Influence of Adolescent Heavy Session Drinking on the Systemic and Brain Innate Immune System

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Abstract — Aims: The aim of the study was to evaluate rat models of intermittent alcohol abuse (heavy session/‘heavy session’ drinking) in relation to inflammatory changes in specific brain regions as well as in the periphery. Furthermore, the study was aimed to assess whether there are inflammatory changes in the blood of human intermittent alcohol abusers who might be associated with changes in neuronal circuitry in the brain, as assessed by functional magnetic resonance imaging (fMRI), which cause adverse effects on memory and learning. Methods: Various regimes of intermittent alcohol administration have been used in rat models, which vary with respect to the dose and duration of ethanol administration as well as the time of abstinence. Immunohistochemical methods were used to identify activated microglia in specific brain regions. The response of isolated alveolar macrophages to in vitro stimuli was assessed by the assay of nitric oxide and the pro-inflammatory cytokines IL-6 and TNFα. Blood samples were collected from university students who had been heavy session drinkers for 2 years to assess whether there was an inflammatory cytokine profile that correlated with cognitive test scores as well as fMRI findings. Results: The extent of microglia activation appears to depend on the doses and duration of ethanol administration. In addition, there is activation of phagocytic cells in the periphery, e.g. alveolar macrophages, in the rat models of heavy session drinking. Changes in the plasma levels of pro- and anti-inflammatory cytokines were present in heavy session drinking students, although no changes were identified in specific cognitive tests (which may be because of compensatory changes in the prefrontal cortex, as identified by fMRI). Conclusion: Changes in the cytokine levels induced by intermittent ethanol abuse may provoke inflammatory pathways in specific brain regions, such as hippocampus and prefrontal cortex (particularly during the stage of active neurogenesis in the adolescent brain), which might induce cognitive impairment in susceptible individuals.

INTRODUCTION

Heavy session drinking (sometimes termed ‘binge drinking’) is the dominant form of alcohol misuse in individuals aged between 13 and 24. The age at which alcohol is first used is (a) a significant predictor of lifetime alcohol dependence, (b) an indicator of an earlier incidence of neurotoxicity and neurodegeneration and (c) an enhancer for the severity of later alcohol dependence (Hingson et al., 2006a,b). Such vulnerability in young heavy session drinkers is related to the fact that there is fine-tuning of specific neuronal connections, via synaptic pruning, during this adolescent period (Spear, 2000). Two brain regions show particularly marked ontogenetic alterations during adolescence. Firstly, the prefrontal cortex, where considerable remodeling occurs within regions that form an interconnecting network of circuits, i.e. the amygdala and extended amygdala, and other dopamine (DA) mesocorticolimbic terminal regions. Second, the hippocampus, where hippocampal stem cells are present in the subgranular zone inside the dentate gyrus granule cell layer (Palmer et al., 1997). These neural stem cells are linked to hippocampal function, which includes learning, memory and mood (Balu and Lucki, 2009). It is in these two regions where alcohol abuse may have its most profound neurotoxic effect, inducing adverse changes in structural integrity which could result in cognitive deficits (Crego et al., 2010), such as memory impairment (retrieval as well as poor attention), visual learning and memory (Sanhueza et al., 2011), executive function (Goudriaan et al., 2007; Scaife and Duka, 2009) and attention working memory (Hartley et al., 2004; Townshend and Duka, 2005). The adolescents investigated constitute a wide range of individuals from university students to uneducated individuals, and those who have responded to college programs as well as newspaper adverts. Furthermore, some studies have indicated that female heavy session drinkers may show greater cognitive deficits. For example, Scaife and Duka (2009) reported that female heavy session drinkers showed deficits in visual memory tasks, in addition to enhanced cognitive flexibility and working memory tasks, while Townshend and Duka (2005) reported that such women showed impaired spatial working memory and vigilance.

NEURO-PHYSICAL TECHNIQUES TO ASSESS CHANGES IN BRAIN FUNCTION IN INTERMITTENT ALCOHOL ABUSERS

A variety of neuro-physical techniques have confirmed significant changes in specific brain regions of heavy session drinkers when compared with age- and sex-matched controls. Electrophysiological markers have identified slowed cerebral activity associated with heavy session drinking, which was associated with impaired emotional auditory processing (Maurage et al., 2009), attention and decision-making (Maurage et al., 2012). Functional magnetic resonance imaging analyses (fMRI) revealed higher bilateral activity in the pre-supplementary motor area of the dorsomedial prefrontal cortex in heavy session drinkers when compared with age- and gender-matched controls (Campanella et al., 2013). However, these latter changes did not reflect gross changes in cognitive function. Lastly, MRI has identified specific reductions in the volume of the hippocampus and prefrontal cortex (reviewed by Crego et al., 2010), which are associated with the deficits in carrying out tasks involving prefrontal cortex, e.g. working memory, planning attention and decision-making. Such neuro-physical measurements have identified that the prefrontal cortex and hippocampus regions are showing marked differences after a heavy session drinking regime in adolescents.

ANIMAL MODELS OF HEAVY SESSION DRINKING

Despite the identification of such differences between heavy session drinkers and controls, with particular reference to the neuro-physical measurements and the changes in cognitive performance, the biochemical and neurochemical pathways...
underlying such susceptibilities are only now being slowly identified. A variety of animal models have been investigated to ascertain the basis of such neurotoxicity, which show wide variations with respect to species, sex, route of ethanol and dose and frequencies of ethanol administration. In some animal models, low ethanol doses, 2 or 3 g/kg/day, were administered either intraperitoneally (i.p.) or intragastrically (i.g.) for varying time periods—for example, 2 g/kg/day i.p. every other day to alcohol preferring (C57BL/6J, B6) and alcohol non-prefering (DBA2J, D2) (Kapasova and Szumlinski, 2008) and 3 g/kg i.p. on two consecutive days, followed by 2 days of abstinence for a period of 14 days (Pascual et al., 2009), while either 2 or 3 g/kg were administered 3×/day i.g. for 2 days, followed by 5 days of abstinence (Ward et al., 2009). In other animal models, even higher oral doses, 5 g/kg ethanol, were administered every third day for 18 days for varying lengthened time periods after an initial period of abstinence (Szumlinski et al., 2007), or for 2-day on–2-day off regime, from postnatal day P25 to P55 (Vetreno et al., 2013), or i.g. with 50% Vanilla Ensure every 8 h for 4 days (Penland et al., 2001). Whether the higher doses of ethanol induce an animal model more aligned to that of alcohol abuse remains debatable. Withdrawal symptoms, such as alcohol craving and tremor, have not been reported in human heavy session drinking adolescents.

ACTIVATION OF BRAIN INNATE IMMUNE SYSTEM IN ANIMAL MODELS OF INTERMITTENT ALCOHOLIZATION

Despite such variation in the animal models used, there is almost complete consensus that a heavy session drinking regime will activate microglia in specific regions of the brain. Microglia are regarded as the resident immune-competent effector cells for the innate immunity in the brain macrophages of the central nervous system (CNS) which regulate and remove cell debris after neuronal death and control apoptosis. They can communicate with the astrocytes and neurons and with other cells of the immune system by a number of signaling pathways. These glial cells have the capacity to interfere with synaptic turnover, thereby affecting synaptic architecture and function (reviewed by Blank and Prinz, 2013). They survey the microenvironment within the brain by extending and contracting processes into nearby synapses, thereby monitoring the functional state of the synapses, and contribute to plastic changes. In addition, they may regulate the transmitter concentration in the synaptic cleft since they have multiple neurotransmitter receptors (reviewed by Blank and Prinz, 2013). In the majority of these animal models of heavy session drinking, neuroinflammation occurs and microglia become activated (Fig. 1) with the release of pro-inflammatory cytokines, TNFα, IL-1β, IL-6, reactive nitrogen and oxygen species (e.g. changes in the gene expression of various factors involved in the assembly of NADPH oxidase, gp91phox, p67phox (Qin et al., 2008)), all of which will contribute to neuronal loss. The higher the dose and duration of ethanol administration, the more extensive the number of brain regions involved. For example, in our studies, when 2 or 3 g/kg of ethanol was administered three times/day i.g. for 2 days followed by abstinence for 5 days for a period of 4 weeks, microglia activation was present only in the dentate gyrus region of the hippocampus (Ward et al., 2009). In a more intensive ethanol administration (3 g/kg i.p. 2 days on and 2 days off for 14 days), inflammatory changes were present in both prefrontal cortex and hippocampus of male adolescent rats (Pascual et al., 2007), although these rats showed alcohol preference at the conclusion of the ethanol regime. In other studies in which even higher ethanol doses, 5 g/kg i.g. (every 8 h for 4 days 5 g/kg), were administered, a more widespread microglia infiltration was present in many brain regions, including the dentate gyrus and the hippocampus (McClain et al., 2011). However, whether this model is typical of a heavy session drinking regime is questionable. In contrast, in a study in which a dose of 3 g/kg was administered i.g. to male Wistar rats every 8 h for 4 days, postmortem evaluation of seven cytokines in six brain regions, including the frontal cortex and the hippocampus, revealed no significant increases in any of the cytokines in the alcohol rats (Zahr et al., 2010).

What is of interest is that such microglia, once activated, will remain activated throughout the heavy session drinking regime. This will have an adverse effect on the function of other glial cells, i.e. astrocytes and oligodendrocytes, as well as neurons (Monk and Shaw, 2006; Harry, 2013; Crichton and Ward, 2014). This is in contrast to normal circumstances, where these immune cells within the brain are only activated to a limited extent by any endogenous stimuli and rapidly revert to a quiescent state (Evo and Wu, 2013). Astrocyte

Fig. 1. On left-hand side: activated microglia within the hippocampal dentate gyrus region of adolescent female rats after 2 g/kg ethanol 3×/day for 2 days followed by 5 days of abstinence. This was repeated three times. Magnification ×20. On right-hand side: Hippocampal dentate gyrus region of adolescent control rat. Magnification ×20.
function may also be adversely affected, altering the release of neurotrophic factors essential for neuronal function, which may induce neuronal death, initiating neuropathology and neurodegeneration. Exactly why such cells remain activated in the heavy session drinking models is unknown, but may be caused by a continuing pro-inflammatory stimulus.

**ACTIVATION OF THE BRAIN INNATE IMMUNE SYSTEM BY SIGNALING FROM THE SYSTEMIC CIRCULATION AFTER INTERMITTENT ALCOHOL ABUSE**

The stimulus responsible for the activation of microglia after intermittent ethanol abuse remains unknown, but may be related to a direct effect of ethanol or its major metabolite on glial cells, changes in neurotransmitter metabolism, upregulation of toll-like receptors on glial cells (Fernandez-Lizarbe et al., 2009) or an indirect effect, such as cytokine signaling from the peripheral circulation. Pro-inflammatory cytokines released from activated cells in the periphery, e.g. monocytes and macrophages, can access the CNS, via the blood–brain barrier (BBB) and interact with receptors on glial cells, to influence every aspect of brain function from neurotransmitter metabolism, synaptic plasticity and neuroendocrine function as well as the neurocircuity that regulate mood, motor activity and anxiety. Such an increase in cytokine production and other inflammatory mediators within the systemic circulation after a heavy session drinking regime are possibly derived from activated macrophages and monocytes, or as a result of ethanol-induced mucosal injury in the upper gastrointestinal tract and the release of gut-derived endotoxins. Cytokines can cross the BBB through (a) increased leakiness of this barrier, (b) active transporters on brain endothelium, (c) activation of endothelial cells which are responsible for the subsequent release of second messengers, such as prostaglandins, (d) transmission of cytokine signals via afferent nerve fibers, the vates nerve and (e) entry into the brain parenchyma of peripherally activated monocytes (reviewed by Capuron and Miller, 2011).

In our recent studies, we investigated whether adolescent university heavy session drinkers show an altered pro- or anti-inflammatory profile in their blood. There was a significantly increased mean plasma TNFα value in male heavy session drinkers compared with controls, while the female heavy session drinkers showed a significantly lower mean value for TNFα, compared with controls. An inflammatory profile, as assessed by increased and decreased plasma values of IL-6 and IL-10, respectively, was evident in heavy session drinkers, although the mean values did not reach significance (Lallemand et al., 2013). In addition, monocytes isolated from the blood of these adolescents engaged in intermittent alcohol abuse and showed small but non-significant increases in their release of cytokines before and after stimulation with lipopolysaccharide (LPS) (Lallemand et al., 2013). However, gross neuropsychological changes were not identified in our heavy session drinking group, which may relate to the fact that such individuals were university students with high cognitive capacity.

Animal studies have also indicated a pro-inflammatory profile in the systemic circulation. For example, heavy session drinking rats, administered alcohol 3x/day for 2 days, followed by 5 days of abstinence, showed an elevated release of IL-6, TNFα and NO from isolated pulmonary alveolar macrophages before and particularly after in vitro stimulation with LPS (Ward et al., 2009). Such pro-inflammatory cytokines, such as IL-6 and TNFα, secreted by serum monocyte-macrophage-like cells can be transported across the blood–brain barrier into the brain, via saturable transport systems, to interact with receptors on glial cells and induce further release of cytokines, thus enhancing the pro-inflammatory milieu. Furthermore, such cytokines may compromise endothelial function and permeability of the BBB (Rachal Pugh et al., 2001), thereby facilitating the migration of inflammatory cells into the brain to promote neuroinflammation (Terrando et al., 2011) (Fig. 2). It is of interest that the receptors for TNFα and IL-6 are highly expressed in both the hippocampus and prefrontal cortex, which may account for their vulnerability to systemic pro-inflammatory cytokines (Chowdhury et al., 2005). Double knockout mice for TNF receptors 1 and 2 do not show significant brain induction of pro-inflammatory cytokines (Qin et al., 2007). The anti-inflammatory cytokine IL-10 will decrease the expression of several pro-inflammatory cytokines, including IL-1 and TNF-α (Knoblach and Faden, 1998; Csuka et al., 1999), in order to suppress further activation of microglia and astrocytes (Kremlev and Palmer, 2005). It could be hypothesized that the decreases in the levels of IL-10 evident in our heavy session drinking individuals might enhance the plasma inflammatory profile. IL-1β is capable of stimulating the release of other pro-inflammatory cytokines, such as TNF-α, which can bind to two known receptors expressed in the CNS, which induces various effects, including apoptosis possibly via NF-xB activation (Perry et al., 2001). Binding of TNF-α to TNFR2 appears to be involved in signaling pro-inflammatory responses as well as enhancing TNFR1-mediated apoptosis (Perry et al., 2001, 2007). TNF-α will also alter the permeability of the BBB.

It is of interest that chronic alcohol abuse does not have such a pro-stimulatory effect on phagocytic cells. Macrophages isolated from alcoholic subjects (Omivardi et al., 1998) or chronically alcoholized rats secrete less TNFα (Omivardi et al., 1998) and reactive nitrogen species, NO (Greenberg et al., 1994; Standiford and Danforth, 1997; Zhang et al., 1998), after LPS stimulation. Heavy session drinking may therefore have the effect of a priming stimulus within the systemic circulation, which will sensitize microglia to subsequent neurological challenge by successive heavy session drinking episodes.

**NEUROINFLAMMATION IN SPECIFIC BRAIN REGIONS INDUCED BY INTERMITTENT ALCOHOL ABUSE**

The hippocampal region appears to be particularly vulnerable to the toxic effects of heavy session drinking. Interleukin 1-beta (IL-1β), released from microglia, is critical for normal hippocampus-dependent learning and memory; in recent studies, it was shown that neonatal bacterial infection will lead to hippocampal-dependent memory deficits in adults, which were evident after a subsequent immune challenge (reviewed by Blank and Prinz, 2013). Such activation of microglia in the hippocampus, when active neurogenesis is occurring during adolescence, could induce changes in the ability of resting microglia to remove apoptotic new cells through phagocytosis (without necessarily being activated) as well as interfering with the release of unknown factors from microglia to stimulate neurogenesis (Kohman and Rhodes, 2013). Pro-inflammatory
cytokines, such as IL-1β, TNFα and IL-6, released from the activated microglia will affect hippocampal neurogenesis; neurons will show increased inhibitory input from synapses that could potentially alter their function.

**CYTOKINES AND MODULATION OF COGNITIVE FUNCTION AFTER INTERMITTENT ETHANOL ABUSE**

Considerable attention has recently been focused on the relationship between activation of the immune system in the periphery and the brain immune responses, such changes affecting cognitive function (Yirmiya and Goshen, 2011), including learning memory neural plasticity and neurogenesis. The induction or reduction of both pro- and anti-inflammatory mediators will disrupt the delicate balance required for the neurophysiological actions of immune processes and lead to neuroinflammation in various brain regions. Many cytokines are involved in the modulation of learning, memory, neural plasticity and neurogenesis (Yirmiya and Goshen, 2011). Chronic inflammation will affect various stages of memory formation from impairing acquisition to disrupting consolidation and reconsolidation, as well as disrupting certain aspects of long-term potentiation.

Microglia activation has been identified in the prefrontal cortex after intermittent ethanol abuse in some studies. Various cognitive functions are assigned to this brain region, which are adversely affected by chronic alcohol abuse, e.g. spatial working memory (Mackiewicz Seghe et al., 2013), executive function (Parada et al., 2012) and recognition working memory processes (Crego et al., 2010). Sanhueza et al. (2011) showed that young people who consumed ethanol 6 (male) or 8 (female) units/drinking session of 2–3 h, had worse performances than the control group in tasks related to executive functions such as cognitive control, working memory, planning and attention, which was possibly indicative of the damage to the prefrontal system. Goudriaan et al. (2007) showed that decision-making was increasingly impaired by the level of heavy session drinking in adolescents, although it was not related to impulsivity. Interestingly, British heavy session drinking university students show poorer sustained attention, episodic memory and planning ability as well as increased levels of self-rated anxiety and depression (Hartley et al., 2004). Clearly, further studies are needed to ascertain whether such changes in various cognitive functions are associated with a pro-inflammatory milieu in the peripheral circulation.

In conclusion, changes in the inflammatory profiles in the peripheral system of both adolescent heavy session drinkers and animal models of heavy session drinking have been identified, which could be important early markers of activation of the innate immune system in the periphery and brain, which may ultimately lead to neuroinflammation in the brain. This in turn could affect cognitive function. Further studies are needed in different groups of heavy session drinkers to ascertain whether such biomarkers of the innate immune system in the peripheral system may reflect neuro-inflammatory changes in the brain and impaired cognitive performance.

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