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Lesions of the central nucleus of the amygdala only impair flavor aversion learning in the absence of olfactory information

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The amygdala is considered a crucial brain nucleus in different modalities of aversive conditioning, including flavor aversion learning (FAL). The importance attributed to the amygdala and its subnuclei has frequently depended on the different stimuli and procedures used in FAL tasks. In this study, FAL was impaired only in animals that had lesions in the central nucleus of the amygdala (CeA) area and also had their olfactory bulbs removed. However, this task was learned by neurologically intact animals, bulbectomized animals, and rats with lesions exclusively centered in the CeA area alone. These results suggest that the CeA area may be relevant in gustatory-gut associative learning but not in FAL, in which the olfactory system may counteract the deficit produced in taste-visceral convergence.

Key words: flavor aversion learning, gustatory-visceral associative learning, central nucleus of the amygdala, olfactory bulbectomy, gustatory-olfactory stimuli

INTRODUCTION

Flavor aversion learning (FAL) is a modality of learning in which animals associate a flavor with noxious, habitually visceral stimuli (Lamprecht and Dudai 2000). The amygdala has been related to various types of aversive learning (for review, see Everitt et al. 2003, LeDoux 2007) and has been considered, along with other anatomically related centers, as a potential visceral-sensory convergence area that could sustain FAL (Coil et al. 1978, Ottersen 1982, Yamamoto et al. 1994, Sakai and Yamamoto 1999, Barot et al. 2008, Desgranges et al. 2010). In fact, it has been found to be involved in the processing of both gustatory and visceral information (Norgren 1976, Fulwiler and Saper 1984, Cechetto 1987, Jhamandas et al. 1996, Barot et al. 2008) as well as olfactory stimuli (Powell et al. 1965, Scalia and Winans 1975, Ottersen 1982, Price 1990, Desgranges et al. 2010), sensory components that are essential to establish FAL.

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However, researchers have reported contradictory results on the relevance of the amygdala in FAL. Some studies showed that lesions of the whole amygdala interrupt FAL (Nachman and Ashe 1974, Aggleton et al. 1981, Yamamoto and Fujimoto 1991, Sakai and Yamamoto 1999, Rollins et al. 2001) and some observed attenuating effects (Fitzgerald and Burton 1983, Gallo et al. 1992), whereas others found no effects (Lasiter 1982, Simbayi et al. 1986, Simbayi 1987, Dunn and Everitt 1988, Bermúdez-Rattoni and McGaugh 1991).

These discrepancies in results may be explained by differences in the flavor stimuli, lesioned amygdala subnuclei, and tests used (Schafe et al. 1998, Lamprecht and Dudai 2000, Spray et al. 2000, Touzani and Sclafani 2005, Miranda 2012; for review, see Reilly and Bornovalova 2005). Thus, the taste and smell sensations of a flavor (the combined effects of gustatory and olfactory information) can be individually associated with visceral malaise, generating taste and/or olfactory aversion learning (Capaldi et al. 2004).

With regard to the different subnuclei of the amygdala, there is evidence of the sensory processing of gustatory (Norgren 1976, Bernard et al. 1993) and visceral information (Saper and Loewy 1980, Cechetto 1987,

Bernard et al. 1993) in both the central nucleus (CeA) and basolateral nucleus (BLA) (Fulwiler and Saper 1984, Karimnamazi and Travers 1998, Barot et al. 2008). For its part, olfactory information projects towards the CeA (Ottersen 1982) but mainly towards the centromedial subnuclei of the amygdala (Scalia and Winans 1975, Price 1990, Wójcik et al. 2013) and BLA (Powell et al. 1965).

The BLA has long been considered the most important amygdala subnucleus in FAL processes (Nachman and Ashe 1974, Aggleton et al. 1981, Yamamoto et al. 1994, Morris et al. 1999, Rollins et al. 2001, St Andre and Reilly 2007, Barot et al. 2008, Wheeler et al. 2013). Thus, some studies showed that lesions of the BLA, but not of the CeA, interrupt olfactory aversion learning without affecting conditioned taste aversion (Bermúdez-Rattoni et al. 1983, 1986, Hatfield et al. 1992, Ferry et al. 1995, Slotnick et al. 1997, Miranda et al. 2007, Desgranges et al. 2008, Sevelinges et al. 2009) or the sensory processing of olfactory information *per se* (Bermúdez-Rattoni et al. 1986, Hatfield et al. 1992, Ferry et al. 1995, 1999, Hatfield and Gallagher 1995, Ferry and Di Scala 2000). Although most studies have ruled out the relevance of the CeA in FAL, some authors have shown that protein synthesis inhibition in the CeA can block conditioned taste aversion memory (Lamprecht and Dudai 1996, Lamprecht et al. 1997, Bahar et al. 2003).

Evidence on the participation of the amygdala and its various subnuclei in the acquisition, consolidation, and expression of aversive conditionings (Davis et al. 2000, Borszcz and Leaton 2003, Koo et al. 2004, Fanselow and Poulos 2005, Schafe et al. 2005, Wilensky et al. 2006; for review, see LeDoux 2007) prompted the present investigation into the specific involvement of the CeA area in gustatory-visceral associative learning, using bulbectomized animals in which gustatory cues alone would appear to intervene in FAL (Alberts and Galef 1971, Bell et al. 1979, Miranda 2012).

In this study, a concurrent discrimination task was used in which two flavors were simultaneously presented in each session, one associated with the intragastric administration of a noxious stimulus and the other with the administration of physiological saline (PS) (Arnedo et al. 1990; for review, see Mediavilla et al. 2005). Previously, olfactory information was interrupted by lesion of the olfactory bulbs, and the intragastrically administered noxious substance was hypertonic NaCl, an appropriate product to induce FAL (Arnedo et al. 1990; for review, see Mediavilla et al. 2005).

Neurologically intact control animals and anosmic control animals can be expected to learn the discrimination task by preferring the flavor not associated with the noxious substance, while animals exclusively lesioned in the CeA area can be expected to develop olfactory discrimination learning. We hypothesized that anosmic animals with bilateral electrolytic lesions of the CeA would be unable to learn the discrimination task due to their inability to integrate the gustatory-olfactory and visceral-aversive stimuli required to develop this modality of concurrent FAL. In other words, by interrupting gustatory-visceral convergence (CeA lesion) and blocking the olfactory signal (olfactory bulbectomy), the animals would not be capable of discriminating between the two gustatory-olfactory stimuli presented, because they would not be able to identify the flavor associated with gastrointestinal malaise.

METHODS

Subjects

Forty-seven male Wistar rats, weighing 270–330 g at the surgery, were randomly distributed into five groups: intact control group (Intact, n=8) and sham surgery control group (Sham, n=7), which could presumably discriminate the flavors by some of the sensory cues available in the concurrent FAL task (gustatory, olfactory, visual, propioceptivas, place...); group with bilateral electrolytic CeA lesion (CeA, n=11), with the same cues available except for those of gustatory information; control group with olfactory bulbectomy and intracranial electrode without current (Anosmic, n=11), with all cues available except for those of olfactory information; and experimental anosmic group with CeA lesion (Anosmic-CeA, n=10), with all cues available except for those of gustatory and olfactory information. Animals were housed in individual methacrylate cages (15×30×15 cm) that also served as training chambers during the experiment. The sides of the cages were black and opaque, and the front and back were transparent. The front had two 1.6 cm holes at the same distance from the center and edges and at the same height above the floor of the cage. These openings allowed the animal access to spouts attached to cylindrical graduated burettes through which liquid

flavors were delivered (Mediavilla et al. 1998). The laboratory was maintained at 22–24˚C with a 12:12 h light/dark cycle (lights on at 8 AM). Experimental procedures were conducted during light periods with white noise. Animals had free access to food and water unless otherwise indicated.

Surgical Procedure

Bilateral electrolytic lesion of the CeA

CeA lesions were made under general anesthesia (intraperitoneal sodium pentothal, 50mg/Kg) using a stereotaxic device (Bilaney, Mod. SAS-4100) [Coordinates: AP=+6.7 mm, L=±4.0 mm, V=+2 mm (Paxinos and Watson 1998) (Interaural=+6.7 mm)]. Animals received bilateral cathodic electric current (1.2 mA) for 20 s, using a DCLM-5 lesion generator (Grass Instruments, Quincy, MA, USA). An electrode was placed (V=+3.0 mm) in the sham surgery control group, but no current was passed.

Olfactory bulbectomy

Olfactory bulbs were sectioned after amygdala surgery and with the animals still under anesthesia (Van Riezen and Leonard 1990). Briefly, olfactory bulbs were sectioned by introducing a scalpel through two orifices (diameter, 2 mm) made on each side of the middle line, 8 mm anterior to Bregma, applying a slight pressure to the base of the cranium and avoiding damage to the adjacent frontal lobe. The orifices were subsequently blocked with bone wax and incisions were sutured.

Implant of two intragastric cannulas

After the two surgeries described above and with the animal still under anesthesia, two Silastic intragastric cannulas were implanted by making an incision of approximately 3 cm along the medial line of the abdominal wall and carefully exteriorizing the stomach out of the abdominal cavity. Through a small incision (2 mm) on the ventral surface of the cardia of the stomach, the end of a fistula (1×2 mm) was introduced, including a small protuberance made with surgical adhesive (Solyplast, Barcelona, Spain) to prevent stomach detachment after closure of the incision around it (Arnedo et al. 1990).

In a small dorsal opening (immediately behind the head), two subcutaneous tunnels were made (one on each side) through which the free ends of the cannulas were exteriorized, subsequently suturing to close the wound. As a prophylactic measure, 0.1 cc penicillin (Penilevel retard, Level, S.A., Barcelona, Spain) was injected (250,000 UI/ml.). After the surgery, animals were returned to their cages, where they remained for a recovery period of ≥8 days with food and water *ad libitum* (Arnedo et al. 1990).

All behavioral procedures and surgical techniques complied with Spanish legislation [Royal Law (1201/2005)] and the European Community Council Directive (86/609/EEC).

Experimental Procedure

The experimental procedure comprised two stages:

1) Stage I (pre-training): After the recovery period, water was available for the animals for only 10 min from graduated burettes. During this three-day stage, animals were habituated to take water from the burettes on both sides (right and left) to avoid position bias. On day 1, the animal was offered two burettes with water (left and right), on day 2, one burette with water was offered on the left, and on day 3 one was offered on the right. The amount of water consumed for 10 min was recorded on each day. Burettes were removed after the 10 min intake and, after a 30-min interval, the animals were offered 15 g of solid food (Alimento de Laboratorio. Dietas Panlab. Panlab S.L., Barcelona).

2) Stage II (learning): This stage comprised five sessions of a learning task to develop aversion towards a flavor associated with an intragastrically administered aversive stimulus (5% hypertonic NaCl, 0.85 M). For 7 min, the animals were offered two burettes with two different flavors, strawberry (S) and coconut (C) [0.5% S and C extract diluted in water (McCormick Co, INC, San Francisco, CAL)], placed in the left and right holes, respectively.

The intake of one of the flavors (50% of animals) was associated with the simultaneous intragastric administration of hypertonic NaCl (*via* one implanted fistula), whereas the consumption of the other flavor was associated with the intragastric administration of PS (*via* the other implanted fistula) (Table I).

The administration rate of both products (hypertonic and isotonic) was 1 cc/cc of liquid, using a Model

Table I

Diagram showing the balanced experimental conditions in the concurrent flavor aversion learning (FAL) modality during the 5 acquisition days in each study group

	Day 1	Day 2	Day 3	Day 4	Day 5
50% of animals	Strawberry Left+NaCl and Coconut Right+PS (7 min)	=Day 1	=Day 1	= Day 1	=Day 1
50% of animals	Strawberry Left+PS and Coconut Right+NaCl (7 min)	=Day 1	=Day 1	=Day 1	=Day 1

A-98 infusion pump (Razel, USA) to administer the dose at a constant rate. After 7 min, the burettes were removed and the consumption of each flavor was recorded.

Histology

At the end of the experiment, animals were anesthetized with an overdose of sodium pentothal (80 mg/Kg, ABBOTT, Madrid) and intracardially perfused with PS and 10% formaldehyde. Brains were removed and stored in 10% formaldehyde for at least 48 h before the lamination of nervous tissue in 40-µ coronal sections. Sections were stained with Cresyl Violet and examined under an optical microscope (Olympus, CO 11) to determine the localization and extension of the lesions (Fig. 1).

Statistical analysis

The ANOVA/MANOVA module of statistical software (StatSoft, Inc., Tulsa, OK, USA) was used for the data analyses. The intake of the two flavors during the five days was analyzed by means of repeated-measures ANOVAs for each group. All data are expressed as means ±SEM, and statistical significance is set at the 5% level.

RESULTS

Two animals in the Intact Control group and one in the Anosmic group were excluded from the study due to the detachment of an intragastric cannula during the experimental procedure. The final sample sizes for these two groups were therefore 6 and 10, respectively.

After the five sessions, no significant differences were observed between the control groups (Intact and Sham) ($F_{4,44}$=0.32, P<0.85), which were therefore considered together as a single group (Total n=13), finding that the days x substance interaction among the four groups was statistically significant ($F_{12,160}$=2.23, P<0.01). The individual analyses of the repeated-measure ANOVA (days x substance) of the Anosmic-CeA group results demonstrated no statistical significance for the interaction ($F_{4,36}$=1.01, P<0.41). Hence, the anosmic animals with bilateral CeA area lesions did not develop the visceral-gustatory-olfactory associations characteristic of concurrent FAL models (Fig. 2D).

The remaining groups successfully learned the discriminative task, and the days x substance interaction was statistically significant in the Total ($F_{4,48}$=10.79, P<0.001) (Fig. 2A), CeA ($F_{4,36}$=5.76, P<0.001) (Fig. 2B), and Anosmic ($F_{4,36}$=2.75, P<0.04) (Fig. 2C) groups, which developed a progressive rejection of flavors associated with hypertonic NaCl over five sessions.

DISCUSSION

In this study, groups of rats with CeA lesion alone, CeA lesion plus olfactory bulbectomy, and bulbectomized and neurologically intact rats underwent a concurrent FAL task in which a flavor was associated with intragastric hypertonic NaCl or PS administration. An aversion for the flavor was successfully developed within five sessions in all animals except for those with CeA lesion plus bulbectomy.

The amygdala, especially its BLA and CeA subnuclei, has been considered a crucial brain region in aversive learning (Fanselow and LeDoux 1999, Davis 2000, Fanselow and Gale 2003, Pare et al. 2004; for review, see Maren 2005), and each of these subnuclei has been related to specific functions in aversive conditioning (Killcross et al. 1997, Amorapanth et al. 2000, Kruzich and See 2001, Koo et al. 2004; for review, see Sah et al. 2003, LeDoux 2007).

Electrolytic lesions were used in this study, because they provide greater anatomical specificity (although less cellular specificity) for a small nucleus such as the CeA. Hence, it appears likely that lesions of the CeA area interrupted gustatory-visceral convergence (but not FAL), given that both sensory systems are processed in this region of the amygdala (Saper and Loewy 1980, Cechetto 1987, Bernard and Besson 1990, Bernard et al. 1993). These lesions may not have interrupted olfactory-visceral associative learning (CeA-lesioned group). This is because, although direct olfactory connections have been identified between the olfactory bulb and the CeA (Ottersen 1982), the main projections of this sensory system are towards the centromedial subnuclei of the amygdala (Price 1990, Scalia and Winans 1975; Wójcik et al. 2013) and, *via* the piriform cortex, towards the BLA (Powell et al. 1965). One possible explanation for the attenuated effect on FAL observed in some experiments with CeA lesions (Fitzgerald and Burton 1983) is that the main associative afferent connection (gustatory) is interrupted but the olfactory information remains intact and available for utilization by other neural systems to sustain this learning.

In the present experiment, the neurologically intact group had two sensory indexes (gustatory and olfactory) (Fig. 2A); however, the Anosmic (Fig. 2B) and CeA (Fig. 2C) groups possessed only one (gustatory or olfactory, respectively), although it appeared to be sufficient for association with the noxious visceral stimuli (Capaldi et al. 2004). In contrast, the animals in the Anosmic-CeA group (Fig. 2D) lacked the sensory cues (chemoreceptors) necessary to acquire this learning and, importantly, they did not appear to use the propioceptive, place, or space information available to carry out the discriminative task. This appears to confirm previous observations in which gustatory-olfactory stimuli seem to be particularly essential in concurrent FAL (García et al. 1974, Mediavilla et al. 2001).

In summary, CeA area lesions in bulbectomized animals may have blocked the acquisition of the gustatory-visceral associative learning in a concurrent FAL task, given that the gustatory and visceral sensory cues involved in this learning modality are known to converge in this brain area (Saper and Loewy 1980, Cechetto 1987, Bernard and Besson 1990, Bernard et al. 1993).

Previous studies have reported that the CeA, as is the case in other related nuclei (Arnedo et al. 1990, Mediavilla et al. 2000, Hurtado et al. 2014), is involved in aversive conditionings that involve visceral-sensory associations, whereas the BLA is relevant in the orosensory relationship (Bernal et al. 2009, Dwyer 2011). Likewise, Nakagawa and others (2003) analyzed the involvement of the different amygdala subnuclei in noxious stimulus processing and concluded that the

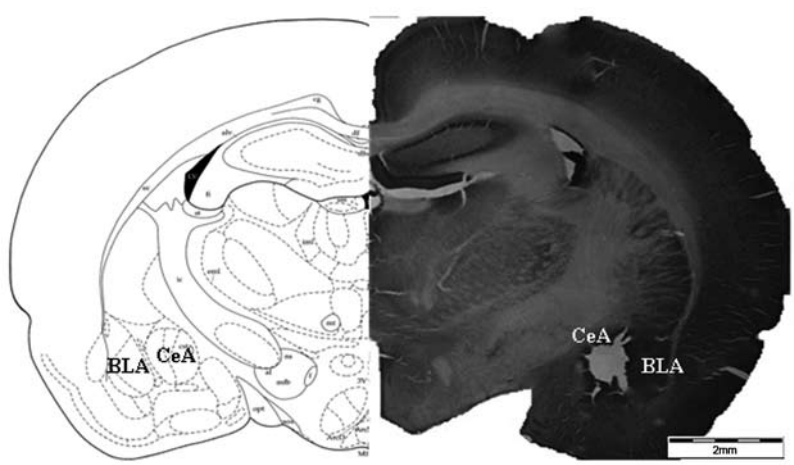

Fig. 1. Anatomical localization of the CeA electrolytic lesion, using the neuroanatomical atlas of Paxinos and Watson (1998) (Interaural=+6.7 mm).

CeA is relevant in processing noxious chemical visceral stimuli (acetic acid) and the BLA in processing somatic stimuli (formalin). These findings are compatible with observations that intragastric hypertonic NaCl administration activates the neurons of the CeA but not those of the BLA (Michl et al. 2001, Mediavilla et al. 2004).

Furthermore, intragastric hydrochloric acid (HCl) administration, which induces FAL (Ervin et al. 1995), produces CeA cell activation and this effect is blocked by vagotomy (Michl et al. 2001), as in concurrent FAL tasks (Arnedo et al. 1993).

The present results are in agreement with previous reports that FAL, besides establishing a gustatory-visceral association, also produces an olfactory-visceral convergence, especially in learning in which the aversive visceral stimulus is administered contiguously with the olfactory stimuli (García et al. 1966, Rusiniak et al. 1979, Durlach and Rescorla 1980, Palmerino et al. 1980, Lasiter et al. 1985, Ferry et al. 1995, Ferry and Di Scala 1997, Dardou et al. 2006, Inui et al. 2006), which is a distinctive characteristic of the concurrent modality used in the present experiment (Arnedo et al. 1990; for review, see Mediavilla et al. 2005).

It appears that olfactory stimuli *per se* may not offer an enduring memory trace, or at least one that is strong enough to be associated with an aversive stimulus. This has led to the proposal that animals acquire a strong aversion to the olfactory stimulus associated with the aversive stimulus only when the latter is related to a gustatory stimulus during the acquisition process (Palmerino et al. 1980, Rusiniak et al. 1979, Durlach and Rescorla 1980). This modality, taste-potentiated odor aversion learning (TPOAL), seems to result from the association between the weak memory trace of the olfactory stimulus and the aversive stimulus, using the presence of the gustatory stimulus to enhance the memory trace during acquisition (Ferry and Di Scala 2000).

The BLA, not the CeA, is considered the essential amygdala subnucleus in TPOAL (Bermúdez-Rattoni et al. 1983, 1986, Hatfield et al. 1992, Ferry et al. 1995, 1999, Hatfield and Gallagher 1995, Ferry and Di Scala 2000, Inui et al. 2006). It is believed that the olfactory cues induced by gustatory stimuli are potentiated in this brain subnucleus for subsequent association with the visceral consequences (Touzani and Sclafani 2005, Inui et al. 2006, Desgranges et al. 2010). In fact, despite being

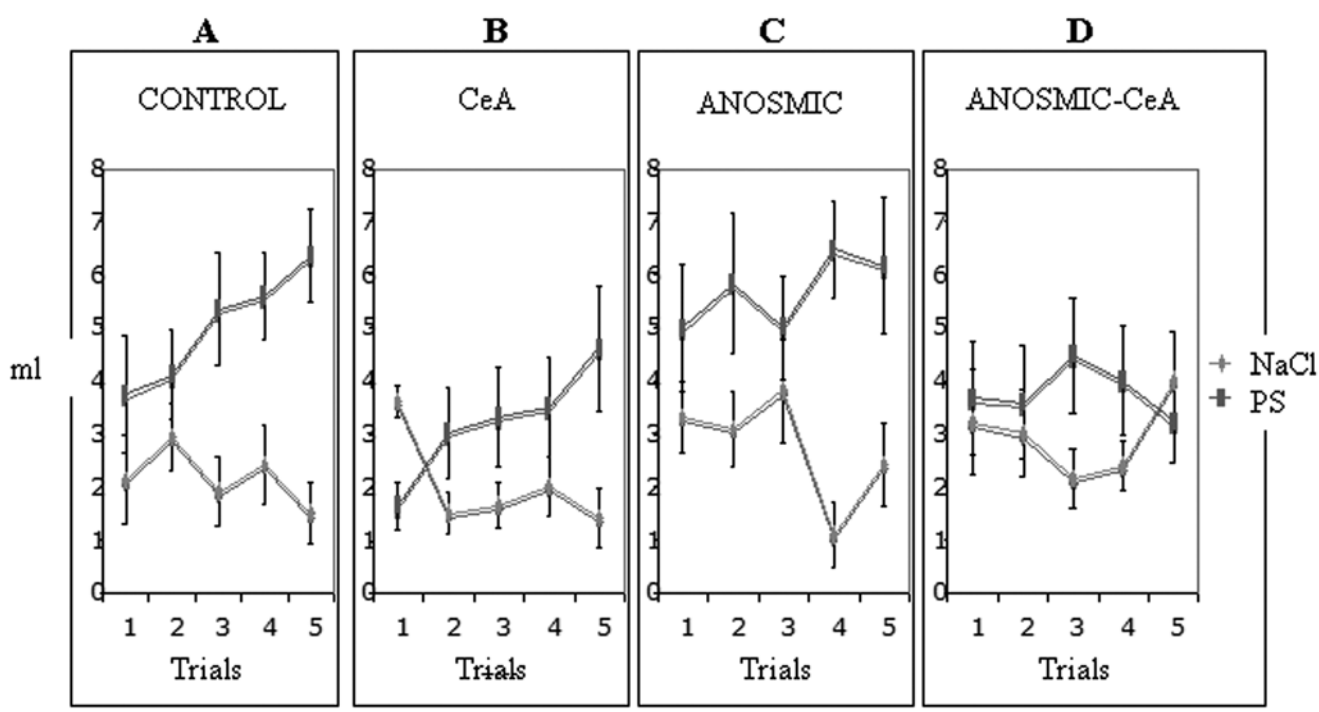

Fig. 2. Daily mean intake (ml) of the flavor associated with hypertonic NaCl (NaCl) and with isotonic physiological saline (PS) in Control (A), CeA (B), Anosmic (C), and Anosmic-CeA (D) group.

a crucial nucleus in visceral noxious signal processing (Bernard et al. 1993, Michl et al. 2001, Nakagawa et al. 2003, Tanimoto et al. 2003, Mediavilla et al. 2004, Bernal et al. 2009, Dwyer 2011), lesions of the CeA do not block TPOAL acquisition (Bermúdez-Rattoni et al. 1983, Hatfield et al. 1992, Ferry et al. 1995), presumably because they are not relevant in gustatory-olfactory associations (Nakagawa et al. 2003).

Although most studies using FAL have verified that the acquisition process is interrupted by BLA lesions but not by CeA lesions (Aggleton et al. 1981, Morris et al. 1999, Nachman and Ashe 1974, Rollins et al. 2001, Sakai and Yamamoto 1999, Schafe et al. 1998), other experiments have shown that the acquisition and consolidation of conditioned taste aversion memory can also be impaired by administering protein synthesis inhibitors or propanolol in the CeA (Lamprecht and Dudai 1996, Lamprecht et al. 1997, Bahar et al. 2003).

CONCLUSION

The present results suggest that the combination of CeA area lesion and olfactory bulbectomy interrupts the acquisition of gustatory-visceral associative learning in a concurrent FAL task in which animals must discriminate between two flavors, one of which is associated with the simultaneous intragastric administration of an aversive substance (hypertonic NaCl). This impairment was not observed in animals with only one of these structures disabled or in neurologically intact animals. These results indicate that the interruption of gustatory and visceral-aversive convergence prevents FAL only in anosmic animals, i.e., in the absence of counteracting olfactory sensory information.

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