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# Time-dependent changes in GLT-1 functioning in striatum of hemi-Parkinson rats

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ABSTRACT

Striatal dopamine loss in Parkinson's disease is accompanied by a dysregulation of corticostriatal glutamatergic neurotransmission. Within this study, we investigated striatal expression and activity of the glial high-affinity $Na^+/K^+$-dependent glutamate transporters, GLT-1 and GLAST, in the 6-hydroxydopamine hemi-Parkinson rat model at different time points after unilateral 6-hydroxydopamine injection into the medial forebrain bundle. Using semi-quantitative Western blotting and an ex vivo D-[$^3$H]-aspartate uptake assay, we showed a time-dependent bilateral effect of unilateral 6-hydroxydopamine lesioning on the expression as well as activity of GLT-1. At 3 and 12 weeks post-lesion, striatal GLT-1 function was bilaterally upregulated whereas at 5 weeks there was no change. Even though our data do not allow a straightforward conclusion as for the role of glutamate transporters in the pathogenesis of the disease, they do clearly demonstrate a link between disturbed glutamatergic neurotransmission and glutamate transporter functioning in the striatum of a rat model for Parkinson's disease.



Glutamate is involved in almost all processes of normal brain functioning while at the same time, when extracellularly present in high concentrations or under conditions of compromised energy and oxygen supply, it can act as a neurotoxin. High extracellular glutamate concentrations will cause excitotoxic damage by overstimulation of glutamate receptors (Choi, 1992) as well as oxidative damage via reversal of the cystine/glutamate antiporter (Lewerenz et al., 2006). Therefore, it is important to keep extracellular glutamate below toxic levels. High-affinity $Na^+/K^+$-dependent glutamate transporters are the sole way of glutamate removal from the extracellular space. This family of reuptake transporters consists of 5 members, i.e. the glial transporters GLAST (Storck et al., 1992; Tanaka, 1993) and GLT-1 (Pines et al., 1992), and the neuronal transporters EAAC1 (Kanai and Hediger, 1992), EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997). Whereas EAAT5 is confined to the retina, the other glutamate transporters are widely distributed throughout the mammalian brain. The bulk of glutamate removal is accomplished by glial transporters, while neuronal transporters might exert other functions, linked to their $Cl^-$ conductance or cysteine reuptake activity (Danbolt, 2001).

The involvement of glial glutamate transporters in neurodegenerative disorders has been amply demonstrated over the last decade (Sheldon and Robinson, 2007). A neurodegenerative disorder that affects 1% of the population by the age of 65 years is Parkinson's disease. The typical motor dysfunction that accompanies this disease is indirectly the result of dopamine shortage in the striatum that originates from the loss of dopaminergic neurons in substantia nigra pars compacta. Consequently, the modulatory interplay between dopaminergic and glutamatergic input in the striatum is disturbed. In 6-hydroxydopamine (6-OHDA) treated rats (Ingham et al., 1998) as well as in Parkinson's disease patients (Anglade et al., 1996), this dysregulation is accompanied by an increased diameter of postsynaptic densities at corticostriatal synapses, which would logically result in hyperactivity of these glutamatergic synapses. However, some controversy exists as to the effect of dopamine deafferentation on striatal extracellular glutamate concentrations. Whereas some investigators report that extracellular striatal glutamate concentrations are unaffected by lesioning of the nigrostriatal pathway (Corsi et al., 2003; Bianchi et al., 2003; Robelet et al., 2004), others observe time-dependent changes, with increases at 3–4 weeks (Lindefors and Ungerstedt, 1990; Meshul et al., 1999; Jonkers et al., 2002; Walker et al., 2009) and decreases 12 weeks after 6-OHDA lesioning (Meshul et al., 1999). Similar, some inconsistency exists regarding the effect of dopamine

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deafferentation on striatal expression of glutamate transporters. Whereas some groups did not detect changes in striatal expression of GLT-1 (Liévens et al., 2001), others report downregulations of GLT-1 and GLAST after 6-OHDA injection into the nigrostriatal pathway (Chung et al., 2008). In the acute MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model, a decrease in striatal GLT-1 immunolabelling was observed (Holmer et al., 2005).

Within this study, striatal protein distribution and expression as well as activity of GLAST and GLT-1 were examined in the 6-OHDA hemi-Parkinson rat using immunohistochemistry, semi-quantitative Western blotting and an ex vivo D-[$^3$H]-aspartate uptake assay, respectively.

## 1. Materials and methods

### 1.1. Animals

Protocols for animal experiments described in this study were performed according to national guidelines on animal experimentation and were approved by the Ethical Committee for Animal Experimentation of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel.

All rats (male albino Wistar rats, Charles Rivers Laboratories, France) used for this study were housed under standard laboratory conditions. Some of the animals included in this study were already used for a previous study (Massie et al., 2008b, 2010).

### 1.2. 6-OHDA lesioning of the medial forebrain bundle

Rats weighing 175–200 g were anaesthetized with a mixture of ketamine:diazepam (90.5:4.5 mg/kg i.p.) and placed in a stereotaxic frame. The skull was exposed and a burr hole was drilled through the skull at the appropriate location. A 6-OHDA solution (containing 4 μg/μl 6-OHDA (Sigma–Aldrich, St. Louis, MO, USA) in 0,01% ascorbic acid, pH 5.0) was stereotactically injected into the medial forebrain bundle (MFB) at coordinates L −1.5, AP −3.2, DV 8.7, relative to Bregma, according to the atlas of Konig and Klippel. A total volume of 4 μl 6-OHDA was injected at a flow rate of 1 μl/min. The syringe was left in place for 2 min and then slowly removed over a 1–2 min time period. The skin was sutured and the rats received 4 mg/kg ketofen (Merial, Brussels, Belgium) i.p. to provide post-operative analgesia. Rats were killed with a lethal dose of Nembutal (pentobarbital, i.p.) (Sanofi sante, Brussels, Belgium) 3, 5 or 12 weeks after 6-OHDA injection. Sham operated rats were injected with vehicle only, whereas control rats did not undergo any surgical procedure.

### 1.3. Immunohistochemistry

Immunohistochemistry was performed as described previously (Massie et al., 2008a). Briefly, after transcardial perfusion with a physiological solution followed by freshly depolymerized 4% paraformaldehyde (Sigma–Aldrich) in 0.15 M phosphate buffered saline (pH 7.42), brains were removed and post-fixed in the same fixative overnight, rinsed in tap water for 24 h and stored in 0.015 M phosphate buffered saline at 4 °C. Free floating 50 μm frontal sections were made with a vibratome and stored in serial order in 0.015 M phosphate buffered saline at 4 °C.

All rinsing steps and incubations of the staining procedure were performed in Tris–saline (0.01 M, 0.1% Triton X-100 (Sigma–Aldrich), pH 7.4) at room temperature, and under gentle agitation. Incubation steps were separated by 3 rinsing steps of at least 5 min. The sections underwent a permeabilizing treatment consisting of incubation in 0.1% trypsin (Fluka, Buchs, Switzerland) for 1 h at 37 °C prior to a blocking step with normal goat serum (diluted 1/5, 45 min; Millipore, Temecula, CA, USA). Thereafter the sections were incubated overnight with the immunoaffinity purified polyclonal GLT-1 (1/10,000; raised in rabbit against residues 500–525, generously provided by Dr. M. Watanabe; Yamada et al., 1998) or GLAST antibodies (1/5000; raised in rabbit against residues 520–543, generously provided by Dr. M. Watanabe; Shibata et al., 1997). The next day, sections were processed by the avidin–biotin method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and immunoreactivity was visualized, after a final rinsing step with acetate buffer, using the glucose oxidase-diaminobenzidine-nickel method (Shu et al., 1988). Immunohistochemical stainings at the level of the striatum, using anti-tyrosine hydroxylase antibodies (raised in rabbit, diluted 1/2000, Millipore), were performed as a control for correct lesioning. Photomicrographs were made of the stained sections and processed using Adobe Photoshop 7.0.

### 1.4. HPLC analysis of dopamine content

After decapitating the rats, brains were removed, snap-frozen and 100 μm cryosections were made at the level of the striatum. Striatal tissue of 4 serial sections was collected and homogenized in 100 μl of antioxidant solution (0.05 M HCl, 0.5% $Na_2S_2O_5$, 0.05% $Na_2EDTA$). The homogenate was centrifuged, the supernatant diluted in 0.5 M acetic acid and analyzed for its dopamine content on a narrow bore (column C18 5 μm, 150 mm × 2.1 mm; Unijet, Bioanalytical Systems, West Lafayette, IN, USA) liquid chromatography system with an electrochemical detector (Intro, Antec, Leiden, The Netherlands). The mobile phase consisted of 0.1 M Na-acetate, 20 mM citric acid, 1 mM Na-octanesulphonate, 1 mM dibutylamine and 0.1 M $Na_2EDTA$, adjusted to pH 3.8. Methanol was added as an organic modifier (3%, v/v).

### 1.5. Western blotting

For protein extraction, striatal tissue was collected from 12 cryosections (see above), located between Bregma +1.6 and −0.4, and homogenized in 300 μl of extraction buffer (2% sodium dodecyl sulphate, 60 mM Tris base, pH 6.8, 100 mM dithiothreitol and 1 mM $Na_2EDTA$). After homogenization, samples were incubated for 30 min at 37 °C. Next, samples were processed 4 times through 20 gauge needles, once through a 26 gauge needle and spun at 10,000 × g at 4 °C (Massie et al., 2003). Supernatants were stored at −20 °C. Protein concentrations were determined using the RC/DC kit (Biorad Laboratories, Nazareth, Belgium). Equal concentrations of protein were loaded on the gel. Standard curves were generated to determine the linear range of the optical density to protein concentration relationship.

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (4–12% gel; Invitrogen, Groningen, The Netherlands) under reducing conditions and transferred to a nitrocellulose membrane using the iBlot system (Invitrogen). Non-specific binding was blocked by incubating the membrane for 1 h at room temperature in 5% ECL Membrane Blocking Agent (GE Healthcare, Roosendaal, The Netherlands). Blots were incubated overnight at room temperature with the immunoaffinity purified polyclonal antibodies to GLT-1 (1/30,000; raised in rabbit; Yamada et al., 1998), GLAST (1/4000; raised in rabbit; Shibata et al., 1997), glial fibrillary acidic protein (GFAP, 1/3000; raised in rabbit; Dako, Glostrup, Denmark) or tyrosine hydroxylase (1/4000, raised in rabbit, Millipore). The next day, after a 30 min incubation with horseradish-peroxidase-conjugated anti-rabbit antiserum (1/4000, Dako), immunoreactive proteins were visualized using enhanced chemiluminescence (ECL Plus kit, GE Healthcare). Next, the membranes were rinsed thoroughly and stripped by incubating them for 45 min in a buffer containing 2% sodium dodecyl sulphate, 1.5% dithiothreitol, 62.5 mM Tris, pH 6.7, at 50 °C. After the membrane was rinsed very thoroughly, non-specific binding was blocked again for 1 h before the membrane was incubated overnight with primary monoclonal antibodies against β-tubuline (1/250; raised in mouse; Santa-Cruz Biotechnology Inc., Santa Cruz, CA, USA). Next the membrane was incubated with horseradish-peroxidase-conjugated anti-mouse antiserum (1/4000, 30 min; Dako) and the immunoreactive signal revealed using the ECL Plus kit (GE Healthcare). All washing and dilution steps were performed with Tris–saline (0.01 M, pH 7.4). The MultiMark Multi-Colored Standard (Novex, Invitrogen) was used as molecular weight standard. Negative controls included the omission of the immunoaffinity purified antibody and the secondary antibody, respectively. Densitometric analysis of the immunoreactive bands was performed using the ImageJ software (National Institute of Health, USA). Densities of glutamate transporter immunoreactive bands were normalized to densities of β-tubuline immunoreactive bands, detected on the same membrane. All samples that were being compared were always loaded on 1 gel and experiments were repeated 3–5 times for each post-lesion time.

An arbitrary chosen sample of the control group was set as reference (100%) and relative expression levels of the glutamate transporters in the control group as well as the lesioned groups are expressed as a percentage of this reference. Expression levels are expressed as means ± standard error of the mean (SEM). Statistical analysis of data was performed using a Student's *t*-test or a one-way ANOVA followed by a Tukey's test for multiple comparison ($\alpha = 0.05$).

### 1.6. Preparation of striatal synaptosomes

All procedures were carried out at 4 °C. After decapitating the rats, striata were surgically removed and immediately homogenized in 4 ml of a cold 0.32 M sucrose solution using 10 up-and-down strokes of a pre-chilled Teflon/glass homogenizer. The homogenate was centrifuged at 1000 × g for 10 min and the supernatant was carefully collected and stored at 4 °C. The pellet was resuspended in 5 ml of cold 0.32 M sucrose solution and centrifuged again at 1000 × g for 10 min. The two supernatants were pooled and centrifuged for 30 min at 17,500 × g. After discarding the supernatant, the final pellet containing the synaptosomes was resuspended in 1 ml of ice-cold Krebs–Ringer buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 25 mM $NaHCO_3$, 6 mM glucose, 1.3 mM $CaCl_2$, pH 7.6). Protein concentration was determined and samples diluted in Krebs–Ringer to a protein concentration of 50 μg/ml.

### 1.7. [$^3$H]GBR12935 binding assay in striatal synaptosomes

Specific binding of [$^3$H]GBR12935 (Perkin-Elmer NEN, Zaventem, Belgium), a potent and selective dopamine reuptake inhibitor, to striatal membranes was measured at 22 °C in plastic tubes containing 25 μg protein resuspended in a final volume of 1 ml. Binding buffer contained 50 mM Tris–HCl (pH 7.5), 1 mM EDTA,