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A retinal-binding protein mediates olfactory attraction in the migratory locusts

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ABSTRACT

Cellular retinaldehyde-binding protein (CRALBP) is abundantly expressed in retina and its mutations are associated with visual impairments. The functions of CRALBP are less known in extra retinal tissues. Herein, we study the function of CRALBP in modulating olfactory behaviors in gregarious and solitary locusts. The expressions of *cralbp* mRNA and protein were enriched in locust brains and antennae. RNAi knockdown of *cralbp* in gregarious locusts decreased their attractive response to gregarious volatiles. RNA-seq and quantitative PCR confirmed that *cralbp* mRNA and protein expression levels were upregulated and downregulated after octopamine receptor α1 (OctαR1) activation and inhibition, respectively. Gene network analysis revealed that *cralbp* is the core hub gene in the interactive network among differentially expressed transcripts (DETs) resulting from activating and inhibiting OctαR1. Moreover, *cralbp* RNAi knockdown inhibited the induction of olfactory attraction by octopamine (OA)-OctαR1 signaling. CRALBP helped to transmit OA signals to mediate olfactory attraction response to guaiacol and veratrole, which are two odorant components in gregarious volatiles. This study suggested that CRALBP may act as a novel effector protein in OctαR1 signaling to mediate olfactory attraction. This study indicated that CRALBP modulates olfactory attraction in extra retina tissues and retinaldehyde metabolism may be crucial for olfactory attraction modulation.

1. Introduction

Cellular retinaldehyde-binding protein (CRALBP) is a water soluble protein that belongs to the CRAL-Trio famlily (Saari and Crabb, 2005; Wu et al., 2004). CRALBP functions in the visual cycle by modulating isomerization of all-*trans*- to 11-*cis*-retinaldehyde, and it has been reported to express in ciliary body, cornea, pineal gland, optic nerve, and brain in mammals (Saari and Crabb, 2005). Mutations of the human gene encoding CRALBP are associate with visual impairments characterized by night blindness and progressive photoreceptor degeneration (Saari et al., 2001; Maw et al., 1997). Other studies have found that CRALBP is expressed in oligodendrocytes and glia cells in the brain and pinal gland (Saari et al., 1997). Apparently, these studies suggested that CRALBP may play roles in the brain other than in eyes. However, the functions of CRALBP are less known in extra retinal tissues.

CRALBP is a conservative protein and expresses in animals from invertebrates to vertebrates. In flies, genetic linkage analysis identified the association of genetic locus of *cralbp* with odor-guided behavior (Anholt and Mackay, 2001). In mice, *cralbp* mRNA has been shown to be expressed in striatum, glomerular layer and inner granular layer of the olfactory bulb, olfactory nerve, and the cells clustered near the hypothalamus (Tsuda et al., 2003; Zetterström et al., 1999). Moreover, this gene is identified as a potential contributor to ethanol preference in mice (Treadwell et al., 2004). The functions of CRALBP in regulating behaviors are less well known until now.

The migratory locust is a representative animal model showing the traits of olfactory attraction and repulsion (Guo et al., 2011; Ma et al., 2016). This insect exhibits phase polyphenism, which includes gregarious and solitary phases, in response to the fluctuation in population density. In high population density, gregarious locusts are active and exhibit an attractive response to their gregarious conspecifics. By contrast, in a low population density, solitary locusts are sedative and show an aversive response to their siblings (Pener and Simpson, 2009; Wang and Kang, 2014). Gregarious volatiles are vital for aggregation of the migratory locusts, and those volatiles elicit two different behavioral responses in gregarious and solitary locusts, namely, olfactory attraction and olfactory repulsion (Guo et al., 2011, W. Guo et al., 2018; X. J. Guo et al., 2018; Ma et al., 2015). The choice between these two forms of behaviors is considered as sensational decision dependent on population density (Ma et al., 2016). This heavy reliance on olfactory guidance has bestowed locusts the attractive advantage to dissect molecular mechanism underlying olfactory sensation. The choice between

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Abbreviations

CRALBP	cellular retinaldehyde-binding protein
DETs	differentially expressed transcripts
dsRNAs	double-stranded RNA
EAG	Electroantennography
FISH	fluorescence in situ hybridization
GPCR	G protein-coupled receptor
MMLV	Moloney murine leukemia virus
OA	octopamine
OctαR1	octopamine receptor α1
RA	retinoic acid

olfactory attraction and aversion in locusts will provide a powerful behavioral model to study the underlying molecular mechanism. Thus, we intend to use this insect as an animal model to study the possible functions of CRALBP in regulating olfactory behaviors.

In this study, we found that CRALBP mediates olfactory attraction to gregarious volatiles in the migratory locusts, and also mediates electrophysiology response to odorant chemicals in the natural mixture of their own volatiles. CRALBP is the effector protein in octopamine receptor α1 (OctαR1) signaling, and *cralbp* RNAi knockdown inhibits the function of octopamine (OA)-OctαR1 signaling in modulating olfactory attraction. This study firstly reported that the *cralbp* protein mediates olfactory attraction other than in eyes and acts as the downstream effector protein in the OctαR1 signaling pathway for olfactory perception.

2. Materials and methods

2.1. Animals

Locust samples were collected from a colony maintained at the Institute of Zoology, Chinese Academy of Sciences, Beijing, China. Gregarious locusts were cultured at a density of 500–1000 insects in per box (40 cm × 40 cm × 40 cm) for at least three generations before experimentation. Solitary locusts were isolated from the gregarious colony and cultured individually in white metal boxes (10 cm × 10 cm × 25 cm) for at least three generations before experimentation. Charcoal-filtered compressed air was supplied continually. The locust colony was maintained under a 14 h light/10 h dark cycle at 30 ± 2 °C and fed on fresh wheat seedlings and bran (Ma et al., 2011).

2.2. RNA preparation and qRT-PCR assay for mRNA

Total RNA was extracted from the brain tissue according to the RNeasy Mini kit (Qiagen) before using DNAase to eliminate DNA contamination. To analyze the expression levels of target genes in this study, we reverse transcribed 2 μg of total RNA from every sample following the manufacturer's instructions using Moloney murine leukemia virus (MMLV) (Promega, Madison, USA). PCR amplification was conducted in Roche Light Cycler 480 using a Real Master-Mix (SYBR Green) kit (Tiangen, Beijing, China). The amplification procedure was initiated with 95 °C for 15 min followed by 40 cycles of 95 °C for 20s; 58 °C for 20 s; 68 °C for 20 s. *RP-49* was used as an endogenous control for mRNAs. The amplification procedure was following the manufacturer protocol of Kits and the melting curve was detected to confirm the amplification specificity of the target genes. All PCR amplicons were sequenced to verify the specificity of the primers. The primers for qRT-PCR assay are provided in Table S1.

2.3. RNA-seq and data processing

After injecting 25 ng of double-stranded RNA of OctαR1 (dsOctαR1) or GFP (dsGFP) into the brains of the fourth-stadium gregarious locusts, we collected the brain samples of the gregarious locusts for RNA extraction 72 h later. OA (69 nl, 5 mM) was injected into the brains of solitary locusts, and brain samples were collected at 1 h after injection. Three independent replicates were prepared for each group, and each replicate contained 10 brains (5 males and 5 females). Total RNA was extracted according to TRIzol protocol (Life Technologies), and RNA quality was confirmed by agarose gel electrophoresis. We prepared the cDNA libraries according to Illumina's protocols and performed paired-end sequencing. The raw reads were cleaned and then assembled by Trinity software (version 2011-08-20) to obtain reference transcripts with respect to locust genome data (Grabherr et al., 2011). We used Bowtie and RSEM modules in Trinity software (version 2011-08-20) for read alignment and abundance estimation. The differentially expressed transcripts (DETs) were analyzed using edgeR software (Robinson et al., 2010), and those transcripts with a fold change > 1.5 and $P < 0.05$ were selected as DETs. Blast2GO software was used to annotate and enrich the DETs (Conesa et al., 2005). Mint and Cytoscape software were used for network prediction and illustration (Meyer et al., 2008; Shannon et al., 2003).

2.4. Phylogenetic analysis of cralbp

To confirm *cralbp* and its relationship with other species, we cloned sequences of *cralbp* by referring to the putative sequences in genome and transcriptome database of the migratory locusts (Wang et al., 2014). The sequences for phylogenetic analysis were downloaded from the NCBI database. Multiple sequence alignments in *cralbp* were performed in Clustal W and curated in MEGA5.34 (Tamura et al., 2011) to define *cralbp*. Neighbor-joining analysis was performed using MEGA 5.34 software with 1000 bootstrap replicates.

2.5. In situ hybridization of cralbp

We performed fluorescence in situ hybridization (FISH) to analyze the spatial expression of the *cralbp* mRNA in the locust brain. The *cralbp* fragment (229 bp) was prepared for antisense and sense probes. We blasted these fragments against genome database of the migratory locusts to detect homologies and avoid nonspecificity in hybridization. The primers for the *cralbp* fragment were described in Table S2. After fixing the dissected brains in 4% formaldehyde for 2 h at room temperature, we washed those dissected brains 2 times for 15 min each in 0.1M phosphate-buffered saline (PBS, pH 7.4) and treated with 20 μg/ml Proteinase K in PBS for 2 h at 37 °C. The treated brains were then washed for 15 min in 0.1 M PBS (pH 7.4) for 3 times and refixed before incubation in prehybridization solution (Boster, Wuhan, China) for 2 h at 65 °C. The prehybridization solution containing 3 μg/ml digoxigenin–labeled probes was used for overnight hybridization in a humidified chamber at 65 °C. Then, washing was carried out in 4 ×, 2 ×, 1 ×, and 0.2 × saline-sodium citrate (SSC) buffer at 65 °C for 60 min for each wash. To detect the presence of hybrids, the brains were incubated with mouse anti–digoxigenin antibody conjugated with Dylight 488 (Jackson ImmunoResearch, 1:300 dilution) overnight at 4 °C. Hybridization was performed on the whole-mount brains, and the hybridized tissues were examined using a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany).

2.6. Western blotting (WB) analysis

The locust brains with associated treatments were collected and homogenized in TRIzol reagent (Life Technologies) for protein extraction according to the manufacturer's instructions. Six independent biological samples were prepared for all the WB experiments (8–10

individuals/sample). Affinity-purified polyclonal antibodies against *cralbp* (rabbit) (BGI, Beijing, China) were developed (Beijing Protein Innovation Co., Ltd., BPI). The protein samples (80 μg) were separated by gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Millipore). Bovine serum albumin (5%) was used to block the nonspecific binding sites on the membranes. The primary antibody rabbit anti-CRALBP serum (1:500) was incubated with the protein-transferred membrane in Tris-buffered saline-Tween (TBS-T) overnight at 4 °C. After overnight incubation, the membranes were washed, and then incubated with an anti-rabbit IgG secondary antibody (1:5000) (CWBIO, China) for 1 h at 25 °C. Highly sensitive 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (CWBIO, China) was used for immunological detection. The GAPDH antibody (CWBIO, China) was used to calibrate the relative expressions of target protein. The intensities of WB signals were quantified using densitometry.

2.7. RNAi and behavioral assay

To inhibit *cralbp* expression, we designed the fragment of the *cralbp* sequence for RNAi and blasted the target fragments against the *Locusta* genome database to detect sequence homologies. Then, we used the fragment without any homologies in genome database to avoid non-specific RNAi knockdown (Wang et al., 2014). Double-stranded RNA (dsRNA) was synthesized using the T7 RiboMAX Express RNAi system (Promega, Madison, USA). The primers for RNAi are described in Table S3. Before injecting 25 ng dsRNA into the brains of fourth-stadium gregarious locusts, the fourth-stadium locusts were placed in a Kopf stereotaxic frame that was specifically adapted for locust surgery. All injections were performed under a dissecting microscope using a Nanoliter injector 2000 (World Precision Instruments, Sarasota, FL, USA) with a glass micropipette tip. The injected gregarious locusts were housed in a cage with gregarious locusts for an additional 3 days before the behavioral assay. The efficiency of RNAi on mRNA expression was investigated by qRT–PCR after 3 days of injection.

2.8. Behavioral pharmacology after RNAi knockdown

To validate the roles of OA in modulating the behavior state of gregarious locusts by *cralbp*, we directly injected OA (100 μg, 25 μg/μl) (Sigma–Aldrich) into the thoracic cavities of gregarious locusts treated with *cralbp* RNAi knockdown (Ma et al., 2015), and their behaviors were assessed 1 h later. The dsGFP-injected groups received the same volume of saline and were used as controls for the behavioral assay.

2.9. Olfactory behavior assay in a Y-tube

The olfactory behavior was examined in a Y-tube. The size of this Y-tube is referred to our previous work (Ma et al., 2015; Guo et al., 2018b). The assays using the Y-tube were arranged in a room with the sole purpose for an olfactory behavioral assay. A Y-tube was placed in an enclosed and sound-insulated box with a transparent window to avoid disturbing by observers.

Each locust was observed for 4 min and examined only once. Whenever a locust moved greater than 4 cm into the volatile or empty air arm within 4 min, the individual locust was recorded considering the first choice for either arm. To quantify the choice behavior, we directly calculated the percentage of locusts that chose either the volatile arm or the air arm.

The odorants consisted of the volatiles (including volatiles from the body and feces) from 30 fourth-stadium gregarious locusts. We used the device we reported previously to collect the volatiles (Ma et al., 2015; Guo et al., 2018b). The volatiles were delivered to either arm to eliminate the possibility of spatial bias. In addition, the air flow was set at 300 ml/min.

Moreover, we analyzed the olfactory response of locusts to guaiacol, veratrole, 1-pentanol and benzaldehyde (Sigma-Aldrich), which are the odorant components in gregarious volatiles (Wei et al., 2017). These chemicals were dissolved in mineral oil with gradient concentration for the behavioral assay. Mineral oil has no obvious effects on olfactory behaviors of the migratory locusts, and we used this oil as the empty control (Wei et al., 2017).

We assessed olfactory preferences by providing each locust with a single choice between gregarious volatiles and air in the Y-tube, and each locust was considered a replicate. For statistical analysis, we pooled all tested insects and calculated the significance using the *G*-test for independence. To quantify the choice behavior, we directly calculated the percentage of locusts that selected the volatile arm or the odorant arm among all tested locusts.

2.10. Electroantennography (EAG)

To investigate the neurophysiological responses in the olfactory periphery of the migratory locusts to the odorant chemicals, EAG tests were conducted using the entire antenna of the fourth-stadium gregarious locusts with or without RNAi knockdown. Antennal recordings were conducted using Ag–AgCl glass electrodes filled with saline solution. An antenna was freshly amputated at the base from the live locusts and suspended between the two electrodes. The tip of the terminal process of the antenna was removed to ensure good contact with a high-impedance amplifier (UN-06; Ockenfels Syntech GmbH, Kirchzarten, Germany). The base of the antenna was conducted to a ground electrode. The odorants were diluted with mineral oil and dropped on the filter paper filling inside of a standard Pasteur pipette as the odor source. Air or filter paper with mineral oil were used to correct the values before each measurement. The airflow velocity used for gas stimulation was 30 m/s. When a switch triggered the gas to stimulate the antennae, the instrument recorded the generated potential in the tested locusts. Ten replicate antennae from 10 locusts were used per odorants. For statistical analysis, we pooled all tested insects and calculated the significance using Student's *t*-test (mean ± SEM).

2.11. Statistical analysis

Probabilistic metrics of solitariness (*P-sol*) for behavioral state evaluation were analyzed by the Mann–Whitney *U* (MWU) test to detect the significance of phase change. The expression levels of *cralbp* in gregarious and solitary locusts were analyzed by Student's *t*-test. Statistical analysis of olfactory preference was conducted according to previous studies on locust aversive learning (Simões et al., 2012) and locust olfactory decisions (Ma et al., 2015). Statistical comparisons between treatment and control groups with respect to volatile preference were analyzed by *G-tests* for independence, and all tested locusts were included in this analysis. When the counts of olfactory response are low, we chose Yate's continuity correction to avoid the overestimation of significance for volatile preference (Sokal and Rohlf, 1998). The standard error of the volatile preference of locusts was calculated as $\sqrt{p}$ $(1\text{-}p)/n$, where p is the proportion of locusts that were attracted or repulsed by the volatiles and n is the number of tested locusts (Collett, 2002).

3. Results

3.1. CRALBP is possibly associated with olfactory behaviors

CRALBP has been suggested to be linked with odor-guided behavior (Anholt and Mackay, 2001). Migratory locusts show olfactory behavioral attraction and repulsion (Guo et al., 2011; Ma et al., 2015). Thus, we intended to apply the migratory locust as an animal model to analyze the linkage of CRALBP with olfactory behaviors. To distinguish locust *cralbp* from other species and validate its function, we performed a phylogenetic analysis using 15 representative sequences and found

the conservative relationship of *cralbp* in the migratory locusts with those in other species (Fig. 1A). Locust CRALBP has CRAL_TRIO domain and SEC14 domain (Fig. S1). To explore the elegant function of *cralbp* associated with olfactory perception in locusts, we first analyzed *cralbp* expression levels in different tissues. We found that *cralbp* did not show differential expressions at the mRNA levels among the detected tissues, but the *cralbp* protein showed differential expression patterns among them (Fig. 1B and C). Most strikingly, the *cralbp* protein is relatively enriched in antennae, brains and optic lobes (Figs. 1C and S2). Immunofluorescent assays also indicated that *cralbp* is located in antennal lobes (Fig. 1D), suggesting the possible function of *cralbp* in olfaction in migratory locusts.

3.2. CRALBP affects olfactory attraction in gregarious locusts

Gregarious locusts show olfactory attraction, whereas solitary locusts show olfactory repulsion (Guo et al., 2011; Ma et al., 2015). We therefore investigated the functional association of *cralbp* mRNA and protein levels in the brains of gregarious and solitary locusts with their olfactory responses. The RNA-seq analysis in the comparison between gregarious and solitary locusts also showed the expression level of *cralbp* is higher in brains of gregarious locusts than those in solitary ones (Guo et al., 2018b). The qRT-PCR analysis showed that *cralbp* is expressed at higher levels in the antennae and brains of gregarious locusts than that of solitary locusts (t = 2.721, $P < 0.05$ for antennae; $t = 2.854$, $P < 0.01$ for brain) (Fig. 2A and S3). Similarly, *cralbp* protein is also expressed at a higher level in the antennae and brains of gregarious locusts than that in solitary ones ($t = 10.436$, $P < 0.01$ for antennae; $t = 1.84$, $P < 0.05$ for brain) (Fig. 2B and S3). The *cralbp* expression pattern in gregarious and solitary locusts indicated a positive correlation with olfactory behaviors, suggesting that the attractive response of gregarious locusts to their own volatiles coincides with the increased *cralbp* mRNA expression level in their brains.

Next, we aim to determine whether functional deficiency of *cralbp* prevents locusts from acquiring olfactory attraction to gregarious volatiles. A cDNA fragment clone of *cralbp* from transcriptome and genome databases of the migratory locusts was used as a template to generate a dsRNA construct. Injection of this dsRNA in gregarious locust brains caused a reduction of approximately 90% in the *cralbp* mRNA expression level at 3 days after injection ($t = 6.006$, $P < 0.001$) (Fig. 2C). The protein expression level was also decreased significantly after *cralbp* knockdown ($t = 1.89$, $P < 0.05$) (Fig. 2D and E). Furthermore, we applied a Y-tube to analyze the olfactory response of RNAi-knockdown locusts to gregarious volatiles. The results indicated that the RNAi-knockdown gregarious locusts showed a decrease in the percentage of locusts attracted to their own volatiles ($G = 30.69$, $P < 0.001$) (Fig. 2F). These results suggested that *cralbp* affects olfactory attraction in gregarious locusts.

3.3. Olfactory behaviors in locusts is not affected by cralbp in optic lobes

Because *cralbp* abnormalities or mutations lead to visual deficiency, we aimed to investigate whether olfactory attraction in locusts is associated with *cralbp* expression and its function in optic lobes. In this study, three days after dsRNA injection, we analyzed the *cralbp* mRNA expression level in the optic lobes and found that the expression level of *cralbp* mRNA decreased by over 90% as compared with dsGFP-injected controls (gregarious locusts: $t = 3.522$, $P < 0.01$; solitary locusts: $t = 3.675$, $P < 0.01$) (Fig. S4A). Moreover, the *cralbp* protein expression level also showed a similar pattern after stable RNAi knockdown (Figs. S4B and C).

To simulate the effects of *cralbp* deficiency on optic lobes, we painted and covered the complex eyes for visual deficiency. Visual deficiency in gregarious and solitary locusts did not affect their original

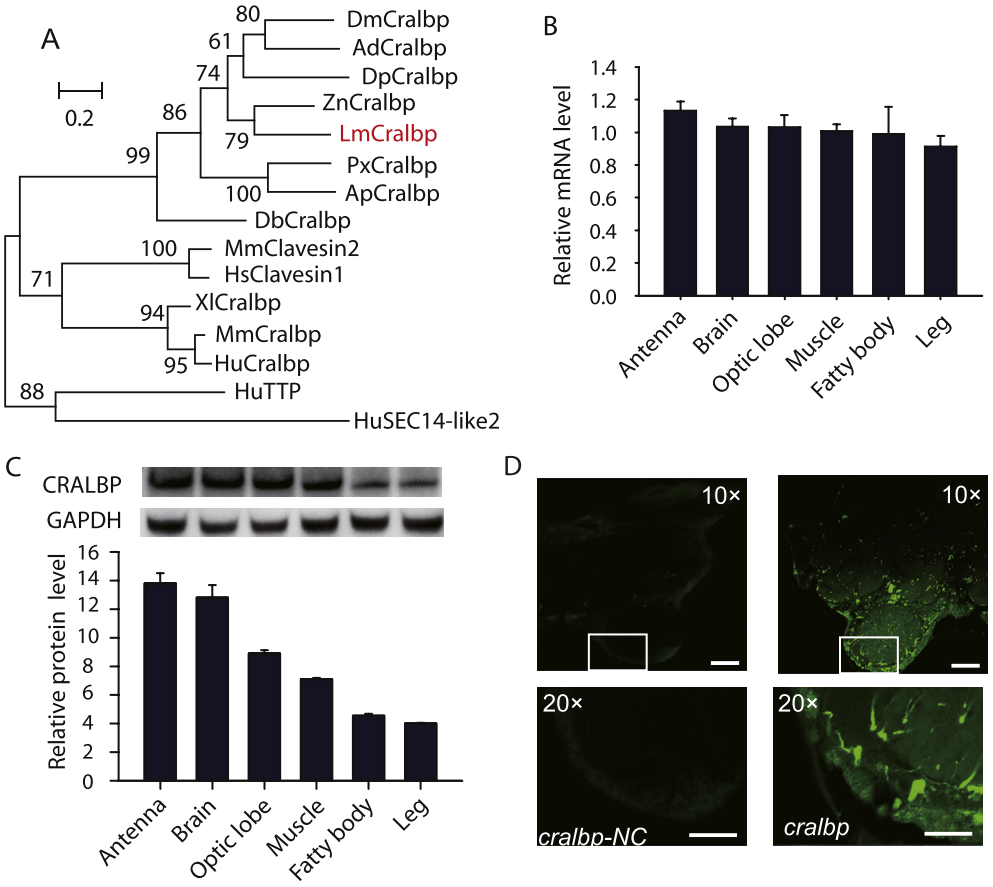

Fig. 1. *cralbp* is likely associated with olfactory perception of migratory locusts. (*A*) Phylogenetic analysis of the CRALBP superfamily in animals. (*B*) The *cralbp* mRNA expression level in various tissues of gregarious locusts (n = 6, mean ± SEM). (*C*) The CRALBP protein expression levels in various tissues of gregarious locusts (n = 6, mean ± SEM). (*D*)The localization of *cralbp* in antennal lobes in gregarious locusts. Abbreviation: NC, negative control.

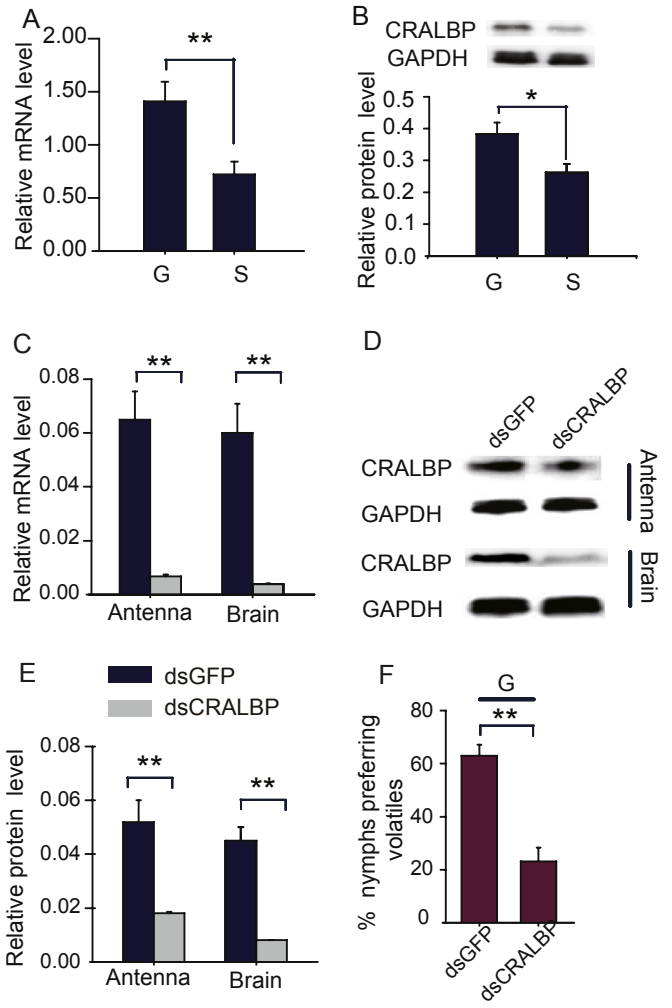

Fig. 2. *cralbp* is associated with olfactory perception. (*A*) The relative expression level of *cralbp* mRNA in the brains of gregarious and solitary locusts (n = 8). (*B*) Western blots show the expression level of CRALBP in the brains of gregarious and solitary locusts (n = 6). (*C*) The expression level of *cralbp* mRNAs in the brains and antennae after *cralbp* RNAi knockdown (n = 8). (*D*) Western blots show the expression level of CRALBP protein in the brains and antennae after *cralbp* RNAi knockdown. (*E*) The expression levels of *cralbp* mRNA in the brains and antennae after *cralbp* RNAi knockdown (n = 8). (*F*) *cralbp* RNAi knockdown significantly decreased the percentage of gregarious locusts for olfactory attraction (n = 56). The asterisks outside the strip indicate the significant differences between controls and the treatment groups by Student's *t*-test (mean ± SEM) (*A*, *B*, *C* and *E*) and *G*-test for independence (*F*). *, $P < 0.05$; **, $P < 0.01$. Abbreviations: S, solitary; G, gregarious.

group-living behaviors (MWU: gregarious, $U = 348$, $P = 0.263$; solitary, $U = 278$, $P = 0.509$) (Fig. S5). Moreover, after covering the eyes for visual deficiency, gregarious locusts still preferred gregarious volatiles and showed a similar behavioral preference to normal gregarious controls ($G = 0.05$, $P = 0.97$). Furthermore, visual deficiency of the solitary locusts also did not change the proportion of the locusts avoiding gregarious volatiles ($G_2 = 4.36$, $P = 0.11$) (Fig. S4D). Therefore, although a significant reduction in the *cralbp* expression level was identified in the optic lobe, visual deficiency did not affect the intrinsic olfactory preferences of solitary and gregarious locusts.

3.4. *cralbp* is a crucial target gene in OctαR1 signaling for olfactory attraction

OctαR1 acts as a membrane G protein-coupled receptor (GPCR) and meditates olfactory attraction in the migratory locusts. Activation of OctαR1 by octopamine in the solitary locusts induces the olfactory attraction (Ma et al., 2015). RNAi knockdown of OctαR1 in the gregarious locusts resulted in the loss of olfactory attraction (Ma et al., 2015; Xu et al., 2017). Thus, upon confirming the efficiency of OctαR1 RNAi knockdown (Student's *t*-test, $t = 15.134$, $P < 0.001$, dsGFP vs. ds OctαR1) (Fig. 3A), we analyzed the transcript expression levels after activation and inhibition of this receptor and intended to identify the possible association between *cralbp* and OctαR1.

After OctαR1 activation, 231 transcripts including *cralbp* were significantly expressed. After OctαR1 inhibition, 257 transcripts including *cralbp* were expressed differentially. Among of these transcripts, 32 transcripts including *cralbp* were simultaneously affected by activation and inhibition of OctαR1 ($P < 0.05$) (Fig. 3B; Fig. S6). Moreover, hierarchical cluster analysis classified these 32 transcripts into 4 clusters. The expression levels of the transcripts in clusters *a* and *c* showed a reversed expression pattern (Fig. 3C). Although RNA-seq method detects expression patterns of those transcripts with relatively higher expression levels, a couple of olfactory-related transcripts (*csp2*, *takeout1*, *takeout2* and *JHBP*) were identified. To identify the critical target transcript potentially mediated by OctαR1, we performed the prediction of gene network and pathway analysis. The gene network analysis showed that besides the olfactory-related transcripts *csp2* and *takeout1* in this network, *cralbp* is the crucial hub target that potentially interacts with other olfaction-related genes after activation and inhibition of OctαR1 (Fig. 3D). After OctαR1 activation, the expression level of *cralbp* was upregulated. After OctαR1 inhibition, the expression level of *cralbp* was downregulated. Moreover, we performed Fisher's exact test to identify potential signaling pathways affected by OctαR1 signaling pathway underlying olfactory preference of the migratory locusts. The results showed that the process of retinal and retinoid binding was significantly enriched ($P < 0.05$) (Fig. 3E and F). The expression of these genes in retinoid transport was activated by OA signaling and repressed after OctαR1 knockdown, respectively. Retinal and retinoid transport processes are the most significant pathways correlated with OctαR1 signaling in the migratory locusts (Fig. 3E and F). Therefore, these results suggest that *cralbp* may be the crucial target gene in OctαR1 signaling.

Moreover, we confirmed the expression of *cralbp* mRNA after activation and inhibition of this receptor, respectively. Three days after OctαR1 inhibition, the *cralbp* mRNA expression level was decreased by 75% in the brains, compared with dsGFP-injected controls ($t = 8.3$, $P < 0.01$) (Fig. 4A). In contrast, after OctαR1 activation, the *cralbp* mRNA expression level was significantly increased by approximately 50% in the brains of solitary locusts ($t = 6.4$, $P < 0.01$) (Fig. 4A). These results were consistent with the expression patterns of *cralbp* detected by RNA-seq. Moreover, Western blot analysis detected the similar expression pattern of *cralbp* protein to those detected by qRT-PCR (OctαR1 inhibition, $t = 7.367$, $P < 0.05$; OctαR1 activation, $t = 6.98$, $P < 0.05$) (Fig. 4B and C). These results suggested that OctαR1 signaling is correlated with the expression of *cralbp* at mRNA and protein levels, implicating that *cralbp* mRNA and protein levels are controlled by OctαR1 signaling.

Considering that *cralbp* is the crucial target gene in OctαR1 signaling, we further analyzed the functional association of *cralbp* with OctαR1 signaling for olfactory attraction. The behavioral assay showed that *cralbp* knockdown inhibits the inductive function of OA in olfactory attraction ($G = 16.68$, $P < 0.001$) (Fig. 4D), suggesting that *cralbp* is the downstream regulator associated with OctαR1 signaling for olfactory attraction in the migratory locusts.

3.5. *cralbp* acts as the effector in OctαR1 signaling to affect the attractive response to odorants

cralbp protein mediates olfactory attraction to gregarious volatiles, which are the volatile mixtures from body faces and fecal of locusts. Therefore, we analyzed the olfactory response of gregarious locusts to

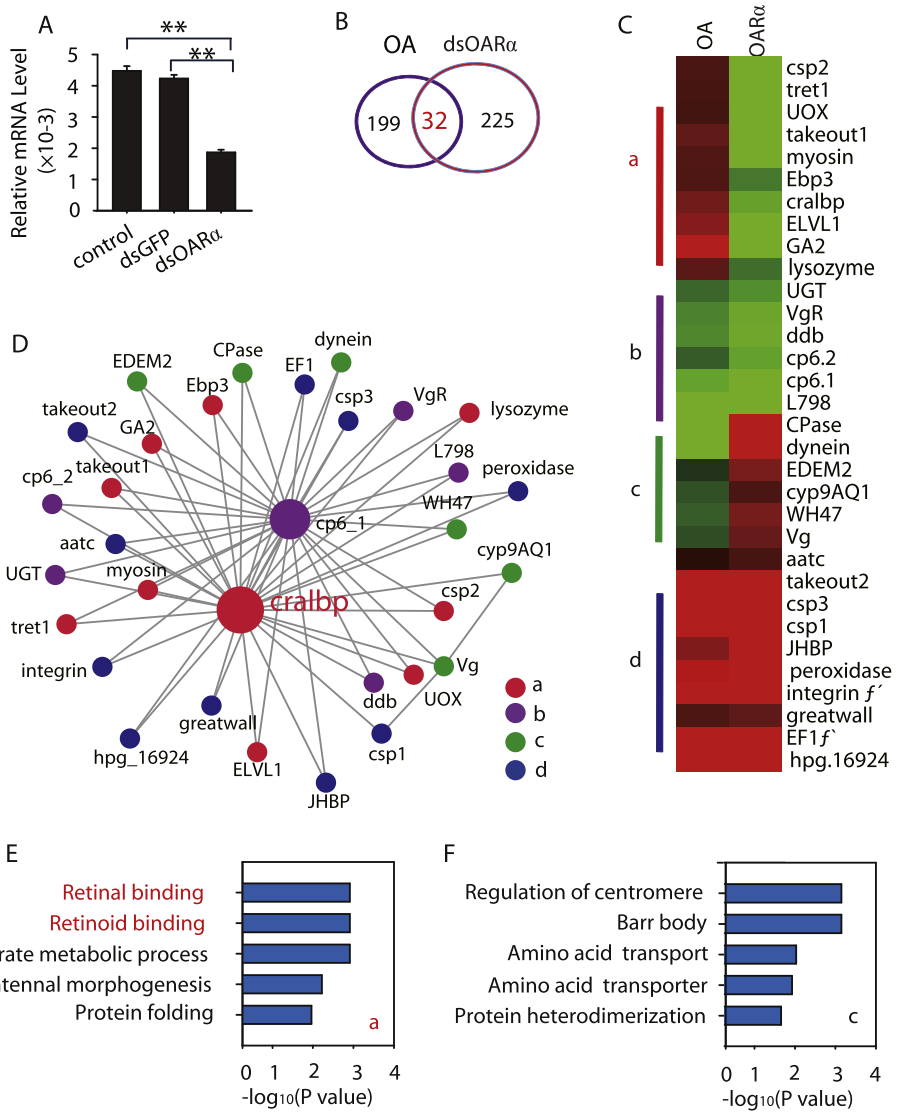

Fig. 3. *cralbp* is the potential target of OctαR1 signaling in the migratory locusts. (*A*) *OctαR1* RNAi knockdown significantly decreased the mRNA expression level in brains (n = 8). (*B*) The number of differentially expressed transcripts (DETs) after OctαR1 activation and inhibition ($P < 0.05$). (*C*) Hierarchical clustering analysis of DETs after activation and inhibition of OctαR1. (*D*) *cralbp* is the crucial hub gene among the network of the DETs ($P < 0.05$) affected by OctαR1 signaling. (*E*, *F*) Functional clusters of the DETs in clusters *a* and *c* determined by gene ontology (GO) enrichment. The asterisks outside the strip indicate the significant differences between the control and the treatment groups determined by Student's *t*-test (mean ± SEM). **, $P < 0.01$. Abbreviations: OA, octopamine; SA, saline.

these chemicals (guaiacol, veratrole, 1-pentanol and 1-benzaldehyde) in gregarious volatiles. The Y-tube behavioral assay indicated that the migratory locusts showed attractive responses to guaiacol and veratrole ($G = 8.85$, $P < 0.05$ for guaiacol; $G = 6.91$, $P < 0.05$ for veratrole) (Fig. 5A, B), whereas the migratory locusts did not show olfactory responses to 1-pentanol and 1-benzaldehyde (Fig. S7). Considering that *cralbp* protein was also enriched in the brains and antennal lobes, we analyzed antennal EAG responses of the gregarious locusts to those two chemicals, guaiacol and veratrole. The results showed that the antennal EAG responses in gregarious locusts were significantly higher than those in controls after exposure to guaiacol and veratrole ($t = 6.965$, $P < 0.01$ for guaiacol; $t = 6.532$, $P < 0.01$ for veratrole) (Fig. 5C). After knocking down *cralbp* in the migratory locusts, the EAG responses of the antennae in gregarious locusts were decreased significantly ($t = 5.53$, $P < 0.01$ for guaiacol; $t = 4.77$, $P < 0.01$ for veratrole) (Fig. 5D), suggesting that CRALBP in the antennae mediates the olfactory sensation of guaiacol and veratrole. Furthermore, knocking down *cralbp* in gregarious locusts significantly reduced their attractive response to guaiacol and veratrole ($G = 13.31$, $P < 0.01$ for guaiacol; $G = 11.25$, $P < 0.01$ for veratrole) (Fig. 5E), and the knockdown of *cralbp* also inhibited the attractive response elicited by the activation of OctαR1 signaling ($G = 11.56$, $P < 0.01$ for guaiacol; $G = 10.68$, $P < 0.01$ for veratrole) (Fig. 5F). These results suggested that CRALBP acts as an effector protein in OctαR1 signaling for olfactory attraction regulation.

4. Discussion

In this study, we found that *cralbp* modulates olfactory attraction to gregarious volatiles. OctαR1 signaling controls *cralbp* mRNA and protein expression levels, and *cralbp* is the critical target of OctαR1 signaling. A deficiency in *cralbp* function inhibits the roles of OctαR1 signaling for modulating olfactory attraction. Moreover, *cralbp* may act as an effector protein in OctαR1 signaling to mediate the electrophysiological response to odorants in gregarious volatiles.

The function of OA in regulating olfactory attraction for the aggregation of locusts is closely associated with the effectors in OctαR1 signaling (Ma et al., 2015; Xu et al., 2017). The level of OA in the brains of group-living gregarious locusts was greater than that in the brains of solitary locusts. In the development of gregarious locusts from the third to the fourth stadium, calmodulin is one of the components in OctαR1 signaling that mediates olfactory attraction (Xu et al., 2017). However, it is unknown whether and how OA binds with OA receptors to mediate the effector expression in the downstream signal transduction system. The novel mechanism by which OctαR1 modulates *cralbp* expression extends our understanding of the mode of OA action, and provides a more comprehensive understanding of OA function in modulating olfactory attraction.

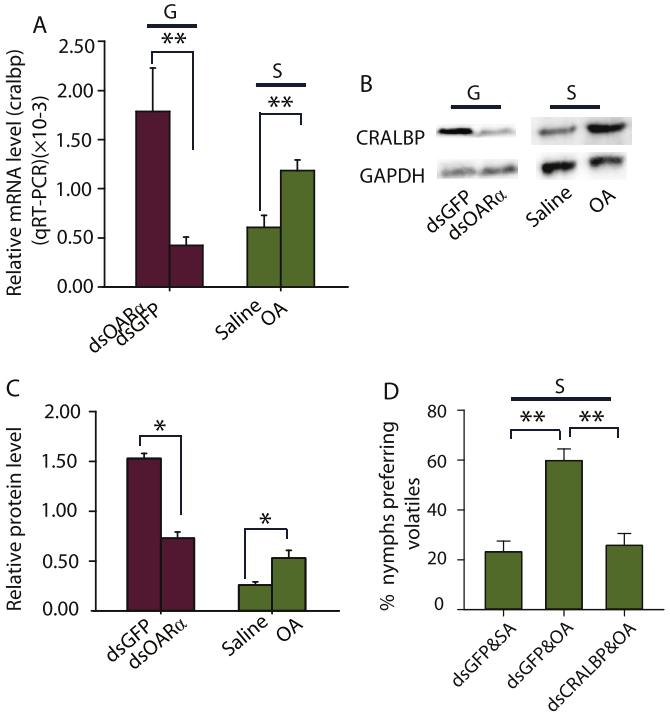

Fig. 4. CRALBP is the target of OctαR1 signaling in modulating olfactory attraction. (*A*) The relative expression levels of *cralbp* mRNA after inhibition or activation of OctαR1 (n = 8). (*B*) Western blots show the expression levels of CRALBP protein after inhibition or activation of OctαR1. (*C*) The relative CRALBP expression levels after inhibition or activation of OctαR1 (n = 6). (*D*) After *cralbp* RNAi knockdown, OA can not induce olfactory attraction in solitary locusts. The asterisks outside the strip indicate the significant differences between the control and treatment groups determined by Student's *t*-test (mean ± SEM) (*A*, *C*) and *G*-test for independence (*F*). *, $P < 0.05$; **, $P < 0.01$. Abbreviations: S, solitary; G, gregarious; SA, saline; OA, octopamine.

Neurotransmitters bind with their membrane receptors and activate the cAMP-PKA or calcium-PKC-Akt secondary messenger system to induce the downstream mRNA expression (Keefe and Horner, 2016). The activation of mRNA expression may require the release of other cooperative transmitters to activate the downstream signaling pathway. Despite the increased understanding of neurotransmitters and receptors that regulate gene expression in the striatum and central nervous system, the functional significance of these changes in gene expression remains unknown to a great extent. Although OA-OctαR1 signaling induces olfactory attraction for the aggregation of locusts (Ma et al., 2015), the downstream effectors that are regulated by OctαR1 signaling are unknown. In this study, we found that OA-OctαR1 signaling affects the changes in mRNA expression networks, and that *cralbp* is crucial for mediating olfactory attraction. The identification of *cralbp* as the crucial regulator in OA-OctαR1 signaling suggests the novelty of this pathway in modulating olfactory attraction and provides a novel link between neurotransmitter-GPCR signaling and the regulatory mechanism of mRNA expression. This study confirmed that *cralbp* is a critical modulator of olfactory attraction in OA-OctαR1 signaling.

The *cralbp* protein in brains binds retinaldehyde for the synthesis of retinoic acid (RA) for olfactory behavior modulation. RA acts as a chemical for brain physiology and behavioral regulation (Lane and Bailey, 2005). In flies, *cralbp* mRNA has been shown to be specifically enriched in neurons closely associated with olfactory sensation (Anholt and Mackay, 2001). Moreover, *cralbp* is confirmed to express in the oligodendrocytes of the optic nerves and brains, and may control ethanol preference in mice (Treadwell et al., 2004). Similar to these findings, *cralbp* mRNA in locusts is enriched in peripheral cells in antennal lobes, consistent with the localization of OctαR1 in antennal lobes (Ma et al., 2015). More importantly, *cralbp* mRNA is not localized in glomerus regions that enrich projection and in local neurons that process olfactory stimuli, suggesting that the *cralbp* protein acts as the effector regulator in OctαR1 signaling for olfactory attraction. The expression of *cralbp* is an indicator of the presence and distribution of RA in tissues (Asson-Batres et al., 2003), and promotes the isomerization of all-*trans*- to 11-*cis*-retinol for RA synthesis. The expression and localization of the *cralbp* may implicate the function of RA in modulating olfactory attraction for the aggregation of locusts.

On the other hand, it seems unlikely that CRALBP acts as an odorant binding protein (OBP) for behavioral regulation. OBPs are usually small proteins with about 14–30 kDa in size. They show lipocalin function domain and belong to the family of pheromone binding proteins (PBP) and general-odorant binding proteins (GOBP) (pfam01395, PBP/GOBP family) (Guo et al., 2018a). By contrast, Locust CRALBP protein (39 kDa in size) has CRAL-TRIO domain and SEC14 domain for chemical binding. CRALBP is unlikely the odorant binding proteins in the migratory locusts. Thus, we speculate that CRALBP does not act as an odorant binding protein in modulating olfactory attraction. The *cralbp* protein is well known to bind retinaldehyde for RA synthesis in visual pigment Müller cells, and its mutation is genetically associated with visual impairments characterized by night blindness and photoreceptor degeneration (Saari et al., 2001; Maw et al., 1997). However, its function outside the visual system is not well characterized. Moreover, the visual deficiency caused by *cralbp* knockdown did not affect the olfactory preferences of gregarious and solitary locusts for gregarious volatiles, although *cralbp* knockdown resulted in visual deficiency. Therefore, the effects of *cralbp* knockdown in the brains of gregarious locusts on olfactory attraction does not result from the roles of *cralbp* in visual signaling.

In this study, OctαR1 was shown to promote the mRNA and proteins expressions of *cralbp*, whereas blockade of this signaling was shown to reduce *cralbp* protein and mRNA expression levels, suggesting that OctαR1 may mediate *cralbp* expression at transcriptional and post-transcriptional levels. The mechanism by which OctαR1 signaling mediates the expression of the critical target gene *cralbp* has not been reported until now. OctαR1 belongs to the superfamily of membrane GPCRs (Zeng et al., 1996), and this transmembrane receptor mediates its target genes through intermediate regulators. OctαR1 is a receptor that coupled with cAMP and Ca^{2+} secondary messenger pathway. OctαR1 activation results in a transient or sustained increase in intracellular Ca^{2+} concentration and an oscillatory increase in the intracellular Ca^{2+} concentration ($[Ca2+]_i$) in insects (Xu et al., 2017). Calmodulin is also a signaling intermediate in OctαR1 signaling. Therefore, *cralbp* expression may be affected by Ca^{2+}-calmodulin secondary messenger pathway.

The expression levels of mRNAs were controlled by OctαR1 signaling, suggesting that transcription factors may be involved in the activation of *cralbp* expression. The transcription factor pax6 has been reported to mediate *cralbp* mRNA transcription in mice (Boppana et al., 2012). PaX6 regulates morphogenesis and neurogenesis in olfactory epithelium and bulb and is coexpressed with *cralbp* in nervous system (Holm et al., 2007; Nomura et al., 2007). *cralbp* may be the direct downstream target of PaX6 in the development of mouse forebrain (Collinson et al., 2003). In addition, the activity of pax6 is regulated by the calcium-activated p38 mitogen-activated protein kinase (MAPK) pathway (Mikkola et al., 1999). OctαR1 may activate the p38 MAPK signaling pathway by calcium to activate pax6 for *cralbp* expression.

In this study, we found CRALBP affects olfactory attraction in locusts, and it also acts as the effector protein in OA-OctαR1 signaling to mediate the attractive response of gregarious locusts to their volatiles. The finding that CRALBP modulates olfactory behaviors in the brain other than in eyes implicates its functions in extra retinal tissues. The role of CRALBP in OctαR1 signaling indicate functional connection between retinal transporting and GPCR signaling pathway in the nervous system, suggesting an even broader neuroscience focus on CRALBP

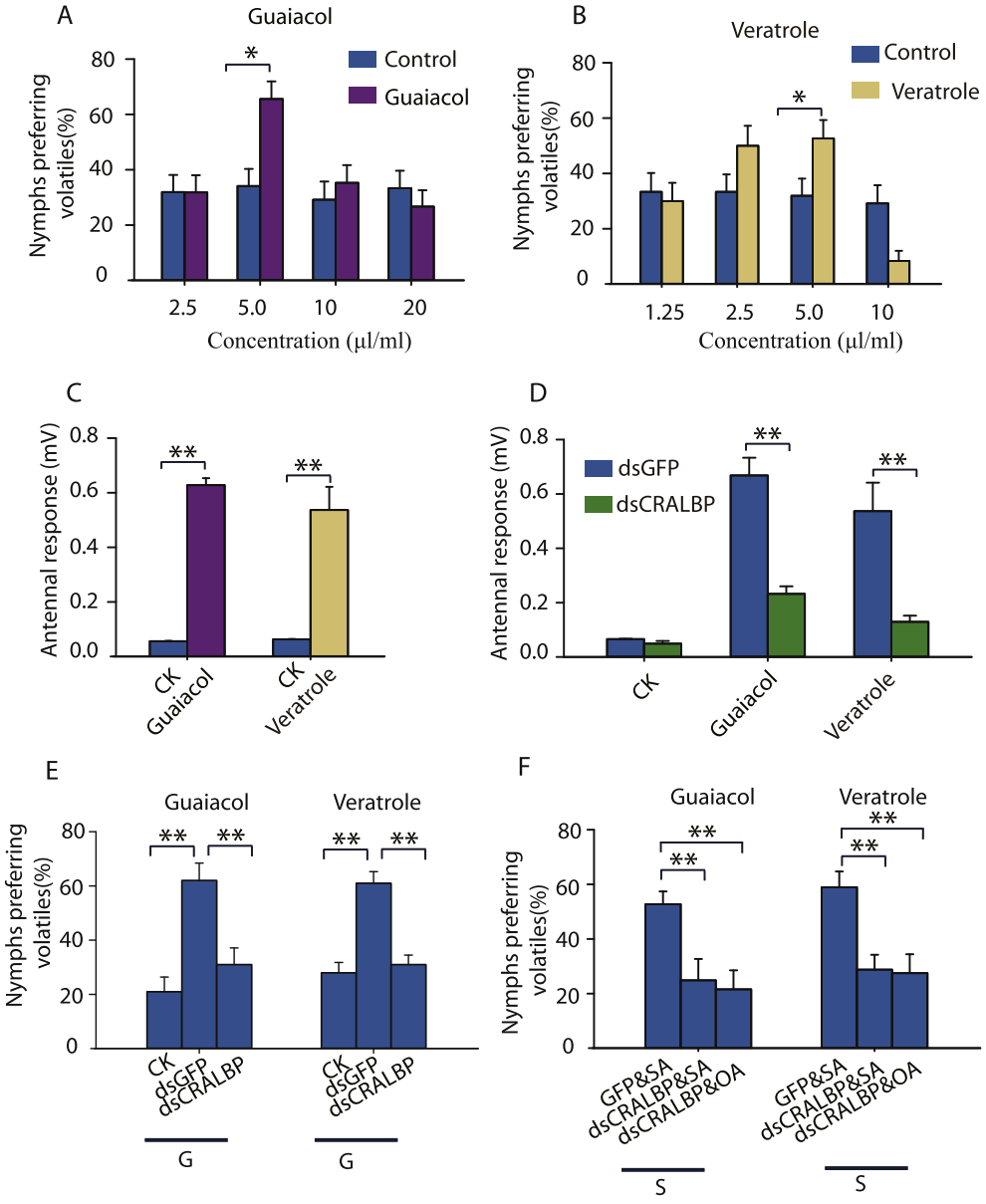

Fig. 5. *cralbp* mediates olfactory attraction to the odorant guaiacol and veratrole. (*A*, *B*) Olfactory responses of the migratory locusts to guaiacol and verratrole in different concentrations (n = 80). (*C*) The antennal responses of gregarious locusts to guaiacol and veratrole (n = 20). (*D*) *cralbp* RNAi knockdown reduced the antennal responses of gregarious locusts to guaiacol and veratrole (n = 20). (*E*) *cralbp* RNAi knockdown inhibited olfactory attraction in gregarious locusts (n = 56). (*F*) *cralbp* RNAi knockdown inhibited the induction of olfactory attraction by OA in solitary locusts (n = 50). The asterisks indicate the significant differences between the control and treatment groups determined by *G*-test for independence (*A*, *B*, *E* and *F*) and Student's *t*-test (mean ± SEM) (*C*, *D*). *, $P < 0.05$; **, $P < 0.01$. Abbreviations: S, solitary; G, gregarious; OA, octopamine; SA, saline.

in the future.

Author contributions

The author(s) have made the following declarations about their contributions. ZM conceived projects, wrote manuscripts, did experiments and data analysis. JL performed EAG and behavioral assay. XG helped collected samples for RNA-seq and finish in situ hybridization of *cralbp*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103214.

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