

RESEARCH ARTICLE

Odour discrimination learning in the Indian greater short-nosed fruit bat (*Cynopterus sphinx*): differential expression of *Egr-1*, *C-fos* and PP-1 in the olfactory bulb, amygdala and hippocampus

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ABSTRACT

Activity-dependent expression of immediate-early genes (IEGs) is induced by exposure to odour. The present study was designed to investigate whether there is differential expression of IEGs (*Egr-1*, *C-fos*) in the brain region mediating olfactory memory in the Indian greater short-nosed fruit bat, *Cynopterus sphinx*. We assumed that differential expression of IEGs in different brain regions may orchestrate a preference odour (PO) and aversive odour (AO) memory in *C. sphinx*. We used preferred (0.8% w/w cinnamon powder) and aversive (0.4% w/v citral) odour substances, with freshly prepared chopped apple, to assess the behavioural response and induction of IEGs in the olfactory bulb, hippocampus and amygdala. After experiencing PO and AO, the bats initially responded to both, later only engaging in feeding bouts in response to the PO food. The expression pattern of *EGR-1* and *c-Fos* in the olfactory bulb, hippocampus and amygdala was similar at different time points (15, 30 and 60 min) following the response to PO, but was different for AO. The response to AO elevated the level of *c-Fos* expression within 30 min and reduced it at 60 min in both the olfactory bulb and the hippocampus, as opposed to the continuous increase noted in the amygdala. In addition, we tested whether an epigenetic mechanism involving protein phosphatase-1 (PP-1) acts on IEG expression. The observed PP-1 expression and the level of unmethylated/methylated promoter revealed that *C-fos* expression is possibly controlled by odour-mediated regulation of PP-1. These results in turn imply that the differential expression of *C-fos* in the hippocampus and amygdala may contribute to olfactory learning and memory in *C. sphinx*.

KEY WORDS: Fruit bat, Olfactory learning, Immediate-early genes, Protein phosphatase-1, Hippocampus, Amygdala

INTRODUCTION

Odour cues appear to play a significant role in olfactory memory when learned within a social/biological context. Among fruit bats, a preference is shown for food odours signalling beneficial nutrients, whereas aversive behaviour is a response to odours signalling the presence of harmful/toxic compounds (Willander and Larson, 2007; Ventura and Worobey, 2013). In general, olfactory information is

transferred directly from the olfactory bulb to the amygdala and then to the hippocampus (Wilson et al., 2004; Mouly and Sullivan, 2010). Depending on the context, the learning experience triggers neurotransmitter release (Loving, 2010) and activates a signalling cascade through protein kinase A (PKA), extracellular signal-regulated kinase-1/2 (ERK-1/2) (English and Sweatt, 1997; Yoon and Seger, 2006; García-Pardo et al., 2016) and cyclic AMP-responsive element binding protein-1 (CREB-1), which is phosphorylated by ERK-1/2 (Peng et al., 2010). Activated CREB-1 induces expression of immediate-early genes (IEGs), such as early growth response gene-1 (*Egr-1*) (Cheval et al., 2012; Charra et al., 2013; Chawla et al., 2013; Veyrac et al., 2014) and the transcription factor *C-fos*, which in turn induce the transcription of various late-response genes that alter the cellular process and synaptic plasticity (Josselyn and Nguyen, 2005; Minatohara et al., 2016). In addition, expression of *C-fos* in the olfactory bulb (Monstag-Sallaz and Buonviso, 2002; Mukilan et al., 2015), amygdala (Lüscher Dias et al., 2016) and hippocampus (Guzowski, 2002; Huff et al., 2006; Mamiya et al., 2009) has been reported to indicate learning experience and memory formation (Navarro et al., 2000; Hadamitzky et al., 2015; Minatohara et al., 2016). In contrast, induction of serine/threonine protein phosphatase-1 and -2A (PP-1 and PP-2A) is known to regulate the process of memory formation negatively (Oberbeck et al., 2010), via an epigenetic mechanism (Koshibu et al., 2011).

The Indian greater short-nosed fruit bat, *Cynopterus sphinx*, feeds on a variety of fruits, flowers and leaves (Bhat, 1994; Rajan et al., 1998). Volatile compounds originating from the food source offer a basis for fruit-eating bats to learn which plant fragments are palatable and which are non-palatable (Sánchez et al., 2006; Elangovan et al., 2006; Hodgkinson et al., 2007). We have shown that odour stimulates the 5-HT_{1A} receptor, when the olfactory bulb serotonin (5-hydroxytryptamine, 5-HT) level is optimal, and then activates the ERK-1/2–CREB IEG signalling pathway (Ganesh et al., 2010, 2012). We have also shown that microRNA-132 and -148a participate in the process of olfactory memory formation in *C. sphinx* (Mukilan et al., 2015). In the present study, we examined odour cues and memory formation at different levels, and tested two hypotheses: (i) bats respond positively to a preferred odour (PO) and negatively to an aversive odour (AO), and over time the number of feeding attempts in relation to PO increases, whilst that for AO decreases; and (ii) PO and AO differentially induce expression of IEGs (*Egr-1*, *C-fos*) along the olfactory pathway (from the olfactory bulb via the amygdala to the hippocampus), as a response to learning experience and the generation of synaptic plasticity. To test these hypotheses, we performed behavioural experiments and examined the expression pattern of IEGs in the olfactory bulb, amygdala and hippocampus regions of *C. sphinx*, after bats had been exposed to either PO or AO. In addition, we tested whether

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PP-1 is a molecular suppressor of olfactory memory that acts differentially on IEG expression to regulate PO/AO memory and synaptic plasticity.

MATERIALS AND METHODS

Animals

We performed all of our experiments on the greater short-nosed fruit bat, *C. sphinx* (Vahl 1797) (family Pteropodidae), which is classified under the IUCN's Least Concern category (Bates et al., 2008), and causes major damage to commercial crops such as guava (*Psidium guajava*), mango (*Magnifera indica*), banana (*Musa* spp.) and grape (*Vitis vinifera*) across the Indian subcontinent (Srinivasulu and Srinivasulu, 2001, 2002). We captured male *C. sphinx* ($n=12$; forearm length 67 ± 4 mm, body mass 51.6 ± 9.0 g) using a mist-net (9×2 m; Avinet, Portland, ME, USA), in a guava orchard 2 km from the Bharathidasan University Campus, Tiruchirappalli, India ($10^{\circ}16'N$, $78^{\circ}15'E$). We tagged these bats with plastic neck collars consisting of light-reflective coloured tapes, and recorded their morphometric details (Rajan and Marimuthu, 1999). We then kept the bats in an animal house facility in a free-flight chamber ($2.2\times1.3\times2.1$ m), under standard conditions (temperature $30\pm3^{\circ}C$, relative humidity $85\pm3\%$ and 12 h:12 h light:dark cycle). Food, consisting of fruits such as papaya (*Carcia papaya*), sapota (*Achras sapota*) and guava (*P. guajava*), and water were provided *ad libitum*. The Bharathidasan University Wild Animal Ethics Committee approved the entire experimental protocol, which was in compliance with the laws in India.

Behavioural analysis

Although bats were kept in the free-flight chamber, which also served as a roosting chamber, the behavioural experiments were conducted in an experimental chamber ($2.1\times2.4\times2.4$ m) that was connected to the free-flight chamber via a window, which allowed for undisturbed transfers by the bats. We habituated the bats for 14 days in the free-flight chamber, assessing their health status by inspecting fur, bite wounds and infections. We excluded from the study inactive bats and those declining to eat, releasing them at their capture site. In the course of the behavioural tests, we permitted only one bat at a time to remain in the experimental chamber, and used a computerized activity monitor (Electronic Engineering Corporation Inc., Mumbai, India) to record each bat's activity, also incorporating an infrared (IR) receiver-transmitter and a mass-sensitive platform (with the food tray). There were two platforms separated by a distance of 1.8 m in the experimental chamber, as sources of the PO and the AO, respectively. We changed the locations of platforms randomly every day to prevent spatial learning, while keeping the distance from the perch and the distance between platforms constant (at 1.8 m). To train individuals, we offered all the bats in the free-flight chamber fresh pieces of chopped apple as a control (on one platform) and either PO [pieces of chopped apple (50 g) mixed with freshly prepared cinnamon powder (0.8% w/w)] or AO [pieces of chopped apple (50 g) mixed with citral (Sigma-Aldrich, St Louis, MO, USA; C8, 300-7) (0.4% w/v)] (on the other platform). We ran the experiment for 3 h per day (20:00 h–23:00 h), at the same time each day for 4 days. These PO and AO food items formed a dietary combination not available in the bat's natural habitat (Ratcliffe et al., 2003; Ratcliffe and ter Hofstede, 2005). After the PO/AO exposure, we provided all bats in the free-flight chamber with fruit, such as *A. sapota*, *C. papaya* and *P. guajava*, to avoid food deprivation and allow the animals to maintain their energy budgets.

Four days after an odour exposure, bats were individually tested for their preference in the experimental chamber. Bats were

transferred to the chamber 1 h before the behavioural test commenced. We conducted all experiments under red light (0.09 ± 0.02 lx) to minimize the visual cue (Xuan et al., 2012; Shafie et al., 2014). While the memory test was running, we simultaneously provided PO (on one platform) and AO (on the other platform). We recorded the responses of individual bats to PO and AO as: (1) flights out – short flights from the perch (indicating that bats are active); (2) attempts – approaches to the food tray (novel odour), alighting on the platform, but returning without chopped fruit being picked up; and (3) feeding bouts – direct landings on the food tray, with chopped fruit being picked up (Ganesh et al., 2010, 2012; Mukilan et al., 2015). We recorded the behavioural responses of bats between 20:00 h and 23:00 h, training each bat individually and comparing its learning performance with respect to PO/AO on a day-to-day basis for four consecutive days.

Tissue collection and sample preparation

We decapitated individuals representing each group ($n=6$), first dissecting out the olfactory bulb tissues, then removing the hindbrain and separating the two hemispheres by sagittal incision. The samples were placed cortex-side down and non-cortical and meningeal tissues were removed, before dissecting out tissues of the hippocampus and amygdala using a fine scalpel (Glowinski and Iversen, 1966; Kalin et al., 1994). We used half of the tissue from each sample to prepare genomic DNA, and the remaining tissue to prepare protein. We isolated genomic DNA from the samples using an Ultraclean[®] Tissue and Cells DNA isolation kit, following the manufacturer's instructions (Mo Bio Laboratories, Inc., Qiagen, Hilden, Germany; 12334-250), and stored it at $-80^{\circ}C$. Processing isolated DNA involved the bisulphite modification of genomic DNA, following the manufacturer's instructions (EpiTect Bisulfite Kit; Qiagen; 59104). Homogenization of the olfactory bulb, hippocampus and amygdala tissues was carried out in 400 μ l of ice-cold lysis buffer (150 mmol l⁻¹ NaCl, 50 mmol l⁻¹ Tris-HCl pH 7.5, 5 mmol l⁻¹ EDTA, 0.1% v/v NP-40, 1 mmol l⁻¹ DTT, 0.2 mmol l⁻¹ sodium orthovanadate, 0.023 mmol l⁻¹ PMSF) containing protease inhibitor cocktail mixture (10 μ l ml⁻¹; Sigma-Aldrich; catalogue number P8340). This was followed by incubation on ice for 30 min, prior to centrifugation at 9660 g for 30 min at $4^{\circ}C$. We then collected the supernatant in a fresh tube and again centrifuged at 9660 g for 30 min at $4^{\circ}C$. Aliquots were stored at $-80^{\circ}C$. Estimates of DNA and protein sample concentrations were made using a BioPhotometer plus (Eppendorf, Hamburg, Germany).

Quantitative real-time PCR

We quantified levels of methylated/unmethylated DNA in the PP-1 promoter using quantitative real-time PCR (qPCR). qPCR was carried out in a 10 μ l reaction volume containing SSo Advanced[™] SYBR[®] green super mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), specific primers for PP-1 (Miller and Sweatt, 2007) [unmethylated FOR 5'-GAGGAGAGTTTGGTGTTTATAAGATGGT-3' and REV 5'-TCCTCCAAAACCTCAACTCAAACAA-3', and methylated FOR 5'-GGAGAGTTTGGTGTTTATAAGATGGC-3' and REV 5'-CGAAAACCTCGACTCGAACGA-3' (10 μ mol l⁻¹ each)] and DNA (1 μ g). We ran the qPCR reactions under standardized conditions (initial denaturation at $94^{\circ}C$ for 30 s, denaturation at $94^{\circ}C$ for 5 s, annealing at $60^{\circ}C$ for 5 s and extension at $72^{\circ}C$ for 5 s), and performed each reaction in triplicate. The data are presented as relative expression levels (CFX Manager[™] version 2 software; CFX-96 Touch[™] Real-time PCR Detection System; Bio-Rad Laboratories, Inc.).

Western blot analysis

We resolved equal concentrations (30 µg) of total proteins on 10% SDS-polyacrylamide gel, then transferred separated proteins electrophoretically (SD 20; Cleaver Scientific Ltd, Rugby, UK) onto polyvinylidene difluoride (PVDF) membranes (Millipore India Pvt Ltd, Bangalore, India). Membranes were blocked with 5% non-fat milk at 25°C for 3 h then incubated at 4°C for 8 h with a specific primary antibody, i.e. affinity purified anti-rabbit polyclonal EGR-1 (Santa Cruz Biotechnology, Inc. Dallas, TX, USA; SC-189; 1:250), total-c-Fos [Cell Signaling Technology (CST), Danvers, MA, USA; 4384; 1:2000], phospho-c-Fos (Ser 32) (CST; 5348; 1:2000), PP-1α (CST; 2582; 1:2000) or β-actin (Santa Cruz Biotechnology, Inc.; SC-130656; 1:2000) antibody. We then incubated the membranes with alkaline phosphatase (ALP)-conjugated goat anti-rabbit (621100180011730; 1:2000; Merck, Bangalore, India) secondary antibody for 4 h to detect membrane-bound primary antibodies. We then washed the membranes twice with 1× TBS with 0.1% Tween-20 and once with 1× TBS without 0.1% Tween-20. Finally, we incubated the membranes in a nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate to detect the specific level of proteins. Image acquisition was done using Image Lab 2 software (Gel Doc XR+ System; Bio-Rad Laboratories, Inc.) and we calculated the trace quantity for each band.

Immunohistochemistry

To gain insights into the specific c-Fos expression pattern, we exposed bats to either PO or AO for a period of 60 min in the same way as in the training session, before proceeding to decapitate them. We dissected out the brain, and then the olfactory bulb, hippocampus and amygdala regions, subsequently processing and fixing them in paraffin wax. We used a microtome to prepare paraffin-embedded olfactory bulb, hippocampus and amygdala tissue sections (6 µm) (Weswox optik, Haryana, India; MT-1090A). We deparaffinized the sections with xylene at 60°C and then dehydrated them using isopropanol; endogenous peroxidase activity was blocked by incubating the sections for 30 min in a solution containing 10% H₂O₂ and 10% methanol in 1× PBS, prior to treatment with 0.1% trypsin in 0.1% CaCl₂ at 37°C for 10 min. We then incubated the sections with 2 mol l⁻¹ HCl at 37°C for 45 min to denature the DNA, before proceeding with 2% BSA treatment for 1 h at 4°C in order to block non-specific staining. We incubated the sections with anti-rabbit polyclonal c-Fos antibody (CST; 4384; 1:1500), overnight at 4°C, and then washed them with PBS-Tween three times before incubating them for 2 h with goat anti-rabbit IgG-HRP (SC-2030; 1:1000) secondary antibody. We stained sections using DAB (peroxidase development kit, SK-4100; Vector Laboratories, Inc., Burlingame, CA, USA) as a substrate, counterstaining sections with haematoxylin. We mounted prepared slides with DPX (HiMedia, Mumbai, India), and took photomicrographs using a light microscope (Nikon Eclipse 80i) with an attached Cmx-5000 digital camera (Euromex Microscopen bv, Arnhem, The Netherlands).

Statistical analysis

Tests for differences in behavioural response and expression patterns of IEGs entailed multivariate and two-way ANOVA. After performing ANOVA, we subsequently tested the same using the Bonferroni *post hoc* test (SPSS software version 22.0). We used one-way ANOVA to examine differences between the groups (control versus PO; control versus AO; PO versus AO) using Sigma Stat software (version 3.5). Data are presented as means ± s.e.m., and plotted with KyPlot (version 1.0).

RESULTS

Behavioural responses of *C. sphinx* during PO/AO learning

The behavioural test provided evidence that *C. sphinx* responded differently to PO and AO (Fig. 1). *Cynopterus sphinx* feeding attempts differed significantly between odours ($P<0.001$) and between days ($P<0.001$), and for the interaction between odours and days ($P<0.001$). Similarly, our comparisons of feeding bouts revealed significant differences between odours ($P<0.001$). We found no statistical difference between days ($P=0.84$) within a given

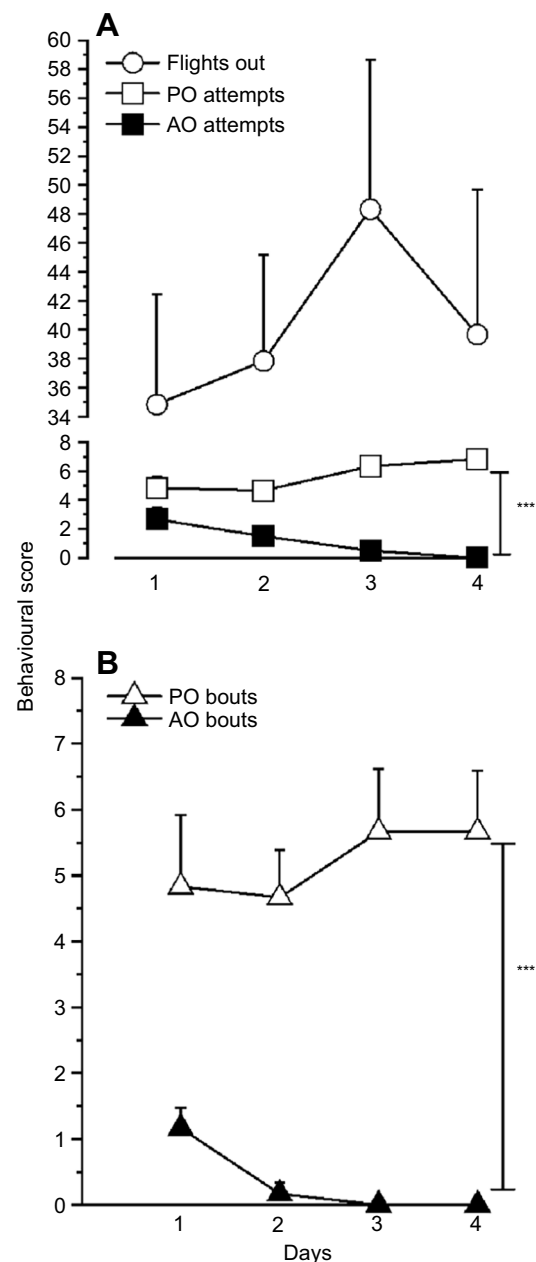


Fig. 1. Exposure to preference odour (PO)/aversive odour (AO) leads to memory formation in *Cynopterus sphinx*. Behavioural responses of *C. sphinx* showed that bats initially responded to both PO and AO, and later attempts were high for PO, but low for AO. Observed behavioural scores indicate that *C. sphinx* developed memory for both PO and AO. Data are shown as means ± s.e.m. There were significant differences (***) with respect to feeding attempts between odours, feeding attempts between days, feeding attempts between odours × days and feeding bouts between odours.

odour, or in the case of the interaction between odour and days ($P=0.37$) (Table S1).

Induction of IEGs in the olfactory bulb during PO/AO learning

We tested whether PO and AO differentially induce the expression of IEGs (*Egr-1*, *C-fos*) in the olfactory bulb of *C. sphinx*. This was found to be the case with the expression of EGR-1 in the olfactory bulb (Fig. 2A). The observed level of expression differed significantly between odours ($P<0.001$) and time intervals ($P<0.001$) and with regard to the interaction of odour \times time intervals ($P<0.001$) (Fig. 2B; Table S2). *Post hoc* analysis showed that AO induced *Egr-1* expression more rapidly than PO at the 15 and 30 min time points (in both cases, $P<0.001$). Interestingly, the level of EGR-1 continued to increase in trials involving PO, but was already significantly lower in the presence of AO after 60 min ($P<0.001$). Examination of the level of expression of c-Fos revealed no significant difference between odours ($P=0.30$), but we did find a significant difference between time intervals ($P<0.001$), as well as with regard to the odour \times time interaction ($P<0.001$) (Fig. 2C; Table S2). *Post hoc* comparisons between PO and AO revealed that the former induced significantly greater c-Fos expression than the latter at 15 min ($P<0.001$), but not at 30 min ($P=0.11$). Sixty minutes into trials, PO continued to induce elevated *C-fos* expression, whereas that in the presence of AO had already decreased ($P<0.001$). These observations suggest that *Egr-1* and *C-fos* respond differently to PO and AO in the olfactory bulb of *C. sphinx*.

Level of PP-1 in the olfactory bulb during PO/AO learning

The expression pattern of PP-1 was similar in the olfactory bulb after the bats had been exposed to PO or AO, with the level of

PP-1 decreasing gradually from the 15 to the 30 min time interval, and further from 30 to 60 min (Fig. 3A). We did not find significantly different levels of expression in the comparison between odours ($P=0.58$), but did note a significant difference between the time intervals ($P<0.001$), and with regard to the odour \times time interaction ($P<0.001$) (Fig. 3B; Table S3). In comparisons between levels at different time points, the *post hoc* test showed a significant difference at 15 min ($P<0.01$) and at 60 min ($P<0.05$), but not at 30 min ($P=0.28$). We were unable to confirm the cause of the significant differences as levels of unmethylated (control versus PO: $P=0.53$; control versus AO: $P=0.90$; PO versus AO: $P=0.51$) and methylated (control versus PO: $P=0.84$; control versus AO: $P=0.53$; PO versus AO: $P=0.50$) PP-1 remained unchanged (Fig. 3C; Table S4). This analysis showed that the PP-1 level was altered by PO and AO in the olfactory bulb of *C. sphinx* but the methylation pattern was unchanged.

Induction of IEGs in the hippocampus during PO/AO learning

The level of EGR-1 expression increased in the hippocampus after the bats had been exposed to PO or AO (Fig. 4A), albeit with the level of expression differing significantly between odours ($P<0.01$) and also between time intervals ($P<0.001$), and with regard to the odour \times time interaction ($P<0.05$) (Fig. 4B; Table S2). In addition, *post hoc* analysis revealed that the AO-induced level of *Egr-1* was significantly higher than that induced by PO at 15 min ($P<0.001$), but with no difference between AO and PO at 30 min ($P=0.27$) and 60 min ($P=0.39$). The level of c-Fos expression differed significantly with regard to odour ($P<0.001$), time ($P<0.001$) and

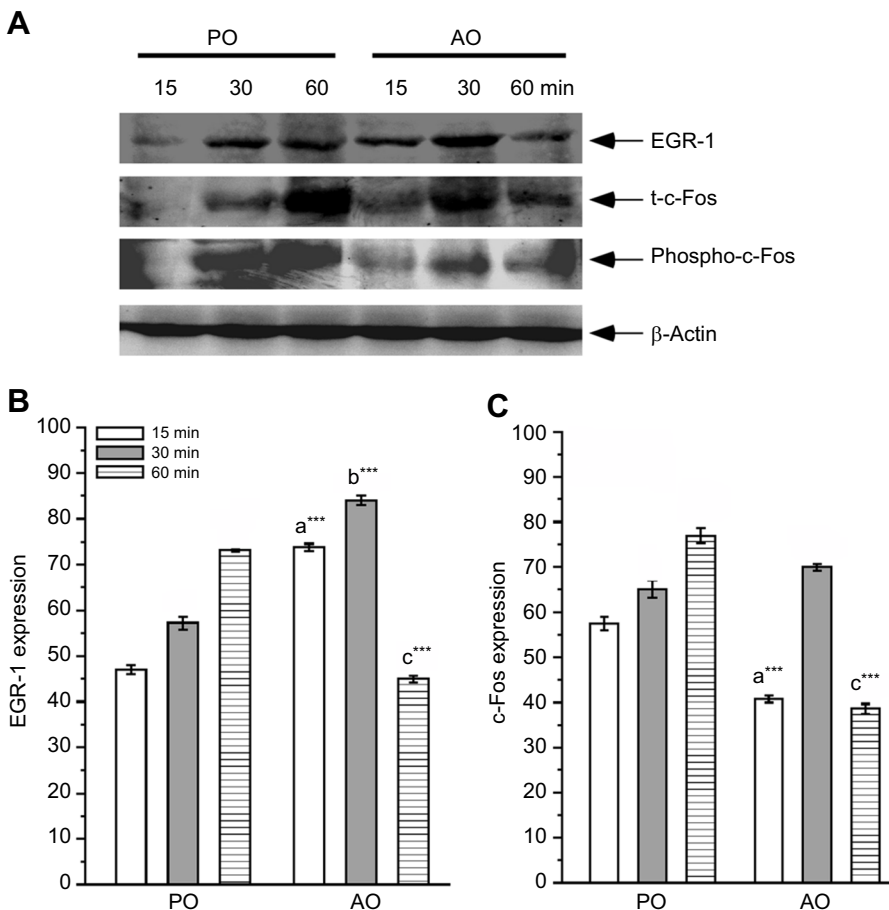


Fig. 2. PO and AO differentially induce the expression of immediate-early genes (IEGs) [EGR-1, total (t)-c-Fos/phospho-c-Fos] in the olfactory bulb (OB) of *C. sphinx*.

(A) Representative western blots showing the expression pattern of IEGs in the OB. (B) Quantitative analysis showing that EGR-1 expression was significantly elevated from 15 min to 60 min during PO learning, but returned to the basal level at 60 min during AO learning. (C) A similar pattern of expression was observed for c-Fos. Data are shown as means \pm s.e.m.; asterisks indicate significant differences ($***P<0.001$), with respect to comparison of (a) AO 15 min versus PO 15 min; (b) AO 30 min versus PO 30 min; and (c) AO 60 min versus PO 60 min.

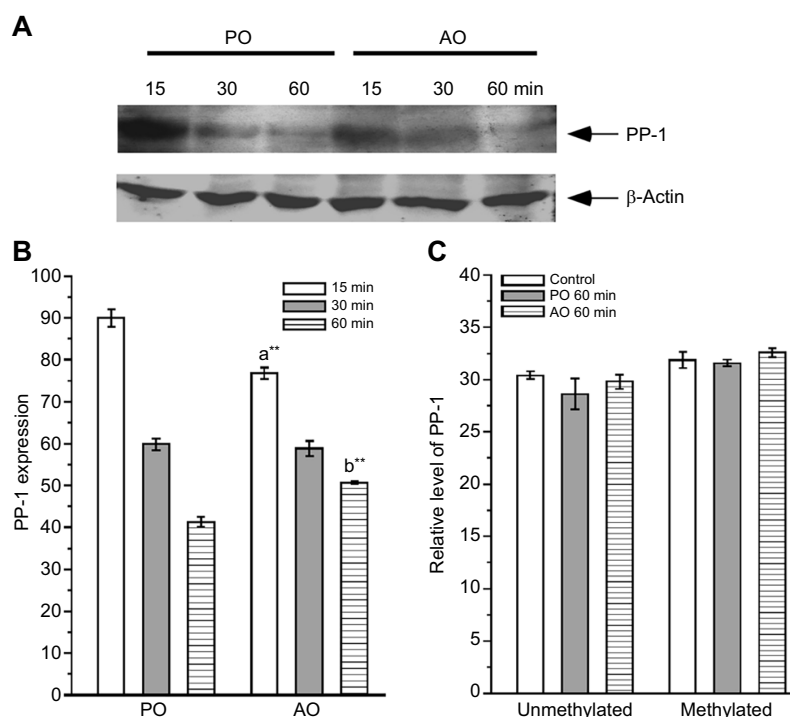


Fig. 3. Expression pattern of PP-1 in the OB of *C. sphinx* after training to PO or AO. (A) Representative western blots showing the expression pattern of PP-1 in the OB of *C. sphinx*.

(B) Quantitative analysis showing that the level of PP-1 was significantly decreased from 15 min to 60 min after the bats were trained to PO or AO. (C) qPCR results showing no significant differences between control, PO and AO in the level of methylated/unmethylated PP-1. Data are presented as means \pm s.e.m.; asterisks indicate significant differences (** $P<0.01$), with respect to comparison of (a) AO 15 min versus PO 15 min; and (b) AO 60 min versus PO 60 min.

the interaction between them ($P<0.001$). *Post hoc* analysis showed that the expression of c-Fos was significantly greater in AO than in PO at 15 min ($P<0.001$), but not at 30 min ($P=0.87$). Notably, the level of c-Fos was significantly lower at 60 min after AO exposure

than after PO exposure ($P<0.001$) (Fig. 4C; Table S2). These observations suggest that PO/AO induces the expression of c-Fos differently from that of EGR-1 in the hippocampus of *C. sphinx*, and at different time points.

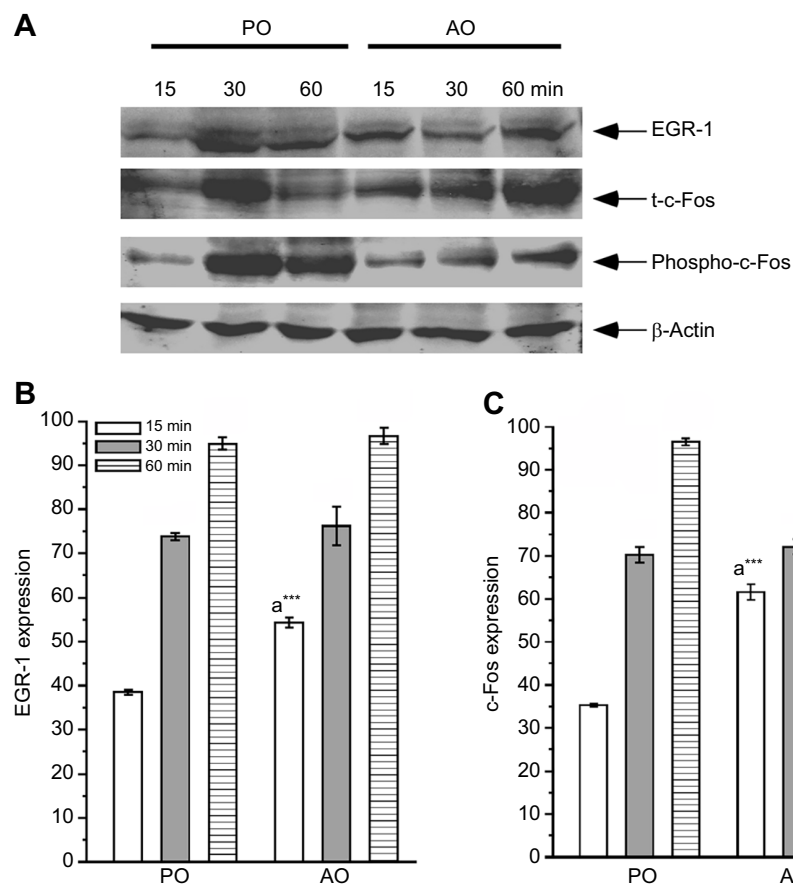


Fig. 4. Expression pattern of IEGs (EGR-1, t-c-Fos/phospho-c-Fos) in the hippocampus of *C. sphinx* after training to PO or AO. (A) Representative western blots showing the expression pattern of IEGs in the hippocampus.

(B) Quantitative analysis showing that EGR-1 expression gradually increased from 15 min to 60 min after the bats were trained to PO or AO. (C) The level of c-Fos expression increased from 15 min to 60 min after training to PO, but decreased after 30 min in bats trained to AO. Data are presented as means \pm s.e.m.; asterisks indicate significant differences (***) ($P<0.001$), with respect to comparison of (a) AO 15 min versus PO 15 min; and (b) AO 60 min versus PO 60 min.

Differential PO/AO inducement of PP-1 in the hippocampus

Our analysis showed that the PP-1 expression pattern differed between the odours at different time intervals (Fig. 5A). These differences were statistically significant with respect to odour ($P<0.001$), time ($P<0.001$) and the odour \times time interaction ($P<0.001$) (Fig. 5B; Table S3). The results of the *post hoc* analysis also demonstrated that PP-1 expression was at a significantly higher level after the bats had been exposed to PO as opposed to AO, albeit only at 15 min ($P<0.001$) and 30 min ($P<0.001$). By 60 min, the level of PP-1 was lower again in the case of both PO and AO, although the level of the latter was significantly higher than that of the former ($P<0.01$). In parallel, we estimated the methylated and unmethylated levels of PP-1 in the control and 60 min post-PO/AO exposure. No significant difference in any comparison of unmethylated PP-1 levels could be found (control versus PO: $P=0.11$; control versus AO: $P=0.45$; PO versus AO: $P=0.09$). Similarly, the level of methylation did not vary (control versus PO: $P=0.59$; control versus AO: $P=0.59$; PO versus AO: $P=0.49$) with regard to PO/AO exposure (Fig. 5C; Table S4). Evidently, AO and PO did not alter the methylation status of PP-1 in the hippocampus of *C. sphinx*.

Inducement of IEGs in the amygdala during PO/AO learning

The expression patterns and levels of EGR-1 in the amygdala of *C. sphinx* varied between PO and AO (Fig. 6A). The level of expression of EGR-1 differed significantly between odours ($P<0.001$), time ($P<0.001$) and the odour \times time interaction ($P<0.001$) (Fig. 6B; Table S2). *Post hoc* tests showed that AO induced EGR-1 expression significantly more than PO at 15 min ($P<0.001$), but the elevated level was not significantly different between the AO and PO trials at the two other time points

(30 min, $P=0.95$; 60 min, $P=0.18$). Similarly, the level of c-Fos expression differed significantly between odours ($P<0.001$), time ($P<0.001$) and the odour \times time interaction ($P<0.001$) (Fig. 6C; Table S2). *Post hoc* analysis revealed that the level of c-Fos was significantly higher after AO (as opposed to PO) at 15 min ($P<0.001$) and 30 min ($P<0.001$), but not at 60 min ($P=0.69$). This analysis therefore showed that PO and AO are responsible for differential activation of IEGs (*Egr-1* and *C-fos*) in the amygdala. The induction was also stronger with AO than with PO.

Differential activation by PO/AO of PP-1 in the amygdala

The expression patterns and levels of PP-1 differed with PO/AO in the amygdala (Fig. 7A). The level of PP-1 expression differed significantly in relation to odour ($P<0.01$), time ($P<0.001$) and the odour \times time interaction ($P<0.001$). Regarding the induction of PP-1 at different time points, the results of the *post hoc* test revealed that this level was significantly higher at 15 min ($P<0.01$) in the case of AO, had decreased at 60 min ($P<0.01$) and did not differ at 30 min ($P=0.18$) (Fig. 7B; Table S3). In general, levels of methylated and unmethylated PP-1 showed that both PO and AO alter methylation status significantly (Fig. 7C; Table S4). The basal level of methylated PP-1 was significantly higher than that of unmethylated PP-1 ($P<0.05$) in the control group. Similarly, the level of methylated PP-1 was significantly higher in the amygdala of *C. sphinx* experiencing either PO ($P<0.01$) or AO ($P<0.01$). Interestingly, the level of methylated PP-1 was significantly higher for AO ($P<0.05$) and PO ($P<0.01$) than the corresponding level of unmethylated PP-1. This suggests that PP-1 methylation responds differently to AO and PO in the amygdala.

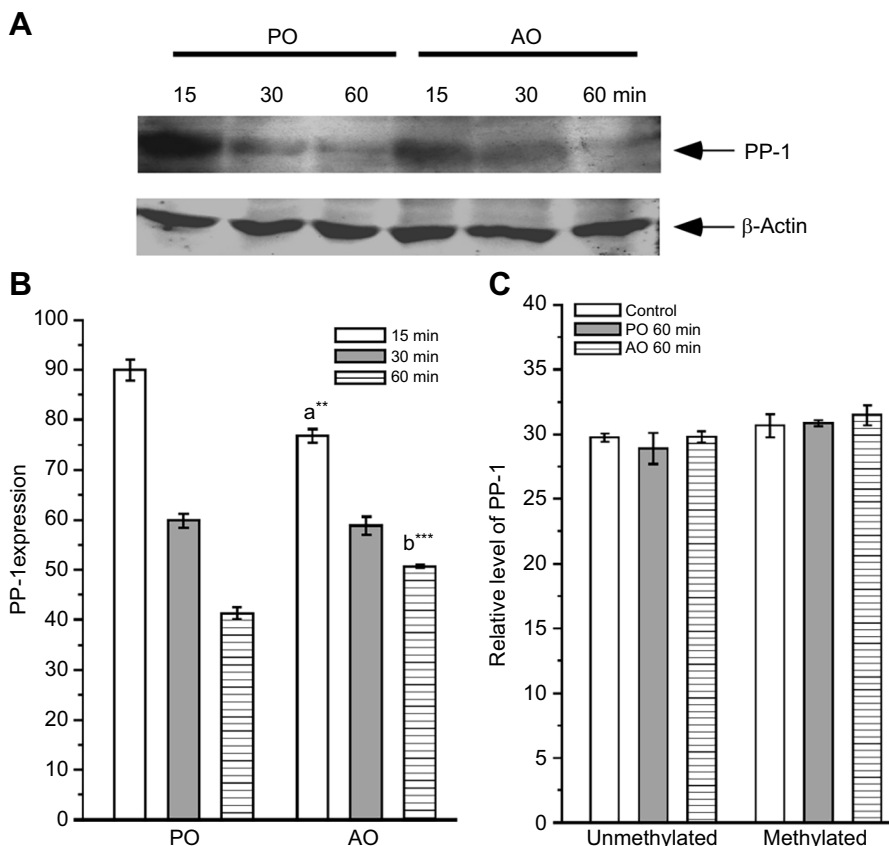


Fig. 5. Expression pattern of PP-1 in the hippocampus of *C. sphinx* after training to PO or AO. (A) Representative western blots showing the expression pattern of PP-1 in the hippocampus of *C. sphinx*. (B) Quantitative analysis showing that the level of PP-1 significantly increased from 15 min to 30 min and was maintained at the same level in bats that were trained to PO, but increased from 15 min to 30 min and decreased in bats that were trained to AO. (C) qPCR results showing that the level of methylated/unmethylated PP-1 was not altered after the bats were trained to PO or AO. Data are presented as means \pm s.e.m.; asterisks indicate significant differences (** $P<0.01$, *** $P<0.001$), with respect to comparison of (a) AO 15 min versus PO 15 min; and (b) AO 60 min versus PO 60 min.

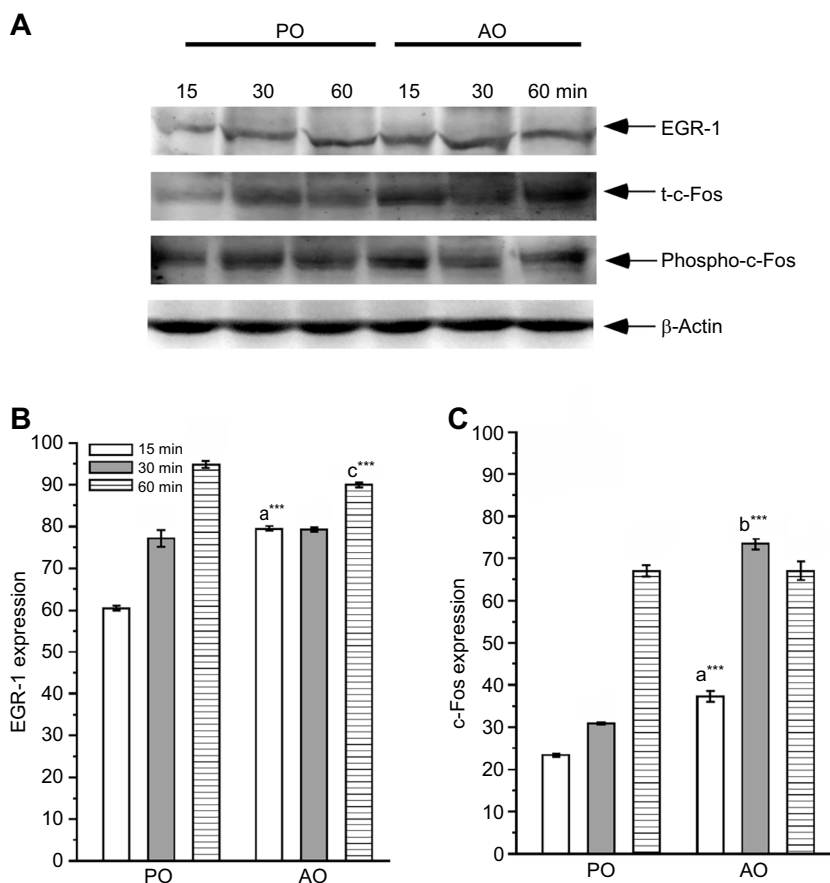


Fig. 6. Expression pattern of IEGs (EGR-1, t-c-Fos/phospho-c-Fos) in the amygdala of *C. sphinx* after training to PO or AO. (A) Representative western blots showing the expression pattern of IEGs in the amygdala. Quantitative analysis showing that the expression of both (B) EGR-1 and (C) c-Fos gradually increased from 15 min to 60 min after the bats were trained to PO or AO. Data are presented as means±s.e.m.; asterisks indicate significant differences (** $P<0.001$), with respect to comparison of (a) AO 15 min versus PO 15 min; (b) AO 30 min versus PO 30 min; and (c) AO 60 min versus PO 60 min.

Immunohistochemical analysis of c-Fos expression in the olfactory bulb, hippocampus and amygdala

Our immunohistochemical examination of c-Fos expression in different brain regions (i.e. the olfactory bulb, hippocampus and

amygdala) revealed an increase in the presence of either PO or AO. However, c-Fos expression in the olfactory bulb (glomerular cell layer, GL; mitral cell layer, ML) of *C. sphinx* was stronger for PO than for AO (Fig. 8). It was also raised in the hippocampus

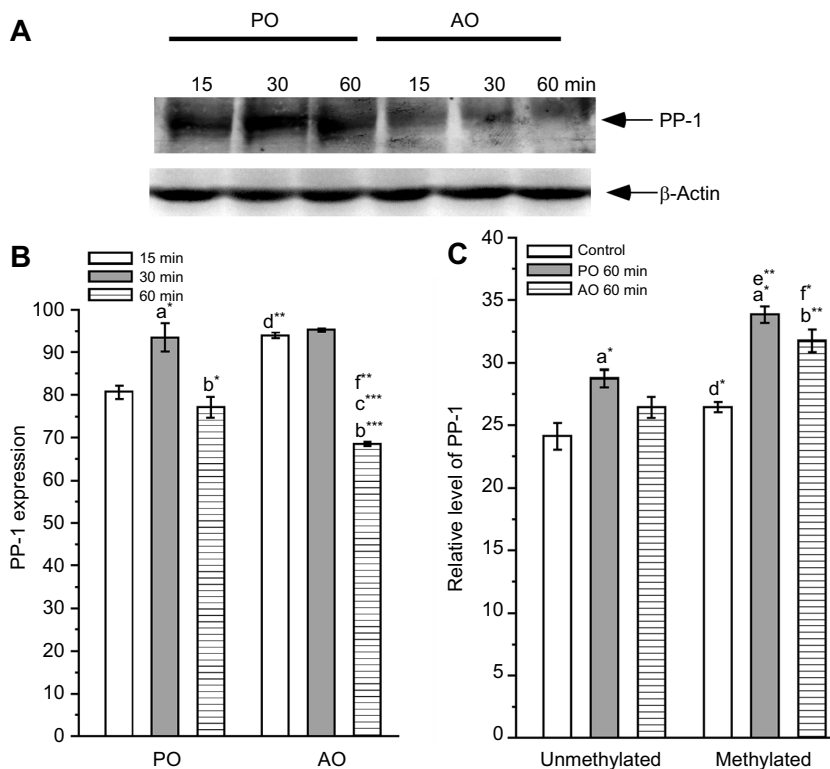


Fig. 7. Expression pattern of PP-1 in the amygdala of *C. sphinx* after training to PO or AO. (A) Representative western blots showing the expression pattern of PP-1 in the amygdala of *C. sphinx*. (B) Quantitative analysis showing that the level of PP-1 increased from 15 min to 30 min and decreased at 60 min after training to PO, but was maintained at the same level up to 30 min and decreased after training to AO. (C) qPCR results showing that methylated/unmethylated PP-1 significantly increased after training to PO and AO; however, this increase was significantly lower for AO compared with PO. Data are presented as means±s.e.m.; asterisks indicate significant differences (* $P<0.05$, ** $P<0.01$, *** $P<0.001$) with respect to comparison of (a) control versus PO 60 min; (b) control versus AO 60 min; (c) PO 60 min versus AO 60 min; (d) unmethylated versus methylated control; (e) unmethylated versus methylated PO 60 min; and (f) unmethylated versus methylated AO 60 min.

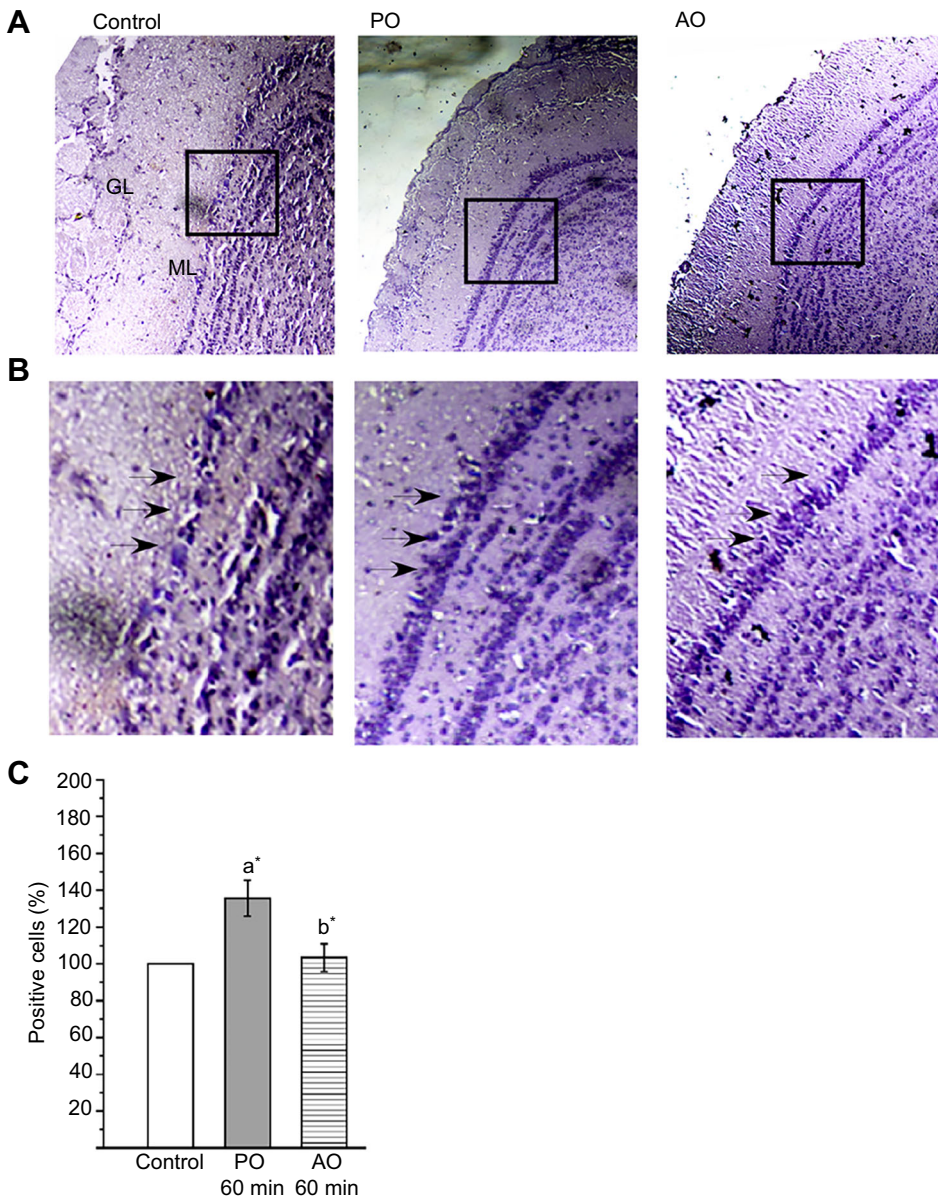


Fig. 8. Representative photomicrographs showing the expression pattern of c-Fos in the OB of *C. sphinx* after training to PO or AO. Images at (A) 10× and (B) 20× magnification. c-Fos expression increased after training to PO in the OB compared with AO and control (GL, glomerular cell layer; ML, mitral cell layer). (C) The number of c-Fos immunoreactive cells is shown in relation to the control (\pm s.e.m.); asterisks indicate significant differences ($*P < 0.05$) with respect to comparison of (a) control versus PO 60 min; and (b) PO 60 min versus AO 60 min.

following exposure to PO/AO. The intensity in the ventral hippocampus, and especially in the granule cell layer of the dentate gyrus, was higher in bats experiencing AO and PO than in the control group (Fig. 9). Similarly, exposure to PO/AO induced the expression of c-Fos in the amygdala. However, the level of expression was higher in the amygdala of bats exposed to AO as opposed to those exposed to PO or controls (Fig. 10). These analyses suggest that there was a differential response to AO/PO in different brain regions (Table S5). In both the hippocampus and the amygdala, there was higher-intensity expression of c-Fos with AO than with either PO or the control.

DISCUSSION

In behavioural experiments, we observed that *C. sphinx* responded to both PO and AO controlled conditions, but displayed a steadily increasing number of feeding attempts from day 1 to day 4 in relation to PO, as compared with a decreasing number in the case of AO. Such a preference may be developed on the basis of odours associated with an oral sensation or taste, or a positive post-digestive

experience. Following learning, subsequent exposure no longer elicits a novelty response, and in fact becomes preferable, being associated positively with energy, taste and odour (Bures et al., 1998; Sclafani, 2001; Yamamoto and Ueji, 2011).

The odour-associated stimuli are connected through multiple regions of the brain, including the olfactory bulb, hippocampus and amygdala (Wilson et al., 2004; Mouly and Sullivan, 2010). Expression of IEGs is known to strengthen synapses during memory formation. In our research, we recorded similar expression patterns of *Egr-1* and *C-fos* in the olfactory bulb, hippocampus and amygdala following the response to PO, but different patterns after exposure to AO. Earlier studies have reported that the expression of IEGs in the olfactory bulb is odour specific, and may depend on the afferent olfactory input (Sallaz and Jourdan, 1996; Monstagn-Sallaz and Buonviso, 2002). However, we did not record any variation in the expression pattern of PP-1 with respect to PO and AO, and PP-1 may act as a molecular constraint on IEG expression (Koshibu et al., 2009; Peters et al., 2009). This suggests that differential expression of IEGs in the GL and ML of the

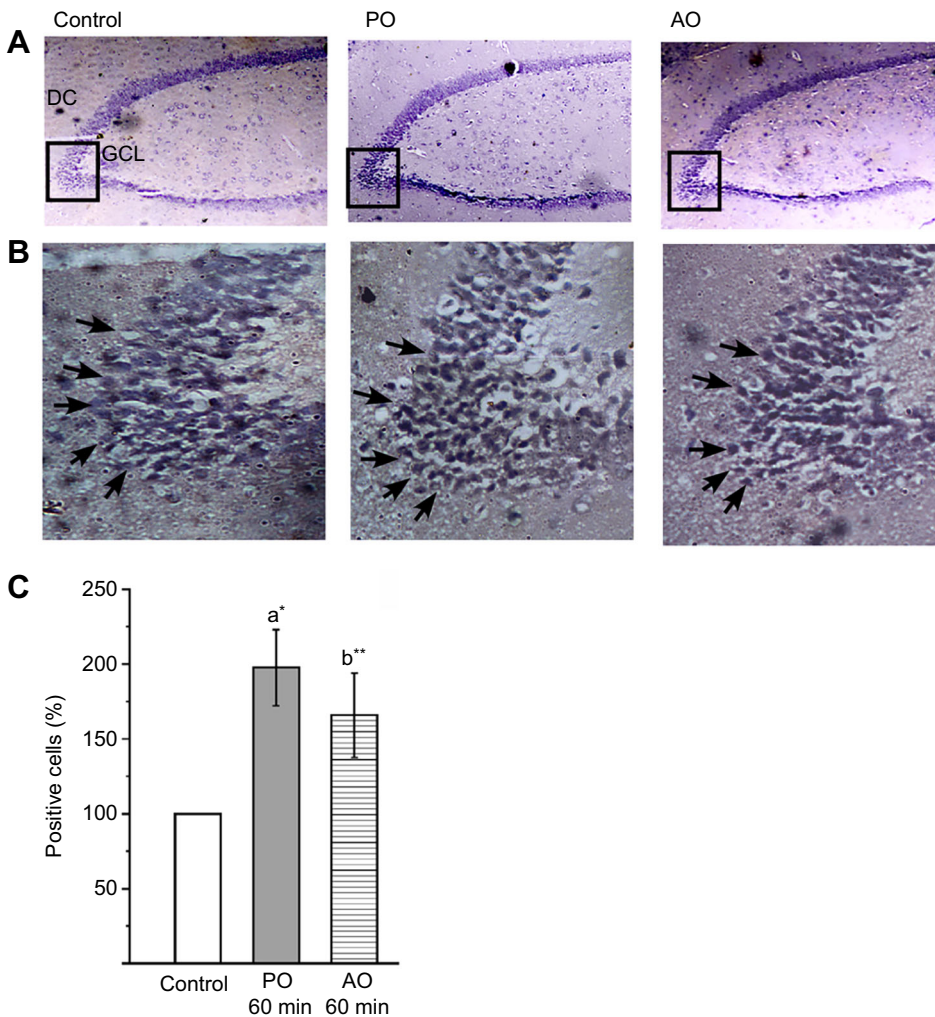


Fig. 9. Representative photomicrographs showing c-Fos reactivity in the granule cell layer (GCL) of the dentate gyrus (DG) in the hippocampus of *C. sphinx* after training to PO and AO. Images at (A) 10 \times and (B) 20 \times magnification. In both cases, an increase in c-Fos expression was observed. (C) The number of c-Fos immunoreactive cells is shown in relation to the control (\pm s.e.m.); asterisks indicate significant differences (* P <0.05, ** P <0.01) with respect to (a) control versus PO 60 min; and (b) control versus AO 60 min.

olfactory bulb may be associated with plasticity, as well as olfactory learning and memory. GL and ML cells receive input from sensory neurons in the olfactory bulb, and transmit olfactory information to other brain regions (Sosulski et al., 2011; Pacifico et al., 2012). The observed difference in the expression of c-Fos in GL and ML of the olfactory bulb may contribute to the development of PO and AO memory in *C. sphinx*.

We also tested the activity pattern of IEGs to understand the level of neuronal activation with respect to PO/AO exposure in the hippocampus. The expression pattern of EGR-1 after exposure to PO and AO was remarkably similar, but there was a differential level of expression at different time points. The sharp elevation (at 15 min) and reduction (at 30 min) of c-Fos expression for AO, but not for PO, may be linked to the acquisition of contextual aversive information and hippocampus activity (Ploghaus et al., 2000). Differences in c-Fos expression have been ascribed to connectivity with inputs from other regions (Dong et al., 2009; Segal et al., 2010).

The expression of c-Fos in neurons observed in the granule cell layer, in relation to PO and AO exposure, showed that granule cell neuron populations responded to odour stimuli (Jung and McNaughton, 1993; Ramírez-Amaya et al., 2005), with the activation capable of contributing to an encoding of memories and neural plasticity (Kee et al., 2007; Hunsaker and Kesner, 2008; Aimone et al., 2011). It is worth mentioning that the dentate gyrus is critically involved in memory formation (Gilbert et al., 2001; McHugh et al., 2007; Deng et al., 2010). In line with the results of

Saddoris et al. (2009), the activation of *C-fos* in granule cell layer neuron populations in the dentate gyrus suggests that these neurons are involved either directly or indirectly in PO and AO memory formation in *C. sphinx*. We speculate that a unique set of neurons in distinct brain regions may produce the specific epigenetic change relating to PO/AO odour, which may maintain the mechanism in line with neuronal function. At this point, to form new and subsequent memories, the hippocampus may allow the epigenetic mechanism to reset after being encoded once (Okuno, 2011; Liu et al., 2012; Hadamitzky et al., 2015). The variations in the expression pattern may be regulated by PP-1 during olfactory memory formation (Windling et al., 2011; Yang et al., 2015). However, along with PP-1, some other modulatory mechanism ensures the reduction of c-Fos at 60 min, which could be related to the aversive memory (Goosens, 2011). Our observations suggest that *Egr-1* responds similarly to PO/AO, whereas *C-fos* behaves differently, possibly on account of some other mechanism which may regulate PO/AO learning in the hippocampus (Huff et al., 2006; Dong et al., 2009; Segal et al., 2010).

In the amygdala, despite similarities in the expression pattern of EGR-1 and c-Fos, we observed a sharp increase in c-Fos expression at the 30 min time point in the case of AO. This suggests that there may be two different mechanisms for PO and AO in the amygdala. This view is supported by Herry et al. (2008), who proposed that there are probably two distinct neuronal populations in the basolateral amygdala: 'fear' and 'extinction' neurons. Fear neurons are possibly

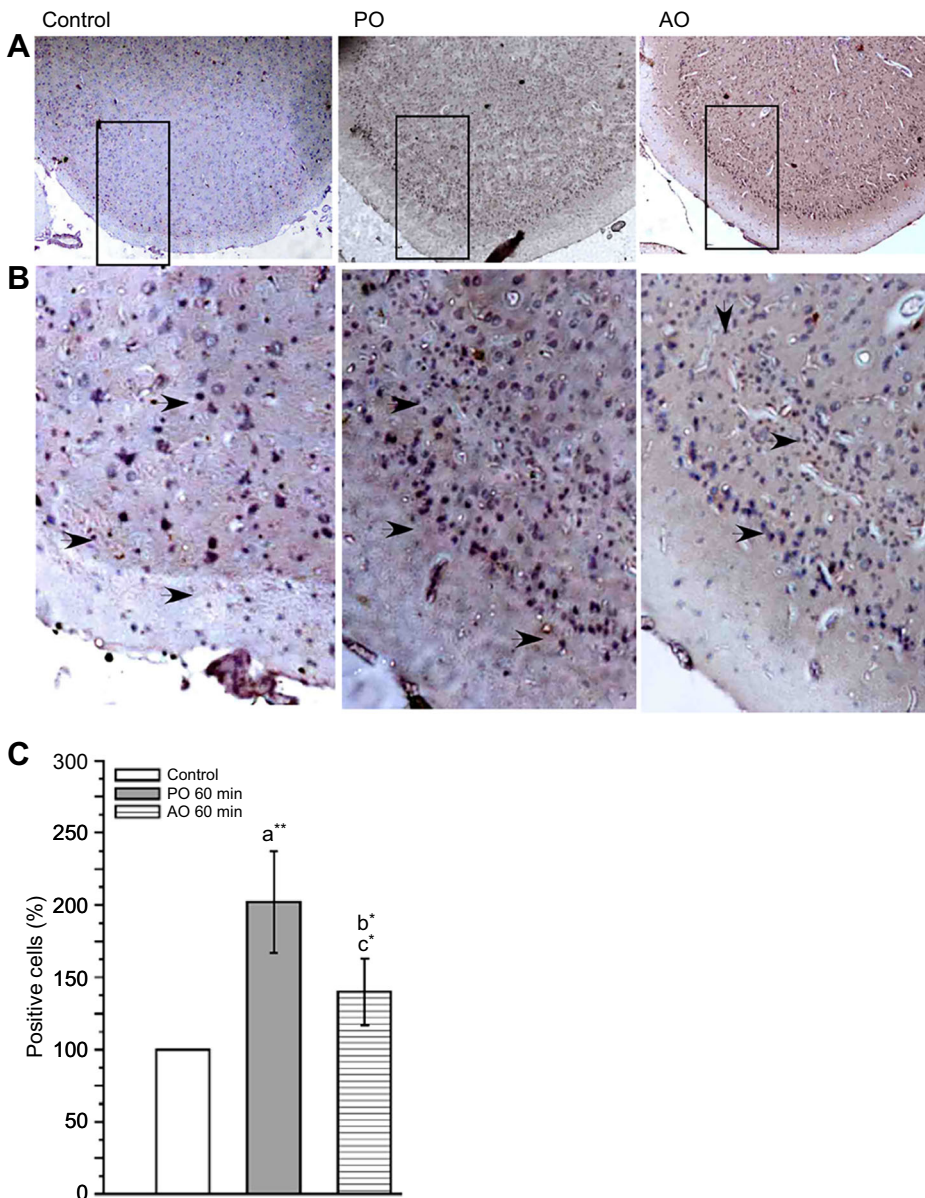


Fig. 10. Representative photomicrographs showing the expression of c-Fos in the amygdala of *C. sphinx* after training to PO or AO. Images at (A) 10× and (B) 20× magnification. c-Fos expression was higher in the amygdala of bats which were exposed to AO compared with PO and control. (C) The number of c-Fos immunoreactive cells is shown in relation to the control (\pm s.e.m.); asterisks indicate significant differences (* P <0.05, ** P <0.01) with respect to (a) control versus PO 60 min; (b) control versus AO 60 min; and (c) PO 60 min versus AO 60 min.

excited in the case of fear or aversion. When *C. sphinx* have been trained to AO, these neuronal populations are possibly activated, reaching a high level of IEG expression at 60 min after the beginning of the experiment. In this population, the epigenetics of PP-1 is controlled tightly, as in the case of AO, and we estimated a greater level of methylated PP-1 than in the case of PO or the control. There may be differential epigenetic regulation (H3K9 dimethylation and H3K4 trimethylation) other than that of PP-1, which may promote the induction of *C-fos* expression during AO learning (Gupta-Agarwal et al., 2012; Cortés-Mendoza et al., 2013). In addition, the level of glucocorticoid input in the amygdala is possibly greater with the aversive experience during AO learning, which also enhances *C-fos* expression and in turn synaptic plasticity and the consolidation of AO memory (Donley et al., 2005; Roozendaal et al., 2009). Dwyer and Killcross (2006) described the basolateral amygdala as the region responsible for aversive (odour–taste) learning. Another study claimed that the basolateral complex might be important for gustatory reward (Johnson et al., 2009). Our observations suggest that the amygdala is involved in olfactory learning by connecting the

olfactory bulb and hippocampus. Our behavioural data show that bats initially responded to AO, which suggests that olfacto-gustatory learning could also be involved.

In summary, our experiments reveal the involvement of IEG expression in the olfactory bulb, hippocampus and amygdala for olfactory learning, suggesting that differential expression of IEGs may contribute to neural plasticity in the frugivorous bat *C. sphinx*. Moreover, the expression pattern noted for *C-fos* and the granule cell layer in the hippocampus and amygdala is possibly associated with the storage, retrieval and extinction of PO/AO memory, and elucidates the role of PP-1 in PO and AO memory formation. Further study is required to reveal how PP-1 activates/controls the target proteins according to the odour stimulus.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.E.R.; Methodology: G.M., K.E.R.; Formal analysis: M.M.; Investigation: M.M.; Resources: K.E.R.; Writing - original draft: K.E.R.; Writing - review & editing: W.B., G.M.; Supervision: K.E.R.; Project administration: K.E.R.; Funding acquisition: K.E.R.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.175364.supplemental>

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Table S1. Analysis showing the difference in behavioural response to preference/aversive odour

Behaviour	Comparison	df	Two way ANOVA	
			F	P
Feeding attempts	Between odours (a)	1,48	130.17	< 0.001
	Between days (b)	3,48	8.14	< 0.001
	Between odours X days (c)	3,48	7.73	<0.001
Feeding bouts	Between odours (d)	1,48	107.11	<0.001
	Between days	3,48	0.48	0.84
	Between odours X days	3,48	1.07	0.37

Table S2. Analysis showing the expression pattern of marker genes (*Egr-1*: *C-fos*) in different brain region responding to preference/aversive odour

Brain regions	Marker	Comparison	df	Two way ANOVA	
				F	P
OB	<i>Egr-1</i>	Between odours	1,35	200.36	< 0.001
		Between time intervals	2,35	117.9	< 0.001
		Between odours X time intervals	2,35	862.04	<0.001
	<i>C-fos</i>	Between odours	1,35	1.17	0.3
		Between time intervals	2,35	466	<0.001
		Between odours X time intervals	2,35	387.68	<0.001
HP	<i>Egr-1</i>	Between odours	1,35	18.56	< 0.01
		Between time intervals	2,35	291.45	< 0.001
		Between odours X time intervals	2,35	8.69	<0.05
	<i>C-fos</i>	Between odours	1,35	107.39	< 0.001
		Between time intervals	2,35	35.52	<0.001
		Between odours X time intervals	2,35	46.93	<0.001
AMY	<i>Egr-1</i>	Between odours	1,35	73.11	< 0.001
		Between time intervals	2,35	333.08	< 0.001
		Between odours X time intervals	2,35	116.59	<0.001
	<i>C-fos</i>	Between odours	1,35	71.25	< 0.001
		Between time intervals	2,35	242.04	<0.001
		Between odours X time intervals	2,35	774.21	<0.001

OB: olfactory bulb, HP: hippocampus, AMY: amygdala

Table S3. Analysis showing the differential expression of PP-1 in different brain region responding to preference/aversive odour

Brain regions	Marker	Comparison	df	Two way ANOVA	
				F	P
OB	PP-1	Between odours	1,35	0.31	0.58
		Between time intervals	2,35	571.8	< 0.001
		Between odours X time intervals	2,35	41.98	<0.001
HP	PP-1	Between odours	1,35	1746.64	< 0.001
		Between time intervals	2,35	1223.38	< 0.001
		Between odours X time intervals	2,35	303.81	<0.001
AMY	PP-1	Between odours	1,35	9.87	< 0.01
		Between time intervals	2,35	118.55	< 0.001
		Between odours X time intervals	2,35	42.22	<0.001

OB: olfactory bulb, HP: hippocampus, AMY: amygdala

Table S4. Differential methylated/unmethylated state of PP-1 in different brain region responding to preference/aversive odour

Brain regions	Marker	Comparison	df	One way ANOVA	
				F	P
OB	Unmethylated PP-1	Con Vs PO 60'(a)	1,11	0.45	0.53
		Con Vs AO 60'(b)	1,11	0.01	0.90
		PO 60' Vs AO 60'(c)	1,11	0.50	0.51
	Methylated PP-1	Con Vs PO 60'(a)	1,11	0.04	0.84
		Con vs AO 60'(b)	1,11	0.45	0.53
		PO 60' Vs AO 60'(c)	1,11	0.53	0.50
HP	Unmethylated PP-1	Con Vs PO 60'(a)	1,11	3.95	0.11
		Con Vs AO 60'(b)	1,11	0.66	0.45
		PO 60' Vs AO 60'(c)	1,11	4.7	0.09
	Methylated PP-1	Con Vs PO 60'(a)	1,11	0.33	0.59
		Con Vs AO 60'(b)	1,11	0.03	0.59
		PO 60' Vs AO 60'(c)	1,11	0.55	0.49
AMY	Unmethylated PP-1	Con Vs PO 60'(a)	1,11	13.32	<0.05
		Con Vs AO 60'(b)	1,11	2.97	0.16
		PO 60' Vs AO 60'(c)	1,11	4.48	0.10
	Methylated PP-1	Con Vs PO 60'(a)	1,11	21.43	<0.01
		Con Vs AO 60'(b)	1,11	33.55	<0.01
		PO 60' Vs AO 60'(c)	1,11	3.60	0.13
		Con (unmethyl Vs methyl) (d)	1,11	8.54	<0.05
		PO 60'(unmethyl Vs methyl) (e)	1,11	28.28	<0.01
		AO 60'(unmethyl Vs methyl) (f)	1,11	18.87	<0.05

OB: olfactory bulb, HP: hippocampus, AMY: amygdala, Con: control, PO: preference odour, AO: aversive odour

Table S5. Differential expression of *C-fos* in different brain region responding to preference/aversive odour

Tissue Sections	Marker	Comparison	df	One way ANOVA	
				F	P
OB	<i>C-fos</i>	Con Vs PO 60'(a)	1,11	11.36	<0.05
		Con Vs AO 60'(b)	1,11	0.12	0.73
		PO 60' Vs AO 60'(c)	1,11	10.04	<0.05
HP	<i>C-fos</i>	Con Vs PO 60'(a)	1,11	23.80	<0.01
		Con Vs AO 60'(b)	1,11	9.73	<0.05
		PO 60' Vs AO 60'(c)	1,11	2.38	0.19
AMY	<i>C-fos</i>	Con Vs PO 60'(a)	1,11	28.2	<0.01
		Con Vs AO 60'(b)	1,11	16.24	<0.05
		PO 60' Vs AO 60'(c)	1,11	12.74	<0.05

OB: olfactory bulb, HP: hippocampus, AMY: amygdala, Con: control, PO: preference odour, AO: aversive odour