Full title:

The action of orexin B on passive avoidance learning. Involvement of neurotransmitters.

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Abstract

The extensive projection of orexigenic neurons and the diffuse expression of orexin receptors suggest that endogenous orexins are involved in several physiological functions of the central nervous system, including learning and memory. Our previous study demonstrated that orexin A improves learning, consolidation and retrieval processes, which involves α- and β-adrenergic, cholinergic, dopaminergic, GABA-A-ergic, opiate and nitrergic neurotransmissions. However, we have little evidence about the action of orexin B on memory processes and the underlying neuromodulation. Therefore, the aim of the present study was to investigate the action of orexin B on passive avoidance learning and the involvement of neurotransmitters in this action in rats. Accordingly, rats were pretreated withthe selective orexin 2 receptor (OX2R) antagonist, EMPA; the γ-aminobutyric acid subunit A (GABA-A) receptor antagonist, the bicuculline; a D2, D3, D4 dopamine receptor antagonist, haloperidol; the nonselective opioid receptor antagonist, naloxone; the non-specific nitric oxide synthase (NOS) inhibitor, nitro-L-arginine; the nonselective α-adrenergic receptor antagonist, phenoxybenzamine and the β-adrenergic receptor antagonist, propranolol. Our results demonstrate that orexin B can improve learning, consolidation of memory and retrieval. EMPA reversed completely the action of orexin B on memory consolidation. Bicuculline blocked fully; naloxone, nitro-L-arginine, phenoxybenzamine and propranolol attenuated the orexin B-induced memory consolidation, whereas haloperidol was ineffective. These data suggest that orexin B improves memory functions through OX2R and GABA-ergic, opiate, nitrergic, α- and β-adrenergic neurotransmissions are also involved in this action.

keywords: orexin B, passive avoidance learning, neurotransmitter

1. Introduction

The 33-amino acid orexin A (also known as hypocretin-1) and the 28-amino acid orexin B **(**also known as hypocretin-2) were described in the hypothalamus in 1998. Both neuropeptides are derived from the common precursor peptide (preproorexin) [1, 2]. Orexin-expressing neurons are predominantly localized in the lateral hypothalamus area (LHA) and posterior hypothalamus and extensively project to the entire neuroaxis, including the cerebral cortex, olfactory bulb, thalamus, anterior and posterior hypothalamus, diagonal band of Broca, amygdala, hippocampus, bed nucleus of the stria terminalis, septum, brainstem and spinal cord [1-6].

Orexins activate at least two distinct G-protein coupled receptors, the orexin 1 receptor (OX1R) and the orexin 2 receptor (OX2R). OX1R has a tenfold greater affinity for orexin A than orexin B, whereas OX2R has nearly equal affinity for both neuropeptides [2, 7]. OX1R and OX2R are located on both pre- and post-synaptic processes, as well as on cell bodies [8, 9]. Prominent expression of OX1R mRNA was found in the locus coeruleus (LC) and to a lesser extent in the dorsal raphe nucleus, hippocampal formation and tenia tecta. On the other hand, OX2R mRNA was identified mainly in the cerebral cortex, nucleus accumbens, paraventicular thalamic and subthalamic nuclei [10, 11].

The extensive projection of orexigenic neurons and the diffuse expression of orexin receptors suggest that endogenous orexins may be involved in several physiological functions of the central nervous system (CNS), such as regulation of blood pressure [12, 13], body temperature [14], drinking behavior [15], food intake [2], nociception [16], sleep-waking cycle [17, 18], sleep disorder [19], reward and drug addiction [20], stress and arousal [21, 22], LH secretion [23, 24], prolactin secretion [25], and thyroid function [26]. The presence of numerous orexigenic terminals in the memory related brain regions, such as the hippocampus and amygdala suggests the involvement of orexins in learning and memory.

In our previous study, we have demonstrated that orexin A improves learning, consolidation and retrieval processes [27]. Several other studies provided further evidence about the memory enhancing action of orexin A [28, 29] and about the role of OX1R in memory [30-32]. In contrast, the effect of orexin B on memory processes has not been elucidated. Additionally, there are indications that the orexins might act as neurotransmitters per se or in concert with other monoaminergic structures [33, 34]. In our previous study, we demonstrated that a number of transmitters could be involved in the action of orexin A on memory consolidation [27]. However, we have little evidence about the underlying neuromodulation of the presumable effect of orexin B on learning and memory. Therefore, the

first aim of the present study was to investigate the action of orexin B on passive avoidance learning and memory formation in rats. The second aim of our study was to investigate the involvement of neurotransmitters in mediating the action of orexin B on memory consolidation, thus the animals were pretreated with transmitter receptor antagonists prior to peptide administration.

2. Methods and Materials

2.1. Experimental animals and ethics statement

Male Wistar rats, weighing 150-250 g were used. The animals were maintained and treated during the experiments in accordance with the instructions of the Ethical Committee for the Protection of Animals in Research of the University of Szeged (Szeged, Hungary), which specifically approved this study. The rats were kept in their home cages at a constant temperature (23 °C) on a standard illumination schedule with 12-h light and 12-h dark periods (lights on from 6:00 AM). Commercial food and tap water were available ad libitum. To minimize the effects of nonspecific stress, the rats were handled daily. All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

2.2. Surgery

For intracerebroventricular (icv) administration, the rats were implanted with a stainless steel Luer canulla (10 mm long) aimed at the right lateral cerebral ventricle under Nembutal (35 mg/kg, intraperitoneally, ip) anesthesia. The stereotaxic coordinates were 0.2 mm posterior; 1.7 mm lateral to the bregma; 3.7 mm deep from the dural surface, according to the atlas of Pellegrino et al., 1979 [35]. Cannulas were secured to the skull with dental cement and acrylate. The rats were used after a recovery period of 5 days.

2.3. Treatments

Orexin B and the selective OX2R antagonist EMPA (N-ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulphonyl)-amino]-N-pyridin-3-ylmethyl-acetamide) were purchased from Bachem Inc., Switzerland. Orexin B was applied via the icv cannula in a dose of 0.5 or 1.0 µg/animal. For combined treatment, only 1.0 µg orexin B was used. Orexin B was administered 30 min before the learning trial (learning), after the learning trial (consolidation) and 30 min before the 24 h test (retrieval). EMPA was injected icv in a dose of 2.0, 4.0 or 8.0 µg/animal.

EMPA and the receptor blockers were applied immediately after the learning trial, followed 30 min later by orexin B administration.

The following receptor blockers were used: bicuculline methiodide (Sigma, St Louis, USA), 1 mg/kg ip; haloperidol (G. Richter Budapest, Hungary) 10 µg/kg ip; naloxone hydrochloride (Endo Lab., New York, USA), 0.3 mg/kg ip; nitro-L-arginine (N-w-nitro-L-arginine, N-NA, Sigma-Aldrich, Budapest, Hungary), 10 µg/2 µl icv; phenoxybenzamine hydrochloride (Smith, Klein and French, Herts, UK), 2 mg/kg ip and propranolol hydrochloride (ICI, Macclesfield, UK), 10 mg/kg ip. The doses of the receptor blockers were selected on the basis of our earlier experience as being effective when administered with other neuropeptides, but not affecting the paradigm per se [27, 36].

2.4. Behavioral testing

2.4.1. Passive avoidance test

One-trial learning, step-through passive avoidance behavior was measured according to Ader et al., 1972 [37]. The apparatus consists of two separate chambers connected through a guillotine door. One of the chambers was illuminated, while the other was dark. Rats were placed on the illuminated platform and allowed to enter the dark compartment. Since rats prefer dark to light, they normally entered within 5 s. Two additional trials were delivered on the following day. After the second trial, unavoidable mild electric footshocks (0.75 mA, 2 s) were delivered through the grid floor. The guillotine door was closed immediately after the rat entered the dark chamber and the animals could not escape the footshock. After this single trial, the rats were immediately removed from the apparatus and were treated. The consolidation of passive avoidance behavior was tested 24 h later. When the action of the peptide was tested on acquisition processes, the animals were treated before the single trial. When the action of the peptide was tested on retrieval processes the animals were treated with the peptide 30 min before the 24 h testing. For consolidation, the animals were treated with the peptide following the learning trial or first with the receptor antagonist and 30 min later with the peptide for combined treatment. In the 24 h testing each rat was placed on the platform and the latency to enter the dark compartment was measured up to a maximum of 300 s.

2.5. Statistical analysis

Statistical analysis of the behavioral testing was performed by two-way analysis of variance (two-way ANOVA), which was followed by Tukey's post hoc comparison test. Only

the mean percentages were plotted and the standard error of the mean (SEM) is given in the figure captions. The differences between groups were examined by Tukey's post hoc comparison test, and a probability level of 0.05 or less was accepted as indicating a statistically significant difference.

3. Results

The action of orexin B on learning was studied when the peptide was administered 30 min prior to the learning trial. Orexin B lengthened significantly the latency of the avoidance response in the dose of 1 μg, whereas the 0.5 μg dose was ineffective [$F(2, 53) = 4.87$]; $p < 0.05$ (Fig. 1). Thus orexin B facilitated the learning and consolidation in a dose-dependent manner.

The effect of orexin B on consolidation of the passive avoidance response was investigated when the peptide was given immediately following the learning trial. Orexin B lengthened significantly the latency of the passive avoidance response in the dose of 1 μg, while the dose of 0.5 μg was not effective [$F(2, 88) = 6.03$]; $p < 0.01$ (Fig. 2). Therefore orexin B facilitated the consolidation of the passive avoidance response in a dose-dependent manner.

EMPA alone in a dose of 2.0, 4.0, 8.0 μg did not change the passive avoidance learning, however in combination with orexin B (1 μg) blocked fully the orexin B-induced facilitation in consolidation of passive avoidance learning [$F(5,54) = 4.84$]; $p < 0.05$ (Fig. 3).

The action of orexin B on retrieval of passive avoidance learning was investigated when the peptide was administered 30 min before the 24-h test. Orexin B lengthened significantly the latency of the passive avoidance response in the dose of 1 μg, whereas the dose of 0.5 μg was ineffective [$F(2, 39) = 4.08$]; $p < 0.05$ (Fig. 4). Thus orexin B facilitated the retrieval of passive avoidance learning in a dose-dependent manner.

In the bicuculline-treated group, orexin B (1 μg/2 μl i.c.v.) facilitated the consolidation of passive avoidance learning [$F(3,92) = 6.29$]; $p < 0.01$. Bicuculline (1 mg/kg i.p.) itself had no action, while bicuculline pretreatment fully blocked the action of orexin B on this consolidation (Fig. 5).

In the naloxon-pretreated group, orexin B (1 μg/2 μl i.c.v.) facilitated the consolidation of passive avoidance learning [$F(3,42) = 4.39$]; $p < 0.01$. Naloxon (0.3 mg/kg i.p.) itself had no effect and attenuated but did not fully block the action of orexin B (Fig. 6).

In the nitro-L-arginine-treated group, orexin B (1 μg/2 μl i.c.v.) facilitated the consolidation of passive avoidance learning [$F(3,81) = 4.19$]; $p < 0.01$. Nitro-L-arginine (10

μg/2 μl i.c.v.) itself had no effect and attenuated but did not fully block the action of orexin B (Fig. 7).

In the phenoxybenzamine-treated group, orexin B (1 μg/2 μl i.c.v.) facilitated the consolidation of passive avoidance learning [$F(3,41) = 4.56$]; $p < 0.01$. Phenoxybenzamine (2 mg/kg i.p.) itself had no effect and attenuated but did not fully block the action of orexin B (Fig. 8).

In the propranolol-pretreated group, orexin B (1 μg/2 μl i.c.v.) facilitated the consolidation of passive avoidance learning [$F(3,64) = 5.44$]; $p < 0.01$. Propranolol (10 mg/kg i.p.) itself had no effect and attenuated but did not fully block the action of orexin B (Fig. 9).

In the haloperidol-pretreated group, orexin B (1 μg/2 μl i.c.v.) facilitated the consolidation of passive avoidance learning [$F(3,43) = 8.08$]; $p < 0.01$. Haloperidol (10 μg/kg i.p.) itself had no effect and did not block the action of orexin B significantly (Fig. 10).

4. Discussion

Orexin A and orexin B are proteolytic derivates of a common precursor polypeptide (preproorexin) and activate 2 distinct G-protein coupled receptors (OX1R and OX2R). Orexin-producing neurons and orexin receptors are widely distributed in the entire neuraxis and involved in several physiological functions of the CNS, including learning and memory [1-6]. Our previous study showed that orexin A is able to improve learning, consolidation of memory and retrieval processes in a passive avoidance paradigm in rats [27]. Other studies provided further evidence that orexin A can induce long-term potentiation (LTP) in the dentate gyrus in anesthetized rats [38] and could enhance memory in the mouse model of Alzheimer's disease [28]. It has also been reported that OX1R play a prominent role in spatial learning and memory by using a selective OX1R antagonist (SB-334867-A) in rats [39]. The present study demonstrates for the first time that orexin B also can improve learning, consolidation of memory and retrieval in a passive avoidance paradigm in rats. This action is mediated through OX2R, since the selective OX2R antagonist EMPA reversed completely the orexin A-induced memory consolidation. EMPA alone in the doses used showed only a tendency to change the baseline learning, which may indicate that the endogenous orexin B is not involved prominently in the action described. The results of our previous study suggested that α- and β-adrenergic, dopaminergic, GABA-A-ergic, opioid receptors and nitrogen-monoxide (NO) play role in the action of orexin A on consolidation of memory [27]. The results of the current study show that α- and β-adrenergic, GABA-A-ergic, opioid receptors and nitric oxide (NO) are involved in the effect of orexin B on consolidation of memory.

The neurotransmitter γ-aminobutyric acid (GABA) plays a prominent role in the mediation of physiologic memory processes [40] and in the pathogenesis of neurologic disorders, such as schizophrenia [41]. Several prior behavioral and biochemical studies revealed correlations between the GABA-ergic and the orexigenic systems. We have demonstrated previously that GABA is involved in the mediation of the orexin A-induced memory consolidation in a passive avoidance test in rats [27]. Electrophysiological and histological investigations showed that orexigenic neurons of the LHA activate the GABA-ergic neurons of the medial septum/diagonal band of Broca (MSDB) and increase the release of GABA locally via OXR2 [42]. MSDB, through its GABAergic and cholinergic projections to the hippocampus, modulates the hippocampal theta rhythm and associated learning and memory functions during exploratory behavior and REM sleep [42-44]. Additionally, intra-CA1 infusion of orexin A increased the GABA and glutamate release in the hippocampus [45]. On the other hand, optogenetic activation of GABA-ergic neurons in hypothalamic preoptic area (POA) resulted in rapid inhibition of the orexigenic neurons of the LHA [46]. Our present study demonstrates for the first time that GABA-ergic neurotransmission is involved in the action of orexin B on memory consolidation since the selective GABA-A receptor antagonist, bicuculline blocked fully the foregoing consolidation.

The adrenergic system is also important in memory functions. The primary source of forebrain norepinephrine (NE) is the LC, which is known to be implicated in attention and memory [47]. The highest density of orexin-fiber projections and OX1R are also found in noradrenergic neurons of the LC [5, 48]. An in vitro study showed that orexin B can increase the frequency of action potentials in rat and monkey brain slices containing the LC [34]. The hippocampus is one target of LC projections and also involved in learning and memory. It has been shown that adrenergic system can modulate different forms of synaptic plasticity in the hippocampus [49]. Though the hippocampus has a significant noradrenergic innervation, particularly in the dentate gyrus [50], it has only a modest orexigenic input [4, 6] and orexin receptor density [11]. In vitro studies revealed that intra-LC infusion of orexin A can produce NE-LTP in the dentate gyrus and NE release in the hippocampus in anesthetized rats [29]. Amygdala is implicated in the formation of emotional memory and represents another target of LC projections, along with a prominent orexigenic innervation and OX1R expression [32]. A recently published study demonstrated that the orexin fibers originating in the perifornical region of the hypothalamus directly depolarize LC neurons through rapid corelease of orexin and glutamate. The orexin activity in the LC, likely via OX1R, can drive NE signaling through β-adrenergic receptors in the lateral nucleus of the amygdala to enhance threat

memory formation [51]. Our present study demonstrates for the first time that both α- and β-adrenergic neurotransmission is involved in the action of orexin B on memory consolidation since both the non-selective α-adrenergic receptor antagonist, phenoxybenzamine and the non-selective β-adrenergic receptor antagonist, propranolol attenuated this consolidation.

The opioid system is also involved in memory as well as in reward and addiction [52]. Previous studies using OX1R and OX2R antagonists revealed that the orexigenic system is implicated in morphine withdrawal [53, 54]. On the other hand, investigations with opioid receptor antagonists showed that the action of orexin A on memory consolidation [27] or feeding [55] is mediated through opioid receptors. Our present study demonstrates for the first time that opioid neurotransmission is also involved in the action of orexin B on memory consolidation since both the μ and to a lesser extent δ and κ receptor antagonist naloxone attenuated this consolidation.

NO is another type of neurotransmitter which is associated with synaptic plasticity, learning and memory [56]. There is morphological evidence that NO interferes with orexins. NO and orexin-coexpressing neurons were identified in several nuclei of the hypothalamus, such as the arcuate, the dorsomedial, the ventromedial and the perifornical nuclei [57]. A prior study using nitro-L-arginine, a non-specific nitric oxide synthase (NOS) inhibitor revealed that NO participates in mediation of vasopressor/vasodepressor effects of orexin A and orexin B [58]. In addition, our previous study showed that NO plays role in the action of orexin A on memory consolidation as well [27]. Our present study demonstrates for the first time that nitrergic neurotransmission is also involved in the action of orexin B on memory consolidation since nitro-L-arginine attenuated the foregoing consolidation.

The mesolimbic dopaminergic system plays role in reward, drug addiction and memory [59]. It has been shown that dopaminergic and GABA-ergic neurons of the ventral tegmental area (VTA) receives orexigenic afferents from the LHA [60]. In vitro studies revealed that dopaminergic neurons of the VTA can be activated by both orexin A and B [61]. In vivo studies showed that intra-VTA administration of orexin A or orexin B enhances the release of dopamine in the nucleus accumbens (NAc) [62]. Our previous study showed that dopamine is involved in the mediation of the orexin A-induced memory consolidation in a passive avoidance test in rats [27]. Our present study demonstrates that dopaminergic neurotransmission may not be involved in the orexin B-induced consolidation of memory, as the D2, D3, D4 dopamine receptor antagonist, haloperidol did not block this consolidation. This observation can be interpreted by the differences between orexin A and orexin B in terms of mechanism of action in the VTA. On one hand, orexin A can increase the firing rate of

VTA GABA-ergic neurons, which may dampen the effect on dopaminergic neurons [63]. On the other hand, the effect of orexin B is seems to be secondary to a direct control of the local glutamatergic neurotransmission in the VTA [64].

5. Conclusion

The present study demonstrates that orexin B can improve learning, consolidation of memory and retrieval in a passive avoidance paradigm in rats. Our results also show that α- and β-adrenergic, GABA-ergic, nitrergic, and opiate neurotransmissions are involved in the orexin B-induced improvement in memory consolidation.

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Figure legends

Fig. 1. The effects of different doses of orexin B on passive avoidance learning. The peptide was given 30 min before the learning trial. Orexin B (0.5 μg/2 μl, icv), orexin B (1.0 μg/2 μl, icv) *$p < 0.05$ vs. control. The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

Fig. 2. The effects of different doses of orexin B on the consolidation of passive avoidance learning. The peptide was given immediately after the learning trial. Orexin B (0.5 μg/2 μl, icv), orexin B (1.0 μg/2 μl, icv) *$p < 0.05$ vs. control. The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

Fig. 3. The effects of the selective OX2R antagonist, EMPA on orexin B-induced consolidation of passive avoidance learning. Orexin B (1.0 μg/2 μl, icv) *$p < 0.05$ vs. control, EMPA (2.0 μg/2 μl, icv), EMPA (4.0 μg/2 μl, icv), EMPA (8.0 μg/2 μl, icv), OreB 1.0 μg/2 μl icv + EM 2.0 μg/2 μl icv. Abbreviations: OreB = orexin B, EM = EMPA. The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

Fig. 4. The effects of different doses of orexin B on the retrieval of passive avoidance learning. The peptide was given 30 min before the 24-h testing. Orexin B (0.5 μg/2 μl, icv.), orexin B (1.0 μg/2 μl, icv) *$p < 0.05$ vs. control. The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

Fig. 5. The effects of the γ-aminobutyric acid subunit A (GABA-A) receptor antagonist, bicuculline on orexin B-induced consolidation of passive avoidance learning. Orexin B (1.0 μg/2 μl, icv) *$p < 0.05$ vs. control, bicuculline (1 mg/kg, ip), combined (bicuculline 1 mg/kg ip + orexin B 1.0 μg/2 μl, icv). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

Fig. 6. The effects of the nonselective opioid receptor antagonist, naloxone on orexin B-induced consolidation of passive avoidance learning. Orexin B (1.0 μg/2 μl, icv) *$p < 0.05$ vs. control, naloxone (0.3 mg/kg, ip), combined (naloxone 0.3 mg/kg ip + orexin B 1.0 μg/2 μl, icv). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

Fig. 7. The effects of the non-specific nitric oxide synthase (NOS) inhibitor, nitro-L-arginine on orexin B-induced consolidation of passive avoidance learning. Orexin B (1.0 μg/2 μl, i.c.v.) *p < 0.05 vs. control, nitro-L-arginine (10 μg/2 μl, icv), combined (nitro-L-arginine 10 μg/2 μl icv + orexin B 1.0 μg/2 μl, icv). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

Fig. 8. The effects of the nonselective α-adrenergic receptor antagonist, phenoxybenzamine on orexin B-induced consolidation of passive avoidance learning. Orexin B (1.0 μg/2 μl, i.c.v.) *p < 0.05 vs. control, phenoxybenzamine (2 mg/kg, ip), combined (phenoxybenzamine 2 mg/kg, ip + orexin B 1.0 μg/2 μl, icv). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

Fig. 9. The effects of the β-adrenergic receptor antagonist, propranolol on orexin B-induced consolidation of passive avoidance learning. Orexin B (1.0 μg/2 μl, i.c.v.) *p < 0.05 vs. control, propranolol (10 mg/kg, ip), combined (propranolol 10 mg/kg, ip + orexin B 1.0 μg/2 μl, icv). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

Fig. 10. The effects of the D2, D3, D4 dopamine receptor antagonist, haloperidol on orexin B-induced consolidation of passive avoidance learning. Orexin B (1.0 μg/2 μl, i.c.v.) *p < 0.05 vs. control, haloperidol (10 μg/kg, ip), combined (haloperidol 10 μg/kg, ip + orexin B 1.0 μg/2 μl, icv). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.