Contributions of the Ventrolateral Orbital Cortex to Pain-Related Negative Emotion

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Abstract: Painful stimuli evoke pain sensation and unpleasant emotional feelings. Recent studies demonstrated that the anterior cingulate cortex (ACC) and amygdala play a crucial role in pain-related negative emotion using a rat formalin-induced conditioned place aversion (F-CPA) model, a nociceptor-driven, associative avoidance-learning assay, which was considered as a direct reflection of negative emotion of pain. In this study, we further revealed the role of the ventrolateral orbital cortex (VLO) in pain-related negative emotion. Electrolytic or chemical lesion of the bilateral VLO abolished F-CPA. Interestingly, this lesion affected neither acute formalin-induced two-phase spontaneous nociceptive behaviors nor low-intensity electric foot shock-induced CPA (S-CPA). Furthermore, we showed that formalin nociceptive conditioning induced a persistent (>24 hrs) cAMP response element binding protein (CREB) phosphorylation (pCREB) and an accompanying increase in Fos expression in the bilateral VLO. Re-exposing rats to the nociceptive conditioning context for retrieval of pain experience produced upregulation of pCREB and Fos expression in the bilateral VLO also. These results provided direct evidence indicating that the VLO is required for pain-related aversion, and also suggested a role for CREB phosphorylation in the induction of pain-related aversion and reconsolidation of pain-related aversive memory following pain experience retrieval. Taking the present study together with the previous reports, it is reasonable/advisable to accept the proposal that a neural network of the VLO with the ACC, amygdala and perhaps some other limbic structures contributes to the negative emotion of pain. Thus, a multidisciplinary integrated approach to preventing chronic pain-induced emotional disturbance might be raised.

Keywords: Ventrolateral orbital cortex (VLO); VLO lesion; pain, pain-related negative emotion; Formalin-induced conditioned place aversion (F-CPA); F-CPA retrieval; formalin-induced acute nociceptive behavior; cAMP response element binding protein (CREB); fos; rat.

INTRODUCTION

The pain experience includes the sensory-discriminative aspect and the affective-motivational aspect [1]. Although the neural structures, pathways, physiology and biochemistry associated with "pain sensation" have been relatively well established in the past four decades, the underlying mechanisms of "pain affect" remain unclear.

Accumulating evidence indicated that the ventrolateral orbital (VLO) cortex is related to pain, memory, and emotion [2-5]. Anatomical studies have established that the VLO receives projections from thalamus nucleus submedius (Sm) that receives projections from lamina I spinothalamic tract neurons in the dorsal horn of spinal cord [6-9]. Electrophysiological studies showed that most of the neurons in the VLO responded to a variety of modalities of noxious stimuli (e.g. skin and viscera), especially prolonged stimuli, and had very large and bilateral receptive fields [2, 10]. Electrical stimulation of C-fibres in the sciatic nerve or the perfusion of bradykinin into the lower extremity elicited an increase in local cerebral blood flow in the VLO [11]. Formalin s.c. injection into a forepaw induced increase in local glucose utilization rates in the bilateral VLO [12]. Animal behavioral studies indicated that bilateral anaesthetization of the sulcal prefrontal cortex, a region near the VLO (caudal-lateral to the VLO), of the rat decreased the aversive response to noxious stimulation [13]. Our previous studies demonstrated that electrical stimulation of the VLO or intra-VLO of glutamate depressed the radiant heat-evoked tail-flick reflex and jaw-opening reflex [4, 14, 15]. Taken together, these data suggested that the VLO may be involved in processing of both pain sensation and pain affect.

Considering the similarities between synaptic plasticity in the hippocampus and nociceptive central sensitization in the spinal cord, we assumed that pain-related negative emotion may share common neuronal mechanisms with nociceptive sensitization and learning/memory. Several lines of evidence have suggested that cAMP response element-binding protein (CREB) activation is necessary for the long-term potentiation in hippocampus and the formation, consolidation, and reconsolidation of long lasting memories [16-20]. Also, in the long-term facilitation of spinal cord neurons after inflammation and injury, CREB signaling has been proposed to play a crucial role [21-23].

In the present study, we first demonstrated that bilateral lesions of the VLO significantly suppressed pain-related

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negative emotion. Furthermore, we measured phosphorylation of CREB （pCREB）in VLO neurons following formalin nociceptive condition and retrieval of F-CPA using a specific antibody [21]. In addition, we also compared pCREB induction with that of Fos, the protein product of an immediate-early gene (IEG), *c-fos*, and the expression of which can be regulated by pCREB [24].

MATERIALS AND METHODS

Animals

Experiments were performed on adult (weighting 220-250g) male Sprague-Dawley rats. Rats (From Experimental Animal Center of Fudan University) were on a 12: 12 light-dark cycle with a room temperature of 23 ± 1 ^{o}C, and received food and water *ad libitum*. Before experimental manipulations, the animals were given a period of 7 days to adjust to the new surroundings and handled daily. All experiments were carried out in according with the guidelines of the International Association for the study of pain, and were approved by the Shanghai Animal Care and Use Committee.

Brain Lesion

Rats were anesthetized with an intraperitoneal injection of Chloral hydrate (40 mg/kg), and securely placed into a sterotaxic device with Bregma and Lambda at a horizontal level. For electrolytic lesion, an insulated tungsten electrode (shank diameter 200 μm, exposed tip 250 μm) was stereotaxically placed in the VLO (3.2-3.7 mm rostral to Bregma, 1.5-2.5 mm lateral, 4.5-5.0 mm below cortical surface) on both sides [25], and 0.45 mA anodal DC current was passed for 20-40 s on each side. Indifferent electrode was inserted subcutaneously into the wound margins. Sham VLO lesion controls received inserting the lesion electrode into the bilateral VLO without anodal DC current passing.. For chemical lesion, a 1 μl Hamilton syringe filled with quinolinic acid (QA, 200 nmol/μl) or sterile normal saline (NS) was lowed into the VLO. Five minutes after lowering the syringe to the target coordinates, QA or NS (0.6 μl) was slowly microinjected over a period of 10 min. The syringe remained in place for 5 min after each injection to prevent the spread of the agent to the surface of the brain. This procedure was then repeated in the opposite hemisphere. A recovery period of 7 days was allowed before additional experimental procesure. At the end of the experiment, brains were sectioned and Cresyl violet stained to verify the location and size of the lesions.

Conditioned Place Avoidance (CPA)

CPA was conducted as described previously [26] with slight modifications. The place conditioning apparatus consists of three opaque acrylic compartments. Two equal-large ones are conditioning compartments (30×30×30 cm) and a smaller one is a neutral choice compartment (15×20×30 cm, long × width × height). The conditioning compartments are placed in parallel and separated by a wall with a square door (10×10 cm). The neutral compartment is laid in front of the two conditioning compartments with two doors (10×10 cm) to them. A movable transparent ceiling covers each compartment. The two conditioning compartments are both painted black, but one is decorated with a transverse white band and contains an odor produced by 1.0 % acetic acid; the other is decorated with a white vertical band and has an odor of cinnamon. The floors of the conditioning compartments are also different. One of the floors is made from plexiglas, and the other is a polyester board with a metal band on it, which can provide an electric shock. Thus, the two conditioning compartments have distinct visual, olfactory and tactile cues. The neutral compartment is white, absents distinctive odor and has a solid acrylic floor with a slope.

The experimental process consists of three distinct sessions: a pre-conditioning session, a conditioning session and a test (post-conditioning) session. CPA task processing takes 4 days. Day1 is preconditioning day. At the beginning, the rat was placed in the neutral compartment. After habituating for about 2 minutes, the entrance to each conditioning compartment was opened. When the rat entered any conditioning compartment, the doors connecting neutral and conditioning compartments were closed. The rat was allowed to explore the two conditioning compartments freely for 15 min. A timer automatically recorded the time spent in each of the compartments in a blind fashion. Rats that spent more than 80 % (720 sec) on one side on Day 1 were eliminated from the subsequent experiments. Day 2 and 3 are conditioning days. On day 2, the rat received nothing and was randomly confined to one of the conditioning compartments for 45 min. On day 3, for formalin-induced CPA (F-CPA), the rat was given a unilateral hindpaw intraplantar (i.pl.) injection of 5% formalin (50 μl) or NS (control), and then restrained in the other conditioning compartment for 45 min. For electric foot-shock-induced CPA (S-CPA), the rat received an electric shock (0.5 mA for 2 s) every 10–12 min in the other conditioning compartment during the 45-min training session. Both the treatments (formalin, shock, NS, or no treatment) and the compartments were counterbalanced. Day 4 is postconditioning day. The procedure is the same as day 1. The time spent in each compartment was measured.

F-CPA Retrieval

F-CPA retrieval rats received the same training trails in days 1-3 of conditioning procedure as F-CPA, but on day 4, they were placed into the formalin-paired compartment for 10 min to retrieval formalin-induced pain experience. Non-retrieval rats received the same training trails as F-CPA retrieval, but on day 4, they were allowed to remain in their home cages without 10 min F-CPA retrieval before they were perfused.

Formalin Test

Rats were placed in a 20×20×20 cm plexiglas chamber and allowed to habituate for about 15 min. A mirror was positioned below a chamber at a 45°C angle for unobstructed observation of the rat's paws. The rat was given a unilateral hindpaw intraplantar (i.pl.) injection of 5% formalin (50 μl) and placed into the chamber. The responses to formalin injection were monitored by measuring the amount of time the animal spent with the injected paw elevated or licking and biting the paw per 5 min during the 45-min observation period. A weighted pain score for each animal was calculated using the following formula [27].

Formalin pain score = [time spent with injected paw elevated + 2 (time spent licking or biting injected paw)]/-300.

Immunohistochemistry

After defined survival times, rats were killed by overdose of Chloral hydrate (80 mg/kg) and perfused intracardially with NS followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were then removed, post-fixed in the same fixative for 6 hrs at 4°C, and immersed from 10 % to 30 % gradient sucrose in PB for 24-48 hrs at 4°C for cryoprotection. Coronal sections (30 μm) were cut in a cryostat (Leica 1900, German) and processed for immunohistochemistry according to the ABC method [28]. Briefly, the sections were blocked with 10 % goat serum in 0.01M PB saline (PBS, pH 7.4) with 0.3 % Triton X-100 for 1 hr at RT and incubated overnight at 4°C with rabbit anti-pCREB (1:3000; Upstate Biotechnology) and rabbit anti-Fos (1:5000, Oncogene Science, Uniondale, NY) primary antibody in PBS with 1 % normal goat serum and 0.3 % Triton X-100. Following three 15 min rinses in PBS, the sections were incubated for 90 min at RT with the biotinylated secondary antibody (1:200) and subsequently with the ABC complex (1:100; ABC Kit, Vector Laboratories, Burlingame, CA). The reaction product was visualized with 0.05 % DAB/0.006 % hydrogen peroxide in 0.1 M acetate buffer, pH 6, containing 2 % ammonium nickel sulfate for 5 min and then rinsed in acetate buffer, air-dried, dehydrated, and coverslipped. Omission of primary antibody served as negative control. For pCREB/NeuN, pCREB/OX-42, pCREB/GFAP, or pCREB/Fos double immunofluorescence, the sections were incubated with a mixture of rabbit anti-pCREB (1:3000) and mouse anti-NeuN (1:5000, Chemicon, Temmecula, CA), mouse anti-OX-42 (1:5000, Serotec, Oxford, UK), mouse anti-GFAP (1:3000, Sigma), or goat anti-Fos (1:200, Santa Cruz Biotechnology, CA) overnight at 4°C. Following three 15 min rinses in PBS, the sections were incubated in rhodamine-conjugated donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch), fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA), or FITC-conjugated donkey anti-goat IgG (1:200, Jackson ImmunoResearch) for 2 hrs at 4°C. The sections were cover slipped with a mixture of 50 % glycerin in PBS, and then observed with a Leica SP2 confocal laser-scanning microscope (Mannheim, Germany). The specificity of the staining and antibodies has been tested in previous studies [29-32].

Quantification and Statistics

In the case of CPA experiments, the CPA scores represent the time spent in the treatment-paired compartment on the pre-conditioning day (day 1) minus the time spent in the same conditioning compartment on the post-conditioning day (day 4). The differences in CPA scores among treated groups were compared using one-way ANOVA followed by *post hoc* Dunnett's test when comparing more than two groups. In addition, the absolute time spent in the treatment-paired compartment on the pre-conditioning day versus the post-conditioning day was compared in training (F-CPA, S-CPA), sham training, lesion or sham lesion animals using Paired *t-test*. In the case of formalin-induced nociceptive behaviors, formalin pain scores per 5 min were analyzed using a two-way (treatment × time) ANOVA with a *post hoc* comparison for analysis of the differences at each 5-min test time period. For the quantification of pCREB and Fos immunoreactive signals, immunohistochemically stained tissue sections were examined under a light microscope at 10× magnification and the images were captured with a CCD spot camera. The numbers of pCREB and Fos positive nuclei were counted in a region of interest (a square box, 1×1 mm) within the VLO, using a computerized image analysis system (Image J). The threshold of positive-immunoreactive staining was set by the experimenter, who was blind with respect to treatment, and was determined by eye as cellular staining darker than the surrounding neuropil. The mean number of pCREB and Fos positive nuclei per section was determined from six non-adjacent randomly selected sections (30 μm) through the VLO (3.2-4.2 mm rostral to Bregma) for each animal. Four rats were included in each group for quantification of immunohistochemistry results. Differences between groups were compared using one-way ANOVA followed by *post hoc* Dunnett's test or using student's *t-test* if only two groups were applied. All data were expressed as mean ± SEM, and the accepted level of statistical significance for all experiments was $p<0.05$.

RESULTS

The VLO Lesion Impairs Acquisition of F-CPA

Dilute formalin selectively activates nociceptive Aδ and C-fiber primary afferent nociceptors [33] and that s.c. formalin injection is painful to humans [34]. In rats, hindpaw formalin injection produced both acute nociceptive behaviors and CPA, indicating that formalin is aversive to the animal in a manner resembles the response to noxious stimuli in humans. When unilateral intraplantar injection of 5 % formalin was paired with a particular compartment in the place conditioning apparatus, the rats spent significant less time in this compartment on the post-conditioning day compared with the pre-conditioning day (237 ± 35 s post-conditioning vs. 474 ± 38 s pre-conditioning, paired t-test, $p<0.01$, n=8), which means that CPA was produced (Fig. **1A** and **B**). Intraplantar injection of normal saline (NS) did not produce CPA (454 ± 43 s post-conditioning vs. 485 ± 26 s pre-conditioning, paired t-test, $p>0.05$, n=8). The differences in the CPA scores between formalin- and NS-treated rats were statistically significant (Student's t-test, $p<0.01$) (Fig. 1C). Following the bilateral VLO lesion (either electrolytic or chemical lesion), rats failed to produce obvious CPA. There was no significant difference in the time spent in the formalin-paired conditioning compartment between the post-conditioning day (397 ± 43 S) and pre-conditioning day (459 ± 30 s) (Paired t-test, $p>0.05$, n=8). Sham lesion had no effect on F-CPA (Fig. 1B and 1C). Group comparisons of F-CPA scores analysis revealed a significant effect of treatment (one way ANOVA, $F_{2, 23} = 8.863$, $p<0.01$, n=8). The locations and size of the lesion in the bilateral VLO are shown in Fig.(**2**).

The VLO Lesion Fails to Change Formalin-Induced Acute Nociceptive Behaviors

It's possible that the VLO lesion simply suppressed formalin-induced persistent pain during the conditioning trails, leading to a failure in F-CPA acquisition. To address

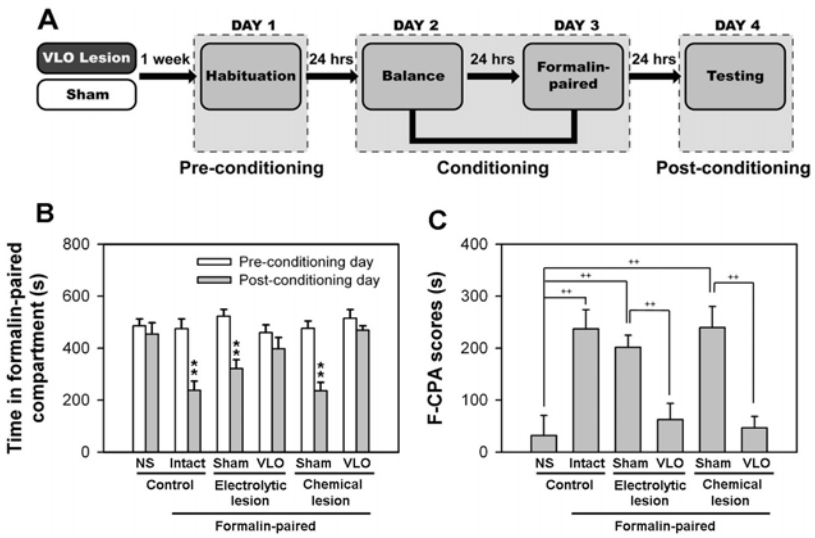

Fig. (1). The VLO contributes to the induction of F-CPA. **A**, Schematic of the protocol for behavioral testing. **B, C**, Histograms showing formalin-induced CPA and the effects of VLO lesion on F-CPA, indicated by time spent in treatment (intraplantar normal saline, NS, or formalin)-paired compartment on pre- and post-conditioning day (**B**), and CPA scores (**C**, the time spent in the treatment-paired compartment on the pre-conditioning day minus that on the post-conditioning day). ** $p<0.01$ versus pre-conditioning day. ++ $p<0.01$. Values are expressed as mean ± SEM (n=8).

this issue, we examined the effects of VLO lesion on formalin-induced nociceptive responses. Intraplantar injection of formalin elicited characteristic biphasic nociceptive behavioral responses including lifting, licking, shaking and biting. An early response (phase I) lasting for about 5 min was followed by a 5-10 min period of decreased activity, and then a late response (phase II) lasting for about 40 min. The bilateral lesions of the VLO did not change formalin-induced acute nociceptive behaviors (Fig. **3**). Two-way ANOVA revealed no significant effect of VLO lesion ($F_{1,\ 89}$ = 2.623, $p>0.05$), and no significant interaction between VLO lesions and time ($F_{8,\ 89}$ = 0.36, $p>0.05$). No obvious motor dysfunction (such as circling behavior, head weaving or limb paralysis) was produced by the VLO lesion.

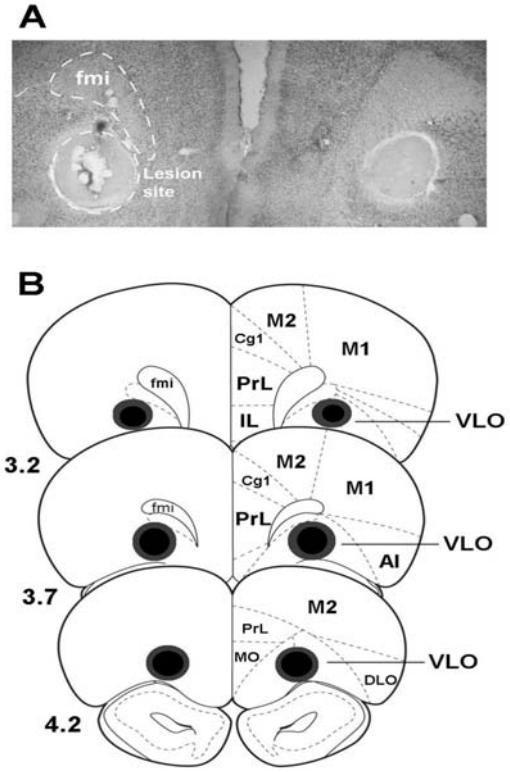

Fig. (2). Photomicrograph of coronal section showing the locations and size of the electrolytic lesion in the bilateral VLO (**A**). Histological reconstraction showing the electrolytic location and extent of the lesions (shadel area). Coronal sections taken between 3.2 and 4.2 anterior to Bregma (**B**). Cg1, coingulate cortex, area 1; M1, primary motor cortex; M2, secondary motor cortex; Prl, prelimbic cortex; fmi, forceps, minor of the corpus callosum.

The VLO Lesion Fails to Affect the Acquisition of S-CPA

To assess the effects of VLO lesion on general aversive learning, we next examined the effects of VLO lesion on mild foot shock-induced fear conditioning (S-CPA). When a lower intensity electric foot-shock (0.5 mA, 2 s) that has been demonstrated previously to fail to evoked nociceptive responses in spinal dorsal horn neurons [26], was paired with a particular compartment in the place conditioning apparatus, CPA was elicited. The time spent in the foot-shock-paired conditioning compartment was significantly different between the post-conditioning day (79 ± 16 s) and pre-conditioning day (413 ± 35 s) (Paired t-test, $p<0.01$, n=8). The bilateral lesions of VLO did not abolish the S-CPA (Fig. **4A**). The differences in the S-CPA scores among VLO lesion, sham VLO lesion, and VLO intact groups were not significant (One-way ANOVA, $F_{2,\ 21}$ = 0.168, $p>0.05$, n=8) (Fig. **4B**).

Formalin Nociceptive Conditioning Up-Regulates pCREB and Fos Expression in the VLO

The level of CREB phosphorylation in the VLO neurons was analyzed after a single unilateral injection of formalin in a hindpaw with contextual conditioning exposure. CREB phosphorylation was detected using a specific antibody raised against the serine-133-phosphorylated form of CREB (pCREB). Rats received the same training trial in days 1-3 of conditioning procedure as F-CPA but remaining in formalin-

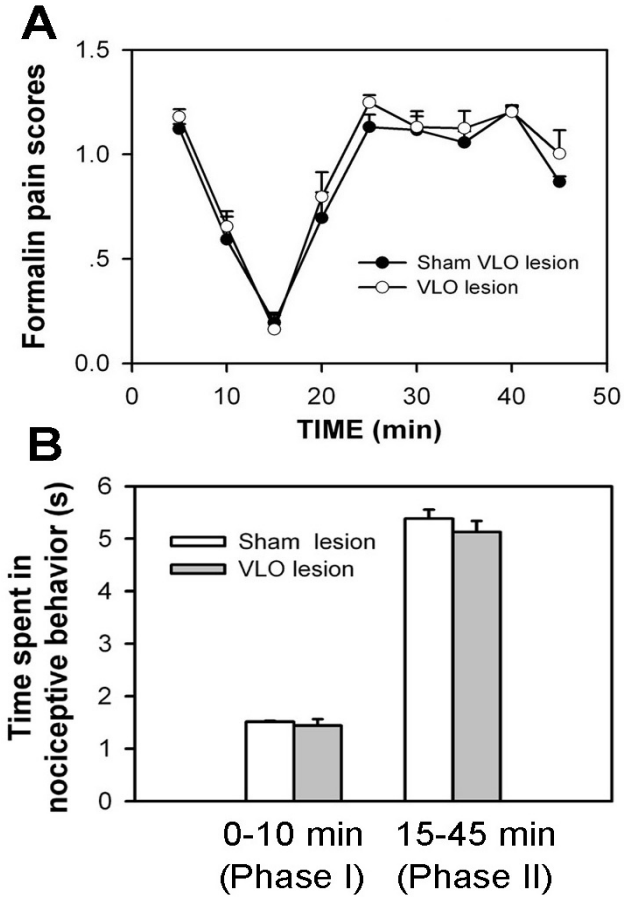

Fig. (3). Effects of VLO lesion on formalin-induced biphasic nociceptive responses. A, formalin-pain scores [(time spent with injected paw elevated + 2 × time spent licking or biting injected paw)/300]. B, Results from (**A**) were grouped into two phases.

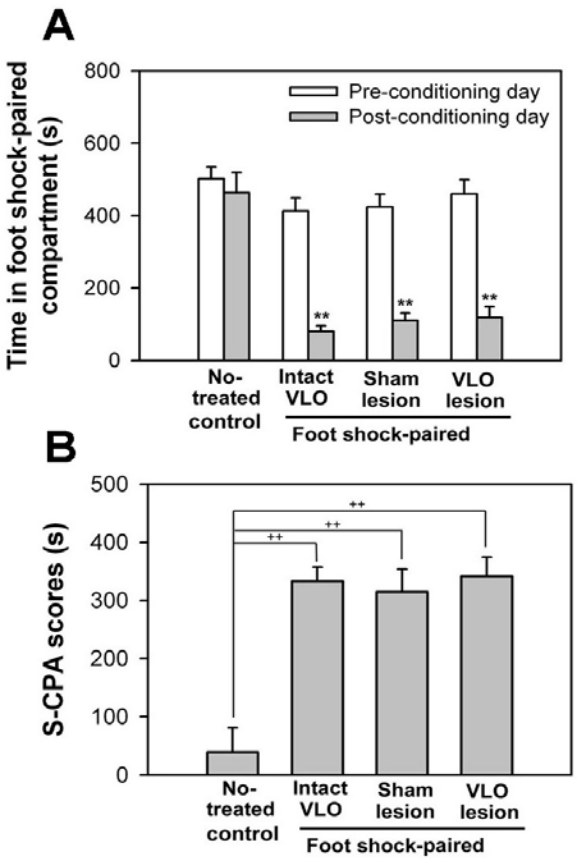

Fig. (4). Histograms showing low intensity electric foot shock-induced CPA and the effects of VLO lesion on S-CPA, indicated by time spent in treatment-paired compartment on pre- and post-conditioning day (**A**), and CPA scores (**B**). ** $p<0.01$ versus pre-conditioning day. ++ $p<0.01$. Values are expressed as mean ± SEM (n=8).

paired compartment for 45 min, then scarification after pre-defined times on day 3 (Fig. **5A**). In no-treated control rats, there is basal pCREB expression. In control rats, basal pCREB expression was present and restricted to nuclei (Fig. **5B** and **6**). Formalin nociceptive conditioning induced a significant increase in pCREB-positive nuclei in the bilateral VLO with a peak at 45 min and 2 hrs after formalin injection. The number of pCREB-positive nuclei showed a clear decrease at 6 hrs, but still remained higher than baseline after 24 hrs (One-way ANOVA, Ipsi. $F_{5, 22} = 48.38$, $p<0.01$; Contr. $F_{5, 22} = 40.49$, $p<0.01$). Intraplantar injection of NS did not induced significant pCREB upregulation in the VLO (Fig. **5B** and **5C**).

Formalin-induced pCREB colocalized with the neuronal marker NeuN, indicating that pCREB confined to the VLO neurons (Fig. **6A**). We found no evidence of pCREB in astrocytes and microglia, although some GFAP (an astrocytic marker)- or OX-42 (a microglia marker)-positive cells were positioned in close content with pCREB-positive nuclei with overlapping processes (Fig. **6A**).

Since CREB phosphorylation may regulate transcription of c-fos, and formalin injection results in an increase of Fos-positive neurons in the anterior cingulate cortex (ACC) and some intralaminar thalamic nuclei that project to the VLO [35, 36], we therefore examined formalin nociceptive conditioning-induced c-fos expression in the VLO. In no-treated control rats, only a few Fos-positive neurons could be detected in the VLO. Formalin nociceptive conditioning induced a significant increase in c-fos expression level in the bilateral VLO, with a similar time course to pCREB (Fig. **7**). The total number of Fos-positive neurons in the VLO was much lower than that of pCREB-positive neurons, and almost all Fos-positive neurons were also pCREB positive (Fig. **6B**, and **7**).

F-CPA Retrieval Activates CREB and c-fos in the VLO

To investigate whether the pain experience retrieval induces pCREB and Fos expression in the VLO during arouse of pain-related memory, rats were re-exposed to the conditioning context (formalin-paired compartment) for 10 min on day 4 of the F-CPA paradigm (Fig. **8A**). A significant increase in both pCREB and Fos expression in the bilateral VLO was observed 2 hrs after F-CPA retrieval. Rats that received the same treatment in day 1-3 of conditioning procedure as F-CPA but remaining in their home cage instead of re-exposure on day 4 (non-retrieval controls) showed a significant lower pCREB and Fos expression level, as compared with re-exposed rats (Student's t-test, $p<0.01$, n=4-5) (Fig. **8B** and **8C**).

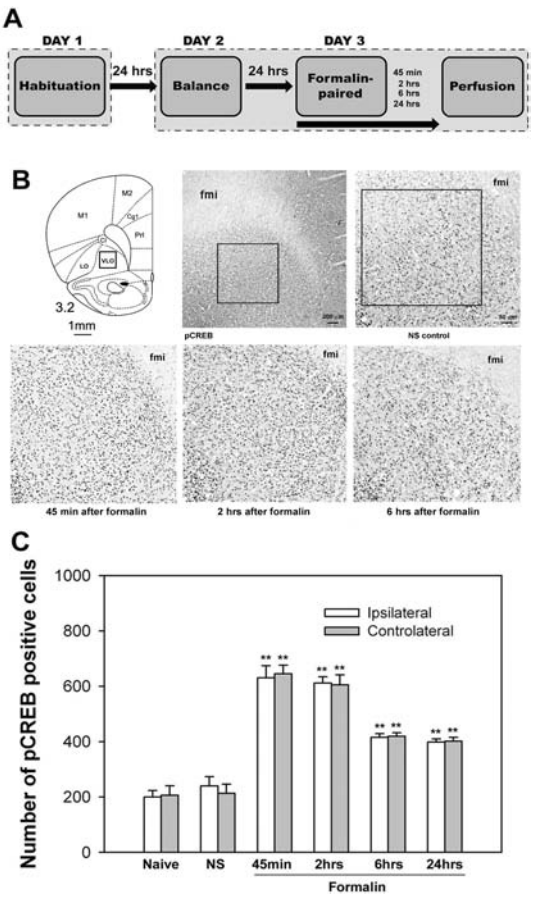

Fig. (5). Time course of CREB phosphorylation in the VLO evoked by unilateral intraplantar (i.pl.) injection of formalin with contextual conditioning exposure. **A**, Schematic of experimental designed in subsequent studies. **B**, Photomicrographs showing pCREB immunoreactivity in the VLO at 2hrs after i.pl. NS, and 45 min, 2 hrs, and 6 hrs after formalin injection. **C**, Numbers of pCREB-positive nuclei in the bilateral VLO are significantly greater in formalin treated rats than that of NS controls. ** $p<0.01$ versus NS control. Values are expressed as mean ± SEM (n=4). Cg1, coingulate cortex, area 1; M1, primary motor cortex; M2, secondary motor cortex; Prl, prelimbic cortex; LO, lateral orbital cortex; fmi, forceps, minor of the corpus callosum.

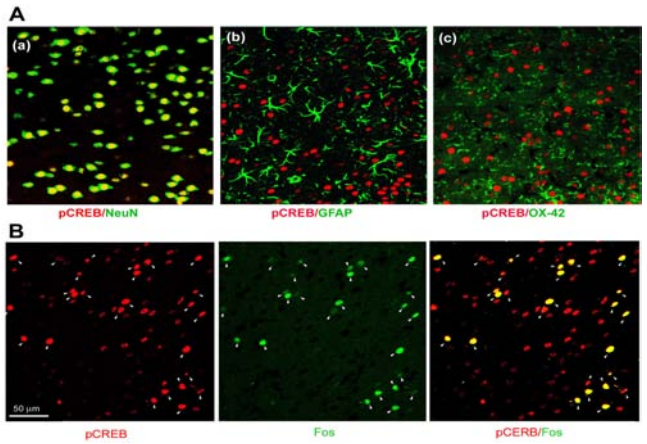

Fig. (6). Double immunofluorescence reveals that pCREB (red) co-localizes with NeuN (**Aa**, green) and Fos (**B**, green), but dose not co-localize with GFAP (a marker of astrocyte; **Ab**, green) and OX-42 (a marker of microglia; **Ac**, green) in the VLO after formalin injection. Arrowheads indicate pCREB and Fos double labeled nuclei.

DISCUSSION

There are numerous reports that in chronic pain patient, prefrontal lobotomy can relieve suffering without abolishing the "sensations" of pain [37]. The orbital area is a main component of the prefrontal cortex comprising the orbital surface of frontal lobe [38, 39]. Anatomically, the VLO receives projections from the nucleus submedius (Sm) of medial thalamic nuclei, a region which has been consider to subserve the affective-motivational aspect of pain [40], and has strong connections with other cortical and subcortical areas, such as the amygdala, anterior cingulate cortex (ACC), insular cortex, perirhinal cortex, inferior temporal cortex, and olfactory cortical areas, which are all implicated in emotion, cognition, memory, behavioral control and pain [41-45]. Electrophysiological studies in rats [10, 46, 47] and cats [2] have demonstrated that the majority of neurons in the VLO respond to noxious somatic and/or visceral stimulation. The activated VLO neurons have large and bilateral peripheral receptive fields, and the responses of some neurons outlast the application of the stimuli by many minutes. Thus, the absence of information concerning modality and stimulus location, and the prolonged neuronal responses to transient noxious stimuli in the responses of VLO neurons supported the proposal of Craig *et al.* [7] that the VLO might be involved in the affective aspect of pain [2].

In the present study, we revealed for the first time that bilateral electrolytic or chemical lesion of the VLO abolished F-CPA. This effect is unlikely to be related to a non-specific reduction in supraspinal nociceptive processing, because the VLO lesion did not affect acute formalin-induced nociceptive behaviors, despite that the VLO has been demonstrated to play an important role in pain modulation, especially in mediating Sm stimulation-induced anti-nociceptive effect [4, 14, 48]. In support of the present results, our earlier study showed that either electrolytic lesion of the VLO or microinjection of GABA into the VLO did not affect radiant heat-evoked tail flick (TF) reflex in lightly anesthetized rats [15, 49]. In contrast, Baliki *et al.* [50] reported that selectively blocking the VLO by microinjection lidocaine, electrolytic lesion, or kainic acid chemical lesion significantly depressed the neuropathic pain in spared nerve injury (SNI) rats. Also, clinically, surgical lesion of the VLO and surrounding cortical tissue gave the patient relief from chronic pain [51]. This discrepancy may result from difference in types of pain. It appears that the VLO lesion depresses pathological or clinical pain rather than acute pain, such as tail-flick reflex and formalin-induced acute inflammatory pain.

Several lines of evidence suggested that the VLO is involved in both ascending and descending nociceptive pathways. An ascending spino-Sm-VLO pain pathway has been mentioned above. Unlike the remaining parts of the prefrontal cortex, the VLO sent efference descending to the

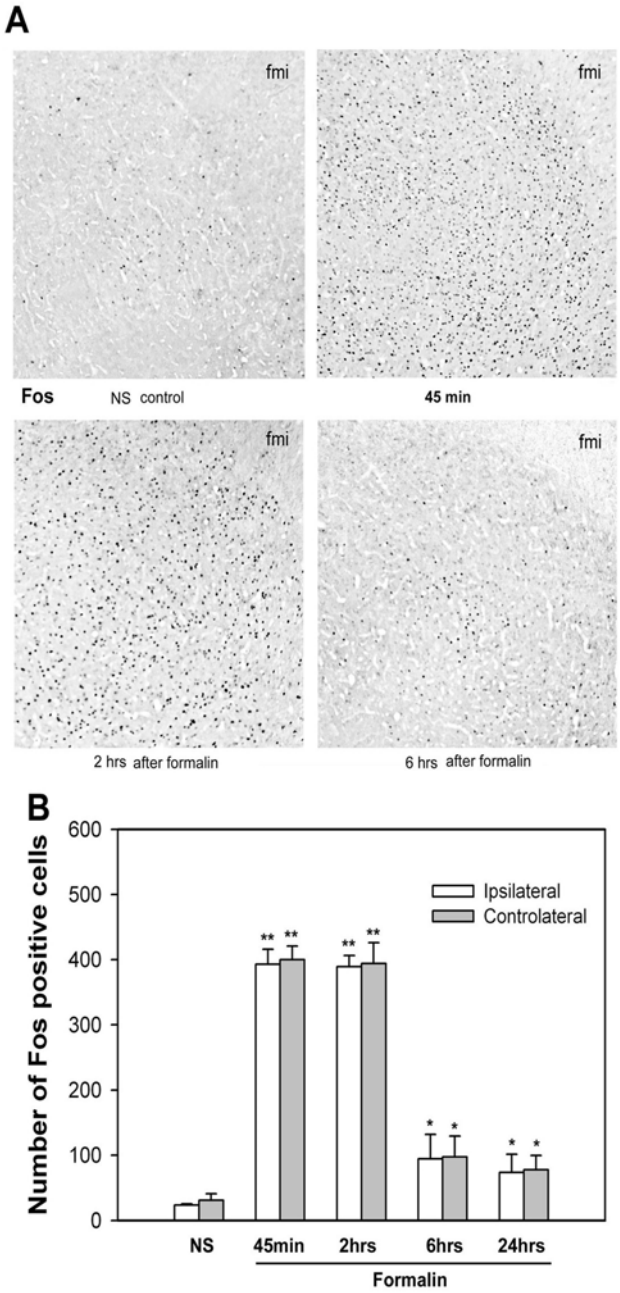

Fig. (7). Time course of Fos expression in the VLO evoked by unilateral intraplantar injection of formalin with contextual conditioning exposure. **A**, Photomicrographs showing Fos immunoreactivity in the VLO at 2hrs after i.pl. NS, and 45 min, 2 hrs, and 6 hrs after formalin injection. **B**, Numbers of Fos-positive nuclei in the bilateral VLO are significantly greater in formalin treated rats than that of NS controls. * $p<0.05$ and ** $p<0.01$ versus NS control. Values are expressed as mean ± SEM (n=4).

ventrolateral parts of the periaqueductal gray (PAG) [7, 52, 53]. Stimulation of the VLO can influence the firing of neurons in the PAG and rostral-ventro-medial medulla (RVM) [54]. Studies from Tang's laboratory suggested that this descending pathway may be a substrate for the anti-nociceptive effect of the VLO [4, 14]. Taking these findings together with our present study, it suggests the involvement of the VLO in either pain-related aversive emotion or pain modulation.

Given that F-CPA paradigm is based on the associative learning between painful stimulation-induced aversive emotion and environmental context, an inevitable question is whether the VLO lesion causes a disruption in neural processing relating to associative learning rather than reducing pain-related aversion. Actually, the VLO has been implicated in acquisition of some physical and social behaviors. For example, Tranel *et al.* [3] have reported a case of "acquired sociopathy" following bilateral lesion of the VLO. The fundamental characteristic of sociopathy is a reduced capacity in learning in response to either socially or physically administered negative reinforcement. Although we can not completely rule out this possibility, it is important to point out that the VLO lesion did not abolish the acquisition of CPA evoked by a low-intensity of electric

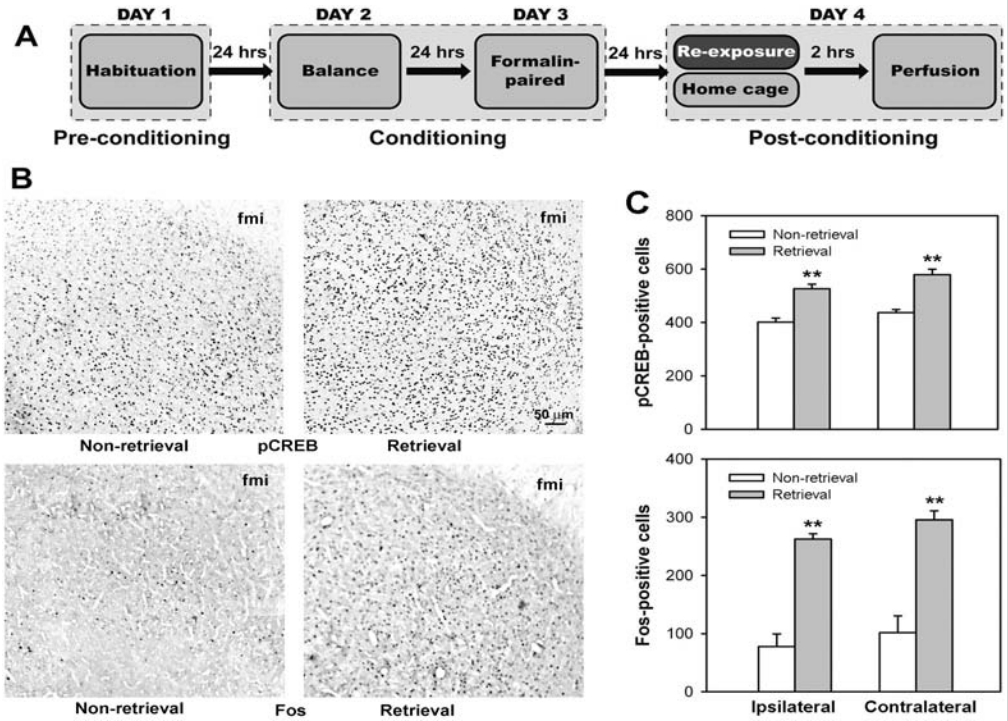

Fig. (8). CREB phosphorylation and Fos expression in the VLO evoked by F-CPA retrieval. **A**, Schematic of experimental design used in subsequent studies. **B**, Immunohistochemistry for pCREB (upper) and Fos (low) in the VLO representative of control (non-retrieval) and 2 hrs after re-exposure to the conditioning context (retrieval). **C**, Numbers of pCREB- and Fos-positive nuclei in the VLO are significantly greater in F-CPA retrieval rats than that of non-retrieval rats. ** $p<0.01$. Values are expressed as mean ± SEM (n=4).

foot-shock (0.5 mA, 2s) that has been demonstrated previously to fail to evoke nociceptive responses in spinal dorsal horn neurons [26]. Thus, it is reasonable to conclude that the VLO is particularly important for pain-related negative emotion and pain-related aversive learning rather than general aversive learning.

In addition to the VLO, there are some other brain regions which are thought to be involved in affective responses to pain. The ACC is a notable candidate related to the negative emotion of pain. Studies from our and other laboratories have shown that the ACC activation is necessary for the induction of pain-related negative emotion [26, 35, 55-58]. Another candidate related to affective pain is amygdala. Bilateral lesion of the amygdala has been reported to abolish F-CPA [26, 27]. Since there are direct and indirect connections among the VLO, ACC and amygdala [41-43], it is likely that the neural network of these structures is important for the negative affect of pain.

Both the formation of new long-term memories and memory retrieval is known to require gene expression and protein synthesis [59, 60]. The importance of CREB in transcriptional regulation and long lasting memories has been demonstrated *in vivo* [16, 19, 20, 61, 62]. *c-fos* is one of the first identified CREB target genes, whose expression is induced in a CRE-dependent manner in response to certain stimuli that activate CREB [24, 63]. In the present study, another important finding is that not only formalin nociceptive conditioning but also retrieval of formalin pain experience induced CREB phosphorylation and *c-fos* expression in the VLO. This result suggests that molecular events underlying gene expression and protein synthesis during the induction of pain-related aversion are re-activated during retrieval of pain experience. Our previous studies have shown that F-CPA retrieval induced Fos expression in the ACC, and the manipulation that eliminated F-CPA also blocked F-CPA retrieval-induced Fos expression in the ACC [35, 36]. Like Fos expression, CREB phosphorylation could serve as a marker for the activation of VLO neurons under an affective pain condition.

Traditionally, retrieval has been related to the hippocampus [64-66]. However, studies from Izquierdo's laboratory have shown that memory retrieval requires a coordination of several brain regions, including the hippocampus, basolateral amygdala, entorhind, posterior parietal, and ACC [67-69]. The present results further suggest the involvement of the VLO in the induction of pain-related negative emotion and pain experience retrieval.

Sustained activation of CREB phosphorylation has been suggested to be required for the activation of gene transcription [70, 71]. The present results showed a persistent (>24 hrs) up-regulation of pCREB in the VLO after formalin injection with contextual conditioning exposure. An accompanying increase in Fos, the protein product of a CREB-regulated gene, may also be indicative of such sustained CREB phosphorylation. pCREB, therefore, may regulate the expression of other immediate-early genes (IEGs) and late response genes, such as *zif 268 and BDNA* [19, 62]. However, target genes that are regulated by CREB-mediated transcription in the VLO and the role of these genes in pain affect remain to be investigated.

In summary, our data indicated that bilateral electrolytic lesion of the VLO abolished the F-CPA, but did not affect

acute formalin-induced nociceptive behaviors and low-intensity of electric foot shock-induced aversive learning. Furthermore, a sustained CREB phosphorylation concurrent with increased Fos expression in the VLO was observed following nociceptive conditioning. Also, pain experience retrieval produced increases in pCREB and Fos expression levels. We conclude that the VLO contributes to both induction and retrieval of pain-related aversion.

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