Expression of neuronal nitric oxide synthase/NADPH-diaphorase during olfactory deafferentation and regeneration

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Abstract

Neuronal nitric oxide synthase (nNOS) expression can be regulated under natural or experimental conditions. This work aims at elucidating whether the expression of nNOS or its related NADPH-diaphorase (ND) activity are modified by manipulation of the normal inputs to neurons. We used the olfactory bulbs from two mouse strains, BALB and CD1, because they show divergences in their synapse patterns, and these differences affect periglomerular cells, interneurons expressing tyrosine hydroxylase or nNOS/ND. The olfactory inputs to these neurons can be disrupted by inhalation of methyl bromide. The effect of this gas on olfactory axons, as well as the synaptic features in both mouse strains, were studied using electron microscopy. The changes in expression were analysed qualitatively and quantitatively at different times after lesion to nine topographical regions of the olfactory bulb. Methyl bromide inhalation induced a degeneration of olfactory axons in both strains, but had different effects on the expression of nNOS/ND and tyrosine hydroxylase. In BALB mice, where periglomerular cells do not receive direct inputs from olfactory axons, no changes were detected in tyrosine hydroxylase or in ND expression. In CD1 periglomerular cells, where olfactory axons establish direct synapses, a significant down-regulation of both markers was observed. These changes were observed differentially across the olfactory bulb, being more pronounced in rostral regions and more acute for ND than for tyrosine hydroxylase. Our results indicate that the synaptic inputs influence the expression of ND activity related to nNOS and that the activation of the enzyme is more severely affected than its protein expression.

Introduction

Nitric oxide (NO) is formed from l-arginine by nitric oxide synthases (NOS; EC 1.14.13.39; Abu-Soud et al., 1997). There are three known isoforms: neuronal (NOS I or nNOS), endothelial and immunological (Förstermann et al., 1995). Neuronal NOS (nNOS) is expressed in some neurons and astrocytes and its localization in the nervous system of vertebrates has been well documented in situ hybridization studies, immunohistochemistry, and through the related NADPH-diaphorase (ND) histochemical activity (for review see DellaCorte et al., 1995; Morris et al., 1997; Alonso et al., 2000).

Because NO itself rapidly interacts with target molecules, regulation of its activity must be carried out on the enzymes themselves, regarding either their expression or their catalytic activities (Dinerman et al., 1994; Southan & Szabó, 1996; Cowart et al., 1998). Despite evidence of the up- and down-regulation of nNOS expression (see Dawson et al., 1998), little is known about the relationship between nNOS/ND expression and the maintenance of a normal input pattern onto the neurons that normally localize the enzyme.

The olfactory system is specially suited for neuronal plasticity studies because: (i) the olfactory bulb (OB) is a laminar region where changes are readily detected; (ii) its synaptic circuitry is well-known; (iii) the neurochemical phenotypes have been described in considerable detail; (iv) the system maintains a dynamic organization due to the continuous turnover of peripheral afferent inputs; and (v) these inputs can be experimentally manipulated with relative ease (see Brunjes, 1994; Shipley & Emms, 1996).

One subpopulation of periglomerular (PG) cells of the olfactory bulb is nNOS/ND-positive. Immunohistochemistry for the protein and ND histochemistry colocalize to all neurons from this subpopulation under specific technical conditions (Kishimoto et al., 1993; Weruaga et al., 1998). The PG cells establish reciprocal synapses with dendrites of projection neurons (i.e. mitral and tufted cells) but also receive inputs from axons arriving from the olfactory mucosa (Pinching & Powell, 1971; White, 1972; Toida et al., 1994; Kosaka et al., 1997, 1998). This general model of rodent glomerular synaptology has alternative circuitries in two mouse strains: whilst in CD1 mice there are direct synaptic inputs from olfactory axons onto PG cells, this is not the case in the BALB strain (White, 1972, 1973). These circuitries respond selectively to olfactory deprivation: on studying the regulation of the expression of the dopaminergic phenotype of PG cells, experimental elimination of olfactory inputs induces a dramatic reduction in tyrosine hydroxylase (TH) in rats and CD1 mice, but only a very moderate reduction in the BALB strain (Nadi et al., 1981; Kawano & Margolis, 1982; Baker et al., 1984; Baker, 1988). Therefore, the intraspecies synaptic differences between CD1 and BALB mice, together with the experimental

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elimination of olfactory inputs, offers a profitable model for studying the regulation of the expression of the nitricergic phenotype.

The aims of this work are: (i) to confirm the divergence in the synaptology of the two mouse strains, CD1 and BALB, by the use of immunocytochemical markers and electron microscopy; (ii) to establish a reproducible method for olfactory deafferentation in the mouse; (iii) to evaluate methyl bromide injury by examination of the ultrastructure of olfactory axons and determination of quantitative changes in TH expression in PG cells of both mouse strains; and (iv) to assess the effects of gas-induced lesions and the ensuing olfactory deafferentation on the expression of nNOS/ND.

Materials and methods

**Animals**

Virgin female mice (*Mus musculus*) from the strains CD-1 (CD1) and BALB/cByJc6 (BALB) were obtained from Griffa S.A. (Barcelona, Spain). They were maintained in the animal facilities of the University of Salamanca at constant humidity and temperature, a 12 h light/dark cycle, and fed and water *ad libitum* (Rodent toxicology diet, B & K Universal SJ, Barcelona, Spain). At the end of the experiments, all animals were deeply anaesthetized with chloral hydrate (5% w/v in saline, 10 μL/g body weight i.p.) and killed by perfusion. All animal manipulations were performed under the prescriptions of the directives of the European Communities Council (86/609/EEC) and current Spanish legislation (BOE 67/8509–121988) for animal care and experimentation.

**Olfactory lesions**

Groups of 12 or 18 conscious mice of 10 weeks of age were placed in a PVC box measuring 30 × 25 × 10 cm and exposed for 7 h to a mixture of synthetic air (20% O₂ in N₂) and 330 p.p.m. of methyl bromide (BrMet; Sociedad Española de Carburos Metálicos, Valladolid, Spain) at a flow rate of 4 L min⁻¹, sufficient for correct ventilation of all animals (Altman & Dittmer, 1974). Survival times after gas treatment were 10, 15, 20, 25 and 60 days (G10, G15, etc.), each group consisting of six animals from each mouse strain. Additional experimental groups consisting of two animals from times G1, G4 and G6 were employed to evaluate the short-term effects of gas inhalation on the OB. Eight mice from each strain were similarly handled and exposed to synthetic air without BrMet and killed 20 days later; these were considered control animals.

**Tissue preparation for light microscopy**

After the induction of anaesthesia, animals were perfused through the aorta first with heparinized saline (5 IU ml⁻¹; Byk Leo, Madrid, Spain), followed by a solution containing 4% (w/v) depolymerized paraformaldehyde and 2% (w/v) picric acid in 0.1 M phosphate buffer (PB) pH 7.4 for 20 min. The brains were dissected out and postfixed in the same solution for 2 h at 4 °C. For cryoepreparation, brain blocks were trimmed, rinsed in PB overnight and immersed in 30% (w/v) sucrose in PB until they sank. Coronal sections 30μm thick 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 such as histochemistry or immunohistochemistry as well as for standard neurohistological stainings.

Each one-in-six series of each OB was stained either with Nissl, ND histochemistry, nNOS immunohistochemistry, TH immunohistochemistry or double-labelled for ND and nNOS. The remaining series was reserved for controls and other purposes.

**ND histochemistry and immunohistochemistry**

Free-floating sections were rinsed in PB (5 × 10 min) then processed in a single incubation as described (Wueraga et al., 1998). Briefly, the sections were incubated in a medium containing 1.2 mM β-NADPH (tetrasodium salt; Sigma, St. Louis, MO, USA), 0.1 mM nitroblue tetrazolium (Sigma) and 0.8% (w/v) Triton X-100 (Probus, Badalona, Spain) in phosphate-buffered saline (PBS), pH 8.0. The reaction was controlled under the microscope to avoid undesirable formation of formazan crystals, and stopped with cold PB.

Free-floating sections of OB were immunostained for nNOS or TH, using the avidin-biotin-immunoperoxidase method. (i) Specific primary antibodies were diluted in PBS, pH 7.4, with 5% (v/v) normal serum (Vector, Burlingham, CA, USA) and 0.01% (w/v) Triton X-100 and incubated for 72 h at 4 °C. Polyclonal antisera against nNOS (K205; Herbison et al., 1996) was diluted 1:15000 with normal rabbit serum, and monoclonal antibody against TH (Incstar #29241, Stillwater, MN, USA) was diluted 1:15000 with normal horse serum. (ii) Secondary biotinylated anti-IgG was applied for 60 min (Vector, 1:250 in PBS); antisheep and antimouse IgGs for nNOS and TH detection, respectively. (iii) Avidin-biotin-complex (Vectastain Elite Kit, Vector; 1:250 in PBS) was incubated for 90 min. Between steps, tissue sections were rinsed in PBS (4 × 10 min). Tissue-bound peroxidase was visualized by incubating the sections in 0.003% (v/v) H₂O₂ and 0.02% (w/v) DAB in 0.05 M Tris-HCl buffer, pH 7.6, the reaction being controlled under the microscope.

For double-labelling of ND and nNOS, OB sections were first processed for the histochemical technique and subsequently for the immunodetection of nNOS.

Stained OB coronal sections were mounted onto slides, dehydrated in ethanol, and coverslipped with Entellan™ (Merck, Darmstadt, Germany).

**Morphometric analysis**

All measurements were done on coded preparations by researchers unaware of their experimental history. Rostrocaudal series of stained OB coronal sections were defined, five levels were chosen and medial and lateral regions in each level were delimited as described elsewhere (Wueraga et al., 1999). Briefly, each selected section within a series corresponds to the centre of five equidistant rostrocaudal segments within the OB (I–V). This method allows comparison of similar rostrocaudal levels in OB corresponding to different species or strains, different ages, or experimental treatments in which changes in the size of the OB are suspected (see Brunjes, 1994). At each level, the medial (M) and lateral (L) halves were differentiated. In level V, the main OB is restricted to only medial regions, the lateral half of the section being occupied by the anterior olfactory nucleus and the accessory olfactory bulb. Thus, a total of nine topographical regions were analysed in each series. M and L regions of the external glomerular border were delimited using a 135° angle drawn from the centre of the OB section, thus excluding dorsal and ventral regions. With this procedure, homologous regions from different experimental groups were analysed. TH-positive juxtaglomerular neurons and ND-positive PG cells were counted and the corresponding perimeters of the medial and lateral borders of the glomerular layers were calculated using a semiautomatic image analysis system (MOP Videoplan, Kontron, Munich, Germany). A TH-positive juxtaglomerular neuron was counted only if the soma and a second order dendrite could be visualized using a 40× objective, whilst ND-positive periglomerular cells were counted when the soma and a third-order dendrite could be distinguished. Positive cells lying on the ventral border of the region analysed were
discarded. Only cell profiles that included a nucleus were counted. The analysed sections were at least 180 μm apart. This method assures accurate counting without missing or double-counting cells. The resulting densities of positive cells for each marker were compared among the different experimental groups (control, G10, G15, etc.) by means of the Kruskal–Wallis test. Only when this nonparametric test showed statistically significant differences among experimental groups was the Mann–Whitney U-test employed to compare each experimental group with the respective control values. For all tests, values of \( P < 0.05 \) were considered significant for the differences.

**Electron microscopy**

Additional animals from each strain studied were employed for electron microscopical analysis: four animals of groups G1, G4, G6 and 10 control animals from each strain. Animals were fixed similarly as for light microscopy but 0.2% (v/v) glutaraldehyde was added in the perfusion mixture. Coronal sections of 50 μm thickness were obtained with a vibratome. After thorough rinsing in cold PB, sections from control animals were immunostained for nNOS and for TH as described, but incubation times were doubled, incubation and rinsing were always performed at 4°C, and Triton X-100 was omitted. Sections from rostral regions (levels I and II) from experimental and control mice were processed identically but without performing the immunostaining step. In all cases, the sections were postfixed with 2.5% glutaraldehyde and 1% (w/v) OsO4, both in PB at 4°C and each for 2 h. The tissue was immersed in 1% (w/v) uranyl acetate in 70% ethanol, dehydrated in a graded series of increasing ethanol concentrations and embedded in Epon™ 812 (Fluka, Buchs, Switzerland).

Semithin sections of selected OB regions were obtained with a Reichert-Jung Ultracut E (Nussloch, Germany) and observed and photographed with a Zeiss Axioshot microscope (Oberkochen, Germany). For demonstration of axonal degeneration, resin blocks containing nonimmunostained tissue from the same number of control and experimental animals from each strain were trimmed to select fields in the olfactory nerve layer–glomerular layer boundary. The same number of blocks containing medial and lateral regions were cut into serial ultrathin sections (silver-grey interference colour). Sections were mounted on copper grids and examined at 50kV in an EM902 Zeiss electron microscope to evaluate axonal damage.

For comparison of glomerular synaptology, we selected proper TH-stained slides from three control animals of each strain. Blocks were trimmed around glomeruli with proper TH immunostaining. Ultrathin sections from the surface of the block were obtained to minimize a false negative in the PG cell dendrite staining, due to the limited tissue penetration of the antibodies. Excitatory synapses between olfactory axon terminals and dendrite endings (≤ 1 μm in diameter) were sought (see Results). Thirty excitatory synapses per animal were analysed, with a total of 90 synapses of each mouse strain studied.

**Results**

**nNOS/ND and TH patterns**

The nNOS/ND pattern in the mouse OB has been thoroughly described in a previous work (Wengu et al., 1998). Both markers colocalize in all neurons when a single incubation is performed, and their distribution pattern is similar for both mouse strains studied. After the described fixation and incubation procedures, nNOS and ND appeared only in neurons, identified as a subpopulation of PG cells, superficial and deep short-axon cells, weakly stained granule cells and some centrifugal fibres. ND histochemistry revealed staining-grade differences among PG cells. These differences were not so evident after nNOS staining, which labelled positive PG cells very weakly and even then not the whole of the cells. Thus, ND labelling was considered more useful as a marker for nitricergic PG cells.

The TH pattern in the mouse olfactory bulb of both strains has been described at the light microscopical level by Baker (1986) and our results are in agreement with that author’s findings: most TH-immunopositive neurons in the OB are located in the glomerular layer and are identified as mostly periglomerular cells and a few external tufted cells (Baker, 1986; Crespo et al., 1997). Catecholaminergic neurons in deeper layers of the OB were very scarce and slightly more abundant in BALB than in CD1 mice.

ND- and TH-positive PG cells constituted different subpopulations in the two strains, as demonstrated by double-labelling experiments (data not shown), which matched with existing results in other rodents (Johnson & Ma, 1993; Samama & Böhm, 1996).

**Ultrastructure of the olfactory glomerulus in both mouse strains**

The olfactory glomerulus is flanked mainly by PG cells, which are easily distinguishable by their ultrastructural characteristics at electron microscopical level (Fig. 1A and B). Furthermore, TH labelling resulted in a perfect filling of the PG cell cytoplasm in the soma and dendrites by an electron-dense DAB product (Fig. 1B). Thus, it was possible to distinguish three kinds of profiles (see Pinching & Powell, 1971; White, 1972): (i) numerous, small and densely packed profiles, with natural dense cytoplasm (dark grey), corresponding to olfactory axons and terminals, the latter being full of round synaptic vesicles (Fig. 1C); (ii) larger profiles with a clear cytoplasm (white), corresponding to dendrites and spindles of mitral

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![Fig. 1. Electron micrographs of the olfactory bulb of a CD1 mouse showing the main elements of the olfactory glomeruli. The section has been immunostained for tyrosine hydroxylase using DAB as chromogen. (A) Two somata of immunonegative periglomerular cells (PG) can be distinguished at the periphery of the glomerulus. Their nuclei present a characteristic indentation and a peripheral nucleolus and the cytoplasm is only present as a thin rim. Inside the glomerulus (upper half), groups of olfactory axons (OA; dark cytoplasm) intermingled with dendritic profiles of different OB neurons (black asterisks; light cytoplasm) can be seen. Some of the latter structures are labelled with DAB product (white asterisks on black profiles), thus corresponding to TH-immunopositive PG cells. (B) A TH-positive PG cell is clearly shown. (C) Type I synapses (arrows) from olfactory terminals (AO) onto an unlabelled dendrite are observed. Scale bars, 2μm (A,B), 0.5μm (C).](image-url)
cells, tufted cells and PG cells; and (iii) DAB-filled profiles (black), corresponding only to PG cells, because the rare positive tufted cells were not taken into account during observation under light microscopy of vibratome and semithin sections. Clear excitatory synapses could be identified between olfactory axons and both negative and positive dendrites (Figs 1C and 2). Whereas in the CD1 strain, synapses of olfactory axons onto both negative (~70%) and positive (~30%) dendritic profiles could be seen (Fig. 2A and B), in the BALB mice such synapses could be clearly confirmed onto negative profiles, but almost never onto TH-positive periglomerular cell dendrites (only one out of 90 synapses analysed; Fig. 2C and D).

Effects of BrMet inhalation on olfactory axons
All animals undergoing gas inhalation had transitory (24 h) ocular irritation and diarrhoea, which were slightly more pronounced in mice gassed with BrMet than in those receiving synthetic air. All animals survived until at least 60 days after the lesion.

On analysing the ultrastructure of rostral regions of the olfactory nerve layer of the OB we found some differences in G4 and G6 animals as compared with the control and G1 mice. We did not find any relevant differences between the strains studied: (i) in BrMet-gassed animals there were deformed and very electron-dense structures (picnotic profiles; see Fig. 3) intermingled with olfactory axons bundles; (ii) several axon terminals displayed grouped vesicles with more heterogeneous and larger diameters in comparison with normal synaptic vesicles; and (iii) a higher density of phagocytic cells, with multiple vesicles, was observed. All these morphological features were occasionally found in G1 and control animals, but their density was considerably higher in G4 and G6 animals from both strains. These features matched a stronger axonal degeneration and repair pattern.

TH expression in experimental animals
CD1 strain
In the CD1 strain, the TH-immunostaining pattern was very similar to that of the controls in G10 animals, but from G15 to G25, with a clear peak on G20, TH immunoreactivity decreased dramatically in juxtaglomerular cells (Fig. 4). TH immunoreactivity decreased in dendrites and somata, and in many cells disappeared completely. In some CD1 animals, the labelling was completely lost in the rostralmost levels (I and II). Within these levels, the most affected regions corresponded to medial and dorsomedial locations. The most drastic effects of BrMet lesions were seen in levels located at the rostral tip of the OB. Accordingly, they were not included in the

Fig. 2. Electron micrographs of mouse olfactory bulb inside the olfactory glomerulus immunostained for tyrosine hydroxylase (TH) using peroxidase and DAB. Profiles with more electron-dense and granular cytoplasm correspond to dendrites of TH-positive periglomerular cells. Profiles of intermediate intensity (dark grey) are olfactory axons and axonic terminals. Elements with clear cytoplasm correspond to dendrites of unidentified neurons, all of them TH-negative. (A and B) CD1 strain: it is frequent to observe type I synapses from olfactory axons onto both negative postsynaptic elements (black arrow) and positive postsynaptic profiles (white arrows; TH-positive periglomerular cells). (C and D) Type I synapses in the BALB strain. In this case, we found olfactory axons synapsing onto negative dendrites (arrows) but never onto TH-positive PG cell dendrites (white asterisk), even though both positive and negative profiles are often found in apposition. Scale bar, 0.5 μm.

Fig. 3. Electron micrographs of the olfactory bulb of a CD1 mouse that underwent methyl bromide inhalation and was killed four days later (CD1/G4). Frames correspond to the outermost location of an olfactory glomerulus at the OB region most affected by the experimental treatment: rostralmost levels and the medial region (IM, see Results). (A) Three signs of axonal degeneration can be distinguished: arrows point to picnotic profiles; arrowheads indicate axon terminals with enlarged vesicles, which are more clearly depicted in (B). M indicates a cell with macrophage/microglial features, i.e. a heterochromatic nucleus (white asterisk) and multiple pleomorphic vesicles of heterogeneous content (black asterisks). Scale bars, 2 μm (A), 0.5 μm (B).
quantification. Sixty days after gas lesion, the number of immuno-reactive cells increased considerably and the TH-immunolabelling pattern was similar to that of the control animals.

In control animals, in the comparison between topographical regions of the values of the densities of juxtaglomerular cells labelled with TH, the Kruskal–Wallis test disclosed statistically significant

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**Fig. 4.** Olfactory bulb sections immunolabelled for tyrosine hydroxylase (TH) after experimental inhalation of methyl bromide by CD1 mice. G20 and G60 correspond to gassed animals killed 20 and 60 days after the gas treatment, respectively. (A–C) Panoramic views demonstrating a clear loss of TH immunoreactivity in (B) G20 animals as compared with (A) control animals; (C) the control TH expression pattern had recovered 60 days after gas lesion. (D–F) Higher magnifications of the glomerular layers in medial regions of A–C, respectively. The TH pattern consists of a high density of cells around glomeruli (juxtaglomerular cells). The loss of immunoreactivity at G20 is reflected not only by a decrease in staining in both dendrites and somata, but also by a quantitative decrease in the number of the latter elements (see Fig. 5). Scale bar 1 mm (A–C), 100 μm (D–F).
differences ($P < 0.001$), which means that this parameter is not homogeneous across the normal OB. Thus, each region should be considered an independent element when performing tests for the comparison of experimental groups. The nine plots in Fig. 5 reflect the data corresponding to each region in the six experimental groups of CD1 animals. The Kruskal–Wallis test applied to each topographic region afforded the following $P$-values: I/L, $P = 0.0490$; I/M, $P = 0.0060$; II/L, $P = 0.059$; II/M, $P = 0.001$; III/L, $P = 0.012$; III/M, $P = 0.008$; IV/L, $P = 0.012$; IV/M, $P = 0.006$; V/M, $P = 0.034$. These data indicate that, except for the II/L region, there are significant differences among the experimental groups: treatment with BrMet affects the density of TH-positive juxtaglomerular cells throughout almost the whole OB of CD1 mice. We next performed a Mann–Whitney $U$-test by pairs, comparing each experimental group (G10, G15, etc.) with the respective control group, in each topographical region that was found to have differences with the Kruskal–Wallis test (i.e. all except II/L). The $P$-values are shown in the respective plots of Fig. 5. Eight out of nine regions showed differences in the density of TH-positive juxtaglomerular cells induced by the inhalation of BrMet, with peaks around 15–25 days after the lesion.

**Fig. 5.** Each of the nine charts represents values for a specific topographic region within the olfactory bulb of CD1 mice. On the abscissa axes, experimental groups including controls are shown, and the ordinate axes represent the densities of TH-positive juxtaglomerular cells per mm of bulbar perimeter. Values are presented as means ± SEM of each group ($n = 5$). In all regions except II/L, the Kruskal–Wallis test revealed statistically significant differences among the experimental groups. After this, the Mann–Whitney $U$-test was performed between each experimental group and its respective control. Cases showing significant differences are indicated by the corresponding $P$-value.
Rostral regions were more severely affected. In most regions, 60 days after BrMet treatment the parameter returned to values that were statistically similar to those of the controls.

**BALB strain**

No great changes in TH expression were found in the OB of this mouse strain, except for a slight decrease in the labelling of distal dendrites.

![Fig. 6](image_url). BALB mouse olfactory bulb sections immunolabelled for tyrosine hydroxylase (TH) after experimental inhalation of methyl bromide. G20 and G60 correspond to animals killed at 20 and 60 days after gas treatment, respectively. In this strain, the TH pattern is similar to that of the CD1 animals, although a few more positive elements were found in deep layers of the olfactory bulb (the arrow in F shows a positive van Gehuchten cell). (A–C) Panoramic views of Control, G20 and G60 animals. (D–F) Higher magnifications of the glomerular layer in medial regions of A–C, respectively. In this strain, the inhalation of methyl bromide and the subsequent peripheral deafferentation do not affect TH expression quantitatively (see Fig. 7), although a slight decrease in the labelling of distal dendrites was found. Scale bar, 1 mm (A–C), 100 μm (D–F).
dendrites. Figure 6 offers some examples, including G20 animals, which showed the most striking decrease in TH expression in the CD1 animals. The Kruskal–Wallis test also revealed differences (P < 0.0001) in the density of TH-positive juxtaglomerular cells between different regions of the OB. Therefore, we compared this density among the experimental groups separately within each region. The quantitative comparison is shown in the nine plots in Fig. 7. In this strain, the P-values for the Kruskal–Wallis test were: I/L, P = 0.739; I/M, P = 0.479; II/L, P = 0.871; II/M, P = 0.320; III/L, P = 0.317; III/M, P = 0.229; IV/L, P = 0.653; IV/M, P = 0.285; V/M, P = 0.503. These values indicate that no changes occur in the density of TH-immunopositive cells in the glomerular region of BALB mice after BrMet inhalation.

ND-stained sections displayed changes after BrMet inhalation that were even more dramatic than in OB sections labelled for TH. As early on as G10, a reduction in the ND staining of periglomerular cells dendrites was seen throughout the rostrocaudal axis. In G20, the reduction was clearly more marked than in adjacent TH sections (Fig. 8). In this case, the enzymatic activity was reduced not only in the glomerular region but also in the neuropil of the granule cell layer, where ND labelling almost completely disappeared. In the experimental groups, ND-positive short-axon cells (both deep and superficial) were visible, but were only stained weakly. In G20,

**nNOS/ND expression in experimental animals**

**CD1 strain**

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**Fig. 7.** Each of the nine plots represents values for a specific topographic region within the olfactory bulb of BALB mice. On the abscissa axes experimental groups including controls are shown, and the ordinate axes represent the densities of TH-positive juxtaglomerular cells per mm of bulbar perimeter. Values are presented as means ± SEM of each group (n = 5). The Kruskal–Wallis test did not reveal differences among the experimental groups in any location.
positive granule cells were not visible, despite the loss of the positive neuropil in this layer. Similar to TH expression, the reduction in ND activity differed between the topographical locations: rostral regions were the most severely affected, but more so in lateral regions than in medial ones (Fig. 9). After 60 days (Fig. 8C), the staining patterns returned to those of the control animals, although we observed a

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**Fig. 8.** Olfactory bulb coronal sections from CD1 mice stained for NADPH-diaphorase histochemistry (ND). G20 and G60 correspond to animals gassed with methyl bromide and killed at 20 and 60 days after gas treatment. (A–C) Panoramic views: the deafferentation effect is striking at 20 days postlesion because of the reduction in ND activity in both glomerular and granule cell layers. G60 animals have a similar appearance to control mice. (D–F) At higher magnification it is possible to appreciate that the reduction in staining affects the somata and dendrites of periglomerular cells. Scale bar, 1 mm (A–C), 100 μm (D–F).
slight difference in the shape and orientation of PG cell dendrites. nNOS was also affected by the deafferentation in CD1 mice, but clearly less intensely than the related ND activity (not shown).

ND-positive PG cell densities in control animals pointed to differences between topographical locations (P < 0.0001 for the Kruskal–Wallis test), indicating that this parameter should be compared independently within each region. These comparisons are summarized in Fig. 10. Regarding this parameter, all nine topographical regions showed differences among the experimental groups when compared using the Kruskal–Wallis test: I/L, P = 0.001; I/M, P = 0.001; II/L, P = 0.001; III/M, P = 0.001; IV/L, P = 0.003; IV/M, P = 0.002; V/M, P = 0.038. Accordingly, the post hoc Mann–Whitney U-test was performed in each region, comparing the experimental group with the control group. In general, the enzymatic activity was most affected in G20, where ND-positive PG cell densities were severely diminished. In some animals, the loss of ND-positive PG cells was total at rostral levels; only 7% of the initial number of positive cells (on average) expressed ND activity in the I/L region. Unlike TH, this parameter rapidly recovered, being statistically similar to control values at G25 in most regions.

BALB strain
Like TH, in the BALB strain small differences were found in the distribution pattern of ND labelling (Fig. 11). At most, only in some G20 animals was there a slight decrease in the staining of dendrites in PG cells from rostral regions, whereas the central and caudal regions remained completely unaffected. In G25, the BALB animals did not show these differences. We did not find qualitative differences (even in the G20 group) in nNOS-immunoreactivity patterns.

In control animals, ND-positive PG cell densities also showed differences among the topographical regions (P = 0.002 for the Kruskal–Wallis test). Like TH, there were no differences among the experimental groups in any of the topographical regions in the BALB strain: I/L, P = 0.615; I/M, P = 0.914; II/L, P = 0.635; II/M, P = 0.525; III/L, P = 0.960; III/M, P = 0.370; IV/L, P = 0.751; IV/M, P = 0.228; V/M, P = 0.689. The graphic representation is shown as nine plots in Fig. 12.

All quantitative results for both markers, TH and ND, and both strains, CD1 and BALB, are summarized in Fig. 13. The values are presented as the means for each group relative to the control values (100%) in order to compare the effects of BrMet on the two neural markers.

Discussion
In the present work we have shown that the expression of TH, nNOS and ND in periglomerular cells after lesion of the olfactory mucosa are different in two mouse strains. These strains show variations in the synaptology of their glomeruli. In the BALB strain, the peripheral olfactory inputs have no direct synaptic contacts onto PG cells, but only indirect ones via synapses through mitral and tufted cells. In these BALB animals, when the peripheral olfactory inputs are disrupted no appreciable changes are seen in the TH phenotype, in immunoreactivity for nNOS, or in ND activity. By contrast, the CD1 strain receives afferent inputs directly from olfactory receptor neurons to PG cells. In these animals, the experimental elimination of olfactory inputs elicited a significant reduction in the expression of TH, nNOS and ND activity in PG cells, and was most pronounced in the latter.

Synaptology of PG cell subpopulations in both mouse strains
Synaptic differences within the glomerulus of the BALB and CD1 mouse strains have been described in only two works in the literature reviewed. First, in several rodent species, including the CD1 mouse, White (1973) found direct synapses of olfactory axons onto PG cell dendrites. He did not find this feature within the olfactory glomeruli of the BALB strain. The second work studied the qualitative changes occurring in the expression of TH after olfactory deafferentation in both mouse strains and the reduction was found to be more marked in CD1 as compared with BALB (Baker, 1988). In the work of White (1973), the conclusions were built from serial reconstructions of discrete dendrites at electron microscopical level. By contrast, in our work we distinguished fine dendrite profiles (i.e. endings or spinules) by labelling those corresponding to a specific neuronal type. TH immunolabelling allowed us to demonstrate that the vast majority of PG cell dendrites (if not all of them) from the CD1 strain receive direct olfactory inputs. In the case of BALB mice, labelled dendrites, belonging to the large subpopulation of TH-positive PG cells, do not receive direct olfactory inputs.

In the rat OB, different groups of neurons and their subpopulations have been defined by their chemical phenotype and by their patterns of arborization and synapses (Kosaka et al., 1994, 1997, 1998; Toida et al., 1994, 1998). In elegant experiments combining confocal microscopy, immunocytochemistry and both conventional and high-voltage electron microscopy, these authors concluded that the calbindin-immunoreactive subpopulation of PG cells does not receive direct olfactory inputs, unlike the TH-immunopositive PG cells (a subpopulation largely overlapped by GABAergic PG cells), which do exhibit direct synapses from olfactory axons (Toida et al., 1998). These authors proposed that White (1973), on studying different mouse strains, might have been analysing dendrites of different subpopulations of PG cells, incurring overly definitive conclusions. nNOS/ND-positive PG cells in the rat (Alonso et al., 1993) and mouse (our own observations) are different from the calbindin-immunopositive PG cell subpopulation and probably also exhibit a different synaptology. Thus, from both direct observation of the structures and indirect analyses of the changes in expression after

Fig. 9. Sections of olfactory bulbs from CD1 mice stained for NADPH-diaphorase histochemistry. (A and C) Control animals. (B and D) Methyl bromide-treated mice killed at 20 days after lesion. At G20, the expression of the histochemical activity almost disappeared. This effect is more dramatic at rostral (I/L; C and D) than at central levels (II/M; A and B), where some weakly stained periglomerular cells can be seen (arrows). Scale bar, 50 μm.

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deafferentation, it may be concluded that the TH- and the nNOS/ND-positive PG cell subpopulations have different synaptologies in the two mouse strains, each of them receiving direct olfactory inputs in CD1 mice, but not in BALB animals. These features are reflected in Fig. 14.

**Olfactory lesions**

Most methods employed to produce olfactory lesions in rodents, and the subsequent olfactory deafferentation, are of chemical nature. Surgical disruption of the olfactory nerve in the mouse is not advisable owing to the size of the animal (see Baker, 1988). Chemical deafferentation has largely been employed in rodents: nasal irrigation with zinc sulphate, Triton X-100 or vinblastine (Nadi et al., 1981; Kawano & Margolis, 1982; Baker et al., 1984; Kream et al., 1985). The most extensive lesions are caused by zinc sulphate irrigation, which produces the same effects as nerve transection in rats (Nadi et al., 1981). In preliminary experiments we used the zinc sulphate irrigation method (not shown), but morbidity and mortality were high, rendering this method unusable for statistical purposes. In rats, the inhalation of BrMet has been shown to be destructive to the olfactory epithelium and to avoid mortality when the doses are species-tuned (Honma et al., 1994; Schwob et al., 1994; Miller et al.,

![Graphs showing periglomerular cell density](image)

**Fig. 10.** These nine plots represent the densities of NADPH-diaphorase-positive periglomerular cells (number of cells per mm of bulbar perimeter) in nine topographical regions of the olfactory bulb of CD1 mice. In each plot, the experimental groups are compared. The Kruskal–Wallis test demonstrated significant differences in all cases. Thus, post hoc comparisons using the Mann–Whitney U-test were performed. P-values for the latter test are depicted only when they were statistically significant (P<0.05).
1995; Goldstein & Schwob, 1996; Yougentob et al., 1997). In our experiments, treated animals killed 4 and 6 days after gas lesion showed pronounced signs of axonal degeneration as demonstrated by electron microscopy (Perry et al., 1987; Barron et al., 1990; Eriksson et al., 1993). Moreover, the general features of TH expression in the OB were similar to those observed in rats and mice after zinc sulphate irrigation or surgical olfactory lesions (see Baker, 1988 and Brunjes, 1994): TH immunoreactivity decreases in CD1 mouse and rat

![Figure 11](image_url)

Fig. 11. Coronal olfactory bulb sections of BALB mice labelled with NADPH-diaphorase histochemistry. (A–C) At low magnification, no differences can be appreciated. (D–F) At higher magnification of centro-medial glomerular regions a slight decrease in ND expression is noted at 20 days postlesion, although the pattern is the reverse of the appearance of the controls by 60 days of survival. Quantitative analysis of periglomerular cells densities did not reveal any differences (see Fig. 11). Scale bar, 1 mm (A–C), 100 μm (D–F).
juxtaglomerular cells and the temporal pattern of these reversible changes matches those of previous studies, as commented below. BrMet inhalation destroys almost the whole olfactory epithelium in diet-restricted rats (Schwob et al., 1995), but we did not observe such dramatic results in the histological analyses of mice subjected to slightly higher doses (not shown). This means that, at this dose, gas toxicity probably has an effect only on specific subpopulations of receptor neurons. Whether higher doses of BrMet would differentially affect chemically defined subsets of olfactory receptor neurons remains to be elucidated. Experiments using higher doses would offer a useful approach for gaining further insight into the organizational principles of the olfactory nerve projections.

**TH expression during olfactory deafferentation**

The reduction in TH expression after sensory inactivation by nasal occlusion and after olfactory deafferentation has been previously demonstrated in rat, mouse and gerbil (Nadi et al., 1981; Kawano & Margolis, 1982; Baker et al., 1984; Kosaka et al., 1987; Baker, 1988; Stone et al., 1990, 1991; Baker & Farbman, 1993; Cho et al., 1996; Vallejo-Valdezate, 1996; Philpot et al., 1997b). TH levels decrease

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**Fig. 12.** Each of these nine plots represents values for a specific topographic region within the olfactory bulb of BALB animals. On the abscissa axes experimental groups, including controls, are shown, and the ordinate axes represent the density of NADPH-diaphorase-positive juxtaglomerular cells per mm of bulbar perimeter. Values are presented as means ± SEM of each group (n=5). The Kruskal–Wallis test did not reveal differences among the experimental groups in any location in the BALB strain.
following these manipulations, but the enzymatic activity and other dopaminergic precursors remain unchanged in juxtaglomerular cells, suggesting a shift in cellular phenotype rather than cell death (Baker et al., 1983, 1984; Stone et al., 1991). The general results of our work are in accordance with those of Baker (1988) who also compared the two mouse strains. This author reported that a reduction in TH in BALB mice is apparent but no so dramatic in comparison with the picture in CD1 animals; indeed, while some qualitative changes were

Fig. 13. Each of the nine plots represents the density values of juxtaglomerular cells labelled with tyrosine hydroxylase (TH) or with NADPH-diaphorase histochemistry (ND) in nine different topographical regions of the olfactory bulb of two mouse strains. On the ordinate axis, the six experimental groups are sorted, and the abscissa axis shows the relative densities (% of control values); thus each value is the relativized mean of each group. Triangles and broken lines correspond to CD1 animals, and circles and continuous lines to the BALB mice. Open symbols are values of TH labelling and solid ones represent ND staining. Note the parallelism between the two markers: BALB values were very constant among the experimental groups but the CD1 values were not. This strain shows a decrease at about G20, returning to control values by about G60. The loss in CD1 animals is more dramatic for ND than for TH expression and both are more marked at rostral levels (I, II).
observed in the BALB strain, we did not detect a statistically significant decrease in any of the OB regions of these animals, but we did find them in almost the whole CD1 olfactory bulb.

**nNOS/ND expression during olfactory deafferentation**

ND labelling was much more affected after BrMet injury in comparison with the results obtained with TH immunohistochemistry, although both reductions showed parallel temporal and spatial patterns: both markers were more severely down-regulated in rostral regions and both showed a peak in G20 in all regions analysed. Minor regional differences can be explained in terms of different mechanisms underlying the expression-regulation of the two markers, although differences in the synaptology of the two subpopulations of PG cells cannot be ruled out.

Morris et al. (1997) have demonstrated that ND staining is dependent (as is NOS) on Ca2+ and calmodulin, that specific endothelial NOS activators (such as bradykinin and carbachol) enhance ND staining intensity, and that antagonists of NMDA glutamate receptors decrease the intensity of the histochemical reaction. These authors suggest that the intensity (and presence) of ND staining would be directly related to the activation level of the NOS enzyme at the time of tissue fixation, although this hypothesis remains to be verified. However, some aspects of our work do point in this direction. After BrMet injury, immunoreactivity for nNOS is diminished in PG cells, although still present, and the ND reaction, which in control animals is stronger than nNOS immunolabelling, is completely abolished in several OB regions. This suggests that olfactory deafferentation affects the activation of nNOS instead of its protein expression, although qualitative changes were also observed in nNOS immunoreactivity in the CD1 mice. These data must be confirmed with biochemical assays, i.e. quantification of [3H]-l-citrulline formation (Bredt & Snyder, 1990) or the evaluation of nitrate/nitrite levels (Taskiran et al., 1997; Yamada & Nabeshima, 1997).

We found that the expression of both nNOS and ND can be manipulated under experimental conditions and that their levels can be affected unequally. Indeed, increasing evidence is emerging about the inducibility of nNOS/ND expression, including that due to hormonal factors (Weiner et al., 1994; Sánchez et al., 1998; Rachman et al., 1998), stress (Kishimoto et al., 1996; Ueta et al., 1998) and in pathological conditions (Moncada & Higgs, 1993; Wu et al., 1994; Iadecola, 1997), among others. However, the underlying mechanisms of these manipulations and olfactory deafferentation are too dissimilar for homologies between them to be established. In this sense, assays performed in the spinal cord involving stimulation of different fibres (for a review see Callsen-Cencic et al., 1999) are closer to the experimental denervation studied here. Apart from the opposite results due to the different experimental protocols employed, an important conclusion of those studies was that nNOS immunoreactivity and ND histochemistry are affected unequally (see Hoheisel et al., 1997), in agreement with our results.

ND expression has only been studied experimentally in the OB after olfactory deprivation brought about by nasal occlusion. According to Croul-Ottman & Brunjes (1988), the distribution pattern of the histochemical activity is not altered in the rat, although those authors only made qualitative comparisons. Vallejo-Valdezate (1996) quantitatively compared the densities of labelled PG cells in the gerbil; nasal occlusion produced a decrease in TH-labelled juxtaglomerular neurons but not in ND-labelled PG cells. On comparing these observations with the results of the present work, it appears that different mechanisms underlie the responses to the two types of sensory deprivation, because olfactory deafferentation elicits a more dramatic reduction in ND expression than naris closure.

We used a model in which the elimination of afferent inputs is easily controlled and the natural regeneration of olfactory axons reverses TH and nNOS/ND patterns to control levels (see Fig. 13). Also, the bulb circuitry is well known; mitral cells establish reciprocal synapses with periglomerular cells (Pinching & Powell, 1971; White, 1973) and, at deeper levels, with granule cells (Shepherd, 1972; Jahr & Nicoll, 1982). In the mouse, both types of interneurons express nNOS/ND (Kishimoto et al., 1993; Weruaga et al., 1998).

We have found that nNOS/ND is diminished both in PG cells and in the neuropil of granule cells after olfactory lesion; these changes are very acute in the CD1 strain and not significant in BALB mice. It has been established that mitral/tufted cells show a spontaneous activity that is independent of odourant and that such activity is partially attenuated following naris closure (Philpot et al., 1997a). Under normal conditions we can postulate that the nitricergic phenotype in PG cells is controlled (i) in BALB animals only by the inputs from mitral/tufted cells, which might be stronger than in the CD1 strain; (ii) in CD1 animals by inputs from mitral/tufted cells.
and by direct olfactory afferents. After olfactory lesion, the normal synaptic equilibrium is disrupted; periglomerular cells in CD1 animals lose their olfactory afferents and nNOS/ND expression is down-regulated. PG cells in BALB olfactory bulbs seem to be less clearly affected. We cannot exclude synaptic differences in deeper layers between the two mouse strains, because the expression of nNOS/ND presents a parallel behaviour to what occurs in the glomerular region. Ultrastructural studies on this topic should be undertaken to check this hypothesis.

We conclude that the nitrergic phenotype of periglomerular cells is positively maintained by the direct peripheral inputs, because it is down-regulated when direct olfactory synapses are injured by methyl bromide inhalation in CD1 animals. However, sensory deprivation alone is not sufficient to produce this effect on the periglomerular cells that do not receive such direct inputs, as in the BALB animals, in which nitric oxide production is preserved in these interneurons by other afferent inputs. Current ongoing studies are now addressing the issue of whether or not mitral inputs influence ND expression in periglomerular cells.

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Abbreviations

BrMet, methyl bromide; L, lateral (part of olfactory bulb); M, medial (part of olfactory bulb); ND, NADPH-diaphorase; NO, nitric oxide; NOS, nitric oxide synthase nNOS, neuronal nitric oxide synthase; OB, olfactory bulb; PB, phosphate buffer; PBS, phosphate-buffered saline; PG, periglomerular; TH, tyrosine hydroxylase.

References


