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Avoidance behavior and neural correlates of allergen exposure in a murine model of asthma

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Abstract

Allergic asthma is characterized by intermittent airway obstruction, inflammation, airway hyperreactivity, and increased production of IgE. The pathophysiology of asthma is well understood but little is known about its influences on brain activity and behavior. We recently described the neural correlates of food allergy and its associated modulation of behavior using an experimental model that also generates a T helper type 2 (Th2)-skewed response, with high levels of IgE. Here we show that mice allergic to ovalbumin (OVA) have an increase in the activity of the paraventricular nucleus of the hypothalamus (PVN) and in the central nucleus of the amygdala (CeA) following a single nasal OVA challenge. Moreover, we adapted a classical passive avoidance test using an OVA aerosol as the aversive stimulus. We found that allergic mice avoid entering the dark compartment of the apparatus that had been previously associated with nebulization of the allergen, while their non-immunized controls still move into the dark side of the test box. Thus, allergic mice have increased activity in areas of the CNS commonly associated with emotionality-related behavioral responses, such as the avoidance of a context previously associated with an unpleasant or harmful situation. Moreover, our findings on the avoidance test illustrate that previous experience with an airborne allergen can modify behavior.
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1. Introduction

Asthma is a chronic respiratory disease characterized by intermittent airway obstruction, inflammation, airway hyperreactivity, and increased production of IgE. The underlying mechanisms contributing to the development of asthma are complex and may vary according to individual, social, and geographical influences, but appear to be altogether caused by an intrapulmonary allergen-driven T helper type 2 (Th2) response characterized by increased secretion of type 2 cytokines (IL-4, IL-5, and IL-13) by CD4+ T lymphocytes (reviewed by de Sousa Mucida et al., 2003).

In asthmatic patients, the allergic reaction progresses in two distinct phases: an immediate response mediated by IgE and mast cells, and a late phase, during which Th2 lymphocytes, cytokines, and other cells build an inflammatory milieu that play a major role (Maddox and Schwartz, 2002).

Although the pathophysiology of asthma has been well characterized in humans and experimental models, little is known about its influences on brain activity and behavior. Changes in emotional status and increased levels of anxiety are commonly associated with asthma crises (Lawrence, 2002; Lehrer et al., 1993); on the other hand, the distress associated with a recurrent, chronic disease could be responsible for generating pathological anxiety in patients suffering from long-term asthma (Dahlen and Janson, 2002; Rietveld et al., 2000).

Basso et al. have tackled the importance of immune-to-brain communication in allergic responses using a murine model of food allergy. Mice immunized with ovalbumin (OVA) avoid the consumption of sweetened egg white in tap water, an antigen-containing solution (Basso et al., 2001). It was subsequently shown that OVA given by

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gavage to allergic mice leads to a strong activation of the paraventricular nucleus of the hypothalamus (PVN) and central nucleus of the amygdala (CeA), important areas of the central nervous system (CNS) in the mediation of affective- and emotionality-related behavioral patterns (Basso et al., 2003). The behavioral change and brain activation are dependent on IgE since the induction of immunological tolerance to OVA (for a review, see Russo et al., 2001) or administration of an anti-IgE antibody preclude these responses (Basso et al., 2003).

Although a modulation of behavior was previously described in a model of food allergy, we are not aware of any study focusing on brain activation and behavioral change after airborne allergen challenge. Allergic responses initiated in the gastrointestinal (GI) tract and in the airways might share similarities but a number of morphofunctional differences might also be taken into account. For instance, the GI tract is constantly exposed to environmental antigens; it also displays resident eosinophils, mast cells and has a constant trafficking of lymphocytes through an associated lymphoid tissue (Rothenberg et al., 2001). In contrast, few mast cells and lymphocytes are normally observed in the airways of rodents (Gersch et al., 2002). Nonetheless, the intimate contact between mast cells and nerve endings widely described in the GI tract (Bienenstock et al., 1988a,b; Stead et al., 1989; Williams et al., 1997) also exists in the airways (Bienenstock et al., 1988a,b; Greene et al., 1988; Kiernan, 1990), giving anatomical support for the existence of a similar bi-directional immune-neuroendocrine (INE) interaction in respiratory allergic diseases.

In the present study we adapted a classical passive avoidance test using aerosolized OVA as the aversive stimulus in order to analyze a possible immune-induced change in behavior. Furthermore, we asked whether allergic asthma might have repercussion in the activation of particular brain areas (PVN and CeA) employing a murine OVA model of asthma. Here we show that OVA challenge by the airway route induces an increase in the activity of the PVN and CeA in OVA-sensitized animals. In addition to CNS changes, allergic mice avoid a compartment previously associated with aerosol of OVA, showing the importance of these changes on the development of a behavioral adaptive response, namely avoiding contact with the allergen. Our results show the neural correlates of an immune reaction triggered by an airborne allergen, emphasizing the importance of immune-to-brain communication on adaptive responses.

2. Methods

2.1. Animals

Six- to 8-week-old male BALB/c mice were obtained from the animal breeding unit at the Department of Pathology, School of Veterinary Medicine-USP, São Paulo, Brazil. The animals were housed accordingly in controlled temperature (22–26 °C) and artificially lighted rooms on a 12 h light–12 h dark cycle (lights on at 7:00 am) with free access to rodent chow and water. Experiments were always carried out in the morning (between 9:00 and 12:00) to minimize variation due to circadian rhythmicity; in situations when the proposed number of animals could not be tested in this time frame, the groups were split in order to have animals from each group being tested in different days. Animals were housed and used in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo; these guidelines are similar to those of the National Research Council, USA.

2.2. OVA Immunization, booster and airway challenge

We used two different protocols of immunization and challenge. One protocol consists of immunization by two i.p. injections on days 0 and 7 with 4 μg OVA (grade V) adsorbed to 1.6 mg of aluminum hydroxide gel (alum) suspended to 0.4 ml in PBS (Sigma, St. Louis, Missouri) followed by a single intranasal (i.n.) OVA challenge on day 14 by instillation of 10 μg OVA in 50 μl saline delivered into the nostrils under light halothane anesthesia with the aid of a micropipette. The second immunization protocol consists of a single i.p. injection of 4 μg OVA/1.6 mg of alum on day 0, followed by aerosol challenges on days 12, 13, and 14 by means of two 30-min nebulizations with 2.5% OVA in saline, 30 min apart, in their home cages. These two different protocols were used to fit particular requirements of each experiment. The first immunization protocol ensures more accurate control of the route and dose of allergen delivery. Also, it allows determining the immediate effects of allergen challenge on brain activity. The second protocol resembles natural exposure of allergic patients to airborne allergens and ensures a manipulation-free challenge, therefore minimizing the influence of stressful operator-induced changes in behavior during analysis. Blood samples used for antibody quantification were collected 24 h after the last OVA challenge.

2.3. Determination of OVA-specific IgG1 and OVA-specific IgE antibodies

OVA-specific serum IgG1 antibodies were assayed by sandwich ELISA as previously described (Russo et al., 2001). Briefly, Nunc-Immuno Plates Maxi-Sorb (InterMed, São Paulo, Brazil) were coated overnight at 4 °C with 2 μg OVA per well. After washing with (PBS)-Tween 0.05%, and blocking with 0.25% casein in PBS, serum samples were added and incubated for 1 h at

room temperature. Bound antibodies were revealed by goat anti-mouse IgG1, followed by peroxidase-conjugated rabbit anti-goat IgG (H + L) antibodies (Southern Biotechnology, Birmingham, Alabama). The reaction was developed by a substrate containing 0.5 mg/ml OPD (Sigma, St. Louis, Missouri) and 0.0015% of H_2O_2 in 0.1 M citric acid/sodium citrate buffer (Merck S.A., São Paulo, Brazil), pH 5.0. The reaction was stopped with 4 M H_2SO_4 and the absorbance of the samples determined at 490 nm using a Multiskan EX (Thermo Labsystems, Vantaa, Finland). The concentrations of IgG1 antibodies were estimated by comparison with IgG1 standards run in parallel.

For OVA-specific serum IgE determinations, plates were coated overnight at 4 °C with 2 μg/ml of goat anti-mouse IgE antibodies (Southern Biotechnology). Serum samples were added to the wells followed by biotin-labeled OVA. Bound OVA–biotin complex was revealed by extrAvidin peroxidase conjugated (Sigma) followed by 100 μl/well of substrate as described above. OVA-specific IgE levels of samples were compared to an internal standard obtained from pooled sera of hyperimmunized BALB/c mice that was arbitrarily assigned to 1000 U.

2.4. Activity in the open field

Animals submitted to the second immunization and challenge protocol described above were used in this test. Motor activity was analyzed in a standard open field arena. Ninety minutes after OVA nebulization on day 15, mice were observed in an open field described elsewhere (Palermo Neto et al., 2001). Briefly, the open field consists of a round wooden arena (45 cm of diameter, 20 cm high walls) painted dark gray. Each mouse was individually placed in the center of the apparatus and observed during 5 min for total ambulation. A computer-aided animal tracking system (Ethovision for Windows, version 3.0, Noldus, The Netherlands) was used to estimate the distance covered by each animal in the 5-min trial.

2.5. Passive avoidance test

We adapted a classical passive avoidance test based on the discrimination of a compartment that had been previously paired with an unpleasant stimulus for the evaluation of the behavioral impact of an antigenic challenge to immunized mice, using the second protocol describe above. On day 15 mice were given one training trial, during which they were individually placed in the lit part of our test apparatus that consists of an acrylic box of dimensions $45 \times 20 \times 20$ cm ($l \times b \times h$) divided into two compartments: one illuminated (by a 60 W bare bulb) comprising one third of its total area, and the other dark, constituting the remaining two thirds (adapted from Bourin and Hascoet, 2003); these two chambers are connected by a small sliding door (5×5 cm). The delay for the first entry in the dark compartment was recorded (cut-off 1 min). Upon passage onto the dark compartment, the connecting door was closed and the animal received a 10-min nebulization with 2.5% OVA in saline. On day 16 the mice were given the test trial, i.e., they were reintroduced into the lit compartment of the apparatus described above and the delay for the passage to the dark chamber recorded (cut-off 5 min). Non-immunized animals were sham-injected on day 0 and handled exactly as immunized mice for all other procedures.

2.6. Fos immunohistochemistry

Following the second immunization (using the first protocol described above), animals were gradually habituated to the anesthetic procedure required for nasal instillation of OVA or saline: each animal was daily removed from its home cage, transferred into a similar box without bedding, and restrained by a transparent glass container for 1 min. Immunohistochemistry was performed on samples collected 90 min after nasal OVA challenge. Briefly, mice were deeply anesthetized with xylazine and ketamine and immediately perfused intracardially with 25 ml of 0.1 M phosphate-buffered saline (PBS) followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Their brains were removed and post-fixed for at least 5 h in the same fixative and cryoprotected overnight in 30% sucrose in PB. Coronal, frozen 30-μm sections were cut and collected in PB. Floating sections were incubated overnight with an antiserum directed against the Fos protein (Ab-5, Calbiochem, San Diego, CA). The Fos antibody was used at 1:1000 in a solution of 5% normal goat serum in PB with 0.3% Triton X-100. After washing, the sections were incubated for 90 min with a biotinylated goat anti-rabbit antibody (Jackson, West Grove, Pennsylvania) diluted at 1:200 in PB with 0.3% Triton X-100. Following incubation with the avidin–biotin complex (Vectastain kit, Vector, Burlingame, CA), peroxidase activity was detected using diaminobenzidine as a chromogen and hydrogen peroxide. The sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. Numbers of stained nuclei were estimated using computer-aided image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, Maryland). Control groups included: non-immunized animals (sham-injected on days 0 and 7) receiving OVA intranasally, immunized mice that were given saline, and unmanipulated animals (naive group), withdrawn from their home cages just before euthanasia.

2.7. Statistical analysis

Behavioral data and serum anti-OVA antibodies were analyzed by the *t* test (Welch corrected, when

appropriate). Fos immunohistochemistry data were analyzed by ANOVA followed by Dunnet's post hoc test. Values of $p < .05$ were considered significant for all tests.

3. Results

3.1. Immunization protocols and allergic responses

We first determined the levels of OVA-specific antibodies attained by the two protocols employed. IgE and IgG1 were measured in order to ensure the efficacy of our immunization and challenge procedures for the induction of an anti-OVA Th2-skewed response. In both protocols OVA challenge induced OVA-specific IgG1 and IgE antibodies in OVA-immunized mice but not in non-immunized animals ($p < .0001$). The IgG1, but not IgE production was higher in animals that received OVA aerosol during three consecutive days than in animals receiving a single intranasal OVA challenge ($p < .0001$). These results document that both protocols were effective in inducing anaphylactic antibodies (Fig. 1).

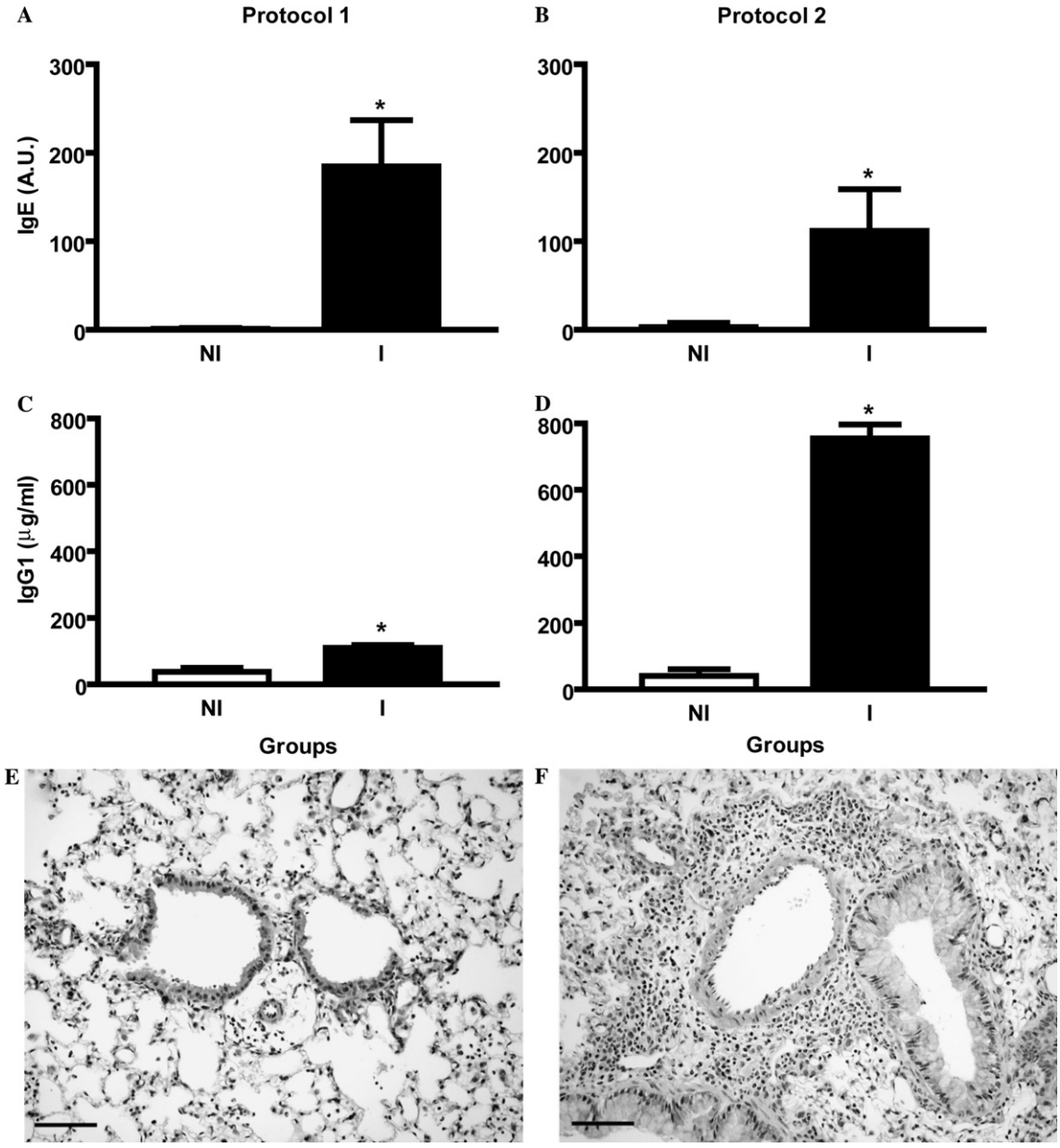

Fig. 1. Serum levels of IgE, IgG1, and lung histology. Ovalbumin (OVA)-immunized (I) or non-immunized (NI) mice were challenged with OVA according to the protocols described in Methods. Protocols 1 and 2 were effective in generating detectable levels of IgE (A,B) and IgG1 (C,D) anti-OVA antibodies. Results are expressed as means ± SD. *Significant difference ($p < .0001$) when comparing I with NI group (9–12 animals per group). Moreover, few morphological changes were observed in lungs of OVA-immunized animals challenged according to protocol 1 (E) while an intense peribronchovascular inflammatory infiltrate was observed in lungs of OVA-immunized mice challenged according to protocol 2 (F). Scale bar = 100 μm.

Histopathological findings revealed that few morphological changes were observed in the lungs collected 90 min after a single i.n. OVA challenge in OVA-immunized animals according to protocol 1 (Fig. 1E), while mice immunized by protocol 2 that received multiple OVA-aerosol challenges presented an intense peribronchovascular inflammatory infiltrate (Fig. 1F).

3.2. Ambulation in the open field is not altered by OVA challenge in immunized mice

To rule out a possible influence of decreased locomotion due to allergy-associated distress, we determined whether OVA-immunized mice challenged with OVA 90 min prior to test in an open field arena display normal levels of ambulation. Fig. 2 shows that allergic mice challenged with OVA aerosol behave like non-immunized animals in the open field (7304 ± 981 cm and 6117 ± 1910 cm of total distance, respectively; $t(16) = 1.93$, $p > .05$; 12–13 animals per group).

3.3. Immunized mice avoid the dark compartment associated with OVA challenge

Having determined that OVA-immunized and challenged animals do not show any decrease in locomotion that could account for changes in other behavioral tests, we then examined whether allergic mice would avoid a place previously associated with OVA nebulization. To verify this point, we adapted a passive avoidance test, using OVA as the aversive stimulus. During the training trial in the light/dark box there was no difference on the delay for the passage from the lit (supposedly aversive) compartment to the dark one (safer for rodents) between immunized and non-immunized mice (34 ± 24 s and 24 ± 18 s, respectively; $t(9) = .75$, $p = 0.47$; 4–7 animals per group). Nonetheless, on the test trial the delay for the first entry of the immunized group was markedly increased when compared to non-immunized animals (194 ± 38 s and 9 ± 9 s, respectively; $t(3) = 9.55$, $p < .01$), or to themselves on the first trial ($t(6) = 7.08$, $p < .01$). Thus, immunized mice avoid the compartment in which they have been challenged with OVA whilst non-immunized animals still quickly move into this compartment (Fig. 3).

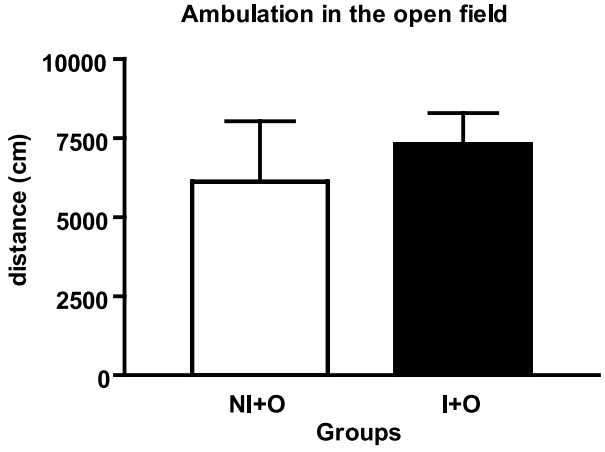

Fig. 2. Ambulation in the open field. The distance covered by mice from non-immunized (NI + O) or immunized (I + O) groups, and challenged with ovalbumin was similar. Results are expressed as means ± SD (12–13 animals per group; $p > .05$).

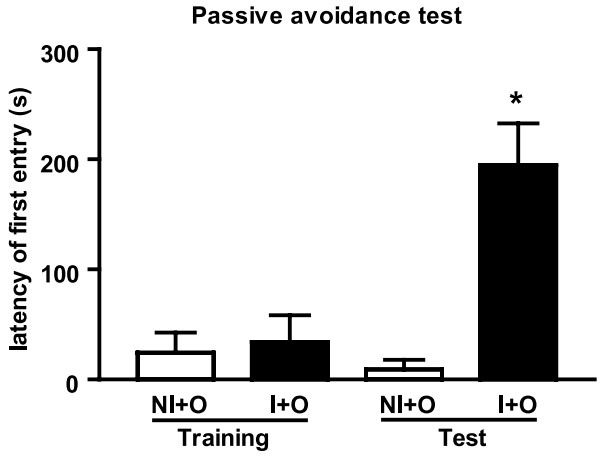

Fig. 3. Passive avoidance test in the light/dark box. During the training session, non-immunized mice challenged with ovalbumin (NI + O) behave like immunized mice that received the allergen (I + O). Nonetheless, on the test session, animals from group I + O stayed in the lit compartment longer than mice from group NI + O or themselves on the training session. Results are expressed as means ± SD. *Significant difference ($p < .01$) when comparing NI + O with I + O groups (4–7 animals per group).

3.4. Immunized mice have increased Fos expression in the PVN and CeA

Fos immunoreactivity (a marker of neuronal activation) in PVN and CeA was subsequently analyzed. Immunized mice challenged with i.n. OVA displayed an increase in the activity of the PVN ($F(3, 12) = 11.69$, $p < .01$; 3–5 animals per group) and CeA ($F(3, 15) = 55.91$, $p < .01$; 4–5 animals per group) when compared with control groups: immunized mice that received saline, non-immunized animals that receive i.n. OVA, or naive mice, withdrawn from their home cages just before perfusion (Figs. 4–6). Therefore, we conclude that only OVA-immunized and challenged animals exhibit strong Fos immunoreactivity in these brain areas.

4. Discussion

One of the early descriptions of an immune-related change in behavior was the so-called 'rose effect' characterized by bronchoconstriction in asthmatic patients

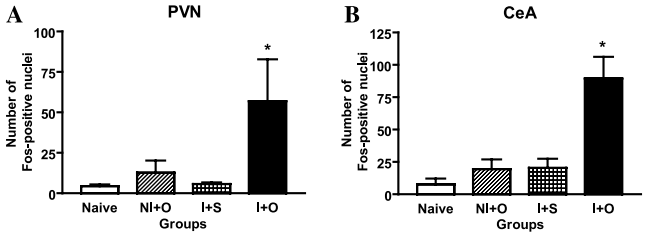

Fig. 4. Fos staining in the paraventricular nucleus of the hypothalamus (PVN) and central nucleus of the amygdala (CeA) of immunized mice challenged with ovalbumin (OVA). Immunized and OVA-challenged animals (I + O) displayed an intense Fos staining in the PVN (A) and CeA (B). Few positive nuclei were observed in naïve mice, or in non-immunized and OVA-challenged (NI + O) or in immunized mice that received saline (I + S). Results are expressed as means ± SD. *Significant difference ($p < .05$) when compared to the naïve group (3–5 animals per group).

facing an artificial rose (Mackenzie, 1886); these patients would obviously avoid the contact with such fake roses, aiming at the maintenance of their health. This fact represents a conditioned behavior developed after an association of the source of antigen (patients allergic to roses) and the image of its source. It was recently shown that OVA-immunized rats display a behavioral repertoire associated with an anaphylactic response when exposed to a conditioned stimulus in the absence of the allergen—in this particular case, a buzz inside the nebulization chamber in which the animals are challenged with OVA. This study provides experimental evidence for the existence of conditioned behaviors associated with allergic reactions (Palermo-Neto and Guimaraes, 2000). Although the authors did not assess histological or immunological parameters to show that rats could mount an allergic response in the absence of the allergen, it had been previously shown that, in immunized animals, secretion of mediators (such as protease II) by mast cells could be conditioned using audiovisual cues (MacQueen et al., 1989). To build a behavior such as the 'rose effect,' an early brain correlate of immune phenomena ought to be established. In the present study we focused on the relationship between airborne allergic reaction and changes in CNS activity involved in behavioral responses such as avoidance and anxiety. Altogether, our results point towards a direct relationship between this allergic response and the expression of an adaptive response, represented here by avoidance.

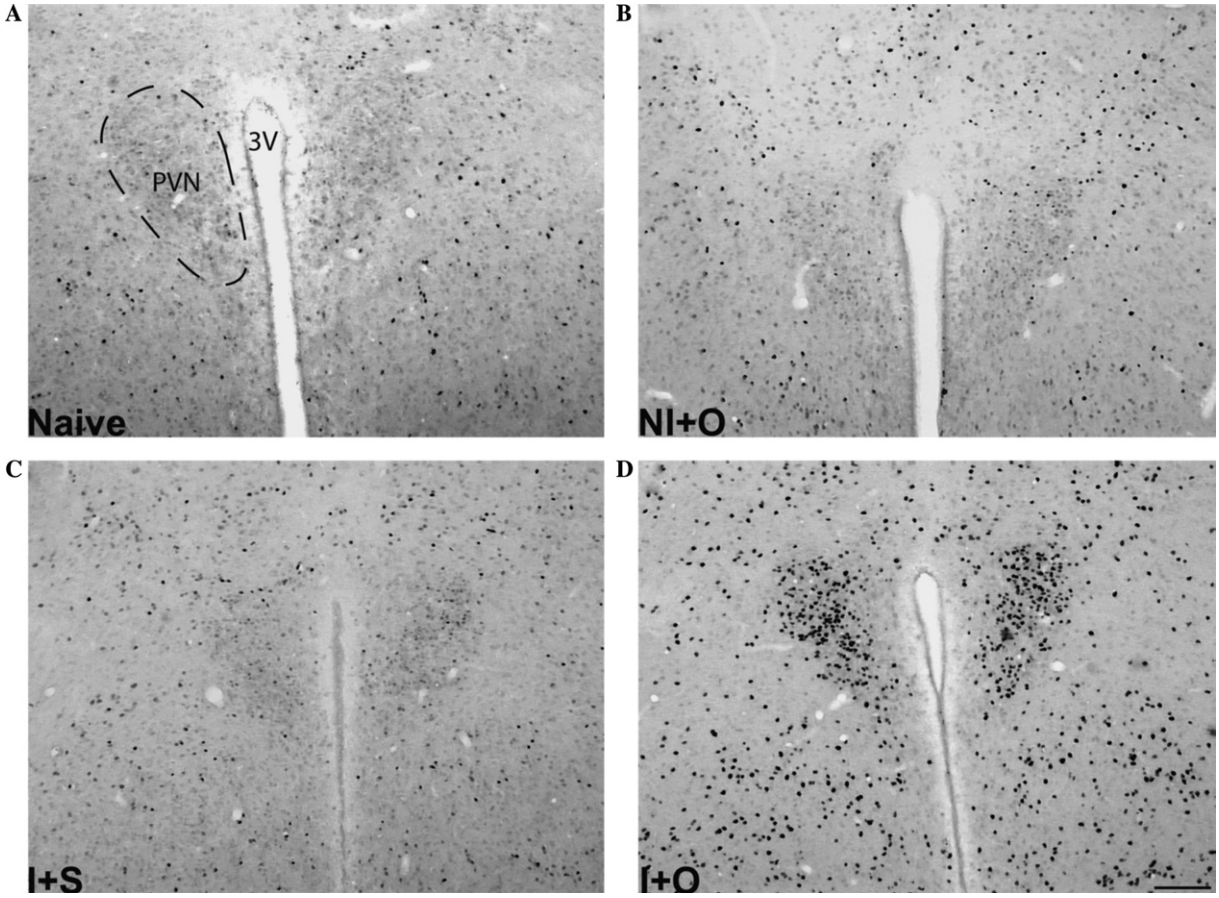

Fig. 5. *c-fos* expression in the paraventricular nucleus of the hypothalamus (PVN). Representative brain coronal sections showing few Fos-positive nuclei in the PVN in naive mice (A), non-immunized mice challenged with OVA (B) or immunized animals challenged with saline (C), and intense Fos-immunoreactivity in immunized and OVA-challenged mice (D). 3V = third ventricle. Scale bar = 100 μm.

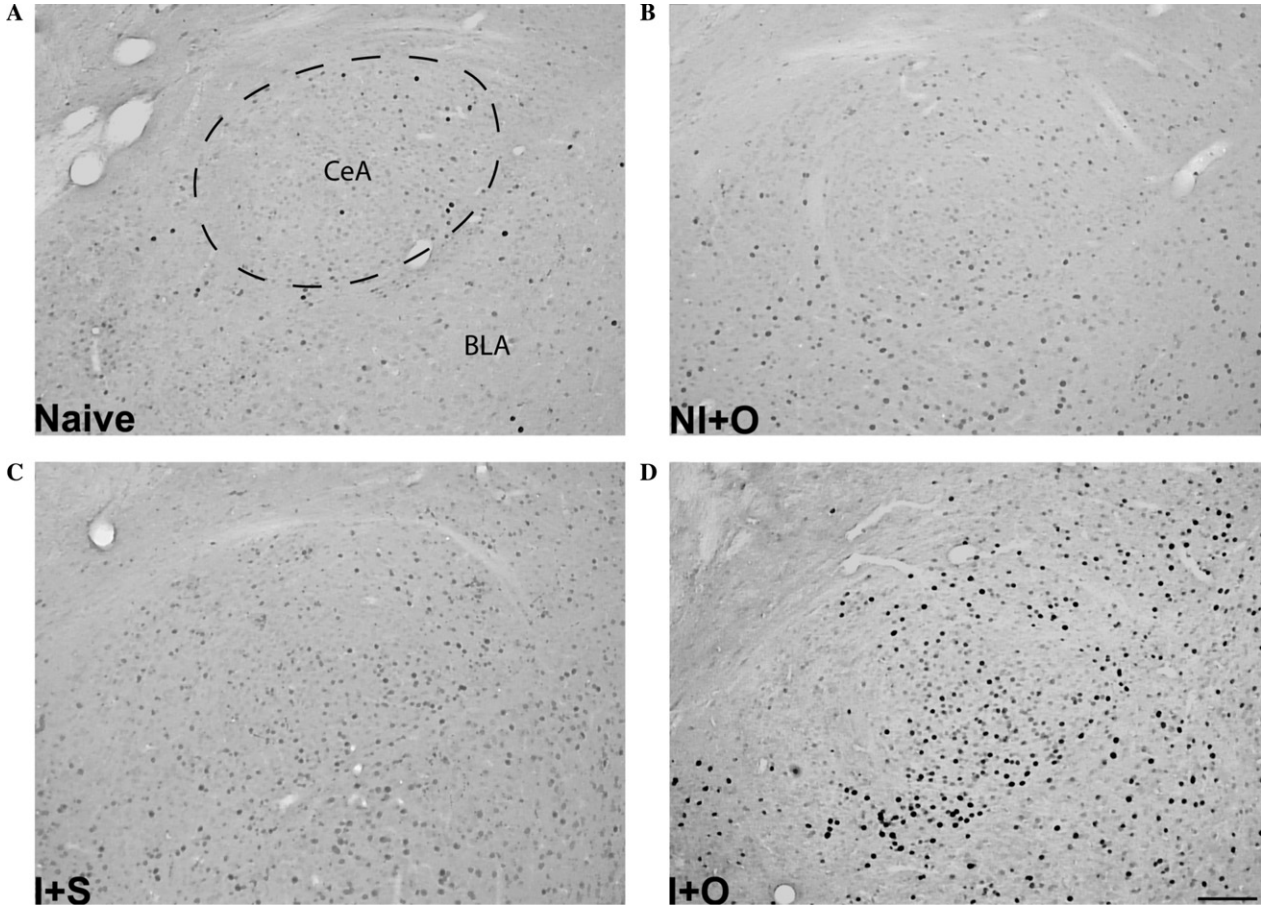

Fig. 6. *c-fos* expression in the central nucleus of the amygdala (CeA). Representative brain coronal sections showing few Fos-positive nuclei in the CeA in naive mice (A), non-immunized mice challenged with OVA (B) or immunized animals challenged with saline (C), and intense Fos-immunoreactivity in immunized and OVA-challenged mice (D). BLA = basolateral amygdala. Scale bar = 100 μm.

We show here that immunized mice avoid a compartment previously associated with presentation of the allergen against which they were immunized. Thus, albeit attracted by the supposedly safer, dark compartment of the apparatus, OVA-immunized mice avoid entering it, preferring the lit (usually aversive) side of the box. The results obtained in this immune-driven passive avoidance test prompted us to study the changes in CNS activity that could underlie this behavioral change. We found that allergic animals presented an enhanced Fos immunoreactivity in the PVN and CeA after airway OVA challenge. These CNS areas are commonly linked to emotional- and affective-related behavioral patterns (Iversen et al., 2000) that comprise important components of the development of conditioned aversive responses, such as the one described here. Animal models used to demonstrate its importance include, for instance, the development of conditioned taste aversion to saccharin using i.p. lithium chloride as the unconditioned stimulus, during which a strong activation of the PVN (Lamprecht and Dudai, 1995) and CeA (Yamamoto et al., 1997) was described. It was subsequently demonstrated the functional link between CeA activation and the development of aversion using an injection of a *c-fos* antisense into this nucleus, resulting in deficient acquisition of conditioned aversion (Lamprecht and Dudai, 1996). In this context, it is noteworthy that the PVN and the CeA are among the most important areas of the CNS in number of corticotropin-releasing factor (CRF)-expressing neurons, of well-known importance as regulators of adaptive stress responses (Gray, 1993; Gray and Bingaman, 1996; Swanson et al., 1983). Regarding the association between immune responses and CNS activity, most of the studies utilized immunization protocols that result in Th1-skewed response. For instance, peripheral stimulation of T lymphocytes with staphylococcal enterotoxin B (SEB) leads to an increased activity of the PVN shown by *c-fos* expression (Goehler et al., 2001) and CRF mRNA (Kusnecov et al., 1999), also found in the CeA by the same group. Furthermore, SEB is capable of eliciting emotionality-related behavioral responses (Kusnecov et al., 1999). These results clearly demonstrate the role of these CNS areas in the elaboration of affective responses.

We have recently shown in a model of experimental food allergy that a Th2-associated response also induces changes in CNS activity and behavior through an IgE-dependent mechanism (Basso et al., 2003). It is known that allergic asthma proceeds through two distinct phases: an immediate response mediated by IgE and mast cells and a late phase, during which the presence of Th2 cells, eosinophils, and other immune cells is evident (Maddox and Schwartz, 2002). Thus, it would be relevant to determine the actual mechanism by which the immune system signals the CNS in our model. Since the activation of the PVN and CeA was detected 90 min after a nasal OVA challenge, therefore in the absence of an overt pulmonary allergic inflammation, it is likely that the immediate hypersensitivity reaction, which is dependent on IgE-mediated mast cell degranulation, plays a major role in brain activation. However, both the early and late phase allergic responses might mediate the avoidance behavior because this response is observed in animals receiving multiple OVA aerosol challenges and consequently presented an ongoing lung inflammatory reaction, besides high levels of IgE.

A possible drawback of the first immunization and challenge protocol is its requirements of unavoidable handling of the animal and anesthesia for nasal challenge. Conversely, it seemed reasonable to use the second immunization protocol on our behavioral test, since it does not include any handling or anesthesia immediately prior to the test. Moreover, an aerosol challenge more accurately represents the human situation of exposure to airborne allergens.

Adaptation to the surrounding environment is crucial for survival and the development of a complex central nervous system represented an important step towards integrating signals from several sensory systems. Evidence from the past decades have shown that the immune system participates along with the CNS in a higher-level network of interactions, turning the immune system in a *diffuse* sensory organ (Blalock, 1984). Considering that the most important adaptive systems of the body, namely the CNS and the immune system, interact with each other forming a wider network (Elenkov et al., 2000), it is anticipated that their cross-talk is responsible for dealing with a multitude of situations that require physiological and behavioral modulation.

The general pattern of brain activation displayed by rodents during stress responses, or facing the choice of avoiding unpleasant or uncomfortable situations, includes hypothalamic nuclei and areas of the limbic system, as seen after OVA challenge in immunized mice. This early activation, seen after a single airway challenge, could be responsible for triggering adaptive responses later on, such as the avoidance of a place associated with the antigen. This represents an immune-driven choice of safe versus dangerous contexts, turning this environmental discrimination an adaptive response directed to the allergen.

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