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·Original Article·

Progressive changes of orexin system in a rat model of 6-hydroxydopamine-induced Parkinson's disease

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Abstract: Objective Sleep disturbance, which is characterized by excessive daytime sleepiness and sleep attacks, is frequently observed in patients with Parkinson's disease (PD). Loss of orexin neurons in hypothalamus and the resultant decreased level of orexin in cerebrospinal fluid (CSF) found in narcolepsy patients may also play an essential role in the pathogenesis of sleep disturbance. The present study aimed to investigate the possible changes in the orexin system during PD progression. **Methods** After the establishment of a rat PD model by injecting 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle, the numbers of orexin-A- and tyrosine hydroxylase (TH)-positive neurons, and the levels of orexin-A fibers and orexin-A in CSF were examined by immunohistochemistry and ELISA assay, respectively. **Results** Compared to the TH-containing neurons that exhibited fast degeneration in response to 6-OHDA, orexin-A-containing neurons were less sensitive to 6-OHDA. The number of orexin-A-positive neurons began to decrease at day 21 after operation, and at day 49, it decreased by 30% of the initial level. The orexin-A level in CSF of PD rats did not show any obvious fluctuations compared to the control, and there was no obvious reduction in the density of orexin-A-positive fibers in brain areas such as tuberomammillary nucleus. **Conclusion** These results reveal for the first time the dynamic changes of orexin system during the progression of PD. This may provide valuable information for drug development to reverse the loss of orexin neurons and sleep disturbance in PD patients.

Keywords: orexin; Parkinson's disease; sleep disturbance; degeneration

1 Introduction

Orexin, also named as hypocretin, is a newly discovered neuropeptide exclusively produced by a small number of neurons located in the lateral and posterior hypothalamus[1]. In 1999, two experimental groups simultaneously found that mutation of the orexin receptor would cause narcolepsy in dogs and that knockout of orexin per se in mice could reproduce the symptoms of narcolepsy[2,3]. Shortly after the report of these findings, it was revealed that there was no detectable orexin in cerebrospinal fluid (CSF) of patients with narcolepsy or cataplexy[1,4]. Since then, orexin has been established as a wake-promoting neuropeptide.

Parkinson's disease (PD) is a neurodegenerative disease mainly characterized by a series of motor symptoms such as resting tremor, rigidity and bradykinesia. Approxi-

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mately 5%-50% of PD patients are reported to suffer from excessive daytime sleepiness with frequent naps[5]. Some PD patients even develop sleep attacks featured by fragmented nocturnal insomnia and distorted rapid eye movement (REM). All of these symptoms are reminiscent of human narcolepsy, a sleep disorder caused by the dysfunction of orexin neurons[6]. Furthermore, many postmortem morphological studies in the function of orexin system have revealed a decreased level of orexin in CSF and reduced number of orexin neurons in PD patients[7-10].

However, it has not yet been addressed how changes in the orexin system are associated with PD progression. In this study, the time-dependent changes of orexin-A neurons were investigated and orexin-A level in CSF was analyzed in a rat model of PD induced by 6-hydroxydopamine (6-OHDA).

2 Materials and methods

2.1 Establishment of the rat PD model Sixty-three male Sprague Dawley (SD) rats weighing 250-300 g were used in this study. All procedures were approved by the Committee of Animal Use for Research and Education at the Fourth Military Medical University. Rats were randomly divided into control (n=21) and test (n=42) groups, and raised in cages under a 12:12 h light/dark cycle with food and water *ad libitum*. After being anaesthetized with sodium pentobarbital (40 mg/kg, i.p.), rats were stabilized in a stereotactic apparatus, followed by slow injection of 6 μL 6-OHDA (4 μg/μL, dissolved in 0.2% ascorbic acid) into 2 sites targeting medial forebrain bundle (MFB) of the right hemisphere (AP: 3.7 and 4.4; ML: 1.7 and 1.2; DV: 8.4 and 8.2). In control group, rats were injected with the same amount of dissolved 0.2% ascorbic acid. From day 14 post operation, apomorphine (0.5 mg/kg) was injected (i.p.) once per week. Rats rotating more than 7 circles per min were regarded as the successful PD model. At day 4, 7, 14, 21, 35, 49 or 63 after injection (6 rats were assigned to the test group at each time point and 3 rats to control group at each time point), the rats were anaesthetized for CSF extraction and sacrificed for immunohistochemical examination.

2.2 Collection of CSF Considering the circadian effect on the concentration of orexin in CSF[11], all samples were collected at the ZT 0 (the time of light on). Under anaesthesia, rats were positioned to a stereotactic apparatus and CSF was collected using a 10 μL syringe without plunger. Samples were allowed to flow out freely without pressure. On average, 80 μL CSF could be collected from each rat. Blood-tinged CSF was excluded from further analysis. CSF was placed on ice immediately after collection and stored at -80 ºC until use.

2.3 ELISA assay After diluting CSF with ELISA buffer at varied ratios, test assay was performed to determine the optimal ratio for each sample. Blank control was used as the denominator to calculate the relative optical density. Mouse anti-orexin-A IgG (Sigma) and horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG (Chemicon) were properly diluted and added into the wells in turn. The OPD/H_2O_2 system was used for color development. The optical density was measured by an assay reader (Fluostar, BMG Labtach, Germany). After averaging the results from duplicated wells, the relative optical density of the sample well was expressed as the ratio of optical densitiy of sample-embedded wells to that of the blank wells (designated as P/N value). The relative orexin-A level was calculated as the ratio of experimental P/N value to the control P/N value.

2.4 Tissue preparation Under anaesthesia with overdose i.p. injection of sodium pentobarbital (60 mg/kg), rats were transcardially perfused with 100 mL phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.4) followed by 500 mL 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB, pH 7.4). Brain was then removed and immersed into 30% sucrose-containing PB until sinking at 4 ºC. Coronal sections of hypothalamus and midbrain were cut by a freezing microtome at a thickness of 30 μm and kept as 6 series in 0.01 mol/L PBS at 4 ºC.

2.5 Immunohistochemical staining Sections containing fornix and substantia nigra (SN) were selected for immunohistochemical staining of orexin-A and tyrosine hydroxylase (TH), respectively. To eliminate the activity of endogenous peroxidase, sections were pre-treated with 0.3% H_2O_2 for 30 min. After being rinsed, they were blocked with normal goat serum (Boster) for 1 h and incubated with mouse anti-orexin-A (Abcam, 1:1 000) or mouse anti-TH (Sigma, 1:2 000) antibody diluted in 0.01 mol/L PBS containing 0.25% carrageenin, 0.3% Triton X-100, 0.05% NaN_3 and 3% normal goat serum (this dilution was designated as NTS) overnight at room

temperature. After being rinsed, sections were incubated with biotinylated goat anti-mouse IgG (Chemicon, 1:200) in NTS for 4 h at room temperature, followed by incubation with avidin-biotin complex (Vector, 1:200) in PBS containing 0.3% Triton X-100 at room temperature for 2 h. Sections were then incubated with 0.04% diaminobenzidine plus 0.003% H_2O_2 in Tris-HCl (pH 7.6) for 30-40 min, thoroughly rinsed and mounted onto glass slides. After being dried, they were dehydrated, cleared and cover-slipped for microscopic observation.

2.6 Cell counting Empirically, orexin neurons are most densely distributed on the plane where the fornix approximately parallels the upper 1/3 height of ventral 3rd ventricle. These sections were selected for counting the number of orexin-A-positive neurons (3-5 sections for each rat), and only cells containing clear nuclei were regarded as the positive neurons. In the case of midbrain, sections with the medial terminal nucleus of the accessory optic tract were defined as the central portion of the SN, and these sections were chosen for counting TH-positive neurons as described[12]. Cells in both injured and uninjured sides were counted separately and the cell remaining percentage was calculated as the ratio of cell number in the injured side to that in the uninjured side.

To evaluate the staining intensity of orexin-A-positive neurons, gray value measurement was performed by the Photoshop CS3 software. Area selection was carefully performed inside the profile of each soma.

2.7 Statistics Data were presented as mean±SEM and analyzed by Student's paired *t*-test. $P < 0.05$ was considered as statistically significant.

3 Results

Following procedure of immunostaining, TH-immunoreactive (-ir) and orexin-A-ir neurons were clearly identified in midbrain and hypothalamus (Fig. 1). In control rats, the number of TH-ir neurons in the designated slice was about 120 and there was no statistical significance between injured and uninjured sides. As shown in Fig. 2, in rats subject to 6-OHDA injection, however, the remaining percentage of TH-positive neurons decreased to (62.0±6.2)% at day 7 post operation, although at day 4, it was approximately comparable to that of control rats. At day 14 post operation, the percentage of TH-positive neurons further decreased to (24.5±3.5)% and it almost fell to zero at day 21 [(2.1±0.4)%, Fig. 2].

The average number of orexin-A-ir neurons in the designated slice was approximately 150 and introduction of PBS into MFB caused a minor fluctuation of this value. In comparison to the rapid degeneration of TH-ir neurons after 6-OHDA treatment, the dynamic change of orexin-A neurons was much attenuated. No obvious cell loss was observed during the first 14 d post operation. At day 21, however, the number of orexin-A-ir neurons at the injured side (right side ipsilateral to the operation) started to decrease, as compared with that at the intact side, and the remaining percentage was (84.1±7.9)%. At day 35 post operation, the percentage dropped to (79.8±7.8)% and it finally reached (72.0±6.0)% ($P < 0.05$ *vs* control) at day 49. This value did not change further at day 63 post operation (Fig. 2).

Meanwhile, the intensity of orexin-A-ir neurons was also measured. Results showed that there was no significant difference in the average gray value of orexin-A neurons between control and tested groups at any time point [e.g., control (57.2±6.8) *vs* PD (60.1±10.1) at day 63, $P > 0.05$]. The concentration of orexin-A in CSF reflects the pathophysiological status of orexin-A neurons. The ELISA assay revealed that there was no significant difference in CSF concentration of orexin-A among various time points post operation (Fig. 3).

While cell bodies are located exclusively in lateral and posterior hypothalamus, orexin-A neurons have extensive projections throughout the whole brain[1]. To verify the results of ELISA assay, fiber staining for orexin-A was further performed in tuberomammillary nucleus (TN), a brain area suggested to be important in the sleep-wakefulness regulation[3]. As illustrated in Fig. 4, orexin-A-ir fibers and endings or varicosities of control and PD rats were similar in density, both in dorsal TN (DTN) and in ventral TN (VTN), at day 49 post operation The gray value measurement revealed that there was no significant difference in fiber density between these 2 groups [control (113.2±10.2) *vs* PD (109.8±12.1) at day 49, $P > 0.05$].

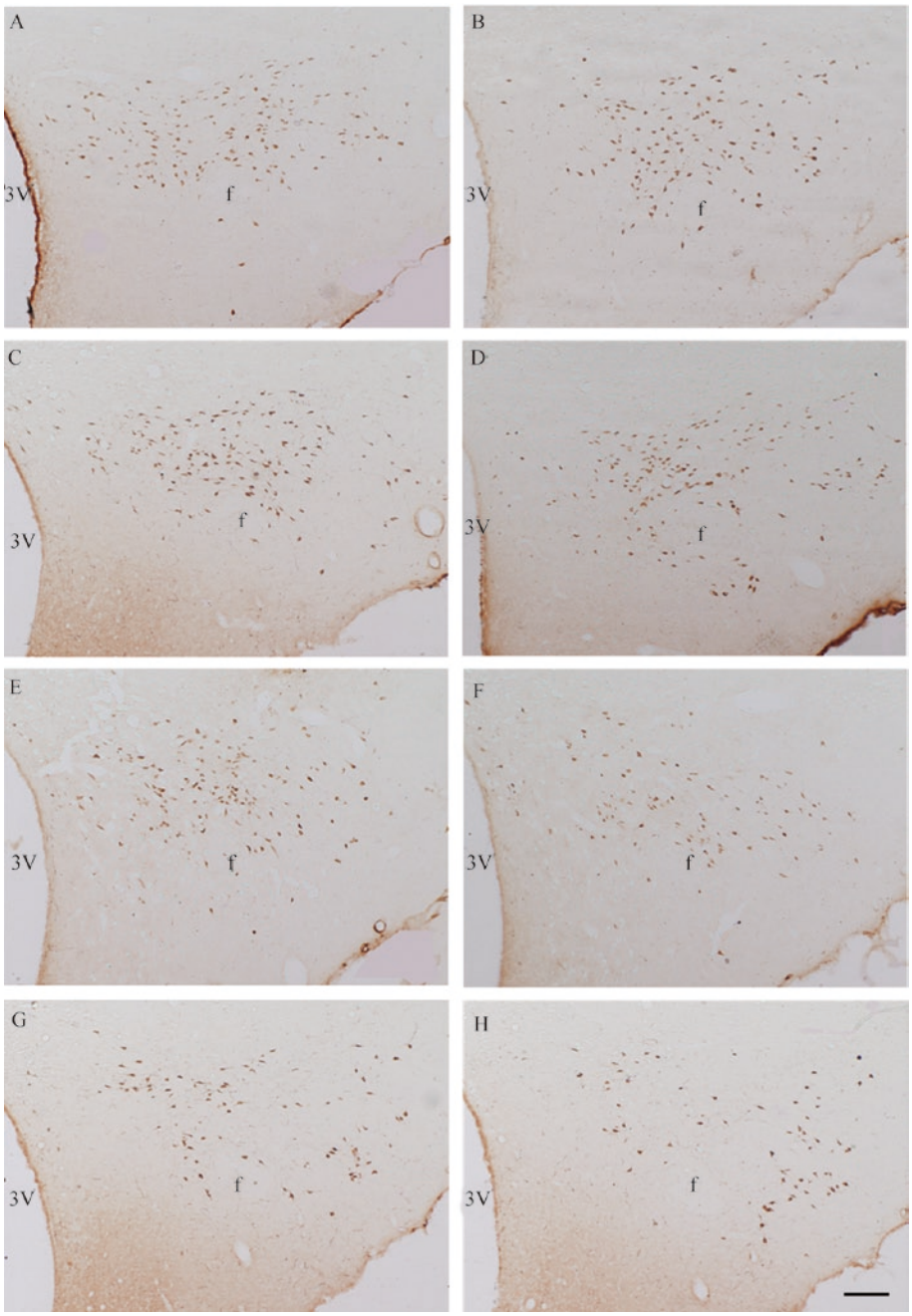

Fig. 1 Immunohistochemical staining of orexin-A neurons in hypothalamus in control (A) and test groups at day 4 (B), day 7 (C), day 14 (D), day 21 (E), day 35 (F), day 49 (G), and day 63 (H) post operation. Results showed the tendency of decrease in orexin-A neurons in hypothalamus during the progression of PD. 3V: the third ventricle; f: fornix. Scale bar, 200 μm.

4 Discussion

Sleep disturbance, which is characterized by excessive daytime sleepiness with frequent naps, often occurs in PD patients[6]. However, the etiology of these symptoms remains unclear. Since dysfunction of the orexin system in hypothalamus is indicated to be the main cause for the primary narcolepsy, numerous studies have been conducted to evaluate the functional status of the orexin system in PD patients. Orexin has 2 isoforms termed orexin-A and -B, which are com-

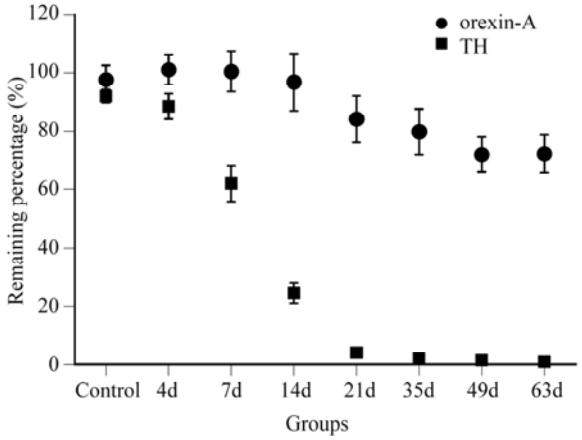

Fig. 2 Percentage of remaining TH- and orexin-A-ir neurons in the injured side of the rat brain subject to 6-OHDA injection. Remaining percentage was defined as the ratio of the number of cells counted in the ipsilateral side of hemisphere to that in the contralateral side (n = 4-6). TH: tyrosine hydroxylase.

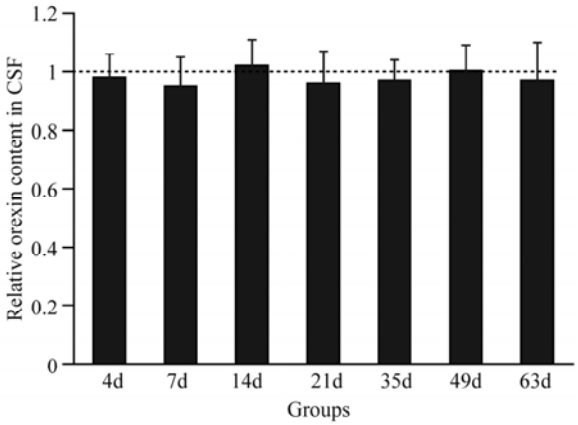

Fig. 3 Relative content of CSF orexin-A among different groups, as revealed by ELISA assay. The relative orexin-A level was calculated as the ratio of experimental P/N value to the control P/N value.

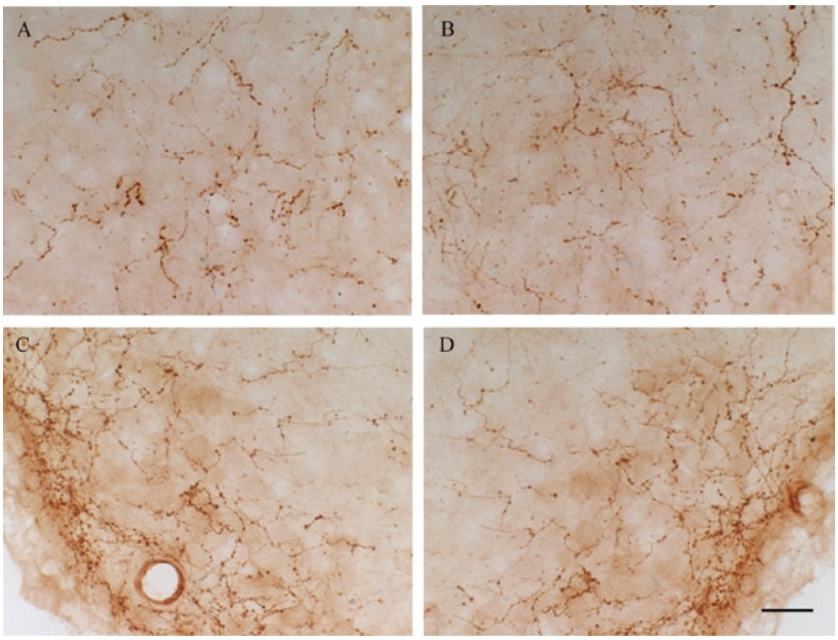

Fig. 4 Immunohistochemical staining of orexin-A-containing fibers in TN of control (A, C) and PD (B, D) rats at day 49 post operation. A, B: dorsal tuberomammillary nucleus; C, D: ventral tuberomammillary nucleus. Scale bar, 50 μm.

posed of 33 and 28 amino acids, respectively[1]. After being synthesized by the neurons located in the lateral and posterior hypothalamus centered by fornix, orexin is thought to be released into the ventricular CSF through elongated axons protruding into the lumens of the third and lateral ventricles[13]. Thus, analyzing the level of orexin in CSF is the most common way to check the function of this system. In an early report by Drouot *et al.*[7], the orexin-A level in advanced PD patients (mean disease duration: 15 years) was only 1/4 of the control level. Actually, this orexin-A level is negatively correlated with the disease severity. In support of this observation, Asai *et al.*[10] have also shown that the decreased

orexin-A level in spinal CSF in PD patients is associated with longer disease duration. Cell number counting in the postmortem brain is a more direct way to observe the function of the orexin system in PD. A recent postmortem study using immunohistochemistry and radioimmunoassay has shown that the total number of orexin-A neurons in 9 PD patients is 50% less than that of the control. Besides, orexin-A level in CSF and tissue concentration of orexin-A in the prefrontal cortex are 25% and 40% lower than the control levels, respectively[8]. By using similar methods, Thannickal *et al.*[9] have also found that neuronal orexin-A loss is positively correlated with the clinical stage of PD (23% and 62% loss for stages I and V, respectively).

Despite the close correlations of orexin level and cell number with the severity or the clinical stage of PD, little information is yet available to elucidate the initial and early developmental dysfunction of the orexin system in PD. By using the rat model of 6-OHDA-induced PD, we systematically investigated the time-dependent loss of orexin-A and TH neurons, and the coincident changes in the orexin-A level in CSF. Our results showed that the number of TH neurons in the midbrain, which was not significantly changed at day 4 after injury, rapidly decreased to approximately 2/3 at day 7 post operation and 1/3 at day 14. At day 21, it almost fell to zero. The time point of half loss was estimated to be approximately at day 9 or 10. This dynamic change of TH neurons after administration of 6-OHDA into MFB is consistent with a previous report[14]. In comparison with this rapid loss of TH neurons, the number of orexin-A neurons started to decrease only at day 21 after surgery, and at day 49, the neuronal loss was about 30%. The number of orexin-A neurons did not further decrease at day 63. However, it seems paradoxical that orexin-A level in CSF in all tested stages did not show obvious changes, and further morphological staining also revealed no essential difference in the density of orexin-A-ir fibers in TN, an area closely involved in sleeping regulation, between control and PD rats. This inconsistency is probably due to the fact that orexin-A level can be partially compensated by the remaining neurons when exposed to the stress of degeneration. After lesioning orexin neurons with neurotoxin orexin-saporin, it was reported that the loss of 73% neurons only caused a 50% reduction in the orexin level of CSF[15], suggesting that the loss of orexin neurons precedes the alteration of orexin level in CSF. This has also been verified in a postmortem study in PD patients, which shows that the 50% loss of orexin neurons is accompanied by its 25% lowered CSF level[8].

Administration of 6-OHDA into SN or MFB is a classic way to establish a chemical lesioned PD model. It is reported that TH neurons would start to die at as early as 12 h after 6-OHDA injection into SN[16]. MFB is composed of projecting axonal fibers from SN to the striatum. Injection of 6-OHDA to MFB could also cause rapid cell degeneration, and the half loss appears within the first 10 d[14]. Apart from the morphological loss of TH neurons, the rats subject to these 2 operations also develop obvious hypokinesia within the first 21 d. Therefore, injection of 6-OHDA into SN or MFB is usually used to reproduce a rat model mimicking the end-stage of PD. Since orexin levels in CSF are reported to be decreased in advanced PD, and the extent of the decrease may be dependent on the disease duration and severity[7,9,10], it is thus not surprising that a detectable orexin-A cell loss occurs at day 21 post operation, the time point preferred by most researchers.

In summary, by using a 6-OHDA MFB injection model, our present study shows that orexin neurons started to die at day 21, the time point when TH neurons were hardly observable. The number of orexin neurons further decreased by approximately 30% at day 49. This is the first study of the dynamic correlations of degenerative changes in orexin-A- and TH-positive neurons in PD models. These findings may provide an important clue for future drug development in the aim of reversing the loss of orexin neurons and thus ameliorating sleep disturbance.

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6-羟多巴诱致帕金森病大鼠模型中 orexin 系统的进行性变化

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摘要：目的 帕金森病(Parkinson's disease, PD)患者经常发生以白天睡眠过多和睡眠猝发为特征的睡眠障碍，其发病机理可能与下丘脑orexin神经元的丢失及其引发的脑脊液中orexin含量降低有关。本文旨在探讨PD进程中orexin系统的动态变化。**方法** 大鼠内侧前脑束中注入6-羟多巴建立PD模型，运用免疫组织化学法对orexin-A阳性和酪氨酸羟化酶(tyrosine hydroxylase, TH)阳性神经元进行染色而后计数，用ELISA法对脑脊液中orexin-A的含量进行检测。**结果** 6-羟多巴能导致中脑TH神经元的快速凋亡，而orexin-A神经元对此变化不敏感。此外，orexin-A神经元数量在术后21天时开始下降，至第49天时下降幅度达到30%左右。与对照组相比，脑脊液中的orexin-A水平并不随PD进程发生明显改变，并且orexin-A阳性纤维在一些睡眠调节相关脑区(如结节乳头体核)内也无明显的数量变化。**结论** 以上结果揭示了orexin系统随PD进程的动态变化情况，对于以逆转orexin神经元丢失和缓解PD病人睡眠障碍为目的的药物研发具有一定的指导意义。

关键词：orexin；帕金森病；睡眠障碍；凋亡