Adolescent Alcohol Binge-Drinking Insidiously Impairs Adult Behavior In Mice

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Abstract

Adolescence is a developmental period characterized by significant changes in brain architecture and behaviors. 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, by interfering with brain maturation, adolescent alcohol exposure (AAE) may have profound and long-lasting behavioral consequences. In this study, we used an AAE model, in which adolescent male and female mice reach high blood alcohol concentration after voluntarily 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 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. Our data suggest that AAE insidiously impairs adult behaviors, without any warning sign in late adolescence.

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 is associated with lack of inhibitory control over behaviors in adolescents (3, 4). Adolescence is typically the period for initial exposition to a number of potentially toxic exogenous agents, including alcohol (7, 8). Indeed, their engagement in risky behaviors and the immaturity of the frontal brain regions heighten the vulnerability of adolescents to long-lasting cognitive and behavioral consequences of excessive alcohol intake.

Alcohol is the most consumed addictive substance among teenagers, with 27% of adolescent worldwide reporting alcohol consumption in the past month (9). Binge-drinking of alcohol, which corresponds to ingestion of at least five alcoholic drinks in males (four in females) within a 2-hour period, has become a common pattern of adolescent alcohol consumption. Binge-drinking leads to high blood alcohol concentrations (above 0.08g/dl) (10-12), and is thought to be particularly harmful for the adolescent brain, as it may interfere with ongoing maturation of frontal circuits. It is believed that adolescent heavy alcohol exposure leads to profound and long-lasting changes on brain function and behaviors. 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 attenuated white matter development (15). AAE is also believed to have deleterious effects on verbal learning and memory, and lead to long-lasting deficits on attentional and executive functions (14, 16). Moreover, AAE significantly increases the risk of developing psychiatric and behavioral disorders later in life, including alcohol addiction (7, 13, 17, 18), and studies have revealed a significant association between the age of drinking onset and the risk of developing alcohol dependence later in life (19, 20). Altogether, it has become clear that, because of the high plasticity of the brain, adolescence is a very sensitive period for the development of alcohol-related behavioral impairments. However, although preventive programs and control policies have showed some success in reducing the detrimental effects of alcohol abuse on the adolescent brain, a better understanding of AAE-induced neurobiological and behavioral impact is needed to improve strategies for prevention, treatment and attenuation of the detrimental neuropsychiatric sequelae (21, 22). Over the past years, rodent models have been used to study alcohol's impact on adolescent brain and consequent behavioral impairments. Despite not fully recapitulating the complex human behavior defects, animal models provided findings partly consistent with human research (14, 18, 23).

Here, we developed a paradigm to model adolescent binge-drinking of alcohol in mice, in which male and female adolescent mice voluntarily consume high amounts of alcohol in a limited time. Following AAE, a battery of behavioral tests was performed at two different time-points, either late adolescence or adulthood, in order to examine whether voluntary adolescent alcohol binge-drinking significantly impacts mice behaviors in short- and long-term.

Results

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

We used a modified version of the Drinking In the Dark (DID) paradigm (24), in which adolescent mice have access to alcohol 20% during 4 hours per day, for 10 sessions between P29 and P40 (Fig 1A). Alcohol intake during adolescence did not alter body weight gain during the treatment period (Fig 1B) and adolescent mice voluntarily consumed high amounts of alcohol (Fig 1C). Females drank significantly more alcohol than males (mean alcohol consumption, 7.25±0.2 and 6.52±0.09 g/kg/4h) and their alcohol intake increased over sessions. Mixed linear model showed a main effect of sex ($\chi^2_{(1)}$=25.17, p<0.001) and session ($\chi^2_{(9)}$=109.04, p<0.001) and yielded a significant sex x session interaction ($\chi^2_{(9)}$=26.86, p<0.01) (Fig 1C). The blood alcohol concentrations (BAC), measured shortly after the last drinking session, positively correlated with alcohol intake (Linear regression, $t_{(1,15)}$=2.44 r^2=0.31, p<0.05) (Fig 1D). Overall, our AAE model shows that adolescent mice reached BAC values comprised between 100 and 280mg/dL, which correspond to binge-drinking values observed in humans (24). As alcohol intoxication can lead to behavioral defects, a battery of behavioral tests was performed at two different time-points following AAE: Short-term effects were evaluated at P42, 48 hours after the last drinking session, whereas long-term effects were evaluated on independent cohorts in adulthood, at P80 (Fig 2A).

Adolescent alcohol exposure increases anxiety-like behaviors in adulthood

Studies have suggested that repeated alcohol exposure in adolescent rats may lead to the development of anxiety-like behaviors (25-27), while other groups have showed AAE-dependent

increased impulsivity in rat models (28-30). AAE- and water-exposed adolescent (P42) and adult (P80) mice underwent the Open Field (OF) and the Elevated Plus Maze (EPM) tests, which are commonly used to assess anxiety-like behaviors in rodents (Fig 2B) (31-33). No significant difference in the percentage of time spent in the center of the OF over three sessions was found between AAE and water-exposed P42 animals (Linear mixed-effect model: main effect of session ($\chi^2_{(1)}$=53.59, p<0.001); Fig 2C). In addition, AAE animals and water controls spent similar percentage of time exploring the open arm of the EPM (Fig 2D), and no difference was observed in the percentage of open arm entries (Fig 2E), suggesting that AAE does not modulate anxiety levels in males or in females, shortly after alcohol exposure. Anxiety-like behavior was further assessed 40 days after the last drinking session. Adult mice that were exposed to alcohol during adolescence exhibited enhanced thymotagsis, as compared to water-exposed littermates (treatment ($\chi^2_{(1)}$=63.77, p<0.001); Fig 2F). Statistics also revealed a significant effect of session ($\chi^2_{(1)}$=59.09, p<0.001) and sex ($\chi^2_{(1)}$=13.73, p<0.001), all groups exhibiting equivalent habituation to the environment over sessions, although males showed higher avoidance of the central square compared to females (Fig 2F). When tested in the EPM, adult AAE animals from both sexes spent less time exploring the open arm (Two-way ANOVA: main effect of treatment ($F_{(1,50)}$=30.97, p<0.001); *post hoc* tests males p<0.001; females p<0.01; Fig 2G), and reduced their percentage of open arm entries (treatment ($F_{(1,50)}$=40.2, p<0.001), *post hoc* tests males p<0.01; females p<0.001; Fig 2H). We measured the potential effect of AAE on locomotion activity or exploratory behavior (S1 Fig). No difference in locomotor activity or locomotor activity habituation was found in the OF between AAE and water mice, neither at P42 or P80 (S1 Fig). Furthermore, the total number of EPM arm entries did not differ between groups, suggesting that AAE does not modify mice exploration behavior (S1 Fig). Together, these data suggest that heavy alcohol use during adolescence results in increased anxiety-like behaviors in adulthood.

Adolescent alcohol exposure enhances depressive-like behavior in adulthood

In order to assess the consequences of AAE on depressive-like behavior, young and adult mice were subjected to the Forced Swimming Test (FST). The FST is widely used to investigate the response to antidepressant treatments and to assess depressive-like behavior in animal models (34-36). Briefly, a period of inescapable swimming leads to a state of despair, in which mice give up actively swimming and remain floating passively (34, 36) (Fig 2I). Forty-eight hours after the last drinking session, AAE and water-exposed adolescent mice exhibited equivalent immobility time, although immobility was increased in males compared to females (Two-way ANOVA: main effect of sex ($F_{(1,43)}$=4.36, $p<0.05$); Fig 2J). Accordingly, no difference was found in the delay before the first immobility between AAE and water mice, with males immobilizing before females (sex ($F_{(1,43)}$=6.17, $p<0.05$); Fig 2K). In contrast, when adult animals underwent the FST, mice that binge-drank alcohol during adolescence exhibited increased immobility time compared to water controls (treatment ($F_{(1,61)}$=35.21, $p<0.001$), *post hoc* tests males $p<0.01$; females $p<0.001$; Fig 2L). In addition, the delay before the first immobility episode was also shorter in AAE animals (treatment ($F_{(1,61)}$=22.98, $p<0.001$); *post-hoc* tests males $p<0.01$; females $p<0.05$; Fig 2M). Those results suggest that AAE increases depressive-like behavior in adult but not adolescent mice.

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

The Novel Object Recognition (NOR) test evaluates the mice' ability to recognize a novel object in their environment. As there is no positive or negative reinforcer in this task, it assesses the

natural preference for novel objects normally displayed by mice, and gives insights about their recognition memory performance (37, 38). Following three days of habituation in the OF, mice were allowed to familiarize with two similar objects for a single 10-minutes session. Twenty-four hours later, one object is replaced by a novel object, and the time spent exploring each object is measured (Fig 3A). Typically, a mouse that recognizes the familiar object will spend more time exploring the novel object (37, 38). Shortly after AAE, mice did not exhibit defects in exploration behavior during the familiarization and test sessions (Fig 3B, C). In addition, AAE adolescent males and females showed similar discrimination index (DI) and familiar object habituation index (FHI) compared to water-exposed controls, suggesting that AAE does not impair recognition memory in adolescence (Fig 3D, E). When adult mice underwent the test, no defect in object exploration behavior was observed between groups (Fig 3F, G). However, AAE mice exhibited significantly decreased DI and FHI compared to water littermates, both in males and females (Two-way ANOVA: main effect of treatment (DI: $F_{(1,60)}=49.86$, $p<0.001$; FHI: $F_{(1,60)}=49.5$, $p<0.001$; *post hoc* tests $p<0.001$; Fig 3H, I). Altogether, these results suggest that alcohol binge-drinking during adolescence does not impact recognition memory performance in adolescence, but leads to dramatically impaired novel object recognition in adulthood.

Alcohol binge-drinking during adolescence does not affect sociability

We investigated the consequences of AAE on social behaviors by performing the three-chambered test social approach task, which examines the amount of time a mouse spent sniffing a social stimulus (a mouse under a wired cup) compared to a non-social stimulus (empty wired cup) (S2 Fig) (39, 40). At P42, all adolescent mice spent significantly more time sniffing the mouse compared to the empty wire cup and no difference was found between AAE and water-exposed mice (Linear mixed-effects model: main effect of stimulus ($\chi^2_{(1)}=86.37$,

p<0.001); S2 Fig). In adulthood, all animals also showed similar preference for the social stimulus (Linear mixed-effects model: main effect of stimulus ($\chi^2_{(1)}$=70.27, p<0.001); S2 Fig), suggesting that AAE does not impact mouse sociability. 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 (41) (S2 Fig). Our results did not show any difference in social novelty preference between AAE and water adolescent mice, although SNI was found slightly higher in males compared to females (Two-ways ANOVA: main effect of sex ($F_{(1,39)}$=4.1, p<0.05); S2 Fig). Furthermore, no difference in SNI was found between AAE- and water-exposed animals in adulthood, and none of the groups of mice exhibited significant preference for the novel mouse (S2 Fig). Those results suggest that AAE does not impact sociability, neither in short- or long-term.

Adolescent alcohol exposure impairs reversal learning in adulthood

Clinical studies have shown that AAE leads to long-lasting deficits in executive functions (14, 15). We sought to identify the effect of AAE on reversal spatial learning by performing the Barnes Maze test (Fig 4A). Learning was first investigated for five days, then reversal learning was evaluated. At P42, during learning sessions, escape time was equivalent in all groups and similarly decreased over days (Linear mixed-effect model: main effect of days ($\chi^2_{(4)}$=753.1, p<0.001); S3 Fig). Accordingly, no difference was found in the number of primary errors (days ($\chi^2_{(4)}$=351.43, p<0.001; S3 Fig). Seventy-two hours after the last learning session, a probe test in absence of the escape tunnel was performed for 80 seconds, and the percentage of time spent in the correct sector was analyzed. No difference was observed between AAE and water animals in both sexes (S3 Fig). Learning was also not affected by AAE in adulthood, as shown by similar escape time (days ($\chi^2_{(4)}$=683.02, p<0.001); S3 Fig) and primary error number (days

($\chi^2_{(4)}$=152.26, p<0.001); S3 Fig) in all groups. Moreover, no difference was found during the probe test (S3 Fig). Those results suggest that AAE has no effect on spatial learning acquisition. Then, in order to assess reversal learning abilities, the escape tunnel location was moved by 180°, and escape time and primary errors were analyzed during five days (Fig 4A). At P42, AAE and water control mice displayed similar escape time, which decreased over days (days ($\chi^2_{(4)}$=363.3, p<0.001)), although males exhibited higher escape time compared to females (sex ($\chi^2_{(4)}$=4.52, p<0.05); Fig 4B). In addition, no difference was found in the number of primary errors between groups (days ($\chi^2_{(4)}$=342.42, p<0.001); Fig 4C). Following reversal learning, 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). Those results suggest that AAE has no effect on reversal spatial learning in adolescence. However, at P80, AAE-exposed mice showed significantly increased escape time compared to water controls (Fig 4F). Linear mixed-effect model showed a main effect of days ($\chi^2_{(4)}$=539.37, p<0.001), treatment ($\chi^2_{(1)}$=47.87, p<0.001) and sex ($\chi^2_{(1)}$=7.92, p<0.001), as well as a significant interaction days x sex ($\chi^2_{(4)}$=24.81, p<0.001), and days x treatment ($\chi^2_{(4)}$=61.63, p<0.001). This suggest that although the escape time decreased over days in all groups of mice, AAE-exposed mice needed significantly more time to find the new escape location (Fig 4F). Interestingly, adult males showed higher escape time compared to females, both in water and AAE groups (Fig 4F). Similar results were observed when analyzing primary errors number, with more errors in the AAE groups compared to water controls, and increased number of errors in males compared to females (days ($\chi^2_{(4)}$=273.91, p<0.001); treatment ($\chi^2_{(1)}$=29.15, p<0.001); sex ($\chi^2_{(1)}$=4.10, p<0.05); interactions days x treatment ($\chi^2_{(4)}$=39.74, p<0.001) and sex x treatment ($\chi^2_{(1)}$=4.51, p<0.05); Fig 4G). Moreover, 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%). Two-way ANOVA showed a main effect of sex

($F_{(1,48)}$=6.01, p<0.05) and treatment ($F_{(1,48)}$=16.62, p<0.001; *post hoc* tests p<0.05) (Fig 4H). Interestingly, AAE animals spent more time in the sector of the previous position of the escape tunnel compared to water controls (Water males 23.16±3.6%, AAE males 28.4±3.6%, Water females 19.15±3.1%, AAE females 26.46±3.3%; treatment ($F_{(1,48)}$=44.19, p<0.001); *post hoc* tests males p<0.01 and females p<0.001; Fig 4I), indicating that AAE animals exhibited increased perseveration behavior compared to water controls. Our result also showed higher perseveration in males compared to females, in both water and AAE groups (sex ($F_{(1,48)}$=9.91, p<0.01); Fig 4I). Thus, while AAE does not change reversal learning in adolescent, it does strongly impair it during adulthood. These results suggest that alcohol binge-drinking during adolescence may reduce behavioral flexibility in adulthood.

Binge-drinking during adolescence increases alcohol consumption and preference in adults, but not in adolescents

Clinical and pre-clinical studies have suggested that AAE increases the risk of developing alcohol use disorders later in life (7, 13, 20). In order to test whether AAE modulates alcohol intake and preference in mice, P42 and P80 mice underwent 5 sessions of intermittent access to 20% alcohol – 2 bottle choice paradigm (Fig 5A) (42, 43). Alcohol intake, alcohol preference and total fluid intake were measured at the end of each 24-hour session. No difference was observed in alcohol intake or preference between P42 AAE mice and water controls (Fig 5B-D). Data also showed that females consume more alcohol than males (Mean alcohol intake males 23.95±2.03 and females 26.92±2.96 g/kg/24h; main effect of sex ($\chi^2_{(4)}$=17.28, p<0.001)), and more fluid in general (Mean fluid intake males 265.9±24.5 and females 310.5±6.12 ml/kg/24h; main effect of sex ($\chi^2_{(1)}$=34.6, p<0.001); Fig 5B-D). In contrast, adult mice which binge-drank alcohol during adolescence consumed 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; mean effect of treatment ($\chi^2_{(1)}$=34.86, p<0.001); Fig 5E). AAE mice also exhibited higher alcohol preference (treatment ($\chi^2_{(1)}$=33.21, p<0.001); Fig 5F), without any difference in total fluid intake (Fig 5G). As for adolescents, adult females significantly consume higher amount of alcohol compared to males (Mean alcohol intake males 16.56±3.4 and females 20.61±5.2g/kg/24h; sex ($\chi^2_{(1)}$=17.28, p<0.001)), and presented higher total fluid intake (Mean fluid intake males 189.7±27 and females 236.6±43 ml/kg/24h; sex ($\chi^2_{(1)}$=21.1, p<0.001); Fig 5E-G). These results thus suggest that AAE dramatically increases alcohol intake and preference in adulthood.

Interestingly, alcohol consumption and preference were also measured four hours after the beginning of each drinking session, as a measure of alcohol binge-drinking (43). Similarly to the 24-hours analysis, no difference was observed in alcohol consumption or preference between AAE- and water-exposed P42 animals (S4 Fig). However, when tested in adulthood, AAE animals showed higher alcohol intake and preference compared to water littermates (S4 Fig), suggesting that AAE also increases alcohol binge-drinking in adulthood.

Then, mice underwent a second drinking paradigm, with intermittent access to sucrose 1% instead of alcohol. We showed that AAE- and water-exposed animals displayed similar sucrose consumption and preference at both P42 and P80 time-points (Fig 5H-M). Females also consumed more sucrose than males (P42; main effect of sex ($\chi^2_{(1)}$=4.78, p<0.05); Fig 5H and P80; sex ($\chi^2_{(1)}$=45.30, p<0.001); Fig 5K), and showed enhanced total fluid intake (P42, main effect of sex ($\chi^2_{(1)}$=6.02, p<0.01; Fig 5J and P80, sex ($\chi^2_{(1)}$=65.14, p<0.001); Fig 5M). Altogether, these results show that while AAE does not modify alcohol or sucrose consumption in adolescents, it dramatically increases alcohol intake and preference in adulthood, without changing sucrose intake.

Discussion

In this study, we used a mouse model of adolescent voluntary alcohol consumption and revealed that adolescent binge-drinking has limited short-term impact on behaviors, but leads to the development of dramatic behavioral impairments in adulthood. In addition, although differences between males and females were observed, the consequences of AAE on long-term behaviors were comparable in both sexes.

AAE induces anxiety-like and depressive-like behaviors in adult mice

Our findings indicate that adolescent binge-drinking only increases anxiety levels during adulthood. The OF and EPM tests explore unconditioned responses and involve mice spontaneous reactions to stressful stimuli that do not involve pain or discomfort (44). Our results are partly consistent with other studies showing that AAE induces anxiety-like disorders in adult rats (25-27) and mice (45, 46). However, those results are in opposition with other findings reporting that exposure to ethanol vapor increases exploration of adult rats in the open arms of the EPM (28-30, 47). OF and EPM are tasks assessing the balance between the innate exploratory drive and the anxiety generated by a novel environment (48). 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 another study by Lee et al, which used a similar mouse model and showed AAE-dependent increased depressive-like behaviors and anhedonia in adult males (49).

AAE decreases recognition memory performance in adulthood

Behavioral tests that evaluate the ability of animals to recognize a familiar stimulus constitute a powerful tool to study recognition memory (37). Among them, the novel object recognition (NOR) test is often 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 (37, 50). Our results showed that mice that were exposed to alcohol during adolescence presented poorer recognition memory performances in adulthood. Indeed, AAE males and females spent almost the same percentage of time exploring the familiar and the novel object during the test session, and therefore exhibited very low preference for the novel object. 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 (50). In order to minimize potential bias caused by increased anxiety-like behaviors of AAE mice, we used a long habituation protocol which reduces the stress associated with the OF (50). In addition, no difference was found between AAE and water mice regarding the exploratory behavior of adult mice, 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 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 impairs sociability when assessed by the 3-chamber test, neither short- or long-term after AAE, as all groups significantly preferred to investigate a novel mouse over an empty wired cup. Interestingly, Sabry et al showed that AAE did not affect sociability in late adolescent male rats, but suppressed sociability when coupled to overcrowding conditions (51). This interesting result suggests that alcohol exposure *per se* may not be sufficient to affect sociability, but may enhance 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, although no difference in novelty preference was found between AAE- and water-exposed animals in adulthood, we cannot conclude on those findings, as 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 (52).

AAE impairs behavioral flexibility in adulthood but not in late adolescence

Several cognitive studies have revealed that AAE has minimal effect on spatial learning and memory tasks (14, 53, 54). However, flexibility impairments have been reported when reversal learning or set-shifting tasks were demanded (14, 46, 47, 55). 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 adult animals previously exposed to alcohol during adolescence significantly lacked behavioral flexibility and exhibited increased perseveration of previously learned behavior. Such defects in flexibility have been interpreted as AAE-dependent loss of executive functions caused by disruption of frontal cortical areas control (8, 14, 56). Here we show that AAE-dependent impairment of behavioral flexibility is a long-term development process, and that frontal brain regions alterations caused by AAE may not be present in late adolescence yet, but rather develop with frontal circuit maturation.

AAE enhances adult but not adolescent alcohol consumption and preference

AAE has been reported to promote alcohol consumption in adulthood (14, 26, 47, 49, 56), although some studies showed opposite results (57-59). Most of those studies have used rats to assess the consequences of AAE on alcohol seeking and drinking behaviors (26, 30, 47, 60). In the present study, we confirmed that voluntary alcohol intake during adolescence 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 do not account for rewarding substances in general. In contrast, no difference in alcohol intake or preference was found shortly after AAE. This is quite surprising to see that naïve, water-drinking adolescent mice consumed the same amount of alcohol than AAE animals. However, 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 20%, it is possible that further increase in alcohol consumption could not be possible. One possibility to identify more subtle changes in alcohol intake and preference in adolescent would be to use a modified version of the IA20%-2BC paradigm, with less concentrated alcohol and/or adapted intermittent access strategy.

Importance of animal model, mode of alcohol administration and timing of behavioral testing

Over the past years, several rodent models have been used to assess the behavioral consequences of AAE, and some provided relative evidence that AAE predicts poorer executive functioning in adulthood (46, 47), increased alcohol-associated compulsive behaviors (8, 61) as well as anxiety-like and depression-like behaviors (26, 27, 45). However, large inconsistencies are found between experimental results regarding the short- and long-term consequences of AAE on rodent behaviors. Such variability can be explained by (*i*) the specie used, (*ii*) the timing of alcohol exposure and behavioral testing, and (*iii*) the mode of alcohol administration. Pre-clinical studies have mostly used rat models of AAE. However, rats voluntarily consume very low amount of alcohol, and they would need several weeks of intermittent access to alcohol to gradually escalate from moderate to excessive alcohol drinking and reach BAC relevant to those obtained in mice (62). Rats models of AAE thus mostly involve repeated intraperitoneal

injections of alcohol, which lead to acute increases in blood alcohol concentrations (14, 26, 27, 46), but also to non-negligible amount of stress, which may interfere with alcohol's effects on behaviors (63). It is thus not surprising that comparable behavioral test gives different results according to the AAE model used. The stress associated with i.p. injections could explain why, in opposition to our findings, some groups observed that AAE-dependent anxiety-like behaviors (26), depressive-like behavior (64-66) and recognition memory deficits (67) are already presents in adolescent rats. In addition, the timing of AAE and behavioral testing seems crucial. For example, in the study of Pascual *et al*, rats received alcohol between P25 and P38, and were tested at P41, corresponding to early/mid adolescence, whereas in the present study, mice received access to alcohol during early/mid adolescence, and were tested in "late adolescence" time-point. Those differences in timing must be taken into account when interpreting behavioral data, especially given that our results show that AAE leads to delayed development of behavioral impairments in mice.

Potential cellular and molecular mechanisms underlying the development of the AAE-induced behavioral impairments

The present study shows that adolescent voluntary alcohol binge-drinking leads to severe and persistent behavioral impairments that are observed only in adulthood. Early/mid adolescence has been shown to be a particularly vulnerable period regarding the consequences of alcohol exposure (14, 56, 61). However, neural consequences of adolescent alcohol exposure are not fully understood yet, although mechanistic findings in brain circuitry underlying AAE-induced adult behavioral impairments are starting to emerge (8, 14, 56, 61). Studies have reported that AAE induces long-lasting induction of neuroimmune signaling, through complex cascades involving high mobility group protein B1 (HMGB1), Toll-like receptors and the release of pro-inflammatory cytokines, which lead to synaptic plasticity disruption and neuropathy. The authors nicely showed that reducing neuroinflammation protected mice against AAE-induced behavioral

consequences (45, 67-69). As most of those studies used repeated i.p. injections of alcohol, it would be of great interest to assess the neuroinflammation in the brain of adolescent mice voluntarily consuming alcohol, in order to decipher whether alcohol consumption *per se* triggers such inflammatory cascades or whether this non-physiological mode of alcohol administration exacerbates the pro-inflammatory effects of alcohol. Besides, AAE has also been shown to disrupt neurotransmitter systems, such as decreased activity of dopamine (DA) projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (14, 70) and reduced levels of DA neuron markers in the PFC (71). In addition, serotonergic and cholinergic systems also appear disrupted after AAE, with alcohol-induced loss of cholinergic neurons in the basal forebrain and consequent decreased acetylcholine efflux (72-74). Moreover, AAE is also believed to decrease neurogenesis, by reducing the expression of the brain-derived neurotrophic factor (BDNF) via changes in DNA methylation and/or acetylation (17, 56). Finally, AAE-dependent alterations in epigenetic programming have been reported (17, 75) and are likely to mediate the long-lasting effects of AAE. Indeed, it is believed that alcohol affects epigenetic pathways, modifying chromatin remodeling, gene expression, dendritic spines morphology and synaptic plasticity, to ultimately affect neurocircuits function and behaviors (17, 75). Epigenetic changes could explain the progressive development of impaired behaviors that we observed after adolescent binge-drinking, and further research to unravel AAE-induced epigenetic reprogramming and its relationship with long-lasting behavioral effects of alcohol exposure is definitely required.

Conclusions

This study demonstrates 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 show 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 in adulthood. 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.

Experimental Procedures

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 were weaned at P28, and underwent a modified version of the Drinking in the Dark paradigm (76) between P29 and P40. They were group-housed, excepted during ethanol access. At 9:30, mice were weighted and single-housed, with water and food ad libitum. At 12:00, water bottle was replaced by 20% ethanol solution for four hours. At 16:00, alcohol was removed and mice were group-housed until the next day. Control

adolescent mice underwent the same treatment, but received only water. Mice received alcohol access for 5 consecutive days (P29-P33), then had access to water only for two days (P34-P35) and underwent a second 5-days session with access to alcohol bottle (P36-P40). Mice were used for behavioral testing at P42 or P80. AAE and control mice were mixed in cages after the last drinking session. Ninety-one percent of the animals drank more than 4g/kg/4h and were included in the study. Individual drinking data can be found in S1 table.

Blood Alcohol Concentration Measurement

Blood alcohol concentration (BAC) was measured as previously described (77, 78). Immediately at the end of the last alcohol drinking session, mice were euthanized and trunk blood was collected in heparinized capillary tubes. Serum was extracted with 3.4% perchloric acid followed by centrifugation (5 minutes, 420g) and assayed for alcohol content using the nicotinamide adenine dinucleotide redox (NAD+/NADH) enzyme spectrophotometric method. BACs were determined by using a standard calibration curve.

Behavioral tests

A week before the beginning of behavioral experiment, mice were handled twice a day for 2 minutes. One hour before the beginning of the experimental procedure, mice were placed in the testing room. All tests were monitored by a camera and analyzed by blinded experimenter. All apparatus were cleaned with 75% ethanol and dried between each mouse and session. All 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 using the Open Field (OF). Locomotor activity was used as a measure of overall activity, and the same experimental protocol was used two consecutive days to evaluate the locomotor activity and its habituation. On testing days, all mice were placed into the OF (40x40x40 cm, white Plexiglas walls and floor). The distance

travelled (cm) was recorded for 30 min by an activity tracking system, Videotracking® (France, Lyon). A second cohort of mice were used to assess thymotagsis in the OF. Time spent in the center of the OF was recorded on three consecutive days for 5 minutes.

Elevated Plus Maze Test

Animals were placed in the center of the elevated plus maze and allowed to freely explore the arms for 5 minutes. The time spent by the mouse in open/unprotected maze arms, closed/protected maze arms and in center was recorded. Time spent in the center of the maze was similar for all groups of mice (data not shown) and was measured independently of closed arms time. The total number of entries in open and closed arms was used as a measure of the general activity (79).

Forced swimming Test

This experimental model was performed according to the procedures described by Porsolt et al. (80). Mice were forced to swim in a transparent glass cylinder (30 cm in height per 20 cm in diameter) filled with water to a 15-cm depth (25 °C ± 1◦C) and monitored for 6 min using a camera. The immobility time was determined during the last 4 min of the single experimental session. Mice were considered immobile when they ceased struggling and remained motionlessly floating in the water, making only movements necessary to keep their heads above the water surface. After the behavior assessment, the animals were placed on a heating bed until complete drying and then brought back to the cages.

Novel Object Recognition Test

NOR was performed in the OF using the protocol described by Leger et al (50), with a long habituation phase. Mice were placed in the empty OF once a day (10 minutes) for 3 consecutive days for habituation. Twenty-four hours after last habituation session, two identical objects were placed in the box (10 cm from the walls) and mice were allowed to familiarize with both objects

for 10 minutes. The time for reaching 20 seconds of exploration was measured. Mice which did not reach the 20 seconds exploration criterion were excluded from the study (2 /110 mice). Twenty-four hours after the familiarization session, the test session began. One object was replaced by another object (different in shape, color and texture), and mice were allowed to explore both objects for 10 minutes. Time to reach 20 seconds exploration was recorded and mice which did not reach the criterion were excluded from the study (6 /110 mice). The time spent exploring the familiar and the novel object was recorded. Valid exploration was characterized by the nose of the mouse directed toward an object at less than 2 cm of distance. Discrimination index was calculated as (Time exploring the novel object - Time exploring the familiar object) / Total exploration time.

Three Chamber Test

The three chamber test was employed to characterize sociability and preference for social novelty, as described in Moy et al (81). Briefly, after 5 minutes of habituation in the apparatus, a stranger mouse was placed inside a wired cup. Position of the mouse and the empty wired cup were alterned between tested subjects. Mice were allowed to freely move between social and non-social chambers for 10 minutes. Sociability was evaluated by examining the amount of time spent by the mouse sniffing the social stimulus (i.e. the mouse) and the time spent sniffing the non-social stimulus (i.e. the empty wired cup). Then, a novel mouse was introduced in the empty wired cage, and mice were allowed to freely move between chambers for 10 minutes. Social novelty preference index (SNI) was calculated as (Time sniffing new mouse – Time sniffing familiar mouse) / (Time sniffing both mice). Position of the familiar and novel mouse was alternated between tested subjects.

Reversal Learning Test

The reversal learning test was performed by using the Barnes maze, as described in Riedel et al (82) and Pitts et al (83), with slight modifications. The maze consisted of an open circular

area (120cm diameter) with 20 holes (5 cm diameter) regularly spaced, placed 2,5cm from the edge. The maze had no perimeter walls and was elevated 80cm from the floor. The arena was surrounded by four distal cues. Both direct and indirect illumination of the maze (~120 lx) and loud music (~90dB) provided an aversive stimulus to motivate escape. A black opaque target box containing a tissue paper was attached to the underside of the arena under one of the holes and was the only mean to escape from the bright arena. All sessions were recorded by an overhead camera.

Learning

Mice were first habituated to the escape box for 1 minute and to the Barnes maze platform for 5 minutes. Mice which did not enter the escape tunnel were directed toward it by the experimenter. One hour after the habituation, mice started the acquisition trainings. Training consisted of two acquisition trials daily (3 minutes limit per trial, intertrial interval of 1 hour). At the start of each trial, the mouse is placed in an opaque start chamber located in the center of the maze, which is lifted to allow the mouse exploring the maze. The position of the escape tunnel remained the same during the acquisition training. For each trial, a blinded experimenter recorded the delay before entering the escape tunnel, as well as the number of primary errors (number of incorrect holes checked by the mouse). Seventy-two hours after the last learning session, mice underwent a 80-second probe trial, in which the escape tunnel was removed from the apparatus. During the probe trial, time spent in the correct sector (in the quadrant containing the correct hole) was recorded.

Reversal learning

Twenty-four hours after the learning probe trial, mice underwent 10 sessions of reversal learning (2 sessions per day, intertrial interval of 1 hour), in which the escape tunnel location was relocated to the hole that was exactly 180° from the initial location. Time to escape and number of primary errors were analyzed. Seventy-two hours after the last reversal learning session,

mice underwent a probe test, in which time spent in the correct sector and in the sector of the previous location of the escape tunnel were recorded.

Two-bottle choice drinking paradigm

Intermittent Access to 20% alcohol

The intermittent access to 20% alcohol two-bottle choice drinking procedure (IA20%-2BC) was conducted as previously described (43, 84) (Fig 5A). Mice were single-housed and weighted every other day. Mice were given 24 hours of concurrent access to one bottle of 20% alcohol (v/v) in tap water, and one bottle of water. Drinking sessions started at 12:00 on Monday, Wednesday and Friday, with 24- or 48 hours (weekend) of alcohol-deprivation periods in which mice consumed only water. The placement (left or right) of water and alcohol bottle was alternated between each session in order to control for side preference. Water and alcohol bottles were weighted at the beginning of each alcohol session, 4 hours after the beginning of each alcohol session (binge-drinking), and at the end of each alcohol sessions.

Intermittent Access to 1% sucrose

Mice underwent an IA-2BC paradigm as described above but had access to one bottle containing 1% sucrose solution in water, and another bottle containing water.

Statistical tests

Data were analyzed by using the appropriate statistical test, including Two-ways analysis of variance (ANOVA) or linear mixed-effects model, as detailed in the figure legends. Significant main effects and interactions 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.

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Figures legends

Fig 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 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 ($\chi^2_{(9)}=1.18x10^4$, $p<0.001$ $\eta^2=0.65$) 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 x 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. Centerline is the linear regression and dashed lines are the 95% confidence interval. Linear regression $t_{(1,15)}=2.44$, $p<0.05$, $r^2= 0.31$; n=17 mice.

Fig 2: **Enhanced anxiety-like and depressive-like behavior in adulthood but not in late adolescence after adolescent alcohol exposure**

(A) Diagram depicting the timing of behavioral tests following adolescent alcohol exposure. Animals underwent the DID paradigm from P29 to P40. They were tested at P42 (short-term), two days after the last drinking session, or at P80 (long-term), with forty days without any access to alcohol. **(B)** Schematic representation of the Open Field (OF) (left) and the Elevated Plus Maze (EPM) apparatus (right). **(C)** P42; 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 interactions; n=12-14 per group. **(D)** P42; 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)** P42; 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)** P80; Percentage of time spent in the center of the OF. Linear mixed-effects model showed a 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)** P80; 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)** P80; 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)** P42; 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)** P42; 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)** P80; 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)** P80; 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.

Fig 3: **Adolescent binge-drinking decreases Novel Object Recognition performances in adult but not in 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)** P42; Familiarization session: Time to reach criterion (20 seconds of total object exploration). Two-way ANOVA showed no effect of treatment ($F_{(1,42)}$=0.27, p=0.6) or sex ($F_{(1,42)}$=3.52, p=0.07). **(C)** P42; Test session: Time to reach criterion. Two-way ANOVA showed no effect of treatment ($F_{(1,42)}$=0.02, p=0.88) or sex ($F_{(1,42)}$=0.05, p=0.83). **(D)** P42; 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 effect of treatment ($F_{(1,42)}$=0.10, p=0.76) or sex ($F_{(1,42)}$=0.87, p=0.36). **(E)** P42; 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 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)** P80; Familiarization session: Time to reach criterion (20 seconds of total object exploration). Two-way ANOVA showed no effect of treatment ($F_{(1,60)}$=0.03, p=0.87) or sex ($F_{(1,60)}$=0.1, p=0.76). **(G)** P80; Test session: Time to reach criterion. Two-way ANOVA showed no effect of treatment ($F_{(1,60)}$=0.81, p=0.37) or sex ($F_{(1,60)}$=0.39, p=0.53). **(H)** P80; Discrimination index. Two-way ANOVA showed a main effect of treatment ($F_{(1,60)}$=49.86, p<0.001, η^2=0.45) but no effect of 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)** P80; Index of habituation to the familiar object (HFI). Two-way ANOVA showed a main effect of treatment ($F_{(1,60)}$=49.50, p<0.001, η^2=0.44) but no effect of 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 sessions of training 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)** P42; Reversal learning: mean escape time per day across 5 days. Linear mixed-effects model showed a main effect of day ($\chi^2_{(4)}$=363.3, p<0.001, η^2=0.55) and a main effect of sex ($\chi^2_{(1)}$=4.52, p<0.05, η^2=0.086) but no 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)** P42; Reversal learning: primary errors per day across 5 days. Linear mixed-effects model showed a main effect of day ($\chi^2_{(4)}$=342.42, p<0.001, η^2=0.55), but no

effect of treatment ($\chi^2_{(1)}$=2.15, p=0.14) or sex ($\chi^2_{(1)}$=0.32, p=0.57) and no interactions (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)** P42; Percentage of time spent in the correct sector during probe test, 72 hours after the last reversal learning session. Two-way ANOVA showed no effect of treatment ($F_{(1,44)}$=0.18, p=0.67) or sex ($F_{(1,44)}$=1.47, p=0.23). **(E)** P42; Percentage of time spent in the previous sector during probe test. Two-way ANOVA showed no 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)** P80; Reversal learning: mean escape time per day. Linear mixed-effects model showed a 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), 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)** P80; Reversal learning: primary error number. Linear mixed-effects model showed a 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)** P80; Percentage of time spent in the correct sector during probe test, 72 hours after the last reversal learning session. Two-way ANOVA showed a 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)** P80; Percentage of time spent in the previous sector during probe test. Two-way ANOVA showed a 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 (but not sucrose) consumption and preference in adult mice

(A) Scheme depicting the intermittent access to alcohol 20%-two bottle choice paradigm. P42 (B-D) and P80 (E-G) mice underwent the IA-20%-2BC paradigm for 5 sessions. Mice were then switched to an intermittent access to 1% sucrose-2BC. **(B)** P42; Alcohol intake (g/kg/24h). Linear mixed-effects model showed a 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)** P42; Alcohol preference. Linear mixed-effects model showed a 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)** P42; Total fluid intake (ml/kg/24h). Linear mixed-effects model showed a 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**) P80; Alcohol intake (g/kg/24h). Linear mixed-effects model showed a 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)** P80; Alcohol preference. Linear mixed-effects model showed a 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)** P80; Total fluid intake (ml/kg/24h). Linear mixed-effects model showed a

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. **(H)** P42; Sucrose intake (ml/kg/24h). Linear mixed-effects model showed a main effect of session ($\chi^2_{(4)}$=149.38, p<0.001, η^2=0.35) and sex ($\chi^2_{(1)}$=4.78, p<0.05, η^2=0.09) but no effect of treatment ($\chi^2_{(1)}$=0.24, p=0.62) and no interaction (session x treatment $\chi^2_{(4)}$=6.88, p=0.14, sex x treatment $\chi^2_{(1)}$=0.53, p=0.47, session x sex $\chi^2_{(4)}$=1.74, p=0.78). **(I)** P42; Sucrose preference. Linear mixed-effects model showed a main effect of session ($\chi^2_{(4)}$=141.31, p<0.001, η^2=0.34), but no effect of sex ($\chi^2_{(1)}$=1.39, p=0.24) or treatment ($\chi^2_{(1)}$=0.26, p=0.61) and no interaction (session x treatment $\chi^2_{(4)}$=6.65, p=0.16, sex x treatment $\chi^2_{(1)}$=0.16, p=0.69, session x sex $\chi^2_{(4)}$=3.37, p=0.5). **(J)** P42; Total fluid intake (ml/kg/24h). Linear mixed-effects model showed a main effect of session ($\chi^2_{(4)}$=57.78, p<0.001, η^2=0.18) and sex ($\chi^2_{(1)}$=6.02, p<0.01, η^2=0.11) but no effect of treatment ($\chi^2_{(1)}$=0.38 p=0.54) and no interaction (session x treatment $\chi^2_{(4)}$=4.60, p=0.33, sex x treatment $\chi^2_{(1)}$=1.29, p=0.26, s x sex $\chi^2_{(4)}$=0.53, p=0.97); n=12 per group. **(K)** P80; Sucrose intake (ml/kg/24h). Linear mixed-effects model showed a main effect of session ($\chi^2_{(4)}$=33.9, p<0.001, η^2=0.12) and sex ($\chi^2_{(1)}$=45.30, p<0.001, η^2=0.2) but no effect of treatment ($\chi^2_{(1)}$=0.01 p=0.93). This model also showed an interaction session x sex ($\chi^2_{(4)}$=12.14, p<0.05, η^2=0.04) but not sex x treatment ($\chi^2_{(1)}$=0.07, p=0.79) or session x treatment ($\chi^2_{(4)}$=6.26, p=0.18). **(L)** P80; Sucrose preference. Linear mixed-effects model showed a main effect of session ($\chi^2_{(4)}$=39.4, p<0.001, η^2=0.14) and sex ($\chi^2_{(1)}$=5.58, p<0.05, η^2=0.03) but no effect of treatment ($\chi^2_{(1)}$=1.17, p=0.28) and no interaction (session x treatment $\chi^2_{(4)}$=3, p=0.56, sex x treatment $\chi^2_{(1)}$=0.86, p=0.35, session x sex $\chi^2_{(4)}$=3.04, p=0.55). **(M)** P80; Total fluid intake (ml/kg/24h). Linear mixed-effects model showed a main effect of session ($\chi^2_{(4)}$=12.34, p<0.01, η^2=0.04) and sex ($\chi^2_{(1)}$=65.14, p<0.001, η^2=0.25) but no effect of treatment ($\chi^2_{(1)}$=0.39 p=0.53). This model

also showed an interaction session x Sex ($\chi^2_{(4)}$=19.57, p<0.001, η^2=0.07) but not sex x treatment ($\chi^2_{(1)}$=0.59, p=0.44) or session x treatment ($\chi^2_{(4)}$=4.99, p=0.29); n=12 per group.

Supporting Information

S1 Fig: **Adolescent alcohol exposure does not alter locomotion activity**

(A) P42; Mean locomotion activity in the Open Field (OF). Adolescent mice were allowed to explore the OF for 30 minutes and the test was repeated 24 hours later. Locomotor activity (cm) was measured every 5 minutes. Linear mixed-effects model showed a main effect of interval ($\chi^2_{(5)}$=507.7, p<0.001; η^2=0.38) and day ($\chi^2_{(1)}$=41.7, p<0.001, η^2=0.13) but no effect of treatment ($\chi^2_{(1)}$=1.1, p=0.29) or sex ($\chi^2_{(1)}$=0, p=0.96); n= 9-12 per group. **(B)** P42; Total number of arm entries in the EPM. Two-way ANOVA showed no main effect of treatment ($F_{(1,46)}$=2.18, p=0.15) or sex ($F_{(1,46)}$=0.07, p=0.8); n=12-14 per group. **(C)** P80; Mean locomotion activity in the Open Field (OF). Linear mixed-effects model showed a main effect of interval ($\chi^2_{(5)}$=610.6, p<0.001, η^2=0.41), day ($\chi^2_{(1)}$=66.18, p<0.001, η^2=0.19) and sex ($\chi^2_{(1)}$=4.81, p<0.05, η^2=0.07) but no effect of treatment ($\chi^2_{(1)}$=0.01, p=0.93); n=7-10 per group. **(D)** P80; Total number of arm entries in the EPM. Two-way ANOVA showed no main effect of treatment ($F_{(1,50)}$=0.73, p=0.4) or sex ($F_{(1,50)}$=1.23, p=0.27); n=13-14 per group.

S2 Fig: **AAE does not impair sociability**

(A) Schematic representation of the three-chambers sociability test. The experimental apparatus consists in three chambers connected via sliding doors. For sociability assessment, an empty wire cage is placed in the non-social chamber and a cage containing a mouse is placed in the social chamber (left scheme). For social novelty assessment, a new mouse is introduced in the empty wire cage (right scheme). **(B)** P42; Sociability: percentage of time

sniffing the mouse and the empty wire cage. Linear mixed-effects model showed a main effect of stimulus (mouse vs empty cage $\chi^2_{(1)}$=86.37, p<0.001, η^2=0.67) but no effect of sex ($\chi^2_{(1)}$=2.76, p=0.1) or treatment ($\chi^2_{(1)}$=0.12, p=0.72) and no interaction (sex x treatment ($\chi^2_{(1)}$=0.9, p=0.34), sex x stimulus ($\chi^2_{(1)}$=0.0, p=0.98), treatment x stimulus ($\chi^2_{(1)}$=2.03, p=0.15); n= 10-12 per group. **(C)** P42; Social Novelty: social novelty preference index (SNI) was calculated as (Time sniffing new mouse – Time sniffing familiar mouse) / (Time sniffing both mice). Two-way ANOVA showed a main effect of sex ($F_{(1,39)}$=4.1, p<0.05) but no main effect of treatment ($F_{(1,39)}$=0.03, p=0.85) and no interaction ($F_{(1,39)}$=0.07, p=0.79); n= 15-16 per group. **(D)** P80; Sociability: percentage of time sniffing the mouse and the empty wired cage. Linear mixed-effects model showed a main effect of stimulus (mouse vs empty cage $\chi^2_{(1)}$=70.27, p<0.001, η^2=0.54) but no effect of sex ($\chi^2_{(1)}$=3.58, p=0.06) or treatment ($\chi^2_{(1)}$=1.48, p=0.22) and no interaction (sex x treatment ($\chi^2_{(1)}$=0.01, p=0.91), sex x stimulus ($\chi^2_{(1)}$=0.17, p=0.68), treatment x stimulus ($\chi^2_{(1)}$=0.02, p=0.89); n=10-12 per group. **(E)** P80; Social Novelty: social novelty preference index. Two-way ANOVA showed no effect of sex ($F_{(1.57)}$=1.27, p=0.26) or treatment ($F_{(1,57)}$=0.41, p=0.52) and no interaction ($F_{(1,57)}$=0.19, p=0.66); n=15-16 per group.

<u>S3 Fig</u>: AAE does not induce spatial learning defects.

(A) P42; Learning: mean escape time per day (mean of two sessions) across 5 days. Linear mixed-effects model showed a main effect of day ($\chi^2_{(4)}$=753.1, p<0.001, η^2=0.76) but no effect of sex ($\chi^2_{(1)}$=0.24, p=0.62) or treatment ($\chi^2_{(1)}$=0.03, p=0.85) and no interaction (day x sex $\chi^2_{(4)}$=2.20, p=0.7; day x treatment $\chi^2_{(4)}$=0.43, p=0.98; sex x treatment $\chi^2_{(1)}$=0.03, p=0.86). **(B)** P42; Learning: primary errors per day (mean of two sessions). Linear mixed-effects model showed a main effect of day ($\chi^2_{(4)}$=351.43, p<0.001, η^2=0.59), but no effect of sex ($\chi^2_{(1)}$=0.84, p=0.36) or treatment ($\chi^2_{(1)}$=2.92, p=0.09) and no interaction (day x sex $\chi^2_{(4)}$=4.71, p=0.32; day x treatment $\chi^2_{(4)}$=1.09, p=0.9; sex x treatment $\chi^2_{(1)}$=0.02, p=0.88). **(C)** P42; Percentage of time

spent in the correct sector during probe test (72 hours after the last learning session). Two-way ANOVA showed no effect of treatment ($F_{(1,44)}$=0.01, p=0.91) or sex ($F_{(1,44)}$=1.15, p=0.29) and no interaction ($F_{(1,44)}$=0.62, p=0.44); n=12 per group. **(D)** P80; Learning: mean escape time per day. Linear mixed-effects model showed a main effect of day ($\chi^2_{(4)}$=683.02, p<0.001, η^2=0.71) but no effect of sex ($\chi^2_{(1)}$=1.43, p=0.23) or treatment ($\chi^2_{(1)}$=0.15, p=0.7) and no interaction (day x sex $\chi^2_{(4)}$=6, p=0.2; day x treatment $\chi^2_{(4)}$=0.91, p=0.92; sex x treatment $\chi^2_{(1)}$=0.02, p=0.87). **(E)** P80; Learning: primary errors number. Linear mixed-effects model showed a main effect of day ($\chi^2_{(4)}$=152.26, p<0.001, η^2=0.35), but no effect of sex ($\chi^2_{(1)}$=0.13, p=0.72) or treatment ($\chi^2_{(1)}$=0.21, p=0.65) and no interaction (day x sex $\chi^2_{(4)}$=2.81, p=0.59; day x treatment $\chi^2_{(4)}$=1.56, p=0.82; sex x treatment $\chi^2_{(1)}$=0.02, p=0.9). **(F)** P80; Percentage of time spent in the correct sector during probe test (72 hours after the last learning session). Two-way ANOVA showed no effect of treatment ($F_{(1,48)}$=0.60, p=0.44) or sex ($F_{(1,48)}$=2.5, p=0.12) and no interaction ($F_{(1,48)}$=0.34, p=0.56); n= 13 per group.

<u>S4 Fig</u>: Binge-drinking of alcohol is increased in AAE adult but not adolescent mice

(A) P42; Alcohol binge drinking (g/kg/4h). Linear mixed-effects model showed a main effect of session ($\chi^2_{(4)}$=71.59, p<0.05, η^2=0.23), and sex ($\chi^2_{(1)}$=4.14, p<0.05, η^2=0.08) but no effect of treatment ($\chi^2_{(1)}$=0.05, p=0.82) and no interaction (session x treatment $\chi^2_{(4)}$=3.26, p=0.51; sex x treatment $\chi^2_{(1)}$=1.45, p=0.23; session x sex $\chi^2_{(4)}$=4, p=0.41). **(B)** P42; Alcohol preference. Linear mixed-effects model showed a main effect of session ($\chi^2_{(4)}$=94.83, p<0.001, η^2=26), but no effect of sex ($\chi^2_{(1)}$=0.26, p=0.61) or treatment ($\chi^2_{(1)}$=1.33, p=0.25) and no interaction (session x treatment $\chi^2_{(4)}$=7.57, p=0.11; sex x treatment $\chi^2_{(1)}$=1.55, p=0.21; session x s$\chi^2_{(4)}$=6.31, p=0.18). **(C)** P42; Total fluid intake (ml/kg/4h). Linear mixed-effects model showed a main effect of session ($\chi^2_{(4)}$=25.85, p<0.01, η^2=0.1) and sex ($\chi^2_{(1)}$=4.97, p<0.05, η^2=0.09) but no effect of treatment ($\chi^2_{(1)}$=1.55, p=0.21). This model also showed an interaction session x sex ($\chi^2_{(4)}$=9.81,

$p<0.05$, $\eta^2=0.04$) but not session x treatment ($\chi^2_{(4)}=0.37$, $p=0.98$) or sex x treatment ($\chi^2_{(1)}=0.7$, $p=0.4$); n=12 per group. **(D)** P80; Alcohol binge-drinking (g/kg/4h). Linear mixed-effects model showed a main effect of treatment ($\chi^2_{(1)}=25.49$, $p<0.001$, $\eta^2=0.12$), sex ($\chi^2_{(1)}=15.14$, $p<0.001$, $\eta^2=0.07$) and session ($\chi^2_{(4)}=17.22$ $p<0.01$, $\eta^2=0.06$), but no interaction (session x treatment $\chi^2_{(4)}=6.54$, $p=0.16$; sex x treatment $\chi^2_{(1)}=0.46$, $p=0.50$; session x sex $\chi^2_{(4)}=5.65$, $p=0.23$). **(E)** P80; Alcohol preference. Linear mixed-effects model showed a main effect of treatment ($\chi^2_{(1)}=50.40$, $p<0.001$, $\eta^2=0.21$) and session ($\chi^2_{(4)}=37.34$, $p<0.001$, $\eta^2=0.14$) but no effect of sex ($\chi^2_{(4)}=1.41$ $p=0.24$) and no interaction (session x treatment $\chi^2_{(4)}=4.65$, $p=0.32$; sex x treatment $\chi^2_{(1)}=0.5$, $p=0.48$; session x sex $\chi^2_{(4)}=7.74$, $p=0.1$). **(F)** P80; Total fluid intake (ml/kg/4h). Linear mixed-effects model showed a main effect of session ($\chi^2_{(4)}=22.2$, $p<0.001$, $\eta^2=0.08$) and sex ($\chi^2_{(1)}=23.89$, $p<0.001$, $\eta^2=0.11$) but no effect of treatment ($\chi^2_{(1)}=2.72$ $p=0.1$). This model also showed an interaction treatment x sex ($\chi^2_{(1)}=4.42$, $p<0.05$, $\eta^2=0.03$) but not session x treatment ($\chi^2_{(4)}=1.29$, $p=0.86$) or session x sex ($\chi^2_{(4)}=5.09$, $p=0.28$); n=12 per group.

Fig 1

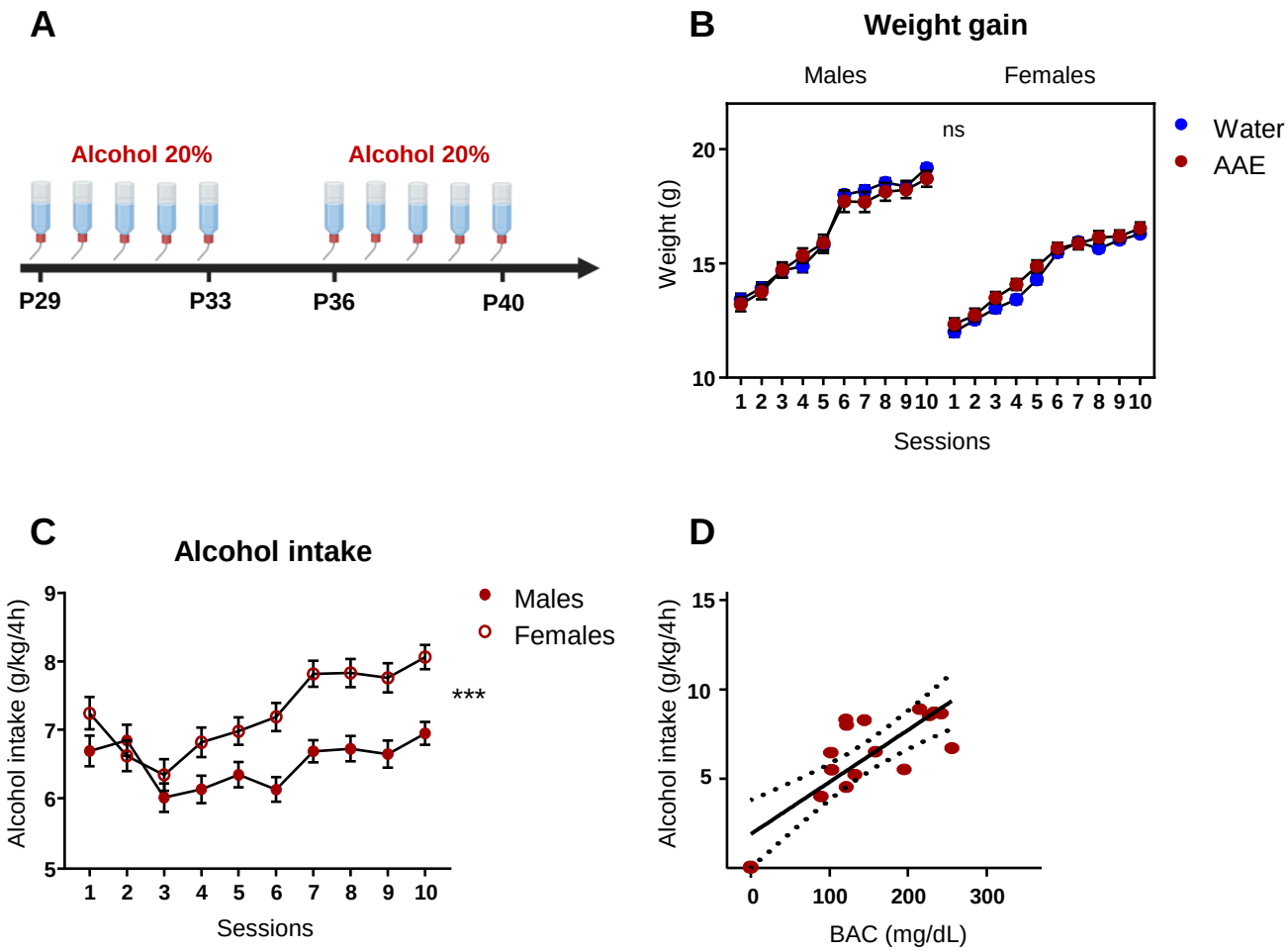

A
Alcohol 20%
Alcohol 20%
P29
P33
P36
P40
B
Weight gain
Males
Females
ns
Water
AAE
Weight (g)
20
15
10
1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10
Sessions
C
Alcohol intake
Males
Females
***
Alcohol intake (g/kg/4h)
9
8
7
6
5
1 2 3 4 5 6 7 8 9 10
Sessions
D
Alcohol intake (g/kg/4h)
15
10
5
0 100 200 300
BAC (mg/dL)

Fig 2

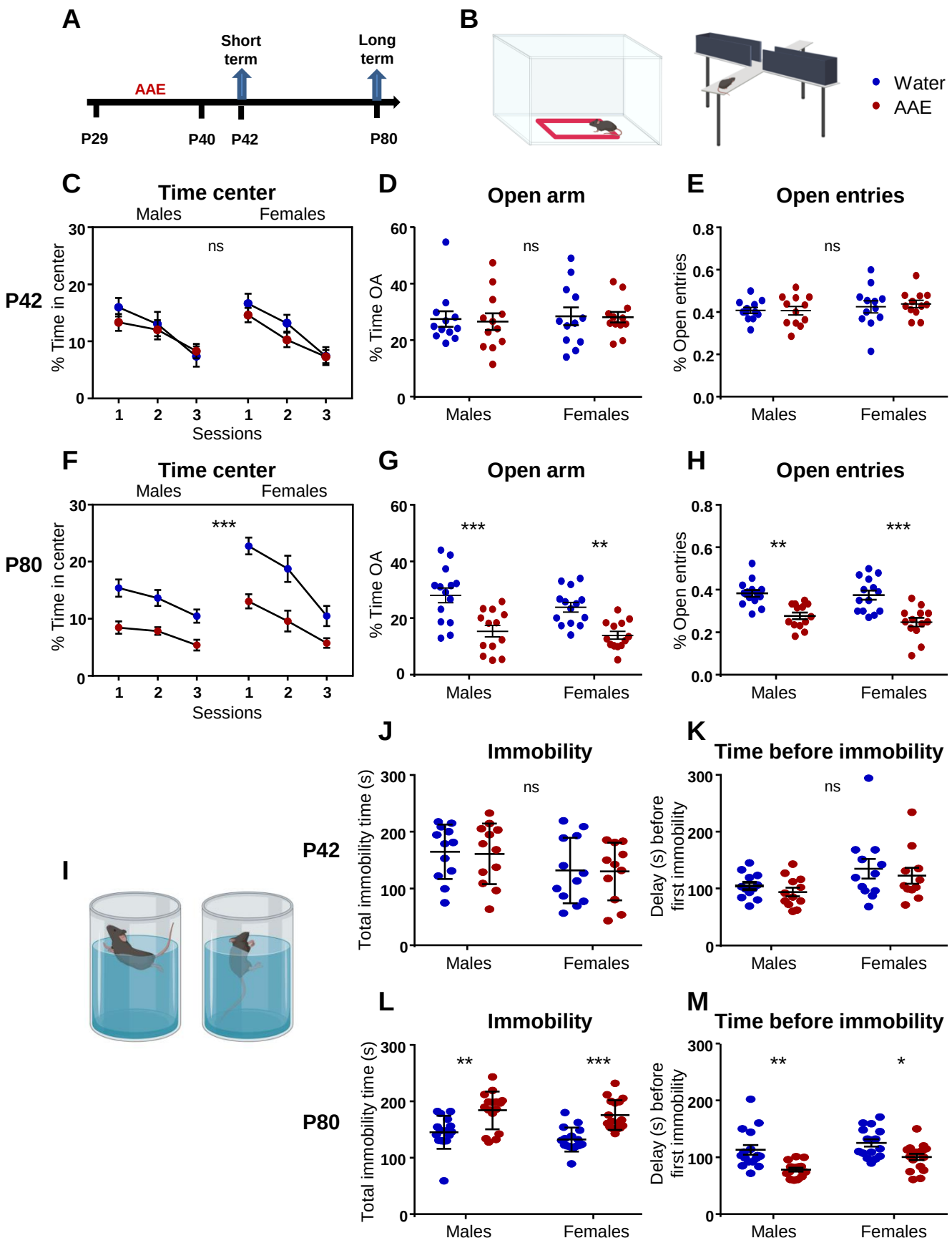

A
Short term
Long term
AAE
P29
P40
P42
P80
B
Water
AAE
C
Time center
Males
Females
P42
% Time in center
ns
Sessions
D
Open arm
% Time OA
ns
Males
Females
E
Open entries
% Open entries
ns
Males
Females
F
Time center
Males
Females
P80
% Time in center
***
Sessions
G
Open arm
% Time OA
***
**
Males
Females
H
Open entries
% Open entries
**
***
Males
Females
I
J
Immobility
P42
Total immobility time (s)
ns
Males
Females
K
Time before immobility
Delay (s) before first immobility
ns
Males
Females
L
Immobility
P80
Total immobility time (s)
**
***
Males
Females
M
Time before immobility
Delay (s) before first immobility
**
*
Males
Females

Fig 3

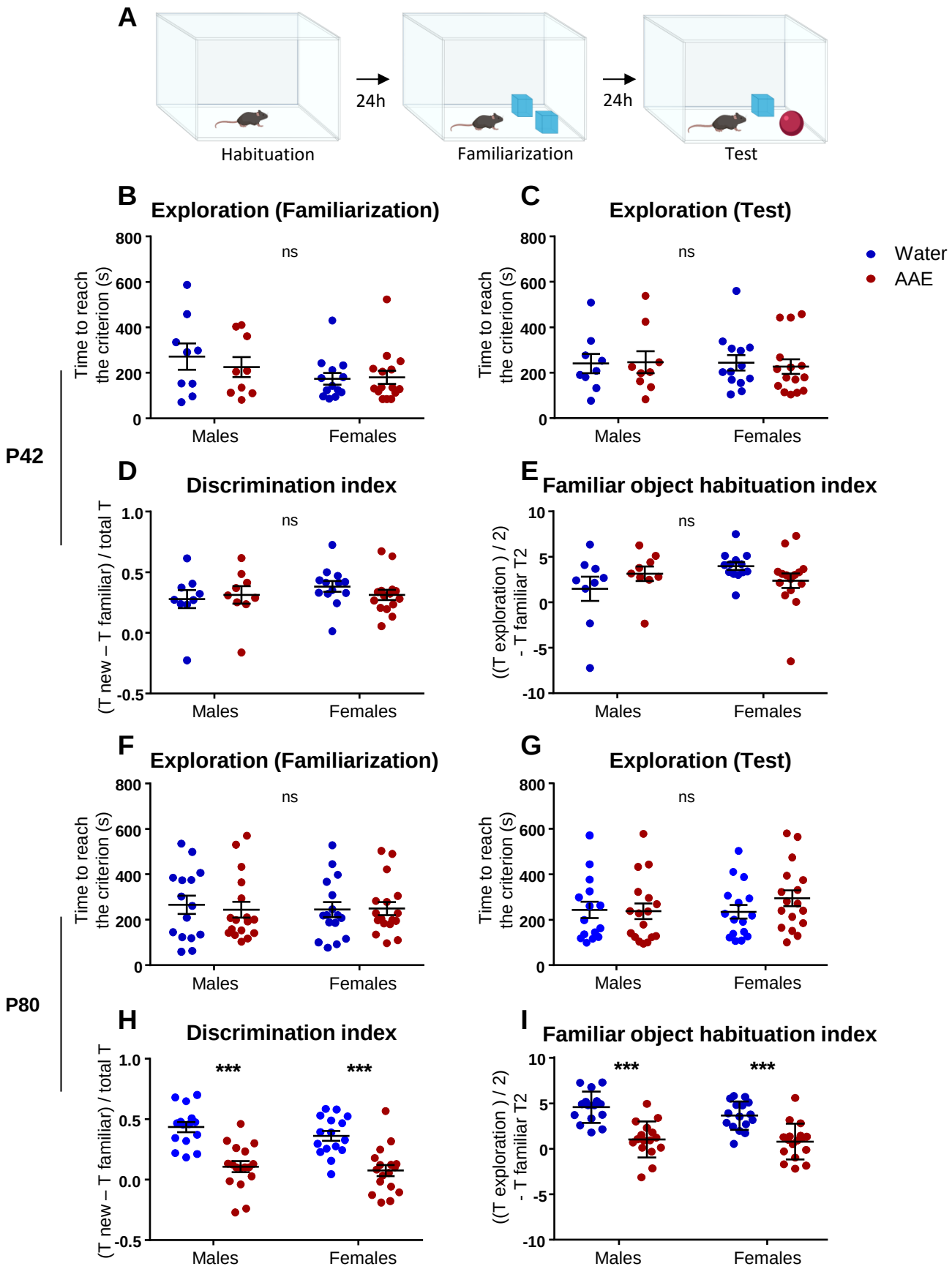

A
Habituation
24h
Familiarization
24h
Test
B
Exploration (Familiarization)
Time to reach the criterion (s)
ns
Males
Females
C
Exploration (Test)
Water
AAE
P42
D
Discrimination index
(T new – T familiar) / total T
E
Familiar object habituation index
((T exploration) / 2) - T familiar T2
F
Exploration (Familiarization)
G
Exploration (Test)
P80
H
Discrimination index
***
I
Familiar object habituation index

Fig 4

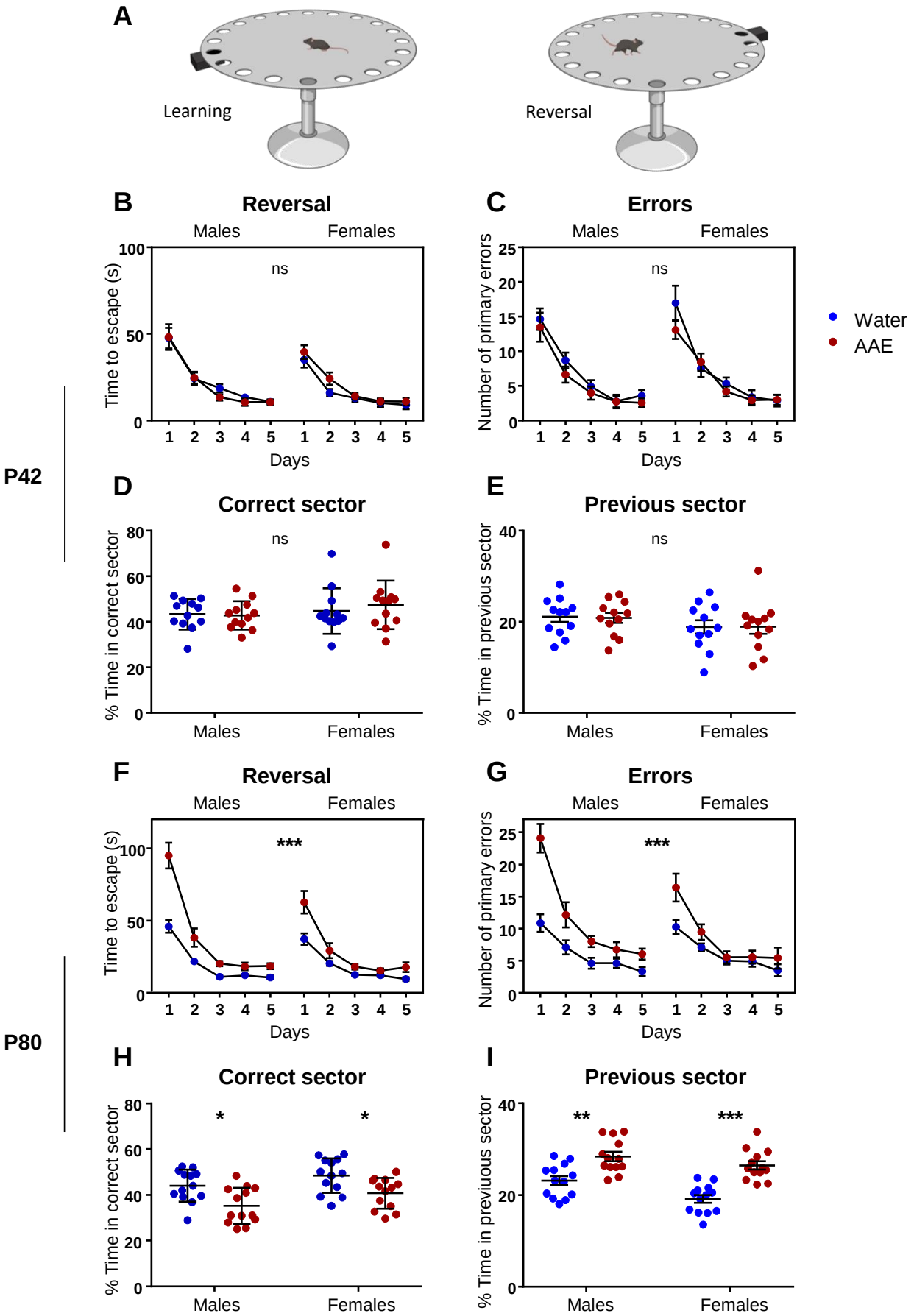
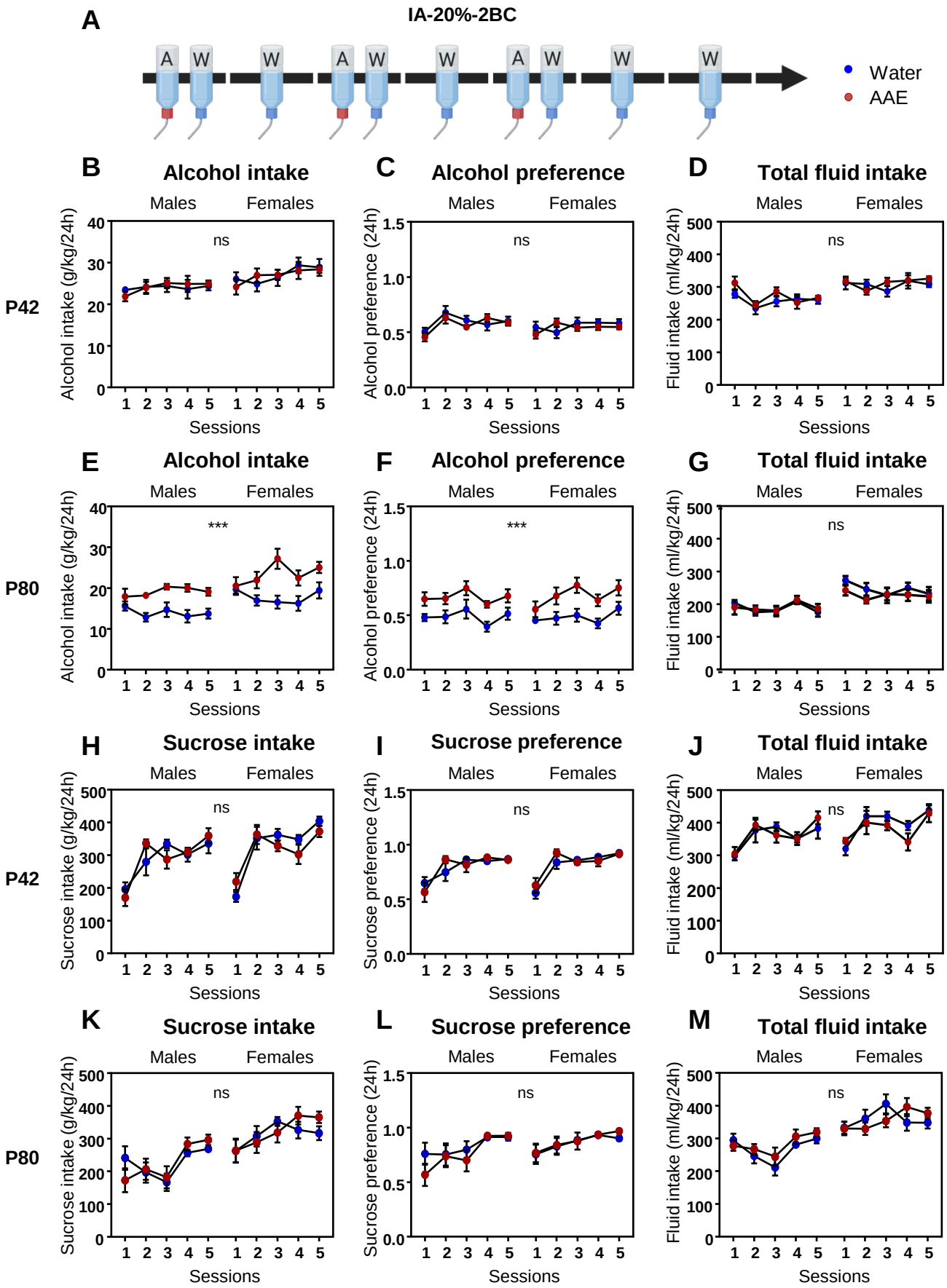
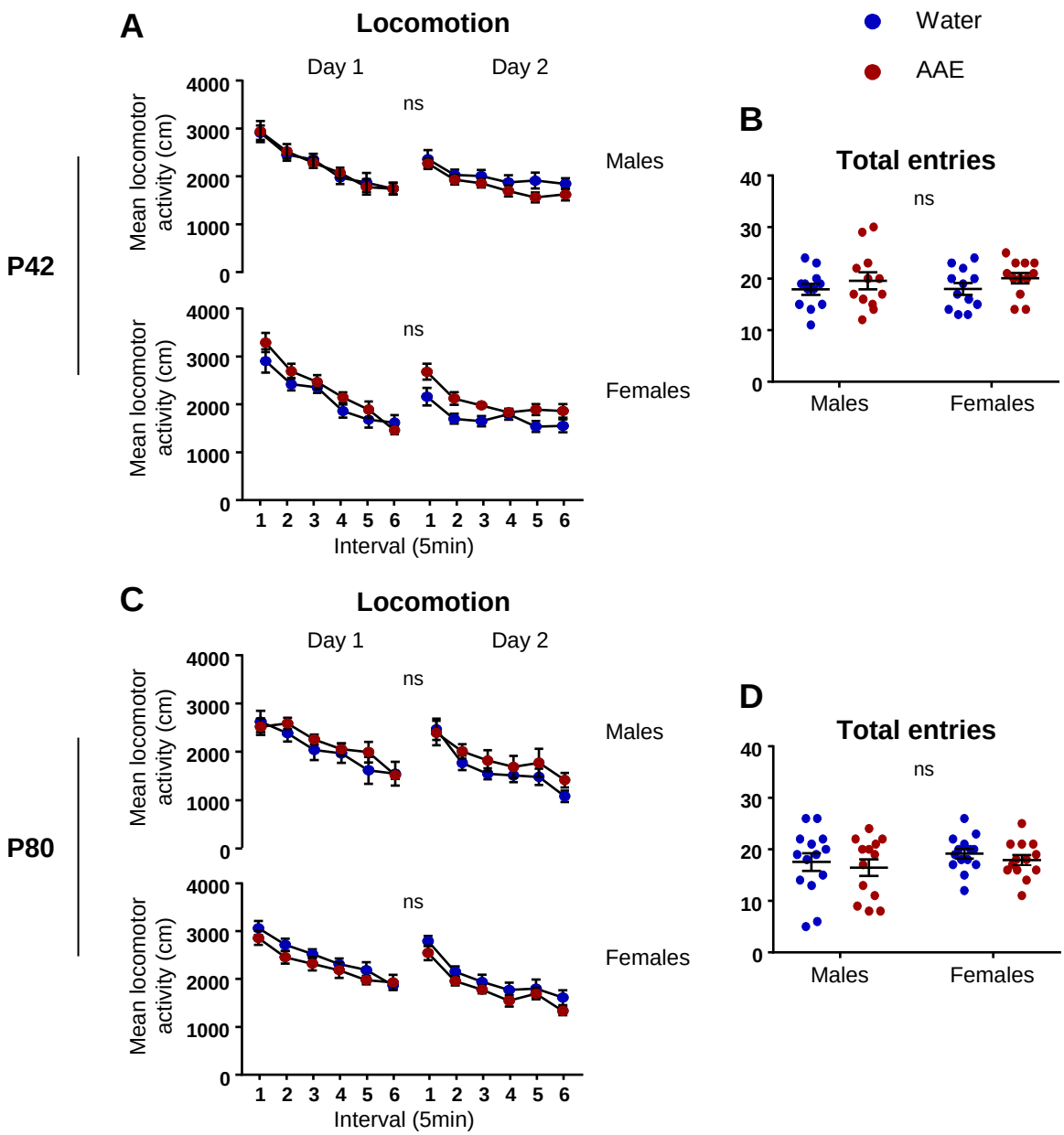

A
Learning
Reversal
B
Reversal
Males
Females
Time to escape (s)
ns
Days
C
Errors
Number of primary errors
Water
AAE
P42
D
Correct sector
% Time in correct sector
E
Previous sector
% Time in previous sector
F
G
P80
H
I
% Time in previuous sector
*
**
***

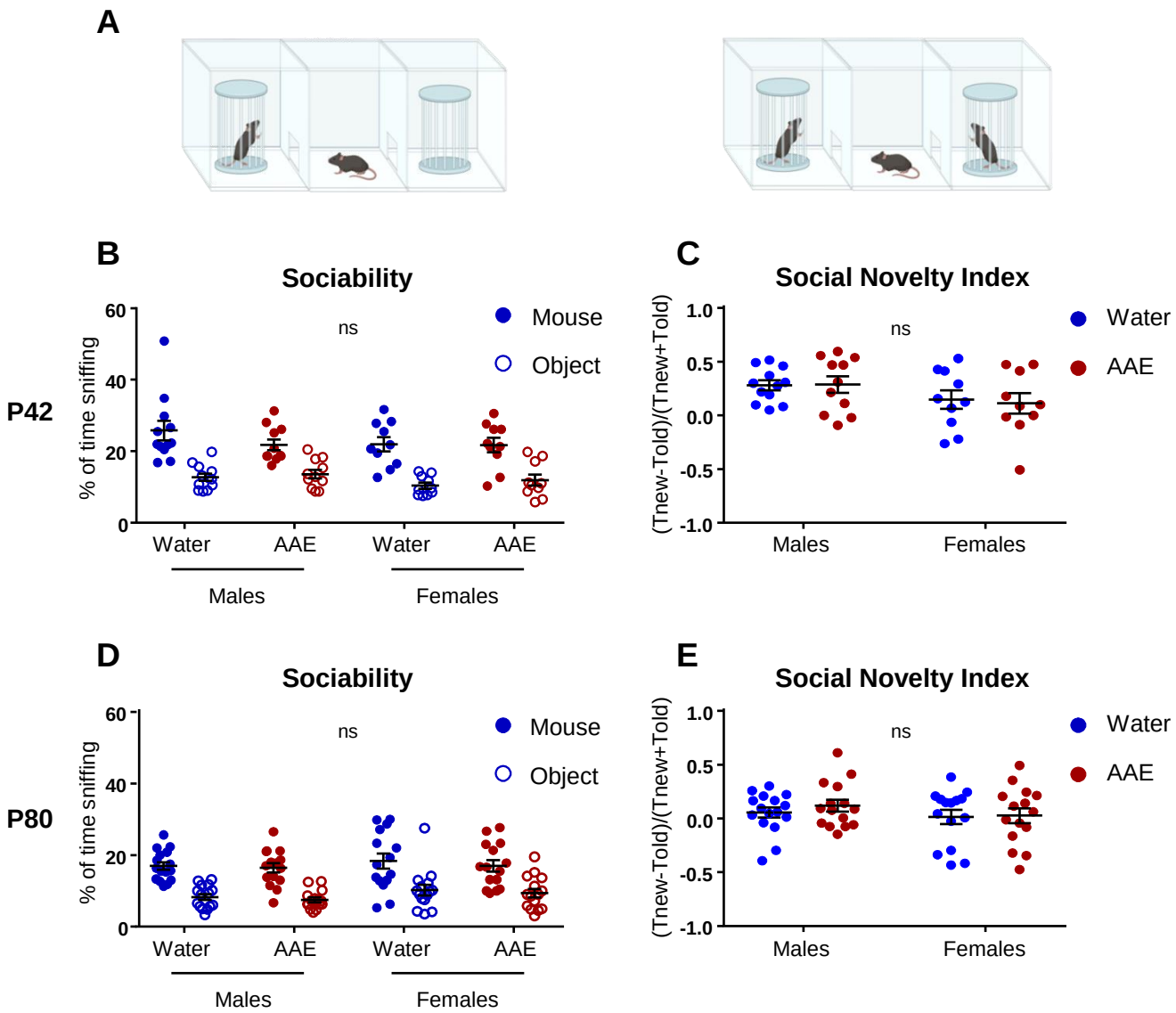
Fig 5


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
E Alcohol intake
F Alcohol preference
G Total fluid intake
H Sucrose intake
I Sucrose preference
J Total fluid intake
K Sucrose intake
L Sucrose preference
M Total fluid intake
P42
P80
Males
Females
ns
***
Alcohol intake (g/kg/24h)
Alcohol preference (24h)
Fluid intake (ml/kg/24h)
Sucrose intake (g/kg/24h)
Sucrose preference (24h)
Sessions

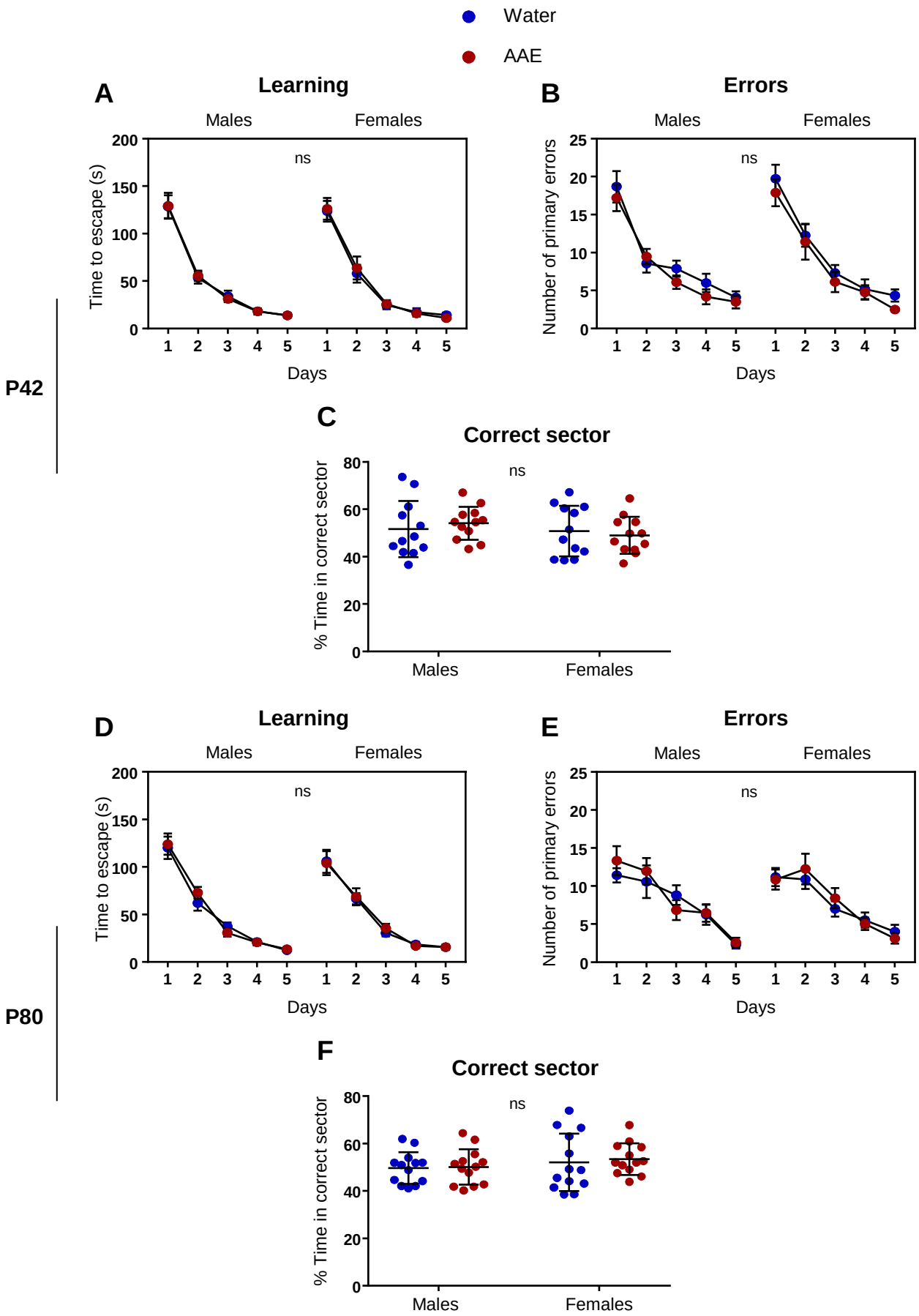
S1 Fig


A
Locomotion
Day 1
Day 2
ns
Mean locomotor activity (cm)
4000
3000
2000
1000
0
Males
Females
P42
1 2 3 4 5 6 1 2 3 4 5 6
Interval (5min)
Water
AAE
B
Total entries
40
30
20
10
0
Males
Females
C
Locomotion
D
Total entries
P80

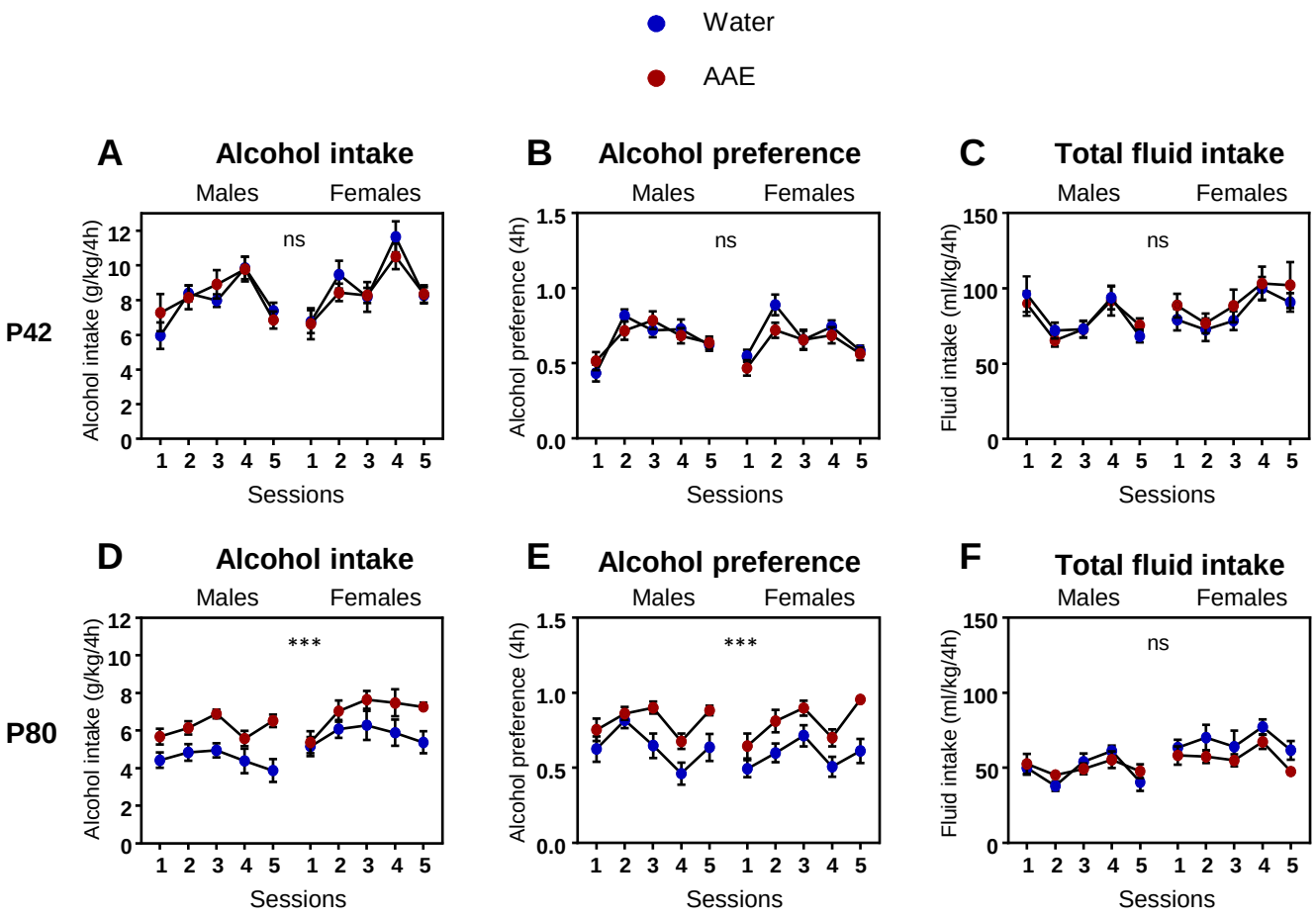
S2 Fig


A
B
Sociability
P42
% of time sniffing
60
40
20
0
ns
Mouse
Object
Water
AAE
Water
AAE
Males
Females
C
Social Novelty Index
(Tnew-Told)/(Tnew+Told)
1.0
0.5
0.0
-0.5
-1.0
ns
Water
AAE
Males
Females
D
Sociability
P80
% of time sniffing
60
40
20
0
ns
Mouse
Object
Water
AAE
Water
AAE
Males
Females
E
Social Novelty Index
(Tnew-Told)/(Tnew+Told)
1.0
0.5
0.0
-0.5
-1.0
ns
Water
AAE
Males
Females

S3 Fig


Water
AAE
A
Learning
Males
Females
Time to escape (s)
ns
Days
B
Errors
Number of primary errors
C
Correct sector
% Time in correct sector
P42
D
E
F
P80

S4 Fig


Water
AAE
A
Alcohol intake
Males
Females
ns
Alcohol intake (g/kg/4h)
Sessions
B
Alcohol preference
Alcohol preference (4h)
C
Total fluid intake
Fluid intake (ml/kg/4h)
P42
D
E
F
P80
***

S1 table

Behavioral test	Sex	Age at test	Number	Mean alcohol consumption (g/kg/4h)
Locomotion	Male	P42	8636	7.35
			8637	8.68
			8638	4.79
			8639	7.69
			8640	6.18
			8641	9.61
			8642	6.27
			8643	8.04
			8644	7.76
			8645	7.94
			8646	6.64
			8647	6.09
				Mean 7.25+-1.3
	Female	P42	8626	8.21
			8627	6.08
			8628	8.01
			8629	7.41
			8630	5.48
			8631	9.15
			8632	8.04
			8633	7.40
			8634	8.06
			8635	8.83
				Mean 7.67+-1.1
	Male	P80-100	6363	7.15
			6364	7.15
			6365	5.85
			6366	5.35
			sb	7.37
			6368	6.22
			6369	5.79
				Mean 6.41+-0.8
	Female	P80-100	6352	7.51
			6353	6.62
			6354	8.46
			6355	6.49
			6356	8.01
			6357	8.72

			6358	6.83
			6361	6.90
			6362	7.03
				Mean 7.4+-0.82
Open Field	Male	P42	4181	6.36
			4176	6.17
			4178	6.24
			4179	5.15
			4173	5.83
			4183	5.26
			4177	6.40
			1932	5.63
			1933	4.86
			1934	8.63
			1935	6.69
			1936	6.70
			8672	7.41
			8671	8.19
				Mean 6.39+-1.1
	Female	P42	4169	7.32
			4164	8.77
			4167	5.55
			4163	5.21
			4170	7.34
			1937	8.21
			1939	4.86
			1940	4.59
			1941	7.43
			1942	7.41
			1944	7.56
			1957	9.21
			8677	8.22
			8675	6.18
				Mean 6.99+-1.5
	Male	P80-100	3517	5.88
			3521	5.07
			3528	6.86
			3518	5.69
			3515	5.95
			3522	6.58
			3520	6.78
			1917	6.37

			1918	6.07
			1921	7.37
			1502	5.32
			1504	6.1
				Mean 6.17+-0.62
	Female	P80-100	3536	7.24
			ss bc	6.87
			3533	5.47
			3543	6.65
			3539	6.53
			3537	6.65
			3534	6.75
			1913	7.08
			1911	7.09
			1909	6.53
			1506	5.77
			1507	6.78
				Mean 6.62+-0.52
	Male	P42	7537	7.32
			7540	5.08
			7542	7.17
			7543	4.48
			7545	4.83
			1968	8.27
			1971	8.77
			1970	7.89
			1973	6.62
			1974	7.07
			1972	7.28
			1975	6.62
				Mean 6.78+-1.36
	Female	P42	7550	6.35
			7561	7.20
			7551	5.22
			7549	6.04
			7563	8.23
			7564	6.12
			1955	8.15
			1953	6.89
			1954	8.85
			1956	11.05
			1957	10.96

Elevated Plus Maze			1959	6.10
				Mean 7.60+-1.9
	Male	P80-100	3517	5.88
			3521	5.07
			3528	6.86
			3518	5.69
			3515	5.95
			3522	6.58
			3520	6.78
			1917	6.37
			1918	6.07
			1921	7.37
			1502	5.32
			1504	6.1
			6100	6.13
				Mean 6.16+-0.63
	Female	P80-100	3536	7.24
			ss bc	6.87
			3533	5.47
			3543	6.65
			3539	6.53
			3534	6.75
			1913	7.08
			1911	7.09
			1909	6.53
			1506	5.77
			1507	6.78
			1508	6.16
			1509	6.53
				Mean 6.57+-0.51
	Male	P42	4103	6.34
			4104	5.29
			4101	6.59
			3000	5.66
			4105	4.31
			4106	6.87
			2999	4.89
			1778	5.8
			1781	7
			1762	6.2
			1763	5.3
			1784	5.5
				Mean 5.81+-0.82

Forced Swimming Test	Female	P42	4112	5.61
			4110	8.71
			4111	7.77
			4109	4.56
			4108	8.77
			1773	7.3
			1772	6
			1768	5.6
			1766	6.1
			1774	7.4
			1760	6.3
				Mean 6.78+-1.35
	Male	P80-100	6683	4.75
			6688	6.90
			6685	4.54
			6696	5.41
			6698	5.26
			7545	4.83
			7540	5.08
			7537	7.32
			1501	7.65
			1503	5.93
			6100	6.13
			1525	6.38
			1523	5.04
			1526	5.45
			1522	7.68
			1527	7.39
				Mean 5.99+-1.1
	Female	P80-100	6690	8.11
			6692	6.96
			6687	6.82
			6686	8.25
			6693	7.60
			7550	6.35
			7563	8.23
			7561	7.20
			7549	6.04
			1511	8.75
			1510	7.43
			1508	6.16
			1536	5.21

			1534	7.60
			1535	8.93
			1537	8.77
			1533	7.34
				Mean 7.40+-1.1
Novel Object Recognition Test	Male	P42	1932	5.63
			1935	6.69
			1936	6.70
			8671	8.19
			8672	7.41
			8673	6.46
			8668	7.87
			8669	7.60
			8670	5.60
				Mean 6.91+-0.93
	Female	P42	1937	8.21
			1939	4.86
			1940	4.59
			1941	7.43
			1942	7.41
			1944	7.56
			1957	9.21
			1943	7.17
			1938	5.43
			8674	9.43
			8676	10.00
			8678	5.93
			8679	9.18
			8680	7.60
			8681	8.50
				Mean 7.5+-1.7
	Male	P80-100	9772	7.09
			9773	5.87
			9774	8.62
			9771	5.79
			1917	6.37
			1918	6.07
			1504	6.10
			1502	5.32
			1503	5.93
			6100	6.13
			1501	7.65

			1523	5.04
			1524	5.44
			1525	6.38
			1526	5.45
			1527	7.39
				Mean 6.29+-0.95
	Female	P80-100	9775	5.96
			9776	7.76
			9780	6.32
			9778	4.78
			9777	7.71
			9779	6.42
			1909	6.53
			1911	7.09
			1913	7.08
			1506	5.77
			1509	6.53
			1510	7.43
			1511	8.75
			1534	7.60
			1533	7.34
			1535	8.93
			1537	8.77
				Mean 7.1+-1.1
	Male	P42	2	5.68
			3	9.52
			4	8.87
			5	9.13
			6	4.72
			7	8.96
			8	8.06
			9	8.35
			11	7.33
			12	5.49
			13	8.12
				Mean 7.6+-1.6
	Female	P42	8682	6.3
			8683	5.4
			8684	7.1
			8685	6.2
			8686	9.1
			8687	10.7

Sociability/Social Novelty			8688	5.2
			8689	10.6
			8690	10.3
			8691	8.6
				Mean 7.9+-1.9
	Male	P80-100	1570	6.98
			1572	5.85
			1573	6.28
			1574	6.85
			1578	6.00
			1579	7.71
			1580	4.72
			1581	5.99
			1582	6.45
			1593	6.48
			1594	5.26
			1595	5.36
			1596	5.41
			1597	4.93
			1598	5.48
				Mean 5.98+-0.82
	Female	P80-100	1583	7.00
			1584	8.67
			1585	8.22
			1586	5.16
			1587	9.69
			1588	10.27
			1589	7.48
			1590	7.42
			1591	7.42
			1592	7.73
			1599	8.57
			1600	6.88
			1701	7.86
			1702	5.96
			1705	8.10
				Mean 7.76+-1.3
			2492	8.83
			2493	5.87
			2494	7.88
			2495	5.51
			2496	7.60

Barnes Maze Test	Male	P42	979	7.21
			980	4.63
			981	6.03
			982	8.45
			983	5.95
			985	6.81
			986	8.95
				Mean 6.98+-1.4
	Female	P42	2481	7.10
			2482	8.43
			2483	8.13
			2484	6.77
			2485	7.56
			2486	7.52
			992	6.82
			993	6.86
			994	5.96
			995	7.00
			996	7.77
			997	9.75
				Mean 7.47+-0.98
	Male	P80-100	1582	6.45
			1580	4.72
			1581	5.99
			1572	5.85
			1570	6.98
			1579	7.71
			1575	6.43
			1571	6.83
			1574	6.85
			1595	5.36
			1573	6.28
			1596	5.41
			1578	6
				Mean 6.22+-0.79
	Female	P80-100	1701	7.86
			1600	6.88
			1702	5.96
			1588	10.27
			1585	8.22
			1592	7.73
			1586	5.16

			1591	7.42
			1584	8.67
			1590	7.42
			1583	7
			1589	7.48
			1587	9.69
				Mean 7.67+-1.4
IA-20% 2BC Test	Male	P42	5363	5.46
			5339	5.39
			5338	4.71
			5367	6.43
			5341	7.59
			5340	3.84
			5365	6.73
			5366	4.35
			6612	7.00
			6611	7.06
			6610	6.00
			6609	9.13
				Mean 6.14+-1.5
	Female	P42	5333	6.24
			5332	6.69
			5336	6.04
			5369	6.87
			5368	7.88
			5331	5.78
			5335	8.28
			8608	8.36
			8606	8.58
			8604	5.05
			8607	8.29
			8605	8.85
				Mean 7.24+-1.3
	Male	P80-100	3053	6.52
			3054	8.65
			3055	5.93
			3065	4.76
			3047	5.30
			3048	4.68
			3049	5.60
			3050	6.09
			3051	5.41

			3052	4.22
			7542	7.16
			7543	4.47
				Mean 5.73+-1.3
	Female	P80-100	3057	7.27
			3058	7.50
			3059	7.66
			3060	7.98
			3067	4.70
			3069	6.02
			3070	4.88
			3071	5.83
			3072	6.61
			3073	5.33
			7551	5.22
			7565	5.96
				Mean 6.25+-1.1