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Effect of orexin B and OX2-R antagonist micro-infusion into the nucleus accumbens on consummatory behaviour in wistar rats

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Abstract
Aims and objectives: To elucidate role of Orexin B in the control of ingestive behaviour mediated through nucleus accumbens. Materials and Methods: In the present study Orexin B was infused into the nucleus accumbens in Wistar albino rats. Fifty four male Wistar rats, divided into three groups of 18 each, were further divided into three subgroups as Control, Sham operated control and experimental group (n=6 each). In the experimental group, Orexin B was administered bilaterally into the Nucleus accumbens in overnight fasted animals, at a dosage of 3nmol/µl (group 1) and 30nmol/µl (group 2) and their food intake and water intake were measured meticulously after 1 hour, 2 hours, 4hours, 6 hours,12 hours and 24 hours. Further to establish the role of Orexin B unequivocally, OX2R antagonist TCS-OX2-29 was infused into NAcc bilaterally in the third group and their feeding behaviour was assessed. Results: In low doses of Orexin B (3nm/µl) there was slight increase in food and water intake. But difference was not statistically significant, except increase in water intake at the end of first hour ($p<0.05$). The high dose of Orexin B (30nm/µl) increased food and water intake significantly at the end of 1, 2, 4, 6 hour, 12 hour and 24 hours, compared to control and sham operated rats. The infusion of the Orexin B antagonist (TCS-OX2-29) resulted in decreased food and water intake significantly at the end of 2, 4, 6, 12 hour and 24 hours.

Keywords: Food intake, Nucleus accumbens, Orexin B, Orexin antagonist, Water intake.

1. Introduction
Orexins are pair of neuropeptides which have long been implicated in the mammalian feeding behaviour due to its origin in the Lateral hypothalamic area (LHA) [1, 2]. Earlier reports also stated that two orexin receptor subtypes, named OX1R and OX2R, of which the former has high affinity for Orexin A and the latter has affinity for both Orexin A and B. [3, 4] Orexinergic neuropeptides were reportedly stimulated feeding. [1, 5] However potential role of orexin in feeding behaviour was evident from the earlier work where administration of Orexin A and Orexin B into the lateral ventricle produced feeding in rats. [6] Some of the research findings suggested that Orexin B modulate feeding behaviour only under nutritional duress. [7] Orexin A enhanced feeding following a 24-h fasting. [8] The observation of Orexin mRNA expression in lateral hypothalamus supports the role of orexin in ingestion. [1] The physiological relevance of the effects of orexin on feeding was further supported by the finding that, the central administration of anti-orexin antibody reduced food intake. [9, 10] Orexin A antagonist, SB-334867 has been shown to inhibit Orexin A driven feeding when given intraperitoneally. [11, 12] The two orexin receptors are widely distributed in the central nervous system, but are regionally selective and non-overlapping [13]. Specifically OX_2R mRNA was expressed abundantly in other parts of brain such as nucleus accumbens, cerebral cortex, subthalmic nucleus, paraventricular thalamic nuclei, anterior pretectal nucleus and raphe nucleus. [14, 15]Also moderate number of orexin fibres exists in the medial nucleus accumbens. [16] But the roles of Orexin B and OX_2 receptor in feeding are still to be established. [11] The nucleus accumbens is a basal forebrain structure situated deep in the grey matter. [17] Although it is evident that Nucleus accumbens is a candidate structure that played prime role in the control of food intake, the distinction between shell and core region of nucleus accumbens has been recognized much later and only recent studies have implicated that the shell sub region of nucleus accumbens (NAcc) had more influence in the mediation of feeding behavior [18, 19].

It is well known that neurons of Nucleus accumbens Shell (AcbSh) area project to Lateral hypothalamic area (LHA), which is considered to be the origin of Orexinergic cell bodies [20] Electrical and chemical stimulation of LHA neurons can induce robust feeding in satiated animals. [21] Therefore it was named feeding center. Due to the high level of expression of Orexin receptors 2 within the Nucleus accumbens (NAcc) [22] and the presence of orexinergic fibers and varicosities within the area, we hypothesized that orexin B may partly regulate feeding behaviour by signalling through this site. Furthermore, to establish a link between Orexin B in nucleus accumbens and OX_2R in feeding, we have taken up orexin antagonist in this study by infusing Orexin B specific antibody, TCS-OX2-29 to nucleus accumbens. The role orexinergic system in the modulation of feeding by the NAcc has not been studied so far and the reports appearing were sketchy. Therefore, in this study, we have investigated, whether orexin B /Orexin B antagonist affected feeding behaviour, by injecting them, specifically to the region of nucleus accumbens, following which the food consumption and water consumptions were monitored. Further, these tests were carried out during calorific challenge, by starving the animals for 24 hours before the experiments, in order to further prove the role of orexin B in these centres on ingestive behaviour compared to control animals.

2. Materials and Methods

Animals

Adult male albino rats of Wistar strain (200-275gms) which were inbred in the institutional animal house were used for the present study. Animals were housed individually in polypropylene cages (29cms x 22cms x 14cms) with paddy husk bedding under normal day-night cycle in temperature controlled room during the experimental period. Food pellets (Amrut laboratory animal feed, Amrut rat and mice pellet. Pranav Agro Industries Ltd, Maharashtra, India), potable tap water was made available to animals *ad. Lib. (Except as mentioned in the experimental requirement).* All experiments were conducted with strict adherence and principles contained in CPSEA guidelines. Institutional ethical committee approval was obtained before the commencement of the animal experiments (IIAEC/KMC/57/2009-2010).

Animals were divided into three groups. Group 1& Group 2 with 18 animals each were again subdivided into control group (n=6; untreated); Sham operated group (n=6; Saline infusion); Treated group. Group 1- served as low dose group (Orexin B treated – 3nmol/µl) and Group 2 served as high dose group - orexin (30 nmol/µl) treatment. 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 a cocktail of ketamine (60 mg/kg body weight) and xylazine (10 mg/kg weight) injected intraperitoneal route. Anaesthetized rats were fixed in the stereotaxic apparatus. For intracranial injection a guide cannula was implanted in place by stereotaxic method with 22 gauge stainless steel cannula. For the study of Nucleus accumbens, cannula was placed (AP= -1.6 mm behind the bregma; V=7.2mm from the skull; L = ±0.8mm from the midline). [23] Bilaterally implanted cannulae were 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 allowed for a week for securing the cannula and recovery from surgery without measuring any parameters. One lakh units of Penicillin was injected intra muscular route (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, convenient for handling. [24] Infusion cannula extends 1 mm beyond the respective guide cannula. Cannula placement was confirmed by post-mortem histological verification. Rats with misplaced cannula were to be 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 with 0.9% saline and solution **was** stored at 4 ºC. Infusion was made on each side with the help of infusion pump (Harvard Pico plus, Harvard apparatus) with 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. Infusion cannula (30G, PLASTIC ONE) was placed extending one mm beyond the tip of the guide cannula (22G) [25]. Neuropeptide Orexin B, in two doses (3 nmol/µl and 30 nmol/µl) was injected in respective groups of unanaesthetised, free moving animals at a flow rate of 0.6µl/min, which took place for over 90 seconds. The injector was left in position for an additional 30s to ensure complete extrusion from the tip and for diffusion. We performed the study by using low dose (3 nm/µl) and high dose (30nm/µl) of orexin B in separate groups. In the control group no procedure was done, whereas the Sham operated controls underwent surgical procedure, but infused with normal saline. The dose of orexin B was selected based on the previous work. [1, 5] Orexin B antagonist, TCS-OX2-29 (Catalog.No.3370; Tocris Bioscience, UK) was infused at a dose of 10 micrograms/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 infusion. Food intake was measured at different time points after drug administration i.e. at the end of 1, 2, 4, 6, 12 and 24 hrs. Food consumed was weighed manually on a digital weighing machine by providing premeasured amount of pellets and weighing the left over including the weight of food spillage (intake is calculated). 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 average of the four readings was taken. Values are expressed in grams of food consumed per 100 g of body weight. At least 72 hours elapsed between consecutive treatment days. All activities in the animal house were carried out between 0900 and 1100 across the groups except for the days of infusion trials, where the rats were monitored the whole day.

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. [26] The cannula was removed carefully, then 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 and were stained with 0.1% Cresyl violet stain. These sections were examined under dissection microscope for verification of implanted guide cannula and the site of infusion.

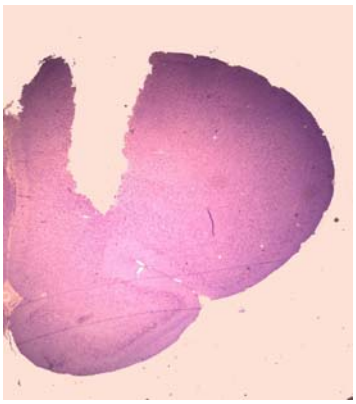

Fig 1: Photomicrograph of the brain slice stained with Cresyl violet showing the site of insertion of guide cannula (Magnification 1.5x).

Statistical Analysis

Food and water intake were expressed as means± S.E.M. The data were not normally distributed, analyzed 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 operated) and orexin B/Orexin B antagonist infused group, considering each hour readings separately (e.g. 1hour Control and Sham control Vs 1hr Orexin B group).

Results

Infusion of Orexin B at low dose (3 nmol/µl; Fig 1a & 1b) marginally increased food intake (Not significant) and water intake ($p<0.05$) in the first hour post infusion. Consumption for the whole day too was increased (NS) compared to the Operated controls and unoperated control animals. Orexin B at dose of 30 nmol/µl produced significant increase in food as well as water intake (Fig 2a & 2b; $p<0.05$ in the hour immediately after the infusion and $p<0.01$ intake per day). In the high dose group, significantly increased food and water intake was observed at the end of 1, 2, 4, 12 and 24 hours (Fig 2a&2b).The infusion of Orexin B antagonist(TCS-OX2-29) resulted in decreased food and water intake significantly at the end of 2,4,6,12 and 24 hours.(Fig 3a&3b)

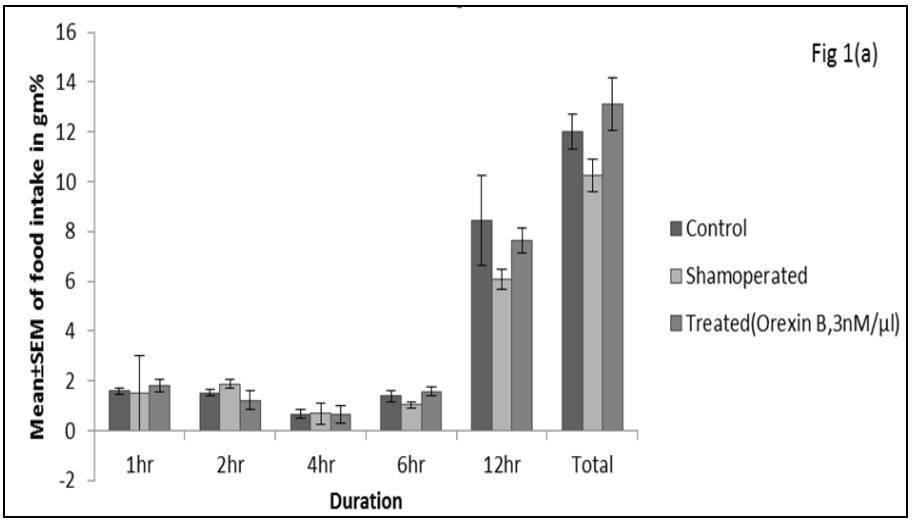

Fig 1(a): Effect of Orexin B (3nanomoles/µl) on food intake at different time periods following infusion into Nucleus accumbens: 1, 2, 4, 6, 12 hours and cumulative food intake (24hrs) (n=6 rats /group) [*p<0.05Experimental vs control/sham operated group]

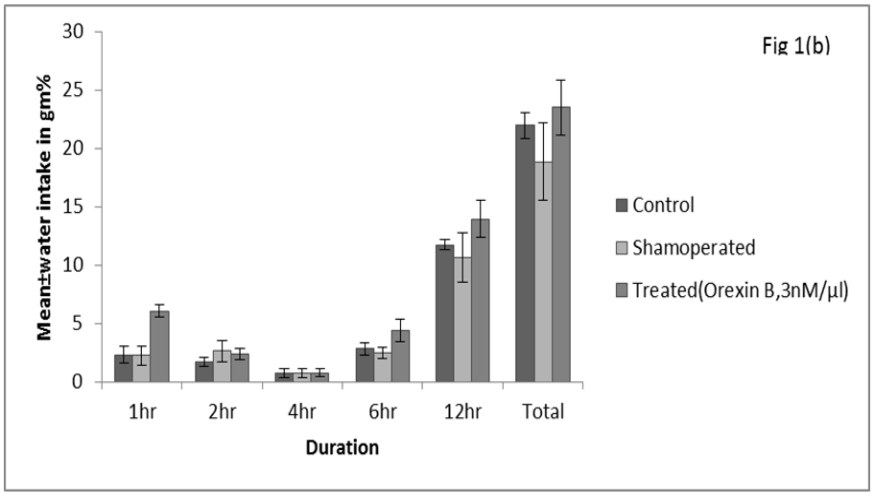

Fig 1(b): Effect of Orexin B (3nanomoles/µl) on water intake at different time periods following infusion into Nucleus accumbens: 1, 2, 4, 6, 12 hours and cumulative food intake (24hrs) (n=6 rats /group) [*p<0.05, Experimental vs control & sham operated group]

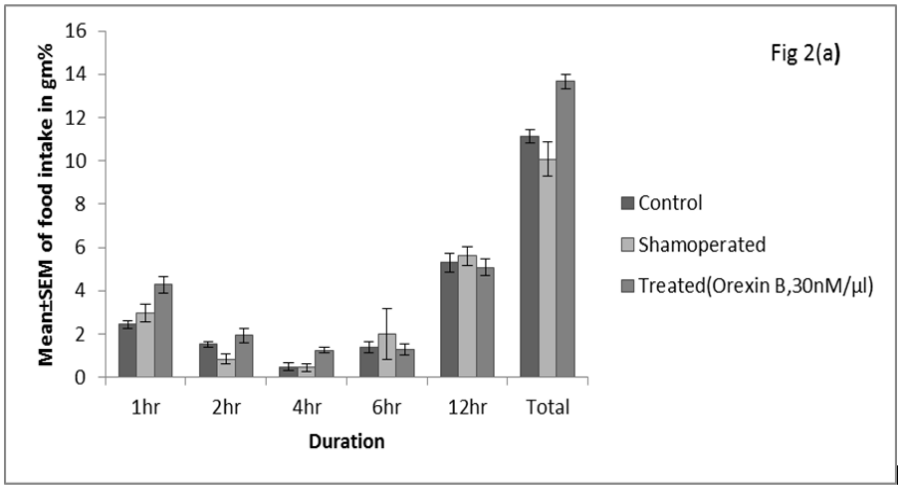

Fig 2(a): Effect of Orexin B (30nanomoles/µl) on food intake at different time periods following infusion into Nucleus accumbens: 1, 2, 4, 6, 12 hours and cumulative food intake (24 hrs) (n=6 rats/group).[*p<0.05,**p<0.01,Experimental vs control & sham operated group]

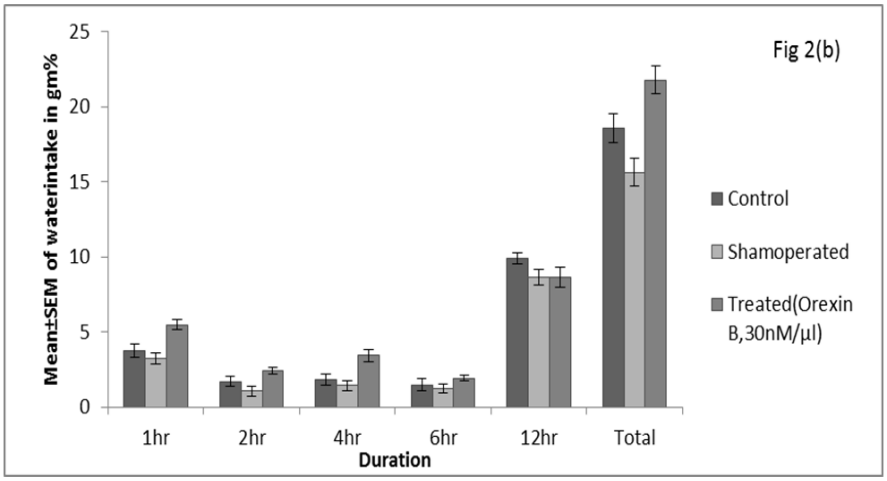

Fig 2(b): Effect of Orexin B (30nanomoles/µl) on water intake at different time periods following infusion into Nucleus accumbens: 1, 2, 4, 6, 12hrs and cumulative food intake (24hrs) (n=6 rats/group).[*p<0.05,**p<0.01, Experimental vs control/sham operated group]

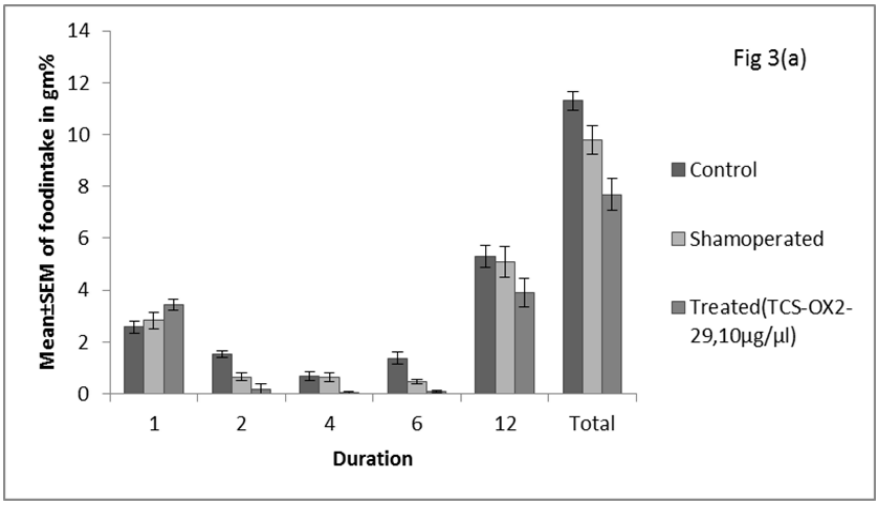

Fig 3(a): Effect of Orexin B antagonist, TCS-OX2-29(10 µg/µl) on food intake at different time periods following infusion into Nucleus accumbens: 1, 2, 4, 6, 12 hrs and cumulative food intake (24hrs) (n=6 rats /group) [*p<0.05, **p<0.01, ***p<0.0001 Experimental vs control/sham operated group]

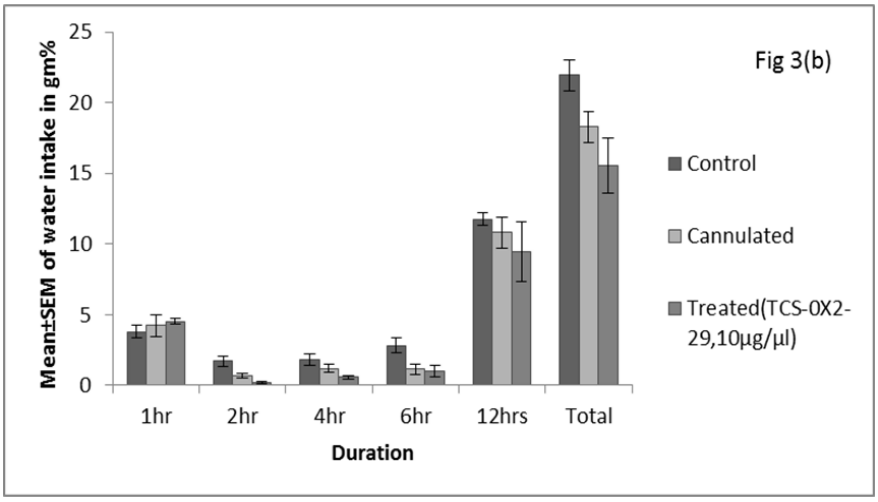

Fig 3(b): Effect of Orexin B antagonist, TCS-OX2-29(10 µg/µl) on water intake at different time periods following infusion into Nucleus accumbens: 1, 2, 4, 6, 12 hrs and cumulative food intake (24 hrs) (n=6 rats /group) [*p<0.05, **p<0.01Experimental vs control/sham operated group]

Discussion

Data obtained in this series of experiments provide sound evidence of a functional relationship between Nucleus accumbens and orexinergic link in the lateral hypothalamus with reference to the control of appetitive behaviour. Although direct reciprocal connections between the nucleus accumbens and lateral hypothalamus have been described previously [27], until now there has been little information about the behavioural correlates of this connection. Previous work from the different laboratories demonstrated that the Nucleus accumbens had a specialized role in modulating feeding behaviour [28-30].

In the present experiments, injection of orexin B into Nucleus accumbens enhanced consummatory behaviour in Wistar rats. Earlier studies also reported increased feeding following micro infusion of Orexin A into nucleus accumbensc [31]. Now we have shown that Orexin B infusion also produced an increase food intake, which is evident at a higher dosage. The increase in food intake was observed in previous studies where Orexin B was administered to Para ventricular nucleus of hypothalamus [5]. Earlier studies have also proved that intracerebroventricular injection of orexin B specifically stimulated food intake in rats [5, 32]. Further to investigate if orexin receptor OX2R was involved, we carried out orexin B receptor specific antagonist infusion of TCS-OX2-29 into NAcc. TCS-OX2-29 significantly decreased food intake and water intake following the infusion. Yamada et al (2000) have demonstrated decreased food intake following central administration of orexin antibodies [9]. The stimulation of drinking behavior in response to orexin B was well documented in rodents [33, 34]. These observations suggest a physiological role for orexin as mediators that regulate drinking behaviour. The orexin signalling may be important in modulating the feeding network under times of nutritional duress [35]. Further Orexin m RNA expression was shown to increase during fasting. [1]

The physiological relevance of the effects of orexin on feeding is supported by our research finding that the administration of OX2-R selective antagonist, TCS-OX2-29 into the nucleus accumbens reduced food intake. This may

suggest the role of OX2 in mediating feeding behavior. According to Yamada et al, OX2R mediate pathways which might be important for energy homeostasis [9, 36] Yet complex behaviour like feeding may depend upon the stimulation of both orexin receptors.

Therefore our study confirms that Orexin B may have a role in mediating the ingestive behaviour. The interconnections of Lateral hypothalamus and nucleus accumbens may be involved in this activity and Orexin B could be a candidate neuro-hormone, which might bring about regulatory aspects of ingestive behaviour. By demonstrating that Orexin B at high dose increased food intake and OX2 Receptor antagonist decreased the same, we hereby confirm a role for Orexinergic system in subcortical control of complex feeding behaviour. However, further studies are required for proving Orexinergic ramifications among these centres and their specific role in other aspects such as food preference and addiction, which also is the domain of nucleus accumbens.

Conclusion

This result suggests that infusion of Orexin B into NAcc bilaterally, led to increase in food and water intake in Wistar rats, which is confirmed by the opposite results obtained from the TCS-OX-29, Orexin B receptor antagonist infusion.

Acknowledgement

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