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Upregulated vimentin suggests new areas of neurodegeneration in a model of an alcohol use disorder

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Abstract

Excessive alcohol intake, characteristic of an alcohol use disorder (AUD), results in neurodegeneration as well as cognitive deficits that may recover in abstinence. Neurodegeneration in psychiatric disorders such as AUDs is due to various effects on tissue integrity. Several groups report that alcohol-induced neurodegeneration and recovery include a role for adult neurogenesis. Therefore, the initial purpose of this study was to investigate the effect of alcohol on the temporal profile of neural progenitor cells using the radial glia marker, vimentin, in a model of an AUD. However, striking vimentin expression throughout corticolimbic regions led, instead, to the discovery of a significant gliosis response in this model. Adult male rats were subjected to a four-day binge model of an AUD and brains harvested for immunohistochemistry at 0, 2, 4, 7, 14, and 28 days following the last dose of ethanol. A prominent increase in vimentin immunoreactivity was apparent at 4 and 7 days post-binge that returned to control levels by 14 days in the corticolimbic regions examined. Vimentin-positive cells co-labeled with glial fibrillary acidic protein, which suggested that cells were reactive astrocytes. A second experiment supported that increased vimentin was not primarily due to alcohol withdrawal seizures and is more likely due to alcohol-induced cell death. As this gliosis was remarkably distinct in regions where cell death had not previously been reported in this model, adjacent tissue sections were processed for FluoroJade B staining for cell death. FluoroJade B-positive cells were evident immediately following the last ethanol dose as expected, but were significantly elevated in the hippocampal dentate gyrus and CA3 regions and corticolimbic regions from 2 to 7 days post binge. Intriguingly, vimentin labeling of astrogliosis is more widespread than FluoroJade B labeling of cell death, which suggests that four-day binge ethanol consumption is more damaging than originally realized.

Keywords

Alcoholism; neural stem cell; adult neurogenesis; astrogliosis; ethanol

Excessive intake of alcohol, the hallmark of an alcohol use disorder (AUD), often results in significant behavioral and cognitive impairments (Sullivan and Pfefferbaum, 2005). Although these deficits are attributed to changes in brain structure, surprisingly, only a weak correlation exists between observations of cell loss and behavioral impairment. For example, human imaging studies of alcoholics detail white and grey matter loss in cortical and

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hippocampal regions (Pfefferbaum et al., 1992; Sullivan et al., 1995; Agartz et al., 1999) that has been confirmed in postmortem examinations in some studies (Bengochea and Gonzalo, 1990), but not others (Kril and Harper, 1989; Harding et al., 1997). Animal models of AUDs have been necessary to show the direct relationship between excessive alcohol intake and neurodegeneration (Walker et al., 1980; Cadete-Leite et al., 1988; Collins et al., 1996; Crews et al., 2000; Zahr et al., 2010). In rodents, corticolimbic neurodegeneration is suggested by amino cupric silver staining for degenerating cells with only limited reports using other measures of neuropathology such as electron microscopy (Collins et al., 1996; Crews et al., 2000; Obernier et al., 2002a; b). Intriguingly, little cell death has been reported in the hippocampus in humans with AUDs, despite consistent reports of hippocampal deficits (Pfefferbaum, 1988; Oscar-Berman and Hutner, 1993; Sullivan et al., 1995; Tivis et al., 1995; Pfefferbaum et al., 1997; Harper, 1998; De Bellis et al., 2000; Beresford et al., 2006; Bartels et al., 2007).

This incongruence between alcohol-induced behavioral impairments and structural deficits lead to recent studies on novel mechanisms that contribute to the integrity of the hippocampus, specifically the effect of alcohol on neural stem cells and adult neurogenesis (Nixon and Crews, 2002; Nixon, 2006; Crews and Nixon, 2009; Hansson et al. 2010). Neural stem cells are now considered critical to the structural integrity and function of regions that have constitutive adult neurogenesis (Imayoshi et al., 2008; Zhao et al., 2008). Indeed, alcohol impairment of adult neurogenesis could be a primary means of neurodegeneration for the hippocampus (Nixon and Crews, 2002; Morris et al., 2010a). Conversely, during abstinence after excessive alcohol intake, a compensatory burst in neurogenesis has been speculated to contribute to brain recovery in regions such as the hippocampus (Nixon and Crews, 2004; Crews and Nixon, 2009). The mechanism of how alcohol abstinence increases neurogenesis is not known, which led to investigating the effect of alcohol on neural stem cells by examining a progenitor cell marker, such as vimentin, in a model of an AUD.

Vimentin is a type III intermediate filament found in radial glia, cells which have been described as potential precursors in adult neurogenesis (Dahl et al., 1981; Pixley and de Vellis, 1984; Gotz et al., 2002). During reactive adult neurogenesis in models of brain damage, radial glia become more prominent and are hypothesized to either guide newborn, migrating neurons after the damaging event and/or are the source of newborn cells (Gotz et al., 2002). However, other studies have shown that vimentin is expressed in reactive astrocytes, which by definition involves the upregulation of intermediate filament proteins (Eddleston and Mucke, 1993; Pekny et al., 2007). As excessive alcohol intake in a model of an AUD results in neurodegeneration and has temporally-specific effects on neural precursors leading to reactive neurogenesis in the dentate gyrus (e.g. Nixon and Crews, 2004), we examined vimentin expression at various time points after binge alcohol exposure in adult rats. The striking upregulation of vimentin immunoreactivity during abstinence, however, led us to consider its role in reactive gliosis and compare it to the pattern of cell death in this model using the FluoroJade B stain for neurodegeneration.

EXPERIMENTAL PROCEDURES

Animal model

Ninety-one adult male Sprague-Dawley rats (295–345g; ~70 days of age; Charles River) were used across two experiments. Rats were individually housed and allowed to acclimate for 3–5 days at either the University of North Carolina at Chapel Hill or University of Kentucky vivariums. Rats were maintained on a 12-h light-dark cycle with free access to food and water unless otherwise noted. Animal use strictly followed the NIH Guide for the Care and Use of Laboratory Animals (NRC, 1996) and all protocols were approved by the

respective Institutional Animal Care and Use Committees prior to experimentation. Alcohol exposure followed a four-day binge model modified from Majchrowicz (Majchrowicz, 1975) as previously reported (Nixon and Crews, 2004). This model was chosen because it produces high blood ethanol levels characteristic of binge pattern intake in alcohol abuse or dependence. Either ethanol diet (25% w/v in, Vanilla Ensure®, Abbott Laboratories, Columbus, OH) or an isocaloric control diet was administered by gavage every 8 h for 4 days. An initial 5 g/kg dose was administered with subsequent doses based on the intoxication state of the rat and scored according to a six-point behavioral intoxication scale: 0, normal rat; 1, hypoactive; 2, ataxia; 3, ataxia with dragging abdomen and/ or delayed righting reflex; 4, loss of righting reflex; 5 loss of eye blink reflex (Knapp and Crews, 1999). Each score was associated with a dose of ethanol between 0 and 5 g/kg such that rats received between 7 – 13 g/kg/day averaging 9.4 ± 0.2 g/kg/day. During binge treatment, chow was removed but rats had ad libitum access to water. Eight hours after the last dose of ethanol, chow was returned. At 10 h after the final dose of ethanol, withdrawal behaviors were observed and scored for 30 m of every hour for 15 h. Withdrawal behavior was scored using a scale modified from Majchrowicz (1975) as reported previously (Penland et al., 2001; Nixon and Crews, 2004; Morris et al. 2010b). In the first experiment, rats (31 control, 33 ethanol) were humanely sacrificed at various time points after binge treatment: T0 (0 days after last dose), T2 days, T4 days, T7 days, T14 days, or T28 days.

Diazepam Treatment (Experiment 2)

Because seizures increase both glial fibrillary acid protein (GFAP) and vimentin expression (Stringer, 1996) and ethanol withdrawal may include seizures, we examined the effect of reducing withdrawal severity (seizure-like behaviors) on vimentin immunoreactivity at T7 days following binge ethanol exposure. Twenty-seven rats were binged exactly as described above. In a 2×2 design, ethanol ($n=15$) and control ($n=12$) groups were subdivided into diazepam (DZ) or vehicle (VEH) administration. At 10 h after the last dose of ethanol, rats received diazepam (10 mg/kg, i.p.) or vehicle (2 drops of tween-20, q.s. to 10 ml w/ saline) respectively every 8 h for 24 h. The dose of diazepam was based on previous studies where 10 mg/kg diazepam reduced the majority of overt withdrawal behaviors, and especially seizure-like behaviors, in this model (Mhatre et al., 2001). To best test this hypothesis, only rats with no observed convulsions in the ethanol+DZ group were used for immunohistochemical analysis. Observations for withdrawal behavior were taken by blinded observers for 30 m of every hour between 10 and 26 h after the last dose as described above. All rats in this experiment were then sacrificed 7 days later.

Blood Ethanol Concentrations

In all ethanol rats, blood ethanol concentrations (BECs) were determined from serum derived from tail blood. On the second day of the binge, 90 m after the afternoon dose (expected peak), tails were nicked and blood was drawn into heparinized microcentrifuge tubes. Samples were centrifuged and then stored at -20°C until analysis. Serum was extracted, and BECs were determined by an Analox Alcohol Analyser (London, UK) based on an external 300 mg/dL standard.

Tissue preparation

At the various timepoints following binge treatment, rats were sacrificed via anesthetic overdose (Nemubutal® 100 mg/kg i.p.) followed by transcardial perfusion with 0.1M phosphate buffered saline (PBS; pH 7.4) and then 4% paraformaldehyde as described (Knapp and Crews, 1999). Brain tissue was extracted then postfixed overnight in 4% paraformaldehyde. Following rinses in PBS, brains were stored in PBS at 4°C until sectioning. A vibrating microtome (Leica VT1000S) was used to cut 12 series of sections at a 40 μm thickness beginning around Bregma 2.4 (random start point) through Bregma -7.0 .

This unbiased collection procedure results in 12 sets of adjacent sections where sections in a well are 480 μm apart for the entire rostral to caudal extent of the hippocampus. Sections were stored in cryoprotectant in 24-well plates at -20°C until processing.

Immunohistochemistry

For 3,3'-diaminobenzidine tetrahydrochloride (DAB) immunohistochemistry, every 12th section was processed in a free-floating procedure at room temperature unless otherwise stated. The specificity and optimal dilution of all antibodies were determined in control experiments, including the elimination of primary and/or secondary antibodies. Vimentin immunohistochemistry was performed in batches by time point. Sections were rinsed in tris-buffered saline (TBS) followed by 20 minutes in 1% H_2O_2 to block endogenous peroxidases, rinsed twice in TBS and then blocked in blocking buffer (10% horse serum / 0.1% Triton-X/TBS) for 30 minutes. Sections were incubated for 24 h at 4°C in mouse anti-vimentin (1:750, Chemicon, Temecula, CA). Following washes in blocking buffer, sections were incubated for 1 h in horse, anti-mouse biotinylated secondary antibody (Vector Laboratories Inc, Burlingame, CA) and 1.5% horse serum. With 3×10 m washes in TBS between each step, sections were incubated in a preformed avidin and biotinylated horseradish peroxidase macromolecular complex for 1 h (ABC; Vector Laboratories, Burlingame, CA), which was then detected using DAB (Polysciences, Warrington, PA) as a chromagen. Sections were rinsed, mounted to glass slides, dried overnight and coverslipped in Cytoseal[®] (Stephens Scientific, Wayne, NJ).

For multiple fluorescent immunohistochemistry, an adjacent series (every 12th section) was rinsed in TBS then blocked (10% goat serum/0.1% Triton-X/TBS) for 30 m. Sections were incubated overnight at 4°C in mouse anti-vimentin (1:400, Chemicon, Temecula, CA) and rabbit anti-GFAP (1:2500, DAKO, Glostrup, Denmark) or rabbit anti-Iba1 (1:400, Wako, Richmond, VA). Following washes in blocking buffer, sections were incubated in fluorescent coupled secondary antibodies, Alexa-Fluor 488 anti-mouse and Alexa-Fluor 568 anti-rabbit. Sections were rinsed in TBS, mounted to glass slides, dried and coverslipped in ProLong[®] antifade mounting medium (Invitrogen, San Diego, CA). All steps from the secondary forward were carried out in an opaque, dark box to prevent photobleaching. Vimentin-positive (vimentin+) cells were assessed for colabeling with GFAP on a laser scanning confocal microscope (Leica TCS SP5; Wetzlar, Germany) with a 63x oil lens. Z plane image stacks (1024×1024 pixels) were taken at $< 1.0 \mu\text{m}$ for each cell analyzed and reconstructed, viewed and analyzed for colabeling within the Leica software similar to that previously described (Nixon et al., 2008; Morris et al., 2010a).

FluoroJade B labeling

FluoroJade B (FJB) staining was conducted following (Schmued and Hopkins, 2000) as published previously (Leasure and Nixon, 2010). In brief, an adjacent series of sections (every 12th section) was mounted to Superfrost Plus[®] slides (Fisher Scientific, Waltham, MA), dried overnight, processed for FJB (Chemicon, Temecula CA), dried and then coverslipped in Cytoseal[®] (Stephens Scientific, Wayne, NJ). All steps from FJB forward were carried out in an opaque, dark box.

Quantification

This manuscript focused on the hippocampus and an associated corticolimbic pathway that is damaged in this model (Collins et al., 1996; Obernier et al., 2002b). Thus, only the hippocampus and limbic cortex with visibly distinct DAB-labeled vimentin immunoreactivity were quantified. Due to the considerable number immunopositive cells, a densitometric approach was chosen to quantify immunoreactivity. We have shown that carefully conducted densitometric approaches produce identical results (percent change) as

stereology or profile counting methodologies (Crews et al., 2004). An image analysis system (Visiopharm, Hørsholm, Denmark) coupled to a BX-51 Olympus Microscope and DP-70 camera (Olympus; Center Valley, PA) collected images at 100x, a region of interest drawn around hippocampal or cortical regions according to Paxinos and Watson (Paxinos and Watson, 2004), and the percent area of staining was obtained. Percent area was converted to and reported as pixels/mm².

FJB-positive (FJB+) cells were counted at 20X on an Olympus BX-51 microscope with epifluorescence (488λ cube for blue light excitation) using a profile counting methodology identical to previous reports (Leasure and Nixon, 2010). A profile counting method was chosen over stereology because of (a) difficulty in accurately detecting section thickness in FJB-stained tissues (b) the few profiles to count and (c) our previous report that profile counting methods result in identical percent change between control and experimental groups (Crews et al., 2004). FJB+ cells are reported as mean number of cells per section ± standard error of the mean (SEM).

Statistical Analyses

All behavioral scores were analyzed by Kruskal-Wallis or repeated measures ANOVA as appropriate. BEC were analyzed via one-way ANOVA. Because vimentin immunohistochemistry was performed in batches, the density of vimentin immunoreactivity in controls varied slightly, but not significantly. Therefore, vimentin data were analyzed by factorial ANOVA followed by Bonferroni posthoc tests. Correlations between continuous (IR) and noncontinuous (behavior) data were conducted using a Spearman rank correlation coefficient. FJB counts in all regions of controls were identical and therefore collapsed across time points and analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison post test. Data were assembled and then analyzed in Excel (Microsoft Office 2004 for MAC, 2007 for PC) or GraphPad Prism 5.0. All data are reported as mean ± SEM with $p < 0.05$ considered a significant difference.

RESULTS

Animal Model data - Experiment 1

All groups (time points) of ethanol-exposed rats were similarly intoxicated (see Table 1). Grand mean intoxication behavior score was 2.0 ± 0.1 , defined as significant ataxia, which resulted in a mean dose per day of 9.4 ± 0.2 g/kg/day of ethanol for all rats in this experiment (individual groups shown in Table 1). This dose is similar to that observed in past studies (Nixon and Crews, 2004; Nixon et al., 2008) and, notably, is similar to voluntary intake in chronic exposure studies (Bell et al., 2009). Accordingly, the mean blood ethanol concentration in this experiment averaged 310.1 ± 12.5 mg/dl across all rats. Rats that experienced withdrawal (all except T0 which were still intoxicated upon sacrifice) were also similar in severity. Mean withdrawal (the mean of all withdrawal scores assigned) was 1.7 ± 0.1 whereas peak withdrawal (the highest withdrawal score assigned) was 3.4 ± 0.1 .

Vimentin immunoreactivity in the hippocampus and cortex after binge alcohol exposure

In controls, vimentin immunoreactivity was faintly observable in what appeared to be blood vessels across most regions, which is consistent with its constitutive expression in the endothelial cells of blood vessels (Baldwin and Scheff, 1996). A few distinct progenitor-like cells in the subgranular zone of the dentate gyrus were observed and the subventricular zone displayed prominent labeling as expected for a pool of progenitors (arrows, Figure 1A; Figure 2A). As vimentin labels radial glia, which may be progenitors in these regions, these cells were expected in controls (Schnitzer et al., 1981; Gotz et al., 2002). In ethanol-treated brains at multiple time points, vimentin immunoreactivity was visually distinct and

strikingly upregulated, especially in hippocampus (Figure 2B–D). Higher magnification images in Figures 1E and 1F show the star-like shape of these vimentin+ cells across hippocampal regions. Furthermore, because vimentin immunoreactivity was visually observable across many regions of cortex in ethanol treated rats, only corticolimbic regions were selected for analysis in this manuscript (cortical regions outlined in Fig 1).

Hippocampus—Densitometric assessment of vimentin immunoreactivity was taken in the CA1 field, the CA2/3 fields, the hilus, molecular layers (ML) and the granule cell layer (GCL) of the dentate gyrus (Figure 2). As immunohistochemistry was conducted in batches by timepoint, we first assessed whether control values were similar between timepoints by ANOVA. There were no significant differences between control groups in any region; therefore we analyzed the data from each region by two-way ANOVA with the independent variables of treatment (control v. ethanol) and time point (time of sacrifice after binge treatment). Two-way ANOVAs indicated a significant interaction between treatment x time point in the CA1 field [$F(5,46)$ 3.39, $p=0.01$], hilus [$F(5,46)$ 4.93, $p=0.001$], GCL [$F(5,46)$ 15.64, $p<0.0001$], and ML [$F(5,46)$ 13.55, $p<0.0001$]. Bonferroni posthoc analyses further revealed that vimentin immunoreactivity in ethanol rats did not differ from controls during the first 48 hours following the last EtOH exposure in any hippocampal region. However, at 4 days there was a significant increase in vimentin immunoreactivity in the CA1, hilus, GCL, and ML relative to controls that remained significantly elevated through 7 days after the last dose. By 14 days and 28 days, vimentin immunoreactivity returned to near control levels as controls and ethanol rats did not differ from each other at these time points. However, it is of note that individual vimentin stained cells are observable, but weakly immunoreactive at these latter two time points. In the CA3 region, two-way ANOVA revealed a main effect of treatment [$F(1, 46)$ 4.500, $p=0.04$] that reflected a slight, but significant increase in vimentin immunoreactivity at 4 days after the last dose of ethanol only. Also of note is the variability in vimentin immunoreactivity in this model. This variability was not unexpected and is similar to that observed in cell death measures using multiple approaches (e.g. Collins et al., 1996; Crews et al., 2000; Leasure and Nixon, 2010) and despite much less variability in dose/day and intoxication behavior.

Cortex—Four regions of limbic cortex within Bregma 2.4 to –7.0 that is typically damaged in this model had visually distinct vimentin immunoreactivity in ethanol treated subjects and were therefore analyzed with densitometry (Figure 3): rostral piriform (rPir), caudal piriform (cPir), agranular-insular cortices (AI), and perirhinal and entorhinal cortex combined (Peri/EntoR). Similar to hippocampal regions, control values were similar across timepoints and therefore data were analyzed by factorial two-way ANOVA. Two-way ANOVAs conducted on each region showed a significant treatment x time point interaction for all four regions, the rostral piriform [$F(5,44)$ 4.701, $p=0.002$], the caudal piriform [$F(5,45)$ 8.360, $p<0.0001$], the agranular insular [$F(5,45)$ 5.718, $p=0.0004$], and the perirhinal-entorhinal cortices [$F(5,45)$ = 6.600, $p<0.0001$]. Similar to the timecourse of staining observed in the hippocampus, there was little evidence of vimentin immunoreactivity in any cortical region in the first 2 days following the last dose of ethanol as revealed by Bonferroni post hoc tests. At 4 days, vimentin immunoreactivity was significantly higher in ethanol treated rats in each cortical region analyzed, which was maintained throughout 7 days and similarly returned to control levels at 14 and 28 days (Figure 3A – D).

No change in vimentin immunoreactivity after diazepam reduced seizure severity

Withdrawal severity, namely seizure-like behavior, has been shown to impact vimentin expression (Khurgel et al., 1992). Therefore, the correlation between withdrawal severity according to the scale of Penland et al. (2001) and vimentin immunoreactivity on Day 7 was examined. Significant correlations were observed for mean withdrawal score (Spearman $r =$

0.80, $p=0.01$; Figure 4) and peak withdrawal score (Spearman $r = 0.70$, $p=0.04$) with vimentin immunoreactivity at day 7.

Because of the significant correlation between withdrawal severity and vimentin expression, a separate study was conducted where diazepam was used to depress seizure-like behavior and vimentin immunoreactivity was examined. Following the standard 4-day binge procedure, diazepam (10 mg/kg) or vehicle was administered every 8 h for 24 h and withdrawal behavior was scored. Intoxication parameters were similar between the ethanol +VEH group ($BEC = 303.8 \pm 60.1$ mg/dl; mean intoxication score = 1.7 ± 0.3) and ethanol +DZ group ($BEC = 311.9 \pm 30.0$ mg/dl; mean intoxication score = 1.6 ± 0.2). In rats where diazepam administration reduced overt withdrawal behavior (Figure 4), diazepam did not significantly reduce vimentin immunoreactivity. A main effect of diet type (control vs. ethanol) was evident [$F(1,23) 11.41$, $p=0.0026$] but the interaction was not significant.

Vimentin colabels with astroglial marker GFAP

Although the original intent of the experiment was to examine the number of progenitor cells after binge ethanol exposure using a progenitor cell marker, the vast vimentin immunoreactivity in non-neurogenic regions lead us to consider that vimentin expression may be in reactive astrocytes. Therefore, to confirm the type of cell where vimentin was being upregulated (Graeber et al., 1988), we examined the co-expression of vimentin and GFAP, an accepted marker of astroglia, by dual fluorescent immunohistochemistry. As shown in representative images from a 7-day ethanol-exposed brain in Figure 5, vimentin colabels with GFAP in a majority of cells, suggesting that the majority of these cells are reactive astrocytes. Conversely, no colabeling of vimentin and Iba-1, a calcium binding protein expressed in microglia, was observed in the cells sampled.

FluoroJade B timecourse

Reactive astrocytosis is a hallmark of degeneration and the striking upregulation of vimentin immunoreactivity observed suggests that significant degeneration is occurring throughout the hippocampus and not just in the ventral dentate gyrus as several studies consistently report (Obernier et al., 2002a; b). Past studies had primarily employed the de Olmos method of amino-cupric silver stain to detect degenerating cells; therefore we sought to examine the faster, simpler and more consistent FJB in a timecourse following binge ethanol exposure. FJB was performed on sections adjacent to that used for vimentin immunohistochemistry and regions with visible FJB staining were quantified, and included hippocampal (Figure 6) and cortical regions within Bregma 2.4 and -7.0 (Figure 7). Controls were practically zero (<0.1 cell/section; data not shown) similar to that reported previously (Leasure & Nixon, 2010; Morris et al., 2010a) and were identical across all timepoints. Therefore, controls were collapsed and the timecourse analyzed via one-way ANOVA followed by Dunnett's Multiple Comparison post test. In the hippocampus, FJB+ cells were observed in the CA3 and dentate gyrus. Although only an occasional FJB+ cell was observed in CA3 sections, ANOVA revealed that the increase was significant [$F(5,54) 18.70$, $p<0.0001$] at 2, 4, and 7 days after binge exposure ($p<0.05$, Dunnett's post test; Figure 6). ANOVA revealed a significant increase in the number of FJB+ cells in the dentate gyrus [$F(5,54) 5.271$, $p=0.0006$] at 2 and 7 days after exposure ($p<0.05$ Dunnett's post test; Figure 6). In both hippocampal regions, FJB+ cells were similar to controls by 14 days post binge exposure. In the cortex, FJB+ cells were observed and quantified in the rostral and caudal piriform cortex, the agranular-insular cortex and the perirhinal/entorhinal cortex (Figure 7). Similar to the hippocampus, control values for all cortical regions were identical (near zero, $p>0.05$) and therefore combined for analysis by one-way ANOVA followed by Dunnett's post test. In the rostral piriform, FJB+ cells were significantly increased only at T0, or immediately following ethanol treatment [$F(5,41) 4.400$, $p=0.0031$; $p<0.05$ Dunnett's post test]. In the

caudal piriform, FJB+ cells remained significantly elevated at 0, 2, and 4 days after ethanol exposure [$F(5,54) 5.602$, $p=0.0004$; $p<0.05$ Dunnett's post test]. Further, ANOVA revealed that FJB+ cells were significantly higher than controls through day 7 of abstinence in the agranular-insular cortex [$F(5,54) 9.679$, $p<0.0001$; $p<0.05$, Dunnett's post test] and perientorhinal cortices [$F(5,54) 11.17$, $p<0.0001$; $p<0.05$, Dunnett's post test].

DISCUSSION

This is the first study to examine vimentin expression in a model of an AUD. The dramatic upregulation of vimentin in corticolimbic regions during abstinence (Figures 2 and 3) coupled with the observation that the majority of vimentin+ cells colabel with GFAP but not Iba-1 (Figure 5) supports that reactive astrogliosis results from four-day binge alcohol exposure. Reactive astrogliosis has been shown to occur after many weeks of alcohol exposure (Franke, 1995; Tagliaferro et al., 2002; Baydas and Tuzcu, 2005; Evrard et al., 2006; Dalcik et al., 2009; Udomuksorn et al., 2011), but to our knowledge not after such a short duration of exposure. The upregulation of vimentin occurs maximally between 4 and 7 days after the last dose of ethanol. Although in most cases, vimentin immunoreactivity statistically returns to control levels by 14 days, faint vimentin+ cells remain visible up to 30 days that were not captured by our densitometry measures.

Upregulation of intermediate filaments such as vimentin is a hallmark of astrogliosis and a well-accepted indicator of structural damage in the central nervous system (Pekny and Nilsson, 2005; Sofroniew and Vinters, 2010). Indeed, vimentin has been used as a sensitive marker of reactive astrocytes in a variety of brain injury models including traumatic brain injury (Baldwin and Scheff, 1996; Moon et al., 2004), seizure (Stringer, 1996; Nakagawa et al., 2004), ischemia (Schmidt-Kastner et al., 1990), neurotoxins (Andersson et al., 1994) and according to these data, alcohol-induced brain damage. Because vimentin expression is consistently described in immature astrocytes, radial glia, and ependymal cells (Schnitzer et al., 1981), we examined the type of cell expressing vimentin in this model by dual fluorescent immunohistochemistry. Cells with high levels of vimentin in these corticolimbic regions at the Day 7 time point are also labeled by GFAP and therefore, are most likely reactive astrocytes. These data suggest that visibly distinct upregulation of intermediate filament proteins in astrocytes occurs at 4 days after the last dose of alcohol which is notably similar to the expression patterns in TBI (Baldwin and Scheff, 1996; Moon et al., 2004). Together, these data suggest that gliotic events have similar timecourses across various types of injury (Sofroniew, 2009; Sofroniew and Vinters, 2010). Although vimentin immunoreactivity at Day 7 correlated to the severity of withdrawal behavior (Figure 4), upregulation of vimentin immunoreactivity continues in the dentate gyrus despite suppression of convulsions. Furthermore, in rats with low or absent withdrawal behaviors, vimentin immunoreactivity was distinctly higher than controls. These data support that vimentin upregulation is due to cell death rather than ethanol withdrawal-induced seizures.

Vimentin upregulation is thought to be indicative of permanent damage (Petito et al., 1990) and a more sensitive indicator of necrosis than GFAP upregulation (Schmidt-Kastner et al., 1990). Accordingly, following binge ethanol exposure, vimentin is upregulated in many of the same regions that show neurodegeneration according to de Olmos cupric silver stain (Collins et al., 1996; Crews et al., 2000; Obernier et al., 2002a; b). However, vimentin was also dramatically increased in hippocampal regions such as the CA fields and hilus where cell death has not been observed or reported in this model (e.g. Collins et al., 1996; Crews et al., 2000; Obernier et al., 2002a; b). Therefore, we examined a timecourse of FJB labeling of neuronal degeneration as it is considered a more reliable indicator of cell death and has not been fully described following binge ethanol exposure (Schmued et al., 1997; Schmued and Hopkins, 2000; Ishida et al., 2004). Consistent with previous reports of binge-induced

neurodegeneration according to silver stain methods (Collins et al., 1996; Obernier et al., 2002a), little to no FJB was evident in hippocampal CA fields, the hilus, or molecular layers of the hippocampus. Only the dentate gyrus granule cell layer had a large number of FJB+ cells and merely an occasional dead cell was observed in the CA3 region (Collins et al., 1996; Figure 6). As persistent vimentin immunoreactivity is observed in regions with neuronal necrosis in other brain injury models (Schmidt-Kastner et al., 1990) and this ethanol exposure model produces necrotic cell death (Obernier et al., 2002a), the major finding of this study is that vimentin immunoreactivity may be a more sensitive indicator of alcohol-induced damage. Altogether, the FJB and vimentin results indicate that a four day binge results in damage that is more pervasive than previously reported or considered.

A second major finding of this study is that in the regions where cell death occurs in this model, degeneration continues across the first week of abstinence. This finding contrasts with the gradual decline in amino cupric silver staining of neurodegeneration reported by others (e.g. (Collins et al., 1996; Crews et al., 2000)). The pattern of FJB in this model indicates that cells begin to die while the animal is still intoxicated (e.g. T0) and continues through withdrawal and abstinence. This timeline of cell death throughout the first week is not surprising if silver stain only labels an “early histopathological state” of degenerating cells when some labeled cells potentially recover from the insult (Ishida et al., 2004). Perhaps it takes cells time to progress from silver stain positive “early degeneration” to an FJB+ “definitely dying” state (Ishida et al., 2004; Poirier et al., 2000; Schmued et al., 1997).

Potential role of vimentin+ cells in adult brain

These data contribute to the growing body of work that aims to elucidate the neuropathological events in models of alcohol use disorders (see Fig 8 for summary). Although gliotic events have a fairly consistent timecourse across models of brain injury, astrogliosis is a graded response and can be as varied as the conditions that produce it (Sofroniew and Vinters, 2010; Sofroniew, 2005, 2009). Originally, reactive astrogliosis was thought to contribute additional pathology through the release of pro-inflammatory cytokines and reactive oxygen species and the formation of glial scars (Sofroniew and Vinters, 2010). However, recent work has shown that some reactive astroglia have beneficial effects. For example, the upregulation of intermediate filaments may contribute to repair by inducing activated cells to migrate and form the scar as a protective barrier against infiltration of inflammatory cells (Bush et al., 1999; Moon et al., 2004). However, only a moderate reactive astrogliosis appears to occur in this model of an AUD. Although intermediate filaments upregulate, astrocytes do not proliferate in this model (Nixon and Crews, 2004; Nixon et al., 2008) and the pattern of vimentin immunoreactivity does not indicate the formation of glial scars around areas of damage. Although the consequences of moderate reactive astrogliosis are not well defined, its observed resolution predicts promotion of recovery (Figure 2, 3). This idea is consistent with the variety of deficits that recover with abstinence, though the mechanism of recovery is not known (Sullivan and Pfefferbaum, 2005; Zahr et al. 2010). Moderately reactive astrocytes could promote neuroprotection by taking up excessive glutamate produced during alcohol withdrawal (Dahchour and De Witte, 1999), producing glutathione in response to alcohol-induced oxidative stress (Nordmann et al., 1990), and/or limiting alcohol-induced inflammation (Qin et al., 2008). In the least, reactive astrocytes are reflecting significant perturbation, if not injury, to the brain by binge ethanol exposure. Furthermore, their recently described graded activation highlights potential mechanisms of repair and recovery that may occur in abstinence (see Sofroniew and Vinters, 2010, for review).

Several groups suggest that reactive astrocytes may be a source of multipotent progenitor cells in an injured brain (Costa et al. 2010; Buffo et al., 2008). In the adult, a GFAP-positive (+)/vimentin+ cell is considered the putative stem cell (Doetsch et al., 1999; Encinas et al.,

2006). Thus, the reemergence of GFAP+/vimentin+ cells in adulthood during damage and astrocyte activation has been interpreted as astrocytes *dedifferentiating* into stem cells (Buffo et al., 2008). However, whether the observed reactive astrocytes dedifferentiate and function as a stem cell is not well supported *in vivo*. Only *in vitro* are isolated reactive astrocytes able to form neurospheres and demonstrate self-renewal and multipotentiality; most reactive astrocytes *in vivo* are lineage restricted due to strictly non-neurogenic microenvironments (Costa et al., 2010). These findings raise the intriguing question of the role of reactive astrocytes in the neurogenic niche of the hippocampal subgranular zone, a region where vimentin immunoreactivity was remarkably increased (note inset of Figure 2C). Currently, it seems more plausible that reactive astrocytes impact neurogenesis through their influence on the neurogenic microenvironment rather than as stem cells themselves, as astrocytes play a critical role in regulating the neurogenic niche (Doetsch et al., 1999; Seri et al., 2001). Indeed, cytokine-activated astroglia may promote neurogenesis (Song et al., 2002; Liberto et al., 2004) and the expression of vimentin in astrocytes is correlated to proliferation of local progenitor cells in neurogenic regions (Alonso, 2001). Based on the timeline of neuronal and glial events that occur following binge alcohol exposure (Figure 8), vimentin+ astrocytes could induce the reactive burst in neurogenesis observed 1 week into abstinence (Nixon and Crews, 2004). In addition, consistent with other models of brain injury, if T0 is the peak of cell death or injury, microglia would become activated first (Akiyama et al., 1994; Nimmerjahn et al., 2005) and produce proinflammatory cytokines, which in turn activate astrocytes (Jensen et al., 1994; Turner et al., 1998). Low-grade microglial activation in this model appears to occur as early as T0 (Marshall et al. 2010; unpublished data) followed by the hallmark of microglia reactivity, microglia proliferation, at T2 days (Nixon et al., 2008; McClain et al., 2011). Thus, alcohol-induced vimentin+ reactive astrocytes in the dentate gyrus in the least, may reflect an increase in the number of proliferating progenitors, or at the most, may be responsible for binge-induced reactive neurogenesis via cytokine or growth factor secretion (Liberto et al., 2004). Of course, these potential roles should be and will be examined in future studies.

In summary, vimentin upregulation revealed a greater extent and timecourse of cortico-limbic neurodegeneration than previously reported or considered in the binge model of an AUD. Importantly, this work and others are showing that relatively short windows of intoxication can have significant effects on brain structure. As several groups propose that neurodegeneration in corticolimbic regions could underlie or contribute to the development of an AUD (Crews and Nixon, 2009), and that drug effects on adult neurogenesis contribute to the development or maintenance of addiction (Noonan et al., 2010), understanding the mechanism of alcohol-induced neurodegeneration and the contribution of neural progenitors is essential for the development of better treatment strategies to prevent progression to an AUD.

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Highlights

- Striking vimentin expression indicates astrogliosis in an alcohol use disorder model.
- Gliosis was observed in regions where cell death has not been observed in this model.
- Astrogliosis underlies scar formation to recovery to influencing adult neurogenesis.
- Binge ethanol exposure may be more damaging than previously realized.

Figure 1. Cortical regions with visually distinct Vimentin expression

Besides the entire rostral to caudal extent of the hippocampus, cortical regions were analyzed for vimentin immunoreactivity as outlined in black (left; Paxinos and Watson, 2004): (A) agranular-insular cortices, rostral piriform, (B) agranular-insular cortices, caudal piriform and (C) perirhinal/entorhinal cortices. Representative low magnification image of binge ethanol-exposed brain at T4 days (right) highlights the vast vimentin expression across numerous, additional cortical regions (*) that will be reported on in a separate manuscript. The arrows highlight subventricular zone vimentin immunoreactivity that is apparent in both controls and ethanol-exposed.

Figure 2. Vimentin is strikingly upregulated in the hippocampus at T4 and T7 days

Representative photomicrographs are shown for (A) controls (Con), and rats exposed to 4-day binge ethanol (EtOH) at (B) T2 days, (C) T4 days, and (D) T7 days. Vimentin+ progenitor cells are apparent along the dentate gyrus subgranular zone as expected in control rats (arrow; A). Higher magnification images of T7 are shown for (E) the CA3 and (F) a cross section of the CA1 to molecular layer (ML). Quantification of Vimentin immunoreactivity for each region (regions shown in A) is shown for (G) Cornu Ammonis (CA)1/2 (CA1/CA2), (H) CA3, (I) dentate gyrus granule cell layer (GCL), (J) hilus, and (K) molecular layer (ML). Scale bar in A = 250 μ m; E, F = 100 μ m. * p <0.05 post hoc test for control versus ethanol at that timepoint.

Figure 3. Vimentin is upregulated in corticolimbic regions

Results from densitometry measures (left side) are shown for (A) rostral piriform, (B) caudal piriform, (C) agranular-insular cortices, and (D) perirhinal/entorhinal cortices.

Representative photomicrographs are shown for agranular-insular and caudal piriform (E – H) for (E) control, (F) T2 days, (G) T4 days, and (H) T7 days and shown for entorhinal-perirhinal cortices (I – L) for (I) control, (J) T2 days, (K) T4 days, and (L) T7 days. Scale bars in G, I = 100 μ m, inset scale bar = 25 μ m. * p <0.05 post hoc test for control versus ethanol at that timepoint.

Figure 4. Although vimentin immunoreactivity correlates with seizure severity, it remains significantly elevated in rats that were not observed to seize
 (A) Because mean withdrawal severity score correlated with vimentin expression ($r=0.80$, $p<0.05$), we examined vimentin immunoreactivity when seizure-like behaviors (convulsions) were reduced by diazepam (DZ). (B) Vimentin immunoreactivity remained elevated in all ethanol treated groups reflected by a main effect of diet type ($*p<0.05$) and no significant interaction.

Figure 5. Vimentin is primarily upregulated in GFAP-labeled astrocytes

Vimentin is expressed in the majority of GFAP labeled astrocytes throughout the hippocampus and corticolimbic regions. Representative dual immunofluorescent labeling of vimentin (A, D), GFAP (B, E) and merged (C, F) is shown in the hippocampal dentate gyrus region (A – C) and a single cell (D – F). Vimentin (G) did not colabel with the microglia-specific marker Iba1 (H) as shown in the merged image (I). GCL = granule cell layer. Scale bar in C = 100 μ m; in F, I = 10 μ m.

Figure 6. Cell death, indicted by FJB, continues for a week in to abstinence in the hippocampus
 In the hippocampus, FJB+ cells were observed only in the CA3 and dentate gyrus, quantified in A (controls were <0.1 and are not shown). Representative fluorescent photomicrographs show the limited number of FJB+ cells in the CA3 (B – D) from (B) controls, (C) T4 days and (D) T7 days. Significant numbers of FJB+ cells were apparent in the dentate gyrus (DG; E – G) region following binge ethanol exposure as shown for (E) controls, (F) T4 days and (G) T7 days. GCL = granule cell layer. Scale bars = 100µm; inset = 10µm. *p<0.05 different from controls.

Figure 7. Cell death, indicted by FJB, continues for a week in to abstinence in corticolimbic regions

In cortex, FJB+ cells were observed and reported for four regions (A). Representative fluorescent photomicrographs show FJB+ cells in the four cortical regions: rostral piriform (rPir; B – D), caudal piriform (cPir; E – G), agranular-insular cortices (AI; H – J) and perirhinal-entorhinal cortices (Per/EntoR; K – M). As can be seen in the top row of images (B, E, H, K), FJB+ cells in control groups were essentially zero and therefore collapsed in the analysis (data not shown). The discovery of continued FJB labeling of degenerating cells is shown for T 4 (middle row: C, F, I, L) and T7 (bottom row: D, G, J, M). Insets highlight individually stained cells. Scale bar in D = 100µm; inset = 10µm. *p<0.05 different from controls.

Figure 8. Neuropathological events in the 4 day binge model of an AUD

Cells begin to degenerate or die as early as 2 days of exposure with a potential peak of cell death occurring on the 4th day of exposure (Obernier et al., 2002a). As shown with FJB, cells continue to die throughout the first week of abstinence and potentially result in a decrease in brain mass (solid dark line; Zahr et al. 2010). The various glial events, including microglia proliferation (dotted line; Nixon et al., 2008) and astrogliosis (dashed line) could counteract that loss of mass although recovery is not in neurons. Neuronal recovery (thin line) begins to occur at day 7 in abstinence with a burst in neural stem cell proliferation (Nixon and Crews 2004). Altogether, these and recent data support that alcohol-induced neuropathological events are still continuing at a week in abstinence.

Table 1

Intoxication parameters for the animal model (experiment 1)

Group	Intoxication Score	Dose/day	BEC	WD-Mean	WD-peak
T0 (n = 6)	2.1 ± 0.1	9.5 ± 0.4	323.4 ± 17.8	n/a	n/a
T2 days (n = 6)	2.1 ± 0.2	9.3 ± 0.5	286.7 ± 25.1	1.2 ± 0.3	3.2 ± 0.4
T4 days (n = 6)	2.0 ± 0.2	9.5 ± 0.5	336.2 ± 19.7 [†]	1.5 ± 0.3	3.3 ± 0.2
T7 days (n = 6)	1.8 ± 0.3	10.0 ± 0.8	311.1 ± 13.4	1.9 ± 0.2	3.5 ± 0.1
T14 days (n = 4)	2.1 ± 0.4	9.3 ± 1.2	252.0 ± 51.5	2.3 ± 0.5	3.6 ± 0.2
T28 days (n = 6)	2.1 ± 0.1	9.1 ± 0.2	332.9 ± 26.0	1.7 ± 0.4	3.3 ± 0.1

[†] bloods from a separate group of T4 days rats due to Analox malfunction. Separate group had nearly identical intoxication scores (2.0 ± 0.1) and resulting dose/day (9.4 ± 0.2) and were run at the University of Kentucky within months of the rats used in this study.