BILATERAL Olfactory DEPRIVATION REVEALS A SELECTIVE NORADRENERGIC REGULATORY INPUT TO THE Olfactory BULB

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Abstract—Unilateral olfactory deprivation in the rat induces changes in the catecholaminergic system of the olfactory bulb. Nevertheless, evidence suggests that unilateral deprivation does not fully prevent stimulation of the deprived bulb. The present report analyses the response of the catecholaminergic system of the olfactory bulb in fully deprived rats obtained by bilateral naris occlusion. The complete deprivation produces more rapid and dramatic changes in both the intrinsic and extrinsic catecholaminergic systems of the olfactory bulb. Intrinsic responses involve a rapid decrease in dopamine-containing cells to about 25% of controls, correlated with a decreased Fos expression in juxtaglomerular cells of all olfactory glomeruli, with the only exception of those of the atypical glomeruli which maintain unaltered expression of both markers. In parallel with these events, there is a progressive increase in the density of extrinsic noradrenergic axons arising from neurons in the locus coeruleus, which show, in parallel, a progressive increase in Fos expression. This model demonstrates plastic changes in the catecholaminergic system of the olfactory bulb forming a valid morphological substrate for lowering thresholds in the processing of olfactory information. In addition to this generalized response, there is another one, directed to a specific subset of olfactory glomeruli (atypical glomeruli) involved in the processing of odor pheromone-like cues related to behavioral responses, that could be responsible for keeping active this reduced and selected group of glomeruli carrying crucial olfactory information.

These results indicate the existence of adaptive changes in the catecholaminergic system of the olfactory bulb as a response to the lack of afferent peripheral stimulation. These changes involve dopamine- and noradrenaline-immunoreactive elements, in a strategy presumably directed at maintaining to the highest possible level the ability to detect olfactory signals. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: atypical olfactory glomeruli, catecholamines, noradrenaline, sensory deprivation, typical olfactory glomeruli.

Afferent activity regulates a variety of developmental, cellular, molecular and physiological events in sensory systems. In the olfactory system, afferent activity is needed for the development and maintenance of the normal structural and biochemical features of the olfactory bulb. This has been demonstrated by olfactory deprivation, a procedure that experimentally alters neural activity in the bulb, inducing striking neuroanatomical, neurochemical and functional changes.

The degree of stimulation modulates the olfactory bulb function, and thus, olfactory deprivation enhances projecting neurons responses to both odors and electrical stimulation of the olfactory nerve. The mechanisms responsible for these modulatory events involve catecholaminergic systems: bulbar dopaminergic juxtaglomerular neurons regulate the strength of primary olfactory input through a presynaptic inhibition of olfactory nerve glutamate release, and noradrenergic central inputs may increase mitral/tufted cells excitability. In this context, olfactory deprivation obtained by unilateral naris closure induces a down-regulation of dopamine synthesis in juxtaglomerular neurons, as demonstrated by a reduction in dopamine content and expression of its biosynthetic enzyme tyrosine hydroxylase (TH) in the olfactory bulb ipsilateral to the occluded naris, whereas at the same time, there is an increase in noradrenaline content.

Although the changes in the bulbar content of these catecholamines are evident after unilateral deprivation, the decrease in dopamine and TH is heterogeneous, the loss of these markers being more dramatic in the rostral and medial areas rather than in caudal and lateral portions of the olfactory bulb, and noradrenaline content shows only a transient increase in the first days after deprivation. These data raise the question of how effective unilateral naris closure is in fully depriving olfactory bulb neurons of odorant stimulation. Although the signal value of the stimulus arriving at the occluded olfactory bulb could somehow be degraded, anatomical, behavioral and immunohistochemical evidence exists for stimulation of the olfactory bulb ipsilateral to the occluded naris in unilaterally deprived animals. On this basis, the current experiments were designed
to investigate the time-course and extent of the reactive changes in the catecholaminergic system of the olfactory bulb after full olfactory deprivation, and to elucidate whether these changes differ from those reported after unilateral deprivation. We hypothesized that more dramatic and rapid adaptive changes would occur in order to compensate for the lack of afferent stimulation that in macroscopic mammals, such as rats, is crucial for life.

**EXPERIMENTAL PROCEDURES**

Thirty-five adult female Wistar rats weighing 250–280 g were used. Animals were housed under constant temperature conditions (22°C) on a 12-h light/dark cycle with food and water available *ad libitum*. All efforts were made to minimize the number of animals used and their suffering during the experimental procedures, in accordance with the guidelines of the NIH, European Communities Council Directive (86/609/EEC) and current Spanish legislation for the use and care of laboratory animals (BOE 67/859-12, 1988).

**Tissue processing**

Animals (*n* = 21) were deeply anesthetized with ketamine (Ketolar, 50 mg/kg body weight) and both external nares were closed by cautery using a bipolar coagulator. Sham-lesioned animals (*n* = 7) were identically treated, except that the cautery was placed on the top of the nose just above the nares. After surgery, xylocaine and antibiotic cream were applied to the wound area to alleviate pain and to prevent infection, respectively. Animals were then returned to their home cage and examined daily. Only those showing complete closure of the nares in all examinations were used for immunohistochemical analysis. Additional animals (*n* = 7) that did not undergo surgery served as controls for each time-period examined. At 12 h, one, two, three, five, seven and 14 days post-surgery (*S1/2, S1, S2, S3, S5, S7 and S14*), three deprived animals, one sham operated and one control were killed for either histochemical or immunohistochemical analysis.

Animals were i.c. perfused under deep anesthesia (Ketolar 50 mg/kg body weight) with 100 ml Ringer solution followed by 400 ml fixative solution made up of 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.3. The brains were removed from the skull, and both the olfactory bulbs and regions containing the locus coeruleus were dissected and postfixed in the same fixative at 4°C for an additional 2 h. Tissue blocks were either sectioned at 50 μm, or cryo-protected by immersion in 30% sucrose in 0.1 M phosphate buffer, pH 7.3, at 4°C until they sank, and were cut at 25 μm thickness using a cryostat. One series of each region was Nissl stained with 0.25% thionin for the demonstration of cell bodies and general histological organization. Adjacent series were processed for either immunohistochemical or histochemical procedures. The sections from animals of the same group (either deprived, sham operated or control) were processed for either immunohistochemical or histochemical procedures. The sections from animals of the same group (either deprived, sham operated or control) were processed for either immunohistochemical or histochemical procedures. The sections from animals of the same group (either deprived, sham operated or control) were processed for either immunohistochemical or histochemical procedures.
Olfactory deprivation and catecholaminergic system

Table 2. Number of Fos-immunoreactive cells in the locus coeruleus unilaterally (mean ± S.E.M.) in a one-in-three series of 25-μm-thick sections at different days post-occlusion

<table>
<thead>
<tr>
<th>Days post-surgery</th>
<th>Fos-positive cells (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>75.61 ± 9.3</td>
</tr>
<tr>
<td>3</td>
<td>173.88 ± 15.6</td>
</tr>
<tr>
<td>7</td>
<td>249.48 ± 27.9</td>
</tr>
<tr>
<td>14</td>
<td>374.22 ± 30.2</td>
</tr>
</tbody>
</table>

are given as the mean number (± S.E.M.) of cells. These data are shown in Table 2.

RESULTS

Animals were surgically treated to obtain a bilateral naris occlusion, and their olfactory bulbs were analysed and compared with olfactory bulbs from both sham-operated and additional animals (“naive”) that did not undergo surgery. Preliminary analysis demonstrated that sham-operated and “naive” animals can be all considered as controls since no differences in bulb morphology, histochemical or immunohistochemical stainings were observed.

Olfactory bulbs from experimental animals demonstrated a decreased number of TH-immunoreactive juxtaglomerular neurons, which fell to 25% of controls by seven days after surgery (S7 animals; Table 1; Fig. 1a–d). This decreased expression of dopaminergic phenotype, although extensive, was not uniform, and selected cell groups demonstrated similar TH immunolabeling as in control animals. These groups were not scattered in the glomerular layer but they were located in specific glomeruli at the caudal half of the olfactory bulb, with identical morphological and topological features in all animals (Fig. 2).

Based on studies reporting the existence in the rat of intrinsic cell types,11–13,54 we tried to identify the glomeruli showing an invariable occurrence of dopaminergic cells. Adjacent sections processed for acetylcholinesterase histochemistry allowed the identification of those glomeruli with invariable density of TH-immunopositive neurons as atypical glomeruli. They demonstrated an unchanged density of TH-immunostained juxtaglomerular cells surrounding them in all animals, at all postsurgical periods (Table 1; Fig. 2).

Since one mechanism of TH transcriptional regulation is through interaction of its promoter regulatory elements with Fos proteins encoded by the proto-oncogene c-fos,30 we examined the relationship between the expression of Fos and TH. Fos immunostaining in control animals revealed a moderate number of labeled cells in the glomerular layer and in the granule cell layer (Fig. 3a). The olfactory bulbs from S1 and S3 animals displayed a slightly higher number of Fos-immunostained cells compared to control animals. From S5 onwards, Fos immunostaining was progressively reduced in the glomerular layer both in number of labeled elements and in their intensity, whereas the staining in the granule cell layer was apparently constant (Fig. 3b). The glomerular layer at S7 and S14 showed scarce Fos-immunostained elements with the exception of abundant Fos-immunoreactive cells in the atypical glomeruli (Fig. 3c, d).

The invariable presence of TH in juxtaglomerular cells of atypical glomeruli (Fig. 2) and their continued expression of Fos proteins (Fig. 3c, d) indicate that these cell groups continue to be activated after bilateral olfactory deprivation, whereas those in typical glomeruli do not. Since olfactory receptor cells projecting to both typical and atypical glomeruli are intermingled in the olfactory mucosa,28,39,54 it is conceivable that the maintenance of cell activity in atypical glomeruli is mediated by other mechanisms distinct from peripheral inputs.

Among the most extensive centrifugal afferents involved in modulation of the olfactory bulb activity (cholinergic, catecholaminergic and serotonergic), we did not find differences in the distribution and staining intensity of labeled fibers after choline-acetyltransferase immunohistochemistry, acetylcholinesterase histochemistry and serotonin immunohistochemistry. However, in parallel with the decrease in the expression of dopamine in bulbar juxtaglomerular neurons, there was a marked increase in the catecholaminergic innervation of the olfactory bulb (Fig. 1e, f), revealed by means of either TH or dopamine-β-hydroxylase immunostaining.

In normal conditions the noradrenergic innervation in the olfactory bulb, is mainly distributed in the granule cell layer; other layers such as the mitral cell layer, the external plexiform layer and the glomerular layer have much less dense innervation (Fig. 1c, e). After deprivation, there is a gradual increase in the density of noradrenergic axons from S3 up to S14 (Fig. 1d, f). Although this occurs in all bulbar layers, it is particularly evident in the granule cell layer, the external plexiform layer and in specific locations of the glomerular layer corresponding to atypical glomeruli.

Correlated with the locations of this maximal increased noradrenergic innervation was the presence of strongly Fos-immunoreactive elements. Thus, at long deprivation times (S7 and S14), strongly Fos-immunoreactive cells were located in the regions of more dense noradrenergic innervation: the granule cell layer, where most, if not all, granule cells were strongly Fos immunolabeled; and the glomerular layer, specifically in the juxtaglomerular cells surrounding the atypical glomeruli. These observations suggest a relationship between noradrenergic innervation and cell activation revealed by the strong expression of early genes.

The only source of extrinsic noradrenergic innervation to the olfactory bulb is the locus coeruleus. This pontine nucleus contains the most important noradrenergic neuronal population of the brain, which projects diffusely to most regions, including the olfactory bulb. We checked for possible changes related to the activation of those neurons after olfactory deprivation and found a progressive increase in the intensity and number of Fos-immunostained cells in parallel with the deprivation...
Fig. 1. Catecholaminergic elements revealed by tyrosine hydroxylase immunohistochemistry in the olfactory bulb of control animals (a, c, e) and after 14 days of bilateral olfactory deprivation (b, d, f). (a, b) Low-power photomicrographs of the olfactory bulb showing the distribution of catecholaminergic cells in control animals (a) and the notable reduction of juxtaglomerular neurons expressing TH in the glomerular layer after 14 days of bilateral deprivation (b). (c, d) High-power photomicrographs of the olfactory bulb showing TH staining in normal condition (c) and after 14 days of bilateral olfactory deprivation (d). Note the dramatic reduction in the number of juxtaglomerular neurons expressing TH in the glomerular layer (GL) and the increased density of catecholaminergic fibers in the remaining layers of the olfactory bulb. (e, f) Dark-field photomicrographs showing catecholaminergic fibers in normal condition (e) and the increased density after 14 days of bilateral olfactory deprivation (f). EPL: external plexiform layer; MCL: mitral cell layer; GCL: granule cell layer. Scale bar (in f) = 1 mm (a, b), 200 µm (c, d), 175 µm (e, f).
The increased expression of Fos in the locus coeruleus can therefore be paralleled by the reinforcement of the noradrenergic innervation observed in the olfactory bulb, and it could represent the anatomical basis for a plastic response of this central modulatory system induced by the lack of stimulation of the olfactory receptors.

**DISCUSSION**

Our results reveal that bilateral naris occlusion leads to more dramatic changes in the catecholaminergic system of the rat olfactory bulb than previously reported after unilateral naris closure. Firstly, the number of intrinsic dopaminergic elements falls to approximately 25% of controls, whereas previous reports indicate that after unilateral naris closure the number of TH-containing neurons is maintained at 40–45% of control levels. Secondly, this dramatic loss of dopamine expression was faster after bilateral deprivation than in unilaterally deprived animals where the decrease in TH expression continues gradually up to four to eight weeks post-occlusion, whereas we found a maximal depletion just seven days after surgery. Thirdly, depletion of dopamine expression affects similarly all typical glomeruli, while after unilateral deprivation, the glomeruli located...
at the lateral and caudal portions of the olfactory bulb are apparently unaffected by deprivation. Fourthly, whereas in unilaterally occluded animals there is a transient increase in noradrenaline content in the first days after occlusion, bilaterally deprived animals demonstrate a progressive increase in the density of noradrenergic fibers.

These findings clearly suggest that unilateral naris closure does not result in a total sensory deprivation. In fact, region-specific levels of maintained activity have been demonstrated in the ipsilateral bulb to the occluded naris in unilaterally deprived animals, indicating that, although at a lower rate, the deprived olfactory bulb continues to receive afferent stimulation. By contrast, bilateral naris occlusion prevents olfactory mucosa stimulation, and although odorants refluxed through the nasopharynx could theoretically reach the olfactory mucosa, our results demonstrate that this pathway, at least in rat, is not effective in activating the olfactory system, as indicated by the large and rapid reduction in TH expression.

Deprivation enhances mitral/tufted cell responses to both odors and electrical stimulation of the olfactory nerve. Despite the underlying mechanism not being clear, it has been suggested that the degree of olfactory stimulation modulates olfactory system function through controlling the level of dopaminergic inhibition.

Dopaminergic juxtaglomerular cells modulate the olfactory sensory input regulating the strength of primary olfactory input, apparently through a presynaptic inhibition of olfactory nerve glutamate release. A large reduction in dopamine synthesis in typical glomeruli as observed in this study would therefore enhance the strength of presynaptic nerve input, thus making odors previously ineffective at activating a particular cell, now over-threshold. In a transitory circumstance, this will result in a partial restoration of olfactory function. In bilaterally deprived animals, by contrast, this down-regulation mechanism would end in the total loss of TH expression, as observed in our material.

Juxtaglomerular neurons of atypical glomeruli did not show, however, this characteristic loss of dopamine expression in any of the deprivation periods analysed. The association between TH and Fos expression in the

Fig. 3. Photomicrographs showing Fos immunoreactivity in the rat olfactory bulb in control and in seven-day-deprived animals. (a) Fos immunostaining in the olfactory bulb of a control animal. (b) After seven days of deprivation. Note the reduction in the number of juxtaglomerular neurons in the glomerular layer. (c, d) Maintained Fos immunostaining after deprivation in the atypical glomeruli (arrows); a reduction in the number of positive elements is evident in typical glomeruli (arrowheads). c, control; d, seven days of deprivation; GL, glomerular layer; MCL, mitral cell layer. Scale bar (in d) = 200 μm.
olfactory bulb, and the fact that Fos detection has proved useful for identification of activated neurons suggest that these glomeruli remain active under deprivation conditions. Taking into account that olfactory receptor cells projecting to atypical glomeruli are intermingled with typical receptor cells in the olfactory mucosa, we must assume that odorants are similarly impeded to access them. Therefore, the apparent normality observed in the juxtaglomerular cells of atypical glomeruli, demonstrated by maintenance of TH and Fos expression, must be mediated through a mechanism not involving peripheral inputs.

The main centrifugal inputs to the olfactory bulb remain apparently unaltered after bilateral sensory deprivation, with the only exception of noradrenergic projection which showed a marked increase in its density. A transient increase in the noradrenaline content in the deprived bulb has been detected after unilateral deprivation in the first three days after surgical treatment. After this adaptatory period, noradrenaline expression seems to be unaffected by the manipulation and returns to normal levels. The initial increase in noradrenaline content can be interpreted as an adaptive mechanism that tends to regulate the odorant detection thresholds in the occluded olfactory bulb. After this first stage of noradrenergic system activation, it is conceivable that the activity of the non-occluded bulb influences indirectly the activity of the occluded bulb through fibers connecting both sides of the olfactory system, whether at the bulbar level such as the interbulbar associational system, or at higher levels of the olfactory pathway. This is supported by the fact that unilaterally deprived animals exposed to odorants demonstrate a centrifugal stimulation in both normal and deprived olfactory bulbs. This mechanism could maintain a certain degree of activity in the occluded bulb, thus preventing, or minimizing, a continued response of the noradrenergic regulatory system. Accordingly, our results suggest that bilateral deprivation would induce a similar reaction of the noradrenergic modulatory system to that found in the first days after unilateral deprivation. However, given the total lack of stimuli in both olfactory bulbs, the reaction of the noradrenergic system cannot be compensated, and its response continues throughout the deprivation time, as demonstrated by the progressive increase in both dopamine-β-hydroxylase- and TH-stained fibers in parallel with the deprivation time.

Activation of the noradrenergic projection, important in the regulation of olfactory response and crucial in neural plasticity throughout the brain, could be correlated to the maintenance of Fos expression in the granule cells and in juxtaglomerular cells at the atypical glomeruli. Although Fos synthesis cannot be considered as a simple marker of spiking neuronal activity, in

![Fig. 4. Progressive increase in the number of Fos-immunostained elements in the locus coeruleus. (a) Control; (b) three days of deprivation; (c) seven days of deprivation; (d) 14 days of deprivation. Scale bar = 100 μm.](image)
many neural systems, including the olfactory system, Fos expression is regulated in association with neuronal activity. In addition, triggering of Fos expression seems to be mediated through β-adrenergic receptors, suggesting that the maintained Fos expression in these cell groups may originate, in this circumstance, from the noradrenaline released by centrifugal afferents.

Several studies have revealed a spectrum of actions for noradrenaline in sensory areas of the brain, which suggests that at the level of single cells, this neuroactive substance enhances the efficacy of synaptic transmission. A consistent finding across a number of systems is that exogenous noradrenaline or activation of the locus coeruleus enhances the signal to noise ratio of evoked responses, typically by decreasing spontaneous activity proportionately more than afferent-evoked activity. This process appears to be mediated by interactions with both α- and β-adrenergic receptors through which noradrenaline can convert weak or sub-threshold stimuli into a threshold response.

In the olfactory bulb, available data demonstrate that at the level of the reciprocal excitatory–inhibitory synapses established between mitral/tufted cells and granule cells, noradrenaline inhibits monosynaptic excitatory post-synaptic potentials recorded on granule cells through inhibition of presynaptic voltage-sensitive calcium channels of mitral/tufted cells, resulting in an inhibitory neuromodulatory mechanism. This effect is mimicked by the β-adrenergic receptor agonist clonidine, but not by the β-adrenergic receptor agonist isoproterenol, thus suggesting that noradrenaline disinhibits mitral cells by reducing mitral cell excitation of granule cells via a presynaptic α-adrenergic receptor mechanism. Therefore, the release of noradrenaline in the olfactory bulb may enhance mitral cell excitability, increasing their sensitivity to weak odors or low stimulation from receptor axons.

The disinhibitory effect of noradrenaline on mitral/tufted cells can also occur at the level of the glomerular layer where these projection neurons establish synaptic relationships with periglomerular cells similar to those established with granule cells in deeper layers. Although the noradrenergic innervation in the glomerular layer is not as extensive as it is in other bulbar layers, our results demonstrate the presence of a population of glomeruli with a dense noradrenergic innervation. These atypical glomeruli are, in addition, characterized by a high cholinergic innervation, and appear to be unaffected by the olfactory deprivation.

A widespread interaction between cholinergic and catecholaminergic circuitries has been demonstrated in the rat CNS. In the olfactory bulb, there is evidence of cholinergic regulation of noradrenaline release from locus coeruleus terminals. Thereby, the noradrenergic innervation directed to the “atypical system”, a privileged pathway responding to a restricted group of signals, with a strong centrifugal control and a limited local modulation that allows a fast and primed input transmission, would result in the maintenance or facilitation of the readiness of this segregated olfactory subsystem. During periods of increased output from locus coeruleus neurons, the response properties of sensory neurons may be enhanced to the point that these cells are able to encode more precisely information from the sensory surround. Then, under sensory deprivation conditions, the modulatory system originated in the locus coeruleus will react in two ways using noradrenaline as a tool: (i) originating a general response, directed to lowering the thresholds of mitral cells through a generalized granule cell inhibition, and (ii) a more selective one, keeping at the highest possible level the readiness to respond to olfactory signals of particular cell circuitries (atypical glomeruli) responsible for the processing of specific cues, crucial for the survival of the animal.

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