SOCIETÀ ITALIANA DI FARMACOLOGIA

RELAZIONE DI FINE PERIODO

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DIPARTIMENTO: Farmacia e Biotecnologie (FaBiT)

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TIPOLOGIA DI BORSA RICEVUTA: Borsa SIF-MSD 6 mesi

TIPOLOGIA DI RELAZIONE: finale

TITOLO DELLA RELAZIONE: "Epigenetic and molecular correlates in addiction neuroplasticity: innate genetic differences of histone deacetylases (HDACs) and their transcriptional changes evoked by prolonged alcohol exposure in the amygdala of Marchigian Sardinian alcohol preferring (msP) rats"

1. Background

The interaction between genes and environment is responsible for altering developmental trajectories, thus lending vulnerability or resilience to mental disease conditions including addictive behavior [Sinha (2001) Psychopharmacology (Berl.) 158:343-359; Agrawal and Lynskey (2008) Addiction. 103:1069-81]. In this regard, the most reasonable model of alcoholic inheritance involves the interaction of multiple genes and environmental factors, and the hypothesis that such interactions lead to alcohol dependence in genetically susceptible individuals is increasingly ascribed [Bierut et al. (2010) Proc Natl Acad Sci U S A. 107:5082-7; Zollanvari and Alterovitz (2017) BMC Syst Biol.11:19. doi: 10.1186/s12918-017-0403-7].

At molecular level, epigenetic studies provide invaluable insights to elucidate the interaction among genome, environment and addictive drugs [Jaenisch and Bird (2003) Nat Genet 33:245-254]. The presence of enduring effects, even after long periods of abstinence, suggests the likelihood that illicit drugs cause persistent molecular changes in the brain engendered by long-lasting epigenetic,

transcriptional, and translational elements [Robison and Nestler (2011). Nat Rev Neurosci.12:623-37]. Epigenetic mechanisms include DNA methylation and various chemical histone modifications, which remain the most recognized and established epigenetic marks. Various enzymes have been identified as able to modify the chromatin steady-state by adding (writers), removing (erasers) or reading (readers) the so-called epigenetic marks. These reversible chemical modifications on histones define the histone code. In particular, the acetylation process is regulated by the activity of two families of enzymes with antagonistic functions: histone acetyl transferases (HATs) and histone deacetylases (HDACs) which promote gene activation and gene repression, respectively [Kuo and Allis (1998) Bioessays 20:615-26].

HDAC family enzymes have been extensively studied, mainly for their role in cancer but also in alcoholism. HDAC is a family of zinc-dependent enzymes divided into four classes (I, IIa, IIb and IV) that differ in size, tissue distribution and mechanism of action [Gregoretti et al. (2004) J Mol Biol 338:17-31]. Notably, class I HDAC comprises HDAC1, HDAC2, HDAC3 and HDAC8 isoforms. A peculiar involvement of this class of enzymes in alcoholism has been proposed, suggesting that the decrease of HDAC activity in the nuclear compartment maybe critical in the chromatin remodeling effects of EtOH [Zou and Crews (2014) PLoS ONE 9,e87915].

The hypothesis of a relevant role for class I HDACs in EtOH effects is also supported by the observation that the selective class I HDACs inhibitor, MS-275, is able to decrease the motivation to consume EtOH and the relapse (by about 50%), thus suggesting the potential therapeutic interest on these specific isoforms [Jeanblanc et al. (2015) Int J Neuropsychopharmacol 18(9).pii:pyv029].

Taking into account the relevance of HDACs involvement in alcoholism, we aimed at evaluating the innate differences of class I HDACs enzymes between Marchigian Sardinian alcohol-Preferring (msP) rats and Wistar strain controls. To our purpose, the use of genetically selected msP rats represents a valid strategy to improve the understanding of innate behavior in alcohol-dependent subjects. In fact, msP animals show innate EtOH preference together with an anxious phenotype that ameliorates following EtOH intake [Ciccocioppo et al. (2006) Addict Biol 11:339-55].

The analysis of class I HDAC enzymes has been carried out in the amygdala (AM) and also in the bed nucleus of the stria terminalis (BNST), since latest evidence agree in identifying the extended amygdala as the site where addiction and stress interact, sharing common neuronal pathways.

2. Methods

2.1 msP rats: chronic intermittent two-bottle free choice

Adult msP and Wistar rats (~ 500 g b.w.) were housed under 12/12 hours light/dark cycles in condition of temperature and humidity. Behavioral experiments were conducted in the laboratory directed by Prof. Roberto Ciccocioppo. Two groups of animals (6 per group - ***Wistar EtOH and msP EtOH***) were exposed to a chronic intermittent EtOH treatment. Rats received a 10% alcohol solution (v/v) in the two bottles free-choice paradigm; EtOH was available for 24 hours every other day for a total of 30 days. Daily EtOH intake was measured as g/kg at the end of every day in which EtOH was available. Two other groups of rats (6 per group - ***Wistar vehicle and msP vehicle***) instead received water as vehicle. On day 31 animals were sacrificed and the amygdala (AM) and the bed nucleus of stria terminalis (BNST) were rapidly harvested and frozen at -80°.

2.2 RNA extraction and real-time qPCR

Gene expression analysis was carried out in the AM of rats. Total RNA was isolated from rat brain tissue using TriZOL (Life Technologies, Grand Island, NY, USA) followed by RNA purification. RNA integrity was checked by 1 % agarose gel electrophoresis and RNA concentrations were measured. Total RNA was then reverse transcribed using random primers and MuLV reverse transcriptase (Life Technologies, Grand Island, NY, USA).

Quantitative real-time PCR was performed using the SYBR Green master mix.

The following primers were used to amplify the genes of interest: HDAC1 Forward 5'-GATCGGCTAGGTTGCTTCAA-3' and Reverse 5'-CAGCACCGAGCGACATTAC-3'; HDAC2 Forward 5'-GCTGTCCTCGAGCTACTGAAA-3' and Reverse 5'-GTCATCACGCGATCTGTTGT-3'; HDAC3 Forward 5'- ATGCTGAAGAGAGGGGTCCT-3' and Reverse 5'-TCATAGAATTCATTGGGTGCTTC-3'; HDAC8 Forward 5'-TGCCCTGCATAAACAAATGA-3' and Reverse 5'- GATAGGCATCAGTGTGGAAGG-3'; BDNF Forward 5'-AAGTCTGCATTACATTCCTCGA-3' and Reverse 5'- GTTTTCTGAAAGAGGGACAGTTTAT-3'. All data were normalized to the reference gene GAPDH using the ΔΔCt method [Livak and Schmittgen (2001) Methods. 2001 Dec;25(4):402-8]. Results are expressed as mRNA fold changes. Class I HDACs mRNA levels were measured in the AM and in the BNST of Wistar compared to msP rats, in both vehicle and EtOH exposed.

3. Results

3.1 EtOH intake in a two bottles free-choice paradigm

msP and Wistar rats were exposed to the chronic intermittent ethanol (CiE) two bottles free-choice paradigm. As shown in **Fig.1** msP rats consumed a higher amount of EtOH compared to Wistar rats, since the early phase of the task. The CiE protocol produced an escalation in alcohol consumption more remarkable in msP rats compared to wistar animals. The daily EtOH intake remained high until the end of the experimental protocol in both strains.

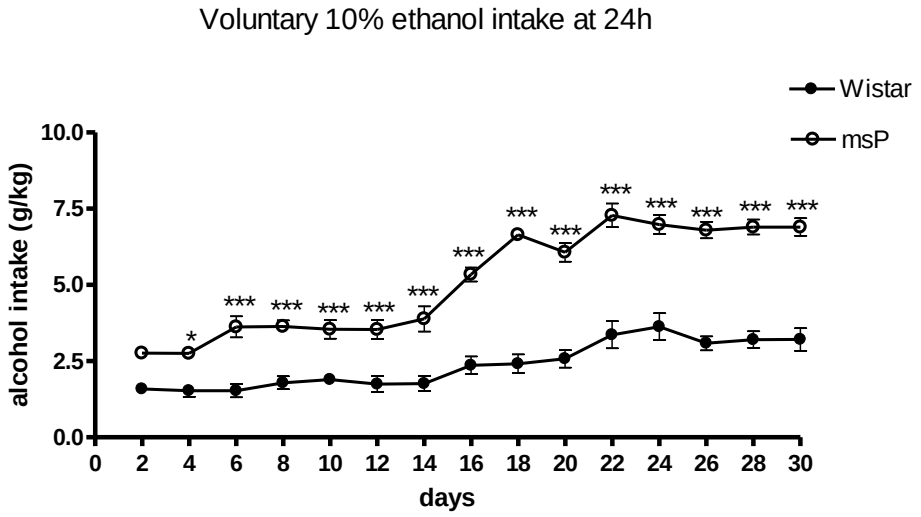

Figure 1. Voluntary CiE intake: Data are presented as mean ± SEM. Differences between groups were analyzed by two-way ANOVA followed by Bonferroni post-hoc test. (* $p < 0.05$; *** $p < 0.001$).

3.2 mRNA levels of class I HDACs in the AM

Significant differences were observed between Wistar vehicle and msP control group in the AM area. The mRNA basal levels of HDAC2, HDAC3 and HDAC8 were significantly lower in the msP vehicle rats compared to Wistar vehicle [**HDAC2**: Wistar vehicle 1.00 ± 0.08 vs msP vehicle 0.70 ± 0.06 $p < 0.01$; **HDAC3:** Wistar vehicle 1.00 ± 0.09 vs msP vehcile 0.56 ± 0.09 $p < 0.01$; **HDAC8:** Wistar vehicle 1.00 ± 0.09 vs msP vehicle 0.47 ± 0.08 $p < 0.001$] (**Fig.2**).

EtOH exposure induced a marked down-regulation in HDAC2, HDAC3 and HDAC8 mRNA levels of Wistar rats only [**HDAC2:** Wistar vehicle 1.00 ± 0.08 vs Wistar EtOH 0.74 ± 0.06 $p < 0.05$; **HDAC3:** Wistar vehicle 1.00 ± 0.09 vs Wistar EtOH 0.66 ± 0.07 $p < 0.05$; **HDAC8:** Wistar vehicle 1.00 ± 0.09 vs Wistar EtOH 0.53 ± 0.05 $p < 0.001$] (**Fig.2**).

Finally, no changes in the class I HDAC mRNA levels were detected in the AM of msP rat following EtOH exposure (**Fig.2**).

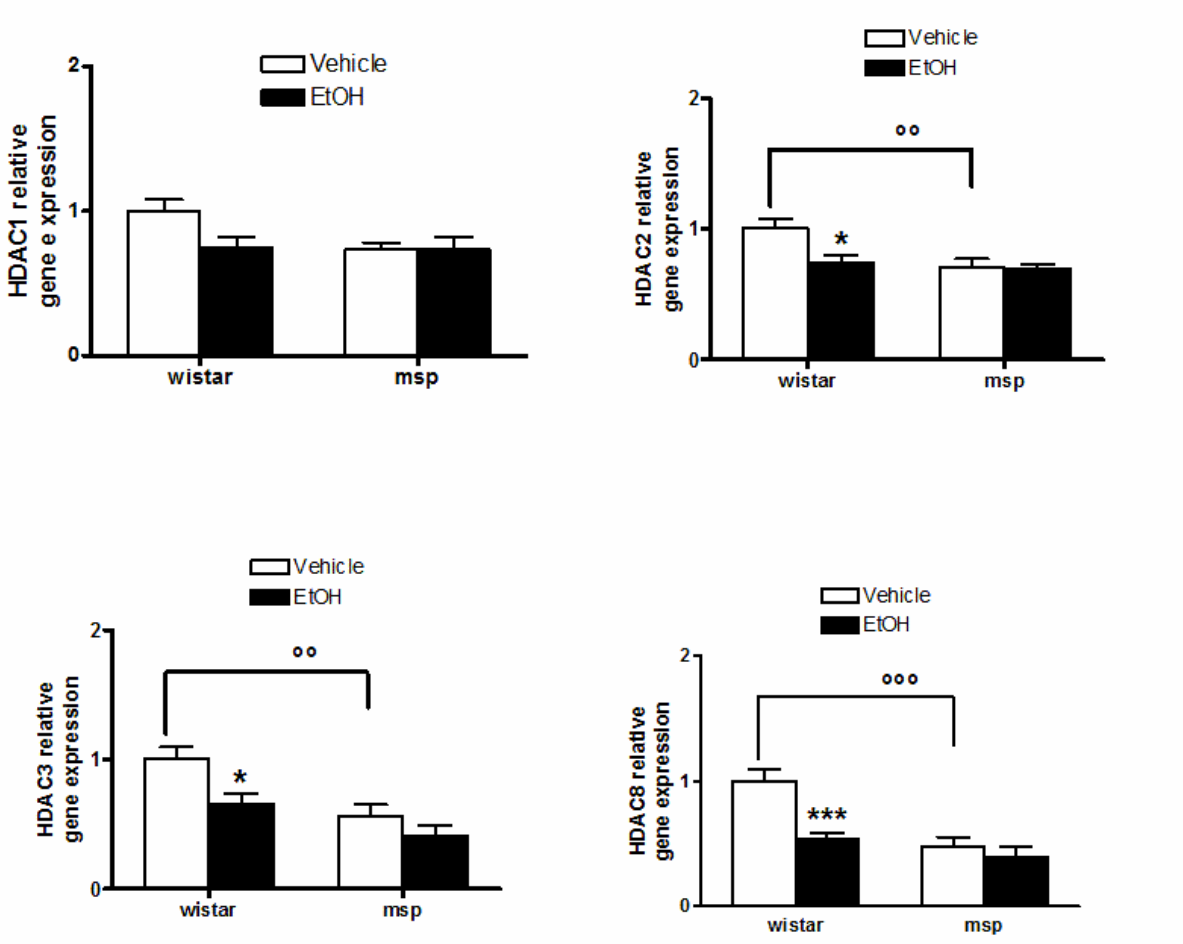

Figure 2. class I HDACs gene expression in the AM. The HDAC1, HDAC2, HDAC3, HDAC8 mRNA levels were assessed in the AM using Real-Time qPCR analysis compared to GAPDH. Data are presented as mean ± SEM (n = 6 rats per group) and analyzed by two-way ANOVA (* $p < 0.05$; *** $p < 0.001$ vs wistar vehicle ; °° $p < 0.01$; °°° $p < 0.001$ vs msP vehicle).

3.3 mRNA levels of class I HDACs in the BNST

Significant differences were observed between Wistar vehicle and msP control group in the BNST area. The mRNA levels of HDAC1 and HDAC2 were significantly higher in the msP vehicle rats compared to Wistar vehicle [**HDAC1:** Wistar vehicle 1.00 ± 0.06 vs msP vehicle 1.96 ± 0.21 $p < 0.001$; **HDAC2**: Wistar vehicle 1.00 ± 0.08 vs msP vehicle 1.56 ± 0.19 $p < 0.01$] (**Fig.3**).

No changes in the mRNA basal levels of HDAC3 and HDAC8 were detected comparing msP to Wistar rats in the BNST.

The EtOH exposure induced a marked up-regulation of HDAC1 gene expression in msP rats only [**HDAC1:** msP vehicle 1.96 ± 0.21 vs msP EtOH 2.55 ± 0.17 $p < 0.05$] (**Fig.3**), since no significant alterations were noticed in the BNST of Wistar rat following EtOH exposure.

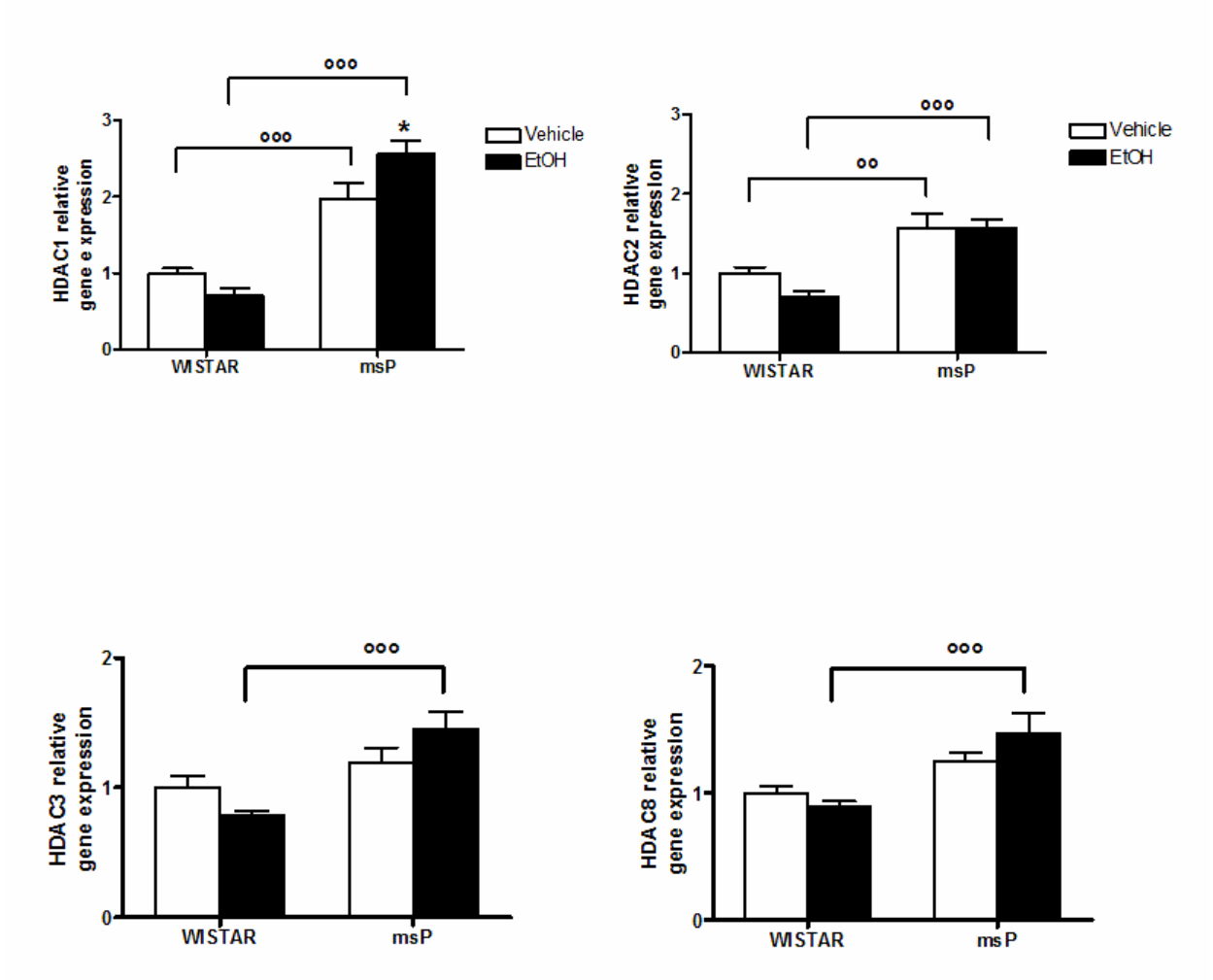

Figure 3. class I HDACs gene expression in the BNST. The HDAC1, HDAC2, HDAC3, HDAC8 mRNA levels were assessed in the BNST using Real-Time qPCR analysis compared to GAPDH. Data are presented as mean ± SEM (n = 6 rats per group) and analyzed by two-way ANOVA (* $p < 0.05$ vs wistar vehicle ; °° $p < 0.01$; °°° $p < 0.001$ vs msP vehicle).

3.4 mRNA levels of BDNF in the AM and in the BNST

The mRNA basal levels of BDNF were significantly lower in the msP vehicle rats compared to Wistar vehicle in the AM [Wistar vehicle 1.00 ± 0.09 vs msP vehicle 0.55 ± 0.12 $p < 0.05$] **(Fig.4a).** In contrast, the BDNF mRNA basal levels in the BNST were significantly higher in the msP rats compared to Wistar [Wistar vehicle 1.00 ± 0.11 vs msP vehicle 1.89± 0.38 $p < 0.01$] **(Fig.4b).**

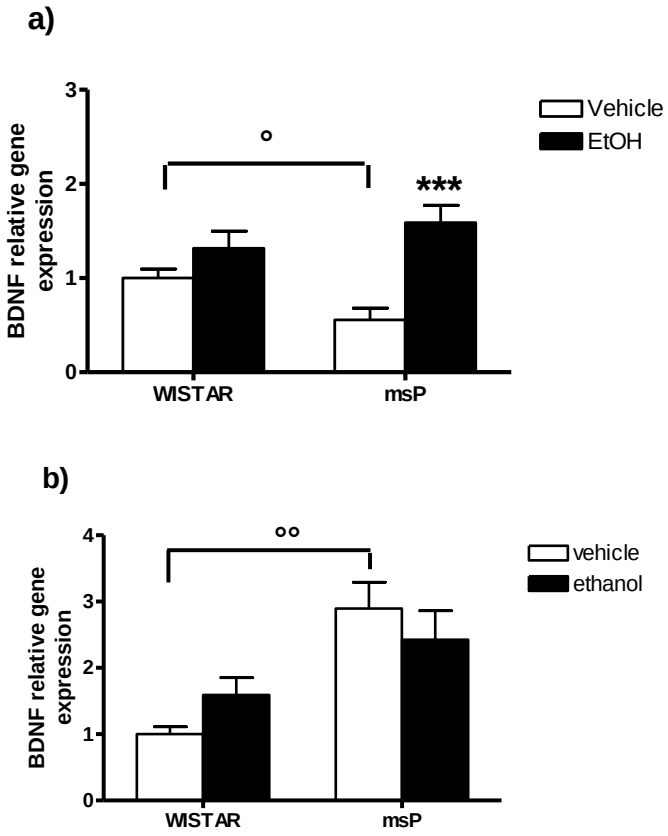

Figure 4. BDNF gene expression in the BNST. The BDNF mRNA levels were assessed in the BNST using Real-Time qPCR analysis compared to GAPDH. Data are presented as mean ± SEM (n = 6 rats per group) and analyzed by two-way ANOVA (*** $p < 0.001$; ° $p < 0.05$; °° $p < 0.01$).

4. Discussion

Similarly to what has been observed for selected neurotransmitters and / or neuromodulators such as dopamine, 5- HT and NPY [Murphy et al. (1987) Pharmacol Biochem Behav. 26:389-92; Ehlers et

al. (1998) Alcohol Clin Exp Res. 22:1778-82], an innate differences in the levels of class I HDACs isoforms between Wistar and msP rats was indicated by RT-PCR analysis. Present data confirm that class I HDACs enzymes displays an important role not only in response to EtOH exposure, but also in the liability to alcoholism. Notably, the detection of lower HDAC levels in the msP compared to Wistar rats is in agreement with our previous results. These last showed that the BDNF heterozygous mice, presenting a genetic predisposition to alcoholism, also display lower levels of class I HDACs in the striatum [Caputi et al. (2015) Drug Alcohol Depend 155:68-75], suggesting that the presence/absence of BDNF allele could be determinant in the modulation of class I HDACs levels. However, it is relevant to note that the basal level of class I HDAC enzymes are lower in the AM but higher in the BNST of msP compared to Wistar rats indicating that these mediators may play specific roles depending on the brain structure. Present findings also suggest that the involvement of the class I HDAC enzymes in the EtOH-induced effects might be dependent on the genotype, since no alterations were observed in in the extended amygdala of msP rats following EtOH exposure.

Since the HDAC1 and HDAC2 core promoter regions contain Sp1 binding sites, recent studies have identified Sp1 protein as a regulator for the class I HDAC enzymes [Yang et al. (2014) FASEB J. 28, 4265–4279]. We previously hypothesized that the reduction of BDNF pathway in the BDNF heterozygous mice could in turn decrease the Sp1 downstream action on HDAC1 and 2 promoters. The absence of one BDNF allele might promote our previous observed reduction of class I HDAC basal levels in the BDNF heterozygous mice [Caputi et al. (2015) Drug Alcohol Depend 155:68-75]. Based on this observation, we also evaluated the BDNF mRNA levels in the AM and BNST of msP rats highlighting that the neurotrophin levels show a similar trend to that of class I HDACs. Indeed, the mRNA basal levels of BDNF were significantly lower in the msP vehicle compared to Wistar vehicle in the AM, whereas they were significantly higher in the BNST corroborating the hypothesis that the correlation between BDNF and class I HDAC levels may play specific roles in alcohol dependent animals depending on the brain structure.

In conclusion, our results contribute to define the peculiar gene expression arrangement of msP rats in which an innate preference for alcohol has been co-segregated with an anxious and depression-like phenotype.