A standardized model for the anatomical division of the rodent olfactory bulb

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SUMMARY

The present study reviews the anatomy of the rodent olfactory bulb and proposes its subdivision along the rostrocaudal axis into five equidistant rostrocaudal levels and the definition of eight topographic locations on each level. This subdivision enables the study of analogous regions when comparing different developmental stages, different experimental conditions or different closely-related species. As examples, we analyze the results for rats and mice describing the patterns of Nissl staining and NADPH-diaphorase histochemical labelling in the olfactory bulb. Different experimental conditions in which the olfactory bulb undergoes size changes are also discussed.

Key Words: Histochemistry - Interspecies comparison - Olfaction - Olfactory deafferentation.

INTRODUCTION

Most sensory systems, including olfaction, have undergone fairly modest changes during the course of vertebrate phylogeny, probably because of the importance of information about smells for the survival of the individual and the species (see Eisthen, 1997). The glomeruli of the olfactory bulb are among the unique and highly interesting structural and functional features of this chemosensory neural system that have persisted throughout the course of evolution, being present in the olfactory system of both invertebrates and vertebrates (Allison, 1953; Andres, 1970; Hildebrand and Montague, 1986; Finger, 1988; Meisami, 1990).

For several reasons the olfactory system is specially indicated for neuronal plasticity studies: a) the olfactory bulb is a laminar (allocortex) brain region in which experimental changes are readily detected, b) it is an encephalic region whose synaptic circuitry is well known, c) the neurochemical phenotypes of the different neural populations have been described in considerable detail, d) the system maintains an intrinsic dynamic organization due to the continuous turnover of the peripheral afferences along the whole of the life span, and e) these peripheral afferences can be experimentally manipulated with relative ease (Brunjes, 1994).

Although the above situation has resulted in a large number of studies on the anatomy, cytoarchitecture, synaptology, and neurochemistry of the main olfactory bulb (OB), in most cases, the descriptions of these characteristics are related only to the central area of the OB or to an "idealized" olfactory bulb, under the incorrect assumption that the OB is a uniform encephalic region along the rostrocaudal, dorsoventral or lateromedial axes.

The aim of this article is to establish a model for the anatomical division of the OB in order to obtain similar and comparable patterns of this brain region for morphological studies on related species, different developmental stages, or after experimental treatments. In particular, we describe methods to achieve a correct anatomical representation along the spatial co-ordinates of the rodent OB.

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Materials and Methods

Five female mice (*Mus musculus*) of each BALB/cByJIco, CD-1, and C57 strains and five female Wistar rats (*Rattus norvegicus*) were obtained from Criffa S.A., Barcelona, Spain. They were maintained in the animal facilities of the University of Salamanca at constant humidity and temperature and under 12/12 hour light cycle, following the directives of the European Communities Council (86/609/EEC) and the Spanish legislation (BOE 67/8509-12, 1988) for animal care and experimentation. At ten weeks of age, animals were deeply anaesthetized with chloral hydrate (4% w/v in saline; i.p. 10 μl.g^{-1} b/w) and sacrificed by perfusion via the ascending aorta, first with heparinized saline (5 I.U.·ml^{-1}; Byk Leo, Madrid), and then by a solution containing 4% (w/v) depolymerized paraformaldehyde and 15% (v/v) of a saturated solution (25°C) of picric acid in 0.1 M phosphate buffer, pH 7.4, (PB) for 20 min. The perfusion flow rate was 10 ml·min^{-1} for mice and 30 ml·min^{-1} for rats. Whole brains were carefully removed, trimmed in three pieces along the coronal plane with a Rodent Brain Matrix and postfixed for two hours in the perfusion solution. For cryoprotection, brain blocks were rinsed in PB overnight and immersed in 30% (w/v) sucrose in PB until they sunk. Thirty μm-thick coronal sections were obtained with a cryostat (Leica Jung, Nussloch, Germany) and collected in order in cold PB on 24-well plates. This method allows easy reconstruction of six serial sections for free-floating procedures such as histochemistry or immunohistochemistry as well as for standard neurohistological stainings. As examples, here we report the results for Nissl staining and NADPH-diaphorase histochemistry (Figs. 3, 4). For Nissl staining, a one-in-six series was postfixed for one week in buffered formaline, mounted in gelatine-coated slides, air-dried, immersed in chloroform-ethanol for four hours for defatting, stained with 0.25% (w/v) thionine at 37°C, and coverslipped with Entellan™. The remaining series were immersed in a solution containing 30% (v/v) glycerol, 30% (v/v) ethylenglycol in PB and kept at -20°C until the samples were processed.

NADPH-diaphorase histochemistry was performed as previously described (Weruaga et al, 1998). Briefly, after thoroughly rinsing in PB, free-floating sections were double incubated in the darkness, with shaking, at 37°C, in A) 1.2 mM β-NADPH (tetrasodium salt), 0.1 mM nitroblue tetrazolium (both from Sigma, St. Louis) and 0.8% (w/v) Triton X-100 in phosphate-buffered saline, pH 8.0, and B) 1.2 mM β-NADPH, 0.8 mM nitroblue tetrazolium, 0.03% (w/v) Triton X-100 in Tris·HCl buffer, pH 8.0. Between both steps, sections were thoroughly rinsed in cold PB, and

the reactions were controlled under the microscope to avoid undesirable formation of formazan crystals. Then, the stained sections were mounted onto slides, dehydrated and coverslipped as for the Nissl series.

Prior to the division of the OB along the rostrocaudal axis, we arbitrarily established landmarks in a coronal series by defining a first (rostral) section and a last (caudal) one (Fig. 1). The first section studied was defined as "the one next to that section in which the mitral cell layer is observed" (Fig. 1A,C). The last coronal studied section was "the one before to that section in which olfactory glomeruli can be observed" (Fig. 1B,D).

The total number (length of the rostrocaudal axis) of sections in each series was divided into five equidistant levels: I, rostral; II, centro-rostral; III, central; IV, centro-caudal; V, caudal. Type sections of each level were obtained according to the following expression:

$$\frac{s}{5}\left[\frac{1}{2}+n\right]$$

where s is the number of sections, and n are the natural numbers.

For the mediolateral and dorsoventral divisions, each coronal section selected was divided into 8 octants as indicated (Fig. 2). Therefore, each coronal section was divided in several topographical regions as follows: D, dorsal; DL, dorsolateral, L, lateral, VL, ventrolateral; V, ventral, VM, ventromedial, M, medial, DM, dorsomedial.

Results

After the described protocol of fixation and sectioning, a one-in-six series consisted of 15-17 (mice) or 28-32 (rats) coronal sections, depending on the intraspecies variability and minor changes in the exact positioning of the block for coronal sectioning. The general shape of the coronal sections from both species studied was similar at each of the levels selected (not shown), although rat OBs were more elongated along the dorso-ventral axis. As a general example, we describe the CD-1 mouse OB (Fig. 3). At the rostral level (I), all layers of the OB were present except the periependymary white matter, which in most cases was still absent. The general shape was triangular with vertices at the dorsal, ventral and dorsolatero-dorsal borders. Olfactory glomeruli were seen to be arranged in a single array or, very rarely, in two. At the centro-rostral level (II) all histological layers of the OB were clearly differentiated, and the perie-
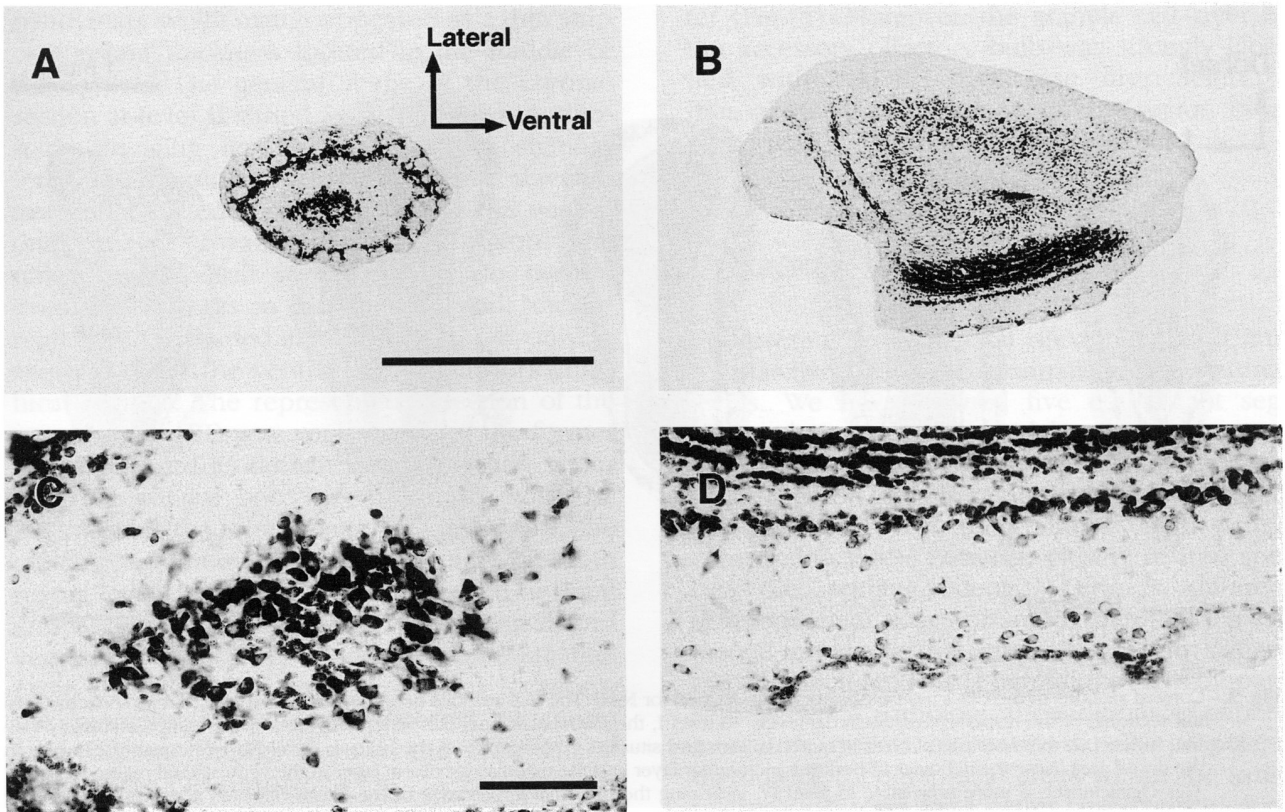
Fig. 1.– Nissl-stained coronal sections of the mouse olfactory bulb used to establish rostral and caudal boundaries in a rostrocaudal series. Sections are 30 μm-thick and correspond to a one-in-six coronal series of an olfactory bulb. **A** shows the first section in which the mitral cell layer is clearly seen, photographed at higher magnification in **C)**. The following section in the series is considered as the first (rostral) section. **B)** is the last section in which olfactory glomeruli are clearly seen (at higher magnification in **D)**, at the medial edge. The former section is considered as the last (caudal) section. Scale bar for **A** and **B** = 1 mm; for **C** and **D** = 100 μm.
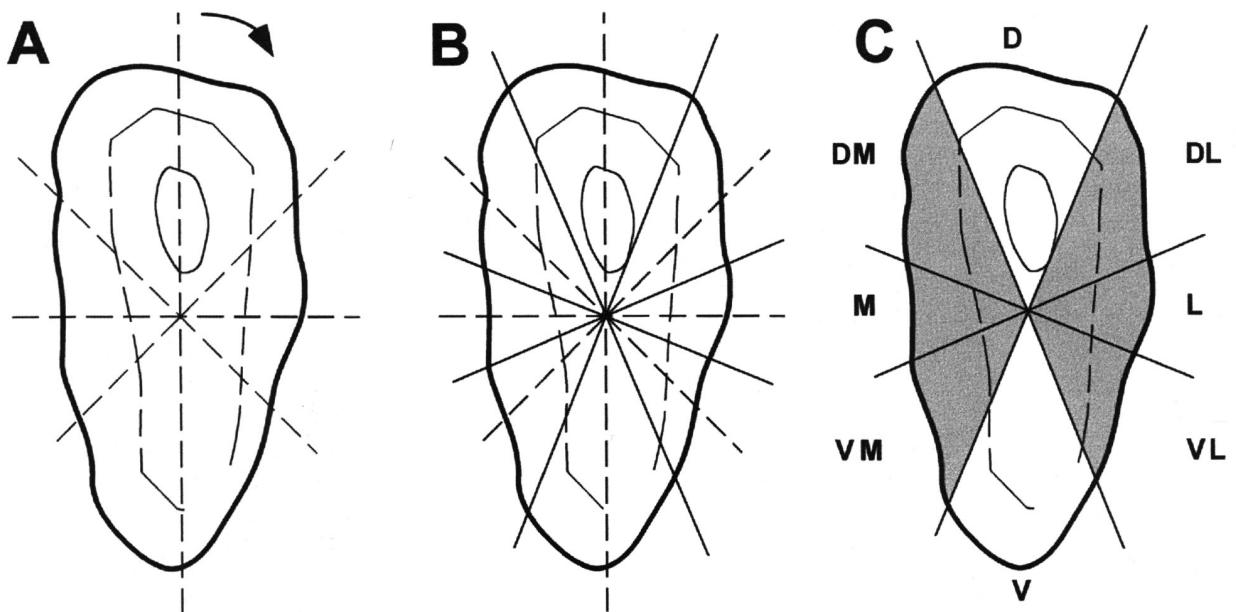

Fig. 2.– Division of an olfactory bulb coronal section into topographic regions. First, each section is divided into 8 octants as obtained by the dorsoventral and lateromedial axes and their respective bisectrices (broken lines in **A**). The resulting angles (defined by broken lines in **B**) are layouts to obtain their medial bisectrices (full lines in **B**), which establish the boundaries for the eight topographical regions. **(C)**: D, dorsal; DL, dorsolateral; L, lateral; VL, ventrolateral; V, ventral; VM, ventromedial; M, medial; DM, dorsomedial. The topographical regions can be studied separately or grouped as "medial regions" (DM + M + VM) and "lateral regions" (DL + L + VL), depicted in the scheme as shaded areas.
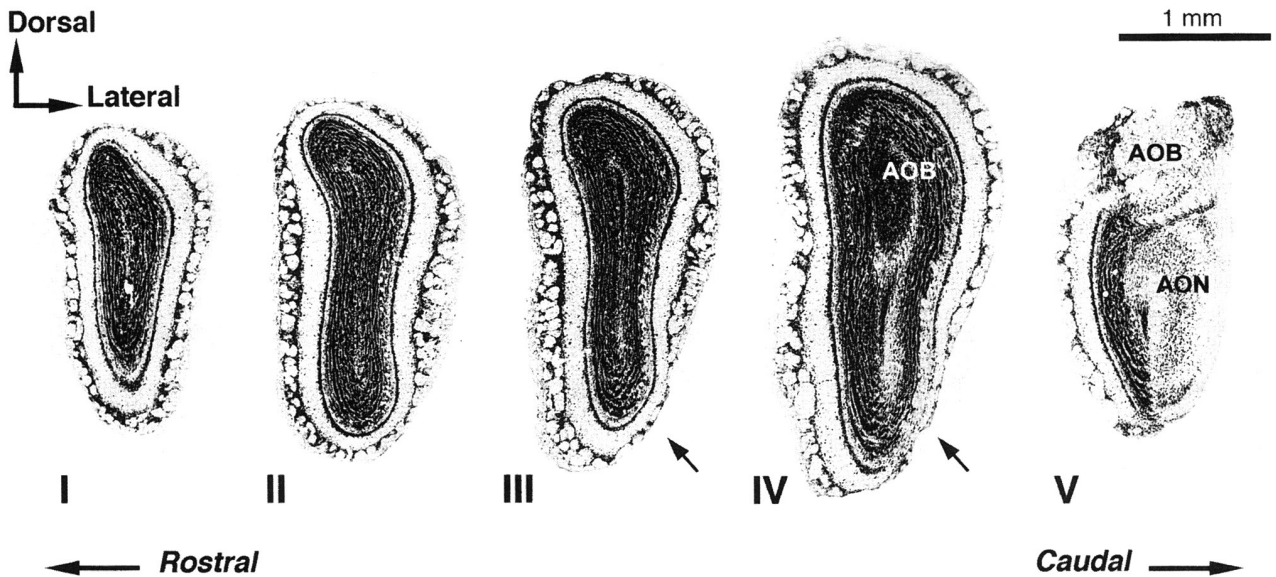
Fig. 3.– Coronal sections of the CD-1 mouse olfactory bulb stained for Nissl. The five sections noted as I-V correspond to type sections (central sections) of the respective rostrocaudal levels. In level I, the rostralmost one, the periependymary white matter is scarcely visible. In level II, this histological layer is quite uniform and situated in the centre of the bulb. In level III, white matter expands in the dorsal area. Note the tightness of both the glomerular layer and the external plexiform layer in the ventrolateral region (arrows). This characteristic is more noticeable in level IV, indicating the proximal appearance of the lateral olfactory tract and the anterior olfactory nucleus (AON). At level V, the caudalmost one, the main olfactory bulb is present only in medial areas, sharing the coronal with the accessory olfactory bulb (AOB) and the AON.
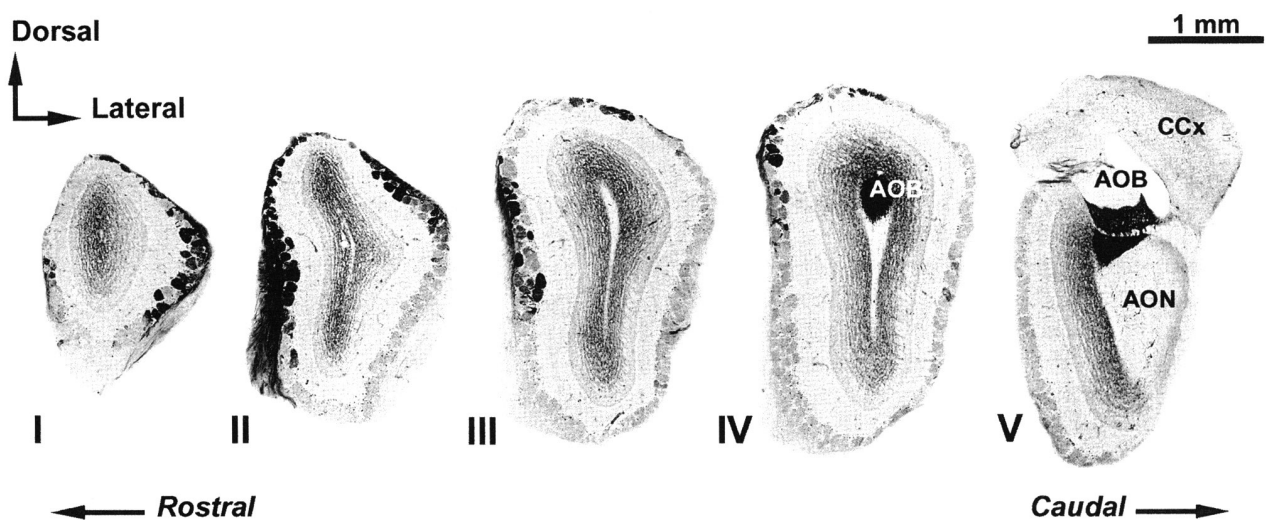

Fig. 4.– Coronal sections of the BALB/c mouse olfactory bulb stained with NADPH-diaphorase histochemistry. The five sections denoted I-V correspond to type sections (central sections) of the respective rostrocaudal levels. Apart from different labelled interneurons (not distinguishable at this magification), note the parallelism respecting the main anatomical structures with the Nissl-stained series in Figure 3. NADPH-diaphorase-positive glomeruli are distributed in different topographical regions within each level and are absent in level V. AOB: accessory olfactory bulb, AON: anterior olfactory nucleus, CCx: cerebral cortex.

pendymary white matter appeared as a thin strip of constant thickness located in the middle of the section. The general shape of the coronal section at level II varied from triangular to ellipsoid, changing its morphology to a sigmoid form, i.e. the dorsal area was slightly directed towards the medial boundary while the ventral one coursed towards the lateral region. The olfactory glomeruli were disposed in two or even three arrays in the "lateral" and "medial regions" (see shaded areas in Fig. 2) but only as single rows in the ventral and dorsal topographical regions. The representative section of the central level (III) was very similar to the former but larger, and its dorsal half was clearly wider than the ventral one, in consonance with the thickness of the white matter. As in level II, the olfactory glomeruli were larger than those in rostral levels, and were disposed in two or three arrays lateral and medially. In the dorsal and ventral locations, the glomeruli were smaller. From this level (III) to more caudal locations, the glomerular layer and external plexiform layer became thinner towards the ventrolateral edge of the OB (arrows in Fig. 3). This noticeable characteristic was more pronounced within the centro-caudal level (IV) due to the eminent sectioning at this anatomical level of the lateral olfactory tract and the anterior olfactory nucleus. This latter anatomical region also began to appear close to the ventral white matter within the granule cell layer of the OB. The accessory olfactory bulb appeared literally embedded in the OB at this level: first the accessory granule cell layer in the dorsal region of the main layer and, thereafter, the plexiform and glomerular layers. The rostral level of the OB (V) was characterized by its presence only at dorsomedial, medial and ventromedial locations, the lateral areas of the sections being occupied by both the accessory olfactory bulb (dorsal) and the anterior olfactory nucleus (central and ventral).

NADPH-diaphorase staining results in a very similar pattern in rats and mice (Davis, 1991; Alonso et al., 1993; Weruaga et al., 1998). This histochemical technique, under the fixation protocol described above, accounts for neuronal nitric oxide synthase-positive elements (i.e. different types of interneurones across the glomerular and granule cell layers). However, some olfactory glomeruli are so strongly labelled that their observation is possible without the use of microscope. NADPH-diaphorase-positive glomeruli do not exhibit neuronal nitric oxide synthase immunoreactivity (see Weruaga et al., 1998), and are localized at defined topographical regions, mainly in levels I-III (Fig. 4), where they were also intermingled with negative ones. Neuropil staining was also evident in the granule cell layer, but not in the periependymary white mat-

ter (Fig. 4). Moreover, the granule cell layer of the accessory olfactory bulb was strongly labelled with NADPH-diaphorase histochemistry, thus allowing visualization of this region even better than with Nissl staining.

DISCUSSION

The present work aims at dividing the OB into standardized, easy-to-compare rostrocaudal levels. We have defined five equidistant segments along the rostrocaudal axis, but the procedure followed also offers a solution for the rapid division of this region when more levels are desired. The method allows us to define comparable levels between closely related species (i.e. within rodents), between different developmental stages or among different experimental protocols that elicit changes in size or cytoarchitecture. This is helpful for any comparisons between pathological conditions or experimental treatments. Moreover, we also propose a division of topographical areas within the same rostrocaudal level for a better characterization of dorso-ventral and latero-medial variations.

The list of works dealing with the anatomical and neurochemical organization of the OB is too vast to be summarized here. Coronal sections and representations are preferred, but a common feature in most authors is to consider the OB as a uniform region, thus choosing the central level of the rostrocaudal axis as representative of the whole structure. However, a fair amount of evidence has disclosed differences across both the rostrocaudal axis and the different topographical regions in the mammalian OB: the R4B1 antigen in rabbits (Mori, 1987) and rats (Schwob and Gottlieb, 1986), carbohydrate antigen reacting with CC2-antibodies (Schwarting and Crandall, 1991), carbohydrates reacting with *Lotus* lectin (Stewart and Touloukian, 1996), the OCAM molecule, related to olfactory projections in both the main and accessory OBs (Yoshihara et al., 1997), and NADPH-diaphorase activity in several vertebrate species (Davis, 1991; Vincent and Kimura, 1992; Alonso et al., 1993; Kishimoto et al., 1993; DellaCorte et al., 1995; Porteros et al., 1996; Weruaga et al., 1998; see Fig. 4). Some of these neural markers are localized only in the rostral most part of the OB, and others, such as a few glomeruli strongly-labelled with acetylcholinesterase histochemistry (Zheng and Jourdan, 1988; Le Jeune and Jourdan, 1991, 1993; Crespo et al., 1996, 1997), and human placental antigen X-P$_2$ (Shinoda et al., 1989, 1990) are exclusively present or selectively abundant in the caudal most region. There is also a subdivision of projecting areas from the olfactory epithelium to the olfactory bulb based on both the expression of

different mRNA for odour receptor molecules (Ressler et al., 1993; Vassar et al., 1993) and tract-tracing methods (Astic and Saucier, 1986; Saucier and Astic, 1986; Astic et al., 1987; Stewart and Pedersen, 1987) that points to the heterogeneity of the olfactory projections to external layers. We consider that the subdivision in rostrocaudal levels and in topographical regions (medial, ventral, etc.) facilitates the location, comprehension and comparison of the distribution pattern of all neural markers.

Another difficulty when studying the olfactory bulb is the comparison of patterns when it undergoes size changes. This phenomenon occurs naturally during development and under certain experimental conditions. Several cases in which experimental manipulation of the olfactory system results in a size reduction of the OB have been reported. As examples, there are two experimental procedures for studying neural plasticity in the olfactory system: nasal occlusion and peripheral deafferentation by injuring the olfactory epithelium or the olfactory nerve. Nasal occlusion in rodents produces a 25% reduction in the original size of the OB (Meisami, 1976; Brunjes, 1994; Vallejo-Valdezate, 1996) due to neuronal and/or glial loss or modification (Meisami and Safari, 1981; Meisami and Mousavi, 1982; Benson et al., 1984; Skeen et al., 1985; Meisami and Nousinfar, 1986; Frazier and Brunjes, 1988). In adulthood, these experimental conditions also result in OB size reduction, although the reduction is less pronounced (Maruniak et al., 1989; Henegar and Maruniak, 1991). The other procedure to eliminate peripheral afferences to the OB is the destruction of the olfactory epithelium by either surgical techniques or nasal irrigation with zinc sulphate or Triton X-100. The size reduction of the bulb in the latter two cases is more dramatic in comparison with nasal occlusion (Margolis et al., 1974; Harding et al., 1977; Nadi et al., 1981; Kawano and Margolis, 1982; Baker et al., 1983, 1984; Brunjes et al., 1985; Kream et al., 1985). In peripheral deafferentation, size reduction results primarily from the loss of axonal arborization of olfactory primary afferences within the glomeruli (Baker et al., 1983).

All these experimental modifications as well as the postnatal development of the rodent OB affect the size and shape of the OB homogeneously, because both the natural enlargement and the destruction of peripheral afferences affect elements distributed homogeneously across the surface of the whole structure. Because of this change in volume, the ensuing modifications in any neurochemical pattern under such conditions should be properly compared with control cases, at different rostrocaudal levels and topographical regions. For this purpose, the selection of absolute coordinates (i.e. Bregma levels) in each case do not correspond to analogous areas

of the organ, thus providing in inadequate conclusions. A solution has been offered by Stewart and co-workers (1979), who chose five levels to study glucose uptake in PCD mutant mice, whose olfactory bulbs are shrunk as compared with normal animals (Mullen et al., 1976; Greer and Shepherd, 1982). These five levels, in contrast to the classification proposed here, are not equidistant, which makes additional representations necessary if they are to be reproduced with accuracy.

We consider that five levels are enough to obtain a representative and comprehensive design of the entire OB. Most anatomical and neurochemical features of the rodent OB expand sufficiently along the rostrocaudal axis to be represented in the proposed anatomical division. For instance, NADPH-diaphorase-positive glomeruli (see Fig. 4) are never located in the last level but they are along the rostral four levels. The accessory olfactory bulb always appears at coronal type level IV, and the anterior olfactory nucleus at level V. Similarly, study of the OB by topographical regions is necessary, since some neural markers are differentially distributed across a given coronal level, even if they are present throughout the rostrocaudal level, such as *Lotus* lectin binding sites (Stewart and Touloukian, 1996). The subdivision of the OB into more than five levels would result of course in a more exact description of the whole organ, but the comparison among levels and among topographical regions of different levels would be harder and would considerably increase the complexity or the analysis.

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