Involvement of Neuropeptide Orexin B in Basolateral Amygdala Mediated Consummatory Behaviour in Male Wistar Albino Rats

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Abstract

The basolateral amygdala has been implicated in the regulation of food intake besides the hypothalamic centres. In the present study, we hypothesized that the Orexin B, a polypeptide identified in the lateral hypothalamic region, may be involved in the modification of the functions of the amygdaloid centres. We therefore studied the effect of infusion of Orexin B and its antagonist (TCS-OX2-29) into Basolateral amygdala to study the feeding behaviour.

Materials and methods: Adult male Wistar albino rats were selected and grouped into control, sham operated control and experimental groups (n=6 each) Orexin was infused in two doses (3 nmol/µl, 30 nmol/µl) and TCS-OX2-29(10 µg/µl) was infused in another group. Sequential Food intake and water intake were measured at 1, 2, 4, 6, 12 hours and intake for the day was also recorded in all groups and the results (mean±SEM) were statistically analyzed by Kruskal Wali’s test and p<0.05 was considered significant.

Results: The food intake and water intake were significantly increased (p<0.01) in the high dose group though the increase in the low dose treated animals was less. Injection of Orexin B antagonist decreased the food and water intake significantly.

Discussion and conclusion: The results suggest that Orexin plays a role in the modulation of feeding behaviour. In the lower doses it did not show significant effect. At higher doses, the effect was marked. The role of orexin in ingestive behaviour is further confirmed by the action of antagonist infusion, which resulted decrease in the feeding activities.

Key words: Orexin, food intake, water intake, basolateral amygdala
Introduction

Orexin A and Orexin B (also known as hypocretin 1 and 2 respectively) are peptides with 33 and 28 amino acid residues, respectively, produced from a common precursor prepro-orexin (1, 2). There are two orexin receptors, OX1 and OX2, of which the former has highest affinity for orexin A and the latter has affinity for both orexin A and B (3, 4). Among the first published reports of orexin by two separate groups of scientists, de Lecea et al and Sakurai et al (5, 6) linked orexin to modulation of feeding behaviour. Orexinergic cell bodies reside within the LHA, which was classically considered as the “feeding centre” and thus orexins were named for the Greek root for appetite (orexis) (3, 7). Hence it was speculated that orexins may play a prime role in the feeding behaviour (8). The orexinergic fibres have its wide projections to different areas of the brain. In the hypothalamus it projects to the Arcuate nucleus (ARC), Paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH) and ventro medial hypothalamus (VMH), which is mainly implicated in feeding behaviour. These neurons also project to the cerebral cortex, limbic system and brain stem. These findings may indicate that orexins are not only involved in the aspect of energy homeostasis of feeding behaviour, but also involved in emotional, cognitive and motivational aspects of feeding behaviour (4).

Some of the research findings suggest that Orexin B modulates feeding behaviour only under nutritional duress (9). Prepro-orexin, the genetic precursor of both peptides, was shown to be up-regulated 2.4-fold after 48 h. of food deprivation (10). Orexin A dose-dependently enhances the action on feeding to a greater extent following a 24-h fast (8). The forebrain structures implicated in the consummatory behaviour included the nuclear groups of amygdala, namely basolateral and central amygdala, and also the lateral and ventromedial hypothalamus. The functional relationship between VMH and BLA was evaluated in our laboratory earlier (11, 12). Immunohistochemical studies have revealed that the nerve terminals of orexin immunoreactive neurons are present in various regions of cerebral cortex, limbic system and brain stem (13, 14). The projection of these neurons suggests a possible relationship between hypothalamus and basolateral amygdala through orexin.

Even though there are many research findings that indicate the role of orexin in feeding behaviour when it is injected to the ventricle, there is a lack of substantial evidence from nuclei specific studies. Thus, we have investigated in this study whether orexin B acts through basolateral amygdala by injecting orexin B, specifically to Basolateral amygdala and also further, modulation of feeding behaviour during metabolic challenge.

Orexin B-specific antagonist, TCS-OX2-29 (N-Acyl 6,7-Dimethoxy-1,2,3,4 tetrahydroisoquinolone) selectively blocks Orexin 2 receptors (15, 16). A review of the work done in this area shows that the role of Orexin B in the Amygdala mediated consummatory behaviour is still unclear. The effects of Orexin B and it’s receptor antagonist receptor antagonist infusion should reveal the nature of the role in feeding behaviour. In this study we have demonstrated for the first time the effect of bilateral micro infusion of Orexin 2 receptor antagonist into the basolateral amygdala. Our findings provide further evidence of the role of Orexin in the regulation of feeding behaviour.

Materials and methods

Adult male albino rats of Wistar strain (200-275 gms) that were bred in the institutional animal house were used for the present study. Animals were housed individually in polypropylene cages (29 cms × 22 cms × 14 cms) with a paddy husk bedding under normal day-night cycle during the experimental period. Food pellets (Amrut laboratory animal feed, Amrut rat and mice pellet. Pranav Agro Industries Ltd, Maharashtra, India) and potable tap water was made available to animals ad.Lib. All experiments were conducted with strict adherence to the CPSEA guidelines. Institutional ethical committee approval was obtained before the commencement of the animal experiments. (IIAEC/KMC/57/2009-2020).
Animals were divided into three groups. Group 1 and Group 2 with 18 animals each were subdivided into control group (n=6; untreated), sham operated group (n=6; Saline infusion) and treated group. Group 1 received a low dose of Orexin B (3 nmol/µl) and Group 2 received a high dose of the same (30 nmol/µl). The third group (Group 3) was infused with TCS-OX-29 (10 micrograms/µl) in the experimental animals and other two subgroups were maintained as in the case of Orexin groups.

**Surgical procedure and cannulation:**

The rats were anaesthetized with Ketamine (60 mg/kg body weight) and Xylazine (10 mg/kg body weight) injected intraperitoneally. Anaesthetized rats were fixed in the stereotaxic apparatus. For intracranial injection, a guide cannula was implanted in place by stereotaxic method. For the central administration, animals were implanted with 22 gauge stainless steel cannula. For BLA studies, cannulae were bilaterally placed (H=–8.5 mm from the surface of the skull; L=±4.8 mm from the midline; AP=–3.14 mm behind the bregma (17). The implanted cannula was secured with the help of screw and dental acrylic. A dummy stylet was placed in the cannula after the surgery and between the injections to prevent blockade. Rats were housed singly during the recovery period of one week without measuring any parameters. One lakh units of Penicillin was injected intramuscularly (Penidure, Hindustan Antibiotics Ltd) in a single dose to prevent infection. Infusion cannula was fabricated from 30G sterile siliconised disposable dental needle (SEPTODENT, France), which has a hub that is convenient for handling. (18) Infusion cannula extends 1 mm beyond the respective guide cannula. Cannula placement was verified by post-mortem histology. Rats with misplaced cannula were excluded from the study. However, there were no significant deviations among the rats in the cannula placement.

**Infusion experiments:**

Orexin B (Catalog no. 06262 Sigma Aldrich, St Louis, USA) was dissolved in 0.9% saline and the solution was stored at 4°C. Infusions were made with the help of infusion pump (Harvard Pico plus, Harvard apparatus) with a Hamilton micro syringe (10 µl, Hamilton Company) attached to polyurethane tubing backfilled with saline. A 2 microliter air bubble separated the drug from the saline. Injectors (30G, PLASTIC ONE) extended 1.00 mm beyond the tip of the guide cannula (22G)(19). Neuropeptide Orexin B (3 nm/µl and 30 nm/µl) was injected into the unanesthetized, free moving animals at a flow rate of 0.6 µl/min over a period of 90 seconds, with the injector left in position for an additional 30s to ensure the complete extrusion of orexin from the tip and its diffusion. We performed the study by using low dose (3 nm/µl) and high doses (30 nm/µl) of orexin B in separate groups. In the control group no procedure was done. The sham-operated controls underwent surgical procedure but were infused with normal saline. The dose of orexin B was selected on the basis of previous work. (6, 10) Similarly Orexin B antagonist, TCS-OX2-29 (Catalog No.3370; Tocris Bioscience, UK) was dissolved in distilled water and infused at a dose of 10 microgm/µl in another group (Group 3) of rats (n=6 each).

Rats were deprived of both food and water for 24 hours prior to drug injection. Food intake was measured at different points of time after drug administration i.e. at the end of 1, 2 4 6, 12 and 24 hrs. Food intake was weighed on a digital scale, correcting the difference of the weight for food spillage. Similarly water intake was measured by providing measured volume of water in the drinking bottle and measuring the left over. The four trials were carried out and the average of the four readings was noted. The values are expressed in grams of food consumed per 100 g of body weight. At least 72 hrs elapsed between consecutive treatment days.

**Histology:**

On completion of the experiments, animals were anesthetized with overdose of diethyl ether in an anaesthesia chamber and then transcardially perfused with 0.9% saline and 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.4 (20). The cannula was removed carefully and the brain was dissected out. Brains were post-fixed in 4% paraformaldehyde solution. The brain tissue was processed and paraffin blocks were made. Seven micron sections were cut
Statistical analysis

Food and water intake data are expressed as mean±S.E.M. The data, which were not normally distributed, was analysed using non parametric Kruskal Wallis test as appropriate, using SPSS 16 version. Differences were considered significant at p≤0.05. Data analysis was done between controls, cannulated (sham control) and orexin B/TCS-OX2-29 infused group, considering each hour readings separately (e.g. 1 hr control and sham control vs. 1 hr Orexin B group).

Results

In low doses of Orexin B (3 nmol/µl) there was a slight increase in food and water intake but the difference was not statistically significant except for the increase in food intake at the end of 4th hour (Table I, p<0.05). In contrast, there was significant decrease in food intake and intake during night time compared to the control/sham operated rats. The high dose of Orexin B (30 nmol/µl) increased food intake significantly at the end of 1, 4, 6 and

<p>| TABLE I : Effect of Orexin B (3 nanomoles/µl) on food intake and water intake at different time periods following infusion into Basolateral amygdala: 1, 2, 4, 6, 12 hours and cumulative food intake (24 hrs) (n=6 rats/group) [*p&lt;0.05 Experimental vs control/sham operated group]. |</p>
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<p>| TABLE II : Effect of Orexin B (30 nanomoles/µl) on food and water intake at different time periods following infusion into Basolateral amygdala: 1, 2, 4, 6, 12 hours and cumulative food intake (24 hrs) (n=6 rats/group) [*p&lt;0.05, **p&lt;0.01. Experimental vs control/sham operated group]. |</p>
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Fig. 1: Photomicrograph of the brain stained with Cresyl violet showing the site of insertion of guide cannula (Magnification 1.5x) and stained with 0.1% cresyl violet stain. These sections were examined under dissection microscope for the verification of implanted guide for the confirmation of the site of infusion.
24 hours and similar response was seen regarding water intake at the end of 2, 4, 6 and 24 hrs (Table II).

Food intake in the second hour after the injection of the Orexin B antagonist decreased significantly (p<0.01, Table III). The water intake significantly decreased in the second hour after the infusion (p<0.05, Table III). However in the subsequent period as well as the intake for the day did not show any significant changes. In the fourth hour, there was decrease in water intake but the food intake did not show significant decrease. Both food intake and water intake showed no significant variation in the 6-24 hour period or total intake for the day.

Discussion

Control of ingestive behaviour is very complex and involves several centres in the basal brain and neurotransmitters. It has been already suggested that orexin-A and orexin-B are physiological mediators of appetite regulation (6). In our study rats showed increase in food and water intake in dose dependent manner after the injection of orexin B in to the BLA. The increase in food intake to orexin B at highest dose was consistent with the previous studies where Orexin B was administered to paraventricular nucleus of hypothalamus (10). Earlier studies have also proved that intracerebroventricular injection of orexin B specifically stimulated food intake in rats (10, 21). Administration of orexin antibodies resulted in decreased food intake (22). The stimulation of drinking behavior in response to orexin B is well documented in rodents. These observations suggest a physiological role for orexin as mediators regulating drinking behaviour (23, 24). Thus we examined the effect of orexin B on water intake and found that orexin B potently increase water intake. The greatest feeding response and dose dependent enhancement of feeding were observed when Orexin B was administered to rats deprived of food for 24 hours. The orexin signalling may be important in modulating the feeding network at times of nutritional duress (25). Orexin-B was administered up to a dose of 30 nmoles, which is the maximum dose examined in the published report on orexin (6, 10).

We also observed that drug-injected rats showed the usual nocturnal habit soon after the infusion; they were awake and active after drug infusion and often showed exploratory behaviors like rearing, grooming and face washing. This was also previously reported when orexin A was administered ICV, chronically for 7 days (26). Orexin may be involved in the maintenance of arousal so that they stay awake during the day time. Orexin modulates arousal and sleep changes through its connectivity to hindbrain nuclei (27). This could be a mechanism by which orexin increases food intake and water intake during the day time. Since orexin neurons densely innervate the cerebral cortex and limbic system, probably it might have roles in cognitive, emotional and motivational aspect of feeding behavior (23).

Our findings suggest that orexin B augment consummatory behavior via activation of OX₁ and OX₂ receptors. To support this, specific Orexin 2 antagonism study with TCS OX2-29 was carried out. Orexin B antagonist produced a decrease in food intake (Table III).
and water intake compared to the control group. The effect was more evident in the second hour and to a lesser extent during 2-6 hrs. Decrease in food and water intake following infusion suggested that blocking Orexin action in BLA leads to decrease in drive for feeding. But appearance of reduction in food intake in the second hour and in subsequent reading as well as not finding an immediate response (in the first hour) needs further study.

The present observations indicate that orexin may be involved in short term, immediate regulation of food intake. Further, they might have role in maintaining arousal and vigilance for normal feeding behavior. These data also throw a light on orexin-signaling that may be specifically modulated for feeding behavior under conditions of nutritional challenge. Before the role of orexin in the appetite-regulation hierarchy can be ascertained, further studies examining the effects of chronic injection and the interactions with known appetite-regulatory peptides and nuclei are required.

Acknowledgements

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References


