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SB-334867-A, a selective orexin-1 receptor antagonist, enhances taste aversion learning and blocks taste preference learning in rats

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ABSTRACT

Lateral hypothalamus (LH) has been proposed as a possible center for the anatomical convergence of gustatory and postingestive information relevant to taste aversion learning (TAL) and conditioned flavor preference (CFP). Orexin, a neuropeptide that mainly originates in neurons in lateral hypothalamic areas, was recently related to learning and memory processes. The present study was designed to analyze a possible relationship between the orexinergic system and taste learning. We studied the effect of intracerebroventricular administration of three doses (3, 6, and 12 μg/1 μl) of the selective orexin-1 receptor antagonist SB-334867-A on the acquisition of TAL induced by a single administration of LiCl. Infusion of SB-334867-A did not block this learning and appeared to enhance TAL in a two-bottle test. However, SB-334867-A (6 μg/1 μl) blocked taste preference learning when a flavor associated with saccharin (CS+) was offered on alternate days against a different flavor without saccharin (CS−), during three acquisition sessions. These results offer evidence of a relationship between the orexinergic system and taste learning; they tentatively suggest the possibility that endogenous orexin and gustatory and postingestive (visceral and oral) signals converge in brain areas relevant to the acquisition of taste learning.

1. Introduction

Organisms appear to have innate mechanisms that lead them to prefer certain flavors and avoid others. The preference or aversion for certain foods can also develop through experience, by the association of tastes with their postingestive consequences. Thus, taste aversion learning (TAL) is manifested in the tendency to reject foods or tastes previously associated with visceral malaise. Indeed, the survival of organisms is highly dependent on their capacity to learn to exclude potentially noxious foods from their diet. Organisms can also learn to prefer a taste associated with positive postingestive consequences, by a process designated flavor preference learning or conditioned flavor preference (CFP). These two learning modalities are not only important in the selection of daily diets but also in certain clinical situations, including anorexia and bulimia, and in patients undergoing chemotherapy (Bernstein, 1999; Capaldi, 2004; Scalera and Bavieri, 2009).

There have been numerous attempts over recent years to determine the neurobiological bases of TAL and CFP. Specifically, studies have been conducted to explore the possible anatomical pathways for transmission of gustatory and visceral data and the structures involved in the convergence and association of this information, above all in TAL (see Bures et al., 1998; Reilly and Schachtman, 2009). There have been fewer studies on the neural pathways involved in CFP, and they have centered on brain regions previously related to TAL (Touzani and Sclafani, 2009). Despite these efforts, the neurobiological mechanisms of TAL and CFP have not yet been determined with precision.

The lateral hypothalamus (LH) has been proposed as one of the anatomical regions in which relevant gustatory and visceral information converge (Bernardis and Bellinger, 1996; Dell and Olson, 1951; Norgren, 1976; Uematsu et al., 2010). Information would reach the LH through its connections with the parabrachial area (Bester et al., 1997). Some authors observed that lesions of the LH are ineffective to block TAL induced by LiCl (Touzani and Sclafani, 2001; Roman et al., 2006), although they were found to impair the learning of a preference for a flavor paired with delayed intragastric (i.g.) maltodextrin (Touzani and Sclafani, 2001). Another study reported that both TAL and CFP were impaired in LH-lesioned animals when there was a long delay between flavor and postingestive consequence (Touzani and Sclafani, 2002). However, the use of lesions to study the role of LH may not be appropriate, since the effects on TAL and CFP may be masked by the severe hypodipsia and hypophagia in animals with LH lesions (Bernardis and Bellinger, 1996).

In this context, there have been recent reports on a new class of neuropeptide, orexins (Sakurai et al., 1998), also called hypocretins (De Lecea et al., 1998), which are produced by a small group of hypothalamic

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neurons and whose actions are mediated by two membrane receptors, orexin-1 (OX_1R) and orexin-2 (OX_2R). Orexinergic neurons have been detected in the perifornical nucleus and in dorsal and lateral hypothalamic areas (Peyron et al., 1998). Despite their localized anatomical origin, they widely project to numerous brain regions, including the cerebral cortex, olfactory bulb, amygdala, septum, hippocampus, thalamus, brain stem, and spinal cord (Nambu et al., 1999; Peyron et al., 1998). This widespread pattern of orexinergic fiber distribution indicates that this peptide system may intervene in numerous functions. In fact, it has been related to a wide variety of brain functions, including feeding and drinking (Kunii et al., 1999; Sakurai et al., 1998), the sleep–waking cycle (Sakurai, 2007), addiction to drugs of abuse (Borgland et al., 2006; Narita et al., 2006), and learning and memory (Akbari et al., 2007; Borgland et al., 2006; Harris and Aston-Jones, 2006). Hence, the orexinergic system may play a role in both appetitive and aversive learning. In fact, double-label immunohistochemistry study demonstrated activation of both orexin and Fos in LH orexin cells from animals showing conditioned place-preference (CPP) for morphine, cocaine, or food (Harris et al., 2005), and CPP was impaired after bilateral excitotoxic lesions of LH orexin neurons (Harris et al., 2007). In addition, pre-proorexin gene knock-out mice and rats administered with a selective orexin receptor antagonist in the ventral tegmental area could not acquire CPP for morphine (Narita et al., 2006). Intracerebroventricular (i.c.v.) administration of orexin-A reinstated cocaine-seeking, probably by induction of a stress state (Boutrel et al., 2005). Orexin-A was also reported to facilitate the learning, consolidation, and retrieval of a passive avoidance task (Telegdy and Adamik, 2002). In contrast to these studies, which suggest that orexin-A would facilitate learning in certain tasks, another group found that i.c.v. orexin-A retarded learning and impaired memory in the Morris water maze (Aou et al., 2003).

LH orexin neurons are known to project towards TAL-related brain regions such as the nucleus of the solitary tract (NST), parabrachial area, and area postrema, among others (Peyron et al., 1998). Moreover, orexins would send visceral information to the brain through the NST (Kirchgessner, 2002), the first area of visceral and gustatory afferent integration needed for TAL acquisition (García et al., 1974). Furthermore, the main afferents of LH orexin neurons come from brainstem areas, such as the lateral parabrachial nucleus (Yoshida et al., 2006), which has been repeatedly studied in relation to TAL and CFP (Agüero et al., 1993; Mediavilla et al., 2000; Reilly and Trifunovic, 2000; Sclafani et al., 2001).

Given this background and the contradictory data on the participation of orexin in learning and memory, the aim of the present study was to evaluate its role in LiCl-induced TAL and saccharin-induced CFP. For this purpose, we examined the effects of the i.c.v. administration of the selective orexin-1 receptor antagonist SB-334867-A, the most potent and selective OX_1R antagonist, on the acquisition of these two learning modalities.

2. Experiment 1: Taste aversion learning induced by a single LiCl administration

Successful TAL can be established after a single taste-aversive postingestive consequence pairing and after a long delay between gustatory stimulus exposure and gastrointestinal discomfort (Bures et al., 1998; Garcia et al., 1955). In this experiment, we examined the effects of the i.c.v. administration of three doses of the selective orexin-1 receptor antagonist SB-334867-A on TAL induced by a single LiCl administration. Animals were i.p. injected with LiCl immediately after the intake and i.c.v. administration of SB-334867-A, with no delay. However, it should be taken into account that the first symptoms of sickness do not appear until 5–10 min after the injection of LiCl (Nachman and Ashe, 1973), implying a minimal inter-stimulus delay.

2.1. Materials and methods

2.1.1. Animals

Male Wistar rats weighing 250–270 g on arrival at the laboratory were provided by Harlan Laboratories (Barcelona, Spain). They were individually housed in methacrylate cages that also served as training chambers during the experiment. Room temperature was maintained at 21–24 °C under a 12:12 light–dark cycle with lights on at 8:00. Food and water were available *ad libitum* except when reported otherwise. All procedures and experimentation were carried out in accordance with guidelines established by the European Union (86/609/EEC) and Spanish Royal Law 1201/2005 and were approved by the Ethical Committee for Animal Research at the University of Granada. All efforts were made to reduce the number of animals used in this experiment.

2.1.2. Drugs

Three doses (3, 6, and 12 μg/1 μl) of the orexinergic antagonist SB-334867-A (1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl-urea hydrochloride, Tocris, Madrid, Spain) were i.c.v administered. The selection of these doses was based on previous reports, including studies on the relationship between the orexinergic system and acquisitive behaviors (Akbari et al., 2006; Borgland et al., 2006; Narita et al., 2006). SB-334867-A was dissolved in 10% dimethyl sulfoxide (DMSO, Sigma, Madrid, Spain) in water. DMSO was used as vehicle, since previous studies found no effects of this substance on learning or memory (Akbari et al., 2006). LiCl (0.15 M, 20 mg/kg, Sigma, Madrid, Spain, i.p.) served as noxious visceral stimulus.

2.1.3. Surgery

For the i.c.v. injection, rats were deeply anesthetized with sodium pentothal (Lab. Abbot, Spain, 50 mg/kg i.p.) and unilaterally implanted with a cannula (Plastic One, 26-gauge stainless-steel guide) in the left lateral cerebral ventricle. Stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson (2005) and were as follows: AP: 0.96 mm posterior to the bregma, L: 1.8 mm from midline, V: 3.2 mm below the skull surface. The incisor bar was placed 3.3 mm below the interaural line. The guide cannula was secured to the skull with two screws and dental cement and closed with a dummy cannula. After the intervention, all animals received an intramuscular injection of 0.1 cc penicillin (Penilevel, Level, S.A., Barcelona, Spain) and started a 7-day recovery period with food and water *ad libitum*. During this period, the rats were handled daily and the dummy cannula was carefully removed and replaced.

2.1.4. Microinjection procedure

SB-334867-A and vehicle were administered through a guide cannula using an injection needle (33 gauge) connected by polyethylene tubing to a 5.0 μl Hamilton micro-syringe driven by an infusion pump (KD Scientific Inc., MA, USA). The injection needle was inserted 1 mm beyond the tip of the guide cannula. Infusions were delivered in a total injection volume of 1 μl over a period of 60 s. After each infusion, the injector remained in place for 60 s to allow diffusion of the solution into the tissue and to minimize reflux along the injection track. The accuracy of cannula placements was verified *in vivo* by observation of an intense drinking response within 5 min of Angiotensin II administration (100 ng/4 μl) (Sigma, Madrid, Spain).

2.1.5. Experimental procedure

Animals were water-restricted and habituated to the ingestion of water for 15 min a day from an inverted graduated cylinder (20 ml, 1-ml gradation) with a sipper spout that extended into the cage. The graduated cylinders were located centrally on the front side of the cage, and their position (left–right) was counterbalanced to prevent a side preference. Food pellets were removed during each drinking session. One hour after ending experimental sessions, all animals received 30 g

of food. At 4 h after the end of the experimental phase, there was a daily 30-min period of rehydration. Water and food intake and animal weight were recorded daily.

After 2 days of training sessions, all animals were offered a saccharin solution (0.15%, Sigma, Madrid, Spain) for 15 min and the amount consumed was recorded. The saccharin intake was then followed by i.c.v. injection of SB-334867-A (3, 6, and 12 μg/1 μl) or a similar volume of DMSO (control group). Animals then immediately received an intraperitoneal (i.p.) injection of either isotonic NaCl (0.9%) or LiCl. After a 24-h recovery period under similar conditions to those of the training sessions, the aversion was tested on day 5 by a two-bottle test (saccharin-water). In this choice test, two graduated cylinders containing water and saccharin, respectively, were offered for 15 min, and the difference in consumption between them was taken as the aversion index. Control groups served to assess the preference for saccharin and the capacity of SB-334867-A *per se* to produce aversion.

The following animal groups were established for the different experimental conditions: A) DMSO + saline (n = 4); B) DMSO + LiCl (n = 6); C) SB-334867-A (6 μg/1 μl) (n = 8); D) SB-334867-A (3 μg/1 μl) + LiCl (n = 5); E) SB-334867-A (6 μg/1 μl) + LiCl (n = 6); and F) SB-334867-A (12 μg/1 μl) + LiCl (n = 6).

2.1.6. Statistical analyses

Values are expressed as means ± SEM, and statistical analyses were performed using Statistica 5.0. Mean intakes during the choice tests (saccharin-water) were analyzed with two-way repeated-measure ANOVA (group × fluid). The index of aversion for saccharin in the choice tests was also calculated as percentage of saccharin intake (100 × saccharin intake/total intake) and group differences were evaluated with one-way ANOVA. The effects of treatments on food intake (pretreatment day *vs.* posttreatment day) were examined by two-way repeated-measure ANOVA (group × day of intake). When a significant *F* was obtained, Newman–Keuls's *post hoc* tests and planned comparisons were performed to assess specific comparisons. Statistical significance was determined at the 5% level.

2.2. Results and discussion

2.2.1. TAL acquisition

The effects of various doses of SB-334867-A on the choice test are shown in Fig. 1. A two-way repeated-measure ANOVA (group × fluid) analysis revealed a significant effect of the group variable ($F_{5,29} = 4.29$, $P < 0.005$) and fluid variable ($F_{1,29} = 31.87$, $P < 0.0001$), and a significant group × fluid interaction ($F_{5,29} = 35.37$, $P < 0.0001$). Newman–Keuls's *post hoc* tests revealed significant differences between saccharin and water intake in every group. All LiCl-treated groups ingested significantly less saccharin than water in the TAL acquisition test (low dose: $P < 0.0001$; intermediate dose: $P < 0.0001$; high dose: $P < 0.0001$; and DMSO + LiCl: 0.0005). In contrast, groups without LiCl administration showed significant preference for saccharin *vs.* water (DMSO group: $P < 0.0005$; SB-334867-A group: $P < 0.005$), demonstrating that the orexinergic antagonist *per se* does not induce TAL. Further comparisons showed no differences among groups receiving different doses of SB-334867-A and LiCl, implying that TAL acquisition is not blocked by SB-334867-A at the doses used in this study. Significant intergroup differences were also found in the percentage of saccharin ingested ($F_{5,29} = 62.75$, $P < 0.000001$). Newman–Keuls's *post hoc* tests revealed a significantly higher percentage saccharin intake in the DMSO + LiCl group than in the groups treated with SB-334867-A + LiCl (low dose: $P < 0.05$; intermediate dose: $P < 0.01$; and high dose: $P < 0.05$). This last finding indicates that the administration of SB-334867-A not only fails to block TAL but may even enhance this learning.

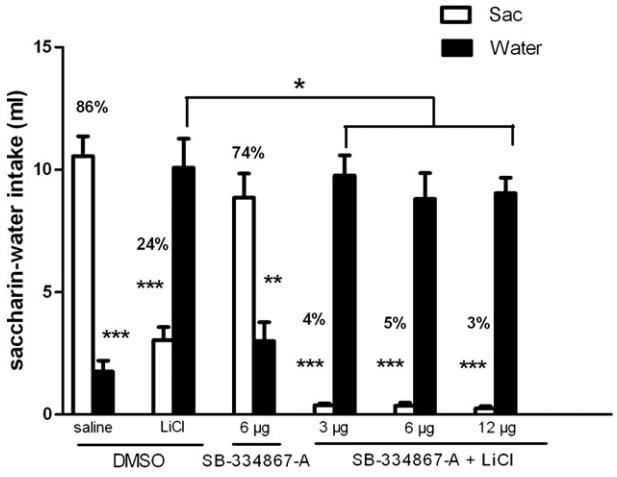

Fig. 1. Saccharin and water intake during the two-bottle choice test in Experiment 1. SB-334867-A i.c.v. administration (3, 6, and 12 μg/1 μl) does not impair TAL acquisition induced by a single saccharin–LiCl pairing. DMSO + LiCl control group also develops aversion for saccharin. In contrast, control groups receiving i.c.v. DMSO or SB-334867-A without LiCl show a preference for saccharin. Data are means ± S.E.M. Numbers above bars indicate mean percentage intakes of saccharin. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

2.2.2. Food intake

The effect of SB-334867-A on food intake was analyzed by comparing the intake on the pretreatment day with the intake at 24 h (Fig. 2). A two-way repeated-measure ANOVA (group × day of intake) revealed significant effects of the day variable ($F_{1,29} = 29.81$, $P < 0.00001$). Newman–Keuls's *post hoc* tests revealed an intake reduction during the first post-administration day in the groups receiving an intermediate ($P < 0.01$) and high dose ($P < 0.01$) of SB-334867-A. Both doses appear to produce a similar effect on intake, since no significant differences were found between these groups. Neither the DMSO + LiCl group nor the low-dose group showed a significant decrease in food intake, implying that the intake reduction of the other experimental groups cannot be attributed to the effects of LiCl. Furthermore, administration of an intermediate dose of SB-334867-A without LiCl did not significantly reduce the food intake. We can therefore conclude that the effects of intermediate or high doses of SB-334867-A combine with the effects of LiCl to significantly reduce intake at 24 h post-administration.

3. Experiment 2: Conditioned flavor preference

CFP is observed when a neutral flavor or food is associated with a previously preferred flavor (e.g., saccharin) or with the i.g. administration of a nutrient. Thus, two procedures are habitually used to develop flavor preferences: flavor–taste learning and flavor–nutrient learning (Capaldi, 2004; Touzani and Sclafani, 2009). Using the flavor–nutrient learning paradigm, Touzani and Sclafani (2001, 2002) demonstrated that the LH is essential when there is a long delay between the flavor and

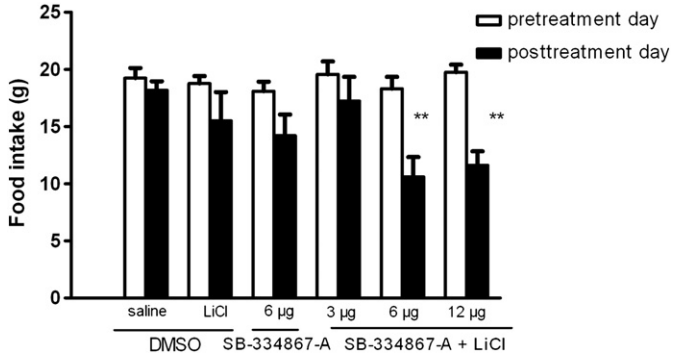

Fig. 2. Effects of the i.c.v. administration of SB-334867-A (3, 6, and 12 μg/1 μl) and i.p. LiCl on food intake at 24 h. Only the higher doses affect intake, implying that the reduction cannot be attributed to LiCl. The intermediate dose of SB-334867-A without LiCl does not significantly reduce food intake. Data are means ± S.E.M. **$P < 0.01$.

the positive postingestive consequences of the i.g. administration of a nutrient (maltodextrin). Other authors recently found that the vagus nerve was necessary for CFP induced by i.g. glutamate, and they proposed that LH is one of the areas where the postingestive signal of glutamate would be integrated with oronasal signals (Uematsu et al., 2010). Hence, the objective of this experiment was to analyze the effect of the administration of orexinergic antagonist SB-334867-A on the development of taste preference by flavor–taste learning, an acquisitive process in which a delay is not possible (Capaldi, 2004).

3.1. Materials and methods

3.1.1. Animals

14 male Wistar rats weighing 250–270 g on arrival at the laboratory were provided by Harlan Laboratories (Barcelona, Spain). Animals were randomly distributed into experimental and control groups and maintained under identical conditions to those reported in Experiment 1.

3.1.2. Drugs

This experiment only used the intermediate dose (6 μg/1 μl) of the i.c.v. orexinergic antagonist SB-334867-A, since the previous experiment demonstrated no learning differences as a function of the dose used. The same volume of DMSO was used as vehicle.

3.1.3. Surgery and microinjection procedure

Implantation of the guide cannula in the left ventricle and the i.c.v. injection procedure were as reported in Experiment 1.

3.1.4. Experimental procedure

Animals were habituated to a water restriction period (2 days), as in Experiment 1, followed by initiation of the sessions to develop taste preferences. CS+ was 0.15% saccharin solution flavored with 0.05% (w/w) non-sweet cherry or grape flavor (Kool-Aid, General Foods, White Plains, NY) and CS− was the flavor diluted with tap water. In three acquisition sessions (6 days), animals were offered, on alternate days, cherry or grape flavor, and the flavor–saccharin pairs were counterbalanced across subjects. The i.c.v. administration of SB-334867-A was also counterbalanced in the experimental group. Thus, i.c.v. SB-334867-A was associated with the grape flavor on odd days and the DMSO with the cherry flavor on even days in half of the rats (n = 4), while SB-334867-A was associated with cherry flavor on even days and the vehicle with grape flavor on odd days in the other half (n = 3). SB-334867-A or DMSO was administered immediately after 15 min of intake and after recording the amount of liquid ingested.

One animal died during the experimental procedure in the control group, which finally comprised 6 animals. In all sessions, control animals received i.c.v. DMSO immediately after 15 min of intake.

On day 7, all animals were subjected to a choice test in which two tubes containing CS+ and CS− were simultaneously presented in the same positions as during the acquisition sessions. In this test, both flavors were presented in water and there was no i.c.v. administration.

3.1.5. Statistical analysis

Values are expressed as means ± SEM, and statistical analyses were performed using Statistica 5.0. Mean intakes during the preference tests (CS+ *vs.* CS−) were analyzed by two-way repeated-measure ANOVA (group × fluid). The CS+ preference index in the choice tests was also calculated as percentage of CS+ intake (100 × CS+ intake/total intake) and group differences were evaluated using one-way ANOVA. The effects of treatments on the body weight of animals and on food intake during the experiment were examined by two-way repeated-measure ANOVA (group × day). When a significant *F* was obtained, Newman–Keuls's *post hoc* tests were performed to assess specific comparisons. Statistical significance was determined at the 5% level.

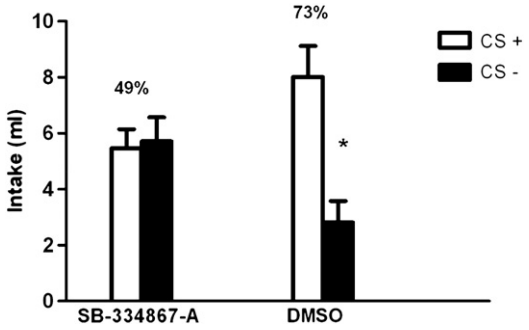

Fig. 3. Effects of the i.c.v. administration of SB-334867-A (6 μg/1 μl) on intake of CS+ and CS− during the two-bottle preference tests in Experiment 2. Data are means ± S.E.M. Numbers above bars indicate mean percentage intakes of CS+. *P<0.05.

3.2. Results and discussion

3.2.1. CFP acquisition

Fig. 3 shows the mean intakes in the choice test by the experimental group (i.c.v. SB-334867-A) and control group (DMSO). Repeated-measure ANOVA (group × fluid) found that the interaction ($F_{1,11}$ = 5.736, P<0.05) was significant but not the group or fluid variable. Newman–Keuls's *post hoc* tests demonstrated that only the control group showed significant differences between CS+ and CS− intake (P<0.05), indicating that the animals in this group were capable of learning the flavor–saccharin association during the acquisition sessions. In contrast, the experimental group consumed similar amounts of both flavors in the test, indicating that they could not acquire a taste preference under these experimental conditions. One-way ANOVA showed also significant differences between the groups in percentage CS+ intake ($F_{1,11}$) = 5.65, P<0.05). Hence, the i.c.v. administration of SB-334867-A appears to block the learning of taste preferences.

3.2.2. Food intake and body weight

The mean food intake during the seven experimental days was analyzed using repeated-measure ANOVA (group × day), which showed the main effect of day to be significant ($F_{6,66}$ = 8.739, P<0.00001), with both groups showing the same increase in food intake.

Repeated-measure ANOVA (group × day) was also used to analyze animal weight during the experimental sessions, finding significance for the main effect ($F_{6,66}$ = 9.720, P<0.000001). Newman–Keuls test results revealed an increase in the weight of experimental animals over the days, which reached significance from day 4 (P<0.005).

In contrast to the acute administration of SB-334867-A in Experiment 1, repeated infusion in three sessions permitted the analysis of its effect on food intake and weight during the 7 experimental days of Experiment 2. These data again appear to rule out a generalized malaise induced by i.c.v. SB-334867-A that impaired the learning.

4. General discussion

This study examined the effect of the i.c.v. administration of the selective orexin-1 receptor antagonist OX_1R SB-334867-A on the acquisition of TAL induced by a single administration of LiCl and on flavor preference induced by saccharin. The main finding was that SB-334867-A blocks CFP acquisition but does not impair LiCl-induced TAL.

Previous anatomical and lesion studies suggested that the LH may have a relevant role in TAL and CFP (Bernardis and Bellinger, 1996; Bester et al., 1997; Touzani and Sclafani, 2001, 2002; Uematsu et al., 2010). This, alongside the fact that orexinergic neurons located in the LH project to regions related to TAL and CFP, such as the NST, parabrachial area, area postrema, and amygdala (Peyron et al., 1998) may indicate the participation of the orexinergic system in taste learning. However, the role of orexin in learning remains controversial, with some authors reporting that orexin-A administration produces a decrease in spatial

learning and memory (Aou et al., 2003) and others finding that it facilitates learning and the consolidation and retrieval of passive avoidance learning (Telegdy and Adamik, 2002).

In the present study, none of the three doses of SB-334867-A (3, 6, and 12 μg/1 μl) impaired TAL acquisition. In fact, animals in these groups may even have developed a greater aversion for saccharin as measured in a two-bottle test. The floor effect observed in these groups hindered confirmation of this possibility and also prevented the finding of significant differences among the different doses used. However, the fact that the percentage saccharin intake was lower in the three experimental groups than in the DMSO + LiCl group may suggest that LiCl-induced TAL was even strengthened by the intervention in the orexinergic system. In this context, we have obtained data (not included) indicating that the administration of SB-334867-A (at 6 and 12 μg/1 μl but not at lower doses) increases the retention of LiCl-induced TAL. Differences in the magnitude of TAL became evident after 1 month, at which time the learning was extinguished in the group receiving the low dose of SB-334867-A (3 μg/1 μl) in the acquisition session.

Some possible aversive property of SB-334867-A might also explain the increase in learning magnitude, but administration of this drug alone (without LiCl) was not sufficient to produce TAL. Therefore, the effect does not appear to be caused by drug-induced malaise or other non-specific toxic effects. In the same line, a recent comparative study on the effect of the administration of equi-anorectic doses of LiCl and SB-334867-A established clear behavioral differences between the visceral discomfort induced by LiCL and the satiating effect of the orexinergic antagonist (Ishii et al., 2004). On the other hand, orexin was reported to promote the overconsumption of sweet and pleasant tastes, e.g., saccharin (Furudono et al., 2006), implying that this orexinergic antagonist may have affected the processing of the gustatory stimulus. However, preference for saccharin in the choice test was evident in animals that receive SB-334867-A without LiCl. Hence, the most plausible explanation is that the combination of SB-334867-A and LiCl acts on the associative process, enhancing the TAL.

Nonetheless, additional experiments may be warranted to rule out other possible explanations. Thus, the use of one-bottle and two-bottle test could be compared, since some authors consider that the double-choice test may strengthen aversion and amplify learning magnitude (Roman et al., 2006). On the other hand, a choice test appeared to be more appropriate because it permits discriminating purely aversive from motivational aspects (Bures et al., 1998), and orexin-A has also been related to motivational processes (Boutrel and de Lecea, 2008; Harris and Aston-Jones, 2006). Assessment of the effects of SB-334867-A on taste preference learning would be useful to rule out the possibility that the present results are due to the mere addition of the noxious visceral effects of both substances.

This possibility was examined in Experiment 2, whose results confirmed that the administration of an intermediate dose (6 μg/1 μl) of SB-334867-A blocks the acquisition of a saccharin-induced taste preference. The fact that the animals consumed similar amounts of both flavors in a choice test again indicated that the orexinergic antagonist is insufficient to produce taste aversion, despite its repeated administration (3 acquisition sessions in Experiment 2). There were no differences between experimental and control groups in food intake, but the experimental animals showed a significantly increase in body weight over the seven experimental days. Obesity despite hypophagia has been reported in narcolepsy patients and mice with chronic orexin deficiency (Hara et al., 2001; Sakurai, 2006). Nevertheless, a dose-response study could be necessary in order to replicate the results of Experiment 2.

Study of the effects of i.c.v. SB-334867-A on food intake and body weight is relevant because of the well-established relationship between the orexinergic system and feeding behavior (Sakurai et al., 1998). Thus, it has been verified that i.c.v. administration of orexin stimulates food consumption, finding a stronger effect with orexin-A than with orexin-B (Haynes et al., 1999; Sakurai et al., 1998). Furthermore, the i.p. administration of antagonist OX_1R SB-334867-A was found to reduce food intake at 60 min and 24 h and block the hyperphagic effect of orexin-A (Ishii et al., 2005; Rodgers et al., 2001). Consequently, orexin may play a role in the short-term control of feeding and may also have longer-term (24 h) effects on food intake. In contrast, although i.p. LiCl (90 mg/kg) also evidences a significant anorectic effect at 1 h, the effect can be undetectable at 24 h. (Ishii et al., 2004). Nevertheless, under the present study conditions, neither LiCl (DMSO + LiCl and low-dose + LiCl groups) nor the orexinergic antagonist (group SB-334867-A without LiCl in Experiment 1 and experimental group in Experiment 2) had a significant effect on food intake, which was only significantly reduced when intermediate or high doses of SB-334867-A were administered in combination with LiCl. Thus, an additive effect of SB-334867-A and LiCl may have produced the food intake reduction observed at 24 h post-administration in Experiment 1. At any rate, these findings allow us to rule out the possibility that data obtained in TAL and CFP were attributable to any generalized or sensory (gustatory) incapacity. Hence, this intervention in the orexinergic system appears to specifically impair the acquisition of a taste preference.

There are various possible explanations of these opposite effects on taste learning. The orexinergic system may play a greater role in appetitive learning than in aversive learning, as in the case of the lateral hypothalamus, which is more directly related to flavor–nutrient learning than flavor–toxin learning (Touzani and Sclafani, 2001). Distinct neurobiological mechanisms may be involved in taste aversion and flavor preference. For instance, the processing of taste and flavor stimuli is different in the amygdala and parabrachial nucleus (Sclafani et al., 2001; Touzani and Sclafani, 2005), and postingestive signals can also use different anatomic pathways (Martin et al., 1978; Uematsu et al, 2010). Alternatively, the intervention in the orexinergic system may have affected both modalities of learning (TAL and flavor preferences) if orexin has a more general role, e.g., in motivation (Boutrel et al., 2005; Boutrel and de Lecea, 2008; Borgland et al., 2010; Boutrel et al., 2010).

It has been proposed that orexin-A neurons can integrate both central and peripheral information on feeding and energy balance (Haynes et al., 1999). Moreover, since LH orexin neurons are activated by stimuli that predict both food and drug reward, they may play an important role in reward-related learning and memory through their anatomical connections with the ventral tegmental area (Cason et al., 2010; Harris and Aston-Jones, 2006; Harris et al., 2005, 2007). Thus, exogenous stimulation of orexin neurons was reported to reinstate extinguished drug-seeking behavior (Boutrel et al., 2005; Harris et al., 2005), and VTA injections of SB-334867-A blocked the development of heroin-conditioned place-preferences (Narita et al., 2006). *In vitro* and *in vivo* studies demonstrated that orexin-A induces the synaptic plasticity and behavioral sensitization to cocaine observed in VTA dopaminergic neurons, thereby linking learning mechanisms with the neural changes produced in addiction to drugs of abuse (Borgland et al., 2006). Aston-Jones et al. (2010) observed that SB-334867-A prevents cue- and context-induced reinstatement of extinguished cocaine-seeking but does not affect the reinstatement of drug-seeking by cocaine itself, indicating that intervention in the orexinergic system does not interfere with the reinforcing properties of cocaine. The above authors consider LH and VTA orexin neurons to be critical regions in drug abuse, principally in reward-based learning and memory (Harris and Aston-Jones, 2006; Aston-Jones et al., 2010).

Orexinergic neurons have mainly been found in the LH (Peyron et al., 1998). The i.c.v. administration of SB-334867-A in the present study prevents the precise localization of the orexinergic neurons affected. However, it has been proposed that orexinergic neurons in perifornical and dorsomedial areas are involved in arousal and waking, whereas those in the LH participate in reward conditioning (Harris and Aston-Jones, 2006). Hence, given the anatomical connections of the two regions (Yoshida et al., 2006), it appears reasonable to attribute the results in TAL and CFP to the inhibition of orexinergic neurons in the LH.

Touzani and Sclafani (2002) recently highlighted the importance of the LH in TAL and CFP when increasing the delay between flavor intake and i.g. administration of the visceral stimulus, proposing that LH lesions may impair flavor memories, especially with nutrient reinforcement. In the present study, the inter-stimulus delay does not appear to have been decisive, and there was no delay in Experiment 2 because it used a flavor–taste paradigm (Capaldi, 2004). It also appears that delay is not relevant when CFP is induced by i.g. glutamate, which would be transmitted to the LH *via* the abdominal vagus nerve (Uematsu et al., 2010). In any case, it could be proposed that the release of orexin enhances the intake of gustatory stimuli associated with positive visceral and oral signals. In the present study, inhibition of the orexinergic system along with noxious visceral information transmitted by the humoral pathway (LiCl) may have reinforced saccharin aversion, thereby strengthening TAL. In the case of CFP, the orexinergic antagonist appears to have deprived animals of the positive reinforcing signals required to develop a taste preference. Although the neural basis of the reward value of taste remains unknown (Yamamoto, 2006), the coexistence of neurons responsible for the sensory processing of taste information with those specialized in the hedonic aspect of taste has been proposed in various structures (Sewards, 2004). Hence, in the present study, SB-334867-A may have prevented associations that are essential for CFP in forebrain structures. Alternatively, the intervention in the orexinergic system may have extinguished signals that reinforce primary afferent information to the brainstem given that orexinergic fibers from the LH terminate in the NST (Peyron et al., 1998; Kirchgessner, 2002).

These findings tentatively suggest the possibility that endogenous orexin and gustatory and postingestive (visceral and oral) cues converge in brain centers relevant to both food intake and taste learning, implying that the orexinergic system may provide an essential signal to reinforce the association between the stimuli required for learning to take place. Further studies are required to determine relevant centers and the precise mechanisms that underlie the present results. Meantime, this study demonstrates for the first time the relationship between the orexinergic system and taste learning. Interventions in this system may offer an alternative to the lesion method for the study of the role of the LH in these two learning modalities.

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