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Ceftriaxone Ameliorates Motor Deficits and Protects Dopaminergic Neurons in 6-Hydroxydopamine-Lesioned Rats

T. C. H. Leung,[†] C. N. P. Lui,[†] L. W. Chen,[‡] W. H. Yung,[§] Y. S. Chan,[∥] and K. K. L. Yung*[,†]

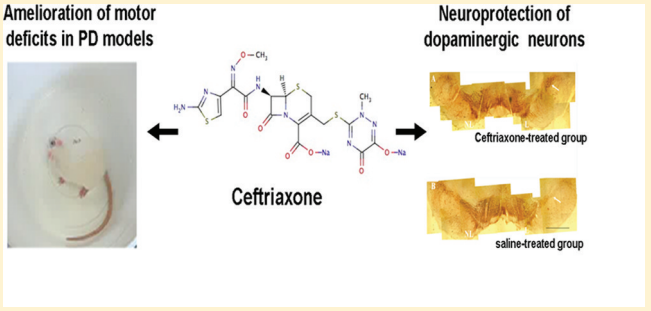
[†]Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong

[‡]Institute of Neurosciences, The Forth Military Medical University, Xian, PR China

[§]School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

[∥]Department of Physiology and Research Centre of Heart, Brain, Hormone and Healthy Aging, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong

ABSTRACT: Parkinson's disease is caused by the degeneration of dopaminergic neurons in substantia nigra. There is no current promising treatment for neuroprotection of dopaminergic neurons. Ceftriaxone is a beta-lactam antibiotic and has been reported to offer neuroprotective effects (Rothstein, J.-D., Patel, S., Regan, M.-R., Haenggeli, C., Huang, Y.-H., Bergles, D.-E., Jin, L., Dykes, H.-M., Vidensky, S., Chung, D.-S., Toan, S.-V., Bruijn, L.-I., Su, Z.-Z., Gupta, P., and Fisher, P.-B. (2005) Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression *Nature 433*, 73−77). In the present study, efficacy of ceftriaxone in neuroprotection of dopaminergic neurons and amelioration of motor deficits in a rat model of Parkinson's disease were investigated. Ceftriaxone was administrated in 6-hydroxydopamine-lesioned rats. Using behavioral tests, grip strength and numbers of apomorphine-induced contralateral rotation were declined in the ceftriaxone-treated group. More importantly, cell death of dopaminergic neurons was found to decrease. In addition, both the protein expression and immunoreactivity for GLT-1 were up-regulated. The present results strongly indicate that ceftriaxone is a potential agent in the treatment of Parkinson's disease.



KEYWORDS: *Animal model of Parkinson's disease, degeneration of dopaminergic neurons, antibiotic, ceftriaxone, glutamate transporter, glutamate transporter subtype 1, basal ganglia*

Parkinson's disease is a serious movement disorder characterized by the massive loss of dopaminergic neurones in the substantia nigra pars compacta.[1,2] In the past decade, the well-known levo-dopa therapy showed the best symptomatic improvement in the parkinsonian patients. However, the patients suffered from a wide range of motor response combinations, such as wearing off, on off, dystonias, and dyskinesia, in the prolonged treatment. Its applicability and effectiveness were thus questioned. Nowadays, the trend of innovative therapy in Parkinson's disease is no longer only focusing on symptomatic amelioration but also on neuroprotection, neurorestoration, and neuroregeneration of the dompaminergic neurons in substantia nigra.

The glutamate excitotoxicity, as a hallmark of Parkinson's disease and associated with dopaminergic degeneration in Parkinson's disease, has been studied for many years. Glutamate antagonists are found to be neuroprotective. According to the Committee to Identify Neuroprotective Agents in Parkinson's (CINAPS), 2 of 16 recent neuroprotective drugs are glutamate antagonists, including Amantidine and Remacemide. Nevertheless, there is no glutamate antagonist can completely halt the disease progression and the intolerable side effects limit the medical application.

The removal of synaptically released glutamate may provide another strategy in the amelioration of glutamate excitotoxic cell death. Rothstein et al. have shown that beta-lactam antibiotics, in particular a common clinically used antibacterial antibiotic called ceftriaxone, can offer neuroprotective effect on some neurological disorders associated with glutamate excitotoxicity.[3,4] Ceftriaxone is able to pass freely via the blood brain barrier and up-regulate the functional expressions of glutamate transporter as well as glutamate transporter subtype 1 (GLT1).[5] Moreover, it is able to conserve cell survival against oxygen-glucose deprivation in dissociated embryonic cortical cultures which is an in vitro model of stroke, [5] and to induce ischemic tolerance in an experimental in vivo model.[6] Glial glutamate transporters are known to be related to the dopaminergic degeneration in Parkinson's disease due to the glutamate excitotoxicity.[7,8] Furthermore, GLT1 has been investigated as the predominant glutamate transporter, which is essential to recycle glutamate and maintain a relatively

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low level of extracellular glutamate. This helps in preventing glutamate excitotoxicity.[10−12] Previous study also reported that down-regulation of GLT1 protein expression levels was found in the lesioned striatum of the 6-hydroxydopamine (6-OHDA) lesioned rodent models.[13]

Based on the properties of beta-lactam antibiotics in up-regulation of functional GLT1 expression, one of the most potent antibiotics in the same group called ceftriaxone was investigated in this study. We demonstrated that the pretreatment of ceftriaxone for 7 consecutive days could improve the Parkinsonian signs and show neuroprotection in rat parkinsonism. The protein expression of GLT1 was analyzed by Western blotting and immunocytochemistry. The capabilities of symptomatic amelioration and dopaminergic neuroprotection were also investigated.

RESULTS AND DISCUSSION

Ceftriaxone Pretreatment Ameliorates Muscular Rigidity and Apomorphine-Induced Contralateral Rotation in 6-Hydroxydopamine-Lesioned Rats. The results of the grasping test showed that the holding times were increased in both the ceftriaxone-treated lesioned rats and the saline-treated lesioned rats (Figure 1A). In the saline-treated group, the grasping time was significantly enhanced from post-lesion day 4 to day 10 while that of the ceftriaxone-treated group was significantly enhanced on post-lesion day 8 and day 10 only. Besides, the average holding time of the ceftriaxone-treated group was significantly lower than that of the saline-treated group, in particular on post-lesion day 4 and day 6. However, no significant difference was found on post-lesion day 2, day 8, and day 10 between two groups.

In the ceftriaxone-treated group and the saline-treated group, an increasing trend of the contralateral rotation number could be observed from post-lesion day 2 to day 10 (Figure 1B). However, significant decreases in the number of contralateral rotations were found in the ceftriaxone-treated animals on post-lesion day 6 and day 8 compared to the saline-treated group (Figure 1B). No significant differences between the two groups were found on post-lesion day 2, day 4, and day 10 (Figure 1B).

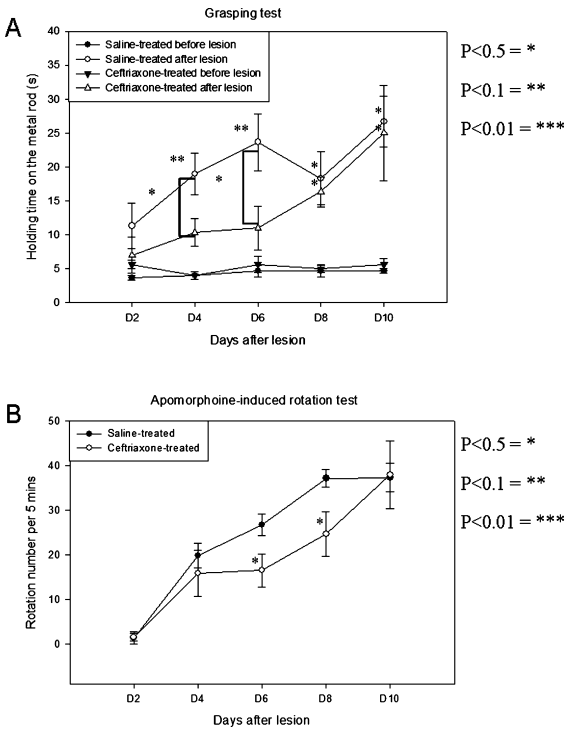

Figure 1. (A) Diagram showing the effect of ceftriaxone on the behavioral changes in the striatum of the 6-OHDA lesion animals. In the grasping test, the holding time was significantly increased on post-lesion day 4, day 6, day 8, and day 10 in the saline-treated animal group, and on post-lesion day 8 and day 10 in the ceftriaxone-treated group. Significant differences of the holding time were found on post-lesion day 4 and day 6. There was no significant difference between the holding times of each group before lesion (n = 3 for each set). (B) In the apomorphine-induced rotation test, an increment trend of the contralateral rotation number was observed from post-lesion day 2 to day 10 in both ceftriaxone-treated and saline-treated animal groups. A significant decrease in rotation number was found on post-lesion day 6 and day 8 in the ceftriaxone-treated group comparing to the saline-treated groups, whereas no significant difference was found between the two groups on post-lesion day 2, day 4, and day 10 (n = 3 for each set).

Neuroprotective Effect of Ceftriaxone on Tyrosine Hydroxylase (TH)-Positive Dopaminergic Neurons in Substantia Nigra. TH-immunoreactive neurons of both ceftriaxone-treated and saline-treated animals were stained and counted under light microscope (Figure 2). Decreasing trends of TH-immunoreactive neurons were observed in the substantia nigra of both saline- and ceftriaxone-treated groups (Figure 3A). However, the number of TH-immunoreactive cells was significantly higher in the ceftriaxone-treated group on post-lesion day 4, day 6, and day 8 when compared with that of the saline-treated group (Figure 3A). No significant differences in the TH-positive cell number were found on post-lesion day 2 and day 10 between two groups.

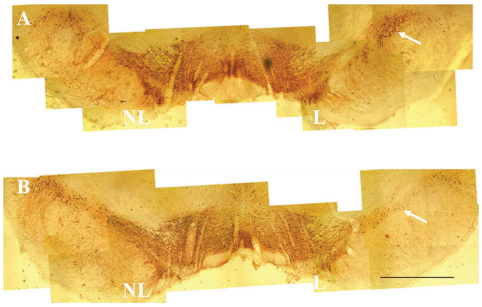

Figure 2. Immunoreactivity for tyrosine hydroxylase was presented in the cell bodies and axon of neurons in both substantia nigra pars compacta and substantia nigra pars reticulata, whereas the fiber bundles were immunonegative. The substantia nigra sections of the animals among different days after lesion were investigated. (A) and (B) represent ceftriaxone-treated animals and saline-treated animals on post-lesion day 6, respectively. The TH-positive neurons were counted by using a light microscope. Scale bar in (B) = 1 mm for (A) and (B); NL = nonlesioned side; L = lesioned side.

Referring to the above results, a significant neuroprotection of TH-immunoreactive neurons, that is, dopaminergic neurons, was provided by the treatment of ceftriaxone at early stages of lesion (on post-lesion day 4 to day 8). Further investigations on the levels of protein expression of TH and GLT1 were done among the selected time points.

Increase in GLT1 Immunoreactivity under the Treatment of Ceftriaxone. The expression level of GLT1 immunofluorescence in striatum was analyzed by the average gray values using the imaging analyzing software (Figure 4). In the saline-treated animals, the levels of GLT1 immunoreactivity were found to be significantly decreased in the lesioned sides

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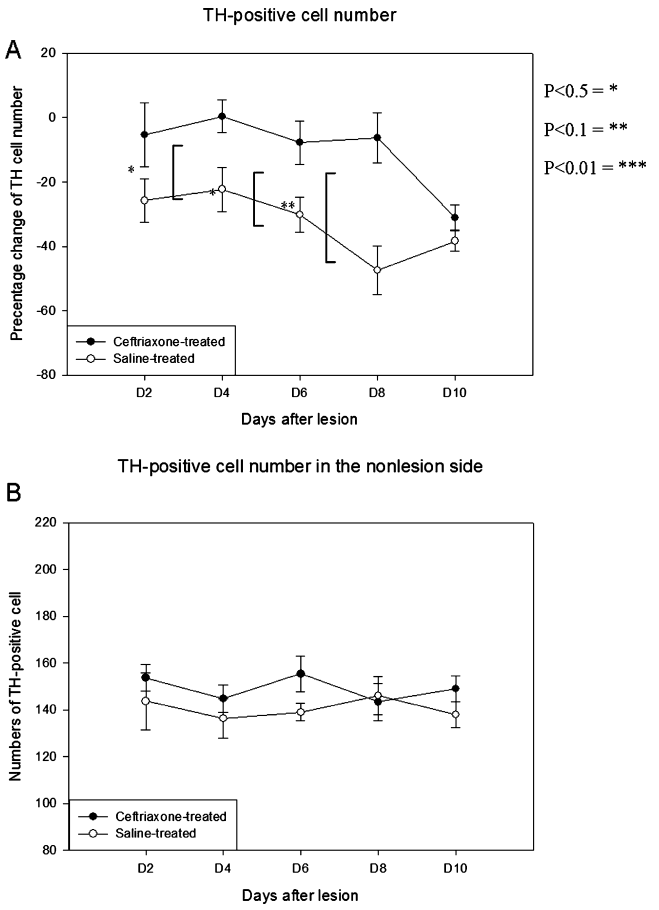

Figure 3. (A) Diagram showing the effects of the ceftriaxone treatment on the TH-positive cell number which was represented as the percentage of the neuronal cell bodies in the lesioned side against nonlesioned side of the substantia nigra. An increment trend of the percentage loss in the TH-positive cell number was noted. It was found significantly on post-lesion day 8 and day 10 in the saline-treated groups, while that was only significant on post-lesion day 10 in the ceftriaxone-treated groups. Moreover, a significant lower level of percentage cell loss was found in the ceftriaxone-treated animals on post-lesion day 4, day 6, and day 8 comparing to that of the saline-treated animals. No significant differences were found on post-lesion day 2 and 10. (B) There was no significant change between the nonlesioned sides of both ceftriaxone-treated and saline-treated animals groups at each time points (n = 3 for each set).

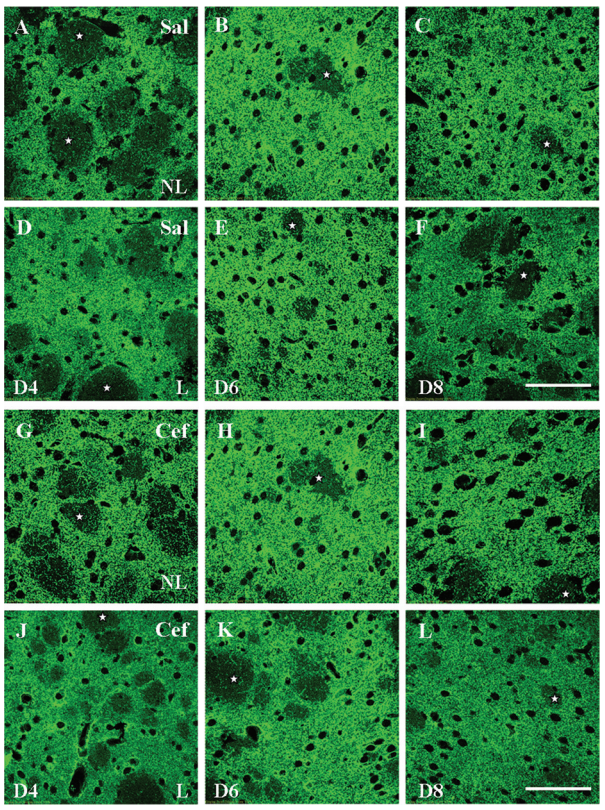

Figure 4. Glutamate transporter subtype 1 immunoreactivity (green; FITC) of the rat striatum was revealed under the laser confocal microscopy. The comparison between both hemispheres of striatum in ceftriaxone-treated and those in saline-treated animals was studied by the fluorescent intensities. The immunofluorescence images of the saline-treated animals are shown in (A)−(F), and those of the ceftriaxone-treated group are presented in (G)−(L). Scale bars in (C) and (I) = 50 μm for (A)−(L).

when compared to the nonlesioned sides on post-lesion day 6 and day 8 (Figure 5). In the ceftriaxone-treated animals, the decrease was significant on post-lesion day 8 (Figure 5). Moreover, the GLT1 level of the lesioned sides in the ceftriaxone-treated animals was significantly higher than that of the saline-treated animals on both post-lesion day 6 and day 8 (Figure 5). Furthermore, there were no significant differences of the GLT1 immunoreactivity of the nonlesioned sides for both groups at all time points (Figure 5).

Protein Expression of GLT1 and Tyrosine Hydroxylase after Ceftriaxone Treatment. Western blotting analysis was employed to examine the levels GLT1 (Figure 6A) and TH (Figure 7A) in the striatum and in the substantia nigra, respectively. In the saline-treated group, a trend of percentage decrease in GLT1 proteins was found from post-lesion day 4 to day 8 in the lesioned side (Figure 6B). In the ceftriaxone-treated animals, the percentage of GLT1 proteins was also found to be significantly increased on post-lesion day 6 and 8 in the lesioned side when compared with that of the saline-treated group.

A decreasing trend of the TH proteins in the lesioned side of the substantia nigra was found in both groups from post-lesion day 4 to day 8 (Figure 7B). The percentage reduction was found to be in a lesser extent in the ceftriaxone-treated group when compared to the saline-treated group on post-lesion day 4 and day 6 (Figure 7B).

Level of Ceftriaxone in Brain after 7-Day Treatment. The total brain level of ceftriaxone was examined after the 7-day injection of the drug. The identification of the compound was done by MS/MS spectra analysis and database searching. Also, the multiple reaction monitoring (MRM) chromatogram shown in Figure 8B indicated that ceftriaxone was able to pass through the blood brain barrier and thus exert its neuroprotective effects, including the up-regulation of GLT1 in the striatum. The calculated concentrations of ceftriaxone found in the rat brains were between 16.0 ng/mL and 54.1 ng/mL (Figure 8).

Ceftriaxone Ameliorates Motor Symptoms of Parkinson's Disease. The present results indicated that increasing striatal GLT1 expression ameliorated symptomatic signs and offered neuroprotection in rat parkinsonism. A continuous loss of dopaminergic neurons from day 2 to day 8 after striatal 6-OHDA lesion in rat parkinsonism and ceftriaxone treatment provided a significant arrest of the degeneration of dopaminergic neurons after lesion. Importantly, the animals

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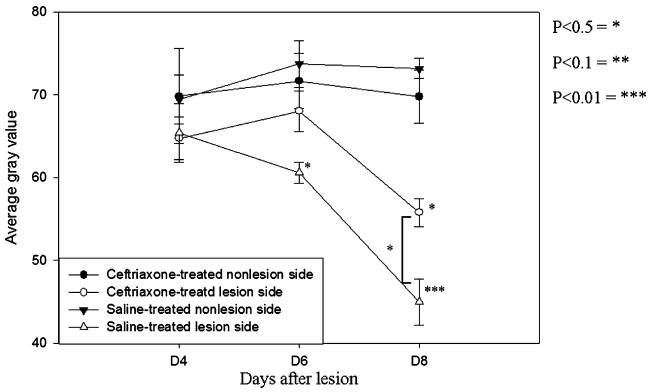

Figure 5. Diagram showing the immunoreactivity of glutamate transporter subtype 1 in the rat striatum of different groups. A decreasing trend of the glutamate transporter subtype 1 immunoreactivity was observed in the lesioned side of the both the ceftriaxone-treated and saline-treated animals. A significant reduction of glutamate transporter subtype 1 immuno reactivity was found on post-lesion day 6 and day 8 in the saline-treated group while that was found on post-lesion day 8 only in the ceftriaxone-treated animal groups. Moreover, the reduction of the glutamate transporter subtype 1 immunoreactivity on post-lesion day 8 was much more significant in the ceftriaxone-treated group than that in the saline-treated group. No significant difference of the glutamate transporter subtype 1 immunoreactivity was found between the nonlesioned sides of two groups (n = 3 for each set).

after ceftriaxone treatment also showed effective improvements in motor symptoms, including muscular rigidity and apomorphine-induced rotation. Both the symptomatic and neuroprotective effects were also found to be on short-term basis for pretreatment of ceftriaxone, that is, until post-lesion day 6.

Two behavioral tests, including grasping test and apomorphine-induced rotation test, were investigated as parkinsonian symptomatic signs of the rodent model. The attenuated number of rotation and holding time implied the symptomatic amelioration activity of ceftriaxone. Although we could not conclusively demonstrate the actions of ceftriaxone or the up-regulation of GLT1 expression in striatum of the basal ganglia microcircuitry, the up-regulation probably contributed to directly mediate the cortico-striatal glutamate transmission. It was relevant that down-regulation of GLT1 protein expression level was found in the lesioned striatum, but not in other basal ganglia regions.[4,13] This thus regulated the activity of other downstream regions, like subthalamic nucleus which played a central part in causing the motor abnormalities of Parkinson's disease,[16,17] in basal ganglia to ameliorate the symptomatic signs. Modulation of signal transmission in subthalamic nucleus, such as by deep brain stimulation, provided significant improvements for motor symptoms.[18] Yet, the mechanism of deep brain stimulation remained unclear.[19−21]

More importantly, no significant differences in both behavioral tests were found between the ceftriaxone-treated and saline-treated groups (data not shown). In addition to the parkinsonian behavioral changes after ceftriaxone treatment, another group also demonstrated that there were no significant behavioral changes, including multiple movements and motor sequences, between ceftriaxone-treated and saline-treated wild-type mice in an open-field activity as a Huntington's disease model.[22,23]

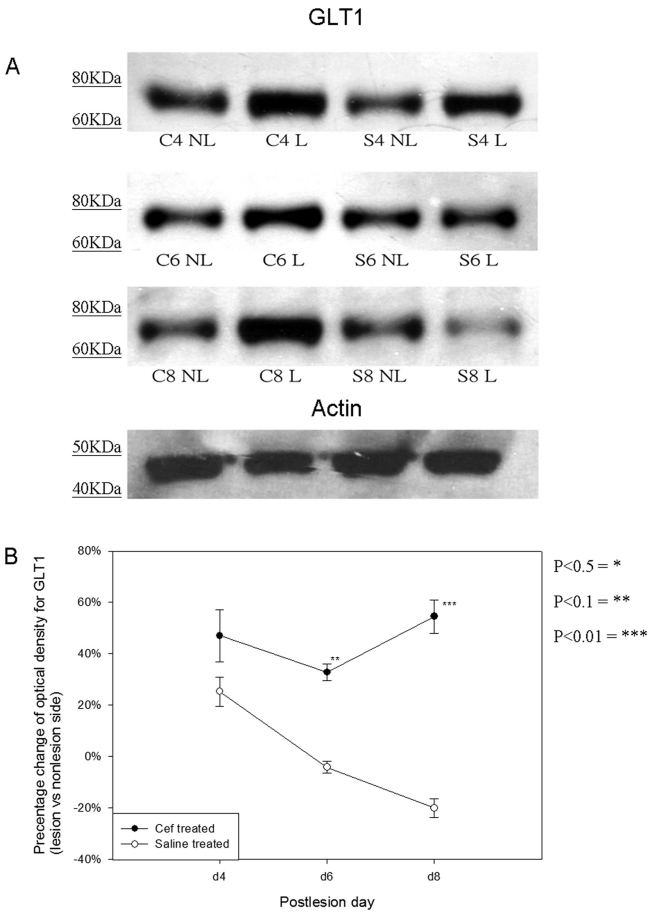

Figure 6. (A) Western blotting analyses of the levels of glutamate transporter subtype 1 proteins in the striatum of both animal groups. Bands of approximately 73 kDa representing the glutamate transporter subtype 1 proteins were observed. The band density of glutamate transporter subtype 1 protein in the striatum was increased in the lesioned side of the ceftriaxone-treated animals comparing to the nonlesioned side and both sides of the saline-treated group. A decreasing trend of the band density could be illustrated in the lesioned side of the saline-treated animals. (B) Diagram showing the band density of glutamate transporter subtype 1 protein in the striatum of ceftriaxone-treated animals and saline-treated groups. The diagram only shows the preliminary data where n = 1 for each set. NL= Nonlesion side, L= lesion side. C4 = Ceftriaxone-treated animal on post-lesion day 4. C6 = Ceftriaxone-treated animal on post-lesion day 6. C8 = Ceftriaxone-treated animal on post-lesion day 8. S4 = Saline-treated animal on post-lesion day 4. S6 = Saline-treated animal on post-lesion day 6. S8 = Saline-treated animal on post-lesion day 8.

Neuroprotective Effects of Ceftriaxone. Many studies in GLT1 expression demonstrated its importance in regulating glutamate homeostasis in brain. The removal of GLT1 site by immunoprecipitation represented more than 90% reduction in the excitatory amino acid uptake in the rat brain.[24] The antisense oligonucleotide specific for GLT1 showed that GLT1 contributed not less than 60% of striatal and hippocampal excitatory amino acid transport, and the inhibition of GLT1 protein expression was associated with neuronal damage.[4,25] A previous report by our team showed that down-regulation of GLT1 protein expression level was found in the lesioned striatum of the 6-OHDA lesioned rodent models.[8] It was relevant that a selective decrease in GLT1 protein expression was associated with the neuronal loss observed in the sporadic form of amyotrophic lateral sclerosis[25] and contributed to the

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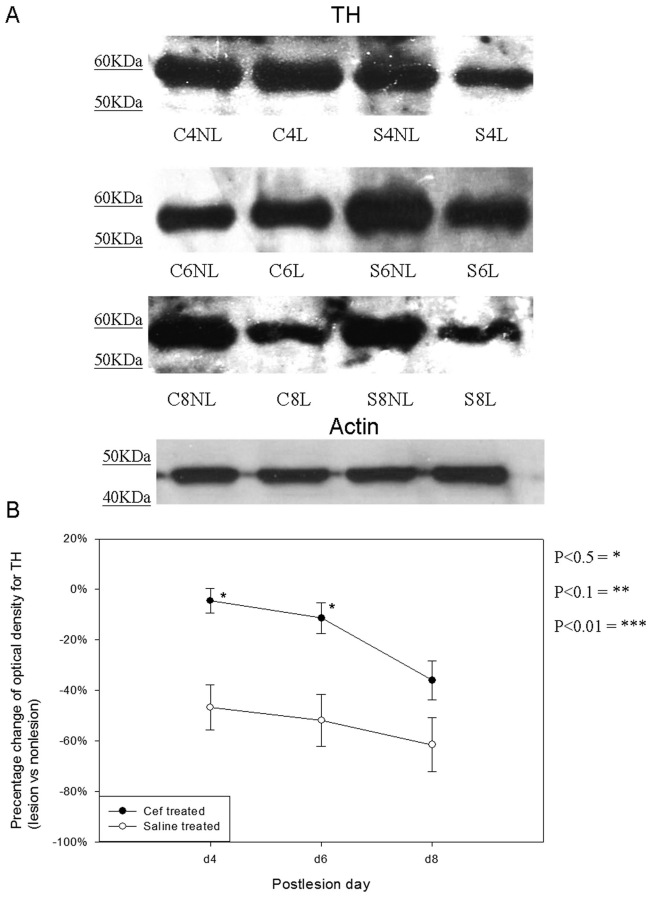

Figure 7. (A) Western blotting analyses of the levels of tyrosine hydroxylase proteins in the substantia nigra of both animal groups. Bands of approximately 60 kDa representing the tyrosine hydroxylase proteins were observed. The band densities of tyrosine hydroxylase protein in the substantia nigra were decreased after 6-hydroxydopamine lesion in the lesioned side comparing to the nonlesioned side of the saline-treated group on post-lesion day 4, day 6, and day 8. For the ceftriaxone-treated group, the band densities declined significantly on post-lesion day 8. The degree of the percentage decrease in the band density was lower in the ceftriaxone-treated animals compared to that of the saline-treated animal. (B) Diagram showing the band density of tyrosine hydroxylase protein in the substantia nigra of ceftriaxone-treated animals and saline-treated group. The diagram only shows the preliminary data where $n = 1$ for each set.

pathophysiological changes in Alzheimer's disease,[26] ischemia, stroke,[27] brain tumors,[28] and epilepsy.[29] The obstruction of GLT1 expression in the parkinsonism model might therefore contribute to the pathophysiological outcome.

Up-regulation of GLT1 expression was found from post-lesion day 2 to day 6 in immunocytochemistry analysis, while that was found until post-lesion day 8 in Western blotting. The results were matched to that of the motor tests and the neuroprotective effect on dopaminergic neurons which showed a reduced response over the post-lesion period. This represented relatively short-term responses of ceftriaxone treatment in the parkinsonism model. Moreover, a longer expression time of GLT1, that is, up to post-lesion day 8, was investigated in Western blotting analysis compared to the result of immunocytochemistry analysis, that is, up to post-lesion day 6. This might implicate that the functional response of ceftriaxone did not correspond to its protein expression level but to the redistribution of GLT1, for example, by removing extrasynaptically released glutamate. Other research demonstrated that cerftriaxone increased not only GLT1 expression but also its function.[23,30]

Besides this property of ceftriaxone, the neuroprotection of the dopaminergic neurons in parkinsonism model might be contributed by other characteristics of the drug. One possible neuroprotective mechanism suggested by this study was the side chain of ceftriaxone, D-α-aminoadipic acid. This side chain was readily carbonylated upon oxidative damage[31] and prevented the carbonylation of endogenous targets, such as DJ-1 protein which was suggested to be linked to sporadic Parkinson's and Alzheimer's diseases,[32] leading to the reduction in oxidative stress and apoptosis.[33] Another possible mechanism was about the *N*-methyl-D-aspartic acid (NMDA) receptor antagonistic properties of ceftriaxone.[34] D-α-Aminoadipic acid side chain was an antagonist of NMDA receptor.[35] Therefore, ceftriaxone might function as a noncompetitive antagonist of the NMDA receptor and thus might attenuate glutamate excitotoxicity. Some recent researchers[6,36] demonstrated that ceftriaxone could reduce inflammation and apoptosis. It could also reduce the expression of matrix metallopeptidase 9 which had a similar property of NMDA antagonist, and tumor necrosis factor-alpha which played a facilitatory role in glutamate excitotoxicity and up-regulate α-amino-3-hydroxyl- 5-methyl 4-isoxazole-propionate receptors expression on synapses.[37,38]

In light of the short-term responses, a long-term administration period was suggested to facilitate and prolong the functional benefit promoted by ceftriaxone treatment, through the functional up-regulation of GLT1 expression. Although the daily dose of ceftriaxone (200 mg/kg) employed in this study was relatively high and adverse side effects might be induced, including candidiasis, diarrhea, nausea, vomiting, and reversible biliary pseudolithiasis,[39] none of these side effects were reported in the study of a Hungtington's disease mouse model under high-dose treatment (200 mg/kg per day).[23] In clinical application, cerftriaxone was used to treat bacterial infections and meningitis. The levels of ceftriaxone in the central nervous system found in the patient were about 0.3−6 μmol/L,[40] which was comparable to the EC50 (3.5 μmol/L) and required to increase GLT1 expression.[3,41]

The potentials of ceftriaxone in the treatment of neurological disorders are gaining interest among many researchers. For example, administration of ceftriaxone was found to attenuate hypobaric hypoxia induced cognitive impairment,[42] decline infarct volumes in both transient and permanent models of cerebral ischemia,[6] and ameliorate Hungtington's disease behavioral phenotype in R6/2 mice. Ceftriaxone or drugs with similar functional properties may therefore provide a novel therapy in Parkinson's disease and other neurological diseases.

METHODS

Animals. All experiments were conducted with female Sprague−Dawley (SD) rats weighting from 200 to 220 g, and a total of 60 rats were sacarified. The animal experimental protocol performed in this study was strictly under the guidelines of the Animals (Control of Experiments) Ordinance, Department of Health, Hong Kong. Also, it conformed to the Committee on the Use of Human and Animal Subjects in Teaching and Research, Hong Kong Baptist University, and the Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985). All experimental animals were provided by the Laboratory Animal Unit, The University of Hong Kong.

Ceftriaxone Pretreatment. The animals were injected with ceftriaxone solution (200 mg/kg/day, i.p., Sigma) for 7 days before

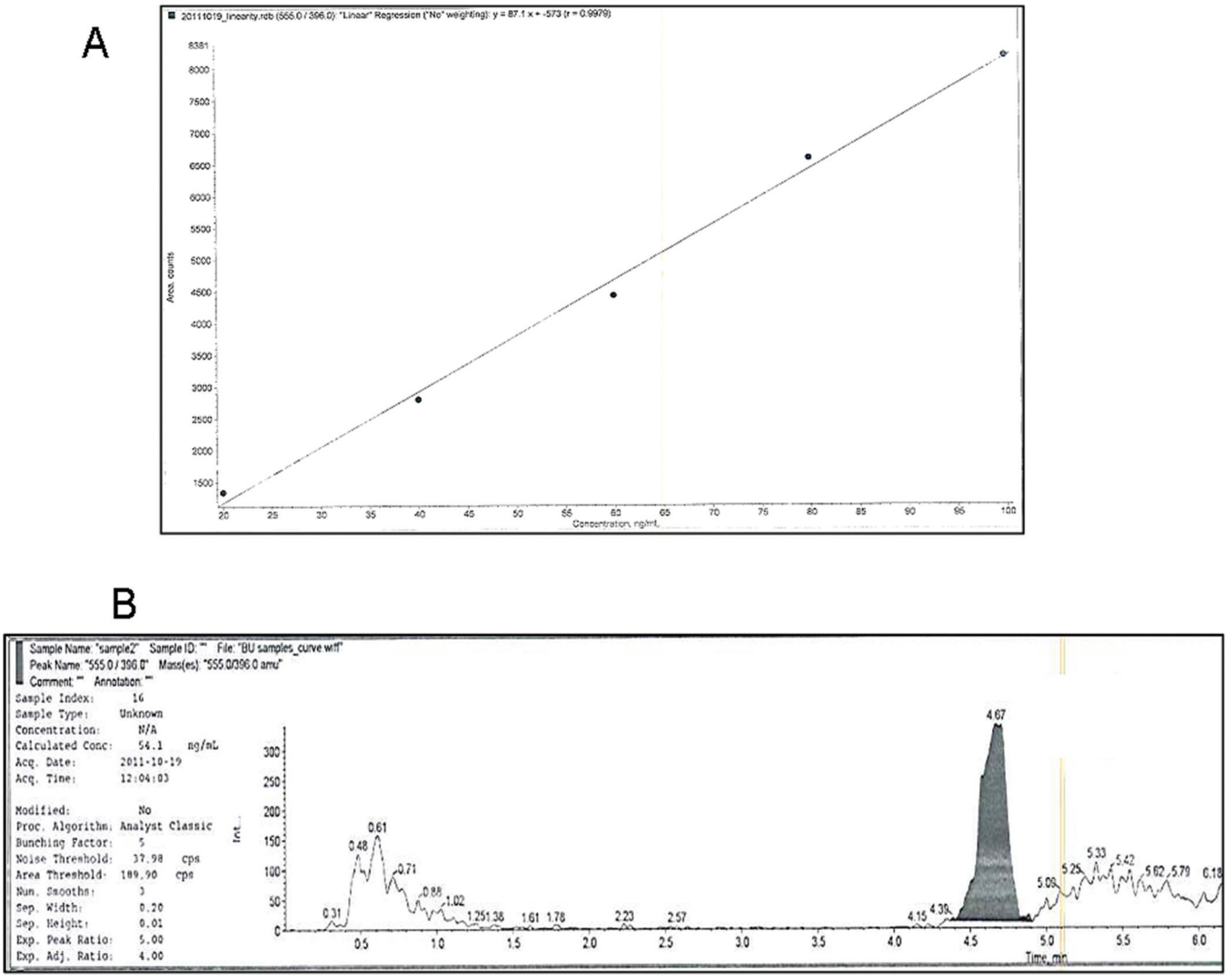

Figure 8. (A) Calibration curve of ceftriaxone. $y = 87.1x - 573$ ($r = 0.9979$). (B) MRM chromatogram indicating the presence of ceftriaxone in the brains of 7-day ceftriaxone-treated rats ($n = 3$).

lesion surgery. For the control animals, 0.9% saline was employed for the 7-day injections.

Striatal Lesion. Before the lesion surgery, the rat was first anesthetized with 2−3 μL of sodium pentobarbital (60 mg/kg, i.p. Sagittal). And 6 μL of a 0.3% 6-OHDA solution (Sigma) dissolved in 0.9% ascorbic acid solution was injected at point one: Bregma: +0.14 cm, Medline: −0.26 cm, Dura: −0.5 cm; and point two: Bregma: −0.04 cm, Medline: 0.38 cm, Dura: −0.5 cm in accordance to the atlas of Paxinos and Watson, (1986).

A period of 10 min was used for the administration in each site, and the needle of the Hamilton syringe (10 μL) was kept in situ for 10 min following the second injection or deposit to prevent backfilling along the injection tract. Injection was performed on the right side only in this study. The animals were allowed to recover and kept in the animal house (Hong Kong Baptist University), with feeding on a daily basis; tap water was available ad libitum.

Apomorphine-Induced Rotation Test. After 6-OHDA lesion, the rats were administrated apomorphine (1 mg/mL, i.p., RBI) for the test of the rotational behavior among different time points, that is, on post-lesion days 2, 4, 6, 8, and 10. The contralateral rotation of each rat within 30 min was counted and the rotation number/min was referred as an indicator to observe the behavioral changes.

Grasping Test. Similarly, the rats were tested with the grasping experiment at different respective time points. The rats were suspended by their forelimbs on a metal rod (diameter: 0.5 cm) and positioned approximately 50 cm above the table. The holding time on the metal rod was recorded.

Tissue Preparation. The rat was perfused after behavioral tests to obtain the brain sections. The rat was first deeply anesthetized with an overdose of sodium pentobarbital (5 −6 μL 60 mg/kg, i.p., Sagittal) and then perfused transcardially with 100 mL of 0.9% saline to remove any blood followed by 250 mL of fixative (3% paraformaldehyde with 0.1% glutaraldehyde solution in 0.1 M phosphate buffer (PB), pH7.4) by using a peristaltic pump. The perfusion flow rate was set to 25 mL/min for saline and 15 mL/min for fixative. The fixed brain was immediately removed from the skull and stored overnight at 4 °C in postfix solution (3% paraformaldehyde in 0.1 M PB, pH7.4). The brain was washed three times with phosphate-buffered saline (PBS; 0.01M, pH7.4) before cutting into 70 μm sections with a vibratome (Vibratome 1000, Technical Products International, St. Louis, MO). All the sections were stored in PBS at 4 °C before use.

Antibody Characterization. Guinea pig polyclonal anti-GLT1 (AB1783, Chemicon/Millipore, Temecula, CA, Bedford, MA; peptide sequence: the last 20 residues of the carboxyl terminus of Swiss-Prot entry P43004) and TH were employed.[13] Previous research by Figiel and Engele[15] demonstrated the specificities of the GLT1 antibody by Western blotting experiments using proteins from primary glial cell culture of rat cortex. And the antibody was found to bind to proteins at 71 kDa. Preabsorption of the antiserum with the corresponding immunogen peptides (AG391, Chemicon/Millipore) for GLT1

(AG377, Chemicon/Millipore) completely abolished the immunostaining in rat brain (manufacturer's technical information). A mouse monoclonal antibody against tyrosine hydroxylase (TH; MAB318, Chemicon/Millipore) and a rabbit polyclonal antibody (AB152, Chemicon/Millipore) were prepared against denatured TH, Swiss-Prot entry P04177, from rat pheochromocytoma. Both antibodies recognized a single band at 60 kDa by Western blotting using whole-brain homogenates and labeled cells with the morphology of neurons (manufacturer's technical information).

Immunocytochemical Staining. For the substantia nigra regions, three sections would be stained for each brain. They were the third section, seventh section, and eleventh section counting from the first section of the substantia nigra region during the sectioning process. Sections of the substantia nigra of the 6-OHDA-lesioned rats were incubated with mouse antibody against TH (1:2000, Chemicon/Millipore) in PBS containing 0.1% Trition X-100 overnight at room temperature. The sections were washed three times with PBS and incubated in a biotinylated secondary antibody (1:200 dilution in PBS; Vector Laboratories, Burlingame, CA) for 2 h at room temperature. The sections were then incubated with the solution containing avidin−biotin complex (Elite ABC kit; Vector Laboratories) for 45 min at room temperature and subsequently reacted with diaminobenzidine (DAB; Vector Laboratories). After being rinsed with PBS, the sections were mounted on gelatin-coated slides, dehydrated in a series of graded alcohol, and coverslipped.

Immunofluorescence Staining. All the striatal sections were incubated and reacted in a single reaction sequence to minimize variation in immunofluorescence. The striatal sections were incubated in primary antibody solutions against GLT1 (guinea pig polyclonal, 1:3000 in PBS supplemented with 2% normal goat serum and 0.1% Triton X-100; Chemicon/Millipore) for 24 h at room temperature with continuous and gentle shaking. After incubation, the sections were washed three times with PBS and incubated in Alexa 488 conjugated secondary antibody solutions (goat anti-guinea-pig IgG or goat anti-mouse IgG; 1:200 in PBS-Triton; Molecular Probes, Eugene, OR) for 2 h at room temperature in the dark with gentle shaking. The reaction was stopped after 2 h by washing the sections with PBS for three times.

Processing for Laser Scan Confocal Microscopy. After immunofluorescence staining, the sections were mounted on clean slides with fluorescent mounting medium (Dako) and covered with coverslips. Semiquantitative analyses of the fluorescence micrographs were performed. Digital images of each group of animals, that is, normal sham-operated striatum of control rats, the nonlesioned striatum of 6-OHDA lesioned rats, and the lesioned striatum of 6-OHDA lesioned rats, were captured under the same parameters with the confocal microscope (LSM 510, Carl Zeiss) at high magnification (using a C-Apochromat 63×/1.20 W Korr objective, Carl Zeiss). The fluorescent intensity of the confocal microscope images was determined by image analyzing software (Metamorph, Universal Imaging, West Chester, PA). The average gray values indicating the levels of GLT1 immunoreactivity in astrocytes were determined. Intensities were expressed as mean ± standard error of the mean. Statistical comparisons between the groups were made by one-way analysis of variance (ANOVA, SPSS software, Chicago, IL). Controls for immunofluorescence were accessed by omission of primary antibody in the original reaction sequences.

Western Blotting. Rats were decapitated. The striatum and substantia nigra tissues were collected. The tissues were homogenized in lysis buffer (250 mM sucrose, 50 mM imidazole hydrochloride, 2 mM sodium EDTA, and 2 mM β-mercatoethanol, pH 7.2) and centrifuged at 3000g for 5 min. The supernatant was collected and centrifuged at 14 000g for 60 min. The final supernatant was stored at −80 °C for further investigation.

The protein concentrations of the supernatants were analyzed by the RC DC protein assay method using the RC DC protein assay kit (Bio-Rad). The supernatants were diluted to 40 ug/lane with sample buffer and heated at 95 °C for 5 min. The protein mixtures were loaded into an SDS-8% PAGE gel for NR1 immunoblotting and an SDS-10% PAGE gel for GLT1 and TH immunoblotting. To transfer the separated protein to the nitrocellulose membrane, electrophoresis was performed and processed in 2× electrophoresis buffer (Tris-base, glycine, SDS, pH 8.3) at 40 V for 30 min followed by 70 V for 4 h at room temperature in the Mini-PROTEAN 3 electrophoresis system (Bio-Rad). The blots were collected, rinsing with TBS-T twice per 10 min and finally with TBS for 10 min. Blocking of nonspecific binding sites was performed by incubating with 5% nonfat milk for 2 h and then overnight at 4 °C or 2 h at room temperature with one of the following antibodies in 2% nonfat milk: anti-GLT1 (1:3000; Chemicon) or anti-TH (1:2000; Chemicon). After incubation, the blots were rinsed with TBS-T for three times per 5 min and finally with TBS for 5 min. The blots were then incubated in horseradish peroxidase conjugated antibody (Zymed Laboratories Inc.) against the primary antibodies in appropriate working concentrations for 1 h at room temperature. The immunoreaction was detected with the Western blotting kit (ECL, LabFrontier). Bands were visualized on a Biomax X-ray film (Kodak, Japan). Each of the antipeptide antibodies detected distinct proteins, that is, anti-GLT1 antibodies binding 73 kDa proteins and anti-TH antibodies binding 59 kDa proteins.

Semiquantitative analyses of the Western blotting were performed. The immunoreactivities for GLT1 and TH in terms of optic density for the Western blotting analysis were analyzed by the software called Metamorph. The values of both sides of striatum and substania nigra for different groups of rats were analyzed by using a statistics program, namely SPSS. And one-way ANOVA was employed to calculate the significant difference between different groups. The densities were expressed as mean ± standard error of the mean. Control for loading was done in revealing actin proteins using either striatal or nigral tissues of the same animals.

Liquid Chromatography Coupled with Tandem Mass Spectrometry. The analysis of the total brain level of ceftriaxone was performed by liquid chromatography coupled with tandem mass spectrometry (LC/MSMS). Rats were decapitated after the 7-day ceftriaxone treatment. The whole brains were collected and homogenized in lysis buffer (250 mM sucrose, 50 mM imidazole hydrochloride, 2 mM sodium EDTA, and 2 mM β-mercatoethanol, pH 7.2). The homogenate of brain tissue was then centrifuged at 3000g for 5 min. The supernatant was collected and centrifuged at 14 000g for 60 min. The final supernatant was stored at −80 °C before use. The study of the MS/MS spectra was for the confirmation of the compound, whereas that of the MRM chromatograms was for the quantitative analysis.

AUTHOR INFORMATION

Corresponding Author

*Fax: (+852) 34115995. Telephone: (+852) 34117060. E-mail: kklyung@hkbu.edu.hk.

Author Contributions

T.C.H.L. and K.K.L.Y. designed the study. T.C.H.L. conducted the experiments, analyzed the data, and drafted the manuscript. W.H.Y., Y.S.C., and K.K.L.Y. discussed the data. C.N.PL. revised the manuscript. All authors read and approved the final version of the manuscript.

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