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High-resolution Magnetic Resonance Spectroscopy of the Mouse Vomeronasal Organ

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Abstract

The chemical composition of the vomeronasal organ (VNO) was investigated by means of *in vitro* proton magnetic resonance spectroscopy (MRS) in prepubertal and adult mice of both sexes. Results demonstrate that MRS detects several chemical constituents in the VNO, showing their age- and sex-associated changes in concentration. Preliminary experiments also suggest the ability of MRS to show compositional changes in the VNO after pheromonal stimulation. MRS can serve as a useful technique to investigate vomeronasal chemoreception.

Key words: accessory olfactory system, analysis, chemoreception, composition, major urinary protein, sex

Introduction

In vitro magnetic resonance spectroscopy (MRS) is a powerful tool to analyse the chemical composition of biological fluids and tissues. Proton (^{1}H) MRS is of special advantage because the ^{1}H nucleus is 100% natural abundance, it shows higher sensitivity and it is found in all metabolites (Petroff, 1988). MRS has been used to investigate changes in brain composition during development (Bates *et al.*, 1989; Zancanaro *et al.*, 2001) and in the adult individual (Kauppinen *et al.*, 1993). Knowledge of the composition of such structures would help understanding their metabolism and function under normal and pathological conditions. In this work, extracts of the vomeronasal organ (VNO) of mice were analysed by means of high-resolution ^{1}H MRS to yield an account of its chemical composition and determine the average concentration of several metabolites therein.

Materials and methods

In this work, a total of 106 mice of both sexes were used. All procedures complied with Italian law (L. 116/92) on animal experiments. Mice were deeply anaesthetized (20 mg/kg body wt xilazine and 75 mg/kg ketamine) and VNOs were surgically excised by cutting the palate just behind the incisor teeth; VNOs were immediately frozen on dry ice and maintained at –70°C until extraction. In order to reduce contamination with non-vomeronasal tissue, the VNO was freed of its bony capsule before freezing; this procedure could not be used in prepubertal VNOs due to the thin, soft bony nature of the capsule, which could only be separated from the VNO at the expense of a significant amount of VNO tissue. MRS is a highly discriminative technique, but it is relatively insensitive and several tens of milligrams of raw biological material are needed to yield a well-resolved ^{1}H spectrum. Therefore, individual VNO (weighing 2.5–3.5 mg) could not be used in this experiment; instead, VNOs taken from adult and prepubertal male and female mice were pooled according to sex and age in order to obtain a suitable amount of extractable material. Adult mice were either 9 (male, n = 19) or 6 months old (female, n = 13) and weighed ~40 g; prepubertal mice were 20/22 days old (male, n = 21; female n = 24) and weighed 15–20 g. For extraction, pooled VNOs were thawed, lightly blotted on filter paper, weighed to the nearest tenth of a milligram and mechanically homogenized in 12% perchloric acid; the homogenate was then centrifuged to remove solid material. The supernatant was neutralized with KOH and precipitated salts were removed by centrifugation. The clear solution was freeze-dried in an Edwards Modulyo apparatus and redissolved in deuterated water for MRS. A known amount of sodiumtrimethyl-silyl propionate (TSP) was added to each tube as a concentration and internal chemical shift reference (0 p.p.m.).

High-resolution spectra were obtained on a Bruker DRX-500 spectrometer operating at 500.13 MHz for ^{1}H. Water-suppressed ^{1}H spectra were acquired with an acquisition time of 3 s; a total of 2 K free induction decays (FIDs) were collected with a flip angle of 90° and a spectral width of 5 KHz. Spectral resonances were identified on the basis of available chemical shift data (Behar and Ogino, 1991; Kauppinen and Williams, 1994; Florian *et al.*, 1996; Govindaraju *et al.*, 2000); where necessary, confirmation of assignments was obtained from two-dimensional sequences, in particular chemical shift correlated spectroscopy (COSY) and total correlated spectroscopy (TOCSY). In nuclear magnetic resonance spectra of biological material it is generally found that the resonances from metabolite present in low concentrations overlap, presenting chemical noise that results in problems in quantification; therefore, only peaks with a signal-to-noise ratio >2 were considered for analysis and the concentration of only those metabolites that could be assigned unambiguously was estimated. The concentration of metabolite was determined by comparing metabolite fit integral with the fit integral of TSP, both scaled according to the number of the corresponding protons. Integrals were determined using the integration software implemented in the spectroscope (XWinNMR; Bruker).

Results were expressed as mg/gww; we refrained from expressing data as mg/mg protein in order to save material for extraction.

In a further pilot experiment, the effect of major urinary protein (MUP), a pheromonal stimulus acting through the VNO to induce the anticipation of puberty onset (Mucignat-Caretta *et al.*, 1995) was investigated. VNOs were taken as described above from prepubertal female mice after a 3 day single nasal administration of MUP (40 µl, 6 mg/ml protein purified by molecular sieve chromatography, n = 15) or saline (n = 14). Administration was started on postnatal day 30; mice were killed 24 h after the last administration (postnatal day 34) and treated as above for MRS examination. In this experiment, the olfactory bulbs (OBs) of mice were also obtained; OBs were cut with a scalpel by lowering the blade immediately anterior to the frontal cortex, behind the accessory olfactory bulb on a coronal plane. In this way, a small part of the anterior olfactory nucleus was included in the section. OBs were pooled according to treatment (MUP or saline) and processed as above for MRS.

To facilitate comparison, all changes in metabolite concentration are referred to in the text as percentage of an arbitrarily chosen reference value as indicated.

Results

Figure 1 (upper panel) shows the high-resolution ^{1}H spectrum of pooled VNOs from adult control females. Several metabolites were unambiguously identified and they are indicated in the legend.

The concentration (mg/gww) of several metabolites found in the VNO of prepubertal and adult, male and female mice is presented in Table 1. It should be emphasized that concentrations were calculated from spectra of pooled VNOs; therefore, figures represent an 'average' value for a given age and sex group. It is apparent that metabolite concentration is generally lower in either prepubertal (80–63%; female = 100%) and adult (95–52%; female = 100%) male mice but for betaine, which does not change and citrate of prepubertal mice, which is higher in males. Comparison of prepubertal (=100%) and adult mice shows a consistent decrease in the concentration of choline (85 and 75%), citrate (27 and 83%), glutamate/glutamine (43 and 54%), *N*-acetylaspartate

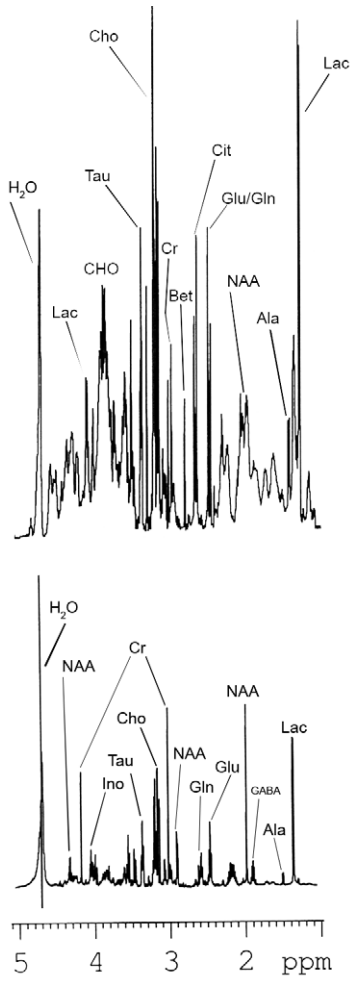

Figure 1 Upper panel: high-resolution, 500 MHz proton spectrum of the perchloric acid extract of pooled adult female mouse vomeronasal organs. Resonances are indicated as follows: Lac, lactate; Ala, alanine; NAA, N-acetyl aspartate; Glu/Gln, glutamate/glutamine; Cit, citrate; Bet, betaine; Cr, creatine; Cho, choline; Tau, taurine. H_2O, residual water signal; CHO, carbohydrates. Lower panel: high-resolution, 500 MHz proton spectrum of the perchloric acid extract of pooled prepubertal female mouse olfactory bulbs. Resonances are indicated as follows: Lac, lactate; Ala, alanine; GABA, γ-aminobutyrate; NAA, N-acetyl aspartate; Glu, glutamate; Gln, glutamine; Cr, creatine; Cho, choline; Tau, taurine; Ino, inositol. H_2O, residual water signal. Note that spectra in the upper and lower panel are scaled differently on the ordinate axis.

Table 1 Concentration (mg/gww) of several metabolites in pooled VNOs of adult or prepubertal male and female mice

Mice	Taurine	Choline	Creatine	Betaine	Citrate	Glutamate	NAA	Alanine	Lactate
Male adult	1.78	0.25	0.56	0.08	0.63	0.60	0.14	0.26	0.79
Male prepubertal	1.70	0.29	0.54	0.07	2.32	1.39	0.22	0.40	1.25
Female adult	1.87	0.31	0.71	0.07	1.25	0.96	ND	0.38	1.02
Female prepubertal	2.48	0.41	0.67	0.06	1.44	1.76	0.28	0.64	1.53

NAA, *N*-acetylaspartate; ND, not detectable.

Table 2 Concentration (mg/gww) of several metabolites in pooled OBs of prepubertal female mice

Taurine	Inositol	Choline	Creatine	Glutamate	Glutamine	NAA	Alanine	Lactate	GABA
3.73	10.62	0.57	4.87	3.03	4.00	2.93	0.49	2.57	3.64

NAA, *N*-acetylaspartate; GABA, γ-aminobutyric acid.

(NAA, 62%), alanine (65 and 59%) and lactate (63 and 66%) in adult male and female, respectively. Creatine was almost unchanged (105 and 106%) in adult males and females, respectively.

Figure 1 (lower panel) shows the high-resolution ^{1}H spectrum of pooled OBs from prepubertal, female mice administered saline in the nose for 3 days. Several metabolites were unambiguously identified and these are indicated in the legend; their concentrations (mg/gww) are reported in Table 2. In comparison with VNO of prepubertal female mice (Table 1, bottom line), betaine and citrate were lacking in the OB; instead, inositol, glutamine and γ-aminobutyric acid (GABA) were found in the OB, but not in the VNO. The concentration of common metabolites is consistently higher in OB than in VNO of prepubertal female mice (150–1000%) but for alanine, which is slightly lower in OB. Chronic (3 days) administration of MUP to prepubertal female mice was found to affect metabolite concentrations in the VNO: taurine (214%), creatine (329%) and alanine (214%) more than doubled in comparison with respective control values (=100%); a decrease was found for choline (66%), citrate (63%) and glutamate (73%). Almost no change in concentration (97–105%) was found in the OB of MUP-exposed mice for the majority of metabolites; a slight increase was found in the concentration of alanine (121%) and GABA (113%).

Discussion

This study investigated the composition of OVN extracts in prepubertal and adult mice by means of MRS. The results demonstrate the following points:

- MRS is able to identify and quantify several relevant chemical components in the OVN;
- differences in the concentration of metabolites are detected between sexes and in prepubertal versus adult mice.

MRS is of vantage in neurochemical research because it allows the *in vitro* simultaneous detection and quantification of several chemical components of neural structures with a minimum of preparative procedures; moreover, *in vivo* MRS yields interesting data on the presence and concentration of major brain metabolites (Tkac *et al.*, 1999). Despite extensive investigation of the VNO in the last several years (review in Halpern and Martinez-Marcos, 2003), data on its chemical composition are generally lacking. A first results of the present study is that ^{1}H MRS is able to detect a number of chemical components in pooled VNOs (Figure 1, upper panel and Table 1), including amino acids, choline and creatine. In particular, a small resolved resonance from NAA was detected in three out of four VNO pools (Table 1). This is of interest, because NAA is a neuronal marker (Urenjak *et al.*, 1992) and should be representative of the relatively small number of neurons present in the VNO.

Individual VNO extracts could not be analysed in this work due to the relatively low sensitivity of MRS; pooled VNOs were used instead, thereby preventing statistical analysis of the data. Therefore, results are assumed to represent the average content of individual metabolites in the VNO for sex and age group. Comparison of male and female mice showed a generally lower concentration of metabolites in pooled VNOs of the former (Table 1), irrespective of age. These data are in accordance with the sexual dimorphism found in the VNO as to the overall volume, neuroepithelial volume and number of bipolar neurons (Segovia and Guillamon, 1982) and the biogenic amines content (Zancanaro *et al.*, 1997); a higher concentration of glutamate/glutamine was found in prepubertal and adult female mice; this is of interest, because glutamate has been suggested to act as a neurotransmitter (Dudley and Moss, 1995; Quaglino *et al.*, 1999).

Therefore, the current results confirm and extend the concept that VNO is sexually dimorphic, albeit the possible

functional role of different metabolites concentrations remain to be determined. When adult mice of both sexes were compared to prepubertal individuals, a general decrease of metabolites concentrations was found in the former. The reasons for that can be manifold: for example, the larger amount of vascular tissue found in adult versus prepubertal VNO could 'dilute' metabolites (results were normalized per gram of wet weight); however, functional correlates can not be ruled out, because the adult VNO is fully competent in chemoreception, whereas the precise activity of prepubertal VNO is still not completely unravelled.

MUP administration with the same time schedule used in this work is able to induce anticipation of puberty onset in prepubertal female mice (Mucignat-Caretta *et al.*, 1995). It should be of interest to highlight possible mechanisms for this effect; therefore, a pilot experiment was carried out to evaluate the effect of MUP or saline exposure on VNO and OB metabolites concentrations in prepubertal female mice. MRS revealed that the ^{1}H spectrum of the mouse OB (Figure 1, lower panel) is comparable with previous findings (Florian *et al.*, 1996) in the rat OB. As expected, the chemical constituents of the OB, which consists almost exclusively of neural tissue, partially differ from the VNO, which is a multi-tissue organ (Table 2, compare with last row of Table 1); however, the concentration of common chemical components in OVN and OB is comparable. Chronic MUP exposure was able to induce several obvious changes in the VNO metabolites concentrations, but left OB concentrations almost unaffected. The most obvious reason for the latter finding is that vomeronasal axons only project to the accessory olfactory bulb which is a small portion of the OB; a further explanation could be that the central relay of the vomeronasal system is mature enough at postnatal day 30 to process the MUP-induced stimulus without obvious changes in neuronal composition.

In conclusion, the *in vitro* MRS results presented in this paper give a first account of the chemical composition of the VNO and its age- and sex-associated changes. It also appears that MRS is able to detect compositional changes in the VNO upon physiological (MUP) stimulation. MRS is a valuable source of information to investigate vomeronasal chemoreception.

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