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CONSEQUENCES OF IMPAIRED PURINE RECYCLING IN DOPAMINERGIC NEURONS

J. C. LEWERS,[a] I. CEBALLOS-PICOT,[b] T. L. SHIRLEY,[a] L. MOCKEL,[b] K. EGAMI[a] AND H. A. JINNAH[a]*

[a]*Department of Neurology, Meyer Room 6-181, 600 North Wolfe Street, Johns Hopkins University, Baltimore, MD 21287, USA*

[b]*Department of Biology, Necker-Enfants Malades Hospital and René Descartes University, Paris 5, France*

Abstract—A unique sensitivity to specific biochemical processes is responsible for selective vulnerability of midbrain dopamine neurons in several diseases. Prior studies have shown these neurons are susceptible to energy failure and mitochondrial dysfunction, oxidative stress, and impaired disposal of misfolded proteins. These neurons also are especially vulnerable to the loss of purine recycling. In the brains of humans or mice with inherited defects of the purine recycling enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT), the most prominent defect is loss of basal ganglia dopamine. To investigate the nature of the relationship between HPRT deficiency and dopamine, the mouse MN9D dopaminergic neuronal cell line was used to prepare 10 sublines lacking HPRT. The mutant sublines grew more slowly than the parent line, but without morphological signs of impaired viability. As a group, the mutant sublines had significantly lower dopamine than the parent line. The loss of dopamine in the mutants did not reflect impaired energy status, as judged by ATP levels or vulnerability to inhibitors of energy production. Indeed, the mutant lines as a group appeared energetically more robust than the parent line. The loss of dopamine also was not accompanied by enhanced susceptibility to oxidative stress or proteasome inhibitors. Instead, the loss of dopamine reflected only one aspect of a broad change in the molecular phenotype of the cells affecting mRNAs encoding tyrosine hydroxylase, the dopamine transporter, the vesicular monoamine transporter, monoamine oxidase B, catechol-O-methyltransferase, and GTP-cyclohydrolase. These changes were selective for the dopamine phenotype, since multiple control mRNAs were normal. These studies suggest purine recycling is an intrinsic metabolic process of particular importance to the molecular phenotype of dopaminergic neurons independent of previously established mechanisms involving energy failure, oxidative stress, or proteasome dysfunction.

*Corresponding author. Tel: +1-410-614-6551; fax: +1-410-502-6737.
E-mail address: hjinnah@jhmi.edu (H. A. Jinnah).

Abbreviations: AEC, adenylate energy charge; ANOVA, analysis of variance; COMT, catechol-O-methyltransferase; DAT, dopamine transporter; DDC, dopa-decarboxylase; DOPAC, dihydroxyphenylacetic acid; FBS, fetal bovine serum; HPRT, hypoxanthine-guanine phosphoribosyltransferase; $HPRT^-$, hypoxanthine-guanine phosphoribosyltransferase deficient; HVA, homovanillic acid; MAO, monoamine oxidase; MTT, 3(4,5-dimethyltiazol-2-yl)2,5-diphenyltetrazolium bromide; qPCR, quantitative PCR; SDS, sodium dodecyl sulfate; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter; 3MT, 3-methoxytyramine; 6TG, 6-thioguanine; 8AG, 8-azaguanine.

Dopaminergic pathways projecting from the midbrain to the forebrain play a key role in regulating multiple functions including motor activity, motivation, mood, and cognition. Dysfunction of these pathways is linked with several neurological and psychiatric conditions, including Parkinson disease and schizophrenia. Because of the importance of these dopaminergic pathways in several diseases, a great deal of effort has been devoted to characterizing unique properties that affect their function and survival.

Dopaminergic neurons have an unusual vulnerability to mitochondrial dysfunction (Cookson, 2005; Moore et al., 2005; About-Sleiman et al., 2006), and they show particularly robust expression of genes encoding proteins involved in energy metabolism (Greene et al., 2004). Their high energy requirements may reflect the ability of dopamine to inhibit mitochondrial ATP production (Ben-Shachar et al., 1995; Gluck and Zeevalk, 2004). Dopaminergic neurons also are unusually susceptible to oxidative stress, as a result of generation of reactive oxidant molecules during the metabolism of dopamine together with high endogenous iron stores (Fahn and Cohen, 1992; Jenner, 2003). Finally, dopaminergic neurons are especially sensitive to the toxic effects of misfolded proteins or impaired proteasome function (Rideout et al., 2005; McNaught et al., 2006). These processes are not independent, as abnormalities of one affect the others.

Another important influence affecting dopamine neurons involves purine recycling. This influence was first revealed in studies of Lesch-Nyhan disease, a rare genetic disorder due to deficiency of the purine recycling enzyme, hypoxanthine-guanine phosphoribosyltransferase (HPRT). This enzyme recycles hypoxanthine and guanine into nucleotides (Jinnah and Friedmann, 2000). The most severe and consistent abnormality in the hypoxanthine-guanine phosphoribosyltransferase–deficient ($HPRT^-$) human brain is loss of basal ganglia dopamine (Lloyd et al., 1981; Ernst et al., 1996; Wong et al., 1996; Saito et al., 1999; Visser et al., 2000; Jinnah et al., 2006). Also reduced are dopamine metabolites, the enzymes responsible for dopamine synthesis, and dopamine transporters (DAT). The loss of dopamine also occurs in $HPRT^-$ knockout mice (Jinnah et al., 1994, 1999). In both $HPRT^-$ humans and mice, these abnormalities are neurochemically selective, with little change in most other neurotransmitters. The abnormalities are also neuroanatomically selective. Midbrain dopaminergic pathways are affected while other dopaminergic pathways are not. These findings imply midbrain dopamine neurons are unusually dependent upon HPRT-mediated purine recycling.

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The mechanisms responsible for dopamine loss in HPRT deficiency are unknown. A popular hypothesis to explain the relationship between dopamine and HPRT is that the absence of purine salvage results in failed development of nigrostriatal axonal arborizations or early degeneration of these axons or neurons (Lloyd et al., 1981; Ernst et al., 1996; Wong et al., 1996; Saito et al., 1999; Visser et al., 2000). This hypothesis has been called into question by recent anatomical studies of the HPRT$^-$ mouse brain, which revealed no anatomical defects despite significant loss of striatal dopamine (Egami et al., 2007). Alternative intrinsic biochemical mechanisms must be considered. Because ATP is a downstream product of HPRT, HPRT deficiency may result in energy limitation (McKeran, 1977; McCreanor and Harkness, 1995; Jinnah and Friedmann, 2000). HPRT deficiency also causes secondary metabolic changes that may increase oxidative stress (Visser et al., 2002; Smith and Jinnah, 2007). Because proteasomes are ATP-dependent and affected by oxidative stress, HPRT deficiency also may impair proteasomal function. To determine if dopaminergic neurons are susceptible to the loss of purine recycling via these mechanisms, several HPRT$^-$ subclones were prepared from the MN9D cell line. This line expresses many characteristics of immature dopamine neurons, including all enzymes responsible for the synthesis and catabolism of dopamine (Choi et al., 1991; Hermanson et al., 2003).

EXPERIMENTAL PROCEDURES

Establishment of MN9D subclones

The MN9D and N18TG2 cell lines were generously provided by Alfred Heller (Chicago, IL, USA) and cultured at 37 °C under an atmosphere of 5% CO_2 and 95% air in DMEM (Sigma, St. Louis, MO, USA) supplemented with 15% fetal bovine serum (FBS), 100 U/mL penicillin, and 50 mg/mL streptomycin. The MN9D cell line is a hybrid that was derived through the somatic fusion of primary midbrain dopaminergic neurons from an embryonic day 14 mouse with the mouse neuroblastoma line N18TG2 (Choi et al., 1991). All experiments conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

To establish HPRT$^-$ sublines, cells were plated at a density of 2×10^4 cells/well in 96-well plates in culture medium supplemented with 30 μM 6-thioguanine (6TG) or 130 μM 8-azaguanine (8AG) according to well-established methods (Nelson et al., 1975). The medium containing these agents was replaced every 3 days for 3–4 weeks until resistant colonies emerged. Colonies were isolated and expanded separately in the same culture medium without 6TG or 8AG, and all subsequent studies were performed in the absence of either agent.

HPRT enzyme activity

HPRT enzyme activity was assessed in live cells by adapting methods previously established for other cells (Wood et al., 1973). In brief, each cell line was grown to ~90% confluency in a T75 flask. Cells were dislodged by trypsinization and pelleted by centrifugation at $1000\times g$ for 5 min. The cells then were resuspended at a concentration of 5×10^6 in 500 μL in six replicates with fresh medium supplemented with 25 μM [^{14}C]-hypoxanthine (40 μCi/mL, Sigma). Cells were incubated for 60 min at 37 °C with continuous rotation to maintain suspension and then pelleted by centrifugation. The supernatant was discarded and the pellet rinsed by resuspension in cold Dulbecco's phosphate-buffered saline. After the rinse, the pelleted cells were disrupted by addition of 50 μL of cold 0.1 M perchloric acid and frozen at −80 °C. After thawing, insoluble materials were pelleted by centrifugation at $10{,}000\times g$ for 10 min, and 20 μL of the supernatant spotted onto microplates with diethylaminoethyl anion exchange paper (Millipore Multiscreen, Millipore, Bedford, MA, USA). The sample was allowed to adsorb for 60 min and each well washed once with 300 μL H_2O and three times with 50% methanol in H_2O by vacuum filtration. The filters were counted using a LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Fullerton, CA, USA). Protein pellets were dissolved in 2% sodium dodecyl sulfate (SDS) and quantified by the Pierce BCA protein assay (Rockford, IL, USA).

Cell growth

Population doubling times were estimated according to established procedures (Freshney, 2000). Briefly, cells were plated in 24-well plates at concentrations of 1×10^5, 3×10^5, and 1×10^4 cells/mL. Cell numbers were determined at regular intervals by direct counting with a hemocytometer after Trypan Blue staining. The numbers of cells were plotted against time in culture, and the population doubling time estimated during the exponential growth phase.

Cell morphology

For morphometric analyses, confluent cultures of the parent MN9D cells and each of the 10 HPRT$^-$ sublines were subcultured in separate six-well plates with a 1:10 split. Within 2–4 days while the cultures were still sparse, a technician blinded to HPRT status obtained 10 random phase-contrast digital (TIFF) photomicrographs from each cell line at 20× magnification. The TIFF images were imported into NeuroLucida (MicroBrightfield, Williston, VT, USA) and a technician blinded to cell line digitally traced at least 50 cells in which all elements could be clearly separated from neighboring elements using Wacom Intuos 2 digitizing tablet. The digital images were imported into NeuroExplorer (MicroBrightField) for determination of several morphometric measures including soma cross-sectional area, number of neurites, and lengths of neurites.

Cell viability

Viability after toxin exposure was evaluated by monitoring the conversion of 3(4,5-dimethyltiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) into a blue formazan dye (Hansen et al., 1989). The parent and each subline were plated in quadruplicate and assessed after timed exposure to varying doses of the toxin. Cells were exposed continuously for 3 days to rotenone, oligomycin, or 2-deoxyglucose. They were exposed for 36 h to epoxomicin, lactacystin, or MG115. They were exposed for 24 h to dopamine. For H_2O_2 and menadione, viability was assessed 24 h after a transient 30 min exposure. These exposure times and doses were based on prior studies of each toxin in other cell lines (Fenteany et al., 1994; Desagher et al., 1996; Ding and Keller, 2001; Kweon et al., 2004). To estimate viability after toxin exposure, 0.5 mg/mL MTT was added in fresh medium and the cells incubated at 37 °C for 3 h. Cells were lysed by adding 100 μL per well of 20% SDS, 50% N,N-dimethylformamide at pH 4.7. Samples were mixed gently on an orbital platform for 24 h prior to reading at 570 nm with a spectrophotometer.

Purines

Each cell line was plated separately in quadruplicate on six-well plates and allowed to grow to confluency. One day after reaching confluency, the medium was replaced with 1.5 mL DMEM con-