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Markers of apoptosis induction and proliferation in the orbitofrontal cortex in alcohol dependence

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Abstract

Background—Alcohol-dependent (ALC) subjects exhibit glial and neuronal pathology in the prefrontal cortex (PFC). However, in many patients, neurophysiological disturbances are not associated with catastrophic cell depletion despite prolonged alcohol abuse. It is still unclear how some relevant markers of a cell's propensity to degenerate or proliferate are changed in the PFC of ALC subjects without major neurological disorders.

Methods—Levels of pro-apoptotic caspase 8 (C8), X-linked inhibitor of apoptosis protein (XIAP), direct IAP binding protein with low pI (DIABLO), proliferating cell nuclear antigen (PCNA), and density of cells immunoreactive (-IR) for proliferation marker Ki-67 were measured postmortem in the left orbitofrontal cortex (OFC) of 29 subjects with alcohol dependence and 23 non-psychiatric comparison subjects.

Results—ALC subjects had significantly higher levels of the 14kDa C8 fragment (C8-14), an indicator of C8 activation. However, there was no change in the levels of DIABLO, XIAP or in the DIABLO/XIAP ratio. PCNA protein level and density of Ki-67-IR cells were not significantly changed in alcoholics, although PCNA levels were increased in older ALC subjects as compared to controls.

Conclusions—Significant increase of a C8 activation indicator was found in alcoholism, but without significant changes in XIAP level, DIABLO/XIAP ratio, or Ki-67 labeling. These results would help to explain the absence of catastrophic cell loss in the PFC of many alcohol dependent subjects, while still being consistent with an alcoholism-related vulnerability to slow decline in glial cells and neurons in the OFC of alcoholics.

Keywords

Alcohol dependence; prefrontal cortex; vulnerability; postmortem; depression

INTRODUCTION

Some areas in the prefrontal cortex (PFC) of chronic alcoholics experience marked functional disruptions (Durazzo et al., 2008, Moselhy et al., 2001). In addition, the morphology and packing density of neurons and glial cells in dorsolateral prefrontal (dlPFC) and orbitofrontal cortex (OFC), both PFC subdivisions, are significantly changed in alcohol-dependent as compared to non-psychiatric control subjects (Miguel-Hidalgo et al., 2002, Miguel-Hidalgo et al., 2006). Density of glial cells in the PFC is reduced in relatively young alcoholics and either remains unchanged or increases with the duration of alcohol dependence (Miguel-Hidalgo et al., 2002, Miguel-Hidalgo et al., 2006). In contrast to glia, neuronal packing density significantly declines towards late life in the OFC of subjects with alcoholism, most likely due to the long duration of alcohol abuse (Miguel-Hidalgo et al., 2006). In alcoholism, other research has revealed significant reduction in neuronal packing density, average size of neurons, and branching of basal dendrites in pyramidal neurons (Harper, 1998) of frontal cortical areas. However, despite those signs of cellular vulnerability to degeneration, many subjects with alcoholism do not show catastrophic neurological symptoms or macroscopically conspicuous neuronal and glial pathology (Zahr et al., 2011). In these so-called “uncomplicated” cases, which can represent as many as 60% of alcoholics (Harper et al., 1988, Torvik and Torp, 1986, Zahr et al., 2011), the uncovering of cellular pathology has required the use of immunohistochemical techniques and meticulous microscopic cell-counting (Miguel-Hidalgo et al., 2010, Miguel-Hidalgo et al., 2006, Miguel-Hidalgo et al., 2002, Korbo, 1999) or proteomic studies localized to specific brain regions (Alexander-Kaufman et al., 2007). In some of the studies above, low counts of glial cells or progressive neuronal depletion would suggest that even in the “uncomplicated” cases there are alterations in the levels of proteins involved in cell survival or proliferation. Alterations involving the balance between markers of cell death and proliferation in relevant cortical areas could result in subtle but significant disturbances of cellular vulnerability to degeneration or slow decline in glial cells and neurons.

Among proteins involved in the regulation of cell survival, we chose to examine the levels of caspase 8, protein X-linked inhibitor of apoptosis protein (XIAP), and direct IAP binding protein with low pI (DIABLO). Caspase 8 is a major intermediary in the extrinsic apoptosis cascade. Its relevance stems from its role as an apoptosis-promoting, cytoplasmic factor that is activated by receptors of the TNF- α receptor family. In particular, TNF- α and other cytokines are elevated in the brain and plasma of alcoholics (Achur et al., 2010) and their activated receptors may lead to increased activation of caspase 8 itself. Apoptosis, including C8-mediated apoptosis (Deveraux et al., 1998), can be inhibited by XIAP, an inhibitor of effector caspases (Maier et al., 2002) that are activated by C8 (Deveraux et al., 1998). In turn, XIAP is blocked by DIABLO, thus facilitating apoptosis progression (Saito et al., 2003). Augmented DIABLO/XIAP ratio likely reflects increased vulnerability to degeneration or apoptosis (Albeck et al., 2008). Moreover, sufficient XIAP expression appears to protect neurons from apoptosis-promoting disturbances (Holcik et al., 2001). In addition to proteins that regulate apoptosis, we examined in situ labeling of cells containing fragmented DNA, a hallmark of ongoing apoptosis, by means of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

To determine possible alterations in proliferative capacity of OFC cells we examined PCNA levels and the packing density of Ki-67-immunoreactive cells. PCNA is a co-factor of DNA-polymerase delta involved in DNA replication, increasing during DNA replication (Kurki et al., 1986), and declining in non-dividing cells, although in some cells persists after cell division. PCNA is also heavily involved in DNA repair (Essers et al., 2005). Ki-67 is a marker for cells undergoing the mitotic cycle, but deemed more specific for proliferative readiness than PCNA (Kee et al., 2002). Accordingly, Ki-67-immunolabeling was used to quantify changes in the density of cells with proliferative potential. It has to be stressed that in the neocortex, most cells with proliferative potential are glia or glial cell precursors (Koketsu et al., 2003). Thus, it is very likely that in the OFC Ki-67 immunoreactive cells are mostly glia or glial cell precursors.

MATERIALS AND METHODS

Human subjects

Human postmortem brain tissue originated from autopsies at the Cuyahoga County Coroner's Office in Cleveland, OH. Collection of postmortem materials was performed according to a protocol approved by the Institutional Review Boards at the University of Mississippi Medical Center and Case Western Reserve University. Information from knowledgeable informants (next-of-kin, significant others) and medical and toxicological records were used to obtain retrospective psychiatric diagnoses of the deceased. Assessment of psychiatric symptoms (or their absence) rested on the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (Association, 1994). Subjects with head trauma or a neurological disease were excluded. Brain tissue from 52 subjects was included: 29 subjects met criteria for alcohol dependence (ALC), and 23 comparison subjects (COMP) did not meet criteria for any Axis I disorder as described in DSM IV. Among the ALC subjects, 13 subjects had been additionally diagnosed with major depressive disorder at some time in their lifespan (ALCD subjects) and the rest did not have diagnosis of depression (ALCND subjects). The presence of any other psychiatric or neurological diagnosis (including but not limited to Wernicke's encephalopathy and Korsakoff's syndrome) was an exclusion criterion for both cohorts. On the basis of the absence of these neurological diagnosis and of cirrhosis, ALC subjects in this study can be considered "uncomplicated" (Harper et al., 1988, Torvik and Torp, 1986, Zahr et al., 2011). Due to limitations in tissue availability, some experiments included fewer subjects per group, as reflected in the degrees of freedom in statistical data. Further information on the subjects is presented in the supplemental material. Blood alcohol concentrations were measured from autopsy samples. Ethanol was detected postmortem in the blood of 14 subjects with alcohol dependence. Eleven of them were in the ALCND subgroup, among which eight were at or over the legal limit (0.08 g/dL). In the ALCD group, ethanol was detected in three subjects, all over the legal limit. There was no significant correlation between ethanol levels from autopsy samples and the levels of proteins or the counts of immunoreactive cells included in this study.

Tissue—Human postmortem brain tissue from Brodmann's area 47 of the left OFC was used in all determinations. Groups were matched to minimize differences in age,

postmortem interval, tissue pH, and gender. There was no significant difference in those variables between alcohol-dependent and comparison subjects except for brain pH. Within area 47, tissue to be studied was rostral to the transverse sulcus and lateral to the medial orbital sulcus in accordance to cytoarchitectonic criteria and the pattern of gyri and sulci (Uylings et al., 2010). Each frozen block was cut into alternating sections with a thickness of 20 μ m and 50 μ m, which were used for immunohistochemistry and western blots, respectively.

Western blotting

Punch samples were taken from frozen 50- μ m sections and spanned from the pial surface to the gray/white matter boundary. Samples were homogenized in 0.01M Tris-HCl containing 1% SDS, 2 mM EDTA, and protease inhibitor. After centrifugation, 40 μ g of supernatant protein were applied per gel lane and run with the Xcell II NuPAGE Bis-Tris Electrophoretic System (Invitrogen, Carlsbad, CA). Proteins were transferred to PVDF membranes that were probed with the following antibodies: rabbit polyclonal anti-C8 (Ab-4; Thermo Scientific, Fremont, CA), mouse monoclonal anti-PCNA (PC10; Invitrogen, Camarillo, CA), mouse monoclonal anti-DIABLO at 1:2000 (56/Smac/DIABLO, BD-Biosciences, San Jose, CA), and mouse monoclonal anti-XIAP at 1:4000 (48/hILP/XIAP, BD Biosciences, San Jose, CA). Some membranes were first incubated overnight at 4 C with the primary antibody to C8 and processed with alkaline phosphatase-conjugated secondary antibody. Chemiluminescent bands were imaged in a Kodak Image Station-440-C. Antibodies were stripped from membranes and the membranes re-probed with anti-PCNA, followed by stripping, incubation and imaging with anti- β -actin. In other experiments, membranes from a smaller number of subjects were incubated with anti-XIAP, processed for chemiluminescence, stripped, processed for anti-DIABLO chemiluminescence, further stripped and probed with anti- β -actin. There were no significant differences in the optical densities of the actin band between the diagnostic groups. Other researchers have not found significant differences in the intensity of western blot actin bands in the PFC (Flatscher-Bader and Wilce, 2008; Erdozain et al., 2014) or locus coeruleus (Karolewicz et al. 2008) of human alcoholics as compared to non-alcohol abusing comparison subjects.

Samples were run in duplicate, altering gel positions to demonstrate replicability. The level of each protein was calculated as a ratio of the optical density of bands of interest to the band of β -actin. Two samples from two designated non-alcoholic comparison subjects from the non-alcohol dependent group (internal control samples) that were approximately in the middle to low range of reaction intensities for COMP subjects were included in all blots. In this manner, relative levels of proteins were calculated by dividing the protein level (determined relative to actin, see above) in a particular subject by the level of the protein in the internal control samples. The amounts of homogenate delivered were calculated so that blot densitometric measurements fell within the linear range of chemiluminescence intensities. Electrophoresis gels had 15 wells of which 11 ran brain protein samples from 11 different subjects and one a standard molecular weight marker. Five of the sample wells had proteins from subjects with major depression (but no alcohol dependence). The corresponding 5 blot lanes were dedicated to a separate study in which they were analyzed with the comparison subject lanes (Miguel-Hidalgo et al., 2014). These lanes are not

included in any figures, results or statistics in the present article. The rest of the lanes in each blot were from the subjects with alcoholism and from the comparison subjects (illustrated in figures 1 and 2) included in the present study.

Immunohistochemistry

Frozen 20- μ m sections were mounted on slides, dried and fixed in 4% paraformaldehyde for 30 minutes. Three sections per brain were washed in phosphate buffer saline (pH 7.4) and processed with prediluted anti-Ki-67 antibody (Zymed). Three days later, sections were washed in 0.1 M Tris-HCl buffer (pH 7.4), incubated with biotinylated secondary antibodies, washed again and the bound antibody was detected using the ABC method (Vector) with 3'3'-diaminobenzidine enhanced with nickel ammonium sulfate as chromogen.

Fragmented DNA was detected by TUNEL as described in the supplemental materials.

Omission of primary or secondary antibodies from sections or membranes prevented specific staining. Specific immunostaining was also absent after incubating the primary antibodies with the corresponding antigens before their use.

Statistics

Differences in protein levels or cell packing densities were assessed by analysis of covariance (ANCOVA) using pH as covariate, because a significant difference in pH was found between groups. Two-way ANCOVAs with diagnosis and gender as factors or diagnosis and mode of death (suicide versus non-suicide) as factors were also performed for all variables.

Correlational analysis of the relationship between age and each of the variables was also conducted using a Pearson correlation matrix and the significance values were adjusted using the Bonferroni correction for multiple comparisons. Partial correlations of the variables with disorder duration or with age of disorder onset were made using age at the time of death as the controlling variable. Since PCNA and Ki-67 are markers for cells with proliferative potential, correlational analysis between PCNA levels and density of Ki-67 immunoreactive cells was also performed for each diagnostic group. All analyses were implemented with the SPSS statistical software, version 19.

RESULTS

1.1 Caspase 8 levels

The antibody to C8 revealed a band at about 50 kDa known to correspond to procaspase 8 (C8-50), and bands at about the 36 kDa and 14 kDa molecular weight markers indicating caspase 8 cleavage. The band near 14-kDa (C8-14) represents a small fragment of processed procaspase 8 (Fig. 1A) and is considered to represent fragments of activated caspase 8 generated by interaction with death domain proteins (Schamberger et al., 2005). Higher levels of short fragments (18-kDa to 10-kDa) signal activation of caspase 8 (Bredesen et al., 2006). Comparison of the ALC and COMP groups using ANCOVA showed a significant difference in C8-14 levels ($F(1, 49)=15.23$, $p < 0.001$) (Fig. 1B) between cohorts. Levels

were higher in ALC than in COMP subjects regardless of the presence of depression (fig. 1C). C8-50 levels were also higher in ALC subjects (Supplemental figure 1) ($F(1,49) = 5.259$, $p = 0.026$), but were not different between ALC subjects with (ALCD) and without depression (ALCND). Analysis of C8-36 band also showed a significant increase in the whole ALC group as compared to COMP subjects ($F(1, 49)=8.057$, $p = 0.007$) and in the ALCND subgroup ($p=0.038$), but no difference between ALCND and ALCD subjects ($p = 1$) or between ALCD and COMP subjects ($p = 0.107$).

1.2 DIABLO and XIAP in the OFC

The DIABLO/XIAP ratio was not significantly different between the groups (ANCOVA, $F(1, 49)=0.13$, $p = 0.722$) (Figs. 2A,B). Likewise, there was no significant difference in either DIABLO ($p=0.889$) or XIAP ($p=0.780$) levels between ALC and COMP subjects (Fig. 2C,D). The DIABLO/XIAP ratio was not significantly different in ALCND as compared to ALCD subjects (Fig. 2E).

1.3 TUNEL staining in the ORB

TUNEL-positive cells were detected in only one or two subjects per group. There was no correlation between TUNEL staining and pH, postmortem delay or age, although these correlation tests lacked the power to detect differences due to the very few positive cells observable in most subjects in all groups.

2.1 PCNA levels

In western blots, anti-PCNA revealed a single band at about 36 kDa (Fig. 3A) and occasionally a weak band above the main PCNA band. Only the 36 kDa band was quantified (Fig. 3A). ANCOVA analysis did not reveal a significant difference in the levels of PCNA between ALC and COMP subjects ($F(1,49)=1.709$, $p=0.197$) (Fig. 3B,C).

2.2 Packing density of Ki-67-positive cell nuclei

Three 20 μm -thick sections of the OFC per subject were processed for DAB-based immunohistochemistry of Ki-67. The packing density of Ki-67-labeled nuclei (Fig. 4A–D) was measured in gray matter using the optical disector applied to a random sampling of counting frames within the gray matter. The density of Ki-67-IR nuclei in the OFC was not different between COMP and ALC subjects (ANCOVA $F(1,49) = 0.226$, $p=0.637$) (Fig. 4C). However, the highest values of the density of Ki-67 immunoreactive cells were in the group of ALC subjects with comorbid depression (Fig. 4D).

3. EFFECT OF AGE

There was a positive correlation between C8-14 and age in COMP ($r=0.422$, $p = 0.045$) (Fig. 5A), but not in ALC subjects (Fig 5B–D).

In COMP subjects, there was a non-significant trend for correlation between PCNA levels and age ($r = 0.405$, $p = 0.055$) (Fig. 6A). However, there was a clear positive correlation of PCNA level with age in ALC subjects ($r=0.613$, $p<0.005$) (Fig. 6B), still significant after splitting the latter group into ALCND and ALCD subjects (ALCND $r=0.690$, $p = 0.003$; ALCD $r= 0.599$, $p=0.03$) (Fig. 6C,D).

Ki-67 packing density was positively correlated with age only in ALCD subjects ($r=0.679$; $p=0.011$) (Fig 4E). When controlling for age, there was no correlation between density of Ki-67 immunoreactive cells and PCNA levels ($r=0.207$, $p=0.290$) in ALC subjects. However, there was a significant negative correlation between those two variables in COMP subjects when controlling for age ($r=-0.624$, $p=0.002$).

Only in ALCND subjects there was a positive correlation between duration of alcoholism and PCNA levels ($r=0.668$, $p=0.005$). However, this correlation was not significant when using a partial correlation test controlling for age ($r=0.203$, $p=0.468$). Age at onset of alcoholism was positively correlated with density of Ki-67 nuclei in ALCND ($r=0.680$, $p=0.004$) even after controlling for age at the time of death, but not in ALCD subjects ($r=0.042$, $p=0.891$). Age at onset of depression and duration of alcoholism were significantly correlated with density of Ki-67 nuclei in ALCD ($r=0.622$, $p=0.023$ and $r=0.751$, $p=0.003$, respectively), although these correlations were not significant when controlling for age at the time of death ($r=0.363$, $p=0.246$ and $r=0.567$, $p=0.055$, respectively).

Neither pH nor PMI were significantly correlated with any of the protein levels or morphometric variables.

4. EFFECTS OF SUICIDE AND GENDER

Several subjects in the ALC group died by suicide. Two-way ANCOVA with diagnosis and suicide as factors and pH as covariate showed no significant effect of suicide or suicide by diagnosis interaction in any of the variables studied. Likewise, 2-way ANCOVA (diagnosis and gender) showed no significant gender effect or gender by diagnosis interaction. Comparisons with gender as factor may lack, however, sufficient power since there were relatively fewer female subjects in the ALCND group (male/female ratio of about 4:1, with only 3 female subjects) than in the group of non-psychiatric comparison subjects (ratio of about 2:1).

DISCUSSION

Increased levels of a small peptide marker of caspase 8 activation (C8-14) and of procaspase 8 (C8-50) in the OFC of subjects with alcoholism suggest a chronic change in the readiness of C8 to initiate or promote cell death. However, the level of XIAP, antiapoptotic factor that blocks the activation of downstream effector caspases by C8, was not different from comparison subjects. Also unchanged were the DIABLO/XIAP ratio and level of DIABLO, which is an inhibitor of XIAP. In addition, there was no significant difference in PCNA levels or the density of Ki-67 immunoreactive cell nuclei, although in alcohol dependent subjects with a diagnosis of depression there was a trend for higher PCNA levels that became significant with increasing age.

PRO-APOPTOTIC MARKERS

Higher levels of small C8 fragments suggesting C8 activation would be consistent with increased risk for cell death or degeneration in OFC cells in ALC. A slow decline in cell numbers due to a potentially increased vulnerability to degeneration might partly explain lowered density of glial cells in young and middle-aged subjects with alcohol dependence or

reductions of neurons in older subjects with alcohol dependence (Miguel-Hidalgo et al., 2006, Miguel-Hidalgo et al., 2002). An early deficit in glial support combined with the functional alterations caused by alcohol exposure itself may result in progressively increased vulnerability of cortical neurons, although this hypothesis needs to be properly supported with further experiments. Since we did not determine the cell types where increased expression of C8 occurred, it was not possible to ascertain whether glial cells, neurons or both were responsible for the increased C8 or whether changes of C8 in glial cells preceded changes in neurons (Miguel-Hidalgo et al., 2006, Miguel-Hidalgo and Rajkowska, 2003).

In contrast to increased levels of C8-14 (a putative marker of caspase 8 activation), very low occurrence of cells with fragmented DNA evinced by a very sparse detection of TUNEL-labeled cells, suggests that lowered glial and neuronal numbers are not due to a significant or catastrophic ongoing cell death in the gray matter. Cell demise in alcoholics might rather be due to a non-catastrophic, slowly progressing deficit in neural cells. It cannot be ruled out that this cellular demise is partly present early in the disease (especially in the case of glial cells, which have lower than normal densities at relatively young ages in alcohol-dependent subjects) and is further complicated by a slow decline in cell numbers over a protracted period of time. As suggested earlier, a plausible scenario would involve increased C8 causing first glial cell decline, and only later slow neuronal depletion.

Alternatively, high C8 level in alcoholism may not be only a sign of imminent cell death. Previous cytological studies of alcoholism reveal few or no cells with morphological apoptosis or signs of necrosis (Ikegami et al., 2003). A postmortem study of TUNEL staining (marker of apoptosis) in the hippocampus in alcoholism has shown that TUNEL-positive cells are scarce and appear to include only some astrocytes. In that study (Ikegami et al., 2003), subjects with alcoholism revealing some apoptosis (7 subjects out of 11) died of causes different from controls, while a majority of controls (7 subjects out of 9) had died by suicide (50). In our study, where none of the comparison subjects had died by suicide, there was no TUNEL-staining-based evidence of marked apoptosis in either ALC or COMP subjects.

Higher levels of C8-14 in alcoholism, rather than being a marker for impending cell death, might indicate the presence of cells at risk for apoptosis or other forms of cell degeneration and stress, but without necessarily being engaged in a terminal process of cellular demise. Augmented signals of degeneration may be related to the TNF- α -mediated activation of death receptors of the TNF- α family in neural cells (Choi and Benveniste, 2004) as TNF- α is elevated in alcoholism (Syapin et al., 2005). TNF- α receptor activation induces C8 activation and, as illustrated by the consequences of traumatic brain injury in mice, activation of those receptors can result in cognitive deficits independently from cell death (Khuman et al., 2011). Similar mechanisms involving TNF- α may result in cognitive disturbances accompanied by increases in neurodegeneration markers such as C8 activation without resulting in conspicuous cell death in the OFC.

Sparse cell death labeling in the OFC of alcoholics could be explained by the absence of significant decrease in XIAP, a factor that inhibits apoptosis progression. XIAP levels might be high enough in alcohol-dependent subjects to counteract apoptosis induction, resulting in

levels of cell death undetectable by standard histological methods. Sufficient expression of XIAP appears to be a major factor impeding neuronal death even when some parts of the neuron degenerate (Cusack et al., 2013). It is worth to note that in both alcohol-dependent and comparison subjects, there is considerable variability in the levels of DIABLO or XIAP (as compared to β -actin), so it is possible that factors other than chronic alcoholism have greater influence on the expression or processing of those proteins. Moreover, it cannot be ruled out that forms of non-apoptotic cell death may have played a role in causing low densities of glia or neurons in the PFC of alcoholics. Programmed non-apoptotic cell death and autophagy are forms of cell death that might not result in TUNEL labeling or in detectable pyknotic nuclei with Nissl staining (Oppenheim et al., 2001, Carloni et al., 2004). On the other hand a recent study points to the lack of increased expression of several components of the intrinsic apoptosis pathway in the dorsolateral PFC of subjects with chronic alcoholism (Johansson et al., 2009).

MARKERS OF CELL PROLIFERATION

In postmortem studies of alcohol dependent subjects, lower-than-normal packing density of glial cells has been found in the hippocampus (Korbo, 1999) and PFC (Miguel-Hidalgo et al., 2002). In vitro, ethanol inhibits proliferation of human astrocytes (Kane et al., 1996) while repeated alcohol binges and even single binge exposure during adolescence can lead to persistent deficits in neurogenesis (Taffe et al., 2010). It was surprising that in “uncomplicated” alcohol dependent subjects, the density of Ki-67 cells was not statistically different from comparison subjects, suggesting no differences in proliferation of neural precursors. A caveat to this suggestion is that among alcohol dependent subjects, 10 subjects with comorbid depression also were under antidepressant treatment. Antidepressants are known to increase neurogenesis (Malberg et al., 2000; Maney et al., 2001). In fact the three highest values of density for Ki-67 occurred in three alcohol dependent subjects with comorbid depression that were under antidepressant treatment. Further experiments are required to validate the suggestion that in alcohol dependent subjects with depression, antidepressant treatment may result in increased proliferation of cortical neural precursors.

The adult neocortex contains neural precursors with the ability to proliferate (Lee et al., 2000). However, unlike the hippocampus, and in the absence of traumatic brain injury, neocortical precursors in adult mammals produce mostly glial cells (Lee et al., 2000, Feliciano and Bordey, 2013). Thus, most if not all Ki-67 immunoreactive cells in the neocortical OFC detected could have been precursors of astrocytes, oligodendrocytes or microglia. Accordingly, it is very likely that alcohol dependence, in the absence of major neurological damage, is not associated with alterations of the proliferation of glial cells as has been suggested by a previous study in the olfactory bulb and subventricular zone in the postmortem brain of chronic alcoholics (Sutherland et al., 2013). Rather, depletion of glial cells relatively early in the disorder, and slow but sustained depletion of neurons might explain lower densities of glial cells and reduced neuronal densities in relatively young subjects only with prolonged duration of alcoholism (Kril et al., 1997, Miguel-Hidalgo et al., 2006). Although cell proliferation may not be affected by alcoholism in “uncomplicated” alcoholics, other markers of glial cell function could be affected to different degrees. For instance, in previous studies we did not find differences in glutamatergic markers of

astrocytes between alcohol dependent subjects without depression and controls (Miguel-Hidalgo et al., 2010). However, these markers were significantly decreased in alcohol dependent subjects with comorbid major depression (Miguel-Hidalgo et al., 2010). In contrast, recently we found that there was a very significant decrease in the levels of connexin 43, the main gap junction forming protein in astrocytes, and in Cx43-immunoreactive gap junctions aggregates in alcohol dependent subjects regardless of the presence of depression (Miguel-Hidalgo et al., 2014).

The present results cannot rule out a significant influence of ethanol dependence on the proliferation of specific populations of glial cells, because we did not use specific markers for those populations (astrocytes, oligodendrocytes or microglia). It is also known that many cell precursors at the stage displaying Ki-67 labeling lose or lack the specific markers of the cell types into which their lineage will eventually differentiate (Englund et al., 2005, Kee et al., 2002). This lack of markers does not permit to unequivocally determine proliferation changes for precursors of differentiated cell types in human postmortem tissue. Animal experiments that allow labeling of precursors with specific markers and following up the markers into the differentiated state will be necessary to determine how long-term alcohol dependence affects proliferation of the various types of glial cells. It must be also pointed out that the absence of significant changes in the numbers of proliferating cells as revealed by Ki-67 immunoreactivity may be in part a reflection of the criteria for inclusion of ALC subjects in this study. These criteria excluded subjects with major neurological disturbances, cirrhosis, Wernicke's encephalopathy, or Korsakoff's syndrome, all of which involve brain damage that might be associated with disturbances in cell proliferation (Vetreno et al., 2011).

Absence of significant increases in PCNA in subjects with alcohol dependence may be due to factors similar to those considered above when explaining the lack of changes in Ki-67. In ALC subjects there was significant correlation between age and Ki-67, age and PCNA and also between density of Ki-67 immunoreactive cells and PCNA levels. However, the correlation of Ki-67 with age was entirely due to subjects with comorbid depression ($r=0.679$, $p=0.011$), and absent in ALCND subjects ($r=-0.05$, 0.984). In contrast, the correlation between PCNA and age was significant in both ALCD and ALCND subjects. In addition, when statistically controlling for age, there was no correlation between Ki-67 and PCNA in the ALC group or their subgroups, and it was negative in the COMP group. Consistently high levels of PCNA in ALC subjects in late life would contrast with unchanged neurogenesis and/or gliogenesis suggested by the unchanged density of Ki-67-immunoreactive cells in late-life in ALCND and COMP subjects. They also are in contrast to studies in human, non-human primate and other mammalian brains that rather suggest age-dependent declines in proliferative potential of neural precursors (Riddle and Lichtenwalner, 2007). Thus, PCNA changes might not be fully related to increased proliferation, but point to the well-known participation of PCNA in DNA repair (Essers et al., 2005, Maga and Hubscher, 2003).

If DNA repair induced by DNA damage results in elevated detection of PCNA (Cooper-Kuhn and Kuhn, 2002) longer durations of alcoholism may be related to augmented probability of DNA damage in the OFC. DNA damage appears to be complicated by age

(Katyal and McKinnon, 2008), which would be consistent with an age-dependent increase in PCNA. In addition, alcohol exposure reversibly increases the incidence of DNA breaks (Brooks, 2000). Thus, alcohol-related, recurring abnormal nervous activity in the OFC may increase DNA damage events and induction of PCNA even in the absence of cell death. It should be considered that, despite the well-known association of DNA breaks with cellular pathology and neurodegenerative processes (Brasnjevic et al., 2008), recent research has shown that specific forms of brain activity, including patterns of non-pathological activity, result in significant, reversible increases in double strand DNA breaks in neurons, which may be further enhanced in neurodegenerative disorders (Suberbielle et al., 2013). Increases in PCNA, particularly in older subjects with alcoholism, may then be related to an attempt to repair double strand DNA breaks and other forms of DNA damage (Maga and Hubscher, 2003). Further research is needed to ascertain whether increased PCNA levels in alcoholism may occur because of increased DNA damage.

Augmented PCNA levels not positively correlated with increased density of proliferating, Ki-67-immunoreactive cells might not be alcoholism-specific. We have recently found that in major depressive disorder (without comorbid alcoholism) there is an age-related increase in PCNA levels as compared to non-psychiatric control subjects (Miguel-Hidalgo et al., 2014) even if these subjects diagnosed with only depression had significantly lower density of Ki-67-immunoreactive cells. Thus, increased PCNA levels may signal not only direct alcohol effects on cellular function, but also altered neural activity patterns. Further investigation is needed to ascertain the relevance of each of these mechanisms to abnormal OFC function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Western blot detection and quantification of caspase 8 (C8). A) Lanes from two different representative western blots of OFC proteins (40 µg per lane), each blot with 5 different subjects with alcohol dependence (total 10 different ALC subjects, split into those without comorbid (ALCND), and those with comorbid (ALCD) depression) and 2 different comparison subjects (total 4 COMP subjects). The blots were probed with an anti-caspase 8 antibody. Bands at the approximate molecular weights of 50, 36 and 14 kDa are singled out. Bands at 14 and 50kDa were quantified against β-actin bands. B) Bar graph representing the normalized levels of C8-14 in COMP (n=23) and ALC (n=29) subjects. C) Same subjects but with the ALC group split into ALCND (n=16) and ALCD subjects (n=13).

Quantification of the 50 kDa band is presented in supplemental figure 1. Whiskers represent the standard error of the mean. *p<0.001.

Fig. 2.

Western blot immunodetection and quantification of the proteins DIABLO and XIAP in the OFC from COMP and ALC subjects (split into ALCD and ALCND, abbreviations as in figure 1). A) Reactive bands in three different western blot membranes, each including subjects of ALC and COMP groups. Subjects in each blot were different from the subjects in the other blots. B) Bar graph of the ratio of the normalized levels of DIABLO to the normalized levels of XIAP in COMP (n=15) and ALC (n=17) subjects. C) and D) Bar graphs representing the mean normalized levels of DIABLO and XIAP, respectively, in the diagnostic groups. E) Bar graph illustrates the mean levels DIABLO/XIAP ratio in the groups of subjects resulting from splitting alcohol dependent subjects into those without (ALCND) and with (ALCD) depression. Whiskers represent the standard error of the mean.

Fig 3.

Western blot immunodetection and quantification of PCNA in the OFC in ALC and COMP subjects (abbreviations as in figure 1). A) Two representative western blots with subjects from the two diagnostic groups; subjects in the left blot were different from the subjects in the right blot. B) Bar graph representing the normalized levels of PCNA in COMP (n=23) and ALC (n=29) subjects. C) Same as B) but with ALC subjects split into ALCND and ALCD subjects. Whiskers represent the standard error of the mean.

Fig. 4.

Immunohistochemistry and cell counting of Ki-67-immunoreactive cell nuclei in the gray matter of the OFC. A) and B) Low power micrographs of immunoreactive nuclei (dark round spots) in the upper cortical layers in COMP and ALC subjects, respectively. C) Bar graph of the average packing density of Ki-67-immunoreactive nuclei in ALC (n=29) and COMP (n=23) subjects. D) Same as B) but with ALC subjects split into ALCND and ALCD subjects. E) Plot of the positive correlation between age and density of Ki-67 immunoreactive nuclei in the ALCD group. LI=cortical layer I; LII=cortical layer II. (Abbreviations as in figure 1)

Fig. 5.

Scatter graphs illustrating the correlation between levels of the 14 kDa fragment of caspase 8 (C8-14) and age at the time of death in COMP (A) and ALC (B) subjects. The correlation between age and C8-14 levels in ALCND and ALCD is illustrated in C) and D), respectively. There was a significant positive correlation in COMP, but not in ALC subjects. (Abbreviations as in figure 1)

Fig. 6.

Scatter graphs illustrating the correlation between levels of PCNA and age at the time of death in COMP subjects (A) and ALC subjects (B). The correlation between age and PCNA levels in ALCND and ALCD is illustrated in C) and D), respectively. There was a significant positive correlation in ALC subjects, but only a non-significant trend for a correlation in the COMP group. (Abbreviations as in figure 1).