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The effects of chronic smoking on the pathology of alcohol-related brain damage

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Abstract

Both pathological and neuroimaging studies demonstrate that chronic alcohol abuse causes brain atrophy with widespread white matter loss but limited gray matter loss. Recent neuroimaging studies suggest that tobacco smoking also causes brain atrophy in both alcoholics and neurologically normal individuals; however, this has not been confirmed pathologically. In this study, the effects of smoking and the potential additive effects of concomitant alcohol and tobacco consumption were investigated in autopsied human brains. A total of 44 cases and controls were divided into four groups: 16 non-smoking controls, nine smoking controls, eight non-smoking alcoholics, and 11 smoking alcoholics. The volumes of 26 gray and white matter regions were measured using an established point-counting technique. The results showed trends for widespread white matter loss in alcoholics ($p < 0.007$) but no effect on gray matter regions. In contrast, smoking alone had no effect on brain atrophy and the combination of smoking and alcohol showed no additional effect. Neuronal density was analyzed as a more sensitive assay of gray matter integrity. Similar to the volumetric analysis, there was a reduction in neurons (29%) in the prefrontal cortex of alcoholics, albeit this was only a trend when adjusted for potential confounders ($p < 0.06$). There were no smoking or combinatorial effects on neuronal density in any of the three regions examined. These results do not support the hypothesis that smoking exacerbates alcohol-related brain damage. The trends here support previous studies that alcohol-related brain damage is characterized by focal neuronal loss and generalized white matter atrophy. These disparate effects suggest that two different pathogenic mechanisms may be operating in the alcoholic brain. Future studies using ultrastructural or molecular techniques will be required to determine if smoking has more subtle effects on the brain and how chronic alcohol consumption leads to widespread white matter loss.

Keywords

alcohol; smoking; human postmortem brain; brain atrophy; neuron density

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Introduction

The deleterious effects of alcohol consumption costs hundreds of billions of dollars per year at the global level with an estimated annual burden of \$15 billion in Australia alone (Collins & Lapsley, 2008). Alcohol abuse is a major public health burden, with a World Health Organisation (WHO) report estimating the global mortality rate attributable to alcohol at 3.3 million per year (WHO, 2014). The majority of the mortality attributable to alcohol is from cardiovascular disease, trauma, gastrointestinal disease, and cancers. In addition, alcohol adversely affects the brain via direct and indirect mechanisms. In terms of its direct effects, amphipathic alcohol rapidly crosses the blood-brain barrier. Once inside the brain, alcohol can disrupt membrane integrity and act directly on receptors such as the N-methyl-d-aspartate (NMDA) and γ -aminobutyric acid (GABA) receptors (Kumar et al., 2009). In addition to these direct effects, comorbid liver damage and nutritional deficiencies, particularly thiamine deficiency, affect the brain and are common in individuals with alcohol-use disorders (Sutherland, Sheedy, & Kril, 2014).

Deficits across a range of cognitive domains such as memory, attention (Kopera et al., 2012), and executive skills (Munro, Saxton, & Butters, 2000; Sullivan, Rosenbloom, & Pfefferbaum, 2000) have been shown to occur in alcoholics. Impairments in higher order functions such as executive skills are indicative of damage to the frontal lobe. Magnetic resonance imaging (MRI) in alcoholics has confirmed this by demonstrating that the most pronounced gray matter (GM) loss is from the frontal lobe (Pfefferbaum, Sullivan, Mathalon, & Lim, 1997; Ratti et al., 1999). In contrast, early pathological studies found more white matter (WM) loss than GM loss (de la Monte, 1988; Harper & Kril, 1985), and further MRI studies have since confirmed the predominance of WM atrophy (Rosenbloom & Pfefferbaum, 2008). A more recent pathological study, using unbiased stereological methods, found only subtle losses in WM volumes in chronic alcoholics that did not reach statistical significance (Krill, Halliday, Svoboda, & Cartwright, 1997). Kril and colleagues reported no changes in GM volumes but there was a significant reduction in neuron number in the prefrontal cortex.

WM atrophy has largely been thought of as secondary to neuronal loss through Wallerian degeneration. However, an alternative hypothesis is that alcohol has a primary effect on the WM with secondary loss of neurons and GM atrophy (Harper, Kril, & Daly, 1988). The reversibility of cognitive deficits following abstinence from alcohol (Kopera et al., 2012) is consistent with WM damage or a disruption of neuronal connectivity (demyelination and neuronal shrinkage) rather than widespread neuronal loss (Harper & Corbett, 1990).

In addition to nutritional deficiencies and liver dysfunction, chronic smoking is a common comorbidity in alcoholics. It has been estimated that approximately 80% of alcoholics are also chronic smokers (Kalman, Morissette, & George, 2005). There are approximately one billion active smokers in the world, with chronic smoking accounting for 9% of the global annual mortality rate or roughly double the effects of alcohol (WHO, 2012). Tobacco smoke is a complex and dynamic mixture of more than 4000 compounds, many of which can have toxic effects (Dome, Lazary, Kalapos, & Rihmer, 2010). As a result, smoking has

widespread effects including cancer of multiple organs, and damage to the cardiovascular system and immune systems (WHO, 2012).

The broad effects of tobacco smoke throughout the body along with known (such as nicotine) and suspected neuroactive components raise the possibility that chronic smoking may cause brain atrophy. Evidence has been accumulating that this is the case, with a number of neuroimaging and cognitive studies reporting findings of brain dysfunction in chronic smokers. While studies into the acute effects of nicotine show increased cognitive performance in smokers (Heishman, Kleykamp, & Singleton, 2010), studies in chronic smokers have revealed cognitive deficits across a range of domains (Durazzo, Cardenas, Studholme, Weiner, & Meyerhoff, 2007) and in a range of different age groups (Deary et al., 2003; Durazzo, Meyerhoff, & Nixon, 2012; Jacobsen et al., 2005). These deficits in brain function are indicative of structural damage, with evidence from MRI studies of both WM and GM loss in chronic smokers. Unlike alcohol, where there is a relatively well-defined pattern of GM loss, there is not a clear picture of GM loss in smokers. While a number of MRI studies have reported GM loss in the frontal lobe (Brody et al., 2004; Fritz et al., 2014; Gallinat et al., 2006), other studies have found GM loss in the temporal lobe, occipital lobe, parietal lobe, cerebellum, and in some subcortical structures (Das, Cherbuin, Anstey, Sachdev, & Easteal, 2012; Gallinat et al., 2006; Gazdzinski et al., 2005; Hanlon et al., 2014; Kühn et al., 2012). A recent meta-analysis of seven MRI studies (213 smokers and 205 non-smokers) found that the only common site with significant GM volume loss was the anterior cingulate cortex (ACC) (Pan et al., 2013).

The inconsistency in results extends to MRI studies of WM with reports of increases, decreases, and no change in regional WM volumes (Fritz et al., 2014; Gallinat et al., 2006; Yu, Zhao, & Lu, 2011; Zhang et al., 2011). Increases in WM have been found in the temporal lobe (Gazdzinski et al., 2005) and in the cingulate cortex (Yu et al., 2011), while decreases in WM have been reported in frontal regions of the brain (Luhar, Sawyer, Gravitz, Ruiz, & Oscar-Berman, 2013; Zhang et al., 2011).

The majority of past studies into the effects of alcohol on the brain have not taken smoking status into account. This raises the possibility that some of the effects of smoking may have been mistakenly attributed to alcohol. A study in alcoholics by Gazdzinski and colleagues found significant GM losses from the temporal, parietal, and occipital lobes (Gazdzinski et al., 2005). These are not regions typically associated with alcohol-related brain damage (ARBD) and the authors postulated that participant smoking status may account for some of the differences. They retrospectively analyzed their results and found significant reductions in temporal, parietal, and occipital lobe volumes in smoking alcoholics (sALC) compared with non-smoking alcoholics (nsALC) (Durazzo et al., 2007). Following on from this, other studies have found evidence of a greater loss of GM and/or WM in sALC compared to nsALC (Durazzo et al., 2014; Luhar et al., 2013).

A mix of both pathological and MRI studies has been used to assess volume changes in ARBD; however, no pathological investigations of smokers have been published. The current study explores the effects of chronic alcohol consumption and smoking in autopsied brain tissue.

Methods

Ethics and case collection

This study was undertaken following ethics approval from the Human Research Ethics Committee of the University of Sydney (HREC #2014/732). Tissue was obtained from the New South Wales Brain Tissue Resource Centre (NSW BTRC), following approval by the National Institute of Alcohol Abuse and Alcoholism Scientific Advisory Board. The study included 16 non-smoking controls (nsCON), nine non-smoking controls (nsCON), 11 smoking alcoholics (sALC), and eight non-smoking alcoholics (nsALC).

Classification

All cases were classified retrospectively based on the donor's clinical and pathological records as previously described (Sutherland et al., 2016). Briefly, the NSW BTRC classifies a subject as 'Alcoholic' when there has been the consumption of greater than 80 g of ethanol per day for the majority of adult life. Lifetime alcohol was calculated using the following formula: number of years drinking $\times$ mean pure ethanol consumed per day (g) $\times$ 365 (days). Any recorded periods of abstinence exceeding 6 months in duration were subtracted from the number of drinking years. Smokers were defined as individuals who had been current smokers who smoked every day (no pack-year threshold). A non-smoker was an individual who had no reported smoking history. One pack-year is equivalent to smoking one pack of cigarettes (20 cigarettes) per day for one year.

Autopsy procedure

The detailed protocol for the processing of the brain tissue has been published previously (Sutherland et al., 2016). Briefly, the right and left hemispheres of each brain were randomly assigned as fixed or frozen. One hemisphere was fixed in 10% formalin for 3 weeks. The length of the hemisphere was determined prior to embedding in agar, and sectioning was done at 3-mm intervals in the coronal plane using a rotary meat slicer. The digital images of serial coronal sections were used for the volumetric aspect of the study. Following imaging, standardized tissue blocks were removed and embedded in paraffin.

Parcellation of the brain into regions of interest

The serial coronal images were manually divided into the WM and GM regions of interest (ROI) using previously described landmarks (Double et al., 1996; Halliday, Double, Macdonald, & Kril, 2003; Kril et al., 1997). The total occipital lobe GM was measured while the parietal, frontal, and temporal lobe GM were first subdivided into smaller subregions. The WM was subdivided into five regions (Fig. 1). More specifically the ROI were defined as follows:

Frontal GM

Frontal pole – all cerebral cortex anterior to the genu of the corpus callosum (CC), excluding the ACC

Orbitofrontal gyrus – cerebral cortex inferior to the CC, anterior to the adherence of the temporal lobe to the frontal lobe

Anterior cingulate cortex (ACC) – all cortex of the cingulate gyrus anterior to the precentral sulcus

Motor cortices – total volume of the cortices of the motor system (premotor cortex, primary motor cortex, supplementary motor cortex, and motor cingulate cortex). The posterior boundary was the central sulcus while anterior and lateral boundaries were as defined by Picard and Strick (Picard & Strick, 1996).

Inferior frontal gyrus (IFG) – the cortex superior to the lateral fissure, inferior to the inferior frontal sulcus, anterior to the precentral gyrus, and posterior to the genu of the CC

Superior frontal gyrus (SFG) – included the middle and superior frontal gyri. The cortex superior to the inferior frontal sulcus, posterior to the genu of the CC bounded medially by the cingulate sulcus and the supplementary motor area.

Temporal GM

Temporal pole – all temporal GM in sections anterior to the adherence of the temporal lobe to the frontal lobe

Temporal gyri – the cortex of superior, middle, and inferior temporal gyri bounded superiorly by the insula and medially by the lateral occipitotemporal sulcus

Medial temporal lobe – all GM medial to the occipitotemporal sulcus in the temporal lobe excluding the amygdala and the hippocampus

Parietal GM

Sensory cortex – the cortex posterior to the central sulcus and anterior to the postcentral sulcus

Superior parietal lobule – the cortex posterior to the postcentral sulcus, anterior to the parieto-occipital notch and superior to the intraparietal sulcus

Supramarginal gyrus – the cortex posterior to the postcentral sulcus, anterior to the posterior ramus of the lateral fissure, superior to the lateral fissure, and inferior to the intraparietal sulcus

Angular gyrus – the cortex posterior to the posterior limit of the superior temporal gyrus and the lateral fissure, anterior to the occipital lobe, and inferior to the intraparietal sulcus

Area 37 – the cortex posterior to the temporal lobe, inferior to the angular gyri, and anterior to the interior occipital sulcus

Posterior cingulate cortex (PCC) – the cortex of the cingulate gyrus posterior to the central sulcus, and anterior to the precuneus

Precuneus – the cortex inferior to the cingulate sulcus, posterior to the cingulate cortex, anterior to the parieto-occipital fissure, and superior to the calcarine fissure

Occipital GM – The cortex posterior to the anterior limit of the calcarine fissure (excluding the posterior aspect of the precuneus)

Additional GM regions

Insula – the cortex within the lateral fissure. The anterior limit was defined as the adherence of the temporal lobe to the frontal lobe and the posterior limit as the posterior portion of the lateral fissure.

Deep GM – all subcortical GM (thalamus, basal ganglia, claustrum, nucleus accumbens, mammillary bodies, hypothalamus) excluding the amygdala and hippocampus

Hippocampus – defined in each section using the boundaries described by Amaral and Insausti (Amaral & Insausti, 1990). The lateral and superior borders are the lateral ventricle and the posterior border is the crux of the fornix.

Amygdala – defined in each section using the boundaries described by de Olmos (de Olmos, 1990)

WM regions

Prefrontal WM – all WM anterior to the amygdala and superior to the lateral fissure

Posterior frontal WM – WM anterior to the central sulcus, posterior to the prefrontal WM, and superior to the lateral fissure

Mid WM – WM posterior to the central sulcus and anterior to the calcarine fissure (excluding temporal WM)

Temporal WM – all WM of the temporal lobe

Posterior WM – all WM posterior to the calcarine fissure

Global measures

Total GM – sum of all regional GM measures

Total WM – sum of all regional WM measures

Total volume – sum of all regional WM and GM measures

Regional volume determination

A validated point-counting procedure was used to obtain volume estimates for each ROI (Krill et al., 1997). Counting reliability was established by achieving <5% variation in duplicate counts of 10% of the total cases. Digital images of each coronal section were printed to scale on A4 paper and counted by overlaying a transparent counting grid (286 × 196 points) (Fig. 2). The crosses within the grid were 0.6 mm apart giving each cross an associated area of 0.36 cm². The grid was randomly placed on each image and all points that fell within a particular ROI were counted. All points within each brain slice were defined as GM or WM. When a point (or more accurately, the area associated with a point) fell across a GM/WM junction, or on the border of the brain slice, the tissue type that occupied the upper right quadrant was consistently used to ascribe GM or WM status to that point. The total number of points within each ROI of each slice gave an area estimate. The ROI in each 3-mm slice were then added together to give the ROI volume. Due to the significant variation in brain size between individuals and between genders, the raw data were normalized by

dividing all ROI volumes by the mean volume of that ROI in the gender controls as previously described (Krill et al., 1997).

Neuronal density

The three cortical regions chosen for neuronal counts, the supramarginal gyrus (Brodmann area [BA] 40), superior frontal gyrus (SFG, BA8), and superior temporal gyrus (STG, BA22), were selected based on the volumetric study above, or in the case of the SFG, the area considered most likely to demonstrate synergistic effects of alcohol and smoking based on previous MRI and pathological studies. Formalin-fixed paraffin-embedded (FFPE) 10-mm sections were stained with cresyl violet and used for the neuronal counts.

Slide preparation

FFPE-mounted sections were incubated for 40 min at 60 °C and then passed through xylene and graded alcohols to rehydrate. The slides were stained with 0.5% cresyl violet for 5 min on an orbital shaker before being rinsed in alcohol to a satisfactory level of staining intensity. Sections were then dehydrated and air-dried, then cover-slipped using dimethylphthalate in xylene (DPX) mounting medium.

Counting procedure

A validated neuronal counting procedure was used in this study (Krill et al., 1997). Briefly each slide was examined at 40X magnification to identify areas that had a clear GM/WM boundary that was parallel to the pial surface and contained no large blood vessels or artifacts. Three cortical strips from the pial surface to the grey-white junction were counted in each section at 200X magnification on an Olympus BX51 microscope. The eyepiece graticule represented an area of 0.25 mm² at 200X magnification. The total neurons counted in the three strips were divided by the total number of graticules counted and expressed as neurons per mm².

Statistical analyses

All statistical analyses were performed using JMP statistical software (Version 10.0.0, SAS Institute Inc., Cary, North Carolina, USA). The α level was adjusted to control for multiple comparisons. For the volumetric analysis, the Bonferroni correction ($\alpha = 0.002$) was modified based on the mean correlation between the 26 ROI ($r^2 = 0.38$) to $\alpha = 0.007$, as described in previous imaging studies (Durazzo et al., 2014). Group differences were assessed by two-way ANOVA with subsequent pair-wise analyses. Pearson correlations were used to explore relationships with smoking pack-years and lifetime alcohol consumption. Multivariate analysis was used to explore potential covariates including age, postmortem interval (PMI), and liver pathology score.

Results

Clinical and demographic information

This study used a well-characterized cohort of 44 (21 female) chronic alcoholics and neurologically normal controls supplied by the NSW BTRC (Table 1). They were divided

into four groups: 16 non-smoking controls (nsCON), nine smoking controls (sCON), eight non-smoking alcoholics (nsALC), and 11 smoking alcoholics (sALC). The sALC had the highest proportion of females (55%) while the nsALC had the lowest proportion (38%). There was a significantly longer postmortem interval (PMI) in the sALC compared with the sCON but otherwise there were no significant differences between groups in age, brain weight, brain pH, RNA integrity number (RIN), or body mass index (BMI). Both mean daily intake and lifetime alcohol consumption were significantly higher in the nsALC compared with the sALC. In contrast, there was no significant difference in pack-years between the sCON and sALC. In later analyses all alcoholics (nsALC and sALC) were compared with controls (nsCON and sCON). Other than the two alcohol indices, the combined groups differed only in PMI that was significantly longer in alcoholics ($p = 0.013$) and in the severity of liver pathology ($p = 0.003$). There was also a trend for smaller mean brain weight in alcoholics ($p = 0.06$) (Table 2).

Combined effects of alcohol and smoking—Overall there were no GM (Table 4) or WM areas that were significantly different between the groups, although both the middle ($p = 0.03$) and total WM ($p = 0.04$) approached significance (Table 4). Multivariate analysis was used to assess the independent effects of alcohol and smoking and whether there were any interactive effects between the two. There were no smoking main effects or interactive effects in any of the 26 ROI examined here (Tables 3 and 4). There were two GM regions (the supramarginal and sensory cortex) that showed a trend toward increased volume in smokers (Table 3). Furthermore there was no association between smoking pack-years volume and WM including total WM ($n = 20$, $r^2 = 0.02$, $p = 0.2$) (Fig. 3A). There were trends for correlations between pack-years for three GM ROIs: frontal pole ($p = 0.03$), temporal pole ($p = 0.04$), and total GM ($p = 0.06$) (Fig. 3B), but none reached significance. In contrast, there was a significant effect of alcohol on total WM (Table 3) as well as trends for volume reduction in several GM and WM regions (Tables 2 and 3).

Neuronal density

Similarly, there were no smoking or interactive effects seen for neuronal density of the SFG, STG, or supramarginal gyrus, but both alcoholic groups had fewer neurons in the SFG than their respective controls (Table 5).

Alcoholics versus controls

Due to the absence of smoking effects, the four groups were merged into alcoholics (nsALC and sALC) and controls (nsCON and sCON) and the volumetric analyses were repeated, including adjustment for the potential confounders: age, PMI, and liver pathology (score). This led to the disappearance of significant alcohol effects, although strong trends ($p < 0.05$) remained for decreased volume of the prefrontal WM, posterior WM, total WM, and total brain volume (Table 6). In terms of neuronal density, there was a 29% reduction seen in the alcoholic SFG and a trend ($p = 0.07$) for reduced neuronal density in the STG, but no change in the supramarginal gyrus (Table 5). However, when the neuronal counts were similarly adjusted for potential confounders, only a moderate trend for reduced neuronal density in the alcoholic-SFG remained ($p = 0.06$; Table 6).

Pearson correlation analyses showed no significant correlations or trends between lifetime alcohol consumption in the alcoholics and ROIs including total WM ($n = 19$, $r^2 = 0.07$, $p = 0.5$) (Fig. 3C) and total GM ($r^2 = 0.004$, $p = 0.8$) (Fig. 3D).

Discussion

The results found no evidence that cigarette smoking has any neurodegenerative effects in either alcoholics or controls. All trends for decreases in regional volumes could be attributed to alcohol alone. Similarly, the decrease in neurons in the SFG (prefrontal cortex) was due to the effects of alcohol. Importantly, all these differences became trends when adjusted for the potential confounders, age, PMI, and degree of liver pathology. These trends are consistent with the widespread WM loss and decreased number of prefrontal cortex neurons seen in previous pathological studies (de la Monte, 1988; Harper, Kril, & Holloway, 1985; Kril et al., 1997). Albeit, Kril and colleagues reported that only prefrontal cortex neuronal density was significantly different between chronic alcoholics and controls (Kril et al., 1997).

There were no regions where smoking had an effect on volume across the four groups, and there were no regional differences between sCON and nsCON. Similarly, there were no smoking effects on neuronal density. The only regions in which there were trends for a difference in volume based on smoking status suggested an increase in volume in the smokers, rather than a decrease. Furthermore, there were numerically lower volumes in the nsALC compared to the sALC across the majority of the ROIs, opposite to the hypothesized neurodegenerative effects of smoking. This is particularly notable given the high mean pack-years (~37 pack-years) in the two smoking groups here, whereas 20 pack-years has been used previously as a threshold for heavy smoking (Brody et al., 2004; Lin, Wu, Zhu, & Le, 2013).

The results of the present study are contrary to the findings of a number of neuroimaging studies in which smoking caused regional brain volume loss. It is unclear why the current results are at odds with some of the previous neuroimaging studies on smoking and in smoking alcoholics (Durazzo et al., 2014). Certainly there is some discordance in the neuroimaging studies, with some studies finding volume increases or no effects. There are the obvious methodological differences but it has been shown that formalin fixation (part of the protocol in pathological studies) causes very little change in brain volume (Double et al., 1996; Harasty, Halliday, Kril, & Code, 1999). Furthermore, the point-counting technique used here has been applied to various neurodegenerative diseases (Gregory, Macdonald, Schofield, Kril, & Halliday, 2006; Halliday et al., 2003; Kril et al., 1997; Kril, Macdonald, Patel, Png, & Halliday, 2005) and normal aging (Double et al., 1996; Piguet et al., 2009). In general, there seems to be a good correlation between *in vivo* MRI and pathologically confirmed atrophy in diseases such as AD (Csernansky et al., 2004; Jack et al., 2002). However, in normal aging, like ARBD, where more subtle atrophy is seen, MRI studies often overestimate GM losses (Piguet et al., 2009). Nevertheless the potential pitfalls inherent to the volumetric technique, including its low resolution (Kril & Halliday, 1999), may still have prevented the detection of volume changes here, particularly in regions with a smaller cross-sectional area such as the cingulate cortex.

Indeed, there were trends for inverse correlations between three GM regions (total GM, frontal pole, and temporal pole) and interestingly, these trends remained among the smoking controls ($n = 9$). However, these correlations may well be spurious, given that there were no group differences – the trends depended heavily on a sALC with the highest pack-years (80), and they disappeared among the smoking alcoholics ($n = 11$).

Taking into account the above caveat, combined with the repeated observations of cognitive impairment and decreased neuronal viability from magnetic resonance spectroscopy studies in chronic smokers, the results suggest that it may require ultrastructural or molecular techniques to tease out smoking effects on the brain. Electron microscopy and matrix-assisted laser desorption/ionization (MALDI) imaging (Yalcin & de la Monte, 2015) are suitable tools for such detailed studies, with the latter recently used to demonstrate perturbed brain lipid profiles in a rat model of chronic alcohol consumption and smoking (Yalcin, Nunez, Tong, & de la Monte, 2015).

In contrast to the results for smoking, the current study provides support for the detrimental effect of chronic alcohol consumption on the brain. There were trends for alcohol decreasing WM volumes across the brain but decreasing neuronal density only in the SFG (prefrontal cortex) of the three areas examined. Albeit, no ROI showed significant volume loss, but these trends are consistent with a previous report of global WM loss (Harper & Kril, 1985). Having said this, other studies have found only regional volume loss (Pfefferbaum et al., 1997) or trends for regional WM volume loss (Krill et al., 1997). Furthermore, the WM loss found in the latter two studies was mainly confined to the frontal lobe where it was thought to be secondary to the loss of the neuronal cell body in adjacent GM. In the present study, there were similar effect sizes in posterior WM and frontal WM. If WM loss were secondary to neuronal loss, then it would be expected to find more severe WM loss within the frontal lobe. This disparity between WM and GM suggests that twin pathogenic mechanisms are in operation, one affecting the WM and one affecting the GM.

Notwithstanding a possible influence from smoking, the pathogenesis of ARBD is thought to involve synergistic interactions between the direct effects of alcohol, thiamine deficiency, and liver damage (Butterworth, 1995; Kril, 1995). The combined alcoholic group included seven individuals with cirrhosis but multivariate analyses showed that liver pathology had no significant effect on brain atrophy. There were trends for lower volumes in alcoholics with liver pathology (cirrhosis and steatosis groups combined) than in alcoholics with no liver pathology. Alcoholics with cirrhosis are more likely to have acute brain changes that affect postmortem tissue (Sheedy, Say, Stevens, Harper, & Kril, 2012). However, previous studies suggest the effects of thiamine deficiency rather than liver pathology have greater effects on brain atrophy (Krill, 1995; Kril & Butterworth, 1997). Of the other confounders examined, there was only a trend toward an inverse correlation between age and the volume of the temporal pole ($p = 0.03$), while PMI had no effects.

An interesting aspect of the results is that, despite a clear association between WM volumes and alcoholic status, there were no correlations with lifetime alcohol consumption. This may reflect the variation in alcohol consumption required to inflict ARBD among different individuals, but a caveat here is that the NSW BTRC relies on review of medical records and

next-of-kin questionnaires to calculate lifetime alcohol consumption. Despite an independent validation of this approach (Koenig, Jacob, & Haber, 2009), it must be regarded as an estimate of true alcohol use.

A potential WM target for alcohol is the oligodendrocyte, as this cell type has been shown to be as vulnerable to alcohol as neurons in an animal model (Creeley, Dikranian, Johnson, Farber, & Olney, 2013). An important aspect of ARBD is the repeated observations of the reversibility of some of the cognitive impairments with abstinence (Mann, Günther, Stetter, & Ackermann, 1999), predominantly in younger individuals (Pfefferbaum, Adalsteinsson & Sullivan, 2006; Pfefferbaum et al., 2014). In particular, reversibility of WM damage has been demonstrated (Gazdzinski, Durazzo, Mon, Yeh, & Meyerhoff, 2010) as well as further WM damage with alcohol relapse (Pfefferbaum et al., 2014). The reversibility could be due to myelin restoration and fiber reorganization (Pfefferbaum et al., 2014) following mature oligodendrocyte functional recovery or replacement by oligodendrocyte precursor cells (Mi et al., 2009). In contrast, the neuronal loss in the frontal lobe could explain the cognitive deficits that persist even with abstinence.

Having suggested that WM and GM are affected by different mechanisms, it must also be noted that regional neuronal counts here have not been exhaustive. Decreases in neuronal density and changes in neuronal morphology have been reported in the prefrontal cortex (Harper & Kril, 1989; Harper, Kril, & Daly, 1987; Kril et al., 1997). However, unlike the subcortex (see Table 10.1, Greenfield's Pathology, 8th Ed.; Harris, Chimelli, Kril, & Ray, 2008), very few other cortical regions have been examined for changes in neuronal density. Three of the previous studies only compared SFG to the primary motor cortex, while the Kril et al., 1989 study involved neuronal counts in the inferior temporal and cingulate cortices in addition to the motor cortex and SFG. The current study extends this work to the supramarginal and superior temporal cortices. Although no effects were seen, there is still the likelihood that neuronal loss in other regions may explain the widespread, but still secondary, WM loss. Such a hypothesis is supported by the fact that the 29% loss of SFG neurons seen here occurred in the absence of any observable SFG-GM volume changes.

The current study, like many human pathological studies, was small with only eight subjects in the nsALC group and the eight controls for each gender. In particular, the high comorbidity of chronic alcohol use and chronic tobacco smoking limited the size of the nsALC group. Notwithstanding the low sample size, the risk of Type I error was attenuated to some extent by the reduction of the α level using a modified Bonferroni procedure (Sankoh, Huque, & Dubey, 1997). Similarly, a study of this size is ill-equipped to explore confounders with multivariate analyses. Nevertheless we felt compelled to include age and liver pathology here, given their known correlation with brain atrophy (Kril, 2003; Piguet et al., 2009) and contribution to ARBD (Kril, 1995), respectively. Adjustments for multiple comparisons have been made in previous neuroimaging studies (Durazzo, Meyerhoff, & Nixon, 2013; Durazzo et al., 2014), but there is also inconsistency in how different research groups have approached this issue. Some previous studies separated the ROIs into groups (such as subcortical GM and cortical GM) and performed analyses separately in each group. The current study adopted the more conservative approach of grouping all ROIs into the same analysis.

An important point of difference between the current study and previous neuroimaging study of smoking alcoholics may be the derivation of cases. A large proportion of the smoking and alcohol literature comes from the United States; in particular studies undertaken in San Francisco by the Meyerhoff group. The participants for their studies are recruited from a substance-abuse center (treatment-seeking alcoholics), with prior drug use other than alcohol or nicotine permitted if it has not occurred within the last 5 years. In contrast, the cohort from the NSW BTRC excludes any individual with a history of drug abuse and also excludes individuals with a neuropsychiatric history. This makes the NSW BTRC cohort one of the 'cleanest' alcoholic cohorts because of the absence of poly-drug users as well as subjects with neurological disorders.

In conclusion, these findings do not support main or interactive effects of smoking on ARBD. They do support the widespread susceptibility of the WM and the specific susceptibility of neurons in the SFG (prefrontal cortex) to the direct effects of chronic alcohol consumption.

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Highlights

1. The first pathological study of smoking in alcoholics and controls
2. No main or interactive effects of smoking on brain atrophy or neuronal density
3. Support evidence for widespread white matter loss in alcohol-related brain damage but only limited and focal neuronal loss

Fig. 1. Parcellation of white matter

A medial view of the left hemisphere shows approximate boundaries of WM regions of interest (ROI) explored in the study. A = posterior WM, B = middle WM, C = posterior frontal WM, D = prefrontal WM, and E = temporal WM.

Fig. 2. Point-counting technique

A transparent counting grid is overlaid across a typical coronal section image of the right hemisphere. Individual crosses are then counted within the various white and grey matter regions. The boundaries between regions of the frontal lobe have been manually drawn (in red).

Fig. 3. Lifetime smoking and alcohol use and brain atrophy

Scatter plots show the Pearson correlations between cigarette smoking pack-years and (A) total white matter (WM) and (B) total gray matter (GM) volumes, and the correlations between lifetime alcohol consumption and (C) total WM and (D) total GM volumes. Plots generated in JMP® Pro 9.0.0.

Table 1

Demographics and clinical characteristics

Parameter Mean (S.D.)	nsCON	sCON	nsALC	sALC
Group size	16	9	8	11
Males/Females	8/8	5/4	5/3	5/6
Mean age (y)	54 (9)	56(5.)	52 (8)	56 (6)
Brain weight (g)	1408 (165)	1401 (121)	1293 (120)	1332 (180)
Postmortem interval (h)	29 (11)	26 [*] (11)	36 (14)	38 [*] (16)
Brain pH	6.6 (0.3)	6.5 (0.3)	6.7 (0.1)	6.6 (0.2)
RNA integrity number	6.8 (1.6)	7.4 (0.5)	7.4 (1.0)	7.3 (1.3)
Body mass index	29.9 (5.7)	29.9 (4.4)	31.9 (18.0)	25.1 (6.4)
Liver score (0,1,2) ^{a, b, c}	8:7:0	4:5:0	0:4:4	4:4:3
Mean daily alcohol (g)	11 (13)	13 (11)	317 ^{**} (204)	189 ^{**} (104)
Lifetime alcohol (kg)	120 (135)	149 (146)	3503 ^{**} (2228)	2314 ^{**} (1478)
DSM-IV (Abuse:Dependence)	—	—	2:6	5:6
Pack-years	—	35 ^{**} (18)	—	39 ^{**} (18)

^{*}
 $p < 0.05$,

^{**}
 $p < 0.01$; compared to nsCON or for all alcoholics compared to all controls

^a Liver pathology score of 0 = normal, 1 = steatosis, and 2 = cirrhosis

^b No liver score was available for one nsCON

^c ChiSquare test unreliable

Table 2

Characteristics of combined Alcoholic and Control groups

Parameter Mean (S.D.)	Controls	Alcoholics
Group size	25	19
Males/Females	13/12	10/9
Age	54.5 (8.0)	53.9 (6.9)
Brain weight (g)	1406 (148)	1316 (153)
Postmortem interval (h)	28 [*] (11)	38 [*] (15)
Brain pH	6.5 (0.3)	6.6 (0.2)
RNA integrity number	7.0 (1.3)	7.3 (1.2)
Body mass index	29.9 (5.1)	28.0 (12.7)
Liver score (0,1,2) ^{a, b, c}	12:12:0	4:8:7 ^{**}
Mean daily alcohol (g)	11 (12)	242 ^{**} (163)
Lifetime alcohol (kg)	131 (137)	2815 ^{**} (1873)
DSM-IV (Abuse:Dependence)	—	7:12
Pack-years	12.6	22.6

^{*}
 $p < 0.05$,

^{**}
 $p < 0.01$; compared to nsCON or for all alcoholics compared to all controls

^a Liver pathology score of 0 = normal, 1 = steatosis, and 2 = cirrhosis

^b No liver score was available for one nsCON

^c ChiSquare test unreliable

Table 3

The effects of alcohol and smoking on GM volumes

Region	Normalized group volumes					two-way ANOVA (<i>p</i> value)		Pairwise comparisons (<i>p</i> value)							
	nsCON	sCON	nsALC	sALC	Overall	ALC main effect	Smoking main effect	ALC × smoking	nsCON vs sCON	nsCON vs nsALC	sCON vs sALC	nsCON vs nsALC	sCON vs sALC	nsALC vs sALC	nsALC vs sALC
Global	1.00	0.99	0.91	0.94	0.20	0.04	0.71	0.56	—	—	—	—	—	—	—
Total volume	1.00	0.99	0.88	0.93	0.07	0.01	0.55	0.44	—	0.02	—	0.04	—	—	—
Frontal	1.00	0.97	0.95	0.91	0.63	0.33	0.53	0.99	—	—	—	—	—	—	—
Frontal pole	1.00	0.97	0.95	0.91	0.63	0.33	0.53	0.99	—	—	—	—	—	—	—
ACC	1.00	1.26	1.08	1.01	0.21	0.37	0.34	0.09	0.05	—	—	—	—	—	—
IFG	1.00	1.02	0.91	0.97	0.77	0.33	0.59	0.80	—	—	—	—	—	—	—
SFG	1.00	1.03	0.96	0.92	0.51	0.18	0.91	0.51	—	—	—	—	—	—	—
Orbit	1.00	1.04	0.96	0.90	0.37	0.12	0.94	0.41	—	—	—	—	—	—	—
Motor cortices	1.00	0.98	1.03	0.98	0.90	0.71	0.50	0.79	—	—	—	—	—	—	—
Temporal	1.00	1.01	0.97	0.93	0.55	0.21	0.75	0.59	—	—	—	—	—	—	—
Total frontal	1.00	0.98	1.03	0.98	0.18	0.06	0.55	0.71	—	0.05	—	—	—	—	—
Temporal pole	1.00	0.98	1.03	0.98	0.18	0.06	0.55	0.71	—	0.05	—	—	—	—	—
Temporal gyri	1.00	0.98	0.86	0.90	0.19	0.03	0.84	0.57	—	—	—	—	—	—	—
Med. temporal lobe	1.00	0.96	0.99	0.95	0.83	0.86	0.41	0.90	—	—	—	—	—	—	—
Parietal	1.00	0.97	0.89	0.90	0.13	0.03	0.77	0.59	—	—	—	—	—	—	—
Total temporal	1.00	0.97	0.89	0.90	0.13	0.03	0.77	0.59	—	—	—	—	—	—	—
Sensory	1.00	1.11	0.98	1.13	0.20	0.98	0.04	0.75	—	—	—	—	—	—	—
Supramarginal gyrus	1.00	1.13	1.02	1.21	0.11	0.46	0.04	0.68	—	—	0.03	—	—	—	—
PCC	1.00	0.88	0.87	0.92	0.21	0.43	0.52	0.10	—	—	—	—	—	—	—
Occipital	1.00	0.94	0.89	0.96	0.33	0.33	0.91	0.13	—	—	—	—	—	—	—
Sup. Parietal lobule	1.00	0.94	0.89	0.96	0.33	0.33	0.91	0.13	—	—	—	—	—	—	—
Angular gyrus	1.00	0.95	0.90	0.88	0.22	0.09	0.46	0.67	—	—	—	—	—	—	—
Area 37	1.00	0.94	0.82	0.92	0.25	0.12	0.77	0.22	—	0.05	—	—	—	—	—
Precuneus	1.00	1.09	1.00	1.19	0.26	0.52	0.09	0.54	—	—	—	—	—	—	—
Other Regions	1.00	0.98	0.90	0.98	0.31	0.22	0.41	0.18	—	—	—	—	—	—	—
Total parietal	1.00	0.98	0.90	0.98	0.31	0.22	0.41	0.18	—	—	—	—	—	—	—
Total occipital	1.00	1.01	0.87	0.94	0.14	0.03	0.35	0.53	—	0.04	—	0.05	—	—	—
Deep GM	1.00	0.99	0.90	0.92	0.214	0.05	0.90	0.64	—	—	—	—	—	—	—
Insula	1.00	1.04	0.92	1.02	0.53	0.35	0.21	0.61	—	—	—	—	—	—	—

Region	Normalized group volumes				two-way ANOVA (<i>p</i> value)		Pairwise comparisons (<i>p</i> value)							
	nsCON	sCON	nsALC	sALC	Overall	Smoking main effect	ALC main effect	ALC × smoking	nsCON vs sCON	nsCON vs nsALC	nsCON vs sALC	sCON vs nsALC	sCON vs sALC	nsALC vs sALC
Hippocampus	1.00	0.88	0.86	0.97	0.24	0.69	0.88	0.05	—	—	—	—	—	—
Amygdala	1.00	1.03	0.98	0.97	0.83	0.41	0.77	0.67	—	—	—	—	—	—

Shading denotes analyses where trends ($p < 0.05$) were found.

Volumes are expressed as a proportion of nsCON.

nsCON = non-smoking control, sCON = smoking control, nsALC = non-smoking alcoholic, and sALC = smoking alcoholic

Table 4

The effects of smoking and alcohol on WM volumes

Region	Normalized group volumes				two-way ANOVA (<i>p</i> value)				Pairwise comparisons (<i>p</i> value)							
	nsCON	sCON	nsALC	sALC	Overall	ALC main effect	Smoking main effect	ALC × smoking	nsCON vs sCON	nsCON vs nsALC	nsCON vs sALC	sCON vs nsALC	sCON vs sALC	nsALC vs nsALC	sCON vs nsALC	nsALC vs sALC
	1.00	1.00	0.87	0.88	0.17	0.03	0.96	0.92	—	—	—	—	—	—	—	—
Prefrontal WM	1.00	1.00	0.87	0.88	0.17	0.03	0.96	0.92	—	—	—	—	—	—	—	—
Post. frontal WM	1.00	1.04	0.91	0.99	0.34	0.13	0.21	0.70	—	—	—	—	—	—	—	—
Temporal WM	1.00	0.99	0.85	0.87	0.10	0.02	0.82	0.75	—	0.05	—	—	—	—	—	—
Mid WM	1.00	0.96	0.77	0.92	0.03	0.08	0.34	0.09	—	0.004	—	0.03	—	—	—	—
Posterior WM	1.00	1.03	0.83	0.92	0.08	0.01	0.28	0.58	—	0.03	—	0.02	—	—	—	—
Total WM	1.00	0.99	0.83	0.91	0.04	0.006	0.45	0.38	—	0.009	—	0.02	—	—	—	—

Shading denotes areas where trends ($p < 0.05$) were found. Bolded text denotes adjusted statistical significance ($p < 0.007$).

Volumes are expressed as a proportion of nsCON.

nsCON = non-smoking control, sCON = smoking control, nsALC = non-smoking alcoholic, and sALC = smoking alcoholic

Table 5

Main effects and group effects on neuronal density measures

Region	Mean neuronal density				Two-way ANOVA (<i>p</i> value)				Pairwise comparisons (<i>p</i> value)							
	nsCON	sCON	nsALC	sALC	Overall	ALC main effect	Smoking main effect	ALC × smoking	nsCON vs sCON	nsCON vs nsALC	nsCON vs sALC	sCON vs nsALC	sCON vs sALC	nsALC vs sALC	nsALC vs sCON	nsALC vs sALC
SFG	120.4	126.0	91.1	98.2	0.02	0.002	0.45	0.93	—	0.02	0.04	0.01	0.03	—	—	—
STG	162.3	177.8	143.2	153.8	0.28	0.07	0.27	0.83	—	—	—	—	—	—	—	—
Supramarginal	227.2	224.3	216.8	234.8	0.75	1.00	0.51	0.35	—	—	—	—	—	—	—	—

Shaded with bolded text denotes statistical significance ($p < 0.05$).

Mean neuronal density expressed in units of neurons per square mm

nsCON = non-smoking control, sCON = smoking control, nsALC = non-smoking alcoholic, and sALC = smoking alcoholic

Table 6

The effects of alcohol on brain volume and neuronal density

Regions	Control	Alcoholic	<i>p</i> value
Prefrontal WM	1.00	0.88	0.02
Posterior WM	1.01	0.88	0.02
Total WM	1.00	0.88	0.03
Total brain volume	1.00	0.91	0.05
Occipital GM	1.01	0.91	0.06
Temporal gyri	0.99	0.89	0.10
Temporal pole	0.97	0.82	0.10
Total GM	1.00	0.93	0.12
SFG neuronal density*	122 (27)	95 (26)	0.06

* Mean neurons per mm² (S.D.)

Adjusted for age, PMI, liver score

Volume expressed as a proportion of nsCON

Controls = nsCON + sCON, Alcoholic = sALC + nsALC

Shaded denotes trends with $p < 0.05$