Increased mRNA Level of Orexin1 and 2 Receptors Following Induction of Experimental Autoimmune Encephalomyelitis in Mice

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ABSTRACT

Orexin A and B are hypothalamic peptides with a wide variety of effects such as anti-inflammation and neuroprotection. Impaired function of orexin system has been reported in some neurodegenerative diseases like Parkinson, Huntington and Alzheimer. In this study, the mRNA expression levels of some hypothalamic peptides were investigated in C57BL/6 female mice with experimental autoimmune encephalomyelitis (EAE).

Animals were randomly divided into two control and EAE groups. EAE was induced by administration of myelin oligodendrocyte glycoprotein (MOG) with complete Freund’s adjuvant and pertussis toxin. Twenty-first days following immunization, mice were decapitated to remove the brains. Then, the expression profiles of prepro-orexin, orexin 1 receptors (OX1R) and orexin 2 receptors (OX2R) in hypothalamic region were assessed using real-time PCR method.

In this study, we found a considerable increase in the mRNA expression of OX1R and OX2R following EAE induction in C57BL/6 mice.

Elevation levels of OX1R and OX2R following EAE induction suggest that alteration in orexinergic system may involve in pathogenesis of multiple sclerosis.

Keywords: C57BL/6 mice; Experimental autoimmune encephalomyelitis; Multiple sclerosis; Orexin 1 receptors; Orexin 2 receptors; Prepro-orexin

INTRODUCTION

Multiple sclerosis (MS) is the most common progressive and irreversible inflammatory
EAE and Orexinergic System

neurodegenerative disease in young people. The clinical presentation of MS is highly variable and main symptoms include impaired vision, extreme fatigue, spasms, sleep disturbance, depression, and anxiety. Cognitive impairments are also observed at all stages of MS such as dysfunction in free recall from long-term memory, speed of information processing, working memory, and abstract reasoning.

To understand the etiology and pathology of MS, it is important to have animal models mimicking MS pathologic and clinical features similar to human. Experimental autoimmune encephalitis (EAE) is a well-established animal model of MS. EAE is an inflammatory neurodegenerative disease of the central nervous system (CNS) and can be induced by immunization of susceptible animals with a number of myelin antigens such as myelin basic protein, myelinproteolipid protein and myelin-oligodendrocyte glycoprotein (MOG).

Orexin A (OX-A) and B (OX-B), produced from proteolysis of prepro-orexin, are hypothalamic peptides with 33 and 28 amino acids, respectively. Orexins act via two types of G protein-coupled receptors, orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R). Both orexin receptors can bind to OX-A and OX-B, but with different affinities; OX1R has higher affinity for OX-A, while OX2R has equal affinity for both agonists. There are functional differences between these receptors, for example OX1R activity is more closely associated with reward and OX2R activity is more closely related to arousal, sleep and food consumption. OX-A and B have wide variety of physiological effects such as feeding behavior, sleep/awake cycle and energy balance. Intracerebroventricular administration of OX-A regulates stress response, food intake, locomotor activity, and induces wakefulness. OX-A also has anti-inflammatory effects through antioxidant activities, increases insulin receptor expression, modulates the production of pro-inflammatory cytokines such as TNF-α and NO and reduces leukocyte infiltration function. OX-A shows neuroprotective effects via activation of hypoxia-inducible factor-1, suppression of post-ischemic glucose intolerance, decrease in caspase 3 and lipid peroxidation.

Several studies have been performed to detect damages to the orexin system in neurodegenerative diseases such as Parkinson, Huntington, Alzheimer and narcolepsy. It appears that loss of orexin neurons or impaired orexin neurotransmission might have a role in neurodegeneration mechanism responsible for these diseases.

We hypothesized that the neurodegenerative process in MS might also affect the orexin system, which could contribute to some symptoms in MS patients such as sleep disturbances, depression, anxiety, and cognitive impairments. However, it has not yet been addressed how changes in the orexin system are associated with EAE. In this study, the mRNA levels of prepro-orexin, OX1R and OX2R were measured in EAE induced female C57BL/6 mice to further clarify the role of orexin system in MS.

MATERIALS AND METHODS

Animals
C57BL/6 female mice (n=14, 8-10 weeks old) were purchased from Rafsanjan University of Medical Sciences. Mice were randomly divided into control (n=7) and EAE (n=7) groups. Mice were housed in cages (2-4 mice per cage) and maintained at a 12 h light/dark cycle (lights on 07:00 to 19:00) with free access to food and water and maintained at 23±2.0°C. All experimental protocols were approved by the institutional animal care and use committee of Rafsanjan University of Medical Sciences.

Induction of EAE
EAE was induced using myelin oligodendrocyte glycoprotein 35–55 (MOG 35-55; M-E-V-G-W-Y-R-S-P-F-S-R-V-H-L-Y-R-N-G-K) along with complete Freund’s adjuvant (CFA) and pertussis toxin. Mice were immunized with subcutaneous injection of 250 μg MOG mixed with 100 μl of complete Freund’s adjuvant (CFA) containing 5 mg/mL Mycobacterium tuberculosis. Mice were boosted day 0 and day 2 with intraperitoneal injection of 500 ng pertussis toxin.

Clinical Evaluation of EAE
The mice were weighed and scored daily for signs of neurological deficit. Disease severity was scored as previously published for EAE mice: grade 0=no signs, grade 1=partial loss of tail tonicity, grade 2=loss of tail tonicity and difficulty in righting, grade 3=unsteady gait and mild paralysis, grade 4=hind-limb paralysis and incontinence, grade 5=moribund or death. Paralyzed mice were given easy access to food and water.
Tissue Preparation

On the 21st day of EAE induction, mice were sacrificed by decapitation and the brains were immediately removed under aseptic condition. The hypothalamic region was microdissected from coronal sections between bregma −0.3 and −2.3 mm according to anatomical landmarks and immediately stored at −80 °C until use.24

RNA Isolation and cDNA Synthesis

Quantitative real-time RT–PCR was used to investigate any differences in the expression of prepro-orexin, OX1R and OX2R genes between the groups. Total RNA was extracted from the frozen samples by Trizol reagent (purchased from Parsstous Company, Tehran, Iran) according to the manufacturer’s guidance. The RNA was quantitated spectrophotometrically at 260/280 nm. The 260/280 ratio >1.8 was considered as an acceptable measure of RNA purity.

Five micrograms of RNA were converted to cDNA using a cDNA synthesis kit (Parstous Co., Tehran, Iran) with both oligo (dT) and random hexamer primers. The reverse transcription step and generation of cDNA were performed using the following steps: 70 °C for 10 min (without reverse transcription enzymes), 20 °C for 1 min (cooling), addition of reverse transcription enzymes, 42 °C for 60 min, and finally held in 95 °C up to 10 min to inactivate the reverse transcription enzymes.

Quantitative Real Time RT–PCR

Real-time PCR was performed, using 10 µL of a SYBR green master mix which was purchased from Parstous Company (Tehran, Iran), combined with 200 ng of template cDNA with 2 µL of appropriate primers (10 pmol stock) (Table 1) in a final volume of 20 µL by a Bio-Rad CFX96 system (Bio-Rad Company, Foster City, USA) using the following program: 1 cycle of 95°C for 15 min, 40 cycles of 95°C for 30 s, and 60°C for 30 s and finally 72°C for 30 s. Primers were designed in house by researchers and synthesized by the Bioneer Company (Korea). Real-Time PCR was carried out in triplicate and the β-Actin housekeeping gene was used for normalization of amplification signals of target genes. The relative amounts of PCR product were determined, using the 2−ΔΔCt formula. The dissociation stages, melting curves and quantitative analyses of the data were performed, using CFX manager software version 1.1.308.111 (Bio-Rad, Foster City, USA).

Statistical Analysis

Statistical analysis was performed, using Excel and SPSS 18 software. All data are expressed as means ± SEM. Differences between the groups were determined using independent sample t-test. For comparison of behavioral scores and weight in different days through study repeated measurement, ANOVA (RMA) was used. A p value lower than 0.05 was considered statistically significant.

RESULTS

Behavioral Score of the Disease

Photographs of two EAE induced mice on the 18th day after immunization with MOG were shown in Figure 1. Arrows show loss of tail tone (a) (score 2) and hind-limb paralysis (b) (score 4). In the EAE group, the first behavioral score of EAE became apparent, on average, 11.3±0.2 days after immunizations. In this group, 19 days following immunization the behavioral scores increased to a peak level of 2.5±0.5 (RMA, p=0.001) (Figure 2).

Table 1. The primers used in real-time RT–PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Orexin receptor 1</td>
<td>Forward: 5’-GTGGGGGAACCTTCCATCTCTG-3’ (Sense)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-AGAGATAATCGGCCACAGG-3’ (AntiSense)</td>
</tr>
<tr>
<td>Orexin receptor 2</td>
<td>Forward: 5’-TCACCCATGTCGTGCTAAAG-3’ (Sense)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CAAGTCATCCGGGTCATGTATAA-3’ (AntiSense)</td>
</tr>
<tr>
<td>Prepro-orexin</td>
<td>Forward: 5’-GCCCTAGACTTCTTGGGTATTT-3’ (Sense)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-AGGGAAACCTTGGTGAAGGAAA-3’ (AntiSense)</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward: 5’-AGAGGGAAATCGTGCNTGAC-3’ (Sense)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CAATAGTGATGACCTGGCCGT-3’ (AntiSense)</td>
</tr>
</tbody>
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Body Weight

The results showed that the mean body weight of mice in the EAE group decreased through the 21 days of study (RMA, \( p<0.001 \)). For the control groups, the mean body weight of animals increased through the 21 days of study (RMA, \( p<0.001 \)) (Figure 3).

mRNA Levels of Prepro-orexin, OX1R and OX2R Following Induction of EAE

The gene expression of prepro-orexin, OX1R and OX2R in hypothalamus of mice were assessed using quantitative real-time RT-PCR. The measured gene expressions were normalized to \( \beta \)-actin, which known to be invariant upon EAE induction.\(^{25}\)

Relative expression of prepro-orexin mRNA did not change in EAE group compared to control (\( p=0.984 \)) (Figure 4). However, mRNA levels of OX1R increased in EAE group compared to control (7.04-fold, \( p=0.006 \)) (Figure 5).

Figure 1. Photographs of two EAE induced mice, demonstrating loss of tail tonicity (a) (score 2) and hind limb paralysis (b) (score 4) in the C57BL/6 mice

Figure 2. Comparison of the behavioral scores between control and EAE groups. In the EAE group, disease onset was 11.3 ± 0.2 days after immunization and reached, a peak level of 2.5 ± 0.5 at day 19 (RMA, \( p=0.001 \)). EAE = experimental autoimmune encephalomyelitis

Figure 3. Comparison of the mean body weight during the 21 days of study indicates significant difference between EAE and control groups (RMA, \( p<0.001 \)).

Figure 4. The effects of EAE induction on prepro-orexin mRNA expression level

Figure 5. The effects of EAE induction on OX1R mRNA expression level. \( ***p<0.01 \) compared with the control group
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For OX2R, similar changes to orexin 1 receptor were observed. In EAE group transcriptional changes of OX2R mRNA level were higher than those in control group (9.87-fold, \( p=0.007 \)) (Figure 6).

**DISCUSSION**

In this study, the aim was to find out the possible relationship between orexin system and animal model of multiple sclerosis (EAE). For this purpose, we measured the expression profile of prepro-orexin, OX1R and OX2R gene in hypothalamus following EAE induction in C57BL/6 female mice. The results demonstrated that mRNA level of OX1R and OX2R increased while expression of prepro-orexin did not change following induction of EAE.

MS is a neurodegenerative disorder with myelin damage, neuronal loss, and atrophy of the CNS that intensify by increasing the stage of the disease.\(^26\) It is clear that EAE can mimic many aspects of MS such as clinical, neuropathological, and immunological features of the disease.\(^27\)

It was reported that some neurodegenerative diseases like narcolepsy, Alzheimer, Huntington and Parkinson, exert deleterious effects on orexinergic system.\(^18,20,28\) Drouot et al., showed that orexin levels were lower in Parkinson’s patients and decreased with the severity of the disease.\(^29\) Fronczeket al., investigated the role of orexin in Alzheimer disease. They reported that in patients that died from advanced Alzheimer, number of orexinergic neurons in hypothalamus decreased by around 40% and also cerebrospinal fluid (CSF) levels of OX-A were lower compared to healthy persons.\(^20\) In another study, Petersén showed similar results in the mouse model of Huntington’s disease. They reported that in the end-stage of the disease both the number of orexinergic neurons in the hypothalamus and the levels of OX-A in the CSF reduced by 72%.\(^28\)

In contrast, there are some studies indicating a normal concentration of orexin in CSF of MS patients. Constantinescu et al., found no significant reduction of OX-A in MS patients.\(^30\) In another study, Papuć et al., measured the concentration of OX-A in CSF of MS patients and healthy controls. Their results showed no significant difference between two groups.\(^31\) Moreover, Knudsen et al., showed that cerebrospinal fluid OX-A was at normal levels during and between attacks in multiple sclerosis patients.\(^32\)

On the contrary, there are several reports that reveal diminished level of orexin in CSF of MS patients. In a case report, Kato et al. showed that CSF concentration of OX-A in a patient with MS was lower than that in an age and sex matched normal subject.\(^33\) In another study, Oka et al., reported a similar case of multiple sclerosis with low CSF level of OX-A which was associated with hypersomnia.\(^34\)

In conclusion, results of this study demonstrated an increase in expression level of OX1R and OX2R genes following EAE induction. To prove the results, pharmacological interventions using OX1R and OX2R selective agonist and antagonists would be helpful in this regard.

**REFERENCES**

5. Gold R, Lington C, Lassmann H. Understanding pathogenesis and therapy of multiple sclerosis via animal


