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Autoradiographic analysis of 5-HT_{2A} binding sites in the brain of Sardinian alcohol-preferring and nonpreferring rats

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Abstract

The density of 5-HT_{2A} binding sites in the brain of Sardinian alcohol-preferring (sP) and nonpreferring (sNP) rats was evaluated, using [^{3}H]ketanserin for quantitative autoradiography. The highest [^{3}H]ketanserin binding levels were found in the anterior olfactory nucleus, prefrontal cortex, medial prefrontal cortex, post-genual anterior cingulate cortex, insular cortex and claustrum. Statistically significant differences between sP and sNP rats were found in prefrontal cortex, medial prefrontal cortex and post-genual anterior cingulate cortex, where sP rats showed about 20% lower [^{3}H]ketanserin binding levels. No significant difference was found in other areas, although some of them showed slightly lower [^{3}H]ketanserin binding density in sP rats. The 5-HT_{2A} receptor agonist, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane hydrochloride (DOI), microinjected into the medial prefrontal cortex, induced a lower number of wet dog shakes in sP than in sNP rats. These results indicate a different density of 5-HT_{2A} binding sites, and a different functional regulation of 5-HT_{2A} receptor mechanisms in discrete brain areas of sP, in comparison to sNP rats. These findings, and those showing lower levels of 5-HT in the frontal cortex of sP rats, suggest that altered 5-HT function in fronto-cortical areas could be linked to the genetic predisposition to high voluntary ethanol intake in these rats.

Keywords: Sardinian alcohol-preferring rat; 5-HT_{2A} receptor; Autoradiography; Wet dog shake

1. Introduction

Genetically selected alcohol-preferring rats represent an interesting animal model to study the neurochemical and behavioural correlates of ethanol abuse. Several alcohol-preferring and nonpreferring rat lines have been selected, including the Sardinian alcohol-preferring (sP) and Sardinian alcohol-nonpreferring (sNP) rats (Gessa et al., 1991). Like other ethanol-preferring rat lines, sP rats show a high preference for 10% ethanol solution and a daily ethanol intake greater than 5 g/kg body weight (Colombo, 1997).

Several lines of evidence suggest an inverse relationship between alcohol abuse and central serotonergic neurotransmission. Deficiency of brain serotonin and its metabolites has been reported in human alcohol abusers and in genetically selected alcohol-preferring rats (Cloninger, 1987; Murphy et al., 1987; Eriksson and Humble, 1990; Zhou et al., 1990; McBride et al., 1993a). Accordingly, drugs that increase 5-hydroxytryptamine (5-HT) neurotransmission reduce ethanol consumption both in humans and in rats (see for review Sellers et al., 1992; McBride et al., 1993a; Overstreet et al., 1994). Altered binding density of 5-HT_{1A} and of 5-HT_{2A} sites in discrete brain areas of genetically selected alcohol-preferring P rats (Lumeng et al., 1977), compared to nonpreferring NP rats, has been observed (McBride et al., 1993b, 1994). McBride et al. (1993b) have reported a lower density of 5-HT_2 binding sites in a number of cortical and mesolimbic regions of P, compared to NP rats. Lower [^{3}H]ketanserin binding levels in cortical, but not in mesolimbic areas of P rats were also reported by Ciccocioppo et al. (1997). However, no differences in 5-HT_{2A} binding levels between alcohol-preferring (AA)

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and nonpreferring (ANA) lines (Kiianmaa et al., 1992) were detected (Korpi et al., 1992; Ciccocioppo et al., 1997).

A previous study by our group has shown that sP rats exhibit fewer behavioural responses to stimulation of central 5-HT_{2A} receptors, in comparison to sNP rats (Ciccocioppo et al., 1995). Using quantitative autoradiography, the present study was carried out to evaluate whether the different response to 5-HT_{2A} receptor stimulation is accompanied by altered 5-HT_{2A} binding density and/or distribution in discrete brain areas of these two rat lines.

2. Materials and methods

2.1. Subjects

Subjects for the autoradiographic analysis were male sP and sNP rats from the colony of the Department of Neurosciences of the University of Cagliari. They were housed in individual stainless-steel cages in a temperature (20°–22°C)- and humidity (45%–55%)-controlled room, with a reverse 12/12 light/dark cycle (lights off at 10:00 a.m.). Rats had free access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy). All animal testing was carried out according to the Italian ethical rules on animal care (law no. 116 of January 27, 1992).

For the autoradiographic study, 2-month-old rats selected for 37 generations were offered 10% (v/v) ethanol and water for a week and their ethanol preference was measured. After the screening test, the rats were kept for 2 months without access to ethanol. The animals were decapitated when they were 4 months old.

For the behavioural study, 4-month-old ethanol-naive rats, genetically selected for 41 generations, were used. The animals were tested for their ethanol intake and preference at the end of the study; the sP rats used took 5–6 g/kg of ethanol/day, showing a percent alcohol preference of over 90, while the sNP rats had a negligible daily ethanol consumption.

2.2. Drugs

[Ethylene-^{3}H]ketanserin, specific activity 80.9 Ci/mmol, was obtained from New England Nuclear. Methysergide maleate and (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane hydrochloride (DOI) were purchased from RBI, Natick, MA, USA.

2.3. Autoradiography

Six sP and six sNP rats were decapitated, their brains were rapidly removed and frozen at −80°C. Frozen brains were placed in a cryostat (−15 to −19°C) and 20-μm sections were cut and thaw-mounted onto gelatin-coated glass slides. The sections were stored desiccated at −80°C until assay. Autoradiography was carried out by a slight modification of the method described by Pazos et al. (1985). Briefly, tissue sections were removed from storage and allowed approximately 30 min to equilibrate to cold room temperature (4°C). The mounted tissue was then preincubated for 15 min at room temperature in 0.17 M Tris–HCl buffer (pH 7.7). Afterward, the sections were transferred to 0.17 M Tris–HCl buffer containing 2.0 nM [^{3}H]ketanserin in the absence (total binding) or presence (nonspecific binding) of 10^{-6} M methysergide for 60 min at room temperature. Immediately following incubation, the slides were washed in ice cold Tris–HCl buffer (2 × 10 min at 4°C) and dipped (1 s) in ice-cold distilled water to remove buffer salt. The sections were rapidly dried in a stream of cold dry air and exposed to Hyperfilm-[^{3}H] (Amersham) in X-ray cassettes together with [^{3}H] standards (Amersham) for 45 days.

Using an AIS Imaging Research System (Brock University, St. Catharines, Ontario, Canada) autoradiographic films were quantitated by reference to a nonlinear calibration curve for gray levels generated from [^{3}H] microscale standards. Hence, binding sites could be expressed as femtomole per milligram tissue wet weight using tissue equivalent and specific activity of the ligand. Specific binding was determined by subtracting nonspecific binding from total binding for adjacent sections. For individual animals, the mean value obtained from bilateral measurement of sections (minimum three per nucleus) was determined. Mean values for equivalent areas in different animals were grouped per rat line and statistically analyzed. Brain areas were identified using the rat brain atlas of Paxinos and Watson (1986).

2.4. Wet dog shakes and head shakes following injection of DOI into the medial prefrontal cortex

The medial prefrontal cortex is a site of action for 5-HT_{2A} receptor agonists to induce behavioural responses, mainly wet dog shakes (Lucki and Minugh-Purvis, 1987; Willins and Meltzer, 1997).

On the basis of the results of the autoradiographic study, eight sP and eight sNP rats were anaesthetized by intraperitoneal injection of 100–150 μl/100 g body weight of a solution containing ketamine (86.2 mg/ml) and acepromazine (1.3 mg/ml), and stereotaxically implanted with bilateral stainless-steel cannulae aimed at the medial prefrontal cortex. The coordinates were: 3.5 mm anterior and ±0.7 mm lateral to the bregma, 2 mm ventral from the surface of the skull. The cannulae were attached to the skull with jewelry screws and dental cement. One week of recovery was allowed before testing began. During this period, the rats were handled and mock-injected to accustom them to the testing procedure.

DOI, 0.5 or 1 μg/side, was bilaterally injected into the medial prefrontal cortex in a volume of 0.5 μl/side, by means of a stainless-steel injector temporarily inserted into

the guide cannula and protruding 2 mm beyond its tip. Immediately after DOI treatment, the rats were placed in a Perspex box and the number of wet dog shakes and head shakes was recorded for 15 min.

2.5. Histological analysis

After completion of the behavioural experiments, the rats were killed with an overdose of anaesthetic. Their

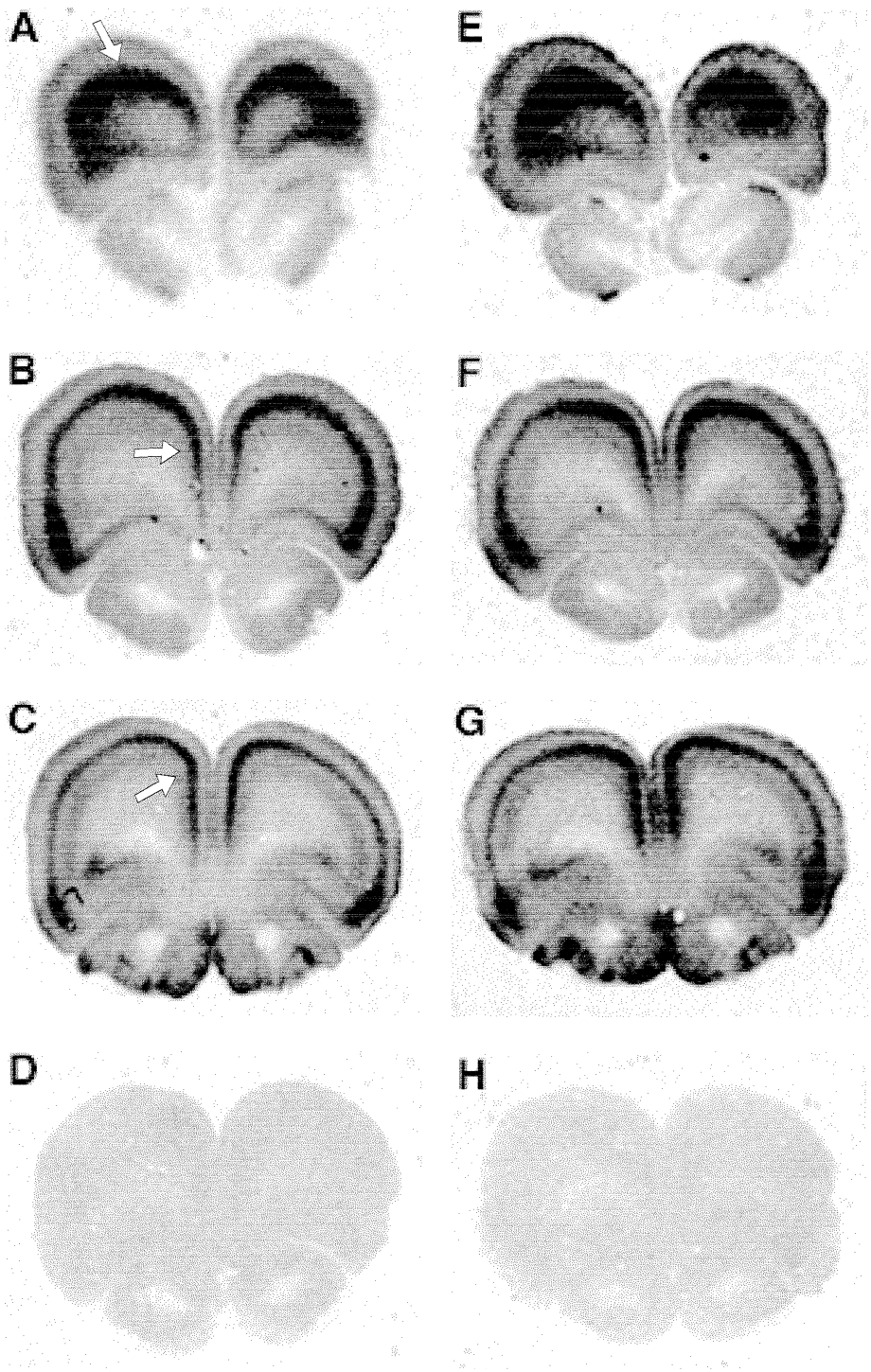

Fig. 1. Distribution of [^{3}H]ketanserin binding sites in the forebrain of sP (A–C) and sNP (E–G) rats. A–C and E–G show the total binding; D and H are examples of nonspecific binding in sP and sNP rats, respectively. The arrows indicate the prefrontal cortex (A), medial prefrontal cortex (B) and post-genual anterior cingulate cortex (C) in sP rats. The results of the quantitative autoradiographic analysis are shown in Fig. 2.

brains were removed and frozen at −80°C. Frozen brains were placed in a cryostat (−15 to −19°C) and 20-μm sections were cut and thaw-mounted onto glass slides. Sections were stained with cresyl violet 0.1% and cannula placement was evaluated. Only data from animals with valid bilateral cannula placement were included in the experimental results.

2.6. Statistical analysis

Binding data were analyzed by means of unrelated two-tailed *t*-tests; the results of the behavioural experiments were analyzed by the analysis of variance (with between-group comparisons for rat line and within group comparisons for drug dose) followed by Dunnett's test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Autoradiography

As shown in Fig. 1, lower densities of [^{3}H]ketanserin binding sites were found in several cortical forebrain areas of sP rats, in comparison to those of sNP rats. Statistically significant differences were found in cortical layer IV of the prefrontal cortex, in the medial prefrontal cortex and the post-genual anterior cingulate cortex (Fig. 2). In these regions, the [^{3}H]ketanserin binding levels were about 20% lower in sP than in sNP rats.

Slightly lower [^{3}H]ketanserin binding levels were also observed in other forebrain areas, such as the frontal cortex, anterior parietal cortex, anterior agranular insular cortex, pyriform cortex, olfactory tubercle and anterior olfactory nucleus, posterior part (Table 1); however the difference was not statistically significant.

Moreover, no significant differences between sP and sNP rats were found in all the other brain regions listed in Table 1.

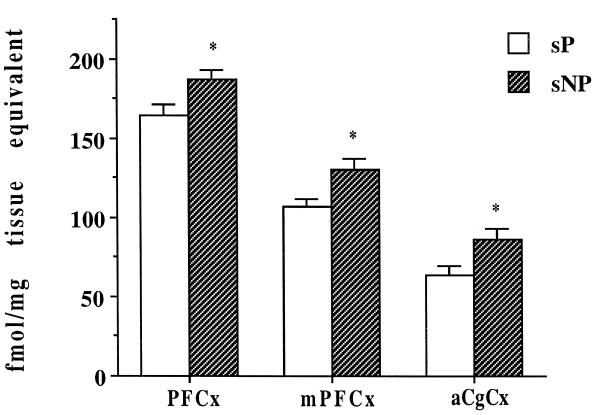

Fig. 2. Density of [^{3}H]ketanserin in the prefrontal cortex (PFCx), medial prefrontal cortex (mPFCx) and post-genual anterior cingulate cortex (aCgCx) of sP and sNP rats. Data are means ± S.E.M. for six rats/line. Statistical different between lines: * $P < 0.05$.

Table 1
Binding of [^{3}H]ketanserin (2 nM) to 5-HT$_{2A}$ sites in several brain regions of sP and sNP rats

Brain regions	Tissue equivalent (fmol/mg)	
	sP	sNP
Frontal cortex (layer IV)	90.4 ± 5.5	100.3 ± 5.7
Ant. parietal cortex (layer IV)	50.9 ± 2.3	58.3 ± 5.5
Post. parietal cortex (layer IV)	53.7 ± 7.2	48.1 ± 5.6
Ant. agranular insular cortex (layer IV)	166.0 ± 6.9	185.3 ± 7.9
Mid. agranular insular cortex (layer IV)	139.6 ± 9.7	126.3 ± 6.5
Post. agranular insular cortex (layer IV)	101.7 ± 5.5	101.8 ± 6.4
Piriform cortex (layer IV)	74.0 ± 3.5	82.3 ± 4.5
Temporal cortex (layer IV)	48.2 ± 6.6	45.9 ± 1.6
Occipital cortex (layer IV)	33.9 ± 4.5	34.7 ± 3.0
Entorhinal cortex (layer IV)	54.5 ± 3.0	52.3 ± 1.8
Olfactory tubercle	48.7 ± 4.9	66.7 ± 9.6
Ant. olfactory nucleus posterior part	164.1 ± 7.3	173.1 ± 9.9
Claustrum	126.4 ± 5.8	111.2 ± 7.2
Ant. nucleus accumbens	23.6 ± 3.8	24.9 ± 2.1
Nucleus accumbens shell	47.6 ± 4.2	43.7 ± 3.6
Nucleus accumbens core	20.6 ± 1.5	19.7 ± 3.9
Caudate putamen head	14.5 ± 1.4	12.1 ± 1.4
Caudate putamen body dorsal part	13.7 ± 1.0	13.6 ± 2.1
Caudate putamen body ventral part	14.1 ± 1.3	14.0 ± 2.2
Caudate putamen tail	31.2 ± 4.2	31.5 ± 1.6
Fundus striati	49.2 ± 3.5	50.2 ± 5.6
Lateral hypothalamus	2.0 ± 0.4	5.1 ± 1.8
Cortical amygdaloid nucleus	54.4 ± 2.9	48.4 ± 5.2
Basolateral amygdaloid nucleus	12.4 ± 1.6	10.2 ± 2.1
Medial amygdaloid nucleus	12.3 ± 0.9	14.0 ± 2.4
Lateral amygdaloid nucleus	14.5 ± 1.6	14.6 ± 2.3
CA$_1$ field of Ammon's horn	2.1 ± 0.9	2.2 ± 0.5
CA$_3$ field of Ammon's horn	5.7 ± 1	7.9 ± 0.9
Dorsal dentate gyrus	1.4 ± 0.8	1.4 ± 0.2
Ventral dentate gyrus	77.8 ± 7.3	82.2 ± 6.5
Supramammillary nucleus	64.3 ± 4.5	58.1 ± 1.6
Medial mammillary nucleus	66.9 ± 5.3	64.1 ± 2.3
Substantia nigra reticular part	2.3 ± 0.9	2.4 ± 0.3
Substantia nigra compact part	2.9 ± 0.8	3.4 ± 0.6
Ventral tegmental area	2.5 ± 1.2	4.0 ± 0.4
Superficial gray layer	9.6 ± 0.5	10.7 ± 1.0
Central periaqueductal gray	5.0 ± 1.3	4.9 ± 0.4

Data are means ± S.E.M. for six subjects for each group. No statistically significant differences between the sP and sNP rats were observed for any of these brain regions.

3.2. Wet dog shakes and head shakes following injection of DOI into the medial prefrontal cortex

Histological analysis revealed a correct cannula placement in all the animals except in one sP rat that was not included in the statistical analysis.

As shown in Fig. 3, sNP rats showed a higher baseline number of wet dog shakes in comparison to sP rats, as observed previously (Ciccocioppo et al., 1995).

Bilateral injections of DOI, 0.5 or 1 μg/side, into the medial prefrontal cortex significantly increased the number of wet dog shakes [$F(2,14) = 6.4$; $P < 0.05$] and head shakes [$F(2,14) = 6.0$; $P < 0.05$] in sNP rats, but not in sP rats [$F(2,12) = 0.3$, $P > 0.05$, and $F(2,12) = 0.09$, $P > 0.05$, respectively]. The analysis of variance revealed a

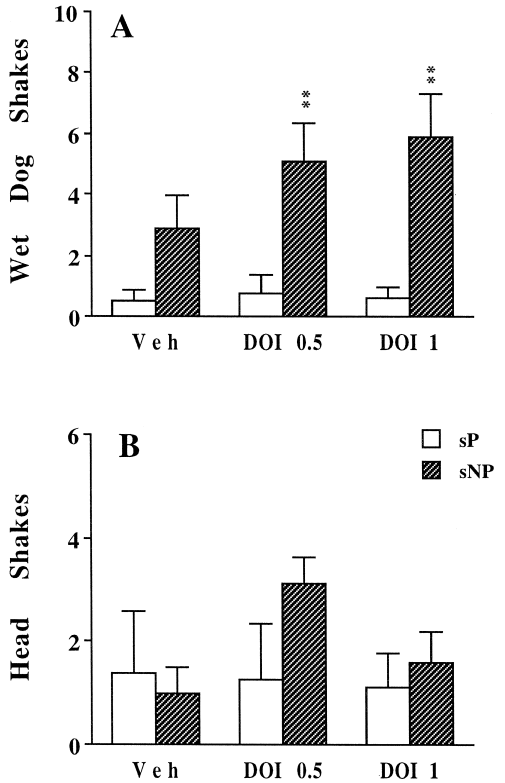

Fig. 3. Wet dog shakes (A) and head shakes (B) in the 15 min following injections into the medial prefrontal cortex of DOI, 0.5 or 1 μg/side, or vehicle in sP or sNP rats. Data are means ± S.E.M. for seven (sP) and eight (sNP) rats. Statistical difference between sP and sNP rats: $^{**}P < 0.01$; where not indicated, difference between rat lines was not statistically significant.

statistically significant difference in the number of wet dog shakes among the two rat lines $F(1,13) = 8.37$; $P < 0.05$]. Although the number of head shakes was lower in sP rats, the difference between rat lines was not statistically significant.

4. Discussion

The present results showed that sP rats exhibited significantly lower [^{3}H]ketanserin binding levels in the cortical layer IV of the prefrontal cortex, in the medial prefrontal cortex and the post-genual anterior cingulate cortex, than do sNP rats; however, the study did not investigate whether differences in binding density are related to different number or affinity of 5-HT_{2A} binding sites.

Moreover, injections of the 5-HT_{2A} receptor agonist, DOI, in the medial prefrontal cortex dose dependently increased the number of wet dog shakes in sNP rats but did not evoke any response in sP rats. These findings reveal a different functional regulation of the 5-HT_{2A} receptor mechanisms in this area.

Interestingly, a 20% decrease in the density of ketanserin binding sites in the medial prefrontal cortex was translated into absence of a response to DOI. In this regard, it could be hypothesized either that such a decrease in 5-HT_{2A} binding sites may be not uniformly distributed among neurons of the medial prefrontal cortex, but may be particularly relevant for a specific population of neurons, or that cellular transduction mechanisms activated by 5-HT_{2A} receptor stimulation also may be altered in sP rats.

Several studies have consistently shown that 5-HT_{2A} receptor antagonism selectively increases dopamine extracellular levels in the prefrontal cortex (Schmidt et al., 1992; Pehek et al., 1993; Schmidt and Fadayel, 1995; Pehek, 1996; Tanda et al., 1996). Thus, 5-HT_{2A} receptors apparently exert a tonic inhibitory control of dopamine release in the prefrontal cortex, and their reduced activity may result in increased dopaminergic neurotransmission in this area. Efferents from the prefrontal cortex make synaptic contact with dopaminergic fibers in the ventral tegmental area and nucleus accumbens (Sesack and Pickel, 1992), and modulate the activity of the mesolimbic dopaminergic system (Murase et al., 1993). In particular, dopaminergic inputs in the prefrontal cortex are inhibitory with respect to subcortical dopamine systems (Louilot et al., 1989; Thompson and Moss, 1995). These findings might allow us to speculate that altered regulation of 5-HT_{2A} receptors in prefrontal cortical areas of sP rats may have relevant consequences for the mesolimbic dopaminergic system of sP rats and, therefore, for the processes of reward and reinforcement (Koob, 1992). It has been reported that, in comparison to the sNP, sP rats exhibit inherent differences in dopamine neurotransmission, as shown by lower numbers of dopamine D_1 and D_2 receptors in limbic structures (Stefanini et al., 1992; De Montis et al., 1993) and a higher sensitivity to the enhancing effect of ethanol on dopamine metabolism (Fadda et al., 1989; Fadda et al., 1990). Thus, an altered functional regulation of 5-HT_{2A} receptors in prefrontal cortical areas may contribute to the abnormal function of mesolimbic dopaminergic neurotransmission in sP rats.

sP rats have been reported to have a lower content of 5-HT and 5-hydroxyindol-3-yl-acetic acid in the frontal cortex (Devoto et al., 1998), but not in the whole brain (Bano et al., 1998), in comparison to sNP rats. These findings, together with those of the present study, suggest that a different 5-HT function in the fronto-cortical areas may be linked to the high ethanol preference and intake of sP rats. Interestingly, Portas et al. (1994) have shown that acute ethanol administration increases 5-HT release in the frontal cortex of sP rats more effectively than in the sNP ones, suggesting that ethanol consumption by sP animals acts as a means to compensate for 5-HT deficiency.

Moreover, in comparison to sNP, sP rats exhibit a longer period of immobility in the forced swimming test, suggesting that they may have a tendency to a depression-like state (Ciccocioppo et al., 1998). Voluntary ethanol intake reduces the immobility score of sP rats to values similar to those of sNP rats, indicating that an elevated

ethanol consumption may represent, for these animals, an attempt at self-medication (Markou et al., 1998). Altered 5-HT function in forebrain cortical areas can be involved in the etiopathology of depression (Mann et al., 1996; Massou et al., 1997). The present findings raise the question of whether the altered regulation of 5-HT_{2A} receptor mechanisms in the medial prefrontal cortex of sP rats may be related not only to their ethanol preference, but also to their depression-like behaviour. Depression and alcohol abuse are characterized by high comorbidity and exhibit a variety of neurobiological similarities (Markou et al., 1998).

5-HT_2 receptor antagonists have been shown to stimulate serotonin and dopamine release in the nucleus accumbens (Devaud et al., 1992) and have been tested, although with conflicting results (see Johnson et al., 1996; Panocka et al., 1996), for their ability to reduce ethanol intake in rats and in humans. However, microinjection of the 5-HT_2 receptor antagonist, ritanserin, in the medial prefrontal cortex failed to reduce ethanol consumption in rats (Panocka et al., 1993), suggesting that blockade of 5-HT_{2A} receptors in this area is not able to attenuate ethanol intake. It would be interesting, in this regard, to evaluate whether stimulation of the same receptors in the medial prefrontal cortex might reduce ethanol intake in sP rats.

In relation to other lines of alcohol-preferring rats, it is noteworthy that the results for sP and sNP rats are to some extent similar to those obtained with P and NP rats (McBride et al., 1993b; Ciccocioppo et al., 1997), where 5-HT_{2A} binding levels in prefrontal cortex are lower than those in NP rats. However, differences in brain 5-HT_{2A} binding levels were not observed between Alko alcohol (AA) and Alko nonalcohol (ANA) lines (Korpi et al., 1992; Ciccocioppo et al., 1997), or between high alcohol-drinking (HAD) and low alcohol-preferring (LAD) rats (Li et al., 1988; McBride et al., 1997). Thus, a different density of 5-HT_{2A} binding sites in forebrain areas is not a common characteristic of rat lines selected for high ethanol preference.

Acknowledgements

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