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Ceftriaxone increases glutamate uptake and reduces striatal tyrosine hydroxylase loss in 6-OHDA Parkinson's model

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Abstract

Excess glutamatergic neurotransmission may contribute to excitotoxic loss of nigrostriatal neurons in Parkinson's disease (PD). Here, we determined if increasing glutamate uptake could reduce the extent of tyrosine hydroxylase (TH) loss in PD progression. The beta-lactam antibiotic, ceftriaxone, increases the expression of glutamate transporter 1 (GLT-1), a glutamate transporter that plays a major role in glutamate clearance in central nervous system and may attenuate adverse

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behavioral or neurobiological function in other neurodegenerative disease models. In association with >80 % TH loss, we observed a significant decrease in glutamate uptake in the established 6-hydroxydopamine (6-OHDA) PD model. Ceftriaxone (200 mg/kg, i.p.) increased striatal glutamate uptake with ≥ 5 consecutive days of injection in nonlesioned rats and lasted out to 14 days postinjection, a time beyond that required for 6-OHDA to produce >70 % TH loss (~9 days). When ceftriaxone was given at the time of 6-OHDA, TH loss was ~57 % compared to ~85 % in temporally matched vehicle-injected controls and amphetamine-induced rotation was reduced about 2-fold. This attenuation of TH loss was associated with increased glutamate uptake, increased GLT-1 expression, and reduced Serine 19 TH phosphorylation, a calcium-dependent target specific for nigrostriatal neurons. These results reveal that glutamate uptake can be targeted in a PD model, decrease the rate of TH loss in a calcium-dependent manner, and attenuate locomotor behavior associated with 6-OHDA lesion. Given that detection of reliable PD markers will eventually be employed in susceptible populations, our results give credence to the possibility that increasing glutamate uptake may prolong the time period before locomotor impairment occurs.

Keywords

Parkinson’s disease; Ceftriaxone; GLT-1; Tyrosine hydroxylase; 6-OHDA; Neuroprotection

Introduction

Glutamate mediates excitatory signal transduction, playing a primary role in brain physiology [1]. However, excessive glutamatergic neurotransmission (excitotoxicity) has been implicated in neurodegenerative disease processes such as Huntington’s disease [2–4], amyotrophic lateral sclerosis [5], and Parkinson’s disease [6]. Although Parkinson’s disease is a disease characterized by the progressive degeneration of dopaminergic neurons [7, 8], concomitant increases in glutamatergic tone have also been observed [9–11]. Indeed, converging literature has shown increased levels of extracellular glutamate [12–16] and increased glutamate receptor expression in Parkinson’s disease models or patients alike [17–19].

Despite evidence that excess glutamatergic excitotoxicity is associated with Parkinson’s disease, it is not clear at what point (if at all) in Parkinson’s disease progression excitotoxicity could contribute to the loss of nigrostriatal neurons. Glutamate increases Ca^{2+} influx through the binding of *N*-methyl-D-aspartate (NMDA) receptors. However, in excess, increased Ca^{2+} influx can trigger the activation of calcium-dependent proteases that promote cell injury and death [20–25]. Calpain is one such protease, and, indeed, there is evidence of increased calpain-related proteolytic activity in postmortem tissue of Parkinson’s disease patients contributing to the loss of dopaminergic neurons [26, 27], while calpain inhibitors can alleviate motor loss in Parkinson’s disease models [26]. There is also evidence that diminishing glutamate receptor activation can mitigate nigrostriatal damage in Parkinson’s disease models [28–30]. Unfortunately, however, the use of glutamate receptor antagonists in the Parkinson’s disease patient have had mixed clinical outcomes [31–33] with some NMDAR antagonists, although showing promise in its ability to block excitotoxicity, can

also inhibit normal excitatory synaptic activity leading to untoward side effects [34, 35]. Therefore, an alternate intervention targeting elevated glutamatergic signaling could prove useful for mitigating nigrostriatal neuron loss, without compromising the essential components of glutamate signaling in neuronal functioning. However, demonstration of evidence that reducing extracellular glutamate could protect loss of nigrostriatal DA neurons is still a necessary first step for advancing prospects that mitigating excitotoxicity is a viable strategy to diminish, if not entirely halt, Parkinson's disease progression.

One possible approach is to reduce excess glutamatergic tone and test the impact upon nigrostriatal loss induction in in vivo Parkinson's disease models. Glutamate transporters, expressed primarily in glial cells and secondarily in neurons, aid in the removal of glutamate from the extracellular space, thereby minimizing its synaptic accumulation [36–39]. The glutamate transporter-1 (GLT-1), predominantly expressed by the glia, may be responsible for the majority of glutamate uptake [39, 40], although GLAST, also expressed in glia, can upregulate in expression when GLT-1 function is compromised [41, 42]. The importance of GLT-1, however, is highlighted by findings indicating GLT-1 dysfunction in amyotrophic lateral sclerosis [43] as well as traumatic brain injury [44] patients. Experimental reduction of the glial transporters increases synaptic glutamate levels, triggering excitotoxic neurodegeneration and paralysis [37, 45].

In Parkinson's disease models, evidence suggests that increasing GLT-1 expression could be a strategy to diminish nigrostriatal loss. Glial cells themselves seem to play a protective role in PD models [46, 47]. Ceftriaxone, a beta-lactam antibiotic, increases GLT-1 expression [43] and could be thus be a feasible venue for augmenting glutamate uptake to reduce excitotixicy. A recent proof-of-concept study indicates that ceftriaxone, when given prior to lesion induction, decreased the degree of nigrostriatal neuron loss [48]. Ceftriaxone may attenuate the Huntington's disease phenotype in the R6/2 mouse [4, 49] and alleviate motor neuron degeneration in an animal model of amyotrophic lateral sclerosis [50]. Obviously, the next step forward is to determine if ceftriaxone could mitigate damage to the nigrostriatal pathway once lesion progression is underway. Thus, once reliable biomarkers for Parkinson's disease are revealed, increasing glutamate uptake could delay the onset of locomotor symptoms that emerge with >70 % striatal DA loss. Given that ceftriaxone requires ≥5 daily injections to increase GLT-1 [43], we determined if tyrosine hydroxylase (TH) loss, already underway in the 6-hydroxydopamine (6-OHDA) Parkinson's disease model, could be mitigated by initiating ceftriaxone treatment at the same time of the lesion induction.

Methods

Animals

Male Sprague–Dawley rats, purchased from Charles River, were used in all experiments. All rats were 4–8 months old, and were housed under controlled lighting conditions (12:12 light/dark cycle) with standard animal chow and water available ad libitum. All animals were used in compliance with federal guidelines and the institutional Animal Care and Use Committee guidelines at LSU Health Sciences Center–Shreveport.

Ceftriaxone Administration

Ceftriaxone (Hospira, Lake Forest, IL, USA) was dissolved in sterile saline (0.9 %) and given by intraperitoneal injection (i.p) at 200 mg/kg. To determine the impact of ceftriaxone on GLT-1 expression and glutamate uptake in naive (not 6-OHDA-lesioned) animals, ceftriaxone was given for seven consecutive days at 200 mg/kg. Control animals were given 0.9 % saline for the same time frame. In order to determine longevity of ceftriaxone effect, 4–8-month-old naive Sprague–Dawley rats were treated with 200 mg/kg of ceftriaxone or vehicle (0.9 % saline) for seven consecutive days. Rats were euthanized, and glutamate uptake was assessed in both groups at either 1 or 4 days and 1 or 2 weeks post last injection (Fig. 1a). In rats receiving 6-OHDA lesion, ceftriaxone was given on the day of lesion and for seven consecutive days thereafter (Fig. 1b). As a control to ceftriaxone injection, the 6-OHDA lesion only group had consecutive daily injections of vehicle.

6-OHDA Lesions

Each animal underwent survival surgery to deliver the neurotoxin 6-OHDA to the medial forebrain bundle as previously described [51]. Rats were anesthetized with 40 mg/kg Nembutal intraperitoneal (i.p.) (pentobarbital Lundbeck Inc, Deerfield, IL, USA) with supplement of 9.0, 0.6, and 0.3 mg/kg ketamine, xylazine, and acepromazine, respectively. Animals were immobilized in a stereotaxic frame to target the medial forebrain bundle at coordinates ML +1.5, AP −3.8, DV −8.0, relative to Bregma, according to Paxinos and Watson rat brain atlas [52]. A total of 16 μg of 6-OHDA in 4 μL in 0.02 % ascorbic acid (concentration of 4 mg/mL) was infused unilaterally at a rate of 1 μL/min. To control for any effects of the lesions surgery alone, the contralateral striatum was also infused with vehicle (0.02 % ascorbic acid) at a rate of 1 μL/min. The syringe was left in place for 10 min before removal to allow for maximal diffusion and to minimize mechanical damage to the tissue. Body temperature was maintained at 37 °C during surgery using a temperature monitor with probe and heating pad (FHC, Bowdoin, ME, USA).

Amphetamine Testing for Lesion Verification

Rotational behavior was assessed for 60 min following amphetamine (2 mg/kg i.p) administration. Rotations ipsilateral to the lesioned side were quantified at 7 days post-6-OHDA infusion (Fig. 1b). Amphetamine-induced rotations increase in response to a lesion and can detect at least a 50 % loss of dopamine [53]. Rats were killed 9 days following 6-OHDA for glutamate uptake analysis, GLT-1, TH, TH ser19 phosphorylation, a Ca^{2+}-dependent process in DA neurons [42, 54], and calpain analyses, 2 days after the amphetamine test (allowing for practically complete clearance of amphetamine).

Preparation of Synaptosomes

Crude synaptosomes were prepared from striatal tissue was homogenized in 5 mL of 0.32 M sucrose solution using 10 up and down strokes of a Teflon/glass homogenizing wand (Glas-Col, Terre Haute, IN, USA) then spun at 1,000×*g* for 10 min. The resulting pellet was stored as the P1 fraction, from which the analysis of total and phosphorylated TH was later conducted by sonicating the pellet in sodium dodecyl sulfate and performing Western blot analysis (we have previously reported the utility of using this fraction in determining the

expression level of cytosolic proteins such as TH Chotibut et al. [51]). The resulting supernatant was spun further at 17,500×*g* for 30 min yielding the P2 fraction. The P2 fraction was used to determine glutamate uptake on the day of preparation, and aliquots were frozen to later analyze GLT-1 and GLAST protein expression. The supernatant was aspirated and resuspended in 1 mL of Kreb's buffer. Protein concentration was determined using a BCA colormetric assay (Thermo Scientific, Rockford, IL, USA). This protocol has been used to determine the reuptake of glutamate [42] and other neurotransmitters endogenous to striatum [55].

Glutamate Uptake Protocol

Synaptosomal P2 fraction contain glial components [56], and ~70 % of the levels of glial fibrillary acid protein are recovered in purified glial plasmalemmal vesicles [57] and thus are adequate for assessment of glutamate reuptake [42]. Synaptosomes were distributed in test tubes at equal protein quantity to prepare for glutamate reuptake, with an aliquot saved for later determination of the protein quantities of GLT-1 TH, ser19 TH phosphorylation, and calpain activity (spectrin breakdown products) [58].

Synaptosomes were used in a quantity of 30 μg of total protein in a 200-μL final volume for glutamate reuptake. In 100 μL, the combination of the synaptosome prep to constitute 30 μg synaptosomal protein and oxygenated Kreb's buffer was prepared at 4°C. The synaptosomes were then placed in a water bath at 35 °C for 5 min, followed by the addition of 100 μL of 10 μM 14C(U)-L-glutamic acid (Perkin-Elmer, specific activity 260 mCi/mmol, catalogue no. NEC290E050UC) to the synaptosome preparations (giving a 5 μM final [glutamate]), allowed to incubate for reuptake for 90 s. The reaction was then terminated with 1 mL of ice-cold Kreb's buffer, and the tubes were reimmersed the tubes into an ice bath. The reuptake time was chosen to be as close as technically and practically possible to the reuptake time of glutamate observed in vivo, which occurs within 10 s [59, 60]. Synaptosomes were washed multiple times in order to remove excess labeled glutamate with equal-osmolarity phosphate-buffered saline through a Brandel M24-TI (Gaithersburg, MD, USA) cell harvester with Brandel GF/C filter paper pretreated with a 2 % polyethylenimine solution to reduce nonspecific binding of label. The filter paper containing the rinsed synaptosomes were then transferred into scintillation vials containing 5 mL of biodegradable scintillation cocktail (Research Products International, Mount Prospect, IL, USA) and counted with a Beckman Coulter LS6500 scintillation counter (Brea, CA, USA).

Quantifying [^{14}C]Glu Uptake into Synaptosomes

To determine the quantity of glutamate reuptake, the percent of glutamate (as the label) recovered in the synaptosomes against the total amount of glutamate (as the label) in the reuptake experiment was first determined. This percentage of reuptake averaged 1.50±0.28 % (mean±SEM, n =7 experiments) using 5 μM glutamate (final concentration) for reuptake studies. In pilot experiments, the percent reuptake was not significantly different using 1 or 2 μM glutamate (n =5 experiments). The picomole of glutamate was then determined based upon this percent of label recovery in the synaptosomes and normalized to mg protein per minute based upon the allotted 90 s uptake time allowed.

The determination of [^{14}C]Glu uptake in the crude synaptosomes from dorsal striatum harvested from the contralateral vehicle-infused and 6-OHDA-infused hemispheres was conducted simultaneously. Each determination was done in triplicate for each assay condition.

Tissue Preparation and Western Immunoblotting

Synaptosome fraction (P1) and the processed preparatory sample (P2) were sonicated in a 1 % sodium dodecyl sulfate solution (pH ~8) using a Branson Sonifier 150 (Danbury, CT, USA). Protein concentration was determined using the bichinchoninic acid colometric assay. Following gel electrophoresis, proteins were transferred for 500 Vh in a Tris/glycine/methanol buffer onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA).

The nitrocellulose membrane was stained with Ponceau S to further normalize staining in each sample lane [61]. These lanes were scanned and quantified by Image J to normalize protein in each sample. This relative total level then served as an additional normalizing value to determine the quantity of each protein assayed [61]. To continue processing, the membranes were blocked in PVP buffer (1 % polyvinylpyrrolidone and 0.05 % Tween 20) for a minimum of 2 h to reduce nonspecific antibody binding. The membrane was soaked in primary antibody for 1–3 h. Specific primary antibodies were as follows: Specific primary antibodies were as follows; GLT-1 (Santa Cruz, Santa Cruz, CA, catalogue no. 15317), TH (Millipore, catalogue no. AB152), GLAST (Novus Biologicals, Littleton, CO, catalogue no. NB100-1869), and serine 19 phosphorylation (PhosphoSolutions, catalogue no. p1580-19). P2 fraction was used to assess GLT-1 expression and P1 fraction used for TH expression Western blot analysis. Nominal protein loads for linear detection of GLT-1 was 20–30 μg total protein 8 μg for TH protein, and ~5–10 μg for serine 19 TH phosphorylation. After primary treatment, blots were exposed to secondary antibody (swine anti-rabbit IgG) for signal enhancement, followed by 1 h incubation with [125I] protein A (PerkinElmer, Waltham, MA, USA).

Statistics

All glutamate uptake studies were done in conjunction with assessment of GLAST and GLT-1, as assessed in aliquots of synaptosomes that were used to determine glutamate uptake. Tissue harvested from the striatum contralateral to 6-OHDA-lesion served as the inherent control to the lesioned striatum for each rat/test subject. We excluded vehicle and ceftriaxone pairs where the vehicle did not have >65 % TH loss (4 of 13 surgery pairs). Surgeries were performed in pairs with the assumption that any irregularities in efficacy of the 6-OHDA lesion may occur in either the vehicle- and ceftriaxone-treated animals. Specifically, surgeries were performed in multiples of two each day to accommodate for the two treatment (Cef and Veh) groups. For instance, on one day, two surgeries were performed and treatment was assigned (Cef, saline). On another day, two additional surgeries were performed and treatment was assigned (Saline, Cef). Thus, any variances that may be encountered on each day of surgery that may affect lesion severity/experiment could be accounted for by analyzing the data using a paired *t* test due to the cef/saline-matched pairing. As a result, a Student's paired two-tailed *t* test was also used to compare glutamate

uptake differences between the two groups and for the lesioned and contralateral striatum, to ascertain differences in TH protein loss between lesioned and contra-lateral striatum, differences in ser19 TH phosphorylation, calpain activity, and GLT-1 expression associated with 6-OHDA lesion in the vehicle-injected control and the ceftriaxone-treatment group. These measures were all conducted from the same tissue sources, thereby obtaining all dependent measures, including rotational behavior, in operationally matched manner. As such, Student's paired two-tailed *t* test was also used for analysis of rotational behavior.

Results

Glutamate Uptake in 6-OHDA Lesioned Rats

We found evidence of decreased striatal glutamate uptake in the 6-OHDA lesioned striatum compared with its contralateral (vehicle-infused) striatum by ~35 % (Fig. 2). To verify the degree of lesion in association with this impairment in glutamate uptake, we determined TH loss in this set of studies to be ~84 % (data not shown), the threshold above which clinical Parkinson's disease symptoms are seen [62].

The decrease in glutamate reuptake capacity, at least when TH loss is at PD levels, suggests the possibility that this impairment could contribute to nigrostriatal neuron loss once 6-OHDA initiates damage to the neuron.

Ceftriaxone Effect on Glutamate Uptake in Naive Rats—Treatment with 200 mg/kg (i.p.) ceftriaxone for 5–7 days has been shown to increase glutamate reuptake capacity [43, 49]. Consistent with these previous observations, we also observed increased GLT-1 expression with a similar regimen 4 days after the last day of administration (Fig. 3).

Longevity of the Ceftriaxone Effect on Glutamate Uptake in Naive Rats—In order to ascertain the longevity of the ceftriaxone effect, we examined glutamate uptake dynamics at 1, 4, 7, and 14 days after five or seven consecutive days of ceftriaxone treatment. We first determined the duration of ceftriaxone-mediated enhancement of glutamate reuptake 1 and 4 days after final (seventh) consecutive day of ceftriaxone administration, representing a time period encompassing the length of time (~9 days) necessary for the 6-OHDA lesions to produce ~70 % loss of TH. Glutamate reuptake was increased ~50 % 1 and 4 days (pooled) after the final injection (Fig. 4a). This increase in glutamate reuptake was also observed between 7 and 14 days after last injection, increasing ~20 % overall (Fig. 4b).

Ceftriaxone Treatment in a 6-OHDA PD Rat Model: Tyrosine Hydroxylase, GLT-1, and GLAST Expression and Glutamate Uptake—With 6-OHDA lesion and at 2 days after seven consecutive injections of ceftriaxone (200 mg/kg), the increase in GLT-1 expression observed in naive rats (Fig. 3) was also observed with 6-OHDA lesion (Fig. 5a). Predictably, this was associated with increased glutamate uptake, with an increase of ~30 % compared to that in the lesioned striatum of the vehicle-injected control group (Fig. 5b). A similar increase in GLT-1 was seen in the unlesioned striatum of ceftriaxone-treated rats compared to the unlesioned striatum vehicle-treated rats (*data not shown*).

There were no significant differences in striatal glutamate–aspartate transporter (GLAST) expression observed between these same 6-OHDA lesioned rats treated with vehicle versus ceftriaxone (*data not shown*).

In comparison to contralateral control striatum, this increased ceftriaxone-mediated glutamate reuptake was associated with a significant attenuation of TH protein loss, with ~3-fold more striatal TH remaining in cef-treated rats compared to that in the vehicle-injected control group (Fig. 6a). A Western blot representing the difference in TH protein loss produced by 6-OHDA between the ceftriaxone-versus vehicle-treated groups is shown,with ~55 % loss seen with ceftriaxone and ~90 % loss seen with vehicle treatment (Fig. 6b).

Rotational Behavior in 6-OHDA PD Rat Model with Ceftriaxone Treatment— Behavioral testing was performed after amphetamine (2 mg/kg) i.p. injection on day 7 after lesion. In addition to attenuating TH loss due to 6-OHDA, the ceftriaxone regimen significantly reduced amphetamine-induced rotation (ipsilateral to the lesion) compared to vehicle treatment by ~50 % in these same rats (Fig. 7). Thus, ceftriaxone treatment also reduced 6-OHDA lesion impact on rotational behavior, an index of nigrostriatal neuron loss.

Mechanism of Ceftriaxone Protection: Evidence for Reduced Ca^{2+}-Influx Serine 19 TH Phosphorylation—Ser^{19} phosphorylation, while not directly affecting TH activity, may alter its confirmation increasing the likelihood for TH degradation [63]. Ser^{19} phosphorylation is also Ca^{2+} dependent [54], and the phosphorylation stoichiometry decreases in association with increased striatal glutamate uptake [42]. Therefore, ser^{19} TH phosphorylation was used as an indirect index of Ca^{2+} influx into DA terminals. The stoichiometry of ser^{19} TH phosphorylation in lesioned striatum was significantly reduced in the ceftriaxone group compared with that in the vehicle group (Fig. 8a, c), without any significant difference between the two groups in the unlesioned contralateral control striatum (Fig. 8b, c).

Discussion

Glutamate excitotoxicty has long since been thought to be a component of various neurodegenerative disorders, as seen in models of Huntington's disease [1, 64, 65] and, more recently, Parkinson's disease [6, 16, 29, 30]. Dopamine and glutamate have reciprocal interaction within the striatum and affect each others' release, thereby playing a role in basal ganglia function and dysfunction [12, 66–71], with both neurotransmitters cooperatively modulating the activity of striatal output (medium spiny) neurons. Indeed, the onset of motor symptoms in Parkinson's disease animal models has been shown to be closely tied with the increase of glutamate levels within the basal ganglia [12–14, 69, 72, 73]. These changes in corticostriatal glutamate release due to dopaminergic denervation imply circuitry imbalance and have been speculated to contribute to further loss of nigrostriatal neurons. The expression of both ionotropic and metabotropic glutamate receptors on nigrostriatal neurons increases its sensitivity to glutamate excitotoxicity [68]. Increased expression of NMDA and mGluR5 glutamate receptors have also been observed in PD patients [17, 18]. Taken together, these scenarios may not only facilitate glutamate excitotoxicty but also play an underlying role in Parkinson's disease progression. The ability to minimize this increase in

glutamate activation in Parkinson's disease pharmacologically remains an elusive goal given the high incidence of side effects from glutamate receptor antagonists [31–35]. Our work here suggests that through ceftriaxone-induced glutamate reuptake, the progression of nigrostriatal neuron loss may be mitigated when administered early in PD pathogenesis.

There is evidence that glutamate can contribute to the demise of nigrostriatal neurons via activation of TH, the rate-limiting enzyme in dopamine biosynthesis [74], further supporting the role that excitotoxicity may be playing in the initiation of Parkinson's disease progression. Additionally, protection against 6-OHDA lesion in mGluR5 receptor knockout mice has also been observed indicating the importance glutamate activation may play in DA denervation [75]. As such, preclinical studies and postmortem analysis of central nervous system (CNS) tissue show evidence that glutamate transporters can provide some measure of protection against excitotoxicity in the CNS, as the main transporter responsible for glutamate reuptake (GLT-1), can increase in expression in response to injury or excitotoxic insult [76, 77]. In line with this, DA denervation may trigger modulations in GLT-1 [9, 78], indicating that GLT-1 dysfunction likely plays a role in Parkinson's disease progression.

In this line, we first demonstrate that there is impaired glutamate uptake, presumably through GLT-1, in the striatum of the 6-OHDA-lesioned rat, supporting the idea of glutamatergic dysfunction in Parkinson's disease, as suggested by others [10–12, 78]. Secondly, when a 7-day treatment period of ceftriaxone is initiated at the time of 6-hydroxydopamine infusion, not only is glutamate uptake increased with increased GLT-1 expression, but TH loss is attenuated by ~3 times compared to vehicle treatment. Amphetamine-induced rotational behavior after lesion also was attenuated by ~50 % after a 7-day regimen of ceftriaxone compared to vehicle, suggesting the potential for ceftriaxone to minimize Parkinson's disease symptoms *after* the lesion has progressed.

TH loss attenuation at 9 days postlesion

Given that the onset of motor symptoms in Parkinson's disease animal models may be associated with glutamate overactivation [72], examining early stages of nigrostriatal pathway loss, wherein intervention may occur remains a clinically relevant approach. As such, ceftriaxone treatment was initiated on the day of 6-OHDA infusion, due to the fact that its ability to increase GLT-1 expression requires a minimum of 5 days of consecutive treatments [43]. For clinical translation, intervention with ceftriaxone could be feasible if a reliable biomarker for Parkinson's disease was detected in a patient, prior to locomotor symptom presentation. The ability for ceftriaxone to attenuate TH loss, after the lesion is well underway, also indicates a potential for the antibiotic to be effective in later stages of Parkinson's disease progression as well. It is not known if our present findings showing TH loss protection may, however, translate into an increase in cell number or protect against their further loss. Thus, subsequent immunohistochemistry studies examining TH-positive cells may reveal an increase in cells or increase in TH per remaining cells and indicate how TH protein loss is mitigated. Current strides in identifying viable Parkinson's disease biomarkers [79] provides the potential for earlier detection, giving further credence to the neuroprotective capability of ceftriaxone in the treatment of Parkinson's disease, especially

from the standpoint of extending the period of time a susceptible individual may realize prior to the manifestation of locomotor symptoms.

Ca^{2+}-Dependent Mechanism: Calpain and Serine 19 Phosphorylation

Ceftriaxone has been thought to increase GLT-1 transcription through the nuclear factor-κβ signaling pathway, implicating increased transcriptional activity to enhance synaptic glutamate clearing capacity. Here, however, we demonstrate a potentially novel mechanism through Ca^{2+}-dependent means by which ceftriaxone is mitigating TH loss. Glutamate-mediated calcium influx through glutamate receptors such as NMDA has long been considered an essential component of glutamate excitotoxicity [22]. The Ca^{2+} overload resulting from the overactivation of glutamate receptors can induce the activation of calpain, a family of cysteine proteases activated by calcium, triggering the cleavage of several downstream substrates leading ultimately, to neuronal death [80]. Although we did not see significant differences in calpain breakdown between treatments of ceftriaxone and vehicle, it does not exclude the possibility of calpain activation given that the striatal tissue analyzed also contains GABAergic, glutamatergic, and glial components. Furthermore, our preparation technique to analyze glutamate uptake in conjunction with all protein measures mandated a homogenization step to produce the crude synaptosomes. This physical stress in the neuronal entities may have affected overall calpain activity to a degree that may have affected the sensitivity of the measures between the striatal samples and treatment groups.

Another calcium-dependent marker, and notably only in catecholaminergic neurons, TH phosphorylation at serine19, was decreased in ceftriaxone-treated compared to vehicle-treated rats by ~30 % in lesioned striatum. NMDA agonists may increase Ser^{19} TH phosphorylation [81], which has been shown to depend upon Ca^{2+} influx [54]. Furthermore, we have recently reported that increased glutamate uptake capacity in striatum may be associated with decreased ser19 TH phosphorylation [42]. In addition, changes in TH phosphorylation may be induced by NMDA receptor activation [81]. In particular, phosphorylation at Ser^{19} has been seen to alter the conformation of TH and may increase TH degradation susceptibility through the ubiquitin–proteasome pathway [63]. Ceftriaxone-mediated reduction of Ser^{19} TH phosphorylation may consequently minimize TH degradation vulnerability and serve as a mechanism through which TH protection is occurring and, importantly, signify less Ca^{2+} influx in the nigrostriatal terminals. Taken together, our data demonstrate the utility of ceftriaxone in protecting from further TH loss when given at the time of 6-OHDA lesion, potentially through a calcium-mediated mechanism.

Transporter Plasticity

Although there is evidence to support that increasing GLT-1 expression can protect nigrostriatal neurons, other glutamate transporters may also provide possible protection of nigrostriatal neurons. For example, EAAC1 function may also be important for nigrostriatal function, due to it being indirectly involved in glutathione biosynthesis [82]. Another glial glutamate transporter, GLAST, may increase function with GLT-1 compromise [41, 42], although ceftriaxone does not seem to affect GLAST expression levels in our hands as well as others [43].

Clinical Translation and Future Work

The establishment of predictive Parkinson's disease biomarkers has been at the forefront of recent research [83, 84]. As such, with the eventual establishment of biomarkers, we show evidence suggesting that ceftriaxone could mitigate or diminish the rate of TH protein loss after a lesion has begun or prior to the emergence of locomotor impairment. If this approach is successfully translated into the clinic, ceftriaxone could be used to treat patients identified to be at high risk for Parkinson's disease due to the presence of such biomarkers, and reduce the rate of dopaminergic neuropil loss in these patients. Although the duration and pharmacological approach for treatment required to protect against Parkinson's disease progression remains to be determined, the preexisting approval of ceftriaxone by the Food and Drug Administration would make clinical translation even more feasible and thus accelerate its availability to Parkinson's disease patients.

The potential of ceftriaxone in Parkinson's disease progression may not be limited to extending the function of nigrostriatal neurons. Ceftriaxone could also conceivably be useful to counteract complications that come with the current gold standard of treatment, L-DOPA. L-DOPA-induced dyskinesia (LID), a debilitating setback for Parkinson's disease patients who rely on L-DOPA for its therapeutic effects occurs in 90 % of patients with 10 or more years of therapy. There is evidence for glutamatergic involvement in LID pathology [85–87], so treatment with ceftriaxone early in Parkinson's disease progression may not only minimize TH loss but also, indirectly, postpone the initial administration of L-DOPA, thereby minimizing or delaying L-DOPA-induced dyskinesia expression. Future experiments will be needed to examine the potential for ceftriaxone to be of benefit in this scenario, as well as examining its potential to prolong the time for attaining protection against or restore TH loss in different Parkinson's disease stages with additional therapies, such as glial cell line-derived neurotrophic factor.

Conclusions

Glutamate excitotoxicity has been long proposed to have some level of involvement in Parkinson's disease progression. Here, we provide evidence to further support a role for glutamate excitotoxicity in the loss of tyrosine hydroxylase, the rate-limiting enzyme for dopamine biosynthesis, in the 6-OHDA model of Parkinson's disease. In association with loss of TH, there is a decrease in glutamate uptake. Pharmacologically induced increases in GLT-1 expression from ceftriaxone (seven consecutive daily administrations) resulted in increased glutamate uptake in the striatum, which was concurrent with attenuating the loss of TH caused by 6-OHDA. This protection was associated with a decrease in ser^{19} TH phosphorylation, serving as evidence that the attenuation of TH loss by 6-OHDA was related to a decrease in Ca^{2+}-influx produced in association with the lesion. The enhanced GLT-1 expression properties of ceftriaxone has been established to take a minimum of five consecutive injections, and we therefore initiated delivery at the time of 6-OHDA, given the rapid rate of TH loss it produces. The translatability of our work may therefore be applicable to a clinical scenario wherein Parkinson's disease progression is suspected prior to presentation of locomotor impairment. Thus, increasing glutamate uptake could serve to prolong the period of time that a susceptible individual could be free from eventual

locomotor impairment, thus increasing the therapeutic window for additional treatments that could further halt or reverse loss of the nigrostriatal neurons.

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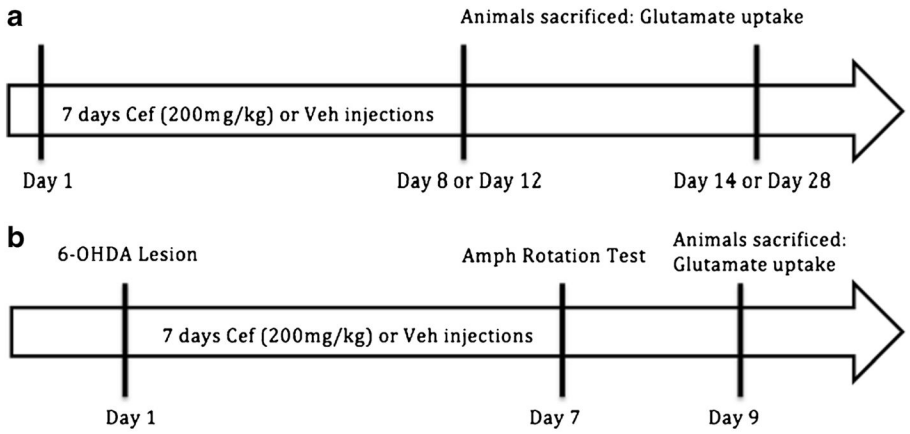

Fig. 1.

Timeline of ceftriaxone regimen. **a** Ceftriaxone longevity experiment: 7 days of 200 mg/kg ceftriaxone or vehicle was injected i.p. in naive male Sprague–Dawley rats. Rats were then killed at various time points after the last (seventh) cef injection: 1 or 4 days after, 1 or 2 weeks after. **b** Ceftriaxone lesion experiment: all male Sprague–Dawley rats were lesioned on day 1. On the seventh consecutive day of ceftriaxone or vehicle (200 mg/kg), amphetamine (*Amph*) induced rotation was assessed. On day 9, rats were killed for glutamate reuptake and biochemical analysis (TH, GLT-1, and GLAST)

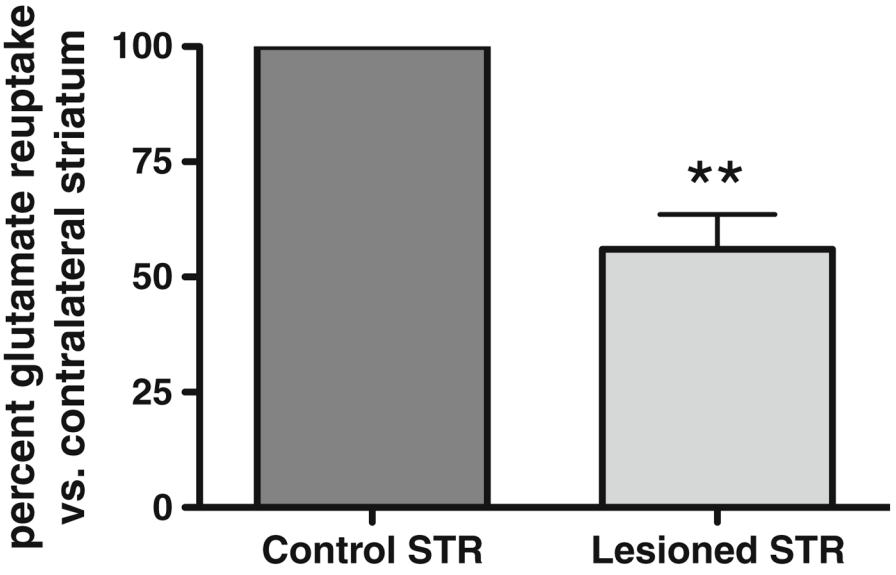

Fig. 2.

Impaired glutamate reuptake in the 6-OHDA lesioned striatum. In association with >80 % TH protein loss (data not shown), after 6-OHDA (16 μg) infusion into the medial forebrain bundle, striatal glutamate reuptake was decreased ~45 % in 4–6-month-old male Sprague–Dawley rats (n =5, Student's paired t test, $^{**}p$ <0.01, t =7.499)

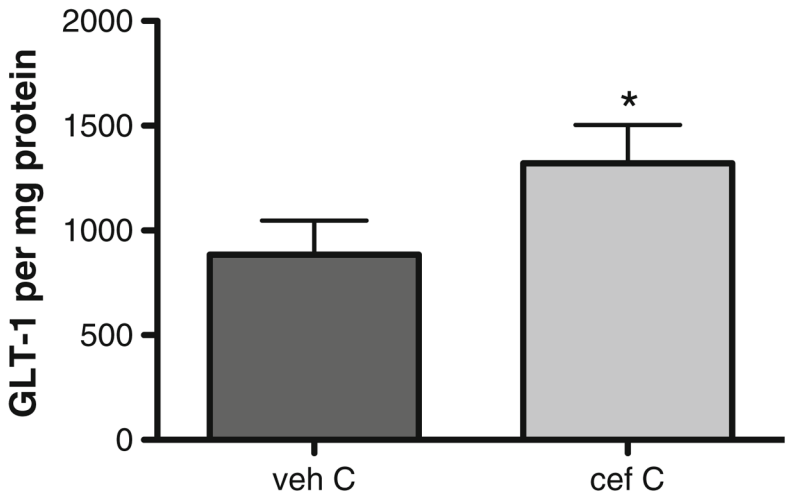

Fig. 3.

Ceftriaxone increases GLT-1 expression in naive rats. Four- to six-month-old male Sprague–Dawley rats were treated with 200 mg/kg ceftriaxone (*cef*) or saline (*veh*) for seven consecutive days. GLT-1 expression was determined 4 days later (n =5, Student's paired t test, *p <0.05, t =3.567)

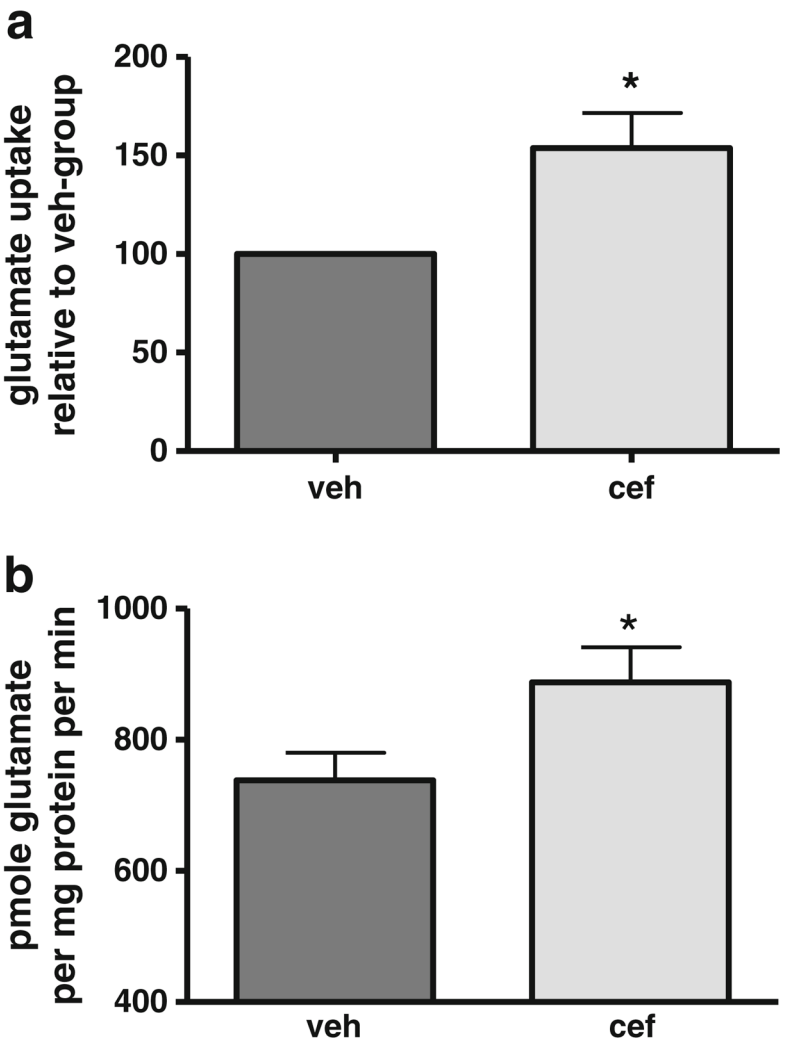

Fig. 4.

Longevity of ceftriaxone enhancement of striatal glutamate reuptake. Four to eight-month-old male Sprague–Dawley rats were treated with 200 mg/kg ceftriaxone (*cef*) or saline (*veh*) for five or seven consecutive days. Glutamate reuptake was assessed and data collapsed for analysis in two time periods post-cef: **a** 1 and 4 days after last cef administration (seven consecutive days). Glutamate reuptake increased ~1.5-fold (154 % of veh) in cef group relative to reuptake in veh group (Student's paired *t* test, **p* <0.05, *t* =2.99, *n* =6). Mean ±SEM increase: after day 1: 176±20 %; after day 4: 131±27 %. Data presented as percent difference in uptake in cef group vs. veh group. **b** 7 and 14 days after last cef administration (five consecutive days). Glutamate reuptake increased ~1.2-fold (122 % of veh) in cef group relative to reuptake in veh group (Student's paired *t* test, **p* <0.05, *t* =3.04, *n* =5). Mean ±SEM increase: after day 7: 118±6 %; after day 14: 127±22 %. Data presented as total glutamate reuptake (picomole glutamate per minute per milligram protein) cef group vs. veh group

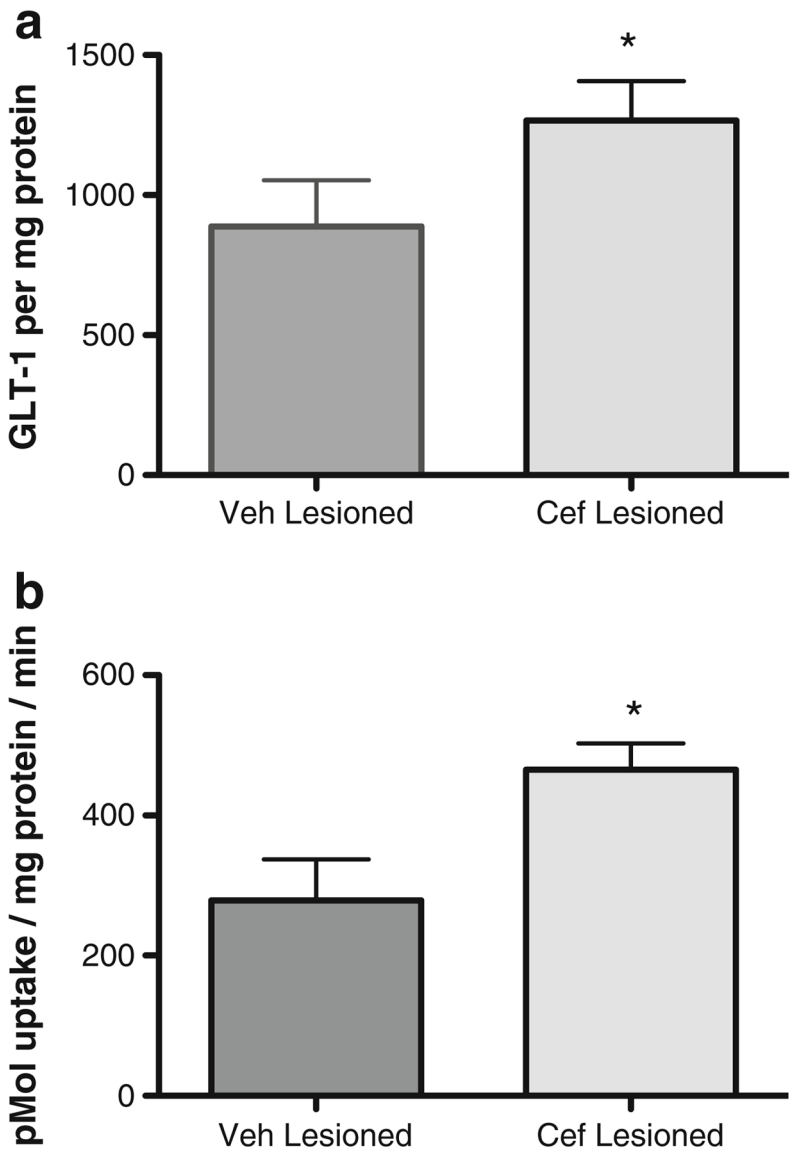

Fig. 5.

Ceftriaxone increases GLT-1 expression and glutamate reuptake in lesioned striatum. **a** GLT-1 per microgram protein (n =5, paired t test, *p <0.05, t =2.954) and **b** glutamate reuptake (n =5, paired t test, t = 4.987, p <0.01) in 6-OHDA-lesioned striatum is increased by ~30 % with seven consecutive injections of 200 mg/kg ceftriaxone (cef) in 4–8-month-old male Sprague Dawely rats

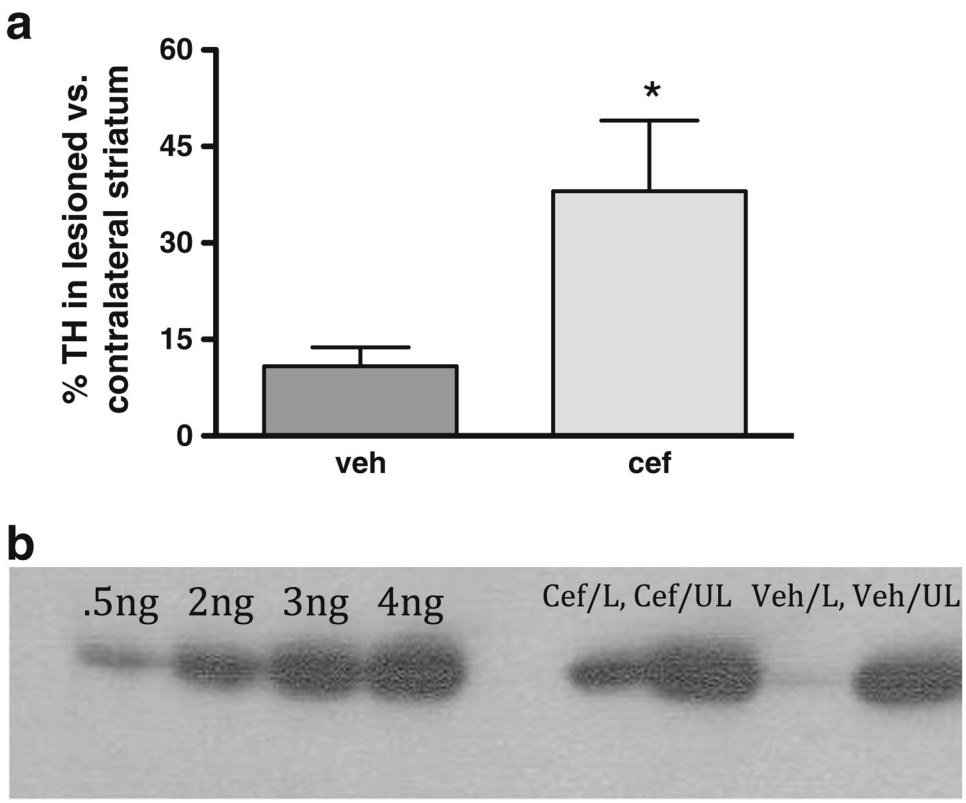

Fig. 6.

Ceftriaxone attenuates tyrosine hydroxylase (*TH*) loss in 6-OHDA lesioned rats. **a** Seven consecutive daily injections intraperitoneally of (200 mg/kg) ceftriaxone starting on the day of 6-OHDA infusion attenuated TH loss by ~30 % in 4–8-month-old male Sprague–Dawley rats, as compared to an average TH loss of vehicle Treated rats was 86 % (n =9, paired t test, t =5.24, p <0.05). **b** Representative Western blot of a (*left to right*): ceftriaxone-treated rat [lesion (*L*), unlesioned (*UL*)] and a vehicle treated rat. Standards of TH shown in nanograms (0.5, 2, 3, and 4)

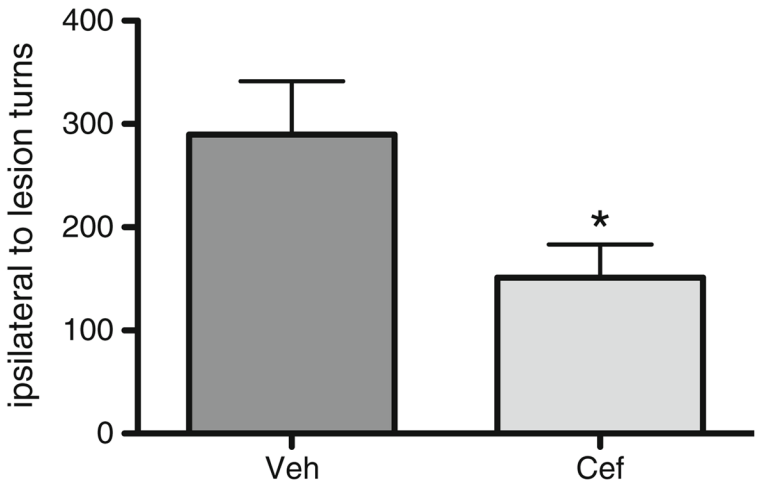

Fig. 7.

Ceftriaxone decreases amphetamine-induced rotational turns in 6-OHDA lesioned rats. Seven consecutive daily injections of ceftriaxone (200 mg/kg, i.p.) starting on the day of 6-OHDA infusion ellicited a reduction in amphetamine-induced rotation compared to vehicle treatment by ~50 % (n =7, paired t test, t =3.552, p <0.05)

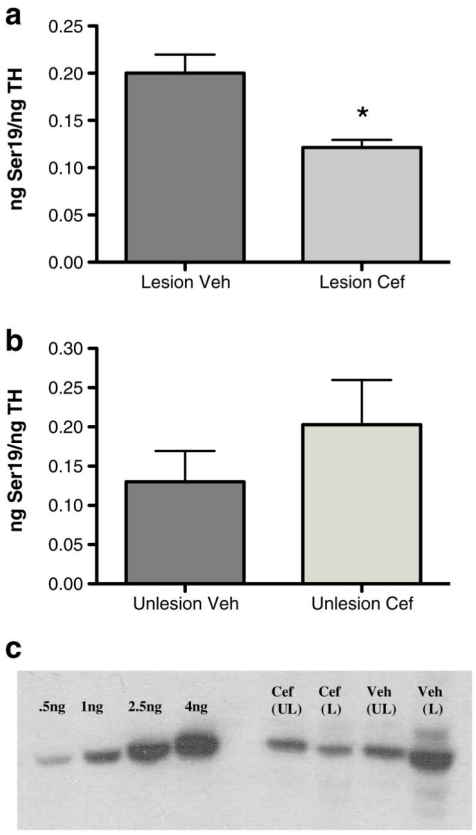

Fig. 8.

Ceftriaxone affects serine19 TH phosphorylation in 6-OHDA lesioned rats. Seven consecutive daily injections of ceftriaxone (200 mg/kg i.p.) starting on the day of 6-hydroxydopamine infusion reduced Ser19 TH phosphorylation in the lesioned striatum (**a**) but did not affect the unlesioned striatum (**b**) (**a** n =7, paired t test, t =5.869, $p < 0.01$). **c** Representative Western blot of a (*left to right*): ceftriaxone-treated rat [unlesion (*UL*), lesion (*L*)] and a vehicle-treated rat (UL, L). Calibrated standards of phosphoSer19 shown in nanograms (0.5, 1, 2.5, and 4), used as previously described [42]