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Short Communication

Chronic restraint stress triggers dopaminergic and noradrenergic neurodegeneration: Possible role of chronic stress in the onset of Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease characterized by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and, to a lesser extent, in the noradrenergic neurons of the locus coeruleus (LC). Most cases of PD are idiopathic and sporadic and are believed to be the result of both environmental and genetic factors. Here, to the best of our knowledge, we report the first evidence that chronic restraint stress (8 h/day, 5 days/week) substantially reduces nigral DA and LC noradrenergic neuronal cell numbers in rats. Loss of DA neurons in the SNpc was evident after 2 weeks of stress and progressed in a time-dependent manner, reaching up to 61% at 16 weeks. This reduction was accompanied by robust microglial activation and oxidative stress and was marked by nitrotyrosine in the SNpc and LC of the midbrain. These results indicate that chronic stress triggers DA and noradrenergic neurodegeneration by increasing oxidative stress, and that activated microglia in the substantia nigra and LC may play an important role in modulating the neurotoxic effects of oxidative stress. Taken together, these data suggest that exposure to chronic stress triggers DA and noradrenergic neurodegeneration, which is a cause of PD.

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1. Introduction

Parkinson's disease (PD) is a late-onset neurodegenerative disease that is characterized by slow movements, tremor, stiffness, and postural instability (Thomas and Beal, 2007) and generally occurs in 1% of the population over the age of 60 years (de Lau and Breteler, 2006). The neuropathological hallmarks of PD include progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). Causes of PD include viral infection, head trauma, herbicides, pesticides, heavy metal exposure, and genetic mutations (Davie, 2008). In particular, mutations in specific genes, such as α-synuclein (SNCA), parkin (PRKN), PTEN-induced putative kinase 1 (PINK1), DJ-1, and ATP13A2, are known to cause PD. However, genetic PD is estimated to account for approximately 5% of the PD cases, and at least 90% of the PD cases are sporadic and have unknown etiology.

Chronic stress has previously been shown to contribute to neuronal loss in the hippocampus (Uno et al., 1989), and experimental evidence suggests that it also contributes to the damage of catecholaminergic neurons. In particular, clinical studies demonstrated that stressful life events may induce PD development. Among these studies, a study involving the prisoners of war reportedly had considerably higher incidence rates of PD development after 35 years of their release (Gibberd and Simmonds, 1980). This finding is consistent with the experimental data indicating that chronic stress decreases dopamine levels in the striatum (STR), nucleus accumbens, and the frontal cortex (Rasheed et al., 2010) as well as reduced the level of dopamine metabolites in the prefrontal cortex (Mizoguchi et al., 2000) and accelerated 6-hydroxy dopamine (OHDA)- or lipopolysaccharide (LPS)-induced DA neurodegeneration in the animal models of PD (Smith et al., 2008; de Pablos et al., 2014). These experimental data suggest that chronic stress preferentially exerts neurotoxic effects on the DA system and accelerates DA cell damage in the brain. However, in those studies, chronic stress is considered to promote the loss of DA neurons,

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which is the primarily triggered by extrinsic neurotoxins; it remains unknown whether prolonged intrinsic stress exposures trigger the loss of DA neurons.

To the best of our knowledge, our study is the first to generate evidence using an *in vivo* model that acute stress can induce the activation of microglial cells in the brain (Sugama et al., 2007). Moreover, similar observations have been made following chronic stress (Tynan et al., 2010; Hinwood et al., 2012) and sub-chronic stress (Wohleb et al., 2012). Microglia participate in neuroinflammation and can produce reactive oxygen species (ROS) that may contribute to neurodegeneration in PD (McGeer et al., 1988). Thus, in this study, we tested whether chronic stress contributes to the degeneration of catecholaminergic neurons by activating microglial cells. In the present experiments, we examined the effects of prolonged chronic stress on the number of tyrosine hydroxylase (TH) neurons, microglial activation, and oxidative stress in the SNpc and locus coeruleus (LC) of rats.

2. Methods

2.1. Animals and treatments

All procedures were approved by the Institutional Animal Care and Use Committee of Nippon Medical School and were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals; animal suffering and sacrifice were minimized. Male Wistar rats (280–320 g, 8 weeks old) were purchased from Japan Laboratory Animals, Inc. (Tokyo, Japan). All animals were housed in a room maintained at 20–22 °C with a 12-h light/12-h dark cycle and food and water were provided *ad libitum*. Repeated restraint stress (RS) experiments were performed as previously described (Watanabe et al., 1992) with minor modifications. Briefly, RS was performed by tightly wrapping rats in the wire net without restricting respiration for 8 h per day (from 8 am to 4 pm) for 5 days per week. The procedure was repeated according to the experimental schedules of 1 day and of 2, 4, 8, and 16 weeks of RS. Control rats were not subjected to restrictions of food and water, and their weights increased by 29.8 ± 1.8 g per week, whereas RS-treated rat weights only increased by 9.0 ± 2.0 g per week. However, the weights of unstressed (RS-untreated) rats, which were deprived of food and water for 8 h per day, did not differ from those of the control rats. Tissues were immediately collected after the last session of stress; unstressed control animals were age matched.

2.2. Immunohistochemistry and immunofluorescence

Immunohistochemistry analyses were performed as previously described with minor modifications (Sugama et al., 2013) using anti-TH rabbit (1:10,000; Calbiochem, SD, USA) and anti-OX-42 mouse (1:500; Serotec, Oxford, UK) antibodies. Immunofluorescent primary antibodies included anti-nitrotyrosine (NT) and polyclonal rabbit antibody (1:200; Millipore, Temecula, USA) and OX-42 (1:200) and were observed using laser scanning confocal microscopy (LSM 710; Carl Zeiss, Germany). To observe Nissl bodies, 40-μm brain sections were mounted on glass slides and were stained with 0.75% cresyl violet and dehydrated using graded alcohols; coverslips were applied using Multi Mount (Matsunami Glass Ind., Ltd., Japan).

2.3. Fluoro-Jade B staining

Fluoro-Jade B (FJB; Millipore) staining was performed according to the manufacturer's instructions. After staining reactions, sections were mounted on gelatin-coated slides and were dehydrated; coverslips were applied using Vectashield D (Vector Lab, Inc., Burlingame, USA) prior to observation using laser scanning confocal microscopy (LSM 710; Carl Zeiss, Germany).

2.4. In situ hybridization

In situ hybridization was performed as previously described with minor modifications (Sugama et al., 2000). Tissues were pre-hybridized in hybridization solution containing 50% formamide, 5% dextran sulfate, 1 × Denhardt's solution, 0.25% SDS, 200-μg/ml *Escherichia coli* transfer RNA, 600-mM NaCl, and 1-mM EDTA. Digoxigenin (DIG)-labeled sense and anti-sense RNA probes were synthesized from the template, and mouse TH cDNA was sub-cloned into pGEM T-Easy vectors (Promega, Madison, USA) using DIG RNA labeling kits (Roche Diagnostics, Manheim, Germany). SP6 and T7 RNA polymerases were used for anti-sense and sense probe labeling with the restriction enzyme-digested plasmids SphI and SpeI, respectively.

2.5. Cell counting of substantia nigra

The total number of TH-immunoreactive (ir) cell bodies in SNpc neurons was counted as previously described (Sugama et al., 2003). Briefly, digital images of TH-ir SNpc neurons were obtained at a magnification of 50× on an Olympus microscope fitted with a video camera (Olympus, Tokyo, Japan). Counting frames were generated using MCID image analysis software (Imaging Research Inc., Ontario, Canada) and were systemically scanned over the outlined SNpc using a motorized stage. Neurons were counted as they appeared within the counting frame. Average neuron densities were calculated by dividing the number of neuron profiles by the calculated volume. The total number of neurons was estimated as the product of neuron densities and SNpc volumes as previously described (Coggeshall, 1992; Gundersen, 1992; Sugama et al., 2003).

2.6. Quantification of immunoreactivity and in situ hybridization

To quantify immunohistochemical and *in situ* hybridization data, mean optical densities in the target area were measured from each section using image analysis software (WinROOF; Sugama et al., 2013).

2.7. Western blot analysis

Western blot analysis was performed as described in our previous report (Sugama et al., 2000) using anti-TH rabbit polyclonal (1:20,000; Calbiochem, SD, USA), anti-inducible nitric oxide synthase (iNOS) rabbit polyclonal (1:2000; Chemicon, CA, USA), and anti-GAPDH rabbit polyclonal (1:4000; Santa Cruz Biotechnology, Inc., Santa Cruz, USA) primary antibodies. Autoradiograms were scanned using the Imaging Densitometer GS-800 (Bio-Rad, Hercules, USA) and were analyzed using Quantity One 4.0 software (Bio-Rad), which calculates relative quantities associated with the background signals of the corresponding lane.

2.8. High-performance liquid chromatography (HPLC)

Rat STR samples were dissected on ice; they were homogenized in ice-cold 5% perchloroacetic acid and centrifuged. Soluble fractions were neutralized with 1 M potassium carbonate, and norepinephrine (NE), dopamine (DA), and 5-hydroxytryptophan (5-HT) concentrations were determined using high-performance liquid chromatography with electrochemical detection (EDT-300; Eicom, Kyoto, Japan), and were quantified using a PowerChrom (AD Instruments, Australia) with external standards (Sigma).

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