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Progressive Parkinsonism by acute dysfunction of excitatory amino acid transporters in the rat substantia nigra

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ABSTRACT

Parkinson's disease (PD) is characterized by the progressive degeneration of substantia nigra (SN) dopamine neurons, involving a multifactorial cascade of pathogenic events. Here we explored the hypothesis that dysfunction of excitatory amino acid transporters (EAATs) might be involved. Acutely-induced dysfunction of EAATs in the rat SN, by single unilateral injection of their substrate inhibitor L-trans-pyrrolidine-2,4-dicarboxylate (PDC), triggers a neurodegenerative process mimicking several PD features. Dopamine neurons are selectively affected, consistent with their sustained excitation by PDC measured by slice electrophysiology. The antioxidant N-acetylcysteine and the NMDA receptor antagonists ifenprodil and memantine provide neuroprotection. Besides oxidative stress and NMDA receptor-mediated excitotoxicity, glutathione depletion and neuroinflammation characterize the primary insult. Most interestingly, the degeneration progresses overtime with unilateral to bilateral and caudo-rostral evolution. Transient adaptive changes in dopamine function markers in SN and striatum accompany cell loss and axonal dystrophy, respectively. Motor deficits appear when neuron loss exceeds 50% in the most affected SN and striatal dopamine tone is dramatically reduced. These findings outline a functional link between EAAT dysfunction and several PD pathogenic mechanisms/pathological hallmarks, and provide a novel acutely-triggered model of progressive Parkinsonism.

Introduction

Parkinson's disease (PD) is a neurodegenerative movement disorder in which cell death mainly affects *substantia nigra* (SN) dopamine (DA) neurons. Neuronal loss is progressive and motor symptoms appear when 50–60% of nigral DA neurons are lost. A number of mechanisms have been implicated in such cell death, including oxidative stress, mitochondrial dysfunction, protein misfolding/aggregation, autophagy, neuroinflammation and excitotoxicity (Ahmed et al., 2012; Cheung and Ip, 2009; Olanow, 2007; Schapira and Jenner, 2011; Yacoubian and Standaert, 2009). Depletion of glutathione (GSH), the main brain antioxidant, is considered as an early and key component of the pathological process. Reduced levels of GSH are measured specifically in the SN of PD patients and incidental Lewy body disease (presumably representing presymptomatic PD), but not in other pathologies affecting DA neurons (Jenner et al., 1992; Pearce et al., 1997; Perry et al., 1982; Sian et al., 1994a, 1994b). Down-regulation of GSH synthesis *in vivo* in rodents impacts mitochondrial complex-I activity and results in nigral DA neuron degeneration associated with protein aggregation (Chinta et al., 2007; Garrido et al., 2011). Dysfunction of excitatory amino acid transporters (EAATs) might link several of these PD pathogenic mechanisms. DA neurons express the neuronal transporter EAAC1 (EAAT3) at high levels (Plaitakis and Shashidharan, 2000; Shashidharan et al., 1997), and pathological conditions leading to excessive neuronal depolarization can affect or even reverse EAAT function by altering the transport driving force (Danbolt, 2001): for example, overactive glutamatergic inputs from the subthalamic nucleus (STN) to DA neurons (Rodriguez et al., 1998), or ATP depletion and subsequent impairment of the Na^+/K^+-ATPase resulting from mitochondrial dysfunction. Neuroinflammation can also affect glial EAAT function (McNaught and Jenner, 2000; Tilleux and Hermans, 2007). Besides clearing extracellular glutamate, EAATs provide substrates for GSH production since they uptake not only glutamate but also cyst(e)ine (Hayes et al., 2005). EAAT dysfunction might then, in turn, sustain excitotoxicity and oxidative stress. Accordingly, oxidative stress on EAAC1 has been involved in MPTP-induced GSH depletion (Aoyama et al., 2008), and EAAC1$^{-/-}$ mice show an age-dependent loss of nigral DA neurons that is prevented by N-acetylcysteine (Berman et al., 2011). Interestingly, the EAAT substrate inhibitor L-trans-pyrrolidine-2,4-dicarboxylate (PDC) triggers preferential DA neuron death *in vitro* through a mechanism involving oxidative stress and excitotoxicity, and can induce early loss of DA neurons when

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injected in the SN (Nafia et al., 2008), whereas it does not induce neuronal damage in other brain regions (Massieu et al., 1995; Montiel et al., 2005).

Here we show that a single unilateral PDC injection in the rat SN initiates a self-sustaining degenerative process selectively affecting DA *versus* non-DA neurons, which progresses up to 120 days after the injection. This provides a novel animal model exhibiting a unique combination of PD hallmarks: i) presumed cell death mode (GSH depletion, oxidative stress, NMDA receptor-mediated excitotoxicity, neuroinflammation); ii) evolution pattern of DA neuron loss from unilateral to bilateral and with a caudo-rostral gradient; iii) development of compensatory mechanisms; iv) appearance of motor deficits.

Materials and methods

Experiments were performed on male Wistar Hannover rats in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and all efforts were made to minimize their number and sufferance. Protocols were approved by the local ethical committee and conformed to the ethical guidelines of the French Ministry of Agriculture and Forests (Animal Health and Protection Veterinary Service).

In vitro electrophysiology

Brains from 4 weeks-old rats (n = 21) were cut in coronal slices (250 μm) by a vibratome in ice-cold solution containing (in mM): 110 choline, 2.5 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 0.5 $CaCl_2$, 25 $NaHCO_3$, 7 glucose, pH 7.4, bubbled with a mix of 95% O_2 and 5% CO_2. Slices were kept in bubbled artificial cerebrospinal fluid (ACSF) at room temperature, composed of (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 11 glucose, and 25 $NaHCO_3$, pH7.4, added with 250 μM kynurenic acid and 1 mM sodium pyruvate. Electrophysiological experiments were done at 35 °C in standard ACSF (without kynurenic acid and sodium pyruvate) flowing at ~2.5 ml/min. Whole-cell patch-clamp recordings were performed by borosilicate micropipettes (4–5 MΩ) filled with a solution containing (in mM): 125 K-gluconate, 10 NaCl, 1 $CaCl_2$, 2 $MgCl_2$, 0.5 1,2-bis (2-aminophenoxy) ethane-N,N,N,N-tetraacetic acid (BAPTA), 19 N-(2-hydroxyethyl)-piperazine-N-s-ethanesulphonic acid (HEPES), 0.3 guanosine triphosphate (GTP), and 1 Mg-adenosine triphosphate (Mg-ATP), pH 7.3. Alexa Fluor 568 (20 μM) was added in the micropipette solution to localize and identify off-line the recorded neurons. 35 DA and 14 non-DA neurons were identified on-line by infrared videomicroscopy (and confirmed off-line by the presence or not, respectively, of Alexa Fluor 568 fluorescence) and recorded by AxoPatch 200B or Multiclamp 700B amplifiers with pClamp10.2 software (Molecular Devices, USA). For slice application, drugs (Tocris-Cookson, UK) were dissolved at the desired concentration in the ACSF. Data were analyzed by pClamp and Prism (GraphPad, USA) software and are expressed as average ± SEM.

Stereotaxic surgery

Surgery was performed in 7–8 weeks old rats (180–200 g). After equithesin anesthesia (4 ml/kg), animals received unilateral (behavioral and morphological studies) or bilateral (biochemical measurements) stereotaxic injection of 300 nmol PDC (5 μl of a 60 mM PDC solution injected at the rate of 1 μl/min) or vehicle (0.9% NaCl; 5 μl at the rate of 1 μl/min) at a point between SN *pars compacta* (SNc) and *pars reticulata* (SNr) (coordinates in mm: AP +2.2, L ±2.0, and DV +3.3, according to de Groot (De Groot, 1959); see Figs. 1A,B). Animals for microdialysis were stereotaxically implanted in the ipsilateral DA-lesioned side or the contralateral un-injected side with a cannula guide CMA/11 (Carnegie Medicine, Stockholm, Sweden), with the tip placed above the striatum (AP: +0.2 mm and L: +/−2.8 mm from bregma; DV: −3.3 mm from dura, according to the stereotaxic atlas of Paxinos and Watson, 1998).

For neuroprotection study, PDC was either co-injected with memantine and/or ifenprodil (2 mM solution) or associated with N-acetylcysteine in drinking water (2 mg/ml started the day of PDC injection until 4 dpi).

Animals were sacrificed at 4 days post-injection (dpi) for biochemical measurements, at 4, 15, 30, 60 or 120 dpi for histology, and at 90 dpi for *in vivo* microdialysis.

Biochemical assays

Anesthetized animals were transcardially perfused with 400 ml of cold PBS pH 7.4 containing 0.16 mg/ml heparin. Immediately after brain removal, striatum and SN were dissected out from 2 mm coronal slices performed using a rat brain matrix, and tissue samples were weighed and homogenized in cold 20 mM HEPES buffer (1/10, w/v), pH 7.2, containing 1 mM EDTA. Homogenates were centrifuged (1600 *g* for 10 min at 4 °C). Part of the supernatant was used to determine

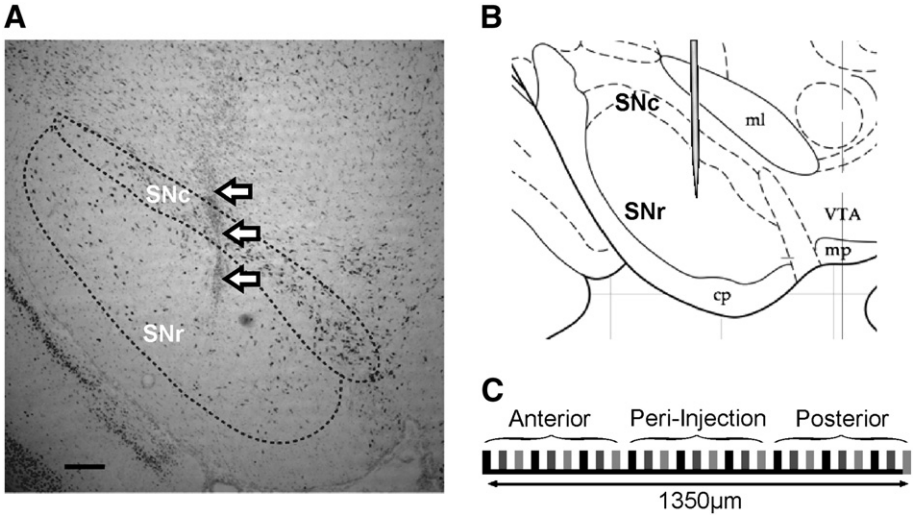

Fig. 1. PDC injection site and procedure of section collection for histological studies. Photomicrograph of a toluidine blue-stained section (A) and scheme (B) (adapted from (Paxinos and Watson, 1998) showing the needle tract (white arrows on the photomicrograph) of PDC injection in the SN. Stereotaxic PDC injection was done between SNc and SNr (abbreviations: cp: cerebral peduncle; ml: medial lemniscus; mp: mammillary peduncle; VTA: ventral tegmental area; scale bar = 100 μm). (C) For each animal, 27 consecutive 50 μm-thick frontal sections (illustrated by black, dark gray and light gray bars) covering an anteroposterior extent of 1350 μm (~90% of SNc extent) of the injected and contralateral SN were carefully collected. SNc was subdivided in three equivalent 450 μm length sub-regions covering, respectively, the anterior, peri-injection and posterior parts. Sections of the same color on the drawing have been used as sample for a given labeling.