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Original Article

Distribution characteristics of vesicular monoamine transporter in brain of mice with Parkinson disease

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Abstract: Objective: To investigate the distribution of vesicular monoamine transporter ($VMAT_2$) in PD model of C57BL mice brain and their relationships with Parkinson disease. Methods: PD model of C57BL mice induced by reserpine or MPTP or reserpine plus MPTP was used respectively. The distribution of $VMAT_2$ and TH in the substantia nigra pars compacta (SNc), the striatum, ventral tegmental ares (VTA) and locus coeruleus (LC) were examined by immunohistochemical staining. Results: The number of $VMAT_2$-positive neurons and the number of TH-positive neurons in SNc were significantly reduced in PD model than the control, but there was no great difference in VTA and LC between PD model and the control. $VMAT_2$-positive neuron fibers and TH-positive neuron fibers in the striatum region were significantly reduced in PD model. The distribution of $VMAT_2$ in the control in SNc was less than that in both VTA and LC. Conclusion: The amount of $VMAT_2$ that has protective effect on the DA neurons in SNc is less than that in both VTA and LC. The week of the protecting function of $VMAT_2$ in SNc may be the important cause of the selective DA neurons loses.

Keywords: Parkinson disease, $VMAT_2$, immunohistochemistry, tyrosine hydroxylase, C57BL mice

Introduction

Parkinson disease (PD) is a relatively common neurodegenerative disease in middle and elderly people with the characteristic pathological change of progressive degeneration and loss of dopaminergic (DA) neurons in the substantia nigra pars compacta in midbrain, but its pathogenesis has been still unknown yet. It was recently found that vesicular transport abnormality of neurotransmitter DA in nigrostriatal system plays an important role in the pathogenesis of PD, while vesicular monoamine transporter ($VMAT_2$) plays a key role in the transport of neurotransmitter DA [1]. $VMAT_2$ can hide 1-methyl-4-phenylpyridinium (MPP^+), an active metabolite of neurotoxin 1-methyl-4-phenyl-tetrahydropyridine (MPTP), in the synaptic vesicles to protect DA neurons [2, 3]. In this study, distribution of $VMAT_2$ in mice model was observed to explore the role of $VMAT_2$ in pathogenesis of PD.

Materials and methods

Animal grouping

A total of 24 healthy male C57/BL brown mice (Supplied by Shanghai Laboratory Animal Center, Chinese Academy of Sciences) at the age of 8 to 10 weeks, weighing (20±3) g, were randomly divided into four groups, including MPTP group, reserpine group, reserpine + MPTP group and control group, with 6 in each group. Mice in the control group were intraperitoneally injected saline at the same volume for 7 consecutive days. The mice acted as usual after injection.

Establishment of MPTP-induced mice model of PD: MPTP (Sigma, USA) was dissolved in saline and intraperitoneally injected at 30 $mg \cdot kg^{-1} \cdot 24\ h^{-1}$ for 7 consecutive days. After administration, movement disorders, such as bradykinesia, tremor, piloerection and poor response to external stimuli, at varying degrees were observed in

all mice. With the increasing of MPTP dosage and duration of administration, movement disorders in mice aggravated and duration of symptoms increased. Except bradykinesia, symptoms completely disappeared after 24 hours.

Establishment of reserpine-induced mice model of PD: Reserpine (Shanghai Hongqi Pharmaceutical Factory) was dissolved in saline and intraperitoneally injected at 1 $mg{\cdot}kg^{-1}{\cdot}24\ h^{-1}$ for 7 consecutive days. After administration, movement disorders, such as bradykinesia, tremor, piloerection and poor response to external stimuli, at varying degrees were observed in all mice. With the increasing of reserpine dosage and duration of administration, movement disorders in mice aggravated and duration of symptoms increased. Except bradykinesia, symptoms completely disappeared after 24 hours.

Establishment of reserpine + MPTP-induced mice model of PD: Reserpine was intraperitoneally injected at 1 $mg.kg^{-1}$. $24\ h^{-1}$ firstly and 2 h later, MPTP was intraperitoneally injected at 30 $mg.kg^{-1}$. $24\ h^{-1}$ for 7 consecutive days. After administration, the movement disorders occurred earlier and also lasted longer.

Sacrifice of mice and specimen collection

Three days after the last injection, mice were anesthetized with intraperitoneal injection of 2% pentobarbital, and heart was exposed through thoracotomy and washed with 50 ml of normal saline through left ventricle. 4% of paraformaldehyde-PB solution (0.1 mol/L, pH 7.4) at 4°C was infused into the heart for maintaining 30 min. Then, the whole brain was taken out rapidly through craniotomy and immersed into 4% paraformaldehyde at 4°C for 24 hours. Based on the mouse brain atlas, corpus striatum, substantia nigra, ventral tegmental area (VTA) and locus coeruleus (LC) were separated in sequence and their coronal sections were made, dehydrated, clearing, embedded in paraffin and sliced with 4 μm thickness.

$VMAT_2$ and TH immunohistochemistry (SP method) [4]

Slices were routinely deparaffinized. After thermal remediation, 3% H_2O_2 was used to block endogenous peroxidase for 10 min and normal goat serum working solution was used to close for 30 min. Then, $VMAT_2$ polyclonal antibody (Chemico, USA) and TH monoclonal antibody (Sigma, USA) at a dilution of 1:100 were respectively dropwise added on the slices and stayed overnight in refrigerator at 4°C. The slices were eluted with PBS for 5 min × 3 times, and secondary antibody (anti-rabbit/mouse IgG) working solution was added on the slices; the slices were incubated in incubator at 37°C for 60 min. The slices were eluted with PBS for 5 min × 3 times, and horseradish peroxidase conjugated streptavidin working solution was added on the slices; the slices were incubated in incubator at 37°C for 60 min. The slices were eluted with PBS for 5 min × 3 times, and were stained by using 0.04% 3,3-diaminobenzidine (DAB), stained contrastively by hematoxylin, dehydrated with alcohol, cleared by xylene and fixed with neutral gum. Observation and radiography were conducted under microscope. PBS was used to instead of primary antibody for negative control and other operations were the same as the above. Result was positive if cytoplasm and processes were tan.

Image analysis

LEICA color pathological image analysis system was conducted to analyze the number of $VMAT_2$ and TH positive cells in brain slices of substantia nigra pars compacta (SNC), VTA and LC during morphological image analysis and data processing. There were 3 brain slices for each part in mice. The counting was conducted under high-power microscope. All positive cells were counted. The total count of positive cell in 3 brain slices in each mouse was calculated to take an average. $VMAT_2$ and TH positive nerve fibers in the striatum area were determined using mean gray value. Meanwhile, mean gray value of positive $VMAT_2$ at each group was determined (Note: The greater the mean gray value was, the lower the intensity of immunostaining was and the lower the content of the material was).

Statistical analysis

Experimental data were expressed as mean ± standard deviation ($\bar{x} \pm s \backslash s$) and the comparison between groups was analyzed using analysis of variance. SPSS10.0 statistical software was employed and $P < 0.05$ was considered statistically significant.

Table 1. Comparison of the number of $VMAT_2$ positive cells in each group

Group	SNC	VTA	LC
Reserpine group	48.38±4.52*	130.35±14.67	87.32±9.48
MPTP group	32.64±5.26*,#	122.19±13.57	83.75±8.68
Reserpine + MPTP group	28.83±5.58*,#	118.72±13.45	81.21±8.56
Control group	79.36±8.76	131.24±14.36	86.63±9.14

When compared with control group, *P < 0.05; when compared with reserpine group, #P < 0.05.

Table 2. Comparison of the number of TH positive cells in each group

Group	SNC	VTA	LC
Reserpine group	45.89±6.78*	133.36±13.67	92.37±9.87
MPTP group	30.42±4.41*,#	128.38±14.16	88.25±10.23
Reserpine + MPTP group	27.63±4.76*,#	122.64±13.87	84.31±9.18
Control group	84.45±9.23	136.58±14.29	90.34±9.68

When compared with control group, *P < 0.05; when compared with reserpine group, #P < 0.05.

Table 3. Comparison of gray values for $VMAT_2$ and TH positive nerve fibers in the striatum area in each group

Group	$VMAT_2$	TH
Reserpine group	160.23±5.81*	159.89±5.07*
MPTP group	170.63±4.84*,#	167.39±4.88*,#
Reserpine + MPTP group	172.89±4.78*,#	169.55±4.83*,#
Control group	146.25±4.72	142.13±5.05

When compared with control group, *P < 0.05; when compared with reserpine group, #P < 0.05.

Table 4. Gray value for $VMAT_2$ in each group

Group	SNC	VTA	LC
Reserpine group	167.38±4.52*	133.35±4.67	122.32±4.48
MPTP group	175.49±5.26*,#	134.19±5.57	123.75±4.68
Reserpine + MPTP group	179.83±6.15*,#	135.72±5.45	124.21±5.26
Control group	155.36±5.76**	132.24±5.36	121.63±5.14

When compared with control group, *P < 0.05; when compared with reserpine group, #P < 0.05; when compared with VTA and LC, **P < 0.05.

Results

Results of $VMAT_2$ immunohistochemical staining

As shown in **Table 1**, the number of $VMAT_2$ positive cells in SNC in experimental group significantly reduced when compared with that of control group (P < 0.05); the number of $VMAT_2$ positive cells in VTA and LC in experimental group showed no significant reduction when compared with that of control group (P > 0.05); the comparisons among reserpine group, MPTP group and reserpine + MPTP group indicated that $VMAT_2$ in SNC reduced more significantly (P < 0.05).

Results of TH immunohistochemical staining

As shown in **Table 2**, the number of TH positive cells in SNC in experimental group significantly reduced when compared with that of control group (P < 0.05); the number of TH positive cells in VTA and LC in experimental group showed no significant reduction when compared with that of control group (P > 0.05); the comparisons among reserpine group, MPTP group and reserpine + MPTP group showed that TH in SNC reduced more significantly (P < 0.05).

Results of gray values for $VMAT_2$ and TH positive nerve fibers in the striatum area

As shown in **Table 3**, the difference of gray values for $VMAT_2$ and TH positive nerve fibers in the striatum area between experimental group and control group was significant (P < 0.05); moreover, the differences among reserpine group, MPTP group and reserpine + MPTP group were also significant (P < 0.05).

Results of gray value for $VMAT_2$ in SNC, VTA and LC

As shown in **Table 4**, the difference of gray value for $VMAT_2$ in SNC between experimental group and control group was significant (P < 0.05); while, the differences of gray value for

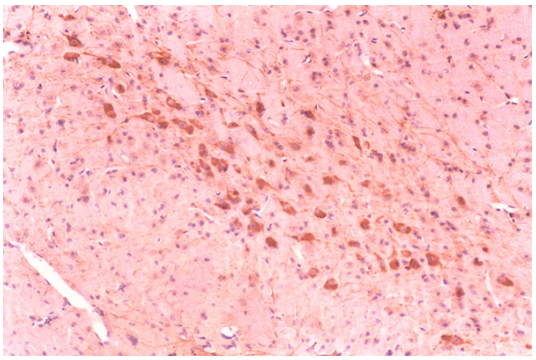

Figure 1. Expression of $VMAT_2$ in mice SNC in control group (Immunohistochemistry SP method, × 200).

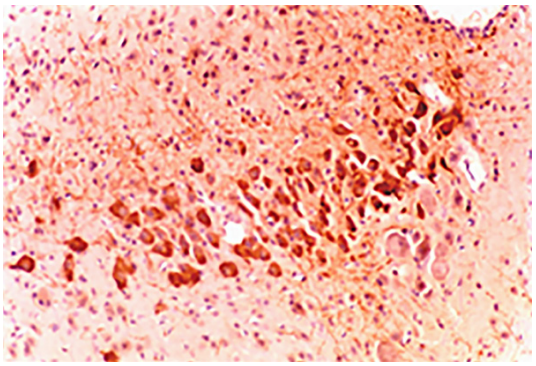

Figure 2. Expression of $VMAT_2$ in mice LC in control group (Immunohistochemistry SP method, × 200).

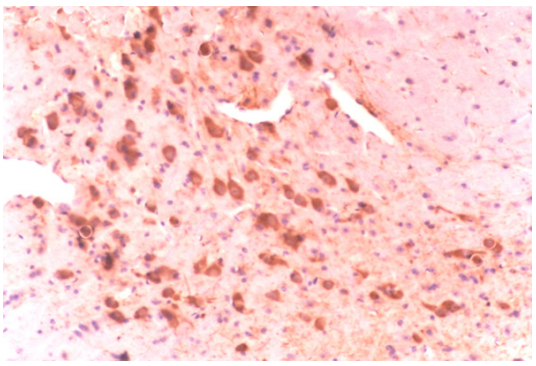

Figure 3. Expression of $VMAT_2$ in mice VTA in control group (Immunohistochemistry SP method, × 200).

$VMAT_2$ in VTA and LC between experimental group and control group was not significant ($P > 0.05$); the comparisons between reserpine group, MPTP group and reserpine + MPTP group indicated that $VMAT_2$ in SNC reduced more significantly ($P < 0.05$); moreover, it was found that the distribution of $VMAT_2$ in control group in SNC was less than that in both VTA and LC ($P < 0.05$) (**Figures 1-3**).

Discussion

There are many theories about the pathogenesis of PD, such as environmental toxins, genetic susceptibility and neurotransmitter theories. But these theories can't provide convincing explanation on PD with selective DA neurons damage and the progressive course of the disease. MPTP is a potent and selective dopaminergic neurotoxin and it has been proved from a large number of studies that MPTP can cause a significant reduction of the content of DA in the striatum area and of the number of dopaminergic neurons in SNC. Neurotoxicity mechanism of MPTP has been demonstrated that MPTP is converted to the active metabolite MPP^+ under the action of monoamine oxidase B and then MPP^+ is actively transported to the mitochondria by the selective uptake of DA neurons, leading to degeneration and apoptosis of DA neurons [5-7]. Systemic administration of MPTP to monkeys and C57BL mice induces neurobiochemical and neuropathological changes, which are very similar to those in humans with PD. Therefore, MPTP model is widely accepted as the ideal animal model for the study of primary PD [8]. As an effective antihypertensive drug that produces side effects similar to depression, Reserpine is a specific and potent $VMAT_2$ inhibitor [9] that depletes catecholamines in the central and peripheral sympathetic nerve endings and competes with the monoamine neurotransmitters for the binding on the recognition site on vesicular transporter at a very low concentration, almost an irreversible binding, so as to effectively inhibit the vesicular transport of monoamine neurotransmitters. Administration of large dose of reserpine can cause a series of symptoms similar to paralysis agitans in laboratory animals, which, therefore, can be applied to establish the mice model of PD [10]. Pathological change of PD is a selective DA neurons damage in substantia nigra. Plasmalemma transporters in DA neurons with dopamine transporter (DAT), adrenergic neurons and serotonin neuron on all cell membranes can transport MPP^+ into cells [11], but only DA neurons in substantia nigra exhibit sensitivity to MPP^+. The reason is that $VMAT_2$ has the function of hiding the transport of MPP^+ and other neurotoxins in cytoplasm

secretory vesicles, so that it can't produce toxic effect on cellular materials outside of vesicles. $VMAT_2$ plays a neuroprotective effect of different intensities in different nerve cells. The more the number of $VMAT_2$ is, the stronger the neuroprotective effect of $VMAT_2$ will be, and vice versa [12, 13]. Sai et al confirmed the anti-toxic effect of $VMAT_2$ [14]. $VMAT_2$ mainly locates at dopaminergic, noradrenergic, adrenergic, serotonergic, histaminergic neurons and corresponding neurons terminals in the central nervous system. SNC and VTA are the main focus of DA neurons and LC is one of the main focus parts of noradrenergic neurons, which has many important physiological functions in human. In the substantia nigra-striatum projection system, 95% of $VMAT_2$ positive fibers are dopaminergic and 5% are serotonergic [15, 16]. Dysfunctional $VMAT_2$ cannot effectively limit the damage of exogenous and endogenous toxicants on mitochondria, which can lead to the degradation of monoamine neurons [17]. Additionally, reduction of $VMAT_2$ in both SNC and striatum area in PD patients is observed [18].

In this study, distribution of $VMAT_2$ in control group in SNC was less than that in both VTA and LC and the result was consistent with that of foreign literature [19]. $VMAT_2$ positive neuron fibers in SNC and striatum area in experimental group significantly reduced than that in control group. Therefore, SNC was the most vulnerable region due to the weakest protective effect of $VMAT_2$. On the contrary, neurons, locating at VTA and LC with abundant number of $VMAT_2$, were hardly damaged. In TH staining, the number of TH positive cells in SNC in experimental group reduced significantly, while in VTA and LC did not significantly reduced. The result of the number of TH positive cells was consistent with the number of $VMAT_2$ positive cells in experimental group of mice, indicating that $VMAT_2$, like the number of TH positive cells, can be used as one of the indicators of pathological examination to determine whether PD animal model was established successfully or not. In this study, both behavioral indicator and pathological indicator in animal model of PD induced by reserpine combined with MPTP were significant than those induced by single reserpine or MPTP. It was analyzed that as a specific and potent inhibitor of $VMAT_2$, reserpine inhibited the protective effect of $VMAT_2$ and exacerbated the toxicity of intracellular MPP + and other toxins [20, 21]; therefore, this model can be used for PD study. In conclusion, it was shown from this study that the change in distribution of $VMAT_2$ in mice model of PD demonstrated that $VMAT_2$ had a protective effect on DA neurons and the distribution of $VMAT_2$ in SNC was less than that in VTA and LC. Meanwhile, the weak protective effect of $VMAT_2$ in SNC was an important cause for PD with selective damage in the substantia nigra.

Disclosure of conflict of interest

None.

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