**ROBERTA JOLY FERREIRA BRAGA**

# ESTUDO DO PAPEL DA SUBSTÂNCIA NEGRA, PARTE COMPACTA, NA MEMÓRIA OPERACIONAL DE RATOS.

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Orientador: Prof. Dr. Cláudio da Cunha

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# SUMÁRIO

**Resumo**

Foram utilizados ratos Wistar machos, com lesão bilateral da parte compacta da substância negra (SNc), induzida pela administração intranigral de 0,5 μmol 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP). Este tratamento resulta em uma perda de células dopaminérgicas seguida de depleção parcial de dopamina estriatal. Os animais foram submetidos ao teste da alternância em um labirinto em Y, onde os mesmos foram treinados por um período pré-determinado ou até atingir o critério de, no mínimo, 80% de acertos. Após lesão da SNc, os animais mantiveram suas porcentagens de acerto inferior ao grupo de animais sham, sendo que estes necessitaram de aproximadamente 2 dias para atingirem novamente o critério de acertos e os primeiros de aproximadamente 6. Os animais treinados somente após a lesão da SNc necessitaram de aproximadamente 18 dias para atingirem o critério, sendo que o grupo sham necessitou em média de 10 dias e os mesmos mantiveram uma média de acertos superior ao grupo lesado. Estes resultados sugerem que a lesão da SNc prejudica a memória de trabalho de ratos no teste da alternância em um labirinto em Y, estando de acordo com os déficits de memória de trabalho relatados em estudos clínicos de pacientes com DP.

**Abstract**

Adult male Wistar rats with bilateral substantia nigra, pars compacta (SNc) lesion induced by intranigral administration of 0.5 μmol 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) were used as a model of early phase Parkinson's disease (PD). This treatment caused loss of dopaminergic cells in the *substantia nigra pars compacta (SNc)* and a partial depletion of striatal dopamine. Animals trained up to 80% correct choices presented significantly worse scores after the SNc lesion compared to sham-operated animals and spent near 6 days to reach this criterion again, while sham-operated animals reached this criterion in about 2 days. When naïve animals had their SNc lesioned before the training, they scored worse than the sham-operated animals and took 18 days to reach the 80% correct choices criterion, while sham-operated reached this criterion after only 10 days. These results suggest that the lesion of the SNc impairs working memory in rats performing this task in agreement with working memory impairment in PD patients reported in clinical studies.

## LISTA DE ABREVIATURAS

DA - Dopamina

DP - Doença de Parkinson

MAO-B - Monoamina-oxidase B

MPP+ - 1-metil-4-fenilpiridina

MPTP - 1-metil-4fenil-1,2,3,6-tetrahidropiridina

SHAM - Falsamente lesado, simulado

SNc - Substância negra, parte compacta

## 1. INTRODUÇÃO

A doença de Parkinson (DP) é caracterizada pela perda progressiva de neurônios dopaminérgicos da substância negra, parte compacta (SNc) (HIRSCH *et al.*, 1988). Distúrbios cognitivos, prejuízos nos processos de informação e déficits na aquisição da memória de curta duração têm sido relatados em pacientes com DP (VALLDEORIOLA *et al.*, 1997). Modelos experimentais animais e estudos clínicos em pacientes demonstram que desordens relacionadas aos núcleos da base resultam em distúrbios cognitivos (Brown *et al*., 1997). Redução da atividade dopaminérgica no estriado e no lobo frontal pode produzir déficits de memória operacional (STEBBINS *et al.*, 1999).

Vários cientistas estudaram o efeito neurotóxico do 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) em modelos animais (BURNS *et al.*, 1983, LANGSTON *et al.*, 1984*,* HEIKKILA e SONSALLA, 1985, HEIKKILA e SONSALLA, 1987). O MPTP é uma potente neurotoxina com efeitos neurotóxicos em neurônios dopaminérgicos nigroestriatais (IRWIN e LANGSTON, 1985, OGAWA *et al*., 1987), produzindo disfunções cognitivas comparáveis às observadas na DP (STERM e LANGSTON, 1985). A administração intranigral de MPTP em ratos reduz a concentração de dopamina na região estriatal assim como seus metabólitos (BARC *et al.*, 2002). A infusão intranigral de MPTP tem sido utilizada como modelo animal para reproduzir alguns eventos celulares da DP (Da CUNHA *et al.*, 2001; GEVAERD *et al.*, 2001; MIYOSHI *et al.*, 2002; Da CUNHA *et al.*, 2003). Da CUNHA *et al*., 2002, demonstraram déficits na memória de ratos após lesão da SNc através da adminitração intranigral de MPTP. Este modelo tem sido utilizado

para estudar a memória operacional no labirinto aquático (MIYOSHI *et al.*, 2002), no labirinto em T (TANILA *et al.*, 1998) assim como na esquiva ativa de duas vias (Da CUNHA *et al.*, 2001; GEVAERD *et al.*, 2001). Prejuízos no teste da alternância espontânea podem ocorrer após lesão de diferentes estruturas cerebrais (LALONDE, 2001).

O objetivo deste estudo foi avaliar o papel da SNc na memória operacional de ratos lesados com MPTP no teste da alternância em um labirinto em Y. Dois diferentes grupos de ratos foram usados. No primeiro grupo os animais foram treinados antes e após o procedimento cirúrgico. No segundo grupo, os animais foram treinados apenas após a cirurgia, tanto após a lesão da SNc com MPTP como o grupo não lesado. Este experimento nos permite estudar o envolvimento da SNc no aprendizado da tarefa assim como o seu papel na memória operacional e na manutenção da memória “per se”.

## 2. REVISÃO DE LITERATURA

### 2.1. DOENÇA DE PARKINSON

Em 1817, James Parkinson descreveu pela primeira vez os sintomas da doença, então chamada Paralisia Agitante, que consistia na presença de movimentos tremulantes involuntários, diminuição da força muscular, alteração da marcha, e inclinação do tronco para frente, sem alterações sensoriais e intelectuais. A DP é hoje classicamente descrita por uma tríade de alterações: bradicinesia (diminuição dos movimentos), rigidez e tremor de repouso, combinado com uma perda do controle postural (MENESES e TEIVE, 1996, STOOF *et al.*, 1999, BLUM *et al*., 2001). É uma doença neurodegenerativa progressiva e severa com quadro clínico manifestando-se geralmente entre 50 e 70 anos de idade (HUGHES *et al.*, 1993). A DP é caracterizada pela morte progressiva dos neurônios dopaminérgicos da SNc, região de onde aferem fibras principalmente para o estriado (HIRSCH *et al*., 1988). Os neurônios dopaminérgicos da SNc são particularmente vulneráveis ao processo neurodegenerativo que ocorre na DP (BARC *et al.*, 2002). A degeneração destas células resultará na diminuição da produção de dopamina (DA) com conseqüente disfunção da via nigroestriatal (MENESES e TEIVE, 1996; LEV *et al.*, 2003). Esta doença é de evolução lenta e a taxa de morte neuronal é muito balxa, isto é, poucas células por dia (LEV *et al.*, 2003). A degeneração do sistema dopaminérgico nigroestriatal é responsável pelas desordens motoras

características desta doença (MARIÉ e DEFER, 2003, McAULEY, 2003). Além das alterações motoras, déficits cognitivos são encontrados em paciente com DP (SCHNEIDER e POPE-COLEMAN, 1995, FAGLIONI *et al.***,** 1997, DUBOIS e PILLON, 1997, LINDNER *et al.*, 1999**).**

Modelos experimentais animais e estudos clínicos em pacientes demonstram que alterações dos núcleos da base resultam em distúrbios cognitivos (BROWN *et al.*, 1997). Além das alterações cognitivas e da memória, 15 a 20% dos pacientes desenvolvem demência no decorrer da evolução da doença (DUBOIS e PILLON, 1997). Lentidão no processamento das informações e prejuízo na aquisição das memórias de curto prazo, segundo VALLDEORIOLA *et al.*, 1997, são alterações cognitivas encontradas em pacientes com DP. Déficits cognitivos que apresentam melhora com tratamento dopaminérgico podem estar relacionados com depleções de DA nos núcleos da base ou no córtex (SKEEL *et al*., 2001). A redução da atividade dopaminérgica no estriado e no lobo frontal pode levar a déficits de memória operacional (STEBBINS *et al.*, 1999). DUBOIS e PILLON 1997, observaram que a memória de longa duração assim como a memória operacional, podem estar alteradas nestes pacientes. A etiologia da DP ainda é desconhecida e o tratamento é basicamente sintomático (LEV *et al.*, 2003).

## 2.2. DOENÇA DE PARKINSON E MPTP

O uso do MPTP na indução do parkinsonismo experimental em animais começou quando se descobriu que esta neurotoxina produzia em humanos as mesmas alterações bioquímicas, histológicas e patológicas encontradas na Doença de Parkinson. Esta descoberta acidental ocorreu em 1982, após o uso ilícito, por jovens, de uma substância derivada da heroína, o 1-metil-4-fenil-propionoxypiperidina (MPPP), um potente agente analgésico estruturalmente relacionado com a meperidina, o qual continha MPTP, um produto formado durante a síntese do MPPP. Após a auto-administração, os jovens apresentaram sintomas clássicos de parkinsonismo tais como tremor, rigidez e discinesia (LANGSTON, 1983).

O efeito neurotóxico do MPTP em neurônios dopaminérgicos nigroestriatais foi primeiramente demonstrado em macacos por BURNS *et al*., 1983. Em 1984, LANGSTON *et al* confirmaram a neurotoxicidade seletiva do MPTP por células da SNc após administração intraperitoneal (i.p.) desta substância em macacos. HEIKKILA e SONSALLA, 1984, demonstraram em seu trabalho que o MPTP apresentava ação neurotóxica em neurônios dopaminérgicos nigroestriatais de camundongos após administração i.p. 1-Metil-4-fenilpiridina (MPP+), produto do metabolismo oxidativo do MPTP pela monoamino-oxidase B (MAO-B), é uma potente neurotoxina que causa destruição das fibras dopaminérgicas em cultura de células produtoras de DA, com conseqüente depleção dos níveis deste neurotransmissor (MYTILINEOU *et al.*, 1985). O mesmo trabalho também

demonstrou que o MPP+ possui maior toxicidade que o MPTP e que este efeito tóxico, em ambas as drogas, é dose dependente. O MPTP é altamente lipofílico atravessando com facilidade a barreira hemato-encefálica. A MAO-B, enzima envolvida na degradação das catecolaminas, converte o MPTP em MPP+ na glia, sua forma ativa e neurotóxica. O MPP+ é captado por terminais dopaminérgicos pelo transportador de dopamina. Concentra-se nas mitocôndrias onde irá inibir a ação da NADH CoQ1 redutase, enzima específica do complexo I da cadeia respiratória mitocondrial. Isto resulta em diminuição da produção de ATP, aumento da produção de radicais livres, diminuição da atividade antioxidante com conseqüente dano oxidativo e morte celular (MOUSSA, 1997, FALL e BENNETT, 1999). A inibição da MAO-B resulta em bloqueio do efeito neurotóxico do MPTP por impedir sua conversão em MPP+ resultando em uma prevenção da neurotoxicidade (NICKLAS *et al.*, 1987). No mesmo trabalho, os autores concluíram que a provável ação tóxica do MPP+ era devido à inibição da respiração mitocondrial. Segundo HEIKKILA e SONSALLA, 1987, os principais déficits bioquímicos, comportamentais e neuropatológicos observados em animais experimentais tratados com MPTP são comparáveis aos apresentados por pacientes parkinsonianos. A neurotoxicidade induzida pelo MPTP pode estar relacionada a vários mecanismos como o estresse oxidativo, deficiência mitocondrial e apoptose (BLUM, 2002). O MPTP produz alterações bioquímicas, histoquímicas e morfológicas típicas da apoptose em culturas de células neuronais ( LEV *et al.*, 2003). Em 1985, IRWIN e LANGSTON demonstraram que, após a administração sistêmica de MPTP em macacos, a concentração de MPP+ na substância negra apresentou-se mais elevada que em outras áreas cerebrais,

resultado sugestivo de uma seletiva toxicidade nigral. HEIKKILA *et al*., 1985, demonstraram a toxicidade do MPP+ através da administração intranigral em ratos. Um estudo mostra que após administração intranigral de MPTP em ratos ocorre uma diminuição de dopamina e seus metabólitos na região estriatal (BARC *et al.*, 2002). OGAWA *et al.*, 1987, observaram que após administração i.p. de MPTP em camundongos os neurônios dopaminérgicos da área tegmental ventral não haviam sido lesados, somente os da substância negra. Embora a 6-hidroxidopamina (6-OHDA) e o MPP+ sejam mais freqüentemente usados na indução da lesão da via nigroestriatal em modelos da doença de Parkinson em ratos, os mesmos apresentam toxicidade também para neurônios noradrenérgicos e serotoninérgicos, resultando em uma lesão não seletiva (MIYOSHI *et al.*, 2002). BOYCE *et al.*, 1984 demonstraram que a administração intra-peritoneal de MPTP em ratos não resulta em uma ação neurotóxica assim como não ocorrem alterações bioquímicas relacionadas aos níveis de dopamina e seus metabólitos estriatais. Trabalhos demonstram que a infusão intranigral de MPTP é mais seletiva e efetiva na lesão de células dopaminérgicas resultando em uma diminuição significativa dos níveis de DA no estriado de ratos (DA CUNHA *et al.*, 2001; GEVAERD *et al.*, 2001; MIYOSHI *et al.,* 2002; DA CUNHA *et al.*, 2003). TANILA *et al*., 1998, demonstraram prejuízo na memória operacional de camundongos treinados na tarefa do labirinto em T após receber MPTP i.p. assim como redução dos níveis de DA no estriado e no córtex. Nosso grupo demonstrou prejuízo na aquisição e retenção da memória na tarefa de esquiva ativa de duas vias em ratos lesados com MPTP na SNc (DA CUNHA *et al.*, 2001; GEVAERD *et al.*, 2001). MIYOSHI *et al.,* 2002 observaram prejuízo no desempenho do labirinto

aquático de Morris nas versões com dica visual e de memória operacional, mas não na versão de memória espacial, em ratos lesados com MPTP.

## 2.3. MEMÓRIA

A memória humana é atualmente considerada um complexo subsistema envolvendo diferentes redes anatomofuncionais, com provável uso de diferentes sistemas de neurotransmissão (DUJARDIN e LAURENT, 2003). Podemos defini-la como um mecanismo pelo qual ocorre à aquisição, retenção e recuperação das informações obtidas pelas experiências diárias (LENT, 2001). Memória operacional ou "*working memory*", tipo de memória classificada segundo a função, cumpre o papel de gerenciadora de nosso contato com a realidade, mantendo as informações por alguns segundos, no máximo de 1 a 2 minutos, variando de instante a instante. Este tipo de memória utiliza poucas vias nervosas, não deixa traços neuroquímicos ou comportamentais e não produz arquivos de registro (IZQUIERDO, 2002). Vários trabalhos sugerem que a principal área funcional cerebral relacionada à memória operacional é o córtex pré-frontal (BADDELEY, 1986, GOLDMANN-RAKIC *et al*., 1990, DUJARDIN e LAURENT, 2003).

Outras memórias são classificadas pelo tempo de duração, entre elas a memória de curta duração. Este tipo de memória estende-se a partir dos primeiros segundos ou minutos até 3-6 horas após o aprendizado, tempo necessário para a formação da memória de longa duração (IZQUIERDO *et al.*, 1999; McGAUGH,

2000). Para que isto aconteça, são necessárias várias fases envolvendo processos metabólicos no hipocampo e outras estruturas cerebrais até a consolidação ou armazenamento da mesma (IZQUIERDO, 2002).

Quanto ao conteúdo, as memórias podem ser classificadas como declarativas e procedurais. Memória declarativa é o tipo de memória que registra fatos, conhecimentos ou eventos, sendo possível relembrar, declarar e relatar conscientemente as informações (HAY, *et al.*, 2002). As memórias declarativas podem ser episódicas, isto é, o indivíduo é capaz de relembrar experiências passadas tais como situações vividas em determinado local e época da sua vida, ou semânticas, memórias relacionadas aos conhecimentos gerais. Comumente somos incapazes de recordar quando e onde tal informação foi adquirida (DUJARDIN e LAURENT, 2003). Memória procedural ou de procedimento pode ser definida como a aquisição gradual de uma habilidade motora ou cognitiva através de regular exposição a um procedimento específico. A aquisição é implícita, isto é, ocorre sem a percepção, e a memória é refletida através da redução do tempo de resposta e dos erros durante a execução repetida do procedimento (DUJARDIN e LAURENT, 2003).

Memória operacional, memória episódica e memória de procedimento mostram-se prejudicadas em pacientes com DP (DUJARDIN e LAURENT, 2003).

## 2.4. MEMÓRIA OPERACIONAL

Termo utilizado primeiramente por Douglas (1967) em seu trabalho sobre funções do sistema hipocampal. Segundo BECKER, 1999, memória operacional ou "*working memory*" pode ser definida como um sistema de componentes que interagem e mantêm a aquisição de novas informações e também a evocação de informações armazenadas, verbais e não verbais, e as disponibilizam para outros processos de informação. A memória operacional encontra-se na intersecção entre a memória, a atenção e a percepção, sendo um sistema necessário para simultaneamente armazenar e manipular as informações (BADDELEY, 1992). A informação é mantida "on line" na memória por um período de tempo necessário para que a mesma seja planejada, resolvida ou decidida. O processamento da memória operacional resulta em poucas alterações bioquímicas dependentes principalmente do córtex pré-frontal e a mesma não deixa traços e nem produz arquivos (IZQUIERDO, 2002). Déficits de memória operacional são encontrados em pacientes com a DP e estes têm sido relacionados a uma deficiência na função do córtex pré-frontal acompanhada de redução de DA nesta região, citado por Williams, 1995. A redução da atividade dopaminérgica no estriado e no lobo frontal na DP pode provocar prejuízo na memória operacional (STEBBINS *et al*., 1999). BROZOSKI *et al*., 1979, demonstraram que a depleção de DA no córtex pré-frontal de macacos resulta em déficits na memória operacional espacial.

A literatura nos mostra contradições em relação às regiões cerebrais envolvidas na memória operacional. Os núcleos da base recebem fibras com

diversas funções de diferentes regiões corticais e aferenta áreas corticais envolvidas com memória operacional e outras funções cognitivas (BROWN *et al.,* 1997). Os receptores dopaminégicos D1 contribuem na função cognitiva por atuação direta em áreas da memória em neurônios pré-frontais, tendo participação na memória operacional (WILLIAMS, 1995). De acordo com SKEEL *et al.*, 2001, na memória operacional podem estar envolvidas diferentes redes neurais em diferentes partes do cérebro. Segundo LALONDE 2001, a indução da lesão em diferentes regiões cerebrais como hipocampo, corpos mamilares, córtex pré-frontal, tálamo, área tegmental ventral, cerebelo, resulta em déficits na alternância espontânea, teste utilizado para avaliação da memória operacional. Em 1990, GARRETT e CRUTCHER concluíram que a arquitetura funcional dos núcleos da base é formada por diferentes circuitos paralelos, participando de funções distintas como processos cognitivos, límbicos e oculomotor. O córtex frontal é anatômica e funcionalmente heterogêneo, constituído de múltiplas subdivisões corticais. Cada subdivisão possui circuitos paralelos, independentes, para circuitos cortico-basais-talamocorticais, resultando no controle de aspectos distintos como, por exemplo, comportamento cognitivo e controle de complexas seqüências de movimento (NAKANO, 2000). Modelos animais e estudos clínicos mostram que as conseqüências comportamentais de disfunções dos núcleos da base resultam em distúrbios dos processos cognitivos (BROWN *et al.*, 1997).

# Lesion of the substantia nigra, pars compacta impairs delayed alternation in a Y-maze in rats

R. Braga[a], I. Kouzmine[a], Newton S. Canteras[b], and C. Da Cunha[a,*]

[a]Laboratório de Fisiologia e Farmacologia do Sistema Nervoso Central, Departamento Farmacologia, Universidade Federal do Paraná, C.P. 19.031, 81.531-990 – Curitiba, PR, Brazil

[b]Departamento de Fisiologia e Biofísica, Instituto de Ciências Biomédicas 1, Universidade de São Paulo, Av. Prof. Lineu Prestes 1524, 05.508-900 São Paulo, SP, Brazil

*Corresponding author. Fax: + 55 41 266-2042. E-mail Address: dacunha@ufpr.br

**Abstract**

Adult male Wistar rats with bilateral substantia nigra, pars compacta (SNc) lesion induced by intranigral administration of 0.5 μmol 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) were used as a model of early phase Parkinson's disease (PD). This treatment caused loss of dopaminergic cells in the SNc and a partial depletion of striatal dopamine. Animals trained up to 80% correct choices presented significantly worse scores after SNc lesion compared to sham-operated animals and spent almost 6 days to reach this criterion again, while sham-operated animals reached this criterion within about 2 days. When naive animals had their SNc lesioned before training, they scored worse than sham-operated animals and took 18 days to reach the 80% correct choices criterion, while sham-operated controls reached this criterion after only 10 days. These results suggest that lesion of the SNc impairs working memory in rats performing this task, in agreement with the working memory impairment in PD patients reported in clinical studies.

**Key words:** Working memory, Y-maze delayed alternation, delayed task, Parkinson’s disease, MPTP, dopamine, substantia nigra.

## Introduction

Parkinson's disease (PD) is characterized by a progressive loss of substantia nigra, pars compacta (SNc) dopaminergic neurons (Hirsch et al, 1988). In addition to motor impairments, cognitive impairments including deficits in working memory have been reported to occur in PD patients (Carbon and Marie, 2003; Costa et al., 2003; Crucian and Okun, 2003; Dubois and Pillon, 1997; Hodgson et al, 1999; Lewis et al., 2003; Owen et al., 1997; Stebbins et al, 1999; Valldeoriola et al, 1997). The idea that the SNc plays a role in cognition has been further substantiated by studies employing animal models of PD (Brown et al, 1997; Da Cunha et al., 2002; Lindner et al., 1999; Ogawa et al., 1987; Schneider and Kovelowski, 1990; Schneider and Pope-Coleman, 1995; Tanila et al., 1998).

Rats with bilateral SNc lesions induced by intranigral infusion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been successfully used as an animal model to study learning and memory impairment related to the early phase of PD (Bellissimo, 2004; Da Cunha et al, 2001, 2002, 2003; Gevaerd et al, 2001a,b; Miyoshi et al, 2002). MPTP is a potent neurotoxin that destroys dopaminergic nigral neurons (Langston et al., 1983; Ogawa et al, 1987). In humans it causes cognitive dysfunction comparable to that observed in PD (Sterm and Langston, 1985). The infusion of MPTP into the rat SNc causes only partial lesion of dopaminergic neurons and a proportional depletion of striatal dopamine (Da Cunha et al., 2002; Harik et al., 1987). This model proved to be ideal for cognitive studies because memory impairments are observed in the absence of gross motor alterations (Da Cunha et al., 2002).

The aim of the present study was to evaluate whether the working memory is affected in this model of PD by studying the effect of bilateral SNc lesions induced by MPTP on the performance of rats in the Y-maze delayed alternation task. The role of the SNc in learning this task was evaluated in rats that had their SNc lesioned before training. Memory retention was studied in another group of animals that were pre-trained before SNc lesion and then retested in the same task.

## Materials and methods

### *Subjects*

Fifty six adult male Wistar rats from our own breeding stock weighing 250–300 g at the beginning of the experiments were used. The animals were housed individually in Plexiglas home cages (30 x 25 x 25 cm) in a temperature-controlled room (22 ± 2°C) on a 12/12-h dark/light cycle (lights on at 07:00) with free access to water and placed under a food restricted diet (70-80% of their free food consumption), except for the time when they were recovering from surgery. Five days after surgery they had free access to a mixture of crumbed food and water. Behavioral experiments were carried out between 14:00 and 18:00. All efforts were made to minimize animal suffering and the guidelines for experimental animal care of the National Institutes of Health and the Brazilian Society for Neuroscience and Behavior (SBNeC) were strictly followed.

*Surgical procedures*

The animals were divided into sham-operated (*N* = 24) and MPTP-lesioned groups (N = 32). The rats received 0.4 mg/kg atropine sulfate (i.p.) and were anesthetized with 3 ml/kg Equitesin (1% thiopental, 4.25% chloral hydrate, 2.13% magnesium sulfate, 42.8% propylene glycol, and 3.7% ethanol in water). MPTP-HCl (0.5 µmol in 1 µl saline, Sigma, St. Louis, MO, USA) was infused into the SNc at a rate of 0.33 µl/min at the following coordinates adapted from the Paxinos and Watson (1986) atlas: anteroposterior (AP) - 5.0 mm from bregma; mediolateral (ML) ± 2.1 mm from midline; dorsoventral (DV) - 7.7 mm from skull. The injection needle was retained in place for an additional 2 min. Sham-operated animals were submitted to the same procedure but 1 µl saline instead of MPTP was infused bilaterally into the SNc. After surgery, the animals were allowed to recover from anesthesia in a temperature-controlled chamber and then placed in individual cages. During the first 5 postoperative days, the solid diet was replaced with a pasty diet to which the animals had free access. The pasty diet consisted of a mixture of the rats' crumbed ration mixed with water. Five MPTP-lesioned animals died before completing the behavioral experiments.

*Y-maze delayed alternation apparatus and procedure*

The apparatus was a wooden black Y-maze. Each arm of the maze was 15 cm wide, 30 cm long, and the walls were 30 cm high. One of the arms was used as the start area and the left and right arms had manually operated guillotine doors. Food pellets (Froot Loops, Kellogg's, Battle Creek, MI, USA) were placed at the end of these arms, behind a 2-cm high wall.

For 2 days the animals were individually confined for 5 min in each arm of the maze to take a food pellet placed at its end. During this time the doors of the arms remained closed. During the next 2 days, the animals were individually placed on the start arm of the maze with the doors of both arms open and allowed to explore for 5 min to freely take one food pellet at the end of the right and left arms. During the following days the animals were trained at 10 trials per day. A rat was placed in the start area with both doors closed. The doors were opened to allow the animal to choose from which arm to obtain a food pellet, available at the end of both arms. After the animal had entered with its four paws in an arm, the door was closed and the animal had 10 s to eat the food pellet. After this time the animal was removed from the maze and remained for 10 s in a small Plexiglas cage. In the next 10 trials this procedure was repeated but these times the food pellet was available only in the arm the animal had not visited in the previous trial. Rats were trained until they made at most 2 errors per day for 3 consecutive days or up to 21 days.

In experiment 1, 12 sham-operated and 14 SNc-lesioned animals were trained up to the criterion before surgery, left to recover from surgery for 5 days and then tested using the same training schedule for a further 12 days. In

experiment 2, 12 sham-operated and 13 SNc-lesioned rats, operated before training, were left to rest for 21 days and then trained in the delayed alternation task up to 21 days as described above.

*Histology and lesion analysis*

After the behavioral tests, the animals were sacrificed by decapitation. The striata were removed and stored at -70ºC and the midbrains were placed in 4% formalin for one week and then in 20% sucrose formalin for 48 h before sectioning. The brainstems of 4 randomly chosen animals in each group were frozen and four series of 30-µm thick sections were cut with a sliding microtome on the frontal plane and collected throughout the levels of the midbrain dopaminergic cell groups. One series of sections was immunostained for tyrosine hydroxylase (TH) using a monoclonal anti-TH antibody raised in mice (Incstar Corp., Stillwater, MN, USA) at a dilution of 1:5000. The primary antiserum was localized with a variation of the avidin-biotin complex system using a commercially available kit (ABC Elite kit, Vector Laboratories, Burlingame, CA, USA). An adjacent series was stained with thionin to evaluate the MPTP injection-induced tissue damage.

*Determination of striatal dopamine levels*

Endogenous dopamine levels of 8 randomly chosen animals in each group were assayed by reverse-phase high performance liquid chromatography with electrochemical detection (HPLC-ED) as described elsewhere (Da Cunha et al., 2001). Briefly, the HPLC-ED system consisted of a C18 reverse-phase column (Shim-pack, CLC-ODS, 250 x 4.6 mm, Shimadzu, Columbia, MD, USA), an amperometric electrochemical detector (Decade, VT-03 flow cell, Antec Leyden, Zoeterwoude, The Netherlands) and a liquid chromatography workstation (CLASS-VP 5032, Shimadzu). The tissue samples were homogenized in 0.2 M perchloric acid with a microultrasonic cell disrupter (Kontes, Vineland, NJ, USA). After centrifugation (12,000 g, 10 min), 20 µl of the supernatant was injected into the column. The mobile phase used at a flow rate of 0.9 ml/min had the following composition: 292 mg NaCl, 15.7 g citric acid, 70 mg octyl sodium sulfate, 20 ml acetonitrile, 10 ml tetrahydrofuran, water (Millique, Millipore, Billerica, MA, USA) completed to 500 ml, and pH adjusted to 3.0 with NaOH. The oxidation potential was + 0.85 V versus an in situ Ag/AgCl reference electrode (Antec Leyden). The peak areas of external standards were used to quantify the sample peaks.

*Statistical analysis*

The percentage of correct answers for the individual trials was averaged by day and analyzed by two-way ANOVA for repeated measures (session day). The

number of days it took to reach the criterion was also analyzed by two-way ANOVA, but, in this case, the pre-/post-surgery results were considered as repeated measures, followed by the Duncan test. Differences in the striatal concentration of dopamine were analyzed by one-way ANOVA. Differences between groups were considered to be statistically significant when $p \leq 0.05$.

## Results

### *Lesion analysis and dopamine depletion*

The administration of MPTP caused neuronal loss which was mainly restricted to the SNc, spreading only modestly to the neighboring brain sites (see Fig. 1). This treatment also caused a significant decrease of striatal dopamine concentration in lesioned animals (mean ± SEM = 1016 ± 569 ng/g tissue) compared to controls (5161 ± 369 ng/g tissue; $F(1,14) = 39.21$, $p < 0.001$, ANOVA).

### *Experiment 1*

As can be seen in Fig. 2, all non-operated animals learned to alternate the entrance in the arms of the Y-maze, reaching the criterion of 80% correct answers

within 10 days. Two-way ANOVA showed that animals in both groups significantly improved their scores ($F(18,432) = 14,91$, $p \leq 0.01$), with no significant difference between groups ($F(1,24) = 0.21$, $p \geq 0.2$) and no significant interaction between group X day factors ($F(18,432) = 1.13$, $p \geq 0.3$). After surgery, the SNc-lesioned group presented significantly lower scores compared to the sham-operated group ($F(1,24) = 8.30$, $p \leq 0.01$). Both groups improved their scores during these post-surgery retesting days ($F(11,264) = 5.89$, $p \leq 0.001$), and no significant lesion X day interaction was observed ($F(11,264) = 1.27$, $p \geq 0.2$). However, sham-operated rats achieved the 80% criterion again in almost 2 days, while the SNc-lesioned rats took almost 6 days to re-reach this criterion, with the difference being significant ($F(1,24) = 4.44$, $p \leq 0.05$, lesion factor; $F(1,24) = 46.02$, $p \leq 0.001$, pre-/post-surgery factor; $F(1,24) = 2.80$, $p = 0.10$, interaction lesion X pre-/post-factors, two-way ANOVA).

*Experiment 2*

As shown in Fig. 3, naive rats submitted to SNc lesion before training in the Y-maze delayed alternation task presented worse training scores than sham-operated animals. Two-way ANOVA showed a significant effect of the lesion ($F(1,22) = 5.28$, $p \leq 0.05$) and day factors ($F(20,440) = 6.66$, $p \leq 0.001$), and a non significant interaction between lesion X day factors ($F(20,440) = 1.54$, $p = 0.06$). SNc-lesioned animals reached the 80% correct choices criterion only after 18 days of training, a number significantly higher than the 11 days spent by the sham group to reach this criterion ($F(1,22) = 13.60$, $p \leq 0.01$, one-way ANOVA).

## Discussion

The selective bilateral lesion of the rat SNc induced by MPTP caused histological, neurochemical, and physiological alterations which were in many aspects similar to the early phase of PD, in agreement with previous studies (Da Cunha et al., 2002; Langston et al, 1983). Cognitive deficits have been reported to occur during all phases of PD (Dubois and Pillon, 1997; Faglioni et al, 1997).

The present study reports impairment of the ability of bilateral SNc-lesioned rats to alternate between the left and right arms in order to find a food pellet in a Y-maze. Since there was a delay of 10 s between trials, the animals had to maintain on line the information of which arm they had previously visited in order to make the correct choice when returning to the maze. This result suggests that the SNc plays a role in working memory, in agreement with previous studies reporting that bilateral (Miyoshi et al., 2002) or left SNc-lesioned rats (Bellissimo et al., 2004) failed to perform a spatial working memory version of the Morris water maze. It is also in agreement with other studies suggesting working memory impairment in mice (Tanila et al., 1998) and monkeys (Schneider and Kovelowski, 1990; Schneider and Pope-Coleman, 1995) after mild MPTP treatment, and with studies reporting visuospatial working memory impairment in PD patients (Carbon and Marie, 2003; Costa et al., 2003; Crucian and Okun,

2003; Dubois and Pillon, 1997; Hodgson et al, 1999; Lewis et al., 2003; Owen et al., 1997; Stebbins et al, 1999; Valldeoriola et al, 1997).

Alternation between the arms of a maze after a short-term delay has been proposed as a model of working memory (Lalonde, 2002). The more popular version of this task uses a T-maze in which rats spontaneously alternate arm choices on successive trials (Dember and Fowler, 1958, Lalonde, 2002). However, other kinds of mazes, including the plus-maze (Stefani et al., 1999) and the 8-arm radial maze (Brito and Brito, 1990), have also been used. Closer to the version used in present study, some studies forced the alternation by blocking one of the arms in the first trial, rewarding alternation or punishing perseveration during multiple trials (Means, 1971; Thomas, 1972; Thomas and Spafford, 1984). More specifically, the spontaneous alternation task is considered to be a model of spatial working memory that depends on hippocampal integrity (Johnson et al., 1977; Kirkby, 1967; Lalonde, 2002; Means et al., 1971; Roberts et al., 1962; Stevens and Cowey, 1973). Therefore, our finding that SNc-lesioned rats are impaired in the Y-maze alternation task may correspond to a model of the spatial working memory impairment reported to occur in PD patients (Costa et al., 2003; Crucian and Okun, 2003; Dubois and Pillon, 1997; Hodgson et al, 1999; Owen et al., 1997). However, the hippocampal/spatial nature of this task should be taken with caution since dorsal hippocampus-lesioned rats can learn the forced version of the T-maze alternation task (Dalland, 1976). In the forced version and in the version in which alternation is rewarded with food, the animals have to learn an alternation rule.

As they choose between only two options, this gradual learning can be considered to be a kind of stimulus-response learning in which the animals need to discriminate the arms based on visual information. Rats can also use kinesthetic or egocentric information, since they present normal alternation even after removal of their retina (Dember, 1958; Dember and Roberts, 1958). In agreement with this, lesions of structures supposed to mediate stimulus-response learning, like the ventral tegmental area in rats (Taghzouti et al., 1988) and the caudate nucleus in monkeys (Batting et al., 1960; Butters and Rosvold, 1968; Divac et al., 1967; Rosvold et al., 1958), reduce two-choice alternation.

The use of rats with bilateral lesions of the SNc induced by MPTP proved to be a good model for the study of memory impairments associated with PD. As shown in the TH-immunohistochemistry photomicrographs of the present study (Fig. 1), this treatment resulted in partial lesion of the SNc and a proportional depletion of striatal dopamine, in agreement with previous studies (Bellissimo, 2004; Da Cunha et al., 2001, 2002, 2003; Harik et al., 1987; Miyoshi et al., 2002). This picture models the early phase of PD when learning and memory impairments are observed in the absence of motor alterations (Dubois and Pillon, 1997). Previous studies have shown that after MPTP SNc-lesioned rats had recovered from surgery, they presented no gross sensorimotor disturbances, were not aphagic or adipsic, and scored like controls in the open field and rota-rod tests (Da Cunha et al., 2001; Miyoshi et al., 2002). Nevertheless, working memory studies in the water maze task involved the problem that impairment was deduced from latency or swimming path to find a

hidden platform (Bellissimo, 2004; Miyoshi et al., 2002). Since PD is characterized by motor impairments, although many controls were included to rule out interference of motor impairments in MPTP SNc-lesioned rats, one would suspect that a longer latency or path resulting from locomotor behavior in an animal model of PD would be reflecting more motor than true working memory impairment. The present study suggests that this was not the case since here the animals were scored by percentage of correct choices. Therefore, no matter the time spent to make the choice or the path chosen, i.e., sinuous or straight to the right or left arms, only the correctness of the choices was scored. In order to make a correct decision the animals simply had to remember the arm visited 10 s before. Therefore, the fact that these SNc-lesioned animals scored worse than controls in this task strongly suggests that the SNc plays a critical role in working memory.

If learning the delayed alternation rule was impaired in SNc-lesioned animals, this does not necessarily imply working memory impairment. Furthermore, for animals submitted to SNc lesion after they had learned to alternate in the Y-maze, impairment would mean that they had forgotten the delayed alternation rule instead of working memory impairment. We solved this dilemma by showing that SNc lesion impaired delayed alternation in both naive or trained rats. Naive rats were first submitted to SNc lesion and then trained to alternate in the Y-maze. Trained rats learned to alternate in the Y-maze prior to surgery and were then retested in the delayed alternation task. The latter experiment suggested that SNc lesion impaired retention of the alternation rule but, even after

extensive retraining, the rats continued committing more choice mistakes than controls, suggesting working memory impairment (see Fig. 2B). In the former experiment, naive SNc-lesioned rats needed more training sessions to learn the delayed alternation rule, a fact suggesting impairment to learn this rule. However, even after 21 days, these animals continued to score worse than controls. Therefore, they probably presented working memory impairment. Considering the compelling body of evidence suggesting that SNc lesioned animals (Bellissimo et al., 2004; Miyoshi et al., 2002; Schneider and Kovelowski, 1990; Tanila et al., 1998) and PD patients (Carbon and Marie, 2003; Costa et al., 2003; Crucian and Okun, 2003; Dubois and Pillon, 1997; Hodgson et al, 1999; Lewis et al., 2003; Owen et al., 1997) present working memory deficits, the present results can be viewed from a different angle: working memory impairment was probably the cause of impaired acquisition and retention of the alternation rule in SNc-lesioned rats.

In summary, the present study provides further evidence corroborating the theory that SNc plays a key role in working memory, whose impairment can be observed in many animal as well as clinical studies.

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Figure legends

Fig. 1. A, B, C - Brightfield photomicrographs illustrating a tyrosine hydroxylase-immunostained section obtained from an animal unilaterally injected with 0.5 µmol MPTP. D – Brightfield photomicrograph of an adjacent thionin-stained section showing small neuronal loss surrounding the injection site in the SNr. Abbreviations: VTA – ventral tegmental area; SNc – substantia nigra pars compacta; SNr – substantia nigra pars reticulata. Scale bars = 200 µm.

Fig. 2. Effect of SNc lesion after training in the spontaneous alternation task. Data are expressed as percent correct choices (entering the alternate arm of the Y-maze). Two-way ANOVA showed a significant difference ($p \leq 0.05$) between SNc-lesioned (open circles) and sham-operated animals (closed circles). * $p \leq 0.05$ compared to sham; + $p \leq 0.05$ compared to the same group before surgery (Duncan test).

Fig. 3. Effect of SNc lesion before training in the spontaneous alternation task. Data are expressed as percent correct choices (entering the alternate arm of the Y-maze). Two-way ANOVA showed a significant difference ($p \leq 0.05$) between SNc-lesioned (open circles) and sham-operated animals (closed circles). * $p \leq 0.05$ compared to sham (Duncan test).

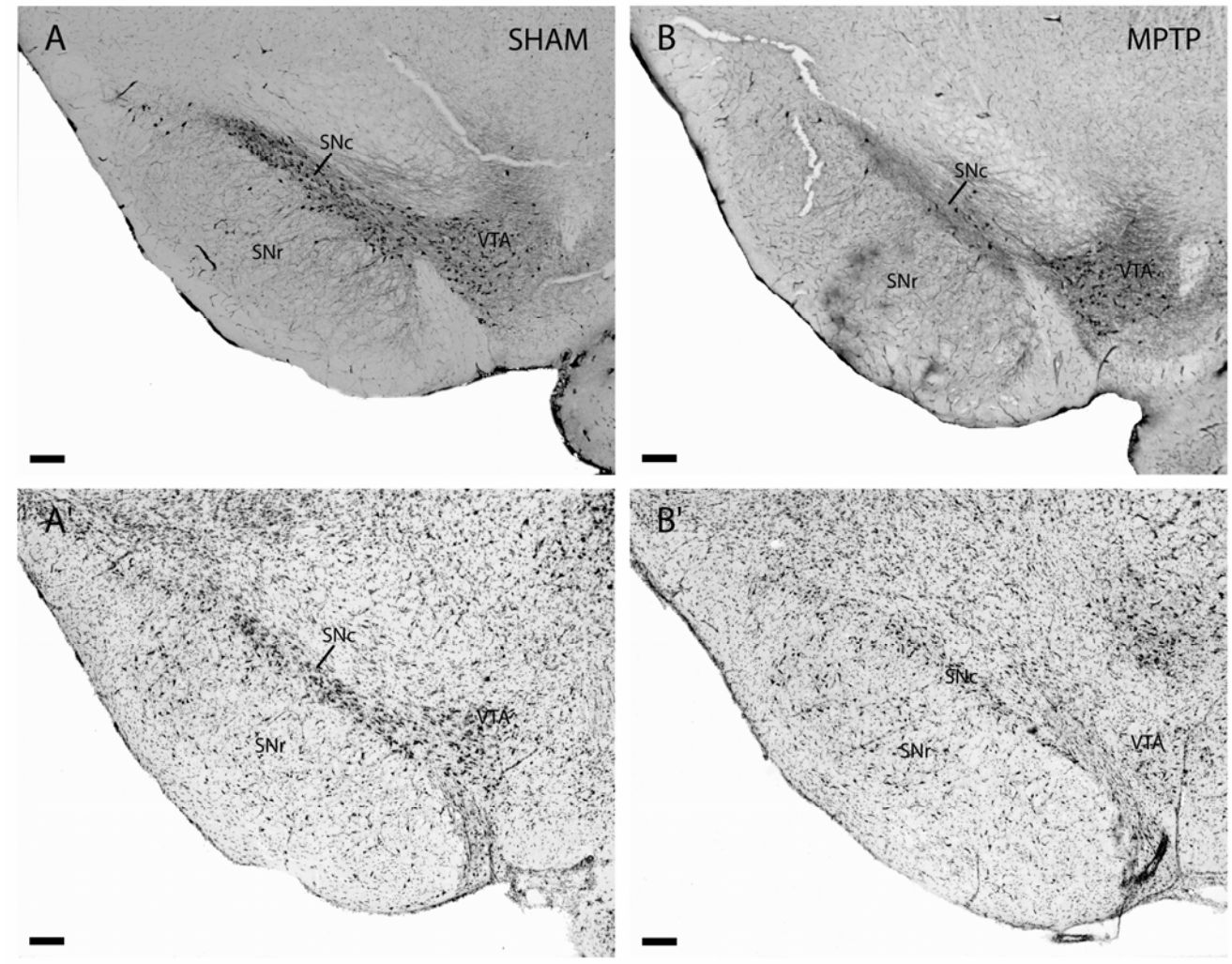


**Figure 1**

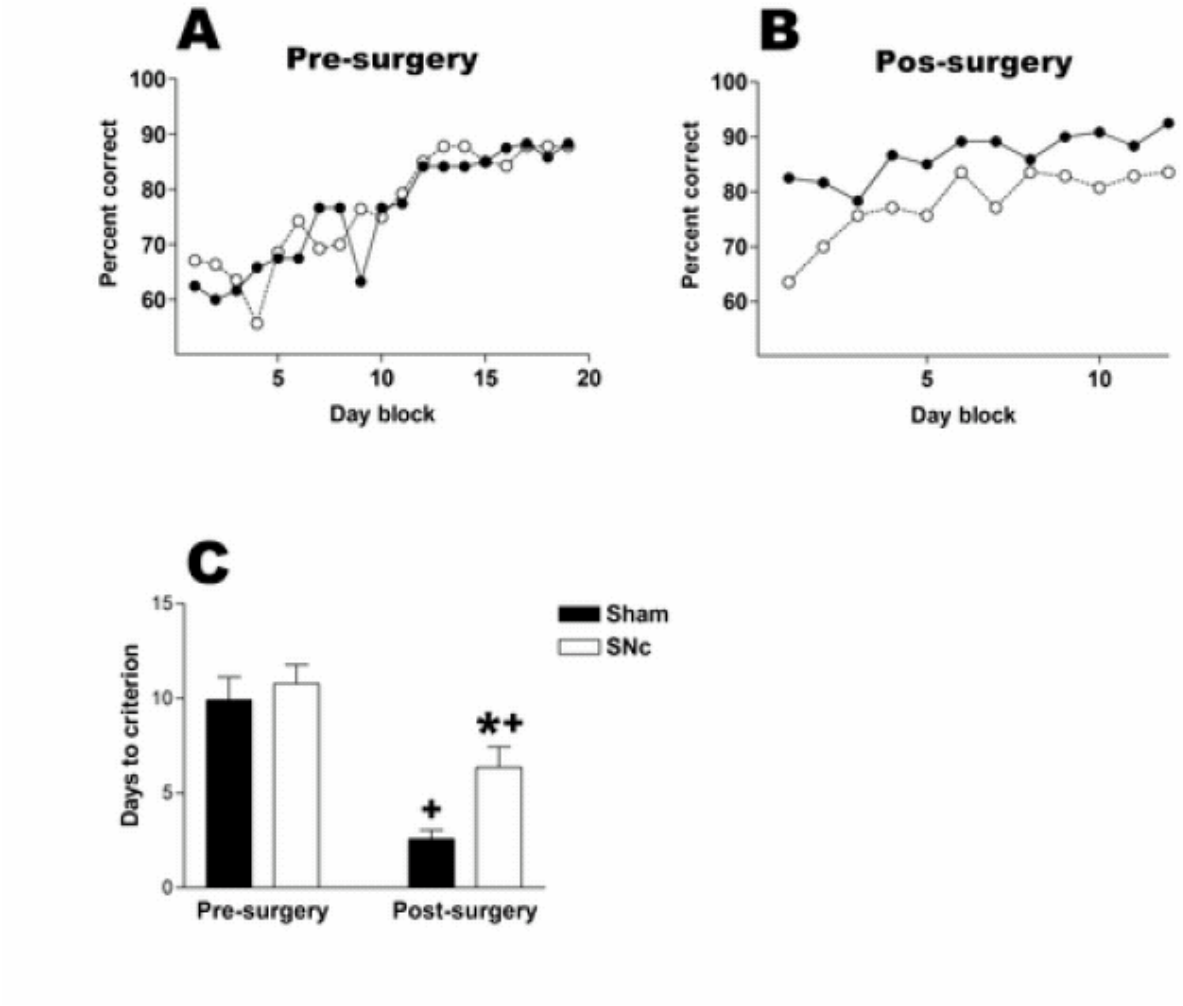
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**Figure 2**

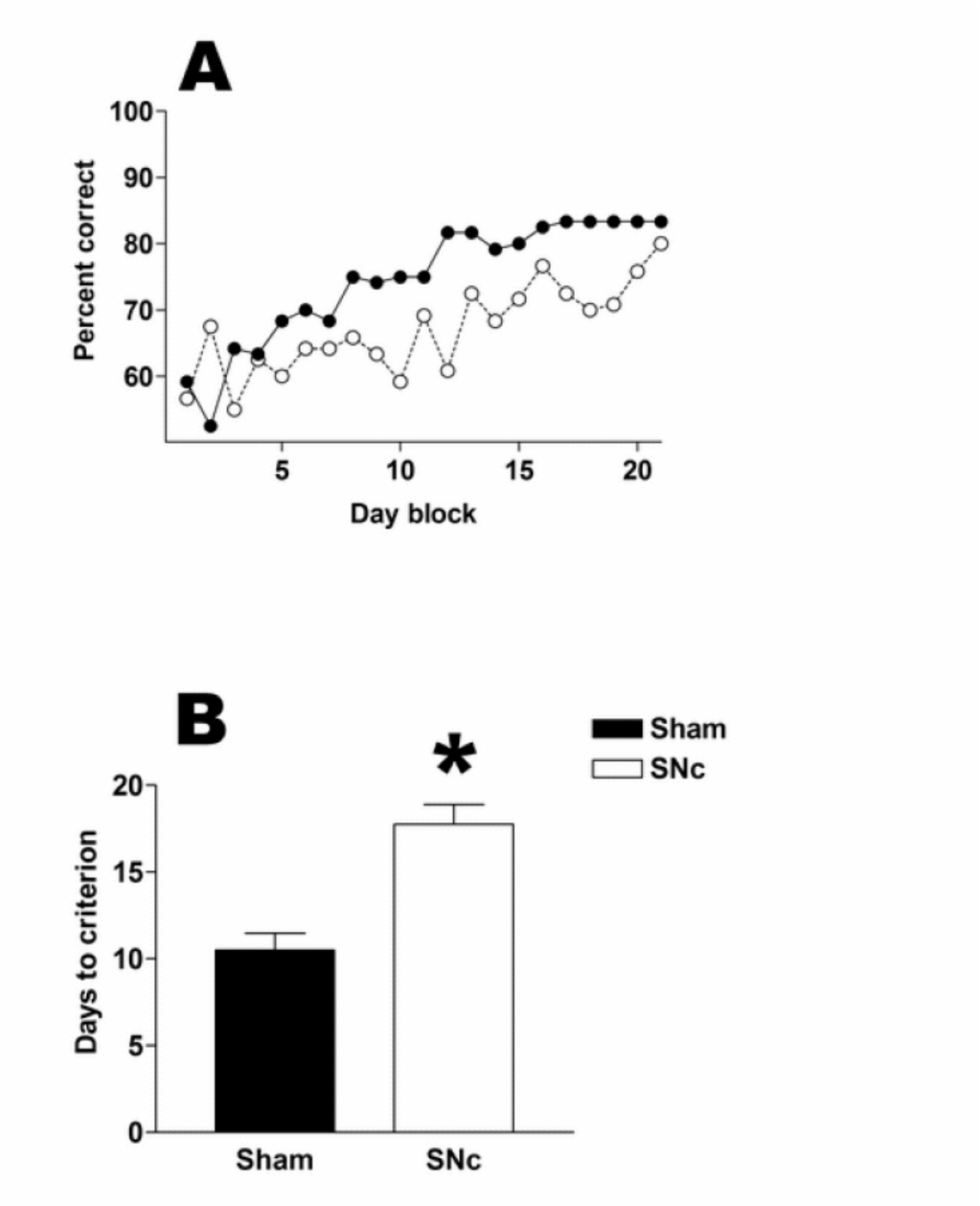
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**Figure 3**

## 4. CONCLUSÃO

Os resultados deste estudo nos mostraram que a lesão induzida por MPTP na SNc produz déficits de memória operacional em ratos submetidos ao teste da alternância em um labirinto em Y. Isto nos sugere que a SNc assim como a via nigroestriatal podem estar envolvidas neste tipo de memória.

Ratos lesados com MPTP na SNc podem ser utilizados como modelo experimental da DP para estudar déficits de memória operacional, uma vez que estas alterações também são encontradas em pacientes com DP.

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