Differential effects of infusion of Orexin A and Orexin B into basolateral amygdala on feeding behaviour in Albino rats

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Abstract

Background: Orexin A and B are two peptides with 33 amino acid and 28 amino acids discovered in the hypothalamus. These were believed to have a role in the regulation of ingestive behaviour. In the present study, we infused orexin A and B and their specific receptor antagonists (Orexin A - SB334867, Orexin B - TCS-OX2-29) into the basolateral amygdala (BLA) and measured the food intake and water intake in the following time durations. Materials and methods: Wistar albino male rats (n=48) were grouped into Orexin A/Orexin B infusion. Further, subgroups were done to infuse different dosages. Results: The food intake and water intake did not show any significant alteration in the Orexin injected animals in both dosages. However, there was a small increase in the food and water consumption in the Orexin A antagonist infused group. There was significantly increased food intake and water intake following the Orexin B infusion, which appeared to be dose dependent. Conversely, treatment with the Orexin B antagonist, decreased the parameters. Discussion: The Orexin A does not appear to be involved in the modulation of food and water intake by BLA, though there was a small variation following the antagonist infusion, which lasted only for the first hour after infusion. Orexin B, showed a dose dependent increase in the parameters, which was reversed on the infusion of its antagonist. Therefore, it can be concluded that the orexinergic inputs to the BLA are from the hypothalamic centers. Further studies are needed to analyze the interaction between amygdala and hypothalamus, and involvement of orexinergic system in the mechanism.

Key Words: Orexin A, Orexin B, Orexin antagonist, food intake, water intake, Infusion

Introduction

Orexins were discovered in the late 90s from hypothalamic regions and were believed to be actively involved in the regulation of food intake due to the location of their occurrence. There are two types identified namely; Orexin A and Orexin B, with 33 aa and 28 aa, respectively -derived from the same precursor molecule with 130 aa. Orexin secreting neurons have been located in lateral hypothalamus and distributed widely.

There has been evidence to suggest that Orexin interacted with the Leptin and other agents concerned with the preference to sucrose solution. Recently, Terrill S J et al. have demonstrated that orexinergic inputs to the VTA neurons were involved in the consumption of palatable and hedonic food and fluid.

Medial prefrontal cortex receives orexinergic inputs from the lateral hypothalamus with a rostrocaudal gradient. These connections may be important for the role of orexinergic system in cognitive aspects. Orexins may augment the role of VTA dopaminergic precursors.

How to cite this article: B Ganaraja, K S Rashmi, Mayannavar S. Differential effects of infusion of Orexin A and Orexin B into basolateral amygdala on feeding behaviour in Albino rats. M/JMS. 2017; 2(1): 17-24.
action of medial prefrontal cortex, affecting stimulus – reward system. This observation was also proposed by other workers. Orexin receptors have been identified in many parts of brain. It extends from anterior forebrain regions to ventral tegmental area. Plenty of orexin receptors have been located in nucleus accumbens also. Orexins have been implicated in reward process, pain sensation. The antinoceceptor actions of the Orexin has been reported by Yazdi F et al., The antinociceptor actions of the Orexin has been reported by Yazdi F et al., (2016). Orexins have been implicated in reward process, pain sensation. The antinociceptor actions of the Orexin has been reported by Yazdi F et al., (2016). Orexins have been implicated in reward process, pain sensation. The antinociceptor actions of the Orexin has been reported by Yazdi F et al., (2016). The autonomic control of the cardiovascular functions were also reported to be influenced by them. The amygdaloid nucleus also received significant inputs from the orexinergic system and the orexin infusion. Considering the distribution of orexins and their receptors the diversity of their actions could be very vast. An orexinergic system has been implicated in sleep, wakefulness, and alertness. The orexins are believed to be arousal stabilizing agents and cause wakefulness, and found to be high in the awake state and it heightened the activity levels. The narcoleptic patients had very little of Orexins in their CSF, suggesting that normal wakefulness needs orexins. Extensive research on these molecules were carried out regarding their role on ingestive activities. Nucleus accumbens, Dopamine levels and leptin action involve Orexinergic neurons in the lateral hypothalamus, which are concerned with ingestive activities. Further, in Orexin knockout mice, the intake of sucrose, which is rewarding taste has been found to be reduced. An interaction of Galanin, leptin and Orexinergic neurons have been suggested to fine control of ingestive activities in Nucleus accumbens dopamine and the orexinergic neurons in lateral hypothalamus. The orexinergic system was also implicated in the neuropeptide Y mediated anorexic effect in chicks. Studies have revealed changes in ingestive behaviour following intra cerebroventricular infusion of orexin in zebra fish, which resulted in increased food intake. It was reported that orexin was involved in the regulation of ingestion of rewarding sweetened solutions by intra-cerebroventricular injection. The results are a mixed bag of information. In an attempt to conclusively understand the role of two orexins on their actions in basolateral amygdala (BLA) in controlling ingestive behaviour, we devised experiments in which we carried out the infusion of Orexin A and B separately, into BLA bilaterally in the rats. Subsequently, we infused the specific antagonists into the same nucleus and recorded the parameters to obtain confirmatory evidence for the observations. Following infusion, we measured the changes in the food and water intake sequentially, and the results are discussed here.

**Materials and methods**

Wistar albino male rats (n=36), weighing about 200±20 g at the time of selection were procured from the central animal house and housed individually in Plexiglas cages with ad-lib food and water, in the standard animal house. All procedures and animal care was done according the highest ethical standards. Before beginning the experiments, clearance was obtained from institutional ethical committee for animal experiments (Institutional Animal Ethics Committee letter dated: No: IAEC/KMC/57/2009-10; dated 10/9/2009). Cannula were implanted in the nucleus accumbens bilaterally, by method developed in our laboratory explained in our previous paper. They were anaesthetized by injecting a cocktail of Ketamine (60 mg) and xylazine (6 mg) per kg body weight. Stereotoxic coordinates were selected using Paxinos and Watson stereotoxic atlas for rat brain. The coordinates for BLA were: AP = - 2.1 mm; L = ± 4.8 mm; V = 8 mm.

**Grouping**

**Orexin A group** (n=18): Group 1, 2, and 3 were respectively control (saline treated); low dose (100 Pico mole/ Microliter) and high dose (250 Pico mole/ Microliter) groups.

**Orexin A antagonist**: Group 1, 2, 3 and 4 were respectively control (saline treated); low dose (3 ng/ Microliter) and high dose (6ng/ Microliter) groups. The fourth group was infused with antagonist.

**Orexin B group**: Orexin B group: Group 1, 2, 3, and 4 were respectively control (saline treated); low dose (3 nano mole/ Microliter) and high dose (30 nano mole/ Microliter) groups, and the group 4 was infused with Orexin B antagonist.
The guide cannula was implanted and secured by screws and dental cement so that the tip remained just one mm above the target nucleus. They were left for a week for the healing and hardening of the implant. Subsequently, the infusion procedure was carried out in conscious free moving animals in their home cage after fasting for 24 hours before the trials. Harvard Pico Plus (USA) infusion pump was used to deliver the drug.

Orexin A and Orexin B (Sigma Chemicals, USA) were dissolved in 0.9% saline before first use and when not in use, the solution was stored at 4 °C for a maximum of three weeks. 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). A cocktail of Ketamine (60 mg/kg; NEON Laboratories limited, Thane, M.S) and xylazine (10 mg/kg; Indian immunological ltd. Hyderabad) were used for anaesthesia.

Orexin A was infused at 100 picomol/micro litre (1 microlitre for 1.33 minute) and Orexin-A 250 picomol dose into BLA bilaterally, and Orexin A antagonist, SB334867 (From: Tocris bioscience) infused in two doses at 3 ng/microlitre (Group 2 and 3) and 6 nano grams/microlitre over about 87 sec to infuse 1 microlitre. The infusion cannula was left in place for 20 seconds following the infusion.

Orexin B was infused at a dosage one microliter solution of 5 nmole/microliter and 30 nmole/microliter over a period about 90 sec, to infuse 0.6 microlitre per minute. The internal cannula was left in place for an additional 20 seconds for the complete transfer of fluid. Orexin B antagonist, TCS-OX2-29 (Catalog No.3370; Tocris Bioscience, UK) was dissolved in 2% cyclodextran in sterile water, was infused at a rate of one microliter of solution containing 10 microgram/microliter.

Three trials were carried out, and the average scores were tabulated. Separate groups of rats were infused with Orexin A; B or their receptor antagonist into basolateral amygdala. Following the infusion, food intake and water intake were measured meticulously for hours and represented for hours post-infusion. Consumption in the first hour, the second hour, the fourth hour, and intake for the whole day (24 hours) of the infusion trial were recorded. The measurements were performed by providing the animals with premeasured quantities of food and water, and re-measuring the remaining after the time period. The wastage was also measured and computed to calculate the actual consumption and represented as ‘g’ or ‘ml’ of consumption for food and water, respectively.

All procedures were followed in the control animals, except that the infusion was done with saline and not the chemicals. The confirmation of accuracy of site of infusion was done by carefully collecting the brain, which was preserved in formalin, processed to prepare wax blocks for sectioning. They were sectioned into 5 micron sections and stained by cresyl blue stain and observed under light microscope at 10X magnification for the location of cannula. Only the data from rats receiving proper cannula implantation position are tabulated and statistically analyzed.

Figure 1: Histopathology confirmation of the location of the guide cannula location in the basolateral amygdala

Results of Orexin A and B and their antagonist infusion into BLA

Experiment 1: Effect of Orexin-A infusion into BLA on food and water (Table 1)

A total of 18 animals were divided in to three groups (n=6). Group 1: control, received normal
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saline - 0.9%. Group 2 and 3 received orexin-A infusion at 100 picomol and Orexin-A 250 picomol dose respectively into bilateral BLA. Post infusion, food and water consumption were measured in all the groups (Table 1).

Food and water intake following Orexin-A infusion into BLA, showed no significant difference between the groups. ANOVA of results obtained at regular time intervals were as follows:

1st hour $[F(2, 15) = 0.97, p = 0.908]$, 
2nd hour $[F(2, 15) = 1.399, p = 0.277]$, 
4th hour $[F(2, 15) = 3.994, p = 0.061]$ and 
24th hour $[F(2, 15) = 1.571, p = 0.240]$.

Water intake:
1st hour $[F(2, 15) = 0.105, p = 0.901]$, 
2nd hour $[F(2, 15) = 0.022, p = 0.979]$, 
4th hour $[F(2, 15) = 1.221, p = 0.323]$ and 
24th hour $[F(2, 15) = 0.481, p = 0.627]$.

Experiment 2: Effect of Orexin-A antagonist (SB-334867) infusion into BLA on food and water intake (Data in Table 2)

Food intake: Compared to control, at the 1st hour SB334867 post infusion showed a significant increase ($F(2, 15) = 12.803, p = 0.001$) in the food intake [i.e. group -1 Vs. group-2 or group-3, **p< 0.001, 0.001]. whereas, no significant change was noticed at 2 hr. ($F(2, 15) =0.076$ p=0.927); 4 hr. ($F(2,15)=0.000$, p=1.00); Total food intake was increased (24 hr) at post-infusion time intervals $[F(2,15)= 8.463$, p=0.003, group-1 Vs. group-3, p<0.01].

Water intake: SB334867 infusion has increased water intake at 1 hr. $[F(2, 15) =3.700, p=0.049$, group-1 Vs. group-3, p< 0.05]; and at total (24 hr.) $[F(2,15)= 3.851, p=0.045]$ compared to control $\text{group-1 Vs. group-3, p <0.05}$ on the contrary there was no effect at 2nd hour. $[F(2,15)= 0.16, p=0.853]$; 4 hr. $[F(2,15) = 0.052, p = 0.949]$. 

| Table 1: Food and water intake following Orexin A infusion at 1st, 2nd, 4th and 24th hour time intervals |
|----------------------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                                      | Food intake (grams) | Water intake (ml)   |
|----------------------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                                       | 1 hr                | 2 hr                | 4 hr                | 24 hr               | 1 hr                | 2 hr                | 4 hr                | 24 hr               |
| Group-1 (0.9% saline)                  |                     |                     |                     |                     |                     |                     |                     |
|                                       | 4.23±0.21           | 0.50±0.09           | 1.53±0.14           | 21.63±2.52          | 4.67±0.30           | 1.66±0.66           | 1.16±0.38           | 40.83±1.13           |
| Group-2                                | 4.37±0.25           | 0.91±0.27           | 1.76±0.33           | 21.86±2.56          | 4.83±0.40           | 1.50±0.71           | 2.16±0.44           | 41.60±2.05           |
| Group-3                                | 4.35±0.26           | 0.96±0.24           | 0.80±0.30           | 22.84±0.43          | 4.91±0.45           | 1.50±0.56           | 1.58±0.53           | 39.00±1.21           |
| ANOVA significance (p value)            | 0.908               | 0.277               | 0.061               | 0.240               | 0.901               | 0.979               | 0.323               | 0.627               |

All data was expressed in mean ± SD, P< 0.05 was considered significant, showed no significant change in food and water intake.

| Table 2: Effects of SB-334867 on food and water intake at 1st, 2nd, 4th and 24th hr time period |
|----------------------------------------|---------------------|---------------------|---------------------|---------------------|
|                                      | Food intake (grams) | Water intake (ml)   |
|----------------------------------------|---------------------|---------------------|---------------------|
|                                       | 1 hr                | 2 hr                | 4 hr                | 24 hr               | 1 hr                | 2 hr                | 4 hr                | 24 hr               |
| Group-1 (0.9% saline)                  |                     |                     |                     |                     |                     |                     |                     |
|                                       | 4.23±0.21           | 0.50±0.09           | 1.53±0.14           | 21.63±2.52          | 4.66±0.30           | 1.66±0.66           | 1.16±0.38           | 40.83±1.13           |
| Group-2                                | 6.36±0.37***        | 0.43±0.20           | 1.53±0.14           | 23.32±2.51          | 4.91±0.50           | 1.50±0.42           | 1.16±0.38           | 41.33±2.00           |
| Group-3                                | 6.35±0.40***        | 0.50±0.09           | 1.52±0.24           | 24.60±0.49**        | 6.50±0.67*          | 1.25±0.44           | 1.33±0.49           | 44.67±1.20*          |
| ANOVA significance (p value)            | 0.001               | 0.927               | 1.000               | 0.003               | 0.049               | 0.853               | 0.949               | 0.045               |

Data are presented as mean values ± S.E.M. Analysis by ANOVA significance (post hoc Tukey HSD, n=6 per group)

1st hr food: Group-1 Vs Group-2 or Group-3, ***p< 0.001, **p<0.001.
24 hr food: Group-1 Vs Group-3 * p< 0.01.
1st hr water: Group-1 Vs Group-3 * p< 0.05.
24 hr water: Group-1 Vs Group-3 * p< 0.05.

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[Table 1: Food and water intake following Orexin A infusion at 1st, 2nd, 4th and 24th hour time intervals]

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<thead>
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<th>Food intake (grams)</th>
<th>Water intake (ml)</th>
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[Table 2: Effects of SB-334867 on food and water intake at 1st, 2nd, 4th and 24th hr time period]

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<td>ANOVA significance (p value)</td>
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Experiment 3: Effect of bilateral micro infusion of orexin B and its antagonist-TCS-OX2-29 into BLA on feeding behavior. (Table 1)
At 3nm/µl of orexin B infusion, there was slight increase in food and water intake. But differences were not statistically significant, except increase in food intake (2.37±0.25) at the end of the 4th hour compared to control (1.55±0.29) (Figure 1; p<0.05). Water intake increased at the end of 4 hr (6.82±0.78) and 12 hr (14.16±1.35) compared to controls (3.58±0.43 & 10±0.51) (Figure 2; p<0.001 and p<0.01), respectively. At 30nm/µl, food intake is significantly increased at the end of 1 hr (11.1±1.19), 2 hr (6.3±0.30), 4 hr (6±0.52), 12 hr (24±4±2.08), and 24 hr (43.8±2.21) when compared to their respective controls (5.16±0.38, 3.1±0.20, 1.55±0.29, 11±1.21 and 20.8±0.71) (Figure 3; p<0.001, p<0.001, p<0.001, p<0.01 and p<0.001, respectively). Increase in water intake was observed at the end of 1 hr (12±0.81), 2 hr (5.13±0.77), 4 hr (9.2±0.69), 12 hr (21.65±1.01) and 24 hr (47.98±1.31), when compared to their respective controls (6.9±0.40, 2.65±0.41, 3.58±0.43,10±0.51 and 23.13±0.94) (Figure 2; p<0.01, p<0.01, p<0.001 and p<0.001, respectively) at 30nmol/µl. Orexin B antagonist did not produce significant changes both in food and water intake except increase in food intake at the end of second hour (1.9±0.19) compared to control (3.1±0.20) (Figure 1 and 2; p<0.05).

Discussion
The basic control of ingestive behaviour and the calorific balance has been vested largely in the hypothalamic areas proposed by Brobeck and Anand way back in the 1950s. They proposed the theory of lateral hypothalamus (LH) as the feeding centre, and ventromedial hypothalamus (VMH) as the satiety center, which has stood the test of time. However, there have been several additions to this basic information by way of further research. The regions in the hypothalamus itself, namely the Paraventricular area (PVN), the septum have been also shown to influence different parameters of the feeding paradigm. Subsequently, the amygdaloid area was shown to influence the hypothalamic activity as a modulator. Further, the basolateral amygdla was shown to be more involved in the hedonic quality of food selection.

Several neurotransmitters have been implicated in the regulation of food intake. They include Acetylcholine, leptin, glutamate, and Dopamine. Apart from all these, several other newly discovered chemical agents also influence the behaviour of food selection and consumption. In this regard, the Orexins were tested in the present study. Since Orexin A and B were first discovered in the hypothalamus, these agents were speculated to have a potent role in the control of ingestion. However, more information regarding their activity has emerged on sleep wakefulness and alertness.
In the present study, we investigated the specific role of Orexin A and Orexin B by infusing them into basolateral amygdala in separate groups of rats through indwelling cannula. Further, we tested the actions of the antagonists of Orexin A and Orexin B. The free moving rats with indwelling cannula were subjected to infusion after overnight fasting. Orexins were reportedly more potent in the made-to-fast animals. Orexin A was infused into BLA at two concentrations, viz. 100 picomoles/ml and 250 picomoles/ml. The food intake and water intake were measured sequentially on: 1st hour, 2nd hour, 4th hour and 24th hour post infusion. We found that there were no significant changes in the food and water consumption in animals after orexin A infusion, either in low dose or high dose (Table: 1 and 2). In order to investigate if the Orexin A antagonist (SB-334867) produce any influence on the said parameters, we infused it to the BLA in the same animals following the same procedure. Two concentrations were used here, namely 3 ng and 6 ng/ml. We found an increase in food intake as well as water intake. The increase was more in 6 ng concentration than that in the 3 ng group. This action of antagonist appeared dose dependent. It will be quite clear that that even though the orexin A does not produce effect, its antagonist infusion resulted in the increase in food and water intake. Since we have infused a pharmacological dose of these antagonist, it is possible that it will cause some effects on similar receptors. This question will have to be addressed, which requires further enquiry.

Further, we proceeded with the infusion of Orexin B in other group of animals. The infusion was also done in two doses. In this case, we measured for the 1st, 2nd, 4th, 12th and the 24th hour measurements. This modification was done in order to clearly elucidate the role of Orexin B on food and water intake, after we encountered an increased parameter reading in orexin A antagonist. We found increased food and water intake in both low dose and high dose or Orexin B in all time periods. In higher dose, the effect was more. This evidence suggested a stimulatory role for Orexin B on food intake and water intake. We tested the findings by infusion of Orexin B antagonist. We found contrary result, which led to decreased food and water intake in the infused animals. Therefore, we could conclude a stimulatory effect for orexin B in feeding behaviour at large. The time line of response is also of significance. The increase in intake was very much more in the first hour after the infusion, while the data showed lower increase in the subsequent hours. This is could be possibly due to the half-life period of injected orexins, which is about 29 minutes. Subsequently the effects faded, however the total consumption for the whole day (24 hours) showed a significant increase.

We have traditionally believed the basic concept of feeding centre and satiety centre of hypothalamic areas to be the fundamental controllers of ingestive behaviours. Since the hypocretins/Orexins have been first identified in hypothalamus, especially in the lateral hypothalamic region, the present findings provide valuable information in expanding the scope of involvement of other regions and neurotransmitters in the regulatory mechanism. In this regard, the Orexin B in particular, appears more potent in influencing the ingestive behaviour. Numerous neuronal fibres emerge from hypothalamus and are distributed in the anterior forebrain regions, including the amygdala. Since the basolateral amygdala is one of the nodal centers in the reward mechanism, the hypothalamo-amygdaloid orexinergic pathway could be an important pathway. Moreover, the BLA has been shown to influence the taste selection, the food reward and hedonic values. They also may play an important role in the function of this pathway.

Further studies are required to elucidate the roles of this system of neuronal connections, and the connecting pathways on the control of complex ingestive behaviour unequivocally. Neurotransmitter studies and other studies involving hedonic food selections could throw light on the functioning of this neuro-circuitry.

Acknowledgement

We acknowledge the liberal funding for this project by Department of Biotechnology, Ministry Science and Technology, Government of India.

We acknowledge the support of Kasturba Medical College, Mangalore and Manipal University, Manipal for the completion of this work.
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