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Functional brain networks underlying latent inhibition of conditioned disgust in rats

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Abstract

The present experiment examined the neuronal networks involved in the latent inhibition of conditioned disgust by measuring brain oxidative metabolism. Rats were given nonreinforced intraoral (IO) exposure to saccharin (exposed groups) or water (non-exposed groups) followed by a conditioning trial in which the animals received an infusion of saccharin paired (or unpaired) with LiCl. On testing, taste reactivity responses displayed by the rats during the infusion of the saccharin were examined. Behavioral data showed that preexposure to saccharin attenuated the development of LiCl-induced conditioned disgust reactions, indicating that the effects of taste aversion on hedonic taste reactivity had been reduced. With respect to cumulative oxidative metabolic activity across the whole study period, the parabrachial nucleus was the only single region examined which showed differential activity between groups which received saccharin-LiCl pairings with and without prior non-reinforced saccharin exposure, suggesting a key role in the effects of latent inhibition of taste aversion learning. In addition, many functional connections between brain regions were revealed through correlational analysis of metabolic activity, in particular an accumbens-amygdala interaction that may be involved in both positive and negative hedonic responses.

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Abbreviations

Adenosine triphosphate (ATP)

Amygdala, basolateral nucleus (BLA)

Amygdala, central nucleus (CeA)

Bed nucleus of stria terminalis (BNST)

Cingulate cortex (Cg)

Conditioned stimulus (CS)

Cytochrome c oxidase (CO)

Infralimbic cortex (IL)

Insular cortex (IC)

Intraoral (IO)

Lithium Chloride (LiCl)

Medial prefrontal cortex (mPFC)

Nucleus Accumbens (NAcb)

Parabrachial nucleus (PBN)

Prelimbic cortex (PrL)

Sodium Chloride (NaCl)

Taste reactivity (TR)

Unconditioned stimulus (US)

Ventral tegmental area (VTA)

Ventralis posterolateral thalamic nucleus (VPL)

Ventralis posteromedial thalamic nucleus (VPM)

1. Introduction

Nausea and vomiting resulting from chemotherapy treatments for cancer is reported as the most distressing side-effect that can cause patients to discontinue treatment [1,2]. In addition to the effects directly induced by the drug-induced nausea, patients often develop anticipatory nausea and vomiting to contextual stimuli related to the hospital environment, and effect that can be explained in terms of classical conditioning [3,4]. The cues that constitute the clinic (smell, sounds, and even the staff) can become associated with the nausea-inducing treatment and, as a consequence of the formation of such association, these stimuli may become able to evoke a response (the conditioned response, CR) that is nausea and vomiting [5]. Theoretical analysis of the conditioning processes involved in the acquisition of context aversions in animals suggests possible behavioral strategies that might be used in the alleviation of anticipatory nausea and vomiting [6,7]. In particular, one effective way to attenuate anticipatory nausea is preventing the formation of context-nausea association by giving extensive prior exposure to the contextual cues, a conditioning phenomenon referred as the latent inhibition effect [8,9].

The initial goal of the current study was to examine the brain regions involved in latent inhibition of nausea-induced conditioned disgust. In the taste aversion paradigm, the latent inhibition effect is defined as a retardation of conditioning due to extensive prior exposure to the flavor that is to be paired with a drug with emetic properties (e.g., lithium chloride, LiCl). Therefore, the contribution of flavor novelty/familiarity in the development of conditioned disgust can be examined with this paradigm. Whilst the latent inhibition effect has been extensively evaluated using voluntary fluid ingestion, the current study aims to evaluate this effect by measuring conditioned disgust reactions elicited by a nausea-paired flavor. Flavors

can be administered directly into the rat´s mouth through a cannula implanted in the oral cavity. This procedure allows the orofacial reactions displayed by the rats during the intraoral infusion of the fluid to be recorded and analyzed, a method termed the taste reactivity (TR) test [10]. The TR method measures changes in the hedonic value of a flavor due to aversive visceral experience. In particular, rodents display conditioned gaping reactions (a disgust reaction selective to nausea) during re-exposure to the conditioned flavor. TR is a particularly suitable procedure for measuring conditioned taste aversion because it is selective to disgust, in contrast to decreases in the voluntary intake of the flavor which may simply reflect taste avoidance without a change in hedonic responses [11,12,13]. In recent studies of our laboratory using taste reactivity and licking analysis methods we found that flavor preexposure attenuates the effects of taste aversion learning on both flavor consumption and taste palatability [14]. Based upon these findings, in the present study we explored the neural networks involved in latent inhibition of conditioned disgust by measuring changes in metabolic activity through cytochrome c oxidative (CO) histochemistry in brain regions generally accepted as being involved in conditioned taste aversion. These brain regions may be divided into two systems, one related to the processing of taste qualities and the other to the reward system. The taste recognition system includes the nucleus of the solitary tract, parabrachial nucleus, the ventralis posteromedial thalamic nucleus, and the insular cortex. The reward systems would include mainly the ventral tegmental area and the accumbens. Prefrontal cortex and amygdala are the candidates to be the interface regions between these two systems.[15,16,17] Despite the evidence that flavor familiarity influences neural activity in conditioned taste aversion as assessed by consumption tests, it is unclear what brain regions are activated in response to hedonic shifts in this learning paradigm. In addition to the fact that latent inhibition is of interest in its own right, the fact that pre-training exposure to the taste stimulus retards conditioning affords a direct comparison of the brain effects of

identical taste-nausea parings between animals that form strong aversions (i.e. the non-exposed group) with those that will not form a strong aversion (i.e. the exposed group).

The neuronal underpinnings of conditioned taste aversion have been widely examined using c-fos immunocytochemistry [18,19,20]. However, the special functional properties of taste aversion learning may require alternative methods to fully explore the physiological bases of this learning. In contrast to other forms of learning, taste aversions are well known to form in a single trial and can be formed despite long-delays between the flavor and the illness [21, 22]. Therefore, a method that assess long term changes in the brain activity would be particularly valuable. Due to the close relationship between the electrical activity of neurons and oxidative energy metabolism, an alternative way to study brain activity is by using histochemical techniques such as the mitochondrial enzyme cytochrome c oxidase (CO) [23,24]. Cytochrome c oxidase is a mitochondrial enzyme implicated in the oxidative phosphorylation process in which adenosine triphosphate (ATP) is generated. This histochemical technique identifies structures involved in long-term metabolic changes such as increased levels of enzymes or neurotransmitters, membrane protein synthesis, morphological changes (high postsynaptic density) and neuronal electrical increased activity, reflecting other process that also require ATP in the cell. CO histochemistry has been used in studies of learning and memory in a variety of animal species [25,26,27,28,29]. The use of CO histochemistry provides a range of advantages over the use of early gene expression as c-Fos immunohistochemistry, in particular c-Fos methodology indicates acute (seconds to minutes) transient changes in neural activation evoked by a stimulus as a result of a particular intervention and thus might not fully capture the processes involved conditioned taste aversion where conditioning can occur with long delays between flavor exposure and the induction of illness. In addition, CO histochemistry allows the assessment of cumulative changes throughout the learning process, which is critical in understanding the processes

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underpinning latent inhibition, where non-reinforced initial exposure can occur a matter of days before conditioning and testing. CO activity provides an accurate assessment of the metabolic history of brain regions because sustained increased or decreased in neuronal activity lead to corresponding changes in CO activity [30,31,32]. As such, it reflects the temporal dynamics of brain metabolism over the acquisition and retrieval of learning, not only in specific brain regions but also in terms of their functional connectivity [33].

Therefore, the goal of the present study was to examine, using CO histochemistry, changes in brain metabolic activity following latent inhibition of conditioned disgust. In particular, because CO activity is sensitive to manipulations between the day of measurement and up to a week before [27], this will reflect the impact of the whole behavioral preexposure, conditioning, and test procedure performed here. More specifically, we attempt to determine the functional connectivity between different brain regions involved in the development of nausea-induced conditioned disgust.

2. Materials and methods

2.1. Subjects

The subjects were 31 male Wistar rats weighing 190-339 g (258 g mean weight) at the start of the experiment. Upon arrival, they were individually housed in opaque plastic cages in a room maintained at 21° C with a 12/12h light-dark cycle. All experimental manipulations were performed during the light portion of the cycle. Food and water available in the home cages throughout the experimental procedures. All experimental procedures were in accordance with guidelines for the care and use of laboratory animal of the Spanish (RD 12301/2005) and European (86/609/EEC) regulations concerning animal experimentation.

2.2. Cannulation surgery

In order to implant intraoral cannula, the rats were anesthetized with ketamine (50 mg/kg) combined with metedomidine hydrochloride (0, 15 mg/kg). A 3-cm square of fur was shaved on the back of the neck and the area was then wiped with an antiseptic product (Betadine). A thin-walled 15-gauge stainless steel needle was inserted at the back of the neck, routed underneath the skin, and brought out inside the mouth behind the first molar. The cannula was inserted through the needle, which was then removed. Cannulae consisted of intramedic polyethylene tubing with an inner diameter of 0.86 mm and an outer diameter of 1.27 mm. To prevent the cannula from moving out of place, two elastic discs were placed over the tubing and drawn to the exposed skin at the back of the neck for the purpose of stabilizing the cannula. After the cannulation surgery, the rats were monitored for three days. The cannulas are cleaned daily with distilled water and the rats were injected subcutaneously with an antibiotic (enrofloxacin 0.3 mg/kg) and anti-inflammatory (ketoprofen 1.5 mg/kg).

2.3. Apparatus

For the purpose of fluid infusion, the cannula was connected to an infusion pump (Kd Scientific Inc., MA) by slipping the tubing of the cannula inside a second polyethylene tubing (inner diameter 1.19 mm; outer diameter 1.79 mm) attached to the infusion pump. Intraoral infusions were carried out in a conditioning chamber that was situated in a room without natural light. The chamber consisted of a wooden frame painted black (150 x 64 x 50 cm) with two points of light (50w) one on each side of the chamber. The top of the apparatus consisted of a sheet of transparent plastic (Plexiglas) and a mirror beneath the chamber (60 x 45 cm) inclined at 45 degrees to facilitate viewing of the ventral surface of the rat during fluid infusion. The rats were placed in the top of the conditioning apparatus within a square compartment (22.5 x 26 x 20 cm) made of Plexiglas. This compartment had a lid of dark Plexiglas with a central hole through which we introduced the plastic tube connected to the

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infusion pump. The other end of the plastic tube was connected to the oral cannula during the IO infusion. The sessions were recorded with a video camera (Sony Handicam) connected to a computer that enabled recordings to be directly stored (see Grill [34] for a schematic representation of the apparatus for videotaping taste reactivity).

Orofacial reactions made by the animals during the infusion of fluids were recorded manually quantified and scored by two raters with the software The Observer XT 9.0 (Noldus Information Technology). The behaviors scored included the frequency of the disgust reactions of gaping (rapid, large-amplitude opening of the mandible with retraction of the corners of the mouth), chin rubbing (mouth or chin in direct contact with the floor or wall of the chamber and body projected forward), and paw treading (forward and backward movement of the forepaws in synchronous alternation). These scores were summed to provide a total disgust reaction score. The ingestion reactions of tongue protrusions (extension of the tongue out the mouth) and mouth movements (movement of the lower mandible without opening the mouth) were also scored. The number of seconds that the rats displayed the reactions of tongue protrusions and mouth movements was used as the appetitive reaction score.

2.4. Behavioral procedure

After recovery from the cannula implantation surgery, rats were randomly assigned to four groups: preexposed LiCl-paired (PE-CTA, n=8), non-preexposed LiCl-paired (NPE-CTA, n=8), preexposed LiCl-explicitly unpaired (PE-CONT, n=7) and non-preexposed LiCl-explicitly unpaired (NPE-CONT, n=8). Experimental sessions were initiated at 10:00 AM each day. Two days after the cannulation surgery the rats were habituated to the infusion procedure, receiving a 5 ml water infusion (rate of infusion 1 ml/min) in the conditioning apparatus and returned to their home cages. The next 4 days constituted the flavor prexposure

phase (see Table 1). During these sessions, the rats in groups PE-CTA and PE-CONT were intraorally infused with 0.1% saccharin for 5 min at a rate of 1 ml/min whereas those in groups NPE-CTA and NPE-CONT received water infusions. On the next day, the conditioning trial was carried out. This trial consisted of a single session of an IO saccharin infusion (0.1%) for 5 min at a rate of 1 ml/min. Immediately following the saccharin infusion, rats from groups PE-CTA and NPE-CTA were injected with LiCl (20 ml/kg, 0.15 M) whereas groups PE-CONT and NPE-CONT received the LiCl injection 24-h after the saccharin infusion. Subsequently, the rats were returned to their home cages in order to recover from the LiCl effects. After a recovery day in the home cages, the TR test was carried out. This test consisted of an intraoral administration of 5 ml of saccharin (0.1%) at a rate of 1 ml/min in the conditioning chamber while the rat's orofacial reactions were videotaped.

TABLE 1

2.5. Cytochrome oxidase histochemistry

The changes in metabolic activity were analyzed using CO histochemistry. The procedure followed was described by Wong-Riley [23] based on certain modifications proposed by Gonzalez-Lima and Jones [24]. The structures were selected for analysis using the atlas of Paxinos and Watson [35]. Ninety minutes after the taste reactivity test, the rats were sacrificed by decapitation. Their brains were rapidly removed and frozen by immersion in isopentane (Sigma-Aldrich, Spain) at -70º C for two minutes and stored at -40º C to prevent tissue damage with loss of enzyme activity.

Subsequently, the brains were sectioned coronally with a microtome cryostat (Microm International Gmblt, HM 505E, Heidelberg, Germany) in sections (30 µm thick) that were placed on slides previously cleaned with alcohol at 100%. The sections were fixed with 0.5 % (v/v) glutaraldehyde and 10% (W/V) sucrose in 0.1 M phosphate buffer, pH 7.6. After that,

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sections were rinsed in 0.1 M phosphate buffer with 10% (W/V) sucrose. Following this procedure, they were immersed in 0.05 M Tris buffered solution, pH 7.6 containing 275 mg/l cobalt chloride, 10% (w/v) sucrose and 0.5 % (v/v) dymethylsulfoxide. The sections were incubated in darkness for 2 h at 37º C in a staining bath containing 0.06 g cytochrome c, 0.016 g catalase, 40 g sucrose, 2 ml dimethylsulfoxide and 0.4 g diaminobenzidine tetrahydrocloride (Sigma-Aldrich, Spain) dissolved in 800 ml of phosphate buffer (pH 7.6; 0.1 M). Finally, the sections were fixed at 4% (v/v) buffered formalin with 10% (w/v) sucrose, dehydrated in alcohol chain and coverslipped with Entellan (Merck, Germany). A series of sections of rat brain cut at different thicknesses (10, 30, 50 y 70 μm) were included together with brain tissue in each bath. These sections were used as standards to control for staining variability across different incubation baths, and were obtained from rat brain homogenates. The tissue homogenates were obtained from the brains of 12 adult male Wistar rats killed by decapitation and homogenized on ice. To establish comparisons in the staining of sections from different staining baths, measurements were taken from CO stained brain homogenate standards. These activity values allowed for the construction of a linear regression curve to convert measured values of optical density of the selected structures in cytochrome oxidase activity units (1 unit: 1 μmol of cytochrome c oxidase/min/g tissue wet weight at 23° C), and were calculated for each incubation bath.

Using an image processing system (Leica Q550, Germany) with a light source (LeicaDM-RHC) connected to a camera CCD (Lohu, Japan) and image analysis software (Leica Q-Win), relative optical density (OD) readings were obtained from the brain regions of interest: medial prefrontal cortex (mPFC) including cingulate (Cg), prelimbic (PrL) and infralimbic cortex (ILc); insular cortex (IC); bed nucleus of stria terminalis (BNST); central and basolateral amygdala (CeA and BLA); ventral tegmental area (VTA); parabrachial nucleus (PBN); ventralis posteromedial thalamic nucleus (VPM) and core and shell nucleus

of accumbens (NAcb). Figure 1 represent microphotographs showing examples of coronal CO stained sections and schematic representations of the selected brain regions. A total of 25 measurements were taken per region. The relative optical density was measured by taking 5 non-overlapping regions in each section for 5 consecutive sections by using a square-shaped sampling window previously adjusted for each region size (see example of PrL sampling on Figure 1). Then, measures were averaged to obtain one data point per region for each animal (group data shown in Table 2).

FIGURE 1

2.6. Statistical analysis

The data were analyzed using SPSS version 19.0 (SPSS Inc., Chicago, USA). Data for behavior scored in taste reactivity test were analyzed by a 2 (preexposure) x 2 (conditioning) analysis of variance (ANOVA). In all cases, a significance criterion of $p < 0.05$ was used. The inter-rater reliability (r's > 0.87) for each behavior scored was highly significant. Differences in CO staining of each brain region between the selected groups were analyzed by a similar analysis of variance (ANOVA). In order to examine functional connectivity between brain regions, pairwise correlations in CO activity were analyzed by calculating Pearson´s product moment correlation coefficients ($p < 0.05$) with CO activity normalized values (for each animal, CO activity of each region divided by mean activity in all regions). A "Jackknife" procedure [36] was used such that pairwise correlations were first calculated including all animals in the analysis. Then, one subject was removed each time and correlations were calculated anew. Only correlations present in all comparisons made without one of the subjects (and all subjects) are reported as significant. This procedure minimizes the risk of spurious correlations being produced by outliers in the data.

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3. Results

3.1. Taste reactivity test

Figure 2 (top panel) presents the mean number of conditioned disgust reactions displayed by rats during the 5-min intraoral infusion of saccharin in the TR test. It can be seen that rats in Group NPE-CTA showed a higher rate of aversive reactions than animals in the remaining groups, indicating that non-reinforced exposure to saccharin attenuated the establishment of LiCl-induced conditioned disgust. The two-way ANOVA conducted with these scores revealed significant main effects of preexposure [$F(1,27) = 8.44$; $p = 0.007$], conditioning [$F(1,27) = 23.05$; $p < 0.001$], and a significant preexposure x conditioning interaction [$F(1,27) = 7.74$; $p = 0.010$]. Simple effects analysis revealed that Group NPE-CTA displayed a higher rate of aversive reactions to the saccharin infusion than Group PE-CTA [$F(1,27) = 16.75$; $p < 0.001$], and that non-conditioned groups did not differ from one another ($F < 1$). These results confirm that repeated exposure to saccharin solution retards the development of conditioned nausea to that flavor. Figure 2 (bottom panel) shows the mean duration of appetitive reactions displayed by the rats during the TR test. The two-way ANOVA conducted with these data revealed a main effect of preexposure [$F(1,27) = 15.32$; $p = 0.001$] and conditioning [$F(1,27) = 13.27$; $p = 0.001$], and a significant preexposure x conditioning interaction [$F(1,27) = 7.79$; $p = 0.010$]. Simple effects analysis revealed that Group NPE-CTA displayed a lower rate of appetitive reactions to the saccharin infusion than Group PE-CTA [$F(1,27) = 23.29$; $p < 0.001$], and non-conditioned groups did not differ from one another ($F < 1$). These results show that prior exposure to the to-be conditioned flavor not only attenuates the development of conditioned disgust, but also protects against the reduction in positive hedonic reactions.

TABLE 2 & FIGURE 2

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3.2. CO activity

Table 2 shows the CO activity measured in the brain areas of interest. The 2

(preexposure) x 2 (conditioning) ANOVA conducted on these data revealed a significant

main effect of conditioning in mPFC subregions [cingulate, prelimbic and infralimbic; Fs

$(1,27) > 4.59$; $ps < 0.041$], in bed nucleus of stria terminalis [$F(1,27) = 12.55$; $p < 0.001$],

basolateral amygdala [$F(1,27) = 4.28$; $p = 0.048$], ventral tegmental area [$F(1,27) = 11.064$; p

$= 0.003$] and PBN [$F(1,27) = 4.07$; $p = 0.05$], but no main effect of preexposure or

preexposure x conditioning interaction ($Fs < 1$), with the exception of the PBN region which

had a significant preexposure x conditioning interaction [$F(1,27) = 5.06$; $p = 0.033$]. Simple

main effects analysis revealed lower CO activity in PBN for the latent inhibition group; that

is, Group PE-CTA [$F(1,27) = 4.85$; $p = 0.036$] compared to NPE-CTA group. There was also

no differences between Group PE-CONT and Group NPE-CONT [$F(1,27) = 1.01$; $p = 0.326$].

This result shows that a conditioned change in the hedonic value of a flavor is related with

long term changes in PBN activity, while the pairing of the flavor with illness resulted in

lower activity in a number of brain regions irrespective of whether this resulted in a hedonic

shift.

Interregional correlations of CO activity were analyzed in order to establish different

brain networks in each group. A network is defined by a set of structures linked by

correlations and independent of correlational links with other structures. Figure 3 shows

significant cross-correlations of CO activity for the different groups (r values). These

correlations revealed two brain networks for Group PE-CONT. The first one included the

PBN and ventral tegmental area with a positive correlation. The second network comprised a

prefrontal cortical region, the cingulate cortices, which showed a positive correlation with the

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nucleus accumbens core. In turn, NAcb shell had a positive correlation with CeA. This latter area also had a negative correlation with the ventral posterolateral thalamus, which showed a negative correlation with the prefrontal cortex and NAcb shell.

FIGURE 3

For the NPE-CONT group, correlations also revealed two brain networks. First, there was a positive correlation between the basolateral amygdala and PBN region. Subsequent correlations show a brain network that comprised cingulate cortex, which had a positive correlation with the thalamus and with the NAcb core and shell. NAcb shell also showed a negative correlation with the ventral tegmental area.

Groups which experienced saccharin-lithium pairings (PE-CTA and NPE-CTA) showed few positive correlations between brain regions. In particular, the PE group showed positive correlations between prelimbic and infralimbic, NAcb core and thalamus, and finally between the PBN and tegmental areas. NPE-CTA group had positive correlations between the prefrontal areas (Cg, PrL and Ilc), NAcb shell and central amygdala, and between PBN and tegmental areas.

4. Discussion

The aim of the present study was to examine the functional brain networks involved in nausea-induced conditioned disgust reactions. For this purpose, a latent inhibition paradigm was used to measure, at both the behavioral and neuronal level, the effect of non-reinforced flavor exposure on the subsequent establishment of a conditioned taste aversion. Behavioral data revealed that non-reinforced exposure to a saccharin solution prior to its pairing with nausea attenuates the development of conditioned disgust reactions to the flavor. This effect

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is consistent with previous latent inhibition studies using consumption tests [8,9] and from our own laboratory using TR [14] or licking microstructure [37] procedures.

With respect to overall differences in regional CO activity, there were reductions in brain CO activity in BLA, BNST, PBN, VTA and prefrontal areas in groups which experienced saccharin-lithium pairings (PE-CTA and NPE-CTA) regardless of whether these pairings resulted in a change in the hedonic reaction to the taste. The only brain region displaying changes in activity relating to the change in taste reactivity was the PBN. Because the taste reactivity change was determined by the presence or absence of latent inhibition, this result highlights the role of the PBN as a key mediator of the way that latent inhibition in attenuates or prevents CS-US associations forming in conditioned taste aversion.

Turning to the analysis of the connectivity between brain regions, our data shows a positive correlation between the PBN and VTA in both conditioned groups, that is, groups PE-CTA ad NPE-CTA, regardless of whether latent inhibition prevented the development of conditioned disgust reactions. It should be noted that groups PE-CTA and NPE-CTA showed decreased CO activity in the PBN and VTA compared to the remaining groups. As already indicated, PBN is the candidate structure for CS-US associations, whereas VTA is involved in fluid reward (regardless of negative or positive hedonic fluid value) [38]. In addition, the data show a positive correlation between the mPFC subregions for the novel CS-US association, and a PrL- Ilc subregions correlation in familiar CS-US associations. This may indicate that conditioned taste aversion learning requires the involvement of all subregions of the mPFC for taste memory formation. This is consistent with other lesion studies in which an impairment of conditioned taste aversion is observed as a result of mPFC injures [39, 40]. In addition, we found a negative correlation between NAcb and thalamus nuclei in group PE-CTA, indicating that both structures may mediate the hedonic shift from positive to negative, and the data implicate the involvement of NAcb in this shift [22]. Perhaps more interesting is

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the amygdala-NAcb interaction found in Group NPE-CTA – the only group to show a

behavioral taste aversion. It is known that both NAcb and amygdala regions are essential for

expression of conditioned taste aversion [41]. Some studies have associated the amygdala

with the processing of visceral information, given that LiCl injections increase c-fos activity

in this region [38], linking its function to the recognition of US information. However, other

studies suggest a central role for this structure in the formation of CS-US association, relating

BLA with aversive conditioning [42] and with the formation of fear learning in general. But

CeA is the main output route for other brain regions related to the expression of specific

emotional responses [43], and its specific role remains unclear. Our data may link this region

to the activity of the NAcb in the formation of taste aversion memory. However, since a

similar relation was found in the PE-CONT group, amygdala-NAcb connections may be

involved in both appetitive and aversive rewards, given that this interaction has been found in

the group for which the saccharin was highly appetitive (PE-CONT) or highly aversive

(NPE-CTA).

Considering group PE-CONT, which had repeated experience with a pleasant taste, in

more detail, the analysis revealed two main brain networks (see Figure 3). First, a positive

correlation was shown between the PBN and VTA. This is consistent with the suggestion that

palatable fluids such as saccharin or sucrose activate VTA neurons [44, 45] and its lesions

selectively reduce consumption of a preferred sucrose solution [46]. This relationship may be

due to the PBN axons that pass through the VTA [47] and the fact that this area is also

connected with other limbic structures [48]. Indeed, the PBN might influence limbic

structures such as the NAcb via direct connections with the VTA [49].

The second network comprised the NAcb (shell and core), CeA and Cg interactions, all

of which showed negative correlations with the thalamus nuclei. Several studies have shown

that NAcb receives afferent inputs from mPFC and amygdala structures (see for instance,

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[41]). Indeed, long-lasting increase in dopamine release in NAcb is indirectly modulated by amygdala-induced dopamine responses in mPFC [50]. NAcb has been associated with reinforced learning, i.e. an association between pleasant flavors (or unpleasant) and its consequences. In particular, NAcb activity increased with intraoral infusions of pleasant flavors [51, 52]. Our data suggest that brain regions involved in reward (NAcb), regions that send information of sensorial properties of flavors (thalamus) to the main candidates for integrate reward and sensory information (amygdala and mPFC) [22], were functionally paired when a familiar and pleasant flavor is intraorally administered.

Brain networks involved in the processing of familiar pleasant solutions differ from those involved in processing novel solutions. For group NPE-CONT, two brain networks may be established, a PBN-amygdala interaction, and a second network that comprises the VTA, NAcb, Cg and thalamus. These results are compatible with other research studies [50] in which the neural pathway of the brain reward system is described as being mainly comprised of the VTA and NAcb. As previously mentioned NAcb is interconnected with PFC which is associated with feeding control and receives afferents from the thalamus via IC. Thalamus (VPM) on the other hand, is related to the processing of novel flavors, given that its lesions disrupt neophobia [22]. Taken together, our results may suggest that information from the reward pathway (VTA and NAcb) and sensorial information (thalamus) regarding flavor novelty may converge in the PFC to be integrated for the formation of flavor memories. Additionally, we have found a positive correlation between PBN and BLA. Previous studies revealed that exposure to a novel, but not familiar, saccharin solution induced robust increases of c-fos activity in the PBN and CeA but not in the BLA [53]. However, the BLA may be involved in neophobia [54], and therefore in the processing of flavor novelty, given that injuries in this area disrupts CS-US associations with a novel, but not familiar CS ([55] for a review). It is also accepted that initial integration of gustatory and visceral signals takes

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place in PBN [56, 57, 41] and as well as the amygdala, PBN plays a crucial role in the processing of novel flavors and the consequences derived from their intake [58, 59, 60]. This hypothesis is supported by our data, in which the PBN reduce its activity as a result of the latent inhibition of the conditioned flavor.

It is important to emphasize that the CO histochemistry technique assesses changes throughout the learning procedure. This means that in contrast to other techniques (early gene expression, for instance) in which activity changes are measured at a specified point in time, the CO technique allows for the measurement of accumulative changes. This may result in unchanged activity in brain regions traditionally related to latent inhibition in conditioned taste aversion, or alternatively, these brain regions may not elicit long-term changes in CO activity.

5. Conclusion

The present study showed that non-reinforced exposure to saccharin prior to the acquisition of a LiCl-induced saccharin aversion attenuates the development of conditioned disgust reactions. Also, it provides the first demonstration of oxidative metabolic activity in brain regions involved in nausea-induced conditioned disgust. In addition, we found novel activation patterns of brain networks thought to be involved in the processing of flavors and their hedonic value. We highlighted an accumbens-amygdala interaction that may be involved in both appetitive and aversive reward, and a central role of the PBN nucleus in processing latent inhibition in conditioned taste aversion learning.

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Figure legends

Figure 1. Microphotographs showing some examples of coronal CO stained sections and schematic representations of the selected brain regions. The relative optical density of each region was measured takin five non-overlapping reading per region, in five consecutive sections using a sampling window adjusted for each regions size (an example is shown for the PrL). As microphotographs show, CO have the advantage of been a method with a high-resolution outcome allowing more accurate and automated measurement of activity in specific brain regions.

Figure 2. Mean number of disgust reactions (top panel) and mean duration (in seconds) of appetitive reactions (bottom panel) elicited during the TR test for the different groups: preexposed (PE-CTA), non-preexposed (NPE-CTA), preexposed control (PE-CONT) and non-preexposed control (NPE-CONT). Error bars represent the standard error of mean (SEMs).

Figure 3. Schematic representations of interregional correlations of CO activity for the different groups. Solid lines represent high positive pairwise Pearson´s correlations ($p < .05$), whereas negative correlations are represented by broken lines. Each quadrant represents a group correlation, namely, preexposed (PE-CTA), non-preexposed (NPE-CTA), preexposed control (PE-CONT) and nonpreexposed control (NPE-CONT). A network is defined when regions are linked by correlations and are independent link from other structures. Specifically, a simple network between PBN and VTA was found in all groups with the exception of NPE-CONT. In this group, results show two main networks, one between PBN and AMY, and other established by thalamus, mPFC and NAcb Additionally, PE-CONT

group show a more intricate network between mPFC, NAcb, AMY and thalamus. We highlight a NAcb-AMY network in PE-CTA group, as well as correlations within mPFC regions. Finally, a simple network between thalamus and NAcb was found in NPE-CTA group.
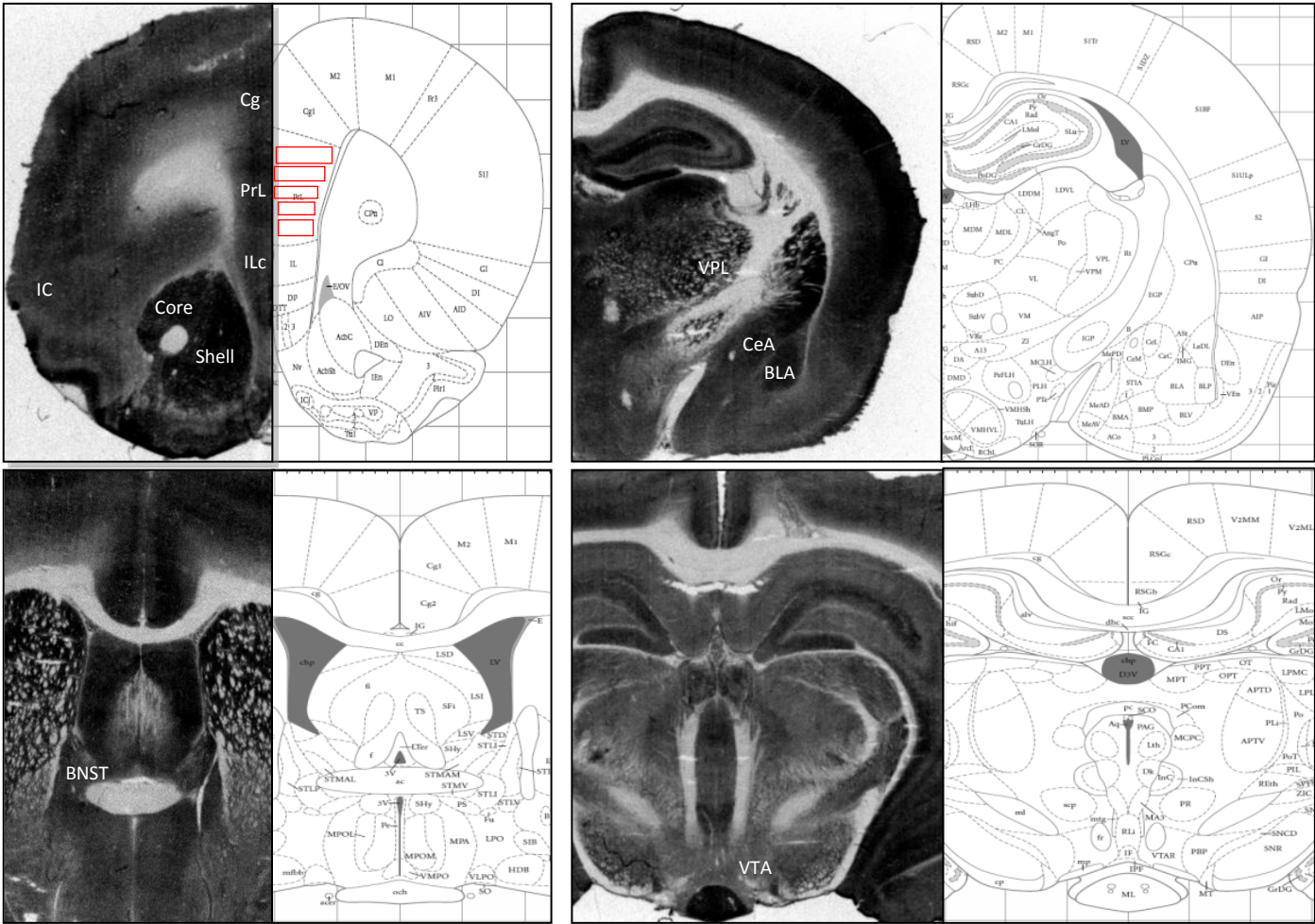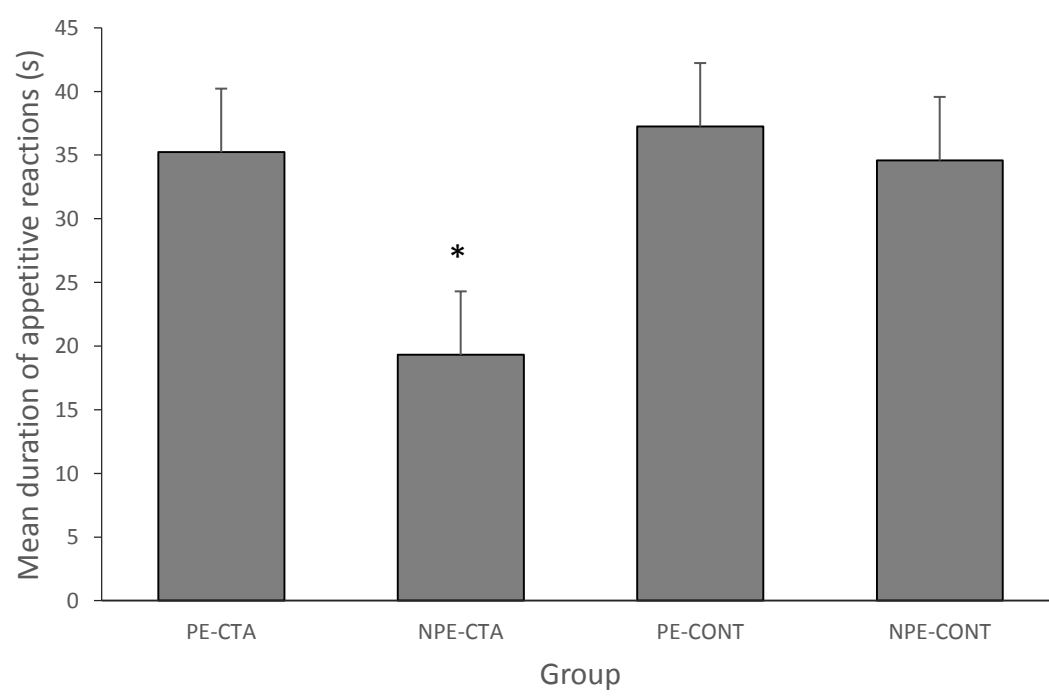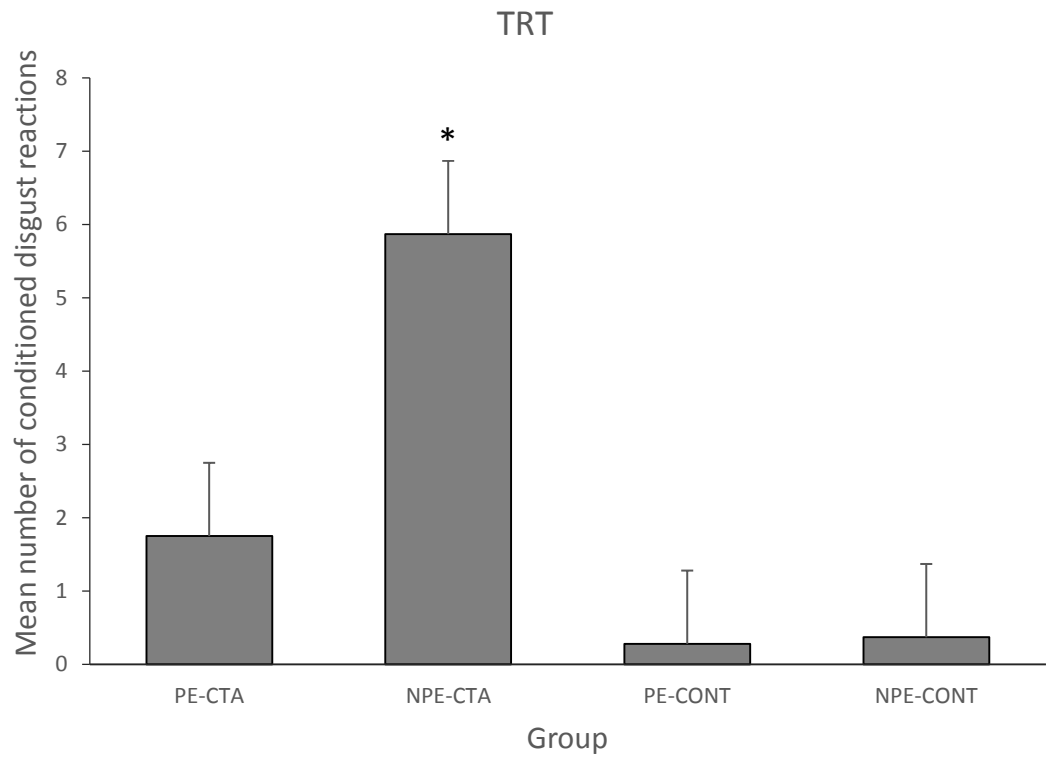
Figure(s)

FIGURE 1

FIGURE 2

TRT

FIGURE 3
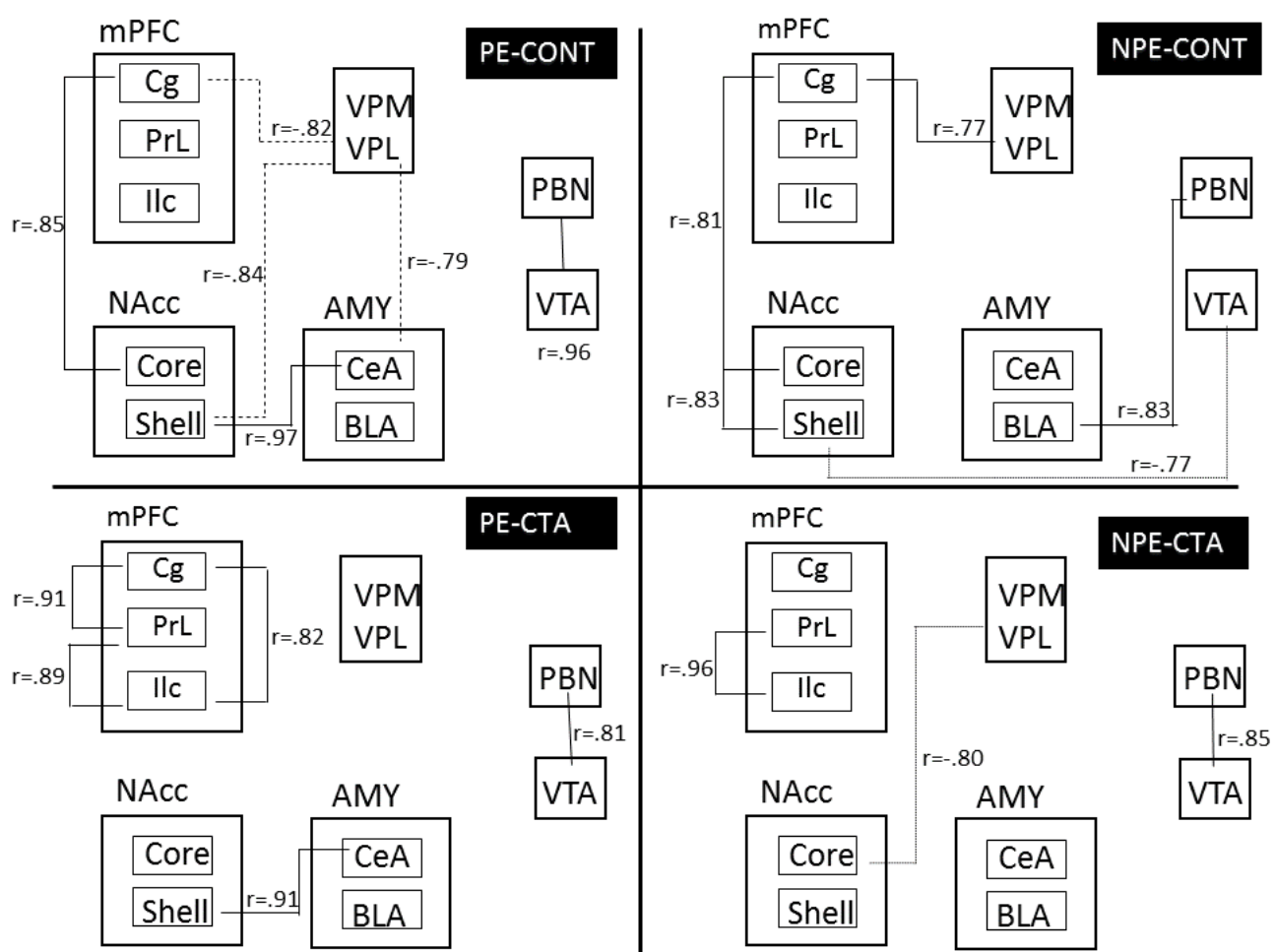
Table 1

TABLE 1. Design of the experiment

Group	Preexposure	Conditioning	TR test
PE-CTA	4 x Sac (IO)	Sac (IO) → LiCl	
NPE-CTA	4 x Water (IO)		Sac (IO)
PE-CONT	4 x Sac (IO)	Sac (IO) / LiCl	
NPE-CONT	4 x Water (IO)		

Note: Taste aversion groups, PE-CTA (preexposed) and NPE-CTA (non-preexposed), were given a LiCl injection during conditioning of saccharin, and control groups, PE-CONT (preexposed) and NON-CONT (non-preexposed), received the LiCl injection on the day following the conditioning session. Sac = saccharin; IO = intraoral infusions; LiCl = lithium chloride injection.

TABLE 2. Cytochrome oxidase activity values of the measured regions

Regions	PE-CTA	NPE-CTA	PE-CONT	NPE-CONT	PE factor		CTA factor		PE *CTA Interaction	
					F	p	F	p	F	p
mPFC										
Cingulate	26.91 ± 1.28	27.51 ± 1.05	32.07 ± 0.80	33.13 ± 2.44	0.28	.603	**11.74**	**.002**	0.02	.883
Prelimbic	27.76 ± 1.84	27.81 ± 0.96	30.96 ± 0.50	30.19 ± 0.96	0.08	.768	**5.29**	**.029**	0.12	.737
Infralimbic	26.26 ± 1.6	26.72 ± 1.01	29.05 ± 0.63	28.99 ± 1.01	0.03	.866	**4.59**	**.041**	0.05	.831
Accumbens										
Core	34.38 ± 1.70	36.97 ± 1.82	34.76 ± 0.97	36.07 ± 3.00	0.89	.353	0.02	.897	0.09	.764
Shell	36.67 ± 1.71	37.36 ± 1.27	38.42 ± 1.08	41.52 ± 4.15	0.59	.451	1.43	.242	0.24	.630
IC	24.62 ± 1.56	27.76 ± 1.72	27.89 ± 0.56	26.29 ± 1.01	0.33	.571	0.45	.507	3.12	.089
BNET	25.75 ± 0.90	23.86 ± 1.00	28.26 ± 0.44	27.48 ± 0.89	2.40	.133	**12.55**	**.001**	0.41	.528
AMY										
CeA	29.76 ± 1.50	28.81 ± 1.16	29.84 ± 0.54	29.98 ± 2.18	0.07	.792	0.17	.684	0.13	.722
BLA	25.96 ± 2.41	27.57 ± 0.96	29.79 ± 0.57	31.13 ± 1.35	1.64	.212	**4.29**	**.048**	0.17	.683
VTA	12.61 ± 1.00	15.40 ± 1.00	17.82 ± 1.04	17.51 ± 1.28	1.28	.269	11.06	.003	1.99	.170
PBN	7.72 ± 0.79	9.16 ± 1.49	13.91 ± 1.63	11.82 ± 1.71	0.66	.425	**4.07**	**.050**	**5.06**	**.033**
Thalamic n.										
VPM	18.75 ± 1.79	21.99 ± 0.95	24.32 ± 0.95	21.09 ± 2.02	0.04	.848	**5.08**	**.033**	0.67	.420
VPL	28.71 ± 2.94	26.20 ± 2.83	21.10 ± 0.90	22.65 ± 2.34	0.60	.297	2.27	.143	4.35	.056

Note: Data represent Cytochrome oxidase values (mean ± SEM); 1 unit: 1 µmol of cytochrome c oxidase/min/g tissue wet weight at 23° C. F values and p values are displayed for preexposure (PE) and conditioning (CTA) factors as well as for the interaction between this two factors. A significant conditioning effect was found for the mPFC, BNET, BLA and VTA. Additionally, a significant preexposure and conditioning interaction was found in the PBN. mPFC = Prefrontal cortex; IC = Insular Cortex; BNST = bed nucleus of estria terminalis; AMY = amygdala (BLA = basolateral, CeA = central); VTA = ventral tegmental area; PBN= parabrachial nucleus; VPM = ventral posteromedial thalamic nucleus; VPL = ventral posterolateral thalamic nucleus. PE-CTA = preexposed group; NPE-CTA = non-preexposed group; PE-CONT = preexposed control group; NPE-CONT = non-preexposed control group.