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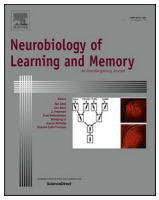

Frustrative nonreward: Detailed c-Fos expression patterns in the amygdala after consummatory successive negative contrast

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ABSTRACT

The amygdala has been implicated in frustrative nonreward induced by unexpected reward downshifts, using paradigms like consummatory successive negative contrast (cSNC). However, existing evidence comes from experiments involving the central and basolateral nuclei on a broad level. Moreover, whether the amygdala's involvement in reward downshift requires a cSNC effect (i.e., greater suppression in downshifted animals than in unshifted controls) or just consummatory suppression without a cSNC effect, remains unclear. Three groups were exposed to (1) a large reward disparity leading to a cSNC effect (32-to-2% sucrose), (2) a small reward disparity involving consummatory suppression in the absence of a cSNC effect (8-to-2% sucrose), and (3) an unshifted control (2% sucrose). Brains obtained after the first reward downshift session were processed for c-Fos expression, a protein often used as a marker for neural activation. c-Fos-positive cells were counted in the anterior, medial, and posterior portions (A/P axis) of ten regions of the rat basolateral, central, and medial amygdala. c-Fos expression was higher in 32-to-2% sucrose downshift animals than in the other two groups in four regions: the anterior and the medial lateral basal amygdala, the medial capsular central amygdala, and the anterior anterio-ventral medial amygdala. None of the areas exhibited differential c-Fos expression between the 8-to-2% sucrose downshift and the unshifted conditions. Thus, amygdala activation requires exposure to a substantial reward disparity. This approach has identified, for the first time, specific amygdala areas relevant to understand the cSNC effect, suggesting follow-up experiments aimed at testing the function of these regions in reward downshift.

1. Introduction

The way mammals respond to a reward often depends on the relative value of the reward rather than on its absolute value. Reward relativity occurs when behavior in a situation involving a particular reward depends on other rewards previously experienced (successive contrast), currently present (simultaneous contrast), or about to occur (anticipatory contrast), in the same or similar context (Flaherty, 1996; Torres & Papini, 2017). The research reported in this article deals with successive negative contrast (SNC), a procedure in which an unexpected reduction in reward magnitude induces a transient disruption of appetitive behavior, whether under consummatory (cSNC; Vogel et al., 1968), instrumental (iSNC; Crespi, 1942), or Pavlovian procedures (pSNC; Conrad & Papini, 2018).

In the cSNC procedure used in the present experiment, 10 daily preshift sessions of access to 32 % sucrose were followed by one session in which the concentration was unexpectedly downshifted to 2 % sucrose. Under these conditions, rats typically exhibit less licking behavior than unshifted controls always exposed to 2 % sucrose—the cSNC effect (Hagen et al., 2023). cSNC is accompanied by behavioral, physiological, and neuroendocrine correlates, such as rejection of the reduced reward, escape responses, activation of the hypothalamic–pituitary–adrenal axis, and changes in aggressive, sexual, and pain-related responses (see Papini et al., 2015).

Papini (2022) suggested that reward relativity demonstrations, such as the cSNC effect, require the presence of negative emotion. Following Amsel (1958, 1992), the unexpected reduction or omission of a reward is called frustrative nonreward (FNR). An alternative phrasing of this conclusion in the context of cSNC would state that animals showing evidence of sensitivity to reward magnitude in their preshift

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performance (e.g., responding more to the large reward than to the small reward), but failing to exhibit the cSNC effect should show no signs of FNR. This is supported by the notion that there needs to be a significant disparity between the values of the obtained and expected rewards to produce evidence of frustration (Papini & Pellegrini, 2006). However, the absence of the cSNC effect on a purely behavioral level does not guarantee a lack of negative emotion as an internal state. Thus, the present experiment incorporated both a large reward disparity (32-to-2 % sucrose) and a small reward disparity (8-to-2 % sucrose) to compare evidence of frustration at both the behavioral and neurobiological levels. A small 8-to-2 % sucrose disparity is expected to produce some behavioral suppression, but not a cSNC effect in that licking would not differ from the level of an unshifted control. To test whether an 8-to-2 % sucrose downshift provides neurobiological evidence consistent with FNR, we assessed amygdala activation as indexed with the protein c-Fos, which is expressed in recently depolarized neurons (Chung, 2015). There is extensive evidence that a variety of situations involving aversive emotional activation, including cSNC, lead to amygdala activation as assessed by c-Fos expression (e.g., Jin et al., 2004; Kovács et al., 2018; Moscarello et al., 2009; Numa et al., 2019; Pecoraro & Dallman, 2005; Soto et al., 2017; Weinberg et al., 2010).

Converging lines of evidence point to the amygdala as a critical component of the neural circuit underlying the cSNC effect. Pecoraro and Dallman (2005) measured c-Fos expression and found significant brain activation after the first session involving a 32-to-4 % sucrose downshift relative to 4 % unshifted controls. Among the regions involved were the basolateral (BLA) and the medial amygdala (MeA). In line with these results, the expression of the pCREB protein (phosphorylated cAMP response element-binding protein), a marker of synaptic plasticity, increased in both the BLA and central amygdala (CeA), after a 32-to-4 % sucrose downshift (Glueck et al., 2015). Excitotoxic lesions of the BLA eliminated the cSNC effect without affecting unshifted controls (Kawasaki et al., 2017). Furthermore, lesions (Becker et al., 1984), lidocaine inactivation (Kawasaki et al., 2015), and chemogenetic inhibition (Guarino et al., 2020) of the CeA reduced or eliminated the cSNC effect relative to sham/vehicle controls, without affecting the behavior of unshifted controls. Thus, the BLA and CeA have been proposed to play a central role in reward comparisons and the suppression of consummatory behavior, respectively, during the downshift (Kawasaki et al., 2017; Ortega et al., 2017). These studies focused on the role of major amygdala nuclei (e.g., CeA, BLA), but failed to provide insights into the role of more restricted regions along an anterior-posterior axis. The present study was designed to provide for the first time a more detailed analysis of the role played by the amygdala's various regions in the cSNC effect.

In addition to this more detailed analysis of various regions of the amygdala, this experiment tested three hypotheses. First, behaviorally, there will be evidence of cSNC in a comparison between 32-to-2 % vs. 2-to-2 % sucrose, but not between 8-to-2 % vs. 2-to-2 % sucrose. Second, given evidence reviewed previously, there should be more neural activation in at least some amygdala regions (e.g., CeA, BLA) in animals exposed to a 32-to-2 % sucrose downshift than in unshifted controls (2 % sucrose). Third, assuming there is a dissociation between cSNC and amygdala activation (as an index of negative emotion), then c-Fos expression should be (from highest to lowest): 32-to-2 % > 8-to-2 % > 2-to-2 % sucrose. However, if reward relativity effects require the presence of negative emotion (Papini, 2022), then the expected absence of cSNC in the comparison between Groups 8–2 vs. 2–2 should be accompanied by a lack of evidence for differential amygdala activation between these two groups.

2. Method

2.1. Subjects

Eighteen 90-day-old female, experimentally naive Wistar rats with ad libitum weights between 250–300 g served as subjects. Flaherty and Rowan (1989) reported no sex differences in consummatory behavior after a 32-to-4 % sucrose downshift, although a more detailed analysis of sex differences in the cSNC task remains to be done. Rats were bred from parents purchased from Charles River Laboratories (Wilmington, MA) according to an IACUC-approved protocol. Animals were weaned at 21–24 days of age, group housed until around 40 days of age, and individually housed thereafter with an enrichment retreat device (Bio-Serv K3245, Fisher Scientific, Waltham, MA). The animals had free access to standard rat chow until 90 days of age. Rats were housed in a colony room with a 12-h light–dark cycle (lights on at 07:00 h), constant temperature (22–23 °C), constant humidity (50–64 %), and free access to water during all their life. After ad libitum weights were recorded, food was gradually restricted until each animal reached 81–84 % of their average ad libitum weight. This weight level was maintained throughout the duration of the experiment by feeding a controlled amount of rat chow each day at approximately the same time, at least 30 min after the end of behavioral sessions. This ensured a relatively constant motivational state. Training took place at approximately the same time, 7 days a week.

2.2. Behavioral apparatus

Behavioral sessions were conducted in 6 aluminum and Plexiglas conditioning boxes (MED Associates, St. Albans, VT), measuring 29.3 × 21.3 × 26.8 cm (Length × Height × Width). Each box was inside a sound-attenuating cubicle containing a speaker delivering masking white noise and a fan providing air circulation. The speaker and fan produce noise with an intensity of 80.1 dB (SPL, C scale). Masking noise was always present during training sessions. A diffuse light (GE 1820) was placed in the center of the roof of each box. The floor was made of steel rods parallel to the feeder wall and separated by 1.2 cm from center to center. A tray with corn cob bedding was placed beneath the floor to collect feces and urine. The chambers were wiped with a damp paper towel before and after each session, feces were removed, and bedding sawdust was replaced as necessary. In the feeder wall there were three holes, each 1 cm wide, 2 cm long, and 4 cm from the ground, equidistant from each other and from the edge of the wall. Sipper tubes 1 cm in diameter and fitted with a ball bearing to minimize leakage were inserted through the central hole and flush with the outside of the feeder wall to deliver sucrose solutions from attached bottles. Rats were moved from the colony to a holding room in a transport rack and in their own cages.

2.3. Behavioral procedure

Animals were randomly assigned to three groups based on the concentration of sucrose received during 10 preshift sessions. Group 32–2 ($n = 6$) had access to 32 % sucrose; Group 8–2 ($n = 6$) had access to 8 % sucrose; and Group 2–2 ($n = 6$) had access to 2 % sucrose. All animals received access to 2 % sucrose during the final postshift session 11. Each rat was randomly assigned to a conditioning box and was always trained in the same box. Each session lasted 5 min starting from the first recorded contact with the sipper tube. The sipper tubes were presented 30 s after the start of the session and were withdrawn 30 s before the end of the session. Session start and end were marked by the turning on and off of the house light. Licking responses were detected by a circuit involving the rods in the floor and the sipper tube. All events were controlled by a computer located in an adjacent room, which also recorded licking responses. Sucrose solutions were prepared by weight (e.g., for 32 %: 32 g of sucrose per 78 g of deionized water). Pilot research indicated no differences in licking between solutions diluted in deionized vs. distilled water. In preparation for perfusions scheduled to occur 90 min after the end of session 11, rats started and ended the training in a staggered manner. The 18 rats were randomly assigned to 3 squads of 6 rats each and each squad had 2 rats from each group. The

second squad started training a day after the first squad, and the third squad started training a day after the second squad. This procedure distributed perfusions at the end of session 11 over a 3-day period. Squad 1 started training at 10:00 h, squad 2 started at 11:00 h, and squad 3 started at 13:00 h. Once training sessions were completed, rats were returned to their cage and taken back to the colony.

2.4. Perfusion

Perfusions started 90 min after postshift session 11. This interval was selected for maximum c-Fos expression (Morgan & Curran, 1991). Rats were anesthetized with an ip injection of sodium pentobarbital and heparin. Once fully anesthesized, rats were perfused transcardially with 0.01-M phosphate buffer saline (PBS) followed by 4 % paraformaldehyde (PFA) in 0.01-M PBS (pH 7.4) via perfusion pump. After full fixation, brains were extracted and kept at 4 °C in 4 % PFA for at least 2 days and then placed in 30 % sucrose for cryoprotection for at least 2 days.

2.5. Histology

Brains were washed in 0.01-M PBS and then sectioned with a cryostat (Leica Biosystems, Buffalo Grove, IL) in 40-μm thick coronal sections between A/P coordinates −1.56 mm and −3.48 mm relative to Bregma (Paxinos & Watson, 2013). These coordinates were chosen with the aim of obtaining rostral, medial, and caudal sections of each amygdala nucleus included in the study. Table 1 and Fig. 1 provide a list of the regions explored and the A/P coordinates. Two out of 3 consecutive sections were collected, one of which was used for immediate analysis and the other for longer term storage. Sections were placed in serial order and maintained free floating in 0.01-M PBS at 4 °C for subsequent immunohistochemistry.

2.6. Immunohistochemistry

For the immunohistochemical analysis of c-Fos, brain sections were first incubated in a 3 % hydrogen peroxide solution in 0.01-M PBS (pH 7.4) for 30 min at room temperature with gentle agitation to inhibit endogenous peroxide activity. Sections were washed with 0.01-M PBS and then incubated for 2 h at room temperature and with gentle shaking in a PBS solution containing 0.3 % Triton X100 and 2 % normal goat serum to minimize nonspecific binding. Sections were then incubated for 48 h at 4 °C with a rabbit polyclonal antibody against the c-Fos protein (ab190289, 1:10.000, Abcam, Cambridge, UK) diluted in a 0.3 % Triton X100 solution in 0.01-M PBS (0.01 M) and 0.25 % bovine serum albumin under gentle agitation. Then, sections were washed and incubated with a biotinylated anti-rabbit antibody (31.820, 1:400, Invitrogen, Carlsbad, CA) diluted in 0.01-M PBS for 2 h at room temperature with gentle shaking. Sections were washed and then incubated with an avidin–biotin–peroxidase complex (Vectastain ABC kit, PK-6100, Vector Laboratories, Burlingame, CA) for 30 min at room temperature with gentle shaking. Sections were then washed two times, 5 min each, in 0.01-M PBS and washed two times, 5 min each, in 0.1-M Tris-buffer (pH 6.0) at room temperature with moderate shaking. Sections were incubated in a 3,3-diaminobenzidine (DAB) substrate kit (SK-4.100, Vector Laboratories, Burlingame, CA). All reactions were carried out for 4 min before being stopped by rinsing of the sections twice, 5 min each, in 0.01-M PBS at room temperature and moderate shaking. Sections were then mounted on gelatinized slides and allowed to dry for at least 24 h. Sections were subsequently dehydrated and rinsed with alcohol of increasing concentrations (80 %, 90 %, and 100 %) and xylol (I and II) for 1 min in each solution. Sections were mounted and covered with DPX, and dried for at least 24 h.

Sections were visualized using a Nikon Eclipse 90i microscope equipped with a DS-Fi1 digital camera (Nikon, Melville, NY). Histological counting of c-Fos positive cells in the regions of interest was obtained using the ImageJ 1.54b software (National Institute of Mental Health, MD). The experimenter counting cells had no knowledge of the experimental conditions. c-Fos-positive cells were automatically identified by the software by thresholding objects with 0.9–1.00 circularity value, matching c-Fos positive nuclei. To minimize background noise and equalize all the microphotographs they were previously converted into 8-bit-type images, at a size of 1 to 10 pixels and lightened (150.0 pixels). Counts were obtained from both hemispheres and then averaged to reach a final count for each region (anterior, medial, and posterior) and for each animal. Counts were then divided by the area of each region, calculated by marking regions of interest in the ImageJ 1.54b software using boundaries set by the neuroanatomical atlas of Paxinos and Watson (2013), superimposing their transparent figures on the photos of each region of interest using Photoshop (Adobe, San José, CA). Data for amygdala regions were quantified according to the coordinates specified in Table 1.

Table 1
Amygdala regions included for c-For analysis with their A/P coordinates. For each region, c-Fos expression was recorded in an anterior, medial, and posterior section. The coordinates come from Paxinos and Watson (2013). BLA: basolateral amygdala. CeA: central amygdala. cCeA: capsular central amygdala. lB: lateral basal of the BLA nucleus. lCeA: lateral central amygdala. mCeA: medial central amygdala. lA: lateral amygdala of the BLA nucleus. mB: medial basal of the BLA nucleus. MeA: medial amygdala. adMeA: antero-dorsal medial amygdala. avMeA: antero-ventral medial amygdala. pdMeA: postero-dorsal medial amygdala. pvMeA: postero-ventral medial amygdala.

		Anterior	Medial	Posterior
	lA	−2.4	−3.0	−3.48
BLA	lB	−1.92	−2.4	−3.48
	mB	−1.92	−2.4	−3.48
	cCeA	−1.92	−2.4	−3.36
CeA	lCeA	−1.92	−2.4	−3.0
	mCeA	−1.92	−2.16	−2.4
	adMeA	−1.56	−1.92	−2.4
MeA	avMeA	−1.92	−2.16	−2.4
	pdMeA	−2.64	−3.0	−3.48
	pvMeA	−2.64	−3.0	−3.48

2.7. Statistics

The dependent variables were lick frequency (total licks recorded for each animal in each 5-min session in the cSNC task) and density (cells/mm^2) for c-Fos quantification. These variables were subjected to analysis of variance with Group and Session as factors for the cSNC task, and Group by A/P axis for c-Fos analysis. Sessions and A/P axis were repeated-measure factors. Pairwise Bonferroni comparisons were used to identify the source of significant interactions. In all statistical tests, $p < 0.05$ was used for inferences and partial eta squared, η_p^2, was used to assess effect size. Only statistical data for significant effects are reported in the Results. All statistics were run on the IBM SPSS Statistics 27 package.

3. Results

3.1. Behavior

Fig. 2 shows the consummatory behavior (lick frequency) of each group during preshift (mean of sessions 8–10) and postshift (session 11) sessions. One animal assigned to Group 2–2 was excluded from all the analyses because its consummatory behavior was inconsistent with very low lick frequency, therefore leaving this group with 5 subjects. A Group (32–2, 8–2, 2–2) by Session analysis indicated significant effects for both main effects and interaction, $Fs > 16.75$, $ps < 0.001$, $\eta_p^2 s > 0.70$. Pairwise Bonferroni comparisons derived from the main analysis provided the following information. For preshift performance, lick frequency in Group 2–2 was significantly lower than in Groups 32–2 and 8–2, $ps <$

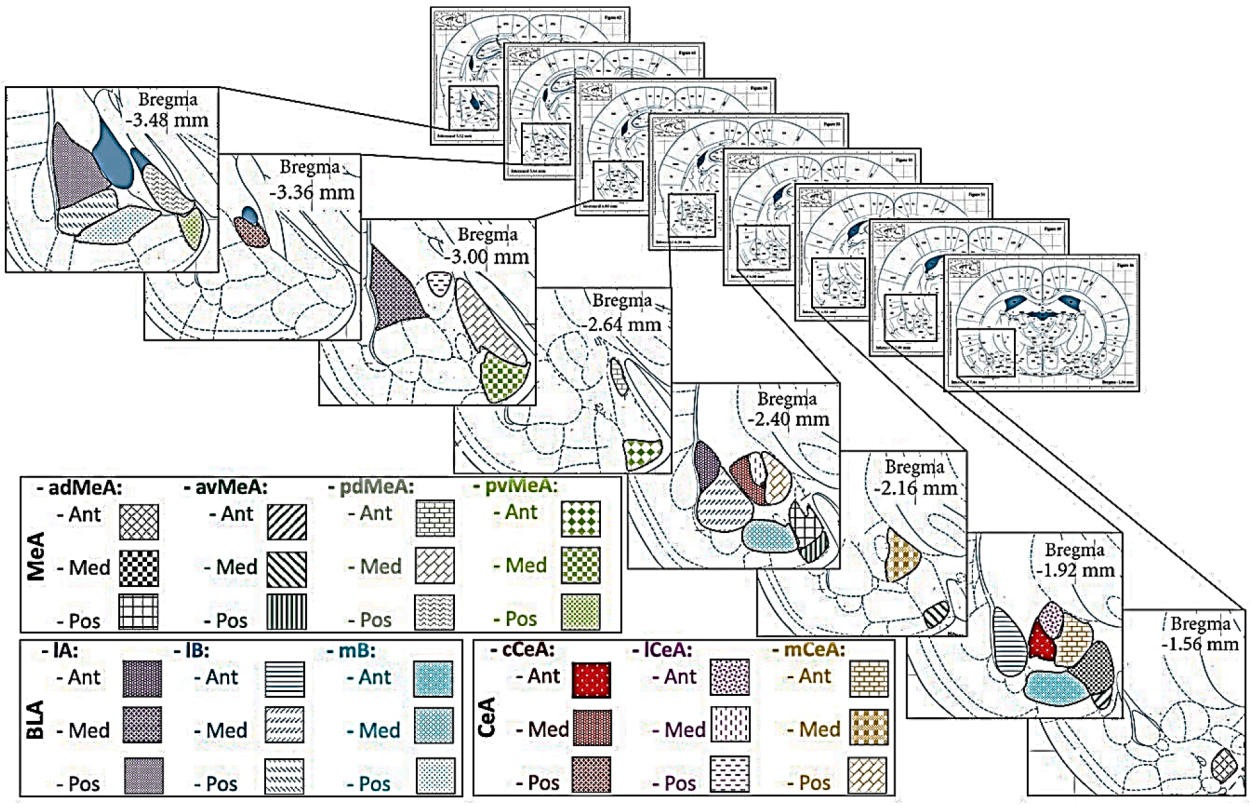

Fig. 1. Amygdala areas surveyed in the present experiment. A/P coordinates from the Paxinos and Watson (2013) atlas. See Table 2 for area acronyms.

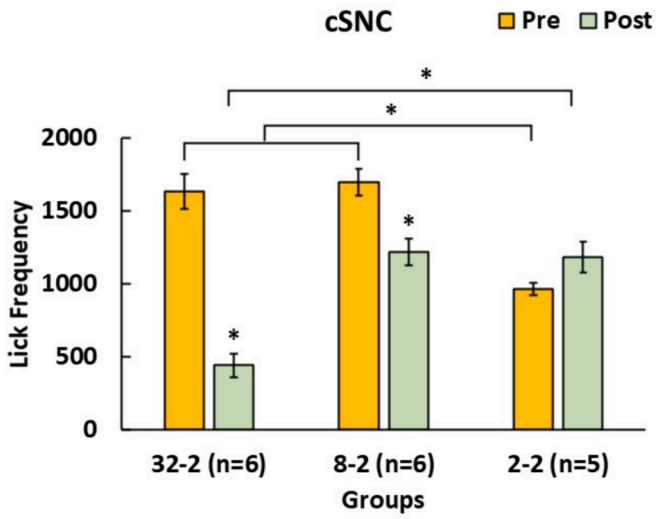

Fig. 2. Mean (±SEM) lick frequency during preshift sessions (Pre, mean of sessions 8–10) and postshift (Post, session 11) in groups that differed in the sucrose concentration during preshift sessions (32 %, 8 %, or 2 % sucrose), but had access to the same concentration during a single postshift session (2 % sucrose). Asterisks: at least $p < 0.006$. All other pairwise comparisons were nonsignificant.

0.001, but the latter did not differ from each other. In terms of preshift vs. postshift differences, there was significant suppression of licking in Group 32–2, $p < 0.001$, and 8–2, $p < 0.006$, but not in 2–2. Importantly, although there was a significant cSNC effect in a comparison between Groups 32–2 vs. 2–2 in their postshift behavior, $p < 0.001$, there was no evidence of a difference in postshift performance between Groups 8–2 vs. 2–2. Thus, the choice of training parameters yielded the expected results, namely, a cSNC effect after a 32-to-2 % sucrose downshift, but not after an 8-to-2 % sucrose downshift, in both cases relative to the 2–2 unshifted control.

3.2. c-Fos expression

Fig. 3 shows the results in terms of c-Fos-positive cells for each section of the A/P axis and for each group (see Table 1 for area labels). These results are grouped in terms of the BLA region (A), CeA region (B), and MeA (C). Group by A/P axis analyses were calculated for each component of these three regions. Figs. 4-7 show c-Fos expression in selected brain slices for regions that were differentially activated among the three groups of this experiment (see below).

In terms of the BLA (Fig. 3-A), the lB region showed group differences in c-Fos expression in the anterior, $F(2, 16) = 5.82$, $p < 0.02$, $\eta_p^2 = 0.45$, and medial regions, $F(2, 16) = 7.66$, $p < 0.007$, $\eta_p^2 = 0.52$. Pairwise Bonferroni comparisons indicated that in both cases, Group 32–2 exhibited significantly more expression than Groups 8–2 and 2–2, $ps < 0.05$. There was also a significant group effect in the posterior region of the mB, $F(2, 16) = 4.07$, $p < 0.05$, $\eta_p^2 = 0.37$. In this case, pairwise comparisons determined that this effect was due to higher expression in Group 32–2 than 8–2, $p < 0.05$.

The cCeA region also produced a significant group difference, $F(2, 16) = 6.04$, $p < 0.02$, $\eta_p^2 = 0.46$ (Fig. 3-B). In this case, the effect was due to higher expression level in Group 32–2 relative to 2–2, $p < 0.02$. The group effect for the mCeA approached statistical significance, $F(2, 16) = 3.71$, $p = 0.051$, $\eta_p^2 = 0.35$.

Three regions of the MeA also showed significant group effects (Fig. 3-C). The significant group difference in the anterior avMeA, $F(2, 16) = 10.16$, $p < 0.003$, $\eta_p^2 = 0.59$, was due to a difference between Group 32–2 and each of the other two groups, $ps < 0.02$. The significant group difference in the posterior region of the avMeA, $F(2, 16) = 4.98$, $p < 0.03$, $\eta_p^2 = 0.42$, was due to a difference between Groups 32–2 and 2–2, $p < 0.04$. The group effect in the posterior region of the pdMeA, $F(2, 16)$

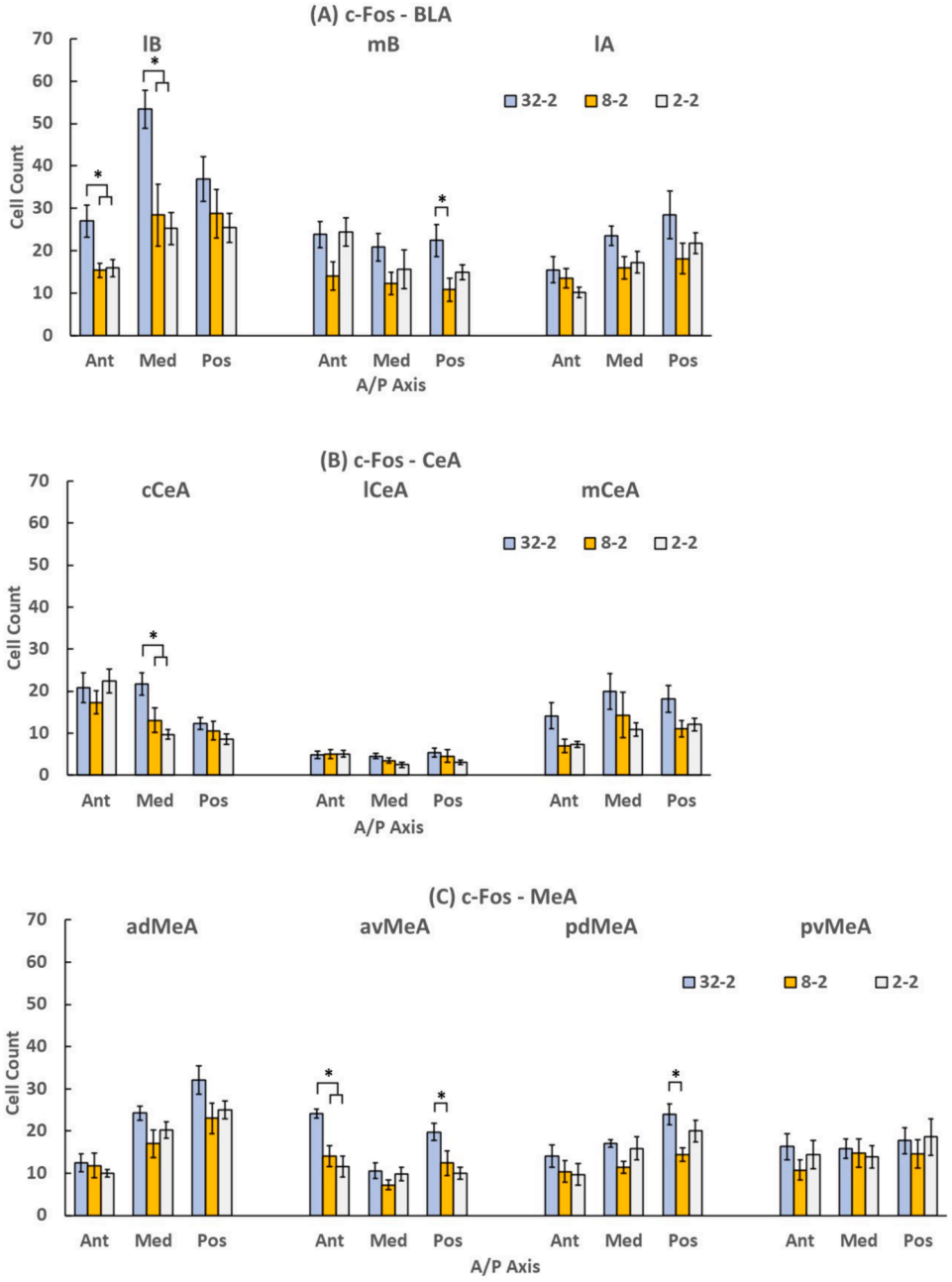

Fig. 3. Mean (±SEM) of c-Fos-positive cells in the basolateral (top), central (middle), and medial regions (bottom). c-Fos-positive cells were recorded in the anterior (Ant), medial (Med), and posterior (Pos) sections of each region along the A/P axis, for animals in Groups 32–2, 8–2, and 2–2. **(A)** lB: lateral region of the basal nucleus. mB: medial region of the basal nucleus. lA: lateral amygdala. **(B)** cCeA: capsular region of the central amygdala. lCeA: lateral region of the central amygdala. mCeA: medial region of the central amygdala. **(C)** adMeA: anterio-dorsal medial amygdala. avMeA: anterio-ventral medial amygdala. pdMeA: posterio-dorsal medial amygdala. pvMeA: posterior-ventral medial amygdala. Asterisks: at least $p < 0.05$.

$= 4.98$, $p < 0.03$, $\eta_p^2 = 0.42$, was caused by a significant difference between Groups 32–2 and 8–2.

3.3. Correlations

It was expected that lick frequency on session 11 (downshift) would be negatively correlated with c-Fos expression in several amygdala regions, but especially in the four regions that showed significant differences between Groups 32–2 and Groups 8–2 and 2–2. This expectation is based on the assumption that amygdala activation reflects the intensity of the frustrative response, which should translate into greater suppression of licking. Table 2 shows the results of Pearson's correlation coefficients computed for all pairwise comparisons between lick frequency and each of the regions assessed. Given the clear prediction concerning the relationship between licking and c-Fos expression, one-tailed tests were used for inferences of significance. Only one correlation was positive (and nonsignificant), out of 30 correlations computed. So, independently of the amygdala's specific involvement in reward downshift, neural activity in the amygdala tends to increase as lick frequency decreases. Importantly, the correlations involving the four regions with significantly higher c-Fos expression in Group 32–2 were all significant. Fig. 8 shows these results for the anterior lB (A), medial lB (B), medial cCeA (C), and anterior avMeA regions (D).

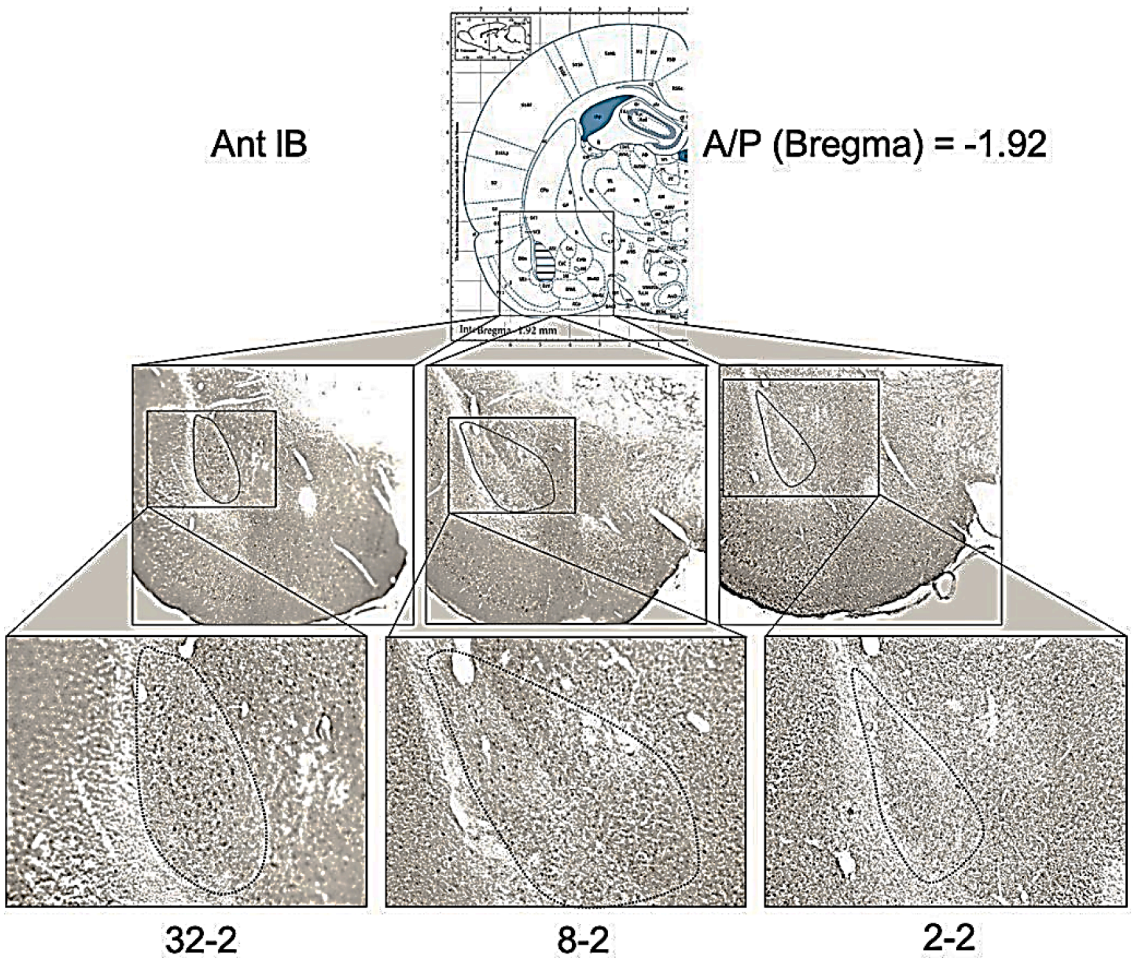

Fig. 4. Photomicrographs of representative sections of the anterior portion of the lateral region of the basal amygdala (Ant lB) in each group. Enhanced c-Fos expression after reward downshift was detected in this region. Anterior/posterior (A/P) coordinate from Paxinos and Watson (2013).

4. Discussion

The present experiment was designed to test three hypotheses: (1) that a cSNC effect would be present only with the large, 32-to-2 % sucrose downshift; (2) that amygdala activation would be present with the large, 32-to-2 % sucrose downshift; and (3) that amygdala activation after the small, 8-to-2 % sucrose downshift would be intermediate between that observed in the other two groups. Unlike previous studies, the present experiment took a more neuroanatomically detailed assessment of the function of the amygdala during the initial response to a 32-to-2 % sucrose downshift, aimed at identifying specific regions selectively involved in the cSNC effect.

The first hypothesis was confirmed by the behavioral results (see Fig. 2). Only animals experiencing the large disparity between obtained and expected rewards exhibited the cSNC effect (32-to-2 % relative to 2-to-2 % sucrose). Furthermore, although the 8-to-2 % sucrose downshift also showed consummatory suppression, licking in these animals was not suppressed below the level observed in unshifted controls. The expected pattern of behavioral results was quite specific, so it is reassuring that it was obtained despite the relatively small sample sizes. These results agree with previous data suggesting that the degree of consummatory suppression is a function of the ratio between the postshift and preshift sucrose concentrations. Generally speaking, when this ratio is below 0.19, there tends to be evidence of the cSNC effect (Papini & Pellegrini, 2006). The ratios for the two discrepancies included in this experiment were 0.062 (for the 32-to-2 % sucrose downshift that exhibited cSNC) and 0.25 (for the 8-to-2 % sucrose downshift that showed an adjustment of behavior without contrast). Thus, reward devaluation is necessary but not sufficient to induce a cSNC effect. Therefore, the behavioral conditions were appropriate to test whether the cSNC effect is necessary to observe FNR, as assessed in terms of amygdala activation (Papini, 2022).

The second hypothesis, that amygdala activation would be present after a 32-to-2 % sucrose downshift, was confirmed in several amygdala regions (see Fig. 3). However, the third hypothesis, that amygdala activation after the 8-to-2 % sucrose downshift would be intermediate between that observed in the other two groups, was contradicted by the present results. In general, c-Fos activation after an 8-to-2 % sucrose downshift was similar to that observed in unshifted controls always exposed to 2 % sucrose. Thus, a substantial amount of consummatory suppression is required for rats to exhibit evidence of FRN in the cSNC task. The lack of evidence for differential amygdala activation in a comparison between Groups 8–2 vs. 2–2, which also showed no evidence of cSNC, is consistent with the hypothesis that negative emotion is necessary to observe reward relativity effects (Papini, 2022).

Several lines of research suggest that the amygdala is linked to negative emotions (see introduction). The detailed assessment used in the present experiment has identified four amygdala regions that exhibited enhanced c-Fos expression after the first downshift session from 32 % to 2 % sucrose, relative to unshifted controls: anterior and medial lB, medial cCeA, and anterior avMeA. Three of these four areas coincide with Kim et al.'s (2016) identification of genetically distinct populations of neurons that respond selectively to stimuli with positive (sucrose) and negative (quinine) valence in mice. Of interest in the

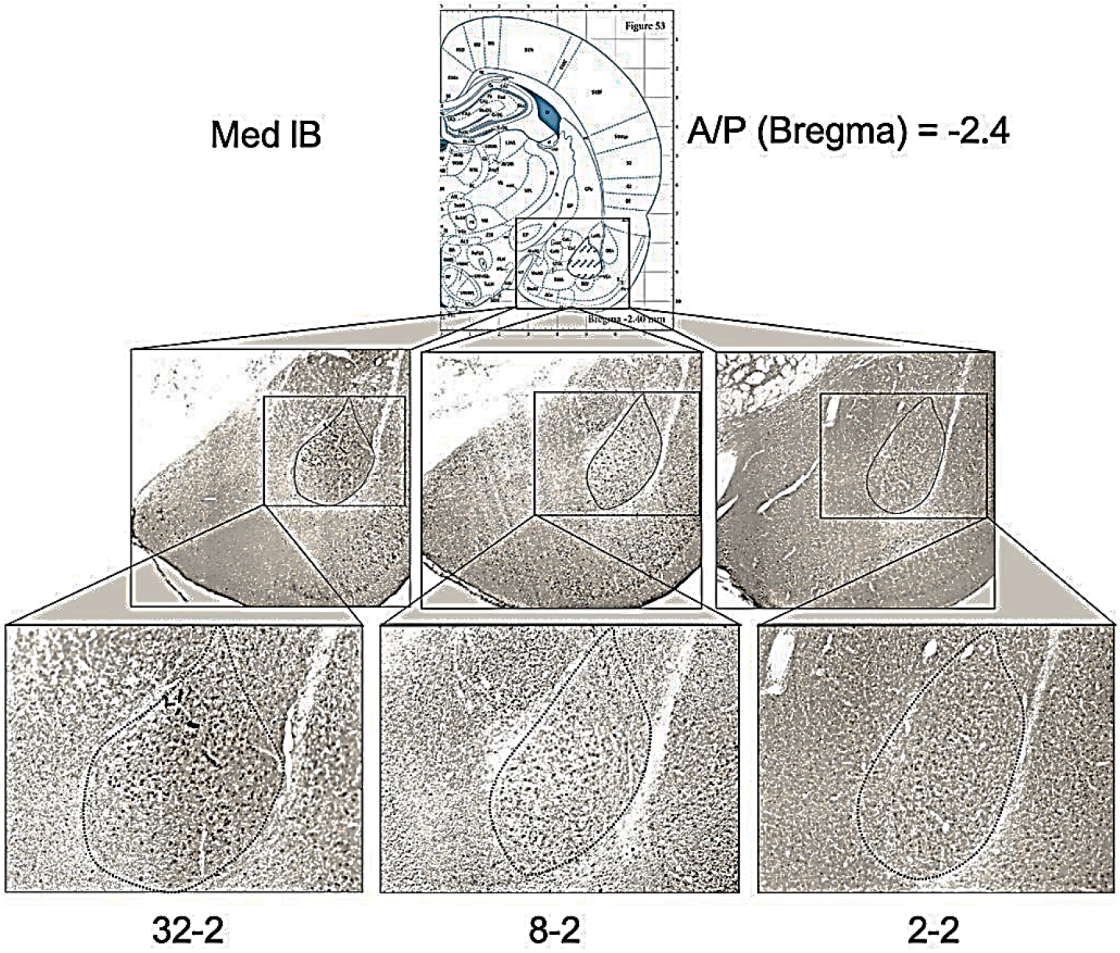

Fig. 5. Photomicrographs of representative sections of the medial portion of the lateral region of the basal amygdala (Med lB) in each group. Enhanced c-Fos expression after reward downshift was detected in this region. Anterior/posterior (A/P) coordinate from Paxinos and Watson (2013).

present context are those populations that respond to stimuli with negative valence since cSNC is accompanied by negative emotion (e.g., Papini et al., 2015). Kim et al. (2016) identified the anterior BLA's connection with the cCeA as key in negative valence (exposure to electric shock or quinine), which is consistent with the increased c-Fos activity in the anterior and medial lB amygdala and medial cCeA found in the current study with reward downshift (see Fig. 3A-B). The extent to which these relatively small amygdala regions are involved in cSNC remains to be assessed using techniques with greater spatial resolution than DREADDs (e.g., optogenetics, unit recordings).

None of the available evidence on the function of the amygdala in reward downshift has a degree of selectivity that maps into these restricted areas, but these three more comprehensive areas (lB, CeA, and MeA) have been implicated in the cSNC effect. For example, Kawasaki et al., (2017; see also Becker et al., 1984) reported that excitotoxic lesions of the BLA eliminated the cSNC effect and also the anticipatory negative contrast (ANC) effect, without affecting autoshaping extinction or open field activity. ANC involves access to two sucrose solutions in each session such that the first solution becomes a signal for the second solution. Thus, rats exposed to 4 % sucrose followed by access to 32 % sucrose consume less 4 % sucrose than rats exposed to 4 % sucrose on both trials (Flaherty et al., 1994). There is no evidence that manipulations designed to influence negative emotion, including treatment with anxiolytics (e.g., chlordiazepoxide, buspirone) affect ANC, suggesting that FNR is not involved in this effect (Flaherty, 1996). Furthermore, two tasks included in Kawasaki et al.'s (2017) experiment that were spared by BLA lesions have been linked to negative emotions: appetitive extinction and open field activity. Appetitive extinction is modulated by anxiolytics and opioids (Norris et al., 2009; Norwood et al., 2011) and open field activity, especially in the central area, is also linked to unconditioned fear of open spaces in rodents (Suarez & Gallup, 1981). Based on this pattern of results, Kawasaki et al. (2017) suggested that the common aspects of cSNC and ANC, the two tasks affected by BLA lesions, is to compare the value of the current reward with the value of a remembered (cSNC) or anticipated (ANC) reward, suggesting that the BLA's function in cSNC is one of reward comparison. In line with this interpretation, the cSNC effect is disrupted by lesions of various regions in the gustatory pathway connecting with the BLA (Price, 2003), including the parabrachial nucleus (Grigson et al., 1994), gustatory thalamus (Reilly & Trifunovic, 1999), and insular cortex (Lin et al., 2009). As a reward comparator, some regions of the BLA should always be active in the cSNC task, even when the obtained and expected rewards match. However, this activity may not be detected by c-Fos expression given that it has been shown that c-Fos expression diminishes after repeated stimulus exposure potentially masking the presence of neural activity (Melia et al., 1994; Ryabinin et al., 1999). Interestingly, activity in the anterior and medial regions of the lB was significantly enhanced after a 32-to-2 % sucrose downshift in the present experiment. One possibility is that neurons in these regions are gating information in the direction of response suppression if the disparity is negative (presumably to the CeA) or they are preserving ongoing behavior when obtained and expected rewards match (possibly influencing neurons in the NAc). Matching of obtained and expected rewards occurs during preshift sessions, but also after recovery of consummatory behavior following the reward downshift. The present results suggest that neural manipulations restricted to the anterior and medial portions of the BLA

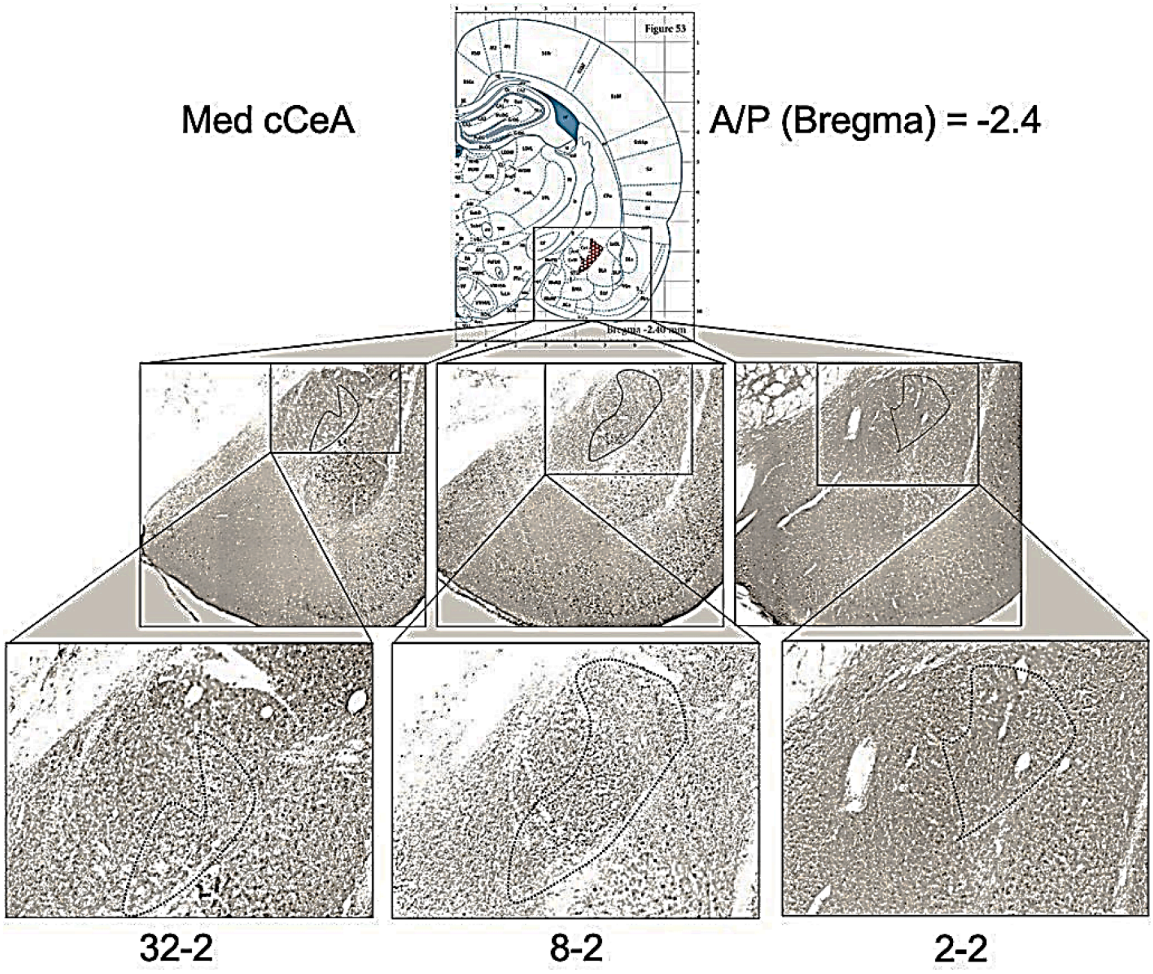

Fig. 6. Photomicrographs of representative sections of the medial portion of the capsular region of the central amygdala (Med cCeA) in each group. Enhanced c-Fos expression after reward downshift was detected in this region. Anterior/posterior (A/P) coordinate from Paxinos and Watson (2013).

are needed to further test this hypothesis. Moreover, perhaps controls not exposed to reward comparisons, even when obtained and expected rewards match, should be included to test whether regions of the lB are active even in unshifted controls. Although, as mentioned previously, it would still be possible that no differences would arise due to the disconnect between repeated neural activation and c-Fos expression, so that other measurements of neural signaling would be required.

CeA activity is critical for the expression of fear through its limbic and brainstem projections (Carvalho et al., 2018), it regulates reward-related behaviors (Seo et al., 2016), and it receives gustatory and visceral inputs (e.g., Agüera & Puerto, 2015; Norgren, 1976). Furthermore, the CeA seems to assign negative emotional valence to aversive events (Nakagawa et al., 2005). Thus, it is not surprising that the CeA plays a key role in cSNC (Becker et al., 1984; Guarino et al., 2020; Kawasaki et al., 2015; Liao & Chuang, 2003; Pecoraro & Dallman, 2005). For example, chemogenetic inhibition of the CeA eliminated the cSNC effect by reducing consummatory suppression after a 32-to-2 % sucrose downshift (Guarino et al., 2020), a result consistent with the hypothesis that the CeA tags the reward downshift event as emotionally aversive. The present results suggest a more specific localization for the effect reported by Guarino et al. (2020), namely inhibition of neurons located in the medial region of the cCeA; this conclusion requires empirical testing.

Additional evidence bears on the comparison between the functions of the BLA and CeA-MeA in the cSNC task. Kawasaki et al. (2015) observed that lidocaine inactivation of the CeA-MeA region (which included the medial region of the cCeA; A/P = -2.4) showed different effects than those observed after BLA lesions (Kawasaki et al., 2017). Lidocaine inactivation of the CeA-MeA region eliminated the cSNC effect and enhanced activity in the open field, but did not affect the ANC effect. Such a pattern is consistent with an emotional function of the CeA since, as mentioned previously, the cSNC and open field tasks involve negative emotion, but there is no evidence that the ANC task has an aversive emotional component. Furthermore, intra CeA administration of the benzodiazepine diazepam eliminated the cSNC effect (Liao & Chuang, 2003) and intra-amygdala administration of the benzodiazepine lorazepam reduced conflict in the punished licking test (Scheel-Krüger and Petersen, 1982). These effects are consistent with a relatively high density of benzodiazepine receptors in the lateral portion of the CeA, which includes the cCeA (Fujimura et al., 2005), thus being a candidate for a region mediating the anxiolytic properties of benzodiazepines (Griessner et al., 2021). As in the case of the BLA, selective intervention in the cCeA could be used to test its involvement in the cSNC effect suggested by the present c-Fos results.

Unlike the case of the BLA and CeA, there is no available research evaluating the effects of restricted MeA manipulations in the cSNC or other tasks involving reward downshifts. Still, available evidence suggests a link between MeA activity and the cSNC effect. MeA neurons play a role in mediating escape from the open arm of a T-maze and also escape induced by stimulation of the dorsal periaqueductal gray (Herdade et al., 2006), and in inducing defensive behavior in rats exposed to predator odor (Blanchard et al., 2005). Consummatory reward downshift also induces escape responses (Norris et al., 2009), therefore supporting the hypothesis that the MeA c-Fos activation observed in the

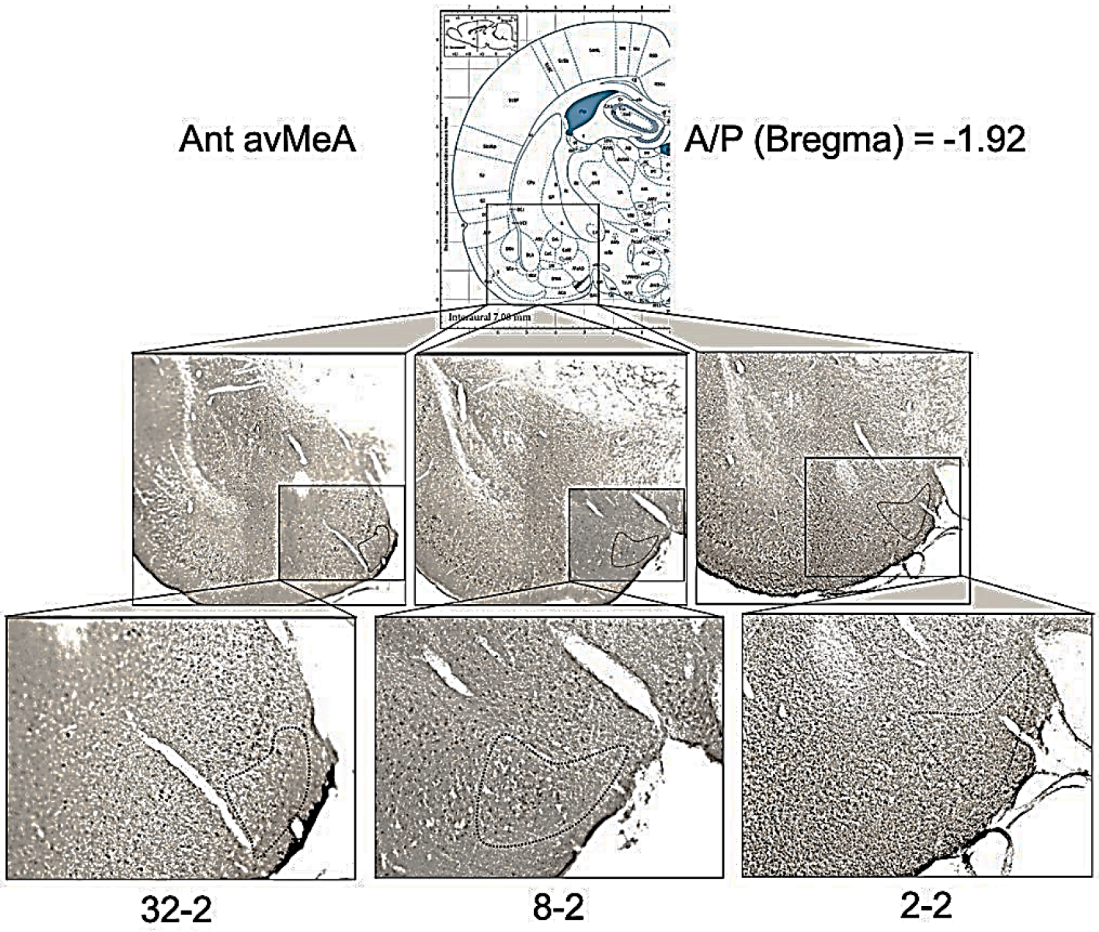

Fig. 7. Photomicrographs of representative sections of the medial portion of the anterio-ventral region of the medial amygdala (Ant avMeA) in each group. Enhanced c-Fos expression after reward downshift was detected in this region. Anterior/posterior (A/P) coordinate from Paxinos and Watson (2013).

Table 2

Pearson's correlation coefficients for lick frequency on session 11 (downshift) and c-Fos expression in each amygdala region assessed. Bold areas are those in which Group 32–2 was significantly higher than Groups 8–2 and 2–2 in terms of c-Fos expression. These four correlations are also presented in Fig. 8. Significance values were computed using one-tailed tests. Ant: anterior region. avMeA: anterior-ventral medial amygdala. cCeA: capsular central amygdala. lA: lateral amygdala. lB: lateral basal amygdala. lCeA: lateral central amygdala. MeA: medial amygdala. mB: medial basal amygdala. mCeA: medial central amygdala. Med: medial region. Pos: posterior region. pdMeA: posterio-dorsal medial amygdala.

BLA	*r*(15)	*p*=	CeA	*r*(15)	*p*=	MeA	*r*(15)	*p*=
Ant lB	**−0.427**	**0.044***	Ant cCeA	−0.027	0.458	Ant adMeA	−0.008	0.488
Med lB	**−0.580**	**0.007***	**Med cCeA**	**−0.473***	**0.028***	Med adMeA	−0.407	0.053
Pos lB	−0.383	0.064	Pos cCeA	−0.069	0.396	Pos adMeA	−0.401	0.055
Ant mB	−0.450	0.035*	Ant lCeA	0.124	0.318	**Ant avMeA**	**−0.609**	**0.005***
Med mB	−0.323	0.103	Med lCeA	−0.477	0.026*	Med avMeA	−0.286	0.133
Pos mB	−0.512	0.018*	Pos lCeA	−0.077	0.384	Pos avMeA	−0.668	0.002*
Ant lA	−0.368	0.073	Ant mCeA	−0.356	0.080	Ant pdMeA	−0.280	0.139
Med lA	−0.444	0.037*	Med mCeA	−0.213	0.205	Med pdMeA	−0.380	0.066
Pos lA	−0.361	0.078	Pos mCeA	−0.372	0.071	Pos pdMeA	−0.561	0.010*
						Ant avMeA	−0.456	0.033*

present experiment (more specifically in the anterior avMeA region) may reflect the instigation of escape responses induced by the aversive valence of the 32-to-2 % sucrose downshift. Again, this merits further study.

Three further considerations limit the interpretation of the current c-Fos results in terms of FNR induced by reward downshift. First, c-Fos expression could be sensitive to differential levels of locomotor activity after reward downshift. The early reaction to reward downshift involves an increase in activity (e.g., Flaherty et al., 1978; Pellegrini & Mustaca, 2000) that has been interpreted as searching for the "missing reward" (Elliott, 1928). Because activity and licking are incompatible, this might be one cause underlying behavioral suppression in the cSNC task. Therefore, it is possible that a greater increase in locomotor activity after the reward downshift is responsible for the higher expression of c-Fos in the amygdala regions observed in the present experiment. Tracking activity trajectories correlated with amygdala activation patterns could provide useful information. Whether c-Fos activation is an artifact of increased locomotor activity could be tested using a procedure that prevents locomotion during a cSNC task. In one experiment (López-Seal et al., 2013), rats were trained to voluntarily walk into a restraint tube to have access to sucrose solutions. Under these conditions, they could not move around, but they still rejected the solution after a 32-to-4 %

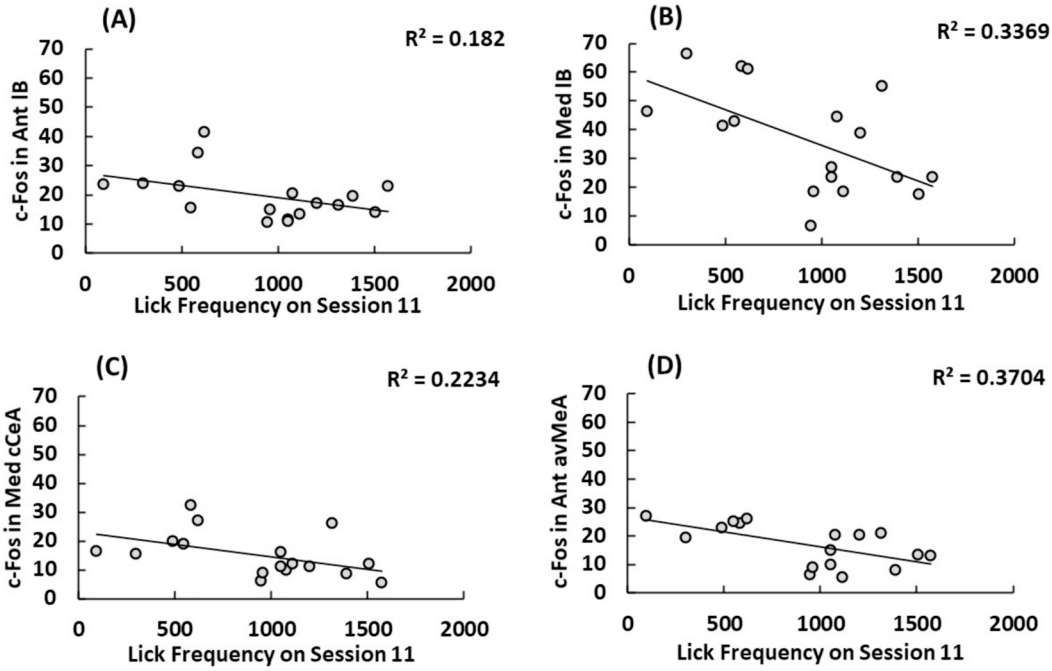

Fig. 8. Correlational analysis between c-Fos expression in the four regions with significant group effects and lick frequency on postshift session 11. The postshift lick frequency values are those used in Fig. 2. Coefficients of determination (R^2) are shown for each correlation; see Table 2 for statistical details. **(A)** Ant lB: anterior portion of the lateral region of the basal amygdala. **(B)** Med lB: medial portion of the lateral region of the basal amygdala. **(C)** Med cCeA: medial portion of the capsular region of the central amygdala. **(D)** Ant avMeA: anterior portion of the anterio-ventral region of the medial amygdala.

sucrose downshift. Would these animals still exhibit increased c-Fos expression in the same amygdala regions as those allowed freedom of movement, as in the present experiment?

Second, FNR may not be exclusively activated by reward downshift. An intriguing possibility was suggested by Crespi (1942), namely, that a food-restricted rat receiving an insufficient amount of food, as it would be the case in unshifted controls receiving access to 2 % sucrose, is on a state of frustration. Crespi (1942, p. 498) reported that rats under these conditions, but not when receiving large rewards or no rewards at all (i.e., in extinction), exhibited "halting excursions back and forth" and "purposeful attempts to escape from the apparatus by jumping near the starting box." Crespi's interpretation of these responses was as follows: "Eating the small incentive serves to stimulate and increase desire or tension in the rat without, however, improving the chances for obtaining more food. […] This state of heightened tension is unpleasant. Therefore, though food is present and the animal has had no food for some twenty-two hours, the situation is labelled 'frustrating'" (p. 498). According to Crespi's hypothesis, it seems possible that the lack of differential c-Fos expression between an 8-to-2 % sucrose downshift and the unshifted controls simply reflects reduced sensitivity of the c-Fos assay to relatively low levels of frustration. This hypothesis could be tested by adding a control condition designed to minimize aversive emotional responses, including animals fed ad libitum and perhaps even maintained in their home cages with a minimum amount of handling. Crespi's hypothesis would be consistent with elevated levels of c-Fos expression in some regions of the amygdala in animals exposed to 2 % sucrose after food restriction relative to these "cage controls.".

Third, a priori, it seems plausible that enhanced c-Fos expression in Group 32–2 is the result of exposure to a context associated with 32 % sucrose, rather than an emotional effect resulting from reward downshift. There is evidence that the cSNC effect is reduced when sucrose downshift occurs in a context previously paired with morphine (Ruiz-Salas et al., 2022). Whereas this result suggests that consummatory behavior during a downshift can be modulated by contextual stimuli, other results suggest that contextual control is limited at best. For example, the within-subject version of cSNC based on exposure to downshifted and unshifted conditions in discriminably different contexts is a rather weak phenomenon (Daniel et al., 2008). Assessing c-Fos expression in animals always exposed to 32 % sucrose could determine whether the effects described in this experiment reflect activation from exposure to a context paired with a highly palatable sucrose reward.

5. Conclusions

The present results uncovered for the first time the potential function of specific regions within the amygdala that might be more actively engaged during episodes of reward downshift, including the anterior and medial lB, medial cCeA, and anterior avMeA. These findings also suggest various manipulations designed to clarify the functions of these regions in the cSNC effect. Thus, c-Fos is a useful tool to ask questions about the role of restricted amygdala regions in reward downshift.

CRediT authorship contribution statement

David Arjol: Writing – review & editing, Investigation, Formal analysis, Data curation. **Antonio D.R. Agüera: . Christopher Hagen:** Writing – review & editing, Investigation, Data curation, Conceptualization. **Mauricio R. Papini:** Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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