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Changes in the expression of genes encoding for mGlu4 and mGlu5 receptors and other regulators of the indirect pathway in acute mouse models of drug-induced parkinsonism

Milena Cannella [a], Marta Motolese [a], Domenico Bucci [a], Gemma Molinaro [a], Roberto Gradini [b], Valeria Bruno [a, c], Ferdinando Nicoletti [a, c], Giuseppe Battaglia [a, *]

[a] I.R.C.C.S. Neuromed, Pozzilli, Italy
[b] Department of Experimental Medicine, University "Sapienza", Rome, Italy
[c] Department of Physiology and Pharmacology, University "Sapienza", Rome, Italy

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ABSTRACT

Neuroadaptive changes involving the indirect pathway of the basal ganglia motor circuit occur in the early phases of parkinsonism. The precise identification of these changes may shed new light into the pathophysiology of parkinsonism and better define the time window of pharmacological intervention. We examined some of these changes in mice challenged with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), or with the dopamine receptor blocker, haloperidol. These two models clearly diverge from Parkinson's disease (PD); however, they allow an accurate time-dependent analysis of neuroadaptive changes occurring in the striatum. Acute haloperidol injection caused a significant increase in the transcripts of mGlu4 receptors, CB1 receptors and preproenkephalin-A at 2 and 24 h, and a reduction in the transcripts of mGlu5 and A_{2A} receptors at 2 h. At least changes in the expression of mGlu4 receptors might be interpreted as compensatory because haloperidol-induced catalepsy was enhanced in mGlu4$^{-/-}$ mice. Mice injected with 30 mg/kg of MPTP also showed an increase in the transcripts of mGlu4 receptors, CB1 receptors, and preproenkephalin-A at 3 d, and a reduction of the transcript of A_{2A} receptors at 1 d in the striatum. Genetic deletion of mGlu4 receptors altered the functional response to MPTP, assessed by counting c-Fos^{+} neurons in the external globus pallidus and ventromedial thalamic nucleus. These findings offer the first evidence that changes in the expression of mGlu4 and mGlu5 receptors occur in acute models of parkinsonisms, and lay the groundwork for the study of these changes in models that better recapitulate the temporal profile of nigrostriatal dysfunction associated with PD.

1. Introduction

Human and animal studies suggest that compensatory mechanisms in the basal ganglia motor circuit occur in the presymptomatic stage of Parkinson's disease (PD), in which motor signs (i.e., bradykinesia, rigidity and resting tremor) are not yet manifest in spite of extensive degeneration of nigrostriatal dopaminergic neurons (reviewed by Bezard et al., 2003; Brotchie and Fitzer-Attas, 2009). Unraveling the molecular nature and temporal profile of these mechanisms may shed new light into the pathophysiology of parkinsonism, and lay the groundwork for new therapeutic interventions aimed at delaying the clinical onset of the disorder. Two different categories of compensatory mechanisms have been identified in early parkinsonism, i.e. those enhancing the activity of surviving dopaminergic neurons, and those restraining the activity of the indirect pathway of the basal ganglia motor circuit (reviewed by Brotchie and Fitzer-Attas, 2009). Examples of compensatory changes influencing the activity of dopaminergic neurons include enhanced levels of tyrosine hydroxylase and L-aromatic amino acid decarboxylase, and reduced levels of the high affinity dopamine transporter (Zigmond et al., 1984; Uhl et al., 1994; Lee et al., 2000; Sossi et al., 2007). Interventions aimed at amplifying these mechanisms may accelerate degeneration of dopaminergic fibers

* Corresponding author. I.R.C.C.S. Neuromed, Località Camerelle, 86077 Pozzilli (IS), Italy. Tel.: +39 0865915201; fax: +39 0865927575.
E-mail address: giuseppe.battaglia@neuromed.it (G. Battaglia).

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because of the production of reactive oxygen species from endogenous dopamine (see Brotchie and Fitzer-Attas, 2009). Changes occurring within the indirect pathway are more "safe" and perhaps occur earlier in the course of the disease. The indirect pathway connects the input station, i.e. the neostriatum, with the output stations of the basal ganglia motor circuit (the internal globus pallidus and the substantia nigra pars reticulata) *via* the external globus pallidus (GP_{ext}) and the subthalamic nucleus (STN). In the indirect pathway, striatal GABAergic projection neurons make synaptic contacts with GPext neurons, which, in turn, send GABAergic projections to the STN. STN neurons send excitatory axons to the output stations, thereby restraining the activity of ventral motor thalamic neurons projecting to the cerebral cortex (reviewed by Conn et al., 2005).

In the neostriatum, the activity of projection neurons of the indirect pathway is negatively modulated by D2 dopamine (DA) receptors and positively regulated by A_{2A} adenosine receptors, NMDA receptors, and mGlu5 metabotropic glutamate receptors. GABA release at the synapses between striatal projection neurons and GP_{ext} neurons is negatively modulated by mGlu4 receptors, as well as by MOR opiate receptors activated by enkephalins that are released from the terminals of striatal projection neurons (reviewed by Conn et al., 2005). CB1 cannabinoid receptors are co-localized with D2 receptors in striatal projection neurons of the indirect pathway, and are also expressed on axon terminals in the GP_{ext} (Gerfen et al., 1990; Mailleux and Vanderhaeghen, 1992; Szabo et al., 1998; Hermann et al., 2002; Mátyás et al.,. 2006; Crespo et al., 2008; Martín et al., 2008; Van Waes et al., 2012). The overall function of CB1 receptors is to inhibit the indirect pathway (Blume et al., 2013).

The following changes have been described in early stages of experimental parkinsonism: (i) a reduced membrane availability of the GluN2B subunit of NMDA receptors expressed by striatal projection neurons (Hallett et al., 2005); (ii) a reduced catabolism of endocannabinoids leading to an increased activation of CB1 receptors (Kreitzer and Malenka, 2007); and (iii) an enhanced production of enkephalins in axon terminals of striatal neurons projecting to the GP_{ext} (Herrero et al., 1995; Asselin et al., 1994; Nisbet et al., 1995; Gudehithlu et al., 1991). No data are available on the expression of mGlu4, mGlu5, A_{2A} and CB1 receptors, in spite of the importance of these receptors as drug targets in the treatment of parkinsonism (Jones et al., 2013; Gasparini et al., 2013; Amalric et al., 2013).

Here, we report that parkinsonism induced by acute injection of haloperidol or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice is associated with transient changes in the expression of genes encoding for mGlu4 receptors, mGlu5 receptors, A_{2A} receptors, CB1 receptors, and pre-proenkephalin-A (the peptide precursor of enkephalins), in the striatum.

2. Materials and methods

2.1. Animals

C57BL mice (22–24 g, body weight) were purchased from Charles River (Calco, Italy). $Grm4^{+/-}$(B6.129-Grm4tm1Hpn/J) mice, on a C57/BL6 background, were purchased from The Jackson Laboratory (Bar Harbor, ME). The $Grm4^{-/-}$ offspring of heterozygotes were used to establish colonies of $Grm4^{-/-}$ mice (see Fallarino et al., 2010). Mice were kept under environmentally controlled conditions (ambient temperature, 22 °C; humidity, 40%) on a 12 h light/dark cycle with food and water *ad libitum*. All experiments were carried out according to the European (86/609/EEC) and Italian (D. Lgs. 116/92) guidelines of animal care. The experimental protocol was reviewed by the internal animal care and use committee and approved by the Italian Ministry of Health (D. Leg. 227/2011-B). All efforts were made to minimize animal suffering and the number of animals used.

2.2. Treatments

Mice were treated systemically with a single injection of haloperidol (Haldol, Janssen). Haldol solution (2 mg of haloperidol/ml) was further diluted in saline to obtain concentrations of haloperidol of 10 or 20 μg/100 μl. Haloperidol was administered s.c. at doses of 0.5 or 1 mg/kg. Control mice were injected s.c. with an equal volume of saline. Haloperidol-treated mice and their controls were killed at 2 h, 1, 3, and 5 d after the injection (n = 3–5 per group).

Additional groups of mice were treated systemically with MPTP hydrochloride dissolved in saline (Sigma-Aldrich; Milwauke, WI, USA). Some groups were injected only once with 36 mg/kg of MPTP hydrochloride corresponding to 30 mg/kg of MPTP free-base. Other groups were injected three times (inter-dose interval = 2 h) with 24 mg/kg of MPTP hydrochoride (corresponding to 20 mg/kg of MPTP free-base) for a cumulative dose of 60 mg/kg of MPTP free-base. Control mice were injected with saline. MPTP-treated mice and their controls were killed at 1, 3, 7, and 14 d after the injection (n = 3–5 per group in the experiment with 30 mg/kg of MPTP; and 4–5 per group in the experiment with 60 mg/kg of MPTP). Independent experiments were performed for biochemical, behavioral, and immunohistochemical analysis.

2.3. Haloperidol-induced catalepsy

Mice were challenged with a 0.5 or 1 mg/kg of haloperidol (n = 4 per group); catalepsy was assessed at different time points, as described by Lipska and Weinberger (1993). Mice were handled gently twice a day in the two days preceding the experiments. Following haloperidol injection, both forepaws were placed on a 2.5-cm elevated horizontal bar. The time spent by the mice with both forepaws on the horizontal bar was recorded for 5 min at each time point.

2.4. RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was extracted from the striatum in Trizol reagent according to manufacturer's protocol. The RNA was further treated with DNAse. Single strand cDNA was synthesized from 1-2 μg of total RNA using superscript III and random hexamers. Gene expression data were obtained by using Power SYBR Green Master Mix on an Applied Biosystems Step-One instrument. Thermal cycler conditions were as follows: 10 min at 95 °C, 40 cycles of denaturation (45 s at 95 °C), and combined annealing/extension (1 min at 58 °C). Real-time PCR was performed by using the following primers: β-actin, forward: 5′-gttgacatccgtaaagacc-3′ and reverse: 5′-tggaaggtggacagtgag-3′; mGlu4 receptor, forward: 5′- ctccagccgcacgcttgaca-3′ and reverse: 5′- gtaggccgagtcctgcccga-3′; preproenkephalin A, forward: 5′- agccaggactgcgctaaat-3′ and reverse: 5′- ttgcaggtctcccagatttt-3′; CB1 receptor, forward: 5′-cttgtagcagagagccagcc-3′ and reverse: 5′- tgagaaagaggtgccaggag-3′; mGlu5 receptor, forward: 5′- acgaagaccaaccgtattgc-3′ and reverse: 5′- agacttctcggatgcttgga-3′; A_{2A} receptors, forward: 5′- gcagagttccatcttcagcc-3′ and reverse: 5′- ccttcatacccgtcaccaag-3′. Absolute amounts of mRNA were calculated from serially diluted standard curves simultaneously amplified with the samples and normalized with respect to β-actin mRNA levels.

2.5. Immunohistochemical analysis

Mice were anesthetized with an i.p. injection of ketamine (100 mg/kg)/xylazine (10 mg/kg) and perfused with 4% paraformaldehyde. Brains were carefully removed, fixed in 4% paraformaldehyde overnight at 4 °C, washed in distilled water for 10 min, embedded in paraffin and sectioned at 20 μm. Deparaffinized sections, regularly spaced every 160 μm through the extension of the GP_{ext}, were processed for immunohistochemical detection of c-Fos protein. After blocking with normal rabbit serum (10% in Tris buffer solution), tissue sections were incubated overnight at 4 °C with the anti-c-Fos antibodies (1:100, catalog #: AB1584, Millipore, Temecula, CA), and then for 1 h with secondary biotinylated anti-sheep antibodies (1:200; Vector Laboratories, Burlingame, CA). 3,3-Diaminobenzidine tetrachloride was used for detection. Control staining was performed without the primary antibodies.

2.6. Stereological counts of c-Fos-positive cells in the GP_{ext}

The number of c-Fos^+ cells was calculated by a stereological technique and the optical fractionator, using a Zeiss Axio Imager M1 microscope, equipped with a motorized stage and focus control system (Zeta axis), and a digital video camera. The software Image-Pro Plus 6.2 for Windows (Media Cybernetics, Inc., Bethesda, MD) equipped with a Macro was used for the analysis of digital images. The Macro was obtained by Immagine and Computer, Bareggio, Italy and the characteristics of this Macro are published (King et al., 2002). The optical fractionator technique (adapted to 20 μm thick sections) was used to obtain estimates of total number of c-Fos^+ cells within the GP_{ext}. A systematically sampled series of sections every 160 μm spanning the entire extent of GP_{ext} was selected for quantification. In each stained section, the area was identified and outlined at 2.5× magnification. After outlining the regions of interest, a sampling grid of known dimensions (75 × 75 μm) was positioned over each area and counting was carried out using a 100× oil immersion lens. The total number of c-Fos^+ cells per each rostro-caudal level was computed from the formula: $N = \Sigma(n) \times 1/SSF \times 1/ASF \times 1/TSF$ where "n" is the total number of neurons counted on each disector; "SSF" (fraction of sections sampled) is the number of regularly spaced sections used for counts divided by the total number of sections through the entire extent of GP_{ext} (=1/5); "ASF" (area sampling frequency) is the disector area divided by the area between disectors [=(5625 μm^2 × disectors number)/region area] and "TSF" (thickness sampling frequency) is the disector thickness divided by