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Neuroimmune circuits involved in β-lactoglobulin-induced food allergy

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ABSTRACT

Several antigens can act as allergens eliciting IgE-mediated food allergy reactions when fed to sensitized animals. One of them is ovalbumin (OVA) which is the main allergen in egg white. Allergic mice develop aversion to OVA consumption. This aversive behavior is associated with anxiety, and it can be transferred to non-sensitized mice by injection of serum of allergic mice. However, it is yet to be determined whether altered behavior is a general component of food allergy or whether it is specific for some types of allergens. Cow's milk allergy is the most prevalent food allergy that usually begins early in life and β-lactoglobulin (BLG) is the milk component with the highest allergenicity. In this study, we investigated behavioral and neuroimmune circuits triggered by allergic sensitization to BLG. A neuroimmune conflict between aversion and reward was observed in a model of food allergy induced by BLG intake. Mice sensitized to BLG did not present aversive behavior when BLG was used for sensitization and oral challenge. Mice allergic to BLG preferred to drink the allergen-containing solution over water even though they had high levels of specific IgE, inflammatory cells in the intestinal mucosa and significant weight loss. When sensitized to OVA and challenged with the same antigen, mice had increased levels of neuron activation in the amygdala, a brain area related to anxiety. On the other hand, when mice were sensitized to OVA and received a mixture of BLG and OVA in the oral challenge, mice preferred to drink this mixture, despite their aversion to OVA, which was associated with neuron activation in the nucleus accumbens, an area related to reward behavior. Thus, the aversive behavior observed in food allergy to OVA does not apply to all antigens and some allergens may activate the brain reward system rather than anxiety and aversion. Our study provides novel insights into the neuroimmune conflicts regarding preference and avoidance to a common antigen associated with food allergy.

1. Introduction

Food allergy is an abnormal immune response against dietary antigens (Johansson et al., 2004). Allergen-specific IgE produced by plasma cells in circulation binds to FcεRI receptors expressed on the membrane of mast cells and basophils, which upon a second allergen exposition, a massive degranulation is observed with the release of various inflammatory mediators and the development of an anaphylactic shock (Bischoff and Crowe, 2005). Importantly, a response triggered by the immune system during food allergy can affect the central nervous

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system through direct signals from sensory nerves present in the intestine (van der Kleij et al., 2010). Moreover, mast cells may establish contact with nerves through cell adhesion molecules type 1 (CADM-1) (Furuno et al., 2012; Hagiyama et al., 2011) and act directly on neurons of the enteric nervous system (Voisin et al., 2017a). As a response, neurons secrete mediators such as neuropeptides and neurotransmitters that bind to cognate receptors expressed on the immune cells involved in food allergy (Dantzer, 2018). Probably, this bidirectional neuroimmunological interaction occurs early, having a great impact on allergic inflammation (Voisin et al., 2017b). Due to this complex net of neuroimmune signaling, symptoms of allergy may range from a slight inconvenience to life-threatening reactions such as anxiety (Sicherer, 2003). Anxiety reactions have been the most common behavioral pattern associated with food allergy (Costa-Pinto and Basso, 2012). Allergic patients in a state of anxiety often have their daily tasks compromised due to a feeling of imminent risk (Sampath et al., 2018).

Among the most important allergens, ovalbumin (OVA) is the major protein component from avian egg-white and one of the primary allergens along with ovomucoid for infants with egg white allergies (Huntington and Stein, 2001). OVA is a potent allergen and when used with $Al(OH)_3$ to immunize mice it induces a strong allergic response with high levels of serum specific IgE, followed by augmented numbers of goblet cells, high levels of secretory IgA as well as recruitment of eosinophils to the gut mucosa after oral challenge with the antigen in the drinking water. Our group demonstrated that this experimental mouse model of food allergy elicited by OVA is associated with weight loss (Saldanha et al., 2004) and that mice previously sensitized to OVA avoid drinking the allergen-containing solution even when it contains a palatable component such as saccharin (Cara et al., 1994). OVA-sensitized mice orally challenged with the allergen present increased levels of anxiety evidenced by shorter time of exploration in the open arms of an elevated plus maze (EPM) and strong activation of specific brain areas involved in anxiety such as the paraventricular nucleus of the hypothalamus (PVN) and the central nucleus of amygdala (CeA) (Salgado et al., 2004). Furthermore, it has been shown that sensitized mice avoid a compartment previously associated with presentation of the allergen against which they were sensitized (Costa-Pinto et al., 2005). In a model of food allergy to OVA, the expression of mRNA for the calcitonin gene-related peptide (CGRP) was increased in the colon of mice whereas the distribution of nerve fibers did not change, suggesting that CGRP release may be increased during allergies (Lee et al., 2013). Furthermore, Basso and coworkers have reported that a Th2-associated response induces changes in central nervous system activity and behavior through an IgE-dependent mechanism (Basso et al., 2003), suggesting that IgE may play an important role in the aversive behavior observed in experimental food allergy. Consistent with this, passive transfer of hyperimmune serum or adoptive transfer of splenocytes from mice allergic to OVA into naïve mice transferred food aversion (Cara et al., 1997). However, little is known about behavioral aspects in food allergy induced by other allergens rather than OVA.

Several dietary antigens, including cow's milk proteins have already been described as potent allergens. Cow's milk contains approximately 30–35 g/L of proteins and the class of lactoserum proteins (whey) represents 20% of total cow's milk protein. β-lactoglobulin (BLG) is the most immunogenic and abundant among whey proteins (approximately 50%) (Jo et al., 2014; Stöger and Wüthrich, 1993; Wal, 2004). One of the most classic models of cow's milk allergy was described by Li et al. (1999). In this model, three-week-old female C3H/HeJ mice were given different doses of cow's milk proteins accompanied by cholera toxin as an adjuvant for six weeks. The oral challenge was performed using two intragastric (i.g.) administrations of these proteins with a 30-min interval between them. As important parameters of allergy, anaphylaxis index, vascular permeability, serum IgE levels, histamine and cytokine production and small intestine histology were evaluated (Li et al., 1999). Another group developed a model using 4- to 5-week-old female C3H/HeJ mice sensitized intragastrically with BLG and cholera toxin as an adjuvant. The oral challenge was performed by the same route with 100 mg of BLG in a single dose and anaphylaxis index, vascular permeability, levels of IgE, IgG1 and IgG2a, cell proliferation and production of the cytokine IL-10 were evaluated (Frossard et al., 2007).

Previous work showed that mice previously sensitized via intraperitoneal injection with a solution of BLG and $Al(OH)_3$ as adjuvant and posteriorly challenged by oral route with whey solution developed signs of food allergy similarly to those observed in OVA models, such as elevated levels of serum immunoglobulin (Ig) E, loss of body weight and eosinophilic inflammation (Gomes-Santos et al., 2015).

In the present study, we aimed to investigate whether mice with BLG-induced food allergy had altered behavior and the neuroimmune circuits underlying this change. We found that mice sensitized to BLG developed a partial aversion to BLG consumption that was further lost despite the high levels of anti-BLG IgE and weight loss, which is a remarkable difference from OVA-sensitized mice challenged with OVA. Moreover, mice sensitized to OVA that received a mixture of BLG and OVA during the oral challenge, preferred to drink this mixture, despite their aversion to OVA. This preference was associated with neuron activation in the nucleus accumbens, a brain area related to the reward system. Thus, different allergens induce different responses, which is likely a result from the cerebral region they activate.

2. Material and methods

2.1. Animals

Male BALB/c mice at 7 to 8 weeks-of-age were obtained from the Federal University of Minas Gerais (UFMG, Brazil) animal facility and maintained under specific pathogen free conditions. All procedures were in accordance with the ethical principles in animal experimentation, adopted by the Ethics Committee in Animal Experimentation of our institution (CEUA - UFMG) that approved our study under the Protocol number 144/2019. Mice were kept in a temperature-controlled room with free access to water and standard chow diet until the oral antigen challenge (24 h prior to experiments).

2.2. Mice sensitization and oral challenge

Food allergy induction was performed according to the model described by Gomes-Santos and coworkers (Gomes-Santos et al., 2015). Briefly, mice were sensitized by intraperitoneal (i.p.) injection of 0.2 ml saline (0.9%) containing 1 mg alum hydroxide ($Al(OH)_3$) as an adjuvant and 20 μg BLG (Sigma, St. Louis, MO, USA). After 14 days, sensitized mice received an i.p. booster immunization with saline containing 20 μg soluble BLG. Seven days later, mice were orally challenged with 20% whey protein solution in the drinking bottle as their only source of liquid for a period of either 7 or 14 days. Mice that were previously sensitized and posteriorly challenged by oral route with BLG are referred as the allergic group, while those that were previously sensitized and received water instead of BLG are referred as sensitized. Mice that were sensitized and boosted by an immunization with soluble antigen are referred as immunized. Drinking solutions used for oral challenge were replaced daily. Whey protein hydrolysate was obtained from EDETEC (80% BLG). Alternatively, mice received 1 mg/ml BLG, OVA (Sigma, St. Louis, MO, USA) or a mixture of both. For each condition, 5 mice per group were used according to sample size calculation performed in the OpenEpi statistical software (Minn M. Soe and Kevin M. Sullivan, Emoryl University). The doses of BLG and whey used were chosen based on the ones standardized and described by our group in a previous study (Gomes-Santos et al., 2015).

Food allergy induction to ovalbumin (OVA) was performed according to the model described by Saldanha and coworkers (Saldanha et al., 2004) using the same dose standardized in the study. Mice were sensitized by intraperitoneal (i.p.) injection of 0.2 ml saline (0.9%) containing 1 mg alum hydroxide ($Al(OH)_3$) as an adjuvant and 20 μg OVA

(Sigma, St. Louis, MO, USA). After 14 days, sensitized mice received an i.p. booster immunization with saline containing 20 μg soluble OVA. Seven days later, mice were orally challenged with either 1 mg/ml OVA solution or a solution containing a mixture of 1 mg OVA + 1 mg BLG in their drinking bottle as their only source of liquid for a period of either 7 days.

2.3. Liquid intake

Liquid consumption was measured during experimental analysis by checking the remaining quantity of liquid in the bottle and the quantity offered in the previous day. The result obtained was the average consumption per mouse/day.

2.4. Two bottle liquid preference test

A two-bottle liquid preference test was performed to evaluate the choice for BLG solution over water. Three days before the test was conducted, mice were individually separated in conventional cages for acclimation. On the same day, two identical, transparent, glass-tipped bottles containing water were placed for each animal to choose between the bottles. In the liquid preference day test, two bottles were placed per cage: one containing the antigenic solution (OVA or BLG or the mixture of both) and the other containing only water. Sweetened water was a solution of water containing 1% saccharin sodium. Liquid consumption was measured every 4 h during a 24-h period, which occurred on the first day of the oral challenge. After each measurement, bottles were filled with the liquid, however the position was changed to avoid conditioning by their location in the cage.

2.5. Measurement of serum specific anti-BLG antibodies

To measure the concentration of anti-BLG IgE, capture-enzyme-linked immunosorbent assay (ELISA) in which plates coated with rat anti-mouse IgE was used, as previously described by our group (Gomes-Santos et al., 2015). Briefly, serum samples were obtained from all mice after the last oral antigen exposure. Fifty μl total serum, biotinylated BLG, and HRP-labeled streptavidin were added individually to a 96-well plate. Subsequently, the reaction was developed by adding H_2O_2 with orthophenylenediamine (OPD, Sigma, St. Louis, MO, USA) as previously described (Russo et al., 2001; Gomes-Santos et al., 2015). Results obtained were reported in arbitrary units using a positive reference serum (1000 U).

To measure the concentration of anti-BLG IgG1, plates were incubated with 100μl/well of a BLG solution (2μg/well) diluted in carbonate buffer pH 9.6 and kept overnight at 4 °C. Next day, after three washes with PBS solution, plates were blocked by adding 200μl/well 0.1M phosphate buffer casein solution (pH 7.4) for at least 1 h at room temperature. Diluted serum was added at 1: 400, and serial dilutions were performed. After plates were incubated at 37 °C for 1 h and washed, peroxidase-labeled anti-isotype antibodies (Goat anti-mouse IgG1-HRP; Southern Biotechnology Associates Inc.) were added at 1:15.000 dilution. The reaction was developed by adding H_2O_2 with OPD.

To measure the concentration of anti-BLG secretory IgA (SIgA), small intestines from mice were collected and rinsed with 10 ml cold 0.1M phosphate buffer solution (PBS, pH 7.4). Intestinal lavages obtained were centrifuged at 12,000g for 20 min at 4 °C. Supernatants were then collected and secretory IgA concentration was determined by ELISA (Gomes-Santos et al., 2012).

2.6. Measurement of proximal jejunum cytokines

Measurement of proximal jejunum cytokines was performed as described previously (Canesso et al., 2018; Miranda et al., 2019). Proximal jejunum samples were weighed and homogenized in PBS containing 0.05% Tween-20, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KIU Aprotinin A using a tissue homogenizer (100 mg tissue/ml buffer). Suspensions were centrifuged at 12,000g for 20 min at 4 °C and the supernatants were transferred to microtubes and stored at −80 °C until analysis. Concentrations of IL-4, IL-5, IL-10 were measured by ELISA.

2.7. Intraepithelial lymphocytes (IEL) counting

The intestinal epithelium was examined on histological slides stained with hematoxylin-eosin (H&E) in 20× magnification under an optical microscope. IELs were identified by their characteristic localization: basal to the nuclei of the enterocytes and small clear halo of cytoplasm around their dense and regular spherical nucleus. For each fragment, 500 epithelial cells were counted, not including goblet cells, as described previously (Ferguson and Murray, 1971). The final number of IELs was expressed as IEL per 100 counted epithelial cells.

2.8. Elevated Zero Maze (EZM)

The maze is a device made of acrylic plastic that has two open arms and two closed arms (Cruz et al., 1994) and is elevated 100 cm from the floor. The arms (open or closed) are positioned on opposite sides. In the EZM test, each mouse is placed individually in the central area of the maze, where they can explore its arms freely for 15 min. The time spent exploring the open and closed arms is recorded by video, as well as the number of entries and exits on each arm. The percentage of time spent in the open arms of the maze is inversely proportional to the level of anxiety in the test.

2.9. Quantification of c-Fos in the brain by immunohistochemistry

Ninety minutes after exposure to the maze, mice were anesthetized with urethane and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed and fixed for 2 h in paraformaldehyde and stored for at least 30 h in 30% sucrose for cryopreservation. 40 μm-thick coronal sections were cut with a freezing microtome and were cryopreserved in duplicate in the cryostat. Sections were first processed for c-Fos labeling by immunohistochemistry, as previously described (Beijamini and Guimarães, 2006; de Oliveira et al., 2000). Finally, after dehydration in xylene diaphonization, slides were mounted in Entellan®.

2.10. Histological analysis of proximal jejunum

Animals were sacrificed and their proximal jejunum collected and opened longitudinally for histological analyses. Proximal jejunum was fixed with 10% formalin in the neutral buffer, and embedded in paraffin. Histological sections were previously deparaffinized and then stained with H&E or Periodic acid-Schiff and analyzed by light microscopy (Olympus BX41). The histological changes were evaluated in a double-blinded fashion.

2.11. Statistics

Results were reported as the mean ± standard deviation. One-way ANOVA with Tukey post-hoc or Bonferroni's test analyses were used for multiple comparison. P-values under 0.05 were considered significant as compared to the control group. Graphs and statistical analyzes were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. BLG induced food allergy

To investigate whether BLG induced food allergy, mice were first

sensitized to BLG and then challenged with whey protein solution (containing 80% of BLG) for either 7 or 14 days (Fig. 1A). We found that mice sensitized and challenged with whey protein at both experimental times lost weight (Fig. 1B), which is an important feature of food allergy. Similarly, a marked reduction of epididymal adipose tissue mass in mice challenged with whey protein was observed (Fig. 1C). We also quantified chow consumption to verify whether the weight loss related to a reduced consumption of chow and found that all groups had a similar chow consumption (Fig. 1D).

Next, we measured the serum levels of specific IgE. Serum anti-BLG IgE levels were elevated in mice that were previously sensitized to BLG and subsequently challenged with whey protein solution, but not in non-sensitized mice (Fig. 1E). BLG-sensitized mice that received water throughout the experiment, but were not challenged orally with whey protein, had higher levels of anti-BLG IgE than non-sensitized animals; however, these levels were still significantly lower as compared to mice that were sensitized to BLG and orally challenged with whey protein. Moreover, mice sensitized to BLG and orally challenged with whey protein for 14 days had higher levels of anti-BLG IgE than mice challenged for 7 days. Furthermore, SIgA in small intestinal lavage fluids was increased in mice previously sensitized with BLG when they were challenged for 14 days, but not for 7 days, with whey protein (Fig. 1F).

We also measured cytokines known to participate in allergic responses such as IL-4, IL-5 and IL-10 in the proximal jejunum of mice. Oral challenge with whey protein in sensitized and orally challenged mice induced increased levels of IL-4 (Fig. 1G) and IL-5 (Fig. 1H) during oral challenge, but IL-10 levels were decreased only after 14 days of oral challenge with whey protein (Fig. 1I). Moreover, ingestion of whey protein induced an increase in the numbers of goblet cells (Fig. 1J), which produce and secrete mucus (Birchenough et al., 2016), and inflammatory cell infiltration in the gut of sensitized mice 7 and 14 days after oral challenge. As part of this inflammatory reaction, the number of eosinophils was higher in sensitized mice challenged with whey protein (Fig. 1K-L). Intraepithelial lymphocytes (IEL) were also increased in the intestinal mucosa of BLG-sensitized mice that were challenged orally for 7 and 14 days (Fig. 1M–N). Thus, mice sensitized with BLG and orally challenged with whey protein containing BLG develop food allergy and are referred to as the allergic group.

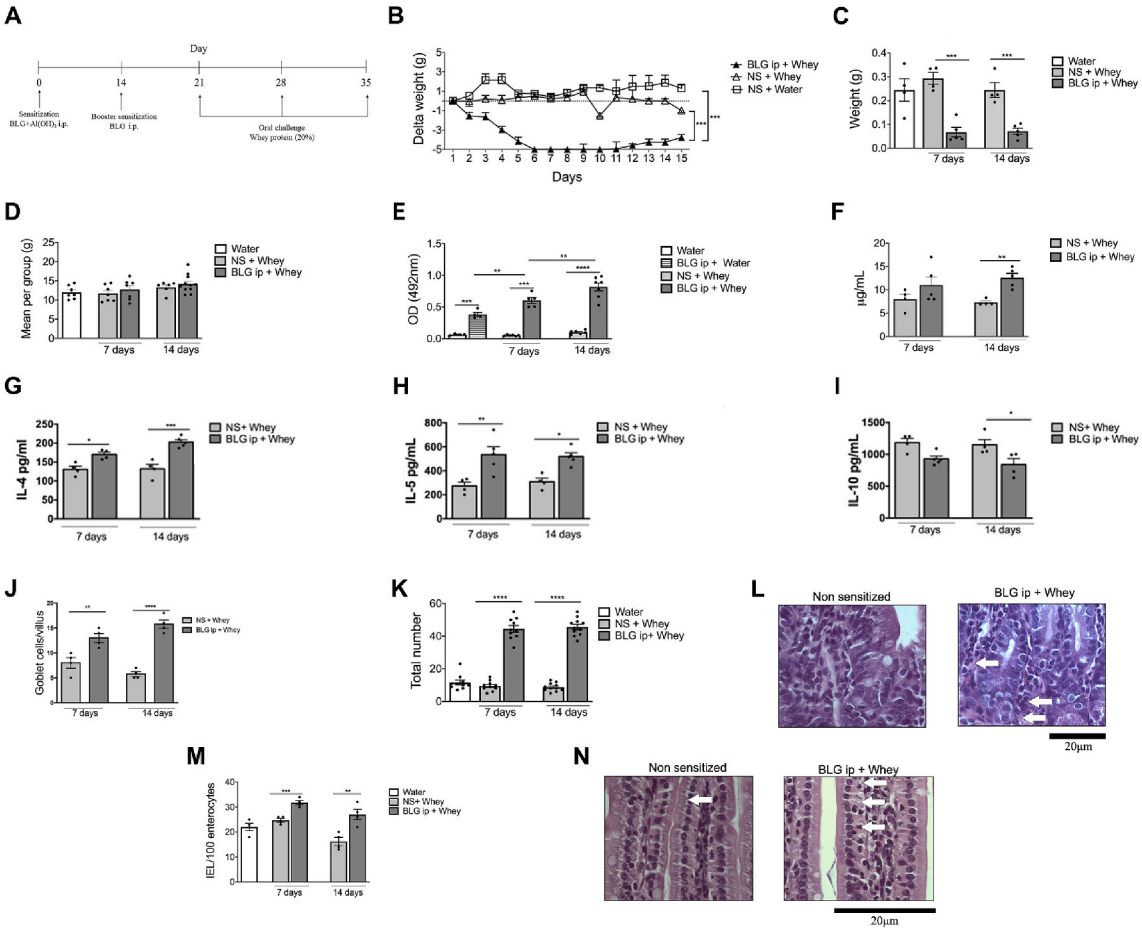

Fig. 1. Food allergy induced to β-lactoglobulin. A) Mice were sensitized with 20 µg BLG ip adsorbed in 1 mg of $Al(OH)_3$. After fourteen days, mice received an immunization booster with soluble BLG ip. One week later, mice were orally challenged with 20% Whey protein solution (containing BLG) diluted in water or BLG 1 mg/ml for either 7 or 14 days being the only source of liquid, then mice were euthanized. B) Body weight of mice represented by delta weight during 14 days of oral challenge. C) Weight of epididymal adipose tissue after oral challenge. D) Chow intake among groups during oral challenge. E) Anti-BLG IgE levels were measured in serum by ELISA as previously described in methods. Data was represented as means ± SEM of non-sensitized and sensitized mice in both 7 and 14 days of oral challenge with whey protein solution. F) Secretory IgA levels were measured in serum by ELISA as previously described in methods. Type-2 cytokines IL-4 (G), IL-5 (H) and IL-10 (I) were measured in proximal jejunum using ELISA. J) Sections of intestinal mucosa were included in histological slides, staining with H&E and analyzed under optical microscope at 40× magnification to examine Goblet cells, eosinophils and intra-epithelial lymphocytes (IEL). Goblet cells of proximal jejunum were counted, and total number was expressed as mean ± SEM. K) Eosinophils counted in random fields of histology slides. L) Representative photomicrographs of H&E-stained eosinophils. M) Number of IEL counted in the proximal jejunum of mice after oral challenge in histology slides. N) Representative photomicrographs of H&E-stained IEL. Data represents 2 independent experiments with 4–6 mice/group and as means ± SEM of non-sensitized and sensitized mice in both 7 and 14 days of oral challenge with whey protein solution. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Data was analyzed using ANOVA-Tukey.

3.2. Mice allergic to BLG developed a partial aversive behavior

The aversive behavior occurs when mice avoid consuming solutions or diets containing the antigen to which they are allergic (Cara et al., 1994). To investigate whether mice allergic to BLG showed differences in consumption of a solution containing whey protein suggesting aversion, we assessed their daily consumption of whey protein (Fig. 2A) and found that both non-allergic (non-sensitized) and allergic (BLG-sensitized) mice consumed significantly more whey protein solution than the control group that was only exposed to water (Fig. 2B). However, allergic mice consumed less whey protein solution than non-allergic (non-sensitized) mice during 12 days, suggesting that mice allergic to BLG developed an aversive behavior. Interestingly, this aversive behavior was lost after 12 days of challenge (Fig. 2B). To address if the aversive behavior was a temporal phenomenon, we compared the intake of whey protein during two different periods (7 and 14 days) (Fig. 2C). BLG-sensitized mice that were challenged with whey solution (allergic mice) consumed less antigen than non-sensitized group during both 7 and 14 days; however, they consumed more whey than the non-sensitized group that was offered water, suggesting a partial aversive behavior and a preference to consume whey over water.

To confirm the preference for the solution containing whey protein, we performed an experiment in a "two-bottle test" format (Mirotti et al., 2010), in which mice were offered the choice between ingesting water or a solution containing the allergen for which they were previously sensitized. The bottles were identical and there was a change of position between them to avoid preference guided by location. Interestingly, we found that sensitized mice preferred whey protein (Fig. 2D) even though they had high levels of anti-BLG IgE in the serum (Fig. 2E). Thus, the aversive behavior observed in BLG-allergic mice can be considered as partial since allergic mice still consumed more whey protein than control mice exposed to water and this effect was not definitive as previously shown for OVA-allergic animals (Cara et al., 1994).

3.3. Consumption of BLG induced a reduction in anxious behavior and in the activation of brain areas related to anxiety

Anxiety is usually associated with the aversive behavior in mice allergic to OVA (Basso et al., 2003; Costa-Pinto et al., 2005). To investigate the association between aversion and anxiety in mice sensitized with BLG and orally challenged with whey, we performed a Zero Maze test in which each mouse was placed individually in the central area of the maze, where they could explore its arms freely for 15 min (Fig. 3A). The percentage of time spent in the open arms of the maze as well as the number of entries in the open arm are inversely proportional to the level of anxiety in the test. We found no difference among groups in the frequency of entries in the closed arms of the maze (Fig. 3B). Non-sensitized mice that only ingested whey protein solution for 7 days showed more entries in the open arms of the maze, although no difference in the number of entries or in the time spent in the open arms among groups was observed (Fig. 3C-D). Moreover, there was no difference in the distance traveled in the maze among groups, showing no impairment in locomotion (Fig. 3. E).

Expression of c-Fos expression has been described as an effective acute marker of neuronal activity, and its overexpression occurs during behavioral stress in brain areas related to anxiety, such as paraventricular nucleus of hypothalamus (PVN) and central nucleus of amygdala (CeA) (Dragunow and Faull, 1989). To correlate behavior with activation of distinct brain areas, we measured c-Fos expression in the paraventricular neurons (PVN), and found that non-sensitized mice drinking whey protein solution had lower levels of c-Fos expression in the PVN, revealing that fewer neurons were activated in this region (Fig. 3F–J). Mice sensitized and challenged with whey protein containing BLG had higher levels of c-Fos expression when compared to non-sensitized animals, but significantly lower levels of c-Fos than sensitized mice that received water during the oral challenge period.

It has been described that mice have a strong preference for sweetened solutions (Cara et al., 1994, 1997). Thus, we hypothesized that BLG could produce the same effect of sweetened drinking water. To investigate this, we performed a two-bottle liquid preference test in non-sensitized mice to determine whether mice preferred BLG over either water or a saccharin-containing solution, whose effect has already been described in previous studies (D. C. Cara et al., 1994), regardless of sensitization. Consumption of the BLG-containing solution was comparable with saccharin-containing solution, and significantly greater than water or OVA-containing solution (Fig. 4A). We next investigate whether purified BLG could reproduce the allergic reaction and behavior previously triggered by whey protein, mice were sensitized to

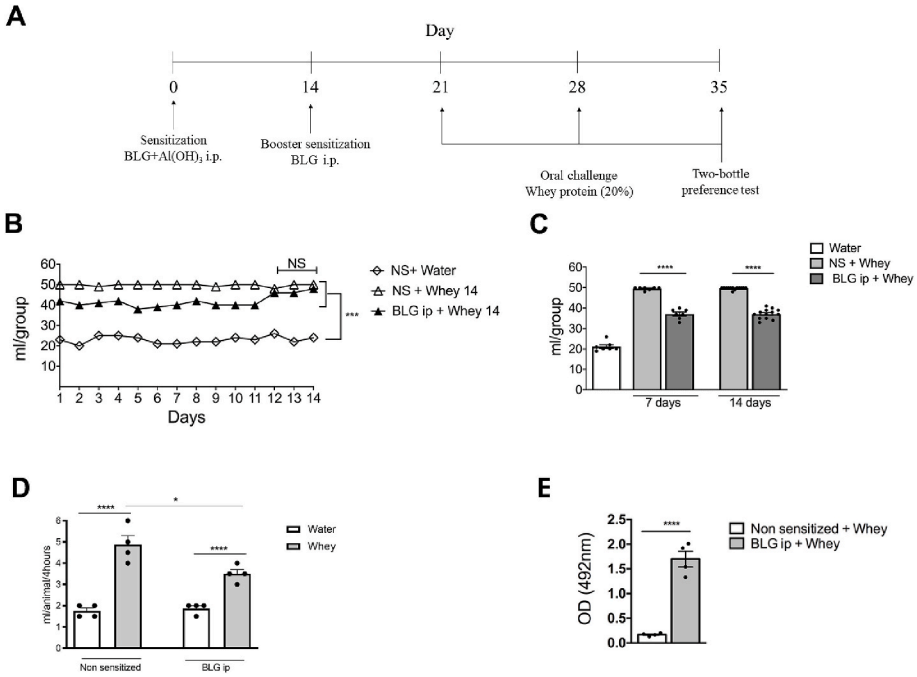

Fig. 2. β-lactoglobulin induced partial aversion to consumption of whey-containing solution. A) Food allergy induction followed by 14 days of oral challenge and two-bottle liquid preference test. B) Liquid intake of whey protein (containing BLG) among sensitized and non-sensitized groups for 14 days measured daily. C) Mean of the total liquid intake among groups during oral challenge. D) Two-bottle liquid preference test of non-sensitized or sensitized BALB/c mice using water or whey solution (containing BLG). Mice were sensitized twice with BLG/Al(OH)$_3$, on days 0 and 14. Control animals received only PBS. On day 21, animals were submitted to the liquid preference test during 24h. E) Levels of anti-IgE BLG were measured in serum after the liquid preference test. Data represents mean ± SEM of two independent experiments with 4–6 mice/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Data was analyzed using ANOVA-Tukey.

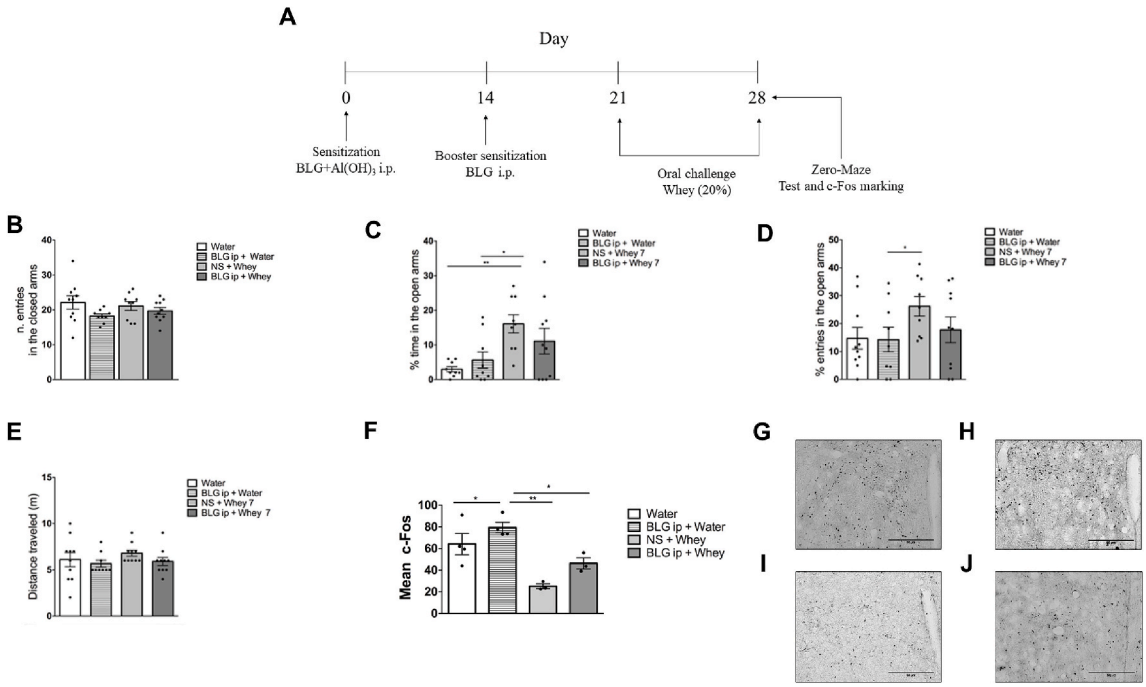

Fig. 3. Analysis of the behavior of sensitized and non-sensitized mice in a Zero Maze test. (A) BALB/c mice sensitized with BLG and non-sensitized mice were placed one by one in a Zero Maze type labyrinth for 15 min. (B) The number of entries in the labyrinth's closed arms, (C) the percentage of time spent by the animals inside the open arms, (D) the number of entries in the maze's open arms (E), and the total distance walked by the animals were evaluated. Activated neurons in the paraventricular nucleus of the hypothalamus of BLG-sensitized mice. (F) Sensitized mice challenged or not with whey protein for 7 days were tested in the Zero Maze labyrinth for 15 min at the end of the oral challenge. Ninety minutes after the labyrinth test, mice were euthanized and perfused with saline and paraformaldehyde 4% and had their brains removed for subsequent slide preparation and immunohistochemical procedure. Neurons labeled with cFos in the PVN region were counted. (G) Representative image of cFos-marked neurons in the PVN area of non-sensitized mice. (H) Representative image of cFos-marked neurons in the PVN area of mice sensitized with BLG and orally challenged with water. (I) Representative image of cFos-marked neurons in non-sensitized mice receiving whey protein solution, containing BLG, for 7 days. (J) Representative image of cFos-marked neurons in mice sensitized with BLG and orally challenged with whey protein, for 7 days. Data represents mean ± SEM of two independent experiments with 6 mice/group. *$p < 0.05$, **$p < 0.01$; Data was analyzed using ANOVA-Tukey.

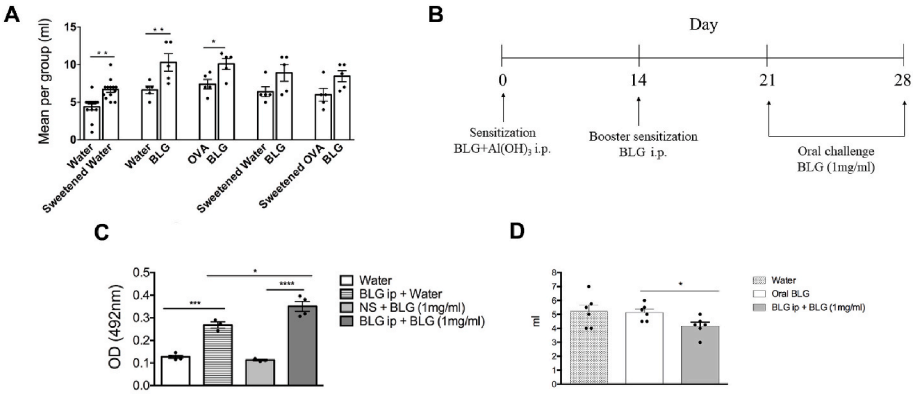

Fig. 4. Preference test for solutions containing BLG. (A). Non-sensitized mice were divided into the following groups according to solutions that were offered in same amounts: water x BLG (1 mg/ml), OVA (1 mg/ml) x BLG (1 mg/ml), Sweetened water (1% saccharin sodium) x BLG (1 mg/ml) and Sweetened OVA (1 mg/ml + 1% saccharin sodium). Mean consumption of liquid in the preference test per group. (B) BALB/c mice were sensitized i.p. with BLG + $Al(OH)_3$, received a booster immunization after 14 days with BLG solution and were challenged with BLG solution (1 mg/ml) for 7 days. (C) Levels of anti-BLG IgE in serum samples after oral challenge was measured by ELISA. (D) Liquid intake among groups during oral challenge was measured. Data represents mean ± SEM of two independent experiments with 5 mice/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Data was analyzed using ANOVA-Bonferroni's multiple comparisons test.

BLG, received a booster immunization with BLG and were challenged with purified BLG (1 mg/ml) rather than whey protein for 7 days as depicted in Fig. 4B. We measured levels of anti-BLG IgE in serum of mice sensitized to BLG and challenged with purified BLG (1 mg/ml) and these mice produced higher levels of IgE than sensitized mice that were not challenged with BLG (Fig. 4C). Moreover, we measured the consumption of BLG-containing solution in these mice and found no difference with non-sensitized mice that received water, although BLG-sensitized mice drank less BLG-containing solution than non-sensitized mice challenged with BLG (Fig. 4D), indicating that mice challenged with purified BLG reproduce our behavioral findings of partial aversion with whey protein challenge.

Taken together, the less activation of brain areas related to anxiety and the strong preference to both whey protein and purified BLG over water or OVA-containing solution suggest that BLG may activate reward-associated brain areas that can potentially overcome the aversive behavior.

3.4. BLG activated the brain reward system

Since non-sensitized mice had a natural preference for BLG-containing solution, we used a classic OVA allergy model, in which

aversion is clearly established (Cara et al., 1994; Saldanha et al., 2004), to verify whether the presence of BLG in the drinking solution used for oral challenge could alter the aversive behavior to OVA. The experimental design is depicted in Fig. 5A. As expected, we found that mice sensitized to and challenged with OVA alone or both OVA and BLG had high levels of anti-OVA IgE (Fig. 5B). Furthermore, mice sensitized to OVA and challenged with OVA consumed less OVA, which characterizes an aversive behavior, but mice challenged with a solution containing BLG alone or both OVA and BLG, consumed significantly more solution than mice sensitized and challenged with OVA solution (Fig. 5C), suggesting that BLG triggered an effect similar to the one observed with sweetened solutions. This result suggested a conflict between aversion (triggered by OVA) and preference (triggered by BLG) behaviors and may indicate that BLG consumption activated reward-related areas in the brain.

We then hypothesized that there were reward-related brain circuits involved in the behavior triggered by BLG consumption. It has been reported that the central nucleus of amygdala (CeA) is involved in the brain reward system, interacting with cortical and subcortical structures including the nucleus accumbens (NAc) (Baxter and Murray, 2002). Previous work showed that the stimulation of synapses between NAc and hippocampus are required for formation of reward-related memories, while the interruption of those synapses led to disruption in that formation, suggesting a strong correlation between NAc and reward behavior (LeGates et al., 2018). Therefore, our aim was to investigate whether mice sensitized to and challenged with BLG show differences in neuronal activation in CeA and NAc during BLG consumption. We found that mice sensitized to and challenged with OVA showed more activation of neurons from the CeA. No activation was observed in mice sensitized to BLG and challenged with BLG. Surprisingly, mice that were sensitized to OVA and challenged with a solution containing BLG alone or both OVA and BLG showed less activated neurons in this area (Fig. 5D). On the other hand, BLG consumption induced a strong activation of neurons from the NAc, even when mice were challenged with OVA, indicating that BLG indeed activated the reward system, which is likely the reason why mice allergic to OVA lose the aversive behavior when exposed to oral BLG (Fig. 5E).

4. Discussion

β-lactoglobulin (BLG) is considered to be the most immunogenic protein among cow's milk allergens (Lindholm Bøgh et al., 2013). Among the many clinical alterations typical of food allergy, behavioral changes such as aversion and anxiety have been described in allergic individuals (Costa-Pinto and Basso, 2012). In mice, the connection between food allergy and behavior has been shown to be allergen specific because mice sensitized to peanut or wheat when offered a mixture of the grains *in natura* chose to ingest grains to which they were not previously exposed (Teixeira G. 1995). The same behavioral changes were also demonstrated by using OVA as the allergenic protein (Basso et al., 2003; Cara et al., 1994). However, the mechanisms underlying these behavioral alterations in food allergy are still elusive. Thus, in the present study, we sought to investigate whether behavioral alterations occur in a model of food allergy induced by BLG, and the neuro-immunological mechanisms involved in this effect.

We showed that BALB/c mice sensitized to BLG and later challenged with a 20% whey protein (80% BLG) solution developed a food allergy characterized by weight loss and small intestine mucosa inflammation, which was associated with increased frequency of eosinophils, goblet cells and intraepithelial lymphocytes. In addition, allergic mice had higher secretory IgA levels after being challenged with whey protein-containing solution. As for the cytokine profile, IL-4 and IL-5 were increased in mice sensitized to and challenged with BLG accompanied by a decrease in IL-10, which is consistent with previous food allergy models (Strid et al., 2005). The decrease of IL-10 levels is associated with reduction of the frequency of induced regulatory T cell (iTreg) development in the gut, resulting in greater propensity to develop inflammatory immune responses to allergens (Curotto de Lafaille and Lafaille, 2009). Moreover, BLG allergic mice had elevated levels of serum anti-BLG IgE. However, contrary to previous reports on OVA-induced food allergy using the same experimental protocol (Batista et al., 2014), prolonged exposure to BLG (14 days) further increased IgE levels as compared to 7 days of oral challenge. Importantly, while OVA allergic mice showed the characteristic aversive behavior when exposed to OVA, mice allergic to BLG had only a partial aversion to BLG, which was abrogated by day 12 post challenge despite the progressive increase of serum anti-BLG IgE levels. This is intriguing because it has been described that the aversive behavior depends on the production of IgE. Depletion of IgE by anti-IgE antibodies prior to oral challenge prevents the development of aversion (Basso et al., 2003). Moreover, this effect was dependent on mast cell degranulation, since cromolyn, a mast cell stabilizer, prevents the aversive behavior to OVA in mice and completely

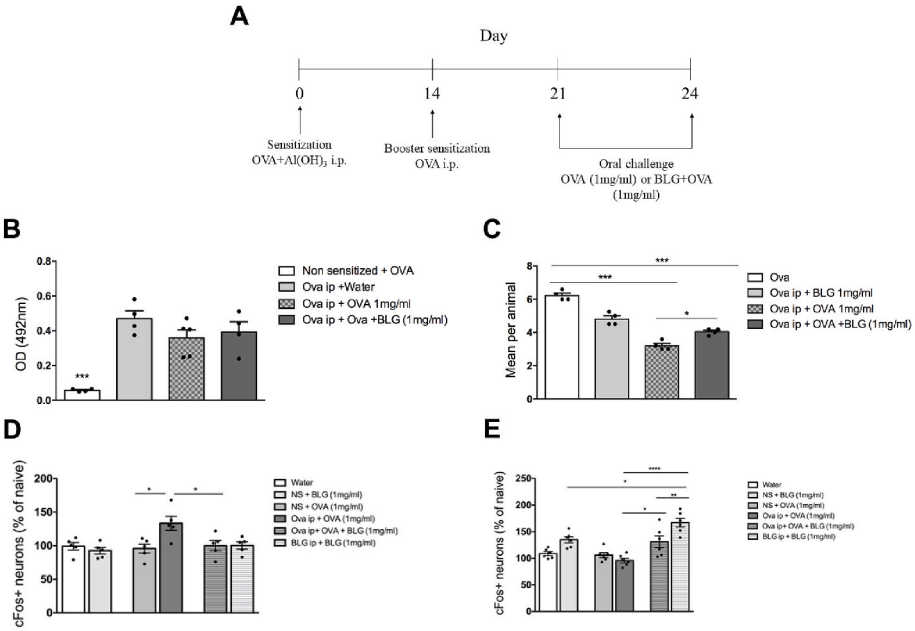

Fig. 5. BLG modified aversion to OVA in a food allergy model to OVA. (A) BALB/c mice were sensitized with OVA i.p. and challenged with OVA solution (1 mg/ml) for three days or with a solution containing a mixture of OVA (1 mg/ml) and BLG (1 mg/ml). (B) OVA-specific IgE levels in serum expressed as absorbance at 492 nm was measured by ELISA. (C) Liquid intake was measured during oral challenge for 3 days. (D) cFos expression in the central nucleus of the amygdala (CeA) was evaluated. (E) cFos expression in nucleus accumbens (NAc) was evaluated. Data represents mean ± SEM of two independent experiments with 5 mice/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Data was analyzed using ANOVA-Tukey.

blocked c-Fos expression in brain areas associated with anxiety and aversion, such as the paraventricular nucleus of the hypothalamus (PVN) and the central nucleus of the amygdala (CeA) (Costa-Pinto et al., 2007). Our hypothesis is that BLG might activate the brain reward system overcoming the aversive behavior induced by allergy. We confirmed this hypothesis by showing that (i) consumption of the BLG-containing solution was comparable with a sweetener-containing solution, and significantly greater than water or OVA. Taste preference for sweetened solutions are known to be associated with the activation of the brain reward system (Low et al., 2014); (ii) unlike OVA, BLG decreased c-Fos expression in neurons from the PVN and CeA, suggesting that BLG has anxiolytic properties; and (iii) BLG increased c-Fos expression in neurons from the nucleus accumbens (NAc), a critical area in the brain belonging to the reward system that is involved in processing incentive salience, pleasure, positive reinforcement and reinforcement learning (Goto and Grace, 2005). Thus, our data suggest that different allergens induce distinct taste preference behaviors, and the outcome is related to neuronal components triggered by these allergens.

Although we have clearly shown, using the same experimental protocol for food allergy induction, that BLG, unlike OVA, activates neurons from reward brain areas, the mechanism underlying this effect remains unknown. Enzymatic digestion of natural proteins, including those derived from milk, generates a range of bioactive peptides that may interact with the nervous system and exhibit anxiolytic activity. Alpha-lactotensin (His-Ile-Arg-Leu, HIRL) for example, is a peptide that was isolated from chymotrypsin digestion of BLG (Yamauchi et al., 2003) and has anxiolytic effect in mice in behavioral tests using a high cross maze (Hou et al., 2011). It is interesting that some bioactive peptides derived from food components such as whey proteins have inhibitory activity for angiotensin converting enzyme (ACE) that regulates blood pressure. Inhibition of this enzyme drives anxiolytic effects in rats subjected to behavioral tests (Welderufael et al., 2012). Thus, it is possible that BLG acts as an anxiolytic through the action of its peptides.

Another plausible possibility is the communication between the digestive system and the brain. It is known that the aversive behavior to OVA, as mentioned above, is associated with neuronal activation in the PVN and CeA, which are brain areas related to emotional and affective behavior. Moreover, PVN and CeA are among the main regions containing neurons expressing the corticotropin-releasing hormone (CRH), which is a key peptide in controlling behavioral, neuroendocrine and autonomic responses to stress, anxiety and depression (Arborelius et al., 1999; Heinrichs et al., 1995; Holsboer and Ising, 2008). Consistent with this, mice with food allergy display higher levels of anxiety and increased serum corticosterone levels. Thus, the CRH-corticosterone axis may play an important role in the anxiety observed during food aversion.

Moreover, stabilization of mast cells by cromolyn prevented the aversive behavior to OVA (Costa-Pinto et al., 2007), suggesting that the early phase of an immediate allergic response is critical for the aversion development. Mast cells secrete a variety of mediators including growth factors, cytokines, histamine and serotonin (Galli et al., 2008; Kalesnikoff and Galli, 2008). Interestingly, mast cells are closely apposed to nerve endings from the vagal nerve in both humans and rodents (Stead et al., 1989) giving anatomical support for mast cell-secreted mediators to interact with neurons. Consistent with this, treatment of neonatal mice with capsaicin, a neurotoxin derived from chilli pepper that selectively promotes the dysfunction of the sensory fibers named C-fibers, blocked c-Fos expression in the PVN and reduced food aversion to OVA-sensitized mice (Basso et al., 2001, 2004). Furthermore, selective antagonism of the serotonin 5-HT3 receptors, which are expressed in C-fibers (Lang et al., 2019) decreased the aversive behavior in rats (Zarzana et al., 2009). Thus, food aversion may occur due to the release of mast cell factors upon IgE/allergen binding that stimulate sensory fibers of the vagal nerve, which in turn drive this peripheral information to the central nervous system.

Additionally, since IgE receptor (FcεRI) has been detected on sensory neurons from mice (Andoh and Kuraishi, 2004; van der Kleij et al., 2010), a direct binding of IgE on vagal nerve sensory fibers may represent an alternative neuronal activation independent of mast cells. However, the circuits involved in the gut-activated brain reward system have remained elusive until recently when Han and coworkers demonstrated that optical activation of gut-innervating vagal sensory neurons recapitulated the classical effects of stimulating brain reward neurons (Han et al., 2018).

Thus, we believe that the reward behavior induced by BLG is a consequence of the stimulation of specialized sensory fibers in the vagal nerve responsible for activating brain areas associated with pleasure and reward such as the NAc.

In summary, we showed that the aversive behavior observed in food allergy to OVA does not apply to all antigens. More importantly, the aversive behavior towards potentially threatening antigens can be overcome by a reward effect when mice consume solutions containing BLG. Mice allergic to BLG, although presenting high serum IgE levels and several signs of inflammation in the intestinal mucosa, ingested solutions containing the allergen even when they were offered other options. Our data suggest that BLG has special characteristics that could be related to its taste or to neurologically active peptides present in its structure, that can subvert the evolutionarily preserved behavior of rejection to potentially toxic substances triggered by allergic reactions dependent on IgE.

Further investigation is important to unravel the biological roles that BLG has in mice and potentially in humans regarding behavior and brain activation of the brain reward system. These results also open novel avenues of investigation on the specific neuroimmune circuits that are triggered by distinct classes of allergens. A complex combination of their potentially harmful effects as allergens with their taste properties may create behavioral scenarios that would be relevant for clinicians in general and pediatricians in particular during treatment for food allergy disorders.

Ethical approval and informed consent

This project was approved by the Ethics Committee on Animal Use at Federal University of Minas Gerais (CEUA/UFMG) with protocol no. 114/19, related to the present study is in agreement with the Ethical Principles in Animal Experimentation.

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Author contributions

LL and HCA performed all the experiments, helped to design the experimental strategies and wrote the manuscript. D.S.R., J.L.A., M.C.C.C., B.K.S.M. T.G.M. contributed in the analysis of the experiments and elaboration of graphs, M.A.M. and L. M. performed cFos staining and analyses, supervised by B.R., L.A.B. and J.G. contributed in the behavior tests, supervised by D.A. D.C.C. contributed to the histological analysis. A.C.G.S. and A.M.C.F. designed the experiments, supervised the work and revised the manuscript.

Data availability statement

All datasets generated or analyzed during the current experimental study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare no conflict of interest.

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