A stereological study on the neuroprotective actions of acute Modafinil treatment on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced nigral lesions of the male black mouse

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Abstract

The effect of an acute administration of the vigilance-promoting drug modafinil ([(±)diphenyl-methyl]-sulfinyl-2 acetamide; Modiodal) on the nigrostriatal dopamine system was studied after damage induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) by means of immunohistochemistry for tyrosine hydroxylase (TH) and a stereological method. MPTP (40 mg/kg) reduced from 24 380 ± 902 to 13 501 ± 522 and from 37 868 ± 3300 to 20 568 ± 1270, respectively, the number of TH immunoreactive (IR) and non-TH IR nigral neurons. Co-administration of Modafinil restored to normal the number of these neuronal populations. MPTP treatment induced also a reduction in the volume of TH IR neurons, which was counteracted by Modafinil administration. The data provide morphological evidence, based on unbiased stereological analysis, for a potential neuroprotective role of Modafinil, not only in dopaminergic neurons, but also with a similar magnitude in the non-DA nerve cell population of the substantia nigra after MPTP lesion. These results suggest that Modafinil has a neuroprotective role in the substantia nigra via a still undefined mechanism in which a crucial role of DA uptake blockade should be excluded. Modafinil may therefore have a therapeutic potential in neurodegenerative processes such as those occurring in Parkinson’s disease. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is known to cause chronic Parkinsonism [2]. Similar brain lesions as found in humans after MPTP abuse were experimentally induced in monkeys and in the black mouse [7]. After systemic administration, MPTP readily crosses the blood-brain barrier, where it is converted to 1-methyl-4-phenylpyridinium ion (MPP⁺) by monoamine oxidase B. This process appears to occur in astrocytes. The plasma membrane DA transporter then selectively takes up MPP⁺ into dopamine neurons [1] which produces neurotoxic effects by inhibiting the complex I of the respiratory chain. Cell death would result from a complex interplay among various phenomena such as mitochondrial dysfunction, oxidative stress, energy failure and excitotoxicity [14]. It has been demonstrated that the MPTP-induced degeneration of the nigrostriatal dopamine (DA) neurons is also associated with a reduction of nigral γ-aminobutyric acid (GABA) stores [11].

Modafinil ([(±)diphenyl-methyl]-sulfinyl-2 acetamide; Modiodal) is a well known drug which promotes vigilance and wakefulness in mammals including man [3]. It induces less tolerance and fewer cardiovascular effects than d-amphetamine in conscious monkeys [8] and is used in the treatment of narcolepsy. Importantly, studies in volunteers and patients indicate that Modafinil has less abuse potential than dexamphetamine or methylphenidate [12]. Modafinil has been demonstrated to protect against MPTP-induced...
DA toxicity in vivo [5] and in vitro [9]. Furthermore, Modafinil has been shown to reduce GABA release in several brain regions, including the substantia nigra (SN), when a high dose (300 mg/kg) is used [15]. It has been demonstrated in vitro that Modafinil binds with a low affinity to the DA uptake carrier [13].

In the present paper MPTP treated black mice were used to analyze the effect of Modafinil on the neuronal degeneration induced by MPTP using stereological methods. The total number of non-TH and TH immunoreactive (IR) neurons of the whole SN and the mean volume of TH IR neurons were estimated after MPTP and Modafinil alone or in combination. The results give evidence for a protective action of Modafinil on both DA and non-DA nigral neurons.

Twenty-five specific-pathogen-free male C57Bl/6 mice (10-week-old, body weight 25 g) were purchased from B&K Universal (Sollentuna, Sweden). The mice were kept under controlled temperature and lighting conditions (lights on at 06:00 h and off at 18.00 h) with free access to tap water and food pellets. MPTP hydrochloride (Research Biochemical, Natick, MA) dissolved in 0.9% NaCl was given subcutaneously (s.c., 40 mg/kg). Modafinil (L. Lafon, Maisons-Alfort, France) was administered (i.p., 100 mg/kg) at the same time as MPTP in a suspension of 0.5% gummi arabicum [5]. The mice were divided into four groups: (1) saline plus Modafinil-vehicle (n = 6); (2) saline plus Modafinil (n = 6); (3) MPTP plus Modafinil-vehicle (n = 6) and (4) MPTP plus Modafinil (n = 7). All MPTP-treated animals were protected from hypothermia and received a solution of isotonic saline and 5% glucose (i.p., 1 ml, twice daily). After 1 week, all animals were anaesthetized with sodium pentobarbital (100 mg/kg, i.p.) and intracardially perfused with 25 ml ice-cold 0.9% NaCl followed by 150 ml of fixation fluid (4°C) for 6 min. The fixative consisted of 4% paraformaldehyde (w/v) and 0.2% picric acid (v/v) solution in 0.1 M sodium phosphate buffer (pH 7.4; PBS). The brains were dissected out, postfixed during 90 min in the same fixative and cryoprotected in sucrose (10% for 24 h followed by 30% for 1 week, at 4°C). The midbrain was cut into coronal, serial 35-μm thick sections on a cryostat (Micron, HM 500 M, Walldorf, Germany). With a random start, every fourth section through the entire SN (levels: bregma −2.54 to −4.04 mm) was sampled into three series of sections. (f1 = 4: first sampling fraction for the stereological analysis). The sections were processed free-floating. Endogenous peroxidase activity was removed by incubating the sections with 0.3% H2O2 for 10 min. After blocking with 1% normal goat serum (Sigma, St. Louis, MO) (40 min, room temperature) the sections were incubated with a mouse monoclonal IgG1 antibody to rat PC 12 TH (Incstar, Stillwater, MI) diluted 1:1500 (4°C, overnight) in 0.1 M PBS containing 0.3% Triton X-100 (Sigma, St. Louis, MO). The immunostaining was performed according to the ABC method using the Vectastain kit (Vector, Burlingame, CA). The chromogen used was 0.03% 3-3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and 0.03% fresh H2O2 in 0.05 M Tris–HCl (pH 7.4). Sections from all animals were processed simultaneously. After mounting on gelatin-chromalum coated slides, the sections were counterstained with cresyl violet (Sigma, St. Louis, MO), dehydrated and coverslipped with Mountex (Histolab, Gothenburg, Sweden). Nucleoli and Nissl substance were stained with cresyl violet without interfering with the brownish TH immunostaining. The fractionator and optical disector methods [6] were used to estimate the total number of three cell populations of the SN: TH IR neurons, non-TH IR neurons with stained Nissl substance and non-neuronal nuclei labeled by cresyl violet. An Olympus BH2 microscope (Olympus, Denmark) was interfaced with a computer (DGC systems, Stockholm) and a color video camera (CCD-iris, Sony, Japan). The CAST-Grid software package (Olympus, Glostrup, Denmark) generated sampling frames with a known area (frame) and directed the motorized X-Y stage (Lang, Huttenberg, Germany) and a microcator (MT12, Heidenheim, Germany), which monitored the movements in the Z-axis with a resolution of 0.5 μm. One side of the entire SN (zona compacta, zona lateralis and zona reticulata) was defined using a 4× objective. With a random start, the software created counting frames on the images generated by a 100× oil objective (NA = 1.25) within the previously defined SN. The distance between the sampled fields on each section (X, Y steps) was 160 μm. The counting frame (frame) was either 1553 μm2 or 388 μm2, for neurons and non-neuronal cells, respectively, giving the second sampling fraction (f2 = X step length × Y step length/area). The sampling volume (disector) in the Z-axis extended 7 μm deep (height of the disector) after excluding 3–5 μm from the top and from the bottom of the section. Staining with cresyl violet allowed the visualization of nucleoli within neurons and the nuclei of non-neuronal cells. Those largely varied in shape and staining, ranging from round and blue pale to irregular and pyknotic nuclei. The location of nucleoli (neurons) or nuclei (non-neuronal cells) within the height of the disector and into the frame area were used as criteria for counting [10]. The total thickness of the sections was also measured, giving the third sampling fraction (f3 = (height of the section)/height of the disector). After having counted the objects (ΣQ−) fulfilling the criteria for being sampled, the total number of TH IR, non-TH IR and non-neuron cells (Ntotal) in the nigral region was estimated: Ntotal = ΣQ− × f1 × f2 × f3 [6]. The coefficient of error (CE) for each estimation and animal, ranged from 0.01 to 0.05. The total CE of each group (CE group value) ranged from 0.02 to 0.04. The mean volume of TH IR neurons was estimated by means of the point sampled intercept method, which is based on Cavalieri’s principle [6]. Estimations of the total number of cells by means of the fractionator are independent of shrinkage during tissue processing. However, estimations of mean volume are obviously influenced by shrinkage. In this experiment, shrinkage in the Z-axis was close to 50%. A one-way analysis of variance (ANOVA) was applied to the parametric data.
using the Fisher’s PLSD post-hoc test for statistical significance between groups.

In the (NaCl + vehicle) group the total number of TH IR neurons, non-TH IR neurons and non-neuronal cells on one side of SN were 24,400 ± 900, 37,900 ± 3300 and 156,000 ± 27,000, respectively (mean ± SEM, Fig. 1A,B; Table 1). A single dose of Modafilin (100 mg/kg) did not alter the number of TH or non-TH IR neurons (Fig. 1A,B). MPTP (40 mg/kg) induced a 43 and 45% reduction in both the number of TH IR and non-TH IR nigral neurons to 13,500 ± 500 and 20,600 ± 1300, respectively and co-administration of Modafilin counteracted these reductions (24,400 ± 2300 and 40,100 ± 1300, respectively; Fig. 1A,B), as evaluated 1 week after MPTP. The mean volume of individual TH IR neurons was 1000 ± 20 μm$^3$ in the (NaCl + vehicle) group. MPTP treatment induced a reduction in volume (820 ± 50 μm$^3$), which was counteracted by Modafilin administration (1000 ± 50 μm$^3$) (Table 1). Modafilin did not alter the mean volume of TH IR neurons in animals not treated with MPTP. The number of non-neuronal cells in SN increased significantly by 27% after Modafilin treatment (156,000 ± 27,000 to 213,000 ± 11,000) and was not altered by MPTP administration, as evaluated 1 week later (Table 1).

The findings show that Modafilin prevented the neuronal damage of TH and non-TH IR neurons of the SN when simultaneously administered with MPTP. Evidence for a protective effect of Modafilin on the DA neurons of the SN in the MPTP mouse model has previously been provided by immunohistochemical and biochemical data [5]. The presence of DA transporter (DAT) on the plasma membrane of nigral neurons is considered a main factor for explaining the preferential DA neurotoxicity of MPTP, which is not specially involve DA uptake inhibition but more likely involves prominent neurotrophic actions [5].

![Fig. 1. Effect of Modafilin (100 mg/kg, s.c.) on the MPTP (40 mg/kg, i.p.) induced reduction of the total number of neurons on one side of the mouse substantia nigra.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Volume (μm$^3$) of TH IR neurons (mean ± SEM)</th>
<th>CE group</th>
<th>Non-neuronal cell nuclei (x10$^5$) (mean ± SEM)</th>
<th>CE group</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl + vehicle</td>
<td>6</td>
<td>1000 ± 20</td>
<td>0.05</td>
<td>156 ± 27</td>
<td>0.02</td>
</tr>
<tr>
<td>NaCl + Modafilin</td>
<td>6</td>
<td>980 ± 50</td>
<td>0.05</td>
<td>213 ± 11*</td>
<td>0.02</td>
</tr>
<tr>
<td>MPTP + vehicle</td>
<td>6</td>
<td>820 ± 50*</td>
<td>0.04</td>
<td>170 ± 18</td>
<td>0.03</td>
</tr>
<tr>
<td>MPTP + Modafilin</td>
<td>7</td>
<td>1000 ± 50**</td>
<td>0.06</td>
<td>200 ± 6</td>
<td>0.02</td>
</tr>
</tbody>
</table>

a For treatment, see text. One-way analysis of variance (ANOVA) and Fischer’s PLSD post-hoc test. *P < 0.05 vs. NaCl + vehicle, **P < 0.01 vs. MPTP + vehicle. CE group represents the coefficient of error in each group for the estimated volume of TH IR neurons or the number of non-neuronal cells.
cells. Thus, Modafinil may rescue not only DA neurons but also probably GABAergic neurons of SN. It is improbable that Modafinil would block the internalization of MPP⁺ in those cells via DA uptake inhibition [13], since they are not provided with DAT. Their rescue may therefore reflect true neuroprotective actions of Modafinil. It is known that GABA is released from striatongrial projections to act on nigral GABAergic neurons in the zona reticulata. GABA would inhibit the activity of these neurons, partially protecting them against excitotoxic stimuli. However, in the dose used Modafinil does not modulate GABA release in SN [4] and this mechanism can therefore not be involved in the neuroprotective actions, especially since Modafinil in several other brain regions reduces GABA release [4].

Unbiased estimates of mean volume of nigral TH IR nerve cells have been calculated following the Cavalieri’s principle [6]. The TH IR nerve cell volumes obtained here were estimates of mean volume after a 50% shrinkage induced by the tissue processing. After MPTP the mean volume of TH IR neurons was reduced by 18%, a decrease counteracted by Modafinil also supporting a neuroprotective activity of Modafinil. In agreement, increases of the mean size of the DA cell bodies of the zona compacta following chronic Modafinil treatment in MPTP-induced injury was previously reported [5]. There was no indication of gliosis following the neuronal degeneration induced by MPTP. However, a small increase in the number of non-neuronal cells, presumably mainly glial cells, took place within SN 1 week after a single dose of Modafinil. The significance of this phenomenon is unclear and requires further studies. Nevertheless, it may reflect an increase of glial trophism within SN.

Taken together, acute Modafinil treatment appears to protect against MPTP-induced injury of DA and non-DA nigral neurons, as evaluated 7 days later, suggesting that the neuroprotective action of Modafinil in the substantia nigra cannot be explained by DA uptake inhibition.

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