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From Cell Biology to Tissue Engineering

Morphologic changes within the cerebellar cortex in the unilateral 6-hydroxydopamine lesioned rat model for Parkinson disease

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Summary. Parkinson's disease (PD) is a common neurodegenerative disorder caused by the progressive loss of dopaminergic neurons in the substantia nigra. Most investigations have focused on the cerebral regions such as the basal ganglia, thalamus, or the substantia nigra, but whether there is pathologic impairment within the cerebellum has rarely been assessed. Synapsin and neurofilament as the inner markers of neurons and synapses reflect the functional state by their distribution or expression. Significant morphologic changes at the cellular level have been demonstrated directly or indirectly in multiple neurodegenerative diseases. The purpose of this study was to determine whether the behavioral abnormalities that accompany PD are associated with the cerebellum using an *in vivo* 6-hydroxydopamine lesioned rat model. Forty-two rats were divided into three groups, the Parkinsonian group (N=22), sham group (N=10) and control group (N=10). The dopaminergic lesion was determined by immunohistochemical analysis for tyrosine hydroxylase-immunopositive cells. Immunohistochemical studies showed that the density of synapsin I in the granular layer of the cerebellum on both sides of the Parkinsonian -model was not statistically significantly different compared to the control and sham groups. However, expression of neurofilament H in the cortex within bilateral paramedian lobule (PML) and Crus 2 of the ansiform lobule (C2AL) in cerebellum posterior lobe of Parkinsonian rats was decreased compared with controls ($P<0.05$), especially in the loss of Purkinje cells and the presence of morphologic abnormalities in the cell nucleus. The study suggested that loss of neurons and synapses may take place in the cerebellar cortex of Parkinson's disease, and might play an important role in the pathologic mechanism of PD.

Key words: Parkinson's disease, 6-hydroxydopamine, Rat, Cerebellum

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Introduction

Parkinson's disease (PD) is associated with different changes in multiple brain areas, such as motor cortex, basal ganglia, or cerebellum (Houck and Wise, 1995). Investigations of the pathologic mechanism of PD have concentrated mainly on the nigrostriatal system (Jellinger, 2002). For decades, a classic model has demonstrated the role of basal ganglia in modulating cortical function through striatal-thalamo-cortical (STC) circuits (Lewis et al., 2011; Yu et al., 2013), the dysfunction of which may lead to many PD motor symptoms, including bradykinesia and rigidity. It is also known that the cerebellum is an important component in motor control and influences cerebral cortical activity via cerebellar-thalamo-cortical (CTC) circuits (Stoodley and Schmahmann, 2010). It has been postulated that the CTC circuits may provide a potential compensatory mechanism in PD to overcome the deficits in the STC circuits (Cerasa et al., 2006; Lewis et al., 2007), such as

resting tremor (Kassubek et al., 2002). Moreover, the cerebellum has been proven to be correlated with varying degrees of cognitive impairment in PD by several recent functional magnetic resonance imaging studies showing abnormal activation associated with cognitive impairment in different regions of the cerebellum (Yu et al., 2007; Wu et al., 2009; Stoodley et al., 2012). However, the role of the cerebellum in PD pathophysiology is not well understood.

Establishing relevant animal models for human neurodegenerative disorders is crucial for a better understanding of disease pathophysiology and the development of efficient therapies. Currently, the most common animal models of PD are the 6-hydroxydopamine (6-OHDA) rat model and the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) mouse model. These have become the most extensively used tools, and have provided considerable insight into the pathological process of loss of dopamine neurons in the mesencephalic substantia nigra (Faull and Laverty, 1969; Kondoh et al., 2005; Khan et al., 2010). Previous studies have shown that α-synuclein is a structural component of glial cytoplasmic inclusions (GCIs) and Lewy bodies, which are also found in the cerebellar dentate nuclei, in addition to the cerebral cortex and brainstem (Iwanaga et al., 1999), and are a neuronal inclusion prerequisite for a diagnosis of Parkinson's disease (Orimo, 2008; Da Costa et al., 2011). Furthermore, other α-synuclein-positive doughnut-shaped structures have been found occasionally in the cerebellar molecular layer in some patients with PD. This has generated a new disease concept, that of α-synucleinopathies, which may be related to the pathogenesis of many neurodegenerative diseases such as PD (Piao et al., 2003). On the basis of these studies, we believe that the cerebellum has a close relationship with the dyskinesia of PD. Therefore, we hypothesized that the cellular structure within the cerebellum of patients with PD may be disrupted in other ways. The purpose of the present study was to discover the pathological changes at the cellular level in the cerebellar cortex using synapsin (SYP) and neurofilament (NF) as important intrinsic markers of neurons and synapses to evaluate the association both with these alternations by establishing a rat model of PD.

Materials and methods

Animals

Adult Sprague-Dawley rats (male or female, n=66), weighing 220-260 g at the beginning of the experiments, were housed at 20-22°C, 50-60% air humidity, under a 12-hour dark/light cycle. Food and water were given *ad libitum*. Animal care followed the Chinese Community Standard for care and use of laboratory animals, and the protocols for animal experimentation were approved by the Animal Care and Use Committee of the China Medical University.

Surgical procedures

Rats in a Parkinsonian and a sham group were anaesthetized with chloral hydrate (10%, 5 ml/kg), and then placed on a stereotaxic apparatus (ALC-H, Shanghai) with the skull held level between bregma and lambda. A hole 2 mm in diameter was drilled through the skull over the 6-hydroxydopamine (6-OHDA) injection site. According to Paxinos and Watson's rat stereotactic atlas (Franklin and Paxinos, 2007), the coordinates of the right compact part of the substantia nigra are defined as AP- 6.5 mm posterior to bregma, R-2.4 mm from the midline and D- 7.6 mm under the skull surface. The rats in the surgical group (N-p=22) were injected stereotactically with a single injection of 8 µl of 6-OHDA (2 µg/µl dissolved in 0.9% sterile saline containing 0.02% ascorbic acid solution (Sigma, H116). The rats in the sham-operated group (N-s=10) were injected with an equivalent amount of 0.9% saline. The injection micropipette was kept in place for an additional 10 min before removal to minimize back flow and facilitate proper diffusion of drugs. An additional group of animals (N-c=10) was not injected with any drugs and served as a control.

Behavioral test

After the operation, each rat was injected intraperitoneally from 2 to 8 weeks with apomorphine (Sigma) at a dose of 0.5 mg/kg per week. The animal was placed in a 30-cm diameter cylindrical chamber, and motor disturbance was assessed by counting 360° turns per min toward the non-lesioned side over 60 min. Those with levo-spontaneous rotation above 7 per min were considered to be appropriate for the experiment. The control and sham-operated animals showed no preference for counter-clockwise rotations.

Immunohistochemistry

16 weeks after the 6-OHDA injection (N-p=12,N-c=12), the rats were deeply anaesthetized with 10% chloral hydrate, and then perfused transcardially with 0.9% saline and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) by intubating directly into the aorta through the left ventricle after thoracotomy, meanwhile cutting the right atrial appendage using scissors. Brains were carefully removed from the skull, and post-fixed overnight in 4% paraformaldehyde. The cerebellum and the midbrain tissue were stripped out carefully and embedded in paraffin. The tissues were cut into 5 µm thick coronal sections from front to back of the whole cerebellum in a manual rotary microtome (CUT4062, Germany). The sections were incubated in a 1:200 monoclonal anti-Tyrosine Hydroxylase (Abcam, EP1533Y), a 1:500 dilution of primary polyclonal anti-

synapsin I (Millipore, AB1543,USA)/PBS and a 1:200 polyclonal anti-neurofilament H (200kDa) (Millipore, AB1989, USA)/PBS overnight at 4°C. The next day, immunostaining was visualized with either fluorescent secondary or the EliVisionTM plus Test Kit (Maixin Bio, Fuzhou, China). After each procedure, all sections were rinsed 3 times for 5 min in 0.1 M phosphate buffered saline (PBS) in addition to the procedure of the goat serum blocking. Reactions were visualized using 3, 3-diaminobenzidine as a chromogen. For detection of the immunofluorescence staining FITC-goat anti rabbit Ab (1:1000, KAIJI, China) was used as secondary antibody in combination with the primary antibodies. Nuclei were stained with a DNA intercalator named DAPI Staining Solution (C1005, beyotime, China). Finally, tissues were mounted on gelatin-coated slides, air-dried, dehydrated and cover slipped. The stained sections were observed under a light microscope. For each animal, we assessed five sections from the cerebellum (200 μm apart) and three sections from the SN pars compacta of Ventral midbrain tissue (100 μm apart) to cover the tissue partly. Mean optical density of positive staining was measured using the image analyzing computer program Image-Pro Plus Version 6.0 (Media Cybernetics, Bethesda, MD, USA).

Western blot

24 rats were used for the detection in the experiment, the posterior lobe was cut off from the cerebellum and suspended in 800 μl lysis buffer (50 mM Tris- HCI, pH 7.4; 175 mM NaCl; 5 M EDTA, pH 8.0; and protease inhibitor [Roche]) and homogenized totally on ice for 20 seconds, 2 or 3 times at 30-second intervals. Each sample was centrifuged at 12000 rpm for 15 min twice at 4°C, and the supernatants were stored at -80°C for subsequent protein measurements. Lysates from each group were pooled (n=3 per group), and protein concentrations were determined by the BCA Protein Assay Kit (Beyotime, China). Blue/Orange 6× Loading Dye (Promega, WI, USA) was added and boiled at 70°C for 10 min, and the samples were subjected to electrophoresis in a 7.5% SDS-polyacrylamide gel and transferred electrophoretically to a Hybond-P pure nitrocellulose membrane (Wu et al., 2013). The membranes were probed at 4°C overnight with primary rabbit anti- synapsin I antibody (AB1543, Millipore, 77 kda) 1:800 and neurofilament H (AB1989, Millipore, 200 kda) 1:300, and exposed the next day to horseradish peroxidase-conjugated secondary antibodies for 1h. Signal development was achieved using an ECL plus Detection kit (mixed Super Signal West Pico Luminol/Enhancer Solution and Super Signal West Pico Stable Peroxide Solution) The bands of these two targets were computer-processed into binary images for consequent statistical analyses. In order to improve the reliability of the results, we pooled 3 samples of the cerebellar tissue extracts from animals in each group at 16 weeks after injection. Four repeat tests were fully conducted.

Statistical analysis

Data were presented as mean ± S.E.M. The Statistical Package for the Social Sciences (SPSS17.0, Chicago, IL, USA) software was employed. The results of SYP and NF were analyzed statistically using a one-way ANOVA, and a two-sample t-test model was used for comparisons between groups. A Paired-sample T test analysis was used in the Statistical evaluation of the loss of optical density of positive staining for tyrosine hydroxylase (TH) in the SN. A P value of <0.05 was considered statistically significant.

Results

Loss of tyrosine hydroxylase in the substantia nigra

We used the tyrosine hydroxylase (TH) staining of the substantia nigra to determine whether or not lesions had been established successfully in the animals in the surgical group. Animals with the same quantity of positive expression of TH compared to the non-lesioned side suggested failed or misplaced injections of 6-OHDA and were excluded from the analysis. Only animals with marked loss of TH-positive dopaminergic neurons in the lesioned side of the substantia nigra (SN) compared to the non-lesioned side were enrolled in the study (Fig. 1A,B), and a quantitative statistical evaluation of TH-positive neurons was shown in Fig. 1E. At high magnification, the positive expression of dopamine cells and nuclei staining by immunofluorescence are displayed more clearly on both sides (Fig. 1C,D). This is consistent with the Research of Kondoh et al. (2005).

HE staining of the cerebellum

The molecular layer (ML), the Purkinje cell layer (PKL) and the granular layer (GL) that are arranged from outside to inside of the cerebellar cortex could be clearly displayed by hematoxylin-eosin staining in normal rats of the control group. (Fig. 2). Different cerebellar lobe structures were distinguished under microscope according to the stereotaxic atlas of the rat brain, including the vermian lobule (VL I-X), flocculus (FL), paraflocculus (PFL), copula of the pyramis (COP), simple lobule (SML), paramedian lobule (PML), crus 1 of the ansiform lobule (C1AL) and the crus 2 of the ansiform lobule (C2AL).

Immunohistochemistry of the cerebellum

We evaluated the positive expression of SYP and NF on both sides of the cerebellar hemispheres in the Parkinsonian rat model and the control rats. The expression of SYP was concentrated mainly in the granular layer of the cerebellar cortex, but was minimal

in the molecular layer and the Purkinje cell layer. Overall, the average amount of SYP in cerebellar tissue was not statistically significantly different between the Parkinsonian model rats (0.0526±0.0102) and the controls (0.0609±0.0089) using a two-sample t-test (P>0.05) (Fig. 3B,C). The quantification result is shown in Fig. 4A.

Another antibody we detected was NF, which is also distributed at different layers of the cerebellar cortex. PKL is the main area of staining, where the positive sites are located in the somas or the proximal axons of Purkinje cells in the PKL, and the dendrites extend regularly to the ML. After analysis, we discovered that the quantification of NF-positive sites in PKL within the cerebellum in the Parkinsonian model rats (0.07256± 0.0155) was decreased compared with the sham group

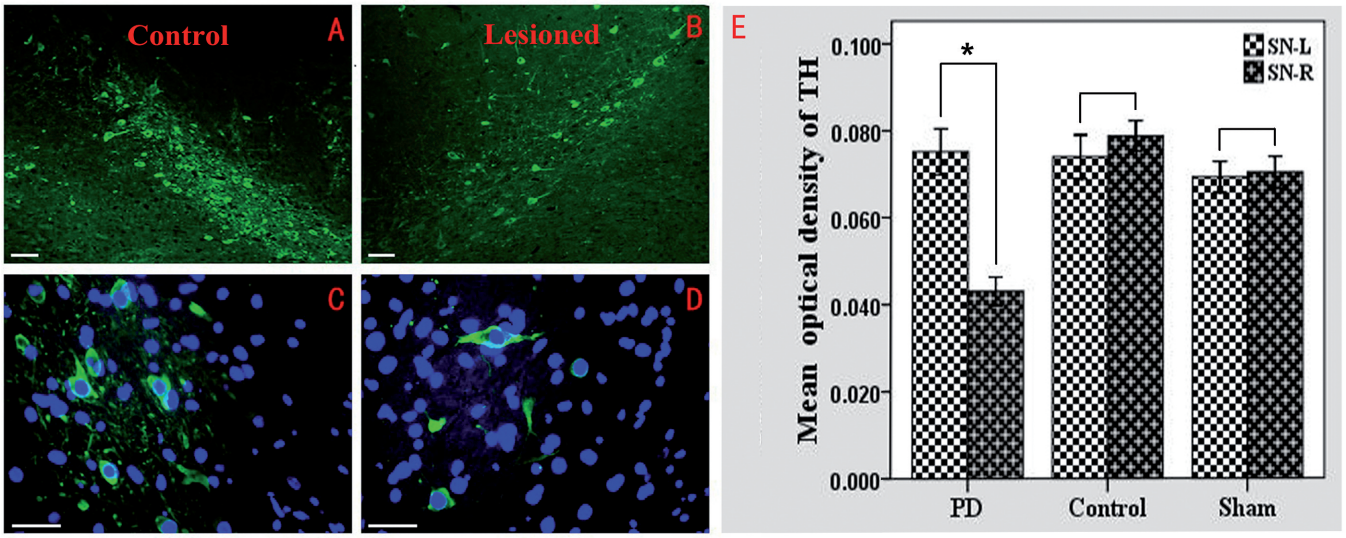

Fig. 1. Cellular localization of tyrosine hydroxylase in the substantia nigra on both sides of the brain. 6-hydroxydopamine-induced lesions resulted in a significant loss of TH-positive neurons in the ipsilateral side **(B)** compared to the contralateral side in Parkinsonian group **(A)**. Merged images of dopamine cells and cell nucleus are shown at high magnification in **C** and **D**, respectively representing the contralateral side of injection and the ipsilateral side. The analysis result of mean optical density of TH is shown in **E**, Data are presented as means ± SEM.*: P<0.05. Scale bars: A, B, 100 μm; C, D, 50 μm.

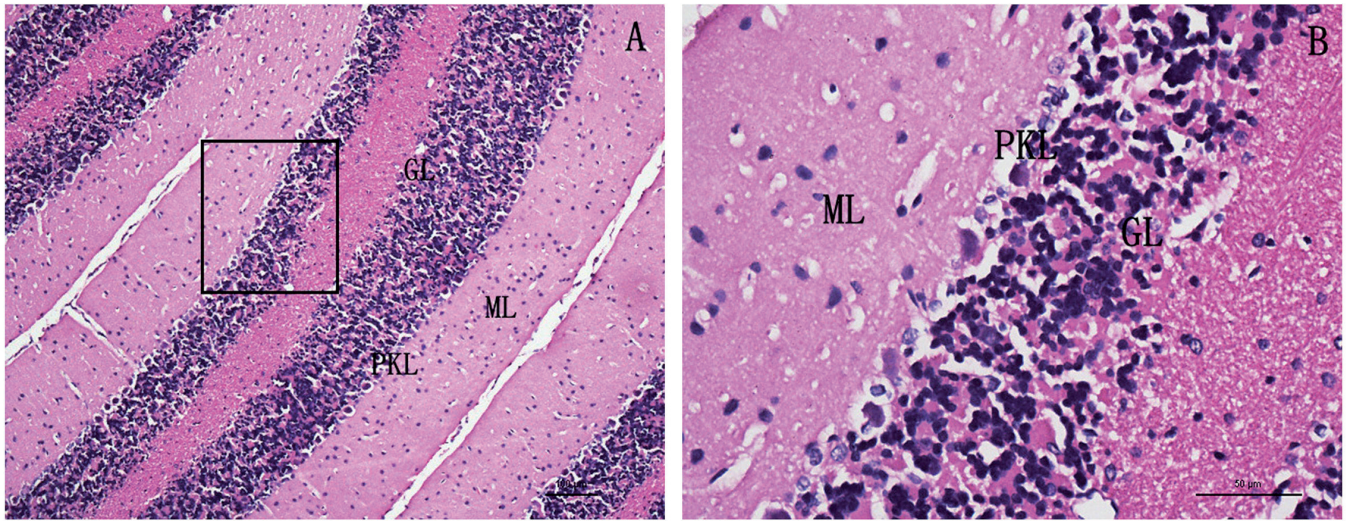

Fig. 2. HE staining of the cerebellum. The staining on coronal sections in the control group showing the molecular layer (ML), granular layer (GL) and Purkinje cell layer (PKL). Scale bars: A, 100 μm; B, 50 μm.

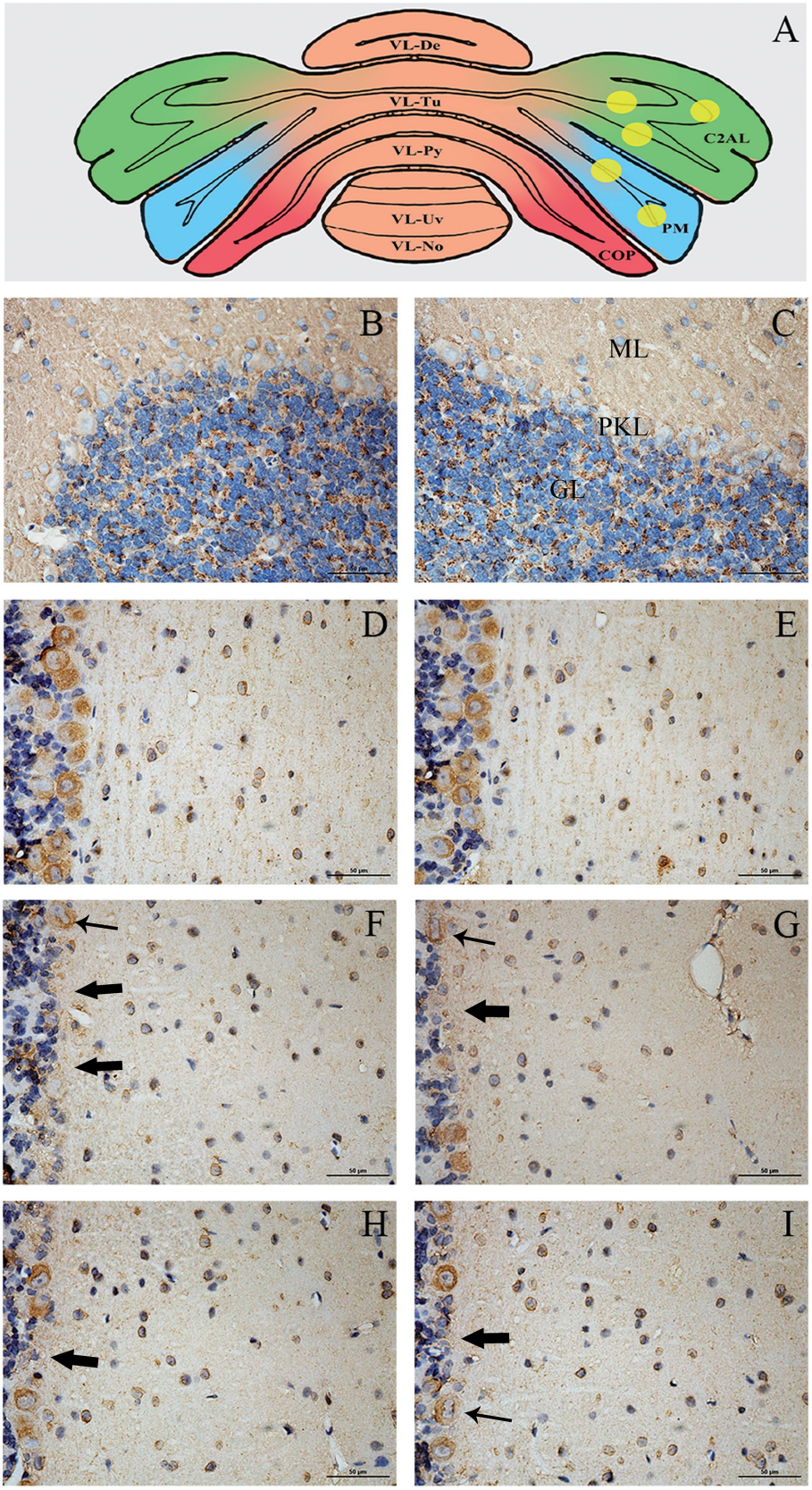

Fig. 3. Immunohistochemistry of rat cerebellar cortex with SYP and NF. **A.** Schematic drawing of the rat cerebellar posterior lobe showing the abnormal regions (Yellow circle). Positive expression of synapsin (SYP) in the granular layer (GL) of the Parkinsonian group **(B)** and control **(C)**. Positive sites of neurofilament H (NF-H) in the ML and PKL in the cerebellar cortex of Parkinsonian rat model and control groups **(D-I)**. **D, F, H,** and **E, G, I** showing NF staining in PML and C2AL of different groups respectively (**D, E**: control group**; F, G, H, I**: PD group),which show loss of PK cells (thick black arrow) and abnormal morphology in cell nuclei (thin black arrow). Scale bars: 50 μm.

(0.0937±0.0187) and controls (0.0964±0.0165) ($P<0.05$), but mainly in the cerebellar posterior lobe, especially in the bilateral paramedian lobule and the right Crus 2 of the ansiform lobule (Fig. 3A). Losses of the Purkinje cells and abnormal cell morphology have taken place in these regions (Figs. 3F-I, 4B). Although the positive sites of NF (0.0164±0.0038) in ML of the Parkinsonian group were sparse compared with the control group (0.0171±0.0033), the difference was not statistically significant (Fig. 4B).

Expression of SYP and NF

Finally, we quantified the expression of SYP and NF in cerebellar posterior lobe under control and experimental conditions. Quantitative Western blot analysis of SYP expression was performed 16 weeks after 6-OHDA injection. As we expected, the level of SYP was not altered in the posterior lobe of cerebellum of Parkinsonian model rats compared with controls (Fig. 5A). By contrast, the expression level of NF detected in the Parkinsonian group was mildly decreased compared to the control group, which was consistent with the results of immunohistochemistry (Fig. 5B).

Discussion

The cerebellum has traditionally been considered as an important brain area that plays a pivotal role in posture, balance, the coordination of movement, and even emotion and cognition (Schutter and Van Honk, 2005). It is functionally divided into several parts that include the flocculonodular lobe, anterior lobe and posterior lobe, inside of which there are a vast number of synaptic neurons that are extensively interconnected (Ito, 2006). It has been suggested that this neural network connects with the cerebral cortex through different deep cerebellar nuclei (DCN) and neural circuits to regulate motor behavior (Sanchez-Campuzano et al., 2007; Brunamonti et al., 2014). Numerous studies have shown that cerebellar dysfunction plays a specific role in many neuropsy-chiatric disorders, such as autism, schizophrenia, anxiety disorders, and even Parkinson's disease (Hoppen-brouwers et al., 2008). It has been found that the cerebellum is involved in the progression of Parkinson's disease through the cerebellar-thalamo-cortical (CTC) circuits as a compensatory mechanism for the defective basal ganglia, and that the dentate nucleus of the cerebellum is particularly important. Research in these areas is concentrated mainly in functional magnetic resonance imaging or positron emission tomography studies, which have demonstrated that the cerebellum showed enhanced or decreased connectivity with bilateral cerebral regions using a variety of motor tasks or in the resting state in patients with PD (Stoodley, 2012), but the pathological changes in the cerebellum in PD are not well understood.

Studies have confirmed that SYP and NF can be used as internal markers that reflect neuronal and synaptic functional states (Bloom et al., 2003; Vasin et al., 2014). Abnormalities of SYP and NF have been demonstrated directly or indirectly in multiple neurodegerative diseases. Abnormalities of both in neurodegenerative disorders are a hallmark of neuronal

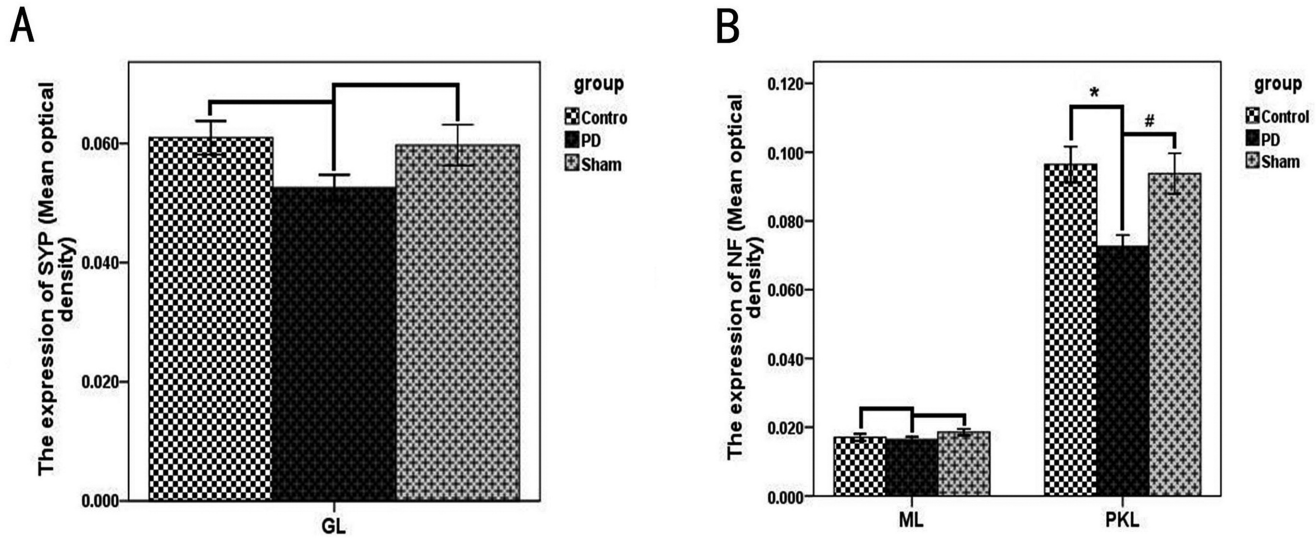

Fig. 4. Quantitative analysis of SYP and NF in different parts of the cerebellum. **A.** Expression of SYP in GL within the rat cerebellar cortex of the three groups. **B.** Quantification analysis shows the NF expression level in PKL and ML within the cerebellum of Parkinsonian, sham and control group, denoting statistical differences between the groups (N=6 per group), Data are presented as means ± SEM. *$P<0.05$ vs. control and #$P<0.05$ vs. sham group. GL, granular layer; ML, molecular layer; PKL, Purkinje cell layer.

dysfunction, especially axonal degeneration. Synapsin protein localizes to presynaptic terminals of neuronal cells, and regulates neurotransmitter release by modulating the assembly of a soluble acetylcholine-sensitive factor attachment protein receptor complex. Neurofilaments are intermediate filaments in neurons composed of three subunits (NF-L, NF-M and NF-H) which play an important role in determining neuronal shape and axonal caliber, and maintain the structural integrity of neurons (Nixon and Sihag, 1991; Perrot and Eyer, 2009). The three NFs are differentially expressed during development. NF-L and NF-M are expressed during the early stages of synaptogenesis and axon targeting, while NF-H is predominantly expressed in the postnatal brain and specifically during circuit stabilization (Carden and Trojanowski, 1987). In the cerebellum cortex, NF is expressed in Purkinje cells (Demilly et al., 2011), while the SYP is mainly in the granular layer zone. NF abnormalities have been shown to be an important factor in the cellular disruption observed in several neurodegenerative diseases by means of the abnormal phosphorylation of neuronal cytoskeletal proteins, which may also affect NF function in turn (Louis et al., 2012).

In our study, the results of immunohistochemistry showed that positive sites staining for SYP in the molecular layer (ML) and granular layer (GL) were not statistically significantly different between the Parkinsonian group and the sham or the control groups. As we know, Synaptophysin is located in the presynaptic terminal, which is a specialized subcellular structure containing synaptic vesicles that facilitates vesicle fusion and the release of neurotransmitters into the synaptic cleft under the condition of receiving Activation signals. This staining result is due to the fact that ML and GL contain a large number of synapse structures, including basket cells, stellate cells and Golgi cells (Li et al., 2010). In the related study, we only found a decrease in number of Purkinje cells, but not any other types of cells changing in morphology, thus leading to the decreased expression of NF in the PKL of the Parkinsonian rat model. Why was that? The reason we considered is that the damage to the substantia nigra by 6-hydroxy-dopamine has destroyed a direct connection between mesencephalic dopaminergic neurons and the cerebellar cortex, or it could be compensatory mechanisms with the cerebellum for motor symptom changes induced by denervation of the substantia nigra. The rats in the Parkinsonian group of our experiment have shown a series of slight movement disorder symptoms including ataxia and balance disorder about five months after the surgery. Abnormal phospho-rylation, oxidative stress, inflammation or other mechanisms might take place with the neuronal cytoskeletal proteins, which is considered to lead to disturbed NF assembly in Purkinje cells, and it may also affect NF functions during circuit stabilization (Petzold, 2005; Rudrabhatla et al., 2010). In some animal experiments, basal ganglia and cerebellar input show decreased neuronal firing in the ventral thalamic areas after dopamine depletion (Rolland et al., 2007), and the level of dopaminergic neuronal loss in substantia nigra pars compacta correlated with a persistent hyper-excitation of the Purkinje cells in the cerebellar cortex (Heman et al., 2012). The Purkinje cells are GABAergic

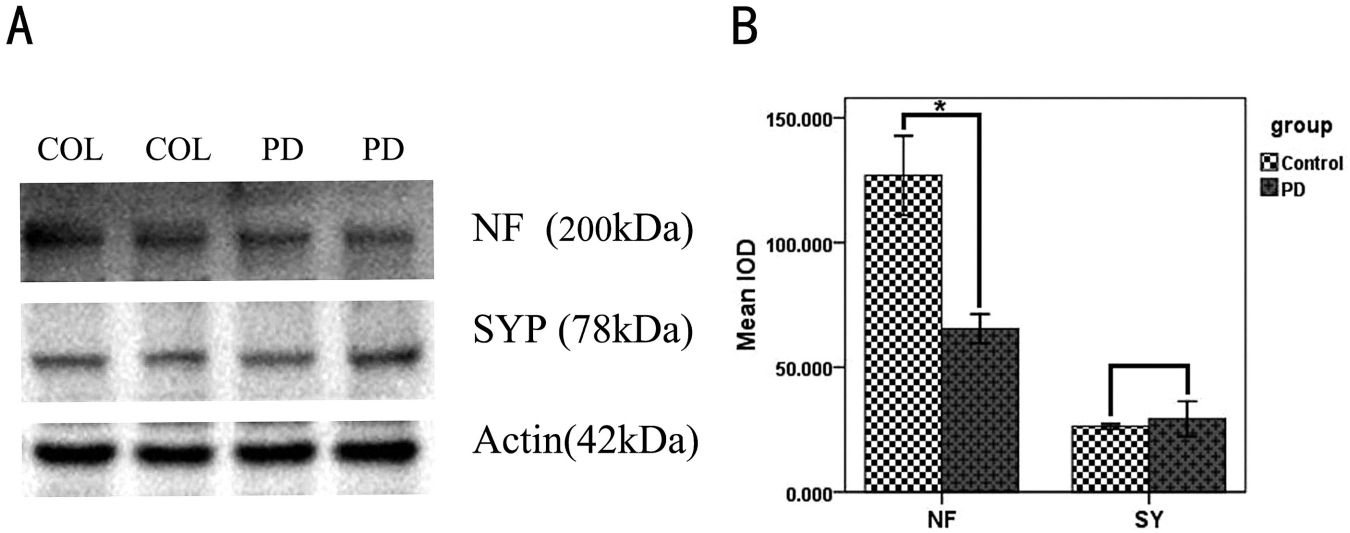

Fig. 5. Quantitative Western blot analysis of NF and SYP expression in different groups. Pulled samples were extracted from the rats at 20 weeks after injection. **A.** Expression level of NF in the Parkinsonian group decreased compared to the control group, but SYP was not significantly different between each group. **B.** Mean IOD measured using the image analyzing computer program Image-Pro Plus Version 6.0 (N=4 per group). Data are presented as means ± SEM.*: P<0.05 vs. control.

and they are the main output cells of the cerebellum, the loss of which may seriously impair cerebellar-thalamo-cortical communication. An inhibitory effect from Purkinje cells projects to the primary motor cortex (M1) via the dentate nucleus and ventral lateral thalamus reduces the excitatory output to the motor cortex that leads to modification of motor control (Holdefer et al., 2000). That is, a reduction of Purkinje cells is expected to result in disinhibition of the DCN, which leads to increased excitatory output to the thalamus with a concomitant increase in thalamo-cortical output (Belmonte et al., 2004a,b). Also, It is known that a protein named DARPP- 32 that is regulated by dopamine and adenosine 3-5-monophosphate (cAMP) is expressed in the cerebellar Purkinje cells, which is involved in the regulation of a variety of neurotransmitters and signal pathway transduction (Alder and Barbas, 1995). Therefore, we deduce that the decrease of Purkinje cells has resulted in unusual behavioral changes in the rat model of Parkinsonian and these pathological changes might be associated with the motor impairments of Parkinson's disease. Another phenomenon that has attracted our attention is that the loss of Purkinje cells in posterior lobe of the cerebellum were mainly concentrated in bilateral paramedian lobule (PML) and Crus 2 of the ansiform lobule (C2AL). A range of deficits that from motor to nonmotor symptoms caused by lesions of the cerebellum often depend on the location of the lesion (Strick et al., 2009). Many reports support the theory that CPL is widely used to adjust nerve function such as cognition, emotion and coordinate movement (Desmond and Marvel, 2009; Stoodley and Schmahmann, 2010), and especially the PML of the CPL is accepted that receives somatosensory information from the spinal cord and is involved in the control of limbs movement (Ito, 1984), so the abnormality of these areas are likely to affect motor impairments in the later stage, and suggest that the cerebellum may be recruited to overcome denervation of striatal networks.

Compared with the control group, the detection of the SYP expression in the GL of the parkinsonian group was not significantly statistical different, while, a new concept- α-synucleinopathies, which were occasionally found in the cerebellar ML of some PD patients with α-synuclein-positive doughnut-shaped structures, may have relationship with the pathogenesis of Parkinson's disease (Piao et al., 2003). One of the main limitations is that animal models could only mimic parts of the Parkinsonism symptoms, which attribute to the different pathogenesis and disease duration between animal experiments and PD patient. The unilateral 6-hydroxydopamine lesioned rat model just proved that the degeneration of the nigrostrial connection could result in dramatic changes in the dopaminergic pathway. Therefore, it may help to explain the pathogenesis of Parkinson disease to some extent. Because pathologic material is difficult to obtain from patients with PD, so the researches of animal experiments are becoming the best alternatives.

In this study, we chose the same batch of rats in the experiment in order to avoid the problem that the amount of SYP and NF protein may decrease with age in rats, so improving the reliability of the results. Because this was a preliminary study, we only found morphological changes of the PKL in some parts of the bilateral cerebellar cortex in the rats. Further research is still needed to determine whether or not there is some correlation between the injured side of the cerebellum and the contralateral side with the specific symptoms of PD. We speculate that Purkinje cell zone might be a key step required for the construction of functional cerebellar circuits, which may provide a new developmental milestone on the road of exploring the Pathogenesis of Parkinson's disease.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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