




Voluntary alcohol binge-drinking in adolescent C57Bl6 mice induces delayed appearance of behavioural defects in both males and females

Laura Van Hees¹ | Vincent Didone² | Manon Charlet-Briart¹ |
Théo Van Ingelgom² | Alysson Alexandre¹ | Etienne Quertemont²  |
Laurent Nguyen¹  | Sophie Laguesse¹ 

¹GIGA-Stem Cells, Interdisciplinary Cluster for Applied Genoproteomics (GIGA-R), University of Liège, Liège, Belgium

²Psychology and Neuroscience of Cognition Research Unit (PsyNCog), Animal models of cognition, University of Liège, Liège, Belgium

Correspondence

Sophie Laguesse and Laurent Nguyen, GIGA-Stem Cells, Interdisciplinary Cluster for Applied Genoproteomics (GIGA-R), University of Liège, Avenue Hippocrate CHU Sart-Tilman, bat B36 +1, Liège 4000, Belgium.
Email: slaguesse@uliege.be; lnguyen@uliege.be

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Abstract

Adolescence is a developmental period characterized by significant changes in brain architecture and behaviour. The immaturity of the adolescent brain is associated with heightened vulnerability to exogenous agents, including alcohol. Alcohol is the most consumed drug among teenagers, and binge-drinking during adolescence is a major public health concern. Studies have suggested that adolescent alcohol exposure may interfere with the maturation of frontal brain regions and lead to long-lasting behavioural consequences. In this study, by using a slightly modified version of the Drinking in the Dark paradigm, adolescent C57Bl6 mice reach high blood alcohol concentration after voluntary binge-drinking. In order to assess short- and long-term consequences of adolescent alcohol exposure (AAE), a battery of behavioural tests was performed during late adolescence and during adulthood. We showed that AAE had no short-term effect on young mice behaviour but rather increased anxiety- and depressive-like behaviours, as well as alcohol consumption during adulthood. Moreover, alcohol binge-drinking during adolescence dramatically decreased recognition memory performances and behavioural flexibility in both adult males and females. Furthermore, we showed that voluntary consumption of alcohol during adolescence did not trigger any major activation of the innate immune system in the prefrontal cortex. Together, our data suggest that voluntary alcohol binge-drinking in adolescent mice induces a delayed appearance of behavioural impairments in adulthood.

KEYWORDS

adolescence, alcohol, behaviour, mice

1 | INTRODUCTION

Adolescence is a crucial developmental phase highly conserved across mammalian species and typically defined as a transitional

Laurent Nguyen and Sophie Laguesse have equal contribution to the work.

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period between childhood and adulthood. This transition period involves significant changes in brain architecture, including cortical grey matter volume decline via synaptic pruning and increased white matter volume due to continued myelination of axons.¹ Adolescence is also characterized by complex developmental changes in neural processing systems and unique behavioural characteristics including increased impulsivity, novelty-seeking and desire of risk-taking.^{2,3} Brain maturation typically begins in posterior brain regions and continues towards more anterior higher-order regions until ~25 years old.¹ The prefrontal cortex (PFC), which is implicated in executive functions,⁴ is one of the last brain regions to become fully mature, and immaturity of this brain region in adolescents is associated with lack of inhibitory control over behaviours.^{2,3} Moreover, adolescence is typically the age for initial exposure to a number of potentially toxic exogenous agents.^{5,6} Alcohol is the most consumed addictive substance among teenagers, with 27% of adolescents worldwide reporting alcohol consumption during the past month.⁷ Binge-drinking of alcohol, which corresponds to ingestion of at least five drinks in males (four in females) within a 2-h period, has become a common pattern of alcohol consumption among teenagers. Binge-drinking leads to high blood alcohol concentration (BAC) (above 0.08 g/dl),^{8,9} which can be harmful to adolescent brain, as it may interfere with ongoing maturation of its frontal circuits. Clinical studies reported that adolescent alcohol exposure (AAE) is associated with brain structure changes, comorbid psychopathology and detrimental neurocognitive consequences.^{5,10,11} Indeed, binge-drinking in adolescent has been associated with thinner cortical and subcortical structures, including the PFC, and reduced white matter development.¹² AAE is also believed to have deleterious effects on verbal learning and memory, attentional and executive functions,^{11,13} as well as to increase the risk of developing psychiatric and behavioural disorders later in life, including alcohol addiction.^{5,10,14,15} Altogether, it has become clear that, because of high brain plasticity, adolescence is a sensitive period for the development of alcohol-related behavioural impairments. Over the past years, rodent models have been used to study alcohol's impact on the adolescent brain and have provided findings consistent with human research.^{11,15,16} Indeed, several studies demonstrated short- and long-term defects in executive functions and behaviours induced by AAE in rodents.^{11,15} Interestingly, most of these studies used a 'binge-drinking-like' administration of alcohol, involving repeated passive exposure to high levels of alcohol (i.p. injections or gavage), and showed that AAE induced activation of the innate immune system in frontal cortical regions as well as short- and long-term behavioural defects.¹⁷⁻²⁰ Acute passive administration and voluntary binge-drinking of alcohol constitute very different experimental procedures, leading to differences in absorption profiles and blood concentrations, and few studies have examined the behavioural consequences of voluntary alcohol consumption during adolescence. Here, we seek to determine whether voluntary binge-drinking of alcohol during early/mid adolescence in the alcohol-preferring mouse strain C57Bl6 leads to the emergence of behavioural defects through the induction of neuro-inflammation in the PFC.

2 | MATERIAL AND METHODS

Detailed information can be found in the Supporting Information.

2.1 | Animals

Males and females C57BL/6J (Janvier Labs, Saint Berthevin, France) were treated according to the guidelines of the Belgian Ministry of Agriculture in agreement with the European Community Laboratory Animal Care and Use Regulations (86/609/EEC) for care and use of laboratory animals under the supervision of authorized investigators (ethical file 18-2004). Experimental animals were bred in-house and maintained with ad libitum access to food and constant temperature (19–22°C) and humidity (40–50%) under a reversed light/dark cycle (lights on at 22:00, off at 10:00).

2.2 | Adolescent alcohol exposure

Adolescent males and females underwent a modified version of the Drinking in the Dark (DID) paradigm²¹ from P29 to P33 and from P36 to P40 (Figure 1E). The age for AAE was chosen during early/mid adolescence.²² All mice were group-housed in order to prevent the emergence of social isolation stress, except for the 4-h alcohol drinking sessions. At 9:30, mice were weighted and transferred to single cages with water and food ad libitum. At 12:00, water was replaced by ethanol (20% in tap water). At 16:00, alcohol was removed, and mice were group-housed until the next day. Control mice received only water and alternated between single- and group-housing accordingly. Different cohorts of mice underwent the behavioural tests either 72 h after the last drinking session (P43, middle adolescence) or after 40 days of abstinence (P80, adulthood). Ninety-one percent of the animals drank more than 4 g/kg/4 h and were included in the study. Animals that drank less than 4 g/kg/4 h for more than two sessions were excluded. The threshold of 4 g/kg/4 h was chosen because it represents the amount of alcohol ingested leading to minimal binge-drinking BAC values, as previously described.²³ Individual drinking data can be found in Tables S1 and S2.

2.3 | BAC measurement

BAC was measured in trunk blood immediately after the last drinking session (P40) by using the NAD⁺/NADH spectrophotometric method, as previously described.²⁴

2.4 | Behavioural tests

Mice were handled twice a day for 2 min for 1 week before behavioural test. Mice were placed in the testing room 1 h before the beginning of each experimental procedure. All tests were monitored by a

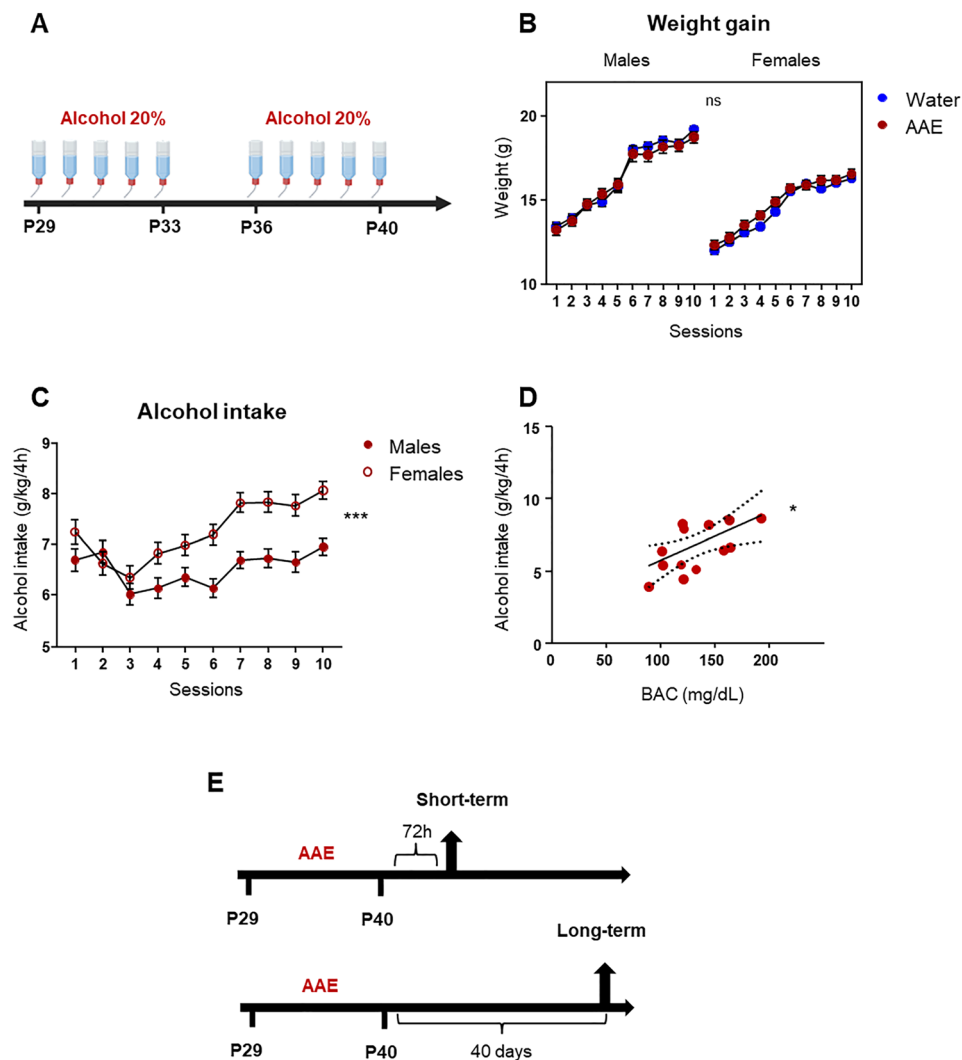


FIGURE 1 Adolescent mice voluntarily consume high amounts of alcohol and reach binge-drinking-related blood alcohol concentrations. (A) Model of alcohol exposure. Adolescent males and females have access to alcohol 20% for 4 h per day, for 10 sessions (from P29 to P33 and from P36 to P40). (B) Weight gain was measured over the course of the treatment and compared with water control littermates. Linear mixed-effects model revealed a main effect of session ($\chi^2_{(9)} = 1.18 \times 10^4$, $p < 0.001$, $\eta^2 = 0.65$) and sex ($\chi^2_{(1)} = 51.41$, $p < 0.001$, $\eta^2 = 0.36$) but no effect of treatment ($\chi^2_{(1)} = 0.11$, $p = 0.74$); $n = 23$ per group. (C) Voluntary alcohol intake. Linear mixed-effects model showed a main effect of sex ($\chi^2_{(1)} = 25.17$, $p < 0.001$, $\eta^2 = 0.08$), session ($\chi^2_{(9)} = 109.04$, $p < 0.001$, $\eta^2 = 0.03$) and a significant interaction sex \times session ($\chi^2_{(9)} = 26.86$, $p < 0.01$, $\eta^2 = 0.007$); $n = 153$ males, 162 females. (D) Scatter plot showing the relationship between alcohol intake and blood alcohol concentration values. Centre line is the linear regression, and dashed lines are the 95% confidence interval. Linear regression $F_{(1,15)} = 7.2$, $p < 0.05$, $r^2 = 0.36$; $n = 15$ mice. (E) Diagram depicting the timing of behavioural tests following adolescent alcohol exposure. Animals underwent the Drinking in the Dark (DID) paradigm from P29 to P40. They were tested 72 h after the last drinking session (short term) or after forty days of abstinence (long term)

camera and analysed by a blinded experimenter. Apparatus were cleaned with 75% ethanol and dried between mice and sessions. Raw data and movies can be found in Mendeley dataset (DOI: 10.17632/gtnmrbtmt4.1).

2.5 | OF test

Locomotion and anxiety-like behaviour were evaluated by conducting the open field (OF) test as described in Fitzgerald et al.²⁵

Mice were placed into the 40 \times 40 \times 40-cm OF and were allowed to explore it freely for 5 min (thymotaxis) or 30 min (locomotion).

2.6 | EPM test

Elevated plus maze (EPM) test was conducted as described in Himanshu and Sarkar.²⁶ Animals were placed in the middle of the EPM and were allowed to explore the maze during 5 min.

2.7 | Forced swimming test

Forced swimming test was performed according to the procedures described in Kraeuter et al.²⁷ Mice were forced to swim for 6 min in a glass cylinder filled with water, and immobility time was recorded during the last 4 min of the test.

2.8 | NOR test

Novel object recognition (NOR) was performed as described in Leger et al.,²⁸ with a long habituation phase. Mice underwent 3 days of habituation (10 min per day), followed by one session of familiarization on Day 4, where mice were allowed to explore freely two copies of the same object for 10 min. On Day 5, one copy of the familiar object was replaced by a novel object, and mice were allowed to freely explore their environment for 10 min.

2.9 | Three-chamber test

The three-chamber test was performed as described in Moy et al.²⁹

2.10 | Reversal learning test

The reversal learning test was performed by using the Barnes maze, as described in Riedel et al.,³⁰ with slight modifications. Learning was assessed for 5 days (two sessions per day, intertrial interval 1 h). Seventy-two hours after the last learning session, mice underwent the 80-s learning probe trial in which the escape tunnel was removed from the apparatus. Twenty-four hours after the probe test, mice underwent 5 days (two sessions per day) of reversal learning. Seventy-two hours after the last reversal learning session, mice underwent the reversal learning probe test.

2.11 | Two-bottle choice drinking paradigms

Intermittent access to 20% alcohol (IA20%-2BC) or 1% sucrose (IA1% suc-2BC) two-bottle choice drinking procedures are described in Laguesse et al. and Ron and Barak^{23,31} (Figure 5A).

2.12 | Immunohistochemistry

Immunohistochemistry was conducted as previously described.³² Fifty- μ m-thick brain sections were incubated in goat anti-Iba1 antibody overnight at 4°C (1/500, Abcam [Cambridge, UK] #ab5076). Donkey anti-goat AlexaFluor 564 was used as secondary antibody. Images were acquired with Nikon A1 confocal microscope on medial sections of the PrL and IL cortices. Morphological characteristics of Iba1+ microglial cells were analysed by using FIJI Software (NIH), by

measuring the cell body size and the size of the ramified processes. The cell body to cell size ratio (%) was determined and utilized as a measurement for microglial activation.³³

2.13 | Western blot analysis

Western blot analysis was conducted from PFC extracts as previously described.³² Membranes were probed with primary antibodies (rabbit anti-HMGB1 1/1000, abcam #ab18256; rabbit anti-TLR4 1/500, Proteintech [Rosemont, IL, USA] #19811; mouse anti-actin 1/5000, Sigma-Aldrich [Saint Louis, MO, USA] #A3854) overnight at 4°C, then probed with HRP-conjugated secondary antibody for 1 h at room temperature. Membranes were developed using enhanced chemiluminescence (ECL), and images were obtained with ImageQuant LAS 4000 camera system (GE Healthcare, Chicago, IL, USA). Band intensities were quantified using ImageJ software (NIH).

2.14 | Enzyme-linked immunosorbent assay

IL-1 β levels were determined from PFC extracts by using the IL-1 β enzyme-linked immunosorbent assay (ELISA) kit (ThermoFisher Scientific, #88-7013) following the manufacturer's protocols.

2.15 | Statistical tests

Data were analysed by using two-way analysis of variance (ANOVA), linear mixed-effects model or student *t* test, as detailed in the figure legends. Significant main effects of ANOVA and linear mixed-effects model were further investigated with Tukey post hoc test, and statistical significance was set at $p < 0.05$. For results homogeneity purpose, all effect sizes were converted in η^2 and indicated in figure legends. The number of subjects is indicated in each of the figure legend.

3 | RESULTS

3.1 | Adolescent mice voluntarily consume high amounts of alcohol

Adolescent mice underwent a modified version of the DID paradigm³⁴ and were given access to a bottle of alcohol 20% for 4 h per day, for 10 sessions between P29 and P40 (Figure 1A). Alcohol intake did not alter body weight gain (Figure 1B), and adolescent mice voluntarily consumed high amounts of alcohol (Figure 1C). Our results further showed that females significantly drank more alcohol than males (mean alcohol consumption 7.25 ± 0.2 and 6.52 ± 0.09 g/kg/4 h, respectively) and exhibited escalation of alcohol consumption (Figure 1C). BAC, measured immediately after the last drinking session, positively correlated with alcohol intake (Figure 1D). Overall, we

showed that adolescent mice voluntarily consumed high amounts of alcohol and reached BAC values comprised between 100 and 200 mg/dl, which correspond to binge-drinking values observed in humans.³⁴ Following AAE, a battery of behavioural tests was performed at two different time points on independent cohorts of animals: Short-term effects were evaluated 72 h after the last drinking session, whereas long-term effects were evaluated on adult animals after 40 days of abstinence (Figure 1E).

3.2 | Voluntary adolescent alcohol binge-drinking leads to long-term development of anxiety-like and depressive-like behaviours

Studies have suggested that heavy alcohol exposure in rats leads to the development of anxiety-like behaviours.^{35–37} Following AAE, mice were tested in the OF and the EPM apparatus, which are commonly used to assess anxiety-like behaviours in rodents²⁶ (Figure 2A,B). Seventy-two hours after the last drinking session, no significant difference in the percentage of time spent in the centre of the OF was found between AAE and water-exposed animals over three sessions (Figure 2C). In addition, AAE animals and water littermates spent similar percentage of time exploring the open arm of the EPM (Figure 2D) and exhibited similar percentage of open arms entries (Figure 2E).

Anxiety-like behaviour was further assessed on independent cohorts of mice after 40 days of abstinence. Our data revealed that adult abstinent mice that were exposed to alcohol during adolescence exhibited significantly enhanced thymotaxis in the OF as compared with water littermates, in both sexes (Figure 2F). Indeed, despite exhibiting habituation across the three consecutive sessions, AAE animals spent significantly less time than the water controls in the centre of the OF (Figure 2F). In addition, AAE animals spent less time exploring the open arms of the EPM (Figure 2G), and the percentage of open arm entries was reduced as compared with water animals (Figure 2H). Importantly, no significant difference in locomotor activity or habituation was found between AAE and water mice (Figure S1A,C), and the total number of EPM arm entries did not differ between groups, suggesting that AAE did not impact exploration behaviour (Figure S1B,D). Together, these data suggest that although voluntary binge-drinking of alcohol during adolescence did not alter anxiety levels in adolescence, it promoted the long-term development of anxiety-like behaviours.

We then assessed the consequences of AAE on depressive-like behaviour by using the forced swimming test (FST), which is widely used to investigate the response to antidepressant treatments and assess depressive-like behaviour in animal models²⁷ (Figure 2I). Seventy-two hours after the last drinking session, AAE and water adolescent mice exhibited equivalent immobility time and delay before the first immobility, both in males and in females (Figure 2J,K). Interestingly, adolescent males exhibited higher immobility time and decreased delay before first immobility, as compared with females (Figure 2J,K).

In contrast, when the FST was performed 40 days after the last alcohol drinking session, mice exposed to AAE exhibited significantly

increased immobility time compared with water littermates, in both sexes, but no difference between sexes was observed (Figure 2L). In addition, the delay before the first immobility episode was shorter in AAE animals (Figure 2M). Interestingly, similarly to adolescent mice, males exhibited shorter time before immobility compared with females (Figure 2M). Together, these results suggest that AAE induces the development of depressive-like behaviour long-term after alcohol consumption.

3.3 | Decreased novel object exploration in adult mice exposed to alcohol binge-drinking during adolescence

The NOR test assesses the natural preference for novel objects normally displayed by mice and gives insights about their recognition memory performance (Figure 3A).³⁸ Shortly after AAE, all groups of adolescent mice showed similar exploration behaviour (Figure 3B,C). In addition, no significant difference in discrimination index (DI) and familiar object habituation index (FHI) was found between animals (Figure 3D,E).

However, 40 days after the last alcohol exposure, despite similar exploration behaviour, AAE mice exhibited significantly lower DI and FHI compared with water littermates, both in males and females (Figure 3F–I). Those findings suggest that although AAE did not impact recognition memory performance in adolescence, it dramatically impaired NOR in adulthood.

3.4 | Adolescent alcohol binge-drinking does not affect mouse sociability

We investigated consequences of AAE on social behaviours by performing the three-chambered social approach task²⁹ (Figure S2A). All mice showed similar preference for the social stimulus, suggesting that AAE did not impact mice sociability (Figure S2B,D). Social novelty preference was also investigated by calculating the social novelty preference index (SNI) after introduction of a stranger mouse in the empty wired cup (Figure S2A), but no significant difference in social novelty preference between AAE and water adolescent mice was observed, either short- or long-term after AAE (Figure S2C,E). However, in contrast to adolescents, adult mice failed to exhibit significant social novelty preference (SNI not significantly different from zero). Overall, those results suggest that AAE had no major impact on mouse sociability.

3.5 | Impaired reversal learning long-term after adolescent alcohol binge-drinking

Clinical studies have reported that AAE may lead to long-lasting deficits in executive functions.^{11,12} We sought to unveil the consequences of AAE on reversal spatial learning by performing the Barnes

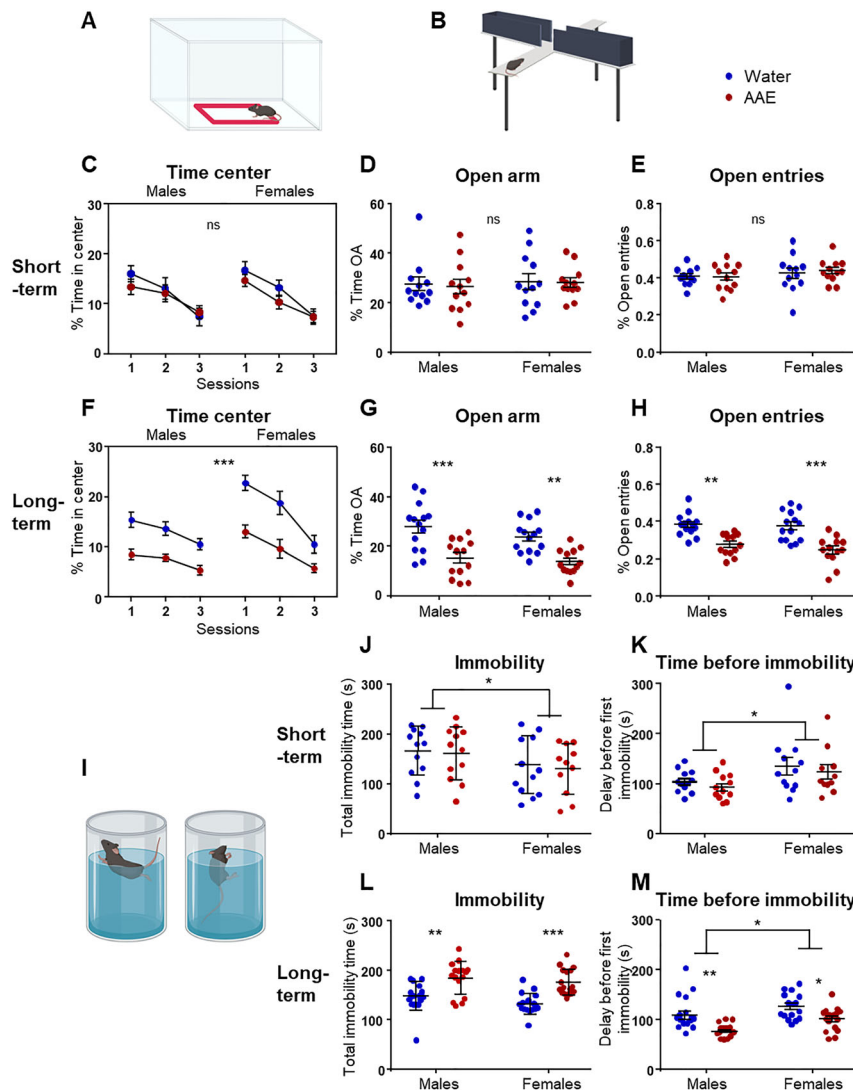


FIGURE 2 Enhanced anxiety-like and depressive-like behaviours in adulthood but not in adolescence after adolescent alcohol exposure. (A,B) Schematic representation of the open field (OF) (A) and the elevated plus maze (EPM) apparatus (B). (C) Short term, percentage of time spent in the centre of the OF. Linear mixed-effects model showed a significant main effect of sessions ($\chi^2_{(2)} = 53.59$, $p < 0.001$, $\eta^2 = 0.33$), but no effect of treatment ($\chi^2_{(1)} = 1.70$, $p = 0.19$) or sex ($\chi^2_{(1)} = 0.00$, $p = 0.98$) and no interaction; $n = 12$ – 14 per group. (D) Short term, percentage of time spent in the open arm of the EPM. Two-way analysis of variance (ANOVA) showed no main effect of treatment ($F_{(1,46)} = 0.14$, $p = 0.71$) or sex ($F_{(1,46)} = 0.66$, $p = 0.42$). (E) Short term, percentage of open arm entries. Two-way ANOVA showed no main effect of treatment ($F_{(1,46)} = 0.01$, $p = 0.94$) or sex ($F_{(1,46)} = 2.52$, $p = 0.12$); $n = 12$ – 14 per group. (F) Long term, percentage of time spent in the centre of the OF. Linear mixed-effects model showed a significant main effect of treatment ($\chi^2_{(1)} = 63.77$, $p < 0.001$, $\eta^2 = 0.57$), sex ($\chi^2_{(1)} = 13.73$, $p < 0.001$, $\eta^2 = 0.22$) and sessions ($\chi^2_{(2)} = 59.09$, $p < 0.001$, $\eta^2 = 0.44$) and a significant interaction sex \times session ($\chi^2_{(2)} = 10.08$, $p < 0.01$, $\eta^2 = 0.07$); $n = 12$ – 14 per group. (G) Long term, percentage of time spent in the open arm of the EPM. Two-way ANOVA showed a main effect of treatment ($F_{(1,50)} = 30.97$, $p < 0.001$, $\eta^2 = 0.38$) but no main effect of sex ($F_{(1,50)} = 0.89$, $p = 0.35$) and no interaction ($F_{(1,50)} = 0.09$, $p = 0.77$). Post hoc Tukey test detected a significant difference between water and adolescent alcohol exposure (AAE) in males ($p < 0.001$) and in females ($p < 0.01$). (H) Long term, percentage of open arm entries. Two-way ANOVA showed a main effect of treatment ($F_{(1,50)} = 40.2$, $p < 0.001$, $\eta^2 = 0.44$) but no main effect of sex ($F_{(1,50)} = 1.10$, $p = 0.30$) and no interaction ($F_{(1,50)} = 0.39$, $p = 0.54$). Post hoc Tukey test detected a significant difference between water and AAE in males ($p < 0.01$) and in females ($p < 0.001$); $n = 12$ – 14 per group. (I) Schematic representation of the forced swimming test (FST). Episodes of active swimming (left) and immobility (right) were recorded and analysed. (J) Short term, total immobility time. Two-way ANOVA showed a main effect of sex ($F_{(1,43)} = 4.36$, $p < 0.05$, $\eta^2 = 0.09$) but no effect of treatment ($F_{(1,43)} = 0.03$, $p = 0.87$) and no interaction ($F_{(1,43)} = 0.003$, $p = 0.96$). (K) Short term, delay before first immobility. Two-way ANOVA showed a main effect of sex ($F_{(1,43)} = 6.17$, $p < 0.05$; $\eta^2 = 0.12$) but no effect of treatment ($F_{(1,43)} = 1.05$, $p = 0.31$) and no interaction ($F_{(1,43)} = 0.01$, $p = 0.94$); $n = 11$ – 12 per group. (L) Long term, total immobility time. Two-way ANOVA showed a main effect of treatment ($F_{(1,61)} = 35.21$, $p < 0.001$, $\eta^2 = 0.36$) but no effect of sex ($F_{(1,61)} = 2.39$, $p = 0.13$) and no interaction ($F_{(1,61)} = 0.11$, $p = 0.74$). Post hoc Tukey test revealed a significant difference between AAE and water mice, both in males ($p < 0.01$) and in females ($p < 0.001$). (M) Long term, delay before first immobility. Two-way ANOVA showed a main effect of treatment ($F_{(1,61)} = 22.98$, $p < 0.001$, $\eta^2 = 0.25$) and sex ($F_{(1,61)} = 7.77$, $p = 0.007$, $\eta^2 = 0.08$) but no interaction ($F_{(1,61)} = 0.66$, $p = 0.42$). Post hoc Tukey test revealed a significant difference between AAE and water mice, both in males ($p < 0.01$) and in females ($p < 0.05$); $n = 16$ – 17 per group

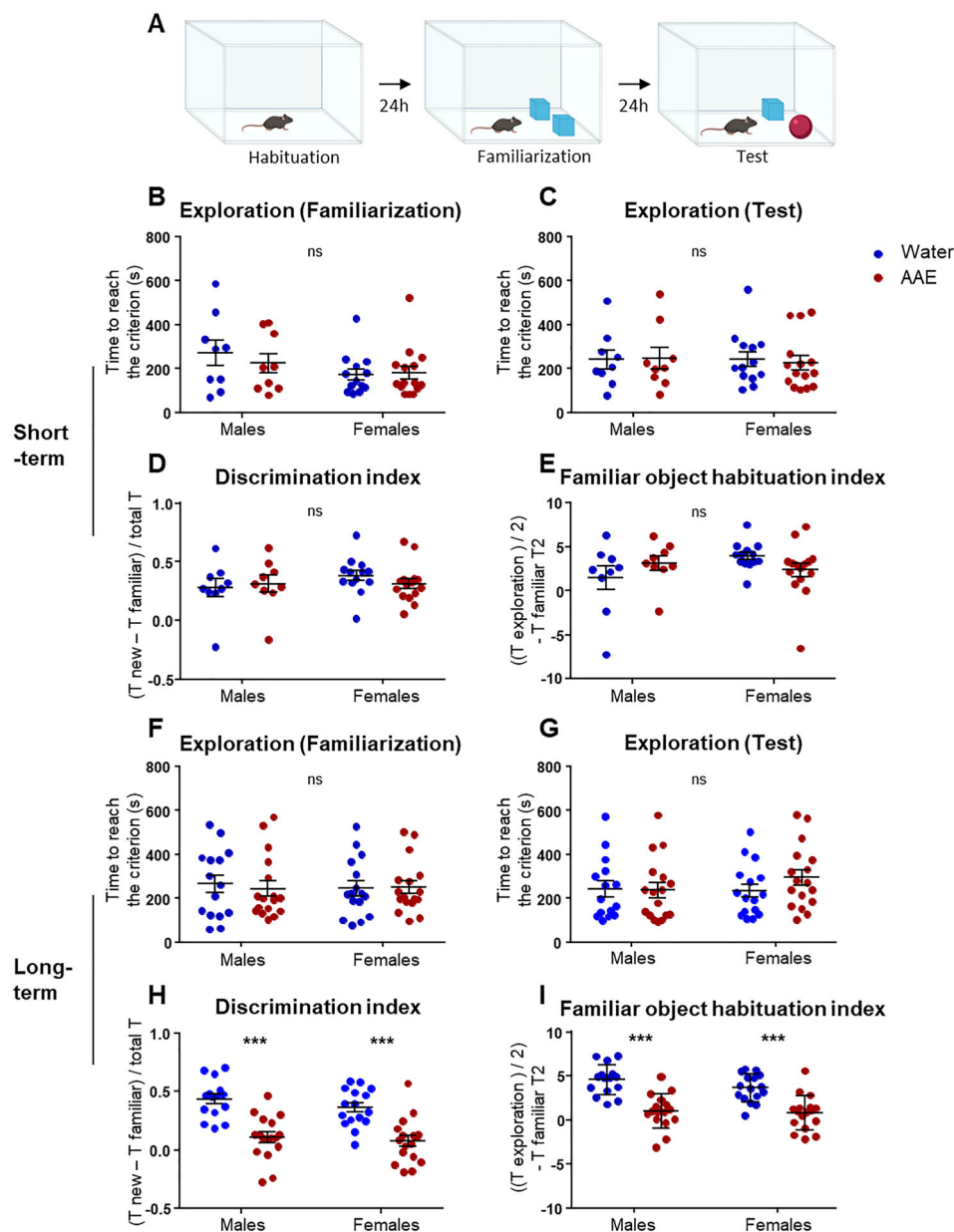


FIGURE 3 Adolescent binge-drinking decreases novel object recognition performances in adult but not adolescent mice. (A) Schematic representation of the novel object recognition (NOR) test. Following 3 days of habituation in the open field, mice were allowed to familiarize with two copies of the same object for 10 min. Twenty-four hours later, one copy of the familiar object is replaced by a novel object and exploration time is recorded. (B) Short term, familiarization session: time to reach criterion (20 s of total object exploration). Two-way analysis of variance (ANOVA) showed no main effect of treatment ($F_{(1,42)} = 0.27, p = 0.6$) or sex ($F_{(1,42)} = 3.52, p = 0.07$). (C) Short term, test session: time to reach criterion. Two-way ANOVA showed no main effect of treatment ($F_{(1,42)} = 0.02, p = 0.88$) or sex ($F_{(1,42)} = 0.05, p = 0.83$). (D) Short term, discrimination index (DI), calculated as the time exploring the novel object minus the time exploring the familiar object, divided by the total exploration time (~20 s). Two-way ANOVA showed no main effect of treatment ($F_{(1,42)} = 0.10, p = 0.76$) or sex ($F_{(1,42)} = 0.87, p = 0.36$). (E) Short term, index of habituation to the familiar object (FHI), calculated as the time exploring both objects during familiarization/2, minus time exploring familiar object during test session. Two-way ANOVA showed no main effect of treatment ($F_{(1,42)} = 0.12, p = 0.73$) or sex ($F_{(1,42)} = 1.06, p = 0.31$); $n = 9$ –15 per group. (F) Long term, familiarization session: time to reach criterion (20 s of total object exploration). Two-way ANOVA showed no main effect of treatment ($F_{(1,60)} = 0.03, p = 0.87$) or sex ($F_{(1,60)} = 0.1, p = 0.76$). (G) Long term, test session: time to reach criterion. Two-way ANOVA showed no main effect of treatment ($F_{(1,60)} = 0.81, p = 0.37$) or sex ($F_{(1,60)} = 0.39, p = 0.53$). (H) Long term, DI. Two-way ANOVA showed a significant main effect of treatment ($F_{(1,60)} = 49.86, p < 0.001, \eta^2 = 0.45$) but not sex ($F_{(1,60)} = 1.15, p = 0.29$) and no interaction ($F_{(1,60)} = 0.33, p = 0.57$). Post hoc Tukey test revealed a significant difference between AAE and water-exposed animals in both males and females ($p < 0.001$). (I) Long term, index of habituation to the familiar object (HFI). Two-way ANOVA showed a significant main effect of treatment ($F_{(1,60)} = 49.50, p < 0.001, \eta^2 = 0.44$) but not sex ($F_{(1,60)} = 1.61, p = 0.21$) and no interaction ($F_{(1,60)} = 0.55, p = 0.46$). Post hoc Tukey test revealed a significant difference between adolescent alcohol exposure (AAE) and water-exposed animals, in both males and females ($p < 0.001$); $n = 15$ –17 per group

Maze test (Figure 4A). Learning abilities were first investigated. Seventy-two hours after the last drinking session, escape time was not different between AAE and water animals and similarly decreased over days (Figure S3A). Accordingly, the number of primary errors also followed a similar decrease trend over sessions in all groups (Figure S3B). Three days later, a probe test session in absence of the escape tunnel was performed, and AAE and water animals spent similar percentage of time spent in the correct sector of the maze (Figure S3C). Long-term after AAE, similarly to adolescent mice, learning abilities of adult mice were not affected by AAE, and similar decreasing escape time and primary errors were observed in all groups (Figure S3D,E). Moreover, no difference was found during the probe test (Figure S3F). Together, these results suggest that AAE had no effect on spatial learning acquisition.

We next assessed reversal learning abilities by rotating the escape tunnel location by 180° (Figure 4A). Adolescent AAE and water mice displayed similar escape time, and no difference was found in the number of primary errors (Figure 4B,C). Furthermore, the probe test did not reveal any difference in the percentage of time spent in the correct sector (Figure 4D) or in the previous sector (Figure 4E).

However, when reversal learning was assessed in adulthood, AAE-exposed mice showed higher escape time and made more errors as compared with water controls, in both sexes (Figure 4F,G). Interestingly, the mean escape time and primary error number were also significantly higher in males compared with females, regardless of their alcohol treatment (Figure 4F,G). In addition, during the probe test, AAE mice spent significantly less time in the correct sector as compared with water controls (water males 44.04 ± 1.94%, AAE males 35.24 ± 2.18%, water females 48.44 ± 2.09%, AAE females 40.75 ± 1.86%) (Figure 4H) and more time in the sector corresponding to the previous position of the escape tunnel (water males 23.16 ± 3.6%, AAE males 28.4 ± 3.6%, water females 19.15 ± 3.1%, AAE females 26.46 ± 3.3%), supporting an increased perseveration behaviour in AAE groups (Figure 4I). Altogether, our results suggest that although AAE did not alter reversal learning short term after the last drinking session, it strongly impaired it in adult mice, long term after alcohol exposure.

3.6 | Adolescent alcohol binge-drinking enhances alcohol consumption in adulthood

Clinical and preclinical studies have suggested that AAE increases the risk of developing alcohol use disorders later in life.^{5,10} In order to decipher whether AAE modulates alcohol intake and preference, mice underwent five sessions of intermittent access to 20% alcohol-2 bottle choice paradigm (Figure 5A).^{31,32} Alcohol intake, alcohol preference and total fluid intake were measured at the end of each 24-h session. Short term after AAE, no significant difference was observed in alcohol intake or preference between groups (Figure 5B–D). Interestingly, data revealed that adolescent females tend to consume more alcohol than males (mean alcohol intake 26.92 ± 2.96 and 23.95 ± 2.03 g/kg/24 h, respectively) and more fluid in total (mean fluid intake 310.5 ± 6.12 and 265.9 ± 24.5 ml/kg/24 h, respectively) (Figure 5B–D).

In contrast, adult mice that were exposed to alcohol during adolescence consumed significantly more alcohol than water littermates in both sexes (mean alcohol intake: water males 14.0 ± 2.7; AAE males 19.12 ± 1.54; water females 17.78 ± 3.2; AAE females 23.45 ± 5.3 g/kg/24 h) (Figure 5E). AAE mice also exhibited higher alcohol preference (Figure 5F), without any difference in total fluid intake (Figure 5G). Similarly, females significantly consumed higher amount of alcohol compared with males (mean alcohol consumption 20.61 ± 5.2 and 16.56 ± 3.4 g/kg/24 h, respectively) and presented higher total fluid intake (mean fluid intake 236.6 ± 43 and 189.7 ± 27 ml/kg/24 h, respectively) (Figure 5E–G).

Furthermore, sucrose consumption was measured short and long term after AAE, and no difference in sucrose intake or preference was found between AAE animals and water controls in both sexes (Figure S4).

3.7 | Voluntary alcohol binge-drinking during adolescence does not induce major neuro-inflammation in the PFC

Several studies using binge-like administration of alcohol during adolescence reported alcohol-induced activation of innate immune signalling in the frontal cortex and promoted behavioural alterations similar to those observed in this study.^{18–20,39} In order to decipher whether voluntary alcohol binge-drinking in adolescent mice induces neuro-inflammation in the PFC, we first assessed the number and activation state of microglial cells in the prelimbic and infralimbic subregions of the PFC. Microglial activation is classically described as a graded modification in cell morphology, with increased cell body size and retraction and thickening of the ramified processes.^{33,40} As shown in Figure 6A–E, there was no difference in the number of microglial cells in the prelimbic cortex of all groups of animals when analysed 72 h after the least drinking session. In addition, the mean cell body/cell size ratio of Iba1+ cells did not differ between groups, suggesting that AAE did not modulate the activation state of microglial cells (Figure 6F). The same analysis was performed in the prelimbic PFC of adult animals, and no alcohol-dependent activation of microglia was observed (Figure S5). Moreover, number and activation state of microglial cells were similar between groups in the infralimbic region of the PFC, either in late adolescence or in adulthood (data not shown). As passive administration of alcohol in adolescent rodents has been shown to increase the expression of the High Mobility Group protein B1 (HMGB1) and the Toll-like receptor 4 (TLR4) in the PFC,^{41,42} we further assessed their expression after voluntary alcohol binge-drinking. Surprisingly, as shown in Figure 6F,G, no significant increase in HMGB1 or TLR4 expression was observed in the PFC of AAE animals compared with water controls, neither in males or females, short term or long term after AAE. Finally, we assessed the concentration of interleukin-1β in the PFC of AAE and water mice and did not find any difference between groups, neither 3 nor 40 days after AAE (Figure 6H,I). Altogether, our data suggest that our model of voluntary binge-drinking of alcohol in mice did not induce major neuro-inflammation in the PFC.

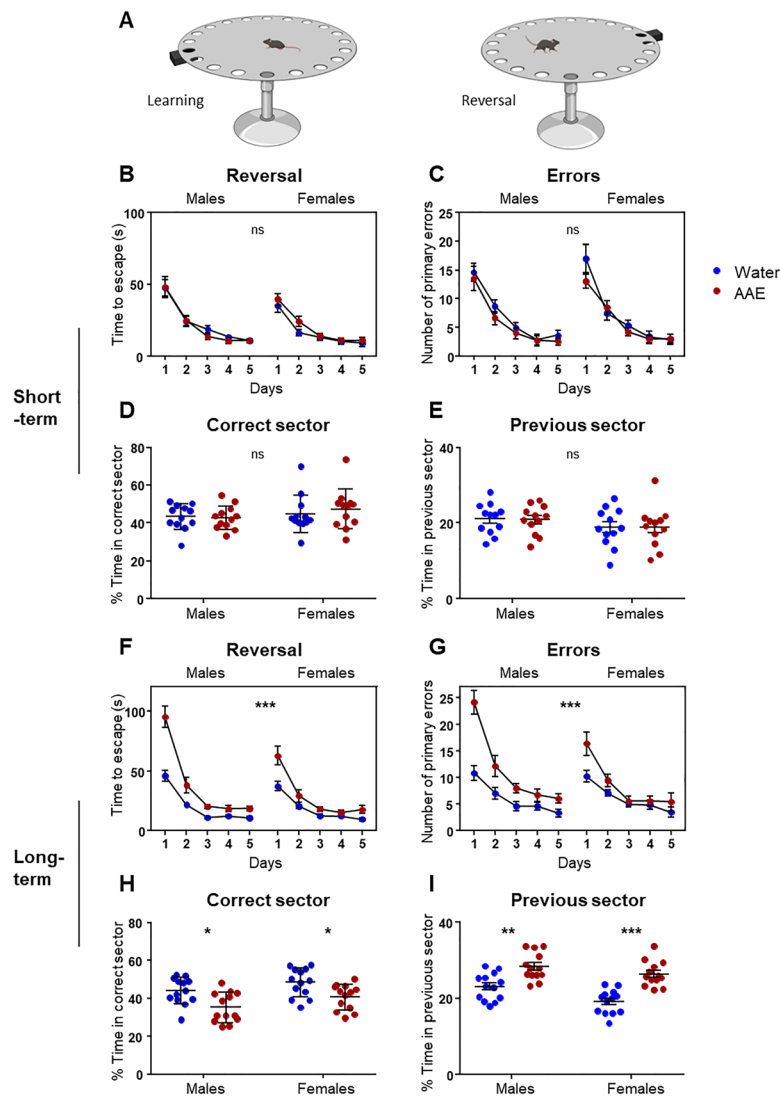


FIGURE 4 Behavioural flexibility is impaired long term after adolescent alcohol exposure. (A) Schematic representation of the Barnes maze test. During learning, mice are given 10 training sessions in order to learn the position of the escape tunnel (left, see Figure S3). Then, the position of the escape tunnel is modified, and mice are given 10 reversal learning sessions (right). (B) Short term, reversal learning: mean escape time per day across 5 days. Linear mixed-effects model showed a significant main effect of day ($\chi^2_{(4)} = 363.3$, $p < 0.001$, $\eta^2 = 0.55$) and sex ($\chi^2_{(1)} = 4.52$, $p < 0.05$, $\eta^2 = 0.086$) but no main effect of treatment ($\chi^2_{(1)} = 0.26$, $p = 0.61$) and no interaction (day \times sex $\chi^2_{(4)} = 7.90$, $p = 0.1$; day \times treatment $\chi^2_{(4)} = 3.37$, $p = 0.5$; sex \times treatment $\chi^2_{(1)} = 1.64$, $p = 0.2$). (C) Short term, reversal learning: primary errors per day across 5 days. Linear mixed-effects model showed a significant main effect of day ($\chi^2_{(4)} = 342.42$, $p < 0.001$, $\eta^2 = 0.55$) but no main effect of treatment ($\chi^2_{(1)} = 2.15$, $p = 0.14$) or sex ($\chi^2_{(1)} = 0.32$, $p = 0.57$) and no interaction (day \times sex $\chi^2_{(4)} = 0.54$, $p = 0.97$; day \times treatment $\chi^2_{(4)} = 3.11$, $p = 0.54$; sex \times treatment $\chi^2_{(1)} = 0.01$, $p = 0.91$). (D) Short term, percentage of time spent in the correct sector during probe test, 72 h after the last reversal learning session. Two-way analysis of variance (ANOVA) showed no main effect of treatment ($F_{(1,44)} = 0.18$, $p = 0.67$) or sex ($F_{(1,44)} = 1.47$, $p = 0.23$). (E) Short term, percentage of time spent in the previous sector during probe test. Two-way ANOVA showed no main effect of treatment ($F_{(1,44)} = 0.01$, $p = 0.93$) or sex ($F_{(1,44)} = 2.49$, $p = 0.12$); $n = 12$ per group. (F) Long term, reversal learning: mean escape time per day. Linear mixed-effects model showed a significant main effect of day ($\chi^2_{(4)} = 539.37$, $p < 0.001$, $\eta^2 = 0.64$), treatment ($\chi^2_{(1)} = 47.87$, $p < 0.001$, $\eta^2 = 0.22$) and sex ($\chi^2_{(1)} = 7.92$, $p < 0.001$, $\eta^2 = 0.13$), as well as a significant interaction day \times sex ($\chi^2_{(4)} = 24.81$, $p < 0.001$, $\eta^2 = 0.08$) and day \times treatment ($\chi^2_{(4)} = 61.63$, $p < 0.001$, $\eta^2 = 0.17$) but not treatment \times sex ($\chi^2_{(1)} = 3.42$, $p = 0.06$). (G) Long term, reversal learning: primary error number. Linear mixed-effects model showed a significant main effect of day ($\chi^2_{(4)} = 273.91$, $p < 0.001$, $\eta^2 = 0.46$), treatment ($\chi^2_{(1)} = 29.15$, $p < 0.001$, $\eta^2 = 0.16$) and sex ($\chi^2_{(1)} = 4.10$, $p < 0.05$, $\eta^2 = 0.07$), as well as an interaction day \times treatment ($\chi^2_{(4)} = 39.74$, $p < 0.001$, $\eta^2 = 0.11$), sex \times treatment ($\chi^2_{(1)} = 4.51$, $p < 0.05$, $\eta^2 = 0.03$) but not day \times sex ($\chi^2_{(4)} = 8.78$, $p = 0.07$). (H) Long term, percentage of time spent in the correct sector during probe test, 72 h after the last reversal learning session. Two-way ANOVA showed a significant main effect of treatment ($F_{(1,48)} = 16.62$, $p < 0.001$, $\eta^2 = 0.24$) and sex ($F_{(1,48)} = 6.01$, $p < 0.05$, $\eta^2 = 0.09$) but no interaction ($F_{(1,48)} = 0.07$, $p = 0.79$). Post hoc Tukey test revealed a significant difference between adolescent alcohol exposure (AAE) and water animals in males ($p < 0.05$) and in females ($p < 0.05$). (I) Long term, percentage of time spent in the previous sector during probe test. Two-way ANOVA showed a significant main effect of treatment ($F_{(1,48)} = 44.19$, $p < 0.001$, $\eta^2 = 0.43$) and sex ($F_{(1,48)} = 9.91$, $p < 0.01$, $\eta^2 = 0.1$) but no interaction ($F_{(1,48)} = 1.19$, $p = 0.28$). Post hoc Tukey test revealed a significant difference between AAE and water animals in males ($p < 0.01$) and in females ($p < 0.001$); $n = 13$ per group

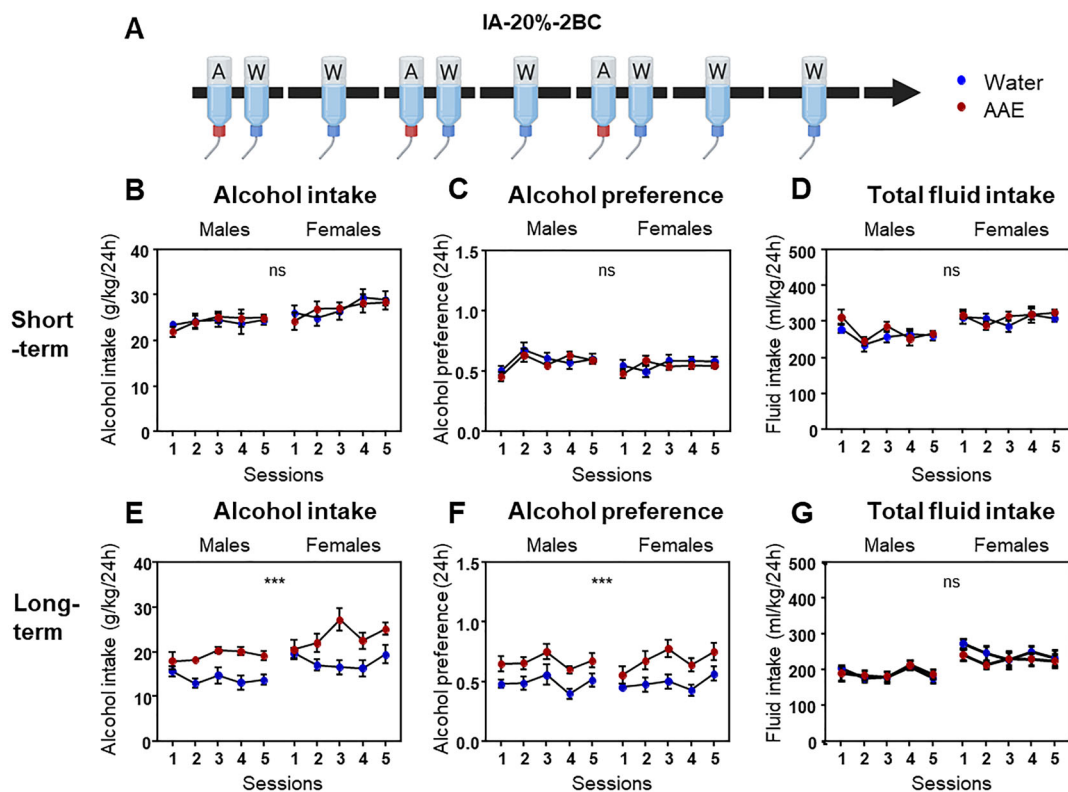


FIGURE 5 Binge-drinking during adolescence increases alcohol consumption and preference in adult mice. (A) Scheme depicting the intermittent access to alcohol 20%-two bottle choice paradigm. Short-term (B–D) and long term (E–G) after adolescent alcohol exposure (AAE), mice underwent the IA-20%-2BC paradigm for five sessions. (B) Short term, alcohol intake (g/kg/24 h). Linear mixed-effects model showed a significant main effect of session ($\chi^2_{(4)} = 11.3$, $p < 0.05$, $\eta^2 = 0.04$) and sex ($\chi^2_{(1)} = 17.28$, $p < 0.001$, $\eta^2 = 0.27$) but no effect of treatment ($\chi^2_{(1)} = 0.1$, $p = 0.76$) and no interaction (session \times treatment $\chi^2_{(4)} = 1.4$, $p = 0.85$; sex \times treatment $\chi^2_{(1)} = 0.08$, $p = 0.78$; session \times sex $\chi^2_{(4)} = 2.88$, $p = 0.58$). (C) Short term, alcohol preference. Linear mixed-effects model showed a significant main effect of session ($\chi^2_{(4)} = 22.42$, $p < 0.001$, $\eta^2 = 0.07$) but no effect of sex ($\chi^2_{(1)} = 1.86$, $p = 0.17$) or treatment ($\chi^2_{(1)} = 0.85$, $p = 0.36$) and no interaction (session \times treatment $\chi^2_{(4)} = 4.57$, $p = 0.33$, sex \times treatment $\chi^2_{(1)} = 0.00$, $p = 0.96$, session \times sex $\chi^2_{(4)} = 9.35$, $p = 0.051$). (D) Short term, total fluid intake (ml/kg/24 h). Linear mixed-effects model showed a significant main effect of session ($\chi^2_{(4)} = 15.12$, $p < 0.01$, $\eta^2 = 0.05$) and sex ($\chi^2_{(1)} = 34.6$, $p < 0.001$, $\eta^2 = 0.42$) but no effect of treatment ($\chi^2_{(1)} = 1.78$, $p = 0.18$) and no interaction (session \times treatment $\chi^2_{(4)} = 5.53$, $p = 0.24$, sex \times treatment $\chi^2_{(1)} = 0.27$, $p = 0.6$, session \times sex $\chi^2_{(4)} = 7.78$, $p = 0.1$); $n = 12$ per group. (E) Long term, alcohol intake (g/kg/24 h). Linear mixed-effects model showed a significant main effect of treatment ($\chi^2_{(1)} = 34.86$, $p < 0.001$, $\eta^2 = 0.15$) and sex ($\chi^2_{(1)} = 17.28$, $p < 0.001$, $\eta^2 = 0.09$) but no effect of session ($\chi^2_{(4)} = 8.53$, $p = 0.07$). This model also showed an interaction session \times treatment ($\chi^2_{(4)} = 14.8$, $p = 0.01$, $\eta^2 = 0.02$) but not sex \times treatment ($\chi^2_{(1)} = 0.00$, $p = 0.98$) or session \times sex $\chi^2_{(4)} = 3.37$, $p = 0.50$). (F) Long term, alcohol preference. Linear mixed-effects model showed a significant main effect of treatment ($\chi^2_{(1)} = 33.21$, $p < 0.001$, $\eta^2 = 0.15$) and session ($\chi^2_{(4)} = 19.6$, $p < 0.001$, $\eta^2 = 0.06$) but no effect of sex ($\chi^2_{(1)} = 0.04$, $p = 0.84$) and no interaction (session \times treatment $\chi^2_{(4)} = 2.17$, $p = 0.7$, sex \times treatment $\chi^2_{(1)} = 0.01$, $p = 0.92$, session \times sex $\chi^2_{(4)} = 3.32$, $p = 0.51$). (G) Long term, total fluid intake (ml/kg/24 h). Linear mixed-effects model showed a significant main effect of session ($\chi^2_{(4)} = 14.63$, $p < 0.01$, $\eta^2 = 0.04$) and sex ($\chi^2_{(1)} = 21.1$, $p < 0.001$, $\eta^2 = 0.1$) but no effect of treatment ($\chi^2_{(1)} = 0.3$, $p = 0.58$) and no interaction (session \times treatment $\chi^2_{(4)} = 2.69$, $p = 0.61$, sex \times treatment $\chi^2_{(1)} = 1.04$, $p = 0.31$, session \times sex $\chi^2_{(4)} = 3.39$, $p = 0.49$); $n = 12$ per group

4 | DISCUSSION

4.1 | AAE induces anxiety and depressive-like behaviours in adult mice

We report that voluntary adolescent alcohol binge-drinking promotes the development of anxiety- and depressive-like behaviours in adulthood, without any warning sign in adolescence. Such results are consistent with other studies showing that repeated passive exposure to alcohol during adolescence induces the development anxiety-like behaviours in adult rodents.^{35–37,43,44} However, opposite findings

have been reported, showing that ethanol vapour exposure during adolescence increased exploration of adult rats in the open arms of the EPM, and such results have been interpreted as AAE-dependent increased impulsivity.^{45–47} OF and EPM are tasks assessing the balance between the innate exploratory drive and the anxiety generated by a novel environment.²⁶ Therefore, it remains challenging to decipher whether behavioural difference results from change in anxiety and/or impulsivity, and data should be carefully interpreted. Furthermore, our results also suggest that AAE, while not impacting adolescent behaviour in the FST, led to the long-term development of depressive-like behaviours. Such results are in line with a study

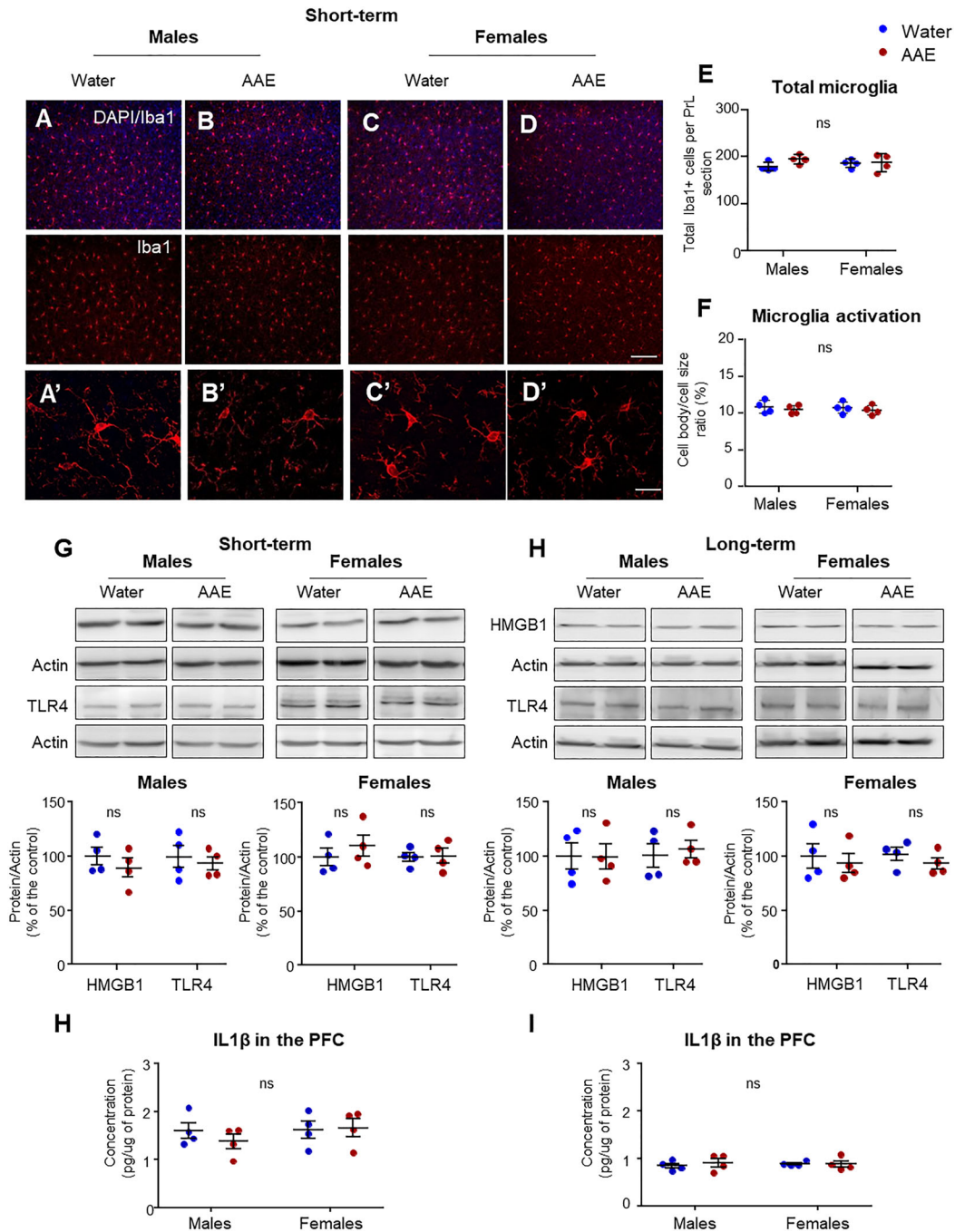


FIGURE 6 Voluntary alcohol binge-drinking during adolescence does not trigger innate immune system activation in the prefrontal cortex. (A–D) Short term, morphological analysis of microglial cells expressing Iba1 (red) in the prelimbic prefrontal cortex of males water (A), males adolescent alcohol exposure (AAE) (B), females water (C) and females AAE (D) by immunofluorescence; bar scale 100 μ m. (A'–D') Representative high magnification image of Iba1+ cells in the PrL; bar scale 20 μ m. (E) Short term, total number of microglial cells per prelimbic prefrontal cortex (PFC) section (mean \pm S.E.M.). Two-way analysis of variance (ANOVA) showed no main effect of treatment ($F_{(1,12)} = 1.87, p = 0.2$), sex ($F_{(1,12)} = 0.002, p = 0.97$) and no interaction ($F_{(1,12)} = 1.27, p = 0.28$); $n = 4$ animals per group. (F) Short term, mean cell body/cell size ratio (%) of Iba1+ cells per prelimbic PFC section (mean \pm S.E.M.). Two-way ANOVA showed no main effect of treatment ($F_{(1,12)} = 0.85, p = 0.37$) or sex ($F_{(1,12)} = 0.04, p = 0.84$) and no interaction ($F_{(1,12)} = 0.004, p = 0.95$). $n = 4$ animals per group, 20–23 cells per animal. (G,H) HMGB1 and TLR4 protein expression were determined by western blot analysis short term (G) and long term (H) after adolescent alcohol exposure. ImageJ was used for optical density quantification. Data are expressed as the average ratio \pm S.E.M. of HMGB1 or TLR4 to actin and are expressed as percentage of water control. Significance was determined using two-tailed unpaired t test. $n = 4$ per group. (I,J) IL-1 β concentration (pg/ μ g of protein) in the PFC was determined short term (I) or long term (J) after adolescent alcohol exposure. (I) Short term, data are represented as the mean concentration \pm S.E.M. Two-way ANOVA showed no main effect of treatment ($F_{(1,12)} = 0.29, p = 0.6$), sex ($F_{(1,12)} = 0.78, p = 0.39$) and no interaction ($F_{(1,12)} = 0.61, p = 0.45$). (J) Long term, data are represented as the mean concentration \pm S.E.M. Two-way ANOVA showed no main effect of treatment ($F_{(1,12)} = 0.25, p = 0.62$), sex ($F_{(1,12)} = 0.03, p = 0.87$) and no interaction ($F_{(1,12)} = 0.35, p = 0.57$). $n = 4$ per group

conducted by Lee et al. in which the authors used a similar mouse model and showed AAE-dependent increased depressive-like behaviours and anhedonia in adult males.⁴⁸

4.2 | AAE decreases recognition memory performances in adulthood

The NOR test is commonly used for assessing the effects of a drug on memory performance because no reward or reinforcement are needed; as such, the test relies primarily on the rodent's innate exploratory behaviour.^{28,38} As environmental familiarization may modulate novel object interaction, increased anxiety levels in AAE animals could participate in the AAE-dependent impaired recognition memory.²⁸ In order to minimize potential bias, we used a long habituation protocol that reduces the stress associated with the OF. Importantly, no difference was found between AAE and water mice regarding their exploratory behaviour, and no preference for one object was evidenced. Intriguingly, Pascual et al. showed reduced performances in the NOR test in both late adolescent and adult rats after AAE,¹⁹ and they recently reported AAE-induced decreased recognition memory in late adolescent mice.⁴⁹ In opposition, the present study reports impaired recognition memory in adult mice only. Such discrepancy may arise from experimental set-up differences, including the animal model (mice vs. rats), the timing of behavioural testing and the mode of alcohol administration. Indeed, in our model, adolescent mice had access to alcohol between P29 and P40 and were tested at P43, whereas in Pascual et al.'s study, mice were tested at P46.⁴⁹ Adolescence is a relatively short developmental period, characterized by a rapid maturation of the frontal brain regions, and is commonly divided into three distinct periods: early (PN21–34), middle (PN34–46) and late adolescence/early adulthood (PN46–59).²² It is thus possible that the defects observed in recognition memory at P46 are not yet detectable at P43. Moreover, different administration routes lead to large differences in alcohol pharmacokinetics. Indeed, alcohol is more rapidly absorbed after i.p. as compared with oral ingestion, and alcohol accumulation in the brain is also lower after oral administration.⁵⁰ Such differences must also be taken into account when comparing different results, and it is possible that the timing of appearance of behavioural defects depends on the final alcohol concentration administered to the brain.

4.3 | AAE does not lead to sociability defects

Very few studies examined the effects of AAE on sociability. In the present study, we found that AAE does not impair sociability when assessed by the 3-chamber test, neither short- or long-term after AAE. Interestingly, Sabry et al. showed that AAE did not affect sociability in adolescent male rats but suppressed sociability when coupled to overcrowding conditions.⁵¹ This suggests that alcohol exposure per se may not be sufficient to affect sociability but rather enhances the development of social issues when associated with social stress.⁵¹ We also showed that AAE does not alter the social novelty preference in

adolescence. However, in adult mice, as none of the groups of mice exhibited significant social novelty preference, no conclusion can be drawn from our findings. This is probably due to the mouse strain used in our study, as it has been shown that adult C57Bl6 mice lacked to demonstrate social novelty preference in the three-chamber apparatus.⁵²

4.4 | AAE impairs behavioural flexibility in adulthood but not in late adolescence

Several cognitive studies have suggested that AAE has minimal effect on spatial learning and memory tasks.¹¹ However, flexibility impairments have been reported when reversal learning or set-shifting tasks were demanded.^{11,44,47} Accordingly, we showed that AAE has no effect on spatial learning but significantly impairs reversal learning for spatial tasks in adulthood. Indeed, all mice were able to learn the initial position of the escape tunnel, but when the task required a more flexible strategy, AAE animals significantly lacked behavioural flexibility and exhibited increased perseveration of previously learned behaviour. Such defect in flexibility has been interpreted as a loss of executive functions caused by disruption of frontal cortical areas control.^{6,11,39} Here, we show that AAE-dependent impairment of behavioural flexibility is a long-term developmental process and that frontal brain region alterations may not be present in late adolescence yet but rather develop along with frontal circuit maturation.

4.5 | AAE enhances adult but not adolescent alcohol consumption and preference

AAE has also been reported to promote alcohol consumption in adult rats,^{11,36,39,47,48} although opposite results have been reported.^{53,54} In the present study, voluntary adolescent binge-drinking promoted alcohol consumption and preference in adult males and females but not in adolescents. This is quite surprising to see that naïve, water-drinking adolescent mice consumed the same amount of alcohol than animals previously exposed to alcohol, and those results should be carefully interpreted. Indeed, we cannot exclude the possibility that the absence of significant difference between AAE and water-exposed mice might be due to a plateau effect as adolescent C57Bl6 mice voluntarily consume very high amounts of alcohol. Interestingly, sucrose consumption and preference were not affected by AAE, suggesting that the mechanisms triggered by alcohol in the adolescent brain are not shared by all rewarding substances.

4.6 | Delayed appearance of behavioural defects: Potential mechanisms

This study reports that although voluntary alcohol binge-drinking during adolescence does not lead to short-term behavioural alterations, it dramatically impairs adult executive functions and behaviour. This

suggests that alcohol may slowly perturb brain maturation thereby leading to the progressive development of behavioural defects, which would only emerge during adulthood. Importantly, all the behaviours assessed in this study involve the PFC, together with several interconnected brain regions such as the hippocampus, the amygdala, the striatum, the ventral tegmental area and the orbitofrontal cortex. It is believed that prefrontal brain regions are particularly vulnerable during adolescence as their peak of maturation is observed during this developmental period.^{2,5} Although involving additional brain regions, the AAE-induced impaired behaviours observed in adulthood are likely to arise, at least partly, from PFC malfunction, which may result from alcohol-dependent interference with its maturation.^{11,39,55} The precise consequences of AAE on PFC maturation and function are not fully understood yet. However, accumulating mechanistic data obtained during the past years suggest a disruption of frontal brain circuitry as one driver of behavioural impairments after AAE.^{6,11,39,55}

Several studies have reported that repeated passive exposure to high levels of alcohol during adolescence leads to the long-lasting induction of innate immune signalling through signalling cascades involving RAGE, HMGB1, Toll-like receptors and pro-inflammatory cytokines.^{19,20,41,43,56} They further showed that alcohol-dependent innate immune signalling induction led to synaptic plasticity disruption, brain damages in frontal regions and long-lasting neurobehavioural consequences.^{17,20,39,57} Furthermore, in an elegant study, Montesinos et al. reported that TLR4 KO mice were protected against the alcohol-induced behavioural alterations.⁴³ Surprisingly, in the present study, despite severe behavioural defects following AAE, no major induction of neuro-inflammation was observed in the mouse PFC. Experimental differences, such as the alcohol administration mode, may explain discrepancies between studies. Indeed, alcohol absorption profiles appear very different in the two mouse models of AAE: In the voluntary alcohol consumption model, mice drank around 7 g/kg of alcohol in a period of 4 h, whereas i.p. injections involved a single acute administration of 3–5 g/kg of alcohol. As elimination of alcohol is done at a constant rate,⁵⁸ the maximal concentration of alcohol after i.p. injection is significantly higher as compared with voluntary drinking. It is thus possible that alcohol reaches a toxicity threshold and triggers the induction of neuro-inflammation in frontal brain regions, which is not observed after voluntary alcohol drinking. In addition, it is important to question the role of stress, which is likely to be associated with gavage or i.p. administration of alcohol. Indeed, repeated stress exposure during adolescence has been associated with adverse neurobehavioural consequences.⁵⁹ Moreover, multiple interactions between stress and alcohol have been shown,⁶⁰ and it is possible that elevated stress going along forced alcohol administration exacerbates the latent pro-inflammatory effects of alcohol exposure. Further research is required in order to better understand the relationship between alcohol exposure model, stress, induction of neuro-inflammation in the rodent PFC and the appearance of behavioural defects.

AAE has also been shown to disrupt neurotransmitter systems and reduce neurogenesis, by altering the expression of the brain-derived neurotrophic factor (BDNF) via changes in DNA methylation

and/or acetylation.^{14,39} AAE-dependent alterations in epigenetic programming have also been reported and may be responsible for the delayed appearance of the long-lasting behavioural effects of AAE.^{14,61} Indeed, it is believed that alcohol affects epigenetic pathways, modifying chromatin remodelling, gene expression, dendritic spines morphology and synaptic plasticity, to ultimately affect neurocircuits function and behaviour.^{14,61} Epigenetic and synaptic plasticity modulation by alcohol during adolescence could explain the progressive development of impaired behaviours observed in the present study, as well as the absence of AAE-induced behavioural defects in adolescent mice. Further research is required to unravel AAE-induced epigenetic reprogramming and its relationship with the delayed appearance of behavioural effects.

5 | CONCLUSIONS

This study shows that voluntary alcohol binge-drinking during adolescence leads to severe behavioural impairments in adult mice in both sexes. Indeed, AAE severely increases anxiety-like behaviours, depressive-like behaviours and alcohol consumption in adulthood, while impairing recognition memory and behavioural flexibility. Although differences were noted between males and females, our data showed that AAE similarly affects their behaviours. Surprisingly, adolescent behaviours were not affected by alcohol binge-drinking, suggesting that AAE-dependent alteration of behaviours is a progressive and insidious process, whose consequences only emerge during adulthood. In this view, our findings are of great importance regarding the major public health issue that is adolescent binge-drinking and could help refining the prevention strategies against harmful alcohol use in youth.^{62,63} Finally, we reported that in opposition with models of passive exposure to alcohol, voluntary binge-drinking in adolescent mice did not induce a major activation of neuro-inflammation in the PFC.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

S.L., L.N. and L.V.H performed the conceptualization; S.L. and V.D performed the formal analysis; L.V.H., S.L., A.A., M.C.B., T.V.I. and V.D. performed the investigation; S.L., L.V.H., V.D. and E.Q. performed the methodology; L.N. and S.L. helped in funding acquisition; L.N. and E.Q. performed the supervision; S.L. and L.V.H. wrote the original draft; L.N., V.D. and E.Q. did the writing—editing.

DATA AVAILABILITY STATEMENT

Raw data and movies can be found in Mendeley dataset (DOI: 10.17632/gtnmrbtmt4.1).

ORCID

Etienne Quertemont  <https://orcid.org/0000-0002-9859-0779>

Laurent Nguyen  <https://orcid.org/0000-0002-8560-3008>

Sophie Laguesse  <https://orcid.org/0000-0002-9217-0087>

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SUPPORTING INFORMATION

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