Adolescent alcohol binge-drinking compromises behaviors during adulthood without triggering neuroinflammation

Running title: adolescent binge-drinking impairs adult behaviors

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Abstract

Adolescence is a developmental period characterized by significant changes in brain architecture and behaviors. The poor maturity 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 (AAE) may interfere with the maturation of frontal brain regions and lead to long-lasting behavioral consequences. In this study, we used a mouse model of AAE in which adolescent male and female mice reach high blood alcohol concentration after voluntary binge-drinking. In order to assess the short- and long-term consequences of AAE, a battery of behavioral tests was performed during late adolescence and during adulthood. We showed that AAE had no short-term effect on young mice behaviors but rather increased anxiety- and depressive-like behaviors, as well as alcohol consumption during adulthood. Moreover, alcohol binge-drinking during adolescence dramatically decreased recognition memory performances and behavioral 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 (PFC). Together, our data suggest that voluntary alcohol binge-drinking in mice insidiously impairs adult behaviors, without inducing major neuroinflammation in the PFC.

Introduction

Adolescence is a crucial developmental phase highly conserved across mammalian species, and typically defined as a transitional period between childhood and adulthood. This transition period involves significant changes in brain architecture, including cortical gray matter volume decline via synaptic pruning and increased white matter volume due to continued myelination of axons (1, 2). Adolescence is also characterized by complex developmental changes in neural processing systems and unique behavioral characteristics including increased impulsivity, novelty-seeking and desire of risk-taking (3, 4). Brain maturation typically begins in posterior brain regions and continues towards more anterior higher-order regions until ~25 years old (2, 5). The prefrontal cortex (PFC) which is implicated in executive functions and decision-making processes (6), is one of the last brain region to become fully mature, and immaturity of this brain region in adolescents is associated with lack of inhibitory control over behaviors (3, 4). Moreover, adolescence is typically the age for initial exposure to a number of potentially toxic exogenous agents (7, 8). Alcohol is the most consumed addictive substance among teenagers, with 27% of adolescents worldwide reporting alcohol consumption during the past month (9). Binge-drinking of alcohol, which corresponds to ingestion of at least five drinks in males (four in females) within a 2-hour period, has become a common pattern of alcohol consumption among teenagers. Binge-drinking leads to high blood alcohol concentrations (above 0.08g/dl) (10-12), which can be harmful to the 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 (7, 13, 14). Indeed, binge-drinking in adolescent has been associated with thinner cortical and subcortical structures, including the prefrontal cortex, and reduced white matter development (15). AAE is also believed to have deleterious effects on verbal learning and memory, attentional and executive functions (14, 16), as well as to increase the risk of developing psychiatric and behavioral disorders later in

life, including alcohol addiction (7, 13, 17, 18). Altogether, it has become clear that, because of the high plasticity of the brain, adolescence is a sensitive period for the development of alcohol-related behavioral impairments. Over the past years, rodent models have been used to study alcohol's impact on the adolescent brain. Despite not fully recapitulating the complex human behavior defects, animal models provided findings partly consistent with human research (14, 18, 19). Indeed, several studies demonstrated short- and long-term defects in executive functions and behaviors induced by AAE in rodents (reviewed in (14, 18)). Interestingly, most studies used a "binge-drinking-like" administration of alcohol, involving repeated i.p. injections or gavage, and showed that AAE induced activation of the innate immune system in frontal cortical regions (20-23). Here, by using a model of voluntary binge-drinking of alcohol in adolescent mice, we reported that while not impacting adolescent behaviors, AAE strongly impairs behaviors during adulthood, similarly in males and females, without inducing major neuroinflammation in the PFC.

Material and Methods

Detailed information can be found in supplementary material.

Animals

Males and females C57BL/6J (Janvier Labs, Saint Berthevin, France) were housed under standard conditions and were treated according to the guidelines of the Belgian Ministery 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 number 18/2004). Mice were maintained with access to food *ad libitum* and kept at a constant temperature (19° to 22°C) and humidity (40 to 50%) under a reversed light/dark cycle (lights on at 22:00, off at 10:00).

Adolescent Alcohol Exposure

Adolescent male and female mice underwent a modified version of the Drinking in the Dark paradigm (24) from P29 to P40. Mice were group-housed. 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 solution (20% in tap water). At 16:00, alcohol was removed and mice were group-housed until the next day. Control mice received only water. Mice were given alcohol from P29 to P33, and from P36 to P40 (Fig. 1E). Different groups of mice underwent the behavioral tests at two different time-points: Seventy-two hours after AAE (P43) or after 40 days of abstinence (P80). Ninety-one percent of the animals drank more than 4g/kg/4h and were included in the study. Adolescent mice which drank less than 4g/kg/4h for more than 2 sessions were excluded from the study. The threshold of 4g/kg/24h was chosen because it represents the amount of alcohol ingested leading to minimal binge-drinking BAC values, as previously described (25, 26). Individual drinking data can be found in Tables S1 and S2.

Blood Alcohol Concentration Measurement

Blood alcohol concentration (BAC) was measured in trunk blood immediately after the last drinking session (P40), as previously described (27, 28). Further information can be found in supplementary material.

Behavioral tests

Mice were handled twice a day for 2 minutes for one week before behavioral test. Mice were placed in the testing room one hour before the beginning of each experimental procedure. All tests were monitored by a camera and analyzed by a blinded experimenter. All apparatus were cleaned with 75% ethanol and dried between each mouse and session. Raw data and movies can be found in Mendeley data, V1, DOI: 10.17632/gtnmrbtmt4.1

Open field test

Locomotion and anxiety-like behavior were evaluated by conducting the Open Field (OF) test as described in (29). Detailed information can be found in the supplementary material.

Elevated Plus Maze Test

Elevated plus maze test was conducted as described in (30, 31). Further information can be found in the supplementary material

Forced swimming Test

Forced swimming test was performed according to the procedures described in (32). Further information can be found in the supplementary material.

Novel Object Recognition Test

NOR was performed as described in (33), with a long habituation phase. Further information can be found in the supplementary material.

Three Chamber Test

The three chamber test was performed as described in (34). Detailed information can be found in the supplementary material.

Reversal Learning Test

The reversal learning test was performed by using the Barnes maze, as described in (35, 36), with slight modifications. Learning was assessed for 5 days (2 sessions per day, inter-trial interval one hour). Seventy-two hours after the last learning session, mice underwent the 80-second learning probe trial, in which the escape tunnel was removed from the apparatus. Twenty-four hours after the probe test, mice underwent 5 days (2 sessions per day) of reversal learning. Seventy-two hours after the last reversal learning session, mice underwent the reversal learning probe test. Further details can be found in the supplementary material.

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 (26, 37) (Fig 5A). Further information can be found in the supplementary material.

Immunohistochemistry

Immunohistochemistry was conducted as previously described (38). Fifty µm brain sections were incubated in the primary antibodies overnight at 4C (Goat anti-Iba1 1/500, abcam (Cambridge, UK) #ab5076, and rat anti-CD68 1/500, Merck (Kenilworth, NJ, USA) #mab1435). Donkey anti-rat AlexaFluor 488 and anti-goat AlexaFluor 564 were used as secondary antibody for 4 hours at room temperature, with DAPI. Images were acquired on a Nikon A1 confocal microscope and NIS-Element Imaging software at 20x magnification. Prelimbic and infralimbic medial sections (636,4µm x 636,4µm) were imaged in z-stack (1µm, 7-11 images per stack) and analyzed by blinded observer. Microglial cell activation state was defined according to their shape: microglia presenting a small nucleus and numerous and ramified extensions were considered as resting, whereas amoeboid-shaped microglial cells with a large nucleus and smaller extensions were considered as activated.

Western blot analysis

Western blot analysis was conducted from prefrontal cortices extracts as previously described (38). 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 4C, then probed with HRP-conjugated secondary antibody for one hour at room temperature. Membranes were developed using ECL and images were obtained with ImageQuant LAS 4000 camera system (GE Healthcare, Chicago, IL, USA). Band intensities were quantified using ImageJ software (NIH).

ELISA

Prefrontal cortices were homogenized in tissue lysis buffer (20 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, protease inhibitors cocktail; pH8) and centrifuged for 10 min at 10 000g at 4C. Supernatants were collected and protein concentrations were determined by using the BCA assay kit (Thermofischer Scientifics, Waltham, MA, USA). IL-1β levels were determined by using the IL-1β ELISA kit (ThermoFisher Scientific, #88-7013) following the manufacturer's protocols.

Statistical tests

Data were analyzed by using two-ways 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 Tuckey *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.

Results

Adolescent mice voluntarily binge-drink alcohol and reach high blood alcohol concentrations

Adolescent mice underwent a modified version of the Drinking In the Dark (DID) paradigm (39), and were given access to a bottle of alcohol 20% for 4 hours per day, for 10 sessions between P29 and P40 (Fig. 1A). Alcohol intake did not alter body weight gain (Fig. 1B) and adolescent mice voluntarily consumed high amounts of alcohol (Fig. 1C). Our results further showed that females significantly drank more alcohol than males (mean alcohol consumption, respectively 7.25±0.2 and 6.52±0.09 g/kg/4h) and exhibited escalation of alcohol consumption (Fig.1C). As

shown in Figure 1D, blood alcohol concentrations (BAC), which were measured immediately after the last drinking session, positively correlated with alcohol intake. Overall, we showed that adolescent mice voluntarily consumed high amounts of alcohol and reached BAC values comprised between 100 and 200mg/dL, which correspond to binge-drinking values observed in humans (39). Following AAE, a battery of behavioral tests was performed at two different time-points: Short-term effects were evaluated seventy-two hours after the last drinking session, whereas long-term effects were evaluated on independent cohorts of adult animals after forty days of abstinence (Fig. 1E).

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

Studies have suggested that heavy alcohol exposure in rats may lead to the development of anxiety-like behaviors (40-42). Following AAE, adolescent and adult mice were tested in the Open Field (OF) and the Elevated Plus Maze (EPM) apparatus, which are commonly used to assess anxiety-like behaviors in rodents (43-45) (Fig. 2A, B). Seventy-two hours after the last drinking session, no significant difference in the percentage of time spent in the center of the OF was found between AAE and water-exposed animals over three sessions (Fig 2C). In addition, AAE animals and water controls spent similar percentage of time exploring the open arm of the EPM (Fig. 2D), and exhibited similar percentage of open arms entries (Fig. 2E).

Anxiety-like behavior 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 thymotagsis in the OF as compared to water-exposed littermates, in both sexes (Fig. 2F). In addition, AAE animals spent less time exploring the open arms of the EPM (Fig. 2G), and the percentage of open arm entries was also reduced as compared to water control animals (Fig. 2H). Importantly, no significant difference in

locomotor activity or habituation was found between “AAE” and “water” mice (Fig. S1A, C), and the total number of EPM arm entries did not differ between groups, suggesting that AAE did not impact exploration behavior (Fig. S1B, D). Together, these data suggest that although voluntary binge-drinking of alcohol during adolescence did not alter anxiety levels in late adolescence, it promoted the long-term development of anxiety-like behaviors.

We then assessed the consequences of AAE on depressive-like behavior by using the Forced Swimming Test (FST), which is widely used to investigate the response to antidepressant treatments and assess depressive-like behavior in animal models (46-48) (Fig. 2I). Seventy-two hours after the last drinking session, AAE and water-exposed adolescent mice exhibited equivalent immobility time and delay before the first immobility, both in males and in females (Fig. 2J, K). Interestingly, adolescent males exhibited higher immobility time and decreased delay before first immobility, as compared to females (Fig. 2J, K).

In contrast, when the FST was performed 40 days after the last alcohol drinking session, mice that binge-drank alcohol during adolescence exhibited significantly increased immobility time compared to water controls, both in male and in female groups (Fig. 2L). In addition, the delay before the first immobility episode was shorter in AAE animals (Fig. 2M), suggesting that AAE induces depressive-like behavior long-term after alcohol consumption.

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

The Novel Object Recognition (NOR) test assesses the natural preference for novel objects normally displayed by mice, and gives insights about their recognition memory performance (Fig. 3A) (49, 50). Short-term after AAE, all groups of mice showed similar exploration behavior during the familiarization and test sessions (Fig. 3B, C). In addition, no significant difference in

discrimination index (DI) and familiar object habituation index (FHI) was found between AAE and water control, both in male and female groups (Fig. 3D, E).

However, forty days after the last alcohol exposure, despite similar exploration behavior, AAE mice exhibited significantly lower DI and FHI compared to water littermates, both in males and females (Fig. 3F-I). Altogether, these results suggest that although AAE did not impact recognition memory performance shortly after alcohol exposure, it dramatically impaired novel object recognition long-term after alcohol consumption.

Adolescent alcohol binge-drinking does not affect mouse sociability

We investigated the short- and long-term consequences of AAE on social behaviors by performing the three-chambered social approach task (51, 52) (Fig. S2A). All mice showed similar preference for the social stimulus, suggesting that AAE did not impact mice sociability (Fig. S2B, D). Social novelty preference was also investigated by calculating the social novelty preference index (SNI) after introduction of a novel mouse in the empty wired cup (53) (Fig. S2A). Our results did not show any significant difference in social novelty preference between AAE and water adolescent mice, either short- or long-term after AAE (Fig. S2C, E). However, in adult animals, SNI was not significantly different from zero in all groups, suggesting that none of the mice exhibited significant social novelty preference. Overall, those results suggest that AAE had no major impact on mouse sociability.

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 (14, 15). We thus sought to unveil the consequences of AAE on reversal spatial learning by

performing the Barnes Maze test (Fig. 4A). Learning abilities were first investigated. Shortly after the last drinking session, no significant difference of escape time was found between AAE- and water-exposed animals, which similarly decreased over days in all groups (Fig. S3A). Accordingly, the number of primary errors was similar in all groups of mice (Fig. S3B). Three days later, a probe test was performed (in absence of the escape tunnel) and no significant difference in the percentage of time spent in the correct sector was observed between AAE and water animals in both sexes (Fig. S3C).

Learning was also investigated long-term after AAE. We reported that learning abilities of adult mice were not affected by alcohol intake during adolescence, as shown by similar escape time and primary error number in all groups (Fig. S3D, E). Moreover, no difference was found during the probe test (Fig. 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° (Fig. 4A). In late adolescence, AAE and water control mice displayed similar escape time and no difference was found in the number of primary errors between groups (Fig. 4B, C). Furthermore, the probe test did not reveal any difference in the percentage of time spent in the correct sector (Fig. 4D) or in the previous sector (Fig. 4E).

However, when reversal learning was assessed in adulthood, AAE-exposed mice showed higher escape time and made more primary errors as compared to water controls, in both sexes (Fig. 4F, G). Interestingly, the mean escape time and primary error numbers were also significantly higher in males compared to females, regardless of their alcohol treatment (Fig. 4F, G). In addition, during the probe test, AAE mice spent significantly less time in the correct sector compared to 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%) (Fig. 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%), suggesting increased perseveration behavior in AAE groups (Fig. 4I). Altogether, our results suggest that while 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.

Adolescent alcohol binge-drinking enhances alcohol consumption in adulthood.

Clinical and pre-clinical studies have suggested that AAE increases the risk of developing alcohol use disorders later in life (7, 13, 54). In order to decipher whether AAE modulates alcohol intake and preference in mice, mice underwent 5 sessions of intermittent access to 20% alcohol – 2 bottle choice paradigm (Fig. 5A) (37, 38). Alcohol intake, alcohol preference and total fluid intake were measured at the end of each 24-hour session. Short-term after AAE, no significant difference was observed in alcohol intake or preference between groups (Fig. 5B-D). Interestingly, data revealed that females consumed more alcohol than males (respectively 26.92±2.96 and 23.95±2.03 g/kg/24h), and more fluid in general (respectively 310.5±6.12 and 265.9±24.5 ml/kg/24h) (Fig. 5B-D).

In contrast, when analyzed long-term after AAE, adult mice which were exposed to alcohol during adolescence consumed significantly more alcohol than water controls, 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/24h) (Fig. 5E). AAE mice also exhibited higher alcohol preference (Fig. 5F), without any difference in total fluid intake (Fig. 5G). Similarly, females significantly consumed higher amount of alcohol compared to males (respectively 20.61±5.2 and 16.56±3.4 g/kg/24h) and presented higher total fluid intake (respectively 236.6±43 and 189.7±27 ml/kg/24h) (Fig. 5E-G).

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

Voluntary alcohol binge-drinking during adolescence does not induce major activation of the innate immune system in the prefrontal cortex of mice

Several studies using binge-like administration of alcohol during adolescence have showed that alcohol activates innate immune signaling in the frontal cortical regions, which induced alterations in behaviors (20-23, 55). In order to decipher whether voluntary alcohol binge-drinking in adolescent mice induces neuroinflammation in the prefrontal cortex, we assessed the number and activation state of microglial cells in the prelimbic region of the PFC by immunofluorescence. Microglial cells with small nucleus, numerous ramified extensions and low expression of the lysosomal marker CD-68 were considered as resting, whereas amoeboid-shaped microglial cells with a large nucleus, smaller extensions, and strong CD-68 expression were considered as activated (Figure S5A, B). As shown in Figure 6A-F, there was no difference in the number of activated microglial cells between AAE and water animals when analyzed 72 hours after the least drinking session (Figure 6E, F). When the same analysis was performed on adult mice, long-term after AAE, no alcohol-dependent activation of microglial cells was observed neither in males nor females (Figure S5C-H). The analysis was repeated in the infralimbic region of the PFC, where no alcohol-dependent activation of microglial cells was observed, neither short- or long-term after AAE (data not shown). We further assessed the expression of the High Mobility Group protein B1 (HMGB1) and the Toll-like receptor 4 (TLR4) in the PFC by western blot analysis. As shown in figure 6G and 6H, no significant increase in HMGB1 or TLR4 expression was observed in the PFC of AAE animals compared to water controls, neither in males or females, short-term or long-term after AAE. Finally, we did not find

any difference in interleukin-1β concentration between AAE and water animals, neither 3 days nor 40 days after AAE (Figure 6I, J). Altogether, our data show that voluntary binge-drinking in mice did not induce major activation of the innate immune signaling in the PFC.

Discussion

In this study, we used a model of voluntary alcohol consumption in adolescent mice and reported that binge-drinking of alcohol during adolescence has limited short-term impact on behaviors but leads to the development of dramatic impairments during adulthood. In addition, although differences between males and females were observed, the consequences of AAE on long-term behaviors were comparable with both sexes. Finally, we reported that voluntary alcohol binge-drinking during adolescence did not trigger major activation of the innate immune system in the prefrontal cortex.

Adolescent alcohol exposure insidiously impairs adult behaviors in mice

We first reported that adolescent binge-drinking induced the development of anxiety- and depressive-like behaviors in adulthood, without any warning sign in late adolescence. Such results are consistent with other studies showing that repeated passive exposure to alcohol during adolescence induces anxiety-like behaviors in adult rodents (40-42, 56, 57). However, opposite findings have been reported and showed that exposure to ethanol vapor 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 (58-61). OF and EPM are tasks assessing the balance between the innate exploratory drive and the anxiety generated by a novel environment (62). Therefore, it remains challenging to decipher whether such behavioral

differences result from change in anxiety and/or impulsivity, and data should thus be carefully interpreted.

Besides, our results also suggest that AAE, while not impacting adolescent mouse behavior in the FST, leads to the long-term development of depressive-like behaviors. Those results are in line with a study conducted by Lee *et al*, in which the authors used a similar mouse model and showed AAE-dependent increased depressive-like behaviors and anhedonia in adult males (63). In addition, recognition memory performances were also affected long-term after AAE. The novel object recognition (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 behavior (33, 49). As environmental familiarization may modulate novel object interaction, increased anxiety levels in AAE animals could play a role in the observed AAE-dependent impaired recognition memory (33). In order to minimize potential bias caused by increased anxiety-like behaviors in AAE mice, we used a long habituation protocol which reduces the stress associated with the OF (33). In addition, no difference was found between AAE and water mice regarding their exploratory behavior, and no preference for one of the two objects was evidenced. Our results are consistent with the study of Pascual *et al*, which showed that rats exposed to alcohol during adolescence presented a reduced discrimination index in the NOR test.

AAE has also been reported to promote alcohol consumption in adult rats (14, 41, 55, 61, 63), although some studies showed opposite results (64-66). In the present study, we confirmed that voluntary adolescent binge-drinking promotes alcohol consumption and preference in adult male and female mice. 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. In contrast, AAE did not increase alcohol consumption in adolescents. This is quite surprising to see that naïve, water-drinking adolescent mice consumed the same amount of alcohol than AAE animals, and those results should be carefully interpreted. Indeed,

we cannot exclude the possibility that the absence of significant difference between AAE and water-exposed adolescent mice might be due to a plateau effect: as adolescent C57Bl6 mice voluntarily consume very high amounts of alcohol, it is possible that further increase in alcohol consumption cannot be observed.

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 late adolescent male rats, but suppressed sociability when coupled to overcrowding conditions (67). This interesting result 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 (67). We also showed no difference in social novelty preference in adolescence. However, in adult mice, none of the groups of mice exhibited significant social novelty preference. 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 (68).

Finally, several cognitive studies have suggested that AAE has minimal effect on spatial learning and memory tasks (14, 69, 70). However, flexibility impairments have been reported when reversal learning or set-shifting tasks were demanded (14, 57, 61, 71). 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 efficiently learn the initial position of the escape tunnel, but when the task required a more flexible strategy, we observed that AAE animals significantly lacked behavioral flexibility and exhibited increased perseveration of previously learned behavior.

Altogether, we show that voluntary alcohol binge-drinking during adolescence does not lead to behavioral alterations in adolescent mice, but dramatically impairs adult executive functions and

behaviors. This suggests that alcohol-dependent behavioral impairments are a progressive and insidious process, which only emerge during adulthood.

Voluntary alcohol binge-drinking in mice does not trigger long lasting activation of the innate immune system in the prefrontal cortex

Importantly, all the behaviors assessed in this study involve the PFC, which is critical for the executive functions (72, 73). In particular, reversal learning and behavioral flexibility have been shown to mainly depend on PFC function (74, 75). In this view, the AAE-dependent impaired behaviors observed during adulthood may arise from PFC malfunction. Adolescence is a vulnerable age period regarding the consequences of alcohol exposure (14, 55, 76). Some pathophysiological mechanisms underlying AAE-induced behavioral impairments are starting to emerge (8, 14, 55, 76). Among them, several studies have reported that repeated passive exposure to high levels of alcohol during adolescence induced the long-lasting induction of innate immune signaling through complex cascades involving Receptor for Advanced Glycation End (RAGE), high mobility group protein B1 (HMGB1), Toll-like receptors as well as the release of pro-inflammatory cytokines, which in turn led to synaptic plasticity disruption and neuropathy in frontal brain regions (22, 23, 56, 77, 78). In addition, in an elegant study, Montesinos *et al* showed that TLR4KO mice were protected against the alcohol-dependent behavioral alterations after AAE (56). Surprisingly, in the present study, although we reported severe behavioral impairments following AAE, no major induction of neuroinflammation was observed in the mouse PFC. Such discrepancy may arise from experimental set up differences, including the animal model (mice vs rats), the timing of behavioral testing and the mode of alcohol administration. Along this line, the large majority of studies showing AAE-dependent induction of the innate immune system used acute i.p. injections of alcohol while our AAE model involves voluntary alcohol drinking. Different administration routes lead to large differences in alcohol

pharmacokinetics. Indeed, alcohol is more rapidly absorbed after i.p. as compared to oral ingestion, and alcohol accumulation in the brain is also lower after oral administration (79). In addition, absorption profiles appear also very different: in the voluntary alcohol consumption model, mice drank around 7g/kg of alcohol in a period of 4 hours, whereas i.p. injections involved a single acute administration of 3-5g/kg of alcohol. As elimination of alcohol is done at a constant rate and independently of alcohol concentrations (80), the maximal concentration of alcohol after i.p injection is significantly higher as compared to voluntary drinking. It is thus possible that after passive exposure by i.p. administration alcohol reaches a toxicity threshold, which triggered the induction of neuroinflammation in frontal brain regions. Besides, it is important to question the role of stress, which may be associated with passive alcohol administration procedures. Indeed, i.p. injections or gavage procedures are likely to lead to higher amount of pain and stress, as compared to voluntary alcohol binge-drinking. We thus cannot exclude that acute i.p. administration of alcohol to rodents exacerbates possible latent pro-inflammatory effects of alcohol. Further research would be needed in order to better understand the relationship between exposure model, stress, and the alcohol-dependent induction of neuroinflammation in the rodent PFC.

Conclusions

This study demonstrated that voluntary alcohol binge-drinking during adolescence leads to severe behavioral impairments in adult mice. Indeed, AAE severely increases anxiety-like behaviors, depressive-like behaviors and alcohol consumption in adulthood, while impairing recognition memory and behavioral flexibility. Although differences were noted between males and females, our data showed that AAE similarly affects their behaviors. Surprisingly, adolescent behaviors were not affected by alcohol binge-drinking, suggesting that AAE-dependent alteration of behaviors is a slow 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 (81, 82). 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 the innate immune system in the PFC.

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Author contributions

Conceptualization (S.L., L.N. and L.V.H); Formal analysis (S.L. and V.D); Investigation (L.V.H., S.L., A.A., M.C.B., T.V.I., V.D.); Methodology (S.L., L.V.H., V.D., E.Q.); Funding acquisition (L.N. and S.L.); Supervision (L.N. and E.Q.); Writing – original draft (S.L. and L.V.H.); Writing-editing (L.N., V.D., E.Q.).

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Figures legends

<u>Fig 1</u>: 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 hours per day, for ten 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 ($\chi^2_{(9)}$=1.18x10^4, p<0.001 η^2=0.65) and sex ($\chi^2_{(1)}$=51.41, p<0.001, η^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 η^2=0.08), session ($\chi^2_{(9)}$= 109.04, p<0.001, η^2=0.03), and a significant interaction sex x session ($\chi^2_{(9)}$=26.86, p<0.01, η^2=0.007); n=153 males, 162 females. **(D)** Scatter plot showing the relationship between alcohol intake and blood alcohol concentration values. Centerline 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 behavioral tests following adolescent alcohol exposure. Animals underwent the DID paradigm from P29 to P40. They were tested 72 hours after the last drinking session (short-term), or after forty days of abstinence (long-term).

Fig 2: **Enhanced anxiety-like and depressive-like behaviors in adulthood but not in late 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 center of the OF. Linear mixed-effects model showed a significant main effect of sessions ($\chi^2_{(2)}$=53.59, p<0.001, η^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 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 center of the OF. Linear mixed-effects model showed a significant main effect of treatment ($\chi^2_{(1)}$=63.77, p<0.001, η^2=0.57), sex ($\chi^2_{(1)}$=13.73, p<0.001, η^2=0.22) and sessions ($\chi^2_{(2)}$=59.09, p<0.001, η^2=0.44), and a significant interaction sex x session ($\chi^2_{(2)}$=10.08, p<0.01, η^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, η^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* Tuckey test detected a significant difference between Water and 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, η^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* Tuckey 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 analyzed. **(J)** Short-term; Total immobility time. Two-way ANOVA showed a main effect of sex ($F_{(1,43)}$=4.36, p<0.05, η^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; η^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, η^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* Tuckey 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, η^2=0.25) and sex ($F_{(1,61)}$=7.77, p=0.007, η^2=0.08) but no interaction ($F_{(1,61)}$=0.66, p=0.42). *Post-hoc* Tuckey 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.

<u>Fig 3</u>: Adolescent binge-drinking decreases novel object recognition performances in adult but not adolescent mice

(A) Schematic representation of the Novel Object Recognition test (NOR). Following 3 days of habituation in the open field, mice were allowed to familiarize with two copies of the same object for 10 minutes. 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 seconds of total object exploration). Two-way 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 seconds). 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 seconds 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; Discrimination index. 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, η^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 AAE and water-exposed animals, in both males and females (p<0.001); n=15-17 per group.

<u>Fig 4</u>: Behavioral 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 supplemental figure 3). 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, η^2=0.55) and sex ($\chi^2_{(1)}$=4.52, p<0.05, η^2=0.086) but no main effect of treatment ($\chi^2_{(1)}$=0.26, p=0.61) and no interaction (day x sex $\chi^2_{(4)}$=7.90, p=0.1; day x treatment $\chi^2_{(4)}$=3.37, p=0.5; sex x 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, η^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 x sex $\chi^2_{(4)}$=0.54, p=0.97; day x treatment $\chi^2_{(4)}$=3.11, p=0.54; sex x treatment $\chi^2_{(1)}$=0.01, p=0.91). **(D)** Short-term; Percentage of time spent in the correct sector during probe test, 72 hours after the last reversal learning session. Two-way 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, η^2=0.64), treatment ($\chi^2_{(1)}$=47.87, p<0.001, η^2=0.22) and sex ($\chi^2_{(1)}$=7.92, p<0.001, η^2=0.13), as well as a significant interaction day x sex ($\chi^2_{(4)}$=24.81, p<0.001, η^2=0.08), and day x treatment ($\chi^2_{(4)}$=61.63, p<0.001, η^2=0.17) but not treatment x 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, η^2=0.46), treatment ($\chi^2_{(1)}$=29.15, p<0.001, η^2=0.16) and sex ($\chi^2_{(1)}$=4.10, p<0.05, η^2=0.07), as well as an interaction day x treatment ($\chi^2_{(4)}$=39.74, p<0.001, η^2=0.11), sex x treatment ($\chi^2_{(1)}$=4.51, p<0.05, η^2=0.03) but not day x sex ($\chi^2_{(4)}$=8.78, p=0.07). **(H)** Long-term; Percentage of time spent in the correct sector during probe test, 72 hours after the last reversal learning session. Two-way ANOVA showed a significant main effect of treatment ($F_{(1,48)}$=16.62, p<0.001, η^2=0.24) and sex ($F_{(1,48)}$=6.01, p<0.05, η^2=0.09) but no interaction ($F_{(1,48)}$=0.07, p=0.79). *Post-hoc* Tukey test revealed a significant difference between 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, η^2=0.43) and sex ($F_{(1,48)}$=9.91, p<0.01, η^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.

<u>Fig 5</u>: 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 AAE, mice underwent the IA-20%-2BC paradigm for 5 sessions. **(B)** Short-term; Alcohol intake (g/kg/24h). Linear mixed-effects model showed a significant main effect of session ($\chi^2_{(4)}$=11.3, p<0.05, η^2=0.04), and sex ($\chi^2_{(1)}$=17.28, p<0.001, η^2=0.27) but no effect of treatment ($\chi^2_{(1)}$=0.1, p=0.76) and no interaction (session x treatment

$\chi^2_{(4)}$=1.4, p=0.85; sex x treatment $\chi^2_{(1)}$=0.08, p=0.78; session x 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, η^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 x treatment $\chi^2_{(4)}$=4.57, p=0.33, sex x treatment $\chi^2_{(1)}$=0.00, p=0.96, session x sex $\chi^2_{(4)}$=9.35, p=0.051). **(D)** Short-term; Total fluid intake (ml/kg/24h). Linear mixed-effects model showed a significant main effect of session ($\chi^2_{(4)}$=15.12, p<0.01, η^2=0.05) and sex ($\chi^2_{(1)}$=34.6, p<0.001, η^2=0.42) but no effect of treatment ($\chi^2_{(1)}$=1,78 p=0.18) and no interaction (session x treatment $\chi^2_{(4)}$=5.53, p=0.24, sex x treatment $\chi^2_{(1)}$=0.27, p=0.6, session x sex $\chi^2_{(4)}$=7.78, p=0.1); n=12 per group. **(E**) Long-term; Alcohol intake (g/kg/24h). Linear mixed-effects model showed a significant main effect of treatment ($\chi^2_{(1)}$=34.86, p<0.001, η^2=0.15) and sex ($\chi^2_{(1)}$=17.28, p<0.001, η^2=0.09) but no effect of session ($\chi^2_{(4)}$=8.53 p=0.07). This model also showed an interaction session x treatment ($\chi^2_{(4)}$=14.8, p=0.01, η^2=0.02) but not sex x treatment ($\chi^2_{(1)}$=0.00, p=0.98) or session x 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, η^2=0.15) and session ($\chi^2_{(4)}$=19.6, p<0.001, η^2=0.06) but no effect of sex ($\chi^2_{(1)}$=0.04 p=0.84) and no interaction (session x treatment $\chi^2_{(4)}$=2.17, p=0.7, sex x treatment $\chi^2_{(1)}$=0.01, p=0.92, session x sex $\chi^2_{(4)}$=3.32, p=0.51). **(G)** Long-term; Total fluid intake (ml/kg/24h). Linear mixed-effects model showed a significant main effect of session ($\chi^2_{(4)}$=14.63, p<0.01, η^2=0.04) and sex ($\chi^2_{(1)}$=21.1, p<0.001, η^2=0.1) but no effect of treatment ($\chi^2_{(1)}$=0.3 p=0.58) and no interaction (session x treatment $\chi^2_{(4)}$=2.69, p=0.61, sex x treatment $\chi^2_{(1)}$=1.04, p=0.31, session x sex $\chi^2_{(4)}$=3.39, p=0.49); n=12 per group.

<u>Fig 6</u>: Voluntary alcohol binge-drinking during adolescence does not trigger innate immune system activation in the prefrontal cortex.

(A-D) Short-term; Immunofluorescence analysis of microglial cells expressing Iba1 (red) and CD-68 (green) in the prelimbic prefrontal cortex of males water (**A**), males AAE (**B**), females water (**C**) and females AAE (**D**); Bar scale 100µm. **(E)** Short-term; Number of activated Iba1+/CD68+ microglial cells per prelimbic PFC section (mean ± S.E.M.). Two-way ANOVA showed no main effect of treatment ($F_{(1,12)}$=0.47, p=0.5), sex ($F_{(1,12)}$=0.05, p=0.82) and no interaction ($F_{(1,12)}$=0.22, p=0.65). **(F)** Short-term; Total number of microglial cells per prelimbic PFC section (mean ± S.E.M.). Two-way 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 per group. **(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 ± S.E.M. of HMGB1 to actin and 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-1β concentration (pg/µg of protein) in the PFC, short-term (**I**) or long-term (**J**) after adolescent alcohol exposure. **(I)** Short-term; Data are represented as the mean concentration ± 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 ± 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.

Figure 1

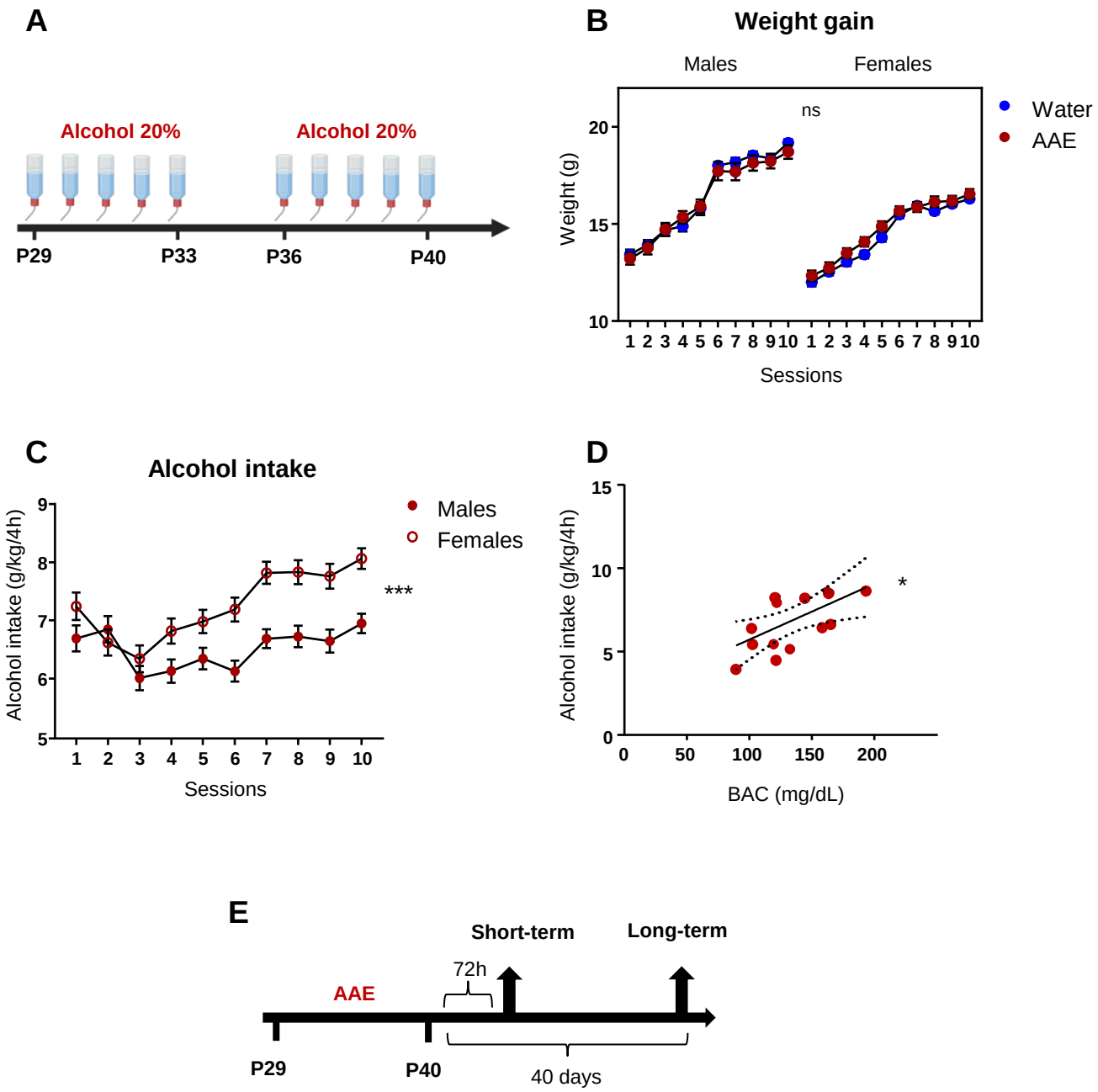

A
Alcohol 20%
Alcohol 20%
P29
P33
P36
P40
B
Weight gain
Males
Females
ns
Water
AAE
Weight (g)
20
15
10
1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10
Sessions
C
Alcohol intake
Males
Females
***
Alcohol intake (g/kg/4h)
9
8
7
6
5
1 2 3 4 5 6 7 8 9 10
Sessions
D
*
Alcohol intake (g/kg/4h)
15
10
5
0
0 50 100 150 200
BAC (mg/dL)
E
Short-term
Long-term
72h
AAE
P29
P40
40 days

Figure 2

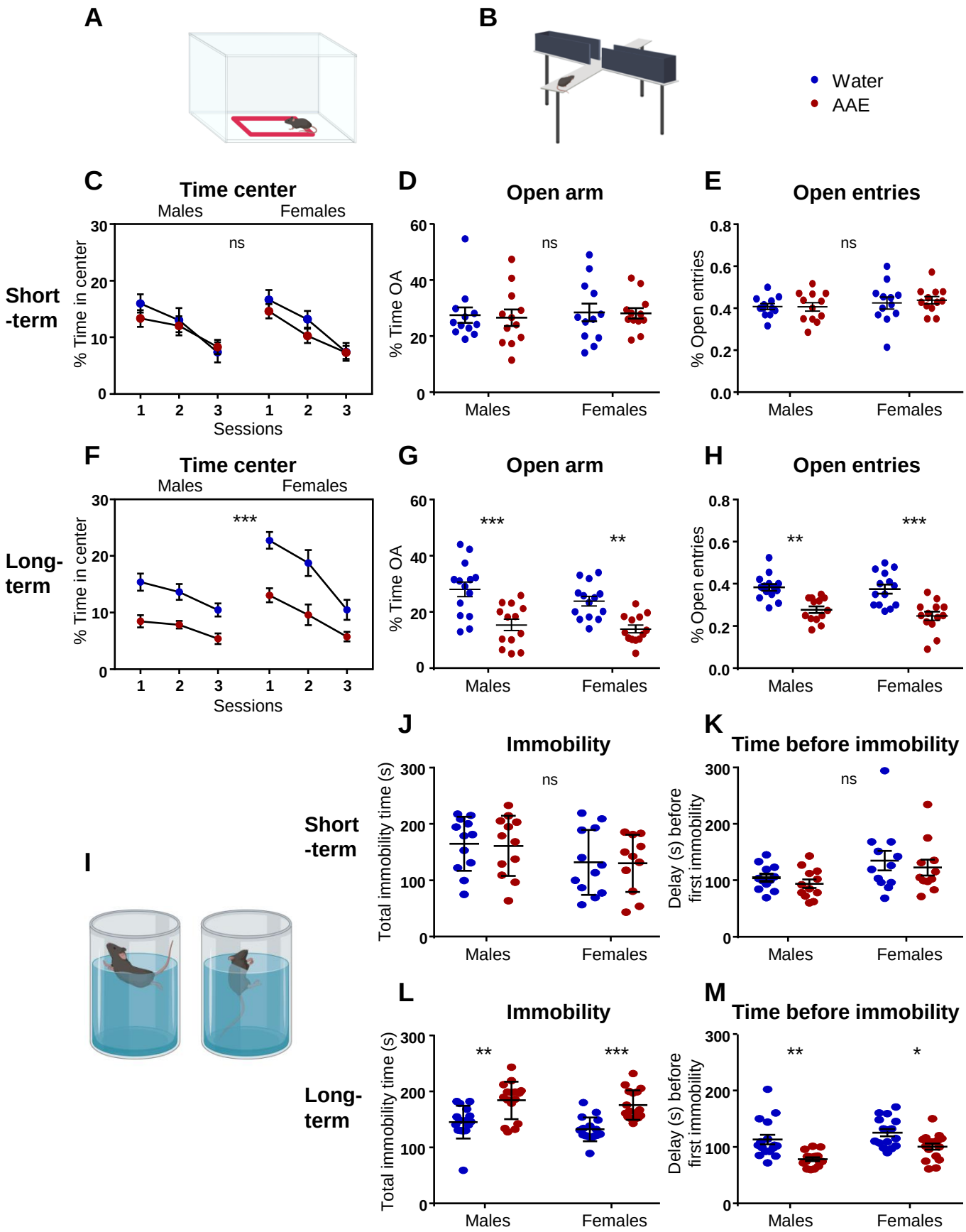

A
B
Water
AAE
C
Time center
Males
Females
% Time in center
ns
Sessions
D
Open arm
% Time OA
ns
Males
Females
E
Open entries
% Open entries
ns
Short -term
F
Time center
Males
Females
% Time in center
***
Sessions
G
Open arm
% Time OA
***
**
Males
Females
H
Open entries
% Open entries
**
***
Long- term
I
J
Immobility
Total immobility time (s)
ns
Short -term
K
Time before immobility
Delay (s) before first immobility
ns
L
Immobility
Total immobility time (s)
**
***
Long- term
M
Time before immobility
Delay (s) before first immobility
**
*

Figure 3

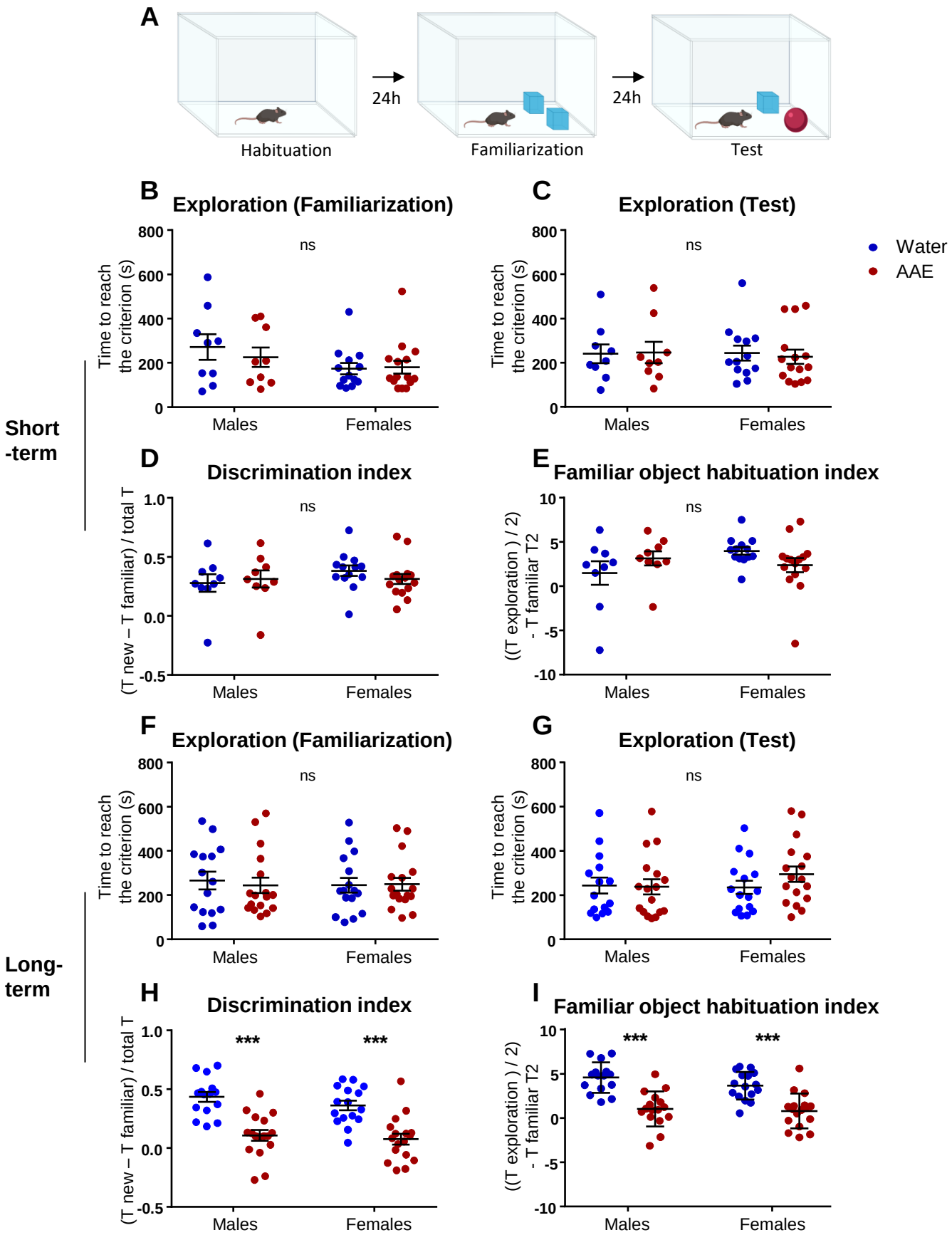

A
24h
24h
Habituation
Familiarization
Test
B
Exploration (Familiarization)
C
Exploration (Test)
Water
AAE
Time to reach the criterion (s)
ns
Males
Females
Short -term
D
Discrimination index
(T new – T familiar) / total T
E
Familiar object habituation index
((T exploration) / 2) - T familiar T2
F
Exploration (Familiarization)
G
Exploration (Test)
Long-term
H
Discrimination index
***
I
Familiar object habituation index

Figure 4

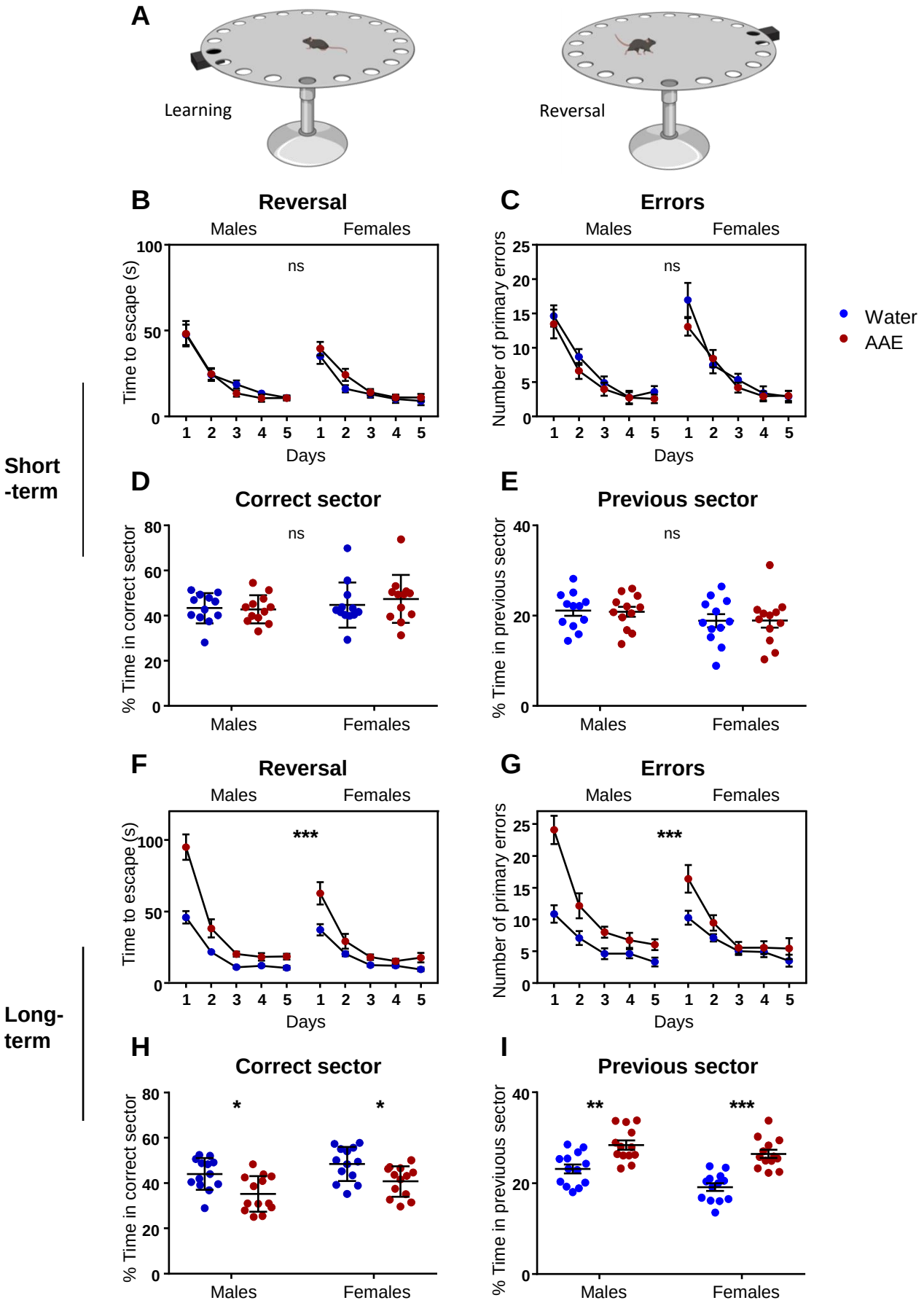
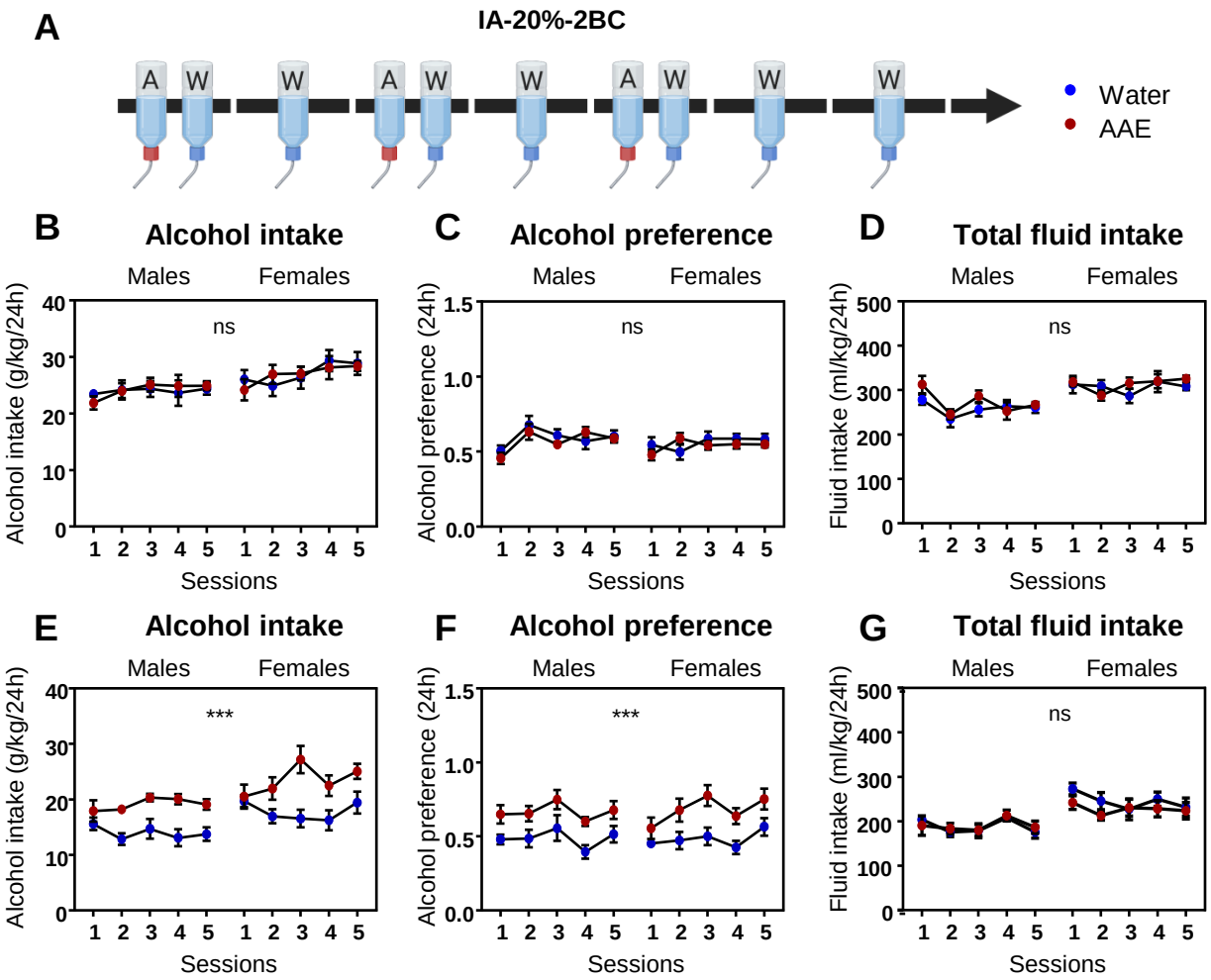
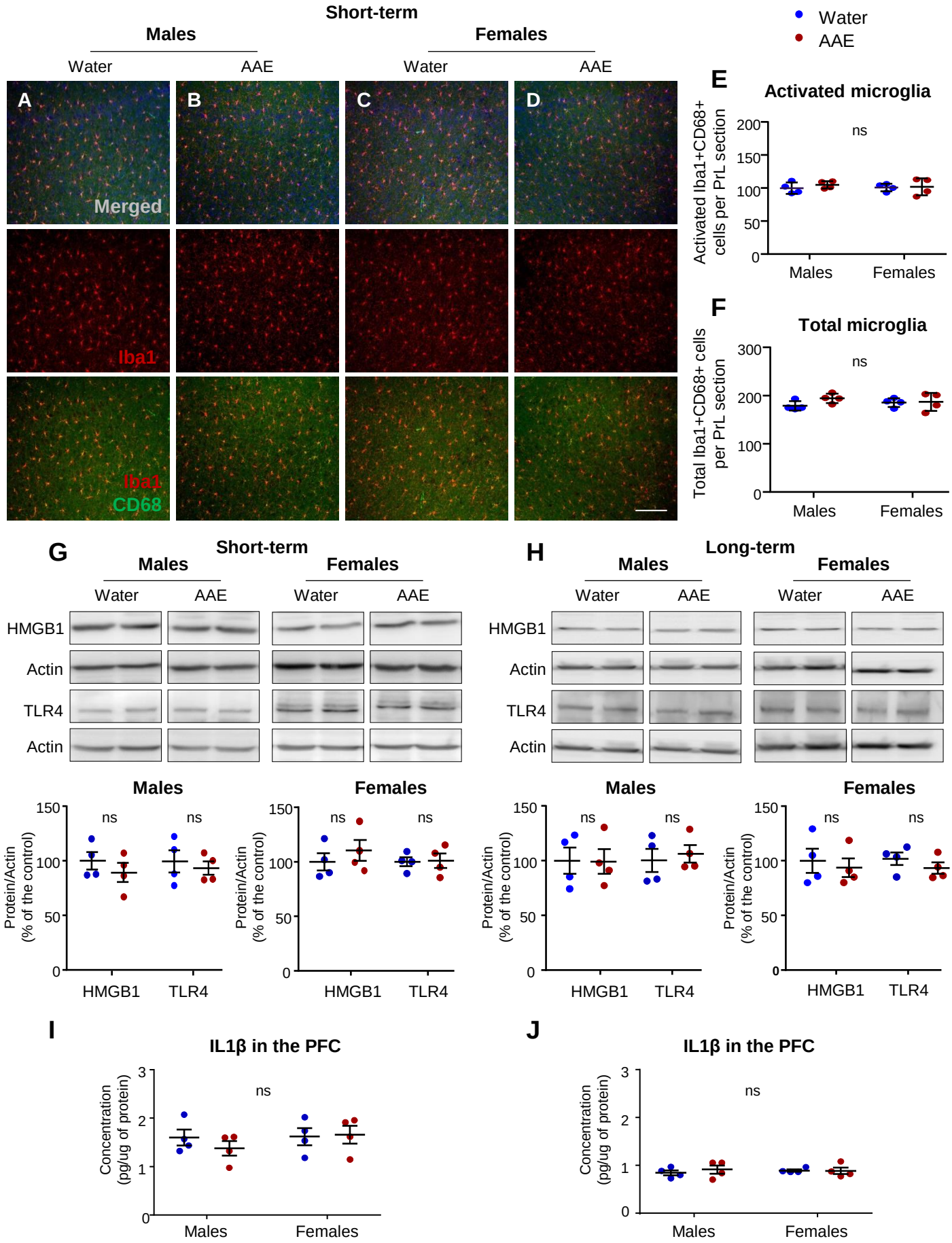

A
Learning
Reversal
B
Reversal
Males
Females
Time to escape (s)
ns
Days
C
Errors
Number of primary errors
Water
AAE
Short
-term
D
Correct sector
% Time in correct sector
E
Previous sector
% Time in previous sector
F
G
Long-
term
H
I
% Time in previuous sector
***
**
*

Figure 5


A
IA-20%-2BC
A W W A W W A W W W
Water
AAE
B
Alcohol intake
C
Alcohol preference
D
Total fluid intake
Short -term
Males
Females
ns
Alcohol intake (g/kg/24h)
Alcohol preference (24h)
Fluid intake (ml/kg/24h)
Sessions
E
Alcohol intake
F
Alcohol preference
G
Total fluid intake
Long-term
***


Short-term
Males
Females
Water
AAE
Water
AAE
A
B
C
D
Merged
Iba1
Iba1
CD68
Water
AAE
E
Activated microglia
Activated Iba1+CD68+ cells per PrL section
ns
Males
Females
F
Total microglia
Total Iba1+CD68+ cells per PrL section
ns
Males
Females
G
Short-term
Males
Females
Water
AAE
Water
AAE
HMGB1
Actin
TLR4
Actin
H
Long-term
Males
Females
Water
AAE
Water
AAE
HMGB1
Actin
TLR4
Actin
Males
Females
Protein/Actin (% of the control)
ns
HMGB1
TLR4
I
IL1β in the PFC
Concentration (pg/ug of protein)
ns
Males
Females
J
IL1β in the PFC
Concentration (pg/ug of protein)
ns
Males
Females