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The effects of chronic alcoholism on cell proliferation in the human brain

G.T. Sutherland [a,*], P.J. Sheahan [a], J. Matthews [a], C.V.P. Dennis [a], D.S. Sheedy [a], T. McCrossin [a], M.A. Curtis [b,c], J.J. Kril [a,d]

[a] Discipline of Pathology, Sydney Medical School, The University of Sydney, NSW 2006, Australia
[b] Department of Anatomy and Radiology, University of Auckland, Private Bag 92019, Auckland, New Zealand
[c] Centre for Brain Research, University of Auckland, Private Bag 92019, Auckland, New Zealand
[d] Discipline of Medicine, Sydney Medical School, The University of Sydney, NSW 2006, Australia

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ABSTRACT

Neurogenesis continues in the human subventricular zone and to a lesser extent in the hippocampal subgranular zone throughout life. Subventricular zone-derived neuroblasts migrate to the olfactory bulb where survivors become integrated as interneurons and are postulated to contribute to odor discrimination. Adult neurogenesis is dysregulated in many neurological, neurovascular and neurodegenerative diseases. Alcohol abuse can result in a neurodegenerative condition called alcohol-related brain damage. Alcohol-related brain damage manifests clinically as cognitive dysfunction and the loss of smell sensation (hyposmia) and pathologically as generalized white matter atrophy and focal neuronal loss. The exact mechanism linking chronic alcohol intoxication with alcohol-related brain damage remains largely unknown but rodent models suggest that decreased neurogenesis is an important component. We investigated this idea by comparing proliferative events in the subventricular zone and olfactory bulb of a well-characterized cohort of 15 chronic alcoholics and 16 age-matched controls. In contrast to the findings in animal models there was no difference in the number of proliferative cell nuclear antigen-positive cells in the subventricular zone of alcoholics (mean ± SD = 28.7 ± 20.0) and controls (27.6 ± 18.9, $p = 1.0$). There were also no differences in either the total ($p = 0.89$) or proliferative cells ($p = 0.98$) in the granular cell layer of the olfactory bulb. Our findings show that chronic alcohol consumption does not affect cell proliferation in the human SVZ or olfactory bulb. In fact only microglial proliferation could be demonstrated in the latter. Therefore neurogenic deficits are unlikely to contribute to hyposmia in chronic alcoholics.

Introduction

Alcohol abuse is the world's third leading cause of disease and disability (World Health Organisation, 2011). In the United States 6.7% of the population over 12 years of age were classified as heavy drinkers while 23% of the population had participated in binge drinking (Substance Abuse and Mental Health Services Administration, 2010). In Australia, 13% of all adults currently indulge in high-risk alcohol consumption (Australian Bureau of Statistics, 2006) and the societal cost is estimated to be $15 billion per annum (Collins and Lapsley, 2008).

One of the more serious consequences of chronic alcohol consumption is alcohol-related brain damage (ARBD). ARBD is characterized by impairment of cognitive functions (Green et al., 2010), including working memory deficits (Pfefferbaum et al., 2001) and smell sensation (hyposmia) (Rupp et al., 2003). On postmortem examination the brains of chronic alcoholic are mildly atrophic, largely due to white matter (WM) deficits (Harper et al., 1988; Pfefferbaum et al., 2009), but also focal neuronal loss (Harper et al., 1987). Previous work from our group has shown cortical neuronal loss from the prefrontal cortex of alcoholics, whereas other areas such as the primary motor cortex show no neuronal loss (Kril et al., 1997). Fundamental questions remain about how the chronic abuse of alcohol leads to ARBD, an area that is clouded by the concomitant effects of alcohol on other organs such as the liver, on nutrition, on risk-taking behavior potentially associated with head injury and the close association of alcoholism with other lifestyle choices such as smoking (Brust, 2010).

Adult mammalian neurogenesis, a recently identified paradigm in the adult human brain, occurs in the subgranular zone (SGZ) of the hippocampus and subventricular zone (SVZ) of the wall of the lateral ventricle (Eriksson et al., 1998). Neuroblasts derived from the SVZ migrate tangentially to the olfactory bulb (OB), via the rostral migratory stream (RMS), where most of the survivors become integrated into the granule cell layer (GCL) as interneurons (Altman, 1969; Doetsch and Alvarez-Buylla, 1996; Lois and Alvarez-Buylla, 1994). Animal studies suggest that these interneurons contribute to odor discrimination (Enwere et al., 2004; Sakamoto et al., 2011).

Neurogenesis is known to be dysregulated in neurological (epilepsy) (Liu et al., 2008), psychiatric (depression) (Lucassen et al., 2010), neurovascular (stroke) (Jin et al., 2006; Marti-Fabregas et al., 2010) and neurodegenerative diseases (reviewed by Curtis et al.(Curtis et al.,

* Corresponding author at: Discipline of Pathology, Rm. 504, Blackburn Building D06, University of Sydney, NSW 2006, Australia. Fax: +61 2 93513429.
E-mail address: g.sutherland@sydney.edu.au (G.T. Sutherland).

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2007a) and Thompson et al. (Thompson et al., 2008)). The latter include Alzheimer's (Crews et al., 2010), Huntington's disease (HD) (Curtis et al., 2003, 2005) and possibly Parkinson's disease (Hoglinger et al., 2004; van den Berge et al., 2011).

ARBD is also a neurodegenerative disease and animal models of both binge and chronic alcohol consumption have shown a decrease in hippocampal (SGZ) (He et al., 2005; Nixon and Crews, 2002; Taffe et al., 2010) and SVZ neurogenesis (Hansson et al., 2010), although a further study suggested that chronic alcohol exposure only decreased SGZ neurogenesis with no effect on the OB (Herrera et al., 2003).

Recent studies showing minimal neurogenesis in the adult human SGZ (Low et al., 2011; Lucassen et al., 2010) combined with work from our own laboratory showing no hippocampal neuron loss in chronic alcoholics (Harding et al., 1997) suggests that SGZ neurogenesis is unlikely to play a major role in ARBD.

In contrast there are relatively high levels of proliferation in the human SVZ (Low et al., 2011) and chronic alcohol consumption could well reduce adult born interneurons in the OB and contribute to the hyposmia associated with ARBD. Here we test this hypothesis by comparing proliferative events in the SVZ and OB of 15 chronic alcoholics and 16 controls.

Materials and methods

Case selection

Prior approval for this project was obtained from the Human Research Ethics Committee of the University of Sydney (HREC #13027). Tissue was obtained from the New South Wales Tissue Resource Centre (NSW TRC), a member of the Australian brain Bank Network and part-funded by the National Institute on Alcohol Abuse and Alcoholism (R24AA012725) to provide brain tissue for alcoholism research. The brain donor program of the NSW TRC is approved by the University of Sydney's Human Research Ethics Committee (HREC #X11-0107) and their banking procedures have been previously described (Sheedy et al., 2008). NSW TRC supplied information on age, gender, cause of death, post-mortem interval (PMI), brain pH, alcohol consumption, smoking status, liver pathology and macro- and microscopic neuropathology. The latter included a detailed assessment for metabolic (hepatic) encephalopathy (HE)(Harris et al., 2008), scored as none, mild (1 or more Alzheimer Type II astrocytes (At2a) per 200× field), moderate (2–3 At2a per field) or severe (mostly At2a). Lifestyle factors, including alcohol consumption and a Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) diagnosis were based on next of kin questionnaires and the available medical records. Mean daily alcohol consumption rates were recorded as grams of ethanol per day based on the number of standard drinks using Australian National Health and Medical Research Council guidelines (http://www.nhmrc.gov.au/your-health/alcohol-guidelines). Lifetime consumption (kg) was calculated using mean daily consumption rate × number of years drinking × 365. Chronic alcoholics were defined as those individuals who had consumed greater than 80 g of alcohol (ethanol) per day for the majority of their adult life (usually > 30 years) while controls had consumed less than 20 g of alcohol per day. Cigarette use was described in mean pack years where 1 mean pack year equals 1 packet of (20) cigarettes per day for one year.

Immunohistochemistry

Tissue blocks (20 mm × 15 mm × 3 mm) incorporating the wall of the lateral ventricle and corpus callosum at the level of the head of the caudate were obtained from fixed brain slices of 15 alcoholics and 16 controls. The 3 mm slices had been previously stored in 15% formalin for periods ranging from 4 to 11 years. Blocks of tissue were cryo-protected for two days in 30% sucrose in 0.1 M TBS (pH 7.4) with 0.1% (v/v) sodium azide, before embedding in OCT compound (Tissue-Tek, Torrance, CA, USA), freezing using CO_2 and sectioning into thick (48 μm) sections using a freezing microtome (Leica microsystems, Wetzlar, Germany). Thick sections were also cut from the available olfactory bulbs (OB) of eight controls and five alcoholics. Antigen retrieval for PCNA immunohistochemistry was performed on free-floating thick sections incubated overnight at 60 °C in 10 mM sodium citrate (pH 8.5). Sections were then incubated in 50% ethanol containing 1% (v/v) hydrogen peroxide for 30 min, followed by 10% normal horse serum (NHS) in 0.1 M TBST containing 0.1% Triton X-100 for 30 min. The PCNA primary antibody (PC-10; sc-56, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:750 (or 1:1000 for some OB sections) was applied for 1 h at room temperature (RT), then overnight at 4 °C, followed by 1 h at RT. The sections were incubated in a biotinylated secondary antibody (1:200; Vector Laboratories Inc., Burlingham, CA,) for 1 h, an avidin/biotin/peroxidase complex (1:100; Vectastain Elite ABC, Universal) for 1 h and diaminobenzidine (DAB) in the presence of 5% hydrogen peroxide for 20 min. In addition 7 μm thick sections of the middle lateral ventricle wall (level of nucleus accumbens) were cut from a formalin-fixed paraffin-embedded (FFPE) block of one of the controls (64-year old male) where the tissue had been fixed for three weeks before embedding. The thin sections were immunostained for PCNA and a second proliferative marker Ki-67 (MIB1, M7240, Dako, Carpinteria, CA). Here a harsher antigen retrieval regime was used with sections in 10 mM Tris/1 mM EDTA (TE buffer; pH 9) heated to 120 °C for 30 min in a decloaking chamber (Biocare Medical, Concord, CA) and incubated with PCNA (1:750) or Ki-67 (1:100). Cresyl violet (CV) and luxol fast blue (LFB) staining of thick and thin sections from selected individuals was performed according to standard protocols to demonstrate the cytoarchitecture.

FFPE thin sections were also cut from an OB of an additional neurologically normal control (49-year old male). This OB had been fixed for eight weeks before embedding. Antigen retrieval was performed in 10 mM Tris 1 mM EDTA buffer pH 9.0 using a decloaking chamber set at 95 °C for 20 min. The neuronal density in the GCL was investigated using the pan-neuronal marker NeuN (rabbit polyclonal (ABN78), 1:200, Merck Millipore, Billerica, USA) as described above.

Immunofluorescent microscopy experiments were also performed on these OB FFPE thin sections. PCNA antibody was used in a cocktail with either beta III tubulin (rabbit polyclonal, 1:250, GR79976, Abcam, Cambridge, UK), GFAP (glial fibrillary acidic protein; rabbit polyclonal, 1:200, Z0334, Dako), Iba1 (ionized calcium-binding adapter molecule 1, rabbit polyclonal (019-19741) 1:150, Wako Chemicals USA Inc), Olig2 (rabbit polyclonal, 1:100, GR80025-1, Abcam), NG2 (a chondroitin sulfate proteoglycan, rabbit polyclonal, 1:200, AB5320, Merck Millipore) or NeuN (1:100) antibodies.

Sections were incubated with the secondary antibodies: AlexaFluor 488, goat anti-rabbit IgG, A-11008 and AlexaFluor 594, goat anti-mouse IgG, A-11005 (Invitrogen) at 1:200 dilution, for 30 min. Sections were then incubated in 0.1% sudan black B (B.D.H Laboratory Chemicals Group) in 70% ethanol for 4 min, and counterstained with DAPI (3 μM; Invitrogen, D306). Sections were viewed with a fluorescent microscope (Olympus AX-70) and images taken with an Olympus DP70 camera and imported using DP controller v2.2 software. Images were also obtained using confocal microscopy (Leica SPE-II microscope with Leica LAS AF software platform, Leica Microsystems, Wetzlar, Germany) at the Advanced Microscopy Facility, Bosch Research Institute, University of Sydney. Contrast and brightness were minimally altered using GNU Image Manipulation Program (GIMP) v2.8 and co-localization images were created using either DP Manager v2.2 or Leica LAS AF software (confocal images). Images were annotated in Photoshop CS5 (Adobe) and GIMP v2.8.

Quantification

The combination of rare events and poorly defined boundaries meant that standard stereological methods could not be applied here.