Up-Regulation in Expression of Vesicular Glutamate Transporter 3 in Substantia Nigra but Not in Striatum of 6-Hydroxydopamine-Lesioned Rats

E.K.Y. Chung^a L.W. Chen^b Y.S. Chan^c K.K.L. Yung^a

^aDepartment of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong; ^bInstitute of Neurosciences, Fourth Military Medical University, Xian, and ^cDepartment of Physiology and Research Center of Heart, Brain, Hormone and Healthy Aging, University of Hong Kong, Hong Kong, PR China

Key Words

Animal model of Parkinson's disease • Neurotoxin • Degeneration of dopaminergic neurons • Vesicular glutamate transporters • Basal ganglia

Abstract

Overactivity of the glutamatergic system is suggested to be closely related to the onset and pathogenesis of Parkinson's disease. Vesicular glutamate transporters (VGLUT1, T2 and T3) are a group of glutamate transporters in neurons that are responsible for transporting glutamate into synaptic vesicles and they are key elements for homeostasis of glutamate neurotransmission. The present study was aimed to investigate the expression of VGLUT1, T2 and T3 proteins after the onset of Parkinson's disease. A rat model of Parkinson's disease, the 6-hydroxydopamine-lesioned rat, was employed. Immunocytochemistry revealed that VGLUT1, T2 and T3 immunoreactivity was not modulated in the striatum of the lesioned rat. Western blotting analyses also showed that there was no change in the expression of T1, T2 and T3 proteins in the striatum. In contrast, no VGLUT1 protein was detected in the substantia nigra. After the lesion, levels of VGLUT2 immunoreactivity and protein were not modulated. Significant increase of VGLUT3 immunoreactivity was observed in the perikarya of GABAergic substantia nigra pars reticulata neurons (+14.7%) although VGLUT3 protein was not modulated

in the nigral tissues. VGLUT3 in GABAergic neurons is suggested to play a role in GABA synthesis. The present results may therefore implicate that VGLUT1 and T2 are not modulated in the striatum and the substantia nigra of the 6-hydroxydopamine-lesioned rat and only VGLUT3 plays a role in pathogenesis of Parkinson's disease.

Copyright © 2007 S. Karger AG, Basel

Introduction

Parkinson's disease (PD) is a progressive neurological disorder and is characterized by a degeneration of dopaminergic neurons projecting from the substantia nigra pars compacta to the striatum of the basal ganglia. Previous studies [6, 7, 13, 17] have shown that changes in glutamate transmission may be involved in the pathogenesis of PD. One of the damaging effects after dopamine denervation is the alteration of the functional circuitry of the basal ganglia. The change is associated with an imbalance in the activity of the so called 'direct' and 'indirect' pathways [2, 3, 13]. The imbalance in fact reflects the changes in GABAergic and glutamatergic pathways of the basal ganglia. Thus the manifestation of PD symptoms has been shown to be related to the over-activity of the glutamatergic pathways [6, 7, 13, 16].

By receiving excitatory cortical and thalamic inputs, the striatum is the input nuclei of the basal ganglia [38]. Release of glutamate in the striatum is modulated by nigrostriatal dopaminergic projections [10, 30]. Because of the differential responses of the dopamine receptors on distinct populations of striatal projection neurons to the denervation, a complex alteration in the functional circuitry of the basal ganglia resulted in favor of the indirect pathway [2, 3, 13]. Activation of the indirect pathway, which involves the pathway from striatum to the output nuclei via the globus pallidus and the subthalamic nucleus, leads to increased firing of neurons in the output nuclei and causes inhibition in targets of the basal ganglia, thus constituting the underlying cause of most of the symptoms that characterize PD [2, 3, 13].

It is recognized that transport of glutamate into synaptic vesicles in glutamatergic axonal terminals are regulated by a group of vesicular glutamate transporters, namely VGLUTs [1, 5, 14, 15, 18, 28, 41–43]. VGLUT-mediated glutamate uptake activity is dependent on the vesicular proton electrochemical gradient generated by the vesicular proton ATPase [4, 26, 31]. Previous studies have shown that the expression of the three vesicular glutamate transporters are highly differential in the basal ganglia, in which VGLUT1 and VGLUT2 are expressed in terminals immunostained by synaptophysin whereas VGLUT3 are expressed in glutamatergic, GABAergic, cholinergic and monoaminergic neurons in various regions in the brain [18, 24, 29, 36, 37, 39].

Increases in glutamate levels have been found in the striatum following dopamine denervation in an animal model of PD [6, 7, 17]. Overactivity in glutamatergic systems is then implicated in the pathogenesis of PD [6, 7, 16, 17]. The status of expression, i.e. alteration in expression of VGLUTs, if any, is one key element to understand the reasons of the onset of overactivity of the glutamatergic pathways in parkinsonian conditions, and also provides a new target for anti-parkinsonian therapies in the future. A previous study has found that there is no change in expression of VGLUT1 and VGLUT2 in the striatum after the onset of PD [34]. The objectives of the present project were to study the changes of expression of VGLUT1, VGLUT2 and VGLUT3 in an animal model of PD, the 6-hydroxydopamine (6-OHDA)-lesioned rat. Changes in VGLUT proteins were investigated using immunofluorescence and Western blot analyses.

Materials and Methods

Animals

In the present study, 15 adult Sprague-Dawley rats (females, 150–200 g, University of Hong Kong) were used. Female rats were used in accordance to a previous study [48]. The handling of rats and all procedures involving the use of animals were approved in accordance with the Animals (Control of Experiments) Ordinance, Department of Health, Hong Kong and also approved by Committee on the Use of Human and Animal Subjects in Teaching and Research, Hong Kong Baptist University, and conformed to the *Principles of Laboratory Animal Care* [NIH publication No. 86–23, revised 1985]. All procedures performed by the investigators were aimed to minimize both animal number and suffering of the animals.

6-Hydroxydopamine Lesion

Rats were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p., sagittal). The rats then received unilateral stereotaxic injections of 6-hydroxydopamine (6-OHDA; 4 μ l, 3 mg/ml in 0.9 % saline containing 0.2 mg/ml ascorbic acid) in the right medial forebrain bundle (coordinates bregma: –0.45, midline: –0.09, dura: –0.75) [32]. The injection was administered within a period of 12 min and the needle of the Hamilton syringe was kept in situ for 10 min following deposit to prevent back filling along the injection tract. The animals were allowed to recover after surgery and kept in the animal house, Hong Kong Baptist University.

Rat Rotation Tests after 6-OHDA Lesion

One week after the 6-OHDA lesion, rats were tested for motor deficits in accordance to classical reports by Ungerstedt and co-workers [44, 45]. Apomorphine (1 mg/ml in saline, i.p., RBI) was injected to the rats. The numbers of rotations in a 30-min test were noted for each rat. Those rats that turned with an average of over 7 turns per min were regarded as successfully lesioned animals.

Tissue Preparation

Rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p., sagittal). They were perfused transcardially with 50–100 ml of normal saline followed by 200 ml fixative (3% paraformaldehyde and 0.01% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4). The brains were quickly removed from the skull after the perfusion and postfixed in 3% paraformaldehyde (in 0.1 M PB, pH 7.4) for a few hours at 4°C. Sections of the striatum and the substantia nigra (70 μ m) were cut on a vibrating microtome (Vibratome 1000, Technical Products International). The sections were collected in phosphate-buffered saline (PBS; 0.01 M, pH 7.4).

Immunocytochemistry

Immunocytochemistry was performed as previously described [23, 27]. All the striatal and nigral sections for comparisons were incubated and reacted in a single reaction sequence to minimize variation in immunofluorescence. The striatal and nigral sections were incubated in primary antibody solutions against VGLUT1, VGLUT2 and VGLUT3 (all guinea pig origin, 1:3,000 in PBS supplemented with 2% normal goat serum and 0.1% Triton-X 100 (PBS-Triton); Chemicon International, Temecula, Calif., USA) for 24 h at room temperature. After incubation,

the sections were washed ($3 \times$ PBS) and incubated in Alexa 488-conjugated secondary antibody solutions (goat anti-guinea pig IgG, 1:200 in PBS-Triton; Molecular Probes) for 2 h at room temperature in dark. The sections were then washed ($3 \times$ PBS), mounted in mounting medium (Vectashield, Vector Labs) and examined on a confocal microscope (LSM 510, Zeiss).

In control experiments for immunofluorescence, where the primary or secondary antibodies were omitted from the reaction sequence, no VGLUT immunoreactivity was observed in those sections (data not shown).

Semiquantitative analyses of the fluorescence micrographs were performed in accordance to previous studies [22, 40]. Digital images of each group of animals, i.e., normal sham-operated striatum and substantia nigra from control rats, the nonlesioned striatum and substantia nigra from 6-OHDA-lesioned rats, and the lesioned striatum and substantia nigra from 6-OHDA-lesioned rats, were captured under the same parameters in the confocal microscope at high magnification (using a $63\times$ lens). At least three sections of each animal were used for semiquantitative analyses. The fluorescent intensity of the confocal microscope images was determined by using an image analyzing software (Metamorph, Universal Imaging). The average grey values indicating the levels of VGLUT1 and VGLUT2 immunoreactivity in neuronal elements and the level of VGLUT3 immunoreactivity in the cytoplasm were determined. Intensities were expressed as mean $\pm$ SEM. Statistical comparisons between the groups were made by one-way analysis of variance (ANOVA, SPSS software).

In control experiments, the above reaction sequences were performed with the omission of either the primary or secondary antibody solutions or each of the primary antibodies in turn, in the reaction sequence.

Western Blot Analysis

The animals that were subjected to Western blot analysis were decapitated and the tissues of the striatum and substantia nigra were dissected as previously described [25]. The tissues were homogenized in a homogenizing buffer (250 mM sucrose, 50 mM imidazole hydrochloride, 2 mM sodium EDTA and 2 mM β -mercaptoethanol, pH 7.2) and centrifuged at 300 g for 10–15 min. The supernatant was further centrifuged at 14,000 g for 15 min. The final supernatant was stored at -80°C . The quantity of proteins in the supernatant was determined using a commercially available kit (Biorad protein assay kit). Proteins (60 μg /well) were submitted to SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes using a commercially available system (Bio-Rad Mini-protein 3). The membrane blots were subjected to Western blot analysis. Nonspecific-binding sites were blocked with 5% nonfat milk for 2 h. The blots were then incubated overnight at 4°C with one of the following primary antibodies in 2% non-fat milk: VGLUT1 (1:2,000 in Tris-buffered saline (TBS) supplied with 2% non-fat skim milk, 0.05 M, pH 7.4; Chemicon), VGLUT2 (1:2,000 in TBS; Chemicon) and VGLUT3 (1:1,000 in TBS; Chemicon), or β -actin (mouse monoclonal; 1:1,000 in TBS; Calbiochem). The membranes were then incubated in appropriate secondary antibodies (horseradish peroxidase-conjugated goat-anti-guinea pig IgG, 1:1,000 in TBS with 2% non-fat skim milk or goat-anti-mouse IgG, 1:500 in TBS with 2% non-fat skim milk, Zymed Laboratories) for 1 h at room temperature. Bands in the membranes were visualized using chemiluminescence detection (ECL Western blotting detection reagents;

Fig. 1. Results of the apomorphine-induced rotation tests in rats before and after the 6-hydroxydopamine lesions are shown. No rotation was detected in rats before the lesion. Rotations were detected in rats after the lesion.

Amersham Pharmacia). Images of the bands were developed on films (Biomax X-ray film, Kodak) and captured by a computer with a scanner. Optical density of each band was then semiquantified by software (Metamorph) and statistically analyzed by one-way ANOVA. Distinct immunoreaction products for each VGLUT were revealed: VGLUT1 (approximately 60 kDa) [1], VGLUT2 (approximately 65 kDa) [28] and VGLUT3 (approximately 60 kDa) [15].

Control for loading for Western blotting was performed in revealing β -actin proteins using either striatal or nigral tissues of the same animals. Specific bands of 45 kDa were revealed in each of the trials.

Results

Rat Rotation

No contralateral rotation was detected in rats before being 6-OHDA-lesioned (fig. 1). After the lesion, rotations were detected (fig. 1).

No Change in Expression of VGLUTs in the Striatum after Dopamine Denervation

At low magnification, VGLUT1 immunoreactivity was seen in the neuropil of the striatum (fig. 2). The fiber bundles and neuronal cell bodies were immuno-negative (fig. 2). No difference was found in the expression pattern and intensity of VGLUT1 between the striatum from control animals, nor from the non-lesioned side and lesioned side of the 6-OHDA-lesioned animals (fig. 2c). At higher magnification, VGLUT1-immunoreactive puncta were clearly observed throughout the striatal sections. The neuronal perikarya were unstained (fig. 2d–f). Semiquantitative analyses revealed that no significant difference in the intensity of VGLUT1 immunoreactivity of the

Fig. 2. Comparison of VGLUT1 immunoreactivity in the striatum of sham-operated control and unilateral 6-hydroxydopamine-lesioned rats. **a–c** Micrographs at low magnification show that VG immunoreactivity is predominantly found in the neuropil of the striatum. The fiber bundles are immuno-negative (some are indicated by white stars). The levels of VGLUT1 immunoreactivity in control striatum (**a**), non-lesioned striatum (**b**), and lesioned striatum (**c**) are similar. At higher magnification, neuronal cell bod-

ies in the striatum are immuno-negative. Similarly, similar levels of VGLUT1 immunoreactivity are found in three groups of striatal sections. Semi-quantitative analysis shows that there is no significant difference in level of VGLUT1 immunoreactivity between the control and lesioned animals (**g**). Mean $\pm$ SEM is shown. Scale bars: 200 μ m (in **a**, for **a–c**); 50 μ m (in **d**, for **d–f**). Con = Control; NL = no lesion; L = lesion.

Table 1. Summarized results of the semiquantitative analysis of levels of immunofluorescence of the vesicular glutamate transporters in the regions of striatum and substantia nigra of the normal and lesioned animals

	Vesicular glutamate transporters	Control	Non-lesioned side (NL)	Lesioned side (L)
Striatum	VGLUT1	22.77 $\pm$ 1.12	21.34 $\pm$ 0.27	21.66 $\pm$ 0.35
	VGLUT2	19.46 $\pm$ 1.55	2.19 $\pm$ 1.18	18.80 $\pm$ 0.65
	VGLUT3	43.41 $\pm$ 2.97	44.34 $\pm$ 1.70	39.25 $\pm$ 2.18
Substantia nigra	VGLUT2	28.11 $\pm$ 3.55	28.41 $\pm$ 1.54	26.46 $\pm$ 2.00
	VGLUT3	28.87 $\pm$ 0.75	32.67 $\pm$ 1.94	37.48 $\pm$ 1.12* (114.72 $\pm$ 5.94%)

A total of 45 images from 15 animals were taken for each transporter in each region. The relative intensity of the images was expressed as the means $\pm$ SEM. The values of the lesioned side were compared with both the non-lesioned side and the control. * $p < 0.05$.

control, non-lesioned and lesioned striatum (number of animals in each group [n] = 5; fig 2g, table 1).

The distribution of VGLUT2 immunoreactivity was also uniformly throughout the striatum and was similar to the immunoreactivity for VGLUT1 (fig. 3). At low

magnification, no observable difference in expression pattern and intensity for VGLUT2 immunoreactivity was noticed between the three groups of striatal sections (fig. 3a–c). At higher magnification, fine VGLUT2-positive puncta were observed throughout the striatum. The

Fig. 3. Comparison of VGLUT2 immunoreactivity in the striatum of sham-operated control rat and unilateral 6-hydroxydopamine-lesioned rat. Similarly, VGLUT2 immunoreactivity is predominantly found in the neuropil of the striatum (**a–c**). The fiber bundles are immuno-negative (some are indicated by white stars). The levels of VGLUT2 immunoreactivity in control striatum (**a**), non-lesioned striatum (**b**) and lesioned striatum (**c**) are similar. At higher magnification, neuronal cell bodies in the striatum are

immuno-negative. Similar levels of VGLUT2 immunoreactivity are found in control (**d**), non-lesioned (**e**) and lesioned striatum (**f**). Semiquantitative analysis shows that there is no significant difference in level of VGLUT2 immunoreactivity between the three groups (**g**). Mean $\pm$ SEM is shown. Scale bars: 200 μ m (in **a**, for **a–c**); 50 μ m (in **d**, for **d–f**). Con = Control; NL = no lesion; L = lesion.

neuronal perikarya in the striatum were immuno-negative for VGLUT2 (fig. 3d–f). Semiquantitative analyses also revealed that there was no significant difference of the intensity of VGLUT2 immunoreactivity in the lesioned side of striatum compared to the control and non-lesioned striatum ($n = 4$; fig. 3g; table 1).

At low magnification, VGLUT3 immunoreactivity was seen to be abundant in the striatum. There were no significant differences in the patterns of distribution and immunofluorescence intensity of VGLUT3 immunoreactivity between the normal, non-lesioned and lesioned striatum (fig. 4a–c). At higher magnification, VGLUT3 immunoreactivity was primarily found in cytoplasm of neurons (fig. 4d–f) that were presumed to be striatal interneurons in accordance to their morphological characters [8]. No significance difference in the intensity of immunoreactivity in the cytoplasm of VGLUT3-immunoreactive perikarya was seen between the three groups ($n = 5$; fig. 4g; table 1).

Results of the Western blot analyses are shown in figure 5. After 6-OHDA lesion, no significant differences in the expression of VGLUT1 (fig. 5a, b), VGLUT2 (fig. 5c,

d) and VGLUT3 (fig. 5e, f) proteins were found, respectively, between the control and the lesioned and non-lesioned striatum.

VGLUT3 Proteins Were Up-Regulated in the Substantia Nigra after Dopamine Denervation

In the substantia nigra, no VGLUT1 immunoreactivity was detected (data not shown). In contrast, VGLUT2 immunoreactivity was found to be abundant in the neuropil of the substantia nigra pars compacta and pars reticulata (fig. 6a–c). Among the three groups of nigral sections, no observable differences in the distribution and intensity of VGLUT2 immunoreactivity were seen (fig. 6a–c). At higher magnification, VGLUT2-immunoreactive puncta were abundant in the pars reticulata where they were found primarily around unstained neuronal perikarya (fig. 6d–f). Semi-quantitative analyses revealed that there was no significant difference in the levels of VGLUT2 immunoreactivity between the three groups of nigral sections ($n = 3$; fig. 6g; table 1).

In contrast, immunoreactivity for VGLUT3 was found in neuronal perikarya that were localized in the substan-

Fig. 4. Comparison of VGLUT3 immunoreactivity in the striatum of sham-operated control rat and unilateral 6-hydroxydopamine-lesioned rat. At low magnification, VGLUT3 immunoreactivity is primarily found in perikarya in the striatum (**a–c**; some are indicated by white arrows). The fiber bundles are immuno-negative. Similar levels of VGLUT3 immunoreactivity have been found in control and in two sides of striatum of the lesioned animals (**a–c**). At higher magnification, perikarya of neurons in the striatum

display intense VGLUT3 immunoreactivity (white arrows). In perikarya of striatal neurons, similar levels of VGLUT3 immunoreactivity are found among the control and the two sides of striatum (**d–f**). Semiquantitative analysis shows that there is no significant difference between the levels of VGLUT3 immunoreactivity (**g**). Mean $\pm$ SEM is shown. Scale bars: 200 μ m (in **a**, for **a–c**); 50 μ m (in **d**, for **d–f**). Con = Control; NL = no lesion; L = lesion.

tia nigra pars compacta and the pars reticulata (fig. 7a–c). Since the dopaminergic neurons in SNc were selectively depleted in the lesioned animals, VGLUT3-positive perikarya were only observed in the pars reticulata of the lesioned side (fig. 7c). More intensely labeled VGLUT3 immunoreactivity was seen in the lesioned substantia nigra when compared to those of the normal animal and the nonlesioned nigra (fig. 7a–c). At higher magnification, a significant higher level of VGLUT3 immunoreactivity was observed in the cytoplasm of neurons in the lesioned substantia nigra (fig. 7d–f). Semiquantitative analyses revealed that there was a significant increase in the level of VGLUT3 immunoreactivity in the reticulata neurons of the lesioned nigra ($+14.72 \pm 5.94\%$; $n = 5$; $p < 0.05$; fig. 7g; table 1).

In contrast, unlike the above results of immunoreactivity, Western blotting analyses showed that there was no significant difference in the expression of VGLUT2 and VGLUT3 proteins between the three groups of nigral tissues ($n = 3$; fig. 8a–d).

Discussion

Dopamine Denervation Does Not Alter the Expression of VGLUTs in the Striatum

The patterns of distribution of VGLUT1 and VGLUT2 immunoreactivity in the striatum is consistent with a previous study [34]. There are regional distributions of VGLUT1 and VGLUT2 in the brain. Previous studies show that VGLUT1 mRNA expression is primarily associated with cortical afferents, whereas VGLUT2 mRNA expression is associated with intralaminar thalamic afferents [19, 28, 46]. The present results also show that VGLUT2 immunoreactivity is predominantly found in the substantia nigra whereas VGLUT1 immunoreactivity is not detected in the nigra.

The striatum is the main target of nigral dopaminergic input and also receives two major glutamatergic projections originating from the cerebral cortex and thalamus [9, 38]. Striatal neurons including medium spiny neurons in the striatum receive convergent synaptic inputs from nigral dopaminergic projections and cortical

glutamatergic projections [9, 38]. Dopamine denervation in PD induces an increase in corticostriatal glutamatergic transmission [6, 7, 10, 16, 17, 30] and an increase in the extracellular glutamate levels in the striatum [34].

Expression of VGLUT1 and VGLUT2 can regulate the quantal size of glutamate synaptic vesicles [12, 33, 47]. Overexpression of VGLUT1 in cultured glutamatergic neurons increases the quantal size of glutamate synaptic vesicles [47]. Therefore, it is believed that the expression of VGLUT1 and T2 may play an important role in controlling the synaptic transmission and also play crucial

roles in regulation of the level of extrasynaptic glutamate.

The present study shows that lesions of nigral dopamine neurons do not significantly affect the expression of either VGLUT1 or VGLUT2 in the striatum. Therefore, it is suggested that the over-spills of glutamate after dopamine denervation may not relate to any modification in the expression of the VGLUTs, thus not relate to the packing of the glutamate in synaptic vesicles. The present results however, do not preclude the possibility that there is change in activity of either the corticostriatal and thalamostriatal pathways.

Fig. 6. Comparison of VGLUT2 immunoreactivity in the substantia nigra of sham-operated control rat and unilateral 6-hydroxydopamine-lesioned rat. VGLUT2 immunoreactivity is predominantly found in the neuropil of the nigra. The levels of VGLUT2 immunoreactivity are similar among all nigral sections. At higher magnification, VGLUT2 immunoreactivity is only observed in the neuropil. Similar levels of VGLUT2 immunoreactivity are

found in the nigral sections (**d-f**). Semiquantitative analysis shows that there is no significant difference in level of VGLUT2 immunoreactivity between the control, non-lesioned and lesioned nigral sections (**g**). Mean $\pm$ SEM is shown. Scale bars: 200 μ m (in **a**, for **a-c**); 50 μ m (in **d**, for **d-f**). Con = Control; NL = no lesion; L = lesion; SNc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata.

There is evidence to show that a large proportion of the increased extrasynaptic glutamate in the striatum after dopamine denervation is not arising from nonvesicular release [34]. The increase is depended on the balance between the release processes and glutamate transporter activities [34]. Previous studies suggest that synaptic release of glutamate from axonal terminals by exocytosis of synaptic vesicles is not the only mechanism supplying the extracellular fluid with glutamate, i.e. most of the released glutamate is of non-vesicular origin [11, 20]. One possible source for the nonvesicular released glutamate is through the cysteine-glutamate exchangers [11, 20].

The present results indicate that VGLUT3 immunoreactivity is primarily expressed by striatal interneurons, the presumed striatal cholinergic neurons [15, 42]. Dopamine denervation also does not modulate the protein expression of VGLUT3 nor the immunoreactivity for VGLUT3 in the region of the striatum or in perikarya of striatal cholinergic neurons. In the striatum, it is suggested that the release of glutamate is modulated by presynaptic acetylcholine receptors and striatal cholinergic interneurons play crucial roles in modulation of the functions of dopamine on medium spiny neurons

[21]. Acetylcholine, from striatal cholinergic neurons, exhibits opposing effects to dopamine on the striatonigral and striatopallidal neurons [21]. The striatal cholinergic interneurons expressing VGLUT3 raised the possibility that glutamate may be simultaneously co-released with acetylcholine [15, 42]. However, the physiological roles of VGLUT3 in cholinergic neurons and their roles in onset of PD symptoms remain to be established.

VGLUT3 but Not VGLUT2 Expression Is Up-Regulated in the Substantia Nigra after 6-OHDA Lesion

The present results also show that VGLUT2 but not T1 immunoreactivity is primarily found in the substantia nigra. It is believed that VGLUT2-expressing glutamatergic synapses may display a higher probability of glutamate release when compared to those of VGLUT1-expressing glutamatergic synapses [14, 18, 46]. Glutamatergic projections from the subthalamic nucleus to the substantia nigra are found to operate exclusively with VGLUT2 [46]. A previous study suggests that the high activity of VGLUT2-expressing glutamatergic projec-

Fig. 7. Comparison of VGLUT3 immunoreactivity in the substantia nigra of sham-operated control rat and unilateral 6-hydroxydopamine-lesioned rat. At low magnification, VGLUT3 immunoreactivity is primarily found in perikarya in the substantia nigra pars compacta and pars reticulata in control and non-lesioned nigra (**a, b**, some are indicated by white arrows). In lesioned nigra, higher levels of VGLUT3 immunoreactivity are found in perikarya of the pars reticulata (**c**, white arrow). At higher magnification, perikarya of neurons in the substantia nigra pars reticulata display intense VGLUT3 immunoreactivity (**d-f**, some are indicated by white arrows).

Similar levels of VGLUT3 immunoreactivity are found in the control and non-lesioned side (**d-f**), whereas a higher level of VGLUT3 immunoreactivity is found in the lesioned side (**g**). Semiquantitative analysis shows that a significantly higher level of VGLUT3 immunoreactivity is found in the lesioned side (**g**). Mean $\pm$ SEM is shown. * $p < 0.05$. Scale bars: 200 μ m (in **a**, for **a-c**); 50 μ m (in **d**, for **d-f**). Con = Control; NL = no lesion; L = lesion; SNc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata.

tions of the subthalamic nucleus to the substantia nigra pars compacta may be implicated as a mechanism driving glutamate excitotoxicity of the dopaminergic neurons [35]. In PD, the loss of dopaminergic neurons in the compacta leads to an overactivity of the subthalamic nucleus which, in turn, causes further excitotoxic damage to vulnerable dopaminergic neurons. As a consequence, an increasing cycle of neuronal loss in the compacta may result in the development of PD [35]. However, the present results have demonstrated that there is no significant change in VGLUT2 expression in the substantia nigra. A homeostatic mechanism may exist to attenuate the excitatory effects of excess glutamate release by controlling the balance between the quantal content and the number of synaptic vesicles [12]. Another previous study demonstrates that an increase expression of VGLUTs in motoneurons leads to an increase in quantal content together with a compensatory decrease in the number of synaptic vesicles released, and these two processes result in a normal level of glutamate [12]. Although no change in the

level of VGLUT2 expression was found in the present study, it does not preclude the possibility that there is an increase in glutamate vesicles released in the synapse.

Interestingly, the present study first demonstrates that there are up-regulations of VGLUT3 immunoreactivity in the reticulata neurons after the onset of PD. In the nigral tissues, no change of VGLUT3 protein is found by Western blotting. This discrepancy is likely due to the change in VGLUT3 is only found in the perikarya of reticulata neurons then the change is not sensitive enough detecting at the whole region level. The modulation of VGLUT3 expression has been found to be parallel to the change of activities of these neurons, i.e. the reticulata neurons are over-active in PD and the expression of VGLUT3 immunoreactivity is found to be upregulated. One possible explanation is that VGLUT3 is known to have a role in regulating intracellular storage pool of glutamate; the intracellular glutamate can be used for the synthesis of GABA [41]. Thus modulations of VGLUT3 expression may associate with changes in GABA synthe-

Fig. 8. Western blotting analyses of the protein levels of VGLUT2 and T3 in the substantia nigra. **a, b** VGLUT2 proteins are revealed (approximately 65 kDa). No significant difference is found between the control, non-lesioned (NL) and lesioned (L) nigral tissues. **c, d** VGLUT3 proteins are revealed (approximately 60 kDa). No significant difference is found between the control, non-lesioned and lesioned nigral tissues. **e** β -Actin proteins were also revealed (approximately 45 kDa) as the control for protein loading.

sis and release in the GABAergic reticulata neurons. The increase in GABA synthesis may in turn relate to he increases in activity of the reticulata neurons. The reticulata neurons are the output neurons of the basal ganglia that their over-activity result in hypokinetic symptoms of PD [2, 3, 13]. Therefore, the increase of VGLUT3 expression in the reticulate may be one key element that leads to the changes of GABAergic pathway in the basal ganglia in PD. VGLUT3 is therefore a target for pharmacological intervention of symptoms of PD.

Acknowledgements

The present work was supported by HKBU 2150/03M Research Grants Council, Hong Kong. Elaine Chung was supported by a postgraduate studentship. The authors would also like to thank Miss L.Y. Man and Miss Louise Ng for technical assistance.

References

- 1 Aihara Y, Mashima H, Onda H, Hisano S, Kasuya H, Hori T, Yamada S, Tomura H, Yamada Y, Inoue I, Kojima I, Takeda J: Molecular cloning of a novel brain-type Na(+)-dependent inorganic phosphate cotransporter. *J Neurochem* 2000;74:2622–2625.
- 2 Albin RL, Young AB, Penney JB: The functional anatomy of basal ganglia disorders. *Trends Neurosci* 1989;12:366–375.
- 3 Albin RL, Young AB, Penney JB: The functional anatomy of disorders of the basal ganglia. *Trends Neurosci* 1995;18:63–64.
- 4 Bai L, Xu H, Collins JF, Ghishan FK: Molecular and functional analysis of a novel neuronal vesicular glutamate transporter. *J Biol Chem* 2001;276:36764–36769.
- 5 Bellocchio EE, Reimer RJ, Freneau RT Jr, Edwards RH: Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science* 2000;289:957–960.

- 6 Blandini F, Greenamyre JT, Nappi G: The role of glutamate in the pathophysiology of Parkinson's disease. *Funct Neurol* 1996;11:3–15.
- 7 Blandini F, Porter RH, Greenamyre JT: Glutamate and Parkinson's disease. *Mol Neurobiol* 1996;12:73–94.
- 8 Bolam JP, Bennett BD: Microcircuitry of the neostriatum; in Ariano MA, Surmeier PJ (eds): *Molecular and Cellular Mechanisms of Neostriatal Function*. Austin, Landes Company, 1995, pp 1–19.
- 9 Bolam JP, Hanley JJ, Booth PA, Bevan MD: Synaptic organisation of the basal ganglia. *J Anat* 2000;196:527–542.
- 10 Calabresi P, Mercuri NB, Sancesario G, Bernardi G: Electrophysiology of dopamine-denervated striatal neurons. Implications for Parkinson's disease. *Brain* 1993;116:433–452.
- 11 Danbolt NC: Glutamate uptake. *Prog Neurobiol* 2001;65:1–105.
- 12 Daniels RW, Collins CA, Gelfand MV, Dant J, Brooks ES, Krantz DE, DiAntonio A: Increased expression of the Drosophila vesicular glutamate transporter leads to excess glutamate release and a compensatory decrease in quantal content. *J Neurosci* 2004;24:10466–10474.
- 13 DeLong MR: Primate models of movement disorders of basal ganglia origin. *Trends Neurosci* 1990;13:281–285.
- 14 Freneau RT Jr, Troyer MD, Pahner I, Nygaard GO, Tran CH, Reimer RJ, Bellocchio EE, Fortin D, Storm-Mathisen J, Edwards RH: The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron* 2001;31:247–260.
- 15 Gras C, Herzog E, Bellenchi GC, Bernard V, Ravassard P, Pohl M, Gasnier B, Giros B, El Mestikawy S: A third vesicular glutamate transporter expressed by cholinergic and serotonergic neurons. *J Neurosci* 2002;22:5442–5451.
- 16 Greenamyre JT: Glutamate-dopamine interactions in the basal ganglia: relationship to Parkinson's disease. *J Neural Transm Gen Sect* 1993;91:255–269.
- 17 Greenamyre JT: Glutamatergic influences on the basal ganglia. *Clin Neuropharmacol* 2001;24:65–70.
- 18 Herzog E, Bellenchi GC, Gras C, Bernard V, Ravassard P, Bedet C, Gasnier B, Giros B, El Mestikawy S: The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. *J Neurosci* 2001;21:RC181.
- 19 Hisano S, Nogami H: Transporters in the neurohypophyseal neuroendocrine system, with special reference to vesicular glutamate transporters (BNPI and DNPI): a review. *Microsc Res Tech* 2002;56:122–131.
- 20 Jabaudon D, Shimamoto K, Yasuda-Kamatani Y, Scanziani M, Gahwiler BH, Gerber U: Inhibition of uptake unmasks rapid extracellular turnover of glutamate of nonvesicular origin. *Proc Natl Acad Sci USA* 1999;96:8733–8738.
- 21 Kaneko S, Hikida T, Watanabe D, Ichinose H, Nagatsu T, Kreitman RJ, Pastan I, Nakanishi S: Synaptic integration mediated by striatal cholinergic interneurons in basal ganglia function. *Science* 2000;289:633–637.
- 22 Lau WK, Lui PW, Wong CKC, Chan YS, Yung KKL: Differential expression of N-methyl-D-aspartate receptor subunit messenger ribonucleic acids and immunoreactivity in the rat neostriatum during postnatal development. *Neurochem Int* 2003;43:47–65.
- 23 Lau WK, Yung WH, Chan YS, Yung KKL: Developmental assembly of GABA receptors in the rat substantia nigra; in Magill PJ, Bolam JP (eds): *Basal Ganglia VIII*. New York, Springer, 2005, pp 229–240.
- 24 Lin LH, Talman WT: Nitroxidergic neurons in rat nucleus tractus solitarius express vesicular glutamate transporter 3. *J Chem Neuroanat* 2005;29:179–191.
- 25 Lui PW, Yeung CW, Yung WH, Shi Y, Chen LW, Chan YS, Yung KKL: Ablation of gene expression of N-methyl-D-aspartate receptor one by antisense oligonucleotides in striatal neurons in culture. *Neurosignals* 2005;14:303–316.
- 26 Naito S, Ueda T: Characterization of glutamate uptake into synaptic vesicles. *J Neurochem* 1985;44:99–109.
- 27 Ng TKY, Yung KKL: Differential expression of GABA_BR1 and GABA_BR2 receptor immunoreactivity in neurochemically identified neurons of the rat neostriatum. *J Comp Neurol* 2001;433:458–470.
- 28 Ni B, Wu X, Yan GM, Wang J, Paul SM: Regional expression and cellular localization of the Na⁺-dependent inorganic phosphate cotransporter of rat brain. *J Neurosci* 1995;15:5789–5799.
- 29 Nickerson PA, Guerci A, El Mestikawy S, Semba K: Vesicular glutamate transporter 3 immunoreactivity is present in cholinergic basal forebrain neurons projecting to the basolateral amygdala in rat. *J Comp Neurol* 2006;498:690–711.
- 30 Onn SP, West AR, Grace AA: Dopamine-mediated regulation of striatal neuronal and network interactions. *Trends Neurosci* 2000;23:S48–S56.
- 31 Ozkan ED, Ueda T: Glutamate transport and storage in synaptic vesicles. *Jpn J Pharmacol* 1998;77:1–10.
- 32 Paxinos P, Watson C: *The Rat Brain in Stereotaxic Coordinates*, ed 2. New York, Academic Press, 1986.
- 33 Pothos EN, Larsen KE, Krantz DE, Liu Y, Haycock JW, Setlik W, Gershon MD, Edwards RH, Sulzer D: Synaptic vesicle transporter expression regulates vesicle phenotype and quantal size. *J Neurosci* 2000;20:7297–7306.
- 34 Robelet S, Melon C, Guillet B, Salin P, Kerkerian-Le Goff L: Chronic L-DOPA treatment increases extracellular glutamate levels and GLT1 expression in the basal ganglia in a rat model of Parkinson's disease. *Eur J Neurosci* 2004;20:1255–1266.
- 35 Rodriguez MC, Obeso JA, Olanow CW: Subthalamic nucleus-mediated excitotoxicity in Parkinson's disease: a target for neuroprotection. *Ann Neurol* 1998;44:S175–S188.
- 36 Schafer MK, Varoqui H, Defamie N, Weihe E, Erickson JD: Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. *J Biol Chem* 2002;277:50734–50748.
- 37 Seal RP, Edwards RH: The diverse roles of vesicular glutamate transporter 3. *Handb Exp Pharmacol* 2006;137–150.
- 38 Smith Y, Bevan MD, Shink E, Bolam JP: Microcircuitry of the direct and indirect pathways of the basal ganglia. *Neuroscience* 1998;86:353–387.
- 39 Stornetta RL, Rosin DL, Simmons JR, McQuiston TJ, Vujovic N, Weston MC, Guyenet PG: Coexpression of vesicular glutamate transporter-3 and gamma-aminobutyric acid markers in rat rostral medullary raphe and intermediolateral cell column. *J Comp Neurol* 2005;492:477–494.
- 40 Sze SCW, Wong CKC, Yung KKL: Modulation of the gene expression of N-methyl-D-aspartate receptor NR2B in the rat neostriatum by a single dose of specific antisense oligodeoxynucleotide. *Neurochem Int* 2001;39:319–327.
- 41 Takamori S, Malherbe P, Broger C, Jahn R: Molecular cloning and functional characterization of human vesicular glutamate transporter 3. *EMBO Rep* 2002;3:798–803.
- 42 Takamori S, Rhee JS, Rosenmund C, Jahn R: Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature* 2000;407:189–194.
- 43 Takamori S, Rhee JS, Rosenmund C, Jahn R: Identification of differentiation-associated brain-specific phosphate transporter as a second vesicular glutamate transporter (VGLUT2). *J Neurosci* 2001;21:RC182.
- 44 Ungerstedt U: 6-Hydroxy-dopamine induced degeneration of central monoamine neurons. *Eur J Pharmacol* 1968;5:107–110.
- 45 Ungerstedt U, Ljungberg T, Steg G: Behavioral, physiological, and neurochemical changes after 6-hydroxydopamine-induced degeneration of the nigro-striatal dopamine neurons. *Adv Neurol* 1974;5:421–426.
- 46 Varoqui H, Schafer MK, Zhu H, Weihe E, Erickson JD: Identification of the differentiation-associated Na⁺/PI transporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses. *J Neurosci* 2002;22:142–155.
- 47 Wojcik SM, Rhee JS, Herzog E, Sigler A, Jahn R, Takamori S, Brose N, Rosenmund C: An essential role for vesicular glutamate transporter 1 (VGLUT1) in postnatal development and control of quantal size. *Proc Natl Acad Sci USA* 2004;101:7158–7163.
- 48 Yamada M, Oligino T, Mata M, Goss JR, Glorioso JC, Fink DJ: Herpes simplex virus vector-mediated expression of Bcl-2 prevents 6-hydroxydopamine-induced degeneration of neurons in the substantia nigra in vivo. *Proc Natl Acad Sci USA* 1999;96:4078–4083.