Published in final edited form as:

Neurobiol Dis. 2007 May ; 26(2): 396–407.

Basal Ganglia Dopamine Loss Due to Defect in Purine Recycling

Kiyoshi Egami¹, Silaja Yitta¹, Suhail Kasim¹, J. Chris Lewers¹, Rosalinda C. Roberts², Mohamed Lehar³, and H. A. Jinnah¹

¹ Department of Neurology, Johns Hopkins University, Baltimore MD 21287

² Maryland Psychiatric Research Center, Department of Psychiatry, University of Maryland School of Medicine, Baltimore MD 21228

³ Department of Otolaryngology, Head and Neck Surgery, Johns Hopkins University, Baltimore MD 21287

Abstract

Several rare inherited disorders have provided valuable experiments of nature highlighting specific biological processes of particular importance to the survival or function of midbrain dopamine neurons. In both humans and mice, deficiency of hypoxanthine-guanine phosphoribosyl transferase (HPRT) is associated with profound loss of striatal dopamine, with relative preservation of other neurotransmitters. In the current studies of knockout mice, no morphological signs of abnormal development or degeneration were found in an exhaustive battery that included stereological and morphometric measures of midbrain dopamine neurons, electron microscopic studies of striatal axons and terminals, and stains for degeneration or gliosis. A novel culture model involving HPRT-deficient dopaminergic neurons also exhibited significant loss of dopamine without a morphological correlate. These results suggest dopamine loss in HPRT deficiency has a biochemical rather than anatomical basis, and imply purine recycling to be a biochemical process of particular importance to the function of dopaminergic neurons.

INTRODUCTION

Parkinson's disease is associated with degeneration of nigrostriatal dopamine neurons. The mechanisms responsible for selective loss of these neurons are not yet understood. Available evidence suggests these neurons are especially sensitive to certain biochemical processes such as mitochondrial dysfunction, oxidative stress, and proteasome dysfunction.

Several rare monogenic forms of parkinsonism provide important insights into biochemical processes particularly important for midbrain dopamine neurons (Cookson, 2005, Moore et al., 2005, Zeevalk et al., 2005, About-Sleiman et al., 2006). The importance of mitochondrial dysfunction is highlighted by parkinsonism resulting from mutations affecting the mitochondrial proteins DJ-1, PINK1, and HTRA2. Studies of DJ-1 and PINK1 similarly have strengthened the idea that dopamine neurons are especially sensitive to oxidative stress. A role for handling of misfolded proteins is emphasized by parkinsonism associated with familial defects of protein folding related to mutations of α -synuclein, and by defects involving the handling of misfolded proteins by the proteasome, such as Parkin and UCHL1. Although these monogenic forms of parkinsonism are rare, they provide valuable experiments of nature

Address Correspondence to: H. A. Jinnah, MD PhD Department of Neurology Meyer Room 6-181 600 North Wolfe Street Johns Hopkins Hospital Baltimore MD 21287 Phone: 410-614-6551 Fax: 410-502-6737 Email: hjinnah@jhmi.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

pointing to specific biological processes of particular relevance to dopamine neurons (Antonarakis and Beckmann, 2006).

Another monogenic disorder associated with selective dysfunction of midbrain dopamine neurons is Lesch-Nyhan disease (Visser et al., 2000). Because dopaminergic systems are affected early in childhood, these patients present with dystonia rather than Parkinsonism (Jinnah et al., 2006). The disease is caused by deficiency of hypoxanthine-guanine phosphoribosyl transferase (HPRT), an enzyme of the purine salvage pathway (Jinnah et al., 2000). The most consistent neurochemical abnormality in the brains of these individuals is 70-90% loss of dopamine in the basal ganglia (Lloyd et al., 1981, Saito et al., 1999). Neuroimaging studies similarly reveal profound loss of [^{11}C]WIN-35,428 binding to the dopamine transporter and [^{18}F]-fluorodopa uptake into dopaminergic fibers in the basal ganglia (Ernst et al., 1996, Wong et al., 1996).

Significant loss of dopamine also occurs in HPRT-deficient (HPRT⁻) knockout mice. These mice have been useful for documenting two important aspects of the dopamine defect. The first is that loss of dopamine is neuroanatomically selective. The striatum is affected but other regions are not (Jinnah et al., 1992b, Jinnah et al., 1994). The second is that loss of dopamine is neurochemically selective. There is no accompanying loss of norepinephrine, serotonin, or GABA (Jinnah et al., 1994, Jinnah et al., 1999, Hyland et al., 2004). The selective neuroanatomical and neurochemical consequences of HPRT deficiency suggest purine recycling is another biological process of particular importance to midbrain dopamine neurons.

The reasons for the loss of striatal dopamine in the HPRT⁻ brain are not fully understood. They are hypothesized to result from failed development or early degeneration of dopamine neurons or their axons (Lloyd et al., 1981, Ernst et al., 1996, Wong et al., 1996, Saito and Takashima, 2000, Visser et al., 2000). This hypothesis was addressed in the current studies via neuroanatomical studies of midbrain dopamine neurons in the HPRT⁻ mouse model and a novel HPRT⁻ tissue culture model based on the dopaminergic MN9D cell line.

METHODS

Animals

The animals used in these studies were HPRT-competent (HPRT⁺) and congenic HPRT-deficient (HPRT⁻) mutant mice bred on a C57BL/6J background for more than 20 generations (C57BL/6J^{HPRT.BM3}) originally obtained from Jackson Laboratories (Bar Harbor, ME). Normal males were mated with heterozygous females to generate HPRT⁻ males and HPRT⁺ littermate controls. Mutants were distinguished from normal mice by an enzyme assay that involved measuring conversion of [^{14}C]-hypoxanthine into [^{14}C]-IMP in a blood sample (Jinnah et al., 1993). All animals were housed in the Johns Hopkins University vivarium, and kept on a 14:10hr light:dark cycle with food and water ad libitum. All procedures conformed to guidelines recommended by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Johns Hopkins Animal Care and Use Committee.

Striatal catecholamines

Monoamines were measured in striatal dissections as previously described (Jinnah et al., 1999) in normal and mutant mice at 3 ages, weanling (3-4 weeks), young adult (3-6 months) and aged (22-26 months). At least 5 normal and 5 mutant mice were evaluated at each age. Their brains were rapidly removed, dissected on ice, and stored at -80°C . The caudoputamen dissection included a 1.5 mm slab of tissue extending from the level of the anterior commissure (Bregma 0.14 mm) rostrally. The cortex overlying the caudoputamen was obtained from this same tissue slab. Tissue was sonicated briefly in 250 μl of 0.1N perchloric acid, and centrifuged

at 10,000g for 10 min to sediment precipitated proteins. The pellet was reserved for protein measurement by the Pierce BCA protein detection kit. The supernatants were further filtered through 0.2 μ m PVDF spin-filter columns (AllTech Associates, Deerfield, IL) at 2,000g before HPLC analysis.

The HPLC system was composed of a 717+ autosampler, a 1525 solvent delivery pump, a flow cell with a 2 mm glassy carbon working electrode with ISAAC reference electrode connected to a 2465 electrochemical detector, and an Empower Workstation (Waters, Milford, MA). Samples were eluted through a C18 reverse-phased MD-150 (150 mm $\times$ 3.2 mm $\times$ 3 μ m) column (ESA Inc., Chelmsford, MA). The buffer ran isocratically at a rate of 0.4 mL/min, and consisted of 75 mM sodium phosphate, 1.7 mM 1-octanesulfonic acid sodium, 25 μ M EDTA, 0.01% triethylamine, 2 mM NaCl, and 10% acetonitrile at pH 2.9.

Striatal DAT and VMAT2

Dopamine transporters (DAT) and vesicular monoamine transporters (VMAT2) were quantified using radioligand binding assays (Darchen et al., 1989, Wilson and Kish, 1996). Freshly dissected striata from HPRT⁻ mutants (n=8) and matched controls (n=8) were Polytron homogenized individually in 15 mL of 100 mM Tris buffer with 120 mM NaCl at pH 7.4. Homogenates were centrifuged at 20,000g for 10 min at 4°C, and the supernatant discarded. Pellets were suspended in 1.3 mL of 20 mM sodium phosphate buffer with 320 mM sucrose at pH 7.4. Binding assays were conducted in 1 mL volumes, using 100 μ L of the suspended pellet and 900 μ L of additional buffer containing the radioligands to make a final concentration of 2 nM [³H]-WIN 35,428 (Amersham Biosciences, Piscataway NJ) for DAT or 2 nM [³H]-methoxytetrabenazine for VMAT2. Non-specific binding was determined by pre-incubating parallel samples for 10 min in 10 μ M GBR-12909 (Sigma-RBI, St. Louis MO) or 2 μ M tetrabenazine (gift from Roche Bioscience, Palo Alto CA) prior to addition of radioligands. The binding reaction proceeded for 60 min at 4°C for DAT and at room temperature for VMAT2. The reactions were stopped by the addition of 3 mL of cold 20 mM sodium phosphate buffer with 320 mM sucrose at pH 7.4, followed by filtration through GF/C filters (Whatman, Florham Park NJ) with a tissue harvester (Brandel, Gaithersburg, MD). Filters were washed 3 times with 100 mM Tris buffer with 120 mM NaCl at pH 7.4 before counting on a LS 6500 scintillation counter (Beckman Coulter, Fullerton CA). Total protein was determined via the Pierce BCA protein detection kit.

Histopathology

Histological stains for degeneration were conducted in serial sections from the brains from 4 HPRT⁻ and 4 HPRT⁺ mice at each of 4 ages (2, 4, 8, and 12 weeks). The entire brain was evaluated, from the olfactory tubercles through the caudal brainstem. The mice were anesthetized by gaseous methoxyflurane and perfused with 1 mL/g of 0.9% saline followed by 4 mL/g of freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. Brains were post-fixed at 4°C for 24 hr in the same fixative, and then stored in 30% sucrose in 0.1 M phosphate buffer pH 7.4 at 4°C for 2-3 days. Serial sections were cut at 30 μ m using a freezing microtome and stored at -20°C in a cryopreservative consisting of 30% glycerol and 30% ethylene glycol in 0.1M phosphate buffer. Every sixth section through each brain was mounted onto glass slides, and dehydrated through graded alcohols and xylenes. After rehydration, a Nissl stained was applied using 0.05% thionine in acetate buffer at pH 4.5. Sections were again dehydrated and coverslipped.

A cupric silver stain was used to identify degenerating profiles in serial sections from the same cohort of animals (De Olmos and Ingram, 1971). Briefly, free floating sections were rinsed in deionized water and incubated for 4 days in an aqueous solution of 2.2% AgNO₃, 0.0066% Cu (NO₃)₂, 3.5% pyridine, and 6.7% ethanol. Sections were then immersed in acetone for 2 min,

diamine-silver solution (0.24% NaOH, 0.67% AgNO₃, and 9% NH₄OH) for 7 min, and reduced in a weak formaldehyde solution containing 0.12% formaldehyde, 0.007% citric acid, and 10% ethanol for 1.5-2 min. The sections were mounted onto glass slides, dehydrated and coverslipped.

Immunostains for glial fibrillary acidic protein (GFAP) were used to identify areas of reactive gliosis in serial sections from the same cohort of animals. Free-floating sections were washed in 50 mM Tris with 150 mM NaCl at pH 7.4, then treated in 0.3% Triton X-100 (TX-100) in the same buffer for 30 min. Endogenous peroxidase activity was suppressed with 3% H₂O₂ for 10 min. Sections were then blocked in 5% normal goat serum and 0.1% TX-100 in buffer for 1 hr, then incubated in antibody to GFAP (1:500, Dako, Carpinteria CA) containing 1% normal goat serum, 0.1% TX-100, and 0.01% sodium azide for 1 day at 4°C. After rinsing, sections were incubated in biotinylated goat anti-rabbit secondary antibody (1:400, Vector Labs, Burlingame, CA) with 1% normal goat serum, 0.1% TX-100, and 0.01% sodium azide for 2-4 hrs at 4°C. The stain was developed using the Vector Elite peroxidase kit with diaminobenzidine (DAB) as the chromogen. The sections were mounted onto glass slides, dehydrated, and coverslipped.

Midbrain neurons

Midbrain dopamine neurons expressing tyrosine hydroxylase (TH) were identified via immunohistochemistry in serial sections from the same cohort of animals used for histopathology. The immunostaining protocol was similar to that described above for GFAP, except that incubations in the antibody to TH (1:800, PelFreez, Rogers AR) were extended to 2-3 days, and incubations in secondary antibody were extended to 1 day. The lengthy incubations in both primary and secondary antibodies improved the uniformity and intensity of staining compared with more commonly used shorter incubations.

Quantitative assessments of stained midbrain sections were conducted on material collected from animals at 12 weeks of age, when the loss of dopamine is fully developed (Jinnah et al., 1999). Estimates of TH-positive cell numbers were estimated from the DAB-stained material using an optical fractionator method similar to that described in other reports (Chadi et al., 1993, Zaborszky and Vadasz, 2001, Chun et al., 2002). Initial studies separated the midbrain population into commonly recognized subdivisions including the ventral tegmental area (A10), substantia nigra (A9) and retrorubral field (A8). However, these regions were combined in the final analysis because we saw no differences between normal and mutant animals in any subregion and the borders between the subregions are not unambiguously defined. Every 6th section from a random starting point was outlined at 5X using an Olympus BX51 microscope (Melville NY) with a Ludl motorized stage (Hawthorne NY) and the Stereologer software package (SPA, Alexandria VA). The material was viewed at 100X using a 14,400 µm² counting grid, and a 1900 µm² frame with 8 µm depth and 0.5 µm top guard zone.

Although the above method for stereological assessment of midbrain cells immunostained for TH with DAB, with or without a simultaneous nuclear counterstain, has been reported frequently in other studies, it suffered several technical limitations. The nucleus was often difficult to identify by optical dissection in the relatively thick sections required for stereology because of the intensely dark stain, the tight cell packing in regions of high cell density, and the many overlapping stained dendrites. Because nuclei were larger than 10% of section thickness and nucleoli could not be visualized reliably, the top edge of the nucleus identified via optical dissection was the counting target. Reducing the staining intensity by adjusting staining parameters (such as antibody concentrations and incubation periods) improved full-thickness visualization of the nuclei, but simultaneously led to drop-out of some more lightly stained cells in less densely packed regions. Because of these technical limitations, the assessment of midbrain dopamine neurons was repeated in an independent group of 5 normal

and 5 HPRT⁻ mice using the same staining protocol except for substituting alkaline phosphatase with Vector Red as the chromogen, and applying a hematoxylin counterstain. This stain improved optical dissection because it provided a translucent red-pink color with a granular quality, with good labeling of soma but sparse labeling of dendrites. Nuclear morphology was readily visualized with the blue-purple hematoxylin counterstain, enabling more reliable identification of nucleoli. For these studies, the region containing stained midbrain neurons was outlined at 10X in every 6th section with the StereoInvestigator system (MicroBrightfield, Williston VT). The sections were viewed at 100X with a 14,400 μm^2 counting grid, and a 2,500 μm^2 frame with 10 μm depth and 1 μm top guard zone. Immunostained cells were counted if the nucleolus fell within the counting frame.

Golgi-Cox histochemistry

Golgi-Cox histochemistry was conducted as previously described (Mikolaenko et al., 2005) to assess the morphology of neurons within the substantia nigra. Briefly, 5 HPRT⁻ mutants and 5 HPRT⁺ controls at 12 weeks of age were anesthetized with gaseous methoxyflurane and perfused with 20 mL of 0.9% saline followed by 100 mL of 4% paraformaldehyde in distilled water at pH 7.4. Brains were removed and placed in an aqueous solution of 5% potassium dichromate, 5% potassium chromate, and 5% mercuric chloride and stored for 14 days in the dark. Tissue was then transferred to 30% sucrose for 2 days in the dark. Vibratome sections were cut at 200 μm , mounted on 2% gelatinized slides, blotted with bibulous paper, and stored in a humidity chamber until staining.

In a dark room, slides were treated in 14% ammonium hydroxide (freshly prepared from a 28% stock solution) for 30 min, and then washed in distilled water. They were next transferred to Kodak Fix for film (diluted 1:1 in distilled water) for 30 min, and washed in distilled water. Slides were dehydrated through graded alcohols, twice each for 5 min, and treated for 15 min in equal parts absolute alcohol, chloroform, and HemoDe (Fisher Scientific, Pittsburgh, PA). After two additional 15 min rinses in HemoDe, slides were coverslipped with Permount and left exposed for 6 months before storing in closed slide boxes.

Three neurons from the pars compacta substantia nigra in each hemisphere of each animal were selected based on previously published criteria (Mikolaenko et al., 2005) by a microscopist blinded to genotype. The selected cells had soma located in the middle third of the section with lengthy and densely stained neurites, and they were free from hindrances to complete visualization. The cells were traced using NeuroLucida software (MicroBrightField, Williston VT) and a Wacom Intuos 2 digitizing tablet at 40X. Soma size, dendrite length, and dendrite branching complexity were analyzed with NeuroExplorer (MicroBrightField, Williston VT).

Light microscopy striatum

The volume of the striatum was estimated in the same cohort of animals used for histopathology and assessment of midbrain neurons. This included 4 HPRT⁻ mutants and 4 HPRT⁺ controls at each of 4 postnatal ages (2, 4, 6, and 12 wks). Every 6th section through the entire striatum was labeled via immunohistochemistry to TH as described above for the midbrain, and the caudoputamen excluding the accumbens and olfactory tubercle was outlined at 2.5X in every 12th section. Volumes were estimated via the Cavalieri method using a 50,000 μm^2 grid size and the Stereologer software package.

A stereological assessment of fiber density was not feasible, since optical dissection of these sections suggested non-uniform staining intensity, with reduced intensity in the deepest parts of the sections. Embedding the stained sections in plastic, and re-sectioning them orthogonal to the original plane of section at 2-5 μm confirmed this impression. Because uniform penetration of staining through the entire thickness of the section is required for accurate

quantification of fiber densities by either stereological or optical density based measures, several attempts were made to obtain more complete and uniform fiber staining over standard protocols. This included the use of thinner free-floating sections (25 μ m), longer incubations in each of the immunostaining reagents, and several different tissue fixation protocols including the commonly used 4% paraformaldehyde at pH 7.5, the 4% paraformaldehyde pH 3-10 shift method, or the addition of glutaraldehyde (0.5-2%) or acrolein (0.5-2%) to 4% paraformaldehyde (Berod et al., 1981, King et al., 1983). The pH shift method and addition of acrolein yielded more intense staining of fibers as previously reported, but with reduced penetration to the interior of the sections. The addition of glutaraldehyde resulted in a less intense stain, but with improved penetration. The conditions providing the most complete and uniform penetration of staining yielded an extremely high fiber density in the striatum that precluded full optical dissection required for stereological assessments of fiber density. The thin plastic sections were therefore evaluated qualitatively.

Electron microscopy

Dopaminergic terminals in the striatum were evaluated in more detail in 4 HPRT⁻ mutants and 4 HPRT⁺ controls at 12 weeks of age by electron microscopy (EM) as previously described (Mikolaenko et al., 2005). For these studies, animals were perfused with 50 mL saline, followed by 100 mL of 4% paraformaldehyde with 0.2% glutaraldehyde in 100 mM sodium phosphate buffer at pH 7.4. Brains were removed and postfixed in 4% paraformaldehyde in buffer overnight, placed in 30% sucrose for 2-3 days, sectioned at 25 μ m on a freezing microtome and stored -20°C in cryopreservative until processing. A 1 mm square was excised from the dorsomedial striatum at the level of the anterior commissure. The tissues were then washed in 50 mM sodium cacodylate, pH 7.4, and exposed to reduced osmium (1% osmium tetroxide and 2.5 mM potassium ferrocyanide in 5 mM sodium cacodylate at pH 7.4) for 1 hr at 0-4°C. Tissue next was washed in distilled water, and counterstained with 2% aqueous uranyl acetate. After another wash in distilled water, tissue was dehydrated in graded alcohols, and rinsed in propylene oxide. Tissue was then embedded in Epon (Ted Pella, Redding CA). The blocks were sectioned at 70 nm with an ultramicrotome and mounted on formvar-coated mesh grids. The grids were then counterstained with 0.3% lead citrate in 0.1N NaOH for 1-5 min. At least 10 images were taken at random at 8.4K magnification on a Jeol TEM 100S electron microscope (Peabody, MA). In each image, a virtual 8 $\times$ 6 μ m box was placed at random, avoiding any areas of staining artifact. An observer blinded to genotype counted the number of symmetric (predominantly dopaminergic) and asymmetric (predominantly glutamatergic) synapses that fell within the box. The criteria for identifying a synapse were parallel pre-synaptic and post-synaptic membranes with a discernable synaptic cleft, a post-synaptic density, and at least three synaptic vesicles in the pre-synaptic terminal. Asymmetric synapses were identified by a thick post-synaptic density, whereas symmetric synapses were identified by a thin post-synaptic density.

Striatal dopaminergic terminals were identified also via nanogold-EM with silver enhancement in 3 HPRT⁻ mutants and 3 HPRT⁺ controls (Bayer and Pickel, 1991). Tissue was prepared as described for EM, and striatal sections at the level of the anterior commissure were selected. Sections were rinsed in 20 mM sodium phosphate with 150 mM NaCl at pH 7.4, and then blocked with 5% normal goat serum in the same buffer for 60 min. Sections were then incubated in antibody to TH with 1% normal goat serum and 0.01% azide in buffer at 4°C for 3 days. Sections were rinsed in buffer, and then incubated in goat anti-rabbit secondary antibody conjugated with 1 nm gold particles (1:50, EMS, Hatfield PA) for 1 day at 4°C. Sections were washed with PBS then post-fixed with 1% glutaraldehyde in buffer for 10 min. They were washed again with distilled water, and fixed with reduced osmium for 1 hr at 0-4°C. After another distilled water wash, the sections were developed in the dark by hydroquinone-silver enhancement (75 mM hydroquinone and 5 mM silver lactate in 30% gum arabic) for 30-40

min. A 2×2 mm tissue square from the most intensely stained region of the dorsomedial striatum was dissected from each stained striatum, counterstained in uranyl acetate, and embedded in Epon as above. The embedded tissue was cut on edge at 70 nm on a Leica Ultracut S ultramicrotome and the plastic sections were mounted on formvar-coated slot grids. Sections viewed on a Hitachi H-7600 TEM (Schaumburg, IL) typically revealed good penetration of the stain for at least 10 μm from the cut surfaces, with incomplete penetration to the deepest parts of the sections. To ensure evaluation of optimally stained material, only the first 10 μm from the edge of each section was evaluated, excluding the first 4 μm to avoid cutting artifacts. At least 20 stained synapses were photographed at 80K magnification from each animal. Stained synapses were defined according to previous guidelines (Nirenberg et al., 1996) as those with at least 3 silver grains. The number of vesicles, vesicle density, and synapse size were assessed semi-quantitatively by an observer blind to genotype using the criteria in Table 1.

Tissue culture model

A cell culture model was developed in which the effects of HPRT deficiency on neurochemical and anatomical characteristics could be rigorously evaluated. The dopaminergic MN9D cell line (Choi et al., 1991) was generously provided by Alfred Heller (Chicago IL) and cultured at 37°C under an atmosphere of 5% CO₂ and 95% air in DMEM (Sigma, St. Louis MO) supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin, and 50 mg/ml streptomycin. HPRT⁻ sublines were established by selective growth in either 6-thioguanine or 8-azaguanine as previously described (Nelson et al., 1975). A total of 10 independent colonies were isolated and expanded separately in culture medium without either selection agent, and each was confirmed to have non-detectable HPRT enzyme function (not shown).

HPRT-competent (HPRT⁺) MN9D sublines were established by limiting dilution. Briefly, cultures of the cells were diluted to a concentration of approximately 3.5 cells/ml, and 200 μL aliquots placed in 96-well Microtest TM culture plates with low-evaporation lids (Beckton-Dickinson, Franklin Lakes, NJ). The cultures were monitored for approximately 2-4 weeks, when individual colonies could be identified in separate wells. Colonies were then collected and expanded separately.

For neurochemical analysis, each cell line was expanded separately in quadruplicate on 100 mm plates. After reaching 80% confluency, the medium was replaced with 10 ml DMEM containing 15% dialyzed FBS. After 24 hours, the cells were collected by trypsinization, centrifuged at 1000 × g for 10 minutes at 4 °C, and disrupted via brief sonication in 0.5 mL of 0.1 M perchloric acid. Samples were stored frozen at -80 °C prior to neurochemical analyses. Samples of conditioned medium and cell pellets were thawed rapidly and centrifuged at 10,000 × g for 10 min at 4 °C to sediment acid-precipitated material. Any remaining insoluble materials were removed via filtration through 0.45 μm PVDF microcentrifuge spin filters (Alltech, Deerfield IL). Monoamines were determined by HPLC with electrochemical detection as above.

RESULTS

Brain monoamines

In the striatum, weanling HPRT⁻ mutants had nearly normal dopamine levels, but adult HPRT⁻ mutants had approximately half normal levels (Table 2). The loss of dopamine was not more prominent in aged HPRT⁻ mutants, arguing against a progressive degenerative process. In the midbrain, the HPRT⁻ mutants exhibited smaller reductions in dopamine. To estimate dopamine turnover, ratios of dopamine to each of its metabolites and to a sum of all metabolites were determined. There was a trend for increased ratios of all metabolites to dopamine for all age groups, although this trend reached significance only in the middle and

older age groups (Table 3). These results imply increased turnover of a smaller total pool of dopamine in the HPRT⁻ mutant animals.

Dopamine levels in the cerebral cortex were normal in the HPRT⁻ mutants (not shown). Minimal changes of norepinephrine or serotonin in the HPRT⁻ mutants were found the striatum, cortex, and midbrain (Table 4). These results are similar to prior reports documenting an age-dependent reduction of striatal dopamine in the HPRT⁻ mice, with smaller or non-significant changes in other brain regions (Jinnah et al., 1994, Jinnah et al., 1999).

Brain VMAT2 & DAT

Prior studies have demonstrated reductions in the enzymatic activities of TH and aromatic amino acid decarboxylase (Jinnah et al., 1994, Jinnah et al., 1999). To determine if DAT or VMAT2 were also reduced in the HPRT⁻ mutants, radioligand binding assays were conducted. These studies revealed a 24% reduction in [³H]-WIN 35,428 binding (normal = 223.2±21.9, mutant = 172.9±23.6, p<0.04), indicating reductions in DAT in striatal homogenates from the HPRT⁻ mutants. There also was a 27% reduction in [³H]-methoxytetraabenazine binding indicative of reduced VMAT2, but the difference fell short of statistical significance (normal = 216.6±28.2, mutant = 157.9±16.3, p<0.07).

Brain histopathology

The most parsimonious explanation for the loss of striatal dopamine and associated markers in the HPRT-deficient brain is that nigrostriatal dopamine neurons fail to develop or undergo early degeneration (Lloyd et al., 1981, Wong et al., 1996, Visser et al., 2000). We therefore performed a detailed histopathological survey of the brains of the HPRT⁻ mice, spanning the 2-6 week time period when the dopamine deficit emerges. Nissl stains revealed a normal histology throughout the brains of the HPRT⁻ mice at 2, 4, 6, and 12 weeks of age (Figure 1A-B).

Silver degeneration stains revealed rare degenerating profiles randomly in different brain regions, occurring equally in the HPRT⁻ and HPRT⁺ mice, predominantly at the earliest developmental times, when the normal postnatal culling occurs (Jackson-Lewis et al., 2000). There was no evidence for abnormal gliosis by GFAP immunostaining in any brain region at any developmental time point (not shown). These results argue against a degenerative process underlying the loss of striatal dopamine and associated markers.

Midbrain neurons

To more precisely characterize histopathology of midbrain dopamine neurons after the dopamine loss was established, immunostains were conducted for TH in animals at 12 weeks of age. The region of the midbrain containing dopaminergic neurons was clearly identifiable after immunostaining. Serial sections through the entire region revealed the HPRT⁻ mutants to have a normal distribution and intensity of stain (Figure 1C-D). The morphology of the TH-positive neurons appeared qualitatively normal in the HPRT⁻ mice (Figure 1E-H).

With the nucleus as a counting target in material TH-immunostained with DAB, normal and HPRT⁻ mutant mice had similar numbers of total midbrain neurons (normal 35,388±2087, mutant 31,953±1597; p=0.22). There were no differences between normal and mutant mice among any of the commonly distinguished subpopulations including ventral tegmental area (A10), substantia nigra (A9), or retrorubral field (A8).

Fewer midbrain neurons were measured with the nucleolus as a counting target in material TH-immunostained with Vector Red, most likely because of the more rigorous criteria for counting. However, normal and mutant mice again had similar numbers of total neurons (normal 15,274

$\pm 1,355$, mutant $17,233 \pm 1,933$; $p=0.38$). These estimates for the total number of TH-immunopositive neurons in the midbrain fall within the ranges previously reported by others using similar methods in normal C57BL/6J mice (Zaborszky and Vadasz, 2001).

Golgi-Cox histochemistry of midbrain

Recent Golgi-Cox studies revealed subtle structural abnormalities among striatal medium spiny neurons in the HPRT⁻ mice, including reduced dendritic lengths and fewer spines (Mikolaenko et al., 2005). This method was used to evaluate the size and morphology of neurons in the substantia nigra pars compacta (area A9) to determine if similar defects might be observed. No differences between the HPRT⁻ mutants and normal controls were detected for soma size, soma shape, dendrite branching, numbers of dendrites, or dendrite lengths (Figure 2). Nigrostriatal axons could not be evaluated because their lengths precluded complete tracing.

Light microscopy of striatum

Because the stereological and morphometric evaluation of midbrain dopamine neurons revealed no structural abnormalities that could account for the reductions of striatal dopamine observed in adult HPRT⁻ mice, we next evaluated the striatal target regions. Nissl stains (Figure 3A-B) and stereological volume estimates (Figure 4) provided no evidence for atrophy of the caudoputamen in the HPRT⁻ mutants at any point during postnatal development.

TH-immunostained sections through the entire caudoputamen and accumbens revealed the HPRT⁻ mutants to have a normal staining intensity and anatomical distribution. High power light microscopy of the 25 μ m sections revealed no apparent loss of TH-immunostained fibers in the HPRT⁻ mutants (Figure 3C-D). The density and morphology of TH-immunostained fibers also appeared normal in 2 μ m plastic sections (Figure 3E-F). Specifically, there was no evidence for reduced axonal numbers, thinning of axons, loss of varicosities, or aberrant axonal morphologies.

Electron microscopy of striatum

Because light microscopic analyses failed to reveal any obvious anatomical defects in the HPRT⁻ mutant mice, we next performed an ultrastructural analysis to search for loss of synapses or other morphological defects. Studies of the striatal neuropil revealed no apparent abnormalities in the HPRT⁻ mutants, such as pathological inclusions or dystrophic neurites (Figure 5). Prior studies have shown dopaminergic synapses to be predominantly symmetric and glutamatergic synapses to be predominantly asymmetric (Pickel et al., 1981, Arluison et al., 1984, Dube et al., 1988, Groves et al., 1994). Both types of synapses were quantified separately. There was no evidence for reductions in either type of synapse, or in the ratio of symmetric to asymmetric synapses in the HPRT⁻ mutants (Table 5).

The ultrastructural morphology of dopaminergic synapses was more precisely evaluated after immunogold labeling of TH. Synapse morphology appeared normal in the HPRT⁻ mutants, with no evidence for reduction in size or vesicle content (Table 6).

Culture model

Extensive anatomical studies of the HPRT⁻ mice provided no evidence for the structural defects thought to underlie the loss of striatal dopamine in the HPRT-deficient brain. The results contradict prevailing hypotheses that the loss of dopamine has an anatomical basis. It is difficult to confidently exclude possible contributions of subtle morphological abnormality not yet found, though it seems unlikely that a defect sufficiently subtle to escape detection in the battery of histological studies conducted could account for a 50% loss of striatal dopamine. The

histological results therefore suggest an alternative hypothesis that loss of dopamine is due to metabolic rather than structural processes. To address this hypothesis, we developed a tissue culture model of HPRT deficiency in which the neurochemical and neuroanatomical characteristics could be rigorously evaluated. The MN9D cell line was chosen because it expresses many biochemical characteristics of dopamine neurons with a relatively simple morphology in the undifferentiated state (Choi et al., 1991, Heller et al., 1996).

Individual sublines derived from an established cell line may exhibit significant variations in both neurochemical and morphological properties, because of genetic drift in culture leading to heterogeneity within the parent population (Koike and Takashim, 1984, Ciccarone et al., 1989, Clementi et al., 1992). To ensure that the characteristics of the HPRT⁻ sublines being evaluated were related to the loss of HPRT and not some idiosyncratic property of an individual subline, 10 independently isolated HPRT⁻ sublines were compared against the parent line.

The intracellular dopamine content of the parent MN9D cells measured under standard conditions in 5 independent experiments over a period of 2 years showed little variation, confirming a stable neurochemical phenotype (Figure 6A). The dopamine content of the HPRT⁻ sublines showed similar minor variation. To control for intra-assay variation, the dopamine content of each HPRT⁻ MN9D subline was compared with a parallel culture of the parent line (Figure 6B). Using this analysis, 8 of 10 had dopamine levels that were significantly lower than the parent line by one-way ANOVA with post-hoc Tukey t-tests. The average dopamine content of the other 2 sublines also fell below that of the parent line, but the difference was not statistically significant.

This traditional statistical approach may not be appropriate for addressing the influence of HPRT deficiency against the background of variability inherent in subcloning such cell lines. One-way ANOVA is useful for detecting significant differences among all the lines, but it is not useful for detecting a consistent change among all of the HPRT⁻ sublines as a group. For any measured variable, increases in some sublines combined with decreases in other sublines is less informative than consistent changes in all of the HPRT⁻ sublines, even if these changes are quantitatively small. In addition, inherent in one-way ANOVA are comparisons that are not relevant for addressing the central question of the significance of HPRT deficiency. Significant differences between individual HPRT⁻ sublines are less important than significant differences between the HPRT⁻ sublines and the control. These limitations of traditional one-way ANOVA could be addressed via a simple probabilistic method, which estimates the likelihood of the direction of change in any variable. Specifically, the probability of all 10 HPRT⁻ MN9D sublines having average dopamine lower than the parent line by chance is $(0.5)^{10}$, or $p < 0.0001$. This simple probabilistic approach seeks consistent changes among the HPRT⁻ sublines as a group, but it does not take into account the variation inherent in the sublines and the magnitude of any differences. One solution to this is the use of a single sample t-test, which compares the HPRT⁻ sublines as a group against the parent line. This method confirms a significant influence of HPRT deficiency on dopamine content in the MN9D cells ($p < 0.0001$). Overall the mutant sublines had about half the dopamine content of the parent line.

To ensure the loss of dopamine could not be attributed to the subcloning process itself, we isolated 16 HPRT⁺ subclones by limiting dilution (Figure 6A). When compared against the mean of the parent line, 9 of the HPRT⁻ sublines were increased and 7 were decreased; and the population average did not differ significantly from the parent line. The single sample t-test revealed no group difference between the HPRT⁺ sublines and the parent control line. These results point to important variation within the MN9D parent line, but show that subcloning itself cannot explain the reductions in dopamine of the HPRT⁻ sublines.

Morphologically the parent MN9D line had cells with heterogeneous morphologies (Figure 7) as previously reported (Choi et al., 1991). Some were small round blast-like forms. Others were larger and flatter, with thin processes suggesting a partially differentiated form. Individual HPRT⁻ sublines each differed slightly in overall morphology, a common result of subcloning established cells lines (Koike and Takashim, 1984, Ciccarone et al., 1989, Clementi et al., 1992). However, there were no obvious morphological abnormalities among the HPRT⁻ sublines; and all appeared healthy with no signs of toxicity such as a high frequency of non-viable cells or debris, or morphological signs of necrosis or apoptosis.

DISCUSSION

These studies confirm an age-dependent, regionally restricted, and neurochemically selective loss of striatal dopamine in the HPRT⁻ mouse brain. The loss of dopamine is accompanied by a loss of multiple related biochemical markers of dopamine neurons. The most parsimonious explanation for the loss of dopamine and its associated biochemical measures is that the dopamine neurons fail to develop or undergo degenerative changes (Lloyd et al., 1981, Ernst et al., 1996, Wong et al., 1996, Visser et al., 2000). This explanation predicts overt morphological abnormalities, such as dystrophy or loss of the neurons or their axons. However, detailed anatomical studies of the HPRT⁻ mice failed to demonstrate any morphological abnormalities of midbrain neurons or their axons. Furthermore, the loss of dopamine could be reproduced in a tissue culture model of HPRT⁻ dopamine neurons, with no apparent morphological correlate. These results demonstrate the loss of dopamine is a cell-intrinsic defect independent from brain connectivity, and secondary to biochemical rather than anatomical processes.

The regionally restricted and neurochemically selective biochemical findings in the knockout mice are similar to those reported for the HPRT⁻ human brain (Lloyd et al., 1981, Ernst et al., 1996, Wong et al., 1996, Saito et al., 1999). These changes in the human brain are generally believed to reflect a loss of dopamine neurons or their axons in the striatum, though histological studies of human autopsy material have failed to detect the presumed defects. Multiple prior studies employing Nissl stains have not revealed significant loss of midbrain dopamine neurons (Jinnah et al., 2006). There is a single report of two brains in which midbrain dopamine neurons were identified by their neuromelanin content or immunostaining for TH (Saito et al., 1999). One brain had reduced numbers of neuromelanin containing neurons, but TH-immunostained neurons were normal in both. The reason for the failure of histological studies of autopsy material to detect any morphological defects among dopamine neurons is perplexing, considering the magnitude of dopamine loss in humans. Though animal and cell models may not reflect the pathology of the human brain, they offer new results that provide for an intriguing explanation for the absence of consistent histological defects in the human brain. Specifically, the results from these models suggest that no such histological abnormalities exist because the loss of dopamine is due to biochemical rather than morphological processes. More careful studies of human autopsy material will be required to address this possibility.

Human HPRT deficiency is often compared with Parkinson disease because of the regional and neurochemical selectivity of dopamine loss (Lloyd et al., 1981, Visser et al., 2000). However, the lack of any apparent morphological defect indicates that the loss of dopamine in the HPRT⁻ brain is not due to a degenerative process analogous to that occurring in Parkinson disease. A precedent for neurochemically specific and regionally selective reductions of striatal dopamine due to non-degenerative processes is found in the dopa-responsive dystonias (DRDs). These disorders encompass heterogeneous etiologies that involve the synthesis of dopamine, such as defects in TH or GTP-cyclohydrolase (Furukawa, 2004). Although the DRDs provide examples of biochemical rather than degenerative causes for selective loss of striatal dopamine, the mechanisms involved appear to be different from those in HPRT

deficiency. Unlike the DRDs, HPRT deficiency is not limited to a loss of dopamine but includes other proteins involved in dopamine handling such as TH, AADC, DAT, and VMAT. Also unlike the DRDs, dopamine replacement therapies do not provide significant benefits in Lesch-Nyhan disease (Jinnah et al., 2006). Most importantly, there are no known pathways linking the functions of HPRT with biochemical pathways involved in dopamine synthesis (Visser et al., 2000). The loss of dopamine in HPRT deficiency appears to involve processes in addition to those involved in dopamine synthesis.

The biochemical mechanisms by which HPRT deficiency leads to a neurochemically and neuroanatomically selective loss of dopamine remain unknown. It remains possible that mechanisms under consideration for Parkinson disease adversely affect the metabolic state of HPRT-deficient dopamine neurons leading to reduced dopamine content without overt degeneration. A link also has been repeatedly established between Parkinson disease and uric acid (Church and Ward, 1994, Davis et al., 1996, de Lau et al., 2005), an end product of purine catabolism that is produced at markedly increased levels in Lesch-Nyhan disease. The overproduction of uric acid may contribute to lower dopamine levels. HPRT deficiency may also result in deficiency of one of its products, GTP. Dopamine synthesis could be impaired because GTP is used in the first and rate-limiting step in the synthesis of tetrahydrobiopterin (Hyland et al., 2004). HPRT-mediated purine recycling may also influence purinergic transmission (Jinnah et al., 1992a), which has a major modulatory influence on dopamine transmission in the basal ganglia (Ferre et al., 1992). Yet another possibility is that HPRT deficiency disrupts the development of the mature biochemical phenotype of dopamine neurons without altering their morphology. Currently, empirical evidence is lacking to discriminate among these possibilities and further studies are needed.

Rare monogenic forms of Parkinsonism have emphasized biochemical processes relevant to the survival of midbrain dopamine neurons, and the DRDs have highlighted biochemical pathways for dopamine synthesis. It is likely that many other biochemical processes of particular relevance to the survival and function of these neurons remain to be discovered. HPRT deficiency provides another example of an inherited disorder in which the neurochemical phenotype of dopamine neurons is selectively altered, providing a window on purine recycling as a biochemical process of particular importance to these neurons. This influence is broader than the defects of dopamine synthesis documented in the DRDs, but without the neurodegenerative changes of Parkinsonism.

Other evidence suggests the existence of similar mechanisms responsible for broad control of the neurochemical phenotype of dopamine neurons. During human fetal development, multiple transcription factors participate in the induction and maintenance of many aspects of the neurochemical phenotype of these cells (Lin and Rosenthal, 2003, Smidt et al., 2003). From early childhood through early adulthood, there is a 2-3 fold increase in striatal dopamine and associated neurochemical measures with a subsequent age-related decline that cannot be explained by changes in the numbers of midbrain dopamine neurons or striatal axon density (Haycock et al., 2003). In aged brains, a subpopulation of nigral neurons has subnormal amounts of TH, with 10% of them having none (Chu et al., 2006). The number of nigral neurons without detectable TH increases to 40-64% in Parkinson's disease and related disorders (Hirsch et al., 1988, Chu et al., 2006). The loss of TH in these brains has been attributed to an "agonal" state, in which sick dopamine neurons are no longer able to maintain expression of their neurochemical phenotype. However, the large numbers of these neurons is not consistent with an "agonal" state, unless this state has a very long duration, with loss of TH occurring well in advance of cell death. These and other observations have led to an increasing appreciation that biochemical derangements of dopamine neurons may significantly affect their function long before overt degeneration becomes apparent.

Mechanisms responsible for dopamine neuron degeneration have received considerable attention because they may guide the rational development of treatments designed to prevent cell death. Fewer studies have addressed regulatory controls of the neurochemical phenotype of the dopamine neuron, and how they might become dysfunctional before overt cell death. Further studies of these processes seem warranted, because restoring function to surviving neurons that have lost their neurochemical phenotype could have an immediate therapeutic impact, and strategies for preventing cell death will have greater impact if the neurochemical phenotype also can be preserved.

Acknowledgements

We thank Michael Kilbourne for providing the ³[H]-methoxytetraabenazine and Dr. Alfred Heller for the MN9D cell line. We also thank Lee Martin for expert advice on histology. This work was supported by NS01985 and NS40470 from the National Institutes of Health and the Lesch-Nyhan Syndrome Children's Research Foundation.

Bibliography

- About-Sleiman PM, Muqit MM, Wood NW. Expanding insights of mitochondrial dysfunction in Parkinson's disease. *Nat Rev Neurosci* 2006;7:207–219. [PubMed: 16495942]
- Antonarakis SE, Beckmann JS. Mendelian disorders deserve more attention. *Nat Rev Genet* 2006;4:277–282. [PubMed: 16534515]
- Arлуison M, Dietl M, Thibault J. Ultrastructural morphology of dopaminergic nerve terminals and synapses in the striatum of the rat using tyrosine hydroxylase immunocytochemistry: a topographical study. *Brain Res Bull* 1984;13:269–285. [PubMed: 6149794]
- Bayer VE, Pickel VM. GABA-labeled terminals form proportionally more synapses with dopaminergic neurons containing low densities of tyrosine hydroxylase-immunoreactivity in rat ventral tegmental area. *Brain Res* 1991;559:44–55. [PubMed: 1685938]
- Berod A, Hartman BK, Pujol JF. Importance of fixation in immunohistochemistry: use of formaldehyde solutions at variable pH for the localization of tyrosine hydroxylase. *J Histochem Cytochem* 1981;29:844–850. [PubMed: 6167611]
- Chadi G, Moller A, Rosen L, Janson AM, Agnati LA, Goldstein M, Ogren SO, Pettersson RF, Fuxe K. Protective actions of human recombinant basic fibroblast growth factor on MPTP-lesioned nigrostriatal dopamine neurons after intraventricular infusion. *Exp Brain Res* 1993;97:145–158. [PubMed: 7907549]
- Choi HK, Won LA, Kontur PJ, Hammond DN, Fox AP, Wainer BH, Hoffmann PC, Heller AC. Immortalization of embryonic mesencephalic dopaminergic neurons by somatic cell fusion. *Brain Res* 1991;552:67–76. [PubMed: 1913182]
- Chu Y, Le W, Kompolti K, Jankovic J, Mufson EJ, Kordower JH. Nurr1 in Parkinson's disease and related disorders. *J Comp Neurol* 2006;494:495–514. [PubMed: 16320253]
- Chun HS, Yoo MS, DeGiorgio LA, Volpe BT, Peng D, Baker H, Peng C, Son JH. Marked dopaminergic cell loss subsequent to developmental, intranigral expression of glial cell line-derived neurotrophic factor. *Exp Neurol* 2002;173:235–244. [PubMed: 11822887]
- Church WH, Ward VL. Uric acid is reduced in the substantia nigra in Parkinson's disease: effect on dopamine oxidation. *Brain Res Bull* 1994;33:419–424. [PubMed: 8124580]
- Ciccarone V, Spengler BA, Meyers MB, Biedler JL, Ross RA. Phenotypic diversification in human neuroblastoma cells: expression of distinct neural crest lineages. *Cancer Res* 1989;49:219–225. [PubMed: 2535691]
- Clementi E, Racchetti G, Zacchetti D, Panzeri MC, Meldolesi J. Differential expression of markers and activities in a group of PC12 nerve cell clones. *Eur J Neurosci* 1992;4:944–953. [PubMed: 12106430]
- Cookson MR. The biochemistry of Parkinson's disease. *Annu Rev Biochem* 2005;74:29–52. [PubMed: 15952880]
- Darchen F, Masuo Y, Vial M, Rostene W, Scherman D. Quantitative autoradiography of the rat brain vesicular monoamine transporter using the binding of [³H]dihydrotetraabenazine and 7-amino-8-[¹²⁵I]iodoketanserin. *Neurosci* 1989;33:341–349.

- Davis JW, Grandinetti A, Waslien CI, Ross GW, White LR, Morens DM. Observations on serum uric acid levels and the risk of idiopathic Parkinson's disease. *Am J Epidemiol* 1996;144:480–484. [PubMed: 8781463]
- de Lau LM, Koudstaal PJ, Hofman A, Breteler MM. Serum uric acid and the risk of Parkinson disease. *Ann Neurol* 2005;58:797–800. [PubMed: 16240356]
- De Olmos JS, Ingram WR. An improved cupric-silver method for impregnation of axonal and terminal degeneration. *Brain Res* 1971;33:523–529. [PubMed: 4109198]
- Dube L, Smith AD, Bolam JP. Identification of synaptic terminals of thalamic or cortical origin in contact with distinct medium-size spiny neurons in the rat neostriatum. *J Comp Neurol* 1988;267:455–471. [PubMed: 3346370]
- Ernst M, Zametkin AJ, Matochik JA, Pascualvaca D, Jons PH, Hardy K, Hankerson JG, Doudet DJ, Cohen RM. Presynaptic dopaminergic deficits in Lesch-Nyhan disease. *N Engl J Med* 1996;334:1568–1572. [PubMed: 8628337]
- Ferre S, Fuxe K, Von Euler G, Johansson B, Fredholm BB. Adenosinedopamine interactions in the brain. *Neurosci* 1992;51:501–512.
- Furukawa Y. Update on dopa-responsive dystonia: locus heterogeneity and biochemical features. *Adv Neurol* 2004;94:127–138. [PubMed: 14509665]
- Groves PM, Linder JC, Young SJ. 5-Hydroxydopamine-labeled dopaminergic axons: three-dimensional reconstructions of axons, synapses and postsynaptic targets in rat neostriatum. *Neurosci* 1994;58:593–604.
- Haycock JW, Becker L, Ang L, Furukawa Y, Hornykiewicz O, Kish S. Marked disparity between age-related changes in dopamine and other presynaptic dopaminergic markers in human striatum. *J Neurochem* 2003;87:574–585. [PubMed: 14535941]
- Heller A, Price S, Won L. Glial-derived neurotrophic factor (GDNF) induced morphological differentiation of an immortalized monoclonal hybrid dopaminergic cell line of mesencephalic origin. *Brain Res* 1996;725:132–136. [PubMed: 8828597]
- Hirsch E, Graybiel AM, Agid Y. Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature* 1988;334:345–348. [PubMed: 2899295]
- Hyland K, Kasim S, Egami K, Arnold LA, Jinnah HA. Tetrahydrobiopterin and brain dopamine loss in a genetic mouse model of Lesch-Nyhan disease. *J Inher Metab Dis* 2004;27:165–178. [PubMed: 15159647]
- Jackson-Lewis V, Vila M, Djaldetti R, Guegan C, Liberatore G, Liu J, O'Malley KL, Burke RE, Przedborski S. Developmental cell death in dopaminergic neurons of the substantia nigra of mice. *J Comp Neurol* 2000;424:476–488. [PubMed: 10906714]
- Jinnah HA, DeGregorio L, Harris JC, Nyhan WL, O'Neill JP. The spectrum of inherited mutations causing HPRT deficiency: 75 new cases and a review of 196 previously reported cases. *Mutat Res* 2000;463:309–326. [PubMed: 11018746]
- Jinnah HA, Hess EJ, Wilson MC, Gage FH, Friedmann T. Localization of hypoxanthine-guanine phosphoribosyltransferase mRNA in the mouse brain by in situ hybridization. *Mol Cell Neurosci* 1992a;3:64–78.
- Jinnah HA, Jones MD, Wojcik BE, Rothstein JD, Hess EJ, Friedmann T, Breese GR. Influence of age and strain on striatal dopamine loss in a genetic mouse model of Lesch-Nyhan disease. *J Neurochem* 1999;72:225–229. [PubMed: 9886073]
- Jinnah HA, Langlais PJ, Friedmann T. Functional analysis of brain dopamine systems in a genetic mouse model of Lesch-Nyhan syndrome. *J Pharmacol Exp Ther* 1992b;263:596–607. [PubMed: 1432691]
- Jinnah HA, Page T, Friedmann T. Brain purines in a genetic mouse model of Lesch-Nyhan disease. *J Neurochem* 1993;60:2036–2045. [PubMed: 8492116]
- Jinnah HA, Visser JE, Harris JC, Verdu A, Larovere L, Ceballos-Picot I, Neychev V, Torres RJ, Dulac O, Desguerre I, Schretlen DJ, Robey KL, Barabas G, Bloem BR, Nyhan WL, Kremer R, G.E. E, Puig JG, Reich SG. Delineation of the motor disorder of Lesch-Nyhan disease. *Brain* 2006;1201–1217. [PubMed: 16549399] manuscript attached in appendix
- Jinnah HA, Wojcik BE, Hunt MA, Narang N, Lee KY, Goldstein M, Wamsley JK, Langlais PJ, Friedmann T. Dopamine deficiency in a genetic mouse model of Lesch-Nyhan disease. *J Neurosci* 1994;14:1164–1175. [PubMed: 7509865]

- King JC, Lechan RM, Kugel G, Anthony ELP. Acrolein: a fixative for immunocytochemical localization of peptides in the central nervous system. *J Histochem Cytochem* 1983;31:62–68. [PubMed: 6187805]
- Koike T, Takashim A. Clonal variability of PC12 pheochromocytoma cells with respect to catecholamine biosynthesis. *J Neurochem* 1984;42:1472–1475. [PubMed: 6707645]
- Lin JC, Rosenthal A. Molecular mechanisms controlling the development of dopaminergic neurons. *Sem Cell Devel Biol* 2003;14:175–180.
- Lloyd KG, Hornykiewicz O, Davidson L, Shannak K, Farley I, Goldstein M, Shibuya M, Kelley WN, Fox IH. Biochemical evidence of dysfunction of brain neurotransmitters in the Lesch-Nyhan syndrome. *N Engl J Med* 1981;305:1106–1111. [PubMed: 6117011]
- Mikolaenko I, Rao LM, Roberts RC, Kolb B, Jinnah HA. A Golgi study of neuronal architecture in a genetic mouse model for Lesch-Nyhan disease. *Neurobiol Dis* 2005;20:479–490. [PubMed: 15908225]
- Moore DJ, West AB, Dawson VL, Dawson TM. Molecular pathophysiology of Parkinson's disease. *Ann Rev Neurosci* 2005;28:57–87. [PubMed: 16022590]
- Nelson JA, Carpenter JW, Rose LM, Adamson DJ. Mechanisms of action of 6-thioguanine, 6-mercaptopurine, and 8-azaguanine. *Cancer Res* 1975;35:2872–2878. [PubMed: 1157053]
- Nirenberg MJ, Vaughan RA, Uhl GR, Kuhar MJ, Pickel VM. The dopamine transporter is localized to dendritic and axonal plasma membranes of nigrostriatal dopaminergic neurons. *J Neurosci* 1996;16:436–447. [PubMed: 8551328]
- Pickel VM, Beckley SC, Joh TH, Reis DJ. Ultrastructural immunocytochemical localization of tyrosine hydroxylase in the neostriatum. *Brain Res* 1981;225:373–385. [PubMed: 6118197]
- Saito Y, Ito M, Hanaoka S, Ohama E, Akaboshi S, Takashima S. Dopamine receptor upregulation in Lesch-Nyhan syndrome: a postmortem study. *Neuropediatrics* 1999;30:66–71. [PubMed: 10401687]
- Saito Y, Takashima S. Neurotransmitter changes in the pathophysiology of Lesch-Nyhan syndrome. *Brain Dev* 2000;22(Suppl 1):S122–S131. [PubMed: 10984673]
- Smidt MP, Smits SM, Burbach JPH. Molecular mechanisms underlying midbrain dopamine neuron development and function. *Eur J Pharmacol* 2003;480:75–88. [PubMed: 14623352]
- Visser JE, Baer PR, Jinnah HA. Lesch-Nyhan syndrome and the basal ganglia. *Brain Res Rev* 2000;32:449–475. [PubMed: 10760551]
- Wilson JM, Kish SJ. The vesicular monoamine transporter, in contrast to the dopamine transporter, is not altered by chronic cocaine self-administration in the rat. *J Neurosci* 1996;16:3507–3510. [PubMed: 8627383]
- Wong DF, Harris JC, Naidu S, Yokoi F, Marengo S, Dannals RF, Ravert HT, Yaster M, Evans A, Rousset O, Bryan RN, Gjedde A, Kuhar MJ, Breese GR. Dopamine transporters are markedly reduced in Lesch-Nyhan disease in vivo. *Proc Natl Acad Sci (USA)* 1996;93:5539–5543. [PubMed: 8643611]
- Zaborszky L, Vadasz C. The midbrain dopaminergic system: anatomy and genetic variation in dopamine neuron number of inbred mouse strains. *Behav Genet* 2001;31:47–59. [PubMed: 11529274]
- Zeevalk GD, Bernard LP, Song C, Gluck JP, Ehrhart J. Mitochondrial inhibition and oxidative stress: reciprocating players in neurodegeneration. *Antioxid Redox Signal* 2005;7:1117–1139.

Figure 1.

Histology of the midbrain in normal (left column) and HPRT⁻ mice (right column). Thionine stains revealed no pathological changes (A,B). Immunohistochemical stains for TH revealed a normal stain intensity and distribution (C,D). Higher power views showed normal appearing neuronal soma in sections TH-immunostained with either DAB (E,F) or Vector Red (G,H).

Figure 2.

Golgi histochemistry of substantia nigra neurons in normal and HPRT⁻ mice. The typical staining quality of a nigral neuron is shown in panel A. Morphometric studies revealed no significant differences between normal and HPRT⁻ mice for the numbers of dendrites at each branch order (B), the numbers of dendrites extending to each 20 μm radius from the soma (C), the total dendritic lengths reaching each target radius, or the numbers of neurons with at least one dendrite reaching the target radius (E). Results show average values ±SEM (n=30 neurons for each measure). Statistical comparisons were conducted via two-way ANOVA, with genotype as the main factor and branch order or radius as a repeated measure (F).

Figure 3.

Histology of the striatum in normal (left column) and HPRT⁻ mice (right column). Thionine stains revealed no pathological changes (A,B). Immunohistochemical stains for TH revealed a normal stain intensity and distribution (C,D). Higher power views of the TH-immunostained sections counterstained with cresyl violet showed a normal density and morphology of axons (E,F).

Figure 4. Striatal volumes in TH-immunostained material from normal (closed circles) and HPRT⁻ (open circles) mice from 2-12 weeks of postnatal development. Results show average values $\pm$ SEM for the caudoputamen, excluding the accumbens and olfactory tubercles (n=4 mice at each point). Two-way ANOVA revealed a significant effect for developmental age, but no significant effect for HPRT status.

Figure 5. Representative electron micrographs of the striatal neuropil from a normal (A) and HPRT⁻ (B) mouse. Typical symmetric (ss) and asymmetric (as) synapses are labeled along with mitochondria (m) and a myelinated fiber (mf).

Figure 6.

Intracellular dopamine in normal and HPRT⁻ MN9D sublines. A) Scatterplot of dopamine levels in the parental MN9D cells under standard conditions collected in 5 independent experiments over 2 years, 16 HPRT⁺ sublines, and 10 HPRT⁻ sublines. For the parent line, inter-assay variability was 5.3% with intra-assay variability of 14.6%. The shadowed areas depict the average of the group $\pm$ SEM. One-way ANOVA revealed significant differences among the groups ($F_{2,28}=7.7$, $p<0.005$). Post-hoc Tukey t-tests revealed no significant difference between the parent line and the LD subclones but a significant difference between the parent line and the HPRT⁻ lines ($p<0.05$). B) To minimize inter-assay variation, dopamine was expressed as the percent difference compared to a parallel culture of the parent MN9D line. A single sample t-test again revealed a significant reduction in dopamine in the mutant lines ($p<0.001$).

Figure 7.

Representative photomicrographs of the MN9D parent line (P) and each of the HPRT⁻ sublines. All cultures included a mixed population of predominantly small and rounded (SR) or larger rounded (LR) cells with few or no processes suggestive of a blast form and a smaller population of larger and more complex forms (CF) with one or more neurites suggesting a more differentiated cell. Subtle morphological differences were apparent in some of the lines, such as relatively smaller or larger soma, changes in the proportion of blast versus differentiated forms, or changes in the tendency to grow in clumps.

Table 1

EM rating scale

Grade	0	1	2	3
Vesicle Number	0-5	5-20	20-50	50+
Vesicle Density	0-25/ μm^2	25-100/ μm^2	100-250/ μm^2	250+/ μm^2
Synapse Size	0	0-0.15 μm^2	0.15-0.3 μm^2	0.3 μm^2 +

Table 2

Brain dopamine content

	Striatum				Midbrain			
	DA	DOPAC	HVA	3MT	DA	DOPAC	HVA	3MT
3-4 weeks								
HPRT+	85.9±3.1	18.5±0.8	13.4±0.5	9.1±0.8	2.3±0.1	1.0±0.1	1.7±0.2	ND
HPRT-	77.7±3.4*	19.1±1.6	14.0±0.8	9.7±0.4	2.2±0.3	0.7±0.1	1.6±0.2	ND
3-6 months								
HPRT+	170.1±4.8	19.8±1.8	16.3±0.7	11.9±0.9	2.0±0.1	1.0±0.1	1.6±0.2	ND
HPRT-	84.5±3.0**	16.9±1.8	14.7±1.1	9.7±0.8*	1.3±0.3	0.8±0.1	1.5±0.2	ND
2 years								
HPRT+	157.7±2.9	30.5±1.4	13.8±0.5	12.8±0.7	4.3±0.3	2.5±0.2	3.1±0.2	ND
HPRT-	94.5±2.0**	24.3±1.3*	12.9±0.4	11.0±0.4	3.0±0.4*	2.4±0.1	3.1±0.4	ND

Results are expressed as ng/mg protein ±SEM from at least 5 mice per group. Results for each brain region were evaluated separately via 2-way ANOVA, with HPRT status and age as factors. To correct for multiple comparisons, a Bonferroni correction for multiple tests (n=24) was used to define p<0.002 as the conservative estimate for statistical significance. Results with 0.002<p<0.05 were considered borderline. For the striatum the effect of HPRT status was significant for dopamine (F=180, p<0.0001), it was borderline for DOPAC (F=5.3, p=0.028) and 3MT (F=4.6, p=0.038), and it was not significant for HVA (F=2.0, p=0.16). The interaction between HPRT and age was significant for dopamine (F=22.6, p<0.0001) but it was not significant for DOPAC (F=1.1, p=0.33), 3MT (F=0.8, p=0.44), or HVA (F=0.2, p=0.79). For the midbrain the effect of HPRT status was borderline for dopamine (F=7.3, p=0.011) and not significant for DOPAC (F=2.2, p=0.14) or HVA (F=0.23, p=0.63). In the midbrain, the interaction between HPRT status and age was not significant for any metabolite. Single asterisks depict a post-hoc Tukey t-test result of 0.002<p<0.05, and double asterisks depict a result of p<0.002. Abbreviations: dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3MT).

Table 3

Brain dopamine metabolism

	Striatum			Midbrain		
	DOPAC/DA	HVA/DA	Σ M/DA	DOPAC/DA	HVA/DA	Σ M/DA
3-4 weeks						
HPRT+	0.24±0.01	0.16±0.00	0.50±	0.49±0.05	0.77±0.07	1.26±0.09
HPRT-	0.28±0.02	0.19±0.01	0.58±0.04	0.35±0.08	0.69±0.15	1.04±0.22
3-6 months						
HPRT+	0.13±0.01	0.10±0.00	0.30±0.02	0.58±0.07	0.84±0.10	1.44±0.16
HPRT-	0.23±0.03*	0.18±0.01**	0.51±0.04**	0.67±0.05	1.12±0.17	1.80±0.22
2 years						
HPRT+	0.22±0.01	0.09±0.00	0.39±0.02	0.65±0.06	0.76±0.03	1.41±0.09
HPRT-	0.29±0.01	0.14±0.00*	0.54±0.02*	0.96±0.10*	1.15±0.22	2.11±0.31

Results from Table 2 were converted to molar values, and ratios calculated individually for each animal. Results for each brain region were evaluated separately via 2-way ANOVA, with HPRT status and age as factors. To correct for multiple comparisons, a Bonferroni correction for multiple tests (n=24) was used to define p<0.002 as the conservative estimate for statistical significance. Results with 0.002<p<0.05 were considered borderline. For the striatum the effect of HPRT status was significant for DOPAC/DA (F=18.2, p<0.0002), HVA/DA (F=53.1, p<0.0001), 3MT/DA (F=37.9, p<0.0001), and Σ M/DA (F=36.8, p<0.0001). The interaction between HPRT and age was significant for HVA/DA (F=5.213, p=0.01), but not significant for DOPAC/DA (F=1.3, p=0.29), 3MT/DA (F=1.8, p=0.18), nor Σ M/DA (F=2.7, p=0.08). For the midbrain the effect of HPRT was borderline for HVA/DA (F=5.3, p=0.03) and Σ M/DA (F=5.3, p=0.03), and not significant for DOPAC/DA (F=3.7, p=0.07). The interaction between HPRT and age was borderline for DOPAC/DA (F=3.6, p=0.04), and not significant for HVA/DA (F=0.8, p=0.47) nor Σ M/DA (F=1.7, p=0.20). Single asterisks depict a post-hoc Tukey t-test result of 0.002<p<0.05, and double asterisks depict a result of p<0.002. Abbreviations are the same as those defined in Table 2, plus Σ M = algebraic sum of all dopamine metabolites (DOPAC+HVA+3MT).

Table 4

Brain norepinephrine & serotonin content

	Striatum		SHIAA	Midbrain	
	NE	5HT		NE	5HT
3-4 wks					
HPRT+	1.7±0.3	5.9±1.3	6.4±1.2	8.1±1.0	24.7±1.6
HPRT-	1.2±0.1*	6.8±0.7	7.4±0.7*	7.8±0.7	20.0±1.8
3-6 mos					
HPRT+	0.9±0.1	11.6±0.5	6.8±0.2	6.8±0.5	23.6±1.2
HPRT-	1.1±0.1	10.4±0.5	8.3±0.8	6.5±0.6	22.8±1.5
2 vis					
HPRT+	1.0±0.1	9.5±0.6	5.1±0.4	13.1±1.1	32.4±2.1
HPRT-	1.3±0.2	9.4±0.4	6.1±0.2	12.9±0.5	31.2±2.1

Results are expressed as ng/mg protein ±SEM from at least 5 mice per group. Results for each brain region were compared separately via 2-way ANOVA, with HPRT status and age as factors. To correct for multiple comparisons (n= 18) in post-hoc tests, a Bonferroni correction was used to define p<0.003 as the conservative estimate for statistical significance. Results with 0.003<p<0.05 were considered statistically borderline. For both the striatum and the midbrain, there were no statistically significant effects of HPRT status for any measure. The interaction between HPRT and age was significant only for norepinephrine (F=4.4, p=0.02). Asterisks depict a post-hoc Tukey t-test result of p<0.05. No comparison yielded p<0.003. Abbreviations: norepinephrine (NE), serotonin (5HT), 5-hydroxyindole acetic acid (SHIAA).

Table 5**Striatal Synapses**

	HPRT⁺	HPRT⁻	p-value
Mitochondria	27.25±0.77	27.40±4.82	0.98
Symmetric synapses	2.83±0.55	2.25±0.37	0.42
Asymmetric synapses	4.95±0.42	4.55±0.50	0.56
Ratio	0.64±0.07	0.67±0.20	0.92

Results show average values ±SEM per micrograph (n= 40 micrographs for each group). The p-value shows the result of Student's t-test.

Table 6

TH Immunogold stained striatal terminals

	HPRT⁺	HPRT⁻	p-value
Terminal size	2.20±0.09	2.08±0.02	0.25
Vesicle number	1.94±0.24	2.17±0.14	0.47
Vesicle density	1.76±0.27	2.24±0.19	0.22

Results show average values ±SEM from 60 immunostained terminals. The p-value shows the result of Student's t-test.