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Research report

Enhancement of antibody production and expression of c-Fos in the insular cortex in response to a conditioned stimulus after a single-trial learning paradigm

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Abstract

Immune responses can be modulated by Pavlovian conditioning techniques. In this study, to evaluate the conditionability of antibody response via a single-trial conditioning paradigm, we used a protein antigen ovalbumin as an unconditioned stimulus (UCS) that was paired with a novel taste of saccharin in a single-trial learning protocol. A significant enhancement of anti-ovalbumin antibody production was observed in the conditioned rats at Days 15, 20 and 25 after re-exposure to the conditioned stimulus. The pattern of conditioned antibody response is similar to that of antigen-induced antibody response. Furthermore, to identify the involvement of a limbic brain structure in the expression of conditioned antibody response, immediate-early gene *c-fos* expression was used as a marker of neuronal activation to detect the functional activation in the insular cortex (IC) in response to the conditioned stimulus. The re-exposure of conditioned rats to the conditioned stimulus resulted in a significant increase of c-Fos immunoreactivity in all three areas of the IC including the agranular, dysgranular, and granular areas, suggesting that IC is involved in the neural mechanism of expression of conditioned immune response.

Keywords: Pavlovian conditioning; Rats; Antibodies; c-Fos; Insular cortex

1. Introduction

Immune responses have been shown to be regulated by Pavlovian conditioning techniques since the pioneering study by Ader and Cohen in 1975 [1,2,4]. Numerous animal and human experiments have examined the conditionability of immunosuppression in various parameters [3,6,16,20,26,31,35]. Studies using an immunostimulating agent as the UCS have reported that natural killer cell (NKC) and cytotoxic lymphocyte (CTL) activity can be augmented by conditioning [18,23–25,30,32]. In contrast, the conditioning of antibody response is of higher specificity, since it uses an antigen rather than immune cell activators or immunosuppressive drugs as the UCS. However, the conditioned enhancement of antibody response is more difficult to model, especially via the single-trial conditioning. Among the first of such studies, multiple training sessions were required to be able to elicit a sufficient elevation in the antibody production, and a low-dose of antigen (the UCS) was necessary to aid the conditioned stimulus (CS) representation [5]. Our previously reported single-trial paradigm—using an antigen ovalbumin (OVA) as the UCS—provided the first evidence that the conditioned enhancement of antibody response can be invoked by re-exposure to the CS alone, via a single conditioning trial [33]. Unfortunately, this paradigm was not replicated successfully [14]. Another research group has reported a single-trial conditioning paradigm in which an enhancement of anti-HEL antibody was induced upon re-exposure to the CS alone [8]. Although most recently this paradigm was documented replicable, in the replicating case the elevation in antibody level elicited by the CS was of lesser magnitude [37]. Therefore, the primary aim of this study is to examine the reliability of conditioned antibody response via the single-trial paradigm.

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Pavlovian conditioning of immune response, by which a previously neutral stimulus gains the immunomodulatory effect, provides new insight into the pathways of communication between the brain and the immune system, plus the potential clinical significance of conditioned alterations in immune function. However, to date, the neurocircuitry underlying the different stages of this particular learning is largely unknown. Insular cortex (IC), a region collectively known as the gustatory, visceral and nociceptive neocortex, makes the essential contribution to the acquisition and storage of different aversively motivated learning tasks such as conditioned taste aversion (CTA), fear conditioning, spatial maze and inhibitory avoidance [9,13,15,17,27,39,53]. Recently, there have been several lesion studies concerning the disruptive effect of damages in the IC on the acquisition of both conditioned immunosuppression (CIS) and conditioned enhancement of antibody response [42–44]. Nevertheless, the functional role of the IC in the expression of conditioned immune response remains unclear and untested. c-Fos, a prototypical immediate-early gene *c-fos* product, has been employed as an almost universal neuronal activity marker [29]. Many different stimuli have been shown to induce the expression of *c-fos* in related brain regions, allowing the Fos immunocytochemistry technique to be successfully applied in mapping the functional activity in the brain [34]. Compared with the lesion method, one advantage of this approach is that changes in c-Fos expression can be used to identify the neural circuits that are activated by a particular stimulus [21].

In the present study, for the sake of evaluating the conditionability of antibody response, a single-trial paradigm using ovalbumin as the UCS was examined. In addition, to investigate the involvement of the IC in the neural circuitry that may contribute to the expression of the conditioned antibody response, we used the c-Fos immunomapping technique to examine the functional activation of the IC in response to the CS re-exposure.

2. Materials and methods

2.1. Subjects

Male Wistar rats, 3 months old at the beginning of tests, were caged individually and maintained on a 12:12 h light–dark cycle. Food and water were provided ad libitum except during the restricted drinking period before conditioning and the test trial days.

2.2. Behavioral procedures

Prior to the start of experiments, three rats were injected intraperitoneally (i.p.) with 80 μg of ovalbumin (Sigma Chemical Co.) in 200 μl PBS (pH 7.4) emulsified in an equal volume of Freund's complete adjuvant. They were bled by an incision in the tail every 5 days; the normal primary anti-OVA IgG response was then monitored by periodical serum assessment with enzyme-linked immunosorbent assay (ELISA). The purpose of this step was to schedule the date of test trial since the testing trial task was predetermined to take place during the declining phase of the primary antibody response.

Rats were randomly divided into various groups and then underwent the single-trial conditioning (described in Table 1 and Table 2). In order to avoid the possible influence of saccharin odor on the unconditioned experimental objects, the conditioned animals exposed to saccharin were kept in a separate room. All experiments were performed during the dark period starting at 9:00 p.m. Five days prior to the conditioning and test trial day, a restricted drinking schedule was set up and the liquid consumption was measured. During this period, two bottles of water were placed on the front of each cage for 15 min (from 9:00 p.m. to 9:15 p.m. daily).

2.2.1. Experiment I: single-trial conditioning of anti-OVA antibody response

Sixty rats were randomly divided into five groups. Besides the experimental group (group CS), four control groups (groups CS_0, CSp, NC, UCS, see Table 1) were generated to control the effects of conditioning, CS, UCS, and the non-specific stress from handling and injection, respectively [7].

During the 5-day restricted drinking period prior to the conditioning day, the CSp group ($n = 10$) was provided with the 0.25% saccharin solution for 15 min at 9:00 p.m. everyday. On the conditioning day (Day 0), rats in groups CSp, CS ($n = 16$) and CS_0 ($n = 10$) were all exposed to 0.25% saccharin for 15 min immediately followed by an

Table 1
Experimental procedure I

Group	Experimental Days			
	−5 to −1 (pre-treatment)	0 (conditioning)	30 (test trial)	35, 40, 45, 50, 55, 60, 67, 74 (samples)
CSp ($n = 10$)	Sac	Sac/OVA	Sac	Blood
CS ($n = 16$)	None	Sac/OVA	Sac	Blood
CS_0 ($n = 10$)	None	Sac/OVA	Water	Blood
UCS ($n = 12$)	None	Water/OVA	Water	Blood
NC ($n = 12$)	None	Water/OVA	Sac	Blood

CS: conditioned stimulus; CS_0: no conditioned stimulus; CSp: CS pre-exposure; NC: non-conditioned; UCS: unconditioned stimulus; Sac: saccharin; OVA: ovalbumin; Sal: saline.

Table 2
Experimental procedure II

Group	Treatment		
	Conditioning day (Day 0)	Test trial day (Day 30)	2 h after the test trial (Day 30)
Vehicle ($n = 4$)	Water/Sal	Water	Perfusion
Sac–Sac ($n = 4$)	Sac/Sal	Sac	Perfusion
UCS ($n = 4$)	Water/OVA	Water	Perfusion
CS_0 ($n = 4$)	Sac/OVA	Water	Perfusion
CS ($n = 4$)	Sac/OVA	Sac	Perfusion

CS: conditioned stimulus; CS_0: no conditioned stimulus; UCS: unconditioned stimulus; Sac: saccharin; OVA: ovalbumin; Sal: saline.

i.p. injection of 80 μg of OVA. At the same time, a bottle of water was also supplied. The rats in groups UCS ($n = 12$) and NC ($n = 12$) were provided with two bottles of water followed by the same i.p. injection of OVA. On the test trial day (Day 30), during the declining phase of the primary antibody response (see Fig. 1), the rats in CS, CSp and NC groups were all given saccharin solution, whereas the rats in CS_0 and UCS groups were given only water. Blood samples were regularly collected by tail incisions 5, 10, 15, 20, 25, 30, 37, and 44 days after the test trial day (on Experimental Days 35, 40, 45, 50, 55, 60, 67, and 74, respectively). Serum was obtained by centrifuging the blood samples at 2500 rpm for 20 min and then stored at −20 °C until determination of anti-OVA antibody was performed by ELISA. Table 1 shows the treatment for each group. Fluid consumption of both saccharin and water was measured by subtracting the post-drinking weight of each bottle from its pre-drinking weight.

2.2.2. Experiment II: c-Fos immunomapping in the IC after the CS re-exposure

Five groups of rats were used in this experiment (see Table 2). The conditioning procedure and the conditioning-test interval were the same as in Experiment I. On the conditioning day (Day 0), the conditioning procedure described before was performed in groups CS ($n = 4$) and CS_0 ($n = 4$). On the test trial day (Day 30), rats of the group CS, but not those of the group CS_0, were re-exposed to the saccharin. To evaluate the effect of repeated saccharin drinking on the Fos production, the group Sac–Sac ($n = 4$) was provided with 0.25% saccharin followed by an i.p. injection of saline on the conditioning day, then retreated with saccharin on the test trial day. The UCS group ($n = 4$) was challenged with OVA antigen on the conditioning day and provided with drinking water during the test trial. Additionally, the Vehicle group ($n = 4$) that received only water drinking and saline injection was used as a control group to measure nonspecific Fos-like immunoreactivity elicited by the handling and injection. On the test trial day, 2 h after the test trial task, the rats from all groups were sacrificed for immunohistochemical analysis of c-Fos expression.

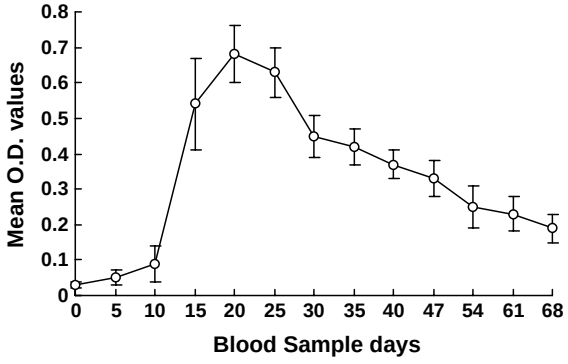

Fig. 1. The level of anti-OVA IgG antibody production (mean ± S.E.M.) after the primary immunization with a dose of 80 μg.

2.3. Assay for IgG anti-OVA antibody by ELISA and statistical analysis

Nunc polystyrene 96-well microtiter plates (Nunc, Denmark) were coated overnight at 4 °C with 100 μl/well of OVA (1 mg/ml in PBS buffer). The wells were then washed twice with PBS containing 0.05% Tween 20 and once with double distilled water then postcoated for 1 h at 37 °C with 100 μl/well of 1% BSA (blocking buffer). The wells were washed again and incubated for 2 h at 37 °C with the test serum diluted 1:200 in the blocking buffer. After washing three times, the plates were incubated for 2 h at 37 °C with 50 μl/well of Peroxidase-conjugated goat anti-rat IgG (Sigma) diluted 1:8000 in blocking buffer. The wells were again washed three times and filled with 100 μl/well of substrate (containing 0.1 M Na_2HPO_4, 0.1 M citric acid monohydrate and 0.04% *O*-phenylenediamine, OPD, 0.03% H_2O_2). After 10 min 50 μl/well of 2 N H_2SO_4 was added to each well to stop the staining process. The optical density (OD) value was then read on a Microplate Reader (Bio-Rad Instruments), using a reference wavelength of 490 nm. Data was analyzed by two-way ANOVA. In addition, one-way ANOVA was used to assess the differences among groups within sample days, using the post-hoc pairwise Scheffe test. The significance level was set at $P < 0.05$.

2.4. Fos immunohistochemistry

The rats were anesthetized with i.p. injection of 10% chloral hydrate and then transcardially perfused with saline (100 ml per rat, 37 °C) followed by 4% paraformalde-

hyde in 0.1 M PBS (500 ml per rat, pH 7.4). Brains were post-fixed for 4 h in the same fixative and then immersed for 24 h in 20% sucrose in PBS. After the tissue was frozen, 40 μm-thick coronal sections were cut with a freezing microtome and were collected every fifth section. Sections were placed in 3% H_2O_2 in 80% methanol for 30 min to eliminate the endogenous peroxidase activity and then saturated with blocking serum containing 3% goat serum, 1% bovine serum albumine and 0.3% Triton X-100 in 0.01 M PBS for 30 min at room temperature. Sections were subsequently incubated with rabbit anti-FOS antibody (1:4000, Santa Cruz Biotechnology Inc.) for 2 h at room temperature, followed by incubation with biotinylated goat anti-rabbit antibody for 12 h (1:500, Vector) then the avidin–biotin–peroxidase complex for 4 h (1:500, Vector). Sections were thoroughly washed between incubation steps. For visualization, a glucose oxidase–DAB–nickel method was used. Finally, sections were mounted, dehydrated, and cover-slipped.

The specificity of the antiserum was confirmed in control sections in which the reaction process was the same as above except the primary antiserum was substituted by normal rabbit serum. There were no immunostained nuclei seen in such cases.

2.5. Quantification and data analysis

Sections in three areas of IC (agranular, AI; dysgranular, DI; granular, GI) were analyzed qualitatively (see Fig. 4). Classification of the brain regions was based on Paxinos and Watson's stereotaxic atlas [41]. Fos-positive cells in selected areas were automatically counted with image analysis software (NIH image 1.62). Nuclei were counted individually and expressed as number of Fos-positive nuclei per mm^2. The counting was performed in all sections for each brain region and the data was analyzed statistically with one-way ANOVA. When significant differences were found, post-hoc analyses were conducted using LSD test.

3. Results

3.1. Experiment I: conditioned enhancement of anti-OVA antibody response via single-trial paradigm

Two-way ANOVA yielded a significant treatment effect ($F(4, 378) = 12.920$, $P < 0.0001$), an effect of days ($F(7, 378) = 8.631$, $P < 0.0001$), and Treatment × Days interaction ($F(28, 378) = 1.774$, $P < 0.05$). One-way ANOVA showed significant differences among groups on Experimental Days 45 ($F(4, 51) = 4.537$, $P < 0.01$), 50 ($F(4, 51) = 6.41$, $P < 0.001$), and 55 ($F(4, 51) = 6.353$, $P < 0.001$). Post-hoc Scheffe analysis revealed that at Days 15, 20 and 25 after the test trial, the antibody response of conditioned rats that were re-exposed to the CS (group CS) was significantly greater than that of the rats in group CS_0 (all time point, $P < 0.05$) and that of the rats in group CSp (all time point, $P < 0.01$). Meanwhile, the level of antibody response in the CS group was significantly higher than the control groups UCS (Day 45, $P < 0.05$; Day 50, $P < 0.01$; Day 55, $P < 0.01$) and NC (Day 45, $P < 0.05$; Day 50, $P < 0.05$; Day 55, $P < 0.01$). The CSp, CS_0, UCS, and

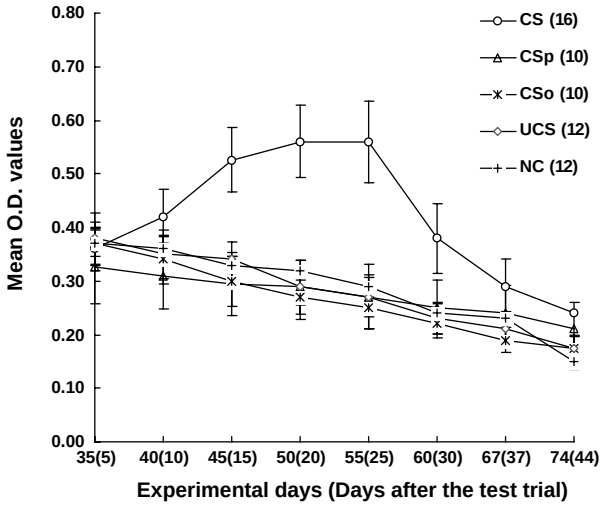

Fig. 2. The level of anti-OVA IgG production (mean ± S.E.M.) in groups CS, CSp, CS_0, UCS and NC. The ordinate axis shows the mean optical densities and the abscissa axis shows the Experimental Days (5, 10, 15, 20, 25, 30, 37, and 44 days after test trial). Post-hoc analysis by Scheffe revealed that the CS group is significantly different from the groups CS_0, CSp, UCS and NC at Days 15, 20, and 25 after the test trial task.

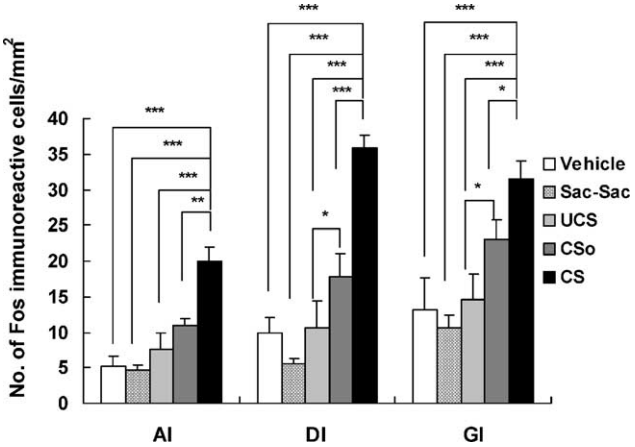

Fig. 3. Number of Fos immunoreactive (ir) nuclei in all three areas of the insular cortex (AI, DI, GI) 2 h after the test trial task. In all three areas of the IC, conditioned rats that were re-exposed to the CS (CS group) presented significantly increased c-Fos expression, in comparison with those that were not provided with the CS (CS_0 group), as well as rats that were unconditioned (UCS group), repeatedly subjected to the saccharin (Sac–Sac group); and treated with saline injection and water drinking (Vehicle group). In the DI and GI areas rats of CS_0 group showed significantly higher levels of c-Fos immunoreactivity than the groups UCS, Sac–Sac and Vehicle. The groups UCS, Sac–Sac and Vehicle did not differ from each other ($P > 0.05$). Statistically significant differences determined by one-way ANOVA: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

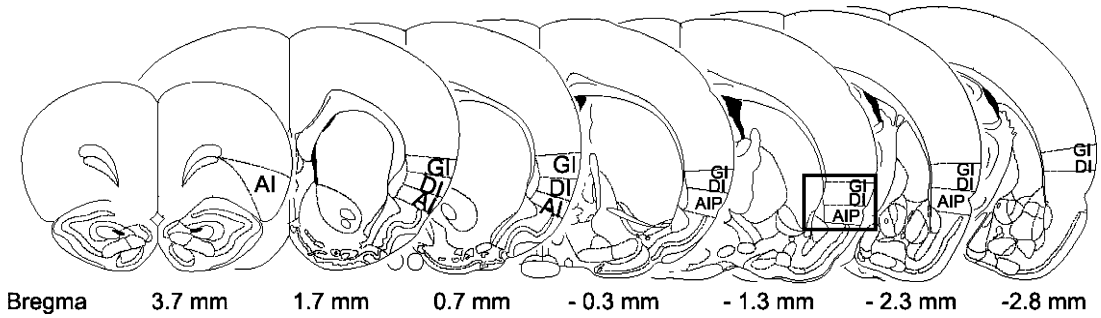

Fig. 4. Schematic representation of coronal sections through the insular cortex showing average area (labeled area) used for quantitative analysis of Fos-ir neurons. Drawing is based on illustrations in the atlas of Paxinos and Watson [41]. The outlined area indicates the area targeted for taking the representative photographs of Fos-ir nuclei in each group. AI, agranular insular cortex; DI, dysgranular insular cortex; GI, granular insular cortex.

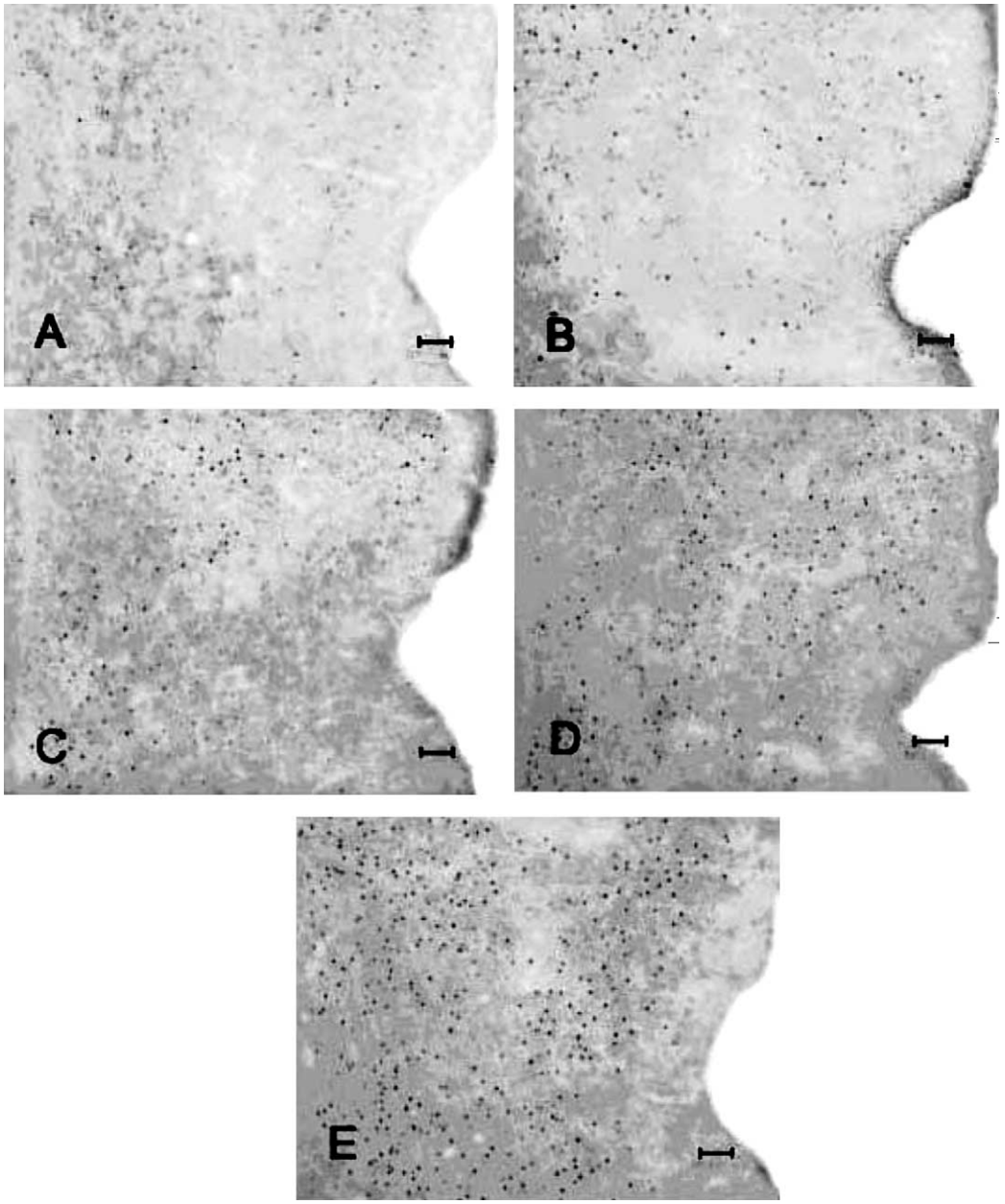

Fig. 5. c-Fos expression in the insular cortex 2 h after the testing trial: (A) group Vehicle, (B) group Sac–Sac, (C) group UCS, (D) group CS_0, (E) group CS. Scale bar: 200 μm.

NC groups did not differ from each other at either time point. Fig. 1 shows the anti-OVA IgG production after the primary immunization with a dose of 80 μg. Fig. 2 shows the level of IgG anti-OVA antibody on 5, 10, 15, 20, 25, 30, 37, and 44 days after the test trial day in which blood samples were taken (on the Experimental Days 35, 40, 45, 50, 55, 60, 67, and 74, respectively). Comparing Fig. 2 with Fig. 1, we found that the pattern of conditioned anti-OVA IgG production was similar to the regular antibody response after primary immunization.

No significant difference in saccharin consumption was found between the different groups.

3.2. Experiment II: expression of c-Fos in the IC after the CS re-exposure

One-way ANOVA revealed significant differences between groups after the test trial task in all three insular subregions: AI ($F(4, 15) = 15.05$, $P < 0.001$); DI ($F(4, 15) = 22.376$, $P < 0.001$); GI ($F(4, 15) = 6.416$, $P < 0.01$). Rats of the experimental group (group CS) produced significantly increased c-Fos production in all insular areas, in comparison with the groups CS_0, UCS, Sac–Sac, and Vehicle. In the DI and GI areas rats of the group CS_0 showed significantly more c-Fos production than those of the group UCS, Sac–Sac and Vehicle ($P < 0.05$). In the rats of groups Vehicle, Sac–Sac and UCS, Fos immunoreactivity in the IC was very modest and no significant difference was found among them ($P > 0.05$). Fig. 3 is a graphical representation of the results obtained in each group. The average area detected for quantitative analysis of Fos-immunoreactive (ir) neurons is depicted in Fig. 4. Moreover, Fig. 5 presents examples of the Fos immunoreactivity in the IC observed in each group.

4. Discussion

The results of the present study showed that by using antigen OVA as the UCS, a conditioned enhancement of antibody response was obtained via a single-trial conditioning paradigm. Our results confirmed the conditionability of antibody response and the reliability of the single-trial conditioning paradigm where re-exposure to the CS alone is sufficient. In addition, a significantly increased c-Fos expression in the insular cortex was observed in the conditioned rats following re-exposure to the CS alone. This is the first demonstration of CS-induced functional activation of insular cortex during the expression of conditioned antibody response.

We previously reported a single-trial paradigm, in which both anti-OVA antibody and T-cell proliferative responses in vitro can be elevated by re-exposure to saccharin alone [33]. In that case, however, the magnitude of conditioned antibody production elicited by the CS alone was relatively small, since the value of statistical significance between the conditioned and unconditioned groups was marginal ($P = 0.048$) despite that fact that a large number of rats was used in each group ($n = 23$). We even attempted to repeat this paradigm by using fewer rats ($n = 12$), but failed to evoke the conditioned antibody response significantly by the CS alone unless a booster nonimmunogenic antigen was coupled with the CS [36]. Recently, conditioned enhancement of anti-HEL IgG and IgM production induced by the CS alone via the single-trial paradigm was demonstrated by Alvarez-Borda et al. and repeated by Ramirez-Amaya, et al. Most recently, it was replicated again with lesser magnitude by Madden et al. [8,37,44]. Thus, using this paradigm for reference, we altered the time for the test trial task, which in the previous paradigm was performed during rapid rise of the primary antibody response. In the current paradigm, the test trial task was scheduled during the declining phase of primary antibody response (1 month after the conditioning). We assume that this delay may place the conditioned response on a lower basal level of primary response and hence magnify the significance of difference between conditioned and unconditioned control groups. Our data demonstrated that by using fewer subjects (12–16 rats in each group) a significant conditioned antibody response was elicited by the CS alone. Additionally, periodic testing of the antibody production revealed that, following the representation of CS, there seemed to be an initial lag phase when no significant elevation of antibody response was detected. This was followed by phases in which the antibody production rose to a plateau and then declined. These results are partially consistent with previous findings [8] and suggest that the characteristic pattern of CS-induced conditioned antibody response is very similar to that of the antigen-induced antibody response. There was not a conditioned taste aversion to saccharin since no significant difference in saccharin consumption was found between the different groups. In addition, pre-exposure to saccharin prevented the conditioned antibody response in the group CSp. These results suggest that the enhancement of antibody response in the conditioned rats was not due to the effect of stress from the behavioral aversive responses or the physiological effects of saccharin. Since the single-trial protocol of the conditioned enhancement of antibody response is antigen-specific and free from non-specific sensory and pharmacological effects, it would be more adequate for investigating the neural circuitry behind the process of the conditioned immune response [44].

In Experiment II, we employed this single-trial conditioning paradigm and c-Fos immunomapping to investigate the functional activation of insular cortex after the CS re-exposure. Results indicated that re-exposure of conditioned rats to the CS resulted in a significant increase of Fos immunoreactivity in all insular areas. This finding is the first c-Fos mapping report suggesting the IC is involved in the expression of conditioned immune response, by showing the functional activation of the IC in response to the CS re-exposure. As is extensively discussed in studies of conditioned taste aversion, insular cortex subserves the acquisition, retrieval, retention and extinction of long-term

gustatory memory [19,22,45,47]. Insular cortex has also been demonstrated to be involved in the neurocircuitry underlying the acquisition of conditioned immunosuppression and conditioned antibody response [42–44]. Our findings further demonstrated that the IC is involved in the expression of conditioned immunity.

However, it still remains unclear as to how the IC mediates the conditioned immunity. The IC is divided cytoarchitectonically into three areas—granular, dysgranular and agranular (areas GI, DI and AI). The cortical taste area in rats is located in the granular and dysgranular insular cortices (GI and DI) [28,40]. Most recently, it has been proposed that the subjective awareness of flavor is most likely due to neuronal activities in the AI area [49]. The AI receives a direct projection from the main olfactory bulb, piriform cortex and endopiriform nucleus [51,52]. Bilateral lesions of the insular cortex can block the association between taste and odor in the rat [48]. It was reported that lesions in the IC disrupted either taste or odor stimuli induced conditioned antibody response [44]. Considering the anatomic and functional participation of IC in the gustatory and olfactory mechanisms, any results regarding the destructive effect of IC lesions on conditioned antibody response induced by either taste or odor stimuli could be interpreted as the effect of the disruption of sensory perception. In the present study, repeated intake of saccharin did not induce the elevated functional activation in the IC, since there was no significant difference of c-Fos expression in the IC between the group Sac–Sac and the basal control group Vehicle. We can therefore infer that the CS-induced increased activation of IC is not due to the mere gustatory information from the CS. This study offered evidence that the involvement of IC in the conditioned immune response is not merely dependent upon the sensory mediation.

Noteworthy, multiple molecular mechanisms for the long-term taste memory involved in the IC are specifically turned on by an unfamiliar taste paired with a reinforcer, but are not turned on by a familiar taste that is not paired with a reinforcer [10–12,46]. The novel taste of the saccharin without pairing with a reinforcer cannot induce increased expression of immediate-early genes *c-fos* in the IC [38]. Therefore, in this study the modest c-Fos expression observed in the IC from the Sac–Sac group can be interpreted as the inactivation of the IC to a familiar stimulus that was not associated with a reinforcer.

It is of interest to note that in the present study the rats of group CS_0, which were subjected to the conditioning but not re-exposed to the CS, showed significant elevated c-Fos production in the DI and GI, in comparison with three control groups of the UCS, Sac–Sac, and Vehicle, which did not receive the conditioning paradigm. These results reflect the remnant neuronal activation in the IC (DI and GI areas) after the conditioning. It was reported that IC lesions made after conditioning prevented the evocation of CIS [42]. Our finding, together with the results from the lesion study, implicates that the IC is also involved in the acquisition and storage of CS/UCS associative learning. Further study should be done to clarify this issue, though.

On the test trial day, although there was still a significant primary antibody response, no significant increase of c-Fos expression in the IC was found in the rats of group UCS, suggesting the elevated c-Fos expression in IC was not related with the antibody response resulted from the unconditioned effects of OVA. This view is reinforced by the findings reported by Ramirez-Amaya and Bermudez-Rattoni in which lesions in the IC did not produce different effects on the normal primary IgG and IgM antibody production [44]. However, to make a definite conclusion of whether IC is involved in antibody production, an additional comparison group to define the unconditioned effects of OVA injected on the test day is needed.

Although IC seems not to play a direct role in the normal antibody response, we cannot rule out the possibility that the IC may receive and process the immune information through some indirect circuitries. In this regard, more brain structures potentially related to the immunity should be tested in the further study, such as the hypothalamus and amygdala, which are anatomically adjacent to the IC and known to receive afferents from the IC [50]. In addition, more sample times to observe brain activation are also needed, which might provide clues as to which of the cascade of immune responses to antigen was associated with changes in brain activity.

In conclusion, we demonstrated that a conditioned enhancement of antibody response can be obtained via a single-trial conditioning paradigm when using the OVA as the unconditioned stimulus. Furthermore, we demonstrated the involvement of the insular cortex in the expression of this conditioned antibody response. To classify the role of insular cortex in cellular and molecular mechanisms of conditioned immunity, further studies are needed.

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