ORIGINAL PAPER

Olfactory learning and memory in the greater short-nosed fruit bat *Cynopterus sphinx*: the influence of conspecifics distress calls

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Abstract

This study was designed to test whether *Cynopterus sphinx* distress calls influence olfactory learning and memory in conspecifics. Bats were exposed to distress calls/playbacks (PBs) of distress calls/modified calls and were then trained to novel odors. Bats exposed to distress calls/PBs made significantly fewer feeding attempts and bouts of PBs exposed to modified calls, which significantly induced the expression of c-Fos in the caudomedial neostriatum (NCM) and the amygdala compared to bats exposed to modified calls and trained controls. However, the expression of c-Fos in the hippocampus was not significantly different between the experimental groups. Further, protein phosphatase-1 (PP-1) expression was significantly lower, and the expression levels of E1A homologue of CREB-binding protein (CBP) (P300), brain-derived neurotrophic factor (BDNF) and its tyrosine kinase B1 (TrkB1) receptor were significantly higher in the hippocampus of control/bats exposed to modified calls compared to distress calls/PBs of distress call-exposed bats. Exposure to the call possibly alters the reciprocal interaction between the amygdala and the hippocampus, accordingly regulating the expression levels of PP1, P300 and BDNF and its receptor TrkB1 following training to the novel odor. Thus, the learning and memory consolidation processes were disrupted and showed fewer feeding attempts and bouts. This model may be helpful for understanding the contributions of stressful social communications to human disorders.

Keywords Olfactory learning · *Cynopterus sphinx* · Distress calls · c-Fos · Brain-derived neurotrophic factor (BDNF)

Abbreviations

ALP	Alkaline phosphatase
bAFM	Broadband arched frequency modulation
BCIP	5-Bromo-4-chloro-3-indolylphosphate disodium
BDNF	Brain-derived neurotrophic factor
CREB-1	Cyclic AMP response element binding protein-1
ERK-1/2	Extracellular-signal-regulated kinase-1/2
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
IEG	Immediate early gene
LTP	Long-term potentiation
nAFM	Narrow band arched frequency modulation
NBT	Nitro-blue tetrazoliumchloride
NCM	Caudomedial neostriatum
PBs	Playbacks
PKA	Protein kinase A
PP-1	Protein phosphatase-1
TrkB1	Tyrosine kinase B1

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Introduction

Acoustic communication plays a significant role in information exchange between conspecifics (Fenton 2003; Gladziola et al. 2012; Hörmann et al. 2020). Conspecific communications are discrete acoustic structures that readily discriminate and transmit the intentional state of emitters to potential receivers, termed "social calls". Earlier studies reported that social calls' acoustic structures are context-specific, such as mother–pup reunion (Knörnschild et al. 2013), foraging coordination (Wright et al. 2014), group recognition (Budenz et al. 2009), mate attraction (Knörnschild et al. 2014) and distress (Huang et al. 2015). Distress calls are produced during stressful situations such as extreme physical stress (Russ et al. 2005; Carter et al. 2015; Walter and Schnitzler 2019) or when being threatened/attacked by a predator (Lima and Ó Keefe 2013; Huang et al. 2015). The

distress call structure is often in the range of low-frequency, high-intensity broadband and is very similar among species, manifesting as "repeated sequences" (Luo et al. 2013; Hörmann et al. 2020). Earlier studies showed that the amygdala plays a central role in neural circuits involved in social vocalization and response (Naumann and Kanwal 2011; Tressler et al. 2011; Gadziola et al. 2012). It is well known that the amygdala receives intrinsically rewarding and aversive input stimuli, which may be associated with acoustic features within calls and context or experience (Ma et al. 2010; Sengupta et al. 2018).

Earlier studies demonstrated that olfactory information is transferred from the olfactory bulb to the amygdala and the hippocampus (Wilson et al. 2004). Depending on the context, olfactory learning activates a signalling cascade through protein kinase A (PKA), extracellular-signal-regulated kinase-1/2 (ERK-1/2), and cyclic AMP response element binding protein-1 (CREB-1) (Peng et al. 2010; Ganesh et al. 2010a). Activated CREB requires CBP/CREB-binding protein (CBP) and its homologue E1A binding protein (p300) to induce a network of genes that regulate learning and memory. Studies have shown that p300 acetylates lysine residues on histones and enhances memory formation (Maddox et al. 2013). Conversely, serine/threonine (Ser/Thr) protein phosphatases (PPs: PP1, PP2A) dephosphorylate CREB to suppress memory formation (Koshibu et al. 2009; Mauna et al. 2011). Activated CREB influences cellular processes and enhances synaptic plasticity (Minatohara et al. 2016) through the induction of *C-fos*. Training-dependent expression of *C-fos* in the amygdala and the hippocampus has been reported as an indication of memory formation and leads to the transcription of late-response genes (Luscher Dias et al. 2016; Minatohara et al. 2016; Mukilan et al. 2018). Furthermore, activation/inhibition of brain-derived neurotrophic factor (BDNF) and its tyrosine kinase B1 (TrkB1) receptor has been known to influence long-term potentiation (LTP), which is likely to contribute to synaptic plasticity, learning and memory (de Deus et al. 2020).

The Indian greater short-nosed fruit bat *Cynopterus sphinx* feeds on a variety of fruits, flowers and leaves, and they can identify and learn about palatability based on compounds originating from food sources (Elangovan et al. 2006). Earlier studies from my laboratory showed that *C. sphinx* produces multiharmonic audible distress calls as a narrowband arched frequency modulation (nAFM) and a broadband arched frequency modulation (bAFM). The specific structural sequence of the call elicits a behavioural response and alters the levels of stress hormones, neurotransmitters and other molecules involved in the stress response in conspecifics (Ganesh et al. 2010b; Mariappan et al. 2013, 2016). The present study was designed to test whether olfactory learning and memory were influenced by a distress call of conspecifics. To test the hypothesis, individuals were

exposed to the distress call/PB of the distress call/modified call and were then trained to a novel odor to test their learning ability. Subsequently, the expression patterns of c-Fos in the caudomedial neostriatum (NCM) region of the auditory nuclei, the amygdala and the expression levels of PP1, P300, BDNF and its receptor TrkB1 in the hippocampus were examined.

Materials and methods

Animals

Male *Cynopterus sphinx* (forearm length 65 ± 7 mm, body mass 54.3 ± 7.0 g) was captured using a mist net ($9 \text{ m} \times 2$ m; Avinet-Dryden, USA) in a guava orchard located 2 km away from the Bharathidasan University Campus, Tiruchirappalli, India ($10° 16′$ N; $78° 15′$ E). Bat's morphometric details were recorded and tagged with plastic neck collars consisting of light-reflective colored tapes (Rajan and Marimuthu 1999) and then transferred to the animal house facility. Bats were maintained in the free-flight chamber (of $2.2 \times 1.3 \times 2.1$ m) under constant conditions (temperature 30 °C $\pm$ 3 °C, relative humidity $85 \pm 3\%$; light:dark 12 h:12 h) at animal house facility and allowed to acclimatize for 5 days. Bats were feed with the commonly available fruits [sapota (*Achras sapota*), papaya (*Carica papaya*), banana *(Musa paradisiaca)* and guava (*Psidium guajava*)] and water ad libitum. The health status of animals was assessed by inspecting fur, bite wounds and infections. Bats that were inactive and declining to eat during the study were excluded and released at their capture site.

Behavioural analysis

Bats ($n = 6$ for each group) were randomly classified into four groups; (1) control (CON); (2) distress call group—bats were exposed to the distress call of conspecifics; (3) playback (PB) of distress call—bats were exposed to playback of distress call and (4) modified call—bats were exposed to PB of modified call. To test whether responses were specific to the distress call, the original distress call as well as the call structure altered by changing the call sequence "modified call" (Mariappan et al. 2016) was used in this study. The distress call was composed of a specific sequence of two types of syllables [narrow band arched frequency modulation (nAFM: 123.33 ± 1.5 ms; 5.75 ± 0.1 kHz) and broadband arched frequency modulation (bAFM: 203.23 ± 2.9 ms; 7.27 ± 0.01 kHz)] alternatively. In the modified call, the sequence of syllables was changed to: nAFM, nAFM, bAFM, and bAFM. Distress and modified calls (Figs. 1, 2) used in this experiment were reported earlier from my laboratory (Mariappan et al. 2016).
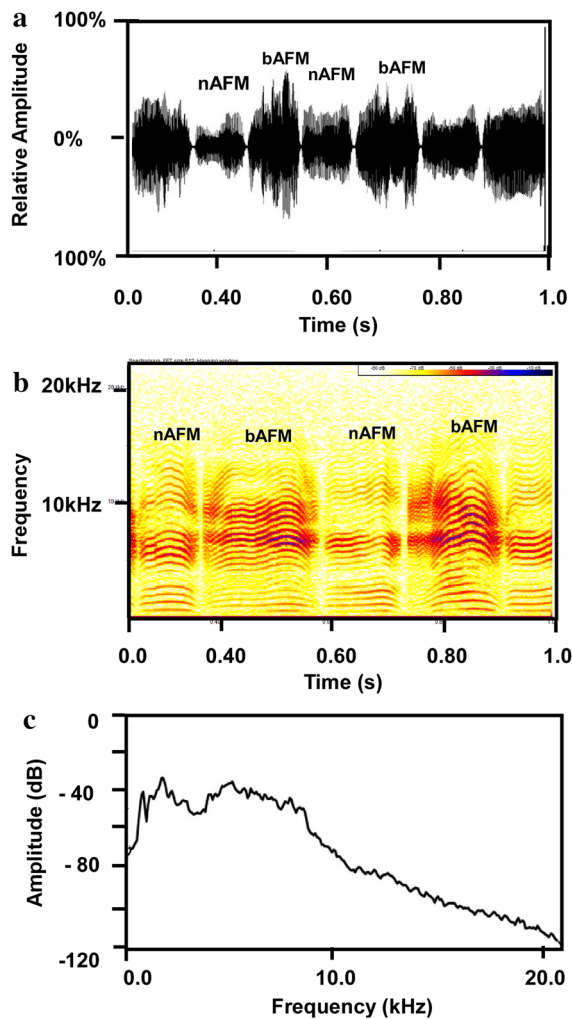
Fig. 1 Free-ranging *C. sphinx* distress call after becoming trapped in a mist net. **a** Oscillogram of a continuous distress call, with narrow-band arched frequency modulation (nAFM) and broadband arched frequency modulation (bAFM) fused together and alternating continuously. **b** Spectrogram of continuous distress calls indicating harmonics. **c** Relative power spectrum depicting peak frequencies of the distress call
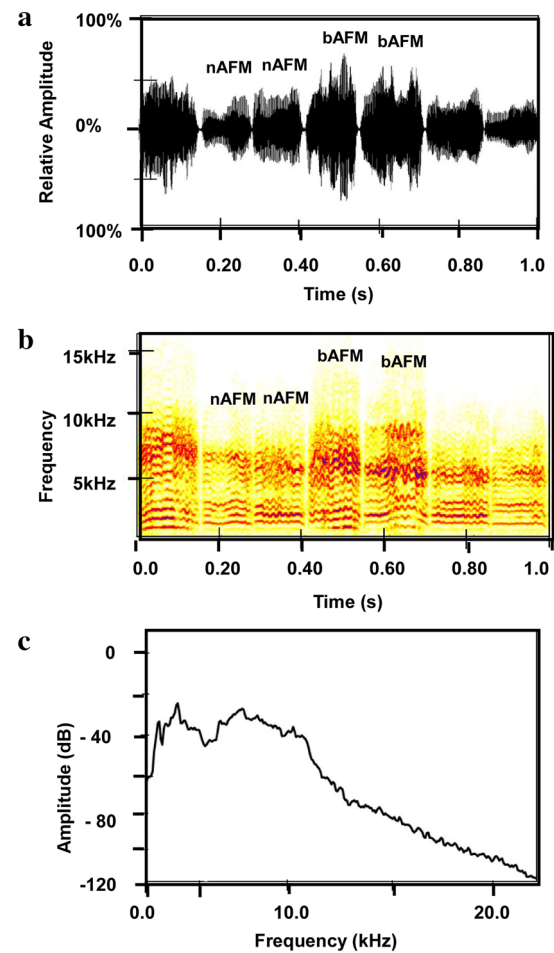

Fig. 2 The modified distress call of *C. sphinx* shows variation in **a** the oscillogram, with nAFM and nAFM fused together and then bAFM and bAFM alternating continuously. **b** Spectrogram of modified calls indicating altered harmonics. **c** Relative power spectrum depicting peak frequencies of the modified distress call

Playback experiment

The experimental facility consists of two chambers: (1) free-flight chamber (2.2 m × 1.3 m × 2.1 m), (2) experimental chamber (2.1 m × 2.4 m × 2.4 m), and the window between chambers facilitates to transfer the bats without disturbances. An hour before the experiment bats were transferred from the free-flight chamber to experimental chamber. Then, distress calls or modified calls were played back (20 s/day) 15 min before the olfactory training (Mariappan et al. 2016). All the experiments were performed under red light (0.09 ± 0.02 lx) to minimize the visual cue (Shafie et al. 2014).

Olfactory training

Bats were individually trained to the novel odor [pieces of chopped apple mixed with freshly prepared cinnamon powder (0.8% wt/wt)] (Ratcliffe and ter Hofstede 2005) and their activities were recorded using a computerized-activity monitor (Electronic Engineering Corporation Inc., India). The activity monitor consists of an infra-red (IR) receiver–transmitter and a mass-sensitive platform (food tray). Their olfactory learning was tested by proving control fruit (fresh pieces of chopped apple) on one platform and fruit with novel odor in the other platform. The constant distance (1.8 m) was maintained between two platforms, as wells between the platforms to perch and location of platforms were changed randomly every day to prevent spatial learning. After acclimatization, bats were individually trained to the novel odor for 5 days (20 min/day), rested for 5 days and then tested their memory for 5 days. Individual bat's responses to novel

odor in terms of: (1) flights out—short flights from the perch (indicating bats are active); (2) attempts—approaches to the food tray (novel odor), but returning without the piece of fruit being picked up; (3) feeding bouts—landings on the food tray and returning with the piece of fruit (Ganesh et al. 2010a, b; Mukilan et al. 2018).

Sample preparation, RNA isolation, and cDNA synthesis

Animals ($n = 4$ from each group) were euthanized and the whole brain was dissected out. The caudomedial neostriatum (NCM), amygdala and hippocampal region were dissected as described elsewhere (Kalin et al. 1994), then bisected for preparation of total RNA and protein. Total RNA was isolated using TRIzol (Merck Specialties Pvt. Ltd., Mumbai, India) and stored with RNase inhibitor (GeNei™; Merck Specialties Pvt. Ltd.) at −80 °C. Total RNA (2.0 µg/sample) was reverse transcribed into cDNA (I Script Reverse Transcription Kit; Bio-Rad Laboratories, Hercules, CA) in accordance with the manufacturer's instructions.

Quantitative real-time PCR

The quantitative real-time PCR (qRT-PCR) reactions were performed using the reaction mixure (20 µL; SSoAdvanced™ SYBR® green supermix; Bio-Rad Laboratories) with specific primers (10 µM) and cDNA (0.1 µg). The specific primers used were: TrkB1 (For 5′-CCAAGAGGC TAAATCCAGTCC-3′ and Rev 5′-CCAGGTTACCAACAT GCTAATA-3′); and GAPDH (glyceraldehydes-3-phosphate dehydrogenase; For 5′-CGGGAAGCTCA CTGGCATGG-3′ and Rev 5′-CCTGCTTCACCACCTTCTTG-3′). The qRT-PCR reactions were performed using the CFX-96 Touch™ Real-time PCR Detection System (Bio-Rad Laboratories Inc.) following the conditions: initial denaturation (92 °C, 30 S); denaturation (92 °C, 5 S), annealing (TrkB1: 61 °C; GAPDH: 58 °C for 5 S), extension (72 °C, 5 S), with 39 cycle repeats and melt-curve analysis (65–95 °C, 0.5 °C increment for 0:05 S). Reactions were performed in triplicates with three fold serial dilution of cDNA to verify the consistency and then normalized with the internal control GAPDH. The data are presented as mean fold change of the relative expression (CFX Manager™ version 2 software, CFX-96 Touch™ Real-time PCR Detection System; Bio-Rad Laboratories Inc).

Western blotting

Samples were homogenised in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–Hcl pH 7.5, 5 mM EDTA, 0.1% V/V NP-40, 1.0 mM DTT, 0.2 mM sodium orthovanadate, 0.23 mM PMSF) with protease inhibitor cocktail (Sigma-Aldrich, USA). Subsequently, homogenates were incubated on ice for 30 min and then centrifuged ($10,000 \times g$) for 30 min at 4 °C. Finally, clear supernatants were collected by centrifuged at $12,000 \times g$ for 15 min at 4 °C, and then stored at −80 °C as aliquots. Protein concentration was estimated using a Biophotometer (Eppendorf Inc., Germany). An equal concentration of protein (60 µg) was mixed with a loading buffer (100% glycerol, 125 mM Tris–HCL pH 6.8, 4% SDS, 0.006% bromophenol blue, 2% mercaptoethanol) and boiled for 5 min, then resolved on 10% polyacrylamide gel (PAGE). Separated proteins were transferred electrophoretically onto the polyvinylidenedifluoride (PVDF) membrane using Turbo-Mini PVDF Transfer Packs (Cat #1704156, Bio-Rad Laboratories Inc, USA) with Trans-Blot® Turbo Transfer System (Cat # 1704150; -Rad Laboratories Inc, USA). The membranes were then pre-blocked with Tris-buffered saline [(10 mM Tris-base pH-7.5, 150 mM Nacl (TBS) containing non-fat dried milk (5.0%) and Tween-20 (0.1%)] for 3 h at room temperature. Membranes were incubated with the any one of the primary antibody, rabbit monoclonal anti-*C-fos* (CST—Cat # 4384, 1:2000)/rabbit monoclonal anti-phospho-*C-fos* (Ser 32) (CST—Cat # 5348, 1:2000)/rabbit monoclonal anti-PP-1α (CST—Cat # 2582, 1:2000)/rabbit polyclonal anti-p300(SC-584; 1:500)/rabbit polyclonal mature anti-BDNF [SC-546 (N-20); 1:500] or rabbit polyclonal anti-β-actin (SC-130656; 1:2000) antibody. The membrane was washed with 1X TBS-T and bound antibodies were detected by incubating for 4 h with goat anti-rabbit (MERK Cat # 62110080011730; 1:2000) alkaline phosphatase (ALP) conjugated antibody. The membrane was washed with 1X TBS-T, and ALP activity was detected with 5-bromo-4-chloro-3-indolylphosphate disodium salt (BCIP)/nitro-blue tetrazoliumchloride (NBT) (AP conjugate substrate kit Cat # 1706432, Bio-Rad Laboratories Inc, USA). The western blot images were acquired using Image Lab 2 software (Molecular Imager ChemiDoc XRS system, Bio-Rad Laboratories, Inc, USA), then the trace quantity of each band was measured and normalized with β-actin.

Statistical analysis

The significant difference was tested using two-way ANOVA for behavioural data and one-way ANOVA for gene expression data and subsequently Bonferroni post hoc test was performed [Sigma Stat software (Ver 22.0)]. Data were presented as a mean ± standard error of the mean (SEM), and plotted with KyPlot (Ver 5.0).
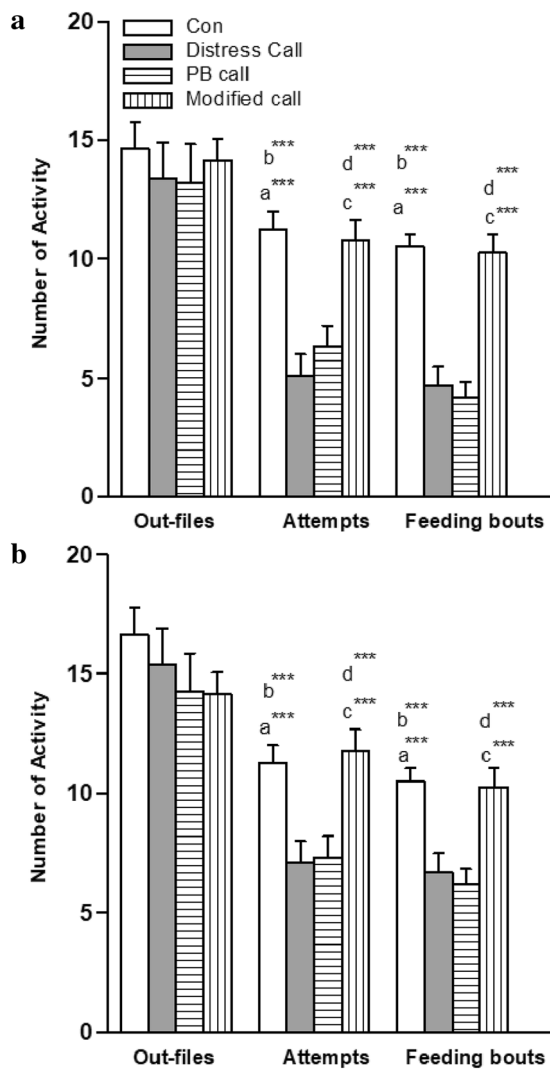
Fig. 3 The specific structure of distress calls influences the olfactory learning and memory of *C. sphinx* conspecifics. Behavioural responses of *C. sphinx* to the novel odour during **a** training and **b** retention tests. Data are shown as the mean ± SEM, *indicates a significant difference (***P 0.001) with respect to comparisons within groups (**a** Con vs Distress call; **b** Con vs PB distress call; **c** modified call vs PB distress call; **d** modified call vs distress call)

Results

Exposure to conspecific distress calls can impair olfactory learning and memory

The observed behavioural response provided evidence that the distress call affects novel odor learning in *C. sphinx* (Fig. 3 During olfactory learning, the *C. sphinx* behavioural response to the novel odor differed significantly between groups ($F_{4,71} = 82.86$; $P < 0.001$) and between behavioural responses ($F_{2,71} = 87.18$; $P < 0.001$), as did the interaction between group × behavioural response ($F_{8,71} = 9.26$; $P < 0.001$). Bonferroni post hoc comparisons revealed significant differences between groups in feeding attempts ($P < 0.001$) and feeding bouts ($P < 0.001$), but not in out-flies ($P = 0.84$) (Fig. 3a).

Similarly, during testing, bat responses to the novel odour were significantly different between groups ($F_{4,71} = 91.62$; $P < 0.001$) and between their behavioural responses ($F_{2,71} = 78.92$; $P < 0.001$), as was the interaction between group × behavioural response ($F_{8,71} = 11.42$; $P < 0.001$). A post hoc test revealed significant differences between groups in feeding attempts ($P < 0.001$) and feeding bouts ($P < 0.001$) but no difference between groups in out-flies ($P = 1.000$) (Fig. 3b). The observed behavioural data demonstrate that the specific structure of the distress call possibly suppresses novel odour learning.

Exposure to conspecific distress calls promotes c-Fos expression in caudomedial neostriatum (NCM)

Expression of c-Fos in the NCM region in the experimental groups was detected, as shown in Fig. 4a. There was a significant difference in the c-Fos expression level between groups ($F_{3,15} = 246.12$; $P < 0.001$). In addition, post hoc analysis revealed that the levels of c-Fos were significantly higher in bats exposed to distress ($P < 0.001$) and PB calls ($P < 0.001$) than in the control group, but there was no difference between the control and bats exposed to modified calls ($P = 0.87$) (Fig. 4b). Similarly, the c-Fos expression levels were significantly higher in bats exposed to distress calls ($P < 0.001$) and PB of distress calls ($P < 0.001$) than in the group exposed to modified calls. The observed c-Fos expression in the NCM of conspecifics suggests that exposure to distress/PB of distress possibly activates neuron in the NCM region.

Exposure to conspecific distress calls promotes c-Fos expression in the amygdala

As shown in Fig. 5a, the level of c-Fos expression in the amygdala differed significantly between the experimental groups ($F_{3,15} = 120.88$; $P < 0.001$). The post hoc analysis showed that the c-Fos expression levels were significantly higher in bats exposed to distress calls ($P < 0.001$) and PB of distress calls ($P < 0.001$), but there was no significant difference between the control and bats exposed to modified calls ($P = 0.246$). Interestingly, when compared with the modified call, the level of c-Fos expression was significantly higher
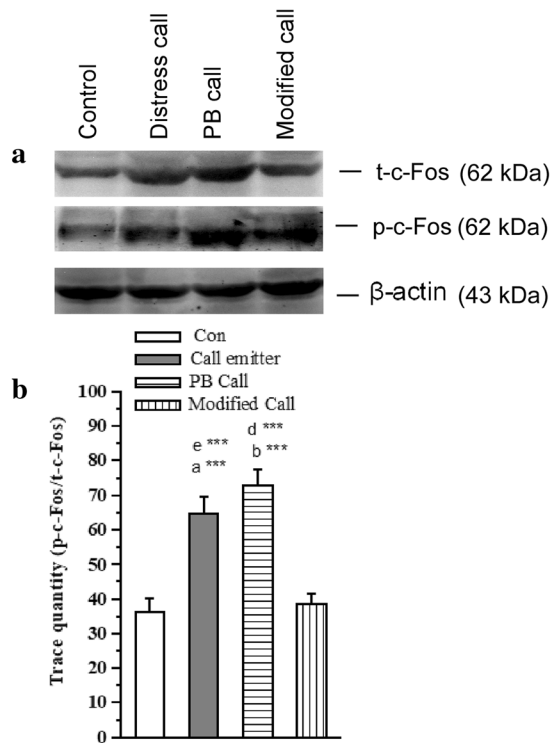
Fig. 4 The specific structure of the distress call promotes c-Fos expression in the caudomedial neostriatum (NCM) of *C. sphinx*. Bats were allowed to listen to the distress call/PB of the distress call/modified call and were trained to a novel odour after 15 min. **a** Representative western blots showing the expression patterns of c-Fos in the NCM regions of experimental groups. Estimated levels of **b** c-Fos expression showing significant differences (*$P<0.05$; ***$P<0.001$) between groups (**a** Con vs Distress call; **b** Con vs PB distress call; **c** distress call vs modified call; **d** PB distress calls vs modified call; **e** Con vs Modified call)

Exposure to conspecific distress calls induces c-Fos expression in the hippocampus

Further analysis showed that the level of c-Fos expression (Fig. 6a) in the hippocampus was not significantly different between the groups ($F_{3,15}=3.12$; $P=0.076$). The results of the post hoc analysis demonstrated that there was no significant difference in c-Fos between the control and bats exposed to modified calls ($P=0.781$). In comparison, there was no significant difference after the bats were exposed to distress calls ($P=0.12$) or PB of distress calls ($P=0.23$) compared with the trained group. Similarly, estimated c-Fos expression levels in bats exposed to a distress call ($P=0.46$) or the PB of a distress call ($P=0.48$) were not significantly different from bats exposed to modified calls (Fig. 6b). This
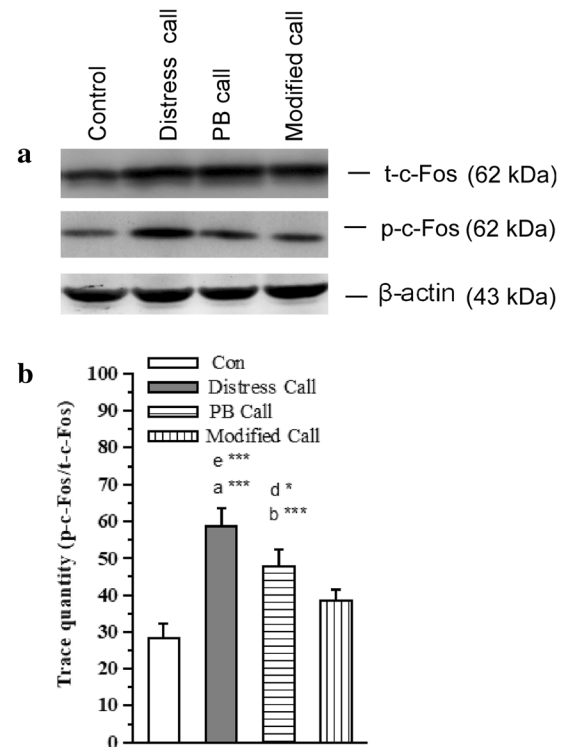
Fig. 5 Conspecific distress calls with specific structures induce the expression of c-Fos in the amygdala of *C. sphinx*. Bats were allowed to listen to the distress call/PB of the distress call/modified call and were trained to a novel odour after 15 min. **a** Representative western blots showing the expression patterns of c-Fos in the amygdala in the experimental groups. Estimated levels of **b** c-Fos expression showing significant differences (*$P<0.05$; ***$P<0.001$) between groups (**a** Con vs distress call; **b** Con vs PB distress call; **c** distress call vs modified call; **d** PB distress calls vs modified call; **e** Con vs modified call)

in the distress call- ($P<0.001$)- and the PB of distress call ($P<0.001$)-exposed groups (Fig. 5b). The observed expression pattern of c-Fos in the amygdala region suggests that the call structure specifically activates the amygdala.

observation demonstrates that a specific call structure differentially induces c-Fos expression in the hippocampus; however, the observed differences in expression were not significantly different.

Exposure to conspecific distress calls selectively regulates protein phosphatase-1 (PP-1) and p300 expression in the hippocampus

A contrasting pattern of PP-1 and p300 expression was observed between the groups (Fig. 7a). The estimated level of PP1 reached significance with respect to the groups ($F_{3,15}=285.48$; $P<0.01$). The post hoc analysis also demonstrated that PP-1 expression was significantly higher after the bats were exposed to distress calls ($P<0.001$) and PBs of distress calls ($P<0.001$) than after the bats were exposed to the trained control group. In comparison, in the modified call-exposed group, the PP-1 expression levels were significantly higher in both the distress call ($P<0.001$)- and the PB of the distress call ($P<0.001$)-exposed groups, but there
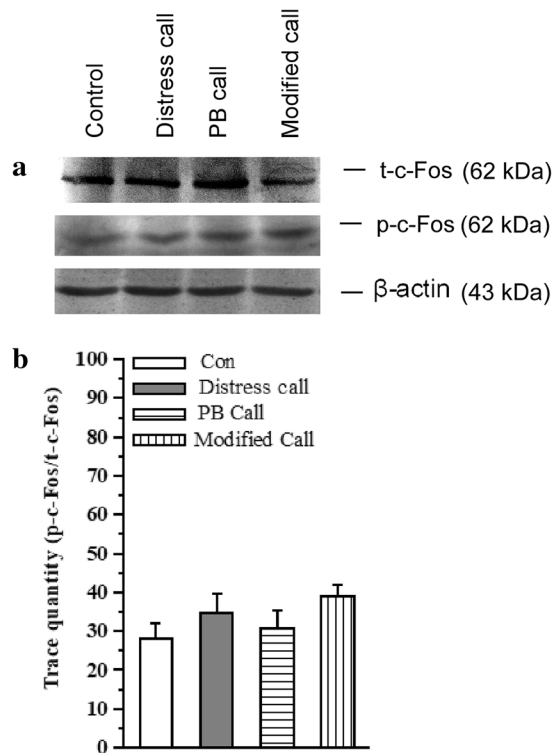
Fig. 6 The specific structure of the distress call induces the expression of c-Fos in the hippocampus of *C. sphinx*. Bats were allowed to listen to the distress call/PB of the distress call/modified calls and were trained to a novel odour after 15 min. **a** Representative western blots showing the expression patterns of c-Fos in the hippocampus of the experimental groups. Estimated levels of **b** c-Fos expression showing no significant difference in any comparison between groups

Fig. 7 Conspecific distress calls with specific structures differentially regulate the expression levels of PP1/P300 in the hippocampus of *C. sphinx*. Bats were allowed to listen to the distress call/PB of the distress call/modified call and were trained to a novel odour after 15 min. **a** Representative western blots showing the expression patterns of PP1 and P300 in the hippocampus of the experimental groups. Estimated levels of **b** PP1 and **c** P300 showing contrasting patterns of expression and significant differences (***$P < 0.001$) between groups (**a** Con vs Distress call; **b** Con vs PB distress call; **c** distress call vs modified call; **d** PB distress calls vs modified call; **e** Con vs modified call)

was no significant difference between the control and modified call-exposed groups ($P = 0.084$) (Fig. 7b). In parallel, the estimated expression levels of p300 were significantly different between groups ($F_{3,15} = 132.46$; $P < 0.001$). Interestingly, post hoc analysis showed no significant difference between the control and modified call-exposed groups ($P = 0.652$). In comparison, p300 levels were significantly lower in the distress call ($P < 0.001$) and the PB of the distress call ($P < 0.001$) groups compared to the control group. Similarly, when compared with the modified distress call group, the p300 levels were significantly lower in the distress call ($P < 0.001$) and the PB of the distress call ($P < 0.001$) groups (Fig. 7c). Evidently, PP1 and p300 were differentially altered in the hippocampus of conspecifics by the specific structure of the distress call.
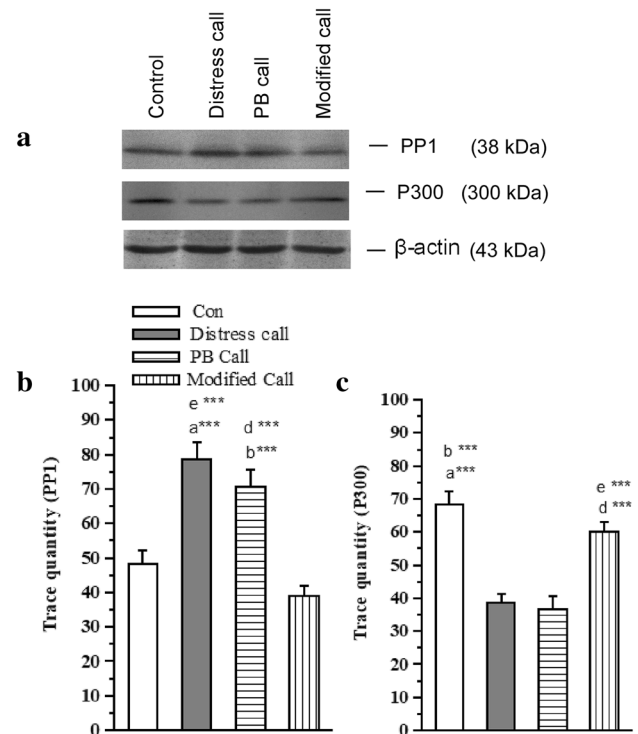
Exposure to conspecific distress calls suppresses the novel odour training-induced expression of brain-derived neurotrophic factor (BDNF) and its receptor tyrosine kinase (Trk) B1 in the hippocampus

Further, the BDNF expression (Fig. 8a) levels were significantly different between the groups ($F_{3,15} = 305.57$; $P < 0.001$). Post hoc analysis revealed that the BDNF levels in the distress call ($P < 0.001$)- and the PB of the distress call ($P < 0.001$)-exposed groups were significantly lower than that of the control group. Furthermore, the observed differences were significantly lower in bats exposed to distress calls ($P < 0.001$) and the PB of distress calls ($P < 0.001$) compared to the modified call-exposed group. However, the expression was not significantly different between the control and modified call-exposed groups ($P = 0.068$) (Fig. 8b). Expression of its receptor TrkB1, which responds to the activation of BDNF, was also significantly different between
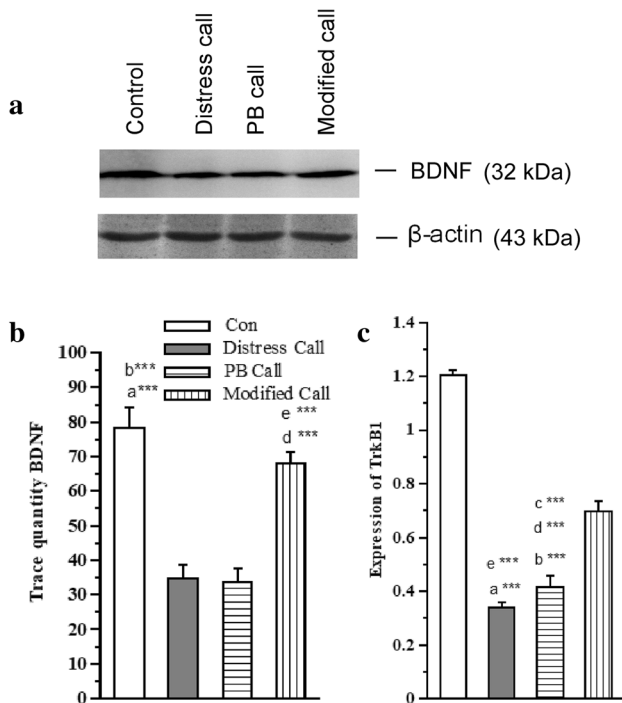
Fig. 8 *C. Sphinx* distress call with a specific structure differentially regulates the expression of BDNF and its receptor TrkB1 in the hippocampus of conspecifics. Bats were allowed to listen to the distress call/PB of the distress call/modified call and were trained to a novel odour after 15 min. **a** Representative western blots showing the expression patterns of BDNF in the hippocampus of the experimental groups. Estimated levels of **b** BDNF and **c** the mRNA levels of TrkB1, showing significant differences (***$P < 0.001$) between groups (**a** Con vs distress call; **b** Con vs PB distress call; **c** distress call vs modified call; **d** PB distress calls vs modified call; **e** Con vs modified call)

Discussion

Earlier studies from my laboratory demonstrated that *C. sphinx* produces distress calls, which trigger call-specific changes in the behavioural, physiological/autonomic state of the receiver (Ganesh et al. 2010b; Mariappan et al. 2013,

2016). The distress call of *C. sphinx* could be a unique syllabi possibly emitted with a distinct temporal pattern (Bohn et al. 2008), could be context-specific and could encode an emotional state (Pfalzer and Kusch 2003; Carter et al. 2015; Walter and Schnitzler 2019). This study was designed to test whether exposure to conspecific distress calls affects olfactory learning in *C. sphinx*.

Conspecific distress call and olfactory learning

After the bats were exposed to the conspecific distress call/ PB of the distress call, they made few feeding attempts and feeding bouts compared to the control/bats exposed to the modified call. Distress calls are known to induce neural responses in the auditory cortex (Martin et al. 2017), amygdala (Gadziola et al. 2016) and hypothalamic–pituitary–adrenal (HPA) axis (Mariappan et al. 2013), and share functional similarities with a human's fearful scream (Hechavarria et al. 2020). Neuronal circuits processing social calls may shape the receptive field selectivity, plasticity and temporal firing pattern (Naumann and Kanwal 2011; Peterson and Wenstrup 2012), possibly due to the contribution of amygdala circuits in aversive or appetitive learning (Namburi et al. 2015; Beyeler et al. 2016). The reconciliation of amygdala activity also depends on the experience, i.e., aversive sound and angry prosody (Andics et al. 2010; Leitman et al. 2010), and raises the possibility that distress call-induced activation may be linked with threatened stimuli-mediated activation of the amygdala associated with aggression or fear (Peterson and Wenstrup 2012; Michael 2019), anxiety-like disorder in humans or social communications with negative emotions (Erkin and Wagen 2007; Sengupta et al. 2018). Therefore, the individuals exposed to distress calls/PBs of distress calls showed fewer feeding attempts and bouts to the novel odour during training and testing. The call sequence of the modified call may be within the acoustic range of *C. sphinx* and activate different populations of neurons in the amygdala (Gadziola et al. 2012) but may not encode stressful information (Mariappan et al. 2016). Thus, bats exposed to modified calls learned to the novel odour and responded during retention.

Distress call-specific syllable sequence induces expression of c-Fos in auditory circuit

The activity-dependent expression of c-Fos is the most commonly used marker to measure neuronal activity, and c-Fos immunoreactivity is elevated in distinct brain regions paired with vocalization or receiving vocal signals compared with silent bats (Ganesh et al. 2010b; Schwart and Smotherman 2011). In this study, a higher level of c-Fos expression was noted in the NCM of bats exposed to distress/PB distress

the groups ($F_{3,15} = 2\,86.17$; $P < 0.001$). Interestingly, post hoc analysis revealed significantly lower expression in individuals exposed to distress calls ($P < 0.001$) and PB distress calls ($P < 0.001$) than in the control group. Similarly, the expression of TrkB1 levels in bats exposed to distress calls ($P < 0.001$) and PB distress calls ($P < 0.001$) were significantly lower than that in the modified call-exposed group. However, the difference in expression between the control and modified call-exposed groups reached significance ($P < 0.001$) (Fig. 8c). The expression pattern of BDNF in the experimental groups showed that different call structures differently activated the expression of BDNF and its receptor.

calls compared to modified calls. This can be interpreted as showing that the specific structure of the distress call/PB of the distress call might promote the induction of c-Fos in the NCM region, as in other animal models (Monbureau et al. 2015), and the expression of c-Fos has been shown to play key roles in synaptic plasticity, learning and memory (Luscher Dias et al. 2016; Mukilan et al. 2018; Kanemoto et al. 2020). Further exposure to distress calls/PBs of distress calls increased c-Fos expression compared to the modified call group. This is possibly due to the specific feature of a distress call that activates auditory cortex neurons and demonstrates experience-dependent plasticity and learning in other bat species (de Hoz et al. 2018; Hörpel and Firzlaff 2019), other animal models and humans (Kanwal and Rauschecker 2007; Behler and Uppenkamp 2020). Earlier studies in other animal models and in humans demonstrated that in the amygdala receiving auditory input (Sander and Scheich 2001), a stressful auditory signal induces the expression of c-Fos in the amygdala and develops fear memory associated with the auditory signal (Nauman and Kanwal 2011; Chaaya et al. 2019). It is well known that amygdala neurons respond to social calls selectively, and their activity is positively correlated with social interactions (Katayama et al. 2009), including fear-related signals in bats (Nauman and Kanwal 2011) and humans (Whalen et al. 2004). In other models, multimodal brain cluster analysis suggest that movement (flight, vocalization, body movement) associated motor control circuits and auditory associated motor control circuits are under general cerebral motor system. However, the activation of movement associated motor circuits did not induce IEGs expression in the hippocampus, and higher auditory brain regions (Nelson 1996; Feenders et al. 2008). Therefore, observed elevated level of c-Fos expression in NCM and amygdala is possibly due to auditory input, which activates emotional reactivity, fear learning and memory circuits embedded in the amygdala (Namburi et al. 2015; Beyeler et al. 2016) and humans (Wiethoff et al. 2009).

Distress call syllable sequences are specifically shifts expression of c-Foss, protein phosphatase-1 (PP-1) and p300 in amygdala, and hippocampus

Odor-associated stimuli are connected through multiple regions of the brain, and input from sensory neurons in the olfactory bulb transmits olfactory information to other brain regions, including the olfactory bulb, amygdala and hippocampus (Wilson et al. 2004; Sosulski et al. 2011). The amygdaloid complex is known to connect two sensory systems (i.e., auditory and olfactory) that are sensitive to stress, which can lead to impairments in learning and memory (Soudry et al. 2011; Kiyokawa et al. 2012). Distress calls induce neuronal activation in the amygdala, resulting in an increase in neuronal excitability that may recall

the fear memory of *C. sphinx* associated with distress calls (Chattarji et al. 2015) or may suppress exploratory behaviour to the novel odor, possibly by inhibiting amygdala output (Colas-Zelin et al. 2012). Thus, the bats exposed to distress calls/PBs of distress calls showed fewer feeding attempts and bouts towards novel odors. However, the modified call acoustic properties are not similar to the conspecific distress call, are unfamiliar to the bats (Mariappan et al. 2016), and may not provide specific information to the receiver (Fallow et al. 2013). Supporting earlier observations, in this study, individuals exposed to the modified calls learned the novel odor and responded during retention.

The reciprocal interaction between the amygdala and the cortex/hippocampus has been known to alter behavioural metaplasticity by aversive/threatening stimuli (Schmidt et al. 2013; Saha et al. 2020). Therefore, the transcription of target genes was examined in the hippocampus. The training-induced expression of p300 was significantly lower in the hippocampus of bats exposed to distress calls/PBs of distress calls. However, p300 expression levels were elevated in control/bats exposed to modified calls after the bats were trained with novel odor, which suggests that p300 was activated by novel odour stimulus (Oliveira et al. 2011). This observation suggests that novel odor-induced stimuli play an agnostic role in p300 expression in the hippocampus and may further activate the transcription of target genes (Oliveira et al. 2011). Conversely, transcription of CREB-targeted genes is suppressed by protein phosphatases (PP1α, PP2A) and phosphorylation of CREB (Koshibu et al. 2009; Mauna et al. 2011), thereby reducing synaptic plasticity. In line with earlier reports, the level of PP1α in the hippocampus was significantly low in bats exposed to modified calls and similar to control bats, but not in bats exposed to distress calls/PBs of distress calls (Koshibu et al. 2009; Mauna et al. 2011). These results suggest that reduction of the PPs and elevated expression levels of CBP/P300 could be linked with bat learning and responses to the novel odor.

Distress call syllable-specific sequences regulates olfactory learning

Activity-dependent expression of BDNF and its tyrosine receptor kinase B1 (TrkB1) has been known to regulate synaptic plasticity, LTP and memory formation (Sakata et al. 2013; Zhong et al. 2016). In this study, the level of BDNF was significantly lower in bats exposed to distress calls/PBs of distress calls than in control bats/bats exposed to modified calls. The observed results suggest that exposure to the distress call/PB of the distress call activates the amygdala and possibly modifies amygdala activity and plasticity in other brain regions, including the hippocampus (Schmidt et al. 2013), which may alter metaplasticity. Thus, exposed bats showed few feeding attempts and bouts to novel odour
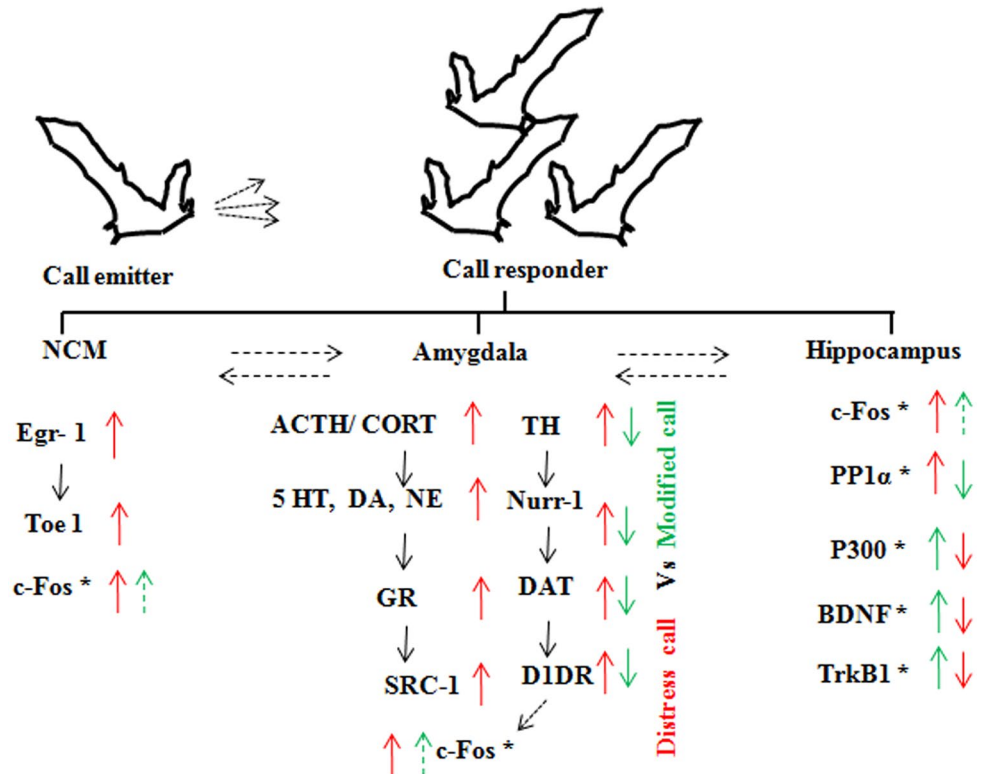
during training and testing. Supporting this observation, earlier studies in other models reported that exposure to stress reduces BDNF expression in the hippocampus (Makhathini et al. 2017). Elevated levels of BDNF in the hippocampus after training with the novel odor in the control/modified call-exposed group could be correlated with a greater number of responses to the novel odor (Sakata et al. 2013; Tong et al. 2018). In addition, it has been demonstrated that elevated levels of BDNF in the hippocampus act through its high-affinity receptor TrkB1 to trigger synaptic plasticity/ long-term memory (Nasrallah et al. 2019). The expression level of TrkB1 was significantly higher in the trained control and the bats exposed to modified calls than in the distress call/PB of the distress call-exposed groups. The observed behaviours of the control/modified call-exposed groups are in line with earlier reports stating that overexpression/ activation of TrkB1 in the hippocampus enhances learning and memory (Karpova et al. 2014; Nasrallah et al. 2019). Reduced levels of TrkB1 possibly disrupt synaptic plasticity; therefore, individuals exhibit very low feeding bouts to novel odors, which is similar to other animal models that have shown impaired learning and memory (Badowska-Szalewska et al. 2010; Ren et al. 2015). These results suggest that BDNF and TrkB1 expression may be tightly controlled by input from the amygdala, which may act as a master switch in learning and memory in different environmental stimuli and biological contexts.

Conclusion

Linking with other studies, distress calls are functionally similar to a human's fearful screams, and the specific structure of the distress call activates the NCM and the amygdala of the receiver. The acoustic structure of the distress call/PB of the distress call may activate neuronal circuits involved in aggressive, fearful and affiliative behaviour embedded in the amygdala, which significantly induces c-Fos expression compared to bats exposed to modified calls. Interestingly, the reciprocal interaction between the amygdala and the cortex/hippocampus could be differentially regulated by the distress call/PB of the distress call/modified call, which further selectively drives the expression of PP1, CBP/P300 and BDNF and its receptor TrkB1 following training to the novel odor. Therefore, the exposure of *C. sphinx* to distress calls/ PBs of distress calls influences conspecific olfactory learning and memory. Furthermore, this model can be used to understand the fear/threatened stimuli related to social communication-induced learning and memory disorders (Fig. 9).

Fig. 9 Schematic representation of the intracellular signalling molecules (Egr-1—early growth response 1 gene; TOE 1—target of Egr 1; 5-Hydroxytryptamine (5-HT-serotonin); ACTH—adrenocorticotropic hormone; CORT—corticosterone; DA—dopamine; DAT—dopamine transporter; GR—glucocorticoid receptor; SRC-1—steroid receptor co-activator; D1DR—dopamine receptor; Nurr-1—nuclear receptor-related factor-1; TH—tyrosine hydroxylase; PP1α—protein phosphatase 1; BDNF—brain-derived neurotrophic factor; TrkB—tropomyosin receptor kinase B) altered by exposure to the distress call or the modified distress call. Observed changes in this study marked with * green arrows indicate the effects of modified calls, and red arrows indicate the effects of distress calls

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