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Neuroimmune Activation and Myelin Changes in Adolescent Rats Exposed to High-Dose Alcohol and Associated Cognitive Dysfunction: A Review with Reference to Human Adolescent Drinking

María Pascual[1], Antoni Pla[1], José Miñarro[2] and Consuelo Guerri[1,*]

[1]Department of Cellular Pathology, Centro de Investigación Príncipe Felipe, C/Eduardo Primo Yúfera 3, 46012 Valencia, Spain and [2]Department of Psychobiology, Facultad de Psicología, Universitat de Valencia, Avda. Blasco Ibáñez, 21, 46010 Valencia, Spain, RTA-Network

*Corresponding author: Department of Cellular Pathology, Centro de Investigación Príncipe Felipe, C/ Eduardo Primo Yúfera 3, 46012 Valencia, Spain. Tel.:+34-96-3289680; Fax:+34-96-328-9701; E-mail: guerri@cipf.es

Abstract — Aims: The aim of the study was to assess whether intermittent ethanol administration to adolescent rats activates innate immune response and TLRs signalling causing myelin disruption and long-term cognitive and behavioural deficits. **Methods:** We used a rat model of intermittent binge-like ethanol exposure during adolescence. **Results:** Binge-like ethanol administration to adolescent rats increased the gene expression of TLR4 and TLR2 in the prefrontal cortex (PFC), as well as inflammatory cytokines TNFα and IL-1β. Up-regulation of TLRs and inflammatory mediators were linked with alterations in the levels of several myelin proteins in the PFC of adolescent rats. These events were associated with previously reported long-term cognitive dysfunctions. Conversely, the same ethanol treatment did not cause significant changes in either inflammatory mediators or myelin changes in the brain of adult rats. **Conclusion:** Activation of innate immune receptors TLRs in the PFC appears to be involved in the neuroinflammation and demyelination processes induced by ethanol exposure during adolescence. The findings support the vulnerability of the juvenile brain to the effects of ethanol and the long-term cognitive consequences of binge drinking. In addition, ethanol-induced PFC dysfunctions might underlie the propensity of adolescents for impulsivity and to ignore the negative consequences of their behaviour, both of which could increase the risk of substance abuse.

INTRODUCTION

Problematic alcohol consumption patterns, including binge drinking, are a constant, evident behaviour in many adolescents across Europe and the USA (Hibell *et al.*, 2009; Johnson *et al.*, 2009). The widespread occurrence in these youngsters of episodes of binge drinking and drunkenness deserves concern since a number of studies published within the last decade have suggested that binge drinking can have long-term adverse consequences during this stage of brain maturation. Studies report not only behavioural and cognitive deficits resulting from alcohol neurotoxicity on the highly vulnerable adolescent brain, but also increased vulnerability to alcohol dependence and addiction in early adulthood (Crews *et al.*, 2000; Zeigler *et al.*, 2005; Pascual *et al.*, 2007; Paus *et al.*, 2008).

Adolescence is a critical developmental period in which certain brain areas undergo important structural and functional changes in synaptic plasticity and neural connectivity (Giedd, 2004, 2008). These remodelling changes are associated with two cellular processes: myelination of axons and overproduction; elimination of synapses, or pruning. Both processes are important for efficient communication between brain regions, higher order cognitive functioning and complex behaviours. Clinical and experimental studies have demonstrated the vulnerability of the adolescent brain to the neurotoxic effects of alcohol (Guerri and Pascual, 2010; Jacobus and Tapert, 2013). Alcohol use by human adolescents is reported to alter white matter integrity as well as reduce myelin fibre tracts with frontal connections (Bava *et al.*, 2013), events that might underlie dysfunctions in the cognitive abilities of learning, memory and executive functions.

How alcohol induces myelin disruption in the developmental adolescent brain (Bava *et al.*, 2013) remains unknown. Using an experimental adolescent binge drinking model (Pascual *et al.*, 2007), we present some data which evidence that ethanol-induced inflammatory mediators might cause neurotoxicity and myelin disruption, events that are associated with the long-term cognitive effects induced by ethanol exposure during adolescence.

BINGE-LIKE ETHANOL TREATMENT, TLR4 AND TLR2 RECEPTORS AND NEUROINFLAMMATORY MEDIATORS IN ADOLESCENT PREFRONTAL CORTEX

Pascual *et al.* (2007) reported that intermittent binge-like ethanol administration to adolescent rats (see Fig. 1) up-regulated inflammatory mediators, such as the COX-2 and iNOS levels, and increased cell death in the neocortex, hippocampus and cerebellum. Chronic ethanol intake in adult mice caused activation of glial innate immune receptors, TLR4 (Fernandez-Lizarbe *et al.*, 2009; Alfonso-Loeches *et al.*, 2010). Activation of TLR4 signalling triggers the stimulation of transcription factor NFκB, which leads to the induction and release of inflammatory cytokines and other inflammatory mediators. Indeed, chronic ethanol intake increases the levels of cytokines and immune mediators in the brain, and causes gliosis and neuronal damage (Alfonso-Loeches *et al.*, 2010). Elimination of TLR4 in mice abolishes ethanol-induced neuroinflammation, gliosis and neuronal damage (Alfonso-Loeches *et al.*, 2010), suggesting the critical role of TLR4 in neuroinflammation and brain damage induced by chronic ethanol abuse in adult mice. Typically these animals are exposed via peritoneal injection to a dose of 3 g/kg (provoking a mean peak blood alcohol level of 190 ± 11 mg⁄dl) on two consecutive days, for 8 days in total with a 2-day interval between exposures.

We therefore evaluated the potential role of TLRs in ethanol-induced inflammatory mediators in the adolescent rat brain. Using the same paradigm of intermittent binge-like ethanol treatment in adolescent rats previously used in our laboratory (see Fig. 1), we demonstrate that ethanol treatment triggers the induction of the mRNA levels of TNF-α and

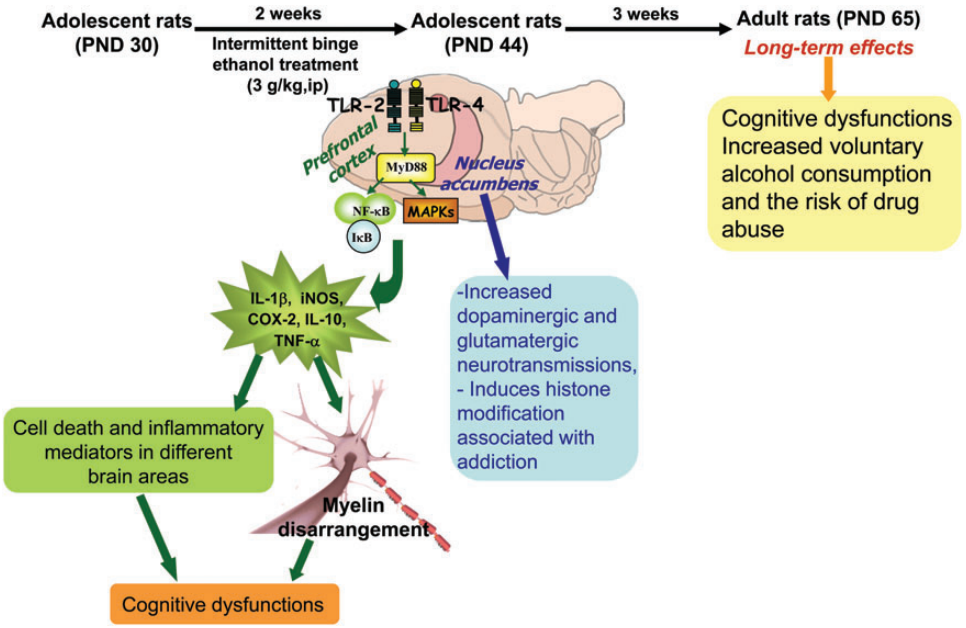

Fig. 1. Schematic representation of the time line of binge-like intermittent ethanol administration to adolescent rats, and the neurochemical and behavioural changes in adolescents' PND (post natal days) 44 and adults (PND 65). Proposed mechanism of ethanol-induced prefrontal cortex (PFC) damage: ethanol, by inducing the TLRs (TLR4 and TLR2) response, triggers inflammatory signalling in glial cells, leading to the release of inflammatory mediators and cytokines (iNOS, COX-2, IL-1β, TNF-α) which cause apoptotic neuronal death, gliosis and myelin disarrangements in the PFC. These events might underlie long-term cognitive and behavioural impairments. Binge-like ethanol administration during adolescence can also activate the mesocorticolimbic dopaminergic circuitry (e.g. *nucleus accumbens*) and alter the glutamatergic and dopaminergic neurotransmission, as well as chromatin remodelling, events that can mediate adolescents' vulnerability to the long-term consequences of alcohol addiction.

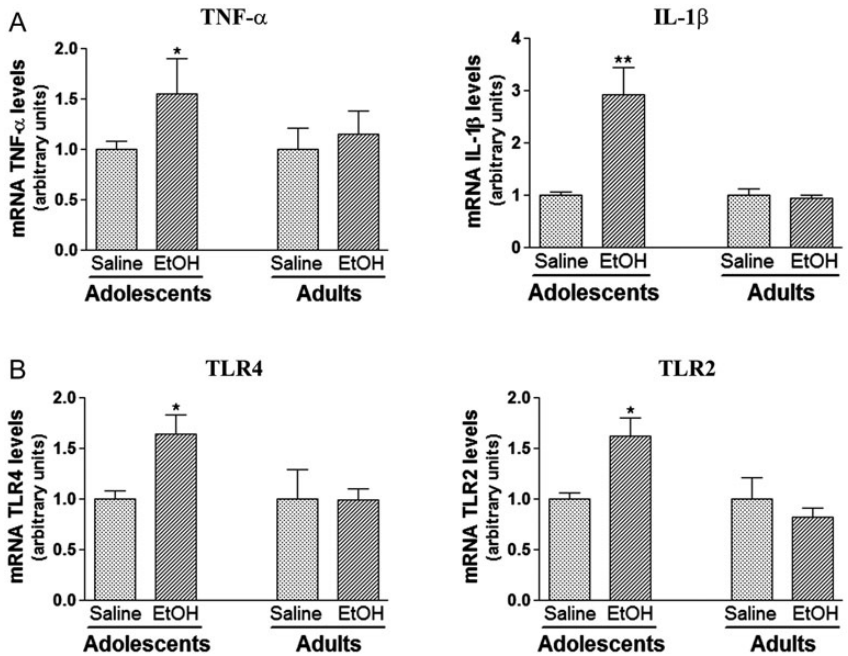

Fig. 2. Binge-like intermittent ethanol administration to adolescent rats increases cytokine levels and the expression of TLR4 and TLR2 in PFC. Adolescent (PND 44) and adult (PND 70) rats received an i.p. injection of ethanol (3 g/kg in 25% (v/v)) or saline in an intermittent ethanol administration pattern (two consecutive days at 48-h intervals over a 14-day period) (Pascual *et al.*, 2007). The peak of blood alcohol levels at 30 min post-injection was 190 ± 11 mg⁄dl. The mRNA levels of TNF-α, IL-1β, TLR4 and TLR2 were obtained by quantitative RT–PCR (see the primer sequences in Table 1), as previously described in Pascual *et al.* (2012). Values represent the mean ± SEM of four independent experiments. *$P < 0.05$ difference in relation to the saline group, according to an unpaired Student's *t*-test.

IL-1β in the prefrontal cortex (PFC) of female adolescent rats. Notably, the same ethanol treatment does not induce significant changes in the mRNA levels of TNF-α and IL-1β in adult rats when compared with untreated adult animals (Fig. 2A and Table 1, M. Pascual and A. Pla unpublished results). Recent data from our laboratory (Fernandez-Lizarbe *et al.*, 2013)

Table 1. mRNA primer sequences

Gene	Forward	Reverse
TNF-α	CTCTTCTCATTCCTGCTCGTGGCG	GCAGCCTTGTCCCTTGAAGAGAACC
IL-1β	TCATCTGGGATCCTCTCCAGTCAGG	AGCTCCACGGGCAAGACATAGG
TLR4	AATCTGGTGGCTGTGGAGAC	CTTGGGCTTGAATGGAGTC
TLR2	CTGATGGAGGTGGAGTTTGAT	GAAAGAGCAGGGAACCAGAA
Cyclophilin A	AGCACTGGGGAGAAAGGATT	AGCCACTCAGTCTTGGCAGT

shows that ethanol can recruit the activation of both TLR4 and TLR2 through its interaction with membrane microdomains *lipid rafts*, leading to a greater inflammatory response. Notably, evidence from our group demonstrated that ethanol treatment up-regulates the mRNA levels of TLR4 and TLR2 in the PFC of adolescent rats when compared with saline-treated control animals (Fig. 2B, M. Pascual and A. Pla unpublished results). However, no significant changes in TLR4 and TLR2 mRNA levels in the PFC were observed in adult rats treated with or without intermittent ethanol treatment. These results suggest that the ethanol-induced activation of TLR4 and TLR2 in the cerebral cortex of adolescent rats can trigger inflammatory cytokines and mediators, which might cause neuronal damage. According to this hypothesis, administration of indomethacin, a COX-2 inhibitor, abolishes the induction of both inflammatory mediators and cell damage in the PFC of the adolescent brain and prevents the long-term cognitive effects observed in adult rats (Pascual *et al.*, 2007). In agreement with our results, activation of TLR4 and TLR3 has been recently reported in the PFC of rats with binge drinking during adolescence (Vetreno and Crews, 2012) and that these effects correlate with the adult neurocognitive dysfunction. Interestingly, using a rat model of adolescent binge drinking and human post-mortem alcoholic brain, Vetreno *et al.* (2013) recently demonstrated that binge drinking during the adolescence upregulates the receptors, advanced glycation end products (RAGE) and TLR4, as well as their endogenous agonist, high-mobility group box 1 (HMGB1) in human and rats and that these effects persist in adulthood animals. The authors suggest that upregulation of RAGE/TLR4-HMGB1 and other neuroimmune genes that persist into young adulthood could contribute to the risk of alcoholism or other brain diseases associated with neuroinflammation. All together, these results provide evidence that the activation of the innate immune response is an important mechanism of ethanol-induced neurotoxicity during adolescence (Vetreno and Crews, 2012; Vetreno *et al.*, 2013).

ETHANOL-INDUCED UP-REGULATION OF TLRS AND ACTIVATION OF THE NEUROIMMUNE SYSTEM AND WITH MYELIN DISRUPTIONS IN THE ADOLESCENT BRAIN

Myelination and myelin integrity play an important role in the acquisition of cognitive functions and complex behaviours during adolescence (Paus *et al.*, 1999). Studies conducted in human adolescents have provided evidence that binge alcohol drinking reduces white matter quality and affects fibre tract integrity linked with frontal connections. These results suggest that ethanol impairs developmental fronto-thalamic tracts, event which might have functional and clinical consequences during young adulthood (Bava *et al.*, 2013).

The mechanisms by which ethanol-induced myelin disruption is caused in both binge-drinking adolescents and alcoholics (Pfefferbaum *et al.*, 2009) remain unclear. However, we recently reported important myelin fibre disruptions in the cerebral cortex and corpus callosum of chronic alcohol adult mice and that these effects are associated with the activation of TLR4 receptors and neuroinflammation. In fact, elimination of TLR4 (TLR4-KO) in mice abolishes ethanol-induced neuroinflammation and myelin disruptions (Alfonso-Loeches *et al.*, 2012), suggesting that ethanol-induced TLR4-dependent proinflammatory environment in the brain could participate in the myelin disruptions and white matter loss observed in human alcoholics.

Considering these results, we asked whether activation of TLR4 and TLR2 and neuroinflammation induced by intermittent ethanol exposure can induce myelin disruptions in the PFC of adolescent rats. Results from our group demonstrated (Fig. 3, M. Pascual and A. Pla unpublished results) that binge-like ethanol treatment significantly reduces the levels of two myelin proteins involved in the structural integrity to the myelin sheath (Lewohl *et al.*, 2005): myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). Conversely, the levels of NG2 proteoglycan and myelin associated glicoprotein (MAG) increase in the PFC of adolescent rats. Nevertheless, no significant changes in any of the myelin proteins analysed were observed in adult animals with the same ethanol treatment (Fig. 3). Notably, NG2 has been shown to promote oligodendrocytes proliferation and to increase in response to injury and demyelination (Kang *et al.*, 2010; Girolamo *et al.*, 2011), while MAG is involved in the protection of axons from acute toxic insults, including inflammatory mediators (Nguyen *et al.*, 2009). Up-regulation of NG2 and MAG observed in the adolescent animals treated with ethanol might be produced to compensate myelin dysfunctions and neural damage.

Alterations in the myelin integrity or reduction in white matter density induced by binge-like ethanol administration during adolescence might underlie some long-term cognitive dysfunctions observed in both human adolescents and experimental animals. Indeed, smaller volumes in prefrontal white matter (De Bellis *et al.*, 2005; Medina *et al.*, 2008) and deficits in attention and spatial working memory (Tapert *et al.*, 1999, 2004) have been reported in human adolescents with heavy alcohol use. Likewise, neuroimaging studies have demonstrated changes in the microstructural and functional myelin integrity in the superior longitudinal fasciculus in human adolescent alcohol users (Bava *et al.*, 2009; Schweinsburg *et al.*, 2010). These effects may represent alcohol-related neurotoxicity in cortical brain regions, which are important for fronto-parietal-temporal networks. Similarly, the alterations in myelin dysfunctions observed in the present study in female adolescent rats exposed to ethanol have been associated with important long-term memory and learning processes (Sircar and Sircar, 2005; Pascual *et al.*, 2007), which continue into

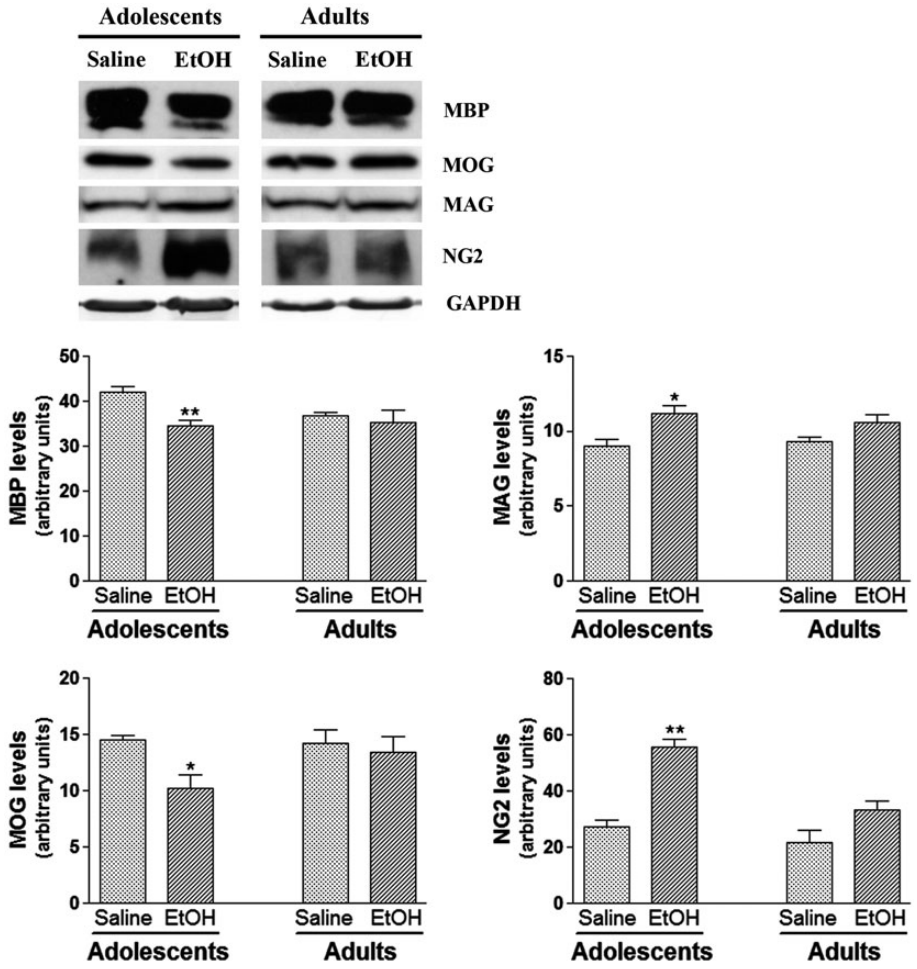

Fig. 3. Intermittent ethanol administration to adolescent rats induces changes in myelin proteins. The levels of the myelin proteins of MBP, MOG, MAG and NG2 were assessed by western blotting in the PFC of adolescent (PND 44) and adult (PND 70) rats after intermittent ethanol administration (3 g/kg, i.p.). A representative immunoblot of each protein is shown. GAPDH was used as a loading control. Values represent the mean ± SEM of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ difference in relation to the saline group, according to an unpaired Student's *t*-test.

adulthood (Sircar and Sircar, 2005; Pascual *et al.*, 2007). It is noteworthy that both clinical and experimental studies demonstrated the vulnerability of female adolescents to the neurotoxic effects of ethanol. Thus, human neuroimaging studies have shown that female adolescents with binge drinking exhibit greater reduction in the PFC and larger white fibre disruptions than male adolescents (De Bellis *et al.*, 2005; Medina *et al.*, 2008; Welch *et al.*, 2013). Studies in experimental animals also support the view that females are more vulnerable than males to the neurotoxic/neuroinflammatory effects of ethanol (Alfonso-Loeches *et al.*, 2013).

In summary, we propose that (see Fig. 1) binge-like ethanol administration during adolescence, by activating TLRs (TLR4 and TLR2) signalling, leads to a neuroinflammatory response and myelin disarrangements, effects that might contribute to long-term cognitive and behavioural impairments.

ADOLESCENCE, A VULNERABLE STAGE OF ALCOHOL AND DRUG ADDICTION

Another important long-lasting consequence of alcohol use during adolescence is the higher risk of developing alcohol abuse and dependence in adulthood. Indeed in both prospective and retrospective human studies, an early onset of alcohol use has typically emerged as a reliable predictor of later problematic use of dependence on alcohol and other drugs (Grant and Dawson, 1997; Hawkins *et al.*, 1997; Labouvie *et al.*, 1997; DeWit *et al.*, 2000).

Adolescence is characterized by increased sensation-seeking, risk-taking behaviours, low levels of harm avoidance, impulsivity and anxiety (Blakemore, 2008). These features are associated with changes in the secretion of gonadal steroids and stress-related hormones (Ceccarelli *et al.*, 2007; Witt, 2007), which might explain the initiation pattern of alcohol and drug consumption. Likewise, the relatively late development of the PFC circuits involved in judgement and inhibitory control, and ethanol-induced damage in this brain region, may also underlie the propensity of adolescents for impulsivity and to ignore negative consequences, both of which could increase the risk of substance abuse.

Experimental studies have also suggested that neurochemical immaturity and heightened neuroplasticity in limbic brain regions might confer greater sensitivity to the addictive drug actions of adolescents. The sensitization and development of the mesocorticolimbic dopamine pathway (Philpot and Kirstein,

2004), along with changes in glutamatergic and dopaminergic neurotransmission and chromatin remodelling (Pascual *et al.*, 2009; Guerri and Pascual, 2010), might mediate adolescents' vulnerability to the long-term consequences of addiction to alcohol or to other drugs. Alcohol intake during adolescence also enhances the effects of other drugs. Indeed, our previous studies demonstrate that exposure to ethanol during adolescence induces long-term behavioural and neurochemical effects which modify the response of mice to methylenedioxymethamphetamine (MDMA or ecstasy) administration in adulthood (Ribeiro Do Couto *et al.*, 2011, 2012). Ethanol exposure during adolescence also enhances the rewarding effects of low doses of MDMA and the susceptibility to priming-induced reinstatement of conditioned place preference in adulthood (Ribeiro Do Couto *et al.*, 2012). Impaired learning has also been observed in the Hebb-Williams maze in mice treated with ethanol during adolescence since they present longer latency scores and need more trials to reach the acquisition criterion score (Vidal-Infer *et al.*, 2012). Together these results suggest that the developing brain is highly vulnerable to the damaging effects of ethanol since mice treated during adolescence with ethanol are less active, exhibit impaired learning and memory (Guerri and Pascual, 2010; Vetreno *et al.*, 2013), show increased behavioural and neurochemical effects of MDMA and are more vulnerable to the addictive properties of MDMA in adulthood.

In summary, several human and experimental studies have demonstrated that adolescence and early adulthood represent a period of much greater vulnerability to addictive drugs. Refinements in the synaptic functions and the neuronal architecture in the adolescent brain are thought to represent important learning-based adaptive processes for developing an adult-like cognitive phenotype. However, this period of heightened neuroplasticity also confers greater vulnerability to addictive drug actions. Sensitization and potentiation of the mesocorticolimbic dopamine pathway along with ethanol-induced neuroinflammation and myelin alteration in the adolescent PFC might not only underlie the cognitive dysfunctions, but also predispose to alcohol abuse and dependence.

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