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Expression of c-fos in Amygdala and Conditioned Immunosuppression *

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Abstract By using *c-fos* protein production as a marker of neuronal activation , the present work attempts to localize the various subdivisions within the amygdala that may participate in the neural mechanism of conditioned immunosuppression (CIS) in a conditioned taste aversion (CTA) paradigm. CTA and CIS were established by intake of saccharin as the conditioned stimulus (CS) with an injection of cyclophosphamide as an unconditioned stimulus (UCS). After animals were re-exposed to CS alone at intervals of 5 or 30 days after two trials of CS-UCS pairing , both CTA and CIS were assessed and the expression of *c-fos* protein in the amygdala nucleus was also observed correspondingly. The results showed that CTA occurred on both day 5 and day 30 after conditioning but CIS occurred only on day 5 after conditioning. Furthermore , it was found dense *c-fos* expression in the amygdaloid central (CeA) in the CS group was observed at the 5 days interval and moderate *c-fos* immunoreactive inductions at the 30 days interval after re-exposure to CS. In the UCS group , low level of *c-fos* immunoreactive productions in the CeA were detected at either interval testing. By comparing the results of the 5th day with the 30th day , it suggests that the CeA is an important nucleus related to the modulation of CIS.

Key words Conditioned immunosuppression , Amygdala , *c-fos* , Rats.

1 Introduction

Since the conditioned immunosuppression paradigm initially developed by Ader and Cohen in 1975[1] , this paradigm has been studied and replicated extensively[2,3]. The majority of studies on conditioned immunosuppression have employed a novel taste solution as the conditioned stimulus (CS) paired with an immunosuppressive agent as the unconditioned stimulus (UCS). After CS/ UCS pairings were made , re-exposure of animals to the CS alone resulted in significant conditioned taste aversion (CTA) and conditioned attenuation of the immune response. Although conditioned immunosuppression (CIS) paradigm reveals interactions between the brain and the immune system , the neural mechanism of CIS remains unclear. It has been known that several brain areas play an important role in regulation of neural-immune interactions , such as hypothalamus , the limbic system and the neocortex .[4] However , there is scarce information regarding the neural mechanism of CIS. Presently , it has been reported that amygdala (AM) lesion made before conditioning trial could lead to disrupt the acquisition of CIS , whereas amygdala lesion made after conditioning trial did not show any effect on CIS , which indicated that AM may be one of mechanisms related to the acquisition of the conditioned immunosuppression .[5] However , due to the amygdala nucleus consists of various subdivisions , which subdivision involved in modulating the response of CIS is not clarified. Therefore , by using *c-fos* protein production as a marker of neuronal activation [6] , saccharin as CS paired with cyclophosphamide (CY) as UCS , the present work attempts to localize the various subdivisions within AM which may participate in modulating the conditioned immunosuppression.

2 Experiment 1

In this experiment , CTA and CIS were examined at two-interval test days after conditioning by using two-trials of CS-UCS pairing

2.1 Materials and Methods

2.1.1 Subjects Fifty male Wister rats (200～250g) obtained from Medicine Department of Peking

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University at 8 weeks of age, were separated into individual cages and maintained under an inverted 12:12hr light-dark cycle (lights on at 7:00 AM～19:00 PM) with food *ad libitum*. Water was available throughout the experiment except during the measurements of scheduled drinking period and saccharin presentation. The temperature was controlled at 22 ± 1 ℃. All experimental rats should be acclimatized to the laboratory for 4 days and to be touched for 2～3 mins each day operated by the laboratory worker. Animals were then introduced to a water restriction regimen in which two bottles containing water were placed on the front of each cage at 8:30 AM for 30 min each day.

2.1.2 Conditioning procedures The experimental rats were randomly distributed into three groups on the conditioning training day. Conditioned animals (CS) were given a total of two CS-UCS pairings for consecutive two days, each consisting of a 30 minute exposure to 0.25% saccharin solution (the CS) and the water, followed immediately by an intraperitoneal (ip) injection of 50mg/kg cyclophosphamide (CY) dissolved in sterile saline (20mg/ml). Unconditioned animals (UCS) were injected with an equal dose of CY by ip after the animals were exposed to two bottles of water for 30 minutes. The animals of conditioned control group (CS_0) were given saccharin as the CS group, but followed by an ip injection of the same dose of saline. The above procedures were operated again next day. Then, at 5 days or 30 days interval after two trials of CS-UCS pairing, animals of the CS and CS_0 groups were re-exposed to a bottle of saccharin and a bottle of plain water for 30 minutes. Animals of the UCS group were re-exposed to two bottles of plain water for 30 minutes followed by an ip injection of the same dose of saline. Twenty-four hours later, all animals were sacrificed and the proliferative responses of spleen lymphocytes to mitogen were measured. Meanwhile, the volumes of saccharin or plain water consumed during the conditioning training day and test day were measured. The details of experimental procedure are outlined in Table 1.

Table 1 Experimental procedure 1

Group	Conditioning training day (two days)	Testing day (5 days after conditioning)	24hr later	Testing day (30 days after conditioning)	24hr later
CS	SAC + CY	SAC + SAL ($n_5 = 9$)	spleen	SAC + SAL ($n_{30} = 9$)	spleen
UCS	WAT + CY	WAT + SAL ($n_5 = 8$)	spleen	WAT + SAL ($n_{30} = 8$)	spleen
CS0	SAC + SAL	SAC + SAL ($n_5 = 7$)	spleen	SAC + SAL ($n_{30} = 9$)	spleen

CS: conditioned stimulus; UCS: unconditioned stimulus; CS_0: no conditioned stimulus; SAC: saccharin; CY: cyclophosphamide; WAT: water; SAL: saline; n: animal number

2.1.3 Lymphocyte proliferation Spleens were collected in RPMI 1640 (Flow Laboratory) and washed through a stainless sieve into PBS containing 5% heat-inactivated fetal calf serum (FCS), 100IU/ml penicillin with 100(g/ml streptomycin, and 0.5% mineral salts. After three washes in the supplemented PBS, the cells were resuspended in RPMI 1640 supplemented with no mineral salts but with an addition of 0.2 mM L-glutamine and 5 ×10M 2-mercaptoethano. They were then cultured in 96-well microtiter plates in the presence of 2.5 μg/ml purified pokeweed (PMW) or 8 μg/ml concanavalin (ConA) (Sigma). All cultures were performed in triplicate with background spleen cell activity stimulated by PBS instead of mitogen. After 48h of incubation at 37 ℃in a 5%CO humidified atmosphere, all cultures were pulsed with 0.5uci[H] thymidine. Following a further 24h incubation, the thymidine incorporation was counted in a scintillation counter after harvesting by a semi-automatic, multiple harvester.

2.2 Results

As can be seen in Fig 1(A,B), the volumes of SAC consumption in animals of the CS and CS_0 groups are the same on the first conditioning day. When animals were re-exposed to SAC alone, the consumption of SAC in the CS_0 group at 5 days interval after conditioning was distinctly increased, however, the CS group showed significantly a reduced saccharin consumption as compared with that of the CS_0 group ($p < 0.0001$, *t*-test). Similar behavioral responses were found in rats of both the CS and CS_0 groups when re-exposed to CS on day 30 after conditioning, that is to say, the consumption of SAC of the animals in CS_0 group approached the volume of SAC consumption as re-exposed to SAC at 5 days interval, whereas the consumption of SAC in animals of the CS group remained significantly less than that of the CS_0 group ($p < 0.0001$, *t*-test).

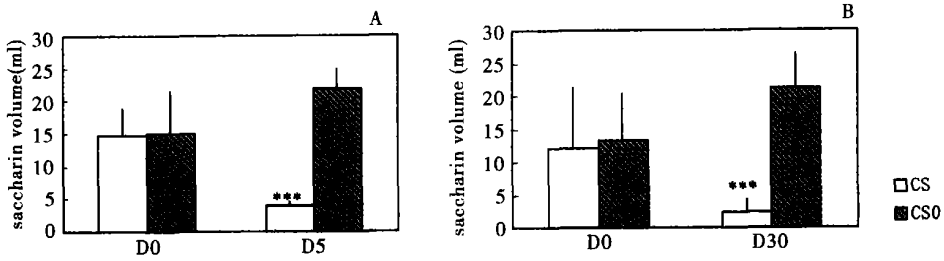

Fig 1 The volume of saccharin consumption on the conditioning day, the test day 5 (A), and the day 30 (B) after conditioning
Note: D0, conditioning day (first day); D5, re-exposure to CS on day 5 after conditioning; D30, re-exposure to CS on day 30 after conditioning *** $p < 0.0001$, compared with CS_0 group.

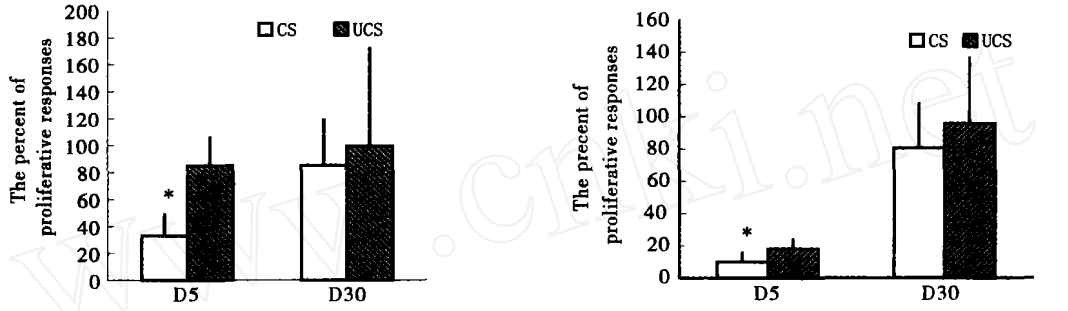

Fig 2A Proliferative responses of spleen lymphocytes to ConA Fig2B Prolifterative responses of spleen lymphocytes to PMW
Note: D5, re-exposure to CS on day 5 after conditioning; D30, re-exposure to CS on day 30 after conditioning
* $p < 0.05$, compared with UCS group

Figure2A and 2B show the results of the proliferative responses of spleen lymphocytes to mitogen. Since there was a great variance in baseline values of the proliferative responses of spleen lymphocytes to ConA and PMW between the 5 days and 30 days interval test after conditioning, the value of the proliferative responses to mitogen measured every time in the CS_0 group was marked as 100 % and the ratio of the CS group or the UCS group to the CS_0 group was analyzed. The results show that the proliferative responses to ConA and PMW were significantly attenuated in the CS group as compared with the UCS group at the 5 days interval test after conditioning ($p < 0.05$, t-test). However, there was no statistical difference in proliferative responses between the CS and UCS groups at the 30 days interval test.

3 Experiment 2

To localize the various subdivisions within AM which may participate in modulating the conditioned immunosuppression, *c-fos* immunomapping technique was used in this experiment to examine the functional activation of the AM in response to the CS re-exposure.

3.1 Materials and Methods

3.1.1 Conditioning procedure Forty male Wister rats (200～250g) obtained from Medicine Department of Peking University at 8 weeks of age. Animals were divided into a conditioned stimulus group (CS, $n = 8$), an unconditioned stimulus group (UCS, $n = 6$) and a conditioned control group (CS_0, $n = 6$) for both tests at 5 days and 30 days interval. The conditioning procedure and the test interval in Experiment 2 was the same as in Experiment 1. The details of experimental procedure are shown in Table 2.

3.1.2 Fos immunohistochemistry Animals were deeply anesthetized with a 10 % chloral hydrate solution (3.5mg/kg) in intraperitoneal injection at 3h both at 5 days and 30 days interval after conditioning. The chest of rat was opened and a cannula was passed into the ascending aorta through the left ventricle. The rats were perfused with 4 % paraformaldehyde (dissolved in 0.1M PBS) for 1h to 1.5h. Brains were removed and soaked into the same fixative for another 3h to 4h, and further soaked in 2 % sucrose (dissolved in 0.1M PBS) at 4 ℃overnight. 24h later, the brains were frozen at 20 ℃and forty micrometer frozen coronal sections were cut on a freezing microtome, and collected in PBS at 4 ℃overnight.

Table 2　Experimental procedure 2

Group	Conditioning training day (two days)	Testing day (5 days after conditioning)	24hr later	Testing day (30 days after conditioning)	24hr later
CS	SAC + CY	SAC + SAL　($n_5 = 8$)	Sacrificed	SAC + SAL　($n_{30} = 8$)	Sacrificed
UCS	WAT + CY	WAT + SAL　($n_5 = 6$)	Sacrificed	WAT + SAL　($n_{30} = 6$)	sacrificed
CS0	SAC + SAL	SAC + SAL　($n_5 = 6$)	Sacrificed	SAC + SAL　($n_{30} = 6$)	Sacrificed

Note: CS: conditioned stimulus; UCS: unconditioned stimulus; CS_0: no conditioned stimulus ; SAC: saccharin; CY: cyclophosphamide; WAT: water; SAL: saline; n: animal number

All floating sections of the brains were washed with 0.01M PBS on the microwave oscillator (3 × 5min), and then were incubated in a solution containing 0.3% hydrogen peroxide and 80% methanol for 30min.

After a rinsing step, they were incubated with 0.3% TritonX-100 (dissolved in 0.1 M PBS) for 20min, blocked with 5% normal goat serum for 10min. Sections were incubated with rabbit polyclonal anti-*c-fos* IgG (Santa Cruz Biotech, USA, diluted 1:4000) at 4 ℃for 24～48h, and incubated at 37 ℃ for another 30min, then rinsed in 0.01M PBS. Sections were incubated at 37 ℃for 40min with a 1∶200 dilution of goat anti-rabbit IgG, rinsed, and incubated with horse radish peroxidused (Vector, USA) at 37 ℃for 40min. The immunal reaction was developed using 0.25% diaminobenzidine (DAB) (dissolved in 0.01M PBS and 0.03% H_2O_2). Sections were mounted onto gelatin-covered slides.

Semi-quantitative image analysis for *c-fos* protein was performed using a system consisting of a *Leica* microscope and camera connected to a Power PC. The planes of sections were standardized as far as possible following the atalas of Paxionos and Watson[7] as well as Wang Pingyu[8].

3.2　Results

Table 3 shows the expression of *c-fos* protein in the amygdaloid nuclei at the 5 days and 30 days interval after conditioning.

Low level of positive expression of *c-fos* protein was detected in the areas of the amygdaloid nuclei in the CS_0 group at the 5 days interval including: anterior amygdaloid area (AA), basolateral amygdaloid nucleus (BLA), basomedial amygdaloid nucleus (BMA) and central amygdaloid nucleus (Ce). However, in the CS group, the positive productions of *c-fos* protein were widely detected. Besides the above mentioned nucleus in the CS_0 group, *c-fos* expression were detected in the areas including the pooterolateral cortical amygdaloid nucleus (PLCo), anterior cortical amygdaloid nucleus(ACo), dense *c-fos* expression in the Ce in particular, and moderate level of *c-fos* expression in the BMA, when compared with that of the CS_0 and UCS groups.

The positive productions of *c-fos* protein were reduced obvioulsy in the Ce in the CS group at the 30 days interval after re-exposure to CS, while the expression of *c-fos* protein in the other nuclei remain the similar level as at the 5 days except the BMA. The expression of *c-fos* protein in the BMA was disappeared at the 30 days interval after re-exposure to CS. In the CS_0 group, *c-fos* immunoreactive productions in the Ce were disappeared. Low level of *c-fos* expression was detected in the Ce of the UCS group (see Table 3 and Fig3).

Table 3　Expression of c-fos in the various subdivisions within amygdala

Groups	Testing day	Amygdala							
		AA	Aco	PLCo	Me	BLA	BMA	La	Ce
CS	5	+	+	+	+	+	+ +	+	+ + +
UCS	5	+	0	0	0	+	+	0	+
CS_0	5	0	0	0	+	+	+	+	+
CS	30	+	+	+ +	0	+	0	+	+ +
UCS	30	0	0	0	0	0	0	0	+
CS_0	30	0	+	+	0	+	0	0	0

Note:. AA: anterior amygdaloid area; Aco: anterior cortical amygdaloid nucleus; PLCo: pooterolateral cortical amygdaloid nucleus; Me: medial amygdaloid nucleus; BLA: basolateral amygdaloid nucleus; BMA: basomedial amygdaloid nucleus; La: lateral amygdaloid nucleus; Ce: central amygdaloid nucleus. Relative densities: + + +. dense; + +. moderate; +. low; +/0. trace or equivocal; 0. below detection limit.

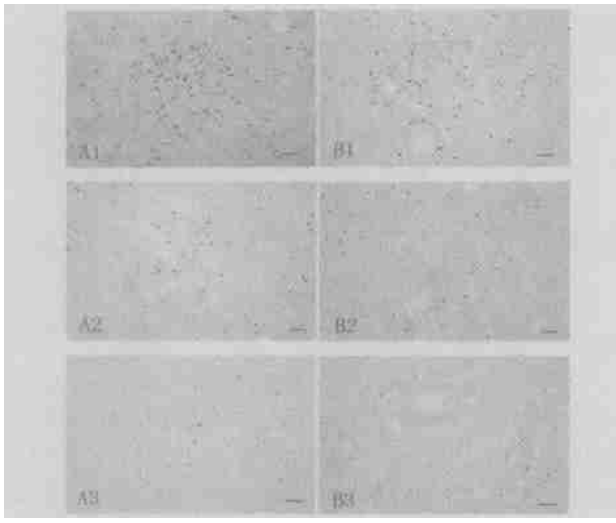

Fig 3 *c-fos* expression in the center amygdala nucleus 2 hr after the test trial.
Note: A, re-exposure to CS on day 5 after conditioning; B, re-exposure to CS on day 30 after conditioning; 1, CS group; 2, UCS group; 3, CS_0 group. Bar = 50um

4 Discussion

Previously, using saccharin as the CS paired with an injection of cyclophosphamide as an UCS in a conditioned immunosuppression paradigm, we found that conditioned taste aversion occurred under either one trial or two trials of CS-UCS pairing, but conditioned immunosuppression was only shown by re-exposure to CS after two trials of CS-UCS pairing [9]. Hence, we used two trials of CS-UCS pairing in the present study. In the first experiment, the results showed that CTA occurred and the level of CTA remained at the every interval testing (5, 30 days) after conditioning. However, CIS occurred obviously only on the 5 days interval after conditioning, and was almost disappeared on the 30 days interval after conditioning. Taken together, it is suggested that there was no association between CIS and CTA. Conditioned behavioral and immunological changes might occur independently. Therefore, we hypothesize that both CTA and CIS may have their own modulation mechanism in the central nervous system. In the second experiment, the results showed that dense *c-fos* expression in the central amygdaloid (CeA) in the rats of the CS group re-exposed to CS at the 5 days interval but obviously reduced expression at the 30 days interval after conditioning. However, in the UCS group, low level of *c-fos* immunoreactive productions in the CeA were detected at either interval testing. By comparing the expression of *c-fos* at the 5 days with the 30 days, it is suggested that CeA might be involved in the modulation of CIS. Nevertheless, CeA may also contribute to CTA learning because *c-fos* expression in the CeA was still in the moderate level when animals reexposed to the CS on day 30 after conditioning.

Numerous experiments have provided evidence concerning the involvement of the CeA in conditioned taste aversion [10~14]. In this regard, our results are consistent with previous reports. However, there is less evidence that the CeA is involved in the modulation of CIS. Our view of the involvement of the CeA in the modulation of CIS is in accordance with the several lines of related research. It is reported that the amygdala is closely associated with the hypothalamus, where endocrine-immune interactions are regulated[15, 16]. It has also been demonstrated that the activation of dopamine D1 receptors within the central amygdala induces selective stimulation of mitogen responsiveness of splenocytes [17]. The central amygdala may represent a critical structure mediating cocaine-induced T cell proliferation[18]. In addition, the CeA may act as a relay pathway in which brainstem catecholamine cells of the nucleus tractus solitarius (NTS) and ventrolateral medulla (VLM) are possibly responsible for the activation of the HPA axis following the immune challenge[19]. The present findings, in conjunction with the above evidence, suggest that CeA as a main nucleus within the amygdala is likely participated in the modulation of CIS.

In the present study, we also detected low Fos-immunoreactive productions in the basolateral amygdaloid nucleus (BLA) at either interval testing. It has been reported that the BLA plays a critical role in learning and modulating the consolidation of memory in CTA[20, 21]. Given that CTA occurred in the animals re-exposed to CS on both day 5 and day 30 after conditioning, it would be suggested that the BLA might be an important nucleus related to CTA.

The present results also showed that *c-fos* expression in the BMA was observed obviously in the rats of the CS group re-exposed to CS at the 5 days interval but not at the 30 days interval after conditioning. These results are consistent with our finding that CS-induced immunosuppression occurred obviously only at the 5 days interval but not at the 30 days interval after conditioning. Since the BMA has wide projections with many brain areas[22], it is probably also directly or indirectly involved in the

general process of CS-induced immmunosuppression.

In summary, the results of the present experiment indicate that both CTA and CIS may have their own modulation mechanism in the central nervous system. The CeA is an important nucleus of the amygdala that is involved in the modulation of both CTA and CIS.

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杏仁核 c-fos 的表达与条件反射性免疫抑制

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摘　要　以糖精水为条件刺激(conditioned stimulus,CS),免疫抑制剂-环磷酰胺为非条件刺激((unconditioned stimulus,UCS),在两次 CS-UCS 结合训练后,观测不同时程再次单独呈现条件刺激对条件反射性免疫抑制和味觉厌恶性行为反应的变化以及大鼠杏仁核各亚核团内 *c-fos* 蛋白表达的影响。结果表明,条件反射性免疫抑制作用在训练后第 5 天较强,第 30 天基本消失,而味觉厌恶性条件反射始终稳定保持到第 30 天。条件反射组大鼠杏仁中央核 *c-fos* 蛋白表达在第 5 天非常密集,而第 30 天明显减少,与细胞免疫功能改变在时程和趋势上具有一致性。通过 *c-fos* 蛋白表达时程差异比较,提示杏仁中央核可能既与条件性的味觉厌恶性行为建立有关,也是参与介导 CS 诱导的免疫抑制效应的重要核团。

关键词　条件反射性免疫抑制,杏仁核,*c-fos*,大鼠。

分类号　B845