

Mechanisms of Neurodegeneration and Regeneration in Alcoholism

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Abstract — Aims: This is a review of preclinical studies covering alcohol-induced brain neuronal death and loss of neurogenesis as well as abstinence-induced brain cell genesis, e.g. brain regeneration. Efforts are made to relate preclinical studies to human studies. **Methods:** The studies described are preclinical rat experiments using a 4-day binge ethanol treatment known to induce physical dependence to ethanol. Neurodegeneration and cognitive deficits following binge treatment mimic the mild degeneration and cognitive deficits found in humans. Various histological methods are used to follow brain regional degeneration and regeneration. **Results:** Alcohol-induced degeneration occurs due to neuronal death during alcohol intoxication. Neuronal death is related to increases in oxidative stress in brain that coincide with the induction of proinflammatory cytokines and oxidative enzymes that insult brain. Degeneration is associated with increased NF- κ B proinflammatory transcription and decreased CREB transcription. Corticolimbic brain regions are most sensitive to binge-induced degeneration and induce relearning deficits. Drugs that block oxidative stress and NF- κ B transcription or increase CREB transcription block binge-induced neurodegeneration, inhibition of neurogenesis and proinflammatory enzyme induction. Regeneration of brain occurs during abstinence following binge ethanol treatment. Bursts of proliferating cells occur across multiple brain regions, with many new microglia across brain after months of abstinence and many new neurons in neurogenic hippocampal dentate gyrus. Brain regeneration may be important to sustain abstinence in humans. **Conclusions:** Alcohol-induced neurodegeneration occurs primarily during intoxication and is related to increased oxidative stress and proinflammatory proteins that are neurotoxic. Abstinence after binge ethanol intoxication results in brain cell genesis that could contribute to the return of brain function and structure found in abstinent humans.

INTRODUCTION: ALCOHOL-INDUCED BRAIN DAMAGE

The characteristic feature of alcohol use disorders is the consumption of dangerous amounts of alcohol despite the knowledge that problems occur during drinking. Excessive levels of intake, the characteristic of binge drinking, leads to the development of tolerance and dependence that then further promotes excessive consumption but is also associated with changes in brain physiology, structure and function. In general, human alcoholics, both men and women, show significant volume loss in cortical and subcortical brain structures that includes both gray and white matter shrinkage. These widespread deficits occur in the absence of major nutritional deficiencies, although nutritional deficiencies can cause neurodegeneration and could contribute to alcoholic degeneration. Both postmortem and *in vivo* imaging studies of brain morphology find abnormal reduced brain volumes of gray and white matter across multiple regions. The frontal lobes are the most insulted region in the alcoholic brain with the superior frontal cortex showing significant neuronal loss (Kubota *et al.*, 2001; Sullivan and Pfefferbaum, 2005). The frontal lobes regulate complex cognitive skills such as working memory, temporal ordering, discrimination and reversal learning that underlie judgment, attention, risk taking, motivation, mood and wanting. Disorders in these behaviors are central if not causal to the consumption of dangerous amounts of alcohol despite the knowledge of negative consequences. Accordingly, chronic alcoholics demonstrate impaired judgment, blunted affect, poor insight, social withdrawal, reduced motivation, distractibility, attention and impulse-control deficits (Parsons, 1987; Oscar-Berman and Hutner, 1993; Sullivan *et al.*, 2000b; Sullivan and Pfefferbaum, 2005). It has been proposed that progressive increases in ethanol consumption lead

to alterations in brain structures that reduce behavioral control promoting further alcohol abuse and neurodegeneration (Crews *et al.*, 2004).

Interestingly, abstinence from ethanol is associated with a recovery in brain volume loss. Pfefferbaum *et al.* (1995) have shown using longitudinal MRI that in recovery or relapse, short-term abstinence (1 month) can increase cortical gray matter volume and long-term (1 year) abstinence shrinks the enlarged third ventricle. Similarly, neuropsychological deficits improve with abstinence particularly working memory, visual-spatial abilities and certain motor abilities. MR spectroscopy reveals improvement in neuronal integrity during abstinence (Sullivan *et al.*, 2000a,b; Parks *et al.*, 2002; Meyerhoff *et al.*, 2004). Regeneration of the brain during abstinence has been proposed to underlie some of these improvements in executive function and impulse control that occur with sustained abstinence (Crews *et al.*, 2004).

The mechanisms of alcohol-induced brain damage and abstinence-induced regeneration are complex. The extent of neurodegeneration and potential for recovery and regeneration varies by brain region and is dependent on many factors, including pattern of intake, genetics and age. This review will focus particularly on a binge model of alcohol dependence first developed to study physical dependence (Majchrowicz, 1975). Later studies indicated that the binge model induced neurodegeneration (Crews *et al.*, 2004), reduced neurogenesis (Nixon and Crews, 2004) and caused cognitive deficits (Obernier *et al.*, 2002b). More recently, studies showed evidence of regeneration during abstinence following binge treatment: striking increases in neurogenesis (Nixon and Crews, 2004) and cell genesis (Nixon *et al.*, 2008) mimic the regeneration of human brain in abstinence. This review will cover these studies and make an effort to relate them to findings in humans.

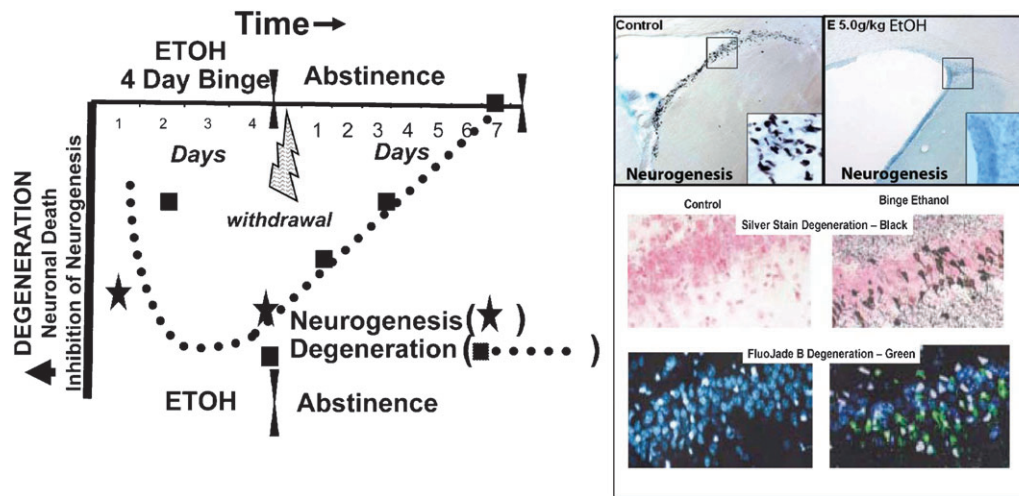


Fig. 1. Ethanol-induced brain damage and inhibition of neurogenesis, and time course of degeneration. Shown on the left is the time course of neurodegeneration (dotted line) with greater damage and loss of neurogenesis as negative downward values. Neurodegeneration (squares, binge ethanol agyrophilic silver stain) and loss of neurogenesis (stars, BrdU+ir) are both plotted during ethanol treatment. Acute treatment with ethanol results in inhibition of neurogenesis and increased apoptotic cells (Obernier *et al.*, 2002a; He *et al.*, 2005). Although little silver stain degeneration is found with acute ethanol treatments, silver stain takes 24–48 h to develop suggesting that cell death may have been missed in acute studies. The 4-day binge shows progressively increased silver stain from Day 2 throughout the 4-day period. Abstinence following the binge results in a progressive loss of cell death silver stain markers with complete clearance after ~1 week (Crews and Braun, 2003). See also Crews *et al.* (2004). Pictures right top: examples of ethanol inhibition of neurogenesis (top left: control, top right: ethanol; 5 gm/kg, i.g.) in adolescent animals. Shown are brain sections of frontal subventricular zone neural progenitor cells (BrdU + IR, black dots on section). Note how acute ethanol has eliminated these progenitors completely [no black dots—low first star in time course; adapted from Crews *et al.* (2006a)]. Pictures right, middle and bottom: degeneration. Brain sections identify 4-day binge ethanol-induced necrotic degeneration in hippocampus visualized by agyrophilic amino cupric silver stain (black—middle photo) or Fluoro-Jade B (green—bottom). Note binge ethanol degeneration, but none in controls. [Adapted from Obernier *et al.* (2002a).]

BINGE MODEL OF ALCOHOL DEPENDENCE

The binge ethanol model of alcohol dependence has been studied widely as a reliable model for ethanol-induced brain damage (Crews *et al.*, 2004). Rats receive ethanol intragastrically three times a day for 4 days. The daily dose of ethanol is ~9 g/kg/day, which in rats results in blood levels between 250 and 400 mg%. The first dose of 5 g/kg causes significant intoxication, and future doses are adjusted depending on the level of intoxication, i.e. the subsequent dose is lowered for those showing intoxication from the previous dose. Tolerance rapidly occurs with most rats showing high blood levels, but little intoxication on Days 3–4. This model mimics the high blood ethanol levels and heavy alcohol consumption common among alcoholics. For example, 4–10% of emergency room patients have blood alcohols over 250 mg% (Urso *et al.*, 1981; Teplin *et al.*, 1989; Cartledge and Redmond, 1990; see also Crews *et al.*, 2000, introduction). Further, binge drinking is one of the most common patterns of intake with 40–60% of alcohol abusers drinking in this manner (e.g. Robin *et al.*, 1998). Thus, the high blood alcohol levels and pattern of intake in the rat binge model are consistent with that commonly found in alcohol abusers.

Many investigators have shown that binge ethanol administration induces brain damage (Crews *et al.*, 2004). Brain damage increases progressively after 2 days beginning in the frontal olfactory bulb followed by additional brain regions showing increasing damage with time. Most of the studies investigating binge ethanol-induced brain damage found peak damage just

after the last of the 4-day doses (Fig. 1). Significant damage in adults is found in limbic association regions, namely agranular insular cortex, anterior piriform cortex, perirhinal cortex, entorhinal cortex and hippocampal dentate gyrus (Fig. 2), particularly ventral dentate gyrus. In these regions, dark cell degeneration, a necrotic form of cell death with shrunken soma, is the predominant form of neuronal death (Obernier *et al.*, 2002a). In addition, binge ethanol treatment reduces hippocampal neurogenesis as discussed later (Nixon and Crews, 2002). In general, brain degeneration in the binge ethanol treatment model is diffused and widespread, similar to that found in human alcoholics. Interestingly, studies examining adolescent rats versus adults (Crews *et al.*, 2004) and a genetic model of alcoholism, the P rat (Crews *et al.*, 2004), have found increased binge-induced brain damage, particularly frontal and limbic cortical damage in both genetic and adolescent models of alcoholic risk. These studies suggest that the binge model is a reasonable model of human alcoholic neurodegeneration.

Cognitive impairments in alcoholics are linked to alcoholic neurodegeneration and alcohol dependence. Following binge alcohol treatment in adult rats, cognitive function has been examined using the Morris water maze (Fig. 3). Although initial learning of this task was similar between controls and binge-treated rats, as was activity, relearning tasks showed deficits in trials to criterion, greater time in the original (or wrong) quadrant and greater entries into the wrong quadrant. These deficits are consistent with disrupted frontal lobe function and perseveration (Obernier *et al.*, 2002b). In addition, analysis

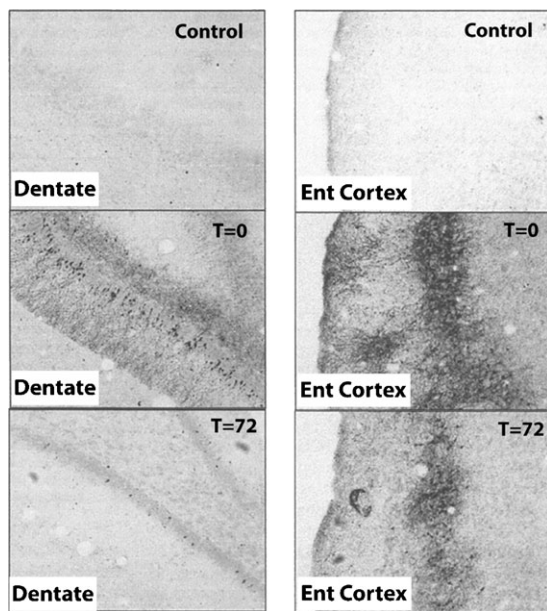


Fig. 2. Binge ethanol-induced brain damage. Shown are brain sections from hippocampal dentate gyrus (left 3 panels) and entorhinal cortex (right 3 panels). Agyrophilic silver stain identifies dying neurons as confirmed by multiple methods showing binge ethanol-induced neurodegeneration (Obernier *et al.*, 2002a). Note little or no cell death in controls (top panels) with significant silver staining in 4-day binge ethanol-treated animals sacrificed just after the last ethanol dose (T = 0; middle 2 panels). The bottom panels show brain sections 72 h after the last ethanol dose. Note the loss of silver stain at 72 h of abstinence. Since silver staining of dying neurons occurs 1–3 days after insults (Switzer III, 2000), it is likely that damage occurs during binge intoxication with little additional damage during abstinence–withdrawal.

of microglial density suggested that damage had occurred according to persistent elevations of PK11195 binding in frontal cortical regions such as the anterior piriform cortex (Obernier *et al.*, 2002b). Studies in alcoholic human brain have also found increased microglial density in cortical regions (He and Crews, 2008). Thus, the rodent binge model shares both neurodegenerative and cognitive deficits found in human alcoholic brain.

MECHANISMS OF ALCOHOL-INDUCED BRAIN DAMAGE: DEGENERATION OCCURS DURING INTOXICATION

A number of studies have focused on the mechanisms of alcohol-induced brain damage. Early studies, mostly *in vitro* culture models, suggested that chronic ethanol inhibited glutamatergic *N*-methyl-D-aspartate (NMDA) receptors that in time resulted in NMDA supersensitivity, an effect only revealed upon the removal of alcohol (Chandler *et al.*, 1993a, 1993b). These *in vitro* studies and others suggested that during

withdrawal, neurotoxicity occurs through the NMDA receptor (Prendergast *et al.*, 2000, 2004). Multiple groups studying the *in vivo* binge ethanol model therefore investigated the effectiveness of NMDA receptor antagonists at blocking or reducing binge-induced brain damage. Various NMDA receptor antagonists, including MK801 (dizocilpine), memantine and DNQX failed to reduce binge ethanol neurotoxicity and surprisingly, some doses even increased degeneration (Collins *et al.*, 1998; Corso *et al.*, 1998; Crews *et al.*, 2004; Hamelink *et al.*, 2005). Other hypotheses suggested that ethanol intoxication increased the brain density of voltage-gated calcium channels, which then contributed to excessive excitability during withdrawal; however, calcium channel blockers did not protect against binge-induced brain damage [see Crews *et al.* (2004) for review]. The *in vivo* binge studies continued to suggest that ethanol-induced brain damage occurred during intoxication and required relatively high blood levels. Blood levels clearly cycle during treatment so the impact of withdrawal was hard to assess. Time course studies of binge-induced brain damage indicated that damage was increasing during ethanol treatment and began to subside with withdrawal (Fig. 1). Most studies used agyrophilic silver stain, which identifies cells irreversibly committed to cell death ~1–3 days after the insult (Switzer III, 2000). Thus silver staining may increase over a several-day course after cell death. In the binge model, silver stain appears after 2 days and peaks just after the last dose of ethanol (Day 4) but then subsides progressively over the first week of withdrawal as silver-stained material is cleared, likely by phagocytes (Fig. 1; Collins *et al.*, 1996, 1998; Crews, 2000; Obernier *et al.*, 2002a; Crews *et al.*, 2004). The progressive loss of cell death markers during ethanol withdrawal over a week suggests that little to no degeneration occurs during withdrawal in this model. Withdrawal hyperexcitability and seizures, if present, occur during the first 24 h of withdrawal. Cell death markers would be expected to appear within days during the following week if damage occurred during withdrawal in this model. Further, the findings that damage occurs during intoxication when ethanol should block NMDA receptors and the lack of effect of NMDA antagonists on damage suggest that the mechanism of ethanol-induced brain damage is not glutamate excitotoxicity. Thus, ethanol-induced brain damage in the binge model occurs during intoxication.

Human studies also support that neurodegeneration occurs during intoxication with most studies supporting a recency and frequency of heavy drinking as the best indicator of alcoholic brain damage (Parsons, 1987; Sullivan and Pfefferbaum, 2005). Over a 5-year interval the degree of excessive drinking in alcoholics corresponded with gray matter loss, particularly in the frontal lobes (Pfefferbaum *et al.*, 1995). Frontal cortical choline-containing compounds measured by MRI were decreased in alcoholics with significant correlations between alcohol consumption in the last 90 days and decreases in anterior cingulate cortex and frontal white matter (Ende *et al.*, 2006). Other studies have suggested that the white matter may recover rapidly in abstinence (Shear *et al.*, 1995; O'Neill *et al.*, 2001) or be more vulnerable to damage during relapse (Pfefferbaum *et al.*, 1995). Further, efforts to relate neurodegeneration to lifetime alcohol consumption have not found a relationship. Thus, recency and frequency of intoxication best predict alcoholic neurodegeneration consistent with damage during intoxication.

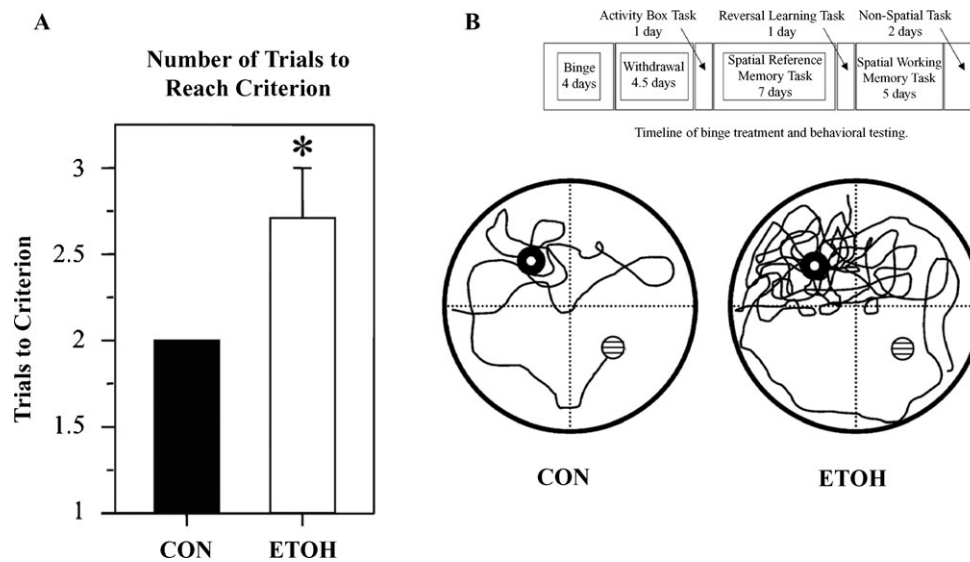


Fig. 3. Reversal learning identifies persistent perseverative repetitive behaviors following binge ethanol administration. Morris water maze learning of platform location was identical in binge ethanol and control animals on 4–11 days of abstinence. Both readily learned the platform location. However, they differed in the reversal-learning task of the Morris water maze. Following the 7 days of place learning, animals are tested in a reversal-learning task. The submerged platform was placed in the quadrant opposite that in which it had been placed during the reference memory task (southwest quadrant). The animals were given four trials, each consisting of a 90-s ceiling and a 60-s intertrial interval. Once the animal reached the platform, it was allowed to remain on the platform for ~10 s. If the animal failed to reach the platform within the trial ceiling, the experimenter gently guided the animal through the water and placed it on the platform where it would remain for 10 s. The animal was then removed to its home cage, which was warmed with a heating pad, to await the next trial. (A) ETOH animals required a significantly greater number of trials to reach the criterion than CON animals [$t(14) = 2.376$; $*P < 0.05$]. (B) Time line of binge treatment and behavioral testing: After a 7-day acquisition of the platform location, the submerged platform was moved to the opposite quadrant and animals were given four trials (12 days' postbinge treatment). A vertical view of the track was taken by a CON and an ETOH rat during the first trial of the reversal-learning task. The open circle represents the location of the submerged platform the animals were trained to and the patterned circle represents the location of the platform during the reversal-learning task. Note the perseverative behavior shown by the ETOH animal with numerous re-entries into the original goal quadrant. The ETOH animal also failed to reach the new platform locations within the 90-s ceiling. [Adapted from Obernier *et al.*, (2002b).]

MECHANISMS OF ALCOHOL-INDUCED BRAIN DAMAGE: OXIDATIVE STRESS AND PRO-INFLAMMATORY CYTOKINES ALTER TRANSCRIPTION FACTORS IN BRAIN

Many findings suggest that ethanol-induced brain damage is related to oxidative stress from proinflammatory enzymes activated during ethanol intoxication. Studies in organotypic hippocampal-entorhinal cortex slice (HEC) cultures have found that ethanol sensitizes neurons to insults. HEC slice cultures maintain all the cellular elements of brain as well as the intact structures and interconnections of brain. Transcription factors, such as cAMP responsive element-binding protein (CREB) and nuclear factor κ B (NF- κ B), regulate the gene expression. During the presence of ethanol, there are changes in protein transcription with increased DNA binding of NF- κ B and reduced DNA binding of CREB (Figs 4 and 5). CREB family transcription factors are activated by phosphorylation and promote neuronal survival, protecting neurons from excitotoxicity and apoptosis through regulating the transcription of pro-survival factors (Lonze and Ginty, 2002; Mantamadiotis *et al.*, 2002). Conversely, NF- κ B is a transcriptional factor that is known widely for its ubiquitous roles in inflammatory and immune responses (O'Neill and Kaltschmidt, 1997). The balance in expression and activation of these transcription factors—and thus the balance of pro-survival versus proinflammatory states—

suggests a mechanism by which alcohol induces brain damage in alcoholic neuropathology.

Due to its pivotal roles in neuronal plasticity and long-term memory, CREB family transcription factors have been implicated in addiction and drug abuse (Lonze and Ginty, 2002; Nestler, 2002). Ethanol activation of oxidative stress coincides with the loss of CREB pro-survival transcription (Figs 4 and Fig. 5). *In vivo*, binge ethanol treatment results in a decrease in pCREB immunoreactivity in brain during ethanol intoxication, an effect that is most pronounced in brain regions showing degeneration (Bison and Crews, 2003). The reduction in pCREB coincides with the peak of neurodegeneration in the *in vivo* binge. In HEC cultures, ethanol reduces levels of the CREB-regulated gene, brain-derived neurotrophic factor (BDNF), with reductions in BDNF coinciding with sensitivity to neurodegeneration (Zou and Crews, 2006). Thus, reduced CREB transcription contributes to ethanol neurotoxicity (Fig. 4). Thus, ethanol loss of trophic signals combined with the induction of oxidative stress proinflammatory signals (detailed below) appears to underlie the degeneration and atrophy that occur in alcoholism. The NF- κ B transcriptional factor is well known for its role in inflammatory and immune response signaling, as well as in control of cell division and apoptosis (O'Neill and Kaltschmidt, 1997). The molecular composition of the NF- κ B family is composed of several members, including p50, p52, p65/RelA, RelB and c-Rel, that form homo- and

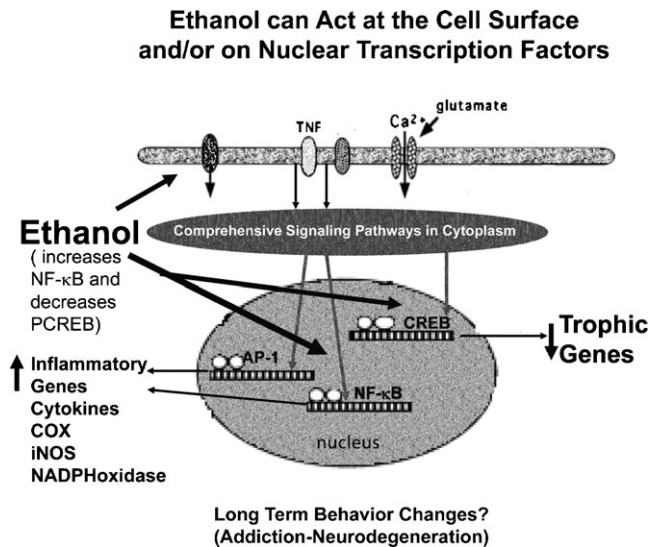


Fig. 4. Schematic representation of ethanol-induced changes in transcription. Ethanol can modulate multiple signaling pathways with convergence on multiple transcription factors, three of which are illustrated: ethanol increased proinflammatory gene expression, the convergence on increased NF- κ B and AP1 transcription initiating and sustaining proinflammatory cascades. CREB is the transcription factor that regulates the transcription of prosurvival target genes, such as *bcl2* and brain-derived neurotrophic factor. Ethanol exposure leads to imbalance between procytokine-oxidative stress and pro-survival gene transcription, causing neuronal atrophy, shrinkage and degeneration and inhibiting neurogenesis. [See Crews *et al.* (2004) and Zou and Crews (2005) for details.]

heterodimers capable of transmitting receptor signals to the nucleus (O'Neill and Kaltschmidt, 1997; Ghosh *et al.*, 1998). NF- κ B is activated by stimuli of oxidative stress, cytokines and glutamate (O'Neill and Kaltschmidt, 1997; Mattson and Camandola, 2001; Hinoi *et al.*, 2002), and is the key in the induction of proinflammatory cytokines and enzymes that promote a proinflammatory cascade by further activating NF- κ B transcription. In the central nervous system, NF- κ B proteins are ubiquitously expressed in neurons and glia (Kaltschmidt *et al.*, 1993; Mattson and Camandola, 2001) where, in addition to regulating physiological processes, they participate in pathological events (O'Neill and Kaltschmidt, 1997; Grilli and Memo, 1999; Pizzi *et al.*, 2002). Increased NF- κ B levels have been reported in dying neurons following trauma or ischemia (Terai *et al.*, 1996a; Bethea *et al.*, 1998; Schneider *et al.*, 1999) as well as in brains of patients with Alzheimer's disease and Parkinson's disease (Terai *et al.*, 1996b; Kaltschmidt *et al.*, 1997). Activation of NF- κ B transcription is associated with increases in pro-inflammatory cytokines with tumor necrosis factor- α (TNF α) being the prototype. Cytokines are large families of secreted proteins that interact in complex cascades or patterns regulating immune, hormonal and nervous systems. Systemic cytokines are transported from serum into brain, particularly for TNF α , IL1[®], NGF, IL6 and IFN[©] (Banks, 2005). A role for cytokines in alcoholic neuropathology is suggested by several studies [see Crews *et al.* (2006b) for review]. Szabo's studies of human monocytes from alcoholics found greater amounts of TNF α spontaneously and in response to challenge (Crews *et al.*, 2006b), and acute ethanol increases cytokine induction by TLR2 and TLR4 ligands (Oak *et al.*,

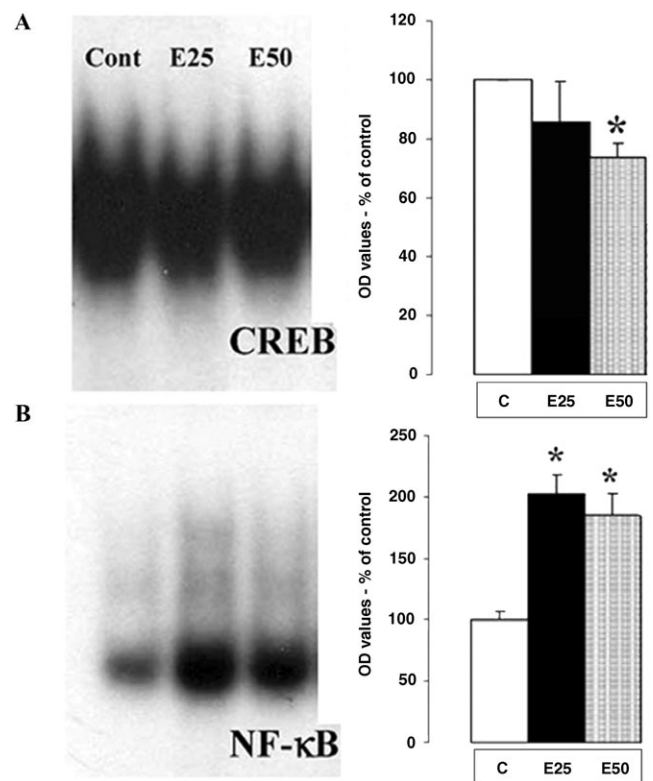


Fig. 5. Ethanol increases CREB and decreases NF- κ B DNA binding. HEC slices were treated with various concentrations of ethanol for 24 h and then removed for processing of nuclear extracts. Nuclear extracts are processed for electrophoretic mobility shift assay (EMSA). EMSA involves an oligonucleotide probe specific for the DNA binding site of each transcription factor with gel separation of transcription factor-bound probe providing an index of transcription factor activation and binding to DNA. (Cont = control; E25 and E50, ethanol 25 and 50 mM, respectively.) Note the decreased CREB binding with increasing concentrations of ethanol and the increased NF- κ B binding (band size) with ethanol addition to brain slices. [Adapted from Zou and Crews (2005).]

2006). Thus, ethanol through both systemic and central nervous system mechanisms induces a proinflammatory cascade in the brain that contributes to neurotoxicity.

Both *in vivo* and *in vitro* evidence support the involvement of a proinflammatory cascade including increased NF- κ B-driven induction of oxidative stress enzymes as a key factor in alcohol-induced brain damage. TNF α can directly potentiate glutamate neurotoxicity by inhibiting glutamate uptake through NF- κ B mechanisms (Zou and Crews, 2005). In human astroglial cells, which normally regulate extracellular glutamate concentrations, ethanol enhances NF- κ B-DNA binding and the induction of iNOS (Davis and Syapin, 2004; Davis *et al.*, 2005). Similarly, we found that ethanol induces COX2 (Knapp and Crews, 1999), iNOS and NADPH oxidase gp91, and increases reactive oxygen species producing enzymes that are downstream of NF- κ B. NADPH oxidase is a multimeric enzyme composed of multiple subunits that in the active enzyme catalyze the transfer of one electron from NADPH to oxygen, giving rise to superoxide. Ethanol significantly increases the brain expression of NADPH oxidase subunits, gp91phox and p67phox, that persists for at least 8 days of abstinence (Qin *et al.*, 2008). Thus, ethanol promotes a proinflammatory and anti-survival

environment through the activation of proinflammatory transcription factors and the inhibition of prosurvival transcription factors.

Considerable support for the proinflammatory mechanism of alcohol brain damage also derives from studies showing protection against alcohol-induced brain damage with antioxidants. Collins' work first suggested inflammation as a mechanism of damage (Collins *et al.*, 1998); however, Eskay clearly established that anti-oxidants protect against binge-induced brain damage (Hamelink *et al.*, 2005). We have also found that administration of the anti-oxidant butylated hydroxytoluene (BHT), during binge ethanol treatment, blocked COX2 induction and neuronal cell death as well as reversing ethanol inhibition of neurogenesis (see Crews *et al.*, 2006a). Furosemide, a diuretic, has been found in some studies to protect against ethanol-induced brain damage and was suggested to act by reducing inflammatory brain swelling (Collins *et al.*, 1998). However, other studies did not find protection by furosemide or the anti-CSF swelling treatment, mannitol (Crews *et al.*, 2004). Eskay's group, in a more thorough study, explained these discrepancies by relating free radical scavenging activity to multiple protective agents including BHT, furosemide and cannabidiol, an anti-oxidant cannabinoid (Hamelink *et al.*, 2005). *In vitro*, we have shown that BHT reduced NF- κ B activation (DNA binding) in HEC slices and reduced ethanol-TNF α , glutamate and/or H₂O₂⁻ stimulated neuronal cell death (Zou and Crews, 2004). We have also tested the hypothesis that ethanol sensitized neurons to insults by decreasing pCREB by experimentally increasing pCREB activation (DNA binding) using phosphodiesterase IV inhibitor rolipram and examining toxicity. Rolipram treatment increased CREB-DNA binding and markedly reduced ethanol-TNF α , glutamate and H₂O₂⁻ stimulated neuronal cell death in HEC slices (Zou and Crews, 2004). Further, rolipram administered with binge ethanol treatment *in vivo* increased pCREB immunohistochemical expression and protected against binge-induced neurodegeneration (Pluzarev *et al.*, 2008). Thus, anti-oxidants that block NF- κ B induction of proinflammatory transcription or block the ethanol reduction in pCREB transcription can inhibit ethanol neurodegeneration. Interestingly, there is crosstalk between these transcription factors since blocking ethanol NF- κ B induction with BHT also blocks the reduction in pCREB by ethanol (Zou and Crews, 2004). Further, adult neurogenesis is inhibited by ethanol and several studies have found that anti-oxidants also blunt ethanol inhibition of neurogenesis (Herrera *et al.*, 2003; Crews *et al.*, 2006a). Thus, inhibitor studies strongly support the ethanol-induced oxidative stress; proinflammatory environment causes alcoholic neurodegeneration.

ETHANOL INHIBITION OF NEUROGENESIS: A MECHANISM OF NEURODEGENERATION

The discovery that alcohol also inhibits the ongoing genesis of neurons and glia has highlighted a new possible mechanism by which alcohol results in brain volume/tissue loss or neurodegeneration. Lack of cell generation may be a key mechanism of neurodegeneration (Nixon, 2006). Indeed, in many neurodegenerative diseases, the lack of ongoing cell generation by stem cells has been hypothesized to contribute to tissue loss (see Armstrong and Barker, 2001). New neurons from neural

stem cells are constitutively produced in at least two regions of the normal, adult brain. Neural stem cells in the subventricular zone of the anterior lateral ventricles produce olfactory bulb neurons (Alvarez-Buylla and Garcia-Verdugo, 2002; Curtis *et al.*, 2007) and neural stem cells (NSCs) or progenitor cells in the subgranular zone of the hippocampal dentate gyrus produce dentate gyrus granule cells (Altman and Das, 1965; Eriksson *et al.*, 1998). Evidence continues to emerge for the role of neural stem cells/adult neurogenesis in neurodegenerative disorders. Early studies on stress showed that increased adrenal steroids both inhibited neurogenesis and resulted in dentate gyrus degeneration (Cameron and Gould, 1994). Studies in a variety of neurodegenerative disorders have shown a direct correlation between inhibition of neurogenesis and dentate gyrus cell or volume loss and most importantly this degeneration often occurs without overt increases in cell death (for review, see Eisch, 2002). Further, by mere inhibition of NSC proliferation/adult neurogenesis, hippocampal-dependent behaviors and functions are impaired (Gould *et al.*, 1999; Nilsson *et al.*, 1999; Shors *et al.*, 2001; Snyder *et al.*, 2001; van Praag *et al.*, 1999a, 1999b), whereas increased neurogenesis improves hippocampus-related behaviors (Santarelli *et al.*, 2003).

Four primary processes comprise the formation of new neurons, or neurogenesis: proliferation, differentiation, migration and survival (Kempermann *et al.*, 2004). Elegant work *in vitro* has shown repeatedly that alcohol alters NSCs, including their ability to form colonies, proliferate, differentiate and survive (Crews *et al.*, 2003; Hao *et al.*, 2003a, 2003b; Tateno *et al.*, 2005), which has been extended to *in vivo* models of alcohol exposure. Most *in vivo* models report that alcohol intoxication inhibits NSC proliferation (e.g. Nixon and Crews, 2002; Jang *et al.*, 2002a, 2002b). The percent inhibition is strikingly similar among studies and exposure patterns with most reporting a 50 \pm 10% decrease in the number of proliferating cells in adults (for review, see Table 1 in Nixon, 2006). Acute alcohol exposure dose dependently reduces NSC proliferation in adolescent rats with high doses completely inhibiting progenitors [see Fig. 1 and Crews *et al.* (2006c)]. Multiple markers covering all stages of neurogenesis have been examined in the 4-day binge model and support the loss of progenitor cell proliferation immediately after the last dose (Nixon and Crews, 2002, 2004; He *et al.*, 2005; Crews *et al.*, 2006a). Several groups have consistently shown that progenitor cell survival is also reduced, which suggests another mechanism by which alcohol reduces neurogenesis in rats (Nixon and Crews, 2002; Herrera *et al.*, 2003; He *et al.*, 2005). Further, ethanol treatment during adult neurogenesis blunts the growth of the progenitor's dendritic arbor (He *et al.*, 2005). Taken together, these studies indicate that ethanol reduces neurogenesis during intoxication contributing to neurodegeneration through loss of cell generation. Intriguingly, inflammatory processes may inhibit neurogenesis (Monje *et al.*, 2003). Thus, ethanol activation of proinflammatory cytokine-oxidative stress cascades likely inhibits neurogenesis as well as mediating the other necrotic degenerative processes (Figs 1 and 4).

GLIA AND ALCOHOLIC NEURODEGENERATION

Neuronal loss may not account for all volume loss across the alcohol-damaged brain (Harper *et al.*, 1987; Harper and Kril,

1989; Kril *et al.*, 1997). Glia may also contribute to alcoholic neurodegeneration. Alcohol causes astroglia to degenerate, leaving a void in trophic and metabolic support, and then neurons degenerate (Kimelberg and Aschner, 1994). With a loss of astroglia comes a reduced ability to take up excess glutamate, buffer K⁺ (ion homeostasis) and eliminate free radicals (e.g. Dringen, 2000). Glia may be more sensitive than neurons to the effects of alcohol (Miguel-Hidalgo *et al.*, 2002). Careful studies in postmortem human hippocampus found a statistically significant loss of 37% of the glial cells in alcoholic hippocampus that included a reduction of astrocytes and oligodendrocytes but no loss of neurons (Korbo, 1999). Studies of astrocyte cultures have found acetaldehyde to be toxic to astrocytes (Holownia *et al.*, 1997, 1999). Long-term chronic alcohol exposure *in vivo* decreases an intermediate neurofilament that is a characteristic of astrocytes, glial fibrillary acidic protein (GFAP), in the cerebellum of male and female rats (Rintala *et al.*, 2001). The loss of GFAP expression suggests a loss of astrocytes (Rintala *et al.*, 2001) consistent with the finding that the number of astrocytes identified by giemsa staining in human hippocampus is reduced in alcoholics (Korbo, 1999; Miguel-Hidalgo and Rajkowska, 2003; Miguel-Hidalgo, 2005; Matsuda-Matsumoto *et al.*, 2007). Alternatively, other studies have reported no effect on glia including stereological studies performed on rat brain tissue immediately after 40 weeks of ethanol-containing liquid diet (Dlugos and Pentney, 2001) and on human alcoholic brains (Fabricius *et al.*, 2007). Findings are complicated by the fact that glia proliferate and/or increase in response to brain damage. The term reactive gliosis refers to damage-induced increases in the proliferation of glia and observable increases in glia markers, coupled with altered morphology and function. Several studies have found that ethanol treatment produced reactive astrocytosis across multiple brain regions as evidenced by increased GFAP immunoreactivity, increased S100 β expression and/or increased glial DNA, all markers of gliosis (Rosengren *et al.*, 1985; Franke *et al.*, 1997; Satriotomo *et al.*, 1999; Baydas and Tuzcu, 2005; Gonca *et al.*, 2005). Thus, the role of glia in alcoholic degeneration and regeneration remains uncertain due to rapidly changing roles and responses.

ABSTINENCE AND BRAIN REGENERATION

Human studies provide substantial evidence that sobriety results in improvement during abstinence (Carlen *et al.*, 1978; Sullivan *et al.*, 2000b). Studies have found improvement in component processes of nonverbal memory, visuospatial abilities, attention, as well as gait and balance within months of sobriety (Sullivan *et al.*, 2000a, 2000b). Longitudinal neuroimaging studies of brain structure find increases in cortical gray matter volume within 1 month of sobriety followed by increases in white matter and reversal of ventricular enlargement with longer abstinence (Pfefferbaum *et al.*, 1995; O'Neill *et al.*, 2001). Very long abstinence has been reported to resolve most neurocognitive deficits associated with alcoholism (Fein *et al.*, 2006). Recent studies have found associations between the recovery of memory and lateral ventricle shrinkage and the recovery of ataxia and fourth ventricle shrinkage (Rosenbloom *et al.*, 2007). Thus in general, studies suggest that recovery of function during abstinence is associated with ultrastructural

changes in brain that include growth of both white and gray matter as well as improved overall function.

In our studies of the binge model of alcohol dependence we found that abstinence results in increased cell proliferation in multiple brain regions (Nixon and Crews, 2004; Nixon *et al.*, 2008; Figs 6 and 7). Cell proliferation increases across multiple brain regions after just 1 day of abstinence (Nixon *et al.*, 2008). After 2 days, cell genesis is increased in cortical regions, including both gray and white matter areas and across many non-cortical brain regions. We found that dentate gyrus proliferation increases from binge ethanol-inhibited levels to control levels after ~1 day of abstinence that is followed by further increases in proliferation several fold more than in controls that persists for more than 1 week. Two bursts of proliferation occur in the first week of abstinence. The first happens around 2 days after the last dose of ethanol and is the most widespread. We have shown that the majority of these cells are proliferating microglia (Nixon *et al.*, 2008), which is a hallmark of microgliosis (Raivich *et al.*, 1999). Interestingly, these new microglia persist for months and migrate to many brain regions. At 7 days after the last dose, a second but regionally specific burst occurs in the dentate gyrus subgranular zone. The burst of proliferation at 7 days in dentate gyrus, as indicated by increased BrdU⁺ cells, results in increased doublecortin expression, a marker of immature neurons, peaking around 14 days of abstinence (Fig. 6). At 5 weeks of abstinence when progenitors have had time to mature into neurons, abstinent animals have more BrdU⁺ cells that have become new dentate gyrus granule cell neurons (Nixon and Crews, 2004). The burst of cell proliferation occurs as the degeneration and fragments of dying neurons clear (Fig. 7). Many brain regions other than the dentate gyrus show increased formation of new cells. Following moderate self-administration, alcohol-preferring rats (P rats) show new cell genesis across the brain including abstinence-induced increases in cell genesis of progenitors in the hippocampus and substantia nigra (He and Crews, 2008). Additional studies are needed to fully understand the mechanisms of increased proliferation and subsequent increase in new progenitors, microglia and neurons during abstinence.

Interestingly, the finding of new progenitors and microglia during abstinence is consistent with recent findings in post-mortem alcoholic brain. Brains of human alcoholics show increased levels of the cytokine MCP1 (CCL2) and increases in brain microglial markers (He and Crews, 2008). Further, studies in animals have reported that chronic ethanol can cause a long-term increase in the density of [³H]PK-11195, a ligand for the peripheral benzodiazepine receptor, that is enriched in microglia (Syapin and Alkana, 1988; Obernier *et al.*, 2002b). Microglia exist in multiple states that can range from phagocytic inflammatory to trophic secretion of growth factors. The 'activated' microglia marker, ED-1, is not expressed in human or rat brain during abstinence. This is supported by the lack of amoeboid-shaped microglia in any of the new abstinence induced microglia (Riikonen *et al.*, 2002; Crews *et al.*, 2006a; Nixon *et al.*, 2008). Although microglial proliferation is a sign of microglial activation, our studies suggest that the abstinence-induced microglia are not activated in a proinflammatory state. Similarly, human alcoholic brain shows increased microglia markers but not signs of increased proinflammatory microglia (He and Crews, 2008). It is possible that the new microglia are

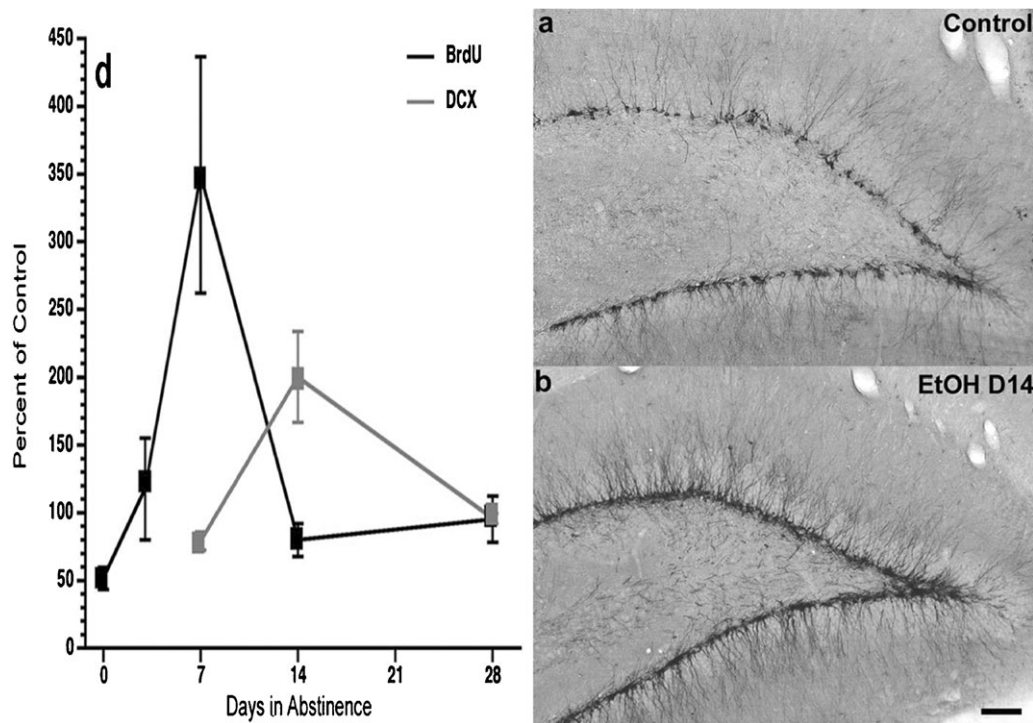


Fig. 6. Bursts of neurogenesis during abstinence from binge ethanol treatment. Left: time course of changes in neurogenesis in hippocampal dentate gyrus during abstinence after the 4-day ethanol binge treatment (Nixon and Crews, 2004). BrdU labels newborn cells providing an index of progenitor proliferation. Each timepoint represents BrdU + immunohistochemistry, 4 h after BrdU dosing. Note the peak in proliferation (BrdU) at 1 week of abstinence. Doublecortin (DCX) is expressed in neural progenitors during differentiation into mature neurons (Brown *et al.*, 2003). Note the peak on DCX expression at 14 days of abstinence. Images of DCX immunohistochemistry in control (a) and 14 days of abstinence after the binge (b) illustrate abstinence-induced neurogenesis. [Adapted from Nixon and Crews (2004).]

trophic microglia that secrete factors that stimulate the brain regeneration during abstinence.

In summary, during the first week of abstinence following a 4-day binge ethanol treatment, there is a burst of brain cell proliferation in many regions of brain, particularly hippocampus and cortex. Proliferating cells labeled with BrdU largely differentiate into microglia in non-neurogenic brain regions. In the dentate gyrus, proliferating cells differentiate over several weeks mostly into neurons. The cell-, microglia- and neurogenesis during abstinence represent a unique and long-term change in brain cell structure that persists for long periods and perhaps permanently.

REGENERATION: MECHANISMS OF ABSTINENCE-INCREASED CELL GENESIS AND BRAIN GROWTH

Regrowth of the brain during abstinence from alcohol likely mimics general mechanisms involved in brain growth and plasticity. Exercise and environmental enrichment are known to protect the brain from neurodegenerative insults, age-related dysfunction and to improve learning ability. Exercise can also block ethanol inhibition of cell proliferation (Crews *et al.*, 2004). One possible mechanism that contributes to increased cell growth during abstinence is the response to cell death, e.g. degeneration stimulated regeneration. Binge-induced degeneration is widespread in cortico-limbic areas; however, postbinge

cell genesis occurs in many more brain regions not previously thought to be damaged by binge ethanol treatment. Studies of cell genesis after brain insults have found cell genesis in regions not directly damaged. For example, entorhinal cortex lesions cause increased cell proliferation 3 days later in the dentate gyrus on both the lesion side and the contralateral hippocampus. This suggests that proliferation occurs in projection areas of the dying neurons as well as in the cell body regions (Hailer *et al.*, 1999). Similarly, focal electrolytic lesions of the dentate gyrus result in increased proliferation on postlesion days 1 and 5 in both the ipsilateral and contralateral dentate gyrus (Ernst and Christie, 2006). The lesion-induced increase in proliferation in the dentate gyrus resulted in more neurons, e.g. increased neurogenesis, at 6 weeks. However, a lesion in the frontal cortex stimulated proliferation, but the cells did not differentiate into neurons (Ernst and Christie, 2006). Some studies have suggested that quiescent progenitors exist across the brain that, when stimulated, differentiate depending upon their surrounding environment, with a special 'neurogenic niche' regulating neurogenesis in the dentate gyrus and subventricular zone where progenitors constantly become neurons (Wurmser *et al.*, 2004). Simple differentiation can stimulate progenitors, as well as NMDA antagonists (Cameron and Gould, 1994). And though differentiation and neurodegeneration occur during alcohol intoxication, cell- and neurogenesis are inhibited during intoxication. Thus, one mechanism of abstinence regeneration may be a response to intoxication-induced cell death that becomes apparent only during abstinence after the alcohol has cleared (Fig. 7).

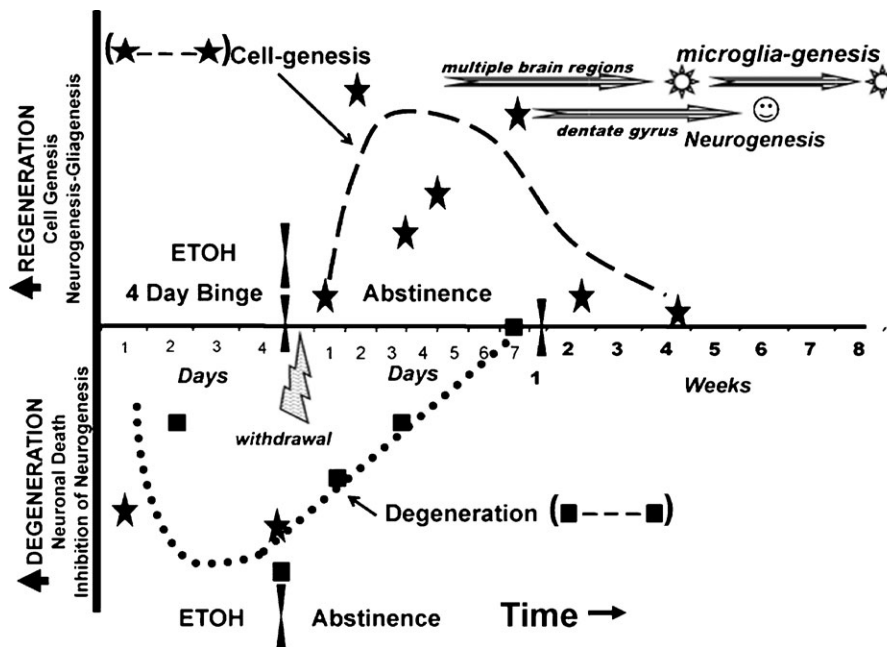


Fig. 7. Temporal relationship of binge ethanol-induced neurodegeneration and abstinence-induced cell genesis. Neurodegeneration is represented as down on the y-axis (solid squares—dotted line). Regeneration is represented as up (cell genesis—solid stars, dashed line). The 4-day binge ethanol treatment has been extensively studied as a model of alcohol dependence and alcohol-induced neurodegeneration. Multiple markers of neuronal cell death, particularly argyrophilic silver stain, have characterized pyramidal dark cell degeneration that shows greater degeneration (down) over the 4-day binge. This neuronal cell death during intoxication corresponds with ethanol inhibition of dentate gyrus neurogenesis (solid stars below midline represent inhibition of neurogenesis). The dotted line represents the course of both markers of degeneration. Symptoms of physical withdrawal occur during the first 24 h (lightning bolt). The bursts of cell genesis (stars above midline) occur in the week following ethanol treatment. After 2 days of abstinence following the binge (T48), a cell genesis burst occurs in multiple brain regions shown to primarily differentiate into microglia at 1 and 2 months of abstinence (upper striped arrow—sun symbol). At a later time, 7 days of abstinence, a hippocampal (T168) cell burst in dentate gyrus results in more new neurons 1 month later (lower striped arrow—smiley face). Studies have shown that cells that survive 1 month tend to persist for long periods in the brain (Kempermann *et al.*, 2004). These studies suggest that abstinence from ethanol has increased new microglia across broad regions of brain.

Another mechanism of abstinence-induced regeneration may be an increase in pCREB transcription that rebounds from suppressed levels during abstinence. A variety of studies have suggested that pCREB transcription increases plasticity and survival of neurons that have been insulted (Walton and Dragunow, 2000; Mabuchi *et al.*, 2001; Hara *et al.*, 2003). Studies have linked increased pCREB formation and transcription to synaptic pro-survival NMDA receptors (Hardingham and Bading, 2002). Stimulation of these synaptic NMDA receptors may occur during the high extracellular glutamate levels characteristic of the alcohol withdrawal hyperexcitable syndrome. This hyperexcitability is associated with increased glutamate release during withdrawal (De Witte *et al.*, 2003) and synaptic glutamate release is associated with increased activation of kinases that increase pCREB formation and drive BDNF synthesis and secretion (Papadia and Hardingham, 2007). This suggests that alcohol withdrawal-induced synaptic glutamate release might activate synaptic NMDA receptors that are trophic. Interestingly, chronic ethanol has been found to increase the sensitivity of NMDA receptors (Chandler *et al.*, 1994), and the dendritic spines and postsynaptic density proteins within excitatory synapses (Carpenter-Hyland *et al.*, 2004; Mulholland *et al.*, 2008). This could increase excitatory synaptic responses stimulating growth. Increased NMDA stimulation will increase neurogenesis (Joo *et al.*, 2007) similar to that found during ethanol abstinence. The binge model of alcohol

dependence shows decreased pCREB histochemistry during intoxication, when NMDA receptors are blunted by ethanol (Fig. 8; Bison and Crews, 2003). Thus, as mentioned earlier, pCREB is reduced during intoxication when degeneration occurs and neurogenesis is inhibited (Fig. 4). Abstinence from ethanol following the binge results in a marked increase in pCREB histochemistry at 3 days of abstinence that is consistent with the cell genesis beginning across brain a few days later (Fig. 8). Thus, it is possible that during abstinence, withdrawal-induced glutamate release stimulates supersensitive synaptic NMDA trophic receptors that increase pCREB transcription resulting in brain growth. The increase in pCREB could increase plasticity, cell growth, cell proliferation and neurogenesis. Thus, another possible mechanism of abstinence-induced increases in neurogenesis, cell genesis, cell growth and plasticity is the increase in synaptic glutamate release and activation of synaptic glutamate receptors during abstinence.

SUMMARY

The binge model of alcohol dependence effectively models the degeneration and regeneration associated with alcohol dependence and recovery as well as alcohol-induced relearning deficits due to perseverative-like behavior, consistent with the loss of executive functions in alcoholism. The binge model

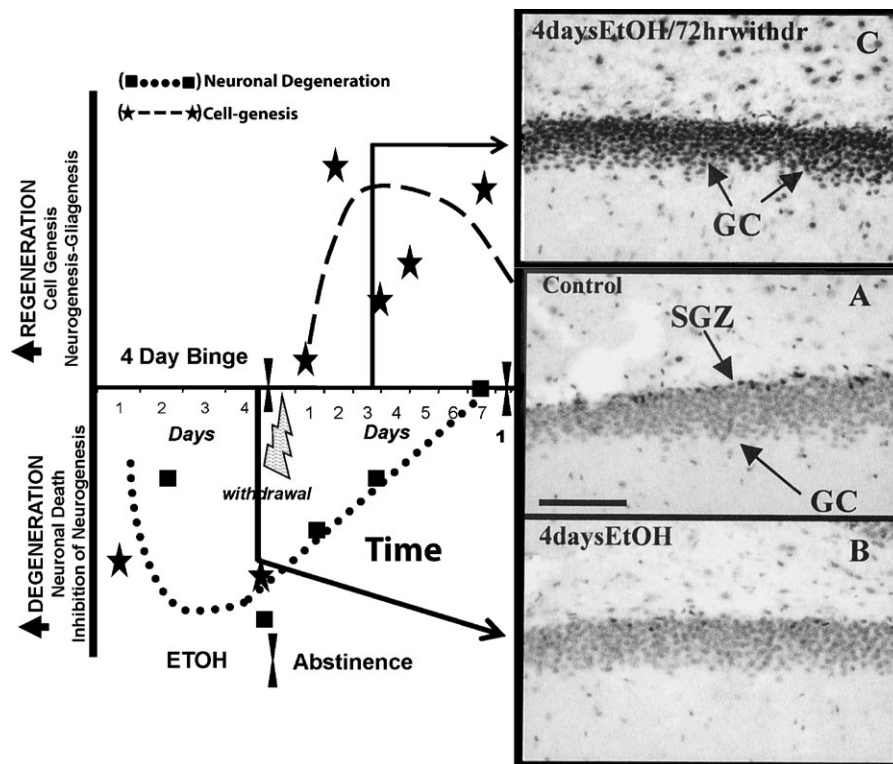


Fig. 8. Regeneration of brain is related to increased CREB transcription, increased neurogenesis and cell genesis. The temporal relationship of binge ethanol-induced neurodegeneration and abstinence-induced cell genesis related to pCREB immunohistochemical staining in dentate gyrus of control, binge ethanol-treated animals (4-day EtOH-T0) and animals at 72 h of abstinence following the 4-day binge (4-day EtOH/72-h withdrawal). Control brain section—middle picture (A) shows pCREB + IR particularly high in the subgranular zone (SGZ) where neurogenesis occurs, but staining is throughout. After 4 days of binge ethanol (4-day EtOH-T0), pCREB staining is decreased when neurogenesis is inhibited and granule cells degenerate. However, after 72 h of abstinence with the first 24 including the excessive excitation of behavioral withdrawal result in a marked increase in pCREB staining (top photo—4-days EtOH/72-h withdrawal) that coincides with or precedes increased cell and neurogenesis and loss of degeneration markers. [pCREB immunohistochemistry adapted from Bison and Crews (2003).]

demonstrates that alcohol-induced degeneration involves brain shrinkage that likely occurs due to multiple factors inhibition ongoing cell generation, cellular atrophy and cell death. The combination of our *in vitro* models followed by *in vivo* experiments have allowed us to show that alcohol shifts the balance of proinflammatory versus pro-survival signaling toward inflammation during ethanol intoxication. Ethanol activation of NF- κ B transcription increases proinflammatory cytokines and oxidative stress inducing enzymes that promote degeneration. Ethanol reductions in pCREB transcription sensitize neurons to insults, including oxidative stress, that likely contribute to the degeneration and loss of neurogenesis. Inhibition of NF- κ B transcription or increased pCREB transcription blocks binge-induced degeneration. Human alcoholics show increased brain cytokines and microglial markers consistent with these processes contributing to the loss of gray and white matter as well as the cognitive dysfunction. Human studies find brain damage associated with the most recent and frequent drinking consistent with the binge studies indicating that ethanol-induced brain damage occurs during intoxication.

Human abstinent alcoholics show a partial reversal of brain cognitive deficits and a regrowth of brain. Similarly, the binge-induced damage clears during abstinence and a burst of new cells signals a regrowth of brain. Cell genesis is found across the brain and results in the formation of new neurons, microglia and progenitors after months of abstinence. Our binge

models also demonstrate that in abstinence, glutamate action on synaptic NMDA receptors shifts the balance of signaling toward survival and growth. Thus, an increase in pCREB transcription was observed and it represents one mechanism of the return of plasticity and increased cell genesis associated with abstinence. Additional studies are needed to fully elucidate the processes that contribute to the degeneration and regeneration associated with dependence and abstinence. It is hoped that understanding these mechanisms will lead to new approaches to block the progression to dependence and improve the success of abstinence.

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