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Chronic intermittent ethanol induced axon and myelin degeneration is attenuated by calpain inhibition

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ABSTRACT

Chronic alcohol consumption causes multifaceted damage to the central nervous system (CNS), underlying mechanisms of which are gradually being unraveled. In our previous studies, activation of calpain, a calcium-activated neutral protease has been found to cause detrimental alterations in spinal motor neurons following ethanol (EtOH) exposure *in vitro*. However, it is not known whether calpain plays a pivotal role in chronic EtOH exposure-induced structural damage to CNS *in vivo*. To test the possible involvement of calpain in EtOH-associated neurodegenerative mechanisms the present investigation was conducted in a well-established mouse model of alcohol dependence - chronic intermittent EtOH (CIE) exposure and withdrawal. Our studies indicated significant loss of axonal proteins (neurofilament light and heavy, 50–60%), myelin proteins (myelin basic protein, 20–40% proteolipid protein, 25%) and enzyme (2′, 3′-cyclic-nucleotide 3′-phosphodiesterase, 21–55%) following CIE in multiple regions of brain including hippocampus, corpus callosum, cerebellum, and importantly in spinal cord. These CIE-induced deleterious effects escalated after withdrawal in each CNS region tested. Increased expression and activity of calpain along with enhanced ratio of active calpain to calpastatin (sole endogenous inhibitor) was observed after withdrawal compared to EtOH exposure. Pharmacological inhibition of calpain with calpeptin (25 μg/kg) prior to each EtOH vapor inhalation significantly attenuated damage to axons and myelin as demonstrated by immunoprofiles of axonal and myelin proteins, and Luxol Fast Blue staining. Calpain inhibition significantly protected the ultrastructural integrity of axons and myelin compared to

Abbreviations: A.U., arbitrary units; CIE, chronic intermittent ethanol; CNPase, 2′, 3′-cyclic nucleotide 3′-phosphodiesterase; deNFP, dephosphorylated neurofilament protein; EM, electron microscopy; EtOH, ethanol; IR, immunoreactivity; LFB, Luxol Fast Blue; MBP, myelin basic protein; PBS, phosphate-buffered saline; PLP, proteolipid protein; NFP, neurofilament protein; NFH, neurofilament heavy; NFL, neurofilament light

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control as confirmed by electron microscopy. Together, these findings confirm CIE exposure and withdrawal induced structural alterations in axons and myelin, predominantly after withdrawal and corroborate calpain inhibition as a potential protective strategy against EtOH associated CNS degeneration.

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1. Introduction

Neuropathological hallmarks of heavy alcohol consumption include shrinkage of gray matter, enlargement of ventricles, and degeneration of the white matter leading to compromised brain structure and associated functional deficits (Harper, 2009; Harper and Kril, 1990; Laas and Hagel, 2000; Sullivan and Pfefferbaum, 2005). Evidence from postmortem human central nervous system (CNS) tissue from alcoholics indicated a loss in brain weight primarily due to the loss of white matter (de la Monte, 1988; Kril et al., 1997). *In vivo* imaging in human alcoholics further revealed perturbation in the microstructure of white matter in brain (Pfefferbaum et al., 2000; Pfefferbaum and Sullivan, 2002; Pfefferbaum and Sullivan, 2005; Pfefferbaum et al., 2006). Disruptions in circuitry identified in diverse brain regions were implicated as underlying causes for the cognitive and motor deficits seen in alcoholics (Chanraud et al., 2009; Colrain et al., 2011; Sullivan and Pfefferbaum, 2005). Importantly, these studies also revealed the protracted presence of altered microstructural profiles in recovering alcoholics (Rosenbloom et al., 2008), thus, warranting the need to address the mechanisms of white matter degeneration associated with chronic alcohol consumption and potential interventional strategies.

Postmortem studies in human alcoholics showed down regulation of several genes associated with axons and myelin as well as degeneration of myelin sheath (Lewohl et al., 2000; Pfefferbaum et al., 2009). Experimental studies *in utero* and in early postnatal days hypothesized that the degenerative effects of ethanol (EtOH) exposure on myelin lead to delayed myelination in cerebral cortex (Jacobson et al., 1979) and decreased number of myelinated axons in spinal cord (McNeill et al., 1991). Findings in an animal model of fetal alcohol syndrome have also confirmed decreased expression of mRNAs of the myelin components including 2′, 3′-cyclic nucleotide 3′- phosphodiesterase (CNPase), and myelin basic protein (MBP) associated with delayed myelination following *in utero* EtOH exposure (Kojima et al., 1994). Postnatal EtOH exposure permanently altered the expression of mRNAs encoding MBP and myelin-associated glycoprotein (MAG) and reduced the expression of selective isoforms of myelin proteins in the cerebellum of adult rodents (Zoeller et al., 1994). Thus, the EtOH exposure is known to cause abnormal effects during the early stages of brain development corresponding to the period of rapid myelination. Recently, the selective vulnerability of myelin to EtOH exposure in adolescent rodent brain was compared to adult and the involvement of TLR-4 was reported as a probable mechanism (Alfonso-Loeches et al., 2012; Pascual et al., 2014). However, less is known about the disruptive mechanisms of alcohol dependence on mature myelin in the adult brain. Disruption in myelin may eventually render the axons vulnerable. Axonal degeneration may also occur following damage to the neuronal cell bodies. Mechanisms by which EtOH trigger damage in brain is only partially understood, hence, this study was undertaken.

The effects of EtOH on the CNS are complex; in any rodent model these effects largely depend on the route of EtOH administration/exposure. Likewise, loss of axonal and myelin integrity in animal models of alcohol dependence, and the underlying mechanism of such degeneration are also subjective and may depend on the model being tested. The present study utilized a standardized chronic intermittent EtOH (CIE) vapor inhalation model that produces escalation in EtOH consumption in adult C57BL/6 J mice (Becker and Lopez, 2004; Griffin et al., 2009a, 2009b; Lopez and Becker, 2005). The model with alternating cycles of EtOH exposure and withdrawal has been extremely well investigated for behavioral cohorts. The model offers a strong platform for mechanistic studies. Further, the extent of EtOH-induced neurodegeneration can have site specificity in brain as reviewed recently (Szabo and Lippai, 2014); we chose to examine the EtOH effects in three regions in brain including hippocampus, corpus callosum, cerebellum and spinal cord - a novel CNS region to study the effects of EtOH.

While multiple factors have been implicated in the loss of axons and myelin in neurodegenerative diseases, and CNS injuries (Das et al., 2008; Geddes and Saatman, 2010; Podbielska et al., 2013; Ray et al., 2011; Samantaray et al., 2008) whether similar mechanisms such as protease activation, inflammatory factors and oxidative stress are also involved in degeneration of axons and myelin following chronic alcohol consumption is not clear. Over-activation of calpain is implicated in neurodegeneration in a wide range of neurological disorders (Bevers and Neumar, 2008; Saatman et al., 2010; Samantaray et al., 2008; Vosler et al., 2008). The challenge is to inhibit the pathological consequences of calpain over-activation while preserving the essential physiological aspects of calpain function. Since, calpain is present in the cytosol and myelin (Banik et al., 1985) and the substrates of two calpain isoforms are similar, and calpain inhibitors, *e.g.*, calpeptin inhibits both the isoforms with similar potency (Geddes and Saatman, 2010; Goll et al., 2003); we tested the efficacy of calpeptin against CIE exposure and withdrawal-induced degeneration axons and myelin *in vivo*.

In a nutshell, the objective of the study was to determine, if CIE exposure and withdrawal perturbs morphological and molecular parameters of myelin, causes axonal degeneration and identify the mechanisms that may regulate the damaging effects of EtOH on CNS axons and myelin. Our findings suggest that EtOH withdrawal causes aggravated degenerative effects on axonal and myelin integrity. In addition, we