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Gender-specific vulnerability to alcohol and depression.
Focus on glutamatergic synapses in the nucleus accumbens

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Introduction

Statistics from the World Health Organization indicate that European adults drink 12.5 litres of pure alcohol per year, corresponding to more than 2 standard drinks of 12 g pure alcohol per day, more than twice than the amount of alcohol consumed per capita globally (Anderson et al., 2015). While statistically there are more men suffering from alcoholism, there is an increasing body of evidence to support a greater impact of alcohol exposure on females as compared to males (Hommer, 2003). The National Institute on Alcohol Abuse and Alcoholism states that 5.3 million women in the United States use alcohol in a way that “threatens their health, safety and general well-being” and that the problems that plague women drinkers are equal to, or greater than, those that affect men (Alcoholism NIOAA, 2008). However, women remain an understudied population, particularly in pre-clinical work. There is a substantial need for the study of neurobiological bases of the sex differences in susceptibility to alcohol harmful effects on stress-integrative circuits. Indeed, reward circuitry and stress-responsive systems are closely intertwined, but while stress and reward normally cause adaptive responses, dysregulation of these systems results in maladaptive long-term changes.

Women and the costs of alcohol use

Even though the prevalence of alcohol use in Europe generally is lower among women compared with men (Anderson et al., 2015), this gap has narrowed (Gruza et al., 2008). Furthermore, although women consume alcohol at lower levels than men, their body composition puts them at higher risk than men of developing some alcohol-related problems, both acutely (because of higher blood alcohol levels from a given amount of alcohol) and chronically (from alcohol-related organ damage). Gruza and colleagues (2008) reported significant increases between 1990–1991 and 2000–2001 in the lifetime prevalence of drinking for women aged 38–47 in the United States. There also was an increase in lifetime prevalence of alcohol dependence among women drinkers aged 38–47. Similar increases were not found for male drinkers, suggesting that the gender gap in alcohol use and dependence is narrowing, at least in these age groups.

Women who become pregnant in their thirties and forties may be more likely to drink during pregnancy than younger women. From 2001 to 2005, 17.7 percent of 35- to 44-year-old women reported drinking during pregnancy, compared with 8.6 percent of pregnant women aged 18–24

(Denny et al., 2009). Among women in eight States who gave birth between 1997 and 2002, 30.3 percent reported drinking during pregnancy, and 8.3 percent reported binge drinking (four or more drinks on one occasion). Whereas 22.5 percent of the women reported drinking during the first month of pregnancy, drinking declined during pregnancy; only 7.9 percent of women reported drinking during the third trimester, and only 2.7 percent reported drinking during all trimesters, although the general advise is to avoid alcohol consumption during pregnancy. Drinking during pregnancy was more prevalent among women over 30 (more than 30 percent drank) than among younger women (Ethen et al. 2009). Understanding the predictors of drinking during pregnancy may help target prevention efforts. The eight-State study by Ethen and colleagues (2009) found that both drinking and binge drinking during pregnancy were predicted by pre pregnancy binge drinking, thus the pre-pregnancy drinking pattern. Drinking and binge drinking during pregnancy also were more prevalent among women who were non-Hispanic whites, who smoked during pregnancy, and whose pregnancy was unintended. A recent review of 14 studies of drinking during pregnancy in nine countries (Skagerström et al., 2011) found that drinking during pregnancy was associated with heavier drinking prior to pregnancy in all seven studies that measured this.

Women's drinking and psychiatric disorders

In addition to physical health risks associated with alcohol use, women's risks of mental health problems also are related to their drinking. It is clear that women's heavy and binge drinking is associated with alcohol use disorders. For example, U.S. data show that among women aged 50 or older, those who engage in binge drinking (four or more drinks on a drinking occasion) have more than three times greater risks of alcohol abuse, and more than five times greater risks of alcohol dependence, than women who drink but do not engage in binge drinking (Chou et al., 2011). However, there has otherwise been limited attention to gender-specific ways in which women's drinking may be related to alcohol use disorders. One exception is that women, like men, are at greater risk of alcohol use disorders if they begin drinking at early ages (Jenkins et al., 2011).

General-population studies often have found links between women's drinking and psychiatric disorders, but the time order and causes of these linkages are often unclear. For instance, a German survey found that women with alcohol abuse or dependence, or women who drank an average of at least 20 to 30 grams of alcohol per day, were more likely than other women to present affective, anxiety, or somatoform psychiatric disorders, and the connections between drinking and disorders were stronger for women than for men (Bott et al., 2005). A Danish survey found that any psychiatric disorders were more likely in women averaging more than three drinks a day, and

anxiety disorders were specifically more likely among women averaging more than two drinks a day, compared with non drinkers (Flensburg-Madsen et al., 2011). In addition, U.S. data on women aged 50 and older showed higher risks of both panic disorder and posttraumatic stress disorder in women who engaged in any binge drinking, compared with non-binge drinkers (Chou et al., 2011). Unlike the preceding studies, which linked drinking patterns to increased risks of general psychiatric comorbidity, most studies of women's alcohol use and psychiatric disorders have focused on comorbidity of specific disorders with alcohol use disorders and risky drinking patterns. Research clearly has established that depressive disorders and symptoms are more likely among people with alcohol use disorders (e.g., Grant et al., 2004), but studies have not always examined this connection specifically among women. However, a large U.S. twin study found that diagnoses of major depression and alcohol dependence were correlated among women (Prescott et al., 2000), and data from the large National Epidemiologic Study on Alcohol and Related Conditions (NESARC) showed that women with major depressive disorder were more likely to report multiple criteria for alcohol abuse and dependence (Lynskey and Agrawal, 2008). Research also has repeatedly found associations of women's depression with binge drinking. For example, in a major Canadian survey, women's binge drinking (five or more, or eight or more, drinks per day) was associated with measures of recent and longer-term depression (Graham et al., 2007), and data from the large U.S. Behavioral Risk Factor Surveillance System surveys showed that lifetime depression was significantly more likely in women who engaged in binge drinking (four or more drinks in a day)(Strine et al., 2008).

Preclinical models of alcohol abuse

Animal models are helpful for elucidating mechanisms associated with drug abuse and provide a better control of external variables occurring within the clinical population. Among the paradigms employed in order to study alcohol abuse and addiction, the two-bottle choice paradigm is a valuable tool to study alcohol consummatory behaviour. In this paradigm, alcohol is consumed through its natural ingestive route for humans, in self-administration free-choice conditions and the drinking behaviour can be evaluated across long time periods. In other terms, this paradigm models the most common alcohol-related behaviour in the general non-addicted population, which freely drink alcoholic beverages during uncontrolled periods, and not always at intoxicating concentrations. Its intermittent variant, the chronic intermittent alcohol access, on the other hand, leads to increased alcohol intake in animals, up to intoxicating doses. Notably, high alcohol doses are usually consumed early during the period of alcohol availability (in the "loading up" hour, Stuber et al., 2008). This pattern of consumption, for schedule and BAC concentrations, has proved to reliably model binge drinking behaviour (Crabbe et al., 2011).

Two-bottle preference

Curt Richter showed that rats allocate their drinking between a water bottle and a bottle containing a dilute ethanol solution, pioneering the two-bottle preference test (Richter and Campbell, 1940). This basic test of preference for 10% ethanol vs water, while both fluids are continuously available, has led to hundreds of publications. Several lines of rats and mice have been selected for high vs low ethanol preference in two-bottle choice paradigms (Crabbe et al., 2011). However, rats and mice studied under continuous access conditions drink in sporadic bouts. They generally do not drink enough to attain BACs that exceed 80 mg% (rats) or 100 mg% (mice). This was noted even in studies of C57BL/6 mice, a high preferring strain (Dole and Gentry, 1984) The reason for this is not understood. Rodents usually appear to limit their consumption of ethanol to sub-intoxicating BACs. Thus, the standard two-bottle preference test is informative of drinking trajectories of alcohol drinking, even along relatively long time windows, and represents a model for moderate alcohol consummatory behaviour.

Chronic intermittent access

Early studies showed that when rats drank ethanol and access to the drug was then suspended, they show increased preference when ethanol was reintroduced (Sinclair and Senter, 1967; Wayner et al., 1972). Wise subsequently showed that intermittent access (every other 24 hr period) to ethanol in rats led to development of drinking patterns that achieved high levels (9 g/kg/day) of alcohol consumption (Wise, 1973). BACs were not measured in any of these studies. The availability of ethanol across the 24 hr cycle with a single daily measurement of intake does not allow estimation of whether significant BACs were ever attained. A recent study offered 4.44% beer for 2 hr to adolescent Wistar rats. Daily access was compared with access once each 3 days. Adolescent rats consumed more beer when it was intermittently available. They reached BACs of 60 to more than 150 mg%. Interestingly, those intermittently exposed adolescents who had been previously exposed to beer for 3 days drank about half as much, and reached lower BACs. The authors characterize this as a “binge-like effect” (Hargreaves et al., 2009). In another study, intermittent access to 20% ethanol vs water (always available) for 24 hr periods on Monday, Wednesday and Friday each week raised ethanol intake of Wistar and Long-Evans rats to 5–6 g/kg/day within 5–6 drinking sessions. P rats reached 7 g/kg/day. Most rats reached maximal BACs of about 60 mg% in samples taken 30 minutes after the start of drinking, i.e., 45 minutes after the start of the dark cycle; these included all 6 P rats, 8/9 Wistar rats, and 7/10 Long-Evans rats. The remaining 4 rats reached BACs of nearly 100 mg% (Simms et al., 2009). Thus, allowing access to ethanol on an intermittent basis offers a methodological route to enhancing alcohol intakes and model binge drinking.

Role of glutamatergic plasticity in the nucleus accumbens in alcohol abuse and mood disorders

Increasing evidence shows that the neuroadaptations resulting from chronic alcohol abuse aggregate into a hyperglutamatergic state, typified by elevated extracellular glutamate levels and alterations in glutamate receptors and transporters (Krystal et al., 2003; Tsai and Coyle, 1998). Elevated levels of excitatory amino acids are reported in the cerebrospinal fluid of alcohol-dependent patients (Tsai et al., 1998) and severity of alcohol dependence, as measured by the Alcohol Dependence Severity Scale, positively correlates with cerebrospinal fluid glutamate (Umhau et al., 2010). Magnetic resonance imaging studies have found that glutamate levels in the hippocampus and anterior cingulate cortex are increased during early withdrawal (Hermann et al., 2012; Mon et al., 2012). This suggests that enhanced glutamate levels might occur during early conditioned withdrawal in abstinent alcohol dependent patients—a hypothesis supported by evidence in animals showing increases in extracellular glutamate in rodent forebrain regions during acute alcohol withdrawal (Rossetti and Carboni, 1995). A recent meta-analysis of rodent microdialysis studies confirmed elevated extracellular concentrations of glutamate in several brain regions and found that these increases, particularly within the nucleus accumbens, strongly correlated with the severity of alcohol withdrawal (Fliegel et al., 2013). Collectively, these findings suggest a central role for glutamate in inducing and maintaining some of the adverse long-term behavioural effects of alcohol abuse.

The NAc is ideally situated to regulate behavior in response to environmental stimuli, and as such, is known to be critically involved in alcohol-seeking behavior following exposure to alcohol-related cues and context (Chaudhri et al., 2010).

Glutamatergic post-synaptic signaling is also enhanced within the NAc following chronic ethanol exposure and withdrawal. Jeanes et al. (2011) observed a transition from NMDA-receptor dependent LTD to LTP in the NAc of mice that underwent chronic intermittent ethanol vapor exposure. This effect is accompanied by increased intrinsic excitability of NAc MSNs and increased AMPA receptor-mediated activity (Marty and Spigelman, 2012a,b; Spiga et al., 2014). This enhancement of AMPA receptor signaling may be due to increased GluA1 expression in the NAc after chronic ethanol exposure (Neasta et al., 2010).

Presynaptically, prolonged ethanol exposure is associated with increased basal glutamate in the NAc. For example, acute withdrawal from chronic ethanol vapor inhalation is associated with significantly elevated NAc glutamate (Dahchour and De Witte, 2003). More recently, this same finding was reported to last well into protracted abstinence (Griffin et al., 2014). In the same study, Griffin et al. (2014) also showed that pharmacological reduction in glutamate signaling in the NAc

reversed dependence-induced escalated ethanol consumption to control levels. Notably, chronic ethanol-induced elevation in NAc basal glutamate levels is not associated with alterations in glutamate transport (Griffin et al., 2015), suggesting that this effect is mediated instead by increased vesicular release of glutamate and/or reduced mGluR autoreceptor activity. In addition, both repeated daily ethanol exposure and binge-like ethanol exposure reportedly increased glutamate release in the NAc following an acute ethanol challenge, indicating that prolonged ethanol exposure sensitizes the glutamatergic system within the NAc to ethanol's effects. These data as well as others support findings from clinical studies suggesting that dependence on alcohol induces a lasting hyperglutamatergic state that facilitates continued alcohol consumption and relapse during abstinence. Indeed, enhanced glutamatergic signaling in the NAc in response to chronic ethanol exposure may facilitate increased salience of alcohol-related cues and promote cue-induced relapse. In addition, enhanced glutamatergic signaling within the NAc may influence dopamine release via its reciprocal connections with the VTA and may play a role in the lasting effects of chronic ethanol exposure on dopamine signaling and the associated increase in reward threshold observed during withdrawal.

To date, however, efforts to understand the effects of chronic ethanol exposure on NAc neuroplasticity have focused largely on male subjects. Moreover, little appreciation for circuit specificity has been paid. The NAc receives glutamatergic input from the PFC, hippocampus, thalamus and amygdala (Phillipson and Griffiths, 1985) yet our understanding of the neuroadaptive changes that take place within each of these circuits as a result of chronic ethanol exposure is still not well understood. This concept is exemplified in findings from an elegant study using *in vivo* optogenetics that demonstrate that stimulation of glutamatergic inputs from the basolateral nucleus of the amygdala to the NAc is both necessary and sufficient to facilitate cue-induced reward seeking behavior (Stuber et al., 2011). In contrast, it was observed that PFC inputs to the NAc are not involved in this behavioral outcome. A greater appreciation for circuit-specificity is evolving as illustrated by recent work by Meinhardt et al. (2013) who used retrograde labeling in combination with laser capture microdissection to examine projection specific changes in glutamatergic signaling following chronic ethanol exposure. This study revealed significant down-regulation of the mGluR2 gene *Grm2* in NAc-projecting neurons of the infralimbic PFC following seven weeks of chronic intermittent ethanol exposure. A similar reduction in *Grm2* expression was observed in post-mortem samples of the anterior cingulate cortex of alcohol-dependent individuals (Meinhardt et al., 2013). The authors then demonstrated a direct link between mGluR2 expression in the NAc and propensity for relapse by showing that viral expression of mGluR2 in NAc-projecting infralimbic neurons of ethanol-exposed rats reduced cue-induced seeking for ethanol. Clearly,

additional work aimed at parsing out the precise role of specific glutamatergic inputs to the NAc and the changes they undergo following alcohol dependence will be critical for uncovering the precise mechanisms underlying dependence-induced changes in behaviour that promote relapse. Dysregulation of NAc glutamatergic transmission is not only involved in alcohol-related behaviours, but it is a key pathophysiological feature of stress and depression susceptibility (Russo and Nestler, 2013; Thompson et al., 2015). Glutamate injection into the NAc dose-dependently decreased swimming time in the forced swim test, whereas the injection of NMDA receptor antagonists resulted in antidepressant activity (Rada et al., 2003). Notably, chronic stress induces functional and structural neuroplasticity at excitatory synapses in the NAc (Christoffel et al., 2011a). Chronic social stress and chronic mild stress altered AMPAR profile decreasing GluA2 and increasing GluA1 protein expressions in the NAc, thus increasing excitatory synaptic strength (Vialou et al., 2010; Toth et al., 2008). Moreover, increased spine density and dendritic length in the NAc was reported following chronic mild stress and chronic social stress (Bessa et al., 2013; Christoffel et al., 2011). On the other hand, epigenetic manipulations that decrease spine density in the NAc reversed the social avoidance of susceptible individuals, a core symptom of depression (Golden et al., 2013). Recent work has identified a family of proteins responsible for vesicular glutamate transport (VGLUTs) as histological markers for glutamatergic axon terminals. Among the three VGLUT isoforms, VGLUT1 and VGLUT2 control glutamate vesicles loading and the pre-synaptic release of glutamate. Importantly, they are largely segregated in the brain: VGLUT1 is primarily found in the neurons of cerebral cortex, hippocampus, basolateral amygdala and cerebellar cortex nuclei. In contrast, VGLUT2 is mainly expressed in the neurons of thalamus, brainstem and deep cerebellar nuclei (Fremeau et al., 2001; 2004). In the NAc, which receives input from both VGLUT1-expressing and VGLUT2-expressing neurons, the projections mainly segregate (Härtig et al., 2003). Chronic stress also affects neuroplasticity at NAc excitatory axon terminals. In particular, chronic social stress increased the expression of VGLUT-2 in the NAc, which reflects an increase of subcortical glutamate input (Christoffel et al., 2015). Indeed, because of their different pattern of expression, VGLUTs isoforms can serve as pre-synaptic markers to evaluate the neuroplasticity of distinct glutamate inputs to the NAc. Interestingly, the rearrangement of synaptic strength, neuronal processes and axon terminals induced by chronic stress in the NAc correlates with the susceptible behavioral phenotype, while opposite changes or no differences were observed in resilient animals with respect to unstressed controls (Vialou et al., 2010; Christoffel et al., 2011; Golden et al., 2013; Christoffel et al., 2015).

Aim of the study

The aim of this study was three-fold: first, to evaluate the effect of the drinking pattern on the behavioural dimensions related to anxiety, depression and affectivity in female animals; second, to individuate female-specific alterations of glutamatergic transmission in the nucleus accumbens, which could represent a useful molecular target for gender-oriented therapeutic interventions in alcohol abuse disorders. Third, assessing the involvement of a circuit-specific glutamatergic input to the nucleus accumbens in inducing depression-like behavioural deficits.

Specific aims

Aim 1: Exploration of the effects of alcohol drinking pattern on emotional and affective processing in female rats

I investigated long-term alcohol intake in female rats by a continuous and intermittent free-choice paradigm from pre-gestational - to pre-weaning time, in order to assess the effect of the drinking pattern on alcohol drinking trajectories in different physiological conditions, including those that apply exclusively to females, such as virginity, pregnancy and lactation. Moreover, the consequences of chronic continuous and intermittent alcohol drinking on emotional and affective processing were explored. In doing so, female animals were tested for behavioural reactivity and exploratory-based anxiety like behaviour in the Open Field and Elevated Plus Maze test; for their response to novelty in the Novel Object exploration and recognition; for their behavioural response to fear in the Fear-Potentiated Elevated Plus Maze test; for their response to natural reward, including saccharin preference and maternal behaviour; ultimately, for their coping with stress in the Forced Swim test, classical screening test for antidepressant drugs.

Aim 2: Assessment of gender-specific alterations of glutamate signaling in the NAc associated with depression susceptibility

Data collected in experiment 1 showed robust deficits in affective-related behaviours associated with chronic alcohol drinking and, in particular, with binge drinking in female rats. Given that increasing evidence points to aberrant glutamatergic neuroplasticity in the NAc as a common

feature of stress and depression susceptibility and alcohol related disorders, I studied sex-specific alterations of glutamatergic transmission in the NAc associated with depression.

Indeed, I examined pre- and post-synaptic aspects of the excitatory synapses in the NAc by using a gender-oriented stress paradigm, able to model females' greater susceptibility to stress (LaPlant et al., 2009; Hodes et al., 2015).

The subchronic variable stress (SCVS) model involves the alternate exposure to unpredictable stressors, such as foot shock, tail suspension and restraint stress, along 6 days. As a result, female mice display a depression-like phenotype in a behavioural battery tailored to capture core symptoms of depression. In particular, female mice show increased passive coping in the forced swim test; decreased hedonic reactivity in the sucrose preference test; decreased motivation to self-groom in the splash test, and to eat in the novelty suppressed feeding, along with no significant differences in exploratory and anxiety-like behaviours. Exposure to SCVS also increases serum corticosterone levels in female mice only and reveals sex-specific alterations of the transcriptome profile in the NAc (Hodes et al., 2015). Importantly, none of these behavioural and neurobiological deficits is observed in male mice, which are resilient to SCVS (LaPlant et al., 2009; Hodes et al., 2015).

I evaluated VGLUT1, VGLUT2 immunofluorescence, as pre-synaptic markers of cortical and subcortical glutamatergic inputs to the NAc, and the post-synaptic density protein PSD95, as a measure of post-synaptic changes induced by SCVS in medium spiny neurons. The VGLUT immunofluorescence represents glutamate transporting vesicles predominately located in glutamate terminals. However, the expression of VGLUT2 has been reported in a subset of dopaminergic terminals from the VTA. The level of co-localization between VGLUT2 and the catecholamine biosynthetic enzyme tyrosine hydroxylase (TH) has been employed to further characterize the neurochemical nature of vGLUT2-positive puncta in the NAc.

Aim 3: Evaluation of the behavioural and functional profile of the thalamic glutamatergic input to the NAc

Data from experiments 1 and 2 suggested the involvement of the increased subcortical (i.e. thalamic) glutamatergic input to the NAc in susceptibility to stress and depression in females and depression can be considered both a cause and an effect of alcohol drinking, representing a permeating characteristic of this phenotype. Thus, I tested the sufficiency of the thalamo-striatal pathway stimulation in inducing behavioural deficits associated with depression. In order to achieve the circuit specificity, I injected a retrograde viral vector for the expression of the light-sensitive cationic channel ChR2 in the NAc. In that, the viral infection of the glutamatergic terminals present

in the NAc propagated retrogradely up to the cell bodies in the projection sources, across 4 weeks. By shining light in the intralaminar thalamus, I was able to activate only the neurons that actually sent their axonal projections to the NAc, were infected by the retrograde viral vector and expressed the light-sensitive cationic channel ChR2.

The effects of the stimulation of the thalamo-striatal pathway were tested on general locomotor activity; on motivation for self-grooming in the splash test; on motivation to eat in the novelty suppressed feeding; on exploratory-based anxiety-like behaviour in the elevated plus maze test. The eventual sustained effect of the optogenetic stimulation was tested on the response to natural reward in the sucrose preference test. Moreover, attempting to characterize cell-specific connectivity in the NAc, the functional activation of total medium spiny neurons and D1-expressing medium spiny neurons was evaluated by immunostaining for the c-fos expression after 60 min from the stimulation.

Materials and Methods

Experiment 1: Exploration of the effects of alcohol drinking pattern on emotional and affective processing in female rats

Animals

Adult female Wistar rats (Harlan, Udine, Italy) (8 weeks, 200-220 g) were housed individually in standard rat cages (40 cm × 60 cm, 20 cm in height) and maintained in a temperature- (22 ± 2 °C) and humidity- (55 ± 5 %) controlled room, with ad libitum access to water and food. The colony was maintained on 12 h light/dark cycle (08:00 to 20:00) and rats were gently handled for 3 min per day for a week before the experimental procedures.

For maternal behaviour testing, twelve female rats from each experimental group were randomly selected and housed, with a single breeder male. The day when the pregnancy was confirmed, designated as gestational day one (GD 1), eight female rats from each experimental group were housed in standard maternity cages, filled with wood shavings. Ethanol consumption was not measured on day 1 and 2, when the male was present in the cage (Allan et al., 2003). Females continued to drink throughout pregnancy, accordingly to the two-bottle choice paradigm. Dams were inspected twice daily for delivery, and the day of parturition was considered as postnatal day 0 (PND 0); dams and litters were kept in a nursery, under proper temperature-controlled conditions (24 ± 2 °C). All the experiments were conducted in accordance with the regulations of the Committee for the Protection and Use of Animals of the University of Palermo, in accordance with the current Italian legislation on animal experimentation (D.L. 116/92) and the European directives (2010/63/EU).

Two-bottle“20% alcohol vs water” choice drinking paradigms

Female rats were matched for body weight, and randomly assigned to one of the three experimental groups (n=24) according to the home-cage two-bottle “alcohol vs. water” choice regimen: continuous alcohol access- rats (CAR, n=24) - 20% v/v alcohol with continuous access (24 h/day, 7 days/7week); intermittent alcohol access-rats (IAR, n=24)- 20% v/v alcohol with intermittent access (three 24 h drinking sessions per week on Monday, Wednesday and Friday) and 2 water bottles for the remaining 4 days; water-drinking controls (CTR, n=24) - receiving two bottles of tap water.

Alcoholic solution (20 % v/v) was daily prepared by diluting ethanol 96° (Carlo Erba Reagenti, Italy) with tap water. Bottles (Tecniplast, Italy) were refilled every day with fresh solution, and presented every day at lights off, in alternative left-right position, to avoid side preference. Alcohol- and water intake were carefully measured by weighing the bottles before delivery, after 1h and

every 24 h (0.1 g accuracy); possible fluid spillage was calculated and subtracted before data analysis according to Loi and colleagues (2014). Data analysed in the present study refer only to alcohol intake during the drinking sessions of either continuous and intermittent-access groups.

During gestation and lactation, female rats were exposed to the two-bottle choice paradigm at the same conditions of pre-gestational period. In particular, alcohol intake was recorded from the GD1 and continued throughout lactation until PND21. Dams' body weight was measured two times a week during gestational and post-partum time, in order to minimize animal distress.

Open field test

Locomotor activity and behavioural reactivity in a novel environment, after 24 h abstinence, were measured in an open field with an automatic video-tracking system, Any Maze (Ugo Basile, Italy). The open field apparatus is a Plexiglas square box, 44 cm long, 44 cm wide, and 20 cm high. The apparatus produces a qualitative mapping of the motor patterns, measuring different parameters simultaneously: total distance travelled (TDT), number of central transitions (NCT) from peripheral to central squares of the arena, and amount of time spent on the central area (TSC). Each experimental session lasted 5 min.

Novel object recognition test

Novel object recognition protocol was modified from Plescia et al., 2014. This test was carried out in the same Plexiglas square box in a dimly lit testing room. After the open field session, which serves as habituation phase, rats were subjected to a 5-min sample session when they were presented two identical, non-toxic objects (i.e. two metal cans) which were placed against a wall in the open field arena. To prevent coercion to explore the objects, rats were released against the opposite wall with its back to the objects. The time spent on exploring each object was recorded using ANY MAZE Video Tracking System, (Ugo Basile, Italy); a 2 cm² area surrounding the object was defined such that nose entries were recorded as time exploring the object. After the sample session, animals were placed in their home cage for 24 h retention interval. Then, following 48 h of alcohol abstinence, animals were returned to the arena containing two objects: one was identical to the familiar one but previously unused (to prevent olfactory cues and the necessity to wash objects during experimentation), the other was a novel object (metal, glass or hard plastic items). Time spent on exploring each object was recorded along 5-min session. Objects were randomized and counterbalanced across animals. The objects and arena were thoroughly cleaned at the end of each experimental session. The recognition index (RI), which is the time spent on investigating the novel object, divided by the total amount of exploration time of the novel and familiar objects, $[RI = TN/(TN + TF)]$, is a measure of novel object recognition and the main index of retention. If RI

percentage is higher than 50%, it indicates more time spent on enquiring into the novel object, whereas less than 50% indicates that time was prevalingly spent on exploring the familiar object, and 50% indicates a null preference.

Elevated plus maze test

The elevated plus maze (EPM) was made from dark-grey PVC and consisted of a plus-shaped platform elevated to a height of 70 cm above the floor. Two of the opposing arms (50 cm × 10 cm) were closed by 40 cm-high side end-walls (closed arms), whereas the two other arms had no walls (open arms). The arms were oriented perpendicular to each other and connected by an open central area 10 cm x 10 cm. At the beginning of each session, rats were placed in the centre of the maze facing one of the open arms. Time spent on each arm and number of entries were recorded by using ANY MAZE Video Tracking System, (Ugo Basile, Italy), along the 5-min test duration. The percentages of time spent on the open arms/total time spent on open and closed arms, and of number of entries in the open arms/number of entries in the open and closed arms, were analysed, as they constitute the primary indices of anxiety-like behaviour. Information concerning treatment effects on general activity can be obtained by examining closed arm entry data.

Fear-Potentiated Elevated Plus Maze test

To further examine the possible difference in anxiety-like behaviour under more stressful conditions, animals were tested for fear-potentiated EPM behaviour. Rats underwent testing after being re-exposed to the compartment where they had experienced an inescapable foot shock 24 h earlier (Korte et al., 1995). Briefly, rats were placed in a waiting Plexiglas (25 x 25 x 30 cm) cage next to the apparatus for 1 min, after which they were entered a compartment where they were allowed to stay for 5 min. This procedure was repeated three times. During the final training trial, an inescapable scrambled foot shock (0.6 mA, AC for 3 s) was given immediately upon entering the compartment. Rats were removed 40s after termination of the foot shock. On the next day, animals were placed in the waiting cage for 1 min; afterwards they were directly transferred into the compartment, but without any further foot shock. Directly after the 5-min period exposure to the compartment in which they had previously been shocked, rats were tested in the elevated plus-maze, located in another sound-proof room.

Saccharin preference test

This validated test is considered as an index of anhedonia (Pucilowski et al., 1993). It measures rat's sensitivity towards natural rewarding stimuli, and is based on the rewarding properties of sweet substances, such as saccharin or sucrose. At week 12, at the end of the last drinking session, 8

cages from each experimental group were supplied with two identical drinking bottles filled with water (habituation phase). On the day of the test, at 24 h alcohol deprivation for both groups, one bottle of water was replaced by a bottle containing a solution with 0.2% (w/v) saccharin (Sigma Aldrich, Italy), and rats were offered to freely choice between the two options over a 24 h period. The location of the two bottles was randomly alternated to avoid location preference bias. The total volume of fluid intake was recorded, and the preference ratio was calculated by measuring the volume of the saccharin solution consumed with respect to total fluid intake.

Forced swim test

At week 12, at the end of the last drinking session, other 8 cages from each experimental group underwent forced swim test. Briefly, the rat was placed individually in a glass cylinder (40 cm high, 18 cm inside diameter) containing 5–6 l of clean water, depending on the rat size. Water temperature was maintained at 22–23 °C. The animal was forced to swim for 15 min on the first day (pre-test). On the second day (48 h alcohol abstinence), each rat was placed into the water and forced to swim for 5 min (test). The session was videotaped and the duration (in seconds) of immobility and swimming, along with the number of climbing episodes, were recorded as measures of depressive-like behaviour. The rat was considered as immobile when it stopped struggling and moved only to remain floating, keeping its head above the water. Climbing was defined by active attempts to climb the walls of the apparatus.

Maternal Behaviour Assessment

Maternal behaviour was assessed by recording dams' spontaneous behaviour in the presence of their offspring in the home cage under undisturbed conditions, by direct periodic observations along 15 days from PND1 to PND21 (from Monday to Friday). An observer, remaining quietly in the room, recorded the measures. Each assessment was performed at the following times: 9:00 am; 11:30 am; 01:30 pm; 03:00 pm, and consisted of 3 trials of 20 sec-observation, for a total of 12 instantaneous observations per animal per day (3 observations x 4 times per day x 15 days = 180 observations/dam). The 20 sec time of observation allows for an exact identification of the ongoing behaviour. The behavioural parameters scored were retrieval, nursing (arched-back, blanket, passive), pup care (licking, anogenital licking), dam self-care (self-grooming, eating, drinking), and others (rearing, moving, resting, standing out of nest). A detailed description of the behavioural categories can be found in Capone et al., 2005. The observations were carried out during diurnal time, when animals behave more maternally (Grota and Ader, 1969, 1974). Original data were recorded using the instantaneous sampling and dichotomous scores (0/1). In particular, score 0 was assigned when the behaviour was not shown in the interval of observation, while score 1 was

assigned when the behaviour was performed. The original dichotomous scores (0/1) were transformed into quantitative variables, adding the original scores of the instantaneous samples within each time point, ranging from 0 (behaviour never shown) to 3, (the maximum number of instantaneous samples within the time point), and along the 4 time points. Thus, a daily score ranged between 0 and 12.

In order to get a general framework of the behavioural measurements, a daily index of overall maternal behaviour (MB-I) was calculated as follows: $(\text{maternal score}) - (\text{non-maternal score}) / (\text{maternal score}) + (\text{non-maternal score})$. The index ranges from -1 (totally non-maternal behaviours) to +1 (totally maternal behaviours).

Statistical analysis

Data on alcohol intake, expressed in g/kg/day, refers to the drinking sessions of both alcohol-drinking groups, and were analysed by a two-way analysis of variance for repeated measures (RM two-way ANOVA), considering "alcohol drinking pattern" as the between subjects factor, and "time" (days, gestational and lactation periods) as the repeated measures factor followed by a Bonferroni post hoc test ($\alpha=0.05$). Data on the total sum of alcohol intake over the drinking sessions were analysed by separate Student's t test for two-tailed unpaired measures. Alcohol drinking behaviour was also analysed comparing maternal alcohol consumption during gestation and lactation to pre-gestational basal intake in both alcohol drinking groups by a one-way ANOVA for paired measures ($n=8$) followed by a Bonferroni post hoc test ($\alpha=0.05$). Data are reported as mean \pm S.D. Statistical significance was set at $p<0.05$.

Data from Open Field Test, Novel Object Recognition, Elevated Plus Maze, Saccharin preference test and Forced Swim test were analysed by using one-way ANOVA. Bonferroni post hoc test was employed, when necessary ($\alpha=0.05$). Data are reported as mean \pm S.D. Statistical significance was set at $p<0.05$.

Data on daily maternal behaviour scores were analysed by RM two-way ANOVA, considering "alcohol drinking pattern" or "treatment" as the between subject factor, and "time" as the repeated measures factor. MB-I scores were evaluated by using RM one-way ANOVA. Bonferroni post hoc test was employed, when necessary ($\alpha=0.05$). Data are reported as mean \pm S.D. Statistical significance was set at $p<0.05$.

Experiment 2: Assessment of gender-specific alterations of glutamate signaling in the NAc associated with depression susceptibility

Animals

C57BL/6J female and male mice (Jackson Laboratory) at 8 weeks of age were employed. Mice were group housed and maintained on a 12 h light/dark cycle with ad libitum access to food and water. Procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the Icahn School of Medicine at Mt. Sinai.

Subchronic variable stress

Subchronic variable stress was performed as described previously (LaPlant et al., 2009; Hodes et al., 2015). Female and male mice (n = 5 - 6 per group) underwent 1-hour unpredictable stress each day for 6 days, consisting of foot shock, tail suspension, or restraint, alternated to prevent stress habituation. Stressors were administered in the following order: 100 random mild foot shocks at 0.45 mA for 1 h (Med Associates, St. Albans, Vermont) (10 mice to a chamber); tail suspension stress for 1 h; restraint stress, placed inside a 50 ml falcon tube, for 1 h within the home cage. The three stressors were then repeated for the next 3 days in the same order. After each stress, animals were returned to their home cage for the duration of the protocol.

Perfusion and tissue processing

24h after the last stress session, all mice were given a fatal dose of 15% chloral hydrate and transcardially perfused with cold phosphate-buffered saline (PBS; pH 7.4), followed by fixation with cold 4% paraformaldehyde in PBS. Brains were dissected and postfixed overnight in the same fixative. Fixed brains were cryoprotected by immersion in 15% sucrose in PBS for 24 hours, followed by immersion in 30% sucrose in PBS until sank. Brains were flash-frozen and coronal sections were prepared on a cryostat (Leica CM1850) at 35µm thickness. Serial slices were collected through the rostral-caudal dimension of the nucleus accumbens (every 6th slice) and stored at 4°C in 0.01% sodium azide in PBS until immunofluorescence processing.

Immunofluorescence staining

Sections (six per animal) were washed in PBS for 30 min and incubated in blocking solution (3% normal donkey serum, 0.3% Triton X-100 in PBS) for 2 h at room temperature under gentle shaking. Sections were then incubated in primary antibody solution overnight at 4 °C under gentle shaking (3% normal donkey serum, 0.3% Tween-20 in PBS, with either guinea pig anti-VGLUT1 (Millipore #ab5905) 1:10,000; guinea pig anti-VGLUT2 (Millipore, #ab2251) 1:10,000; goat anti-PSD95 (Abcam #ab12093) 1:1,000; rabbit anti-TH (Millipore, #ab152) 1:10,000). Sections were

washed in PBS for 1h, incubated in secondary antibody for 2 h under gentle shaking (donkey anti-rabbit Cy2 1:200; donkey anti-goat Cy2 1:200; donkey anti-guinea pig Cy3 1:200 (Jackson ImmunoResearch)). After 1h washing in PBS, slices were briefly incubated with DAPI (1 μ g/ml). All sections were mounted onto superfrost plus slides, allowed to dry overnight, dehydrated in alcohol and cleared in Citrisolv, then coverslipped in DPX (Electron Microscopy Sciences).

Imaging and analysis

VGLUT1-, VGLUT2-, PSD95- and TH- immunofluorescence were analysed in NAc (from bregma 1.70 mm to 0.98 mm, according to Paxinos and Franklin, 2001). For analysis of VGLUT puncta levels, images were acquired on a confocal LSM 780 upright microscope (Zeiss) at 100 \times magnification (Plan-Apochromat 100 \times /1.46 Oil DIC M27; zoom 1.0; pixel size 0.83 μ m), as 2 μ m z-stacks (21 slices, 0.1 μ m interval) and deconvolved using AutoQuant. Analysis of puncta was performed in ImageJ, using the functions 'max intensity' for stacks, 'find maxima' to determine puncta number, and 'colocalization threshold' for colocalization analysis of vGLUT2 and TH puncta.

Statistical analysis

Data were analysed by 2-way ANOVA, including "stress" as the between subject factor and "gender" as the within-subject factor. Bonferroni post test was used for post hoc analysis. All statistical analyses were performed using Graph Pad Prism 6.1 software (Graphpad). Statistical significance was set a $p < 0.05$. Grubbs outlier test was performed and samples that varied 2 SDs from the mean were removed. Data are reported as mean \pm SD.

Experiment 3: Evaluation of the behavioural and functional profile of the thalamic glutamatergic input to the NAc

Animals

8-week aged D1-Td-tomato mice (n=10) were used. Mice were group housed and maintained on a 12-h light/dark cycle with ad libitum access to food and water. Drd1-tdTomato (line 5, Shuen et al, 2008) lines were provided by Dr Nestler (Department of Neuroscience, Icahn School of Medicine at Mount Sinai). Procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the Icahn School of Medicine at Mt. Sinai. Animals that lost the ferrule implant, or that did not showed a correct fiber placement, were removed from the experimental groups.

Injection protocol and coordinates

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and positioned in a small-animal stereotaxic instrument (David Kopf Instruments). The skull surface was exposed and 33 gauge syringe needles (Hamilton) were used to bilaterally infuse 0.5 μ l of the retrograde rAAV2/5-hSyn-hChR2-eYFP (UPenn Viral Core), at a rate of 0.1 μ l /min, into the NAc (bregma coordinates: anteroposterior, 1.6 mm; mediolateral, 1.5 mm; dorsoventral, 4.4 mm; angle 10°). Animals were then unilaterally implanted with chronically implantable optic fibers constructed with 200 μ m core 0.22 NA optic fiber (Thor Labs) threaded through ceramic zirconia ferrules (Precision Fibre Products) in the IL-TH (bregma coordinates: anteroposterior, -2.0 mm; mediolateral, 1.4 mm; dorsoventral, -3.9 mm; angle 10°).

Blue Light Stimulation and Behavioural assessment

4 weeks following the surgery, animals were handled for 3 consecutive days and then underwent optogenetic stimulation during the behavioural assessment. It consisted in 473 nm blue light pulses delivered at a frequency of 20 Hz (20-ms pulse width) and five pulses delivered in a burst, with a burst period of 1 s, using a 100-mW DPSS 498-nm laser (OEM Laser). Intensity of light delivered to ferrule was ~10 mW (Christoffel et al., 2015).

The behavioural assessment included: locomotor activity, splash test, novelty suppressed feeding, elevated plus maze test and sucrose preference test, performed on consecutive days.

Locomotor activity

Mice were acclimated to the testing room 1 h and tested under red light. They were connected to the path cord placed into clear testing chambers (44.45 cm length x 17.78 cm width x 25.4 cm height). Laser was turned on for the stimulated group after the first 15 min of testing and remained off for

control non-stimulated group. The number of photobeam breaks on the x and y planes were measured for 30 min using an automated system (PAS, San Diego Instruments).

Splash test

Testing was based on a published protocol (Isingrini et al., 2010). The test was performed under red light (230 V, 15 W). Mice were habituated to the room for 1 h before testing. Mice were connected to the path cord and sprayed on the back with a 10% sucrose solution 3 times. Laser was turned on for the stimulated group and off for control non-stimulated group. Mice were then placed into an empty housing cage, and behavior for 5 min was recorded. The total amount of time grooming over 10 min period was recorded and hand scored.

Novelty suppressed feeding

Mice were food restricted overnight before testing. On the day of testing, mice habituated to the testing room for 1 h. Under red light conditions, mice were connected to the path cord and laser was turned on for the stimulated group and off for control non-stimulated group. Then they were placed into a plastic box 50x50x20 cm with bedding. A single pellet of food was placed in the center of the box. Mice were placed in the corner of the box, and the latency to eat was scored up to 10 min during testing. Mice were then immediately transferred to their home cage in standard lighting conditions, and the latency to eat was recorded.

Elevated plus maze

Mice were acclimated to the testing facility for 1 h before testing. Animals were mice were connected to the path cord and laser was turned on for the stimulated group and off for control non-stimulated group. Then they were placed in the EPM under red light conditions for 5 min. Each arm of the maze measured 12 x 50 cm. The black plexiglas cross-shaped maze consisted of two open arms with no walls and two closed arms (40 cm high walls) and was on a pedestal 1 m above floor level. Behavior was tracked using an automated system (Noldus Ethovision; Noldus Interactive Technologies).

Sucrose preference test

Testing was conducted according to previously published protocols (Vialou et al., 2010). Immediately after the novelty suppressed feeding test mice were given 2 bottles filled with water for a 24 h habituation period. The following day, immediately after elevated plus maze testing, one of the two 50 ml bottles was replaced with a 1% sucrose bottle for 24 h. The 2 bottles were then weighed, and side position was counterbalanced to avoid side preference. Sucrose preference was

calculated by determining the percentage of total sucrose solution consumption divided by total liquid consumption (sucrose plus water).

Blue Light Stimulation for c-Fos induction

On the last day, mice were connected to the path cord and subjected to 5-min blue light stimulation. Laser was turned on for the stimulated group and off for control non-stimulated group. 60 min after the stimulation, all mice were given a fatal dose of 15% chloral hydrate and transcardially perfused with cold phosphate-buffered saline (PBS; pH 7.4), followed by fixation with cold 4% paraformaldehyde in PBS. Brains were dissected and postfixed overnight in the same fixative. Fixed brains were cryoprotected by immersion in 15% sucrose in PBS for 24 hours, followed by immersion in 30% sucrose in PBS until sank. Brains were flash-frozen and coronal sections were prepared on a cryostat (Leica CM1850) at 35 μ m thickness. Serial slices were collected through the rostral-caudal dimension of the nucleus accumbens (every 6th slice) and stored at 4°C in 0.01% sodium azide in PBS until immunofluorescence processing.

c-Fos immunofluorescence staining

Free floating coronal brain sections were blocked in 3% normal donkey serum (NDS) and 0.3% Triton-X overnight before adding primary antibody. Brain sections were incubated in 1:4000 goat anti-c-Fos (Santa Cruz, sc-52G) in blocking solution for 72h at 4° on a shaker. Sections were rinsed in PBS (3 x 1h) then incubated in 1:100 of donkey anti-goat Dylight 405 (Jackson ImmunoResearch) in PBS for 2 h at room temperature. Subsequently sections were rinsed in PBS (3 x 1h) and incubated again in blocking solution, before incubating them with 1:500 rabbit anti-FoxP1 antibody (Cell Signaling) in blocking solution overnight. Sections were rinsed in PBS (4 x 15 min) then incubated in 1:200 of donkey anti-rabbit Cy5 antibody (Jackson ImmunoResearch) in PBS for 2 h at room temperature. After rinsing in PBS (4 x 15 min), all sections were mounted onto superfrost plus slides, allowed to dry and coverslipped in Vectashield.

Imaging and analysis

c-Fos and FoxP1 immunofluorescence were analyzed in NAc of D1-td tomato mice (from bregma 1.70 mm to 0.98 mm, according to Paxinos and Franklin, 2001). Images were acquired on a confocal LSM 780 upright microscope (Zeiss) at 20 \times magnification (Plan-Apochromat 20 \times /0.8 M27; zoom 1.0; pixel size 0.83 μ m), as 10 μ m z-stacks (11 slices, 1 μ m interval).

c-Fos-FoxP1 and c-Fos-D1 td-tomato positive neurons were counted manually from both hemispheres of ~4-6 brain sections per animals.

Statistical analysis

Data were analysed by Student's t test for unpaired measures. All statistical analyses were performed using Graph Pad Prism 6.1 software (Graphpad). Statistical significance was set a $p < 0.05$. Data are reported as mean \pm SD.

Results

Experiment 1: Exploration of the effects of alcohol drinking pattern on emotional and affective processing in female rats

Alcohol intake along the two-bottle“20% alcohol vs water” choice drinking paradigm

Alcohol consumption expressed in g/kg/day was analysed by RM two-way ANOVA that indicated significant effect of days ($F(35, 1610) = 2728.89, p < 0.0001$), alcohol drinking pattern ($F(1, 46) = 1106.49, p < 0.0001$) and their interaction ($F(35, 1610) = 22.40, p < 0.0001$). Results from Bonferroni post hoc analysis showed a significant increase in alcohol intake in IAR with respect to CAR as indicated in Fig 1A. A two-tailed Student t test on the total amount of alcohol consumed over the 36 drinking sessions showed higher values in IAR ($t=13.56, df=46, p<0.001$), with respect to CAR (Fig. 1 B). Moreover, after the first hour of alcohol exposure during the 36 drinking sessions, IAR displayed higher mean alcohol intake with respect to CAR ($t = 3.139, df = 22, p = 0.0048$) (Fig. 1 C).

The analysis of the drinking behaviour showed that CAR alcohol intake did not exceed the water one (fig. 1 D); on the other hand, IAR consumed higher ml of alcohol than water, starting from the third week onwards (fig. 1 E). Two-tailed Student's t test on mean total fluid intake over the 12 pre-gestational weeks, indicated no significant differences between the two groups ($t=2.015, df=22, p=0.0653$).

Two-bottle“20% alcohol vs water” choice drinking paradigms, during gestation and lactation

Daily alcohol intake in CAR and IAR during gestation is shown in fig. 2 A. The RM 2-way ANOVA on g/kg/day of alcohol consumed by both groups revealed significant effect of gestation ($F(8, 112) = 8.03, p < 0.0001$), alcohol drinking pattern ($F(1, 14) = 17.82, p = 0.0009$) and their interaction ($F(8, 112) = 5.83, p < 0.0001$). In particular, Bonferroni post-hoc test indicated a significant decrease in IAR with respect to CAR, as reported in fig. 2 A. A two tailed Student' t-test performed on the total amount of alcohol consumed over the 9 gestational drinking sessions showed that IAR reduced their alcohol intake during pregnancy ($t=5.497, df=14, p<0.001$), when compared to CAR (Fig. 2 B). Two-tailed Student's t test on mean fluid intake over the 3 gestational weeks, indicated no significant differences between the two groups ($t=1.657, df=4, p=0.1728$) (Fig. 2 E).

Alcohol daily intake in CAR and IAR during lactation is shown in fig. 3 A. The results of a RM 2-way ANOVA performed on g/kg/day of alcohol consumed during lactation revealed significant effect of lactation ($F(8, 112) = 6.65, p < 0.001$), alcohol drinking pattern ($F(1, 14) = 17.22, p = 0.0010$) and their interaction ($F(8, 112) = 4.07, p = 0.0003$). In particular, Bonferroni post-hoc test

indicated that IAR significantly increased their alcohol intake with respect to CAR (Fig. 3 A). A two-tailed Student's t-test performed on the total amount of alcohol consumed over the 9 drinking sessions during lactation, showed that IAR significantly increased their amount of alcohol ingestion ($t=4.867$ $df=14$ $p<0.001$), when compared to CAR (Fig. 3 B).

Two-tailed Student's t test on mean fluid intake over the 3 lactation weeks, indicated no significant differences between the two groups ($t=1.638$ $df=4$ $p=0.1768$) (Fig. 3 E).

Alcohol drinking trajectories were also analysed within CAR and IAR comparing maternal alcohol consumption during gestation and lactation to the respective pre-gestational basal intake during the last 9 pre-gestational drinking sessions. A one-way ANOVA for paired measures performed on CAR cumulative alcohol intake displayed a statistically significant effect ($F(1377) = 28.57$, $p<0.0001$). Bonferroni post hoc analysis showed that during gestation alcohol intake was increased with respect to basal intake ($t=7.508$, $df = 2$, $p<0.001$) while, during lactation it returned to the basal levels ($t=4.518$, $df=2$, $p<0.001$) (Fig. 4 A). When the analysis was performed on IAR drinking habit, the results obtained by a one-way ANOVA for paired measures showed a statistically significant effect ($F(3882) = 46.10$, $p<0.0001$). Bonferroni post hoc analysis indicated that during gestation IAR consumed a lower amount of alcohol with respect to the basal value while during lactation alcohol intake increased reaching the same levels as in pre-gestational time ($t = 9.229$, $df = 2$, $p<0.001$; $t = 6.911$, $df = 2$, $p<0.001$) (Fig. 4 B).

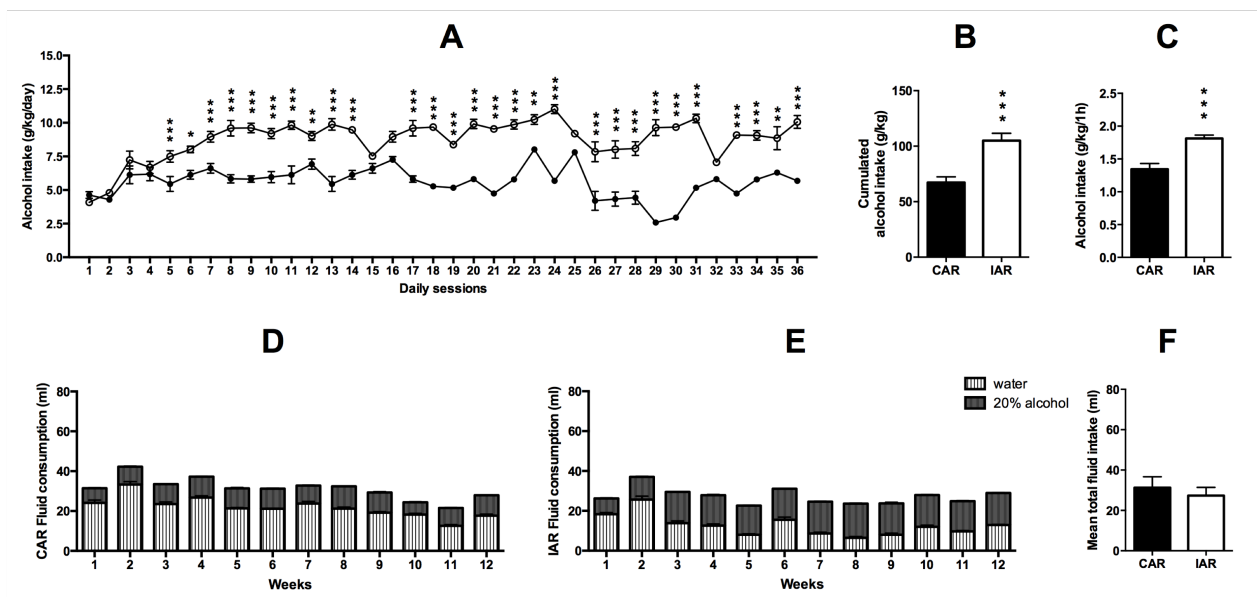


Figure 1. Pre-gestational drinking behaviour. (A) Alcohol intake (expressed in g/kg/day) during the 36 pre-gestational drinking sessions in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access. Results from Bonferroni post hoc analysis showed a significantly higher alcohol intake in IAR, when compared to CAR, on days 5 ($t = 16.125$, $p < 0.001$), 6 ($t = 5.188$, $p < 0.001$), 7 ($t = 6.427$, $p < 0.001$), 8 ($t = 8.561$, $p < 0.001$), 9 ($t = 12.09$, $p < 0.001$) 10 ($t = 8.868$, $p < 0.001$), 11 ($t = 10.11$, $p < 0.001$), 12 ($t = 5.746$, $p < 0.001$), 13 ($t = 12.14$, $p < 0.001$), 14 ($t = 9.174$, $p < 0.001$), 16 ($t = 4.671$, $p < 0.001$), 17 ($t = 10.37$, $p < 0.001$), 18 ($t = 12.05$, $p < 0.001$), 19 ($t = 8.776$, $p < 0.001$), 20 ($t = 11.24$, $p < 0.001$), 21 ($t = 13.16$, $p < 0.001$), 22 ($t = 11.21$, $p < 0.001$), 23, $t = 6.084$, $p < 0.001$), 24 ($t = 14.62$, $p < 0.001$), 25 ($t = 3.794$, $p < 0.001$), 26 ($t = 11.51$, $p < 0.001$), 27 ($t = 20.13$, $p < 0.001$), 28 ($t = 9.102$, $p < 0.001$), 29 ($t = 19.12$, $p < 0.001$), 30 ($t = 18.44$, $p < 0.001$) 31 ($t = 14.12$, $p < 0.001$), 32 ($t = 3.410$, $p < 0.05$), 33 ($t = 11.88$, $p < 0.001$), 34 ($t = 8.977$, $p < 0.001$), 35 ($t = 6.714$, $p < 0.001$) and 36 ($t = 12.02$, $p < 0.001$). (B) Total sum of alcohol consumed over the entire period. (C) Mean alcohol consumption following the first hour of access. (D) Total fluid intake (ml) in CAR over the 12 pre-gestational weeks. (E) Total fluid intake (ml) in IAR over the 12 pre-gestational weeks. (F) Mean fluid consumption over the entire period. Each point or bar is the mean \pm S. D of $n = 24$ rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs CAR.

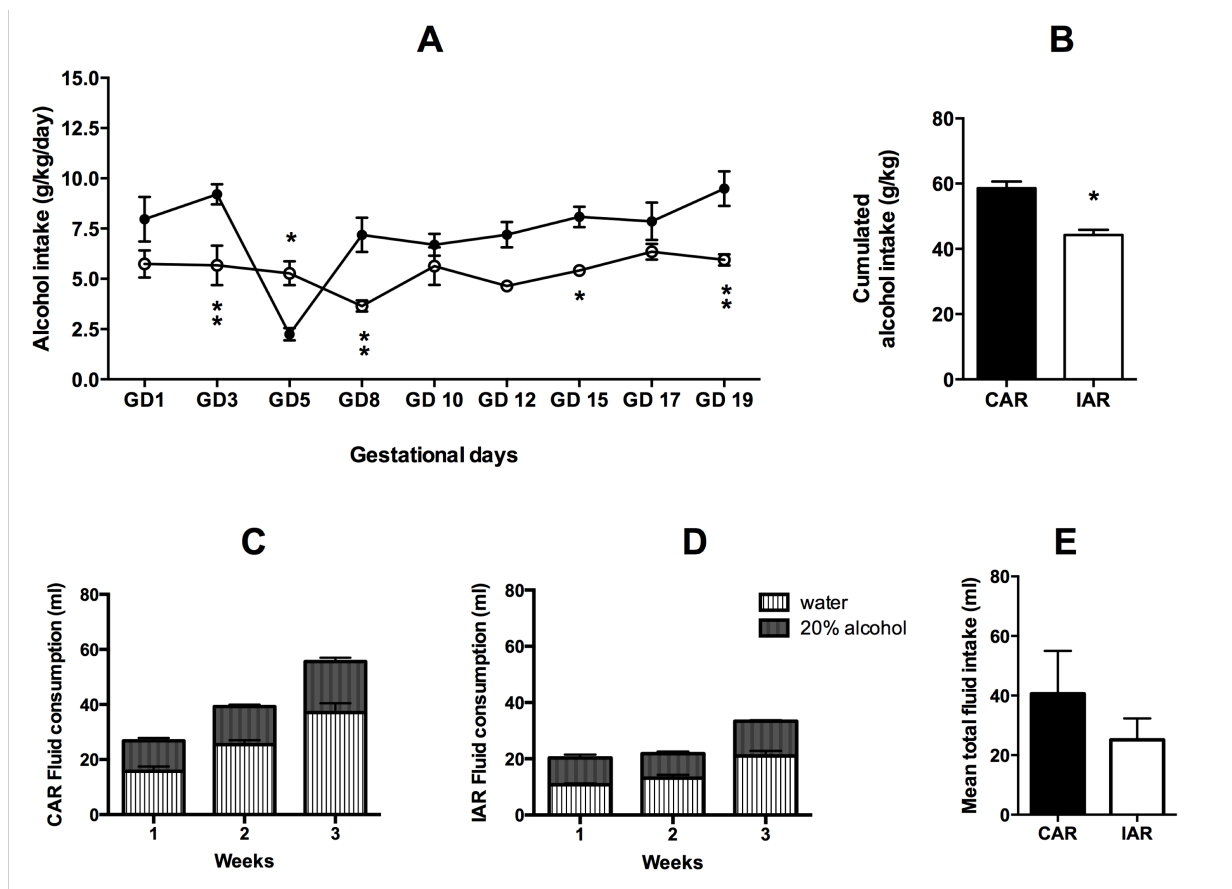


Figure 2. Gestational drinking behaviour. (A) Alcohol intake (expressed in g/kg/day) during gestational days over 9 drinking sessions in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access. Bonferroni post-hoc test indicated a significant decrease in IAR with respect to CAR on GD3 ($t = 3.794$, $p < 0.01$), GD5 ($t = 3.252$, $p < 0.05$), GD8 ($t = 3.779$, $p < 0.01$), GD15 ($t = 2.856$, $p < 0.05$) and GD19 ($t = 3.799$, $p < 0.01$). (B) Total sum of alcohol consumed over the entire period. (C) Total fluid intake (ml) in CAR over the 3 gestational weeks. (D) Total fluid intake (ml) in IAR over the 3 gestational weeks. (E) Mean fluid consumption over the entire period. Each point or bar is the mean \pm S. E. M. of $n = 8$ rats. * $p < 0.05$, ** $p < 0.01$ vs CAR.

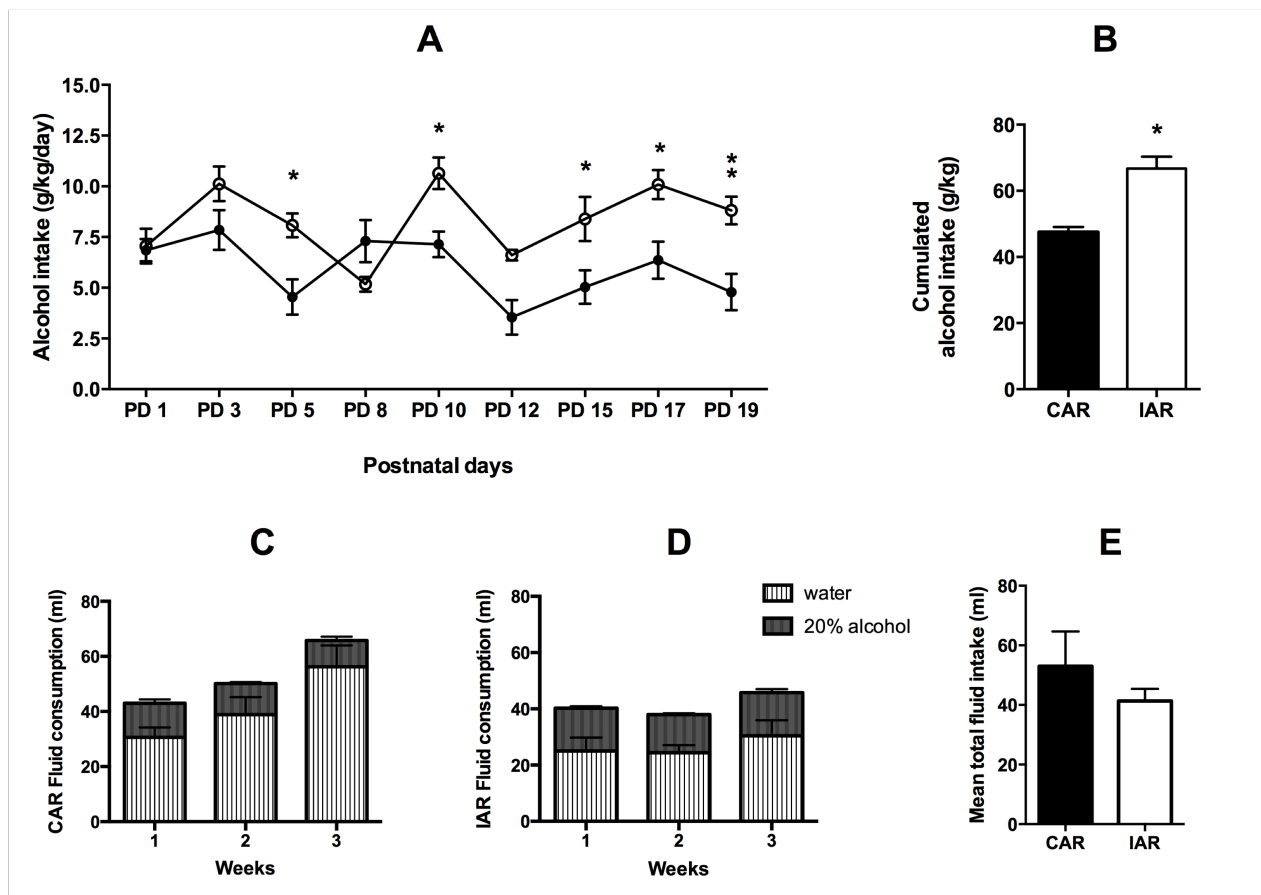


Figure 3. Post-gestational drinking behaviour. (A) Alcohol intake (expressed in g/kg/day) during post-gestational days over 9 drinking sessions in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access. Bonferroni post-hoc test indicated that IAR significantly increased their alcohol intake with respect to CAR on PND5 ($t = 3.161$, $p < 0.05$), PND10 ($t = 3.141$, $p < 0.05$), PND15 ($t = 3.005$, $p < 0.05$), PND17 ($t = 3.336$, $p < 0.05$) and PND19 ($t = 3.602$, $p < 0.01$). (B) Total sum of alcohol consumed over the entire period. (C) Total fluid intake (ml) in CAR over the 3 post-gestational weeks. (D) Total fluid intake (ml) in IAR over the 3 post-gestational weeks. (E) Mean fluid consumption over the entire period. Each point or bar is the mean \pm S. E. M. of $n = 8$ rats. * $p < 0.05$, ** $p < 0.01$ vs CAR.

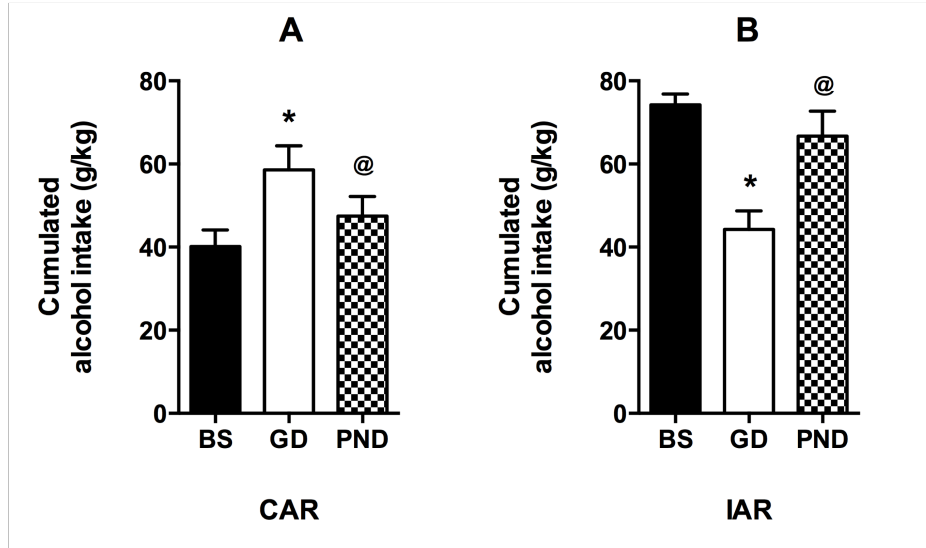


Figure 4. Differences in total sum of alcohol intake (expressed in g/kg) between maternal alcohol consumption during gestation, lactation and baseline (last 3 weeks) intake in CAR and IAR group. Each value represents the mean \pm S.D. of $n = 8$ rats. @ $p < 0.05$, * $p < 0.001$ vs baseline.

Open field test

Animals underwent open field test in order to evaluate locomotor activity and behavioural reactivity in a novel environment following 24 h of alcohol abstinence. Results from a one-way ANOVA showed no significant differences in locomotor activity, in terms of TDT, among the three experimental groups ($F(2, 15)=0.08578$, $p=0.9182$) (fig. 5 A). Statistical analysis indicated significant differences among groups in NCT ($F(2, 15)=4.294$, $p=0.0335$), and TSC ($F(2, 15)=4.271$, $p=0.0340$), with significant decrease in CAR with respect to CTR (NCT: $t=2.775$, $p<0.05$; TSC: $t=2.910$, $p<0.05$) (fig. 5 B).

Novel object recognition test

Following the open field test, rats were subjected to a sample session, when they faced two identical and unfamiliar objects. Statistical analysis showed a significant effect for treatment in time spent by animals on exploring the objects ($F(2, 15)=15.23$, $p=0.0002$). Bonferroni post hoc indicated that both CAR and IAR displayed a statistically significant reduction in object exploration, with respect to CTR (respectively, $t=5.318$, $p<0.001$; $t=3.938$, $p<0.01$) (fig. 6 A).

Animals were tested for novel object recognition 24 h following the sample session, in order to assess declarative memory functioning after 48 h of alcohol abstinence. One-way ANOVA on RI % showed significant differences among the three groups ($F(2, 15)=7.034$, $p=0.0070$). In particular, Bonferroni post hoc test indicated a significant decrease in both CAR ($t=3.588$, $p<0.01$) and IAR ($t=2.741$, $p<0.05$), when compared to CTR (fig. 6 B).

Elevated plus maze test

Animals were tested in the EPM, aiming at investigating on anxiety-like behaviour after 24 h of alcohol abstinence.

One-way ANOVA, performed on data from the percentage of time spent on open arm / time on open and closed arms, showed significant difference among the experimental groups ($F(2, 15)=8.291$, $p=0.0038$). In particular, Bonferroni post hoc test evidenced a significant lower preference for the open arms, in terms of time spent on, in CAR, when compared to CTR ($t=2.912$, $p<0.05$), and IAR ($t=3.921$, $p<0.01$) groups (fig. 7 A). Similar results were draft for data from the percentage of open arm entries, out of total entries: one-way ANOVA showed statistically significant differences among the groups ($F(2, 15)=11.82$, $p=0.0008$). In particular, Bonferroni post hoc analysis showed a significant decrease in CAR group, with respect to CTR ($t=2.964$, $p<0.05$) and IAR ($t=4.821$, $p<0.001$) (fig. 7 B). No significant differences among the three experimental group were observed in number of closed arm entries ($F(2, 15)=0.6339$, $p=0.5441$) (fig. 7 C).

Fear-Potentiated Elevated Plus Maze test

When animals underwent fear-potentiated EPM, statistical analysis indicated that re-exposure to the compartment where they received the foot shock 24 h earlier did not result in an increase in anxiety-like behaviour for IAR rats. One-way ANOVA showed significant differences among the groups in the percentage of time spent on open arms/ time on open and closed arms ($F(2, 15)=8.957, p=0.0028$), when IAR group displayed a significant increase, with respect to CTR ($t=2.712, p<0.05$) and CAR ($t=4.170, p<0.01$) (fig.). Significant differences were observed also in the percentage of open arm entries, out of total entries ($F(2, 15)=10.58, p=0.0014$), with IAR showing a significant increase, when compared with CAR ($t=4.599, p<0.01$) (fig. 8 A). Moreover, one-way ANOVA highlighted differences in the number of closed arm entries ($F(2, 15)=4.414, p=0.0311$), in which IAR group displayed a reduction with respect to CAR ($t=2.873, p<0.05$) (fig. 8 B).

Saccharin preference test

On the day of the experiment, at 24 h alcohol deprivation, animals were subjected to the saccharin preference test, in order to evaluate their response to a natural reward. During the 24 h access to 0.2% saccharin solution, animals consumed the following amounts of saccharin: CTR 520 ± 85 mg/kg; CAR 482 ± 48 mg/kg; IAR 301 ± 59 mg/kg. One-way ANOVA on saccharin solution preference showed significant differences among the three experimental groups ($F(2, 21) = 429.7, p < 0.001$). In details, Bonferroni post hoc test highlighted a significant decrease in saccharin choice in both CAR ($t = 5.992, p < 0.001$) and IAR ($t = 27.85, p < 0.001$) with respect to CTR. Moreover, IAR displayed a significantly lower preference for saccharin compared to CAR ($t = 21.86, p < 0.001$) (fig. 9).

Forced swim test

Depressive-like behaviour was evaluated in CAR and IAR by using the forced swim test. One-way ANOVA showed a significant effect of treatment ($F(2, 21) = 31.40, p < 0.001$) on immobility time. Bonferroni post hoc test highlighted a significant increase in both CAR ($t = 6.492, p < 0.001$) and IAR ($t = 7.183, p < 0.001$) with respect to CTR. Accordingly, a significant effect of treatment was observed on swimming time ($F(2, 21) = 17.57, p < 0.001$), with a significant decrease in both alcohol-drinking groups (CAR: $t = 4.679, p < 0.001$; IAR: $t = 5.491, p < 0.001$), when compared with CTR. Furthermore, one-way ANOVA indicated a main effect of treatment in the number of climbing ($F(2, 21) = 10.67, p < 0.001$). In particular, CAR showed a significant increase with respect to CTR ($t = 4.586, p < 0.001$) and IAR ($t = 2.779, p < 0.05$) (fig. 10).

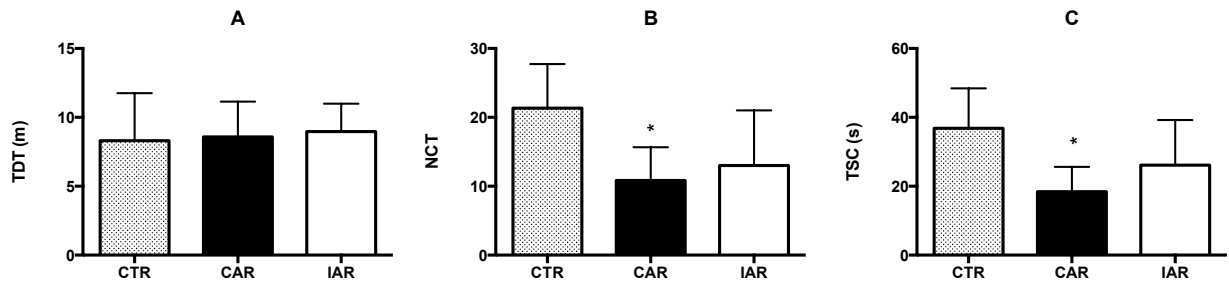


Figure 5. Open field test in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access. Each value represents the mean \pm S.D. of 8 rats. * $p < 0.05$ vs CTR.

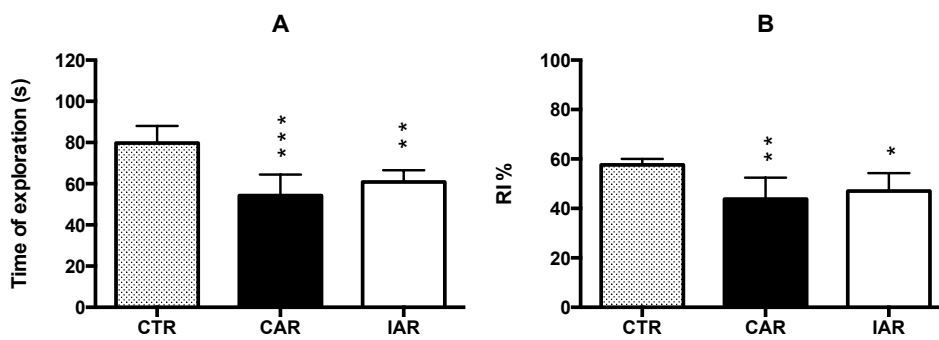


Figure 6. Novel Object recognition test in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access. Each value represents the mean \pm S.D. of 8 rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs CTR.

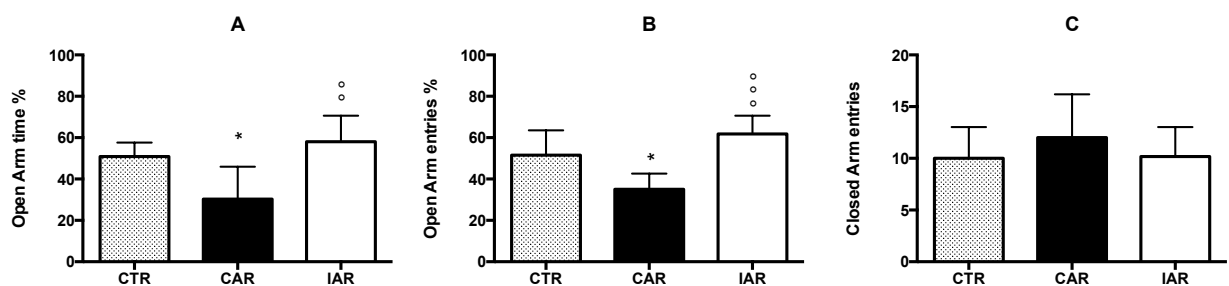


Figure 7. Elevated Plus Maze test in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access. Each value represents the mean \pm S.D. of 8 rats. * $p < 0.05$ vs CTR; ^{oo} $p < 0.01$, ^{ooo} $p < 0.001$ vs CAR.

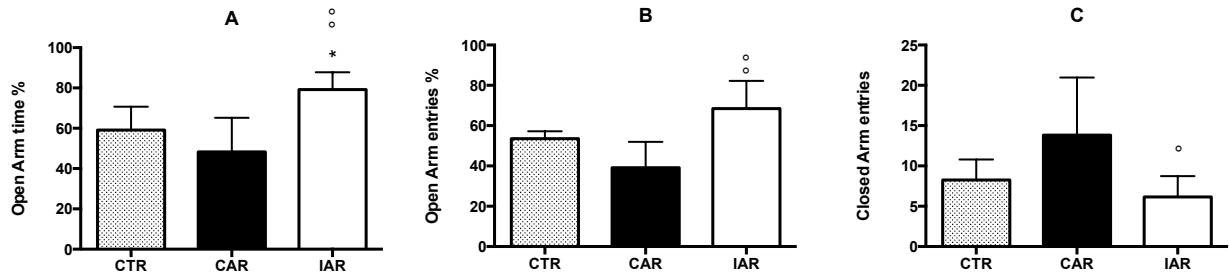


Figure 8. Fear-potentiated Elevated Plus Maze test in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access.. Each value represents the mean \pm S.D. of 8 rats. * $p < 0.05$ vs CTR; ° $p < 0.05$, °° $p < 0.01$ vs CAR.

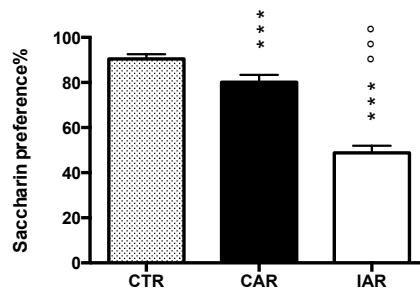


Figure 9. Saccharin preference test in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access.. Each value represents the mean \pm S.D. of 8 rats. *** $p < 0.001$ vs CTR; °°° $p < 0.001$ vs CAR.

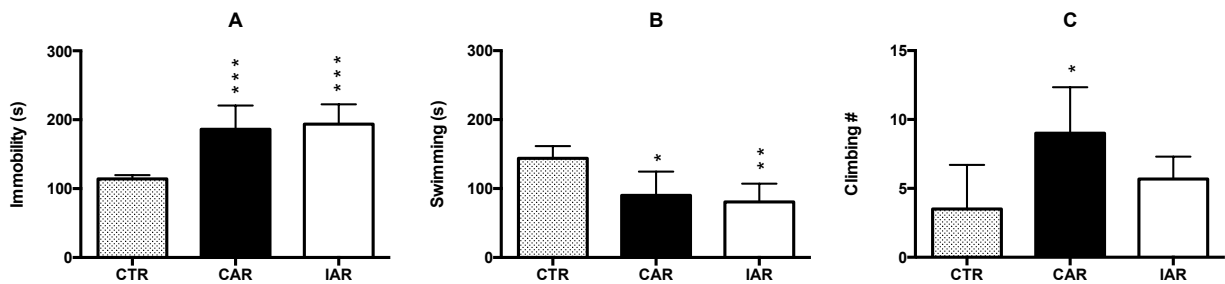


Figure 10. Forced Swim test in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access.. Each value represents the mean \pm S.D. of 8 rats. * $p < 0.001$ vs CTR; # $p < 0.001$ vs CAR.

Maternal Behaviour Assessment

Dam phenotypes from the different maternal and non-maternal categories were recorded 4 times a day, until PND21. Specific behaviours were scored and analysed: retrieval, nursing, pup care, dam self-care and “other behaviours”. Nursing, in terms of arched-back-, blanket- and passive nursing, was the most observed behaviour. The rank of the overall frequency of the behavioural categories over the three weeks of lactation resulted as follows (Fig. 11 A-B):

- CTR: nursing (67.92%) > others (12.36%) > dam self-care (11.39%) > pups care (7.64%) > retrieval (0.69%);
- CAR: nursing (49.39%) > dam self-care (22.78%) > others (18.78%) > pups care (8.20%) > retrieval (0.85%);
- IAR: nursing (40.37%) > dam self-care (31.11%) > others (20.19%) > pups care (7.31%) > retrieval (1.02%).

RM 2-way ANOVA was performed on daily nursing scores, with time as the within subject factor, and treatment as the between subject factor. The analysis showed significant effects of time ($F(14, 294) = 7.841, p < 0.001$), treatment ($F(2, 21) = 52.15, p < 0.001$) and their interaction ($F(28, 294) = 3.917, p < 0.001$). Results from the Bonferroni post hoc test indicated a significant reduction in nursing score in CAR and IAR with respect to CTR, and in IAR with respect to CAR (tab. 1).

RM 2-way ANOVA on dam self-care pattern showed that time ($F(14, 294) = 16.11, p < 0.001$), treatment ($F(2, 21) = 61.40, p < 0.001$) and their interaction ($F(28, 294) = 5.302, p < 0.001$) were statistically significant. Bonferroni post hoc test revealed a significant increase in dam self-care score in CAR and IAR with respect to CTR, and in IAR when compared with CAR (tab. 2).

RM 2-way ANOVA on “other behaviours” showed that time ($F(14, 294) = 5.846, p < 0.001$), treatment ($F(2, 21) = 8.030, p = 0.0026$) and their interaction ($F(28, 294) = 1.584, p = 0.0340$) were statistically significant. Bonferroni post hoc test revealed a significant increase in dam self-care score in CAR and IAR with respect to CTR, and in IAR when compared with CAR (tab. 3). RM 2-way ANOVA on scores from retrieval and pup care (i.e. licking, anogenital licking) scores did not showed significant differences among the three experimental groups (Fig. 11 A-B).

In order to obtain an overall snap-shot of the influence of the drinking patterns on dams' behaviour, RM one-way ANOVA was carried out on MB-I data. The statistical analysis revealed a main effect of treatment on MB-I along the lactation period ($F(1907, 26.69) = 40.71, p < 0.001$), with a significant reduction in CAR and IAR with respect to CTR ($t = 5.818, p < 0.001$; $t = 9.751, p < 0.001$), and in IAR when compared to CAR ($t = 2.935, p < 0.05$). The daily index of overall maternal behaviour for each experimental group is shown in Fig. 11 C.

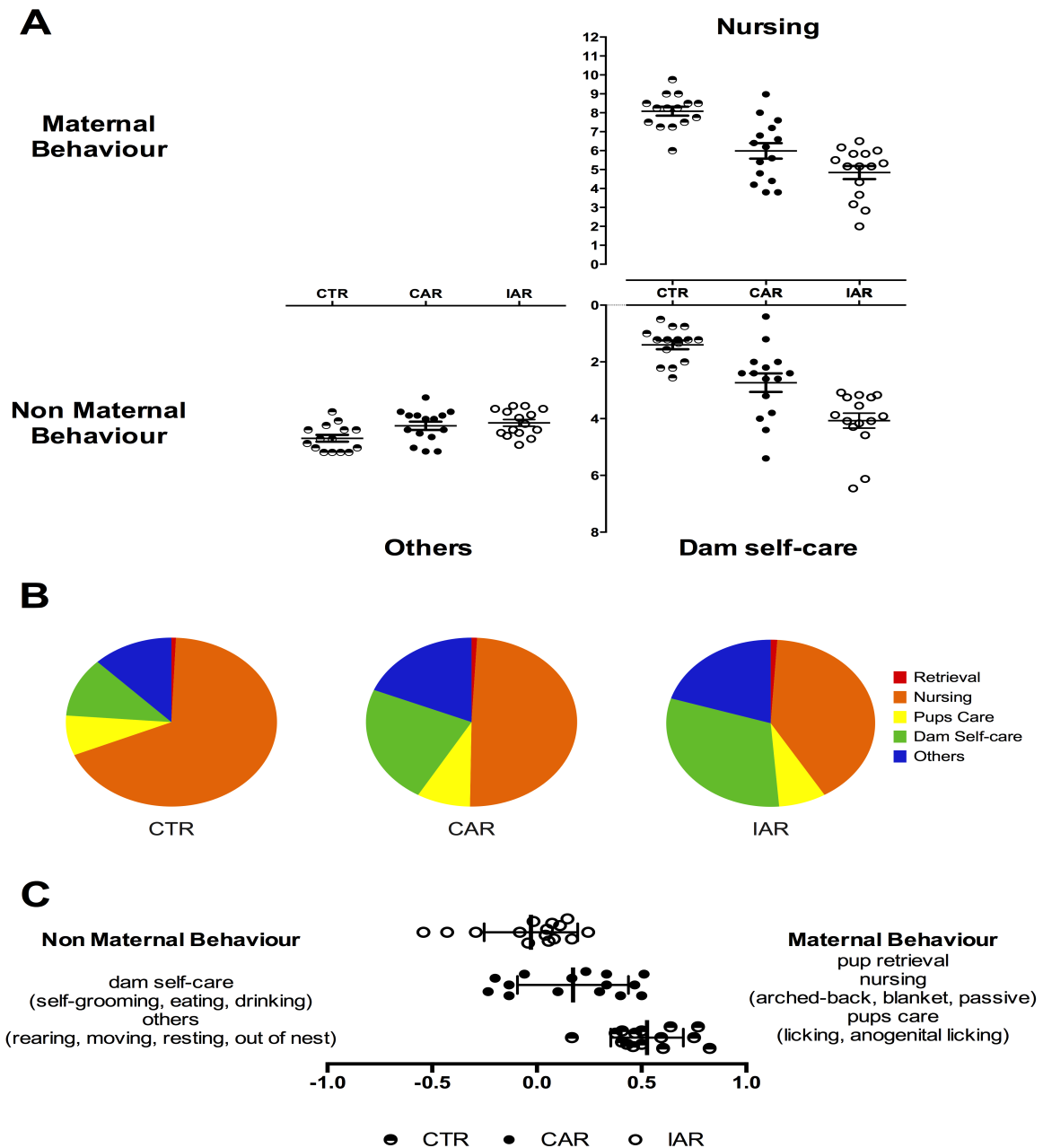


Figure 11. Maternal behaviour in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access. (A) Evaluation of daily nursing (i. e. arched-back, blanket, passive nursing), dam self-care (i. e. self-grooming, eating, drinking) and other behaviour scores (i. e. rearing, moving, resting and standing out of nest). (B) Relative frequency of the maternal and non-maternal behavioural categories observed. (C) Maternal Behaviour Index (MB-I). See text for details and significant differences.

Tab. 1: Evaluation of daily nursing score (i. e. arched-back, blanket, passive nursing) in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access.

	CAR VS CTR	IAR VS CTR	IAR VS CAR
Nursing			
	PND 1 t = 2.829, p = 0.0149	PND 1 t = 6.287, p < 0.001	PND 1 t = 3.458, p = 0.0019
	PND 2 t = 3.595, p = 0.0011	PND 3 t = 4.126, p = 0.0001	PND 10 t = 4.489, p < 0.001
	PND 3 t = 3.301, p = 0.0032	PND 4 t = 3.635, p = 0.001	PND 17 t = 4.205, p < 0.001
	PND 5 t = 3.654, p = 0.0009	PND 5 t = 4.912, p < 0.001	
	PND 8 t = 2.535, p = 0.0352	PND 8 t = 3.831, p = 0.0005	
	PND 12 t = 5.246, p < 0.001	PND 10 t = 2.751, p = 0.0198	
	PND 15 t = 3.419, p = 0.0021	PND 11 t = 3.635, p = 0.001	
	PND 16 t = 4.067, p = 0.0002	PND 12 t = 2.849, p = 0.014	
	PND 19 t = 4.362, p < 0.001	PND 15 t = 3.144, p = 0.0055	
		PND 16 t = 6.189, p < 0.001	
		PND 17 t = 5.501, p < 0.001	
		PND 19 t = 6.287, p < 0.001	

Tab. 2: Evaluation of daily dam self-care score (i. e. self-grooming, eating, drinking) in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access.

	CAR VS CTR	IAR VS CTR	IAR VS CAR
Dam self-care			
	PND 4 t = 2.488, p = 0.0401	PND 1 t = 4.561, p < 0.001	PND 5 t = 2.654, p = 0.0251
	PND 12 t = 4.644, p < 0.001	PND 4 t = 3.317, p = 0.003	PND 10 t = 5.142, p < 0.001
	PND 16 t = 4.810, p < 0.001	PND 5 t = 4.561, p < 0.001	PND 11 t = 4.091, p = 0.0002
	PND 18 t = 3.981, p = 0.0003	PND 8 t = 2.903, p = 0.0119	PND 16 t = 3.483, p = 0.0017
	PND 19 t = 5.391, p < 0.001	PND 10 t = 3.732, p = 0.0007	PND 17 t = 6.303, p < 0.001
		PND 11 t = 4.009, p = 0.0002	
		PND 15 t = 4.561, p < 0.001	
		PND 16 t = 8.294, p < 0.001	
		PND 17 t = 7.879, p < 0.001	
		PND 18 p = 0.0181, t = 2.765	
		PND PND19 t = 6.497, p < 0.001	

Tab. 3: Evaluation of daily “other behaviours” score (i. e. self-grooming, eating, drinking) in female rats exposed to the home cage, 2-bottle “alcohol vs. water” choice regimen, under the following conditions: CAR - 20% v/v alcohol with continuous access; IAR-20% v/v alcohol with intermittent access.

	CAR VS CTR	IAR VS CTR	IAR VS CAR
Other behaviours			
	PND 5 t = 2.022, p = 0.0474	PND 3 t = 3.159, p = 0.0052	PND 9 t = 2.831, p = 0.0148
		PND 4 t = 3.286, p = 0.0034	
		PND 9 t = 2.907, p = 0.0117	

Experiment 2: Assessment of gender-specific alterations of glutamate signaling in the NAc associated with depression susceptibility

VGLUT1-, VGLUT2-, PSD95- and TH- immunofluorescence in the NAc

Confocal imaging revealed dense immunolabeling of VGLUT1- and PSD95-immunoreactive puncta (Fig. 12 A-B) and VGLUT2- and TH-immunoreactive puncta and fibers in the NAc (Fig. 13 A-B) with few incidents of colocalization of TH and VGLUT2 in NAc afferents (Fig. 13 C-F). In particular, the analysis of colocalized voxel revealed that they represented a small percentage of either TH-positive voxels (CTR M: 1.37 ± 0.95 %; CTR F: 1.63 ± 1.58 %; SCVS M: 2.19 ± 0.65 %; SCVS F: 1.05 ± 0.89 %) and VGlut2-positive voxel (CTR M: 0.52 ± 0.58 %; CTR F: 3.14 ± 4.92 %; SCVS M: 1.09 ± 0.38 %; SCVS F: 3.94 ± 5.47 %) and 2-way ANOVA on number of colocalized voxels indicated no significant effect of stress, gender or their interaction.

VGLUT1-im puncta densely surrounds the NAc neurons (fig. 14 A). The analysis of VGLUT1-im puncta per μm^2 showed a significant effect of the interaction between stress and gender ($F(1, 17) = 6.133$, $p = 0.0241$) and Bonferroni post hoc indicated a significant decrease of VGLUT1 puncta only in female mice that underwent subchronic variable stress ($t = 3.086$, $p = 0.0402$), with respect to control females; no difference were found between male and female controls, and between male stressed and unstressed animals (fig. 14 B).

VGLUT2-im terminals discretely innervate the NAc (fig. 15 A). 2-way ANOVA analysis performed on the puncta density data showed a significant effect of stress ($F(1, 17) = 7.762$; $p = 0.0127$) and gender ($F(1, 17) = 11.46$; $p = 0.0035$). Indeed, I observed that the vGLUT2- im puncta density significantly increased only in NAc of female group subject to subchronic variable stress, compared with that of the female control group ($t = 3.172$, $p = 0.0335$), and the male unstressed ($t = 4.462$, $p = 0.0021$) and stressed ($t = 3.605$, $p = 0.0131$) groups (Fig. 15 B). I observed significant levels of PSD95 puncta density in NAc (fig. 16 A). Neither male nor female mice displayed altered PSD95 puncta density in the NAc between unstressed and stressed groups (Fig. 16 B). Additionally, I found dense TH-im punta and fibers in the NAc (fig. 17 A). Statistical analysis revealed no differences in TH-im varicosities density in the NAc among the unstressed and stressed groups (Fig. 17 B).

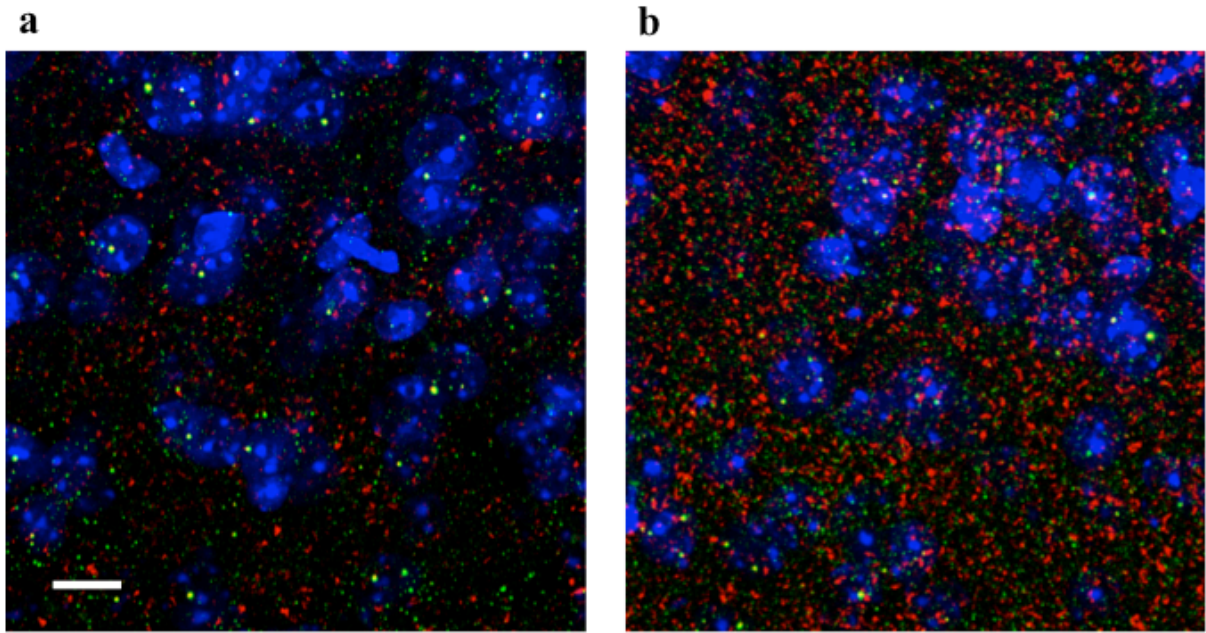


Fig. 12. VGLUT1 (red) and PSD95 (green) puncta in NAc of male (a) and female (b) control mice. 100x, scale bar 10 μ m. Max intensity projections of a 2 μ m z-stack.

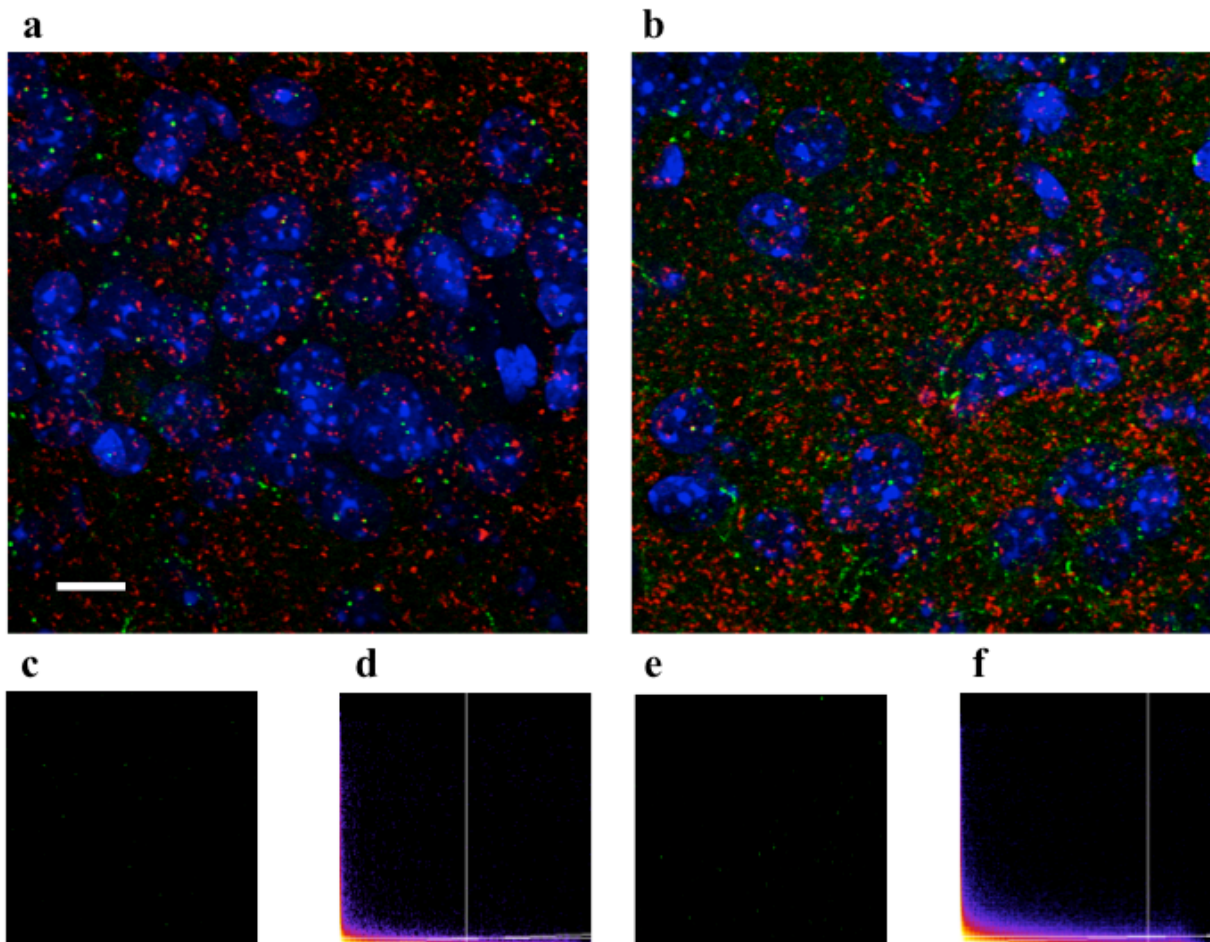


Fig. 13. VGLUT2 puncta (red) and TH puncta and fibers (green) in NAc of male (a) and female (b) control mice. 100x, scale bar 10 μ m. Max intensity projections of a 2 μ m z-stack. Co-localization analysis shows low number of co-localized puncta (c - e) and no correlation (d - f) between VGLUT2- and TH-im.

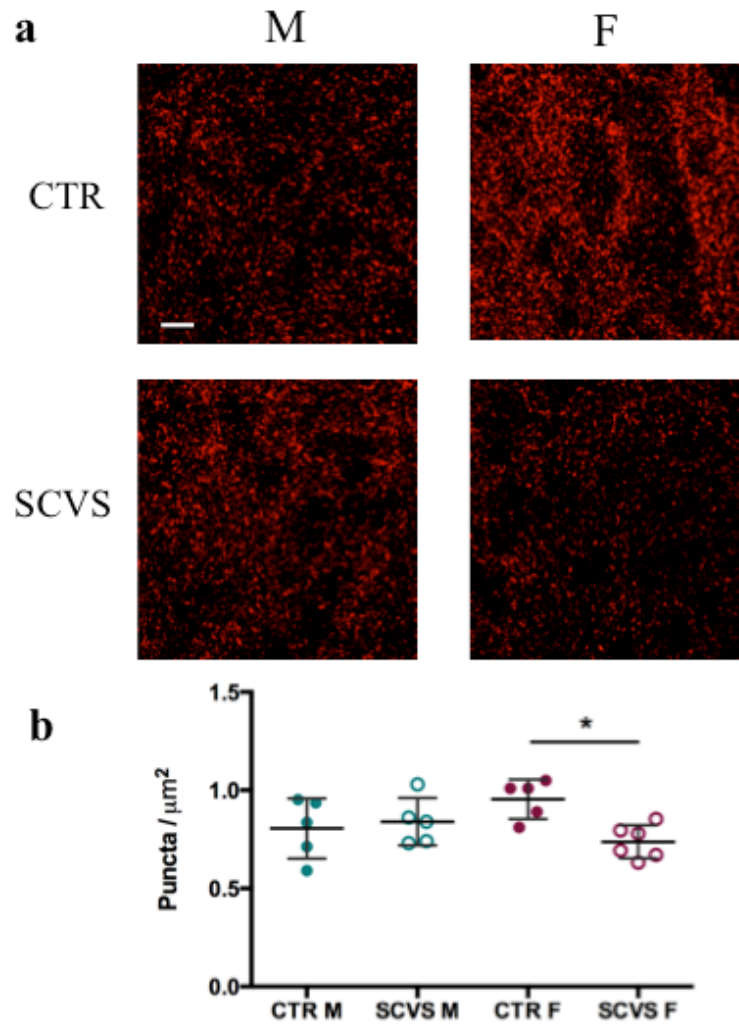


Fig. 14. Effects of SCVS on VGLUT1 puncta in NAc of male and female mice. (a) VGLUT1 puncta in the experimental groups. 100x, scale bar 10 μm . Max intensity projections of a 2 μm z-stack. (b) SCVS decreased VGLUT1 puncta density only in female animals. Values represent the mean of 6 slices. * $p < 0.05$.

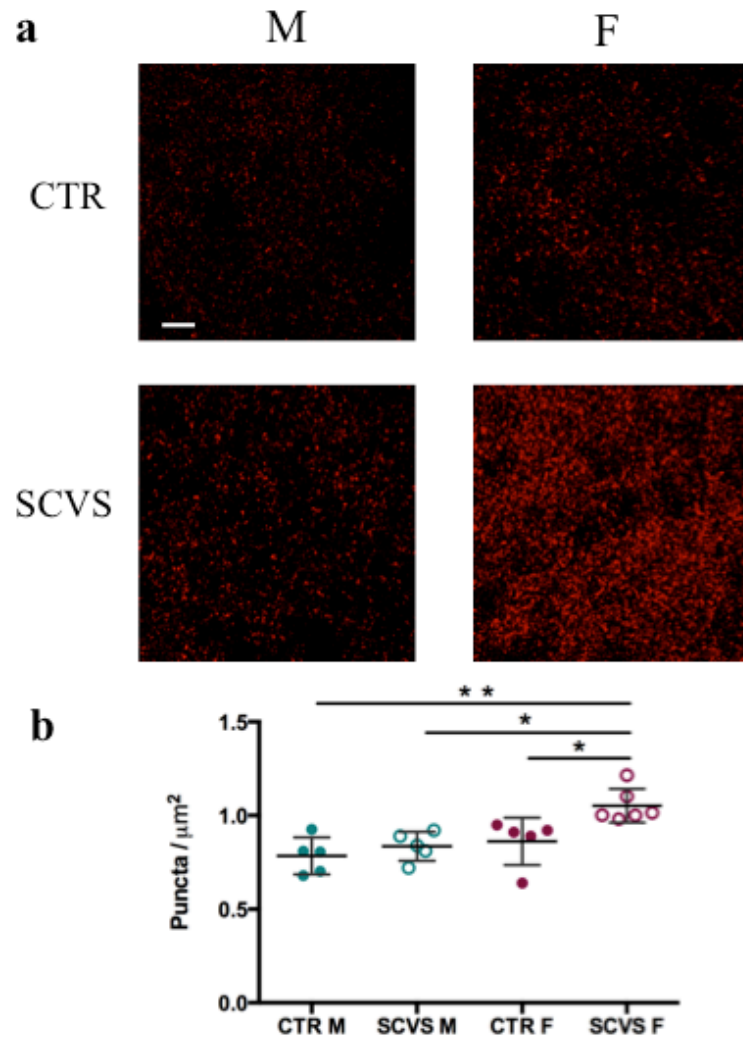


Fig. 15. Effects of SCVS on VGLUT2 puncta in Nac of male and female mice. (a) VGLUT2 puncta in the experimental groups. 100x, scale bar 10 μm . Max intensity projections of a 2 μm z-stack. (b) SCVS increased VGLUT2 puncta density only in female animals. Values represent the mean of 6 slices. * $p < 0.05$, ** $p < 0.01$.

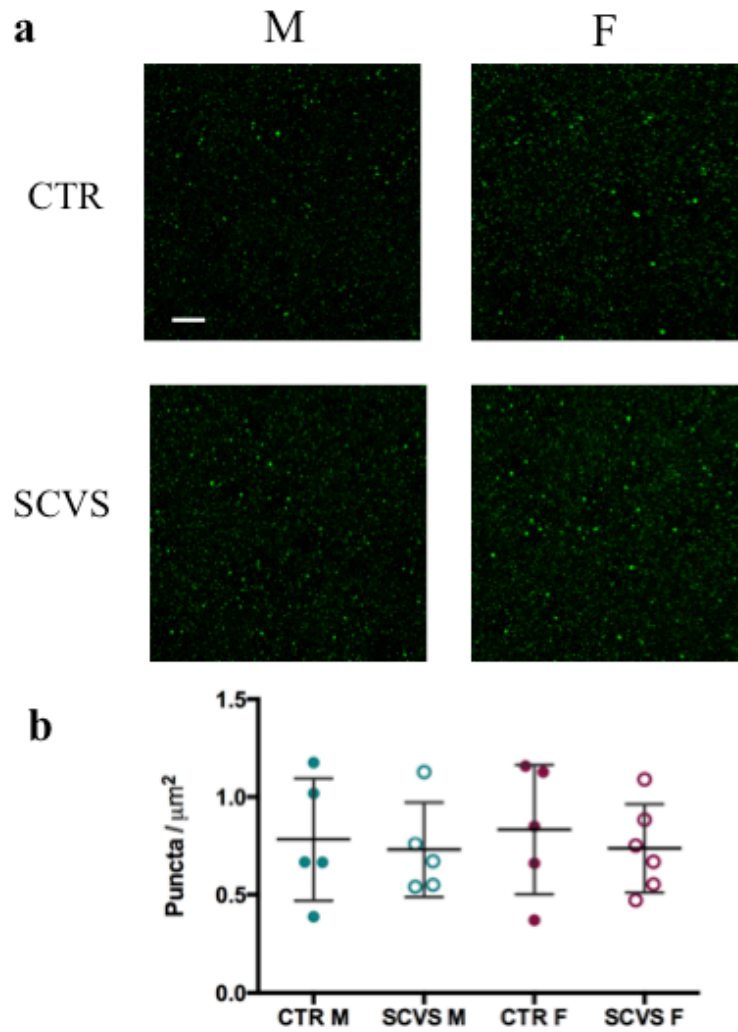


Fig. 16. Effects of SCVS on PSD95 puncta in NAc of male and female mice. (a) PSD95 puncta in the experimental groups. 100x, scale bar 10 μm . Max intensity projections of a 2 μm z-stack. (b) SCVS had no effect on PSD95 puncta density in NAc. Values represent the mean of 6 slices.

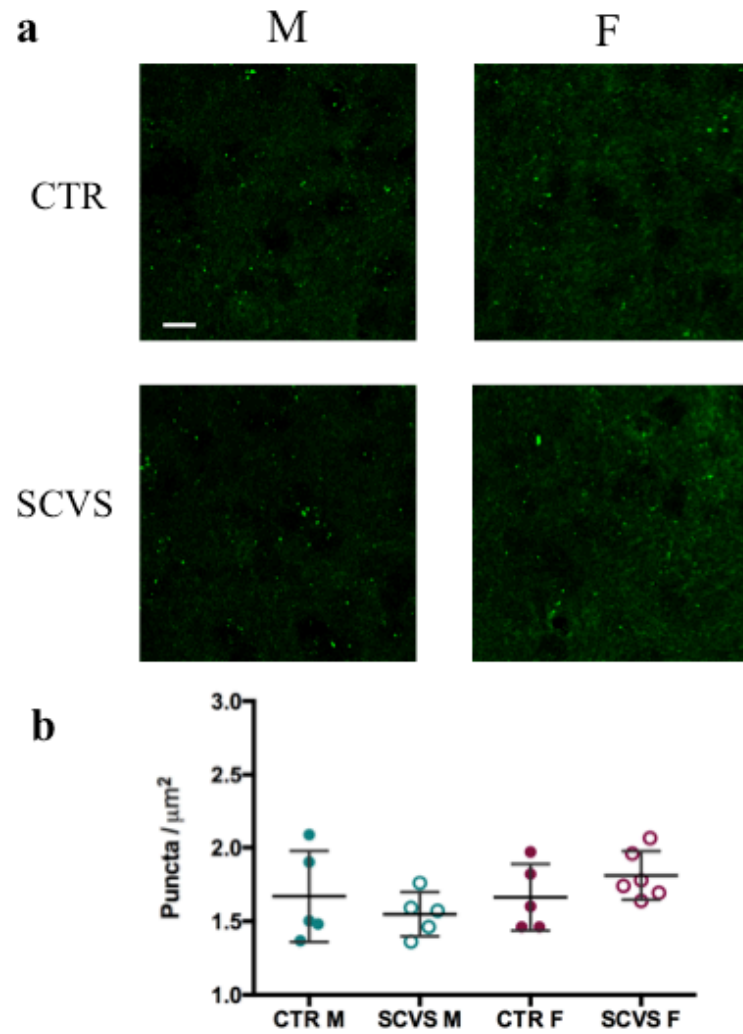


Fig. 17. Effects of SCVS on TH puncta and fibers in NAc of male and female mice. (a) TH immunofluorescence in the experimental groups. 100x, scale bar 10 μm . Max intensity projections of a 2 μm z-stack. (b) SCVS had no effect on TH puncta and fibers in NAc. Values represent the mean of 6 slices.

Experiment 3: Evaluation of the behavioural and functional profile of the thalamic glutamatergic input to the NAc

Locomotor activity

Statistical analysis performed on the data from the locomotor activity test showed that the optogenetic stimulation had no effect on locomotion. In particular, no differences were observed between stimulated and non-stimulated groups, as well as between the first and the second 15 min within the stimulated group, in the total number of beam breaks and in the central beam breaks (fig. 19 A - B - C - D).

Splash test

Data from the splash test showed that neither stimulated nor non-stimulated groups displayed the back grooming behaviour during the first 5 min of test, time window usually employed for the scoring (fig. 20 A). This finding is likely due to the patch cord effect, which probably affected the free moving of the animals in the cage and increased the latency of grooming. However, the analysis of the 10 min period showed no effect of the light stimulation on back grooming duration (fig. 20 B).

Novelty suppressed feeding

Data from the novelty suppressed feeding test showed that the stimulation of the thalamo-striatal pathway had a statistically significant effect on the latency to eat in a novel environment. In particular, two-tailed Student's t test showed a significant increase in the stimulated group, with respect to the non-stimulated one ($t=4.091$, $df=5$, $p=0.0094$). The stimulation induced also an increase in the latency to eat in the home cage ($t=5.385$ $df=5$, $p=0.003$). However, the magnitude of the effect was different. Indeed RM 2-way ANOVA highlighted a significant effect of stimulation ($F(1, 5) = 28.62$, $p = 0.0031$), environment ($F(1, 5) = 58.09$, $p = 0.0006$) and their interaction ($F(1, 5) = 8.848$, $p = 0.0310$). However, Bonferroni post hoc test indicated significant differences only between non-stimulated and stimulated groups in the novel environment ($t= 5.718$; $p=0.0004$) (fig. 21).

Elevated plus maze

Statistical analysis performed on the data from the elevated plus maze test showed that the optogenetic stimulation had no effect on open arm preference. In particular, no differences were observed between stimulated and non-stimulated groups, in the time spent and the number of entries on the open arms, as well as in the time spent and number of entries on the closed arms (fig. 22).

Sucrose preference

Data on the sucrose preference test revealed no sustained effect of the optogenetic stimulation on the sucrose preference. However, the data showed a trend towards a reduction of the sucrose solution consumption in the stimulated group (fig. 23).

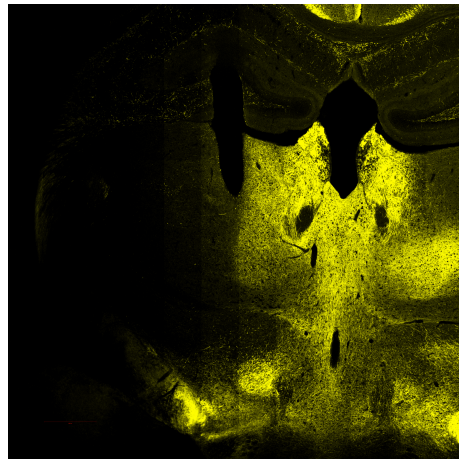


Fig. 18. Fiber placement in the intralaminar thalamus.

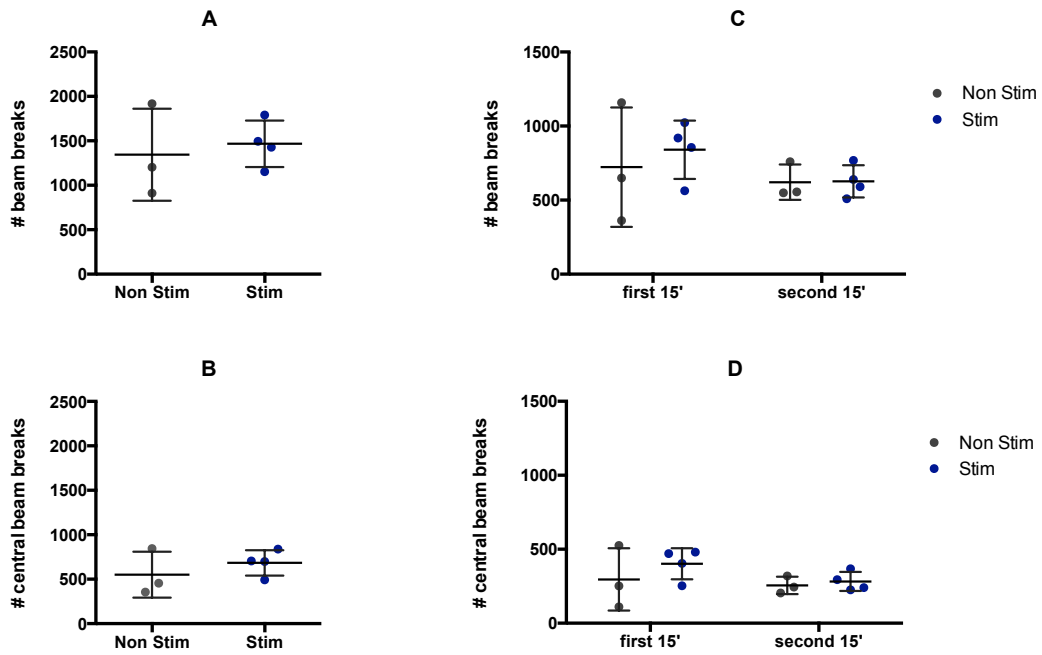


Fig. 19. Locomotor activity in non stimulated and stimulated animals. (A) Total number of beam breaks. (B) Number of beam breaks in the first and second 15 min. (C) Total number of central beam breaks. (D) Number of central beam breaks in the first and second 15 min.

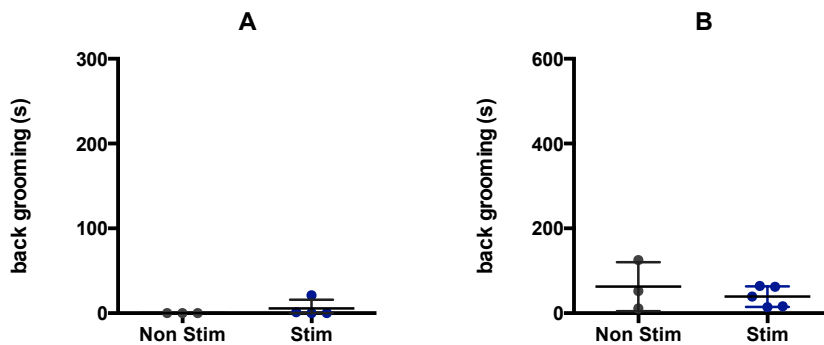


Fig. 20. Splash test in non stimulated and stimulated animals (A) during the first first 5 min and (B) during a 10 min-observation.

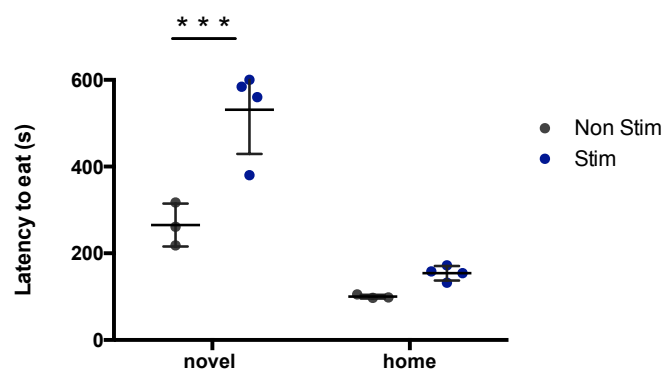


Fig. 21. Novelty suppressed feeding in non stimulated and stimulated animals.

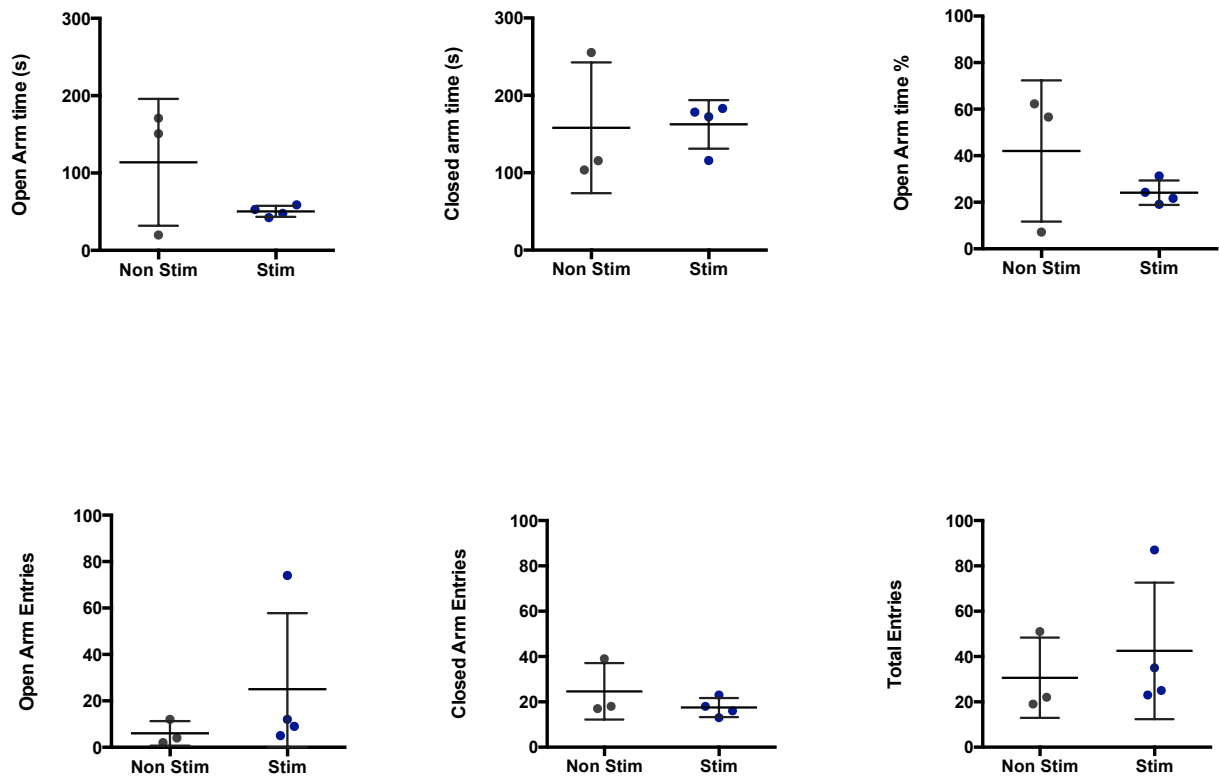


Fig. 22. Elevated Plus Maze test in non stimulated and stimulated animals

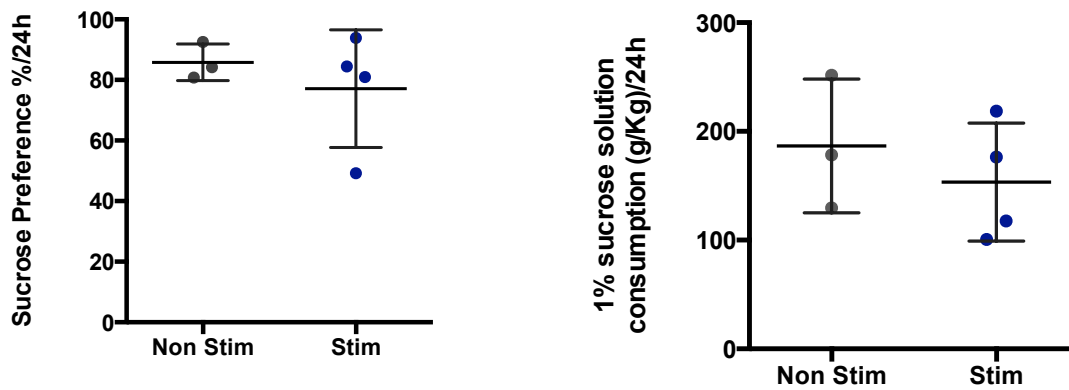


Fig. 23. Sucrose preference test along 24 h after the stimulation.

c-Fos immunofluorescence in the NAc

Data on the functional activation of the medium spiny neurons due to the optogenetic stimulation of the thalamic glutamatergic input showed an extensive c-fos immunofluorescence in the stimulated group in both td tomato-positive and negative medium spiny neurons, as well as in the other uncharacterized cell types. No significant differences were observed. Non-stimulated controls displayed rare c-fos immunofluorescence (fig. 24).

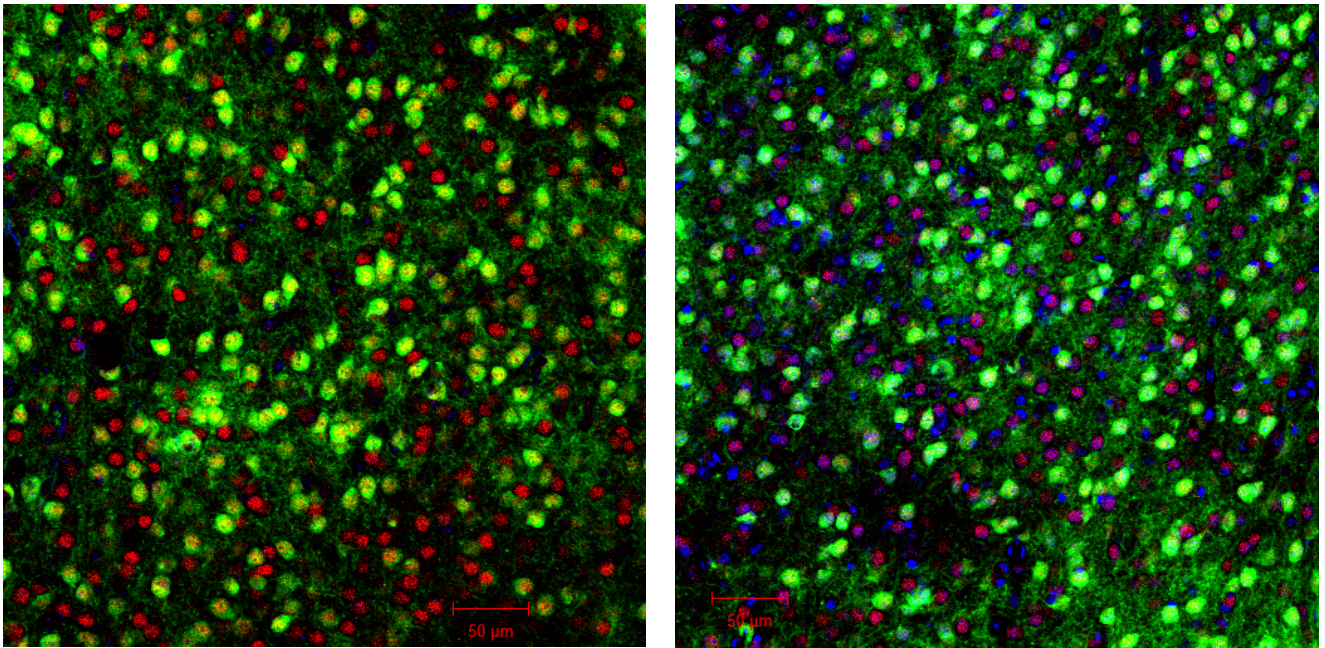


Fig. 24. Non stimulated (left) and stimulated (right) c-Fos (blue) immunofluorescence in MSNs (red) in NAc of D1-td tomato animals (green).

Discussion

Different alcohol drinking patterns induced emotional and affective deficits relevant to depression in female rats

The present study aimed at evaluating the effect of two different models of chronic alcohol consumption, the continuous and the intermittent two-bottle choice paradigms, in female rats, observing pre-gestational-, gestational- and post-partum alcohol drinking trajectories. The consequences of the long-term alcohol intake on emotional and affective processing, including anxiety-like behaviour, behavioural response to novelty, fear and natural rewards and depression-like behaviour were explored, in order to provide an overall snapshot of the effects of alcohol free-consumption during discrete stages of female life and on particular behavioural categories that specifically apply to female, such as maternal care.

The rationale of this study derives from recent reports showing a clear link between alcohol abuse and affective and stress-related disorders, such as anxiety and depression, in women. Binge drinking is increasingly common among women and cross-sectional research suggests that depressive symptoms and heavy drinking are related. Nonetheless, surprisingly little is known about whether depressive symptoms are antecedent of binge drinking, a consequence of binge drinking, or both. Moreover, young women often continue their usual pattern of alcohol consumption into the early weeks of an unplanned pregnancy, and afterwards, thus exposing the foetus to alcohol teratogenicity.

The results show that the continuous- and intermittent alcohol drinking paradigms are associated to differences in the drinking trajectories of female rats, both in pre-gestational time and during pregnancy or lactation; moreover, long-lasting alcohol intake has relevant consequences on affective processing and affect discrete categories of maternal care in a pattern-related manner.

The first evidence of this study regards the occurrence of an escalation in alcohol intake in female virgin rats exposed to the “intermittent access” protocol, from 4.5 g/kg/24 h in the first week to 8-9 g/kg/24h afterwards. The amount of alcohol daily consumed did not display significant variations along the 12 weeks of the pre-gestational drinking sessions, and was significantly higher than that consumed by rats exposed to the continuous pattern of access. This result is consistent with other reports on male rats (Stuber et al., 2008; Hopf et al., 2010) and on Sardinian alcohol preferring female rats (Loi et al., 2014). This data further indicate that the intermittent pattern of alcohol consumption can produce higher alcohol intake than standard continuous access condition, even when the latter includes choice to high alcohol concentration. This evidence confirms the intermittent access induces voluntary consumption of high amounts of alcohol and can reliably

model alcohol abuse and binge-like drinking behaviour in female rats [20]. Indeed, rats and mice studied under continuous access conditions drink in sporadic bouts and they generally do not drink enough to attain pharmacologically relevant blood alcohol concentrations. Blood alcohol concentrations were not measured in this study, but IAR average alcohol intake after the first hour of access is consistent with what Loi and colleagues reported to be sufficient for obtaining intoxicating blood alcohol levels in female rats (2014). The escalation in alcohol intake accounts to loss of control over drinking and seems to be associated to the enhancement in the reinforcing and activating effects of alcohol on the mesolimbic system. In the context of alcohol-related behaviours, I also examined drinking levels during the first hour of alcohol access, as it has been observed that animals exhibit a period of significant drinking or "loading up" during the initial period of alcohol access within a given self administration session (Stuber et al., 2008). The preference for alcohol during the initial hour of access is significantly higher in the IAR group, with respect to CAR, during most of the weeks of access. This finding can be easily related to alcohol deprivation effect, the marked increase in alcohol intake that occurs after forced abstinence periods (Sinclair, 1972). An increased preference for alcohol in IAR group is observed even at the end of the 24 h-session: rats subjected to intermittent access schedule displayed preference for alcohol that exceeded 50% starting from the third week of paradigm, while the group continuously exposed to the alcoholic solution did not develop a higher preference for alcohol, with respect to water, except during the last two weeks of paradigm. A clear preference for alcohol, together with excessive alcohol intake in IAR group, are suggestive for the development of increased salience of alcohol and loss of control over drinking (Robinson and Berridge, 1993). Six-week exposure to voluntary intermittent alcohol intake has been reported to significantly alter glutamatergic input to the ventral tegmental area (VTA), promoting an increase in firing rate of dopaminergic neurons, which may enhance the reinforcing and activating effects of alcohol (Stuber et al., 2008).

After fecundation, alcohol drinking trajectories were evidently modified in that continuous free-access group increased its daily alcohol intake, while intermittent free-access rats decreased their daily consumption. These data are in agreement with previous findings on general female population showing that the reproductive states are able to modulate alcohol intake and preference (Little et al., 1996).

The decrease in gestational alcohol consumption recorded in IAR rats has also been observed in pregnant hamsters and monkeys exposed to alcohol (Means and Goy, 1972; Randall et al., 1980; Forger and Morin, 1982; Carver et al., 1953; Elton and Wilson, 1977) suggesting that intermittent alcohol access allows the onset of maternal adaptive and protective mechanisms for the foetus.

Opposite to IAR, CAR displayed an enhancement in their alcohol intake with respect to the baseline

levels: in these animals chronic continuous alcohol access might be responsible for the induction of neuroadaptive modifications of the neural systems in terms of receptor sensitivity and behavioural tolerance that sustained and promoted alcohol drinking during pregnancy.

Post-partum days are characterized by physiological reduction in the dopaminergic tone, as lactation begins, and notably, alcohol intake in IAR was increased to baseline levels, reproducing the same trajectories as in the pre-gestational period. Also CAR's drinking levels returned to the basal values. Again, the physiological responses of rodents to alcohol are similar to the human ones (Laborde and Mair, 2012) and the neuro-behavioural outcomes of perinatal alcohol exposure have been fairly consistent with clinical and behavioural outcomes in human studies showing that a significant proportion of new mothers resume alcohol consumption and even engage in binge drinking within a year post-delivery (Jagodzinski and Fleming, 2007).

The pattern in which alcohol is consumed has important physiological consequences, and may represent a further vulnerability factor driving alcohol abuse in female subjects, which have higher sensitivity to rewarding effect of alcohol (Torres et al., 2014; Fattore et al., 2014).

Dysfunctions of glutamatergic neurotransmission have been implicated in virtually all alcohol-related behaviours. A recent meta-analysis of rodent microdialysis studies confirmed elevated extracellular concentrations of glutamate in several brain regions and found that these increases, particularly within the nucleus accumbens, strongly correlated with the severity of alcohol withdrawal (Fliegel et al., 2013). The Nucleus accumbens is a key region for affective and motivational processing, involved in mood disorders such as depression. With this in mind, I investigated the consequences of continuous or intermittent chronic alcohol consumption on the affective processing of female rats during acute withdrawal. Animals underwent a sequential behavioural assessment following 24-48 h of alcohol deprivation.

When tested for behavioural reactivity in a novel environment, after 24 h of alcohol deprivation, IAR and CAR groups did not differ from CTR for locomotor activity, while a significant reduction in the exploration of the central area of the open field was observed in CAR, both in terms of number of transitions and time spent on. The open field test is widely used to assess general locomotor activity in a novel environment. As such, it can also yield some information on the rat's emotionality, i.e. avoidance of the exposed central area.

Thus, our findings can be related to altered emotional state, and it is consistent with the increase in anxiety-like behaviour assessed in the elevated plus maze. Indeed, CAR showed lower open arm preference with respect to CTR and IAR, along with no differences in closed arm entries, an important index of locomotor activity in the EPM.

The elevated plus maze test is the most widely used non-conditioned animal model of anxiety,

extensively validated pharmacologically as well as ethologically. However, the animal performance is based on the "endogenous tone" of the animal. Thus I also explored fear-potentiated plus maze performance, which reflects animal's behaviour in an enhanced anxiety state (Korte et al., 1995). In particular, animals had to cope with an environmental challenge, in that they underwent EPM testing after being re-exposed to the compartment where they had experienced an inescapable foot shock 24 h earlier. Thus, animals acquire, process and store information about the emotional significance of the situation. The results show that the drinking pattern is responsible for opposite behavioural alterations when it comes to coping with fear. CAR displayed decreased open arm preference and increased closed arm entries, although not statistically significant, with respect to CTR, thus enhanced anxiety. On the other hand, IAR showed increased preference for the open arm with respect to both CTR and CAR groups, suggestive of impairment in fear processing.

Additionally, I examined the behavioural response to novelty in the novel object test. Novel objects in a familiar environment naturally attract rats, as well as other different species. Moreover, rats recognize an already known object, and spent less time on exploring it, when a new one is presented. Alcohol-drinking rats spent less amount of time visiting the two identical objects, with respect to CTR, during the sample phase. In other terms, they showed decreased motivation to novelty-induced exploration. Furthermore, both alcohol-drinking groups displayed a RI < 50% during the test phase, indicating a deficit in novelty detection. In novelty approach, detection, attention, and motivation processes are involved. Reduction in novelty exploration and increased environmental habituation have been correlated to decreased motivational and rewarding effect of novelty (Fukushiro and Frussa-Filho, 2011).

Accordingly, the saccharin preference test highlighted decreased saccharin preference in both alcohol drinking group, although it was significantly lower in IAR. Dysfunctions of the mesolimbic pathway has been widely proposed as a common neurobiological correlate of withdrawal-related anhedonia (Hatzigiakoumis et al., 2011). However, IAR showed a prominent drop in interest- or pleasure- into naturally rewarding stimuli, that suggests profound alterations in NAc neurotransmissions. Ultimately, animals were tested in the Porsolt forced swim test to evaluate their behavioural coping strategies in an inescapable stressful condition. The results show that both alcohol-drinking groups displayed passive coping and depressive-like behaviour, since they showed longer immobility and lower swimming time than CTR. Nevertheless, CAR displayed increased climbing with respect to both CTR and IAR. Climbing behaviour is associated with increased catecholaminergic neurotransmission (Cryan et al., 2005) and may account for hyper-reactivity to stressful stimuli and prolonged latency to cope with the stressful situation of the inescapable water tank.

Among others, the mother-infant interaction represents a natural reward with a pivotal adaptive value. In clinical studies, analysis of the specific effects of alcohol consumption during pregnancy on maternal care is complicated, due to the difficulty in controlling for confounding factors, such as pattern, dose, and timing of maternal alcohol consumption; alcohol consumption prior to, during and following pregnancy; poly-drug use; maternal age; maternal mental health history; and socioeconomic status.

Conversely maternal behaviour has been studied intensively in the animal model and in the rat in particular (Fleming et al., 1999; Numan and Sheehan, 1997; Meaney, 2001), contributing significantly to the exploration of this field of research. Maternal care in the rat is the result of a complex interplay of hormonal milieu of the dam and the behaviour of both the pup and dam. Studies on the consequences of alcohol consumption during pregnancy on maternal care in the animal model have reported inconsistent results. In some studies alcohol intake during pregnancy did not alter maternal behaviour (Ewart and Cutler, 1979; Abel, 1978) in some others pup retrieval was delayed or reduced (Ness and Franchina, 1990) and the combined exposure to alcohol and nicotine produced increased time away from pups (McMurray et al., 2008).

Evidence from this study shows that chronic alcohol consumption, either continuous and intermittent, yielded significant disruptions in nursing and overall maternal behaviour together with higher frequency of dam self-care and other behaviours throughout the postpartum period. Notably, alcohol consumption in the free-access paradigm during pregnancy and lactation did not change the frequency of retrieval, pup licking and grooming compared with that of naive dams, as reported in other studies (Pepino et al., 2002; Pueta et al., 2008). Deficits in maternal behaviour of alcohol-treated dams could manifest from changes in pups' behaviour toward the mother. Rat pups prenatally exposed to alcohol have a longer latency to nipple attach and reduced ultrasonic vocalizations (Anandam et al., 1980), which could limit alcohol-exposed pups' ability to elicit the same levels of maternal care as controls. Interestingly, the intermittent access to alcohol produced a greater reduction in frequency of nursing, and time spent in the nest with the offspring than the continuous access. At the same time IAR showed an higher frequency of non-maternal behaviours, with a significant increase in dam self-care, that resulted in more time on self-grooming, eating and drinking, than CAR.

Taken together, these data show that chronic alcohol consumption affect affective processing of female rats. In particular, chronic continuous consumption induced a broad spectrum of behavioural deficits, including anxiety-like behaviour. Chronic intermittent access, which models binge drinking, robustly affected hedonic and affective processing of female rats, pointing to prominent dysfunctions of the Nac.

Rewired glutamatergic inputs to the nucleus accumbens are gender-specific neurobiological correlates of females susceptibility to depression

I evaluated the effects of SCVS on glutamate signaling in the NAc of female and male mice. The results showed that SCVS induced synaptic alterations of NAc glutamate neurotransmission only in female animals, which are susceptible to the SCVS paradigm. This data confirm that SCVS is a useful paradigm to study the greater susceptibility to stress and stress-related diseases of females, with respect to males (Marcus et al., 2005; Kessler et al., 1994).

Previous studies demonstrated that male animals are resilient to the 6 day-variable stress procedure, while females display a constellation of signs that mirror depression-like behavior, including passive coping behavior, decreased motivation and low preference for natural rewards (LaPlant et al., 2009; Hodes et al., 2015). Different protocols of chronic unpredictable stress have been successfully employed to study stress-induced depression in male animals, but usually they require several weeks of stress exposure to observe the same behavioral deficits in male animals (Dalla et al., 2010). As a result, the subchronic exposure to variable stressors allows highlighting sex differences in stress susceptibility, which otherwise could not be addressed.

The main finding of this research is that SCVS exerted a substantial rearrangement of glutamatergic inputs to the NAc in female mice only. In particular, the subchronic stress exposure altered the pre-synaptic elements of glutamate signaling in the NAc, without affecting post-synaptic densities and dopaminergic input. Indeed, glutamate signaling takes place between the pre-synaptic neuron, the post-synaptic neuron and the astrocyte, i.e. the tripartite synapse. In the pre-synaptic neuron, glutamate is packaged into synaptic vesicles by the family of VGLUTs. Once released into the synaptic cleft, glutamate may occupy and activate glutamate receptors, located on both neurons and astrocytes. In particular, the post-synaptic density scaffold proteins, including PSD95, in the post-synaptic neuron organize ionotropic glutamate receptors at discrete synaptic locations and regulate the strength of the excitatory synapse.

I found that the levels of VGLUT1 are decreased only in the stressed female group. I did not observed significant baseline differences between males and females, and stress exposure had no effect in male animals. These results are in line with previous reports showing that 6 weeks of chronic mild stress, a protocol able to induce depression-like behavior in male mice, including decreased sucrose consumption and increased immobility in the forced swim test, decreased VGLUT1 protein levels in the hippocampus and frontal cortex (Elizalde et al., 2010). A decrease in VGLUT1 positive puncta, albeit not significant, was also present in the NAc of mice susceptible to chronic social stress (Christoffel et al., 2015). Moreover, mice heterozygous for the VGLUT1 displayed decreased sucrose preference and increased immobility in the forced swim test and they

have been proposed as a genetic model of altered glutamate function linked to depression-like behavior (Elizalde et al., 2010; Balschun et al. 2009). Indeed, VGLUT1 has a key role in glutamate release in the forebrain (Fremeau et al. 2004; Wojcik et al. 2004), and recent clinical studies reported decreased levels of VGLUT1 in the entorhinal cortex of depressed subjects (Uezato et al., 2009).

On the other hand, I found that SCVS significantly increased VGLUT2 levels in the NAc of female mice only. Again, subchronic stress exposure affected the glutamatergic terminals marker in the NAc only in the susceptible group. Indeed I did not observe changes in the male animals, nor baseline differences between males and females.

This data are in agreement with increased VGLUT2 puncta in the NAc of animals susceptible to chronic social stress (Christoffel et al., 2015). In particular, the enhancement of VGLUT2 expression may contribute to increase the pre-synaptic glutamate release and, in turn, to induce post-synaptic dendritic remodeling and spine plasticity which strengthen the synaptic connections of the thalamo-striatal glutamate input. The functional significance, if any, of VGLUT isoforms in determining synaptic plasticity remains to be clarified.

The division of central excitatory neurons into VGLUT1-positive or VGLUT2-positive suggests that these cells might differ in some aspect of the packaging and regulated release of glutamate. For example, the two isoforms could exhibit intrinsic differences in their transport properties such as the speed of transport, with the faster isoform more appropriate for synapses with high firing rates. The expression of VGLUT1 and VGLUT2 mRNA does not, however, correlate with the rates of neuronal firing. VGLUT2 is expressed both at climbing fiber synapses that release glutamate infrequently and in sensory relay pathways that are active at extremely high rates. Indeed, in at least four studies, no consistent difference in the transport activity of VGLUT1 and VGLUT2 has been detected [29,30,32,33]. Rather, the expression of VGLUT isoforms appears to correlate more consistently with the probability of transmitter release, which is generally low at synapses in the hippocampus and parallel fiber synapses in the cerebellum (which use VGLUT1) [45,46] and high at climbing fiber synapses in the cerebellum (which use VGLUT2) [47]. Interestingly, synapses with a low probability of release such as those in the hippocampus have a higher potential for plasticity than those with a high release probability such as climbing fibers. The expression of VGLUT isoform could thus correlate with the potential for synaptic plasticity. At the calyx of Held, more glutamate filling increases the postsynaptic response [48], illustrating the potential for presynaptic mechanisms to alter quantal size. Indeed, several synapses do not saturate with a single vesicle of transmitter.

Clearly, more experiments will be needed to understand if VGLUT expression contributes to the presynaptic regulation of quantal size, and if VGLUT isoforms show differentiating characteristics in short-term plasticity.

In addition, I found no effects of SCVS on the post-synaptic density protein PSD95, a core component of the post-synaptic density scaffold (Nair et al., 2013). PSD95-positive immunofluorescence reflects the total presence of excitatory inputs impinging onto the medium spiny neurons, irrespective of the synapse size or complexity. Taken together, the results suggest that females' susceptibility to SCVS is not associated with altered number of glutamatergic synapses in the NAc. Rather, the data indicate that SCVS induced a substantial rewiring of glutamatergic inputs in susceptible individuals. This finding is in agreement with data showing that chronic unpredictable stress did not alter NAc extracellular glutamate levels in rats (Tata and Yamamoto, 2008). Indeed, medium spiny neurons receive afferents from the hippocampus on proximal dendrites (Meredith et al., 1990), as well as from the amygdala, thalamus, and prefrontal cortex in their more distal arbors (French and Totterdell, 2002, 2003; Moss and Bolam, 2008). Given the substantial segregation between the pre-synaptic excitatory markers VGLUT1 and VGLUT2, I can speculate that stress susceptibility is associated with decreased glutamate input from the prefrontal cortex, basolateral amygdala and hippocampus to the NAc, and increased glutamate input from the thalamus. In the NAc the integration among distinct excitatory input is crucial to shape goal-directed behaviors and it is increasingly recognized that distinct glutamate inputs convey different kinds of information (Tye, 2012).

This concept is exemplified in findings from an elegant study using in vivo dual virus approach and optogenetics that demonstrated that bidirectional modulations of glutamatergic inputs from the intralaminar thalamus to the NAc are both necessary and sufficient to modulate the susceptible phenotype and structural alterations of medium spiny neurons due to chronic social defeat stress (Christoffel et al., 2015). On the other hand, the optogenetic stimulations of the medial prefrontal cortex induced antidepressant effect, and both the activation of the hippocampus and the basolateral amygdala are associated with reward-related behaviors (Covington et al., 2011; Britt et al., 2011; Stuber et al., 2011).

Historically, dopaminergic and glutamatergic projections to the NAc were identified as distinct populations of cells. More recently a subset of glutamatergic neurons has been identified within the ventral tegmental area, whose axons express VGLUT2 and may co-release dopamine and glutamate into the NAc (Tecuapetla et al., 2010; Stuber et al., 2010). Albeit conflicting results have been reported (Been et al., 2013), I performed a VGLUT2-TH double labeling and co-localization analysis in order to obtain further indications on the source of VGLUT2 positive axon terminals in

the NAc. I observed a small co-localization between VGLUT2 puncta and TH-positive fibers. However, we found that SCVS did not affect the number of co-localized VGLUT2-TH puncta nor TH immunoreactivity, i.e. dopaminergic terminals, in the NAc of both sexes. This latter finding confirms previous reports showing no effect of chronic stress on dopaminergic transmission in the NAc of both female and male rats (Dalla et al., 2008). Thus, I can refer the increase in VGLUT2 observed in female mice after SCVS to increased subcortical glutamatergic input, ruling out an effect of SCVS on dopamine projections to the NAc.

In conclusion, the results show that SCVS induce alterations of glutamatergic signaling in the NAc of female animals associated with stress and depression susceptibility.

The rearrangement in levels of pre-synaptic proteins could be related to changes in the strength of distinct glutamatergic input to the NAc and could contribute to the depression-like behavioral deficits induced by SCVS in female mice.

The thalamic glutamatergic input to the NAc controls affective behaviours relevant to depression

In this experiment, I tested the sufficiency of the activation of the glutamatergic input from the intralaminar thalamus to the Nac, for inducing behavioural deficits relevant to depression. The thalamic input represents a relevant subcortical glutamatergic afferent that bears VGLUT2 on its axon terminals in the NAc (Fremeau et al. 2001).

Here I employed a retrograde viral vector, injected in the NAc, and I stimulated the ChR2 expressing neurons in the thalamus, in order to achieve circuit-specificity. Although the experiment represents just a pilot study, since the small number of animals employed, the results are informative of the involvement of the thalamo-striatal pathway in depressive-like behaviour. Indeed, the data show that the optogenetic stimulation during the locomotor activity test had no aspecific effect on locomotion or exploration of the arena. Moreover, I observed no effect of stimulation on the preference for the open arm of the elevated plus maze or on the number of entries. Notably, no differences in closed arm entries and total entries were also observed, confirming the lack of aspecific effect on locomotor activity. When animals were tested in the splash test, in which an ethologically relevant behaviour such as the self-grooming is considered (Kalueff et al., 2015), I failed to observe differences in the grooming duration between the stimulated and non-stimulated groups. For the splash test, animals were sprayed on the back with a sticky 10% sucrose solution, and subsequent grooming behavior was examined; stressed mice groom less than unstressed mice (Surget et al., 2008). Grooming duration in the splash test is primarily modulated by the amygdala-related limbic circuits and reflects the animal affective state. However, stimulated and non-stimulated animals showed very high latency to first back grooming, likely due to the patch cord effect. Indeed, they were tethered to the laser and this negatively affected their free moving.

The stimulation of the thalamo-striatal pathway had a significant effect in the novelty suppressed feeding test. Mice were food deprived overnight and then placed in a novel environment where latency to eat is examined; stimulated animals displayed a longer latency to eat, with respect to non-stimulated ones. This test has been implicated in both anxiety and depression-associated behaviour (Santarelli et al., 2003). Indeed, the performance in this test is sensitive to the modulation of chronic, but not acute, antidepressant treatment (Marcussen et al., 2008).

Moreover, a sustained effect on the response to natural reward was observed. Indeed, the decreased sucrose consumption in the stimulated group was trending, although not significant.

In the attempt to further characterize the cell-specific connectivity of the thalamic axon terminals in the Nac, I performed a c-fos staining in D1-td tomato mice, along with the medium spiny neurons

immunolabeling. The confocal images revealed that the optogenetic stimulation of the intralaminar thalamus induced a wide activation in the Nac, with respect to non-stimulated controls. However, I observe a diffuse c-fos positive staining, including medium spiny neurons and other cell types, with no differences in D1-positive cell activation with respect to D1-negative (i.e. D2) medium spiny neurons. Although a previous report showed a substantial segregation of the thalamic input to the Nac (Papp et al., 2012), the diffuse c-fos expression is likely due to intra-NAc microcircuits activation, since medium spiny neurons send reciprocal collateral projections and afferents to the striatal interneurons. However, the c-fos expression in the stimulated group further validated the circuit-specificity of the optogenetic approach.

Conclusion

The work described above demonstrates a significant link between susceptibility to chronic alcohol-related consequences and depression-like behaviour in female rats.

Chronic alcohol exposure and repeated episodes of withdrawal from alcohol, such as those occurring in chronic intermittent alcohol consumption, are associated with neuroadaptive processes involving glutamate transmission in the NAc.

Notably, a study focusing on presynaptic alterations of glutamate and dopamine terminals in the NAc of alcohol-preferring (P) rats, exposed to continuous and repeated deprivation-schedule of alcohol access, showed that chronic alcohol did not alter the number of TH and VGLUT1-positive terminals in the NAc in either the continuous -and the repeated deprivation-alcohol drinking paradigms. However, the number of VGLUT2-im terminals, was increased in the NAc of the repeated deprivation-alcohol group, compared to the controls. The authors speculated that repeated alcohol deprivation may preferentially increase glutamatergic terminals in the NAc bearing the VGLUT2, which are primarily afferents from the thalamus (

Here I showed that the chronic intermittent alcohol group displayed a more relevant depression-like phenotype, with respect to the chronic continuous alcohol group. Moreover a significant increase in VGLUT2 was present in the NAc of female animals and associated with susceptibility to depression. Ultimately, the optogenetic stimulation of thalamic afferents to the NAc was sufficient for inducing a susceptible phenotype in non-stressed animals.

However, in female animals I observed the rewiring of glutamatergic inputs to the NAc, with decreased cortical excitatory inputs. Although decreased VGLUT1 levels in other brain regions have been associated with depression, this finding can represent a sex-specific glutamatergic alteration. Further studies will be conducted, in order to replicate the findings by Zhou and colleagues (2006) in female animals.

Taken together, these results point to the development of novel compounds targeting the subcortical glutamate projections, namely the VGLUT2, as a novel strategy in the treatment of alcohol and depression comorbidity. Moreover, gender-oriented treatments should be considered of sex-specific alterations of glutamate signaling in the NAc.

Of particular interest will be the development of compounds that exhibit circuit-specific actions and thereby selectively target the thalamo-striatal neurocircuitry.

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