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Original Article

Effect of Orexin-A infusion in to the Nucleus Accumbens on consummatory behaviour and alcohol preference in male Wistar rats

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Abstract

Background: Effect of administration of Orexin-A into nucleus accumbens (NAcc) in relation to the regulation of feeding behavior and alcohol consumption at specific time intervals is relatively unknown.

Materials and methods: In this study, Male Wistar albino rats (n=54) weighing about 250±10 grams were implanted bilaterally with guide cannula (22 gauze) to target NAcc by stereotaxic surgery. Saline (0.9%) for control and Orexin-A for experimental groups (100 pmol or 250 pmol) were infused by Harvard picoplus pump. Food, water and alcohol (10%) consumption were measured at 1, 2, 4 and 24 hours to evaluate the effect of Orexin-A in fasted rats (24 hours). Preference study was carried out by two bottle choice test.

Results: Orexin-A infusion into NAcc showed significant increase in food at 1 hr in all groups compared to controls ($p<0.05$) and alcohol ($p<0.02$) intake. The changes were dose dependent. There was no noticeable preference or alcohol.

Conclusions for: These findings showed that Orexin-A in NAcc could be involved in feeding and drinking but not alcohol preference. The results highlight the effect of Orexin A infusion into NAcc in consummatory behaviour besides other hypothalamic and mesolimbic centres.

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Introduction

The nucleus accumbens (NAcc) has been considered an important mesolimbic structure involved in functions such as reward, reinforcement and motivation (1). When rats were challenged with

addictive drugs such as cocaine, heroin, nicotine, or alcohol levels of dopamine was shown to be increased in NAcc region by micro dialysis technique the extracellular (2). Alcohol is most widely used and abused substance in the world (WHO). Nucleus accumbens has also been shown to have a regulatory role in sweet taste preference and nutritional value among the Wistar rats and also that this function has been vested more in the Shell rather than core of the NAcc (3,4), Food and water intake have also been shown to be affected by other subcortical centres (5, 6).

Orexins A and B, (also named as Hypocretin I & II) are peptide hormones isolated from the rat hypothalamus (7). They are largely found in the regions of brain such as hypothalamus and locus ceruleus (7, 8) and in the spinal cord (9, 10). Intracerebroventricular injection of Orexin-A (hcrt-1) and B (hcrt-2) stimulate food intake and in fasted animals, Orexin mRNA has been shown to accumulate in lateral hypothalamus (7, 11, 12). Further, Kunii et al. reported that central administration of both Orexins, A or B stimulated water intake in a dose-dependent manner, Orexin-A is significantly more potent then Orexin B (13).

A role for Orexins also have been suggested in alcohol preference and consumption. Richards et al. (14) demonstrated that the Ox-R1 antagonist (SB-334867) reduced operant self-administration of ethanol in Long-Evans rats and prevented the cue-induced reinstatement of ethanol-seeking in alcohol preferring rats, which was ratified by other workers too (14, 15).

Orexins appeared to be involved in drug-seeking behaviour (16) and exploration, Poteitated reward which in turn activated orexin neurons of the lateral hypothalamus (17). Lateral hypothalamus and PF N-methyl-d-aspartate (NMDA) or alpha-amino-5-methyl-3-hydroxy-4-isoxazole propionic acid (AMPA) has stimulatory effect on ethyl alcohol consumption mediated by increased orexin (18). Orexin mRNA expression was markedly increased in lateral hypothalamus of alcohol preferring rats (13). But Orexin also was shown to affect several other behavioural activities in animals, such as rearing, grooming etc. after administration of orexin-A in to NACC exhibited exploratory behaviours (19). Orexin A induced firing in locus ceruleusneurons, highlights a potential involvement of the peptide in maintaining arousal state, cells in this region fire maximally during arousal from sleep (20).

Hypocretin-immunoreactive dense fibers were shown to be present in various rat brain regions including nucleus accumbens shell However, others have argued that the Orexin-containing neurons were restricted to sub-region of the hypothalamus, 50% of the Orexin cells were located in the perifornical nucleus at tuberal level (21). The orexin input to the ventral tegmental area (VTA) play a role in cocaine-induced synaptic plasticity and associated compulsive drug-seeking behaviours (22). Intra Ventral Tegmental Area application of orexin increased Fos expression in DA neurons specifically in the caudomedial portion of the VTA (23). VTA orexinergic neurons may be involved in the dopamine induced reward mechanism of Morphine which includes Nucleus accumbens (24) and the medial prefrontal cortex (25).

Further evidence suggested that Orexin administration also significantly increased food consumption, wakefulness, and locomotor activity in other rodent models (19, 26). The evidence available so far which suggested that the role of Orexins in feeding behaviour and in addiction were equivocal and sketchy. We therefore investigated this specific hypothesis that Orexin A may have a modulatory role in ingestive behaviour through nucleus accumbens and since NAcc neurons were also involved in addictive behaviour, this neurochemical could be candidate modulator in this addictive behaviour. In the present study using male Wistar albino rats, we examined for the first time, whether Orexin A could be involved in the role of NAcc on ingestive activity by micro infusion of Orexin to NAcc, bilaterally, through implanted cannula, though several previous studies have been done by infusion into other areas and into cerebroventricles. The present study evaluated feeding and water intake immediately after the infusion in 24 hours fasted animals. We studied ethanol intake and alcohol preference study by two bottle choice test in the cannulated rats

following Orexin A infusion to assess its possible role of Orexin A in addiction. From the results, this study demonstrated for the first time that intra NAcc injection of Orexin A led to increased activity in the ingestive behaviour but may not have a role in alcohol addiction and preference.

Materials and Methods

Animals

Male Wistar albino rats weighing about 250±10 g, 90-120 days old were selected for this study. Fifty four rats were divided into three groups viz. Water group, Alcohol group and two bottle free choice group (n=18 each). They were further subdivided into three subgroups, viz. Group 1 - Control (Saline infusion); Group 2 - Low dose of orexin (100 picomol); Group 3 -High dose (250 picomol) (n= 6 each). Food was provided to all groups *ad lib.*, except where mentioned as overnight fasting.

They were housed in clean polypropylene cages individually and maintained at 27.0±2ºC with normal light and dark cycle.

The experimental protocol was approved by the Institutional Ethical Committee for animal experiments and the study was conducted in compliance with the prescribed guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA) and guidelines of Government of India for the use of laboratory animals.

Drugs and apparatus

Orexin-A (Sigma Chemicals, USA) was dissolved in 0.9% saline before first use and when not in use, solution was stored at 4°C for a maximum of three weeks (11). Harvard Pico plus (USA) infusion pump was used to deliver drug. Tap water was provided in Plastic drinking bottles and rat food pellets (Hindustan Uniliver Ltd.) were provided. Ethyl alcohol (Absolute) was procured (Hayman Ltd. Eastways Park, Witham, Essex, CM83YE, UK) and diluted to make a 10% alcohol (This concentration was selected on the basis of a pilot study on the preference of alcohol concentration). Ketamine (NEON Laboratories limited, Thane, M.S.) and Xylazine (Indian immunological ltd. Hyderabad) were used for anaesthesia.

Surgical procedure

Male Wistar albino rats were anaesthetized by injecting a mixture of ketamine hydrochloride (60 mg/kg), Xylazine hydrochloride (6 mg/kg) and mounted on a stereotaxic apparatus (Inco, India). An incision was made on the shaved scalp, thoroughly disinfected with surgical spirit. The area is cleaned up with hydrogen peroxide. Points were marked on the skull in corresponding areas to reach NAcc shell, taking Bregma and reference point, as per Rat brain atlas of Paxinos and Watson (27). (NAcc: From Bregma–Anteroposterior (AP)=+1.7 mm, Lateral (L) = ±1 mm and Vertical (V) = 7.4 mm). Burr hole was made; Stainless steel sterile guide cannula (22 gauge) was placed, secured with screw and dental acrylic. Stainless dental needle (Septojet Sterile of 30 gauge) which has hub, making it convenient for handling was implanted bilaterally. Guide cannula fitted rat with dummy stylet, to prevent blockage of bore, was allowed to recover for at least 7 days prior to experimentation (28). One lakh units of Penicillin was injected to prevent infection. Prior to the experiments all rats received two training sessions in which they were maintained on 24 hour fasting and then received food pellets, water or alcohol (10%) or both as the case may be.

Experimental procedure

Fasted (24 hours) animals were infused with 0.9% salineor Orexin-A, by using 10 microliter Hamilton syringe connected with polyethylene tube and infusion cannula. This syringe was fitted into the Harvard Pico Plus pump. The stylet in the guide cannula was removed and infusion cannula was inserted. Then the pump delivered 1 microlitre of solution containing 100 picomoles of Orexin A solution into the right and left side of NACCin 1 to 2 minutes (Group 2). In the third group 250 picomoles of Orexin A was delivered (Group 3). In the controls, normal saline was infused (Group 1). At the end of infusion,

the internal cannula was removed and stylet was placed back in position. The experimental procedure was carried out at 9.00 AM followed by the measurements.

Measurements

Pre measured food, water and 10% alcohol were provided in respective groups, and the consumption was meticulously measured. Time was noted. Left over pellets, water and alcohol were removed, weighed at predetermined time intervals of 1 hour, 2 hour, 4 hour and 24 hours and the amount consumed was calculated in respective groups. (Amount consumed= Premeasured quantity supplied-left over quantity). At the end of the study period, the animals were sacrificed by lethal dose of ether and transcardially transfused with formol saline, the brains were dissected out, labelled brains were embedded in wax. 5 micron sections were cut and cresyl violet staining revealed the site of cannula implantation (Figs. 2, 3).

Statistical analysis

Data of consumption (mean±SEM) was analysed by using the statistical software SPSS version 16. One way ANOVA was done to compare the consummatory behaviour in the different groups. Intergroup comparison was done by post hoc Tukey's test for

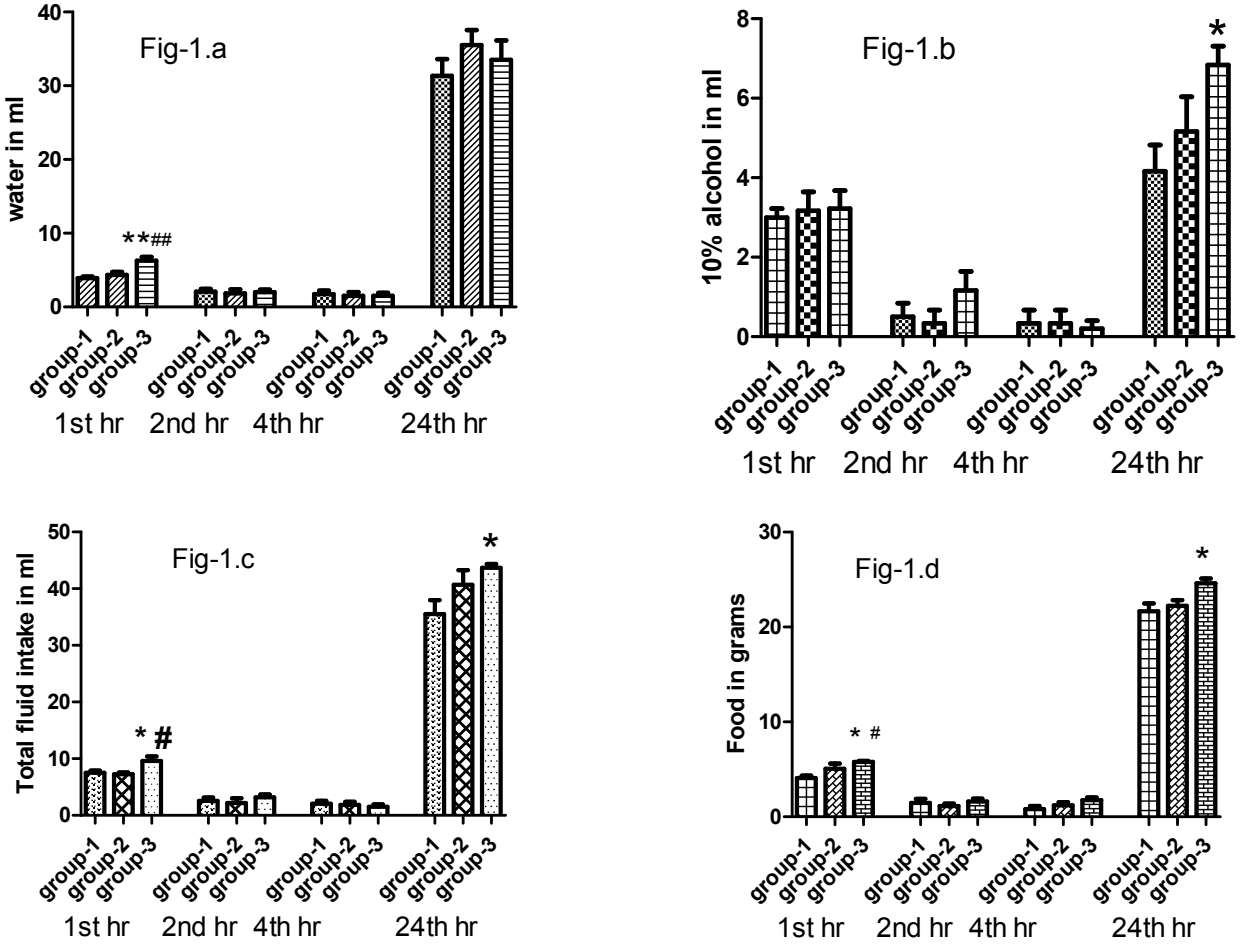

Fig. 1: Orexin A into Nucleus accumbens- Two bottle choice test. Bars represent alcohol, water or food intake of rats, at 1, 2, 4 hours and 24 hour time period with dose of 0 (0.9% saline = group 1) and 100picomol (group 2) or 250 picomol (group 3), (*) indicates significant difference between group 1 Vs group 2 or group 3, (#) indicates, significant difference between group 2 Vs group 3. 1st hour water intake: group-1 Vs group-3, **p<0.002, group-2 Vs group-3, ##p<0.008 (a) 24 hour alcohol intake: group-1 Vs group-3, *p<0.038 (b) 1st hour total fluid intake (water + alcohol): group-1 Vs group-3, *p<0.039, group-2 Vs group-3, #p<0.021, 24 hour total fluid intake (water + alcohol): group 1 Vs group 3 *p<0.038(c). 1st hour food intake: group-1 Vs group-3, *p<0.01, 24 hour food intake: group-1 Vs group-3, *p<0.016, group-2 Vs group-3, ##p<0.05 (d).

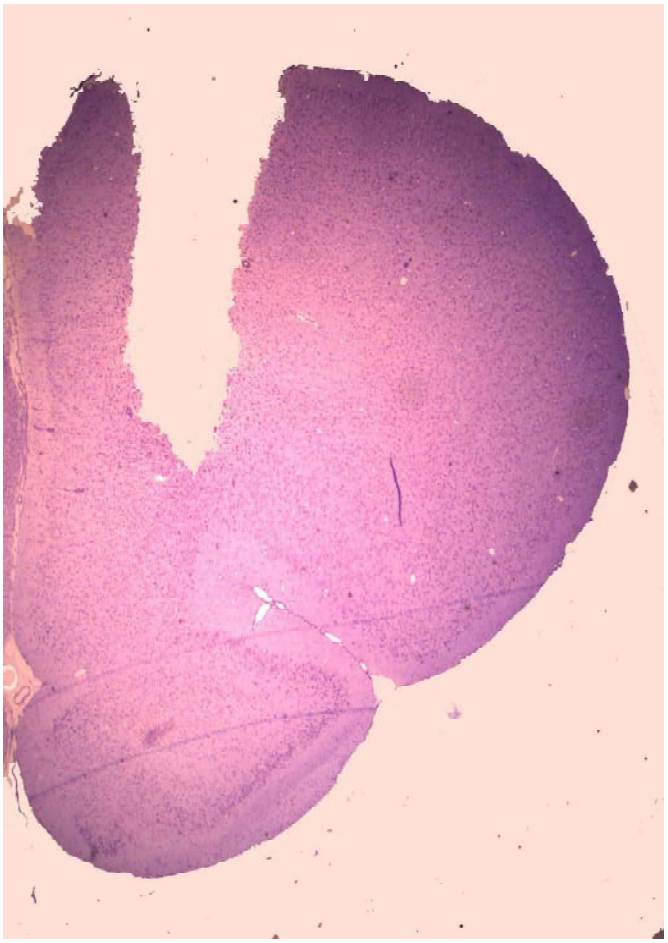

Fig. 2: Histological section (7 micron) showing the site of cannula insertion.

the respective periods of post infusion durations. P<0.05 was considered significant.

Results

Experiment I

In this group (n=18) of animals, Orexin A was infused at dosage of 100 picomol (group 2) and 250 picomol Group 3) into NACC bilaterally. Food and water consumption were measured (n=18, Table I). ANOVA revealed significant increase in food intake following Orexin-A treatment in first hour compared to control group [$F(2, 15) = 3.370$ $p<0.05$]. Post hoc Tukey's analysis showed significant increase in food intake between groups 1 and 3 ($p<0.048$). No significant changes were observed at 2 hr [$F(2, 15) = 1.161$ $p= 0.340$], 4 hr, [$F(2, 15) = 1.033$ $p = 0.380$] and 24 hr [$F(2, 15) = 1.531$ $p = 0.248$] post infusion time intervals. When water intake was compared among control and Orexin-A treated, it showed increase in first hour (Group 1 Vs Group 3 and 2 Vs 3 Tukey's post hoc ($p<0.023$, $p<0.018$)]. There was no significant difference in 2 hour [$F(2, 15) = 0.338$ $p= 0.719$]; 4 hour [$F(2, 15) = 0.379$, $p = 0.691$] readings. Increase in total water intake [$F(2, 15) = 3.971$ $p=0.041$] between group 1 Vs 3 ($p<0.05$) post infusion time intervals was also observed.

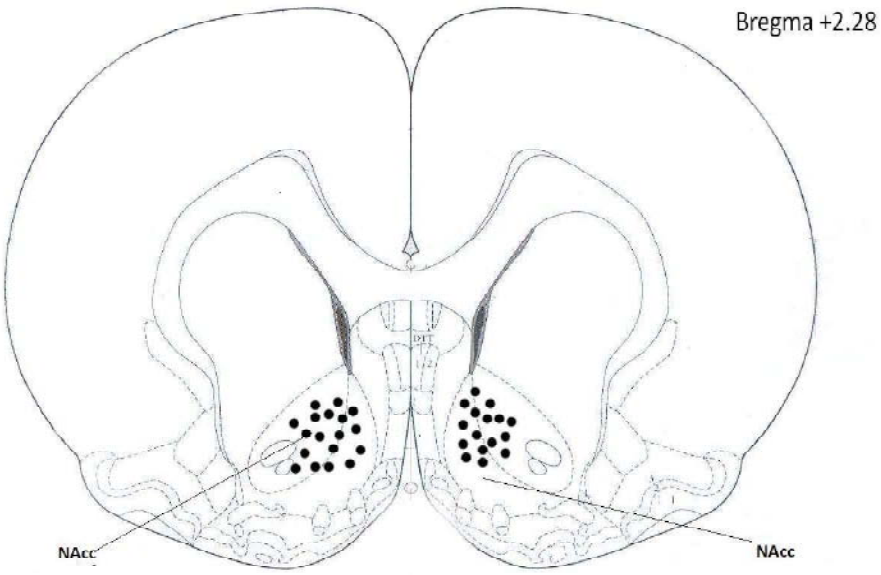

Fig. 3: Diagrammatic representation of site of infusion of Orexin A–Nucleus Accumbens-NAcc.

Experiment II

Alcohol (10%) consumption and food intake were measured (n=18, Table II) in fasted animals. Alcohol (10%) intake showed significantly increased consumption at 1 hour and 2 hour [F(2,16) = 12.988 p<0.000], in high dose Orexin-A treated group (Group 3) compared to control including the consumption for 24 hours [Among the groups p┐ 0.02; group 1 Vs 3 (p<0.039)]. No significant changes were observed at 4 hours consumption. Food intake in Orexin-A treatment group showed significant increase compared to control group at 1 hr [F(2, 16) = 3.690 p=0.048]. Increased food intake was observed between groups 1 and 3 [Tukey's post hoc (p<0.048)]. There were no significant changes in food intake during 2 hr [F(2, 16) = 1.827 p=0.193]; 4 hr [F(2, 16) = 0.546 p = 0.589] and 24 hr [F(2, 16) 0.399 p= 0.677] post infusion time intervals.

Experiment III

Alcohol (10%) and water intake, in two bottle free choice and food intake were measured analysed in this experiment (Fig. 1). In the group 2, where 100 picomol of Orexin A was infused, the data showed slight variation, but it was not statistically significant. In the high dose (250 picomol) infusion group, Water intake was increased in the post infusion period in the first hour after Orexin-A treatment [Group-1 Vs Group-3, p<0.002, Group-2 Vs Group-3, p<0.008]. No significant effect at 2 hour, 4 hour or 24 hour among the groups. In the same animals, alcohol (10%), provided in another bottle was measured and did not show any significant changes in the consumption in 1 hour, 2 hour, 4 hours. However, the consumption was increased for the whole day (p<0.05). Measurement of total fluid intake following Orexin-A treatment was found to be increased in 1 hour [Group-1 Vs Group-3, p<0.039, Group-2 Vs Group-3, p<0.021]. No significant changes were seen at 2 hour, 4 hour but total fluid intake was more in 24 hour infused groups 1 Vs group 3 (p<0.038). Evaluation of food intake Orexin-A (high dose) treatment significantly increased the food intake in

TABLE I: Food (g) and water intake (ml) following Orexin A infusion at 1 hour, 2 hours, 4 hours and 24 hour time period.

	Food intake (grams)				*Water intake (ml)*			
	1 h	*2 h*	*4 h*	*24 h*	*1 h*	*2 h*	*4 h*	*24 h*
Group-1 (0.9% saline)	4.56±0.24	1.48±0.37	1.03±0.29	22.65±0.85	4.25±0.54	3.25±0.89	2.16±0.64	33.33±2.04
Group-2	5.12±0.61	2.02±0.30	1.85±0.62	22.53±0.94	4.08±0.97	2.41±0.74	2.75±0.66	34.08±2.86
Group-3	6.18±0.40*	2.24±0.40	1.68±0.25	24.35±0.62	7.77±0.90*#	2.61±0.57	2.77±0.29	42.11±2.34*
ANOVA significance (p value)	0.05	0.340	0.380	0.248	0.01	0.719	0.691	0.041

Analysis by ANOVA, post hoc Tukey's HSD, N=18 (mean values±S.E.M; * = group 1 Vs group 2 or group 3, # = group 2 Vs group 3)
1st hour food: Group-1 Vs Group-3 *p<0.048.
1st hour water: Group-1 Vs Group-3 *p<0.023, Group-2 Vs Group-3 #p<0.018.
24 hour water: Group-1 Vs Group-3 *p<0.05.

TABLE II: Effects of orexin-A on food and 10% alcohol intake at 1, 2, 4 and 24 hr time period.

	Food intake (grams)				*10% Alcohol intake (ml)*			
	1 h	*2 h*	*4 h*	*24 h*	*1 h*	*2 h*	*4 h*	*24 h*
Group-1 (0.9% saline)	4.95±0.58	0.48±0.24	1.18±0.59	15.68±0.79	5.08±0.27	0.66±0.21	1.16±0.47	25.17±0.40
Group-2	5.41±0.49	1.06±0.19	0.61±0.26	14.96±0.38	4.25±0.34	1.54±0.14*	2.15±0.43	25.42±0.32
Group-3	6.50±0.11*	0.97±0.23	0.68±0.33	16.00±1.07	6.35±0.28* ###	1.71±0.26**	1.50±0.24	27.78±0.98*
ANOVA significance (p value)	0.048	0.193	0.589	0.677	0.000	0.009	0.218	0.027

Data are expressed as mean values±S.E.M. ANOVA significance (Tukey HSD, n=18)
1st food intake: Group-1 Vs Group-3, *p<0.046.
1st hour alcohol intake: Group-1 Vs Group-3, *p<0.02, Group-2 Vs Group-3, ###p<0.000.
2nd hour alcohol intake: Group-1 Vs Group-2, Group-3 *p<0.036, **p<0.01.
24 hour alcohol intake: Group-1 Vs Group-3 *p<0.039.

the first hour and that for 24 hour ($p<0.01$) compared to control and low dose infusion [Group-1 vs Group-3 $p<0.010$]. But there was no significant difference in any time period.

Discussion

From the results obtained after bilateral Orexin A infusion into NACC, we found that there was increase in food and water consumption in rats. Studies carried out by injecting Orexin into cerebral ventricles have earlier indicated that this peptide could be a candidate for the modulation of feeding behaviour (29). In the present study, intra nuclear injection of Orexin-A (OX-A) into NAcc on food intake, Water intake and alcohol intake we found a distinct increase in consumption among the experimental rats, in the first few hours, suggesting a role for Orexin A. We tested the parameters in two doses, viz. low dose of 100 picomol and high dose of 250 picomol. The effect was lower in low dose infusion and more evident in the higher dose. The feeding responses lasted only for one to two hours after single injection. We chose to study the food intake and other measurements in fasting animals, which were not provided with food or water for 24 hours before the infusion day. The infusion was started at 9.00 AM and the readings were noted by measurements, which were done at regular intervals as mentioned in the methodology. In our experiments, intra nuclear infusion produced statistically significant increase in food and water consumption in first hour and second hour. These findings were in agreement with the findings of Thorpe et al (2005) in Sprague-Dawley rats (29). Evidence exists for Orexinergic neuronal bodies found in Lateral Hypothalamus (LH) (29, 30) and perifornical nucleus (PFH) contains 50% of the orexinergic neurons, which have involvement in the neural control of food intake (31). These axons were distributed into several centres including NAcc (21). Therefore our finding could prove that at least partly, Orexin A may have a modulatory role in water and food intake, when infused into NAcc. Our results are in agreement with previous studies on other rodent models, which also reported increased food and water intake following Orexin A infusion (19). Moreover, orexin release also may have a diurnal rhythm since it has a role in sleep, which is closely connected to Suprachiasmatic nucleus (32).

However, NAcc was earlier demonstrated to have role in addictive behaviour (15). Intra peritoneal administration of orexin1 receptor antagonist have been demonstrated to attenuate alcohol seeking behaviour. In order to test whether, orexin A was involved in this activity, we tested the alcohol consumption following orexin infusion into NAcc by providing the rats with 10% alcohol. Though the rats showed increased alcohol intake after the Orexin A treatment, it did not show a higher intake when the results were compared to water intake. To further confirm the specific preference for alcohol we used a Two bottle free choice test, (6) a choice of alcohol and water for the rats. Two bottle choice test revealed an increase in total fluid intake in treated animals, but there was no particular preference for alcohol.

Limitations of this study are first, the one microliter fluid infused may spread to a little more area that we may desire, however, the NAcc is quite a large area, therefore we presume that the mixture was concentrated mostly in this zone. Secondly, infusion for longer duration by prolonging the delivery period may reveal more information. The Orexins were known to be involved in the sleep and activity, therefore our study conducted only in the mornings may not reflect the effects of diurnal changes in the natural endogenous orexin.

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