ELSEVIER

Journal of Neuroscience Methods 159 (2007) 291–299

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

Quantification of MPTP-induced dopaminergic neurodegeneration in the mouse substantia nigra by laser capture microdissection

Diane Stephenson *, Andres Ramirez, Jill Long, Nestor Barrezueta, Eva Hajos-Korcsok, Curt Matherne, Dan Gallagher, Anne Ryan, Ricardo Ochoa, Frank Menniti, James Yan

Pfizer Global Research and Development, Worldwide Safety Sciences and CNS Discovery, MS8274-1348, Eastern Point Road, Groton, CT 06340, United States

Received 26 September 2005; received in revised form 3 July 2006; accepted 25 July 2006

Abstract

The neurotoxin MPTP is widely used to cause damage to the dopaminergic system in rodents and non-human primates to model various aspects of Parkinson's disease. In mice, depletion of striatal dopamine is the commonly used endpoint to assess neuronal damage. However, it has proved technically challenging to quantify dopaminergic cell bodies as an index of neuronal integrity. To meet this challenge, we applied laser pressure catapult microdissection (LCM) of the substantia nigra in combination with quantitative Western blot to provide an index of dopamine neurodegeneration in mice treated with MPTP. Seven days following initiation of MPTP treatment, striatal dopamine depletion was maximal and there was histological evidence of neuronal degeneration in the substantia nigra. To index the integrity of dopamine cell bodies, tyrosine hydroxylase (TH) and β-actin were quantified by Western blot in LCM extracts. In untreated mice, TH was detected in LCM extracts of substantia nigra but was undetectable in equivalently sized extracts of cortex from the same animals. In MPTP-treated mice, there was a significant 70% reduction in TH relative to β-actin in LCM extracts as compared to vehicle-injected controls. This reduction corresponded to decreases in striatal dopamine and loss of immunocytochemically detected TH but not β-actin in the substantia nigra (SN). Thus, this method provides a quantitative means to measure dopamine neuron toxicity in the substantia nigra and, as such has potential application in evaluating regimens that may be neuroprotective or neurorestorative for dopaminergic neurons.

Keywords: Laser capture microdissection; MPTP; Parkinson's disease; Tyrosine hydroxylase; Quantitative Western blot; Dopamine; Immunohistochemistry; β-Actin; Substantia nigra

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by slowness of movement, resting tremor, rigidity and disturbances in balance. These clinical symptoms are caused primarily by the progressive loss of the dopaminergic neurons of the substantia nigra that project rostrally to the striatum. Current therapies that restore dopamine receptor activation in the striatum can alleviate Parkinsonian symptoms, particularly at early stages of clinical manifestation. However, the pathological loss of dopamine neurons is relentless and pharmacological symptom control becomes essentially ineffective as the disease progresses. Thus, there is a clear need for therapies that will slow or stop the underlying progression of neurodegeneration.

The search for disease modifying therapies for Parkinson's disease has been facilitated by the availability of a number of different types of animal models that recapitulate various aspects of the disease (Betarbet et al., 2002; Dawson et al., 2002; Emborg, 2004). One of the most widely used is a model of dopamine depletion in mice caused by the neurotoxin MPTP (Giovanni et al., 1994). MPTP was first identified as a specific dopamine neurotoxin when it was found to be the active agent in producing severe Parkinsonian symptoms in a population of human drug abusers. Subsequent studies in mice and primates indicate that MPTP closely models the oxidative insult and pathophysiology thought to occur in Parkinson's disease (Przedborski and Vila, 2001). Thus, mechanisms that prevent MPTP-induced dopamine

* Corresponding author. Tel.: +1 860 686 6919; fax: +1 860 441 1197.
E-mail address: Diane.t.Stephenson@pfizer.com (D. Stephenson).

0165-0270/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2006.07.027

neuron toxicity represent promising targets for the development of disease modifying therapies for Parkinson's disease.

MPTP has been a commonly used model for more than 20 years. However, the endpoints used to investigate neuroprotective agents vary widely and few studies have employed multiple endpoints. Behavioral endpoints are of great value and utility in primates. In mice, neurobehavioral deficits are not consistently observed and challenges such as reversibility of the deficit make it an unreliable endpoint on its own (Emborg, 2004; Sedelis et al., 2001). The most commonly used endpoint for determination of MPTP induced neurotoxicity of the nigrostriatal pathway is measurement of striatal dopamine and its metabolites. This method is relatively straightforward, is sensitive enough to detect small changes and is specific for detecting changes in this selectively vulnerable neurotransmitter pathway. However, relying on striatal DA as a single endpoint can be problematic. Evaluation of neuroprotective agents in the MPTP model suggests that putative therapeutic compounds can elicit differential effects on the striatum as compared to the substantia nigra (SN) (e.g., Aguirre et al., 2005). Furthermore, investigation of genetically modified mice for their susceptibility to MPTP shows that in some mouse models (e.g. COX-2 and iNOS knockout mice) there is a protective effect on cell bodies in the substantia nigra with no effect observed on striatal dopamine (Feng et al., 2002; Liberatore et al., 1999). Thus, careful evaluation of both the striatum and the SN are important to investigate with any potential neuroprotective agent.

There have been a number of publications reporting protective effects of various treatments on MPTP-induced striatal dopamine loss in mice. However, far fewer have investigated the neuroprotective effects of the treatment on dopamine neuron cell bodies. This is due primarily to technical difficulties in reliably quantifying loss of midbrain neurons caused by MPTP, let alone a neuroprotective response. Assessing neuronal integrity is crucial since truly effective therapies must prevent the loss of dopamine neurons and not just dopaminergic nerve terminals. Several factors contribute to the technical challenges in measuring dopamine neuron loss in the substantia nigra in MPTP-treated mice. First, as in Parkinson's disease, MPTP causes loss of dopaminergic neurons in the substantia nigra pars compacta (SNPc) but largely spares the adjacent ventral tegmental area (Fearnley and Lees, 1991; Seniuk et al., 1990). Complicating matters further, the density of dopamine cell bodies is heterogeneous across the rostro-caudal extent of the pars compacta (Waldeier et al., 2000). These factors necessitate examination of the entire extent of the SNPc for accurate quantitative analysis. Endpoints based on pathologic assessment include semiquantitative image analysis as well as unbiased stereology to quantify the number of remaining TH positive neurons in the ventral midbrain. Because these methods rely on manually counting of TH immunoreactive neurons in histological sections, challenges such as staining variabilities between animals, anatomic heterogeneity of the substantia nigra, inclusiveness of sections throughout the SN, criteria for counting a cell as positive or sampling errors all pose technical hurdles and contribute to variability. In theory, biochemical and molecular techniques offer more quantitative approaches to measuring midbrain neurodegeneration. However, these require precise and consistent isolation of the ventral midbrain region for analyses. This is particularly challenging to apply to brain subregions in small rodent species such as the mouse.

Laser capture microdissection (LCM) has been successfully applied to studies of specific populations of neurons in the CNS (Bohm et al., 2005; Kamme et al., 2003; Sanna et al., 2005; Vincent et al., 2002). Changes in gene expression in the midbrain and in SN neurons captured from brains of MPTP treated animals have been reported using RT-PCR (Chung et al., 2005; Kuhn et al., 2003; Xu et al., 2005). However, it is unknown how changes in gene transcripts translate to changes in protein expression and changes in gene expression have not yet been correlated to functional or pathologic outcome.

In the present study, we measured the effects of MPTP induced neurotoxicity on TH and β-actin expression in substantia nigra extracts isolated by LCM for quantitative analysis of dopaminergic neurodegeneration in mice.

2. Materials and methods

2.1. Animals

Male C57Bl/6 mice weighing 25 ± 1 g were obtained from Charles River Laboratories (Raleigh, NC). Animals were housed 5–6/cage with *ad libitum* access to food and water and maintained at a constant temperature (21–23 °C) and humidity (45–50%) with lights on 07:00–19:00 h. All experimental protocols were conducted in compliance with The Institutional Animal Care and Use Committee.

A total number of 51 mice (9 Naïve, 12 vehicle and 30 MPTP) were evaluated in the present study.

2.2. MPTP administration

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was purchased from Aldrich Sigma (St. Louis, MO). Animals were dosed with either vehicle (0.9% saline) or MPTP (HCl salt dissolved in vehicle). MPTP was administered by intraperitoneal (i.p.) injection at a dose of 30 mg/kg per day for 3 days. MPTP handling and safety measures were in accordance with published guidelines (Przedborski and Vila, 2001). Mice were euthanized at various timepoints after initiation of MPTP dosing for neurochemical, biochemical and histopathologic analyses.

2.3. Dissections/sample collection

Mice were euthanized by rapid decapitation and brains were removed. For animals used for neurochemistry only (MPTP time course study, see below), striatum and frontal cortex were microdissected manually and frozen on dry ice for processing and analysis. Brainstems were fixed and processed into paraffin blocks for histopathology. For the animals used for LCM, the forebrain and midbrains were isolated separately by making a single coronal cut at Bregma −1.0 mm. (Paxinos and Franklin, 2001). The striatum was microdissected manually from the forebrain and frozen on dry ice for neurochemistry.