Published in final edited form as:

Brain Res. 2009 March 9; 1259: 113–122. doi:10.1016/j.brainres.2009.01.063.

PET imaging in rats to discern temporal onset differences between 6-hydroxydopamine and tau gene vector neurodegeneration models

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Abstract

We attempted to monitor the nigrostriatal dopaminergic system in rats with positron emission tomography (PET) during the progression of two experimental disease states. One model was 6-hydroxydopamine (6-OHDA) lesioning and the other was direct gene transfer of the microtubule-associated protein tau to the substantia nigra using an adeno-associated virus vector (AAV9). The PET ligand was 6-[18F]fluoro-L-m-tyrosine (FMT), imaged prior to, and at two intervals after initiating dopaminergic neurodegeneration. The striatum was delineated with the aid of repeated PET imaging (FMT and sodium fluoride for bone), realignment to subsequent computed axial tomography scans, and registration to an atlas, which proved essential to tracking disease progression. The striata on the two sides of the brain were compared over time after unilateral lesioning treatments. 6-OHDA reduced uptake on the ipsilateral side relative to the untreated contralateral side at both 1 and 4 weeks after lesioning, while the AAV9 tau led to reduced uptake of the tracer in the striatum at 4 weeks, but not 1 week after treatment. The amplitude of the loss of FMT uptake in striatum at 4 weeks with either model was subtle relative to the postmortem histological analysis of the tissue, but the multi-modal imaging analysis yielded statistical effects that matched well with the histology in terms of the timing of the loss of dopaminergic markers. Live longitudinal imaging successfully tracked two distinct types of disease progression in individual rats, although the FMT is not a sensitive ligand to monitor the extent of the lesion.

Keywords

adeno-associated virus; gene transfer; microPET; neurodegenerative diseases; substantia nigra; tau

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1. Introduction

In order to longitudinally track and compare the onset and magnitude of deficit in two models of dopaminergic neurodegeneration, we attempted PET imaging of the dopaminergic nigrostriatal pathway in rats. We monitored tracer uptake capacity in the striatum before, and after two distinct unilateral lesioning treatments in order to determine differences in the temporal dynamics of neurodegeneration. We hypothesized that a viral vector gene transfer model of neurodegeneration induced by the microtubule-associated protein tau would have a temporal onset distinct from neurodegeneration resulting from the chemical neurotoxin 6-OHDA, and that the extent of the nigrostriatal degeneration would be greater with the chemical neurotoxin. As previously noted (Strome et al., 2006; Sossi et al., 2007), the longitudinal PET imaging technique is advantageous for studying neurodegeneration in small animals, particularly for genetically modified ones, as in this experiment.

Neurofibrillary tangles, one of the most common neuropathological lesions, are largely made up of microtubule-associated protein tau. Recombinant AAV gene transfer vectors for tau injected into the substantia nigra (SN) of rats induce dopaminergic neurodegeneration (Klein et al., 2005; 2008; 2009). The disease state from tau overexpression in rats mimics human neurodegenerative diseases with neurofibrillary tangles, such as progressive supranuclear palsy, corticobasal degeneration, and frontotemporal dementia with parkinsonism linked to chromosome 17, where there is dopamine neuronal loss and neurofibrillary tangles in the SN (Di Maria et al., 2000; Mirra et al., 1999; Poorkaj et al., 2002; Wakabayashi et al., 1994). The disease state in rats from the AAV tau vectors progresses between 1 and 4 weeks after treatments (Klein et al., 2005; 2008) whereas 6-OHDA injected into the medial forebrain bundle, causes a more rapid and essentially complete loss of dopaminergic markers in the rat nigrostriatal pathway (Kirik et al., 1998). Here we compared the time-course of the tau-induced and 6-OHDA disease states in rats scanned at 3 intervals relative to the lesioning treatments.

Little is known about pre-synaptic changes in the rat striatum after 6-OHDA lesioning as measured by PET, and the resolution of small animal PET scanners is a limitation for visualizing small brain structures of the rat, plus the fact that robust unilateral lesions will obscure the region of interest, the striatum (Sossi et al., 2007). To improve the resolution of the striatum for quantification of uptake, we conducted serial tracer (FMT and sodium fluoride) and serial modality (PET and micro computed axial tomography, CT) imaging which allowed realignment of the images in atlas orientation. The sodium fluoride PET imaging for bone bridges the PET data with the high resolution bone imaging from CT for registration to the atlas. We first attempted PET imaging in rats with 6-[¹⁸F]fluoro-L-dihydroxyphenylalanine (FDOPA) because the ligand was available from syntheses for clinical studies at our institution, but were disappointed with its ability to delineate the striatum in rats. In an attempt to improve labeling, we imaged with 6-[¹⁸F]fluoro-L-m-tyrosine (FMT), and applied serial imaging analysis to delineate and quantify uptake in the striatum. Both FDOPA and FMT have been used reliably in primates (Bankiewicz et al., 2000; Doudet et al., 1999; Elsinga et al., 2006; Jordan et al., 1997; Yee et al., 2001) as a pre-synaptic marker for the integrity of the nigrostriatal pathway. However, a study in mice found that neither tracer clearly visualized the striatum (Honer et al., 2006), although metabolic studies of FDOPA and FMT unequivocally demonstrate that the label accumulates in dopaminergic structures of the rat brain (Jordan et al., 1997; 1998; Barrio et al., 1996). FMT is not metabolized by catechol-O-methyltransferase, avoiding the need to inhibit that enzyme (Barrio et al., 1996), and one study suggests FMT is superior to FDOPA for visualizing the striatum in monkeys (DeJesus et al., 1997). The FMT serves as a tracer of the aromatic l-amino acid decarboxylase (AADC) enzyme, which traps and accumulates the ¹⁸F metabolites in dopaminergic nerve terminals (Jordan et al., 1997; 1998; Barrio et al., 1996; Bankiewicz et al., 2000). While FMT has not been successful previously for PET imaging of nigrostriatal nerve terminals in rats, we investigated whether

the serial imaging analysis would enable quantification of dopaminergic changes mirroring post-mortem histology.

We hypothesized that we could consistently monitor levels of FMT uptake in rat striatum with multi-modal imaging, and that unilateral loss of tracer uptake would be more rapid and more complete in the 6-OHDA model relative to the tau gene transfer model. Histological analyses for tyrosine hydroxylase (TH) were run in parallel with the PET imaging to assess the sensitivity of the imaging technique to detect loss of the dopaminergic marker.

2. Results

2.1 MicroCT imaging

CT results for a single rat scanned at 3 intervals are shown in Fig. 1, which shows the rat's skull. The CT data were used for registration to an atlas to localize the striatum. The cursor lines in Fig. 1 for the top surface of the brain, the midline, and bregma were used for alignment and registration. The rat in Fig. 1 received AAV9 tau vector gene transfer to the SN 1 week after the first scan. A large hole was drilled in the skull over the SN on one side for the tau vector injection. The entire skull for each rat was nearly superimposable over the time-course, and the drill hole appeared similar at both 1 and 4 weeks.

2.2 Micro PET imaging

FMT PET imaging is shown in Fig. 2, which represents an averaged result for each group: 6-OHDA, N = 5; AAV9 tau, N = 6. Brain regions were demarcated with information from the CT scans and atlas registration. The comma shaped striatum was the region of interest. While we could observe specific striatal FMT uptake above background in control tissues, the signal-to-noise ratio was somewhat disappointing. Nevertheless, it was clear to the eye that 6-OHDA caused a side-to-side imbalance in FMT uptake, with reduced signal ipsilateral to the lesion at 1 and 4 weeks (Fig. 2B, C). In the tau vector group, there was no indication of side-to-side differences by 1 week, but by 4 weeks, there was a consistent pattern of reduced FMT uptake ipsilateral to the treatment at 4 weeks (Fig. 2E, F). A single AAV9 GFP control vector rat did not indicate any side-to-side difference in uptake at either interval (not shown). As seen in Fig. 2, there was marked uptake of the tracer in bone, which was also observed in mice (Honer et al., 2006). However, bone activity was not predictably consistent among animals or across time points, thus the need for sodium fluoride imaging to provide bony details for registration.

2.3 Statistical analysis of striatal tracer uptake

Side-to-side differences in the imaging of tracer uptake after unilateral 6-OHDA treatment were subtle, yet consistent. Uptake on the ipsilateral side was decreased at 1 and 4 weeks after treatment (Fig. 3). In contrast, decreased uptake after tau gene transfer occurred at 4, but not 1 week, post-injection. There were 5 rats in the 6-OHDA group and 6 rats in the tau group between -1 to 4 weeks. Two-way repeated measures ANOVA comparing 6-OHDA and tau from -1 to 4 weeks yielded significant effects for treatment ($F_{1, 18} = 25.13$, $P < 0.001$), interval ($F_{2, 18} = 36.08$, $P < 0.0001$), and interaction ($F_{2, 18} = 9.22$, $P < 0.002$), and significant difference between 6-OHDA and tau at 1 week ($P < 0.001$), but not at -1 or 4 weeks, in Bonferroni's post-tests. Analyzing each group over time individually by repeated measures ANOVA yielded a similar pattern of results, with decreased uptake in the 6-OHDA, but not the tau group, by 1 week. In the 6-OHDA group, there was a significant effect of time ($F_{2, 14} = 26.49$, $P < 0.001$), with differences in Bonferroni's post tests at -1 and 1 ($P < 0.01$), and -1 and 4 weeks ($P < 0.001$), but not between 1 and 4 weeks. In the tau group, there was a significant effect of time ($F_{2, 17} = 17.71$, $P < 0.001$), with differences in Bonferroni's post tests at -1 and 4 ($P < 0.01$), and 1 and 4 weeks ($P < 0.01$), but not between -1 and 1 week.

2.4 Histological analyses

After the course of PET imaging, animals were processed for TH immunohistochemistry in the SN and striatum. In the SN, the AAV9 tau gene transfer resulted in almost complete loss of TH immunoreactive cell bodies, as did the 6-OHDA lesioning at 4 weeks (Fig. 4). Clearly, the loss of TH staining was more complete for either disease model at 4 weeks than what was observed with the FMT uptake detected by PET. We previously compared 6-OHDA and AAV9 tau gene transfer for loss of striatal dopamine content, and also evaluated dopaminergic lesions induced by AAV9 tau by stereological assessments of TH neurons in the SN (Klein et al., 2008). In this study, we relied on optical density measurements of TH immunoreactivity in the striatum for a semi-quantitative index of the nigrostriatal pathway. While we did conduct stereology for the SN at 4 weeks, it was obvious that both 6-OHDA and AAV9 tau caused nearly complete loss of dopamine neurons at 4 weeks (Fig. 4). In the striatum, the density of TH immunoreactivity in nigrostriatal axons on the side of either 6-OHDA or AAV9 tau after 4 weeks was less than the contralateral uninjected side which was used as a control (Fig. 5). Expressing the optical density of the TH immunoreactivity on the treated side as a percent of the contralateral untreated side, resulted in $98.6 \pm 0.3\%$ loss with 6-OHDA, $N = 3$, and $86.7 \pm 9.3\%$ loss with AAV9 tau, $N = 6$. There was no difference between the disease models in terms of loss of nigrostriatal axons after 4 weeks. The single AAV9 GFP control vector animal that was imaged by PET had a similar density of striatal TH staining on both sides, consistent with no changes in FMT uptake and the specific loss caused by AAV9 tau.

A small number of rats were analyzed to match the early interval of the PET imaging. At 1 week after treatment, there was a distinct difference between the treatment groups with respect to TH neurons in the SN. While clear loss after 6-OHDA occurred by 1 week, more TH neurons remained 1 week after AAV9 tau (Fig. 4) matching with TH fiber density in striatum at 1 week (Fig. 5), and consistent with the FMT uptake data. Rats receiving the control AAV9 GFP vector demonstrated that levels of transgene product rise from 1-4 weeks (Fig. 6). We used 2 antibodies to detect tau expression, E1 which is specific for human tau, and CP13 for abnormally hyperphosphorylated tau (at serine 202; Jicha et al., 1999). Both antibodies distinguished recombinant tau from background, i.e. no staining in non-transduced tissues (Fig. 7). The tau staining results suggested progressive loss of pars compacta neurons and neurofibrillary pathology. At 1 week, there is both E1 and CP13 immunoreactivity in both the SN pars compacta and below in the SN pars reticulata, while at 4 weeks, staining in the pars compacta is reduced (Fig. 7), suggestive of cell loss in that area and consistent with the TH staining and FMT uptake. We have previously shown that the tau gene transfer results in selective loss of transgene expressing cells in the pars compacta, and loss of both TH and the neuronal marker NeuN (Klein et al., 2008), so reduced tau staining at 4 weeks in the pars compacta probably reflects cell loss. Of note, CP13 staining of pars reticulata neurons displayed a shift from normal to globose (spherical) morphology between 1 and 4 weeks (Fig. 7C, D). Because we previously observed Gallyas silver staining, a method of staining neurofibrillary tangles, in the same neurons with globose CP13 staining (Klein et al., 2004) as in Fig. 7D, the pattern suggests development of tau proteinopathy during this interval.

3. Discussion

The raw results with FMT uptake in the rat striatum were disappointing with respect to the signal-to-noise ratio, but the multi-modal analysis enabled quantification of changes in striatal uptake that mirrored the post-mortem histological results. Under the conditions tested, using carbidopa to block peripheral metabolism, combination CT/PET imaging in rats was successful for observing specific FMT uptake in the striatum, where nigrostriatal axons terminate. Upon unilateral lesioning with 6-OHDA, a significant side-to-side difference in uptake developed by 1 week and remained stable at 4 weeks, further demonstrating that our FMT signal was

specific for the dopaminergic nigrostriatal nerve terminals. In contrast, the microtubule-associated protein tau gene transfer model was slower to develop a side-to-side difference, although a similar loss of tracer uptake occurred in both disease models by 4 weeks. The time-course of the FMT data was consistent with TH immunohistochemistry in the SN and the striatum, with separate animals analyzed after 1 week of treatment. While tau expression occurred by 1 week, the loss of TH immunoreactivity in the SN and striatum required 4 weeks, similar to the FMT changes. In contrast, changes induced by 6-OHDA were more rapid. Again similar to the FMT results at 4 weeks, the extent of loss of TH staining at 4 weeks was the same in both disease models. The tau gene transfer was equally efficacious as 6-OHDA in reducing FMT uptake and TH immunoreactivity by 4 weeks, which was somewhat surprising based on an earlier study using an AAV2 tau vector which produced a partial lesion (Klein et al., 2005). While AAV9 gene transfer levels rose between 1-4 weeks with GFP, in contrast, there was less tau staining at 4 weeks due to the neurodegeneration. The time-course of pathological hyperphosphorylated tau staining matched well with FMT uptake and TH staining, with indication of a shift to a pathological morphology between 1 and 4 weeks, that could potentially be a causative factor in the disease state.

One reason why the FMT or FDOPA PET imaging in rats has a relatively poor signal-to-noise ratio relative to monkeys (Doudet et al., 1999; Jordan et al., 1997; Yee et al., 2001) may be more rapid defluorination in the liver and subsequent uptake in bone, as there was a strong signal in bone with the FMT PET (Fig. 2). Inhibiting defluorination is important for improving PET imaging in rats (Tipre et al., 2006), although we only attempted to block peripheral metabolism of the tracer with carbidopa. The combinatorial imaging protocol that we used may not be of great significance for humans because of the better signal-to-noise ligands, which make realignment and registration less of an issue. However, the combinatorial imaging protocol permitted evaluation of experimental models tailored for rats.

The slower time-course of dopaminergic neurodegeneration with the tau vector relative to 6-OHDA may allow for more efficacious therapeutic intervention that can be observed by imaging, by using small molecules or even gene therapy to block the deficient uptake at 4 weeks in the tau vector model. The vector model is also advantageous to mimic specific familial forms of neurodegenerative diseases, and in particular the diseases with tau pathology and loss of pigmented dopamine neurons in the SN, such as progressive nuclear palsy, corticobasal degeneration, and frontotemporal dementia with parkinsonism linked to chromosome 17 (Di Maria et al., 2000; Mirra et al., 1999; Poorkaj et al., 2002; Wakabayashi et al., 1994).

Despite some degree of success in imaging the integrity of the nigrostriatal pathway in rats with FMT, the imaging did not reflect the amplitude of the lesioning effect. By either the 6-OHDA or the tau vector method, histological analyses revealed a nearly complete loss of dopaminergic markers on the treated side relative to the contralateral untreated side in this study, while the intensity changes in FMT uptake were less remarkable. These decreases in intensity, a 20% reduction, were, however, consistent in both disease models, with no indication of an effect after a control gene transfer treatment. It is possible that the FMT PET is a more functional index of the nigrostriatal pathway than post mortem immunohistochemistry, although the mismatched results could also stem from increased dopamine turnover after lesioning. For example, rats lesioned with 6-OHDA display increased levels of dopamine metabolites relative to dopamine itself (Hefti et al., 1985) which we also observed in our tau gene transfer model (Klein et al., 2005; 2008). Imaging the nigrostriatal pathway in PD patients with ligands for the presynaptic type 2 vesicular monoamine transporter reveals greater losses relative to changes in FDOPA, which could be due to compensatory increases in AADC activity (Martin et al., 2008; Nandhagopal et al., 2008). The small effect on FMT tracer uptake relative to a pronounced effect on dopaminergic cell loss is similar to results using FDOPA in rats (Forsback et al., 2004). In that study, Forsback et al. (2004) found better signal-to-noise with

a ligand for the dopamine transporter relative to FDOPA for delineating the striatum, with a more complete loss of striatal transporter signal correlating with pronounced nigral cell loss. This dopamine transporter ligand has been used successfully in a number of studies in rats (Forsback et al., 2004; Cicchetti et al., 2002; Pellegrino et al., 2007), although dopamine transporter ligands may also be subject to compensatory changes during dopaminergic neurodegeneration (Martin et al., 2008; Nandhagopal et al., 2008). Even targeting the vesicular monoamine transporter 2, which should not be subject to regulatory changes, there is some degree of mismatch between PET data and post-mortem analyses of the nigrostriatal system after 6-OHDA lesions (Strome et al., 2006), underscoring the importance of post-mortem measurements. We plan to pursue improved dopaminergic tracers for rats in future in vivo studies of tau, alpha-synuclein (Klein et al., 2002), or TDP-43 (Tatom et al., 2009) induced neurodegeneration in rats and specific neuroprotective regimens with PET. A combination of FMT PET, sodium fluoride PET, and CT helped to quantify specific FMT uptake in the rat striatum. The small but consistent changes, and the timing of the changes observed in two disease models matched postmortem histological analyses, validating the technique for longitudinal studies of gene products related to neurodegenerative diseases.

4. Experimental procedures

4.1 Tau and GFP AAV vectors

An expression cassette flanked with AAV2 terminal repeats was cross-packaged into recombinant AAV9 (Klein et al., 2008). The promoter/enhancer combination used to drive expression included the hybrid cytomegalovirus/chicken β -actin promoter and the 3' enhancer woodchuck hepatitis virus post-transcriptional regulatory element. Plasmids for the reporter control GFP or the P301L form of human tau including exons 2, 3 and 10 (four repeat microtubule-binding domains, 4R2N) were the same as described previously (Klein et al., 2008).

Human embryonic kidney 293-T cells were co-transfected with one of the transgene plasmids above along with the respective packaging plasmids for AAV9 (Klein et al., 2008). The cell lysate was applied to a discontinuous gradient of iodixanol (OptiPrep, Greiner Bio-One, Longwood, FL) and centrifuged. The AAV was then removed, diluted 2-fold with lactated Ringer's solution (Baxter, Deerfield, IL), and then washed and concentrated by Millipore (Billerica, MA) Biomax 100 Ultrafree-15 units. The final stocks were sterilized by Millipore Millex-GV syringe filters into low adhesion tubes (USA Scientific, Ocala, FL). Encapsidated genome copies were titered by dot-blot. Vectors were aliquoted and stored frozen.

4.2 Animals and stereotaxic injections

Male Sprague-Dawley rats (3 months old, from Harlan, Indianapolis, IN) were anesthetized with a cocktail of 3 ml xylazine (20 mg/ml, from Butler, Columbus, OH), 3 ml ketamine (100 mg/ml, from Fort Dodge Animal Health, Fort Dodge, IA), and 1 ml acepromazine (10 mg/ml, from Boehringer Ingelheim, St. Joseph, MO) administered intramuscularly at a dose of 1 ml/kg, in the case of viral vector gene transfer. Viral stocks were injected through a 27 gauge cannula connected via 26 gauge internal diameter polyethylene tubing to a 10 μ l Hamilton syringe mounted to a microinjection pump (CMA/Microdialysis, North Chelmsford, MA) at a rate of 0.2 μ l/min. The dose of vector injected into the SN for either the AAV9 tau or the AAV9 GFP was 3×10^9 vector genomes in a volume of 3 μ l. The stereotaxic injection coordinates for the SN were 5.3 P, 2.1 L, 7.6 V (Paxinos and Watson, 2004). The needle remained in place at the injection site for 1 additional min before the cannula was removed slowly (over 2 min). The skin was sutured, and the animal was placed on a heating pad until it began to recover from the surgery, before being returned to their individual cages. The 6-OHDA lesioned rats were anesthetized with a combination of pentobarbital sodium (50 mg/

kg, i.p., Abbott, North Chicago, IL) and 0.2 ml/kg of the cocktail described above. The 6-OHDA (Sigma) solution was 4 mg/ml free base in 0.1% ascorbic acid/0.9% NaCl. This solution was injected into the medial forebrain bundle with 2.5 μ l at 2 sites (coordinates: 2.8 and 3.3 P, 1.5 L, 8.5 V). All animal procedures followed protocols approved by an institutional IACUC committee as well as the NIH Guide for Care and Use of Laboratory Animals.

4.3 Animal Imaging

4.3.1 Radiotracer Production—Fluorine-18 labeled F₂ gas was produced by the ¹⁸O (proton, neutron)¹⁸F nuclear reaction using a modification of the ‘two shoot’ method (Nickles et al., 1986). Proton (17–18 MeV) irradiation of [¹⁸O]O₂ (20 μ A for up to 60 min) using the Ion Beam Applications 18/9 cyclotron was followed by cryogenic trapping of the [¹⁸O]O₂. A second irradiation (13 μ A for 10 min) of fluorine gas ([¹⁹F]F₂ 90–100 μ mol) in argon (90–100 μ mol) produces the [¹⁸F]F₂. A custom made electrophilic synthesis unit was used to produce the [¹⁸F]FMT. The [¹⁸F]F₂/Argon gas mixture was bubbled through 10 ml of Freon 11 containing 60 mg of *N,O*-Diboc-TMSn-*m*-tyrosine ethyl ester (VanBrocklin et al., 2004). The solvent was evaporated at 50°C using a gentle stream of helium. Deprotection followed using 3 ml of HBr (48%) at 130°C for 10 min. The reaction was cooled to room temp and partially neutralized with 1.2 ml 4N NaOH and 1ml HPLC eluent (5 mM sodium acetate, 1 mM EDTA, 0.1% acetic acid, 0.01% ascorbic acid). The mixture was then injected onto the semi-prep HPLC column (Phenomenex Gemini C-18, 10 μ m, 10 mm $\times$ 250 mm; Eluent: 5 mM sodium acetate, 1mM EDTA, 0.1% acetic acid, 0.01% ascorbic acid) equipped with matching guard column. The collected fraction was then passed through a 0.22 μ m sterile filter into a sterile vial.

4.3.2 Blocking of peripheral FMT metabolism—Rapid peripheral metabolism of FMT significantly reduces radioisotope delivery to the brain, where uptake is comparatively slow. Carbidopa, which inhibits the enzyme AADC, was used to block peripheral uptake. Early in the experimental design, we compared routes of administration and varying doses of carbidopa, including intravenous, intraperitoneal, subcutaneous, and intramuscular injections. We found that i.p. administration provided sustained absorption of carbidopa in the blood by high performance liquid chromatography, so that peripheral metabolism was inhibited throughout the pre-scan uptake period of FMT (data not shown).

4.3.3 Image Data Acquisition Protocol—Our dual modality imaging protocol was designed to allow realignment of the FMT images in the Paxinos and Watson (2004) atlas orientation so that the striatal regions could be consistently sampled. The protocol utilized three images at timepoints of (-1, 1, 4) weeks relative to intracranial injections: FMT (PET), sodium fluoride (PET), and CT.

4.3.4 PET Imaging—PET images were acquired on a microPET scanner (microPET R4, Concorde MicroSystems, Knoxville, TN) having transaxial full width half maximum resolution of $\sim$ 2 mm in the center field of view. The coincidence window width was set at 6 ns. All images were reconstructed in a single frame using OSEM2D (16 subsets, 4 iterations) with scatter correction and segmented attenuation correction to an image of size 128 $\times$ 128 $\times$ 63 with voxel dimensions of 0.8452 $\times$ 0.8452 $\times$ 1.212 mm³.

Thirty minutes prior to FMT injection, each animal was administered 15 mg/kg carbidopa i.p. 200-300 uCi of FMT was given i.v., followed by a 60 minute uptake time before acquiring a 30 min emission scan. Immediately following the FMT scan, without moving the bed or animal, 200 uCi of sodium fluoride i.v. was administered, and allowed a 10 min uptake time before a 10 min emission scan to provide bone imaging data in the same orientation as the FMT that

could subsequently be aligned with high resolution CT imaging. Animals were anesthetized with isoflurane for all scans and monitored for spontaneous breathing.

4.3.5 CT Imaging—The purpose of the CT imaging was alignment of the FMT/sodium fluoride data for registration to an atlas. CT images were acquired 24-48 hrs after PET imaging, under ketamine/xylazine anesthesia, on a microCT scanner (MicroCAT II, Imtek, Knoxville, TN) using acquisition parameters of 70 kVp voltage, 500 uA current, with 520 projections over 360 degrees and a bin factor of 4. Reconstructed images were $512 \times 512 \times 512$ voxels with a voxel size of 0.109332 mm^3 .

4.3.6 Image Post-processing—Each animals initial CT scan (at week -1) was registered to an MR T2 image already in atlas orientation (Martin et al., 2008) using the normalized mutual information algorithm available in SPM2 (Schweinhart et al., 2003; Ashburner and Friston, 2003) a statistical image processing utility developed for Matlab (Mathworks Inc., Natick, MA). CT scans at later time points were registered to the initial CT. Each NaF image was registered to its corresponding CT, and the same transform was applied to the FMT image. Finally, the FMT image was resliced in the new orientation having cubic voxel dimensions of 2 mm and an origin located at Bregma, then cropped to include only the skull and brain.

To identify regions of interest within the images, previously demarcated regions from a cryosection image (in atlas orientation) (Rubins et al., 2003) were reformatted for use within an atlas/masking tool (Wake Forest Atlas Pickatlas; Maldjian et al., 2003). Masks of the ipsi- and contra- lateral striatum were applied to FMT images and mean voxel intensities were measured. All measurements were expressed as a ratio of mean intensities within the striatal regions. The measurements were made by a trainer observer who was unaware of the experimental groupings.

4.4 Immunostaining

Anesthetized animals were perfused with phosphate-buffered saline (PBS), followed by cold 4% paraformaldehyde in PBS. The brain was removed and immersed in fixative overnight at 4°C. Brains were equilibrated in a cryoprotectant solution of 30% sucrose/PBS at 4°C. Coronal sections (50 μm thick) were cut on a sliding microtome with a freezing stage. Antigen detection was conducted on free-floating sections. Primary antibody incubations were overnight at 4°C on a shaking platform. For immunoperoxidase staining, endogenous peroxidase activity was quenched with 0.1% H_2O_2 /PBS for 10 min. The sections were washed in PBS and incubated for 5 min in 0.3% Triton X-100/PBS, and washed before applying primary antibody. Primary antibodies for immunostaining were TH polyclonal (Pel-Freez, Rogers, AR, 1:2000), E1 monoclonal specific for human tau (provided by Leonard Petrucelli, Mayo Clinic Jacksonville, FL, 1:2000), or hyperphosphorylated tau CP13 monoclonal (Jicha et al., 1999; provided by Peter Davies, Albert Einstein College of Medicine, 1:2000). Biotinylated secondary antibodies for peroxidase staining were from DAKO Cytomation (Carpinteria, CA; 1:2000), incubated for 1 hr at room temperature. The sections were washed with PBS and labeled with horseradish peroxidase-conjugated Extravidin (Sigma, St. Louis, MO; 1:2000) for 30 min at room temperature. The chromogen was diaminobenzidine (0.67 mg; Sigma) in 0.3% H_2O_2 , 80 mM sodium acetate buffer containing 8 mM imidazole and 2% NiSO_4 . After mounting on slides, the sections were dehydrated in a series of alcohol and xylene and coverslipped with Eukitt (Electron Microscopy Sciences, Hatfield, PA).

4.5 Striatal TH analysis

Five sections evenly spaced through the striatum were processed for TH immunohistochemistry. The specific TH staining in the striatum was quantified using the Scion (Frederick, MD) imaging program. The striata were traced and then measured for optical

density of staining (pixels). The ratio of the injected side relative to the contralateral uninjected side was calculated.

4.6 Statistics

Data are expressed as mean $\pm$ SEM. Statistical tests included repeated measures ANOVA/Bonferroni's multiple comparisons.

Acknowledgments

Wynne Schiffer and Dan Rubins provided software and cryosection images used during postprocessing of PET images. David Stout made helpful suggestions for controlling peripheral FMT/FDOPA uptake. Richard Zweig critiqued the manuscript. The study was supported by the Crivant Imaging Award from the Biomedical Research Foundation and National Institute of Neurological Disorders and Stroke R01 NS048450.

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Abbreviations

AAV	adeno-associated virus
AADC	aromatic l-amino acid decarboxylase
CT	X-ray computed tomography
FDOPA	6-[18F]fluoro-L-dihydroxyphenylalanine
FMT, MR	magnetic resonance imaging
6-[18F]fluoro-L-m-tyrosine	
PD	Parkinson's disease
PET	Positron emission tomography
6-OHDA	6-hydroxydopamine
SN	substantia nigra
TH	tyrosine hydroxylase

Fig. 1.

CT imaging of a single rat in the tau AAV9 gene transfer vector group at 3 intervals: -1 wk (pre-treatment), 1 wk after gene transfer (large hole in skull above the injection site, the SN), 4 wk after gene transfer. Coronal, sagittal, and transverse (horizontal) views are shown. The bregma (B) and lambda (L) landmark sutures can be seen on the surface of the skull. The CT scans were used to orient bregma and lambda according to the brain atlas, allowing delineation of the striatum from established stereotaxic coordinates. The cursor crosshairs assist in registering the landmarks to the atlas.

Fig. 2. FMT uptake in rat brains. A-C) Averaged FMT uptake signal from a group of 5 rats that were lesioned with 6-OHDA at intervals of 1 week prior, and 1 and 4 weeks post-lesioning. Brain regions were registered to an atlas and outlined: C, cortex; Cb, cerebellum; H, hippocampus; T, thalamus; S, striatum. Decreased uptake on the left, lesioned striatum is noticeable at 1 and 4 weeks. D-F) Averaged FMT uptake signal from a group of 6 rats that received tau vector gene transfer at intervals of 1 week prior, and 1 and 4 weeks post-lesioning. Decreased uptake on the left, lesioned striatum is noticeable at 4 weeks. The strong signal posterior and lateral to the cerebellum represents tracer uptake in bone, which was observed throughout the study.

Fig. 3.

Changes in FMT uptake in striatum after neurotoxin lesioning or gene transfer. In the 6-OHDA group (N = 5), consistently decreased uptake by approximately 20% was found at 1 and 4 weeks post-lesioning. The data are expressed as a ratio of uptake in the striatum on the injected side relative to the contralateral untreated striatum which was the control. In the tau vector group (N = 6), a consistent decrease in uptake was found at 4 weeks, but not 1 week, after gene transfer, while in 1 rat receiving the GFP control vector, there was no indication of altered uptake at 1 or 4 weeks after gene transfer. Two-way repeated measures ANOVA and post-tests found a difference between the 6-OHDA and tau vector treatment groups over the full time-course ($P < 0.001$) and a difference between 6-OHDA and tau vector at the 1 week interval ($P < 0.001$), but no difference between 6-OHDA and tau vector at 4 weeks, see Results.

Fig. 4.

Tyrosine hydroxylase (TH) neurons in the substantia nigra (SN) after 6-OHDA or tau vector gene transfer. A, B) SN from a rat treated unilaterally with 6-OHDA a week earlier, with numerous dopaminergic neurons and processes on the uninjected side (A), but not on the injected side (B). C) Injected side from a rat treated with 6-OHDA 4 weeks earlier with similar loss of TH as 1 week. D, E) SN from a rat treated with tau AAV9 vector gene transfer a week earlier. TH in the SN is preserved at 1 week. F) By 4 weeks, the tau gene transfer progressed into loss of TH. A-E, bar = 134 μ m.

Fig. 5.

Tyrosine hydroxylase (TH) immunoreactivity in the striatum after 6-OHDA or tau vector gene transfer. Representative striatal section is shown for an uninjected rat (A), 6-OHDA at 1 week (B) or 4 weeks (C), and AAV9 tau gene transfer at 1 week (D) or 4 weeks (E). There is depletion of TH immunoreactivity in the striatum at both 1 and 4 weeks after 6-OHDA, while with the AAV9 tau, TH immunoreactivity is diminished at 4 weeks, but not at 1 week. A-E, bar = 536 μ m.

Fig. 6. Rise in GFP from 1-4 weeks after adeno-associated virus vector (AAV9) gene transfer. A, B) With all conditions matched, there was noticeable increase in GFP in the substantia nigra over the period tested. The 1 week expression also appeared widespread and robust, although requiring longer camera exposures. A, B, bar = 134 μ m.

Fig. 7.

Overexpression of microtubule-associated protein tau and progressive neurofibrillary pathology. A, B) E1 antibody specific for human tau. C, D) CP13 antibody for pathological hyperphosphorylated tau. There was no staining for either antibody in non-transduced tissue. A, C) At 1 week, tau was detected in both the substantia nigra pars compacta (pc) and the pars reticulata (pr). B, D) At 4 weeks, levels of tau immunoreactivity in the pars compacta were diminished, consistent with the loss of tyrosine hydroxylase in that area, while the tau expression persisted in remaining transduced pars reticulata neurons. The globose morphology of the cells in the pars reticulata in D suggests neurofibrillary neuropathology at 4 weeks. A, B, bar = 134 μ m.