Published in final edited form as:
Addict Biol. 2006 September ; 11(3-4): 339–355. doi:10.1111/j.1369-1600.2006.00032.x.

Genetically selected Marchigian Sardinian alcohol-preferring (msP) rats: an animal model to study the neurobiology of alcoholism

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Abstract

The present article provides an up-to-date review that summarize almost 18 years of research in genetically selected Marchigian Sardinian alcohol-preferring (msP) rats. The results of this work demonstrate that msP rats have natural preference for ethanol characterized by a spontaneous binge-type of drinking leading to pharmacologically significant blood ethanol levels. This rat line is highly vulnerable to relapse and presentation of stimuli predictive of alcohol availability or foot-shock stress can reinstate extinguished drug-seeking up to 8 months from the last alcohol experience. The msP rat is highly sensitive to stress, shows an anxious phenotype and has depressive-like symptoms that recover following ethanol drinking. Interestingly, these animals have an up-regulated corticotrophin releasing factor (CRF) receptor 1 system. From clinical studies we learned that alcoholic patients often drink ethanol in the attempt to self-medicate from negative affective states and to search anxiety relief. We propose that msP rats represent an animal model that largely mimics that human alcoholic population that due to low ability to engage in stress-coping strategies drink ethanol as a tension relief strategy and for self-medication purposes.

INTRODUCTION

Genetically selected Marchigian Sardinian (msP) rats have been selected for their high ethanol preference for about 18 years starting from the 13th generation of Sardinian alcohol-preferring (sP) rats originally developed at the Department of Neuroscience, University of Cagliari, Italy (see Colombo *et al*. in press). In 1998, after 20 generations of selective breeding, at the Department of Experimental Medicine and Public Health of the University of Camerino, Italy, these animals have been renamed msP (Ciccocioppo *et al*. 1998). This distinction was made for several reasons: first, when the genetic selection from sP started in Camerino the high alcohol drinking phenotype of the original sP line was only partial. In addition, the two breeding programs were carried out under different husbandry conditions and used slightly different selection criteria. Hence, the genotypic and phenotypic characteristics of sP and msP rats cannot be considered super-imposable. The first publication on the alcohol preferring line breed at the University of Camerino appeared in 1991; since then more than 40 original articles have been published. A large number of them are pharmacological in nature; however, over the years efforts have been made to characterize the behavioral phenotype of msP rats and to provide appropriate validation of this animal model. The present review summarizes all major findings that over the years have been collected using msP rats; the predictive, the face and the construct validity of this

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animal model for human alcoholism is also discussed. Finally, we show here original gene expression data to link some well-documented phenotypic characteristics of msP rats to specific genetic traits.

Altogether, the findings summarized in the present study suggest that msP rats may represent an animal model of genetic predisposition to high ethanol drinking and 'relapse' linked to anxious and depressive-like behavioral phenotype (Ciccocioppo *et al*. 1999a; Hansson *et al*. 2005). High comorbidity between these somatic disorders and alcohol abuse has been clearly identified in a large subset of alcoholic patients (Schuckit & Hesselbrock 1994; Grant *et al*. 2004). In these patients alcohol drinking can be considered as a tension coping strategy and also reflects an attempt to relief from negative mood state associated with anxiety and depression. The msP rats may therefore represent a unique animal model of alcoholism resembling this particular patient subpopulation.

VALIDITY OF ANIMAL MODELS OF ALCOHOLISM

Alcoholism is a chronic relapsing disorder characterized by compulsive drug seeking and use (McLellan *et al*. 2000). Alcohol dependence develops gradually, occurs over the course of years, and requires prolonged and repeated exposure of the brain to significant blood-alcohol levels. As demonstrated by a number of adoption studies the presence of genetic traits provides an important contribution to the development of this pathological condition (Cloninger, Bohman & Sigvardsson 1981; Sigvardsson, Bohman & Cloninger 1996), and recent twin studies estimate the contribution of genetic susceptibility factors to 48–58% (Kendler *et al*. 1997; Prescot & Kendler 1999). Whether genetically encoded vulnerability is present or not, the process of actually developing dependence is influenced by a number of other factors, such as drug availability, environmental conditions, stress (Lê *et al*. 1998; Katner, Magalong & Weiss 1999; Monti *et al*. 1999; Martin-Fardon *et al*. 2000; Rohsenow *et al*. 2000; Ciccocioppo, Angeletti & Weiss 2001). The pathological traits of alcoholism are complex and over the years various theoretical framework have been proposed to explain it. A common consensus has been reached, however, on the concept that alcoholism is polygenic in nature, that exists different typology of patients and that the medication should be optimized according to the patient subgroup treated (Goldman, Oroszi & Ducci 2005; Heilig & Egli 2006).

Translated into preclinical research, all these levels of complexities are such that they cannot be mimicked by univocal experimental protocols or laboratory animal models. Nevertheless, while it is recognized that animal models of alcoholism may not be entirely congruent with the human condition, it should be agreed that there are minimal criteria that must be met for an animal model to be considered valid. Therefore, as discussed for other psychiatric disorders (McKinney & Bunney 1969; Newport, Stowe & Nemeroff 2002; Willner & Mitchell 2002), an animal model must resemble the human condition in several respects: (1) should be sensitive to amelioration or attenuation of the symptoms by treatments effective in humans, and conversely insensitive to those treatments that are inactive in attenuating the human disorder (*predictive validity*); (2) should mimic the fundamental behavioral characteristics of human alcoholism and should be characterized by the same symptoms profile (*face validity*); and (3) the pathology should be triggered by events thought to be important in eliciting the human disorder and should involve similar neurochemical, neurobiological and psychobiological mechanisms (*construct validity*). In the following sections, we summarize the results of our research in msP rats to show that this animal model meets, at least to a large extent, all the three aforementioned criteria.

Predictive validity

In recent years one of the most exciting development in the field of alcoholism treatment is the introduction of effective medications such as naltrexone and acamprosate (Volpicelli *et al*. 1992; Sass *et al*. 1996). These agents proved the feasibility of pharmacological treatment of alcoholism. More recently, other drugs have been tested in humans for their ability to reduce ethanol drinking and relapse. The results of these initial studies showed, for example, that ondansetron, an antagonist of the serotonin 5-HT3-receptor, exerts marked beneficial effects, but did so exclusively in early onset patients (Johnson, Ait-Daoud & Prihoda 2000a, Johnson *et al.* 2000b). Other drugs of interests are those compounds that modulate central GABAergic transmission; among those topiramate, an anti-epileptic drug, and baclofen, a $GABA_B$ receptor agonist, have been proven to have some efficacy in humans (Addolorato *et al*. 2003; Johnson *et al*. 2005). Experiments carried out in genetically selected msP rats demonstrated that these animals are highly sensitive to treatments with at least some of these medications. As shown in Table 1, we have demonstrated that in msP rats naltrexone reduces home cage voluntary ethanol intake both acutely and following subchronic treatment (Perfumi *et al*. 2003; Ciccocioppo *et al*. 2006). Ethanol drinking in these animals was also reduced by administration of baclofen or acamprosate (Table 1). Ondansetron was never tested in these rats; however, administration of MDL72222 another selective 5-HT3 receptor antagonist markedly reduced ethanol intake in these animals (unpublished). All these results provide strong evidence of positive correlation between the efficacy that medications have in reducing ethanol drinking in msP rats and their efficacy in humans.

According to the definition of predictive validity, if a medication is ineffective in humans it should also be inactive in attenuating ethanol drinking in animals. For example the selective serotonin 5-HT2 receptor antagonist ritanserin was shown to be ineffective in controlling ethanol drinking in alcoholic patients (Johnson *et al*. 1996). Consistent with this finding, few years before the first clinical evidence of the lack of the effect of ritanserin in humans a study was published from our laboratory showing that msP rats (at that time named sP) were insensitive to manipulation of the 5-HT2 receptor system by ritanserin (Table 1). In this case, the predictive value of msP rats resulted in higher than that of other animal models because, contrary to msP rats, blockade of 5-HT2 receptors with selective antagonists resulted in inhibition of ethanol drinking in non-selected Wistar rats trained to drink a low (3%) ethanol concentration (Panocka *et al*. 1996), as well in high ethanol drinking rats like the Fawn Hooded, the Finnish AA or the Indiana P rats (Overstreet *et al*. 1997; Roberts *et al*. 1998). Of interest is the unusual case of the Selective Serotonin Reuptake Inhibitors (SSRI). In preclinical research, these drugs showed efficacy in almost all experimental animal models used to investigate their effect on alcohol drinking, including msP rats (Murphy *et al*. 1985; Ciccocioppo *et al*. 1997b; Maurel, De Vry & Schreiber 1999; Rezvani *et al*. 2000). In addition, reinstatement studies demonstrated that fluoxetine reduces also stress-induced relapse in rodents (Lê *et al*. 1999). Contrary to what animal research predicted, treatment with this class of compounds showed very little, if any, efficacy in humans (Garbut *et al*. 1999; Nunes & Levin 2004). Moderate, positive effects on ethanol drinking and on other ethanol-related behaviors were reported only for those patients with a diagnosis of comorbid depression (Nunes & Levin 2004). If we consider that SSRIs markedly inhibit ingestive behavior in general, one could explain this false positive by hypothesizing that the reduction of ethanol drinking in laboratory animals is an epiphenomenon associated to the anorectic effects of these agents. This could be particularly true for genetically selected alcohol-preferring rats because due to their high ethanol consumption (6–8 g/kg day) they retain a considerable amount of calories from alcohol. Hence, their drinking behavior could be highly sensitive to pharmacological manipulation of feeding-related mechanisms. In msP rats an alternative explanation may also be considered. In fact, these animals as well as the original sP line show a higher level of depressive like

behavior in the swimming test; this effect was reversed by repeated intragastric (IG) ethanol administrations or by treatment with the antidepressant drug desipramine (Ciccocioppo *et al*. 1999a). These data suggest that in msP rats ethanol has an antidepressant-like action and this may contribute to their high motivation to drink ethanol for self-medication purposes. This may provide an explanation for which treatment with fluoxetine (Ciccocioppo *et al*. 1997b) or desipramine, removing the depressive-like negative state typical of these animals, may significantly lower their spontaneous ethanol drinking. In other terms msP rats might resemble the population of alcoholics with diagnosis of comorbid depression and that in these animals as in humans fluoxetine could reduce ethanol drinking due to its antidepressant actions.

Face validity

To have face validity, an animal model of alcoholism has to mimic the fundamental behavioral characteristics of human alcoholism and should be characterized by the same symptom profile. In the Diagnostic and Statistical Manual of Mental Disorders – Fourth Edition (DSM-IV), alcohol dependence is defined as a maladaptive pattern of drug use leading to clinically relevant impairment and distress associated with specific phenomena such as drug intoxication, development of tolerance, occurrence of withdrawal, uncontrollable drug seeking, continuous use of the drug despite knowledge of its negative effects. Some of these events are extremely difficult to model in the rat (i.e. drinking despite knowledge of its negative effects); however, behaviors reflecting, at least in part, the human conditions can be described also in rodents.

Studies conducted in msP rats showed that these animals consume pharmacologically relevant daily doses (7–8 g/kg) of ethanol. Alcohol consumption is largely concentrated during the active phase (night) of the light dark cycle during which msP rats drink 80% of their daily alcohol. Drinking is organized in bouts, the largest of which occurs within the first hour after the lights are turned off. The second large drinking episode occurs after 4–5 hours from the first while a third bout is usually registered just before the lights are turned on (Fig. 1). Bouts are characterized by consumption of 8–12 ml of 10% ethanol normally occurring within 30–60 minutes. If the concentration of the ethanol solution is decreased, msP rats compensate for it by drinking larger volumes, but the bout-drinking structure remains similar. Blood alcohol levels (BAL) determined following these drinking bouts average around 70–80 mg/dl but can peak over 100 mg/dl. The msP rats, differently from other animals, do not show spontaneous aversion to alcohol and voluntarily drink large amount of alcohol from the very first day of home cage presentation (Fig. 1). Consistent with this observation in taste reactivity studies aversive reactions to ethanol infused directly into the mouth are almost absent in msP rats (Polidori *et al*. 1998). On the first day of ethanol presentation ethanol intake ranges between 3 and 4 g/kg, almost completely occurs during the 12-hour dark phase, but is not clearly concentrated in bouts yet. Over a week time ethanol intake increases up to 7–8 g/kg and the typical binge drinking leading to high BAL concentration appears (Fig. 1).

Overall these data demonstrate that msP rats seek ethanol and shape their behavior in order to obtain pharmacological effects from the intake of adequate quantities of the substance. This concept is further supported by results of place conditioning studies showing that IG administration of 0.7–1.5 g/kg of alcohol elicits a marked conditioned place preference (CPP) in ethanol-experienced msP rats. Conversely, doses of 0.35 or 2.8 g/kg are not effective. A similar effect is also observed in alcohol naïve msP rats; however, in this case the place preference appears only after administration of 0.7 g/kg (Ciccocioppo *et al*. 1999b).

It is noteworthy that CPP is obtained following IG administration of ethanol concentrations that produced BAL in the range of 45–55 mg/dl following administration of 0.7 g/kg and 110–135 mg/dl following injection of 1.5 g/kg; these doses of alcohol are similar to those obtained following voluntary ethanol consumption in these animals (Fig. 1). The finding that 1.5 g/kg of ethanol induced a CPP in ethanol-experienced, but not in ethanol-naive animals may, in addition, suggests that protracted ethanol experience is associated with the development of tolerance to undesirable effects of high doses of ethanol.

The results of these CPP studies also indicate that msP rats drink ethanol for its post-ingestive rewarding properties and not just for the oral evaluation of the ethanol solution. This is a relevant finding because as shown by Polidori *et al*. (1998) msP rats have a large number of ingestive reactions associated to an intraoral infusion (0.8 ml in 1 minute) of various (10%, 20%, 40% or even 60%) ethanol solutions that suggest an innate positive taste evaluation of ethanol solutions from these animals.

In our studies, we have never observed physical withdrawal symptoms after alcohol is removed from home cages of msP rats. This is not surprising, if we consider that in msP rats the BAL reached following voluntary ethanol intake generally remain below 100 mg/dl, whereas as reported in many research articles, physical symptoms of alcohol withdrawal are evident following intoxication paradigms aimed at reaching BAL of at least 150 mg/dl (Majchrowicz 1975; Roberts, Cole & Koob 1996; Penland *et al*. 2001; Rimondini *et al*. 2002). In humans, however, alcohol withdrawal is also characterized by a number of psychological symptoms that includes agitation, anxiety, depression and dysphoria. Some of these symptoms (i.e. anxiety- and depressive-like signs) can be detected also in laboratory animals, in which they appear after intoxicating doses of alcohol leading to lower BAL compared with those needed to observe physical withdrawal. In one study examining the behavior of msP rats in the forced swimming test it was shown that naïve animals exhibit a longer period of immobility compared with alcohol-experienced msP rats allowed to voluntary drink ethanol for 10 days before the forced swimming test. After 10 days of voluntary 10% ethanol drinking, if alcohol is removed from the home cage for 10 days, immobility score increases again to values similar to that of naïve rats. Voluntary ethanol consumption or IG administration of appropriate doses of alcohol (6.3 g/kg of ethanol given in nine boluses of 0.7 g/kg of ethanol) administered during the 24 hours preceding the swimming test reduced the immobility time (Ciccocioppo *et al*. 1999a). Overall these data show that while ethanol exerts an antidepressant-like action at doses that alcohol-preferring rats voluntary take, an imposed abstinence in alcohol-experienced animals exacerbate depressive-like symptoms (as expected in human abstinent alcoholics).

Another interesting phenomenon that in msP rats is associated to alcohol abstinence is the occurrence of a robust alcohol deprivation effect (ADE). If ethanol-experienced msP rats are withdrawn from ethanol and a period of 10 days is allowed before access to ethanol they show a clear shift toward a higher level of drinking especially during the first hour of access to the alcohol solution (Perfumi *et al*. 2005). The robustness of the ADE in msP rats should be interpreted as the intense motivation of these animals to resume ethanol use following an abstinence period. Alcohol deprivation experiences are recurrent also in human alcoholics during progression of their disease. Like in animals, following abstinence episodes, these individuals often report an increasing urge to drink that normally terminates with an uncontrollable severe alcohol intoxication episode. Owing to these similarities between human and laboratory animals the ADE has been proposed to model some aspects of craving and relapse of alcoholic patients (Boening *et al*. 2001; McBride, Lê & Noronha 2002; Vengeliene *et al*. 2005).

Clinical studies also revealed that conditioning factors and stress may play a major role in facilitating the persistence of addictive behavior and increase relapse in alcohol abuse (Meyer 1996; Koob & Le Moal 1997; O'Brien *et al*. 1998). Conditioning hypotheses are based on observations that relapse is often associated with exposure to ethanol-related environmental stimuli. According to this view, environmental stimuli that have become associated with the subjective actions of ethanol by means of classical conditioning throughout an individual's history of ethanol abuse elicit subjective states that can trigger resumption of drug use. Stress may, instead, result in mood disregulation, disruption of neuroendocrine homeostasis and somatic symptoms such as insomnia that may motivate alcoholic patients to resume drinking to alleviate negative affective states. The msP rats represent an excellent model to reproduce these complex behavioral traits described in human literature. Moreover, like in humans exposure to these vulnerability factors may facilitate relapse even after protracted periods of abstinence (Fig. 2). It has been shown that msP rats trained to operantly self-administer 10% ethanol or water in 30-minute daily session on an FR-1 schedule of reinforcement in the presence of discriminative stimuli (SΔs) associated with the availability of ethanol (S^+) versus water (S^-), following an extinction period resume their lever pressing for ethanol, but not for water-associated cues. Similar behavior was also observed for non-selected Wistar rats; however, remarkable line differences in the magnitude and persistence of the response-reinstating effect of ethanol-associated stimuli can be observed between the two rat lines (Fig. 3c). Specifically, responding for stimuli predictive of alcohol availability on the first reinstatement test is significantly greater in msP than Wistar rats. Moreover, while ethanol-seeking in msP rats shows resistance to extinction over the course of the repeated reinstatement tests, responding progressively decays in Wistar rats (Fig. 3c). The differences in drug-seeking behavior induced by the ethanol-associated stimuli in msP versus Wistar rats closely parallel line differences that are observed in the primary reinforcing effects of ethanol. The msP rats consumed significantly more ethanol than Wistar rats, and ethanol-maintained responding on a schedule of continuous reinforcement during the self-administration training and conditioning phases is significantly greater in msP than in Wistar rats (Fig. 3a). More importantly, the break point for ethanol-reinforced operant responding under progressive ratio schedule is significantly higher in msP rats, indicating that the reinforcing value of ethanol is greater in this alcohol-preferring line compared with non-selected Wistar rats (Fig. 3b). These findings not only confirm that the reinforcing properties of ethanol are increased in rats with a genetic predisposition toward heightened ethanol intake but provide evidence that genetically determined alcohol preference extends to greater responsiveness to the motivating effects of ethanol-associated stimuli. In a recent self-administration study it has been also shown that in an extinction-reinstatement paradigm exposure to intermittent foot-shock stress reinstate lever pressing for ethanol in both msP and Wistar rats. However, msP rats show the highest reinstatement level following administration of 0.3 mA foot-shock current intensity whereas the maximal responses in Wistar animals is observed after exposure to 1.0 mA electric current. At 1.0 mA the locomotor behavior of msP rats was impaired because freezing behavior occurred (Hansson *et al*. 2005). These data suggest that msP rats like Wistar rats show a relapse-like behavior after exposure to stressful stimuli but in msP rats the sensitivity is higher. This reflects the results of several clinical studies that have shown that alcoholic patients have a lower ability to engage in stress-coping strategies and that resumption of alcohol abuse is often a strategy to ameliorate the negative affective state in which they precipitate following exposure to anxiogenic stimuli or stress especially during protracted withdrawal.

Construct validity

An animal model of alcoholism should rely on similar neurochemical, neurobiological and physiological mechanisms and should be sensitive to the same events thought to be

important in eliciting the human disorder in order to have construct validity. Several years of clinical and experimental research have demonstrated that alcoholism is a multifactorial disorder where genetic predisposition associated to environmental factors can contribute to a final level of abuse vulnerability. The fact that genetic selection has led to obtain animal lines (i.e. msP rats) expressing high ethanol drinking phenotype is *per se* an element of construct validity because it shows that, like in humans, vulnerability to abuse ethanol can be inherited. An ideal genetic animal model of alcoholism should carry the same genetic traits that are linked to alcoholism in humans. In recent years a wealth of work has been carried out to understand the genetic basis of alcoholism and a lot of information has been collected. It is now clear that alcoholism is a multigenic disorder and various genetic polymorphisms have been associated to alcohol abuse vulnerability. It is known for example that genes encoding for specific variants of $GABA_A$ receptor (*GABRA2* and *GABRG3*) or muscarinic cholinergic receptor (*CHRM2*) can affect risk for alcohol (Edenberg *et al*. 2004; Wang *et al*. 2004). Polymorphisms at dopamine D2, μ-opioid receptor, and serotonin transporter genes have also been associated with increased vulnerability to develop alcoholism and with a different response to pharmacological interventions (Lawford *et al*. 1995; Oslin *et al*. 2003; Edenberg & Kranzler 2005; Feinn, Nellissery & Kranzler 2005). Finally, gene variants that affect alcohol abuse vulnerability by metabolic mechanisms have been largely described (Chen *et al*. 1999; Whitfield *et al*. 2002).

Recently an extensive gene mapping study, using microarray technology and sequencing analysis, has been undertaken in msP rats aimed at characterizing the genetic traits responsible for the high alcohol drinking phenotype of these animals. The most striking evidence obtained in msP rats is that these animals carry with high correlation two single nucleotide polymorphisms (SNPs) on the promoter region of the gene encoding for the corticotrophin releasing factor 1 (CRF_1) receptor. Combining this finding with the observation that msP rats have a higher expression of CRF_1 receptor mRNA and CRF_1 receptor protein density in various brain regions one may speculate that the gene variant identified in msP rats may be functionally relevant (Hansson *et al*. 2005). This view is further supported by pharmacological data showing that blockade of CRF_1 receptor by antalarmin reduces ethanol self-administration in msP rats but not in non-selected Wistar rats (Hansson *et al*. 2005). Interestingly, in a recent investigation it has been reported that also in humans, polymorphisms at level of the promoter region for the CRF_1 receptor gene are linked to alcohol use disorder. For example, in an adolescent at risk population it was found a significant correlation between two SNPs (Reference SNP IDs-number; rs242938 and rs1876831), binge drinking and lifetime prevalence of drunkenness (Treutlein *et al*. 2006). The same association was found in an independent sample of adult alcohol-dependent patients in which rs1876831 polymorphism was linked to higher level of alcohol drinking (Treutlein *et al*. 2006). Consistent with these clinical observations also msP rats, which seem to carry similar genetic mutations at CRF_1 receptor gene, show a pattern of binge-like drinking (Fig. 1), higher ADE (Perfumi *et al*. 2005), and increased motivation to take ethanol (Fig. 3).

Altogether these findings suggest that msP rats and humans, at least in part, share common genetic predisposing factors to alcoholism. Polymorphisms at CRF_1 receptor gene is one of those, and considering the important function that this system has in the regulation of stress response and mood state, it may be speculated that the high comorbidity between alcohol abuse, anxiety and depression may be linked, at least to some extent, to this polymorphism. Genetically selected msP rats may represent an animal model to mimic a specific alcoholic population in which ethanol abuse is associated to high comorbid anxiety and depression and in which genetic variations at CRF_1 receptor system play an important role.

GENE EXPRESSION PROFILING: COMPARISON BETWEEN MSP AND NON-SELECTED WISTAR RATS

The results reported in Fig. 3 show that, compared with non-selected Wistar rats, msP rats engage in higher rate of ethanol responding under both fixed ratio 1 (FR-1) schedule and progressive ratio (PR) schedule of reinforcement. Whereas in relapse experiments they demonstrate significantly higher vulnerability to resume extinguished ethanol seeking. In addition, in msP rats high ethanol drinking phenotype is associated with elevated emotional reactivity and depressive-like phenotype. These characteristics clearly distinguish this rat line from a non-selected Wistar population from which they were originally derived. To shape the unique phenotype of msP rats environmental influences (i.e. ethanol availability, exposure to stress, etc.) can certainly play an important role. However, genetic background is important to confer vulnerability to these animals that in response to exposure to these environmental predisposing stimuli may engage in the development and maintenance of specific pattern of high ethanol consumption and relapse. Recently, in order to identify the genetic traits subserving these phenotypic differences, we have undertaken an extensive investigation to analyze the gene expression profile of msP rats compared with non-selected Wistar rats. In particular, using Affymetrix technology, we compared the mRNA expression profiles of msP rats and non-selected Wistar rats on the Affymetrix RAE230A chip that was used for the analysis. Among the altered transcripts 216 were down-regulated whereas 392 were up-regulated.

The 608 differentially expressed genes were categorized according to their biological functions and pathways. To this end, we used DAVID (http://apps1.niaid.nih.gov/david/upload.asp) that performs Fisher exact text for enrichment of GO terms and KEGG pathway within groups of regulated genes. The results of the functional annotation using GO term showed 30 distinct statistically significant GO terms associated with gene groups (Table 2, but see also Fig. 4). Among these many terms related to metabolism, including alcohol, lipid, catecholamine and glutamate metabolism were identified. Furthermore, GO categories related to calcium and sodium ion transport, to neuronal functions and synaptic transmission were also identified. Of particular interest was the finding that among the differentially expressed gene pathways the calcium/calmodulin-dependent protein Camk1g and Camk2a kinases resulted differentially expressed. The products of these genes are thought to regulate a variety of neuronal functions. In particular, calcium/calmodulin-dependent protein kinase II, alpha (Camk2a) is one of the most abundant kinases in the brain and plays a critical role in neurotransmission, synaptic plasticity, learning and memory. Previous studies showed, for example, that Camk2a levels were increased in the hippocampus of stressed rats and in anxiety response (Koks *et al*. 2004;Sun *et al*. 2006). We also found differences in expression of gene Ppp3ca (calcineurin A), that is a Ca(2+)/calmodulin-regulated protein phosphatase that like Ca(2+)/calmodulin-dependent serine protein kinase (Cask) plays a role in intracellular Ca(2+)-mediated signaling pathways in the brain takes part to neurotransmission regulation.

We also conducted an analysis to detect the most enriched KEGG pathways (Table 3, but see also Fig. 4). It is noteworthy that differentially expressed genes largely populate the pathway for Mapk cascades showing a possible coordinated different regulation of this pathway in the msP compared with the Wistar line (Fig. 5). Mapk cascades are involved, through extracellular signal-regulated kinase 1 and 2 (Erk1 and Erk2), in cell proliferation and differentiation and in neurons they have a key role in the control of neuronal plasticity. It has been suggested that Mapk signal l transduction pathway is a potential target for ethanol that through this way can influence synaptic plasticity (Roberto *et al*. 2003). In addition, Rimondini *et al*. (2002) showed that repeated cycles of intoxication and withdrawal induces a marked and long-lasting increase in voluntary ethanol intake

associated with an up-regulation of Mapks activity in the cingulate cortex and amygdala of ethanol-treated rats. Based on these data one may speculate that altered regulation of these intracellular kinase cascade in msP rats may be important for the expression of the high ethanol drinking phenotype of these animals. This view is further supported by findings showing that Mapk activities are altered also in the AA rats, as well as in mice lines with different alcohol drinking phenotypes (Arlinde *et al*. 2004;Mulligan *et al*. 2006).

Affymetrix results revealed also other interesting differences between msP and Wistar rats at level of various neurotransmitter system known to play a role in the regulation of alcohol-related behavior. Among these, particularly interesting is the differential expression for genes linked to glutamatergic and GABAergic activities. For example a differential expression was found for the Grm3 that is a metabotropic glutamate receptor involved in glutamate neurotransmission and the solute carrier family 6 (Slc6a1) that is a gamma-aminobutyric acid transporter that in a previous reports was also linked to ethanol sensitivity in mice (Hu *et al*. 2004). Other genes of interest that were found differentially expressed are those for the opioid receptor mu 1 (Oprm1), the opioid receptor-like (Oprl, nociceptin receptor) and the neuropeptide Y receptor 5. Several reports have already established link between these neurotransmitter systems and ethanol abuse (Thorsell *et al*. 1999; Ciccocioppo *et al*. 2000; 2006; Heilig & Thorsell 2002; Schroeder, Overstreet & Hodge 2005).

It is well known that genes affect vulnerability to alcoholism also by pharmacokinetic mechanisms; hence, it is important to point out that in our wide genome scan we also found 11 genes involved in alcohol metabolism that are differentially expressed between msP and Wistar rats (Table 4). The alcohol dehydrogenase 4 (class II), pi polypeptide (adh4) and alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide (adh7) are among them. The alcohol dehydrogenase (ADH) genes are a gene family clustered in a region of chromosome 4q21 in human and chromosome 2q44 in rat. This family of genes encodes enzymes involved in the reversible oxidation of alcohols to aldehydes and they have influence on the risk for alcoholism. For example, polymorphism in the ADH1B human gene can account for a threefold increase in risk for alcohol dependence (Whitfield 1997). Adh4 and Adh7 are both down-regulated in msP strain compared with control Wistar rats. Recent studies showed that the variation in ADH4 gene predisposes to alcoholism (Luo *et al*. 2005;Edenberg *et al*. 2006) and ADH7 enzyme may play a role in the risk for alcoholism (Osier *et al*. 2004).

Moreover, several genes coding for aldehyde dehydrogenase isoforms are differentially expressed between the msP and Wistar (Table 4). More precisely apart from aldehyde dehydrogenase 2 that is down-regulated Aldh1a1, Aldh1a4, Aldh3a2 and Aldh5a1 are up-regulated. The ADH and the aldehyde dehydrogenase enzymes affect the concentration of the acetaldehyde produced during ethanol metabolism. The ADHs produce acetaldehyde from ethanol while the aldehyde dehydrogenases metabolize it. The combination between high activity of ADHs and low activity of aldehyde dehydrogenases bring to a higher steady-state acetaldehyde concentrations after alcohol consumption. This, in turn, produces an aversion to alcohol use. On the contrary a low alcohol-metabolizing ADHs enzyme and an aldehyde dehydrogenase that converts acetaldehyde to ethanol efficiently keep low the aldehyde concentration and, doing so, reduce the aversive effects of ethanol. In addition, low activity ADH may increase alcohol sensitivity. Differences in alcohol metabolism that reduce accumulation of acetaldehyde and increase sensitivity to ethanol have a permissive role that facilitate alcohol drinking (Thomasson *et al*. 1991;Higuchi 1994;Edenberg & Kranzler 2005). Overall gene expression for alcohol and acetaldehyde dehydrogenase systems in msP rats are suggestive of a permissive role of these metabolic pathways toward heightened ethanol drinking (Table 4).

The fact that numerous genes affecting alcohol abuse either by pharmacodynamic or by pharmacokinetic mechanisms have been found differentially expressed is in line with the view that alcoholism is a complex polygenic disorder to which various genetic elements contribute.

THE MSP RAT LINE: LIMITATION AND CAVEATS

As aforementioned a good animal model of alcoholism should incorporate all major criteria listed in the DSM-IV manual for clinical evaluation of alcoholism which is mostly based on interviews and self-report questionnaires to assess quantity, frequency of drinking and the perceived consequences. The obvious impossibility to conduct these type of evaluations in laboratory animals together with the limited significance that physiological measures have for the diagnosis of alcoholism represent a serious limit for the validation of any preclinical model of alcoholism. In effect, the general view is that the perfect animal model of alcoholism does not exist, whereas many different valid models to mimic specific aspects of the human disorders are available. The msP rat is one of those and as any other animal model has several limitations. In addition, various pieces of important information on this rat line are still missing. For example, we have proposed that the msP model has predictive validity because, as in humans, ethanol drinking in these animals is reduced by naltrexone and acamprosate while it is not blocked by ritanserin (Table 1). This is an obvious strength; however, it should be emphasized that in humans naltrexone and acamprosate also reduce relapse rate and naltrexone lowers cue reactivity (Monti *et al*. 1999;Rohsenow *et al*. 2000;Boothby & Doering 2005;Williams 2005). These data are not available in msP rats on which these drugs have not been tested on relapse yet. Moreover, an emerging clinical literature suggests that also other drug treatments seem to be effective in humans (Heilig & Egli 2006). For example the anti-epileptic drug topiramate, the selective 5-HT3 receptor antagonist ondansetron seems to be particularly promising (Johnson 2004;Williams 2005). These drugs still need to be tested in msP rats. On the other hand, one should not expect an animal model to be identically sensitive to all these different treatments. In fact as in humans, also in laboratory animals, being alcohol abuse a multifactorial disorder, it can be triggered by different mechanisms leading to various forms of the disease that not necessarily respond in the same way to all pharmacological manipulations. Another important aspect is that no neurochemical data have been ever published on msP rats, whereas a very limited number of research paper providing some information on the neurochemistry of dopamine, serotonin, acetylcholine and corticotrophin-releasing hormone were published in the progenitor sP line (Fadda *et al*. 1999;Richter *et al*. 2000;De Montis *et al*. 2004). In addition to these limited information, after 40 generations of separate selection of msP and sP rats it is improper to generalize to msP rats the information obtained in the original sP line and vice versa. In future experiments it will be important to fill this gap by running a systematic neurochemical analysis of msP rats, at least for all major neurotransmitter systems involved in the regulation of ethanol-related behaviors.

Another intrinsic limit of msP rats is that they have been selected from the sP line but at that time, the breeding and parallel selection of the non-preferring sNP line was not undertaken. The msP line therefore does not have the non-preferring parental control line. This can limit the possibility to carry out genetic investigations in these animals.

CONCLUSIONS

The results of the studies on msP rats demonstrate that these animals may represent a preclinical model of alcoholism endowed with significant predictive validity and therefore it can be of valuable help to screen new molecules for their potential efficacy in the treatment of alcohol abuse in humans. In addition, msP rats appear to share important common

characteristics with the human disease that confer to them important elements of face and construct validity. The present review also summarized the results of recent gene profiling experiments and *in situ* gene expression analysis in msP rats demonstrating that this rat line carries various genetic differences compared with non-selected Wistar rats that involves Map- and Cam-kinases pathways, alcohol metabolism, and various neurotransmitter systems. The most relevant peculiarity of msP rats is that they are highly sensitive to stress, show an anxious phenotype and have depressive-like symptoms that recover following ethanol drinking. At molecular level these behaviors correlate with a particularly high expression of the gene encoding for CRF_1 receptors and an hyperfunctioning CRF_1 receptor system in various brain areas. We believe that msP rats represent an animal model in which anxiety and depression-like traits have co-segregated with high alcohol preference during the selection leading to the generation of a useful model of genetic susceptibility to alcohol abuse linked to self-medication of negative affective states. In this regard our studies reflect the results of several clinical studies that have shown that a large population of alcoholics have a low ability to engage in stress-coping strategies. In them, resumption of alcohol abuse is often a mechanism to ameliorate the negative affective state in which they precipitate following exposure to anxiogenic stimuli or stress, especially during protracted withdrawal.

Acknowledgments

The study was supported by the EU 5th Framework Program, grant QLRT-2001-01048 (to MH and RC), NIAAA AA01435 (to FW subcontract to RC) and TOK MTKD-CT-2004-509242 (to RC).

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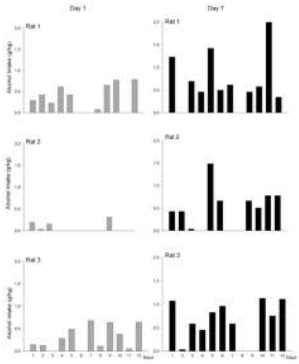

Figure 1.
Typical pattern of 10% ethanol consumption in three different msP rats. Drinking was recorded on the first day (day 1) and seventh day (day 7) of alcohol access. Measurements were carried out every hour during the dark phase of the light/dark cycle. Ethanol intake is expressed as g/kg to reduce the influence of differences in body weight

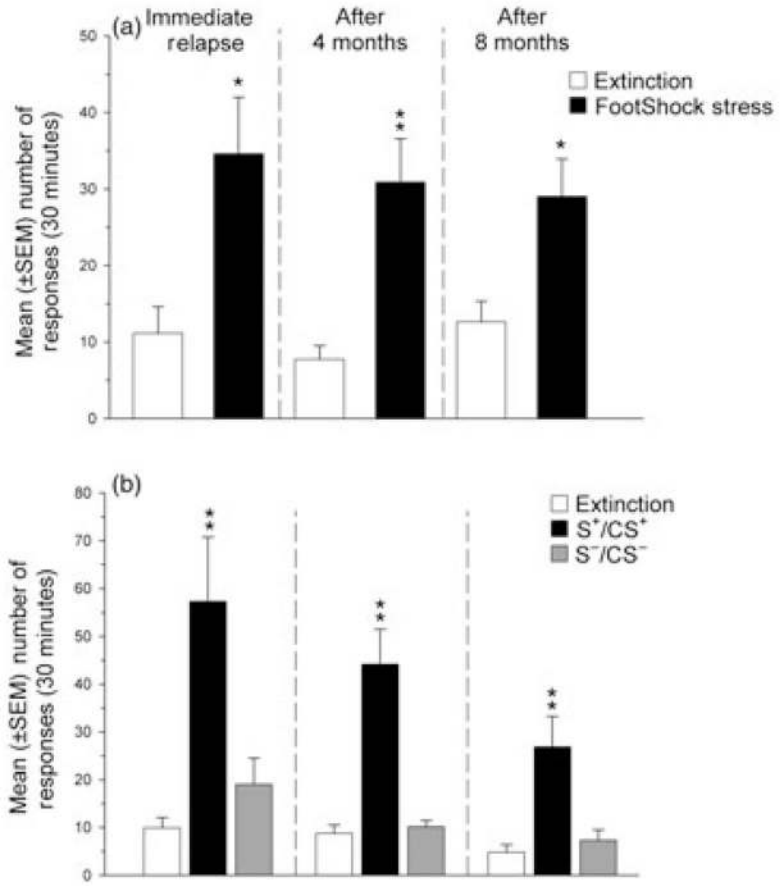

Figure 2.

Alcohol-seeking behavior of msP rats induced by (a) foot-shock stress and (b) re-exposure to the alcohol (CS^+/S^+)- or water (CS^-/S^-)-paired cues. Animals were tested for reinstatement immediately after extinction (Immediate Relapse) or after 4 and 8 months. Between the different relapse the animals were kept in their home cages in the vivarium. Each test was preceded by an extinction cycle to re-establish responding consistent with the extinction criterion. Values represent the mean (± SEM) number of responses at the active lever. Difference from extinction was set at $^*P < 0.05$ and $^{**}P < 0.01$

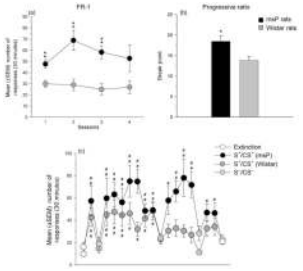

Figure 3.

Performance of msP and Wistar rats on alcohol self-administration under: (a) fixed ratio 1 schedule and (b) progressive ratio schedule. In the progressive ratio the number of responses at the lever required to obtain a single dose (0.1 ml) of 10% w/v ethanol increases progressively. (c) Behavior of msP and Wistar rats under repeated reinstatement tests. Animals were first trained to discriminate the availability of ethanol versus water in the presence of cues predictive of their availability (CS^+/S^+ and CS^-/S^-, respectively). At completion of the discrimination phase, and after an extinction period (no cues present), the CS^+/S^+ and/or CS^-/S^- were re-presented to the animals and their behavior motivated by the stimuli predictive of ethanol (CS^+/S^+) versus water (CS^+/S^+) availability was monitored. For the reinstatement test fluids were not available. Values represent the mean (± SEM) number of responses at the active lever. Statistical difference was set at $^*P < 0.05$ and $^{**}P < 0.01$ between msP and Wistar rats, and $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ from extinction

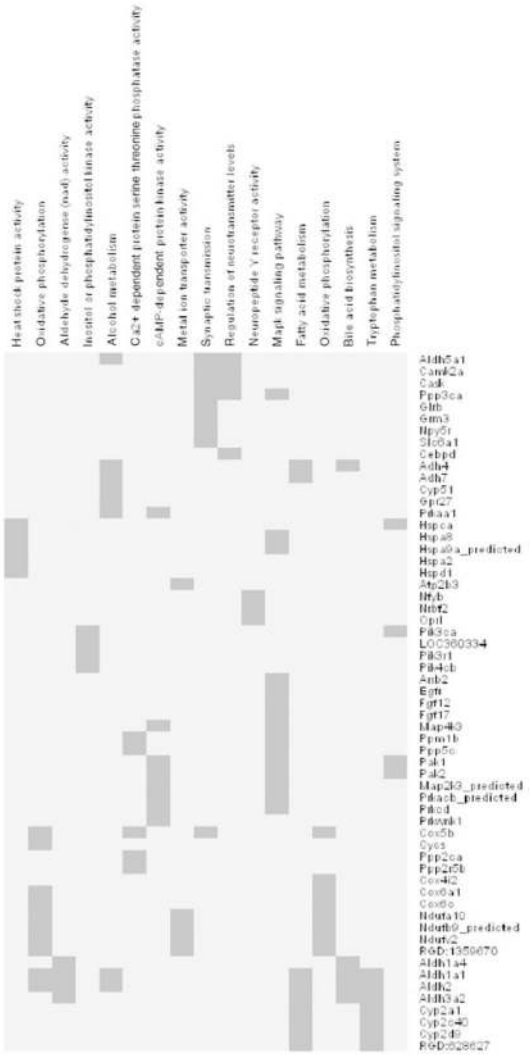

Figure 4.

Heatmap visualization of significant genes related to functional groups. Statistically enriched GO Ontology terms and KEGG pathways were placed against genes differentially between the two strains. Red color indicates the functional categories correlated with the genes

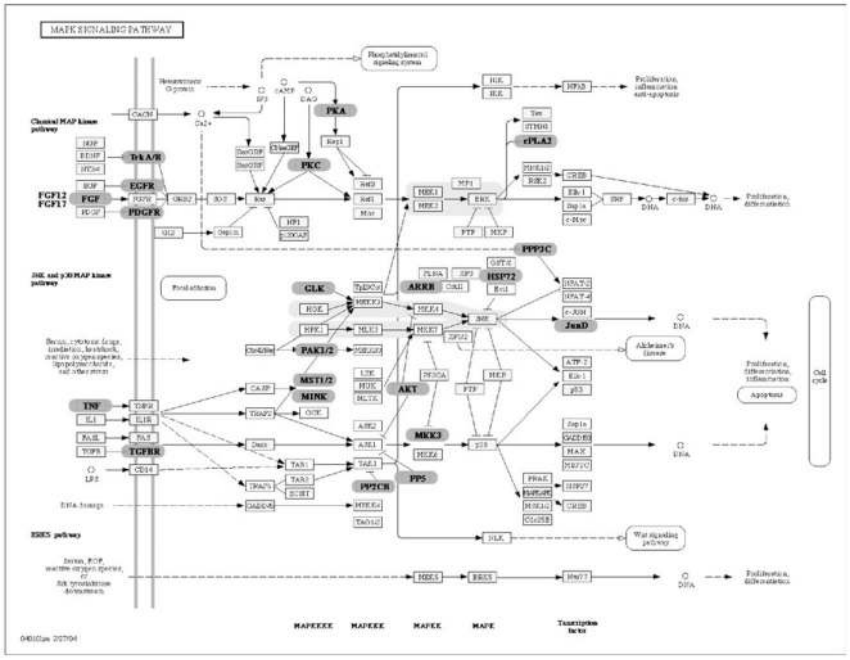

Figure 5.

Mapk signaling pathway from KEGG. Red shaded genes are differentially expressed in Marchigian Sardinian alcohol-preferring (msP) compared with Wistars

Table 1

Summary of the studies evaluating the effects of different drugs on alcohol intake or ethanol-seeking behavior induced by stress or drug-associated cues in Marchigian Sardinian alcohol-preferring (msP) rats.

Drug treatment	Alcohol intake	Stress-induced reinstatement	Cue-induced reinstatement	References
SCH 39166 (D_1 antagonist)	↓	–	–	Panocka *et al*. (1995a)
Haloperidol (D antagonist)	↓	–	–	Panocka *et al*. (1993a)
Ritanserin ($5HT_{2/1C}$ antagonist)	No effect (s.c.)	–	–	Panocka *et al*. (1993a)
	↓ (intraventricular)			Panocka *et al*. (1993b)
Risperidone ($5HT_2$, D_2 antagonist)	↓	–	–	Panocka *et al*. (1993a)
NH2-SENK (NK3 agonist)	↓	–	–	Ciccocioppo *et al*. (1994)
				Ciccocioppo *et al*. (1995)
				Ciccocioppo *et al*. (1997a)
				Polidori *et al*. (1997)
				Ciccocioppo *et al*. (1998)
GR64349 (NK2 agonist)	–	–	–	Ciccocioppo *et al*. (1994)
[Sar^9, $Met(O_2)^{11}$]SP (NK1 agonist)	–	–	–	Ciccocioppo *et al*. (1994)
				Ciccocioppo *et al*. (1997a)
PG-KII (NK3 agonist)	↓	–	–	Polidori *et al*. (1997)
WAY100135 ($5HT_{1A}$ antagonist)	No effect	–	–	Ciccocioppo *et al*. (1997b)
GR113808 ($5HT_4$ antagonist)	↓	–	–	Panocka *et al*. (1995b)
Fluoxetine (SSRI)	↓	–	–	Ciccocioppo *et al*. (1997b)
5-http (5HT precursor)	↓	–	–	Ciccocioppo *et al*. (1997b)
SR141716A (CB1 antagonist)	↓	↓	–	Economidou *et al*. (2006)
Naltrexone, Naloxone (opioid antagonist)	↓	–	–	Perfumi *et al*. (2003)
				Ciccocioppo *et al*. (2006)
N/OFQ (NOP agonist)	↓	↓	↓	Ciccocioppo *et al*. (1999c)
				Martin-Fardon *et al*. (2000)
				Ciccocioppo *et al*. (2002)

Drug treatment	Alcohol intake	Stress-induced reinstatement	Cue-induced reinstatement	References
				Ciccocioppo *et al*. (2003)
				Ciccocioppo *et al*. (2004)
Buprenorphine (MOP, NOP agonist)	↓↑	–	–	Ciccocioppo *et al*. (2006)
Bicuculline ($GABA_A$ antagonist)	No effect	–	–	Perfumi *et al*. (2002)
Baclofen ($GABA_B$ agonist)	↓	–	–	Perfumi *et al*. (2002)
Acamprosate (NMDA antagonist)	↓	–	↓	Ciccocioppo *et al*. (unpublished)
LY379268 (mGlu2/3 antagonist)	↓	–	–	Ciccocioppo *et al*. (unpublished)

Table 2

Most represented Gene Ontology categories for genes differentially expressed between Marchigian Sardinian alcohol-preferring (msP) and Wistar.

GO ID	GO term	No. of genes	P-value
GO:0003773	Heat shock protein activity	9	2.20E-08
GO:0016620	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, nad or nadp as acceptor	8	6.00e-07
GO:0006119	Oxidative phosphorylation	10	1.30e-06
GO:0004029	Aldehyde dehydrogenase (nad) activity	5	4.02e-06
GO:0019957	c-c chemokine binding	5	4.89e-05
GO:0016303	Phosphatidylinositol 3-kinase activity	4	0.00014
GO:0035004	Phosphoinositide 3-kinase activity	4	0.00017
GO:0004428	Inositol/phosphatidylinositol kinase activity	5	0.00021
GO:0004129	Cytochrome-c oxidase activity	5	0.00028
GO:0006066	Alcohol metabolism	11	0.00062
GO:0004723	Calcium-dependent protein serine/threonine phosphatase activity	5	0.00090
GO:0015081	Sodium ion transporter activity	5	0.00090
GO:0015075	Ion transporter activity	11	0.00104
GO:0004691	cAMP-dependent protein kinase activity	8	0.00110
GO:0004025	Alcohol dehydrogenase activity, iron-dependent	3	0.00172
GO:0046873	Metal ion transporter activity	6	0.00277
GO:0019226	Transmission of nerve impulse	10	0.01030
GO:0004685	Calcium/calmodulin-dependent protein kinase activity	3	0.01043
GO:0007268	Synaptic transmission	9	0.01395
GO:0006629	Lipid metabolism	12	0.02026
GO:0004357	Glutamate-cysteine ligase activity	2	0.02106
GO:0006816	Calcium ion transport	4	0.03484
GO:0001505	Regulation of neurotransmitter levels	5	0.04073
GO:0004983	Neuropeptide Y receptor activity	3	0.041915
GO:0006631	Fatty acid metabolism	5	0.06527
GO:0006750	Glutathione biosynthesis	2	0.06644
GO:0000165	Mapkkk cascade	4	0.06734
GO:0042417	Dopamine metabolism	2	0.07355
GO:0006536	Glutamate metabolism	2	0.08760
GO:0004985	Opioid receptor activity	2	0.08817

P-values were computed using Fisher exact test for enrichment of GO terms.

Table 3

Most represented KEGG terms for genes differentially expressed between Marchigian Sardinian alcohol-preferring (msP) and Wistar.

Pathway name	No. of genes	P-value
Mapk signaling pathway	19	1.39E-06
Fatty acid metabolism	9	9E-06
Oxidative phosphorylation	10	0.00027
Bile acid biosynthesis	5	0.00031
Ascorbate and aldarate metabolism	4	0.00090
Tryptophan metabolism	7	0.00116
Glycolysis/gluconeogenesis	5	0.01734
Apoptosis	6	0.02215
Glycerolipid metabolism	5	0.02547
Phosphatidylinositol signaling system	4	0.03413

P-values were computed using Fisher exact test for enrichment of KEGG terms.

Table 4

Expression microarray analysis (RAE230A) in cingulate cortex and amygdala from Marchigian Sardinian alcohol-preferring (msP) and Wistar rats for genes involved in alcohol metabolism.

Affymetrix probeset ID	Gene_title	Gene_symbol	UniGene_ID	msP/Wistars
1369863_at	Alcohol dehydrogenase 4 (class II), pi polypeptide	Adh4	Rn.98159	↓
1369072_at	Alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	Adh7	Rn.42935	↓
1367999_at	Aldehyde dehydrogenase 2	Aldh2	Rn.101781	↓
1387022_at	Aldehyde dehydrogenase family 1, member A1	Aldh1a1	Rn.6132	↑
1368718_at	Aldehyde dehydrogenase family 1, subfamily A4	Aldh1a4	Rn.74044	↑
1368365_at	Aldehyde dehydrogenase family 3, subfamily A2	Aldh3a2	Rn.9113	↑
1371062_at	Aldehyde dehydrogenase family 5, subfamily A1	Aldh5a1	Rn.10070	↑