

A Sexually Dimorphic Group of Atypical Glomeruli in the Mouse Olfactory Bulb

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Abstract

Atypical glomeruli (AtG) are clearly distinguishable from typical ones because of their strong cholinergic innervation. AtG are located in defined positions in the caudal half of the main olfactory bulb of rodents. The AtG partially overlap with other specialized olfactory subsystems, such as the modified glomerular complex, which is close to the accessory olfactory bulb. So far, possible sex differences in these specialised olfactory systems have not been investigated. In this work we have identified AtG in the mouse by means of acetylcholinesterase histochemistry and compared the number and size of these glomeruli between the sexes and also between the two strains that demonstrate intraglomerular synaptic differences, i.e. BALB/c and CD-1 mice. First, we divided the AtG into three types according to their position (I, rostral-most; II, around the accessory olfactory bulb; III, caudal-most) or their reactivity to acetylcholinesterase histochemistry (AtG type II being the least reactive glomeruli). ANOVA analyses revealed differences in the maximum diameter of glomeruli among the three types, but not in their sectional areas, indicating that all three types have different shapes. Moreover, both morphoplanimetric parameters were seen to be different between the two strains studied and also between the sexes: male mice and BALB/c animals had the largest glomeruli. The number of AtG was also significantly different between the sexes and strains, although these factors presented a strong interaction. Thus, the males had higher numbers of AtG in the CD-1 strain whereas in the BALB/c mice males demonstrated fewer AtG than females. These differences in number were largely due to AtG type II. The present work is evidence that AtG type II is a sexually dimorphic group of specialized glomeruli located in the main olfactory bulb.

Introduction

Rodent olfactory function is mediated by two distinct transductional organs and projections, i.e. the main olfactory system and the vomeronasal or accessory olfactory system, the latter being specialized for intraspecific chemical (pheromone) communication (Halász, 1990; Eisthen, 1997). Further, the main olfactory projections can be classified into two functionally different components, termed *generalist* and *specialist* when referring to their sensitivity to odorants (Shepherd, 1994; Ring *et al.*, 1997). According to this classification, the olfactory receptor neurons from the generalist subsystem would be activated by a broad range of signal molecules, in contrast to the specialist system, in which receptor neurons are only adjusted to just a few odorants, perhaps only one (Shepherd, 1994; Mori and Yoshihara, 1995).

One of these specialist systems comprises the 'atypical glomeruli' (AtG), which are structures in the main olfactory bulb (MOB) with a strong and early cholinergic innervation from centrifugal fibres and which have a characteristic ultrastructure and spatial distribution of primary olfactory axons within the glomeruli (Zheng *et al.*, 1987; Zheng and

Jourdan, 1988; Le Jeune and Jourdan, 1991, 1993). They also display marked differences in the phenotype of juxta-glomerular interneurons (Crespo *et al.*, 1996, 1997a,b). This discrete group of glomeruli overlaps with another specialist system, the 'modified glomerular complex' (MGC), which is specifically and selectively activated during suckling and may be tuned to the so-called 'suckling pheromone', isolated from the nipples, saliva and amniotic fluid of parturient rats (Teicher and Blass, 1976, 1978; Teicher *et al.*, 1980; Greer *et al.*, 1982). Using 2-deoxyglucose uptake techniques, the MGC was identified as a discrete group of glomeruli located adjacent to the accessory olfactory bulb (AOB) and the population of AtG was larger but also localized in the caudal half of the MOB.

Morphometric analyses have demonstrated sexual dimorphism within the AOB (Caminero *et al.*, 1991; Segovia and Guillamón, 1993) and this sexual divergence can be modified by hormonal or anti-depressant treatment (Segovia *et al.*, 1991; Valencia *et al.*, 1992; Pérez-Laso *et al.*, 1994). In contrast, it is as yet unknown whether such differences might exist in the main olfactory system. Since the specific

subset of glomeruli referred to above are related to pheromone information (like the vomeronasal system), it seemed justified to search for possible sex differences in its distribution in the MOB. Moreover, it has been demonstrated that 'normal' (typical) glomeruli from two mouse strains (BALB/c and CD-1) differ in their intraglomerular connectivity (White, 1972, 1973; Weruaga *et al.*, 2000) and morphometry (Weruaga *et al.*, 1998).

Here we analyse the number, sectional size and position of the whole population of AtG, identified by means of acetylcholinesterase (AChE) histochemical staining in coronal sections from both male and female mice of the CD-1 and BALB/c strains. The aim of this study was to determine whether there are any differences between the sexes or between the two strains of mice that differ in their synaptic typology in the olfactory glomeruli. Additionally, we propose a morphological subdivision of AtG that should help to relate them to neurochemical properties and specific functions.

Material and methods

Animals

Adult male and virgin female mice (*Mus musculus*) from strains CD-1 and BALB/cByJlco (BALB/c) were obtained from Criffa SA (Barcelona, Spain). A total of 20 animals, five per group (sex and strain), were employed for this study. They were housed in the animal facility of the University of Salamanca at constant humidity and temperature, with a 12/12 h light cycle and fed *ad libitum* (Rodent Toxicology Diet; B&K Universal SJ, Barcelona, Spain). All animals were deeply anaesthetized with chloral hydrate (5% w/v in saline, 10 µl/g body wt i.p.) and killed by perfusion. Manipulations of the mice were performed within the prescriptions of the directives of the European Communities Council (86/609/EEC) and current Spanish legislation (BOE 67/8509-12, 1988) for animal care and experimentation.

Tissue preparation

After the induction of anaesthesia, animals were perfused through the aorta, first with heparinized saline (5 IU/ml; Byk Leo, Madrid, Spain) and then with a solution containing 4% (w/v) depolymerized paraformaldehyde and 0.2% (w/v) picric acid in 0.1 M phosphate buffer, pH 7.4, (PB) for 20 min. The brains were dissected and post-fixed in the same solution for 2 h at 4°C. For cryoprotection, brain blocks were trimmed, rinsed in PB overnight and immersed in 30% (w/v) sucrose in PB until they sank. Thirty micrometres thick coronal sections were obtained with a cryostat (Leica Jung, Nussloch, Germany) and collected in order in cold PB in 24-well plates. This method allows easy reconstruction of six series for free-floating procedures. Stained sections were mounted on gelatine-coated slides, dehydrated through graded alcohol, cleared with xylene and coverslipped with Entellan.

AChE histochemistry

All serial sections were processed for AChE staining as described elsewhere (Crespo *et al.*, 1995). Briefly, they were washed in 0.1 M sodium acetate buffer and placed under shaking in freshly prepared incubation medium at room temperature for 30 min. The incubation medium consisted of 1.7 mM acetylthiocholine iodide (Sigma, St Louis, MO), 0.49 mM sodium citrate, 2.9 mM cupric sulphate, 1.25 mM potassium ferricyanide and 0.2 mM ethopropazine hydrochloride (Sigma), an inhibitor of non-specific cholinesterases, in 0.1 M acetate buffer, pH 6.0.

After rinsing with 0.2 M Tris-HCl buffer, pH 7.6, three times for 10 min each time, AChE activity was visualized using 0.0125% (w/v) 3,3'-diaminobenzidine (DAB) and 0.003% (v/v) hydrogen peroxide in Tris buffer under microscope control. The reaction was stopped by rinsing the sections with Tris buffer (3 × 5 min) and distilled water (2 × 5 min).

Two controls were carried out in three additional CD-1 mice: (i) omission of the substrate acetylthiocholine iodide; (ii) addition of 10⁻⁵ M BW284C51, an inhibitor of AChE activity (Carson and Burd, 1980). No residual activity was observed in either case.

Quantitative analysis

AtG were identified as oval shaped structures with very dense AChE labelling (for details see Results). Each AtG was followed along 2–3 serial sections. Using a camera lucida and a ×40 planapochromatic objective, the maximum areas of AtG were delimited in 30 µm coronal sections. Each glomerulus was identified as type I, II or III, depending on its location in the olfactory bulb (see Results). Sectional areas and maximum diameters of all AtG in all animals studied were measured using a semi-automatic image analysis system (MOP Videoplan; Kontron, Munich, Germany). The total number and the number of each type of AtG were compared between groups using the multi-factorial ANOVA test, with sex and strain as factors. The two planimetric parameters were compared independently using an ANOVA test, the type of glomeruli, sex and strain being the dependent factors. The significance of each test was considered as moderate when 0.05 > *P* ≥ 0.01, high when 0.01 > *P* ≥ 0.001 and very high when *P* < 0.001.

Results

AChE histochemistry in the mouse olfactory bulb revealed a similar pattern to that observed in rat, which is well documented (Le Jeune and Jourdan, 1991). Briefly, in the adult rodent olfactory bulb few AChE-positive cell bodies can be seen distributed across the whole of the surface of the coronal sections, but mostly in the granule cell layer. In addition, positive fibres were heavily stained and were found throughout the volume of the olfactory bulb. These fibres were dense in the inner plexiform layer and surrounded, and

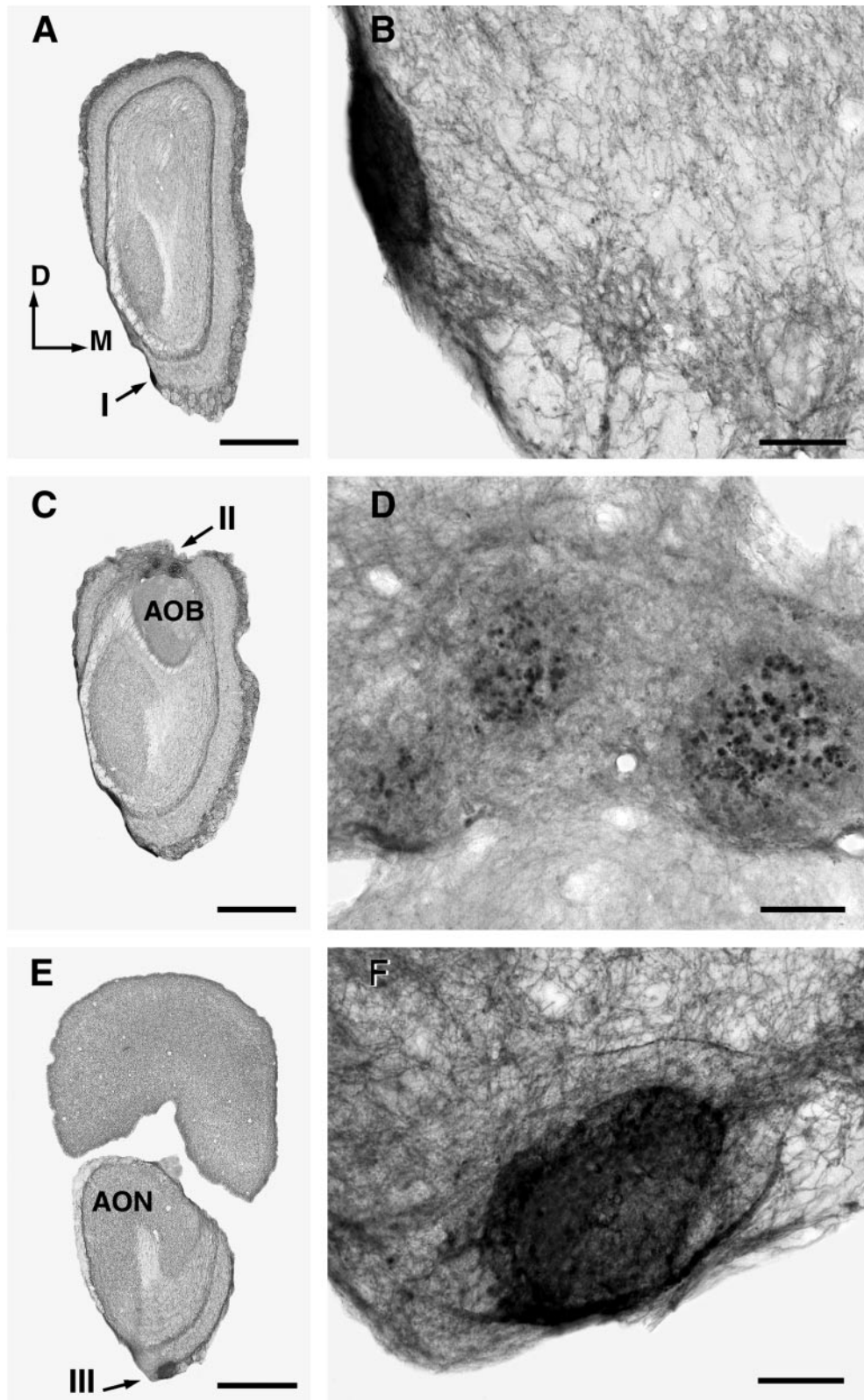


Figure 1 Atypical glomeruli in the mouse olfactory bulb stained with AChE histochemistry in 30 μ m thick coronal sections. These structures are clearly distinguishable because of their strong reactivity. Type I AtG are the rostral-most ones and are located laterally (**A, B**). Type II AtG are located dorsally (**C, D**), close to the AOB. They are less reactive and are more rounded in comparison with the other AtG subtypes. Type III AtG are caudal-most and are located in the medial border (**E, F**). D, dorsal; M, medial; AOB, accessory olfactory bulb; AON, anterior olfactory nucleus. Scale bars: (A), (C) and (E), 1 mm; (B), (D) and (F), 100 μ m.

Table 1 Number of atypical glomeruli in the mouse olfactory bulb

Mouse strain	Type	Male	Female
CD-1	total	20.80 ± 0.58	15.40 ± 1.03
	I	6.20 ± 0.45	5.00 ± 0.63
	II	11.00 ± 1.58	8.20 ± 0.20
	III	3.60 ± 0.40	2.00 ± 0.45
BALB/c	total	14.00 ± 0.32	16.20 ± 0.20
	I	5.60 ± 0.40	5.80 ± 0.66
	II	6.20 ± 0.66	6.60 ± 0.51
	III	2.20 ± 0.37	3.80 ± 0.58

Numbers are mean (± SEM) AtG in the olfactory bulb in two mouse strains (CD-1 and BALB/c) according to sex.

in some cases entered, the ‘typical’ olfactory glomeruli. AtG were readily distinguished because they were literally full of AChE-positive fibres and, in most cases, the DAB product precipitated to form opaque structures (Figure 1). This general pattern was similar in males and in females and in both strains studied, CD-1 and BALB/c.

AtG were located caudally in the olfactory bulb and there were only ~13–22 per bulb. They were slightly larger and usually had an oval shape, whereas typical ones were round. We divided the AtG into three groups. Group I consisted of 4–7 glomeruli ($95.1 \pm 2.3 \mu\text{m}$ maximum diameter) located in the lateral border of the olfactory bulb and was the rostral-most population. In coronal sections, type I AtG appeared at the same level as the anterior olfactory nucleus or even more rostrally. They had a very flattened shape (Figure 1A and B). In group II we included the AtG (4–13 per bulb) visualized in the dorsal half of the MOB, close to the AOB. In this region olfactory glomeruli were very sparse and most of them were atypical. This group contained the smallest AtG ($82 \pm 2.0 \mu\text{m}$ maximum diameter) and they had a more rounded shape. Interestingly, these type II AtG displayed a less dense AChE-positive innervation in comparison with types I and III, but always much denser than typical glomeruli (Figure 1C and D). Finally, type III AtG were very sparse (1–5 per bulb). They were located in the caudal-most level of the olfactory bulb and were always found on its medial or ventromedial border. Their shapes were intermediate between those of type I (flattened) and type II (round) AtG (Figure 1E and F).

The mean numbers of AtG are shown in Table 1 and charts comparing this parameter between sexes and strains and separately for each type of glomeruli are depicted in Figure 2. The multifactorial ANOVA test revealed highly significant differences between the two strains ($P = 0.0002$) and moderately significant between the sexes ($P = 0.0202$). While in the CD-1 strain males had more AtG than females (20.80 ± 0.58 versus 15.40 ± 1.03), in BALB/c mice males had fewer glomeruli than females (14.00 ± 0.32 versus 16.20 ± 0.20). On performing this test separately for each group of

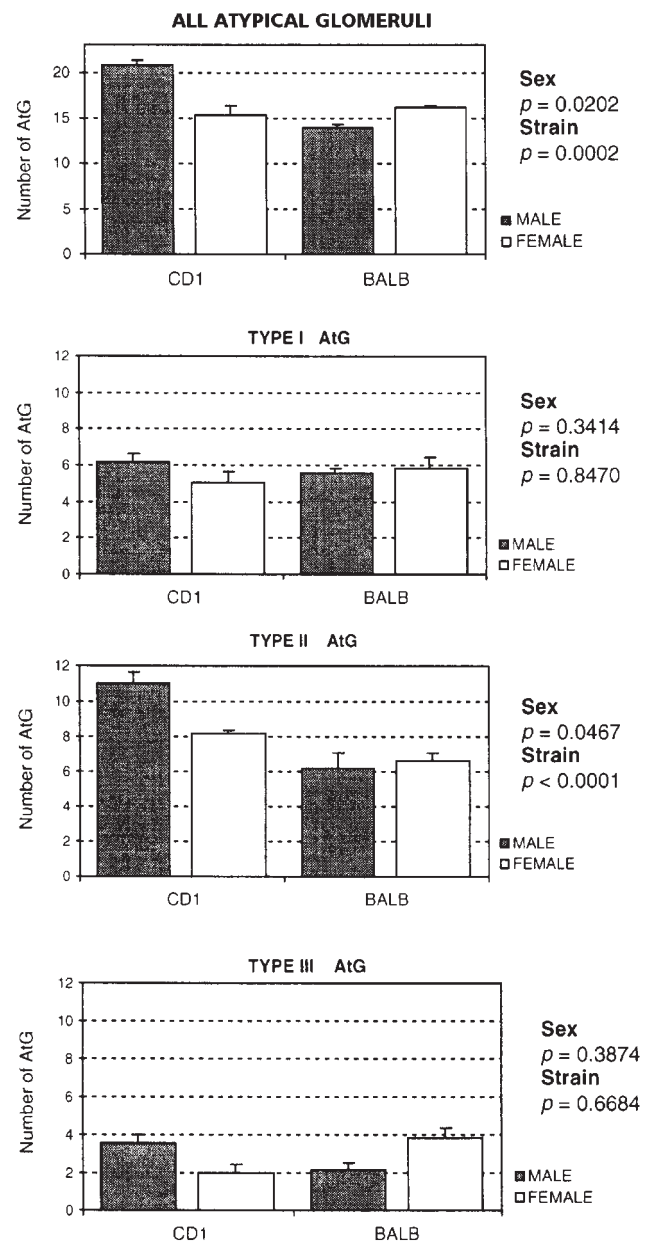


Figure 2 The graphs indicate the number of AtG (mean ± SEM) by strain (CD-1 and BALB/c) and by sex. At the right of each chart the significance for differences between sexes and strains is indicated, as demonstrated by multifactorial ANOVA. The chart at the top shows the results when all AtG are studied and the other three correspond to AtG types I, II and III, respectively.

AtG, we found that the differences both between sexes ($P = 0.0467$) and between strains ($P < 0.0001$) were largely due to group II, since ANOVA could not demonstrate significant differences for these factors in the other two types of AtG, types I and III (Figure 2). Examples of the exact location of AtG from male CD-1 (the group with the highest number) and female BALB/c mice can be seen in Figures 3 and 4.

Tables 2 and 3 show values for the sectional areas and maximum diameters of AtG, respectively. The charts cor-

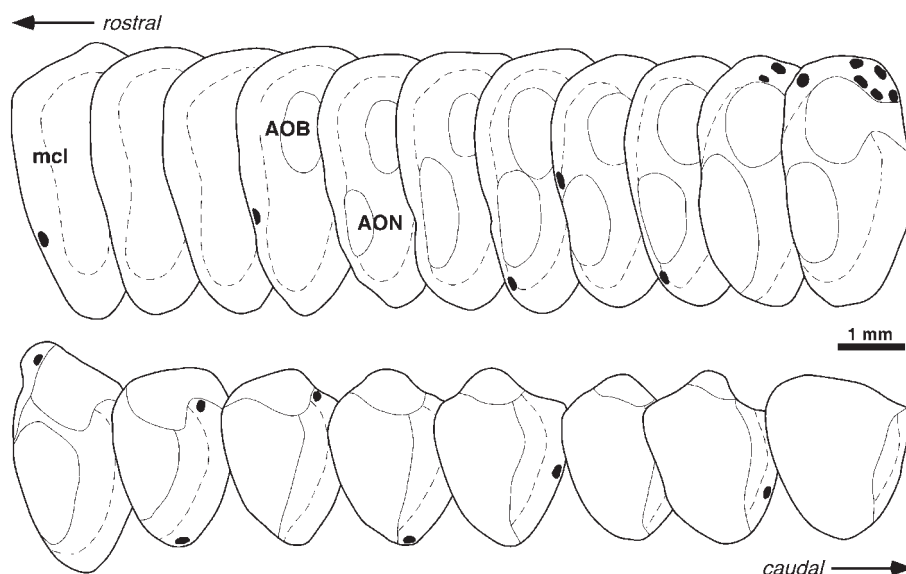


Figure 3 Schematic drawing of serial coronal sections of the mouse olfactory bulb in which all AtG are shown as black structures, as seen in their maximum sectional areas. Each section is 60 μm distant from its neighbour. Male CD-1 mouse sections are represented, which correspond to the group with more AtG. Compare with the example in Figure 4 (female BALB/c). More rostral sections are not depicted since they do not show AtG. AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; mcl, mitral cell layer.

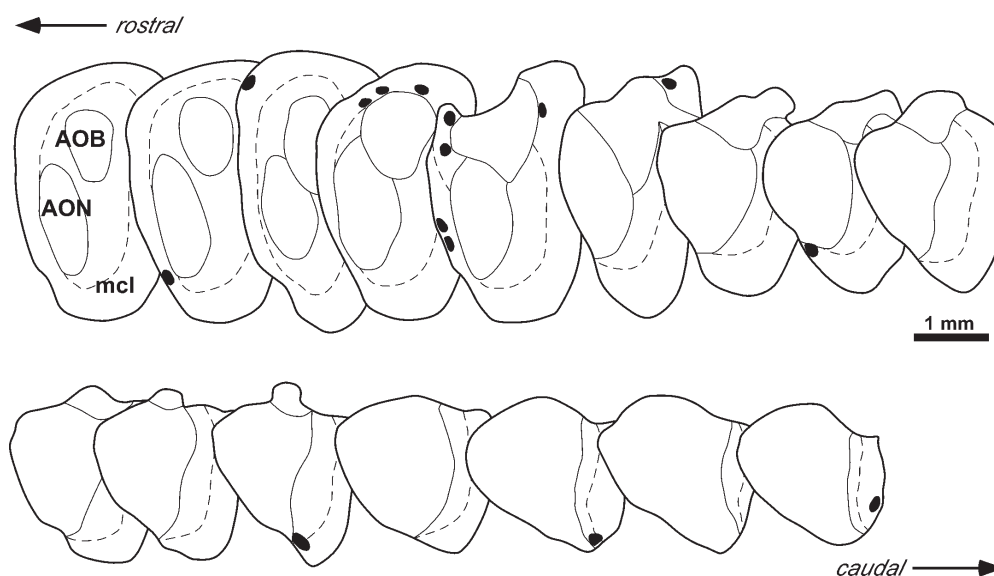


Figure 4 Schematic drawing of serial coronal sections of the mouse olfactory bulb in which all AtG are shown as black structures, as seen in their maximum sectional areas. Each section is 60 μm distant from its neighbour. In this case female BALB/c mouse sections are represented. Compare with the example in Figure 3, corresponding to CD-1 males. AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; mcl, mitral cell layer.

responding to those data are shown in Figures 5 and 6, respectively. The multifactorial ANOVA test revealed that there were highly significant differences among all three types of glomeruli only with respect to the maximum diameter ($P = 0.0002$), but not the sectional area ($P = 0.0703$) of AtG. Type I glomeruli had the largest maximum diameter ($95.1 \pm 2.3 \mu\text{m}$), followed by type III ($88.0 \pm 2.6 \mu\text{m}$) and then type II ($82.1 \pm 2.0 \mu\text{m}$). Since no differences were found in their sectional areas, the three groups of

AtG must therefore have different shapes: while AtG types I and III are flattened, type II are more spherical. Moreover, AtG type II were less reactive structures in comparison with the other types, indicating that these dorsal AtG differ both qualitatively and quantitatively from the rest.

However, highly significant differences were observed with respect to both variables (sectional area and maximum diameter) between the strains ($P < 0.0001$ for both area and maximum diameter) and between the sexes ($P = 0.0002$ for

Table 2 Sectional areas of atypical glomeruli in the mouse olfactory bulb

Mouse strain	Type	Male	Female
CD-1	all	3313.8 ± 148.0	2855.2 ± 161.1
	I	3297.5 ± 259.6	3280.4 ± 184.6
	II	3323.4 ± 213.0	2758.9 ± 269.6
	III	3312.4 ± 348.9	2247.5 ± 170.6
BALB/c	all	5023.6 ± 323.9	3811.6 ± 159.5
	I	4055.8 ± 360.1	3597.2 ± 281.6
	II	6062.0 ± 496.2	3876.5 ± 263.2
	III	4560.5 ± 1034.8	4026.4 ± 270.2

Numbers represent mean (\pm SEM) sectional areas (μm^2) of AtG in the olfactory bulb in two mouse strains (CD-1 and BALB/c) according to sex.

Table 3 Maximum diameters of atypical glomeruli in the mouse olfactory bulb

Mouse strain	Type	Male	Female
CD-1	all	83.3 ± 2.2	77.2 ± 2.6
	I	90.0 ± 4.3	90.1 ± 3.0
	II	80.0 ± 3.0	69.7 ± 4.0
	III	78.8 ± 4.1	76.3 ± 4.5
BALB/c	all	103.9 ± 3.4	88.7 ± 2.0
	I	104.9 ± 6.2	95.3 ± 3.7
	II	103.5 ± 4.7	79.2 ± 2.4
	III	102.4 ± 7.3	95.2 ± 3.3

Numbers represent mean (\pm SEM) maximum diameters (μm) of AtG in the olfactory bulb in two mouse strains (CD-1 and BALB/c) according to sex.

area and $P = 0.0009$ for diameter). As shown in Tables 2 and 3, the BALB/c mice and males had larger AtG glomeruli with respect to both sectional area and maximum diameter. Further analyses of the three different types of AtG separately indicated that AtG type II are the most distinct between the groups (sex or strain), since very highly significant differences ($P < 0.0001$) for sex and strain with respect to both sectional area and maximum diameter were found (Figures 5 and 6). Concerning the other two groups of AtG, the statistical analyses demonstrated differences between the two strains studied: highly significant ($P = 0.0032$) and very highly significant ($P < 0.0001$) for sectional area and maximum diameter of AtG type III, respectively, and moderately significant for maximum diameter of AtG type I ($P = 0.0287$).

Discussion

The main conclusion of the present study is that sexual dimorphism exists in a specific set of glomeruli of the MOB, an aspect that has hitherto not been reported. Furthermore, a divergence can also be detected between the MOB of the

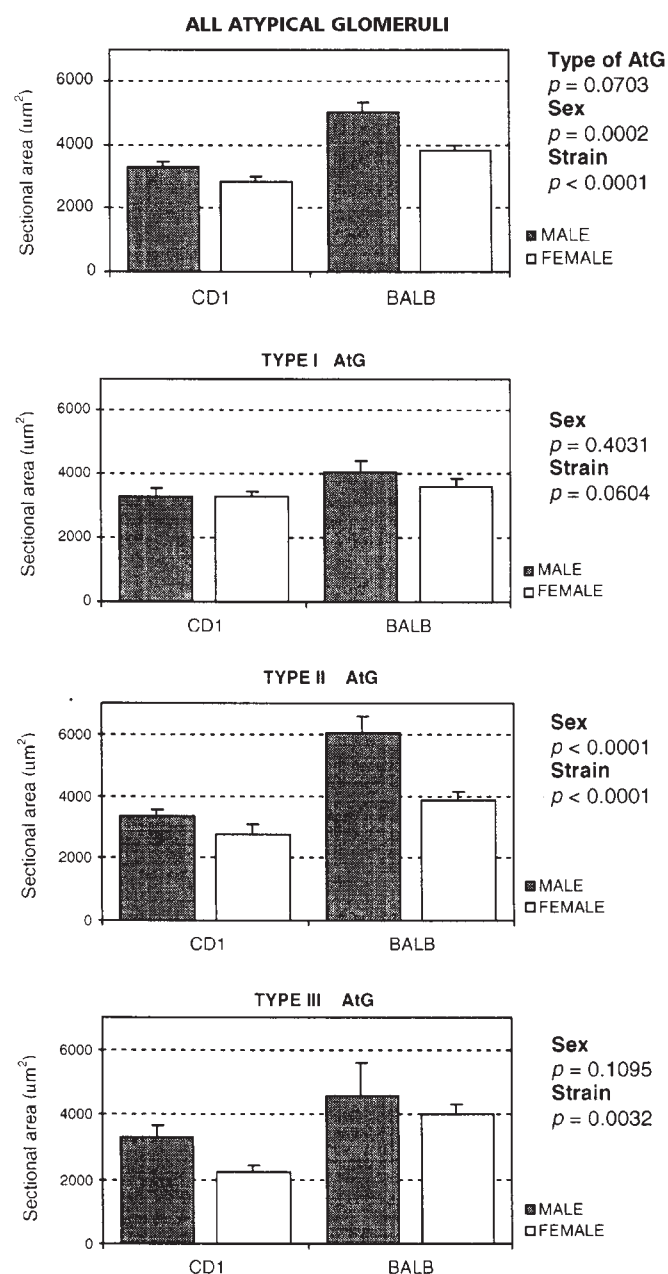


Figure 5 The graphs indicate the sectional areas of AtG (mean \pm SEM) by strain (CD-1 and BALB/c) and by sex. To the right of each chart the significance for differences between sexes and strains is indicated, as demonstrated by multifactorial ANOVA. The chart at the top shows the results when all AtG are studied; the significance for differences between the different types of glomeruli is also depicted at the right. The other three charts correspond to AtG types I, II and III, respectively.

two mouse strains studied, CD-1 and BALB/c. In all these groups of animals AtG had similar locations in the caudal half of the MOB and could be divided into three groups that differ in their shape and staining intensity. Our general description of AtG agrees with previous reports on the MOB of the mouse [as yet undefined as 'AtG' (Carson and Burd, 1980)] and in the rat, in which they have been defined

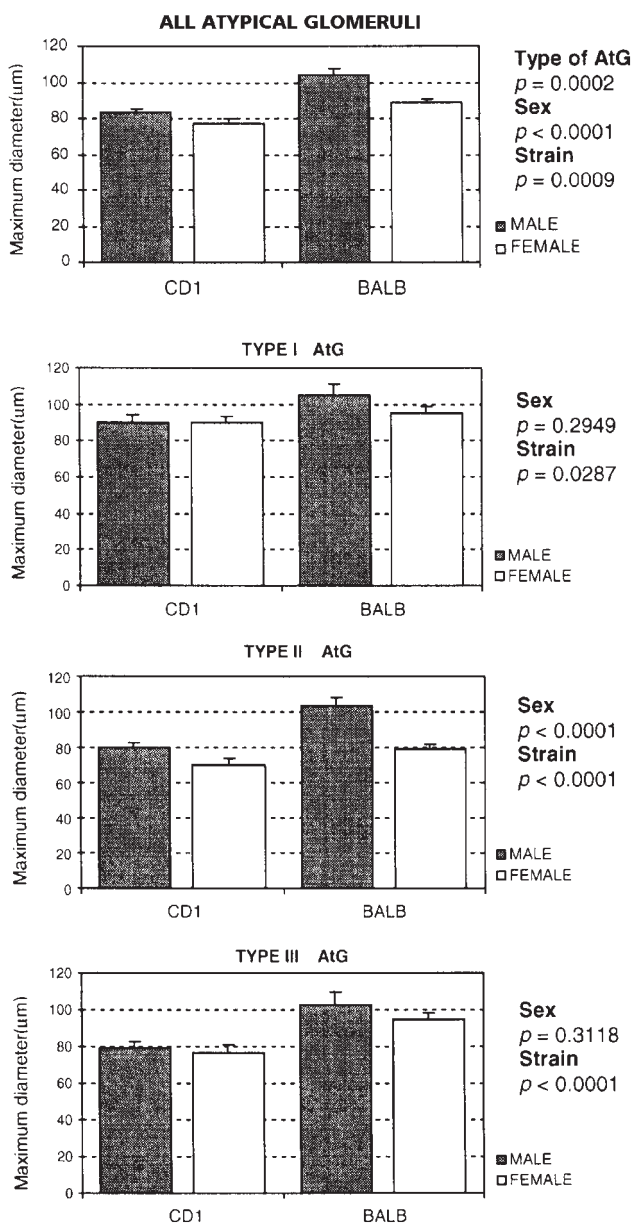


Figure 6 The graphics indicate the maximum diameters of AtG (mean \pm SEM) by strain (CD-1 and BALB/c) and by sex. To the right of each chart, the significance for differences between sexes and strains is indicated, as demonstrated by multifactorial ANOVA. The chart at the top shows the results when all AtG are studied; the significance for differences between the different types of glomeruli is also depicted to the right. The other three charts correspond to AtG types I, II and III, respectively.

by both AChE histochemistry and choline acetyltransferase immunohistochemistry (Zheng *et al.*, 1987; Le Jeune and Jourdan, 1991; Crespo *et al.*, 1996) (personal observations). However, we clearly noted that in the mouse type II AtG are more abundant than in the rat (Carson and Burd, 1980).

The size of the AtG clearly differs between the strains studied: they are larger in the BALB/c olfactory bulbs. The two mouse strains differ in their intraglomerular synaptol-

ogy: while direct synapses from olfactory axons to dendrites of periglomerular cells are found in abundance in CD-1 bulbs, they are practically absent in the MOB of BALB/c mice (White, 1972, 1973; Weruaga *et al.*, 2000). In a previous study using the same mouse strains we found that both groups of animals also differed in the size of their normal (typical) glomeruli, but in the opposite sense: typical glomeruli were larger in CD-1 mice (Weruaga *et al.*, 1998) while AtG were larger in the BALB/c strain (present study).

Not only these morphological characteristics but also neurochemical features indicate that the AtG are indeed different from the rest of the glomeruli of the main olfactory projection. Some divergences have been found in the juxtglomerular neurons around AtG in comparison with normal (typical) ones, such as a transient higher density of neurons expressing neurotensin (Matsutani *et al.*, 1988). Also, the AtG are surrounded by a lower density of neurons expressing either NADPH diaphorase histochemistry (Crespo *et al.*, 1996) or neurocalcin (Crespo *et al.*, 1997a) and a higher density of tyrosine hydroxylase-positive neurons (Matsutani *et al.*, 1988; Crespo *et al.*, 1997b). These data reinforce the idea that AtG must be a specialized olfactory subsystem, with different morpho-functional units, and suggest a different glomerular ultrastructure in the two mouse strains studied, as occurs in typical glomeruli.

The differences in the total number of AtG between sexes is also statistically significant, but this divergence shows a strong interaction with strain, i.e. while male mice have more AtG as compared with females in the CD-1 strain, these structures are more abundant in BALB/c females when compared with BALB/c males. As between strains, this sexual divergence mainly concerns type II AtG. Moreover, the size of AtG (as seen using as parameters their area and maximum diameter) presents a more clear sexual dimorphism, since males have larger AtG in both strains studied. Again, this sexual divergence mainly concerns type II AtG (see Figures 5 and 6), whereas types I and III are considerably more homogeneous. Previously, sexual dimorphism in olfactory function has only been detected in the accessory olfactory system, which is mainly related to pheromone information and reproductive behaviour (Segovia and Guillamón, 1993). This divergence between the sexes has been detected not only in the vomeronasal organ, but also in structures that process vomeronasal input: the AOB, the bed nucleus of the accessory olfactory tract, the bed nucleus of the stria terminalis, the medial preoptic area, the ventromedial hypothalamic nucleus, the ventral region of the premammillary nucleus and the medial amygdaloid nucleus (Collado *et al.*, 1990; Segovia and Guillamón, 1993). In the AOB quantitative studies have demonstrated that males have higher values (in comparison with females) in the somatic area, the degree of branching and the length of dendrites of mitral cells, and these aspects are reversed in both experimentally androgenized females and orchidectomized males (Caminero *et al.*, 1991). The differences

described here for AtG demonstrate for the first time sexual dimorphism in the main olfactory projection. This sexual divergence mainly concerns a specific subset of AtG.

Type II AtG are located in the same region as the cluster of glomeruli termed the MGC (or macroglomerular complex). This group of glomeruli has been proposed as a set of olfactory units related to suckling behaviour (Teicher *et al.*, 1980; Greer *et al.*, 1982; Yagi *et al.*, 1993), however other evidence questions this theory since after lesioning the postero-dorsal bulb the ability of newborn animals to suckle is unaltered (Hudson and Distel, 1987; Risser and Slotnick, 1987). Working with the rat, Shinoda and co-workers reported that a group of interconnected glomeruli around the posterior margin of the MOB express the human placental antigen X-P₂ and form a necklace-like structure around the AOB (Shinoda *et al.*, 1989, 1990). However, these authors (Shinoda *et al.*, 1993) suggested that the X-P₂-expressing glomeruli are not the same as the strongly stained AChE-expressing glomeruli, but only co-localize with some slightly stained AChE structures or may even correspond to AChE-negative ones. Nonetheless, both groups of glomeruli (X-P₂-positive and AtG) seem to be in clear apposition or even interconnected and, at least in the rat, some of them probably correspond to the suckling behaviour-related glomeruli (Shinoda *et al.*, 1993). Additionally, Juilfs and co-workers (Juilfs *et al.*, 1997) have shown that a subset of specific olfactory receptor neurons, and their glomeruli, utilize an unusual cGMP signal transduction pathway (identified by means of the immunohistochemical localization of cGMP-stimulated phosphodiesterase and guanylate cyclase D) and that these glomeruli may correspond to the necklace olfactory glomeruli described by Shinoda *et al.* (Shinoda *et al.*, 1990, 1993). It is likely that olfactory receptor neurons innervating these glomeruli would present a response profile different from that of the rest of non-necklace neurons (Juilfs *et al.*, 1997; Ring *et al.*, 1997), hence triggering a distinct response in the mitral cells they synapse onto.

In addition, two monoclonal antibodies, 2C6 and mAb213, label two discrete subsets of olfactory receptor neurons and their corresponding glomeruli, which occupy reproducible positions along the posterior margin of the MOB and partially overlap the MGC (Ring *et al.*, 1997). 2C6-positive glomeruli do not show an especially dense AChE activity. In contrast, not all but many mAb213-positive glomeruli co-localize with densely AChE-stained structures (AtG) and at least one of them may belong to the MGC, although not all AtG are mAb213-immunopositive (Ring *et al.*, 1997). These data indicate that AtG are not only morphologically (size, number and degree of staining) heterogeneous, but also neurochemically complex. Comparison of these subsets of AtG between sexes could shed further light on these specific sexual divergences in olfaction.

Taking all these anatomical data together, it is clear that

the 'necklace' or dorso-caudal part of the olfactory system is organized quite differently from the rest of the MOB and that the glomeruli of this region are neurochemically very heterogeneous. Considering type II AtG (those lying in the MGC region), our results indicate that these morpho-functional units display sexual dimorphism as regards their number and size, as well as differences between two synaptically distinct olfactory bulbs. While these AtG express a strong AChE activity, autoradiographic studies suggest that these structures are mainly cholinceptive (Le Jeune *et al.*, 1996), suggesting a differential neurochemical modulation of this group of glomeruli as compared with the typical ones. Because AtG type II partially overlap with glomeruli which may be linked to the suckling function, it may also be inferred that this specific behaviour in the mouse could be modulated by cholinceptive afferents to the main olfactory projections and is probably stronger in males.

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References

- Caminero, A.A., Segovia, S. and Guillamón, A. (1991) *Sexual dimorphism in accessory olfactory bulb mitral cells: a quantitative Golgi study*. Neuroscience, 45, 663–670.
- Carson, K.A. and Burd, G.D. (1980) *Localization of acetylcholinesterase in the main and accessory olfactory bulbs of the mouse by light and electron microscopic histochemistry*. J. Comp. Neurol., 191, 353–371.
- Collado, P., Guillamón, A., Valencia, A. and Segovia, S. (1990) *Sexual dimorphism in the bed nucleus of the accessory olfactory tract in the rat*. Devl Brain Res., 56, 263–268.
- Crespo, C., Arévalo, R., Briñón, J.G., Porteros, A., Bravo, I.G., Aijón, J. and Alonso, J.R. (1995) *Colocalization of NADPH-diaphorase and acetylcholinesterase in the rat olfactory bulb*. J. Chem. Neuroanat., 9, 207–216.
- Crespo, C., Porteros, A., Arévalo, R., Briñón, J.G., Aijón, J. and Alonso, J.R. (1996) *Segregated distribution of nitric oxide synthase-positive cells in the periglomerular region of typical and atypical olfactory glomeruli*. Neurosci. Lett., 205, 149–152.
- Crespo, C., Alonso, J.R., Briñón, J.G., Weruaga, E., Porteros, A., Arévalo, R. and Aijón, J. (1997a) *Calcium-binding proteins in the periglomerular region of typical and atypical olfactory glomeruli*. Brain Res., 745, 293–302.
- Crespo, C., Jorge, R.J., Alonso, J.R., Briñón, J.G., Arévalo, R. and Aijón, J. (1997b) *Segregated distribution of TH-immunoreactivity in olfactory glomeruli*. NeuroReport, 8, 2311–2316.
- Eisthen, H.L. (1997) *Evolution of vertebrate olfactory systems*. Brain Behav. Evol., 50, 222–233.
- Greer, C.A., Stewart, W.B., Teicher, M.H. and Shepherd, G.M. (1982) *Functional development of the olfactory bulb and a unique glomerular complex in the neonatal rat*. J. Neurosci., 2, 1744–1759.

- Halász, N.** (1990) The Vertebrate Olfactory System. Chemical Neuroanatomy, Function, and Development. Akadémiai Kiadó, Budapest.
- Hudson, R.** and **Distel, H.** (1987) Regional autonomy in the peripheral processing of odor signals in newborn rabbits. *Brain Res.*, 421, 85–94.
- Juilfs, D.M., Fülle, H.-J., Zhao, A.Z., Houslay, M.D., Garbers, D.L.** and **Beavo, J.A.** (1997) A subset of olfactory neurons that selectively express cGMP-stimulated phosphodiesterase (PDE2) and guanylyl cyclase-D define a unique olfactory signal transduction pathway. *Proc. Natl Acad. Sci. USA*, 94, 3388–3395.
- Le Jeune, H.** and **Jourdan, F.** (1991) Postnatal development of cholinergic markers in the rat olfactory bulb: a histochemical and immunocytochemical study. *J. Comp. Neurol.*, 314, 383–395.
- Le Jeune, H.** and **Jourdan, F.** (1993) Cholinergic innervation of olfactory glomeruli in the rat: an ultrastructural immunocytochemical study. *J. Comp. Neurol.*, 336, 279–292.
- Le Jeune, H., Aubert, I., Jourdan, F.** and **Quirion, R.** (1996) Developmental profiles of various cholinergic markers in the rat main olfactory bulb using quantitative autoradiography. *J. Comp. Neurol.*, 373, 433–450.
- Matsutani, S., Senba, E.** and **Tohyama, M.** (1988) Neuropeptide- and neurotransmitter-related immunoreactivities in the developing rat olfactory bulb. *J. Comp. Neurol.*, 272, 331–342.
- Mori, K.** and **Yoshihara, Y.** (1995) Molecular recognition and olfactory processing in the mammalian olfactory system. *Prog. Neurobiol.*, 45, 585–620.
- Pérez-Laso, C., Valencia, A., Rodríguez-Zafra, M., Calés, J.M., Guillamón, A.** and **Segovia, S.** (1994) Perinatal administration of Diazepam alters sexual dimorphism in the rat accessory olfactory bulb. *Brain Res.*, 634, 1–6.
- Ring, G., Mezza, R.C.** and **Schwob, J.E.** (1997) Immunohistochemical identification of discrete subsets of rat olfactory neurons and the glomeruli that they innervate. *J. Comp. Neurol.*, 388, 415–434.
- Risser, J.M.** and **Slotnick, B.M.** (1987) Nipple attachment and survival in neonatal olfactory bulbectomized rats. *Physiol. Behav.*, 40, 545–549.
- Segovia, S.** and **Guillamón, A.** (1993) Sexual dimorphism in the vomeronasal pathway and sex differences in reproductive behaviors. *Brain Res. Rev.*, 18, 51–74.
- Segovia, S., Pérez-Laso, C., Rodríguez-Zafra, M., Calés, J.M., del Abril, A., de Blas, M.R., Collado, P., Valencia, J.M.** and **Guillamón, A.** (1991) Early postnatal Diazepam exposure alters sex differences in the rat brain. *Brain Res. Bull.*, 26, 899–907.
- Shepherd, G.M.** (1994) Discrimination of molecular signals by the olfactory receptor neurons. *Neuron*, 13, 771–790.
- Shinoda, K., Shiotani, Y.** and **Osawa, Y.** (1989) Necklace olfactory glomeruli form unique components of the rat primary olfactory system. *J. Comp. Neurol.*, 284, 362–373.
- Shinoda, K., Yagi, H., Osawa, Y.** and **Shiotani, Y.** (1990) Involvement of specific placental antigen X-P₂ in rat olfaction: an immunohistochemical study in the olfactory bulb. *J. Comp. Neurol.*, 294, 340–344.
- Shinoda, K., Ohtsuki, T., Nagano, M.** and **Okumura, T.** (1993) A possible functional necklace formed by placental antigen X-P₂-immunoreactive and intensely acetylcholinesterase-reactive (PAX/IAE) glomerular complexes in the rat olfactory bulb. *Brain Res.*, 618, 160–166.
- Teicher, M.H.** and **Blass, E.M.** (1976) Suckling in newborn rats: eliminated by nipple lavage, reinstated by pup saliva. *Science*, 193, 422–425.
- Teicher, M.H.** and **Blass, E.M.** (1978) The role of olfaction and amniotic fluid in the first suckling response of newborn albino rats. *Science*, 198, 635–636.
- Teicher, M.H., Stewart, W.B., Kauer, J.S.** and **Shepherd, G.M.** (1980) Suckling pheromone stimulation of a modified glomerular region in the developing rat olfactory bulb revealed by the 2-deoxyglucose method. *Brain Res.*, 194, 530–535.
- Valencia, A., Collado, P., Calés, J.M., Segovia, S., Pérez-Laso, C., Rodríguez-Zafra, M.** and **Guillamón, A.** (1992) Postnatal administration of dihydrotestosterone to the male rat abolishes sexual dimorphism in the accessory olfactory bulb: a volumetric study. *Devl Brain Res.*, 68, 132–135.
- Weruaga, E., Crespo, C., Porteros, A., Briñón, J.G., Arevalo, R., Aijón, J.** and **Alonso, J.R.** (1998) NADPH-diaphorase histochemistry reveals heterogeneity in the distribution of nitric oxide synthase-expressing interneurons between olfactory glomeruli in two mouse strains. *J. Neurosci. Res.*, 53, 239–250.
- Weruaga, E., Briñón, J.G., Porteros, A., Arevalo, R., Aijón, J.** and **Alonso, J.R.** (2000) Expression of neuronal nitric oxide synthase/NADPH-diaphorase during olfactory deafferentation and regeneration. *Eur. J. Neurosci.*, 12, 1177–1193.
- White, E.L.** (1972) Synaptic organization in the olfactory glomerulus of the mouse. *Brain Res.*, 37, 69–80.
- White, E.L.** (1973) Synaptic organization of the mammalian olfactory glomerulus: new findings including an intraspecific variation. *Brain Res.*, 60, 299–313.
- Yagi, T., Aizawa, S., Tokunaga, T., Shigetani, Y., Takeda, N.** and **Ikawa, Y.** (1993) A role for Fyn tyrosine kinase in the suckling behaviour of neonatal mice. *Nature*, 366, 742–745.
- Zheng, L.M.** and **Jourdan, F.** (1988) Atypical olfactory glomeruli contain original olfactory axon terminals: an ultrastructural horseradish peroxidase study in the rat. *Neuroscience*, 26, 367–378.
- Zheng, L.M., Ravel, N.** and **Jourdan, F.** (1987) Topography of centrifugal acetylcholinesterase-positive fibres in the olfactory bulb of the rat: evidence for original projections in atypical glomeruli. *Neuroscience*, 23, 1083–1093.

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