Research article

NMR IN BIOMEDICINE

Received: 25 July 2011, Revised: 29 February 2012, Accepted: 14 April 2012, Published online in Wiley Online Library: 18 June 2012

(wileyonlinelibrary.com) DOI: 10.1002/nbm.2817

An *in vivo* ultrahigh field 14.1 T ^{1}H-MRS study on 6-OHDA and α-synuclein-based rat models of Parkinson's disease: GABA as an early disease marker

P. G. Coune[a†], M. Craveiro[b†], M. N. Gaugler[a], V. Mlynárik[b], B. L. Schneider[a], P. Aebischer[a] and R. Gruetter[b,c,d*]

The detection of Parkinson's disease (PD) in its preclinical stages prior to outright neurodegeneration is essential to the development of neuroprotective therapies and could reduce the number of misdiagnosed patients. However, early diagnosis is currently hampered by lack of reliable biomarkers. ^{1}H magnetic resonance spectroscopy (MRS) offers a noninvasive measure of brain metabolite levels that allows the identification of such potential biomarkers. This study aimed at using MRS on an ultrahigh field 14.1 T magnet to explore the striatal metabolic changes occurring in two different rat models of the disease. Rats lesioned by the injection of 6-hydroxydopamine (6-OHDA) in the medial-forebrain bundle were used to model a complete nigrostriatal lesion while a genetic model based on the nigral injection of an adeno-associated viral (AAV) vector coding for the human α-synuclein was used to model a progressive neurodegeneration and dopaminergic neuron dysfunction, thereby replicating conditions closer to early pathological stages of PD. MRS measurements in the striatum of the 6-OHDA rats revealed significant decreases in glutamate and N-acetyl-aspartate levels and a significant increase in GABA level in the ipsilateral hemisphere compared with the contralateral one, while the αSyn overexpressing rats showed a significant increase in the GABA striatal level only. Therefore, we conclude that MRS measurements of striatal GABA levels could allow for the detection of early nigrostriatal defects prior to outright neurodegeneration and, as such, offers great potential as a sensitive biomarker of presymptomatic PD. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: 6-OHDA; alpha-synuclein; GABA; Parkinson's disease; proton magnetic resonance spectroscopy; rat model

INTRODUCTION

The availability of sensitive and reliable biomarkers is critical for the development of therapeutic approaches to protect brain cells from neurodegenerative diseases. Early detection of Parkinson's disease (PD), the second most common neurodegenerative disease, could open avenues for preventive approaches and alternatives to current treatments that offer only temporary symptomatic relief. Indeed, PD diagnosis prior to the death of a substantial portion of nigral dopaminergic neurons is essential for developing strategies aimed at slowing down disease progression. Unfortunately, the pathology progresses in nigrostriatal neurons before initial motor symptoms appear, which occurs when at least 50% of striatal dopamine and most nigral neurons have already been lost (1). It has been shown that non-motor perturbations such as impaired olfaction, sleep disorders, gastrointestinal and urinary abnormalities, cardiovascular dysfunction, depression and mood disorders can precede the appearance of typical motor symptoms (2). Although this pre-motor syndrome may represent an opportunity to identify patients at risk of developing PD, efficient diagnosis requires specific early markers of nigrostriatal pathology.

Animal models that mimic human pathology as closely as possible are likely to contribute to the search for disease markers. Until now, mammalian models for PD have been mainly

* *Correspondence to: Prof. Rolf Gruetter, Laboratory for Functional and Metabolic Imaging, Ecole Polytechnique Fédérale de Lausanne, Station 6, CH-1015 Lausanne, Switzerland.*
E-mail: rolf.gruetter@epfl.ch

a *P. G. Coune, M. N. Gaugler, B. L. Schneider, P. Aebischer*
Neurodegenerative Studies Laboratory, Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

b *M. Craveiro, V. Mlynárik, R. Gruetter*
Laboratory for Functional and Metabolic Imaging, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

c *R. Gruetter*
Department of Radiology, University of Lausanne, Lausanne, Switzerland

d *R. Gruetter*
Department of Radiology, University of Geneva, Geneva, Switzerland

† *These authors equally contributed to the study.*

Abbreviations used: *6-OHDA, 6-hydroxydopamine; αSyn, alpha-synuclein; Ala, alanine; Asc, ascorbate; Asp, aspartate; AAV, adeno-associated viral; Cr, creatine; CRLB, Cramér-Rao lower bounds; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; Glc, glucose; Gln, glutamine; Glu, glutamate; Gly, glycine; GPC, glycerophosphocholine; GSH, glutathione; Lac, lactate; MFB, medial forebrain bundle; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MRS, magnetic resonance spectroscopy; myo-Ins, myo-inositol; NAA, N-acetyl-aspartate; NAAG, N-acetyl-aspartyl-glutamate; OD, optical density; PCho, phosphocholine; PCr, phosphocreatine; PCR, polymerase chain reaction; PD, Parkinson's disease; PE, phosphorylethanolamine; Scyllo, scyllo-inositol; SNpc, substantia nigra pars compacta; SNR, signal to noise ratio; SPECIAL, spin echo full intensity acquired localized; Tau, taurine; TH, tyrosine hydroxylase; TU, transducing unit; VAPOR, variable power and optimized relaxation delay; VOI, volume of interest.*

based on the use of neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), which lead to selective and extensive loss of nigrostriatal dopaminergic innervation. However, their mechanisms of action are unlikely to faithfully replicate either molecular or progressive courses of the disease. The precise aetiology of PD remains largely unknown but there is clear evidence that the protein α-synuclein (αSyn) plays a causal role in some familial variants. αSyn may also play a role in the most common idiopathic form of PD (3–5). The implication of the αSyn gene in PD has led to the development of animal models based on adeno-associated viral vectors (AAV) to selectively express human αSyn in adult nigral neurons (6). Over several months, injected rats displayed nigral degeneration similar to early phases of PD with loss of striatal terminals and nigral cell bodies, reduced dopamine transmission and mild motor impairments.

Magnetic resonance spectroscopy (MRS) offers a noninvasive method for investigating animal models to identify potential disease markers. Proton MRS studies have been conducted on toxin-based PD models in monkeys, rats and mice (7–13), and reported changes in striatal levels of several metabolites such as γ-aminobutyric acid (GABA), glutamate (Glu), N-acetyl-aspartate (NAA) and lactate (Lac) have been attributed to the loss of nigro-striatal dopaminergic fibres. In addition, MRS studies have reported variations in NAA levels in the frontal cortex of 6-OHDA lesioned rats (14) and PD patients (15).

This study aimed at using 14.1 T proton MRS to analyze striatal and cortical levels of metabolites in two unilateral rat models of PD. The use of ultrahigh 14.1 T provided a sensitive detection and quantification of about 20 brain metabolites. A complete lesion of the nigrostriatal pathway with 6-OHDA served as a model for advanced neuronal loss, while the progressive degeneration induced by αSyn replicated conditions closer to early pathological stages. Moreover, the unilateral lesion in both models allowed the use of the contralateral hemisphere as an internal control for each animal.

MATERIAL AND METHODS

Animal procedures

Nine-week-old female adult Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 180-200 g were housed in cages with a 12 h light/dark cycle and *ad libitum* access to food and water. Protocols conformed to Swiss legislation and the European Community Council directive (86/609/EEC) for the care and use of laboratory animals.

6-OHDA model

Six rats received injections of 6-OHDA in the medial forebrain bundle (MFB). The animals were anesthetized with a mixture of xylazine/ketamine and placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). Four μl of a 5 mg/ml solution of 6-OHDA in ascorbic-saline (1 mg/ml ascorbic acid in 0.9% saline) (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) were injected in the right brain hemisphere using a 10-μl Hamilton syringe (Sigma) with a 34-gauge blunt tip needle connected to an automatic pump (CMA Microdialysis AB, Solna, Sweden). The following coordinates were used to target the MFB: antero-posterior – 1.9 mm and medio-lateral + 1.9 mm relative to bregma, and dorso-ventral – 7.2 mm relative to skull surface. The injection speed was set at 1 μl/min. The needle was left in place for an additional three min before being slowly withdrawn. Three sham animals received 4 μl ascorbic-saline (1 mg/ml ascorbic acid in 0.9% saline) following the same procedure. MRS measurements were performed in the striatum prior to 6-OHDA injection and in the striatum and primary motor cortex (M1) three weeks later following the same protocol.

AAV-αSyn model experiment

Recombinant pseudotyped rAAV2/6 were produced, purified and titrated as previously described (16). Relative infectious titers were determined by real-time PCR on total DNA extracts from AAV-transduced HEK293T cells. Five rats received 2.8 x 10^7 TU of AAV2/6-pgk-αSyn-WT and six rats received undiluted control non-coding vector whose injected dose was estimated to be 1.5 x 10^7 TU. Using an injection setup similar to the 6-OHDA injections, the *substantia nigra pars compacta* (SNpc) was targeted using the following coordinates: anterio-posterior – 5.2 mm and medio-lateral – 2.0 mm relative to bregma, and dorso-ventral – 7.8 mm relative to skull surface. Two μl of virus preparation was injected in the right brain hemisphere at a speed of 0.2 μl/min. The needle was left in place for an additional five min before being slowly withdrawn. The animals underwent NMR measurements in the striatum 12 weeks after the injection.

NMR measurements and post-processing

All experiments were carried out on an animal 26-cm diameter horizontal-bore 14.1 T magnet (Magnex Scientific, Oxford, UK) interfaced to a Varian INOVA console (Varian Inc., Palo Alto, CA, US). The magnet was equipped with an actively shielded 12-cm-diameter gradient (400 mT/m in 120 μsec; Magnex Scientific, Oxford, UK). A custom-made quadrature surface coil consisting of two geometrically decoupled 14-mm diameter single loops was used as a transceiver.

Rats were anesthetized with 3% isoflurane combined with a mixture of air and O_2, stereotaxically fixed with two earpieces and a bite bar in a holder and placed at the isocenter of the magnet. Throughout the experiment, rats were kept anesthetized with 1.5-2% isoflurane. Breathing and temperature parameters were monitored with an MR-compatible monitor system (model 1025; SA Instruments, Stony Brook, NY, USA) and animal temperature was maintained at 38 °C by circulating warm water. For localization of the volume of interest (VOI), T_2-weighted multislice images were acquired using a fast spin echo technique with a field of view of 25 x 25 mm^2, 256 x 256 data matrix, 0.8 mm slice thickness, effective TE = 52 ms and TR = 5000 ms. Localized ^{1}H-MR spectra were acquired in a 27-μl ($3 \times 3 \times 3$ mm^3) volume from the striatum and in a 13.6-μl ($1.7 \times 2 \times 4$ mm^3) volume from the primary motor cortex (M1) of the right hemisphere using the SPECIAL sequence (17) with TE = 2.8 ms and TR = 4000 ms combined with outer volume suppression and VAPOR water suppression (18). The centre of the voxel located in the primary motor cortex was positioned 0.5 mm posterior and 1.5 mm right (or left for the left hemisphere) from the bregma and -1.5 mm from the skull surface. Voxel coordinates were determined with the help of the Paxinos and Watson atlas (19). Two-hundred and forty averages were acquired for optimal SNR. Static magnetic field homogeneity was adjusted with an EPI version of FASTMAP (20) using first- and second-order shims. As internal controls for the injected hemispheres, spectra were also acquired in the contralateral hemisphere of each animal. Contralateral and

NMR IN BIOMEDICINE

injected hemispheres were randomly scanned to avoid any temporal bias due to anaesthesia. Absolute quantification was performed using the water signal as an internal reference. Water spectra were acquired with the same parameters without water suppression and with eight averaging scans.

In vivo ^{1}H-MR spectra were processed as previously described (21), frequency drift corrected, summed and eddy-current compensated using the water signal from the same VOI. Experimental spectra were fitted with LCModel software (22) using a simulated basis set of metabolites and a spectrum of macromolecules measured *in vivo* using an inversion recovery sequence (21). The metabolite basis set included alanine (Ala), ascorbate (Asc), aspartate (Asp), creatine (Cr), myo-inositol (myo-Ins), GABA, glucose (Glc), glutamine (Gln), Glu, glycine (Gly), glycerophosphocholine (GPC), glutathione (GSH), Lac, NAA, N-acetyl-aspartyl-glutamate (NAAG), phosphocholine (PCho), phosphocreatine (PCr), phosphorylethanolamine (PE), scyllo-inositol (Scyllo) and taurine (Tau).

Immunohistological analysis

Rats were sacrificed and tissues processed as described previously (23). Striatal slices (25-μm thick) were quenched with 0.1% phenylhydrazine in PBS at 37 °C for 1 h, blocked for 2 h at room temperature, incubated overnight at 4 °C with primary antibody TH (tyrosine hydroxylase, rabbit IgG, 1:500, AB152) (Chemicon, Millipore AG, Zug, Switzerland) then incubated 2 h at room temperature in a biotinylated goat anti-rabbit antibody (1:200; Vector Laboratories). Sections were then incubated with avidin-biotin-peroxidase complex (Vector Laboratories Ltd, Peterborough, UK) for 30 min at room temperature and revealed with 3,3′-diaminobenzidine (DAB) solution (Sigma) before mounting on glass slides with Eukitt medium.

Densitometric analysis of striatal dopaminergic innervation

Striatal dopaminergic innervation was analyzed by measuring the optical density (OD; integrated density of gray pixel values corrected for non-specific background) of TH immunoreactivity on 16 striatal sections. Sections were scanned using an Epson Perfection V750 Pro scanner and OD of the striatum was analyzed using ImageJ software (free NIH software: http://rsbweb.nih.gov/ij/). Results are expressed as the percentage of TH immunoreactivity loss with respect to the contralateral non-injected hemisphere.

Stereological quantification of dopaminergic neuronal loss

Midbrain 40-μm thick coronal sections stained for TH were analyzed according to optical fractionator method using Stereo Investigator v.8 software (MBF Biosciences, Magdeburg, Germany). TH positive neurons were counted in every sixth section covering the entire SNpc at 60x magnification (total 12 sections per animal). We applied the following criteria: grid size, 180 × 200 μm; counting frame, 75 × 75 μm; guard zones, 2 μm. Gunderson coefficient of error (m = 1) was inferior to 0.1 for each animal. Since average tissue thickness was measured in both hemispheres for each slice prior to counting, the total number of TH-positive neurons was calculated according to estimated total by mean measured thickness.

Statistical analyses

Immunohistological data statistical analysis was performed using a paired Student's t-test comparing total TH signal ODs from both hemispheres. Statistical analysis of MRS results was performed within each group using a paired Student's t-test, which allows for a comparison between the contralateral and ipsilateral hemisphere in each animal to establish significant changes in the neurochemical profile of the affected hemisphere compared to the healthy one. Differences in metabolite concentration between both hemispheres within each animal of the group were considered significant when $p < 0.05$.

To determine whether potential metabolite level asymmetries between both hemispheres were indeed due to metabolite alterations in the ipsilateral hemisphere and therefore to exclude effects on the results of compensation phenomena between hemispheres, metabolite levels within each hemisphere pre- and post-injection were also compared for metabolites expressing significant asymmetries. Significance of metabolite alteration within each hemisphere between pre- and post-injection stages was determined using a paired Student's t-test.

RESULTS

Typical *in vivo* proton spectra obtained in the striatum and primary cortices of the examined animals are depicted in Figure 1. After shimming, the typical linewidth of the water resonance was 15-18 Hz in the striatum and 18-22 Hz in the cortex. ^{1}H-MR spectra were obtained in both regions with a typical SNR of 30 ± 2 (measured on the NAA singlet). All metabolites included in the basis set except Scyllo were quantified with Cramér-Rao lower bounds (CRLB) lower than 20% in the striatum. In the cortex, Scyllo, Gly, PCho and Ala, which are among the least abundant metabolites, were quantified with CRLB between 20 and 80%. All other metabolites were quantified with CRLB lower than 20%. In both brain regions, the metabolites of interest (NAA, GABA, Glu) were quantified with CRLB lower than 5-10%.

6-OHDA experiment

After the first MRS scan of the striatum as reference, rats received a 6-OHDA or an ascorbic-saline control injection (sham) in the right medial forebrain bundle. Three weeks after the injection, rats were scanned a second time then sacrificed and their brain processed for immunohistochemistry. Analysis of the optical density of the striatal TH staining revealed that all animals injected with 6-OHDA displayed a nearly total striatal denervation, as demonstrated by striatal TH optical density analysis (average decrease = 96.8 ± 0.7% compared to the non-injected side, Fig. 2A) while sham operated animals displayed only marginal innervation loss (average = 6.7 ± 1.4% decrease compared with the non injected side, Fig. 2A). Stereological analysis of the TH positive nigral neurons confirmed the extent of the nigro-striatal denervation induced by 6-OHDA injection with an average loss of 88.8 ± 4.4% compared to the non-injected side (Fig 2B). In comparison, sham operated animals displayed only marginal neuronal loss (average loss = 13.1 ± 11.0% compared to the non-injected side).

Comparison of concentration ratios between lesioned and control striata in the 6-OHDA-treated animals showed a significant increase in GABA concentration (+ 20 ± 5%) while NAA and Glu

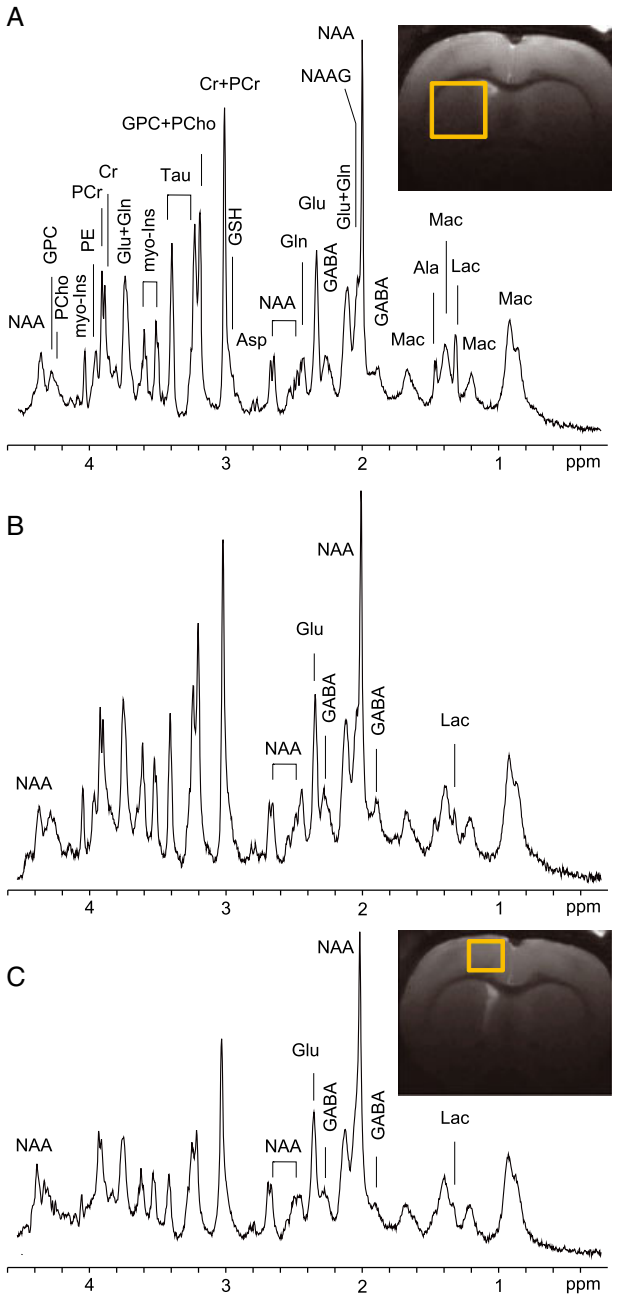

Figure 1. Representative spectra acquired in the ipsilateral striatum of a 12 weeks old rat injected with ascorbic saline (A) or with 6-OHDA (B), 3 weeks after injection, and representative spectrum acquired in the ipsilateral primary motor cortex region of a rat injected with 6-OHDA, 3 weeks after injection (C). The spectra are displayed with a Gaussian function apodisation (gf = 0.2). VOI in both brain regions ($3\times3\times3$ mm^3 in striatum and $1.7\times2\times4$ mm^3 in primary motor cortex) are represented on T_2-weighted images.

concentrations were significantly decreased (- 7 ± 2% and – 6 ± 1%, respectively) in the lesioned striatum (Fig. 3C). Comparison of pre- and post-injection levels performed for the metabolites within each hemisphere demonstrated that significant changes were detected in the ipsilateral striatum while no significant changes were detected between pre- and post-injection stages in the contralateral striatum, allowing for exclusion of any potential effect due to compensation phenomena on the results. No significant differences were observed between the right and left striata in the same animals in the reference scan; i.e. prior to 6-OHDA injections (Fig. 3A). Cortical measurements did not lead to any significant changes (Fig. 3D).

Alpha-synuclein experiments

Animals received an intra-nigral stereotactic injection of either an AAV vector coding for human αSyn or a control non-coding vector. Twelve weeks after the injection, rats were scanned then sacrificed and their brain processed for immunohistochemistry. Immunostaining for TH revealed that all αSyn overexpressing animals displayed a moderate striatal denervation on the injected side (average decrease of 26.1 ± 3.5% compared to the non-injected side, Fig. 2C). Stereological analysis of the TH positive nigral neurons confirmed the extent of the nigro-striatal denervation induced by αSyn over-expression, with an average loss of 27.1 ± 4.6% compared to the non-injected side (Fig 2D). Although expression of human αSyn produced only a moderate axon loss in these conditions, the extent of dopaminergic denervation was clearly different from the animals injected with the empty control vector, with an average loss of 9.6 ± 1.5% compared to the non-injected side (Fig. 2C).

Remarkably, MRS of the striatum of the animals overexpressing αSyn revealed a significant increase in GABA content (+ 12 ± 1%) compared to the non-injected side. We did not observe any other significant metabolite variation, including NAA, glutamate and lactate (Fig 4A). Control animals injected with the empty vector did not show any significant difference in any of the metabolites tested (Fig 4B).

DISCUSSION

This study showed that the two investigated PD models characterized by different levels of neurodegeneration expressed different metabolite alteration patterns but shared an increase in GABA striatal levels. These results were obtained with a 14.1 T ultrahigh field magnet that provided a highly precise measurement of metabolite concentrations in agreement with striatal and cortical values of healthy rat brain regions reported in previous ^{1}H-MRS studies (24,25). Moreover, the increased spectral resolution at 14.1 T was sufficient to partially resolve the GABA resonances.

We investigated the effect of nigro-striatal neuron degeneration within the striatum. Indeed, PD is characterized by the degeneration of the dopaminergic neurons of the SNpc, which is part of deep brain nuclei and is therefore poorly accessible to current MRS procedures. However, the nigral neurons are mostly projecting in the striatum, a large and anatomically homogeneous structure that can be easily measured by MRS. Therefore, nigro-striatal denervation consequences were investigated by measurement of the striatal concentrations of brain metabolites. Moreover, while measurements have been previously conducted in the striata of MPTP mice using a 9.4 T magnet, no previous proton MR spectroscopic study has been performed on a PD rat model at such a high field. Similarly, no measurements of GABA levels using proton MR spectroscopy have been reported in a 6-OHDA model. In addition, the advantage of both models used in our study was that unilateral injections allowed using the contralateral hemisphere as an internal control for each animal.

To interpret the results of our study, it is important to consider the difference between the two models of PD. First described in 1968 (26), intra-nigral injection of 6-OHDA is known to induce an almost complete anterograde degeneration of the dopaminergic nigrostriatal system. Even though it has become a common

NMR IN BIOMEDICINE

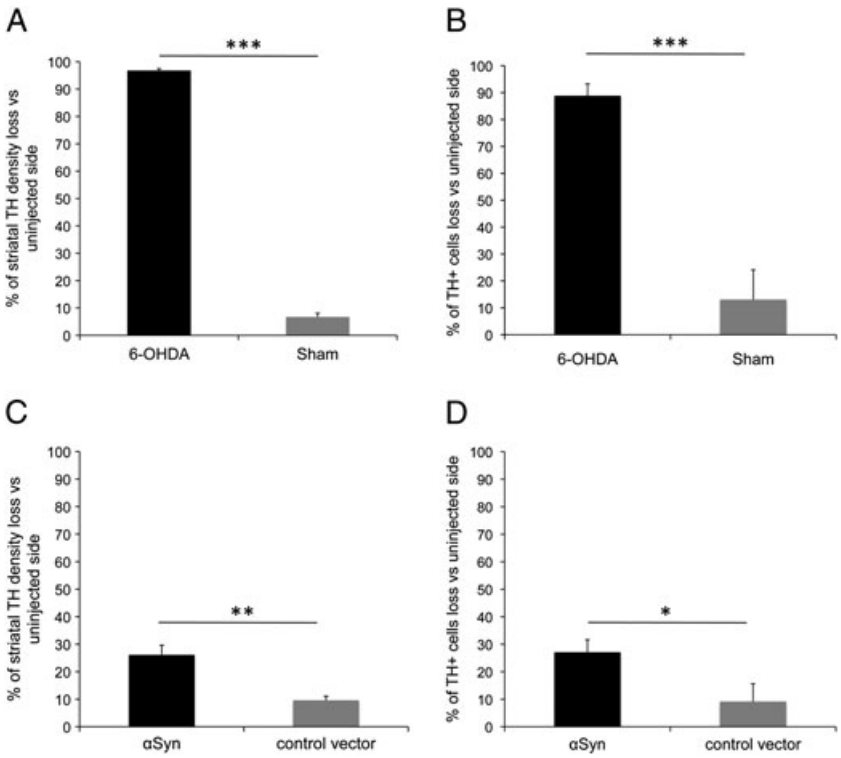

Figure 2. (A) Injection of 6-OHDA led to a nearly complete denervation of the striatum (96.8% of TH signal loss); n = 6 for the 6-OHDA group, n = 3 for the sham group, $^{**}p < 0.01$ compared with sham animals by Student's t-test. (B) Injection of 6-OHDA led to a nearly complete degeneration of the nigral TH positive neurons (88.8% of cell loss); n = 6 for the 6-OHDA group, n = 3 for the sham group, $^{**}p < 0.01$ compared with sham animals by Student's t-test (C) 12 weeks post-injection, α-synuclein over-expressing animals display a modest (26.1% of TH signal loss) but significant striatal denervation; n = 5 for the αSyn group and n = 6 for the empty vector control group. (D) 12 weeks post-injection, α-synuclein over-expressing animals display a modest (27.1% of cell loss) but significant degeneration of the nigral TH positive neurons; n = 5 for the αSyn group and n = 6 for the empty vector control group. Data are presented as group mean ± standard error of the mean. Significance $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p > 0.05$, compared with control animals by Student's t-test.

PD model, the exact mechanism by which 6-OHDA induces dopaminergic cell death has not been entirely elucidated. However it is known to involve increased oxidative stress (27) and impairment of the cellular respiratory machinery (28), and 6-OHDA injections in the nigro-striatal system lead to an acute and nearly total striatal denervation that typically develops over 7 days. Typically, 6-OHDA unilaterally treated animals present strongly asymmetrical motor behaviour in both drug induced and spontaneous testing within three weeks post injection. As such, injection of 6-OHDA in the medial-forebrain bundle aims to mimic end-stage PD by replicating the selective loss of the nigral neurons, which is considered a pathological signature responsible for the most prominent motor symptoms of the disease.

In contrast, our αSyn-based model was developed to induce a functional perturbation in the dopaminergic nigrostriatal transmission. Using an AAV-vector, human αSyn was over-expressed unilaterally in the nigral dopaminergic neurons of the rats. Over twelve weeks, over-expression of human αSyn generated only moderate nigral degeneration. However, the surviving neurons displayed functional impairments leading to decreased striatal dopamine release (6). Despite a significant proportion of surviving nigral neurons in the hemisphere injected with αSyn AAV vector, αSyn overexpressing animals displayed motor deficits in the form of asymmetrical motor behaviour in both apomorphine-induced rotametry and cylinder test. In contrast with 6-OHDA induced motor impairment, αSyn-induced deficit appeared progressively over several weeks and was of moderate extent (6). Overall, αSyn overexpressing rats presented motor impairments that could reflect an early stage of PD. Therefore, this model system appears particularly suited for a rational search of sensitive biomarkers of initial pathological changes. (Tables 1 and 2)

We propose that changes in striatal NAA and GABA levels reflected the difference in the denervation extent induced by these two models for the following reasons. First, NAA is a highly abundant brain metabolite almost exclusively present in neurons. Synthesized in neuronal mitochondria, it is therefore widely accepted as a marker of neuronal integrity (29,30). Moreover, reduced NAA levels have been reported in many neuropathological conditions including Alzheimer's disease, multiple sclerosis and cerebral ischemia (31–33). Indeed, the nearly total dopaminergic denervation observed in the striatum of the 6-OHDA injected animals was paralleled by lower NAA levels in the ipsilateral striata. In contrast, the αSyn overexpressing rats within experimental error did not display any significant difference in NAA. One hypothesis is that, in the absence of a significant effect of αSyn on mitochondrial function consistent with the mild striatal denervation observed, NAA levels would not be significantly affected in contrast with conditions of severe nigro-striatal denervation such as in the 6-OHDA model. It is interesting to note that lower NAA levels have been previously reported in MPTP models of rat, mouse and monkey using MRS (34,35). However, the only study conducted on 6-OHDA-lesioned rats did not report any significant variation in NAA levels (11). Experimental precision could therefore be critical in detecting

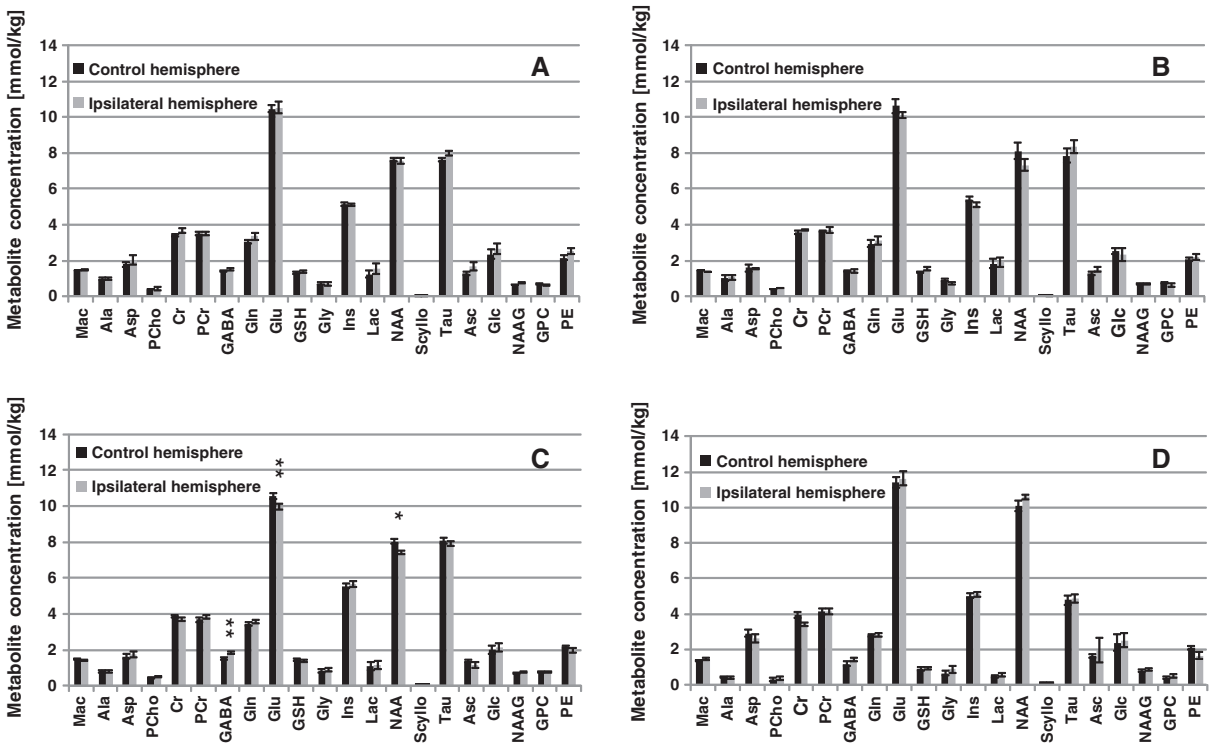

Figure 3. Metabolite concentration measured in the contralateral and ipsilateral striatum of pre-injected animals (n = 6) (A) and injected animals 3 weeks after an injection of ascorbic saline solution (n = 3) (B) or 6-OHDA (n = 6) (C), and cortices metabolite concentration measurements 3 weeks after 6-OHDA injection (n = 6) (D). Error bars represent the standard error of the mean. Significant differences in metabolite level between both hemispheres are indicated with *($p < 0.05$) and **($p < 0.01$) and results are summarized in Table 1.

Table 1. 6-OHDA model. Measured metabolite concentration ratio of ipsilateral (right) and contralateral (left) (C_{right}/C_{left}) hemispheres in the striatum before and 3 weeks after a 6-OHDA or ascorbic-saline injection and in the primary motor cortex 3 weeks after a 6-OHDA injection. Data are presented as group mean ± standard error of the mean. Significance of the asymmetry between both hemispheres in each group was determined with a paired Student's t-test and is displayed with *$p < 0.05$, **$p < 0.01$.

	Striatum before 6-OHDA injection ($n = 6$)	Striatum after 6-OHDA injection ($n = 6$)	Striatum after ascorbic-saline injection ($n = 3$)	Cortex after 6-OHDA injection ($n = 6$)
GABA	1.07 ± 0.03	1.20 ± 0.05**	0.99 ± 0.05	1.27 ± 0.14
NAA	0.99 ± 0.02	0.93 ± 0.02*	0.91 ± 0.04	1.05 ± 0.03
Glu	1.01 ± 0.02	0.94 ± 0.01**	0.95 ± 0.04	1.02 ± 0.05
Lac	1.25 ± 0.13	1.12 ± 0.12	1.05 ± 0.09	1.23 ± 0.27

small variations in NAA levels such as the 7% decrease observed in the present study.

Second, we observed that both 6-OHDA-induced total denervation and αSyn-induced progressive degeneration led to 20% and 12% increases in measured striatal GABA concentrations, respectively. Striatal neurons receiving dopaminergic afferents from the SNpc are GABAergic spiny neurons and it is established that nigral dopaminergic neurons exert a tonic inhibitory control on these GABAergic neurons through D2 receptors (36). Therefore, dopaminergic denervation is anticipated to cause hyperactivity in this subset of neurons. Evidence for such GABAergic hyperactivity can be found in experiments on 6-OHDA-lesioned rats and MPTP-treated-monkeys: striatal denervation leads to a striatal increase in glutamic acid decarboxylase (GAD) mRNA levels and GAD activity (37–39). Moreover, microdialysis studies in 6-OHDA-lesioned rats and MPTP-treated monkeys also showed an increase in extracellular GABA levels following nigrostriatal degeneration (13,37–40). A decrease in dopamine neurotransmission due to functional impairment of the nigral neurons could also affect striatal activity, and therefore GABA levels, independently from nigrostriatal denervation. It has been demonstrated that in conditions of overexpression, αSyn expression can impair neurotransmitter release (41). Such an impairment of dopamine release has been demonstrated in the rat model used in the present study (6). Therefore, the variations in GABA levels observed in the αSyn model could result from a combination of mild neurodegeneration and functional defect in the remaining nigrostriatal neurons. In humans, functional impairments of dopamine transmission can represent an initial step in a vicious circle leading to

NMR IN BIOMEDICINE

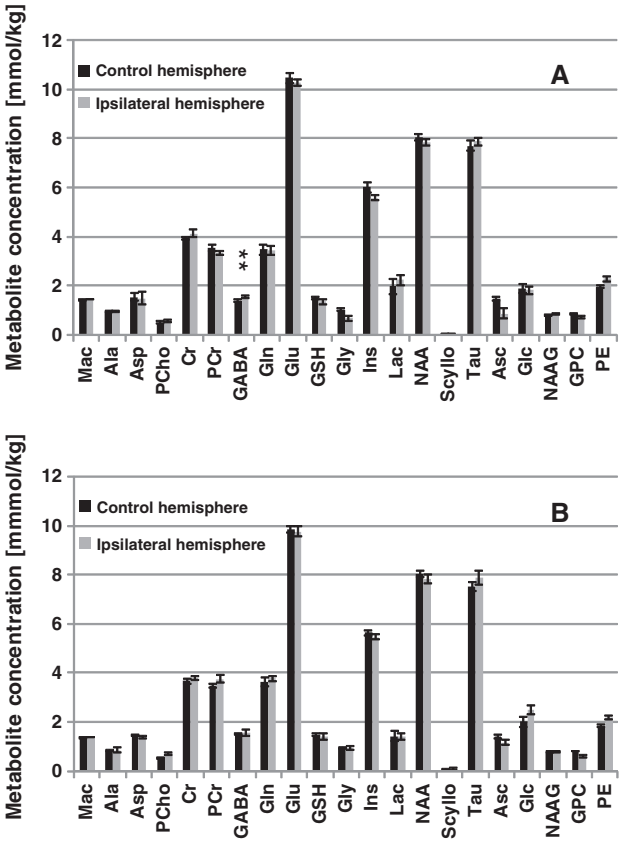

Figure 4. Metabolite concentration measured in the contralateral and ipsilateral striatum of injected animals with AAV vectors coding for α-synuclein (n = 5) (A) and animals of the control group injected with a non-coding vector (n = 6) (B). Error bars represent the standard error of the mean. Significant differences in metabolite level between both hemispheres are indicated with **($p < 0.01$) and results are summarized in Table 2.

Table 2. AAV-αSyn model. Measured metabolite concentration ratio between right and left hemispheres in the striatum after an injection of AAV vectors coding for WT αSyn and after an injection of non coding vectors, 12 weeks after injection in the SNpc. Data are presented as group mean ± standard error of the mean. Significance of the asymmetry between both hemispheres in each group was determined with a paired Student's t-test and is displayed with **$p < 0.01$.

	WT αSyn (n = 5)	Non-coding vector (n = 6)
GABA	1.12 ± 0.01**	1.03 ± 0.08
NAA	0.99 ± 0.02	0.97 ± 0.03
Glu	1.00 ± 0.02	0.98 ± 0.02
Lac	1.26 ± 0.12	1.01 ± 0.08

neurodegeneration. Consequently, measurements of striatal GABA levels could allow for the detection of early pathologic nigrostriatal defects prior to subsequent extensive neurodegeneration and appearance of motor symptoms.

The results obtained in the present study on GABA striatal levels in a 6-OHDA rat model confirm an increase reported in MPTP-treated mice (8). In addition, this increase in GABA appears to be detectable in the αSyn model expressing an early stage of nigrostriatal defects, and could therefore represent a sensitive marker of neuronal dysfunction due to nigrostriatal degeneration. Moreover, GABA seems to be a more sensitive marker of early neuronal defects due to mild nigrostriatal denervation than NAA, which is expected to decrease as a function of neuronal degeneration. Indeed, dopaminergic terminals represent only a small fraction of the complex striatal neuropil, and even an almost complete loss of dopaminergic axons produced by 6-OHDA intoxication only marginally impacts striatal metabolism, yet in a more profound manner than αSyn over-expression.

On the other hand, of the potentially implicated metabolites such as Glu and Lac, we detected a 6% decrease in Glu concentration in the 6-OHDA model. A previous study using ^{1}H-MRS in the same model did not find any significant alteration in glutamate concentration (11). However, the 6% decrease we report is inferior to the 16% detection threshold of glutamate concentration precision shown by Kickler *et al.* and could be detected due to the increased precision provided by the 14.1 T magnet. No significant variation of glutamate concentration was observed in the αSyn model, indicating that such glutamate up-regulation may not be detectable at an early stage of neurodegeneration.

A significant increase in striatal lactate concentration has been reported in a previous MRS study of a 6-OHDA lesion (11). In our study, neither 6-OHDA nor the αSyn based model displayed increased striatal lactate concentration. Interestingly, lactate levels appeared highly variable between hemispheres even prior to any lesion, consistent with isoflurane having dose- and time-dependent effects on brain lactate levels (42). Exposure to isoflurane together with the applied protocol randomizing hemisphere scanning likely explains the observed variability. Additional investigations addressing the role of anaesthesia conditions will be necessary to further characterize potential changes in lactate.

Finally, measurements performed in the cortex of the 6-OHDA lesioned rats did not show any significant concentration changes, though a trend to increased GABA was observed. However, cortex measurements for this metabolite showed a higher variability compared to the striatum. Consequently, the cortex measurements, in contrary to the striatum measurements, did not lead to any significant metabolite alteration and indicate that the striatum, which is the direct projection of the nigral neurons, might be a better investigative target for metabolite alterations due to nigrostriatal denervation.

CONCLUSION

Striatal GABA concentrations could serve as an early marker of Parkinson's disease measurable through noninvasive MRS. The availability of such a marker can serve as a basis for improving the diagnosis of PD and open perspectives for future neuroprotective approaches.

Acknowledgements

This work was supported by the Swiss National Science Foundation (grant 31003_120653), the Merck-Serono EPFL Alliance, the Centre d'Imagerie BioMédicale (CIBM) of the UNIL, UNIGE, HUG, CHUV, EPFL and the Leenaards and Jeantet Foundations.

REFERENCES

1. Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. J. Neurol. Sci. 1973; 20(4): 415–455.
2. Ferrer I, Martinez A, Blanco R, Dalfó E, Carmona M. Neuropathology of sporadic Parkinson disease before the appearance of parkinsonism: preclinical Parkinson disease. J. Neural Transm. 2010; 118(5): 821–839.
3. Li W, Lesuisse C, Xu Y, Troncoso JC, Price DL, Lee MK. Stabilization of alpha-synuclein protein with aging and familial parkinson's disease-linked A53T mutation. J. Neurosci. 2004; 24(33): 7400–7409.
4. Satake W, Nakabayashi Y, Mizuta I, Hirota Y, Ito C, Kubo M, Kawaguchi T, Tsunoda T, Watanabe M, Takeda A, Tomiyama H, Nakashima K, Hasegawa K, Obata F, Yoshikawa T, Kawakami H, Sakoda S, Yamamoto M, Hattori N, Murata M, Nakamura Y, Toda T. Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. Nat. Genet. 2009; 41(12): 1303–1307.
5. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc. Natl. Acad. Sci. U.S.A. 1998; 95(11): 6469–6473.
6. Gaugler M, Genc O, El-Agnaf O, Cantoni M, Bensadoun J-C, Schneggenburger R, Knott G, Schneider B, Aebischer P. Deficits in vesicular dopamine release impair motor behavior upon nigrostriatal alpha-synuclein expression. submitted.
7. Chassain C, Bielicki G, Durand E, Lolignier S, Essafi F, Traoré A, Durif F. Metabolic changes detected by proton magnetic resonance spectroscopy *in vivo* and *in vitro* in a murin model of Parkinson's disease, the MPTP-intoxicated mouse. J. Neurochem. 2008; 105(3): 874–882.
8. Chassain C, Bielicki G, Keller C, Renou J-P, Durif F. Metabolic changes detected *in vivo* by 1 H MRS in the MPTP-intoxicated mouse. NMR Biomed. 2010; 23(6): 547–553.
9. Griffith HR, den Hollander Ja, Okonkwo OC, O'Brien T, Watts RL, Marson DC. Brain metabolism differs in Alzheimer's disease and Parkinson's disease dementia. Alzheimers Dement. 2008; 4(6): 421–427.
10. Griffith HR, Okonkwo OC, Brien TO, Hollander JAD. Reduced brain glutamate in patients with Parkinson's disease. NMR Biomed. 2008; 21(4): 381–387.
11. Kickler N, Lacombe E, Chassain C, Durif F, Krainik A, Farion R, Provent P, Segebarth C, Rémy C, Savasta M. Assessment of metabolic changes in the striatum of a rat model of parkinsonism: an *in vivo* (1)H MRS study. NMR Biomed. 2009; 22(2): 207–212.
12. Koga K, Mori A, Ohashi S, Kurihara N, Kitagawa H, Ishikawa M, Mitsumoto Y, Nakai M. H MRS identifies lactate rise in the striatum of MPTP-treated C57BL/6 mice. Eur. J. Neurosci. 2006; 23(4): 1077–1081.
13. Robertson R. Further investigations into the pathophysiology of MPTP-induced parkinsonism in the primate: an intracerebral microdialysis study of γ-aminobutyric acid in the lateral segment of the globus pallidus. Brain Res. 1991; 563(1): 278–280.
14. Hou Z, Lei H, Hong S, Sun B, Fang K, Lin X, Liu M, Yew DTW, Liu S. Functional changes in the frontal cortex in Parkinson's disease using a rat model. J. Clin. Neurosci. 2010; 17(5): 628–633.
15. Hu MT, Taylor-Robinson SD, Chaudhuri KR, Bell JD, Morris RG, Clough C, Brooks DJ, Turjanski N. Evidence for cortical dysfunction in clinically non-demented patients with Parkinson's disease: a proton MR spectroscopy study. J. Neurol. Neurosurg. Psychiatry 1999; 67(1): 20–26.
16. Dusonchet J, Bensadoun J-C, Schneider BL, Aebischer P. Targeted overexpression of the parkin substrate Pael-R in the nigrostriatal system of adult rats to model Parkinson's disease. Neurobiol. Dis. 2009; 35(1): 32–41.
17. Mlynárik V, Gambarota G, Frenkel H, Gruetter R. Localized short-echo-time proton MR spectroscopy with full signal-intensity acquisition. Mag. Reson. Med. 2006; 56(5): 965–970.
18. Tkác I, Starcuk Z, Choi IY, Gruetter R. In vivo 1 H NMR spectroscopy of rat brain at 1 ms echo time. Mag. Reson. Med. 1999; 41(4): 649–656.
19. Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates 4th edn. Academic Press: United States of America; 1998.
20. Gruetter R, Tkác I. Field mapping without reference scan using asymmetric echo-planar techniques. Mag. Reson. Med. 2000; 43(2): 319–323.
21. Mlynárik V, Cudalbu C, Xin L, Gruetter R. 1H NMR spectroscopy of rat brain in vivo at 14.1Tesla: improvements in quantification of the neurochemical profile. J. Mag. Reson. 2008; 194(2): 163–168.
22. Provencher SW. Estimation of metabolite concentrations from localized *in vivo* proton NMR spectra. Mag. Reson. Med. 1993; 30(6): 672–679.
23. Azeredo da Silveira S, Schneider BL, Cifuentes-Diaz C, Sage D, Abbas-Terki T, Iwatsubo T, Unser M, Aebischer P. Phosphorylation does not prompt, nor prevent, the formation of alpha-synuclein toxic species in a rat model of Parkinson's disease. Hum. Mol. Genet. 2009; 18(5): 872–887.
24. Tkac I, Keene CD, Pfeuffer J, Low WC, Gruetter R. Metabolic changes in quinolinic acid-lesioned rat striatum detected non-invasively by *in vivo* H-1 NMR spectroscopy. J. Neurosci. Res. 2001; 66(5): 891–898.
25. Xin LJ, Gambarota G, Duarte JMN, Mlynarik V, Gruetter R. Direct in vivo measurement of glycine and the neurochemical profile in the rat medulla oblongata. NMR Biomed. 2010; 23(9): 1097–1102.
26. Ungerstedt U. 6-Hydroxy-dopamine induced degeneration of central monoamine neurons. Eur. J. Pharmacol. 1968; 5(1): 107–110.
27. Galindo MF, Jordán J, González-García C, Ceña V. Chromaffin cell death induced by 6-hydroxydopamine is independent of mitochondrial swelling and caspase activation. J. Neurochem. 2003; 84(5): 1066–1073.
28. Hanrott K, Murray TK, Orfali Z, Ward M, Finlay C, O'Neill MJ, Wonnacott S. Differential activation of PKC delta in the substantia nigra of rats following striatal or nigral 6-hydroxydopamine lesions. Eur. J. Neurosci. 2008; 27: 1086–1096.
29. Bates TE, Strangward M, Keelan J, Davey GP, Munro PM, Clark JB. Inhibition of N-acetylaspartate production: implications for 1 H MRS studies *in vivo*. Neuroreport 1996; 7(8): 1397–1400.
30. Narayana PA, Doyle TJ, Lai DJ, Wolinsky JS. Serial proton magnetic resonance spectroscopic imaging, contrast-enhanced magnetic resonance imaging, and quantitative lesion volumetry in multiple sclerosis. Ann. Neurol. 1998; 43(1): 56–71.
31. Cwik VA, Hanstock CC, Allen PS, Martin WR. Estimation of brainstem neuronal loss in amyotrophic lateral sclerosis with *in vivo* proton magnetic resonance spectroscopy. Neurology 1998; 50(1): 72–77.
32. Gideon P, Henriksen D, Sperling B, Christiansen P, Olsen TS, Jorgensen HS, Arlien-Soborg P. Early time course of N-acetylaspartate, creatine and phosphocreatine, and compounds containing choline in the brain after acute stroke. Stroke 1992; 23(11): 1566–1572.
33. Schuff N, Capizzano AA, Du AT, Amend DL, O'Neill J, Norman D, Kramer J, Jagust W, Miller B, Wolkowitz OM, Yaffe K, Weiner MW. Selective reduction of N-acetylaspartate in medial temporal and parietal lobes in AD. Neurology 2002; 58(6): 928–935.
34. Boska MD, Lewis TB, Destache CJ, Benner EJ, Nelson Ja, Uberti M, Mosley RL, Gendelman HE. Quantitative 1 H magnetic resonance spectroscopic imaging determines therapeutic immunization efficacy in an animal model of Parkinson's disease. J. Neurosci. 2005; 25(7): 1691–1700.
35. Jenkins BG, Brouillet E, Chen YC, Storey E, Schulz JB, Kirschner P, Beal MF, Rosen BR. Non-invasive neurochemical analysis of focal excitotoxic lesions in models of neurodegenerative illness using spectroscopic imaging. J. Cereb. Blood Flow Metab. 1996; 16(3): 450–461.
36. Gerfen CR. The neostriatal mosaic: multiple levels of compartmental organization. Trends Neurosci. 1992; 15(4): 133–139.
37. Segovia J, Tillakaratne N, Whelan K, Tobin A, Gale K. Parallel increases in striatal glutamic acid decarboxylase activity and mRNA levels in rats with lesions of the nigrostriatal pathway. Brain Res. 1990; 529(1-2): 345–348.
38. Soghomonian J-J, Laprade N. Glutamate decarboxylase (GAD67 and GAD65) gene expression is increased in a subpopulation of neurons in the putamen of parkinsonian monkeys. Synapse 1997; 27(2): 122–132.
39. Soghomonian JJ, Chesselet MF. Effects of nigrostriatal lesions on the levels of messenger RNAs encoding two isoforms of glutamate decarboxylase in the globus pallidus and entopeduncular nucleus of the rat. Synapse 1992; 11(2): 124–133.
40. Tossman U, Segovia J, Ungerstedt U. Extracellular levels of amino acids in striatum and globus pallidus of 6-hydroxydopamine-lesioned rats measured with microdialysis. Acta Physiol. Scand. 1986; 127(4): 547–551.
41. Nemani VM, Lu W, Berge V, Nakamura K, Onoa B, Lee MK, Chaudhry FA, Nicoll RA, Edwards RH. Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis. Neuron 2010; 65(1): 66–79.
42. Horn T, Klein J. Lactate levels in the brain are elevated upon exposure to volatile anesthetics: A microdialysis study. Neurochem. Int. 2010; 57(8): 940–947.