Full Length Research Paper

Distributional characteristics of vesicular monoamine transporter 2 in human embryonic brain and their correlation with Parkinson’s disease

Xiangyang Tian¹, Zhujuan Sun², Xinsheng Ding³, Min Min³ and Xingzhen Sun⁴*

¹Department of Neurology, Huai’an First People’s Hospital, Nanjing Medical University, 6 Beijing Road West, Huai’an, Jiangsu 223300, P.R. China.
²Department of Orthopedics, Si Chuan Kang Gu Hospital, Cheng Du, Si Chuan 610041, P.R. China.
³Department of Neurology, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, P.R. China.
⁴Department of Pediatrics, Huai’an First People’s Hospital, Nanjing Medical University, 6 Beijing Road West, Huai’an, Jiangsu 223300, P.R. China.

Accepted 17 August, 2012

The aim of this study was to observe the distributional characteristics of vesicular monoamine transporter 2 (VMAT₂), in human embryonic brain tissues and explore the relationship of these characteristics with Parkinson’s disease (PD). The distribution of VMAT₂ and tyrosine hydroxylase (TH) in substantia nigra pars compacta (SNC), ventral tegmental area (VTA) and locus coeruleus (LC) of human embryonic brains of different gestational ages was observed using immunohistochemistry and western blot analysis. VMAT₂ expression in SNC was significantly lower than that in VTA and LC (P < 0.05). VMAT₂ expression in SNC is lower than that in VTA and LC. The weakened protective function of VMAT₂ may be an important cause for the selective loss of dopaminergic neurons in SNC caused by PD.

Key words: Parkinson’s disease, vesicular monoamine transporter 2 (VMAT₂), immunohistochemistry, tyrosine hydroxylase, human fetus.

INTRODUCTION

Parkinson’s disease (PD) is a common degenerative disorder of the nervous system among middle aged and elderly people, which has an incidence of 1% among people of more than 60 years old. This disease is manifested clinically by tremors, rigidity, slowness of movement and difficulty with gait, and its pathology is characterized by the selective degeneration and loss of the dopaminergic neurons in the nigrostriatal pathway. PD is also called idiopathic PD in clinic, but its cause is not yet fully understood. Some theories have been put forward to explain the pathogenesis of PD, such as environmental toxinology, genetic susceptibility, and neurotransmitter dysfunction; however, none of these can satisfactorily explain the selective degeneration of dopaminergic neurons in PD patients and the progressive development of PD. In recent years, it has been discovered that the abnormal behavior of the vesicular monoamine transporter 2 (VMAT₂) in the nigrostriatal system performs an important role in the pathogenesis of PD, and VMAT₂ is crucial for dopamine (DA) transport (Wimalasena et al., 2008; Wimalasena, 2011). VMAT₂ is located mainly in the dopaminergic, norepinephrine (NE), Alzheimer’s disease (AD), 5-hydroxytryptamine (5-HT), histamine neurons and endings of the central nervous system (Nayebi, 2010); DA neurons mainly focused on substantia nigra pars compacta (SNC), ventral tegmental area (VTA) of the midbrain, and locus coeruleus (LC) is the main part of NA neurons focus on, which has many important physiological functions to human body.

1-Methyl-4-phenyl-tetrahydropyridine (MPTP) can lead to PD-resembling clinical symptoms and result in PD-resembling selective damage to dopaminergic neurons, for which it has been used for the establishment of classic PD animal models (Tetrud et al., 1986; Kopin and

*Corresponding author. E-mail: xingzhensun@yeah.net.
MATERIALS AND METHODS

Subjects

A total of 10 spontaneous abortion fresh embryos were collected, including 2 at a gestation age of 3 months, 2 of 4 months, 3 of 5 months, 2 of 6 months, and 1 of 7 months. The present study was conducted in accordance with the declaration of Helsinki, and with approval from the Ethics Committee of Huai’an First Affiliated Hospital of Nanjing Medical University, China.

Specimen handling

The embryos were subjected to open heart surgery, and then washed with physiological saline through the aorta. After perfusion and fixation with 4% polyborate solution (PBS), the brain tissues were taken out. The SNC, VTA, and LC were taken as observational specimens. The specimens were fixed in 4% PBS for 24 h at 4°C, dehydrated with gradient alcohols, and then embedded in paraffin.

Immunohistochemistry

Sections of 4 μm thickness were made and placed onto microscope slides. They were deparaffinized routinely, rehydrated with gradient alcohols, and afterwards incubated with 3% H₂O₂ to block endogenous peroxidase activity and with normal blood serum liquid to block non-specific antigens. The sections were incubated overnight at 4°C with a 1:80 dilution of polyclonal anti-VMAT₂ antibodies (Santa Cruz, USA) and with a 1:100 dilution of monoclonal anti-tyrosine hydroxylase antibody (Sigma, USA). The sections were colored with 3, 3-diaminobenzidine (DAB), and then counterstained with hematoxylin. PBS replaced the antibodies for negative control. Positive cells were those with buffy-stained cytoplasm and processes.

Image analysis

The average grey value of the positive area was determined and positive cells were counted under a high power field using the LEICA image analyzer. A lower grey value indicates higher immunostained intensity and a higher content of the interest.

Three slices of SNC, VTA, and LC were taken, respectively, and five fixed regional positive dyeing parts (upper left, left lower, lower right and the middle) were taken in each slice. The regional grey value was determined at high magnification, and then the average grey values of each specimen slice were determined through statistical analysis.

Western blot analysis

Tissues (100 mg) were taken from different parts of the brain, and proteins were then extracted. Bradford quantification and polyacrylamide gel electrophoresis were performed. The protein samples were transferred to nitrocelulose membranes, and then incubated with polyclonal antibodies against VMAT₂ at a 1:600 dilution. Afterwards, the protein samples were incubated with peroxidase-conjugated anti-IgG antibodies (1:1000 dilution). The membranes were DAB-colored. Absorbance (A) values was calculated.

Statistical analysis

All data were presented as means ± standard error of the means (± s), and analyzed by the SPSS 10.0 software. The normality test used Kolmorogov-Smirnov method. T-test was performed. P < 0.05 was considered statistically significant.

RESULTS

VMAT₂ expression

VMAT₂ expression in SNC (153.05 ± 5.22) was significantly lower than that in the VTA (130.07 ± 5.55) and the LC (118.80 ± 4.79) (P < 0.05). The results are shown in Figures 1, 2 and 3.

Positive cell counts

Immunostaining for VMAT₂ showed that the positive cell counts in SNC, VTA, and LC were 123.06 ± 13.89, 164.13 ± 14.24 and 141.40 ± 13.53, and immunostaining for tyrosine hydroxylase (TH) showed that the positive cell counts in the three areas were 124.67 ± 13.57, 166.13 ± 14.53 and 144.33 ± 16.73, respectively. No significant differences were observed among different areas (P > 0.05).
**VMAT\textsubscript{2} protein expression (A-value)**

Western blot analysis showed that VMAT\textsubscript{2} protein expression in SNC (3245 ± 698) was noticeably lower than that in VTA (4053 ± 953) and LC (6031 ± 732) ($P < 0.05$).

**Morphology of the dopaminergic neurons**

The positive cells in the 3 and 4 months gestation aged brains were round in shape with scant cytoplasm, a relatively large nucleus, and an unclear cell boundary. With the growth of the fetus, most cells turned spindle- or cone-shaped in morphology. The cell body was enlarged, the cytoplasm content was increased, the cell boundary became clearer, and obvious processes were observed. The results are shown in Figures 4 and 5.

---

**Figure 1.** The distribution of VMAT\textsubscript{2} in human fetal SNC (SP, × 200).

**Figure 2.** The distribution of VMAT\textsubscript{2} in human fetal LC (SP, × 200).

**Figure 3.** The distribution of VMAT\textsubscript{2} in human fetal ventral tegmental area (SP, × 200).

**Figure 4.** The morphology of the dopaminergic neurons in the human fetal brain at a gestation age of 3 months. The cells are round or oval in shape with a comparatively large nucleus and an unclear cell boundary (SP, × 400).
**DISCUSSION**

PD is a common degenerative disorder of the nervous system, and its pathology is characterized by the selective degeneration and loss of the dopaminergic neurons in the SNC and stratum of the midbrain. However, its cause remains unknown. Although many theories have been put forward to explain the pathogenesis of PD, none can satisfactorily explain PD-induced degenerative damage to dopaminergic neurons and PD’s progressive development. In recent years, VMAT$_2$ has become one of the hotspots of neuroscience study. The theory of VMAT$_2$ disturbance can offer a better explanation of the mechanism underlying the selective degeneration of the dopaminergic neurons in SNC. According to this theory, VMAT$_2$ might perform a key role in the pathogenesis of PD. VMAT$_2$ is a proton-dependent transporter, which can transport the monoamine neurotransmitters in cytoplasm into synaptic vesicles. The VMAT$_2$-transported neurotransmitters include DA, noradrenaline (NA), histamine, 5-HT and so on, and these transmitters can be inhibited by drugs such as reserpine, tetrabenazine and ketanserin (Peter et al., 1994).

MPTP, a type of neurotoxin, has been used for the establishment of PD animal models because it can lead to PD-reambling clinical symptoms in humans and primates (Markey et al., 1984; Speciale, 2002). The metabolic toxic product of MPTP is MPP$^+$, which can selectively inhibit the mitochondrial function in the dopaminergic neural cells in SNC to cause damage to ATP function, leading to the degeneration and loss of the dopaminergic neurons in SNC (Speciale, 2002). VMAT$_2$ can prevent the degeneration of dopaminergic neurons by transporting MPP$^+$ into vesicles to block its toxic effect on the cellular substances outside the vesicles; VMAT$_2$ has a higher expression in the SNC of rats, in which VMAT$_2$ exerts a protective effect on dopaminergic neurons by transporting MPP$^+$ into synaptic vesicles, than those of monkeys and mice (Staal and Sonsalla, 2000; Staal et al., 2000) (this result indicates that the toxic effect of MPTP varies from species to species). VMAT$_2$ has a great significance in preventing the aggravation of PD and improving its prognosis (Liu et al., 1992). VMAT$_2$ knockout mice die soon after birth (Fumagalli et al., 1999).

A decrease in the distribution of VMAT$_2$ in SNC, whether primary or secondary, may be an important reason for SNC selective damage caused by PD. Nowadays people are more concerned about the treatment and prevention of PD. However, neither medical nor surgical treatment can achieve satisfactory long-term curative effect; furthermore, both methods are costly, and they can lead to various complications. Taking VMAT$_2$ as the departure point, to develop drugs aiming at protecting or even up-regulating, VMAT$_2$ may become a new way to treat PD. In effect, some studies have proved that antioxidant substances such as glutathione (GSH), vitamin E, superoxide dismutase (SOD), and protective microelement selenium (Se) have protective effects on VMAT$_2$ (Drukarch et al., 1996; Kim et al., 1998). GSH removes hydrogen peroxide to protect VMAT$_2$, and Se supplements delay the pathological progression of PD. A few days of extracellular high-K$^+$ chronic stimulation significantly increase the synthesis of VMAT$_2$, and lithium ions increase VMAT$_2$ mRNA expression (Cordeiro et al., 2000). In addition, long-term treatment with clozapine induces an increase in VMAT$_2$ expression (Rehavi et al., 2002). Based on these findings, associated gene therapy has also been attempted: Regulating the transport function of VMAT$_2$ by modifying its protein coding gene improves the symptoms of PD in rats (Lee et al., 1999). However, the study on VMAT$_2$ is still in its infancy, and a lot of work remains to be explored.

PET and SPECT techniques have greatly deepened our understanding of the quantitative changes in neurons at different stages of diseases, but they are restricted by poor spatial resolution, and meanwhile, can be disturbed by some non-specific ligands (Miller et al., 1999). Immunohistochemistry and western blot analysis can localize and then quantitate VMAT$_2$ in SNC using specific polyclonal antibodies against VMAT$_2$. VMAT$_2$ expression in the SNC is lower than that in the VTA and LC of PD mouse models (Liu et al., 1992). Then, is there any congenital factor accounting for the decrease in VMAT$_2$ expression and the weakened function of VMAT$_2$ in SNC? No reports are available in literature. In the present study, a consistent result was also obtained: VMAT$_2$ expression in the SNC of human fetal brains was lower than that in

---

**Figure 5.** The morphology of the dopaminergic neurons in the human fetal brain at a gestation age of 6 months. Most cells are spindle-shaped, with an enlarged cell body, a clear cell boundary and obvious processes (SP, × 400).
the VTA and LC. Therefore, this study can better clarify PD-induced selective damage and the characteristics of the progression of PD. A weakened protective effect on SNC might be an important reason for PD-induced selected damage to dopaminergic neurons in SNC (Liu et al., 1992; Guillot and Miller, 2009). In addition, based on the distributional characteristics of VMAT2 obtained in the current study, the existence of congenital factors that are responsible for a decrease in VMAT2 in SNC is a reasonable presumption.

TH positive cells have been generally considered as a specific marker of dopaminergic neurons. In this study, the numbers of VMAT2 and TH positive cells in SNC, VTA, and LC did not show significant differences after immunostaining. Accordingly, VMAT2 can also serve as a specific marker of dopaminergic neurons as efficient as TH. It can be used to verify the successful or unsuccessful establishment of a PD animal model. Furthermore, the morphology of dopaminergic neurons was observed in this study. The results indicated that the cells in the fetal brain of a 3- and 4-month gestation age were round with scant cytoplasm, a comparatively large nucleus, and an unclear body boundary. With the growth of the fetus, most cells turned spindle- or cone-shaped in morphology, with an enlarged cell body, increased cytoplasm content, a clearer body boundary, and more obvious processes; multipolar neurons were also observed. In nerve cell transplantation, the less poorly transplanted cells are differentiated, the more likely they are to survive in the host after transplantation. On the other hand, their sources will also be greatly restricted given that too poorly differentiated cells cannot synthesize transmitters. The application of human fetal stem cells in the treatment of PD has become a hotspot nowadays.

In conclusion, the results in this study suggest that dopaminergic neurons in fetus at a gestation age of less than 3 or 4 months are immature, and they are suitable to be used as the transplants for PD patients.

REFERENCES


