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Dopamine and its metabolites in cathepsin D heterozygous mice before and after MPTP administration

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HIGHLIGHTS

- CD+/− mice have higher levels of striatal dopamine and metabolites than CD+/+ mice.
- MPTP treatment abolished the observed differences in striatal monoamines.
- There were no discernable behavioral differences between CD+/+ and CD+/− mice.

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ABSTRACT

Cathepsin D (CD) is a lysosomal aspartyl protease which plays an important role in α-synuclein degradation, and neuronal survival. CD knockout mice die by post-natal day 25 ± 1 due to intestinal necrosis. We analyzed the young adult male heterozygous mice, and found no behavior abnormalities in the heterozygous mice compared to wildtype littermates. LC3-II, p62, and α-synuclein levels are similar, while LAMP1 is higher in the striatum in CD heterozygous compared to wildtype mice. Interestingly, we found that dopamine and metabolites in the striatum and olfactory bulbs are at higher levels than wildtype littermates, while the DOPAC/DA and HVA/DA ratio remain similar between wildtype and CD heterozygous mice. In response to sub-chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration, dopamine, DOPAC, and HVA are depleted to similar levels in the striatum in both heterozygous and wildtype mice. Dopamine synthesizing enzyme tyrosine hydroxylase, metabolic enzyme monoamine oxidase, and catechol-O-methyltransferase (COMT) levels are similar in the striatum in wildtype and heterozygous mice. These studies provide valuable information regarding how lysosomal function may contribute to neurochemical homeostasis in animal models.

1. Introduction

The autophagy lysosome pathway has come to the forefront of research in recent years as a signaling pathway which plays an important role in protein and organelle turnover. Perturbations in autophagic function are thought to underlie many disease phenotypes, particularly neurodegenerative disease. Cathepsin D (CD) is a lysosomal aspartyl protease which plays an integral role in maintaining cellular and lysosomal homeostasis. Homozygous CD knockout mice die by post-natal day 25 ± 1 from pathological events which take place in various tissues including intestinal necrosis thromboembolia, lymphopenia and neurodegeneration [15,9]. For this reason, we have focused our studies on comparisons between the CD wild type and heterozygous mice. CD

Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP+, 1-methyl-4-phenylpyridinium; 3-MT, 3-methoxytyramine; MHPG, 3-methoxy-4-hydroxyphenylglycol; DOPAC, 3,4-dihydroxymethylphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; AAV, adeno-associated virus; COMT, catechol-O-methyltransferase; CD, cathepsin D; HVA, homovanillic acid; MAO, monoamine oxidase; PD, Parkinson's disease; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; TFEB, transcription factor EB.

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heterozygotes are viable and fertile, exhibiting no gross physiological defects compared to wild type mice.

Our lab and others have shown that CD is capable of degrading the α-synuclein protein as well as reducing its aggregation and toxicity in both in vitro and in vivo models [17,14]. Aberrant accumulation of α-synuclein is thought to underlie the pathogenesis of several synucleinopathies, the most common of which is Parkinson's disease (PD). In sporadic PD, the α-synuclein protein is thought to be at the root of disease pathogenesis. Through mechanisms which are not yet fully understood, the dopaminergic neurons in the substantia nigra pars compacta (SNpc) exhibit protein aggregates composed primary of α-synuclein [19] and eventually, these neurons degenerate and die.

Because CD is capable of decreasing α-synuclein toxicity, we also wanted to investigate whether CD haploinsufficiency would result in increased pathology in response to PD models which are dependent on the presence of α-synuclein. One such model employs the use of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) compound. MPTP is a PD mimetic which selectively targets the nigrostriatal dopaminergic pathway, causing the death of SN neurons. When given systemically via i.p. injection, MPTP crosses the blood brain barrier within minutes where it is rapidly metabolized by endogenous monoamine oxidase (MAO) to 1-methyl-4-phenylpyridinium (MPP+), the active metabolite [2]. Due to its size and charge, MPP+ can selectively cross into dopaminergic neurons via the dopamine transporter (DAT) [12], where it causes toxicity by binding to mitochondrial complex I, causing increased reactive oxygen species (ROS) and depletion of ATP levels [13]. In lieu of entering the mitochondria, MPP+ can also interact with vesicular monoamine transporters [11], translocate into synaptosomal vesicles [11], or remain in the cytosol where it can aberrantly interact with various cytosolic enzymes [8]. The effects of MPTP toxicity have been shown to be dependent on the presence of α-synuclein, as α-synuclein null mice were found to be highly resistant to MPTP toxicity [4].

We wanted to test whether CD haploinsufficiency results in increased toxicity to MPTP administration compared to wild type controls. To this end, we have employed the use of a sub-chronic MPTP regiment after which striatal biogenic amines were analyzed to assess lesion size in intoxicated animals. We have also used a battery of behavioral assays to determine whether CD+/+ and CD+/− mice exhibit any overt cognitive differences.

2. Materials and methods

2.1. Mice

All animals used in this study were male on a C57BL/6 background and between the ages of 2 and 6 months. Breeding was from both CD+/+ × CD+/− and CD+/− × CD+/− parents. Genotyping was performed using the following primers to distinguish the wildtype allele and the knockout allele: CD WT 5′: AGA CTA ACA GGC CTG TTC CC; CD WT 3′: TCA GCT GTA GTT GCT CAC ATG; CD KO 5′:CTC GTC CTG CAG TTC ATT CA; CD KO 3′: CCC CTC AGC TGT AGT TGC TC. For striatal monoamine analysis, $n = 10$ MPTP-treated mice per genotype were used, and $n = 8$ sham mice per genotype were used. For the striatal monoamine studies which combined sham wild type and heterozygous animals from several studies, a total of $n = 27$ mice per genotype were used. For monoamine studies in the olfactory bulb, $n = 8$ sham mice per genotype were used. For the Morris water maze testing, $n = 20$ mice per genotype were used. We used $n = 5$ mice per genotype in the open field test, and $n = 8$ mice per genotype for the rotarod. Behavioral methods can be found in the Supplemental materials. All mouse experiments were done in compliance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee guidelines.

2.2. MPTP regimen

MPTP-HCl (Sigma) was diluted in saline and a total volume of ∼200 μl was administered per animal per injection. Mice were injected once per day for five consecutive days with 30 mg/kg MPTP-HCl [20]. Control animals were injected with saline on the same schedule as MPTP-injected animals. With this chronic injection schedule, it has been determined that the nigrostriatal lesion is stable 21 d after the last injection [20], at which time animals were sacrificed via pentobarbital overdose. One half of the striatum was removed and flash frozen in isopentane and dry ice. This tissue was sent to the Vanderbilt Neurochemistry Core for HPLC analysis of striatal monoamine content. The other half of the striatum was flash frozen and saved for Western blot analysis. In some studies, olfactory bulb tissue was collected from mice and processed for monoamine analysis.

2.3. HPLC analysis

Striatal and olfactory bulb biogenic amine analysis was performed at the Vanderbilt Neurochemistry Core facility. Tissue Extraction. The brain sections are homogenized, using a tissue dismembrator, in 100–750 μl of 0.1 M TCA, which contains 10^{-2} M sodium acetate, 10^{-4} M EDTA, 5 ng/ml isoproterenol (as internal standard) and 10.5% methanol (pH 3.8). Samples are spun in a microcentrifuge at 10,000 × g for 20 min. The supernatant is removed and stored at −80° [3]. The pellet is saved for protein analysis. Supernatant is then thawed and spun for 20 min. Samples of the supernatant are then analyzed for biogenic monoamines. Biogenic amines: Biogenic amines are determined by a specific HPLC assay utilizing an Antec Decade II (oxidation: 0.4) electrochemical detector operated at 33 °C. Twenty μl samples of the supernatant are injected using a Water 717+ autosampler onto a Phenomenex Kintex (2.6u, 100A) C18 HPLC column (100 mm × 4.60 mm). Biogenic amines are eluted with a mobile phase consisting of 89.5% 0.1 M TCA, 10^{-2} M sodium acetate, 10^{-4} M EDTA and 10.5% methanol (pH 3.8). Solvent is delivered at 0.6 ml/min using a Waters 515 HPLC pump. Using this HPLC solvent the following biogenic amines elute in the following order: noradrenaline, MHPG, adrenaline, DOPAC, dopamine, 5-HIAA, HVA, 5-HT, and 3-MT [10]. HPLC control and data acquisition are managed by Empower software.

2.4. Western blot analysis

Frozen tissue for Western blot analysis was transferred to a glass tube and homogenized by hand with a plastic pestle in RIPA lysis buffer containing the following: 50 mM Tris pH 7.8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS. Protease (Roche) and phosphatase (Sigma) cocktail inhibitors were added to fresh samples. Tissues were incubated on ice for 30 min prior to centrifugation at top speed (∼16,700 × g) in a microcentrifuge at 4 °C. The supernatant was kept for BCA protein analysis and subsequent separation by SDS PAGE. The following primary antibodies were used for protein detection: rabbit anti-TH (Pelfreeze), goat anti-MAOB (C17, Santa Cruz), mouse anti-COMT (BD Biosciences), rabbit anti-LC3 (Sigma), mouse anti-p62 (Abnova), rat anti-LAMP1 (1D4B, Developmental Studies Hybridoma Bank), and rabbit anti-α-synuclein (Santa Cruz). The following secondary antibodies were obtained from Santa Cruz: goat anti-rabbit, goat anti-mouse, donkey anti-rat and donkey anti-goat. Image J was used to quantify the Western band intensities.