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**ESTUDO DA DEPRESSÃO  
ASSOCIADA A MODELOS ANIMAIS DA  
DOENÇA DE PARKINSON**

**MESTRADO FARMACOLOGIA-UFPR  
CURITIBA-PR  
2010**

UNIVERSIDADE FEDERAL DO PARANA

## **Estudo da Depressão Associada a Modelos Animais da Doença de Parkinson**

Dissertação apresentada à Universidade Federal do Paraná – Setor de Ciências Biológicas - Departamento de Farmacologia, como requisito parcial à obtenção do título de Mestre em Farmacologia.

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Curitiba – PR

2010

## **Agradecimentos**

Ao meu marido Marcelo Sato, o amor da minha vida e minha grande inspiração, pelo amor, incentivo, paciência e compreensão, sem você ao meu lado esta caminhada teria sido mais difícil. Te amo.

Aos meus pais e irmãos, que, com muito carinho e apoio, não mediram esforços para que eu chegasse até esta etapa de minha vida.

A minha orientadora Prof<sup>a</sup>. Dr<sup>a</sup> Maria Vital por sua orientação e amizade, obrigada pelos ensinamentos e confiança depositados em minha pessoa.

A minha grande amiga Janaina Barbiero, pela amizade sincera.

Ao Prof. Dr Marcelo Lima pela contribuição, que sem dúvida foi fundamental na conclusão deste trabalho.

Ao Prof. Dr Roberto Andreatini pela sua co-orientação e sugestões sempre construtivas.

Aos professores da especialização, pelo conhecimento transmitido com tanta clareza.

Aos funcionários do setor, em especial a farmacêutica Silvia Genari, pela paciência e dedicação.

Aos colegas da farmacologia, pelos momentos descontraídos no corredor do departamento de farmacologia.

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## RESUMO

Doença de Parkinson (DP) é uma doença neurodegenerativa, cujos sintomas incluem bradicinesia, tremor em repouso, instabilidade postural e muitas vezes depressão. Além de sintomas motores os pacientes também podem apresentar sintomas não motores como a depressão. A prevalência de depressão nesses pacientes varia entre 40% a 70%. O presente estudo teve como objetivo investigar comparativamente o perfil de respostas motoras, depressivas e neuroquímicas produzidas pelas infusões intranigrais das neurotoxinas MPTP, 6-OHDA, rotenona e LPS. Os ratos receberam as toxinas bilateralmente na substância negra através da cirurgia estereotáxica. Para avaliar o comportamento do motor, foi utilizado o teste de campo aberto, para a avaliação de depressão o teste da natação forçada modificado e o teste de preferência de sacarose. Os animais foram divididos aleatoriamente em 6 grupos, controle, sham, MPTP, 6-OHDA, rotenona e LPS. A avaliação da atividade locomotora foi realizada nos dias 1, 7, 14 e 21 após a cirurgia. No teste de natação forçada, foi observado o tempo de natação, escalada e imobilidade dos animais, este procedimento foi realizado 23 após a cirurgia. O teste de preferência de sacarose foi aplicado nos dias 7, 14 e 21 após a cirurgia. No campo aberto os grupos MPTP, rotenona e 6-OHDA apresentaram diminuição da frequência de locomoção e levantar apenas no dia 1 após a cirurgia em comparação com os grupos controle ( $p < 0,05$ ). No teste de natação forçada modificado os resultados indicaram que a infusão de MPTP, 6-OHDA e rotenona reduziu o tempo de natação, além disso, nos ratos do grupo 6-OHDA ocorreu aumento do tempo de imobilidade em relação a grupos controle ( $p < 0,05$ ). No teste de preferência de sacarose os animais lesados por MPTP, 6-OHDA e LPS apresentaram uma redução de preferência no 7 dia após a cirurgia enquanto os ratos dos grupos MPTP, 6-OHDA e rotenona apresentaram redução de preferência no 21 dia após a cirurgia. Estas respostas alteradas comportamentais foram acompanhadas por redução do número de neurônios na substância negra detectada através da coloração de Nissl para todos os grupos experimentais e por diminuição da DA estriatal, ácido homovanílico (HVA) e ácido 3,4-dihidroxifenilacético (DOPAC) restrita aos ratos do grupo 6-OHDA. Além disso, houve redução dos níveis de serotonina hipocampal (5-HT) nos animais lesados por MPTP, 6-OHDA e rotenona. Mais ainda, fortes correlações foram detectadas entre os grupos, quando 5-HT e DA foram correlacionadas com a natação ( $r = 0,97$ ,  $P = 0,001$ ) e imobilidade ( $r = -0,90$ ,  $P = 0,012$ ), respectivamente. Nossos dados indicam que o MPTP, a 6-OHDA e a rotenona, mas não o LPS, foram capazes de produzir comportamentos depressivos acompanhado principalmente por redução hipocampal de 5-HT. Além disso, a DA e 5-HT estão fortemente correlacionadas com a deficiência emocional sugerindo uma importante participação desses neurotransmissores na anedonia e desespero comportamental após lesões nigral promovido pela neurotoxinas.

Palavras-chave: doença de Parkinson, depressão, MPTP, 6-OHDA, rotenona, LPS, ratos

# 1-INTRODUÇÃO

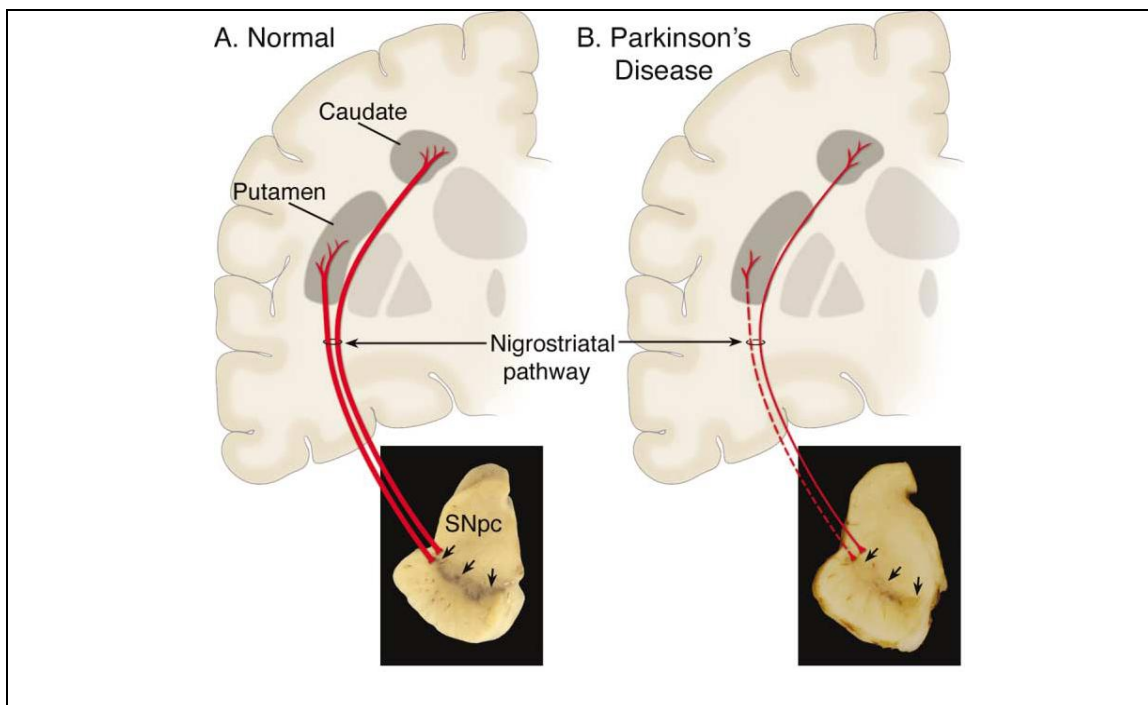
## 1.1 Doença de Parkinson

A doença de Parkinson (DP) foi primeiramente descrita pelo médico britânico James Parkinson, em seu mais famoso artigo, “*An essay on the shaking palsy*”, de 1817, que a denominou “paralisia agitante”. A mudança do nome para Doença de Parkinson foi sugerida por Charcot, em homenagem a descrição clássica de James Parkinson (TEIVE;MENEZES, 2003). .A DP é uma enfermidade neurodegenerativa, progressiva constituída principalmente por alterações de movimento que causam tremores, acinesia e/ou hipocinesia, rigidez muscular, desequilíbrio, instabilidade postural e marcha em festinação (FUCHS et al, 2004; GUIMARÃES;ALEGRIA 2004; LEE;LIU, 2008; FRISINA et, 2009; CICCHETTI et, 2009). É a segunda doença neurodegenerativa mais frequente entre as pessoas idosas ficando atrás somente da doença de Alzheimer (HOWLAND;MYCEK, 2006; DAUER;PRZEDBORSKI, 2003; ELBAZ;TRANCHANT, 2007; BARTELS;LEENDERS, 2009), com prevalência de 1-2% entre esta população (FUCHS et al, 2004; CAMARGO et al, 2004; LIBERMAN, 2006; MANOR et, 2009, LEES et, 2009; BARTELS;LEENDERS, 2009). A idade média de início da doença é de 60 anos. (LEES et, 2009).

A origem neuroquímica da DP foi elucidada em 1960 por Hornykiewicz, que demonstrou que o conteúdo de dopamina (DA) da substância negra *pars compacta* (SNpc) e do corpo estriado em encéfalos *post-mortem* de pacientes com a DP, era muito baixo, e estava associado à redução do número de neurônios dopaminérgicos na via nigroestriatal (GERLACH;RIEDERER, 1996, MEREDITH et al, 2008; HORNYKIEWICZ, 2006), a perda destes neurônios nigroestriatais por sua vez ocasiona a despigmentação da SNpc

(DAUER;PRZEDBORSKI, 2003; LEE;LIU, 2008, BARTELS;LEENDERS, 2009)

como ilustrado na Figura 1.



**Figura 1.** Morte neuronal na DP. A) via nigroestriatal normal, os neurônios dopaminérgicos se localizam na SNpc e se projetam para o estriado (núcleo caudado e putâmen); B) o esquema mostra a via nigroestriatal de um paciente com DP, a via nigroestriatal se degenera com a perda acentuada de neurônios dopaminérgicos, demonstrando também a despigmentação da SNpc devido a perda desses neurônios. Fonte: DAUER;PRZEDBORSKI (2003).

Outra característica fisiopatológica encontrada em encéfalos *post mortem* de pacientes portadores da DP é a presença de corpúsculos de Lewy em diversas regiões mesencefálicas. Esses corpúsculos são caracterizados pela presença de agregados protéicos, que possuem um diâmetro aproximado de 15  $\mu\text{m}$ , compostos por proteínas como: parkina, ubiquitina e principalmente pela proteína sináptica  $\alpha$ -sinucleína (FUCHS et al, 2004; DAUER;PRZEDBORSKI, 2003; ELBAZ;TRANCHANT, 2007; LEE;LIU, 2008; CICCHETTI et al, 2009).

O aparecimento dos sintomas motores está relacionado com a neurodegeneração de aproximadamente 50% dos neurônios dopaminérgicos do mesencéfalo e a perda de 80 a 90% do conteúdo de DA estriatal (HERNÁNDEZ-MONTIEL, 2006; FUCHS et al, 2004; LEE;LIU, 2008; WARRAICH et al, 2009). O início das manifestações motoras quase sempre é insidioso, unilateral e assimétrico (LEES et al, 2009). O paciente parkinsoniano inicialmente relata sintomas pouco específicos como sensação de dor ou dormência, fraqueza ou dores musculares (GUIMARÃES;ALEGRIA, 2004), a natureza e a severidade dos sintomas variam de um paciente para outro. O tremor de repouso tem característica distal, geralmente com início em uma das mãos, como movimentos de contar dinheiro ou enrolar cigarro executados pelo polegar e indicador de forma alternada durante o repouso. Em alguns casos pode acometer primeiramente o membro inferior ou até mesmo a mandíbula (FUCHS et al 2004). A rigidez é caracterizada por ser do tipo “roda dentada” afetando a maioria dos pacientes de forma discreta na fase inicial, por atingir apenas alguns grupos musculares. A marcha em festinação é uma manifestação típica resultante da associação de bradicinesia, rigidez muscular e postura anormal (FUCHS et al 2004).

Como os sinais e sintomas clássicos da doença são fundamentalmente motores, pensava-se que a condição física do paciente era o principal foco de acometimento da DP, contudo, a importância das complicações secundárias que aparecem ao longo da progressão da doença, levando ao comprometimento mental, emocional e social do paciente (CAMARGO et al 2004), vem aumentando com o decorrer dos anos. Dentre os fenômenos não motores podemos destacar as alterações olfatórias, distúrbios do sono,

distúrbio da fala, dificuldade de mastigação, fadiga, perda de peso, alterações cognitivas e principalmente queixas depressivas podem acompanhar a DP (FUCHS et al, 2004; GUIMARÃES;ALEGRIA, 2004; WOLTERS, 2008).

Portanto, a identificação, estudo e tratamento desses distúrbios cognitivos, do sono, do humor, gastrintestinais e cardíacos, compõem uma nova ordem dentro da abordagem e manejo de pacientes com DP (FUCHS et al, 2004; GUIMARÃES;ALEGRIA, 2004; WOLTERS, 2008, FRISINA et, 2009).

## **1.2 Etiologia da DP**

Evidências sugerem que o processo fisiopatológico da DP seja multifatorial (HERNÁNDEZ-MONTIEL, 2006; SING;DIKSHI, 2007; REALE et al, 2009). A patogênese da DP está sob intensa investigação e fatores como a produção de espécies reativas de oxigênio (ROS), a neuroinflamação, a excitotoxicidade, a fosforilação oxidativa, e a apoptose, são discutidas como importantes processos de morte celular (DEHMER, 2004). Já sabemos também que as disfunções no complexo I da cadeia mitocondrial podem contribuir para a degeneração celular neuronal em decorrência do declínio na síntese de trifosfato de adenosina (ATP) (CHEN;LE, 2006).

Algumas das hipóteses propostas para explicar a etiologia da DP idiopática estão descritas nos itens subsequentes.

### **1.2.1 Estresse oxidativo**

O estresse oxidativo é causa de dano neuronal em várias doenças neurológicas incluindo a DP (MOSLEY et al, 2006), é definido como a situação na qual a formação de espécies reativas (de oxigênio, nitrogênio, etc.) excede

a capacidade de defesa antioxidante e de reparo da célula, tendo como consequência o aumento de danos a biomoléculas (DNA, lipídios, proteínas). Estes danos, quando não reparados, acabam comprometendo o funcionamento da célula levando-a a morte por apoptose ou necrose (BARBOSA et al, 2006, ZHOU et al 2009).

Regiões encefálicas ricas em catecolaminas são extremamente vulneráveis a geração de radicais livres (MOSLEY et al, 2006), a DA, por exemplo, durante o seu processo de metabolismo gera radicais livres como o peróxido de hidrogênio ( $H_2O_2$ ), o ânion superóxido ( $O_2^-$ ) e o peroxinitrito ( $ONOO^-$ ). A metabolização da DA pode ocorrer através de enzimas como a monoamino oxidase (MAO) ou por decomposição espontânea através da auto-oxidação, neste caso ocorre liberação do radical livre  $H_2O_2$  e DA-quinona. Níveis elevados de  $H_2O_2$  podem liberar íons férricos de heme proteínas, como a hemoglobina e citocromo, que por sua vez reduzem o  $H_2O_2$  a radical hidroxila ( $HO^-$ ). O  $HO^-$  é o radical mais reativo encontrado *in vivo* capaz de oxidar carboidratos, lipídios, proteínas e DNA. Portanto, o metabolismo da DA pode agravar os danos nos tecidos pelo aumento dos níveis do radical livre  $H_2O_2$  (MOSLEY et al, 2006; BARBOSA et al, 2006).

### **1.2.2 Neuroinflamação**

A inflamação é a primeira linha de defesa do organismo contra lesões teciduais ou infecções, no entanto, uma resposta inflamatória excessiva pode se tornar fonte de uma lesão tecidual ainda maior do que a provocada pelo estímulo inicial. Os neurônios, como resultado de sua grande diferenciação celular, apresentam pouca ou nenhuma habilidade em se dividirem e uma fraca

capacidade de recuperação frente a lesões, tornando-se, portanto extremamente vulneráveis a processos auto-ímmunes e inflamatórios (GAO et al., 2003).

Na resposta inflamatória inicial o sistema imune inato inicia a ativação de uma cascata de eventos que implica no recrutamento do sistema imune adaptativo. Na DP a formação de corpúsculo de Lewy ou a morte de neurônios, ativa a ação neurotóxica do sistema imune inato, compreendido principalmente por células de linhagem mielóide, que são as primeiras a responderem ao dano e cuja função principal é a defesa do encéfalo (MOSLEY et al, 2006). A ativação microglial produz fatores neurotóxicos tal como o óxido nítrico (NO) (MOSLEY et al, 2006, BARBOSA et al, 2006), além de um aumento da expressão da enzima cicloxigenase-2 (COX-2) aumentando conseqüentemente a síntese de prostaglandinas (PGs) (HALD;LOTHARIUS, 2005). Processos inflamatórios associados com um aumento na expressão da enzima COX-2 e elevadas concentrações de PGE2 estão implicadas na cascata de eventos deletérios levando a neurodegeneração. Neste sentido, a expressão de COX-2 tem sido associada com a degeneração de neurônios dopaminérgicos da SNpc tanto em humanos quanto em modelos animais da DP, (LIMA et al, 2006; TEISMANN et al., 2003). A COX-2 esta fortemente associada com a neurodegeneração e a estimulação de um processo inflamatório seguido de morte neuronal (Lima et al., 2006; REKSIDLER et al., 2007).

O lipopolissacarídeo (LPS), uma endotoxina encontrada na membrana externa de bactérias Gram-negativas, é um potente estimulante de células imunes (macrófagos e monócitos) e células da glia, provocando a liberação de várias citocinas pró-inflamatórias e radicais livres, tornando-se uma ferramenta

para o estudo da neuroinflamação e a neurodegeneração dopaminérgica (DUTTA et al, 2008). Lima et al (2006) demonstraram que a produção aumentada de COX-2 induzida pelo LPS gerou um aumento da ativação da microglia na SNpc, que por si só, pode ser interpretado como uma manifestação de danos no sistema dopaminérgico. Considerando esses achados, os autores sugeriram ainda que o LPS pode ser considerado como um modelo neuroinflamatório da DP, em termos de regulação aumentada de COX-2 algumas horas após a exposição (LIMA et al. 2006).

### **1.2.3 Excitotoxicidade**

Com relação à excitotoxicidade, sabe-se que o glutamato é o principal neurotransmissor, responsável por um terço de todas as sinapses no Sistema Nervoso Central (SNC) (HERNÁNDEZ-MONTIEL, 2006 e BARBOSA et al, 2006). Sob certas condições, como insuficiência na captação de glutamato ou despolarização prolongada da terminação nervosa, o glutamato pode se acumular na fenda sináptica e desencadear um processo excitotóxico. Concentrações elevadas de glutamato causam a despolarização excessiva do neurônio pós-sináptico e, conseqüentemente, distúrbios da homeostasia iônica e energética, ativação de enzimas líticas mediada por cálcio ( $Ca^{+2}$ ), geração de radicais livres, lesão mitocondrial e edema osmótico, podendo levar à lise celular e a morte neuronal (HERNÁNDEZ-MONTIEL, 2006; BARBOSA et al, 2006; CHEN;LE, 2006).

#### 1.2.4 Fatores ambientais

Os pesticidas e herbicidas como, por exemplo, a rotenona e o paraquat, inibem a função mitocondrial, levando a uma disfunção no complexo I da cadeia respiratória mitocondrial e a morte celular (HERNÁNDEZ-MONTIEL, 2006). O paraquat é um dos herbicidas mais utilizados na agricultura e apresenta uma estrutura química semelhante ao 1-metil-4-fenilpiridínio (MPP<sup>+</sup>), metabólito ativo do MPTP (DAUER;PRZEDBORSKI, 2003, BARTELS;LEENDERS, 2009). A rotenona, um composto com característica neurotóxica, é um potente membro dos rotenóides, uma família de compostos citotóxicos naturais extraídos de plantas tropicais que foi amplamente utilizada como inseticida e veneno para peixes (CICCHETTI et al, 2009). Roedores intoxicados por ela desenvolvem postura anormal e diminuição de movimentos (DAUER et.al, 2003).

#### 1.2.5 Alteração genética

Pacientes com parkinsonismo familiar representam cerca de 10% a 15% dos casos (GUIMARÃES;ALEGRIA, 2004; HALD;LOTHARIUS, 2005; ELBAZ;FRANCOIS, 2007). Alterações genéticas que afetam genes como os que codificam a  $\alpha$ -sinucleína, a parkina e componentes do sistema proteossômico foram encontrados em alguns casos de parkinsonismo (HERNÁNDEZ-MONTIEL, 2006; ELBAZ;FRANCOIS, 2007; LEE;LIU, 2008). O parkinsonismo familiar ou é autossômico dominante ou autossômico recessivo e mutações em genes específicos foram identificados em 6 dos 8 *loci* que estão relacionados a DP (LEE;LIU, 2008).

### **1.3 Doença de Parkinson e Depressão**

A depressão é o distúrbio neuropsiquiátrico mais comum na DP e mostrou estar mais presente entre os pacientes com DP do que em pacientes portadores de outras doenças crônicas e igualmente incapacitantes (ROJO et al, 2003). A prevalência de depressão em pacientes com DP ocorre entre 25% a 40%, (POEWE et al, 2007; TANDBERG et al, 1997), podendo ocorrer tanto em estágios iniciais quanto finais da doença (SILBERMAN et al, 2004), De acordo com Silberman (2004) e Althaus et al (2008) em cerca de 25% dos pacientes com DP a depressão antecede os sintomas motores, demonstrando uma associação positiva entre depressão e subsequente risco da DP (BRAAK et al 2003).

A depressão pode agravar a evolução da DP (KANDA et al, 2008, FRISINA et al, 2009, COROD-ARTAL et al, 2008). Em estudo realizado por Behari et al (2005) demonstrou-se que a presença de depressão em pacientes com DP, reduz o grau de independência refletindo na qualidade de vida dos pacientes. Nesta linha, Camargo et al (2004) e Silberman et al, (2004) descreveram que pacientes portadores da DP com diagnóstico de depressão apresentam um declínio cognitivo e uma diminuição das atividades de vida diária significativamente maior em relação às pacientes com DP sem depressão. Resultados similares foram observados em outras partes do mundo confirmando que esse distúrbio não motor está diretamente relacionado com a redução na qualidade de vida (COROD-ARTAL et al, 2008).

A causa da depressão em pacientes com DP ainda não está elucidada. A hipótese psicogênica como consequência das limitações motoras (SILBERMAN et al, 2004; KANDA et al, 2008, FRISINA et al, 2009) e a

hipótese da neurodegeneração crônica natural da DP, são cogitadas como responsáveis pelo desenvolvimento da depressão nesses pacientes (CAMARGO et al 2004; NORMAN, 2002; OKUN, 2002; WOLLERS et al, 2008, FRISINA et al, 2009).

A hipótese da neurodegeneração crônica natural da DP é referente à deficiência de neurotransmissores como DA, serotonina (5-HT) e NA (CAMARGO et al 2004; NORMAN, 2002; OKUN, 2002; WOLLERS et al, 2008, FRISINA et al, 2009). A 5-HT tem função inibitória na liberação de DA no estriado, assim, a redução da neurotransmissão serotoninérgica pode ser um mecanismo compensatório relacionado à redução da neurotransmissão dopaminérgica na DP (SILBERMAN et al, 2004; KANDA et al, 2008). Como a redução da neurotransmissão serotoninérgica já esta presente antes do início dos sintomas motores, o risco da depressão anteceder os sintomas motores da DP se torna aparente (SUZUKI et al, 2008; FRISINA et al, 2009). De acordo com Schrag (2004) existem evidências de disfunção dopaminérgica, noradrenérgica e serotoninérgica em pacientes com DP e depressão. Mais ainda, pacientes com DP e depressão apresentaram uma redução no número do receptor 5-HT<sub>1A</sub> cortical em comparação com pacientes não-deprimidos com DP, indicando uma disfunção pós-sináptica do receptor 5HT<sub>1A</sub>. Sugerindo que a depressão possa ser parte integrante da disfunção cerebral na DP. Porém até o momento pode-se afirmar apenas que a interação entre depressão e DP é complexa e bidirecional, ou seja: depressão pode ser um fator de risco para DP, assim como a DP pode ser um fator de risco para depressão (SILBERMAN et al, 2004).

#### **1.4. Modelos animais da DP**

Modelos animais são fundamentais para os avanços das ciências médicas, pois nos permitem o estudo dos mecanismos patogênicos e dos princípios terapêuticos do tratamento e dos sintomas das doenças humanas mimetizadas. Modelos animais refletem as características da doença no homem, simulam as mudanças patológicas, histológicas e bioquímicas da doença e seus distúrbios funcionais. Entretanto a DP é uma doença humana, e não se manifesta espontaneamente em animais, sendo necessária a indução da doença através da administração de agentes neurotóxicos. Infelizmente ainda não existe um modelo que pode, por si só representar fidedignamente todos os sinais da DP (DAUER;PRZEDBORSKI, 2003; LIMA et al., 2006; DA CUNHA et al, 2008).

##### **1.4.1 Modelo do MPTP**

Esta neurotoxina foi descoberta no início de 1980 quando alguns jovens foram vistos na sala de emergência em um hospital da Califórnia com sintomas semelhantes à DP como rigidez, bradicinesia, tremores e instabilidade postural (LANGSTON et al. 1983; LANGSTON;BALLARD, 1983). O aparecimento desta síndrome parkinsoniana nessa faixa etária foi surpreendente uma vez que a DP geralmente se manifesta em pessoas idosas. Após a realização de alguns exames laboratoriais os pesquisadores chegaram à conclusão que os pacientes em questão eram usuários de heroína caseira, e na droga utilizada por eles foi detectada a presença de MPTP (LANGSTON et al. 1983).

O MPTP é uma neurotoxina capaz de produzir mudanças bioquímicas e neuropatológicas semelhantes a que ocorre na DP idiopática

(SCHULZ;FALKENBURGER, 2004), embora raramente ocasione a formação de corpúsculos de Lewy (NEWHOUSE et al., 2004). Devido a estas similaridades bioquímicas e histológicas o modelo do MPTP tem sido muito importante para a compreensão da fisiopatologia da DP (JENNER, 2003, MEREDITH et al, 2008). O MPTP produz, a morte dos neurônios dopaminérgicos da SNpc, com conseqüente redução dos níveis de DA no estriado e perda dos neurotransmissores NA e 5-HT no *locus coeruleus* e no núcleo da Rafe, respectivamente (WATANABE et al., 2005, MEREDITH et al, 2008; MARIEN, et al., 2004). Como característica físico-química o MPTP é altamente lipofílico, uma vez administrado ao animal, atravessa facilmente a barreira hematoencefálica, sendo convertido rapidamente nas células gliais à MPP<sup>+</sup>, radical livre altamente neurotóxico responsável pelo efeito lesivo do MPTP (JENNER, 2003, MATTSON et al, 2008; KUMAR et al, 2009). A produção do metabolito MPP<sup>+</sup> é realizada pela enzima MAO-B nas células gliais. O MPP<sup>+</sup> possui grande afinidade pelos transportadores de dopamina (DAT) ocasionando o seu acúmulo no interior das mitocôndrias dos neurônios dopaminérgicos, uma vez dentro das mitocôndrias o íon MPP<sup>+</sup> inibe o complexo I da cadeia transportadora de elétrons, que consome 100% do oxigênio molecular (SCHULZ;FALKENBURGER, 2004, MATTSON et al, 2008; KUMAR et al, 2009), resultando na menor produção de ATP responsável pelo armazenamento de energia na célula, aumento da concentração de Ca<sup>+2</sup> intracelular e produção aumentada de radicais livres (WATANABE et al., 2005).

Além do estresse oxidativo mitocondrial e deficiência energética causada pelo MPP<sup>+</sup>, esta substância ainda liga-se a vesículas sinápticas através do transportador vesicular monoaminérgico (VMAT2) e é transportado para dentro

da célula, expulsando o neurotransmissor DA para o citosol, o excesso de dopamina citosólica pode sofrer autoxidação e produzir mais ROS, como radicais superóxidos (PRZEDBORSKI, 2005, MATTSON et al, 2008, KUMAR et al, 2009).

De acordo com PRZEDBORSKI (2005), a morte neuronal dopaminérgica pela administração de MPTP esta relacionada com o estresse oxidativo e déficit de energia, porém estes processos não são os principais responsáveis pela morte neuronal. Na verdade, esses processos atuam provocando a lesão inicial e ativando vias moleculares que levam à morte da célula, como por exemplo, a ativação das quinases e vários componentes apoptóticos. Desta forma, embora esses fatores não se encontrem capazes de iniciar a lesão, atuariam amplificando a neurodegeneração.

#### **1.4.2 Modelo da 6-hidroxdopamina (6-OHDA)**

O modelo animal induzido pela toxina 6-HODA foi o primeiro modelo animal associado com a morte de neurônios dopaminérgicos na DP (LUTHMAN et al., 1989, DAUER et al., 2003). A perda maior de neurônios dopaminérgicos é observada na região ventral do encéfalo na SNpc, enquanto os neurônios da região mesocortical são os mais resistentes (JONSSON, 1980; DAUER et al., 2003). As primeiras observações dos efeitos biológicos da 6-OHDA mostraram que este agente foi capaz de induzir uma depleção longa e duradoura de NA em nervos simpáticos no coração. Alguns anos mais tarde foram demonstrados que a injeção de 6-OHDA na SNpc era capaz de causar degeneração anterógrada dos neurônios dopaminérgicos nigroestriatal, gerando assim o primeiro modelo animal de DP. Desde esta descoberta, a 6-

OHDA tem sido amplamente utilizada para mimetizar a neurodegeneração observada na DP. (LIMA et al 2009)

A 6-OHDA, é estruturalmente semelhante à DA e NA e possui uma elevada afinidade para os transportadores de membrana destas catecolaminas (MEREDITH et al, 2008). A 6-OHDA não atravessa a barreira hematoencefálica, portanto deve ser administrada diretamente no encéfalo. É transportada para os neurônios catecolaminérgicos através de mecanismos de transporte específico (p. ex., o DAT e o transportador de noradrenalina), uma vez dentro dos neurônios, é facilmente oxidada e induz a produção de radicais livres como  $\text{ONOO}^-$  e  $\text{OH}^-$  (BETARBET et al., 2002; BLUM et al., 2001; GLINKA et al., 1997; GERLACH;RIEDERER, 1996; UNGERSTEDT, 1968). Após a administração da 6-OHDA na SNpc, os neurônios dopaminérgicos começam a se degenerar em 24 horas (MEREDITH et al, 2008). Quando injetada no estriado, a 6-OHDA causa uma degeneração retrógrada de neurônios nigroestriatais que dura cerca de uma a três semanas. Entretanto, esta toxina não leva à formação de corpúsculos de Lewy (DAUER et. al, 2003, MEREDITH et al, 2008).

#### **1.4.3 Modelo da Rotenona**

A rotenona é uma substância química que pertence à família das isoflavonas, utilizada como um inseticida de amplo espectro (CICCHETTI et al, 2009).A rotenona é altamente lipofílica e não depende do DAT, uma vez dentro da célula a rotenona acumula-se no complexo I mitocondrial (CANNON et al,2009) onde inibe a transferência de elétrons de ferro-enxofre (Fe-S) aumentando a produção de ROS que podem interagir com NO, especialmente

O<sub>2</sub> e HO<sup>-</sup>, levando à formação de ONOO<sup>-</sup>, resultando em danos aos neurônios dopaminérgicos (DAUER et.al, 2003). A rotenona liga-se no mesmo local que o MPP<sup>+</sup> e inibe o complexo I mitocondrial (CICCHETTI et al, 2009).

#### 1.4.4 Modelo do LPS

O LPS é uma endotoxina encontrada na membrana externa das bactérias Gram negativas, sua molécula é composta por uma cadeia lateral de polissacarídeo (antígeno O) ligada à região do *core* a um oligossacarídeo (2-ceto-3-ácido deoxioctônico) que por sua vez está ligado a uma molécula lipídica (lipídeo A) (DUTTA, et al, 2008)

O LPS se liga a proteínas solúveis (LBP) e ao receptor CD14, expostos na membrana plasmática. A transdução de sinal ocorre após a interação entre o complexo LPS (LBP)-CD14, o receptor transmembrana Toll-like-4 (TLR-4) e as proteínas extracelulares MD-2. Esta associação leva à ativação das quinases, de várias vias sinalizadoras intracelulares, transcrição de genes geradores de radicais livres e a estimulação de células da glia (DUTTA, et al, 2008). Por sua vez as células da glia, a partir de um insulto tóxico como o LPS, poderiam produzir substâncias nocivas à célula, tais como citocinas pró-inflamatórias, PGs, ROS e as espécies reativas de nitrogênio (RNS) (FAHN e SULZER, 2004).

De acordo com Mcgeer ;Mcgeer (2004) a ativação microglial pode resultar em aumento da produção de ânions O<sub>2</sub> e outras neurotoxinas, como já demonstrado *in vitro*, podendo contribuir para processos neurotóxicos, incluindo dano às células dopaminérgicas (HIRSCH et al., 2005). A ativação microglial induzida por LPS pode causar ainda um aumento da regulação da

enzima COX-2 aumentando conseqüentemente a síntese de PGs que podem ativar diretamente a caspase-3 ou indiretamente liberar glutamato levando a excitotoxicidade (HALD;LOTHARIUS, 2005). Sabe-se que a injeção intracerebral de LPS pode induzir a expressão de citocinas como, por exemplo, interleucina (IL)-1 $\beta$ , IL-6, IL-12, fator de necrose tumoral alfa (TNF- $\alpha$ ) e óxido nítrico sintase induzido (iNOS). Mais ainda, a injeção intranigral de LPS pode induzir a degeneração dopaminérgica em ratos (ASANUMA et al., 2004; LIMA et al., 2006). Injeções intraencefálicas de LPS no córtex, hipocampo, estriado ou substância negra (SN) de ratos aumentam a morte de neurônios de DA na SN possivelmente porque a densidade de células da microglia no SN é 4-5 vezes maior que em outras regiões (GAO et al, 2002, HERRERA et al, 2000; KIM et al,2000).

## **2- OBJETIVO**

### **2.1 Objetivo geral**

Investigar comparativamente o perfil de respostas motoras, depressivas e neuroquímicas produzidas pelas infusões intranigrais das neurotoxinas MPTP, 6-OHDA, rotenona e LPS.

#### **2.1.1 Objetivos específicos**

- Determinar o comportamento motor induzido pelas neurotoxinas MPTP, 6-OHDA, rotenona e LPS em diferentes períodos de observação após suas administrações (1, 7, 14 e 21 dias)
- Avaliar os parâmetros depressivos, através do teste de preferência de consumo de sacarose, induzidos pelas neurotoxinas MPTP, 6-OHDA, rotenona e LPS, em diferentes períodos de observação após suas administrações (7, 14 e 21 dias)
- Avaliar os parâmetros depressivos, através do teste da natação forçada (versão modificada), induzidos pelas neurotoxinas MPTP, 6-OHDA, rotenona e LPS, após a extinção dos prejuízos motores (23º dia após exposição a neurotoxinas).
- Quantificar o número de neurônios na SNpc através da coloração de Nissl 23 dias após a administração das neurotoxinas.
- Quantificar os níveis de DA e seus principais metabólitos (DOPAC e HVA) na substância negra, através da análise neuroquímica, utilizando a técnica de cromatografia líquida de alta performance (HPLC), 23 dias após a administração das neurotoxinas.

- Quantificar os níveis de 5-HT e NA no estriado, através da análise neuroquímica, utilizando a HPLC, 23 dias após a administração das neurotoxinas.
- Correlacionar os resultados obtidos no teste de natação forçada modificada com os valores encontrados na análise neuroquímica.

**Depressive-like behaviors alterations induced by intranigral MPTP, 6-OHDA, LPS and rotenone models of Parkinson's disease are predominantly associated with serotonin and dopamine**

**Running title:** Depressive-like behaviors induced by models of Parkinson's disease

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**ABSTRACT**

Depression is a frequently encountered non-motor feature of Parkinson's disease (PD) and it can have a significant impact on patient's quality of life. Considering the differential pathophysiology of depression in PD, it prompts the idea that a degenerated nigrostriatal system plays a role in depressive-like behaviors, whilst animal models of PD are employed. Therefore, we addressed the question of whether dopamine (DA) depletion, promoted by the neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), lipopolysaccharide (LPS) and rotenone are able to induce depressive-like behaviors and neurotransmitters alterations similarly that encountered in PD. To test this rationale, we performed intranigral injections of each neurotoxin, followed by motor behavior, depressive-like behaviors, histological and neurochemical tests. After the motor recovery period, MPTP, 6-OHDA and rotenone were able to produce anhedonia and behavioral despair. These altered behavioral responses were accompanied by reductions of striatal DA, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) restricted to the 6-OHDA group. Additionally, decreases on the hippocampal serotonin (5-HT) content were detected for the MPTP, 6-OHDA and rotenone groups. Notably, strong correlations were detected among the groups when 5-HT and DA were correlated with swimming ( $r= +0.97$ ;  $P=0.001$ ) and immobility ( $r= -0.90$ ;  $P=0.012$ ), respectively. Our data indicate that MPTP, 6-OHDA and rotenone, but not LPS were able to produce depressive-like behaviors accompanied primarily by hippocampal 5-HT reductions. Moreover, DA and 5-HT strongly correlated with "emotional" impairments suggesting an important participation of these neurotransmitters in anhedonia and behavioral despair after nigral lesions promoted by the neurotoxins.

**Keywords:** Parkinson's disease; depression; MPTP; 6-OHDA; rotenone

**Abbreviations:** Dopamine (DA); 3,4-dihydroxyphenylacetic acid (DOPAC); dihydroxyphenylglycol (DHPG); ethylenediaminetetraacetic acid (EDTA); electrochemical detection (ED); homovanillic acid (HVA); high performance liquid chromatography (HPLC); 5-hydroxyindoleacetic acid (5-HIAA); noradrenaline (NA); 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); 6-hydroxydopamine (6-OHDA); lipopolysaccharide (LPS); Parkinson's disease (PD); serotonin (5-HT); substantia nigra pars compacta (SNpc).

## INTRODUCTION

Depression is a frequently encountered non-motor feature of Parkinson's disease (PD) and it can have a significant impact on patient's quality of life and may complicate treatment. The estimated prevalence rate of depression in patients with PD ranges from 25-40% (Poewe et al., 2007; Tandberg et al., 1997).

Theories related to the etiology of depressive symptoms in PD argue that depression is "reactive" and secondary to the psychosocial stress of a chronic disease and the associated disability or could be a result from neuroanatomical changes that occur due to the neurodegeneration (Okun et al., 2002). It is important to emphasize that both theories are not mutually exclusive, although the comprehension of the neural network involved in the mood regulation is fundamental for the understanding of depression in PD. Interestingly, different reports suggest that the pathophysiology underlying mood disorders in PD may be different from the mechanisms that account for the behavioral symptoms observed in the general population (Lieberman 2006). In view of that, several studies reported that PD-related neurodegenerative processes of brainstem concern a wide range of involvement of striatal, frontal and limbic dopaminergic, cholinergic, serotonergic, noradrenergic and GABAergic pathways that are thought to be involved in the mood regulation (Blandini et al., 2000; Schrag 2004).

Considering the differential pathophysiology of depression in PD, it prompts the idea that a degenerated nigrostriatal system plays a role in depressive-like behaviors, whilst animal models of PD are employed. Several aspects regarding the neurotoxin-induced animal models of PD have been

further substantiated by studies from our and other groups. Mostly, studies adopting the dopaminergic neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA), have demonstrated motor, cognitive, emotional, neurochemical, molecular and sleep-related disruptions in animals (Da Cunha et al., 2006; Lima et al., 2007; Reksidler et al., 2008; Reksidler et al., 2009; Reksidler et al., 2007; Tadaiesky et al., 2008). In fact, most of the studies regarding depressive-like behaviors in animal models of PD were dedicated to analyze separately the alterations induced by the models. Thus, such approach did not allow the establishment of a clear association between the different behavioral alterations produced by the distinct models and the respective neurotransmitter system involved.

Therefore, we addressed the question of whether DA depletion, promoted by several neurotoxins (MPTP, 6-OHDA, lipopolysaccharide [LPS - a recently postulated animal model of PD (Castano et al., 2002; Dutta et al., 2008; Lima et al., 2006)] and rotenone) are able to induce depressive-like behaviors and neurotransmitters alterations similarly that encountered in PD. To test this rationale, we performed intranigral injections of each neurotoxin, partially destroying dopaminergic neurons located at the substantia nigra pars compacta (SNpc). Our hypothesis predicts an initial alteration in the motor behavior, which possibly will give place to a late impairment of the depressive-like behaviors with markedly neurotransmission impairment. Depressive-like behaviors were assessed by sucrose consumption and forced swimming test. Additionally, motor system function was analyzed through the open-field test. Furthermore, neurochemical analysis of dopamine (DA) and metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA),

noradrenaline (NA) and metabolite dihydroxyphenylglycol (DHPG) and serotonin (5-HT) and metabolite 5-hydroxyindoleacetic acid (5-HIAA) were performed in striatum and hippocampus, areas known to be involved in emotional and motor processes (Owen 2004; Tadaiesky et al., 2008).

## **METHODS**

### **Animals**

Male Wistar rats from our breeding colony weighing 280–320 g at the beginning of the experiments were used. The animals were randomly housed in groups of five in polypropylene cages with wood shavings as bedding and maintained in a temperature-controlled room ( $22\pm 2^{\circ}\text{C}$ ) on a 12-h light-dark cycle (lights on at 7:00 a.m.). The animals had free access to water and food throughout the experiment. The studies were carried out in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals, United States National Institutes of Health. In addition, the protocol complies with the recommendations of Universidade Federal do Paraná and was approved by the University Ethics Committee.

### **Experimental design**

Rats were distributed randomly into 6 groups: control, sham, MPTP, 6-OHDA, LPS and Rotenone. After stereotaxic surgeries that allowed the intranigral injections of neurotoxins, open-field tests were conducted in the subsequent 1, 7, 14 and 21 days. In addition, the same animals were tested in the modified forced swimming test 23 days after the surgeries. Immediately at the end of these tests, the rats were decapitated followed by dissection of the

striatum and hippocampus structures (for neurochemical purposes) that were separated from the intact midbrains which were subsequently used for the histological analysis. Another set of animals underwent analogous randomization, stereotaxic surgery and histological analysis, although those groups were submitted to the sucrose preference consumption test on days 7, 14 and 21 after neurotoxin exposure.

### **Stereotaxic surgery**

Neurotoxins were prepared according to established doses that promote significant dopaminergic neuronal loss as follows: MPTP-HCl (100 µg in 1 µL of artificial cerebrospinal fluid; Sigma, St. Louis, MO, USA) (Lima et al., 2007; Lima et al., 2006; Reksidler et al., 2008), 6-OHDA (6 µg in 1 µL of artificial cerebrospinal fluid, supplemented with 0.2% ascorbic acid; Sigma, St. Louis, MO, USA) (Ferro et al., 2005; Lima et al., 2006), rotenone (12 µg in 1 µL of Polyethylene Glycol 400; Sigma, St. Louis, MO, USA) (Saravanan et al., 2005) and LPS (2 µg in 1 µL of artificial cerebrospinal fluid; from *Escherichia coli*, serotype 0111:B4; Sigma, St. Louis, MO, USA) (Castano et al., 2002; Lima et al., 2006). The animals were anesthetized with equitesin (chlornembutal, 0.3 ml/kg, intraperitoneal - i.p.). Bilateral infusions of MPTP, 6-OHDA, LPS or rotenone were performed through a 27-gauge stainless steel needle, according to the following coordinates: anteroposterior (AP): -5.0 mm from the bregma; mediolateral (ML): ±2.1 mm from the midline; dorsoventral (DV): 8.0 mm from the skull (Paxinos and Watson 2005). The control of the flow of the injections were made by using an electronic pump (Harvard Apparatus, USA) at a rate of 0.33 µL/min, for 3 min, followed by 2 min with the needle in the injection site to

avoid reflux. Sham operations followed the same procedure but sterile artificial cerebrospinal fluid was injected instead.

### **Open-field test**

The apparatus consists of a rectangular box (40×50×63 cm) with a floor divided into 20 (10×10 cm) rectangular units. The animals were gently placed in the right corner of the open-field and were allowed to freely explore the area for 5 min. Three motor parameters were obtained throughout this test: locomotion frequency (number of crossings from one rectangle to the other); rearing frequency (number of times the animals stood on their hind paws) and immobility time (number of seconds of lack of movement during testing). The open-field was washed with a 5% water-ethanol solution before behavioral testing to eliminate possible bias due to odors left by previous rats.

### **Modified Forced swimming test**

The procedure used was a modification of the method proposed by (Porsolt et al., 1978; Reneric et al., 2002). The test was conducted in two sessions. First, in the training session, the rats were placed in a tank (20 × 20 × 40 cm) containing water at a temperature of  $24 \pm 1$  ° C at a depth of 15 cm for 15 minutes. Twenty four hours after the training session, the rats were subjected to the forced swimming test for 5 min, which was video taped for subsequent quantification of the following parameters: immobility (defined as the lack of motion of the whole body consisting only of the small movements necessary to keep the animal's head above the water); climbing (vigorous movements with forepaws in and out of the water, usually directed against the

wall of the tank) and swimming (was coded when large forepaw movements displacing the body around the cylinder, more than necessary to merely keep the head above the water). The water was changed after each animal to avoid the influence of substance and temperature.

### **Sucrose preference test**

Sucrose preference is frequently used as measure of anhedonia in rodents (Papp et al., 1991; Wang et al., 2009). The animals were transferred into single housing cages with free access to food. Each rat was provided with two bottles of water, pre-weighed, on the extreme sides of the cage during the 24 h training phase to adapt the rats to drink from two bottles. After training, one bottle was randomly switched to contain 1% sucrose solution, as described previously (Slattery et al., 2007), and 24 h later, the bottles were reversed to avoid perseveration effects. The sum of water consumption and sucrose consumption was defined as the total intake. The percentage of sucrose intake was calculated by using the following equation ( $\% \text{ sucrose preference} = \text{sucrose intake} \times 100 / \text{total intake}$ ). All the tests were carried out weekly (each Tuesday) between 9:00 and 11:00 am, beginning one week prior to the neurotoxins exposure (to provide baseline values) and finishing 21 days after that. After the sucrose preference test, all the rats received free access to food and water.

## **Determination of dopamine, noradrenaline, serotonin and metabolites concentrations**

The striatum and hippocampus structures were rapidly dissected and stored at -80°C until the neurochemical quantification. The endogenous concentrations of DA, DOPAC, HVA, NA, DHPG, 5-HT and 5-HIAA were assayed by reverse-phase high performance liquid chromatography (HPLC) with electrochemical detection (ED). Briefly, the system consisted of a Synergi Fusion-RP C-18 reverse-phase column (150 x 4.6 mm i.d., 4 µm particle size) fitted with a 4 x 3.0 mm pre-column (Security Guard Cartridges Fusion-RP); an electrochemical detector (ESA Coulochem III Electrochemical Detector) equipped with a guard cell (ESA 5020) with the electrode set at 350 mV and a dual electrode analytical cell (ESA 5011A); a LC-20AT pump (Shimadzu) equipped with a manual Rheodyne 7725 injector with a 20 µL loop. The column was maintained inside in a temperature-controlled oven (25°C; Shimadzu). The cell contained two chambers in series: each chamber including a porous graphite coulometric electrode, a double counter electrode and a double reference electrode. Oxidizing potentials were set at 100 mV for the first electrode and at 450 mV for the second electrode. The tissue samples were homogenized with an ultrasonic cell disrupter (Sonics) in 0.1 M perchloric acid containing sodium metabisulfite 0.02% and internal standard. After centrifugation at 10,000xg for 30 min at 4°C, 20 µL of the supernatant was injected into the chromatograph. The mobile phase, used at a flow rate of 1 mL/min, had the following composition: 20 g citric acid monohydrated (Merck), 200 mg octane-1-sulfonic acid sodium salt (Merck), 40 mg ethylenediaminetetraacetic acid (EDTA) (Sigma), 900 mL HPLC-grade water.

The pH of the buffer running solution was adjusted to 4.0 then filtered through a 0.45  $\mu\text{m}$  filter. Methanol (Merck) was added to give a final composition of 10% methanol (v/v). The neurotransmitters and metabolites concentrations were calculated using standard curves that were generated by determining in triplicate the ratios between three different known amounts of the internal standard. The unit was expressed as ng/g of wet weight.

### **Nissl staining and neuronal quantification**

After the tissue extraction for neurochemical purposes, the rats' midbrains were collected and quickly frozen in dry ice. For each rat 12 sections of 30  $\mu\text{m}$  thick were cut on a cryostat in the coronal plane covering about 360  $\mu\text{m}$  (-4.92 to -5.28 from bregma) of the midbrain (Paxinos and Watson 2005). These coordinates correspond to the maximal extent of the dopaminergic neurons within the SNpc (Reksidler et al., 2008). The sections were mounted onto slides, dried and stained with cresyl violet and coverslipped after dehydration in ascending concentrations of ethanol-xylene solutions. Cell counts were conducted making use of the software Image-Pro Express 6 (Media Cybernetics, USA). Each slice was digitized with a digital camera connected to a microscope IX71 (Olympus Optical Co, Japan). A digital area was created in order to delimitate the boundaries of the SNpc. For each analysis, the same area was adopted. A "manual tag" tool was used to count the neurons inside the area. All the counts were performed in images obtained in 40x magnification. A mean number of neurons in the SNpc were obtained for each group and the results are presented as percentage from the control group.

## Statistical analysis

Differences between groups in the open-field test were analyzed by two-way analysis of variance (ANOVA) with repeated measures followed by the Newman-Keuls test. Forced swimming test was analyzed by one-way ANOVA followed by the Newman-Keuls test. Sucrose preference test was analyzed by two-way ANOVA with repeated measures followed by the Newman-Keuls test. Neurochemical and histological data were analyzed by one-way ANOVA followed by Newman-Keuls test. Pearson correlation coefficients ( $r$ ) were calculated to establish relationships between neurotransmitters concentrations and respective behaviors obtained in the forced swimming test. The level of significance was set at  $P \leq 0.05$ .

## RESULTS

### Open-field test

As can be seen in Table 1, locomotion frequencies obtained after neurotoxin exposures revealed a significant impairment for this parameter in comparison to the control group for the groups MPTP 1 day ( $P < 0.05$ ), 6-OHDA 1 day ( $P < 0.05$ ) and rotenone 1 day ( $P < 0.05$ ), as indicated by the group factor [ $F(5.54) = 4.27$ ;  $P = 0.002$ ], time factor [ $F(3.54) = 17.93$ ;  $P < 0.0001$ ] and interaction [ $F(15.54) = 4.61$ ;  $P < 0.0001$ ]. Moreover, rearing frequencies demonstrated to be significantly reduced for the same groups: MPTP 1 day ( $P < 0.05$ ), 6-OHDA 1 day ( $P < 0.05$ ) and rotenone 1 day ( $P < 0.05$ ), as showed by the group factor [ $F(5.54) = 6.12$ ;  $P = 0.0001$ ], time factor [ $F(3.54) = 9.23$ ;  $P < 0.0001$ ] and interaction [ $F(15.54) = 3.41$ ;  $P < 0.0001$ ]. Regarding the immobility time parameter, the 6-OHDA group exhibited a significant increase

compared to the control group ( $P < 0.05$ ) 1 day after neurotoxin exposure. Interestingly, the rotenone group showed a significant decrease in this parameter in comparison to the control ( $P < 0.01$ ), MPTP ( $P < 0.01$ ), 6-OHDA ( $P < 0.01$ ) and LPS ( $P < 0.001$ ) groups 21 days after neurotoxin exposure as demonstrated by the group factor [ $F(5.54) = 1.79$ ;  $P = 0.13$ ], time factor [ $F(3.54) = 19.06$ ;  $P < 0.0001$ ] and interaction [ $F(15.54) = 1.73$ ;  $P = 0.05$ ].

### **Modified Forced swimming test**

The swimming parameter evidenced that MPTP ( $P < 0.05$ ), 6-OHDA ( $P < 0.01$ ) and rotenone ( $P < 0.05$ ) presented significant reductions in comparison to the control group [ $F(5.53) = 7.801$ ;  $P < 0.0001$ ] (Fig. 1A). By contrast, only the 6-OHDA group showed an increased immobility ( $P < 0.01$ ) compared to the control group [ $F(5.53) = 5.249$ ;  $P = 0.0006$ ] (Fig. 1B). Complementarily, the analysis of the climbing parameter demonstrated that the neurotoxins tested did not exert significant effects in this parameter [ $F(5.53) = 1.672$ ;  $P = 0.1595$ ] (Fig. 1C).

### **Sucrose preference test**

The examination of the sucrose preference test revealed that 7 days after neurotoxins exposure the groups MPTP ( $P < 0.001$ ) and 6-OHDA ( $P < 0.001$ ) and LPS ( $P < 0.05$ ) presented a significant reduction in comparison to the basal and sham group 7 days [ $F(23.9) = 59.11$ ;  $P < 0.0001$ ] (Fig. 2). In addition, the MPTP ( $P < 0.01$ ) and 6-OHDA ( $P < 0.01$ ) groups presented significantly reduced sucrose preference when compared to the LPS group 7 days, as showed in Fig. 2. The analysis of the time-point 14 days indicated that the groups did not differ

statistically. In contrast, 21 days after the surgery the MPTP ( $P<0.001$ ) and rotenone ( $P<0.05$ ) groups exhibited significant reductions in the percentage of sucrose preference, compared to the control group 21 days. Similarly, the 6-OHDA group presented significant reduction ( $P<0.01$ ) for this test in comparison to the sham group 21 days (Fig. 2).

### **Neuronal quantification in the SNpc**

The analysis of the neuronal population resident within the SNpc revealed that all the neurotoxins tested were able to generate a pronounced neuronal loss in the respective area (Fig. 3A). The quantification of the positive Nissl-stained neurons demonstrated that MPTP produced a reduction of 45.2% ( $P<0.001$ ) in the neurons located at the SNpc, as well as 6-OHDA 53.4% ( $P<0.001$ ), LPS 49.6% ( $P<0.001$ ) and rotenone 43.8% ( $P<0.001$ ), [ $F(5.56)=20.79$ ;  $P<0.0001$ ] (Fig. 3B).

### **Determination of dopamine, noradrenaline, serotonin and metabolites concentrations**

Fig. 4A depicts the striatal concentrations of DA and metabolites DOPAC and HVA. DA neurotransmitter was significantly reduced ( $P<0.0001$ ) only in the 6-OHDA group in comparison to the control group [ $F(5.1)=7.825$ ;  $P<0.0001$ ]. Likewise, the 6-OHDA group presented a significant reduction ( $P<0.01$ ) of the DOPAC concentration, compared to the control group [ $F(5.1)=5.907$ ;  $P<0.0001$ ] (Fig. 4B). Moreover, HVA concentration demonstrated to be significantly reduced in the 6-OHDA ( $P<0.01$ ) and rotenone ( $P<0.05$ ) groups compared to the control group [ $F(5.1)=5.784$ ;  $P=0.0001$ ] (Fig. 4C). The

analyses of the neurotransmitters and metabolites within the hippocampus evidenced that 5-HT was found significantly decreased in the MPTP ( $P < 0.05$ ), 6-OHDA ( $P < 0.01$ ) and rotenone ( $P < 0.05$ ) groups, when those groups were compared to the control group 5-HT [ $F(5.43) = 4.848$ ;  $P = 0.0016$ ] (Fig. 5A). Besides, 6-OHDA and rotenone groups demonstrated significant 5-HIAA reductions ( $P < 0.05$ , for both groups) compared to the control group [ $F(5.43) = 3.997$ ;  $P = 0.0052$ ] (Fig. 5B). Hippocampal NA content demonstrated to be reduced only for the 6-OHDA group when compared to the sham group [ $F(5.43) = 2.903$ ;  $P = 0.026$ ] (Fig. 5C). However, only the rotenone group presented a significant reduction ( $P < 0.05$ ) in the DHPG concentration in comparison to the control group [ $F(5.43) = 3.739$ ;  $P = 0.0075$ ] (Fig. 5D).

#### **Neurotoxins-induced neurotransmitters alterations strongly correlated with depressive-like behaviors quantified in the forced swimming test**

Pearson's correlation coefficients revealed a strong positive correlation ( $r = +0.97$ ;  $P = 0.0015$ ) between the hippocampal 5-HT concentration and the swimming parameter for the groups analyzed (Fig. 6A). Additionally, a strong negative correlation ( $r = -0.88$ ;  $P = 0.0021$ ) was found between hippocampal NA and immobility (Fig. 6B). In fact, striatal DA also closely correlated ( $r = -0.90$ ;  $P = 0.012$ ) to the immobility parameter (Fig. 6C). Furthermore, NA weakly correlated ( $r = +0.46$ ;  $P = 0.35$ ) with climbing behavior (Fig. 6D).

## DISCUSSION

The etiology of depression in PD is complex and may result from changed 5-HT brain chemistry that is related with the central dopaminergic deficiency associated with PD motor symptoms (Mayeux 1990; Mayeux et al., 1984). The notion of a DA etiology for depression in PD is not surprising, and is somewhat supported by clinical research showing a high association between mood changes and lesions to the basal ganglia (Mayberg 1994). The basal ganglia receive DA input from the SNpc, which is known to be impaired in PD patients. Thus, observations of pathological features in the SNpc of depressed PD patients, though only trending toward significance appears relevant and bolsters the notion that the nigrostriatal circuit is implicated in the depression of PD (Frisina et al., 2009). Even with such importance there is a lack of studies concerning animal models of PD and depressive-like behaviors in order to elucidate the neurobiology and the interaction between these disorders.

The findings herein indicated that bilateral intranigral infusions of the neurotoxins MPTP, 6-OHDA and rotenone were able to produce depressive-like behaviors assessed through the forced swimming test and the sucrose preference test. Moreover, all the neurotoxins tested produced an average of 48% of neuronal loss confined in the SNpc, suggesting that the intranigral administration of these substances mimic a pathophysiological condition similar to PD. To ensure an unbiased depressive-like behavior response promoted by the neurotoxins we performed the tests after a recovery of the motor behavior, initially impaired due to the dopaminergic damage (Lapointe et al., 2004; Lima et al., 2006; Reksidler et al., 2007). We observed that the motor decline was a rather transitory situation that mostly was manifested 1 day after 6-OHDA ,

MPTP and rotenone exposures. Regarding LPS, this substance is postulated to be a potent dopaminergic neurotoxin able to mimic neuroinflammatory processes in PD (Dutta et al., 2008; Lima et al., 2006; Ling et al., 2006). Such characteristic was corroborated by our data that indicated a significant neuronal loss within the SNpc inflicted by this toxin, although, we did not detect appreciable motor nor depressive-like behavior alterations, as would be expected.

These results also were supported by the neurochemical analyses that indicated a remarkable reduction of striatal DA, HVA and DOPAC restricted to the 6-OHDA group, suggesting a stronger dopaminergic toxicity related to 6-OHDA, compared to the others. In fact, this notion is supported by our histological data which indicates that 6-OHDA was able to produce an 8% and 10%, respectively, larger (but not statistically significant) lesion than MPTP and rotenone did. In addition, previous evidence also indicate the occurrence of more extensive lesions produced by 6-OHDA (Ferro et al., 2005; Segura Aguilar and Kostrzewa 2004). In opposite, rotenone was able to augment striatal HVA content, suggesting an increased DA turnover (Thiffault et al., 2000) and DA receptor sensitization, which is a compensatory mechanism in surviving neurons (Enz et al., 1984). Additionally, decreases on the hippocampal 5-HT content were detected for the MPTP, 6-OHDA and rotenone groups, although 5-HIAA was found reduced for the 6-OHDA and rotenone groups. Contrasting results are reported in the literature regarding the effect of MPTP, 6-OHDA and rotenone lesions on 5-HT brain contents (Pierucci et al., 2009; Saravanan et al., 2005; Unger et al., 2002). It is likely that this variability is probably due to the amount of DA lesion achieved and by the different mechanisms elicited by each

neurotoxin. Although, NA was not modified by the neurotoxins, but rotenone promoted an increased DHPG content, which is compatible with an increase NA turnover.

In agreement with our hypothesis, an initial motor deficit was detected as a result of a massive neurotoxicity to the SNpc neurons. Nevertheless, the subsequent time-points studied evidenced the existence of a compensatory motor effect (more prominently for MPTP and 6-OHDA), probably due to events of neuronal plasticity that balance the neuronal loss. Such adaptation is a well documented characteristic of the early phase models of PD (Bjorklund et al., 1997; Da Cunha et al., 2001; Ferro et al., 2005; Lima et al., 2007; Lima et al., 2006; Reksidler et al., 2008; Reksidler et al., 2007). In view of that, we suggest that the neurotoxins MPTP and 6-OHDA could be more suitable to study depressive-like behaviors without the influence of a motor deficit. Our results support previous data that show anhedonic state after MPTP and 6-OHDA (Kryzhanovskii et al., 1995; Tadaiesky et al., 2008), although the present findings are the first to demonstrate that effect after intranigral neurotoxins injections. Moreover, the anhedonic state was circumscribed at 7 and 21 days after MPTP and 6-OHDA, possibly due to the characteristic of the lesions which are more abrupt than that promoted by rotenone (Ceravolo et al., 2006; Segura Aguilar and Kostrzewa 2004), which provided a more gradual anhedonic state prominently detected 21 days after neurotoxin. Nevertheless, this is a partial explanation because it was detected an increase in the sucrose preference for the MPTP, 6-OHDA and LPS groups tested 14 days after surgery. It is conceivable that the results obtained during the time-point 7 days did not reflect entirely the existence of anhedonia effect, possibly attributable to a residual

locomotor deficit, not detected in the open-field test at this period. Hence, we believe that anhedonia state was fully achieved 21 days after neurotoxin exposure. Complementarily, this state was accompanied, in the 6-OHDA group, by another feature of depressive-like behavior, behavioral despair, which was indicated by an increased immobility time in the forced swimming test, as recently described (Tadaiesky et al., 2008).

Notably, strong correlations were detected among the groups when 5-HT, NA and DA were correlated with swimming ( $r = +0.97$ ) and immobility ( $r = -0.88$ ) and ( $r = -0.90$ ), respectively. That is, the neurotoxins that produced more predominantly DA and 5-HT reductions tended to elicit increases in the immobility and decreases in the swimming parameters, respectively. In opposite, climbing was not modified by the neurotoxins, similar as the hippocampal NA content, indicating that NA weakly correlated with climbing behavior ( $r = +0.46$ ) according to our protocol. These results suggest a strong participation of these monoamines in behavioral despair after nigral lesions promoted by the neurotoxins tested (Kobayashi et al., 2008). In this line, our results are in accordance with others that associate serotonergic and noradrenergic neurotransmissions with swimming and climbing behaviors in the forced swimming test (Detke et al., 1995; Reneric et al., 2002). The aforementioned alterations in dopaminergic and serotonergic systems suggest, therefore, that one or more of these neurotransmitter systems play an important role in depressive-like behaviors in the current models tested, further supporting the involvement of these neurotransmitter systems in PD-related depression (Schrag 2004).

Indeed, there are pathophysiological evidence of 5-HT alterations in patients with PD-associated depression (Schrag 2004), and a hypothesis concerning 5-HT has even been proposed for depression in PD (Mayeux 1990). This hypothesis considers a serotonin-induced dopamine release in the nucleus accumbens which is down-regulated by 5-HT<sub>2C</sub> receptors (Dremencov et al., 2005). As a result, reductions in the 5-HT content or increases in the 5-HT<sub>2C</sub> inhibitory activity could be associated to a decline on dopaminergic neurotransmission in PD patients and subsequent worsening of mood symptoms. In view of that, our findings indicate that 6-OHDA was more harmful to the serotonergic system, compared to the other neurotoxins. In fact, only 6-OHDA was able to decrease DA content, thus it is conceivable that the “5-HT hypothesis” is related to the dopaminergic neurotransmission, particularly in a situation of a larger nigrostriatal dopaminergic lesion (Ferro et al., 2005; Lima et al., 2006).

## **CONCLUSION**

In conclusion, our data indicate that the neurotoxins MPTP, 6-OHDA and rotenone, but not LPS were able to produce depressive-like behaviors accompanied primarily by hippocampal 5-HT reductions. Besides, DA and 5-HT strongly correlated with “emotional” impairments suggesting an important participation of these neurotransmitters in anhedonia and behavioral despair after nigral lesions promoted by the neurotoxins. In fact, more studies are needed to examine the participation of these neurotransmitters in the depressive-like behaviors found in the present study.

**ACKNOWLEDGMENTS**

This work was supported by grants from CNPq, and CAPES that had no further role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. RA and MABFV are recipient of CNPq fellowship. The authors wish to express their gratitude to Dr. Sílvia Marques Zanata for the histological experiment collaboration.

## FIGURE LEGENDS

Figure 1: Depressive-like behaviors elicited by the neurotoxins MPTP, 6-OHDA, LPS and rotenone evidenced by the forced swimming test. (A) Swimming, (B) immobility and (C) climbing. The data were obtained 23 days after neurotoxins exposure. Grey bars represent control or sham groups and black bars correspond to the experimental groups. Values are expressed as mean  $\pm$  SEM (n=8-12/ group). \*P<0.05 and \*\*P<0.01 compared to the control group. ANOVA followed by the Newman-Keuls test.

Figure 2: Percentage of sucrose preference elicited by the neurotoxins MPTP, 6-OHDA, LPS and rotenone. Dashed rectangles represent the significant differences between groups. Values are expressed as mean  $\pm$  SEM (n=10-12/ group). \*P<0.05,  $\Psi$ P<0.05, #P<0.001. ANOVA with repeated measures followed by the Newman-Keuls test.

Figure 3: Histological analysis of the SNpc at the end of the experiments (23 days after neurotoxin exposures). Representative photomicrographs of the Nissl-stained neurons in the ventral midbrain of all the groups are depicted in the panel A (magnification: 4X and 40X for the inset squares). Panel B shows the stereological quantification of the total number of neurons within the SNpc for each group. Values are expressed as percentage of the control group (n=8-12/ group). \*\*\*P<0.01 compared to the control group. ANOVA followed by the Newman-Keuls test.

Figure 4: Neurochemical examination of the striatal concentrations of DA, DOPAC and HVA elicited by the neurotoxins MPTP, 6-OHDA, LPS and

rotenone. (A) DA concentrations, (B) DOPAC concentrations and (C) HVA concentrations. The data were obtained 23 days after neurotoxins exposure. Grey bars represent control or sham groups and black bars correspond to the experimental groups. Values are expressed as mean  $\pm$  SEM (n=10-12/ groups). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 compared to the control group. ANOVA followed by the Newman-Keuls test.

Figure 5: Neurochemical examination of the hippocampal concentrations of 5-HT, 5-HIAA, NA and DHPG elicited by the neurotoxins MPTP, 6-OHDA, LPS and rotenone. (A) 5-HT concentrations, (B) 5-HIAA concentrations, (C) NA concentrations and (D) DHPG concentrations.. The data were obtained 23 days after neurotoxins exposure. Grey bars represent control or sham groups and black bars correspond to the experimental groups. Values are expressed as mean  $\pm$  SEM (n=14-19/ groups). \*P<0.05 and \*\*P<0.01 compared to the control group. #P<0.05 compared to the sham group. ANOVA followed by the Newman-Keuls test.

Figure 6: Depressive-like behaviors closely correlate with the correspondent neurotransmitters systems after the neurotoxins MPTP, 6-OHDA, LPS and rotenone exposure. Pearson's correlation coefficients were calculated considering the following: (A) 5-HT x swimming, (B) NA x immobility, (C) DA x immobility, (D) NA x climbing.

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## Figures

Figure 1 A-B-C

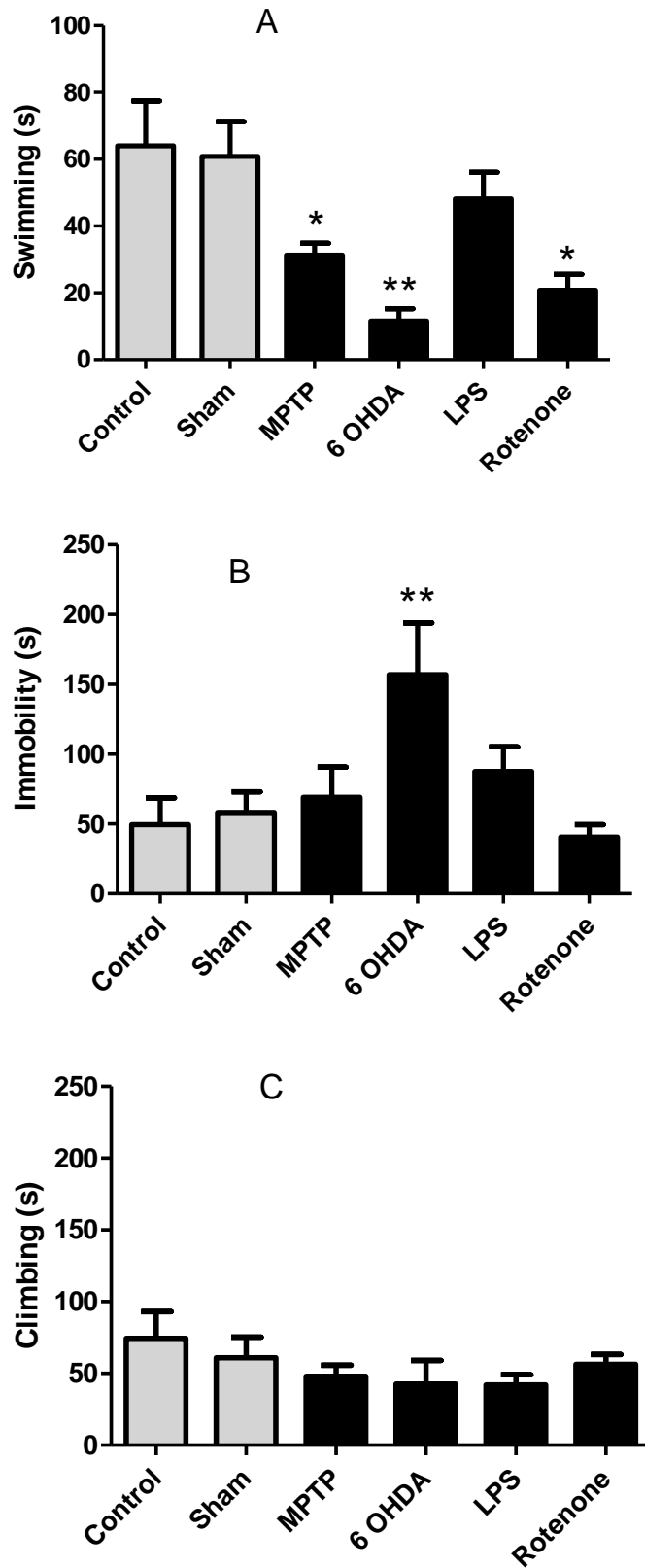


Figure 2

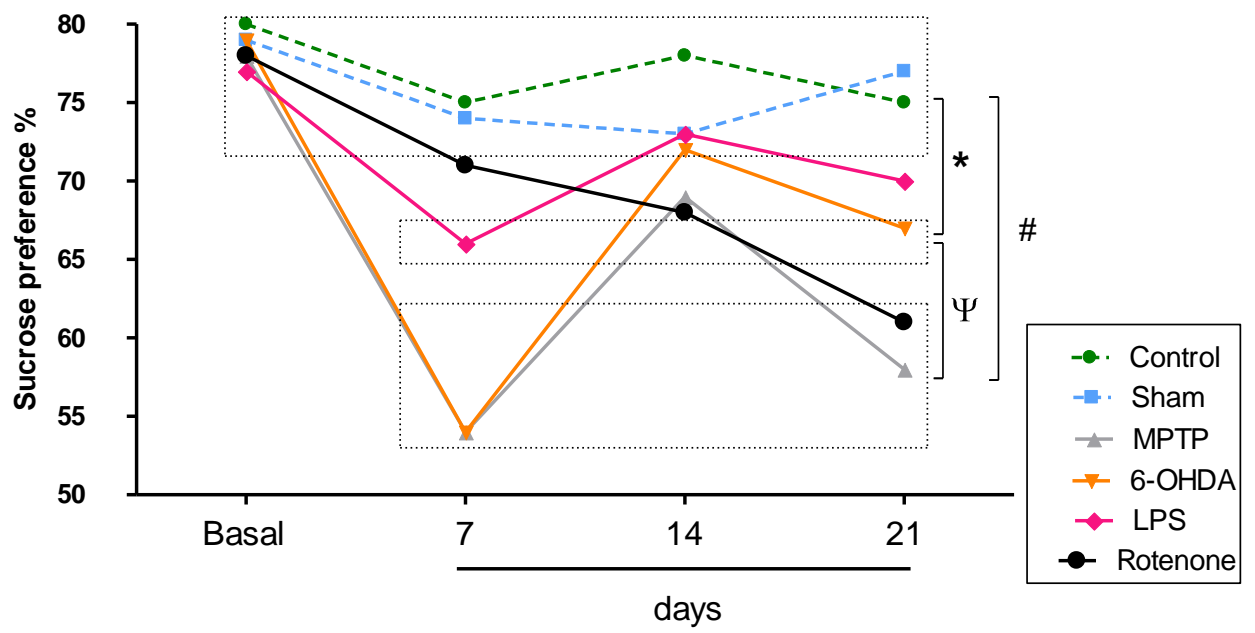
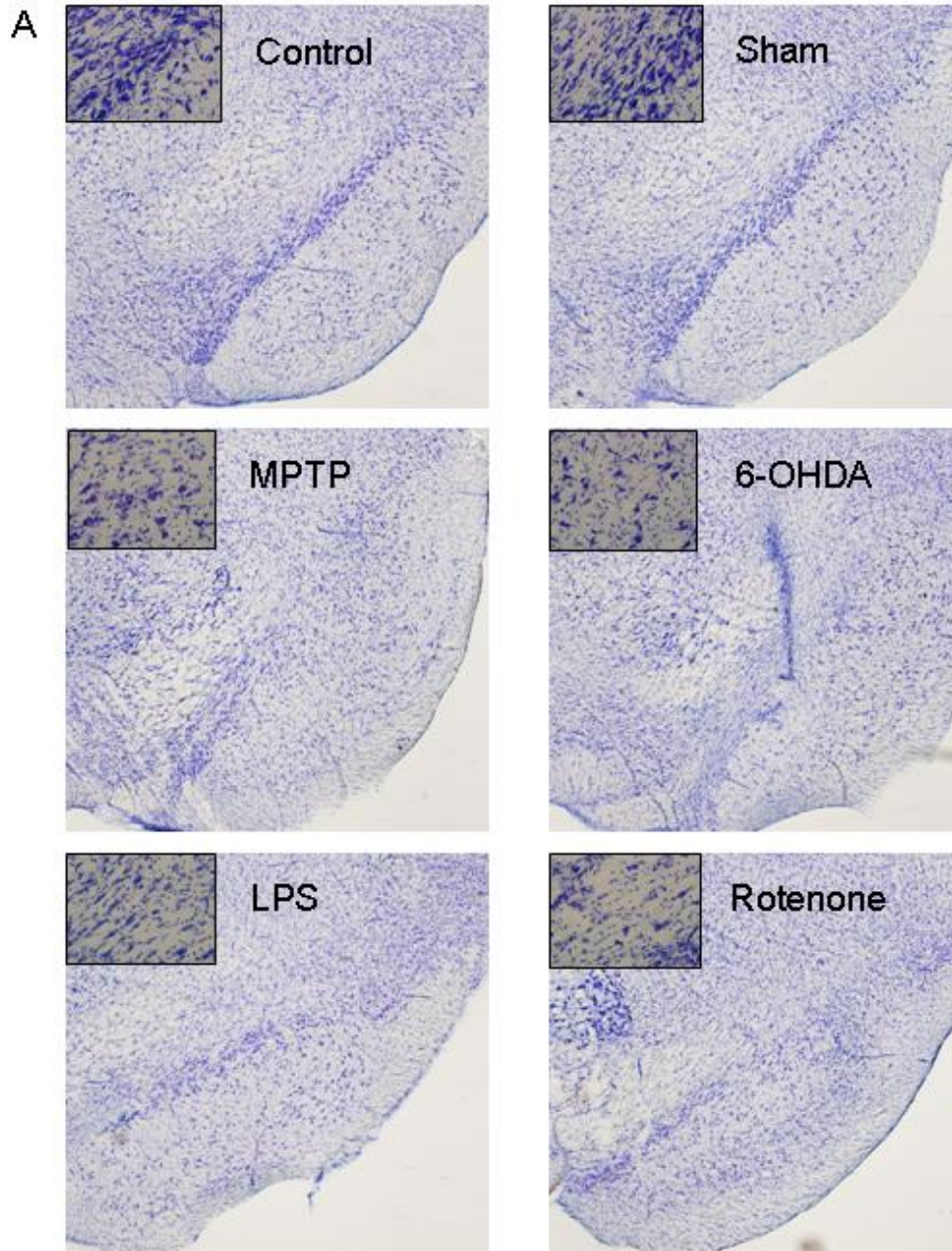


Figure 3 A-B



**B**

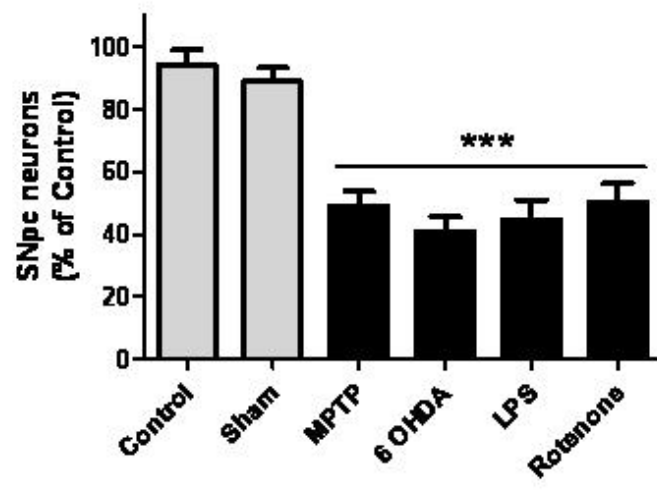


Figure 4 A-B-C

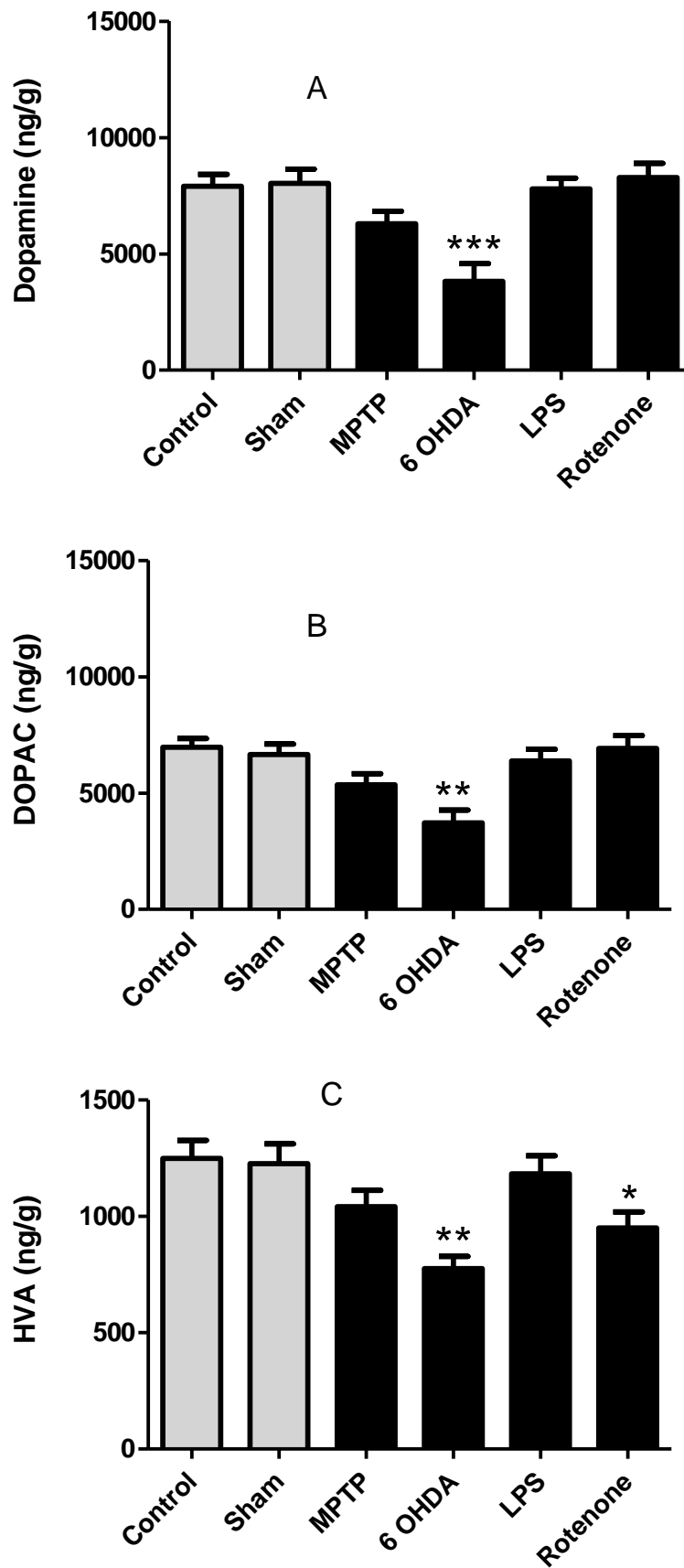


Figure 5 A-B-C-D

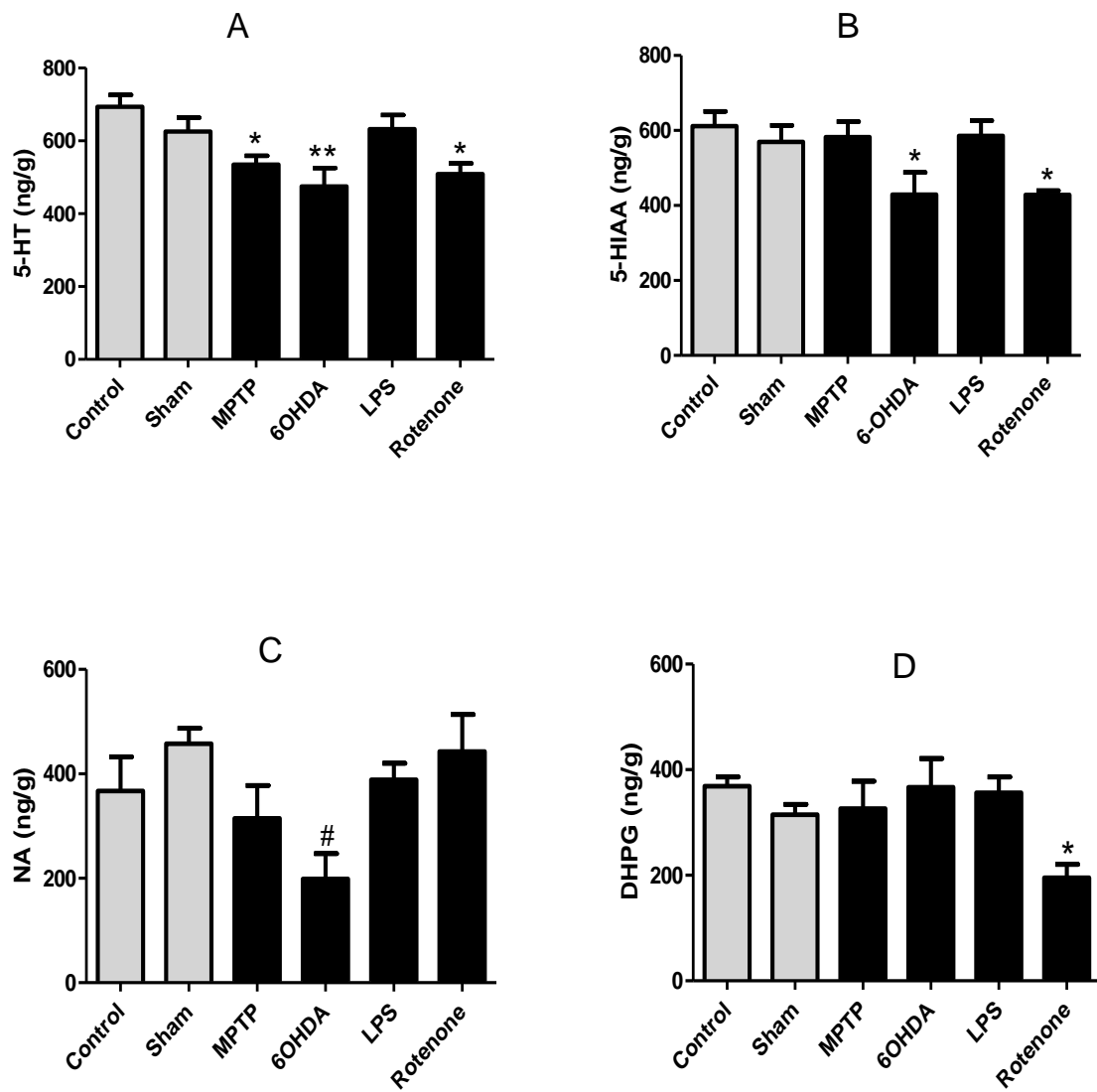


Figure 6 A-B-C-D

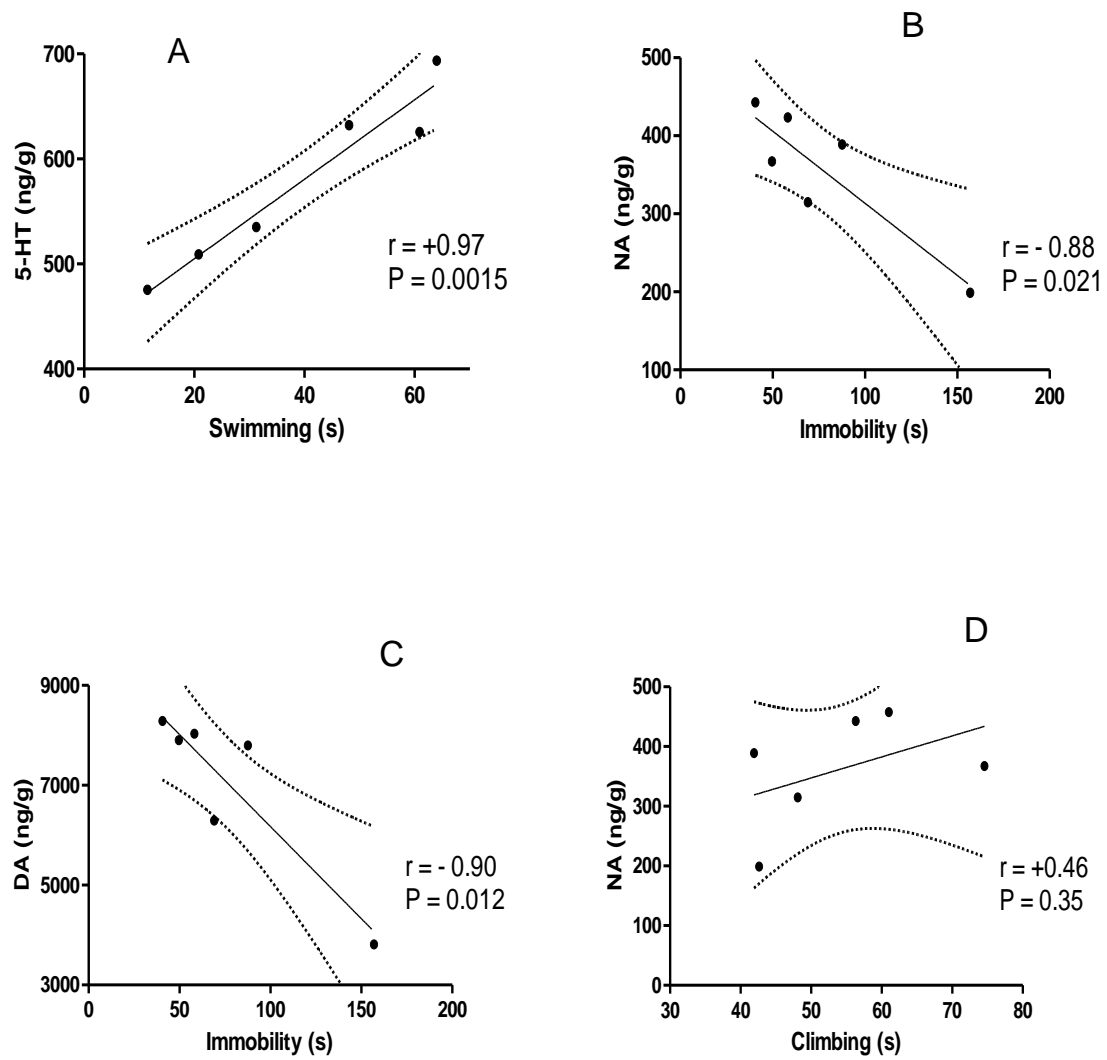


Table 1. Temporal motor behavior alterations quantified after neurotoxin administrations.

Groups	Time-points (days)	Locomotion frequency	Rearing frequency	Immobility time (s)
Control	1	113±10.4	31.5±3.4	10±6.3
	7	82.6±14.1	22.1±2.9	36.2±18.3
	14	55.1±9.2	14.5±2.6	65.8±22.3
	21	59.2±10.3	14.3±2.9	72.6±18.9
Sham	1	115±7.7	26.6±1.5	6±6
	7	83.6±13	20.7±3.1	24.5±12.2
	14	68.3±11.4	19±3.1	41.7±18.1
	21	65.2±12.3	18.8±2.9	56±23.8
MPTP	1	66.8±7.8 <sup>a</sup>	15.7±2.7 <sup>a</sup>	17±7.2
	7	71.6±12.5	14.2±1.9	32.9±14.8
	14	57.3±15.3	10.9±1.4	51.4±20.9
	21	39.7±6.87	10.9±2.1	75.2±22.8
6-OHDA	1	23.6±4.9 <sup>a</sup>	8.3±2 <sup>a</sup>	45.5±12.9 <sup>a</sup>
	7	66.3±6.1	19.7±2.0	15.5±11.7
	14	54.3±8.2	13.6±1.5	34±19.8
	21	32±9.1	8.6±2.1	76±17.8
LPS	1	109.7±8.2	22.9±2.5	15±6.7
	7	66.3±10.8	21±3.6	38.1±14.1
	14	47.3±6.3	12.7±1.7	67.4±17.7
	21	47.1±7.3	12.6±2.4	88.8±17.9
Rotenone	1	67.9±11.8 <sup>a</sup>	15.4±2.8 <sup>a</sup>	7.1±3.3
	7	82.4±8.4	17.8±2.4	8.1±4.2
	14	85.8±9.9	21.7±2.8	30.5±11.8
	21	70.3±6.8 <sup>b</sup>	16.3±2.4	10.4±3.6 <sup>b</sup>

The values are expressed as mean ± SEM (n=10-12/ group). Differences between groups are indicated by letters: <sup>a</sup> different from the control (P<0.05) and sham (P<0.05) groups 1 day after exposure; <sup>b</sup> different from the control (P<0.01), MPTP (P<0.01), 6-OHDA (P<0.01) and LPS (P<0.001) groups 21 days after exposure. Two-way ANOVA with repeated measures followed by the Newman-Keuls test.

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