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Allergic rhinitis induces anxiety-like behavior and altered social interaction in rodents

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Abstract

Epidemiological and clinical studies report higher incidences of anxiety and increased emotional reactivity in individuals suffering from respiratory allergies. To evaluate if respiratory allergies are capable of promoting anxiety-like behavior in rodents, we used models of allergic rhinitis and behavioral evaluations followed by assessment of mRNA for cytokines in relevant brain regions. Mice and rats were sensitized to ovalbumin or pollen respectively following standard sensitization and challenge protocols. After challenge, the animals were evaluated in the open field, elevated plus maze and resident intruder tests. Cytokines and corticotropin releasing factor expression were assessed in several brain regions by real-time RT-PCR and plasma corticosterone concentrations by radioimmunoassay. Mice and rats sensitized and exposed to allergen showed increased anxiety-like behavior and reduced social interaction without any overt behavioral signs of sickness. T-helper type 2 (T_H2) cytokines were induced in both rats and mice in the olfactory bulbs and prefrontal cortex and remained unchanged in the temporal cortex and hypothalamus. The same results were found for CRF mRNA expression. No differences were observed in corticosterone concentrations one hour after the last behavioral test. These results show that sensitization and challenge with allergens induce anxiety across rodent species and that these effects were paralleled by an increased expression of T_H2 cytokines and CRF in the prefrontal cortex. These studies provide experimental evidence that sensitized rodents experience neuroimmune-mediated anxiety and reduced social interaction associated with allergic rhinitis.

Keywords

Intranasal; Anxiety; Prefrontal Cortex; Allergies; Cytokines; CRH; Inflammation; Neuroimmune

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1. Introduction

Allergic rhinitis affects about 20 to 30 % of adults and up to 40% of children (Meltzer, 1997; Sly, 1999; 2002; Berger, 2004; Nathan, 2007; Blaiss, 2007) and contributes to the development of allergic asthma (Meltzer et al., 2004; Meltzer, 2005). A hallmark of allergic disease is the expansion of the type 2 (T_H2) subset of T cells and the generation of IgE antibodies to common allergens. In the sensitized individual following exposure to antigen, there is an early phase hypersensitivity response within 30 minutes, which may be followed by a late phase response 2-6 hours later (Galli et al., 2008). If there is ongoing exposure to antigen, allergic inflammation can persist for days or longer. The early phase primarily results from mast cell degranulation and release of pre-formed and newly synthesized mediators, like proteases, histamine and leukotrienes which effect vascular permeability, and mucus production. Release of chemokines and recruitment of inflammatory cells contribute to the late phase response which is characterized by an influx of eosinophils, and T_H2 lymphocytes which produce T_H2 cytokines such as IL-4 and IL-13 (Galli et al., 2008).

Allergic rhinitis is associated with congestion, nasal discharge, palatal itch and sneezing, however some patients report symptoms of fatigue and changes in mood. The most recognized behavioral complications of allergic rhinitis noted by clinical practitioners are the development of fatigue and sleep disturbances as a consequence of obstruction of the airways (Meltzer, 1997; Schoenwetter et al., 2004; Santos et al., 2006; Woods and Craig, 2006; Sundberg et al., 2007). However, psychiatric complications including anxiety and altered emotional reactivity are also acknowledged (Addolorato et al., 1999; Marshall et al., 2002; Burske-Kirschbaum et al., 2008; Chida et al., 2008; Blaiss, 2008). Nonetheless, the extent to which allergic rhinitis may contribute to mental problems is of relevance since the results of epidemiological and clinical studies consistently reports a higher incidence of anxiety and in some cases depression in individuals suffering from respiratory allergies (Hurwitz and Morgenstern, 1999; Addolorato et al., 1999; Goodwin, 2002; Timonen et al., 2003; Goodwin et al., 2004; Patten and Williams, 2007; Williams et al., 2007; Postolache et al., 2007; Barone et al., 2008; Burske-Kirschbaum et al., 2008; Goodwin and Buka, 2008). However, the possibility of a causal relationship between allergic rhinitis and pathological levels of anxiety is still a matter of controversy. For instance, it has been proposed that high levels of anxiety and further complications resulting in altered emotional reactivity develop as a process of suffering from a chronic condition (Chida et al., 2008). Moreover, it has been suggested that an exaggerated allergic inflammatory response in individuals with high anxiety is the result of hyperactivity of the nervous system and the hypothalamic-pituitary-adrenal (HPA) axis with subsequent stimulation of the immune system (Wright, 2005).

Recent human and rodent studies, however, suggest that there may be a direct relationship between antigen exposure and alteration in brain function that may precipitate high levels of anxiety and emotional reactivity (Rosenkranz et al., 2005; Costa-Pinto et al., 2005; 2006; 2007). Several mice studies showed that exposure to intranasal or aerosolized antigen in sensitized animals induces avoidance behavior and activation of limbic brain regions (Costa-Pinto et al., 2005; 2006; 2007). Moreover, increased anxiety determined by the open field and elevated maze tests was reported during the early phase of the allergic reaction in a rat model of asthma (Palermo-Neto and Guimaraes, 2000). Furthermore, increased anxiety in the open field test and activation of limbic brain regions was reported during the early phase in a mice model of food allergies (Basso et al, 2003; 2004). In humans, increased brain activity in the prefrontal cortex was observed using functional magnetic resonance during the late phase of an asthma episode (Rosenkranz et al., 2005). While these studies provide a foundation in identifying some brain regions and behavioral responses associated with allergen challenge, the notion that these allergies may initiate, perpetuate and exacerbate pathological anxiety

remains controversial and largely unstudied. The scope of the problem may be of significance because allergies and allergic rhinitis in particular are a growing problem in developed countries (Sly, 1999; Schoenwetter, 2000; Galli et al., 2008) and may impose an important immune stressor in a considerable number of people.

In the present study we employed two models of allergic rhinitis to evaluate anxiety-like behavior, social interaction and aggression in mice and rats, behaviors that are indicative of altered emotional reactivity in rodents (Berton et al, 1997). Albumin from chicken egg (OVA) was used in inbred BALB/c mice (Gelfand et al., 2004; Kelly-Welch et al., 2004; Costa-Pinto et al., 2005; Zosky and Sly, 2007) and tree pollen, one of the most common aeroallergens was employed in inbred Brown Norway rats (Steerenberg et al., 1999). These models represent the most accepted approaches to study allergic rhinitis in rodents. We also used real-time RT-PCR to analyze the expression of T_H2 cytokines in the olfactory bulbs, prefrontal cortex (PFC), temporal cortex and hypothalamus. These regions contains several structures shown to be responsive to several models of experimentally-induced allergies (Basso et al, 2003; Rosenkranz et al, 2005; Costa-Pinto et al., 2006). Because of evidence suggesting the involvement of corticotropin-releasing factor (CRF) in allergic processes (Silverman et al., 2004; Theoharides and Kalogeromitros, 2006; Theoharides and Konstantinidou, 2007) and its recognized role in the neurobiology of anxiety (Zoumakis et al., 2006; Ising and Holsboer, 2007; Holsboer and Ising, 2008), we also evaluated the expression of CRF in the same regions. Finally, plasma corticosterone concentrations were determined by radioimmunoassay.

2. Methods

2.1 Allergic Rhinitis to ovalbumin (OVA)

Inbred BALB/c mice (n=40 for behavioral studies and n=12 for PCR determinations) were obtained from Taconic Farms. Because the only existing data on the effects of allergic inflammation on behavior were obtained using this strain (Costa-Pinto et al., 2005), the present studies were carried using these mice. This strain was also chosen for its consistent responses to experimentally induced allergies to OVA and its extensive use in the study of the biology of allergic responses (Gelfand, et al., 2004; Kelly-Welch et al., 2004; Zosky and Sly, 2007). Mice in general do not develop consistent responses to pollen and therefore, OVA is the most appropriate antigen. Allergic rhinitis to OVA was induced as previously described (KleinJan et al., 2006). Briefly, albumin from chicken egg (OVA) was purchased from Sigma (St Louis MO) and absorbed to aluminum hydroxide (Alum) (Pierce) in a 1:1 mixture for 30 minutes under constant shaking at room temperature. Mice were immunized by intraperitoneal injections of Alum alone or with 100 µg/mice OVA/Alum in a volume of 200 µl on day 1 and boosted with the same reagents on day 14. Mice were challenged on day 21, 22 by intranasal instillations with a 1% OVA solution in PBS (allergic mice) or PBS alone (control mice). For this procedure, mice were slightly anesthetized with isoflurane in induction chambers and at the moment of awakening 25 µl of OVA/PBS or PBS alone was delivered with a micropipette on each nostril. Mice were tested for social interaction at day 23 and in the elevated plus-maze test followed by the open field test at day 24 (Figure 1).

2.2 Allergic Rhinitis to pollen

Inbred Brown Norway (BN/NHsd) rats (n = 36 for behavioral studies and n = 16 for PCR determinations) were obtained from Harlan-Sprague Dawley (Indianapolis, IN). This strain was selected based on their susceptibility to develop allergic rhinitis after repeated exposure to pollen (Steerenberg et al., 1999; Motta et al., 2004). This strain has been proposed as a naturalistic model of respiratory allergies and therefore, provides a comparative model for the studies in mice. They were sensitized by repeated intranasal instillations with a mixture of equal parts of tree pollen (oak, cedar, maple and birch) (Greer Laboratories, Lenoir, NC)

dissolved in saline (250 mg/ml pollen mixture) once a day for 5 consecutive days as previously described (Steerenberg et al., 1999). For this procedure, the animals were slightly anesthetized with isoflurane in induction chambers and at the moment of awakening were administered with 100 μ l per nostril of saline solution or pollen mixture. The animals received an intranasal dose at day 15 after the last administration to boost the immune reaction. The rats were challenged at day 22, 23, 24, 25 and 26 for 5 consecutive days with the same solutions. Control animals were treated with saline under the same challenge schedule. Rats were tested on day 27 in the resident intruder test and on day 28 in the elevated plus maze test and open field test (Supplemental figure 1). Weight was monitored daily. Positive allergic reactions were evaluated by the passive cutaneous anaphylaxis technique (Supplemental figure 1).

2.3 Open Field Test

For mice studies, 49 cm \times 49 cm arenas under constant illumination at 35 lux from the top were used (San Diego Instruments, San Diego, CA). The animals were brought to the room and acclimated for at least one hour before behavioral testing. The open field sessions were videotaped from the top of the arenas in 5 minutes session. Data was collected and analyzed with the use of the TopScan system from Cleversys (Reston, VA). The system automatically calculates in cm the distance traveled in the area corresponding to the periphery (75 % of the total area) as well as the percentage of time spent in the center area (25% of the total area) of the arena. For rat studies, experiments were carried out as described for the mice with the following modifications: ambulatory motor activity was monitored in similar 40.1 \times 40.1 cm arenas maintained under constant illumination at 40 lux. The floor was divided in 16 equal squares (12 peripheral and 4 center squares representing 75 and 25 % respectively) and a digital video camera was placed at a top angle and recorded the entire 5 minutes of the sessions. Quantification was carried out by blind experimenters by reviewing the tapes and counting the number of times that an animal crossed a square (counts). Total activity was expressed as total number of counts and further expressed as percentage of activity in the periphery vs the center field. An additional parameter analyzed in both studies was number of fecal boli.

2.4 Elevated Plus-Maze Test

For mice studies, an elevated plus maze instrument with 2 open arms and 2 closed arms specific for mice was used (Stoelting, Wood Dale, IL). The animals were brought to the testing room and acclimated for at least one hour before starting with the sessions. Sessions of 5 minutes held under 5 lux of illumination were recorded with a video camera mounted on top of the maze and the time spent in open arms, total distance travelled and number of entries in open arms was analyzed with the use of the MazeScan system from Cleversys (Reston, VA). For rat studies, experiments were carried out as described for the mice with the following modifications: a fully automated plus-maze instrument (Acuscan, Columbus, OH) with 2 open and 2 closed arms specific for rats was used. Time spent in open arms and total number of crossings between arms was automatically quantified by this system in sessions of 5 minutes. Data was analyzed as a percentage of time spent in the open arms vs time spent in closed arms. Additional parameters analyzed for both rats and mice included number of entries on the open arms and fecal boli.

2.5 Resident Intruder Test

These studies were performed using standard procedures for the resident-intruder test; for mice studies, resident mice were male BALB/c, 9 weeks old at testing that had been housed individually for 4 weeks in our facility. Intruder mice were received one week prior to testing, were group housed, and were 8 weeks of age. Therefore, resident and intruder mice were of same age at the time of testing. All mice were weighed one day before testing and the groups were confirmed to be of comparable weight. Intruder animals were housed in groups of 5 and

a total of 20 intruder mice were used and 2 cages were assigned as intruders for controls and 2 cages as intruders for allergic mice. One week prior to testing the resident mice had their cages changed, with no other cage changes prior to the test. Mice were allowed to acclimate to the testing room one hour prior to the onset of behavioral testing. For the procedure the resident's (isolated mouse) home cage was used, with the food/water bin removed and lid on, and lighting was 30 lux. An unfamiliar group housed animal (intruder non treated mice) was placed in the cage of the isolated animal (resident allergic or control mice) for 10 minutes and behavior was videotaped. After each trial, group housed mice were placed into a new cage (no mouse was placed back into their original cage with test naïve mice). Scoring was done either live, or from video files, using CleverSys Inc Anostar behavioral annotation software. The observer manually enters keyboard strokes to register the incidence and length of events. Latency to initiate contact, duration of social contact (social investigation) and aggressive behavior (attacks and bites) were quantified solely from the resident towards the intruder.

Similarly for rats, intruder rats were of same age and similar weight than resident rats. Intruder rats were housed in groups of 3 and a total of 18 intruder rats were used and 3 cages assigned as intruders for controls and 3 cages assigned as intruders for allergic rats. Resident animals remained in its original home cage placed in the testing area for ten minutes prior to intruder entry. An unfamiliar intruder (untreated rat) was taken from a separate cage and placed in the resident (treated allergic or control rat) cage to begin the test. Tests were videotaped from a horizontal angle during ten minutes session. The same behavioral parameters as those for mice were scored by two observers both during the test, and re-scored on video after the test.

2.6 Corticosterone Determinations

Determinations were done in behaviorally tested animals 1 hour after the final behavioral test. At that time, mice were anesthetized with pentobarbital sodium (50mg/Kg) and blood collected by cardiac puncture. For rats, all animals were sacrificed by rapid decapitation without anesthesia and trunk blood collected in EDTA-containing tubes. Plasma corticosterone levels were determined by radioimmunoassay using a double antibody kit (ImmuChem, MP Biomedicals, Orangeburg, NY) according to manufacturer's instruction. Intra assay variation was less than 8%.

2.7 Real-time RT-PCR

These determinations were done in groups of animal that were treated as shown in Figure 1 but were not behaviorally tested (n = 12 for mice and n = 16 for rats). At the same day corresponding to the last behavioral test, non-behaviorally tested animals were anesthetized by an overdose of pentobarbital sodium and transcardially perfused with physiological saline solution to remove blood from the brain. Brains were then immediately removed and frozen in isopentane until further dissection. Frozen brains were allowed to reach ice-cold temperature and the olfactory bulbs and PFC (approximately Bregma 5.2 mm to 2.7 mm for rats and 3.08 mm to 1.94 for mice) were excised by single cuts. Two additional coronal cuts of the remaining brain portion were performed (approximately Bregma -1.8 mm to Bregma -3.6 mm for rats and -0.82 mm to -2.8 mm for mice) and the temporal cortex and hypothalamus were dissected from this portion. Dissected brain tissue was processed for mRNA extraction using the Trizol reagent (Invitrogen, USA) as described previously (Tonelli, et al., 2004). Total RNA was treated with DNase (Invitrogen, USA) for 15 minutes at room temperature according to manufacturer's instruction. Five hundred ng of total RNA per sample were reverse transcribed into cDNA in a 20 µl reaction using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. Real-time RT-PCR was conducted using the iQ SYBR Green Supermix (Bio-Rad) in a 25 µl reaction using the set of primers listed in Table 1. The PCR products derived from all sets of primers were run on 0.9% agarose gel to confirm a single amplification product. The amplified products were directly cloned into the pCRII-

Topo vector (Invitrogen, Paisley, Scotland, UK) and sequenced to confirm their identity. All the primer pairs were designed using the Accelrys Gene 2.0v software and based on the following criteria: a) primer size between 80 to 180 base pair b) annealing temperature of 55°C for primer vs template c) percentage of G + C content between 40 to 55 % d) unique binding sites.

The real-time PCR reaction was run on a MyiQ instrument (Bio-Rad) with a three step cycling program as follows: an initial hot start for 5 min at 95°C followed by 40 cycles with a denaturation step of 15 sec at 95°C, an annealing step of 30 sec at 55°C, an extension step of 30 sec at 72°C with the optics on at this last step. In preparation of a melt curve, the samples were heated for 1 minute at 95°C then cooled for 1 minute at 55°C, and the melt curve was executed in 10 second increments of 0.5°C with the temperature increasing from 55°C to 95°C with the optics on. All the primers used were selected and tested for the described amplification conditions. Efficiency and consistency of the cDNA synthesis was determined by amplification of the 18S gene as a control. For each round of amplifications, only those samples that were within 1 cycle of difference respect to the mean for 18S were considered for further analysis. For each sample of a specific target gene, each cycle threshold was normalized with respect to the average of 3 control genes including 18S, GAPDH and Actin-β. Relative expression was determined using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008)

2.8 Statistics

Behavioral studies and corticosterone concentrations were analyzed with unpaired t-test. Cytokine and CRF expression were analyzed with two way ANOVAs with treatment and brain regions as factors. Tukey's tests were used for pair comparisons. Significance level was set at $p < 0.05$. Data are expressed as means \pm standard error mean (SEM).

3. Results

3.1 Challenge with allergen in sensitized rodents induces anxiety-like behavior as revealed by the open field test

In both mice and rats, no differences were detected in total motor horizontal activity (Figure 2 A, C) or fecal boli (data not shown) in sensitized animals challenged with allergen with respect to control saline. In mice, a significant difference in the time spent in the center field of the enclosures was detected ($t=2.36$, $p < 0.03$) with OVA-challenged animals showing reduced values with respect to control animals (Figure 2 B). In rats, a significant difference in the activity in the center field was detected in pollen challenged animals which showed a reduced number of counts with respect to saline treated animals ($t=4.29$; $p < 0.001$) (Figure 2 D). Sensitized animals exposed to allergen in two different rodent species consistently displayed reduced activity in the center field of the arena indicating increased anxiety. Moreover, no effect in total motor horizontal activity indicates that these effects are independent of signs of sickness.

3.2 Challenge with allergen in sensitized rodents induces anxiety-like behavior as revealed by the elevated plus maze test

In mice, a significant effect was obtained for percentage of time spent in the open arms ($t=3.2$; $p < 0.01$) with reduced time in OVA challenged animals with respect to control treated mice (Figure 3 B). No differences were observed in locomotor activity measured by the distance traveled within the plus-maze in both open and closed arms indicating that potentially altered motor functions were not driving the main effect (Figure 3 A). No differences were observed in the number of entries in the open arms and in the number of fecal boli. Similarly, rats sensitized and challenged with pollen showed reduced percentage of time in open arms with

respect to saline treated rats ($t= 2.150$; $p<0.05$) (Figure 3 D). No differences were observed in activity measured by the total number of crossings between the 4 segments of the plus-maze suggesting normal motor function (Figure 3 C). No differences were observed in the total number of entries in the open arms and fecal boli. In allergic mice and rats, the reduced time spent in the open arms was the opposite to the sum of the time spent in the closed arms and the center of the maze. These results are consistent across two different rodent species in two different allergens used indicating that sensitization and intranasal challenge with allergen induces anxiety-like behavior as indicated by reduced time spent in the open arms of an elevated plus maze, an effect independent of reduced total activity indicative of sickness behavior.

3.3 Challenge with allergen in sensitized rodents reduces social interaction

These behaviors correspond only to resident animals representing allergic (sensitized and challenged) and control (sensitized not-challenged) treated mice and rats. No data from intruder animals are presented. In mice, a significant effect was detected for latency to initiate social behavior ($t=2.37$, $p<0.04$) (Figure 4 A) and duration of social behavior ($t=2.4$, $p<0.03$) (Figure 4 B) in OVA-challenged animals with respect to control. No differences were observed on the frequency of social contact. No significant differences were observed on measures of aggressive behavior (Supplemental Figure 2). Similarly, in rats, a significant effect was observed for the latency to initiate social contact ($t=2.596$; $p< 0.03$) (Figure 4 C) and in the duration of the contact ($t=3.2$; $p< 0.05$) (Figure 4 D) in pollen-challenged animals with respect to control. No differences in aggressive behaviors were observed between control and pollen treated rats.

3.4 Plasma corticosterone levels and body weight

No significant differences were observed in plasma corticosterone levels between control and sensitized rodents exposed to the allergen 1 hour after the last behavioral tests. Values were 118.4 ± 23.4 ng/ml for control and 133.5 ± 23.7 ng/ml for OVA challenged mice. For rats values were 171.3 ± 19.8 ng/ml in control and 151.3 ± 87.39 ng/ml in pollen challenged animals. Similarly, no significant changes in body weight between control and sensitized rodents exposed to antigen were observed (Supplemental Figure 3).

3.5 Intranasal challenge with allergen induced T_H2 cytokine expression in the prefrontal cortex and olfactory bulbs of sensitized rodents

Single melting curves peaks were obtained for all the genes analyzed in mRNA originated from brain tissue. Amplified products for T_H2 cytokines were further analyzed by gel electrophoresis (Figure 5 A, C). The figure shows final amplified product for T_H2 cytokines in the PFC of OVA-challenged mice (Figure 5 A) and pollen-challenged rats (Figure 5 C). No product was detected when the reverse transcription step was omitted (RT-) confirming that the amplification was specific for mRNA that originated from the prefrontal cortex. The same was obtained for the olfactory bulbs (data not shown). ANOVA analysis detected significant differences in IL-4 expression in mice [$F(1, 24) = 92.59$; $p< 0.0001$]. Post hoc analysis indicated significant increases for OVA treated mice with respect to control in the olfactory bulbs ($p<0.001$) and PFC ($p<0.001$) (Figure 5 B). No differences were observed in the temporal cortex and hypothalamus. Similarly, significant differences were also observed for IL-5 [$F(1, 24) = 80.33$; $p<0.0001$] and IL-13 [$F(1, 24) = 168.6$; $p<0.001$]. As it was observed for IL-4, post hoc comparisons showed significant increases in IL-5 and IL-13 transcription in the olfactory bulbs and PFC (Figure 5 A). No significant changes in IL-5 and IL-13 were detected in the temporal cortex and hypothalamus.

In rats, significant differences were also detected for IL-4 [$F(1, 20) = 17.3$; $p<0.0001$]; IL-5 [$F(1, 20) = 12.6$; $p<0.0001$] and IL-13 [$F(1, 20) = 48.8$; $p<0.0001$] (Figure 5 D). Post hoc analysis showed significant increases in the olfactory bulbs and PFC of pollen treated rats with

respect to controls for the 3 cytokines analyzed (Figure 5 D). No significant differences were observed in the temporal cortex and hypothalamus for any cytokine analyzed.

3.6 Intranasal challenge with allergen increased CRF transcription in the prefrontal cortex and olfactory bulbs of sensitized rodents

Analysis of mRNA expression for CRF in the PFC, temporal cortex and hypothalamus showed a significant effect in mice [$F(3, 24) = 49.75; p < 0.0001$] and rats [$F(3, 21) = 16.02; p < 0.001$]. Post hoc analysis revealed significant increases in CRF expression in OVA treated mice with respect to controls in the PFC and no changes in the temporal cortex and hypothalamus (Figure 6). Similarly, pollen challenged rats showed elevated CRF transcription in the PFC and no changes in the temporal cortex and hypothalamus.

4. Discussion

The present study is the first to report behavioral responses of anxiety and reduce social interaction during experimentally-induced allergic rhinitis and show that these effects are consistent across two rodent species. Moreover, these effects were achieved using two different types of antigens indicating that the host immune response is likely to mediate these effects. In addition, the present results suggest that these effects may be related with the induction of T_H2 cytokines and CRF at least in the PFC and olfactory bulbs. Behavioral responses of anxiety in mice and rats using OVA as an antigen has been described previously during the early phase in experimental models of asthma (Costa-Pinto et al., 2005; Palermo-Neto and Guimaraes, 2000). Together with the present results, these studies show that anxiety is a feature of respiratory allergic processes that occurs during the differences phases and when different antigens are involved. These findings strengthen the idea that the cellular and molecular mediators of allergic inflammatory processes activate common neuroimmune mechanisms that involve emotionally relevant circuits and the induction of anxiety.

The association of allergies with anxiety disorders and increased emotional reactivity has long been recognized and known to be complex. It has been proposed that the distress caused by chronic inflammatory diseases such as allergic rhinitis or allergic asthma could induce pathological anxiety as the result of psychological anticipatory processes of recurrent chronic episodes (Chida et al., 2008). Also, psychological stress and emotion in allergic individuals have been proposed to affect stress responsiveness resulting in exaggerated adrenergic and cortisol responses that may in turn result in aggravation of allergic symptoms (Wright, 2005). In addition, it has been proposed that co morbidity of allergies with anxiety disorders are related with genetic factors that segregate together and influence each other (Wamboldt et al., 1998; 2000). While the relationship between allergic rhinitis and anxiety has been shown to be bidirectional and likely to share genetic and neuroimmune mechanisms, these mechanisms remains undefined. Indeed, the idea that allergen exposure in sensitized individuals can elicit responses of anxiety has received little attention. Two relatively recent reports by the same group using a modified version of the passive avoidance test showed for the first time that sensitized mice display avoidance behavior towards an allergen-associated compartment and that these effects were IgE dependent (Costa-Pinto et al, 2005; 2007). The authors also showed that the avoidance effect occurred during the early phase of the allergic response. In the present study, we used 2 independent tests to evaluate anxiety behaviors during the late phase of the allergic reaction and observed consistent responses of anxiety. In this regard, the elevated plus maze (EPM) test is one of the most validated tests to evaluate anxiety in rodents (Carobrez and Bertoglio, 2005). It is ethologically relevant to the animal involving the natural spontaneous exploration of environments by rodents in the absence of reward (Rodgers et al., 1997; Carobrez and Bertoglio, 2005). The open field test (OFT) is also an ethologically relevant test of anxiety-like behavior (Prut and Belzung, 2003). It is also based on the innate behavior of rodents to

explore in novel spaces that is opposed to the fear of open spaces in the absence of reward or aversive stimulus. The open field test also analyzes total motor horizontal activity which is an important measure in immunologically relevant behavioral studies because they indicate the possibility of effects related to sickness. In the present study, no differences in motor activity were detected in the EPM or OF tests suggesting that sickness did not influenced behavioral responses.

In addition to anxiety disorders, the burden of allergic rhinitis on quality of life has been documented by numerous studies across cultures (Meltzer, 1997; 2001; Nathan, 2007; Blaiss, 2007). The most common reported impairments are sleep disturbances, poor cognitive functioning, absenteeism from work and school and in children difficulties integrating with peers (Marshall et al., 2000; 2002; Berger, 2004; Baiardini, et al., 2006; Santos et al., 2006; Sundberg, et al., 2007; Blaiss, 2007; 2008). While many of the social impairments caused by allergic rhinitis are likely to be related with the symptoms of the disease, the question if allergic rhinitis may induce altered social interactions in an experimental setting was never tested before. We used the resident intruder test that is one of the most commonly used tests to elicit and measure aggression and also analyze social interactions among conspecifics of rodent species. This test is based on agonistic behaviors displayed during encounters between unfamiliar rodents (Mitchell, 2005). After introduction of an intruder rodent into the home cage of a resident rodent, the resident rodent invariably shows investigative behavior and postures that are measures of social behavior or interactions. These behaviors may lead to the expression of aggressive behavior. Although aggressive behaviors were rare in the present experiments, a consistent reduction in social interaction was seen in mice and rats with experimentally induced allergic rhinitis. This indicates that other behavioral components suggestive of altered emotional reactivity are also affected by the inflammatory process of allergic rhinitis. However, it is also possible that reduced social interaction was related or produced by increased social anxiety (Nicolas and Prinssen, 2006). If reduced social interaction in the present study is the resultant of increased anxiety remains to be determined. An important consideration of the present experiments is that the animals were subjected to all of the different behavioral testing. While a minimal effect is expected, it can not be excluded the possibility of an influence of a particular test on any further behavioral testing. In any case, together with the studies of Costa-Pinto et al and Palermo-Neto and Guimaraes showing anxiety-like behaviors during the early phase in a model of allergic asthma, the present study provides additional evidence that similar behavioral alterations reported in humans suffering from respiratory allergies can be mimicked in rodents by experimentally inducing allergic rhinitis or asthma.

Another important consideration to the present studies is that the corticosterone determinations were done in behaviorally tested animals one hour after the last behavioral test and thus comparisons between groups do not represent HPA-axis reactivity. Instead, the reported values are likely to reflect certain degree of recovery following acute activation of the HPA-axis. Transient increases in cortisol levels short after nasal provocation has been reported in allergic patients (Kalogeromitros et al., 2007). Conversely, another study showed blunted cortisol responses to stress in atopic adolescents (Wamboldt et al., 2003). These studies show that the responsiveness of the HPA-axis to allergic processes depends on several parameters that may include the magnitude of the allergic reaction, the timing after the contact with the allergen and also psychological stressors (Palermo-Neto and Guimaraes, 2000). It is notably however, the limited research addressing these issues. Another important consideration of the present study is that allergies have been also associated with increased incidence in depressive disorders (Hurwitz and Morgenstern, 1999; Wamboldt et al., 2000; Timonen, et al., 2003; Goodwin and Buka, 2008), while we have performed studies in rodents using the forced swim test, the results have been so far inconclusive.

The neural circuitry involved in the expression of anxiety and the brain regions compromised in pathological anxiety has been extensively studied (Drevets, 2000; 2003; Bremner, 2004). Studies in humans, non-human primates and rodents implicate the PFC as an important structure that provides modulatory function in the manifestation of anxiety. In addition, functional imaging studies strongly implicate the PFC in providing inhibitory functions for anxiety and depression (Drevets, 2000; 2003; Bremner, 2004). The present study showed that this region responded with increases in the transcriptional activity of T_H2 cytokines and CRF during an allergic inflammatory process in the upper respiratory tract. These results are relevant in the context of the finding reported by Rozenkranz et al (Rozenkranz et al., 2005) showing increased blood flow in the anterior cingulate cortex during the late phase of an allergic reaction when allergic individuals are exposed to antigen. Thus, this region appears to be involved in the neural responses elicited during the late phase of respiratory allergic reactions. Previous studies reported that other regions including the amygdala and hypothalamus are also responsive to respiratory allergies as measured by the expression of c-fos (Costa-Pinto et al., 2005; 2006; 2007). These regions are also involved in the neurocircuitry of anxiety and anxiety disorders. The present study did not reveal any changes in the transcriptional activity of the genes for cytokines and CRF in these regions. This may be related to the approach employed, because the PCR determinations in the temporal cortex and hypothalamus were performed from portions of tissue that contains several distinct neuronal populations of CRF cells, differences in CRF expression may have occurred and were not quantified using our dissection approach. Moreover, if the determinations are made during the early or the late phase of the allergic reaction may also play an important role. Finally, the use of anesthesia prior dissection of the brain regions may have influenced the sensitivity for detection of mRNA changes.

The present study is also the first to report on T_H2 cytokine expression in the brain in response to a peripheral immune challenge. While the expression of IL-4, IL-5 and IL-13 follows the pattern of a T_H2 cell-dependent allergic reaction, the meaning and function of this transcriptional increase in the PFC is unknown at this time. A tempting speculation is that increases in these cytokines may be related at least in part to CRF transcription and that CRF may provide the link between T_H2 cytokine expression in the brain and behavioral changes. The involvement of T-cell dependent processes in influencing behavioral responses has been shown by studies using bacterial superantigens (Rossi-George et al., 2004; Rossi-George et al., 2005; Urbach-Ross et al., 2008). The study of Rossi-George et al., 2004 showed that anxiety-like behavior after activation of the T_H1 subset of T-cells and expression of pro-inflammatory cytokines was reduced in the elevated plus maze test or no evident in other test of anxiety. In this regard is possible that T_H2 immune processes may provide negative regulatory function over a set of pro-inflammatory cytokines and that the behavioral outcomes of allergic inflammation may be the resultant of these interactions rather than the effect of single cytokines. In this regard, decreased IL-2 mRNA in the prefrontal cortex was found associated with increased anxiety in the EPM (Pawlak et al, 2005) further supporting this notion. These effects may be also influenced by the genetic background as well as the specific nature of the immune stressor (Bartolomucci et al, 2003; Lu et al., 1999; Anisman et al., 2008; Dantzer et al., 2008).

In sum, experimentally induced allergic rhinitis in naïve rodents elicits responses of anxiety-like behavior and reduced social interaction on ethologically relevant tests, an effect that is paralleled by increased T_H2 cytokine and CRF transcription in the PFC. These studies indicate that allergic rhinitis is a condition capable of influencing behavioral responses and warrants further studies to deepen the understanding of the complex relationship between atopy, emotional reactivity and possibly mental disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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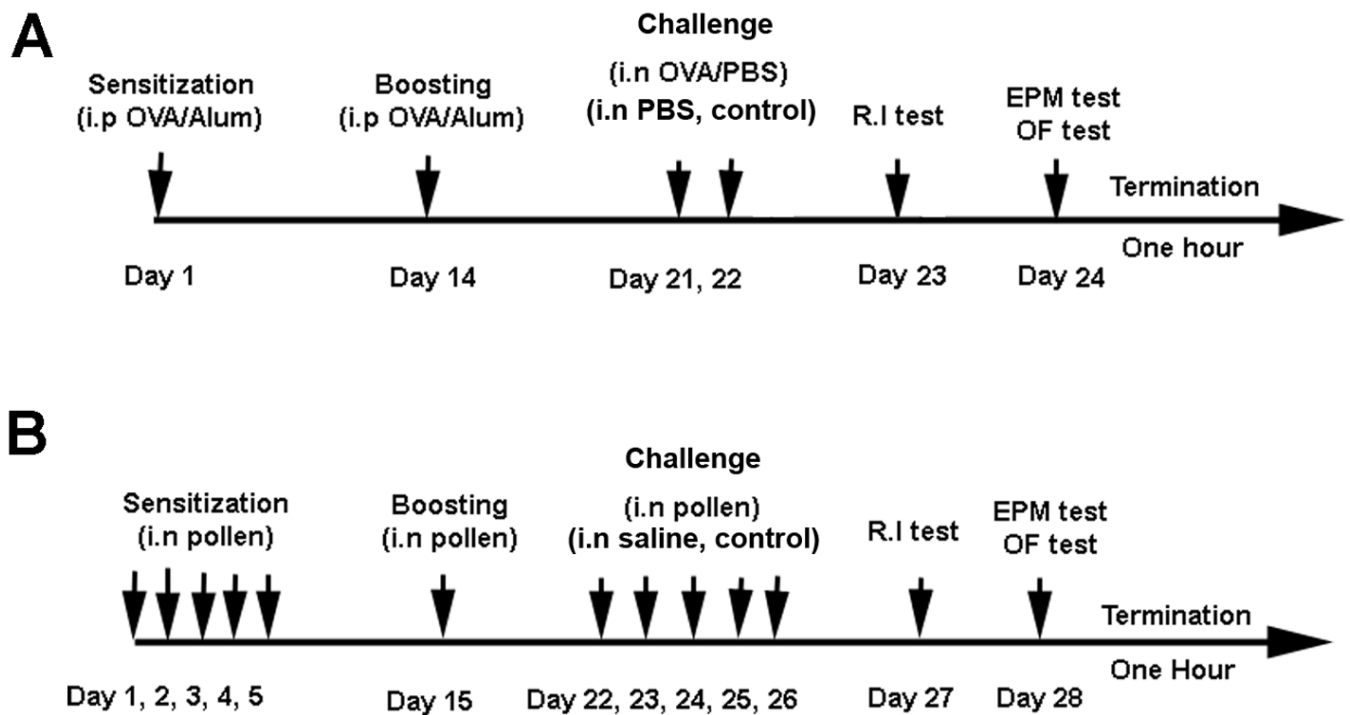


Figure 1. Representative diagram of the sensitization, challenge and behavioral testing schedule in BALB/c mice (A) and Brown Norway (NB/NHsd) rats (B). OVA: albumin from chicken egg; i.p: intraperitoneal; i.n: intranasal; R.I: resident intruder test; EPM: elevated plus maze test; OFT: open field test. Animals used for mRNA quantification were treated as shown and terminated at the same time but were not behaviorally tested.

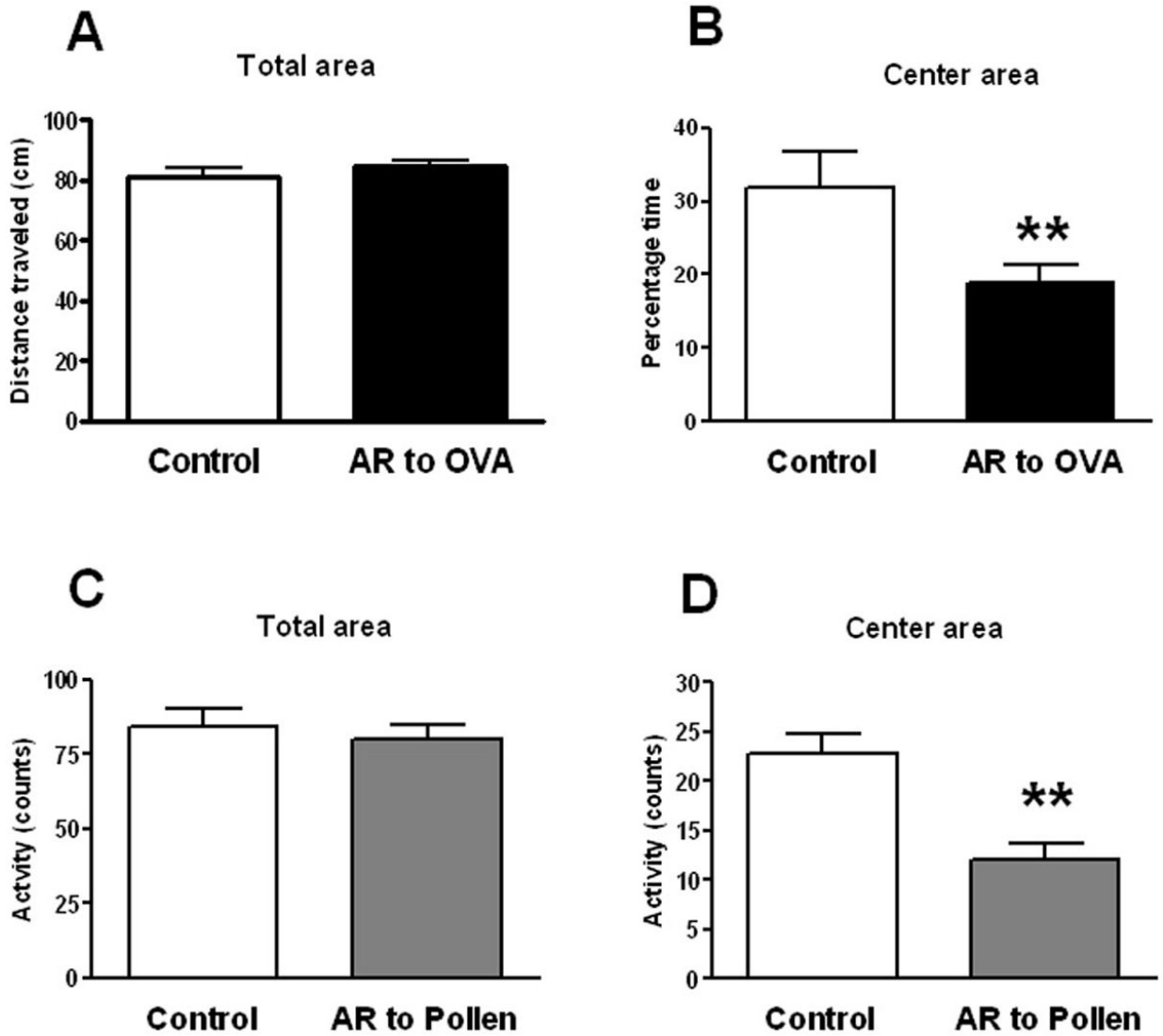


Figure 2. Activity in the open field test of mice (A; B) and rats (C; D) in control condition (sensitized but not challenged) or after intranasal challenge in sensitized animals with ovalbumin (OVA) (A; B) or pollen (C; D) respectively. Activity is expressed as distance travelled (cm) in mice and crossing of squares (counts) in rats. Significant differences ** ($p < 0.001$) were observed in the activity in the center area (B, D) and no differences were observed in the overall activity (A, C). Thus anxiety-like behavior is not the result of reduced motor activity which could be a component of sickness behavior.

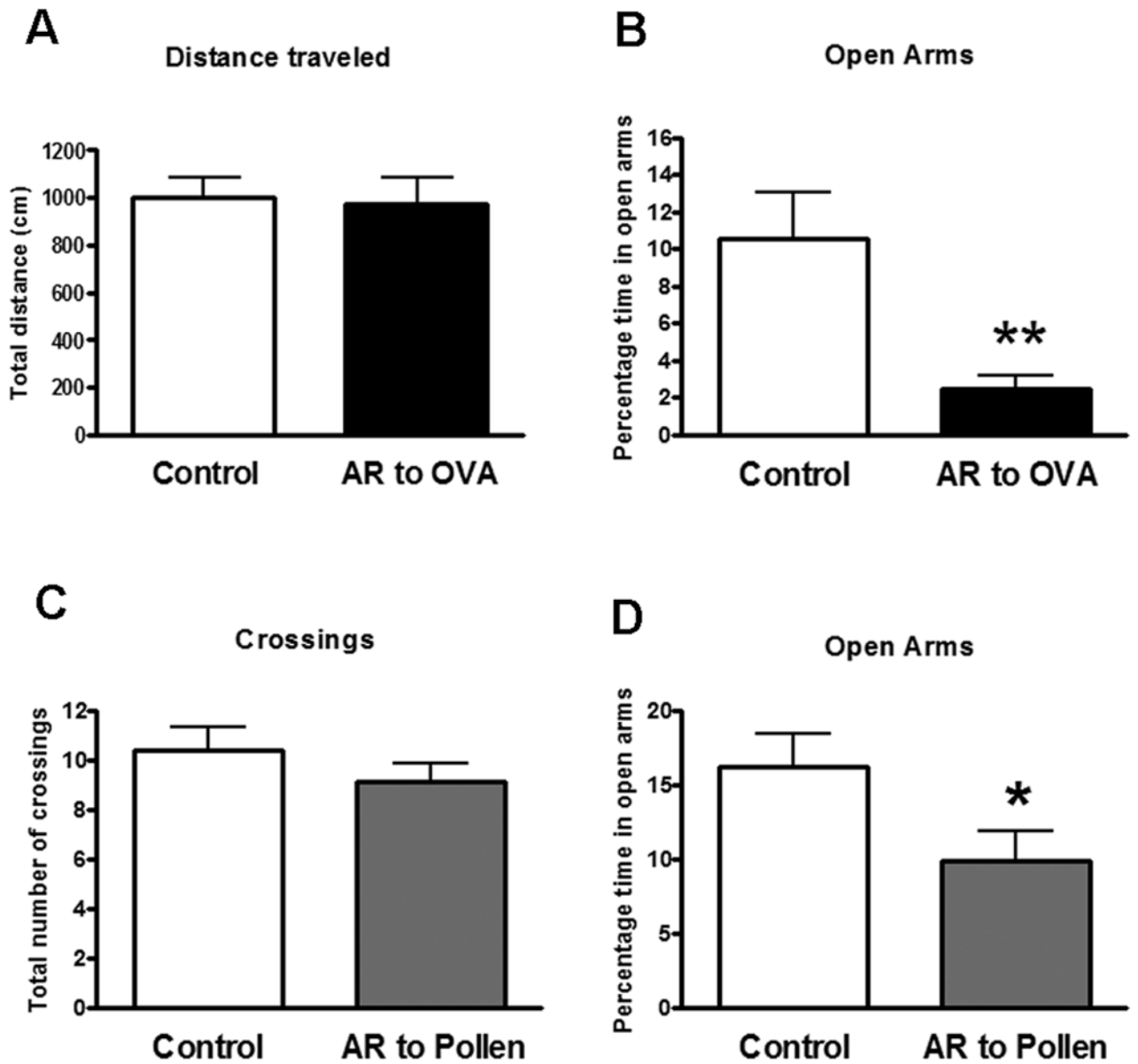


Figure 3. Activity in the elevated plus maze test of mice (A; B) and rats (C; D) in control conditions (sensitized but not challenged) or after intranasal challenge in sensitized animals with ovalbumin (OVA) (A; B) or pollen (C; D) respectively. Overall activity is expressed as total distance traveled in the closed and open arms for mice and number of crossings between the segments of the plus-maze in rats. Activity in the open arms is expressed as the percentage of time spent in the open arms. Significant differences * ($p < 0.05$); ** ($p < 0.001$)

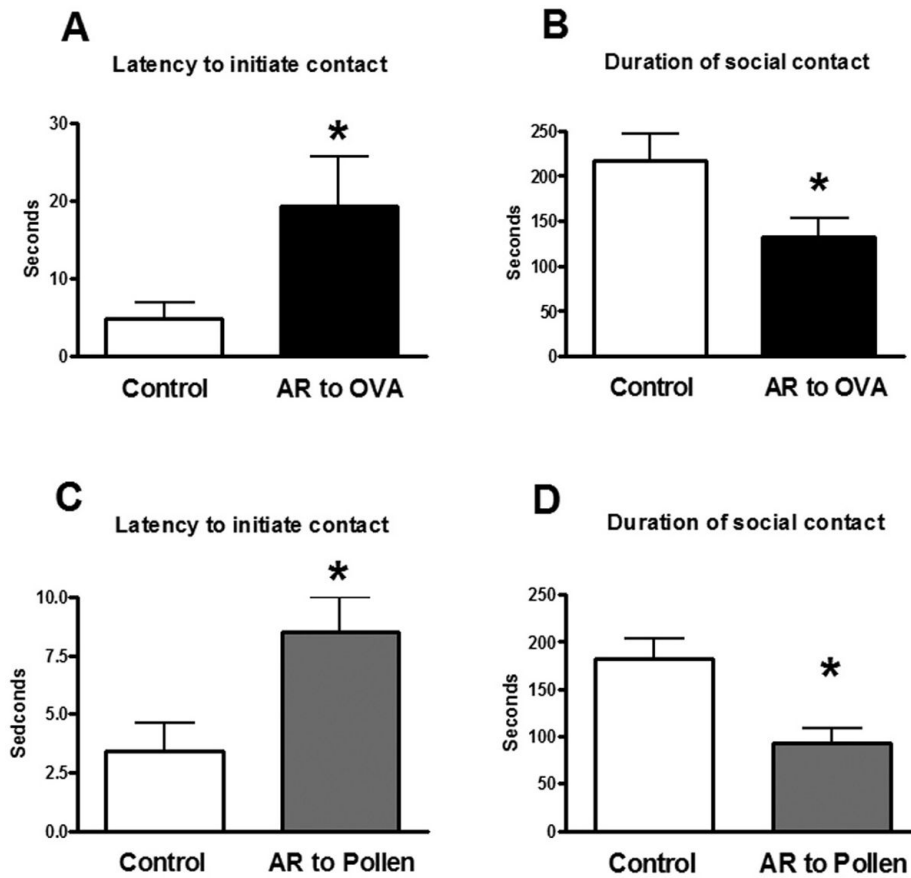
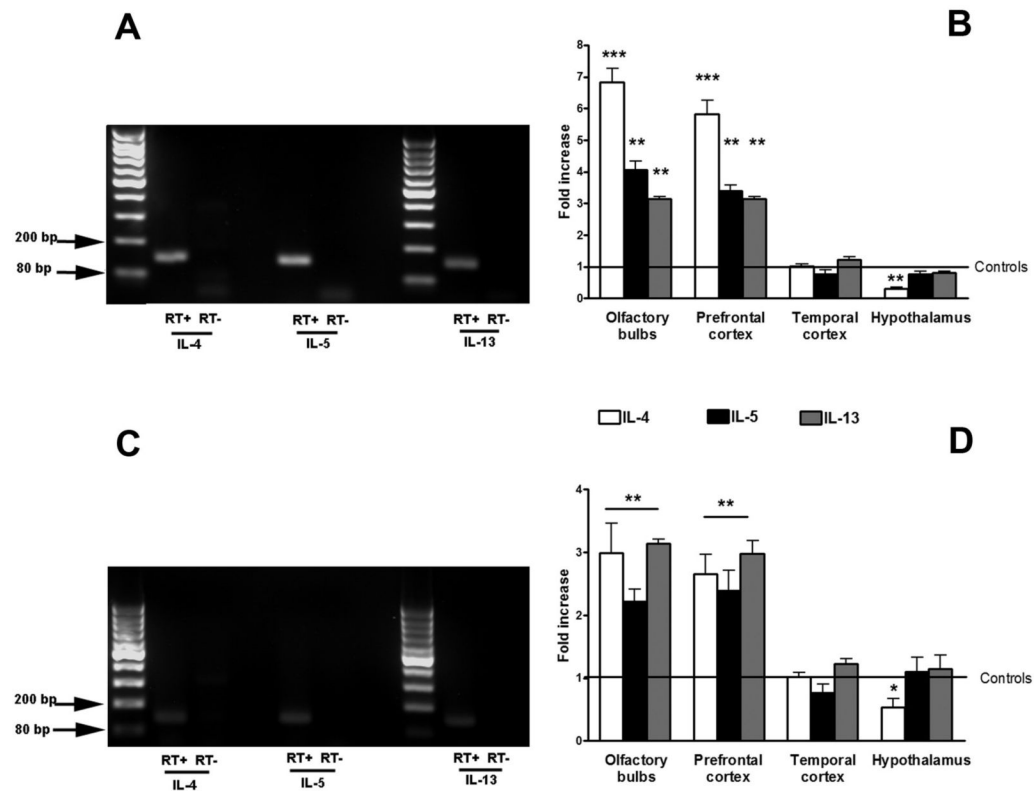


Figure 4. Latency to initiate contact (A, C) and duration of social contact (non-aggressive) (B, D) of mice (A; B) and rats (C; D) in control condition (sensitized but not challenged) or after intranasal challenge in sensitized animals with ovoalbumin (OVA) (A; B) or pollen (C; D) respectively. Significant differences * ($p < 0.05$)

**Figure 5.**

Final products for T_H2 cytokines after 40 cycles of real-time RT-PCR performed from mRNA obtained from the area corresponding to the prefrontal cortex of mice (A) and rats (C) after intranasal challenge in sensitized animals with ovalbumin (OVA) or pollen respectively. No product was detected when the reverse-transcriptase enzyme step was omitted (RT-). Final products were observed between the 80 and 200 base pair standards as expected (see Table 1). Relative expression of T_H2 cytokines (IL-4, IL-5 and IL-13) as determined by real-time RT-PCR (B, D) in different brain regions after the induction of allergic rhinitis to ovalbumin (OVA) (B) or pollen (D). Values are fold increase with respect to the average value of control mice and rats represented by the number 1. Significant differences with respect to control values: ** ($p < 0.001$); *** ($p < 0.0001$).

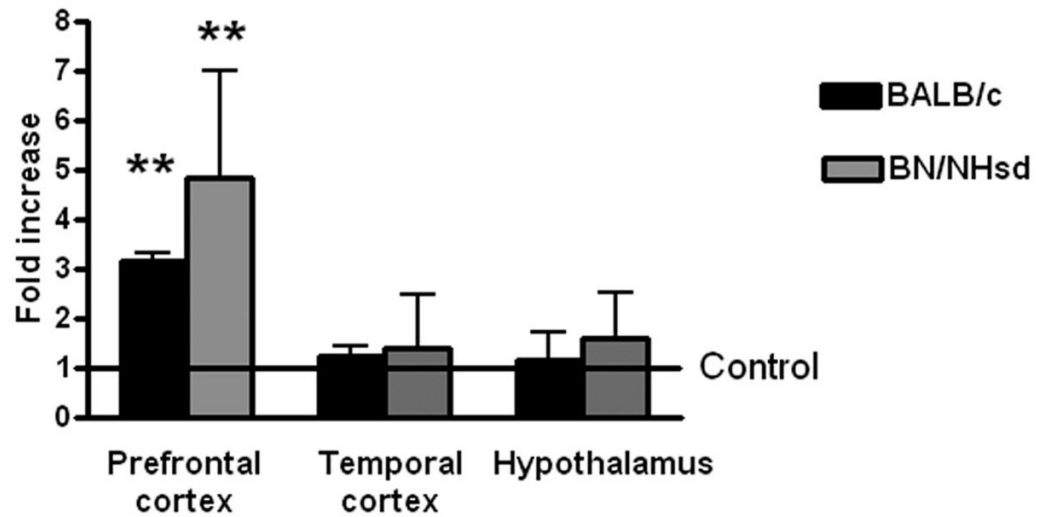


Figure 6. Relative expression of CRF as determined by real-time RT-PCR in the prefrontal cortex, temporal cortex and hypothalamus after the induction of allergic rhinitis to ovalbumin (OVA) in BALB/c mice or pollen in Brown Norway (BN/NHsd) rats. Values are fold increase with respect to the average value of controls treated mice or rats represented by the number 1. Significant differences with respect to control values: ** ($p < 0.001$)

Table 1

Primer sequences used for real-time RT-PCR determinations. Accession: GeneBank accession number for sequences publicly available at <http://www.ncbi.nlm.nih.gov/sites/entrez>. IL-4: interleukin-4; IL-5: interleukin-5; IL-13: interleukin-13; CRF: corticotropin releasing factor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; ACTB: actin, beta isoform; 18S: ribosomal RNA subunit 18S; mus: *mus musculus*; rattus: *rattus norvegicus*

Gene (specie)	Accession	Primer sequence	Region	Product length
IL-4 (mus)	NM_021283	Fwd 5' AGAACACCAAGAGAGTGAG3'	266-417	152
		Rev 5' TGAATCCAGGCATCGAAAAAG3'		
IL-13 (mus)	NM_008355	Fwd 5' TCTTGTGCTTGGCTTGGTGGTC3'	98-237	140
		Rev 5' ATACCATGCTGCCCGTTGCAC3'		
IL-5 (mus/rattus)	NM_010558 (mus) NM_021834 (rattus)	Fwd 5' CATGAGCACA GTGGTGAAG3'	115-252 (mus)	138
		Rev 5' AGCCCTGAAAAGATTCTCC3'	69-206 (rattus)	
CRF (mus/rattus)	NM_205769 (mus) NM_031019 (rattus)	Fwd 5' GGATCTACCTTCCACCTTC3'	633-734 (mus)	102
		Rev 5' CCGATAAATCTCCATCAGTTTCC3'	631-732 (rattus)	
GAPDH (mus)	NM_008084	Fwd 5' CCACTCACGGCAAA TTCAAC3'	196-338	142
		Rev 5' AGACTCCACGACATACATCAG3'		
ACTB (mus)	NM_007393	Fwd 5' TGAGACCTTCAACACCCCCAG3'	452-591	140
		Rev 5' GAGCATAGCCCTCGTAGATG3'		
18S (mus/rattus)	NR_003278 (mus) X01117 (rattus)	Fwd 5' CCAATAAGTGGGGTCAATAAGC3'	1645-1727 (mus)	83
		Rev 5' CCATCCAATCGGTAGTAGCGGAC3'	1650-1732 (rattus)	
IL-4 (rattus)	NM_201270	Fwd 5' TTACGGCAACAAGGAACAC3'	197-336	140
		Rev 5' ACAGAGTTTCTCAGTTTCAACC3'		
IL-13 (rattus)	NM_053828	Fwd 5' GCTGAGCAATCACACAAG3'	117-279	162
		Rev 5' GAGGCCAATCAATATCTCTCTG3'		

Gene (<i>specie</i>)	Accession	Primer sequence	Region	Product length
GAPDH (<i>rattus</i>)	NM_017008	Fwd 5'TACCCACGGCAAGTTCAACCG3'	223-362	140
		Rev 5'ACTCCAGGACATACTCAGCAC3'		
ACTB (<i>rattus</i>)	NM_031144	Fwd 5'TGAGACCTTCAACACCCCCAG3'	453-592	140
		Rev 5'GCCGGTAACCCCTCATAGATG3'		