). A better understanding of the neural circuits that are affected by ethanol and their adaptations during the development of ethanol addiction will provide new opportunities for developing appropriate therapies.

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