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Effects of decreased dopamine transporter levels on nigrostriatal neurons and paraquat/maneb toxicity in mice

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ABSTRACT

How genetic variations in the dopamine transporter (DAT) combined with exposure to environmental toxins modulate the risk of Parkinson's disease remains unclear. Using unbiased stereology in DAT knockdown mice (DAT-KD) and wild-type (WT) littermates, we found that decreased DAT caused a loss of tyrosine hydroxylase–positive (dopaminergic) neurons in subregions of the substantia nigra pars compacta at 3–4 days, 5 weeks, and 18 months of age. Both genotypes lost dopaminergic neurons with age and remaining neurons at 11 months were resilient to paraquat/maneb. In 5-week-old mice, the toxins decreased substantia nigra pars compacta dopaminergic neurons in both genotypes but less in DAT-KD. Regional analysis revealed striking differences in the subsets of neurons affected by low DAT, paraquat/maneb, and aging. In particular, we show that a potentially protective effect of low DAT against toxin exposure is not sufficient to reduce death of all nigrostriatal dopaminergic neurons. Thus, different regional vulnerability of nigrostriatal dopaminergic neurons may contribute to an increased risk of developing Parkinson's disease when multiple factors are combined.

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1. Introduction

The cause and pathologic mechanisms of Parkinson's disease (PD) remain poorly understood. Although the disease may originate outside the brain and affects multiple central and peripheral neurons (Del Tredici and Braak, 2012), a characteristic feature of PD is the progressive loss of nigrostriatal dopaminergic neurons, leading to the motor deficits that define PD. Dopamine is prone to oxidation into reactive molecules that can cause oxidative stress; accordingly, disruption of dopamine homeostasis may contribute to the vulnerability of dopaminergic neurons (Chesselet, 2003; Hastings et al., 1996). The cytoplasmic dopamine transporter (DAT), which recycles extracellular dopamine into dopamine terminals, contributes to the control of intracellular dopamine levels. Mice with overexpression of DAT in dopaminergic cells lose neurons in the substantia nigra pars compacta (SNc) (Masoud et al., 2015), supporting a role for DAT-mediated dopamine transport in modulating the survival of dopaminergic neurons.

Two epidemiological studies have concluded that *DAT/SLC6A3* variations significantly contribute to PD risk in subjects with occupational exposure to pesticides (Kelada et al., 2005; Ritz et al., 2009). These include genetic variants in the 5′ region of the gene together with 9 repeats of a 40-base pair variable number of tandem repeats polymorphism located in the 3′-untranslated region (3′UTR). Although the functional significance of these variants has been debated (Costa et al., 2011; Drgon et al., 2006; Kelada et al., 2005), a large brain imaging study (van de Giessen et al., 2009) revealed that variations in the *DAT/SLC6A3* gene that are associated with increased PD susceptibility, especially the haplotype T-A-9R for the single-nucleotide polymorphisms (SNPs) rs2652511 and rs2937639 and the variable number of tandem repeats, increase the expression of DAT in striatum. A similar conclusion was reached by Faraone et al. (2014) in a large meta-analysis of PET studies. Experimental studies suggest that the synergism between *DAT/SLC6A3* variants and pesticide exposure (Kelada et al., 2005; Ritz et al., 2009) could be in part due to an increased uptake of the herbicide paraquat, as mice with greatly reduced levels of DAT

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(DAT hypomorphs) were resistant to paraquat-induced loss of dopaminergic neurons (Rappold et al., 2011). However, little is known about the role of low DAT expression and environmental toxin exposure across the life span and about their effects on subpopulations of nigrostriatal dopaminergic neurons within the SNc.

In the present study, we examined the effects of low DAT levels on nigrostriatal dopaminergic neurons in young and old DAT knockdown mice (DAT-KD; Zhuang et al., 2001) or wild-type (WT) littermates. In addition, we examined whether low DAT levels modulate the effects of a single exposure to the herbicide paraquat and the fungicide maneb, at different ages. This toxin combination was chosen because it increases paraquat toxicity in animals (Kachroo et al., 2010; Thiruchelvam et al., 2003) and increases PD risk in humans with DAT variants (Ritz et al., 2009). We administered paraquat and maneb to either young adult or middle-aged male DAT-KD and WT mice and examined their effects on different subsets of nigrostriatal dopaminergic cells in the SNc, as well as on dopaminergic terminals in the striatum with histochemical and biochemical approaches.

2. Material and methods

2.1. Animals

Animal care was conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals, and procedures were approved by the Institutional Animal Care and Use Committee at the University of California Los Angeles. DAT-KD mice, in which the insertion of an extra 4-kb DNA sequence in the promoter region resulted in a reduction in DAT expression levels, were obtained from Dr Zhuang, U. Chicago (Zhuang et al., 2001). Heterozygous mice on a 129 Sv/J background were bred to generate WT, heterozygous, and homozygous mutant mice. Male DAT-KD mice at 4 different ages (pups at 3–4 days postnatal; young adult at 5 weeks; middle age adult at 11 months; and aged mice at 18 months) and corresponding littermates were used ($n = 5–10$ in all groups). The genotype of all mice was verified with polymerase chain reaction amplification analysis of tail DNA. Animals were maintained on a reverse 12/12-hour light/dark cycle with lights off at 10 AM. All behavioral testing was performed between 12 and 4 PM during the dark cycle under low light in an allocated room for behavioral experiments adjacent to the animal holding room. DAT-KD mice and WT littermates were tested in parallel in alternating sequence by an investigator unaware of the genotypes. Mice were housed socially (up to 5 mice per cage, conventional housing) with littermates on standard bedding (shredded wood) and material for nest-building (Nestlets, Ancare, NY, USA) was provided. Mice had ad libitum access to water and rodent diet (NIH-31 modified 7013, Harlan). Room temperature in the mouse holding room was 23°C ± 2°C, and relative humidity was about 60%; values were recorded during the daily animal check.

2.2. Toxin exposure

Mice of each litter were assigned to treatment groups to balance litters to avoid any possible litter effect ($n = 5–10$ per group). Paraquat (10 mg/kg) and maneb (30 mg/kg) were dissolved separately in sterile preservative-free saline and injected intraperitoneally (i.p.) in 2 successive injections (first paraquat followed by maneb) in DAT-KD and WT mice at 5 weeks of age. A separate group of DAT-KD and WT at 11 months of age was injected with the same protocol and doses. Respective control mice received 2 saline injections. Body weight and general health were recorded daily. Motoric behavior was assessed 7 days after toxin injection in 5-week-old mice. Mice were sacrificed 7 days (5-week-old mice) or 21 days (11-month-old mice) after toxin injection (Fig. 1A summary study plan).

2.3. Behavior

Spontaneous activity and motor coordination were measured with the cylinder and pole test, respectively, in 5-week-old mice as described (Fleming et al., 2004 and Supplemental information). Sensorimotor responses were measured in 18-month-old mice using the adhesive removal test according to Fleming et al. (2004) and Lam et al. (2011) with small modifications: the cutoff time was set to 2 minutes as the mice of this background (129 Sv/J) required more time to remove the sticker than C57BL/6-DBA2 mice used in the previous study. The intertrial interval was reduced from 2 to 1 minute as the longer latency to remove the sticker overall increased the interval between handling to place the sticker (see Supplemental information for further details).

2.4. Histology

Immunohistochemisty for tyrosine hydroxylase (TH) was performed as described (Fernagut et al., 2007 and Supplemental information). We used unbiased stereological analysis with the optical fractionator probe to estimate the number of TH-positive neurons and the total number of Nissl stained neurons in the SNc as previously described (Fernagut et al., 2007). Briefly, Stereo Investigator software (Microbrightfield, Williston, VT, USA) coupled to a Leica DM-LB microscope with a Ludl XYZ motorized stage and z-axis microcator (MT12, Hendenheim, Traunreut, Germany) was used for stereological sampling. The SNc was delineated at 5X magnification, and TH-positive neurons as well as total neuron counts (TH-positive plus TH-negative/Nissl positive neurons) were performed at 40X magnification. For regional analysis, the SNc was subdivided into medial, lateral, dorsal, and ventral regions as described in Fernagut et al. (2007) and illustrated in Fig. 1B.

2.4.1. Quantification of iron-containing cells in the substantia nigra

Sections at the level of the SNc were processed for Perls' Prussian blue reaction and iron positive cells were counted (Morris et al., 1992 and Supplemental information).

2.4.2. Quantification of TH, DAT, ionized calcium-binding adapter molecule 1 (IBA-1), and alpha-synuclein protein

Striatal sections (40 μm; rostral, medial, and caudal striatum) and sections of the SNc (for alpha-synuclein staining) were processed as described (Supplemental information) with the following primary antibodies: TH, Millipore; DAT, Millipore; anti-IBA-1, Wako, 019-19,741; alpha-synuclein, BD Biosciences, and images were analyzed with a microarray scanner as described (Richter et al., 2014 and Supplemental information).

2.4.3. Quantification of activated versus resting IBA-1-positive microglia in the SN

Immunostaining for IBA-1 was performed as described previously, and analyzed based on differences in diameters between resting and activated microglia (Watson et al., 2012 and supplemental information).

2.4.4. Analysis of aggregated alpha-synuclein

Immunostaining for alpha-synuclein was performed as described previously (Fernagut et al., 2007; Richter et al., 2014) and sections examined for the presence of aggregates; no quantification was performed as no aggregates were detected in this study (see Supplemental information for details).

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