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Animal models of depression: olfactory lesions affect amygdala, subventricular zone, and aggression

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Psychiatric or depressed patients show alterations in both olfactory projection areas and mucosa. In rodents, ablation of olfactory bulbs causes a depression-like syndrome, useful to test antidepressant agents. We studied in mice the behavioral symptoms and neuroanatomical correlates after mucosal damage or ablation of the olfactory bulb. Our results are based on a battery of tests exploiting anxious, aggressive, and depressive behavior, on morphological and immunohistochemical analysis. We found similar results in both sensory-damaged and bulbectomized animals, with a behavioral dissociation concerning different forms of aggression. These findings do not support a simple downregulation of social interactions in damaged mice. The most prominent modifications in the brains of sensory damaged and bulbectomized mice are detected in the subventricular zone (SVZ), the source area of neural stem cells, and in the content of cAMP-dependent protein kinase within the amygdala, suggesting a central role of this structure in the functional modulation of behavior.

Keywords: Olfactory bulbectomy; Rostral migratory stream; Behavior; Depression; Aggression; Neural stem cells

Introduction

Modulation of behavior requires a fine interplay among different cerebral areas. Patients exhibiting mood disorders, depression, or psychiatric syndromes often show alteration in structures related to olfactory function. At a peripheral level, olfactory performance is reduced in depressed patients (Pause et al., 2001) and the differentiation of olfactory neurons is abnormal in persons suffering from schizophrenia (Arnold et al., 2001). Morphological differences are present in olfactory projection areas and noticeably in the amygdala of depressed (Nestler et al., 2002), schizophrenic (Lawrie et al., 1999; McCarley et al., 1999), and psychotic patients (Tebartz van Elst et al., 2002). In depressed persons, the same olfactory projection areas work differently than in control subjects when processing emotional information (Siegle et al., 2002), so that both anatomical and functional deficits are present.

Experimental models of psychiatric diseases are necessary for pharmacological trials but are difficult to implement. In rodents, perturbation of the olfactory system results in marked behavioral and autonomic (Moffitt et al., 2002) deficits. After ablation of the olfactory bulbs and the consequent loss of smell, consistent behavioral modifications ensue, including hyperactivity and alterations in exploration and social behavior (Brunjes, 1992). Noteworthy, bulbectomized mice show poor social behavior without aggression, territorial defense, or social hierarchy (Liebenauer and Slotnick, 1996). The bulbectomized rat or mouse has thus been considered a model of agitated depression (Kelly et al., 1997; Leonard and Tuite, 1981; Lumia et al., 1992) to screen putative useful molecules due to its consistent responses to antidepressants (van Riezen et al., 1977).

The behavioral outcomes of surgical removal of olfactory bulbs are most commonly attributed to Wallerian degeneration, compensatory reorganization after deafferentation, up- or downregulation of neurotransmitters, receptors, or synaptic strength in both olfactory and limbic structures (Brunjes, 1992). To describe the olfactory bulbectomy (BX) model of depression, most literature refers to earlier papers, indicating that the olfactory bulb was not a mere sensory area and suggesting that it could have non-olfactory functions relevant for modulation of behavior (Edwards et al., 1972; Cain and Paxinos, 1974). On the other hand, these studies exploited only one or very few behavioral parameters. Therefore, we examined different behavioral traits in the same mice to ascertain whether olfactory bulbectomy (BX) affects several behavioral parameters and social traits as it happens in human psychiatric patients. From the beginning, the behavioral deficits in BX rodents were attributed to central modifications (Brunjes, 1992; Cain and Paxinos, 1974), and not to peripheral damage. Because in human patients peripheral olfactory deficits have been described (Arnold et al., 2001; Pause et al., 2001), we also tested mice whose olfactory mucosa was previously damaged with zinc sulfate (Zn), but with the olfactory central areas left undisturbed. The results were then compared with BX and control mice (SHAM). Finally, mouse brains were examined to see whether similar behavioral deficits subserve similar modifications in olfactory projection areas of BX and Zn mice.

The aim of the present work is (I) to elucidate the relative contribution of peripheral (Zn) and central (BX) olfactory deaf-

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ferentation in the appearance of previously unreported behavioral deficits, (II) to describe the most prominent changes induced in the brain by both manipulations, and (III) to ascertain whether these changes involve similar areas committed to behavioral control, with the ultimate goal to relate these findings to human diseases to deepen our understanding of neurobehavioral impairments.

Materials and methods

Animals

In all experiments, male Swiss mice were employed. They were kept in a breeding colony on a 12:12 h light schedule (light on at 6:00 a.m.) with food and water ad libitum. They were housed in plastic cages (42 × 27 × 15 cm) at 24 ± 1°C with wood sawdust litter changed every fourth day. Experiments conformed to the Italian law on animal experiments (L. 116/92) and were approved by the Ministry of Health. Three groups of 13 mice each were tested: bulbectomized (BX), sham-operated (SHAM), or zinc-sulfate-treated (Zn). Mice underwent surgical removal of olfactory bulbs after weaning at 18 ± 1 days, an age at which amygdala projection to the frontal cortex has already been established (Bouwmeester et al., 2002). After being anaesthetized with xilazine (20 mg/kg body weight) and ketamine (75 mg/kg body weight), the skin was cut and the skull was opened rostrally to the convergence of venous sinuses. In sham animals, the skin was sutured at this stage, while in bulbectomized animals, both olfactory bulbs were excised by aspiration using a fine pipette (inner diameter: 0.5 mm) up to the rhinal fissure. Care was taken not to damage the whiskers and to leave the rostral pole of frontal lobes intact while removing as much olfactory bulb tissue as possible. Part of the anterior olfactory nucleus was always damaged. The mice were left to recover for 1 day and then returned to their home cage. The destruction of the olfactory mucosa was performed with intranasal irrigation with zinc sulfate (100 μl, 4% in distilled water), rapidly instilled using a 1-ml syringe with a plastic tip in unanaesthetized 60-day-old mice.

Behavioral tests

To describe the behavioral modifications induced by central and peripheral deafferentation, the mice were left to recover for 1 month after surgery, or for 5 days after nasal irrigation, which allowed enough time for nerve degeneration but not for nerve regeneration (Herzog and Otto, 1999). They were then tested during consecutive days. The olfactory function was tested with the Cookie finding test (Hendricks et al., 1994), which operationally defines anosmia as the failure to find a hidden food item within 5 min: the mice were left without food for 12 h and then put for 5 min in a cage (42 × 27 × 15 cm) in which a pellet of food was buried beneath the wood shavings in a variable position but never on the corners or in contact with the lateral walls. The latency to discover the buried pellet was indicative of olfactory function since the presence of a minority of fibers is sufficient for the mouse to retrieve the food (Ducray et al., 2002; Harding et al., 1978).

The mice were then tested in an open field to measure locomotion and exploration in a new environment (Kelly et al., 1997; Stock et al., 2001). The mice were put for 3 min in a clean plastic cage (55 × 33 × 20cm) with opaque walls, with the floor subdivided in 15 squares (11 × 11cm); the number of squares

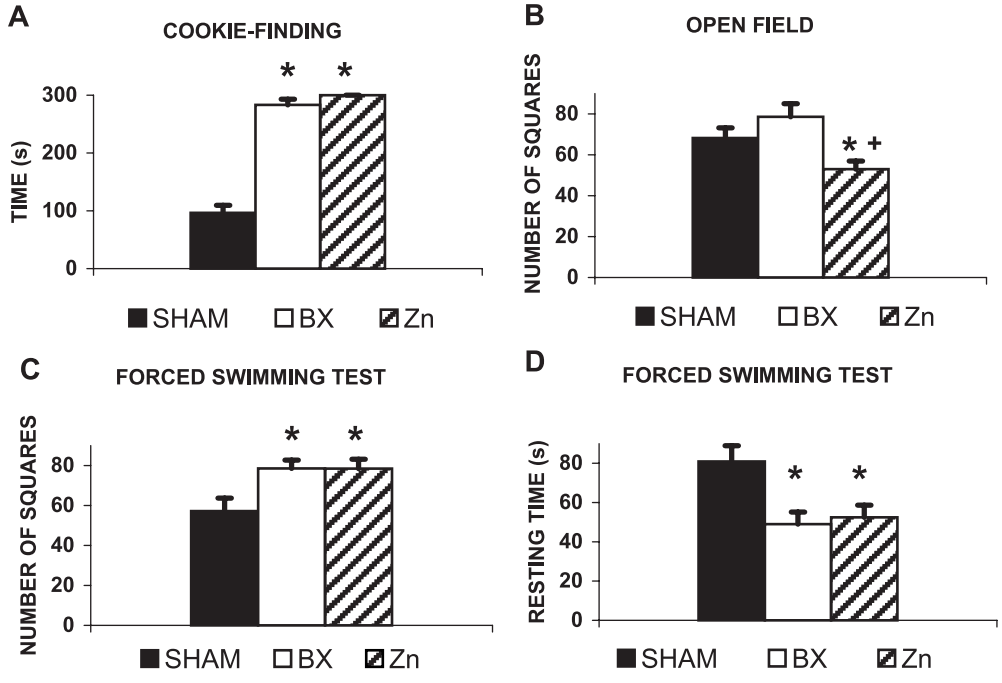

Fig. 1. Behavioral data obtained in the classic tests used to evaluate BX performance as a model of depression. Means + SEM are shown. *$P < 0.05$, different from SHAM. $^{+}P < 0.05$, different from BX. (A) Time to discover the buried pellet in SHAM, BX, and Zn mice. SHAM animals were faster than BX and Zn, which were anosmic. (B) Open field test: number of squares crossed by mice. Zn mice crossed fewer squares than SHAM and BX. (C) Forced swimming test: number of squares crossed by mice. BX and Zn cross a higher number of squares. (D) Forced swimming test: cumulative resting time. SHAM swim less, having a higher resting time than BX and Zn.

crossed with the four paws was scored, and also two indexes of autonomic functions (number of fecal pellets and of urine drops emitted during the test).

The forced swimming test is widely used to test rodent models of depression (Porsolt et al., 1977). The mice were released in the center of a plastic pool (55 × 35 × 30 cm) with vertical walls to prevent escape, filled with water (25°C), 20 cm deep. The floor was subdivided in 15 squares. The mice were left for 3 min in the pool; the latency to the first stop, the total cumulative resting time (in which the mouse does not perform any swimming movement except those movements performed to keep the head above water), and the number of squares crossed were scored. We used a larger pool compared to previously published papers (Porsolt et al., 1977) because this enhances the predictive validity for mouse testing (Lucki et al., 2001) and allows the measure of the traveled distance (as number of squares crossed).

Then three tests of aggressive behavior were done. The first was the resident–intruder test that measures intraspecific aggression towards same-sex, same-age animals. An unknown male mouse was introduced in the home cage of the test mouse and their interactions were recorded for 30 min or until two attacks were released. To decrease the level of aggression of intruders, they were housed in cages with same-sex cagemates and were never chosen among the dominant mice, while resident mice were kept isolated for 48 h before the test. The testing conditions were chosen to be not extremely anxiogenic for the test mouse, that is, it was tested in its home cage in lighting conditions similar to those of the room in which the mice lived.

Olfactory bulbectomy also affects behavior towards pups (Fleming et al., 1979); therefore, a test for infanticide was included to measure noncompetitive intraspecific aggression. This test was designed taking into account the ethical and technical issues posed by Elwood et al. (1991). The mouse was put in a clean cage (25 × 15 × 13 cm) and left undisturbed for 10 min to habituate. Then a mouse pup (up to 48 h after delivery), not familiar to the male, was introduced into the cage and the latency to the first attack was recorded, otherwise the test ended after 5 min. After the first attack, the mouse pup was immediately rescued. If it was seriously harmed, it was euthanized with an overdose of anesthesia (double the dose above). The number of pups used was 39. Three of them were euthanized, the others were returned to their mother that promptly accepted them. No case of rejection was observed.

Behavioral modifications in bulbectomized mice also affect different aggressive behaviors, with a specific enhancement of interspecific aggression (Cain and Paxinos, 1974). Therefore, a measure of predatory aggression was included. The mice were released in a plastic cage (25 × 15 × 13 cm) without wood shavings on the floor and left undisturbed for 10 min to habituate. Then an earthworm (*Lombricus terrestris*) was introduced on the floor and the mouse behavior was observed up to the first attack or for a maximum of 20 min, and the latency towards it was scored.

Data were analyzed with one-way between-subjects ANOVA for the factor group (SHAM, BX, Zn) followed by Newman–Keuls post hoc test. A probability level of $P < 0.05$ was considered significant. All values are presented as means ± SEM.

Histology

All mice were sacrificed after completion of behavioral tests, that is, 7 days after Zn irrigation or 30 days after BX or SHAM surgery. Mice were deeply anesthetized and perfused through the left ventricle with ice-cold phosphate buffer saline (PBS: phosphate buffer 20 mM pH 7.5, NaCl 150 mM) followed by formalin 10% in PBS. Brains were dissected, dehydrated, embedded in paraffin, and sectioned at 7 μm. Every second section was stained with thionine and the areas were identified (Franklin and Paxinos, 2000). Horizontal sections were observed with a Leica microscope equipped with a digital camera. Images of the section in which the rostral migratory stream (RMS) was larger were selected only from mice in which the angle of the cut appeared similar and at a comparable level ($n = 7$ each for SHAM, BX, and Zn), excluding mice whose brain was sectioned tilted at different angles. Images were taken with a 5× objective using a Leica microscope with the resident software at 782 × 582 pixels. Subsequently, the following measures in pixels were calculated with the SCION-NIH program: the maximum width of RMS at the rostral pole and the maximum width of the subventricular zone (SVZ). To correct the artefacts due

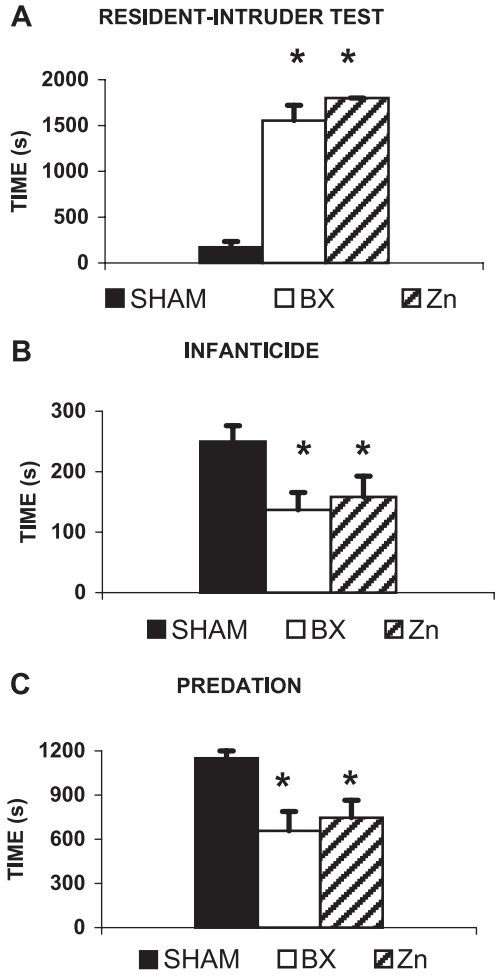

Fig. 2. Behavioral data obtained in tests involving aggressive behavior. The rationale for testing different forms of aggression resides in the fact that different nuclei in the amygdala complex and noticeably those receiving olfactory and vomeronasal inputs mediate the aggressive responses. Means + SEM are shown for the latency to the first attack. *$P < 0.05$, different from SHAM. (A) Resident–intruder test: BX and Zn attack the male intruder slower than SHAM. (B) Infanticide test: counter-intuitively, BX and Zn attack the pup faster than SHAM. (C) Predation test: as in the previous test, both BX and Zn attack faster than SHAM.

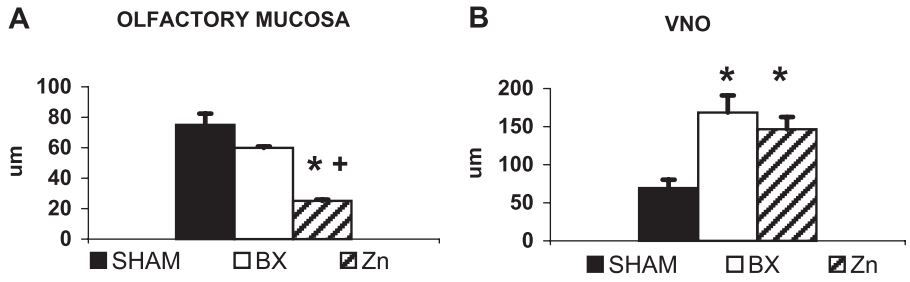

Fig. 3. Height of the olfactory mucosa and of the germinative area of vomeronasal organ. *$P < 0.05$, different from SHAM. $^{+}P < 0.05$, different from BX. (A) The height of the olfactory mucosa is lesser in Zn mice than in SHAM and BX, indicating destruction of the mucosa due to the treatment; no difference is apparent between SHAM and BX. (B) The germinative area is larger in the vomeronasal organ of both BX and Zn mice compared to SHAM.

to fixation shrinkage, a reference length was taken: the distance between the lateral angle of the lateral ventricle, that is, the starting point of RMS, and the interhemispheric scissure, calculated orthogonally to the medial wall of the lateral ventricle. This reference length was not different in the three groups of mice, $F(2,17) = 0.829$, $P = 0.453$, so the SVZ and RMS measures were compared directly. Also, the height of the olfactory mucosa ($n = 6$) and the vomeronasal germinative zone ($n = 7$) that contains the marginal precursor pool (Halpern and Martinez-Marcos, 2003) was measured as above in hematoxylin–eosin-stained sections of decalcified heads with a 40× objective; four measures were taken in different zones from each animal and averaged out. No reliable measures could be taken in the central part of the vomeronasal mucosa that appeared most damaged in Zn mice. Fig. 6 shows the points where the measures were taken in the RMS (Figs. 6a–c), SVZ (Fig. 6c, inset), olfactory mucosa (Fig. 6d), and vomeronasal germinative zone (Figs. 6e and f).

Data were analyzed with ANOVA, as above.

Immunohistochemistry

Representative sections from three mice per group were studied to understand whether mature types of cells were present in the rostral portion of the RMS. Mice were sacrificed by cervical dislocation at the end of behavioral tests (see Histology above), the brains were dissected, frozen, and sectioned with a cryostat at 20 μm and briefly fixed in formalin. Immunohistochemistry was performed with monoclonal antibodies against β3-tubulin (Chemicon), a marker of mature neurons, vimentin (Sigma), a marker of immature neurons, and GFAP (Sigma), a marker of astroglia, or

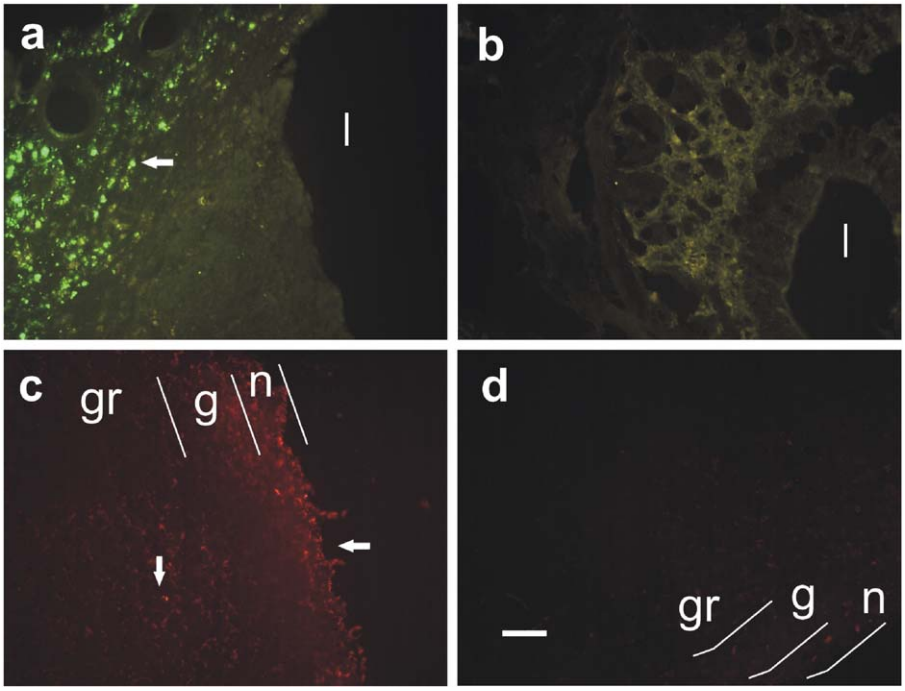

Fig. 4. Modifications observed in the brains of BX and Zn mice. Scale bar = 50 μm. (a and b) Coronal sections through the nose, upper on the top, medial on the left, l = lumen. (a) Fluorescent beads injected in the olfactory bulb reach the olfactory nerve in SHAM but not in Zn mice (b), indicating that the olfactory nerve degenerated; arrow points to labelled olfactory nerve fibers. Fluorescent beads were injected 7 days after Zn treatment; SHAM and Zn mice were sacrificed 7 days after bead injection. (c and d) Coronal sections through the olfactory bulb, medial is on the left for (c) and on the upper left for (d); the pial surface is on the right for (c) and on the lower right for (d). (c) TUNEL reaction (red) showed several apoptotic nuclei in the olfactory bulb of Zn mice, 9 days after Zn treatment. n: Olfactory nerve layer; g: glomerular layer; gr: granular layer. Arrows point to labelled cells. (d) SHAM olfactory bulb, with virtually no apoptotic nuclei, 30 days after SHAM surgery.

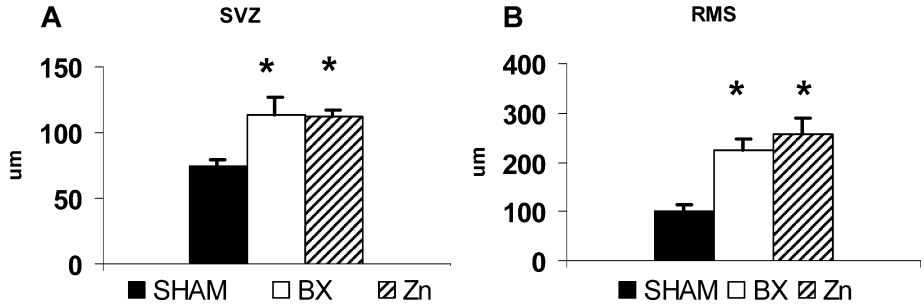

Fig. 5. Modifications in the width of the subventricular zone and of the rostral migratory stream of BX and Zn mice. Means + SEM are shown. *$P < 0.05$, different from SHAM. (A) SVZ is larger in both BX and Zn mice compared to SHAM. This indicates that damage to the olfactory mucosa or to the olfactory bulb induces a wave of proliferation in the SVZ. (B) Also the RMS is larger in BX and Zn mice compared to SHAM.

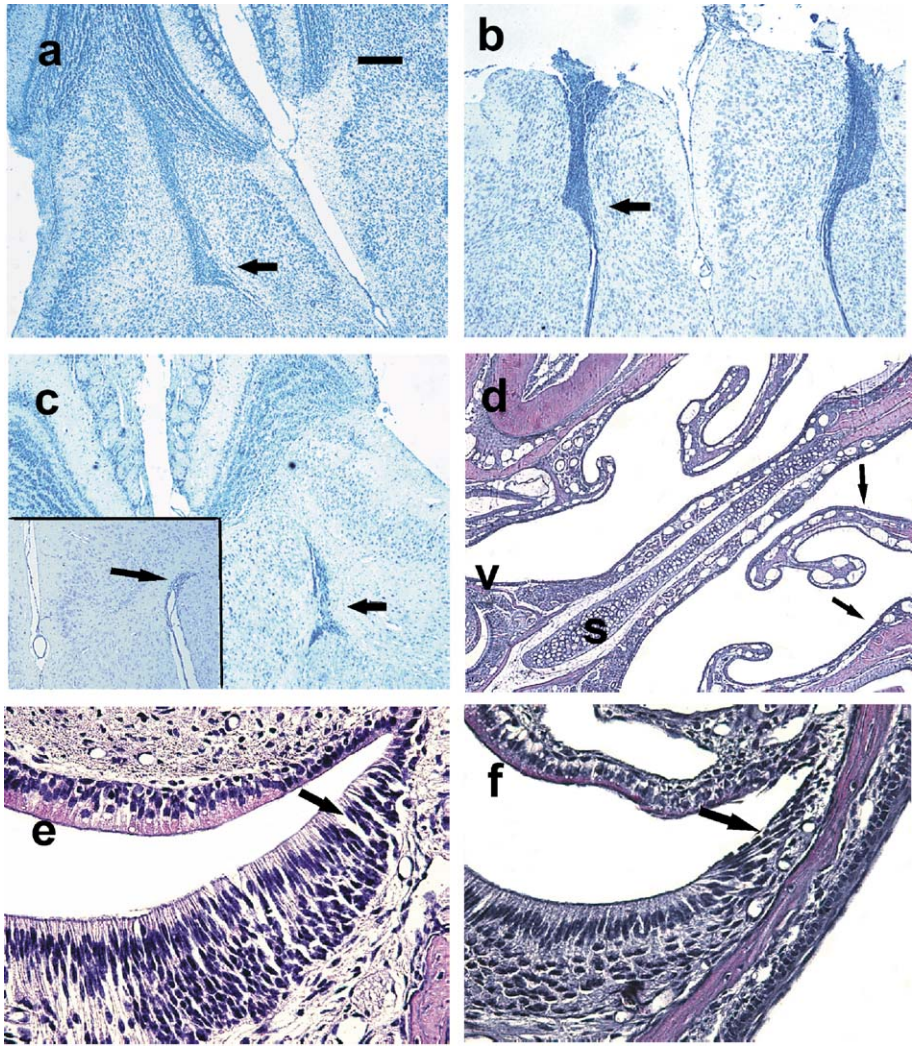

Fig. 6. Modifications observed in the brain and in the nose of BX and Zn mice. Scale bar = 50 μm for (a, b, and c); 200 μm for (d), and 25 μm for (e and f). (a, b, and c) Horizontal sections, Nissl stain. RMS is enlarged in both Zn (a) and BX mice (b), where it ends in the more rostral pole of frontal cortex. (c) SHAM mouse, the inset shows a more caudal field, in which the lateral ventricle with SVZ is evident. The arrow indicates the RMS (and SVZ in the inset) widest point in which measurements were made. (d) Coronal section through the nose, hematoxylin–eosin stain. The arrows indicate two points on different turbinates where the measurements were done, the other two being symmetrical to these. Top on the upper right. V: vomeronasal organ, S: septum. (e) Coronal section through the vomeronasal organ of a Zn mouse, hematoxylin–eosin stain. The arrow points to the lateral germinative zone; cells in the precursor pool are densely packed, the boundary with the neuroepithelium is evident on the lower left. (f) Coronal section through the vomeronasal organ of a SHAM mouse, hematoxylin–eosin stain. The cells of the precursor pool are confined to the most distal portions of the vomeronasal epithelium (arrow).

with polyclonal antibodies against the regulatory subunits of protein kinase A RIα, RIβ, RIIα, and RIIβ (Chemicon). Sections were incubated for 30 min with 0.4% bovine serum albumin and then overnight with the primary antibodies diluted 1:200. Fluorescein-conjugated secondary antibodies (Sigma, 1:200) were incubated for 30 min at 37°C.

TUNEL reaction

TUNEL reaction (Roche) was performed according to the manufacturer's instructions: frozen sections from two mice per group were cut and fixed as above, permeabilized in Triton X-100 0.1%, sodium citrate 0.1% in PBS for 2 min, and finally incubated for 1 h at 37°C in the TUNEL mix. Representative sections were counterstained with DAPI.

Tracer study

To demonstrate the severity of the loss of olfactory nerves in Zn mice, we injected carboxylate-modified latex yellow–green fluorescent beads (0.093- and 0.014-μm diameter, 2.05% solids; Fluospheres, Molecular Probes, Eugene, OR) into the olfactory nerve layer of both olfactory bulb of Zn and SHAM mice (n = 10). Injections were performed in anesthetized mice 7 days after Zn irrigation using a stereotaxic apparatus equipped with a glass capillary mounted on a microinjector; 40 pulses 50 nl each were injected (total: 2 μl). Under visual inspection, injections were made in the anteromedial portions of the olfactory bulbs, behind the lamina cribrosa, where the olfactory nerve layer is larger, by lowering the capillary tube below the pial surface to reach the basal portion of the olfactory bulbs. After 7 days, the mice were tested again (Cookie finding only), sacrificed, and perfused. The heads were decalcified in EDTA 100 mM, cryoprotected in sucrose, and then cut with a cryostat. The nasal mucosa was examined for the presence of beads; no leakage of beads was observed in the brain or outside olfactory nerves in the snout.

Results

Depression-related behavioral changes

Behavioral data (Fig. 1) show that after olfactory bulbectomy the major deficits already described in the forced swimming test can also be observed in our mice. Moreover, similar deficits are also found in mice whose olfactory mucosa has been lesioned.

First, BX and Zn mice are anosmic ($F(2,36) = 145.5$, $P < 0.001$), as shown by longer latencies to discover the buried food in BX and also in Zn compared to SHAM ($P < 0.001$). No difference is apparent between the two lesioned groups.

In the open field, the number of squares crossed is different ($F(2,36) = 6.1$, $P < 0.01$), with Zn mice that cross a significantly lower number of squares compared to SHAM ($P < 0.05$) and BX ($P < 0.05$), while there is no difference between SHAM and BX. This is the only difference that emerged in behavioral tests between Zn and BX. No difference is apparent in indexes of autonomic functions, namely the number of fecal pellets and drops of urine emitted during the test.

In the forced swimming test, the three groups cross a different number of squares ($F(2,36) = 5.53$, $P < 0.01$), with SHAM mice that cross a lower number of squares compared to BX ($P < 0.05$) and Zn ($P < 0.01$). Also the cumulative resting time is different ($F(2,36) = 6.53$, $P < 0.01$), with SHAM resting for a higher amount of time than BX ($P < 0.01$) and Zn ($P < 0.01$). This indicates that both BX and Zn spent less time floating in this test.

Enhanced noncompetitive aggression

Data on social behavior are presented in Fig. 2. Concerning intraspecific competitive aggression, the three groups attack an adult male intruder differently ($F(2,36) = 72.96$, $P < 0.001$), with a shorter latency to attack in SHAM compared to BX ($P < 0.001$) and Zn ($P < 0.001$). There is no difference in latency to attack in the BX and Zn group, so that they both appear less aggressive when facing a competitor present in their home cage.

By testing intraspecific noncompetitive aggression towards a mouse pup, there is a significant difference among groups in the latency to the first attack ($F(2,36) = 5.53$, $P < 0.01$). At variance with the previous test, both BX and Zn groups display shorter latencies to attack compared to SHAM ($P < 0.05$). Again, there is no difference between the two treated groups, but in this case they

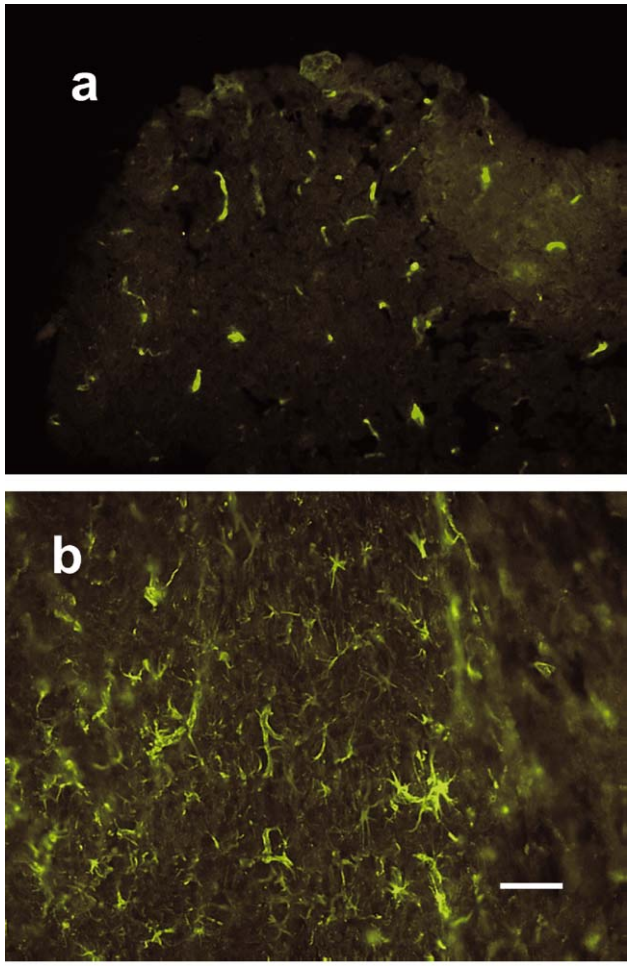

Fig. 7. The rostral pole of a BX mouse, on a horizontal plane. Scale bar = 50 μm. Rostral on the top, medial on the right. Both mature neurons, labelled with neuron-specific β-3 tubulin (a), and astrocytes, labelled with glial fibrillary acidic protein (b), are present in the protruding rostral pole that occupied the cavity left empty after ablation of the olfactory bulbs, but there is no evidence of a cortex-like laminated structure.

consistently appear more aggressive towards the pup. This is not readily predictable, in fact, we expected them to be less aggressive, but it indicates a specific involvement of central areas, mainly amygdala nuclei, mediating some aggressive behavior. This result also excludes that lesion of olfactory mucosa affects the drive to move in new environments, as it could be incorrectly deduced from the open field test.

A similar result is obtained when testing interspecific predatory aggression. A significant difference among groups is apparent ($F(2,36) = 6.12$, $P < 0.01$), with SHAM showing a longer latency to attack the prey than BX ($P < 0.01$) and Zn ($P < 0.05$). There is no difference between BX and Zn, which again predates faster than controls.

Modifications in the subventricular zone and amygdala

The first evaluation was made on the height of olfactory mucosa (Fig. 3A) that was smaller in Zn mice ($F(2,3) = 35.177$, $P < 0.01$). It was also apparent that the vomeronasal organ was affected (Figs. 3B and 6e and f), with a larger lateral germinative zone in both BX and Zn mice ($F(2,4) = 7.975$, $P < 0.05$). This accounts for an involvement of both the main and accessory olfactory system in Zn mice, as is the case for BX, in which both the main and accessory olfactory bulbs were excised.

When beads were injected on the olfactory bulbs of Zn mice, they never reached the olfactory mucosa, as demonstrated by the absence of beads in the nasal cavity rostrally to the lamina cribrosa (Figs. 4a and b).

Because similar behavioral symptoms were present in both Zn and BX, we asked whether similar modifications could be detected in the olfactory projection areas that could subside to these results. Neuronal cell death by apoptosis was examined in all the rostral pole of the frontal lobe including the olfactory bulbs, amygdala, rostral migratory stream, and subventricular zone. Only scarce apoptotic nuclei were detected in all these areas of SHAM, BX, and Zn mice. The only exception was the olfactory bulb of Zn mice, where several apoptotic nuclei were detected, mainly located in the nerve layer and in the granular layer (Figs. 4c and d).

We then evaluated the width of SVZ and RMS (Fig. 5). Both BX and Zn mice showed an enlarged SVZ compared to SHAM

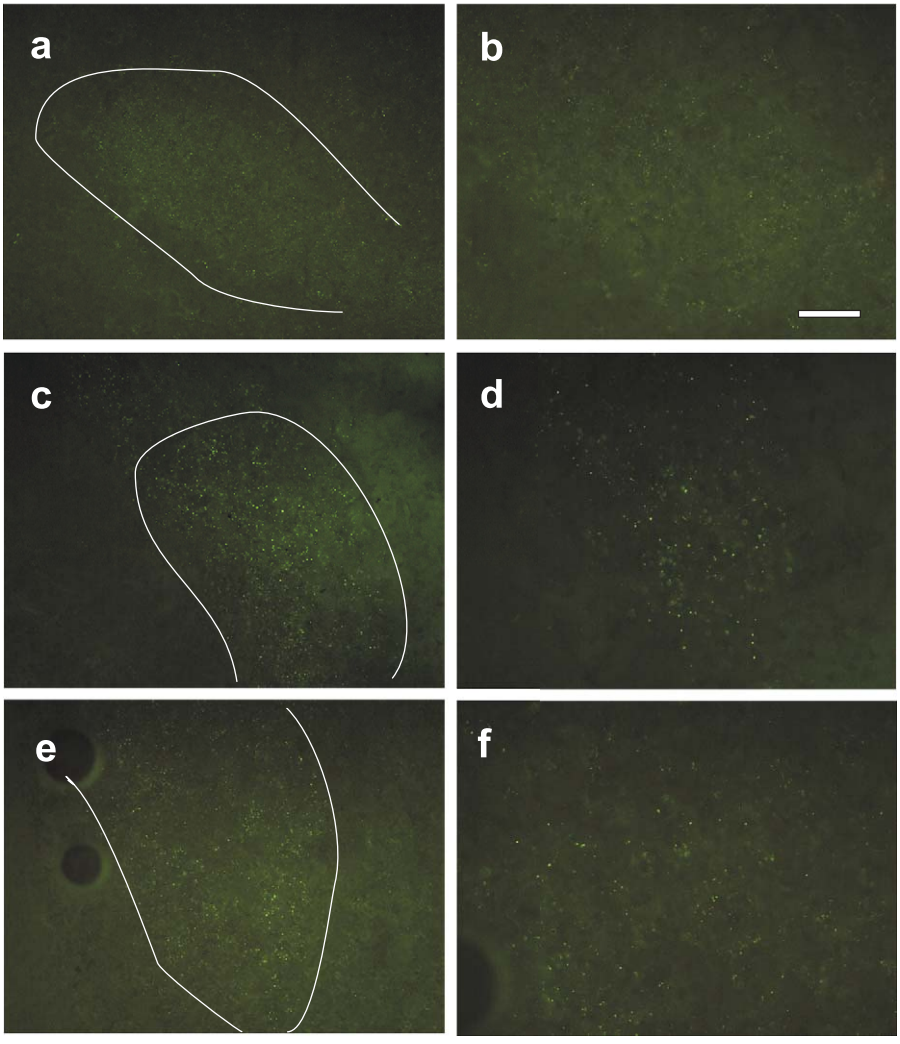

Fig. 8. Distribution of cAMP-dependent protein kinase regulatory subunit RIα in olfactory projection areas. Coronal sections through the amygdala; higher power images of the fields shown in (a, c, and e) are respectively shown in (b, d, and f). Scale bar = 50 μm for (a, c, and e); 25 μm for (b, d, and f), the white segments outline the border of the lateral nuclei of amygdala. Dorsal on the top left, medial on the lower left, the pial surface is on the right. The labelling in the SHAM mouse (a and b) is more faint than in BX (c and d) and Zn (e and f) mice. The external capsule, unlabelled, is on the right (a, c, and e).

($F(2,17) = 7.162$, $P < 0.01$). Also, the RMS was larger in BX and in Zn mice compared to SHAM ($F(2,17) = 12.468$, $P < 0.0005$).

The morphology of the brain was also evaluated. Rostral extensions of the forebrain were first described in mice unilaterally bulbectomized when newborn (Graziadei and Monti Graziadei, 1986). A gross modification, described in adult bilaterally bulbectomized animals (Zigova et al., 1988), was also present in our BX mice, which showed a protruding rostral pole of frontal cortex filling the cavity left empty after bulbectomy. The enlargement of rostral tips of RMS could also be appreciated by inspection of Nissl-stained sections (Fig. 6). The identity of cells within this zone was assessed by immunohistochemistry, which confirmed that both mature neurons, expressing β3 tubulin, and astroglia, expressing GFAP, were present (Fig. 7), but the usual layered appearance of the cortex was not detectable. In a couple of cases, putative ectopic glomeruli were detected in Nissl-stained sections, but we have no proofs of the presence of olfactory axons or of synaptic contacts within them because neither axonal staining nor synapse markers were employed. Under our conditions, the behavior of mice hosting these structures was indistinguishable when compared to the other BX. In these protruding areas (Fig. 6b), the terminal end of the RMS was also located, which could not reach the olfactory bulbs after their elimination.

We also examined the distribution of the regulatory subunits of protein kinase A in the amygdala and found that the major change was evident in RIα subunit, which increased in BX and Zn, compared to SHAM (Fig. 8). This indicates that a functional modulation of amygdala nuclei receiving olfactory and vomeronasal inputs occurs in BX and Zn mice.

Discussion

Behavioral considerations

In these experiments, we used two different ways to perturb the olfactory system, namely the invasive surgical ablation of olfactory bulbs and the less traumatic irrigation of the olfactory mucosa with zinc sulfate. Despite the different traumas, both manipulations resulted in dramatic changes in behavior that extended in a similar way to a wide range of behavioral repertoires. In the open field test, Zn mice were hypoactive, while BX behavior was not affected. Hypoactivity in the open field was already reported for Zn mice (cited in Slotnick and Gutman, 1977). This is the only test in which Zn differs from BX. This result can tentatively be ascribed to the shorter delay from lesion in Zn (7 days) as compared to BX (30 days), a time window that allows the appearance of behavioral anomalies but not the restoring of normal reactivity in the open field, a hypothesis currently under investigation. Mouse behavior is very sensitive to environmental conditions, so the results of open field test are often confounded: for example, a change in light intensity can result in opposite behavior in different laboratory settings (Deprato Primeaux and Holmes, 1999) and hence limits the conclusion that can be drawn. Actually, the total daily activities do not differ between controls and bulbectomized rats (Lumia et al., 1992). Previous literature showed that SHAM and BX behavior does not differ in the open field: hyperactive behavior is elicited in BX mice only by stressful new conditions (van Riezen and Leonard, 1990), like sudden exposure to light (Kelly and Leonard, 1999) or to walls that are not clear (Kelly et al., 1997), or particular hormonal manipulation like gonadectomy (Stock et al., 2000), otherwise no change in line crossing was found (Tiffany et al., 1979; Stock et al., 2000). Noteworthy, the presence of a stressful agent in the environment is sufficient to increase line crossing in both sham and BX animals (Tiffany et al., 1979).

Olfactory bulbectomy in rodents causes marked behavioral symptoms that constitute a model for agitated depression (Brunjes, 1992; Leonard and Tuite, 1981). This definition is based mainly on tests performed under stressful conditions (van Riezen and Leonard, 1990) that underline the reduced ability to adapt to sudden environmental changes. This mimics the trigger of depressive symptoms by peculiar life events in humans. No hyperactivity was induced in BX mice in the open field under our experimental conditions: this can be explained by the material (clear walls) and conditions used in this test, similar to the non-stressful environmental conditions (e.g., light and temperature) present in the vivarium, to which mice were adapted. In the forced swimming test, that is intrinsically more stressful, we have seen a hyperactive behavior in BX and Zn mice for the first time. At variance with other published papers (Kelly and Leonard, 1999), our mice were not adapted previously to the pool, and actually have never touched water before, therefore constituting a more stressful condition. Previous adaptation and data collection limited to the last part of the test (Lucki et al., 2001) can account for the lack of hyperactive behavior in animals tested by other groups. Moreover, antidepressant agents like desipramine and fluoxetine do not reduce immobility in some strains of mice, including Swiss–Webster (Lucki et al., 2001).

Our findings on the forced swimming test suggest that disruption of the olfactory input consistently affects the reactions towards an unavoidable stress and rules out a secondary motor deficit in Zn mice. Treatment with zinc sulfate produces anosmia (McBride et al., 2003) but does not induce transsynaptic degeneration in mitral or tufted cells (Burd, 1993) and does not cause systemic damages (Hansen et al., 1994). Systemic damages caused by inhalation of dangerous substances do not necessarily impair the olfactory function (Sun et al., 1996). Therefore, the behavioral changes in Zn mice imply a selective action of deafferentation on olfactory or limbic projection areas involved in behavioral modulation.

Hypotheses on the neurobiological substrates of behavioral modifications

Bulbectomy also induces a reproducible decrease of defensive behavior (Deprato Primeaux and Holmes, 1999; Stock et al., 2001) and hence a decreased attack rate against intruders (Leonard and Tuite, 1981; Brunjes, 1992) that is consistently modified by antidepressant agents (Leonard and Tuite, 1981; Kelly et al., 1997). This behavior was apparent in our BX and Zn mice, but testing different forms of aggression reveals that BX and Zn mice are not less aggressive in every instance, so the explanation that they do not smell hence they do not recognize and attack an intruder is not supported.

Behavioral modifications can be attributed to different causes. The first obvious consequence of bulbectomy or mucosal destruction is deafferentation. Behavioral and neuroanatomical modifications in BX mice were thought to be independent of olfactory loss. Peripheral deafferentation, involving the destruction of olfactory mucosa, induces loss of sensitivity, but other behavioral modifications were not fully investigated (van Riezen et al., 1977). Conflicting results concerning social behavior are also reported (Edwards et al., 1972; Mayer and Rosenblatt, 1993). The convergent results from our BX and Zn mice indicate that strong,

consistent, and reliable behavioral modifications were present when testing multiple behaviors, and can be tentatively attributed to a perturbation in the olfactory bulb output to the olfactory projection areas.

A second possible cause of behavioral modification is the change in activity of olfactory projection areas, which are interconnected with the limbic system and the frontal cortex, and hence participate in the modulation of social and sexual behavior. The perturbation of the glutamatergic input from olfactory bulbs directly downregulates the function of these areas (Ho et al., 2001; Kelly et al., 1997). Despite the growing body of pharmacological evidence on reversion of BX effects with antidepressant agents, the evidence of neurochemical modification following olfactory bulbectomy is rather scarce, confined to quantitation of choline acetyltransferase (Bobkova et al., 2001), serotonin (Garris et al., 1984; Wrynn et al., 2000), neuropeptide Y (Holmes et al., 1998), and NMDA glutamatergic receptors, often with conflicting results (Robichaud et al., 2001; Webster et al., 2000). Also, neurochemical changes after destruction of the olfactory mucosa are rarely reported (Harding et al., 1978; Mucignat-Caretta and Caretta, 2001), and little is known on such changes in human psychiatric diseases.

The neuroanatomical modifications induced by surgical removal of olfactory bulbs are not entirely clear. After unilateral bulbectomy (Monti-Graziadei and Graziadei, 1992), olfactory axons can reinnervate the telencephalic areas. On the other hand, the SVZ is the source of neural stem cells that migrate through the RMS to the olfactory bulb, where they differentiate (Luskin, 1993; Winner et al., 2002). Anatomical modifications are detected in the RMS after unilateral olfactory bulb lesion (Jankovski et al., 1998; Kirschenbaum et al., 1999): we report that the SVZ and RMS are larger, suggesting an enhancement of proliferation of neural stem cells, in both Zn and BX mice. Preliminary experiments (data not shown) indicated that fluorescent beads injected in the lateral ventricles of 21 BX and SHAM mice labelled cells in the SVZ, RMS, and amygdala; they were also found in the olfactory bulbs of SHAM and in the rostral pole of BX mice. Scarce-labelled cells were found in the olfactory and parietal cortex, and in the hippocampus of BX mice; most probably, these cells came from areas lining the lateral ventricles.

Moreover, we detected similar changes in protein kinase A regulatory subunits in the amygdala of BX and Zn mice, an area directly involved in social recognition (Ferguson et al., 2001). In the amygdala of adult mice, which is of paramount importance for social interaction, a clustered distribution of the RIα subunit is present (Mucignat-Caretta and Caretta, 2002) that appears upregulated after lesioning the olfactory mucosa or bulbs. In addition, the lesion of the olfactory mucosa with zinc sulfate results in a lowered expression of the RIβ subunit in the mitral cells of the olfactory bulb (Mucignat-Caretta and Caretta, 2001): this suggests that the peripheral damage can affect the function of the olfactory projection areas by acting via the cAMP pathway. No data are available up to now in humans, but similar modifications cannot be excluded and should be investigated as they can represent new potential therapeutic targets. Noteworthy, cAMP-dependent protein kinases in the amygdala are involved in behavioral modulation of fear responses and reward-related learning (Jentsch et al., 2002; Moita et al., 2002), and cAMP itself is affected by antidepressant treatment in humans (Lowther et al., 1997).

Surgical or functional perturbation of olfactory input to the limbic areas results in dramatic behavioral modulation that extends to a different context: as is the case for human patients, a strong involvement of the amygdala seems to contribute to the main behavioral symptoms. Although at present we cannot provide a causal link between neuroanatomical modifications and behavioral outcomes but only report the co-occurrence of the two, some indications stem from the present data to be further tested experimentally. A thorough behavioral and neuroanatomical examination should relate human disease and animal models to suggest similarities and to unravel the ultimate causes of behavioral modifications.

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