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Investigação da ativação seletiva de neurônios dopaminérgicos da substância negra pars compacta promovida pela privação de sono REM em ratos

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SUMÁRIO

1. RESUMO

Atualmente, vários estudos abordam a ligação entre o sono e a neurotransmissão dopaminérgica, focando nos mecanismos pelos quais a doença de Parkinson (DP) e o sono podem ser entrelaçados. Por conseguinte, as variações nas atividades durante os ciclos de sono, ou ao nível de corpos celulares dopaminérgicos na área tegmental ventral (VTA) e / ou na substância negra pars compacta (SNPC) podem afetar funções como a memória. Deste modo, foram realizadas quantificações neuroquímicas de DA, serotonina (5-HT) e os seus metabólitos no estriado e no hipocampo de ratos tratados por via intraperitoneal com o haloperidol (1,5 mg / kg) ou piribedil (8 mg / kg) e submetidos a privação de sono REM (REMSD) e sono rebote (REB). Além disso, foram avaliados os efeitos de REMSD no comportamento motor, nos parâmetros cognitivos e a imunorreatividade neuronal da c-Fos na SNPC. Os resultados indicaram que a c-Fos foi fortemente realçada pelo piribedil no grupo REMSD. Uma característica ativação de c-Fos no grupo REMSD foi demonstrada de maneira sinérgica pelo piribedil, indicando uma forte correlação positiva entre os níveis estriatais de DA e ativação de c-Fos nigral. Assim, sugere-se que os processos de memórias foram severamente impactados tanto pelo bloqueio dopaminérgico quanto pela privação de sono REM e ainda mais pela sua combinação. Portanto, a evidência atual reforça que o receptor D2 é uma peça chave na ativação neuronal da SNPC mediada pelo REMSD e como conseqüência dessas mudanças, pode haver impacto direto na cognição e alterações do sono encontrados em pacientes com DP.

Palavras-chave: dopamina, haloperidol, doença de Parkinson, piribedil, privação do sono REM, substância negra pars compacta,

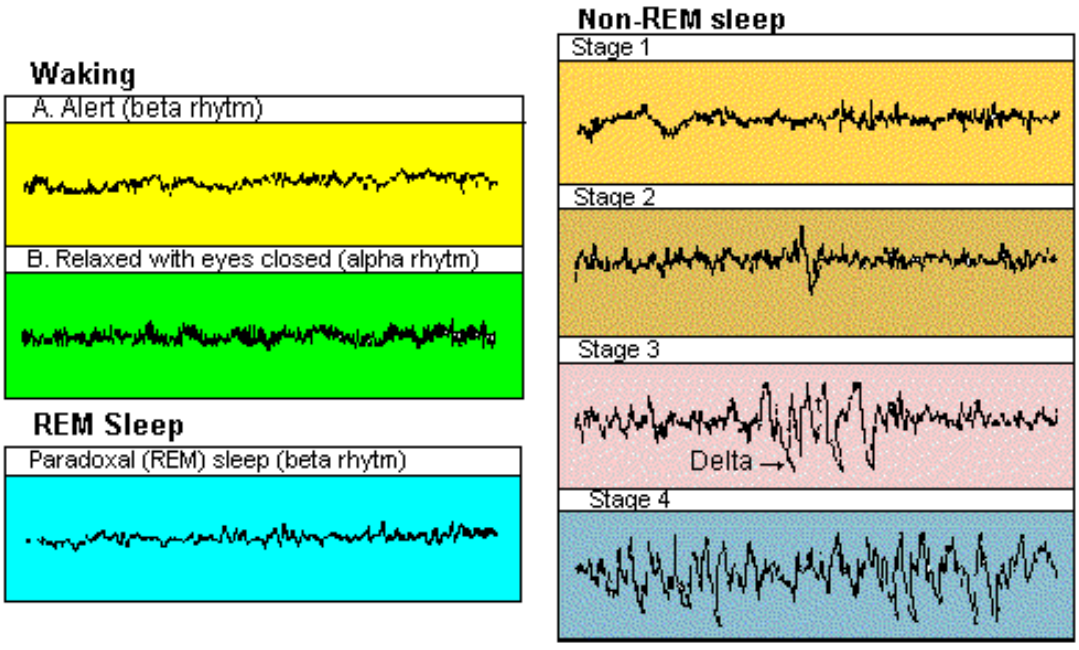
ABSTRACT

Currently, several studies addresses the novel link between sleep and dopaminergic neurotransmission, focusing most closely on the mechanisms by which Parkinson's disease (PD) and sleep may be intertwined. Therefore, variations in the activity of afferents during the sleep cycles, either at the level of DA cell bodies in the ventral tegmental area (VTA) and/or substantia nigra pars compacta (SNpc) or at the level of dopamine (DA) terminals in limbic areas may impact functions such as memory. Accordingly, we performed striatal and hippocampal neurochemical quantifications of DA, serotonin (5-HT) and metabolites of rats intraperitoneally treated with haloperidol (1.5 mg/kg) or piribedil (8 mg/kg) and submitted to REM sleep deprivation (REMSD) and sleep rebound (REB). Also, we evaluated the effects of REMSD on motor and cognitive parameters and SNpc c-Fos neuronal immunoreactivity. The results indicated that DA release was strongly enhanced by piribedil in the REMSD group. In opposite, haloperidol prevented that alteration. A c-Fos activation characteristic of REMSD was affected in a synergic manner by piribedil, indicating a strong positive correlation between striatal DA levels and nigral c-Fos activation. Hence, it is suggested that memories processes were severely impacted by both D2 blockade and REMSD and even more by their combination. Conversely, the activation of D2 receptor counteracted such memory impairment. Therefore, the present evidence reinforce that the D2 receptor is a key player in the SNpc neuronal activation mediated by REMSD, as a consequence these changes may have direct impact for cognitive and sleep abnormalities found in patients with PD.

INTRODUÇÃO

Durante o sono, ao contrário do que já foi preconizado em muitas teorias que propunham uma redução maciça de ativação neuronal, inúmeras regiões encefálicas encontram-se mobilizadas, sendo suas atividades claramente detectadas a partir de estudos eletrofisiológicos ou de neuroimagens [1,2,3]. Alternando-se ritmicamente com o estado de vigília, o sono se desenrola em diversas fases consecutivas que se repetem ciclicamente. Ou seja, um ciclo completo de sono, dentro de um contexto de arquitetura normal é de cerca de 90 minutos em seres humanos e de 15 minutos em ratos [4].

Em seres humanos a caracterização das fases de sono pode ser feita com base em três variáveis eletrofisiológicas que compreendem o EEG, o eletrooculograma (EOG) e a eletromiografia (EMG). Através delas são detectados dois padrões fundamentais de sono: sono de ondas lentas (SOL) e sono com ocorrência de movimentos oculares rápidos (REM) ou também chamado de sono paradoxal. O SOL é composto por quatro fases (I, II, III e IV) onde se observa uma lentificação do ritmo cortical traduzido como um aumento da amplitude e redução da frequência do padrão de ondas (prevalência de ondas gama 33-55 Hz) [5,6]. Por outro lado, o sono paradoxal apresenta uma predominância de ondas teta (5-8 Hz) que também estão presentes durante a vigília (fig. 1) [6,7].



A descoberta dos ciclos de sono despertou considerável interesse pelos mecanismos neurais envolvidos na geração e manutenção do ciclo vigília-sono. Com o desenvolvimento de novas tecnologias, como a análise de expressão gênica e animais transgênicos, grandes progressos foram conquistados no entendimento das vias neurais responsáveis pela vigília, sono de ondas lentas e sono paradoxal [(1)] . Vários neurotransmissores como a noradrenalina (NA), acetilcolina (ACh), serotonina (5-HT), dopamina (DA) e neuropeptídeos como a orexina/hipocretina foram devidamente alocados dentro da circuitaria de regulação do sono [(8,9,10,11)].

O debate atual sobre o papel da DA no ciclo vigília-sono pode ser dividido em duas linhas distintas de pensamento: a primeira assume que a DA é um neurotransmissor diretamente envolvido nos eventos que promovem a vigília; e a segunda linha, mais recente, sugere que a DA é responsável por processos relacionados ao sono, em particular o sono paradoxal [(12)]. Inúmeras dessas evidências têm surgido a partir de estudos clínicos ou em modelos animais da doença de Parkinson (DP) que têm apontado o sistema dopaminérgico e suas áreas relacionadas com sendo chave para a regulação do sono.

Estruturas dopaminérgicas envolvidas na regulação do sono

A dopamina (DA) foi identificada como uma substância essencial na regulação dos estados de sono-vigília [(13,14)]. A DA está intimamente envolvida na regulação dos movimentos complexos e emoções [(14)]. Nos mamíferos, ela está contida em vários grupos celulares distribuído a partir do mesencéfalo caudal para o nível rostral do encéfalo[(14)].

A substância negra pars compacta (SNpc) e a área tegmental ventral (ATV) são regiões heterogêneas do mesencéfalo ventral, responsáveis pela produção de aproximadamente 80% da DA encefálica [(15)]. A SNpc é chamada assim porque seus neurônios apresentam um pigmento escuro chamado neuromelanina, o qual é produzido juntamente com a DA. Nas últimas décadas, pesquisadores fizeram progressos importantes para a compreensão do papel da dopamina e os núcleos da base. [(14)]. Esse grupo de neurônios presentes nos GB (SNpc, caudado/putâmen, núcleo subtalâmico,

globo pálido) têm sido relacionados a uma variedade de comportamentos motores e cognitivos, incluindo seus planejamentos e funções executivas e mais recentemente estas estruturas vêm sendo associadas com a regulação do sono (principalmente do sono paradoxal) [10]. A organização dos neurônios dopaminérgicos decorrentes da SNpc e da VTA é muito mais complexa do que inicialmente proposto. Além de os sistemas nigroestriatal, mesolímbico, e mesocortical, uma série de subsistemas dopaminérgicos distintos que inervam estruturas implicadas no controle do sono e vigília, tem sido reconhecidos [8].

Receptores dopaminérgicos estão presentes no estriado, no núcleo accumbens, tubérculo olfativo, no sistema límbico, no hipotálamo, e no tálamo. O receptor D2 também foi identificado nos neurônios da SNpc e da VTA, onde apresenta uma função de auto-receptor, possivelmente fornecendo feedback negativo para essas áreas. Como resultado, a conectividade recíproca entre o SNpc e VTA com a formação reticular pode exercer uma influência importante sobre o mecanismo de sono REM[16].

Sono E Memória

Existem evidências crescentes de que o sono pode ser importante para o aprendizado e para a memória, enquanto um comprometimento na qualidade do sono resulta em um déficit do desempenho tanto em roedores e humanos [39,40]. No entanto, o papel do sono na formação da memória é complexo e parece depender da natureza da tarefa [41]. Além disso, há dados que indicam que o sono deve ocorrer dentro de uma janela de tempo especifico, na sequência da fase de formação ou de aquisição, a fim de facilitar o aprendizado [43,44,45]. Por outro lado, a relevância fundamental do sono no processamento da memória tem sido questionada [46,47].

A tarefa de reconhecimento de objeto, originalmente desenvolvida para ratos [48] e aplicada em vários estudos [49,50], fornece dados úteis para investigar os efeitos de privação de sono sobre a consolidação e recuperação da memória. Os animais têm de aprender a reconhecer objetos ou a sua localização espacial, e deve ser capaz de fazê-lo quando testado 24 h mais tarde. Foi sugerido que as memórias de objetos em roedores podem ser comparadas com a memória tipo episódicas no humano [50].

Doença De Parkinson

A doença de Parkinson é um dos mais comuns distúrbios neurológicos, afetando 3% da população com idade superior a 65 anos e 4-5% das pessoas acima de 85 anos [51,52]. Pesquisas sobre a patogênese da DP tiveram um rápido avanço com o desenvolvimento de modelos animais que permitem a investigação de novos tratamentos. Alguns destes modelos são alcançados através da utilização de neurotoxinas, em particular 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP), 6-hidroxidopamina (6 -OHDA) e lipopolissacarídeo (LPS). Embora cada modelo mimetize diferentes características neuropatológicas da DP, estudos de múltiplos modelos são necessários para fornecer uma visão mais abrangente da patogênese da DP [53].

Como uma doença neurodegenerativa progressiva, a doença de Parkinson começa com um período pré-sintomático no qual a degeneração está presente, mas a doença não é clinicamente evidente. Isto sugere que deve ser possível prever a DP por exame clínico,através de sintomas, e outros marcadores.

As principais características clínicas incluem bradicinesia, rigidez, tremor de repouso, e os distúrbios do equilíbrio. Estes são o resultado da degeneração dos neurônios da SNpc que leva a uma redução de liberação de dopamina para o estriado. Existem também sintomas não motores, que estão relacionados ao inicio tardio da doença, incluindo diminuição cognitiva, depressão, distúrbios gastrointestinais, bem como alterações do sono [51,54].

- *Alterações Motoras*

A síndrome clínica comum associada à DP e outros tipos de Parkinsonismo inclui problemas motores relacionados com a desaceleração de movimentos (bradicinesia) [51,55], fraqueza [56,57], a rigidez [56,57], tremores, instabilidade postural [56], e a fadiga [51].

A bradicinesia é considerada uma das principais características observada na DP [57]. Este sintoma descreve a lentidão de um movimento voluntário, também se referindo à pobreza de movimentos espontâneos e comumente manifestada como congelamento e aumento do tempo necessário para iniciar um movimento (tempo de reação) [51,54,55,56].

- *Alterações Não- Motoras*

Estudos vêm indicando cada vez mais a presença de sintomas não motores na DP como fatores importantes na determinação da saúde e qualidade de vida relacionada à progressão da doença [58]. Além disso, características não-motoras geralmente não respondem á terapêutica dopaminérgica [59].

As principais manifestações não-motoras da DP incluem:

- Perturbação olfativa
- Ansiedade
- Depressão
- Demência
- Disfunção gastrointestinal
- Distúrbios do sono

Sono Na Doença de Parkinson

A neurobiologia do sono desenvolveu-se rapidamente nos últimos anos, com notável progresso neurofisiológico e conhecimento molecular sobre seus mecanismos[51,69].Uma visão geral da literatura demonstra que a dopamina tem desempenhado um papel importante na regulação do sono[63], como já dito anteriormente.

Praticamente todos os pacientes com DP sofrem de perturbações do sono diversas [69]. Dentro dessas perturbações, apresentam-se os distúrbios do sono grave, como sonolência diurna excessiva, distúrbio comportamental do sono REM, ataques de sono e insônia [51].

Estudos recentes mostram evidências crescentes de que a perturbação do sono e vigília na DP está mais intimamente relacionado com a doença em si e não representam exclusivamente um fenômeno secundário[51,54]. Este efeito primário da DP no sono pode estar ligada ao papel, de dopamina (DA) na modulação dos estados de sono-vigília [55].Além disso,descobertas recentes demonstram que a depleção de DA parcial provoca distúrbios do sono REM sem afetar as funções motoras[57].

Juntamente com a influência de neurotransmissão dopaminérgica na geração de distúrbios do sono,a evidência apóia ainda mais a hipótese de que a via nigroestriatal, especificamente, é fundamental na regulação de padrões de sono[69].

- ✓ Sonolência diurna excessiva

A sonolência diurna excessiva e involuntária e afeta até 50% dos pacientes com DP. Já os ataques de sono, definido como início súbito, com períodos imprevisíveis de sono, semelhante a aqueles de narcolepsia, chegam a afetar até 4% dos pacientes com DP. Esta taxa é muito mais elevada do que a observada na população em geral, na qual 4 a 21% das pessoas podem manifestar sonolência diurna excessiva, pelo menos 3 vezes por por semana[51,70].

- ✓ Ataques de sono

Dentre eles, estão os movimentos periódicos dos membros (PLM), síndrome das pernas inquietas (SPI) e acatisia. Vários estudos têm relatado um aumento de duas vezes na prevalência de síndrome das pernas inquietas e PLM na DP, e ambos os comportamentos são considerados fontes freqüentes de perturbações do sono.

Os PLM e a SPI estão intimamente ligados e são sensíveis a dopamina. Os agonistas dopaminérgicos são as drogas de escolha para o tratamento inicial destes distúrbios [57,70].

- ✓ Distúrbio comportamental do sono REM

Outra síndrome clínica que pode ser um prenúncio da DP é o distúrbio comportamental do sono REM (RBD), uma condição que pode aparecer décadas antes da doença [51,69]. É caracterizada por perda da atonia normal do sono REM, onde os pacientes afetados podem gritar chutar em associação com o conteúdo do sonho [57].

Aproximadamente metade dos pacientes que apresentam RBD desenvolve a DP e metade desenvolve demência. Este alto risco de doença faz da RBD um marcador ideal para a previsão de DP.

Vários grupos de pesquisa têm seguido pacientes com RBD idiopática por algumas décadas, e estudos mostraram que aproximadamente 40% dos pacientes desenvolveu uma síndrome parkinsoniana em uma década e dois terços dentro de duas décadas [57].

- ✓ Insônia

Queixas de insônia são relatadas em aproximadamente 80% de todos os

Pacientes com DP, e os sintomas incluem dificuldade em adormecer,

Dificuldade em manter o sono (ou seja, a fragmentação do sono), má qualidade

Do sono e/ou sono não reparador, os quais resultam em um comprometimento significativo do funcionamento diurno [57, 69,70].

Mecanismos Neuronais de regulação de sono

O uso de agonistas dopaminérgicos, como a apomorfina, mediante modelo animal de privação de sono REM (PSREM), acarreta aumento de responsividade motora, em comparação a animais não privados. Esses resultados foram os primeiros que apontaram em direção da hipótese de que a PSP é capaz de induzir supersensibilidade nos receptores dopaminérgicos [17] .

Nesta mesma linha, outros estudos do mesmo grupo proporcionaram novas informações dando suporte à hipótese de supersensibilidade dopaminérgica [18,19,20]. Verificou-se um intrigante comportamento de agressividade mediante tratamento com apomorfina nos animais privados de sono paradoxal. No entanto, a intensificação das respostas obtidas pelos animais privados não ficou restrita a apomorfina, sendo também observadas através da utilização de outros agonistas dopaminérgicos como a bromocriptina e o piribedil [18,21]. A então hipótese da supersensibilidade dopaminérgica foi reforçada, fato este que foi novamente verificado quando se testou um modelo animal de Parkinsonismo. Verificou-se que animais privados de sono paradoxal por 72 horas, sete dias após sofrerem lesões eletrolíticas na via nigroestriatal, apresentaram melhora significativa em parâmetros motores, tais como ambulação, levantar e latência, em

comparação ao grupo lesado e não-privado [22]. Desta forma é possível inferir que a PSP acarretou supersensibilidade dopaminérgica, e esta por sua vez, foi capaz de gerar um efeito compensatório frente à diminuição do disparo de DA, por parte dos neurônios pré-sinápticos, então lesados. Torna-se claro que existe uma estreita relação entre a via dopaminérgica nigroestriatal e as respostas fisiológicas de vigília-sono. Outros trabalhos mostram que lesões eletrolíticas na SNpc reduzem a resposta mediada pelo estado tálamo-cortical de vigília [23,24]. Ainda, lesões maiores do mesencéfalo ventral, incluindo da SNpc, produzem um acentuado prejuízo no estado de vigília-sono, similar ao que é observado na DP [25].

Neurônios dopaminérgicos do tegmento ventral mesencefálico (VMT) têm sido há muito tempo conhecidos por desempenhar um papel importante na regulação dos estados de sono-vigília [26]. Lesões do VMT diminuíram a excitação [27,28], enquanto que o aumento da dopamina extracelular promoveu vigília e diminuição tanto sono de ondas lentas quanto do sono paradoxal [29]. No entanto, uma diminuição do sono paradoxal e do sono de ondas lentas também tem sido relatado em lesões ou inativação do VMT [30,31].

Tais efeitos diferentes poderiam ser em parte devido aos diferentes papéis desempenhados pelos neurônios da SNpc e da área tegmental ventral (VTA) [26].Também parece provável que esses grupos de células dopaminérgicas poderiam ser diferencialmente ativos durante diferentes estados de sono-vigília, e particularmente durante o sono paradoxal, quando a atonia muscular ocorre simultaneamente com os sonhos, muitas vezes comparado a alucinações [32].

Monoaminas, neuropeptídeos e outros neurotransmissores têm sido implicados em destaque na regulação do sono-vigília. Como dito anteriormente, a dopamina tem se destacado como uma substância chave nessa regulação[33]. Tal envolvimento da DA foi previamente descrito após experimentos utilizando a privação de sono paradoxal, onde se observou a ocorrência de supersensibilidade dos receptores D2 [33,34]. Em estudos anteriores de nosso laboratório, foi analisada a participação do sistema dopaminérgico na regulação do sono [26]. Tomados em conjunto, os nossos

resultados anteriores indicaram que a via nigroestriatal desempenha um papel fundamental no estado de repouso [22,33]. Assim, é provável que a privação de sono REM pode desempenhar um papel relevante na expressão da tirosina hidroxilase (TH) nigral e estriatal, seguida de um sono rebote [26,22].

Os receptores dopaminérgicos D1 e D2 são os receptores mais ampla e abundantemente expressos em regiões do prosencéfalo basal como o estriado (dorsal e ventral) (36) . Observou-se que a administração sistêmica de um antagonista D2 como o haloperidol aumentou significativamente a porcentagem de SOL associado a um bloqueio de expressão de sono REM (8). No entanto, a injeção sistêmica de um agonista do receptor D2 (piribedil), foi capaz de induzir efeitos bifásicos, de tal forma que doses baixas reduziram a vigília, sendo que doses maiores apresentaram o efeito oposto (37,38). Esses achados puderam ser confirmados por meio de outros experimentos utilizando camundongos nocaute para o gene que codifica o transportador de DA (DAT), onde esses animais apresentaram um aumento significativo dos períodos de vigília e consequente redução da porcentagem de SOL (35). Lesões seletivas dos neurônios dopaminérgicos presentes na SNpc provocaram notáveis prejuízos de sono em ratos (12). Tomadas em conjunto estas observações experimentais sugerem que os neurônios dopaminérgicos desempenham um papel importante na regulação dos padrões de sono em ratos, e que distúrbios nestes neurônios produzem complicações maciças em todos os parâmetros analisados, especialmente aqueles relacionados ao sono paradoxal. Outros estudos demonstraram a ocorrência de um aumento robusto da atividade eletrofisiológica dos neurônios dopaminérgicos da ATV durante o sono REM (35). Tais padrões de ativação neuronal também foram detectados mediante protocolos de PSP, onde observou-se um aumento significativo da expressão da proteína c-Fos em áreas dopaminérgicas como a própria ATV (26).

A proteína c-Fos é um proto-oncogene celular que funciona como um ativador da transcrição de inúmeros genes, sendo ele pertencente à família de genes de resposta rápida imediata (immediate early gene). A transcrição de c-Fos é regulada em resposta a vários sinais extracelulares tais como fatores de crescimento (26). Comumente, relaciona-se um aumento de expressão da

proteína c-Fos, ou mesmo de seu transcrito, como marcador indireto da atividade neuronal, pois esta proteína é frequentemente expressa em virtude de aumento do metabolismo neuronal, como numa condição de geração de potenciais de ação [16]. Nesse sentido, também observou-se um aumento seletivo da expressão de c-Fos em neurônios dopaminérgicos da SNpc após a privação de sono total em períodos variando entre uma e três horas de privação de sono total induzido através do método do gentle handling [38]. Entretanto, a literatura aponta uma série de controvérsias relacionando esse aumento de atividade neuronal dopaminérgica como sendo fruto da condição de ausência de manifestação de sono, e ainda o tipo de fase de sono envolvido. Portanto, se faz necessário delimitar o papel das diversas estruturas envolvidas com o sistema dopaminérgico numa tentativa de se identificar quais delas está mais associada com a regulação do sono, em particular do sono REM.

JUSTIFICATIVA

Observou-se que a administração sistêmica de um antagonista D2 como o haloperidol aumentou significativamente a porcentagem de SOL associado a um bloqueio de expressão de sono paradoxal [8]. No entanto, a injeção sistêmica de um agonista do receptor D2 (piribedil), foi capaz de induzir efeitos bifásicos, de tal forma que doses baixas reduziram a vigília, sendo que doses maiores apresentaram o efeito oposto [37,38]. Esses achados puderam ser confirmados por meio de outros experimentos utilizando camundongos nocaute para o gene que codifica o transportador de DA (DAT), onde esses animais apresentaram um aumento significativo dos períodos de vigília e conseqüente redução da porcentagem de SOL [35]. Lesões seletivas dos neurônios dopaminérgicos presentes na SNpc provocaram notáveis prejuízos de sono em ratos [12]. Tomadas em conjunto estas observações experimentais sugerem que os neurônios dopaminérgicos desempenham um papel importante na regulação dos padrões de sono em ratos, e que distúrbios nestes neurônios produzem complicações maciças em todos os parâmetros analisados, especialmente aqueles relacionados ao sono paradoxal. Outros estudos demonstraram a ocorrência de um aumento robusto da atividade eletrofisiológica dos neurônios dopaminérgicos da ATV

durante o sono paradoxal [35]. Tais padrões de ativação neuronal também foram detectados mediante protocolos de PSP, onde observou-se um aumento significativo da expressão da proteína c-Fos em áreas dopaminérgicas como a própria ATV

Tendo isso em vista, espera-se que a administração de uma agonista D2 (piribedil) levaria a um aumento da expressão da proteína c-Fos na SNpc, que seria potencializada pela PSREM, devido aos eventos moleculares, neuroquímicos e comportamentais produzidos pela supersensibilidade dopaminérgica. Em contrapartida, o bloqueio dos receptores D2 (induzido por haloperidol) levaria a uma redução na expressão da proteína c-Fos, que poderia ser revertida pela PSREM.

OBJETIVOS

- **Objetivo geral**

O objetivo do presente projeto é de investigar os efeitos da PSREM sobre o perfil de ativação dos neurônios nigrais mediado por um agonista e um antagonista de receptores D2.

- **Objetivos específicos**

❖ Analisar os efeitos promovidos pela PSREM e rebote sobre o desempenho cognitivo de animais tratados com haloperidol e piribedil, tendo como parâmetro a memória implícita, dependente do estriado, utilizando o teste de reconhecimento de objetos.

❖ Determinar os efeitos promovidos pela PSREM e rebote sobre o desempenho motor de animais tratados com haloperidol e piribedil, utilizando o teste do campo aberto.

- Quantificar através de HPLC os efeitos promovidos pela PSREM e rebote sobre os níveis de neurotransmissores e seus metabólitos na SNpc e estriado de animais tratados com haloperidol e piribedil.

- Quantificar através de imuno-histoquímica os efeitos promovidos pela PSP e rebote sobre a ativação neuronal, indicada pela expressão da proteína c-Fos na SNpc de animais tratados com haloperidol e piribedil.

METODOLOGIA

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±2°C) on a 12-h light-dark cycle (lights on at 7:00 AM). The animals had free access to water and food throughout the experiment.

Ethics statement

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 Federal University of Paraná and was approved by the Institutional Ethics Committee (approval ID #555).

REMSD procedure

REMSD was attained by means of the single platform method, in which each sleep deprived animal is placed onto a cylindrical platform, 6.5 cm in diameter and surrounded by water about 1 cm below the platform surface (Lima et al. 2008). At the onset of each REM sleep episode, the animal experiences a loss of muscle tonus and falls into the water, thus being awakened. When platforms of this size are used, REM sleep is completely eliminated (Machado et al. 2004). Throughout the study, the experimental room was maintained at controlled conditions (22±2 ◦C, 12 h light/dark cycle, lights on 7:00 a.m.). The control group was kept in the same room as the REMSD rats during the study. Food and water were provided *ad libitum* by placing chow pellets and water bottles on a grid located on top of the tank.

Experimental design

The animals were distributed randomly in six groups for each experimental evaluation: control vehicle (n=10), control haloperidol (n=10), control piribedil (n=10), REMSD vehicle (n=10), REMSD haloperidol (n=10), REMSD piribedil (n=10). The rats from the sleep deprived groups underwent 24 h of REMSD and subsequently the respective groups received a single intraperitoneal (i.p.) injection of DMSO/saline 0.9% or haloperidol hydrochloride (1.5 mg/kg; Tocris Biosciences Bristol, UK) or piribedil dihydrochloride (8.0 mg/kg; Tocris Biosciences Bristol, UK) and 60 min after started the behavioral testing, including the open-field and the object recognition tests. At the end of these tests, the rats were allowed to sleep for 24 h (REB period). Afterwards, the groups were re-tested for the same behaviors and immediately decapitated for tissue dissection of striatum and hippocampus for neurochemical purposes or intracardially perfused and the brains were processed for immunohistochemistry to assess c-Fos expression within the SNpc.

Open-field test

The apparatus consists of a circular arena (1 m of diameter) limited by a 40 cm-high wall and illuminated by four 60 W lamps situated 48 cm above the arena floor, providing illumination around 300 lx (Broadhurst 1960). The animals were gently placed in the center of the arena and were allowed to freely explore the area for 5 min. During the experiments, the open-field was video recorded and the measures the locomotion and mean velocity were computed online by an image analyzer system (Smart junior, PanLab, Harvard Apparatus, Spain).

Object recognition test

The apparatus consists of an open box (width x length x height = 80 cm x 80 cm x 50 cm) made of wood and covered with a black opaque plastic film. The illumination on the floor of the box apparatus was around 186 lx. The objects to be discriminated were available in triplicate copies and were made of a biologically neutral material such as glass, plastic or metal. The objects were weighted so that the animals could not move them around in the arena. They

are not known to have any ethological significance for the rats and they had never been associated with a reinforce (Ennaceur and Delacour 1988).

The object recognition test consists of two phases, a sample phase (3 min duration) and a choice phase (3 min duration) with 15 min retention interval between the two phases (Ennaceur et al. 2005). In the sample phase two identical objects are exposed in the back corners of the open box, 10 cm away from the sidewall. The rat is placed in the open box facing away from the objects. The total time spent in exploring the two objects was video recorded. After 3 min of exploration, the rat is removed from the open box and returned to its cage. After a delay of 15 min elapsed the rat is reintroduced to the open box and the choice phase is started for a further 3 min. In the choice phase two different objects are exposed in the same locations that were occupied by the previous sample objects. One of the objects is identical to the object seen in the sample phase and the other is a novel object. The frequencies of approaches of each object are recorded.

The exploration is recorded only when the rat touches the object with its nose or that rat's nose is directed toward an object at a distance ≤2 cm. As a measure of discrimination, "discrimination index (DI)" was calculated by dividing the difference in number of explorations between the two objects (object N-object F) by the total amount of exploration for both objects (object N+object F). DI was then multiplied by 100 to express as a percentage.

Quantification of striatal and hippocampal neurotransmitters and metabolites

The striatum and hippocampus of the rats were rapidly dissected and stored at -80°C until the neurochemical quantification. The endogenous concentrations of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) noradrenaline (NA) and dihydroxyphenylglycol (DHPG) were assayed by reverse-phase high performance liquid chromatography (HPLC) with electrochemical detection.

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 (25ºC). 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 10,000x*g* for 30 min at 4ºC, 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 μm 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.

c-Fos immunohistochemistry

For the immunohistochemical study of the SNpc c-Fos containing-neurons, rats were deeply anesthetized with ketamine immediately after the PSD and rebound, and were intracardially perfused with saline first, then with 4% of the fixative solution formaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed from the skulls and were immersed for 1 week in that fixative solution at 4ºC. Subsequently, the brains were placed in 30% sucrose solution for 48 h before sectioning. Six 30 μm sections per animal were taken between bregma -4.92 mm and -5.28 mm coordinates (Paxinos and Watson

2005). These sections and coordinates were chosen because of their location in the mid-rostrocaudal part of the SNpc, which contains the highest dopaminergic neuronal density (Petroske et al. 2001; Reksidler et al. 2008).

Tissue sections were incubated with primary antibody anti-c-Fos, raised in rabbits, diluted in PBS containing 0.3% Triton X-100 (1:500; Chemicon, CA, USA) overnight at 4°C. Biotin conjugated secondary antibody incubation (1:200 Vector Laboratories, USA), was performed for 2 h at room temperature. After several washes in PBS, antibody complex was localized using the ABC system (Vectastain ABC Elite kit cat # PK6101, Vector Laboratories, USA) followed by 3,3´-diaminobenzidine reaction with nickel enhancement. The sections were then mounted onto gelatin-coated slides and coverslipped after dehydration in ascending concentrations of ethanol-xylene solutions. Cell counts and neuronal density determination were conducted making use of the software Image-Pro Express 6 (Media Cybernetics, CA, USA). The mean number of c-Fos-ir neurons in each hemisphere was considered to be representative of the SNpc neuronal cells in each animal. An adjacent series of sections was stained with cresyl violet to serve as a reference for cytoarchitectural purposes. The selected areas were digitized with a digital camera DP71 of a BX51 Olympus microscope (Olympus Optical Co, Japan).

Statistical analysis

Homogeneity of variance was assessed by the Bartlett test and normal distribution of the data by the Kolmogorov-Smirnov test. Differences between groups in the object recognition test were analyzed by two-way analysis of variance (ANOVA) - with treatment as the between-subjects factor and object as the within-subjects factor - followed by the Bonferroni post hoc test. Open-field test, discrimination index, neurochemical and histological findings were analyzed be one-way ANOVA followed by the Newman-Keuls post hoc test. Pearson's correlation coefficients (r^2) were calculated to establish relationships between neurotransmitters concentrations and respective SNpc neuronal activation. Values are expresses as mean ± standard error of mean (SEM). The level of significance was set at $P \leq 0.05$.

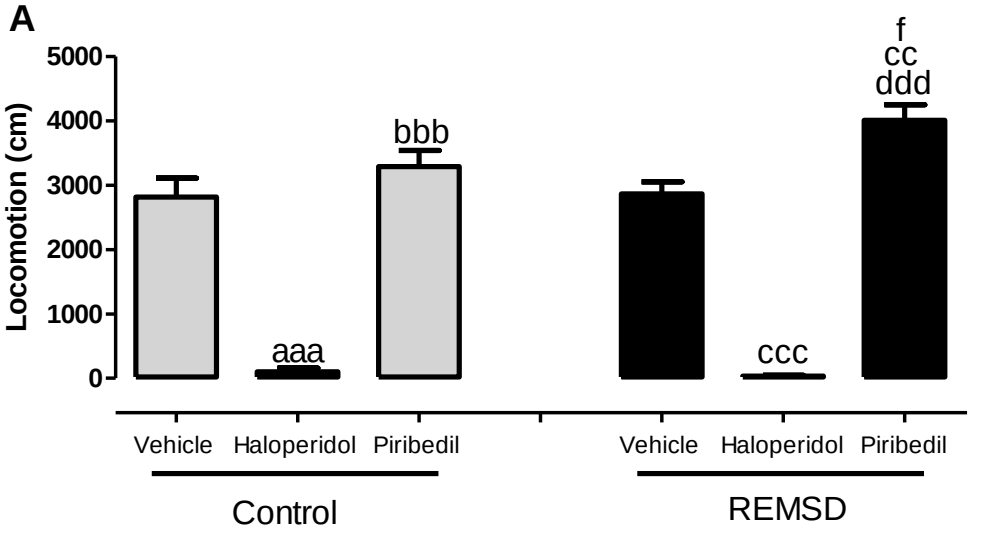
RESULTADOS

Open field test

As can been seen in Fig. 1A the control haloperidol and the REMSD haloperidol groups were equally impaired in comparison to the control vehicle (P<0.001) and REMSD vehicle (P<0.001) groups, respectively [F(5.48)=53.96; P<0.0001]. A similar effect was observed comparing the control haloperidol and the REMSD haloperidol groups to the control piribedil (P<0.001) and REMSD piribedil (P<0.001) groups. However, the REMSD piribedil group exhibited an increased locomotion when compared to the control piribedil (P<0.05) and REMSD vehicle groups (P<0.01). Considering the rebound period (Fig. 1B), the control

Piribedil group showed a higher locomotion in comparison to the control vehicle (P<0.001) and control haloperidol (P<0.001) groups. In addition, the REB haloperidol (P<0.001) and the REB piribedil (P<0.001) groups presented augmented locomotion when compared to the REB vehicle group. Conversely, the locomotion of the REB piribedil was higher (P<0.01) than the REB haloperidol group [F(5.48)=32.31; P<0.0001].

Fig. 1C shows the comparison between the REMSD groups to the REB groups regarding the locomotion. This demonstrated that the REB vehicle exhibited a decrease (P<0.001) in this parameter when compared to the REMSD vehicle group. In opposite, the REB haloperidol group presented an increment (P<0.001) in comparison to the REMSD haloperidol group [F(5.48)=45.38; P<0.0001]. The analysis of the mean velocity obtained from the groups revealed an absolutely equal statistical effect in the groups tested; therefore, these data are not presented in this article.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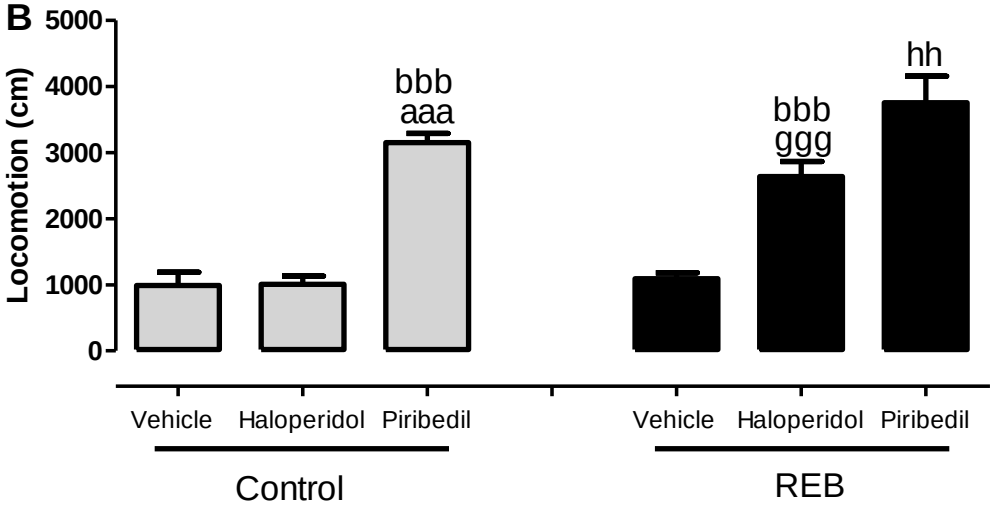
Piribedil
Control
REMSD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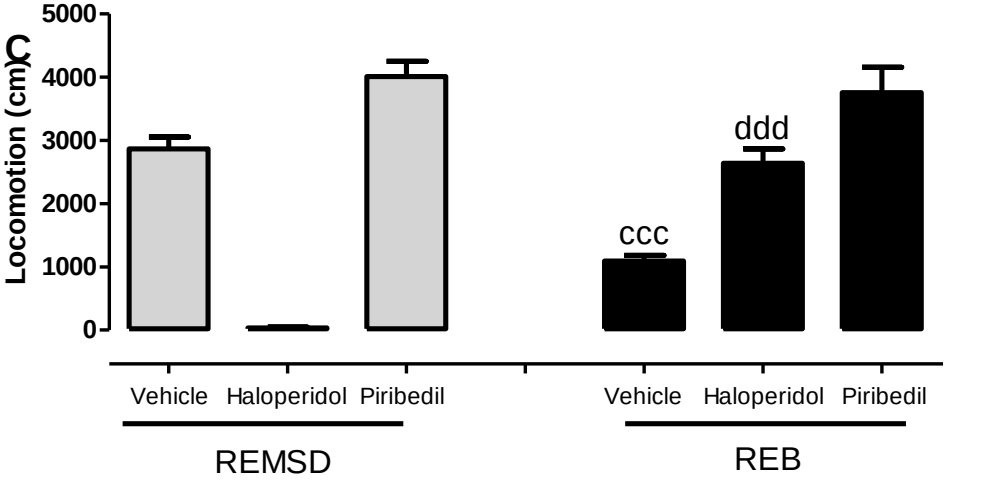
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Haloperidol
Piribedil
REMSD
REB

Figure 1. Locomotion parameter obtained from the open-field test. (A) Comparison between control and REMSD groups; (B) Comparison between control and REB groups; (C) Comparison between REMSD and REB groups. Values are expressed as mean ± SEM. ^{aaa}P<0.001 vs. control vehicle group; ^{bbb}P<0.001 vs. control haloperidol; ^{ccc}P<0.001 vs. REMSD vehicle; ^{ddd}P<0.001 vs. REMSD haloperidol; ^{f}P<0.05 vs. control piribedil; ^{ggg}P<0.001 vs. REB vehicle; ^{hh}P<0.01 vs. REB haloperidol. One-way ANOVA followed by the Newman-Keuls test.

Object recognition test

The results present in Fig. 2A unveil that the control vehicle group spent more time exploring the novel object in comparison to the familiar (P<0.001), during the choice phase, as indicated by the object factor [F(1.46)=21.92; P<0.0001]. A similar result was detected for the control piribedil group which explored more often the novel object (P<0.01) in comparison to the familiar. Conversely, the control haloperidol group demonstrated a similar number of explorations for both objects, indicating impairment in this function. REMSD produced a clear deficit in the object recognition task according since the REMSD vehicle group explored both objects equally. Besides, the novel object was less explored for the REMSD vehicle group when compared to the control vehicle group (P<0.01). In addition, the REMSD haloperidol group demonstrated a comparable diminished exploratory capacity, compared to the control haloperidol group according to the treatment factor [F(5.48)=38.98; P<0.0001]. In contrary, REMSD piribedil group exhibited the same increased exploration to the novel object (P<0.001) apparently reversing the damaged produced by the sleep deprivation, demonstrated by the REMSD vehicle group as indicated by the interaction factor [F(5.48)=11.97; P<0.0001].

Fig. 2B demonstrates the comparison between the control and REB groups after the rebound period. The control vehicle groups demonstrated a preserved capacity of increased exploration of the novel object (P<0.001), compared to the familiar as demonstrated by the object factor [F(1.48)=40.56; P<0.0001]. However, both control haloperidol and control piribedil groups did not show differences in object recognition. Thus, these very groups explored

less frequently the novel object (P<0.001) and (P<0.05), respectively, compared to the control vehicle group. Moreover, the REB vehicle group presented an increased exploration towards the novel object (P<0.001) compared to the familiar. Although, the REB haloperidol and REB piribedil groups did not presented differences in the objects exploration parameter. However, the REB piribedil group exhibited an increased number of explorations of both familiar (P<0.01) and novel objects (P<0.01) in comparison to the REB haloperidol group according to the treatment factor [F(5.48)=19.49; P<0.0001].

As depicted by Fig. 2C the comparison between the REMSD and REB groups indicated a significant effect of the object [F(1.48)=17.52; P=0.0001], treatment [F(5.48)=60.01; P<0.0001] and interaction [F(5.48)=5.22; P=0.0007] factors. The REB vehicle explored more often both, familiar (P<0.001) and objects (P<0.001), compared to the REMSD vehicle group. A similar pattern is observed for the REB haloperidol compared to the REMSD haloperidol (P<0.001; for both objects) and for the REB piribedil compared to the REMSD piribedil group (P<0.001; for both objects).

Lastly, Fig. 2D shows the discrimination index obtained from the number of exploration recorded for each group in the different REMSD and REB paradigms [F(11.94)=11.16; P<0.0001]. Accordingly, the control haloperidol group exhibited a significant reduction in this parameter compared to the control vehicle group (P<0.01). Moreover, the control piribedil group demonstrated an increment in this index, in comparison to the control haloperidol group (P<0.01). As projected by the previous data, REMSD promoted a significant decrease in this index for the REMSD vehicle group when compared to the control vehicle group (P<0.01). Likewise, the REMSD haloperidol group showed a decrease in this parameter compared to the REMSD vehicle group (P<0.01). In opposite, the REMSD piribedil group exhibited a significant increase when compared to the REMSD vehicle (P<0.01) and REMSD haloperidol (P<0.001) groups. Observing the REB period, the results indicated that the discrimination index is increased for the control haloperidol REB group (P<0.001) in comparison to the REMSD haloperidol REB group. However, this parameter was still impaired for the REMSD haloperidol REB group compared to the control haloperidol REB group (P<0.05).

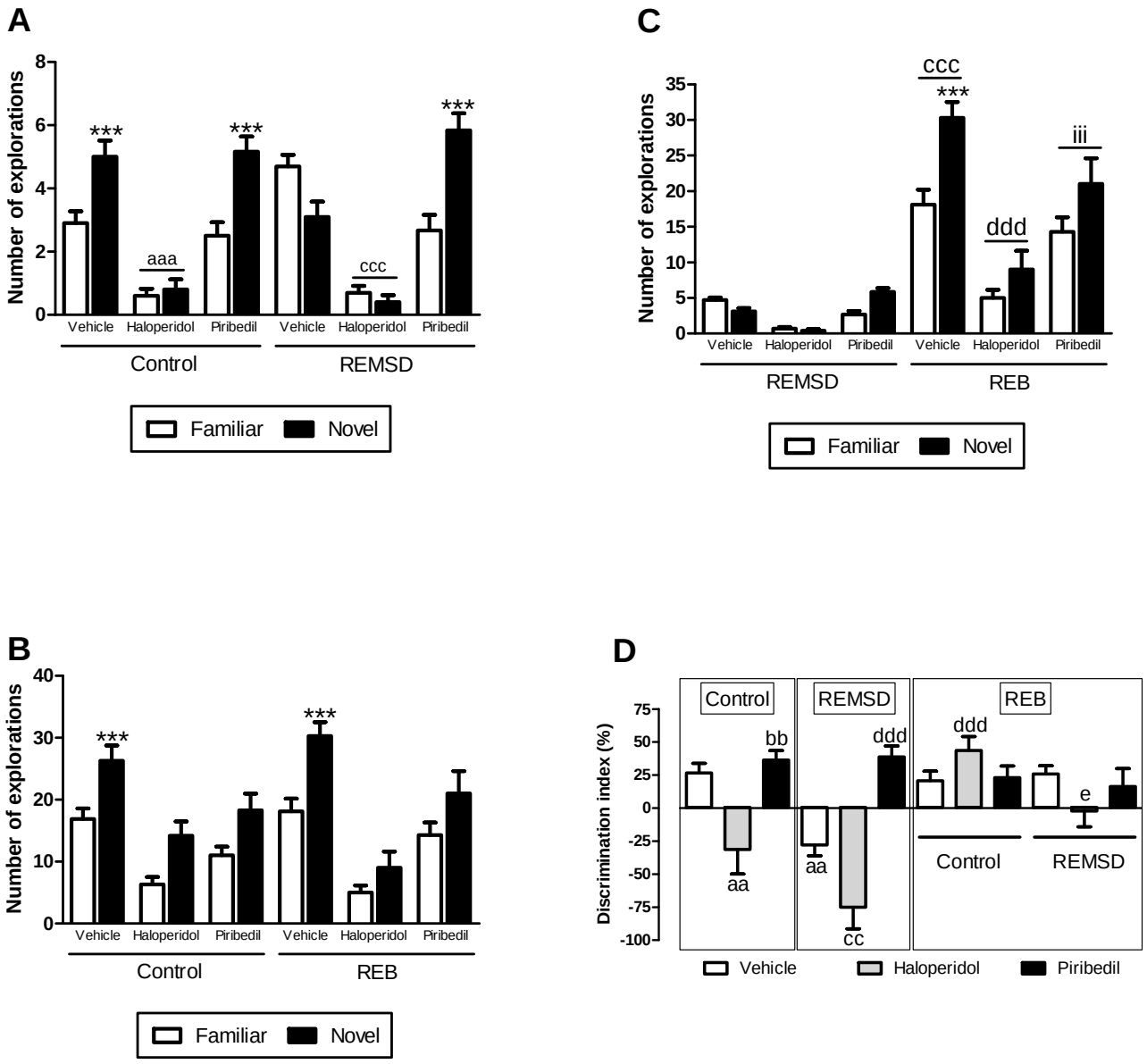

Figure 2. Cognitive effects elicited by haloperidol and piribedil after REMSD and REB. (A) Number of objects exploration after REMSD; (B) Number of objects exploration after REB; (C) Comparison between REMSD and REB periods; (D) Discrimination index. Values are expressed as mean ± SEM. ^{aaa}P<0.001 vs. control vehicle group; ^{ccc}P<0.001 vs. REMSD vehicle; ^{ddd}P<0.001 vs. REMSD haloperidol; ^{iii}P<0.001 vs. REMSD piribedil ***P<0.001 vs. the familiar object. Panels A, B and C were analyzed by two-way ANOVA followed by the Bonferroni test. Panel C was analyzed by one-way ANOVA followed by the Newman-Keuls test.

Quantification of striatal and hippocampal neurotransmitters and metabolites

Fig. 3 shows the alterations in the neurotransmission within the striatum. Accordingly, DA levels (Fig. 3A) were reduced in the REMSD group compared to the control vehicle group ($P<0.05$). Moreover, the control haloperidol group also showed a reduction in the striatal DA content compared to the control vehicle group ($P<0.01$). In opposite the control piribedil group presented an interesting increase of this neurotransmitter level compared to the control vehicle ($P<0.001$) and among all the others treated with vehicle or haloperidol ($P<0.001$). Still of note, the REMSD piribedil group demonstrated a significant increase in the DA levels in comparison to the control piribedil group ($P<0.001$), [$F(5.48)=152.7$; $P<0.0001$]. Considering the striatal DOPAC levels Fig. 3B the REMSD control group presented an increase of this metabolite compared to the control haloperidol group ($P<0.05$). In addition, the REMSD piribedil group exhibited a remarkable increase ($P<0.001$) in this metabolite among all the others [$F(5.48)=19.23$; $P<0.0001$]. An analogous profile of alterations was observed regarding the striatal HVA levels [$F(5.48)=7.17$; $P<0.0001$] data not shown. The calculation of the striatal DA turnover (Fig. 3C) indicated that the REMSD vehicle group presented an increase in this parameter ($P<0.05$), compared to the control vehicle group [$F(5.48)=8.34$; $P<0.0001$]. Besides, this increase was also significant ($P<0.01$) compared to the haloperidol and piribedil treated groups.

Regarding the 5-HT levels detected in the striatum (Fig. 3D) the control piribedil group showed an increase in this neurotransmitter ($P<0.05$) in comparison to the vehicle and haloperidol treated groups. In addition, the REMSD piribedil group presented a significant increase ($P<0.001$) in the 5-HT compared to the control piribedil group [$F(5.48)=8.16$; $P<0.0001$]. In addition, the metabolite 5-HIAA (Fig. 3E) presented a significant increase for both control piribedil ($P<0.05$) and REMSD piribedil ($P<0.001$) groups compared to all other groups. However, Fig. 3F shows the absence of statistical differences among the groups, considering the striatal 5-HT turnover, [$F(5.48)=1.63$; $P=0.17$].

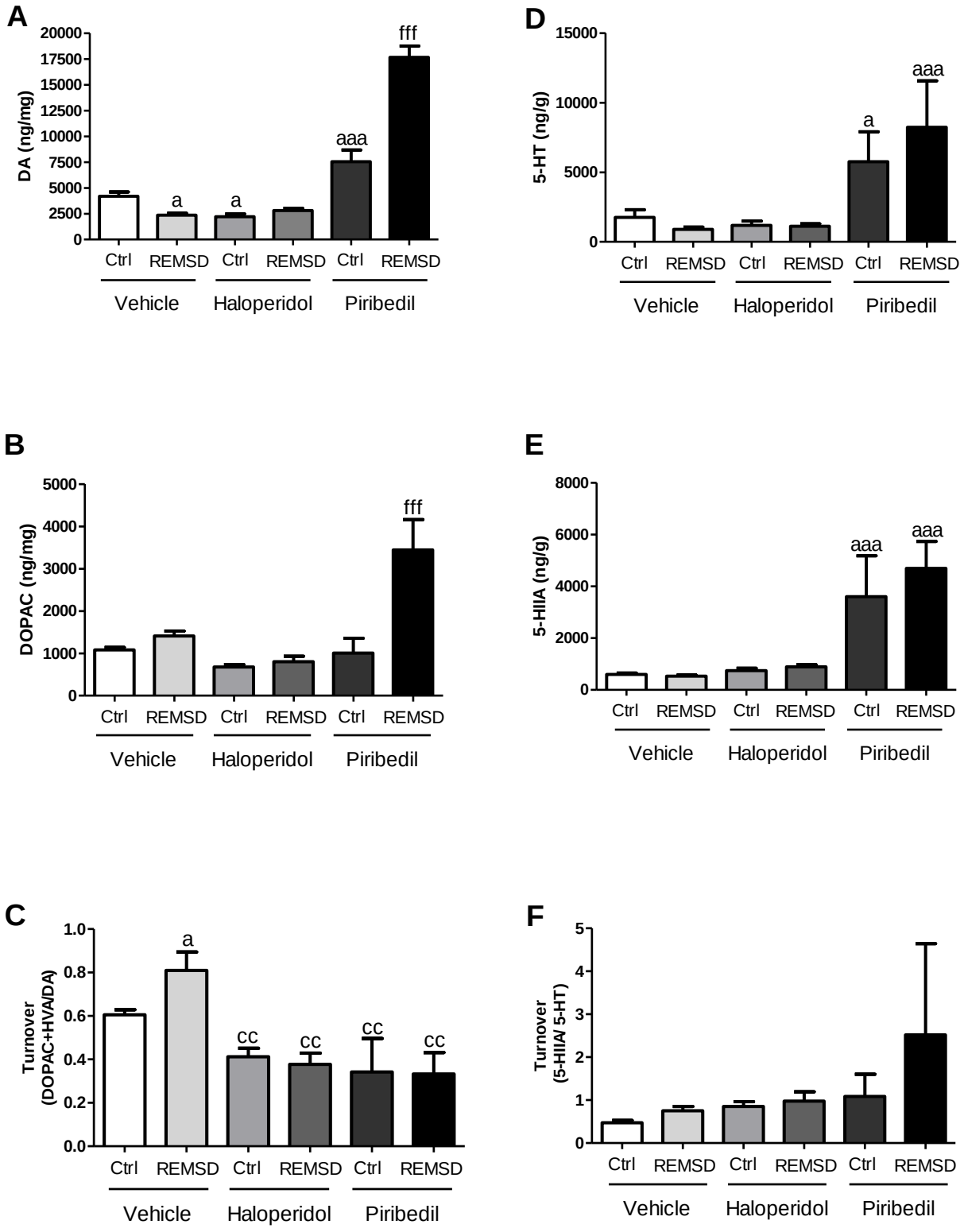

Figure 3. Neurochemical examination of the striatal content of DA, 5-HT and metabolites. (A) DA, (B) DOPAC, (C) DA turnover; (D) 5-HT; (E) 5-HIAA; (F) 5-HT turnover. Values are expressed as mean ± SEM. $^{a}P<0.05$, $^{aaa}P<0.001$ vs.

control vehicle group; ^{cc}P<0.01 vs. REMSD vehicle; ^{fff}P<0.001 vs. control piribedil. One-way ANOVA followed by the Newman-Keuls test.

Fig. 4 shows the alterations in the neurotransmission within the hippocampus. DA levels (Fig. 4A) have been found to be increased for the haloperidol (control and REMSD) and piribedil (control and REMSD) treated groups in comparison to the control vehicle (P<0.001) and REMSD vehicle (P<0.001) groups. Nevertheless, the REMSD piribedil group presented a significant increase (P<0.01) in the hippocampal DA levels compared to the control haloperidol and REMSD haloperidol groups F(5.48)=120.2; P<0.0001]. Furthermore, considering the striatal DOPAC content (Fig. 4B), a quite similar effect is observed. The haloperidol (control and REMSD) and piribedil (control and REMSD) treated groups demonstrated significant increases in the DOPAC levels in comparison to the control vehicle (P<0.001) and REMSD vehicle (P<0.001) groups. Although, the control piribedil group showed higher levels of DOPAC compared to the control haloperidol group (P<0.01). Moreover, the REMSD piribedil group exhibited increased levels of this metabolite (P<0.01) in comparison to the REMSD haloperidol group F(5.48)=112.5; P<0.0001]. Again, a similar profile of alterations was observed regarding the hippocampal HVA levels [F(5.48)=101.1; P<0.0001] data not shown. Regarding the hippocampal DA turnover (Fig. 4C), a massive reduction of this parameter was detected for the haloperidol (control and REMSD) and piribedil (control and REMSD) treated groups in comparison to the control vehicle (P<0.05) and REMSD vehicle (P<0.05) groups [F(5.48)=6.31; P=0.0002].

5-HT levels within the hippocampus (Fig. 4D) demonstrated to be increased for the control piribedil group compared to the REMSD vehicle group (P<0.05). Also, the REMSD piribedil group presented a significant increase (P<0.05) in this parameter compared to the vehicle control and REMSD vehicle groups [F(5.48)=3.71; P=0.0007]. In addition, the 5-HIAA levels (Fig. 4E) were found to be increased for both the haloperidol (control and REMSD) (P<0.001) and piribedil (control and REMSD) (P<0.001) groups [F(5.48)=22.5; P<0.0001]. Finally, regarding the hippocampal 5-HT turnover (Fig. 4F), only the control

haloperidol group presented a significant increase in this parameter (P<0.05), compared to the control vehicle group [F(5.48)=2.91; P=0.02].

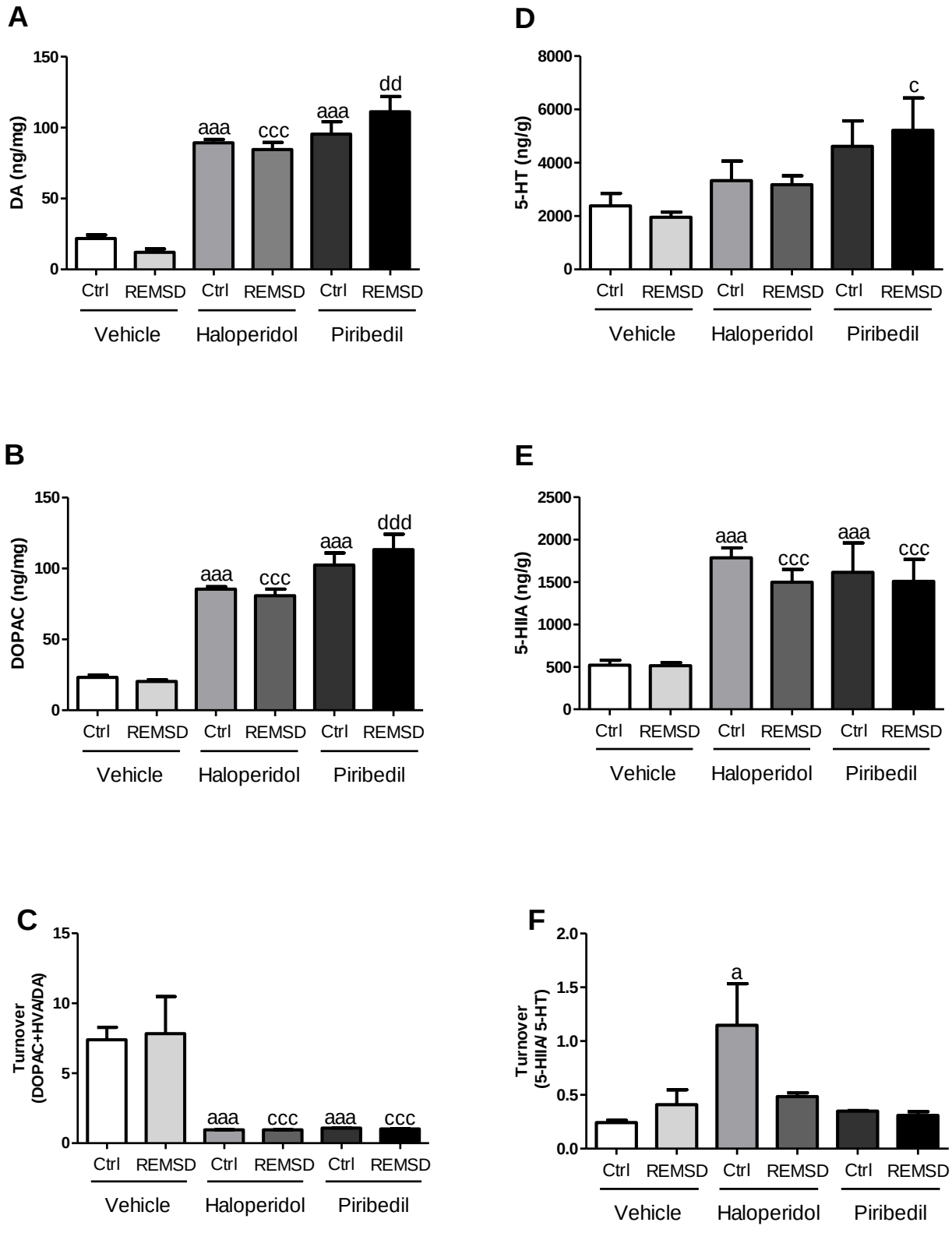

Figure 4. Neurochemical examination of the hippocampal content of DA, 5-HT and metabolites. (A) DA, (B) DOPAC, (C) DA turnover; (D) 5-HT; (E) 5-HIAA; (F) 5-HT turnover. Values are expressed as mean ± SEM. $^{a}P<0.05$, $^{aaa}P<0.001$ vs. control vehicle group; $^{c}P<0.05$; $^{ccc}P<0.001$ vs. REMSD vehicle; $^{dd}P<0.01$; $^{ddd}P<0.001$ vs. REMSD haloperidol. One-way ANOVA followed by the Newman-Keuls test.

c-Fos immunohistochemistry

As depicted in Fig. 5B, SNpc neuronal activation demonstrated to be significantly increased in the REMSD vehicle group in comparison to the control vehicle group ($P<0.01$). In opposite, the control haloperidol group presented a decrement in the nigral c-Fos immunoreactivity within the SNpc when compared to the control vehicle group ($P<0.01$). Interestingly, the REMSD haloperidol group showed an increment in the neuronal activity in comparison to the control haloperidol group ($P<0.001$). Of note, the control piribedil and the REMSD piribedil groups showed increased nigral c-Fos immunoreactivity in comparison to the control vehicle group ($P<0.001$). Thus, the REMSD piribedil demonstrated a significant increment ($P<0.001$) in the c-Fos expression compared to the control piribedil group [$F(5.48)=247.4$; $P<0.0001$].

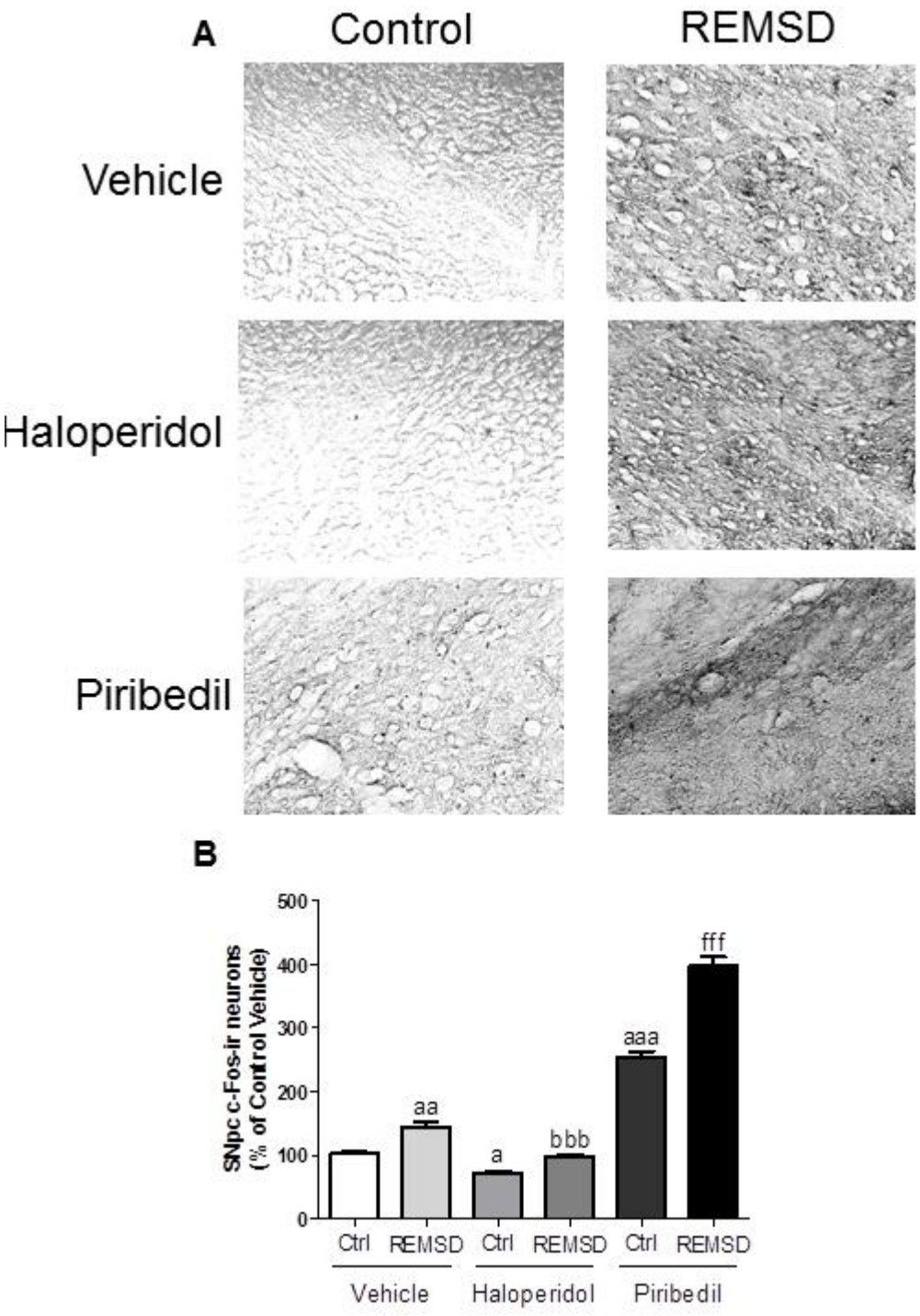

Figure 5. Nigral c-Fos activation after REMSD of haloperidol and piribedil groups. (A) Photomicrograph of representative sections of c-Fos immunoreactive (c-Fos-ir) neurons in the SNpc of the groups (magnification 200x). (B) Quantitative estimation of the SNpc c-Fos-ir neurons of the groups.

[a]P<0.05, [aa]P<0.01 [aaa]P<0.001 vs. control vehicle group; [bbb]P<0.001 vs. control haloperidol; [fff]P<0.001 vs. control piribedil.

Neurotransmitters-induced alterations by REMSD strongly correlate with nigral c-Fos immunoreactivity

Pearson's correlation coefficients revealed a strong positive correlation (r= +0.87; P<0.001) between the striatal DA concentration and the nigral c-Fos immunoreactivity for the groups analyzed (Fig. 6A). Additionally, a moderate positive correlation (r= +0.51; P<0.001) was found between striatal 5-HT and c-Fos immunoreactivity in the SNpc (Fig. 6B). In fact, hippocampal DA also closely correlated (r= +0.45; P=0.01) to the SNpc neuronal activation (Fig. 6C). Furthermore, hippocampal 5-HT levels moderately correlated (r= +0.44; P=0.015) c-Fos neuronal activation within the SNpc (Fig. 6D).

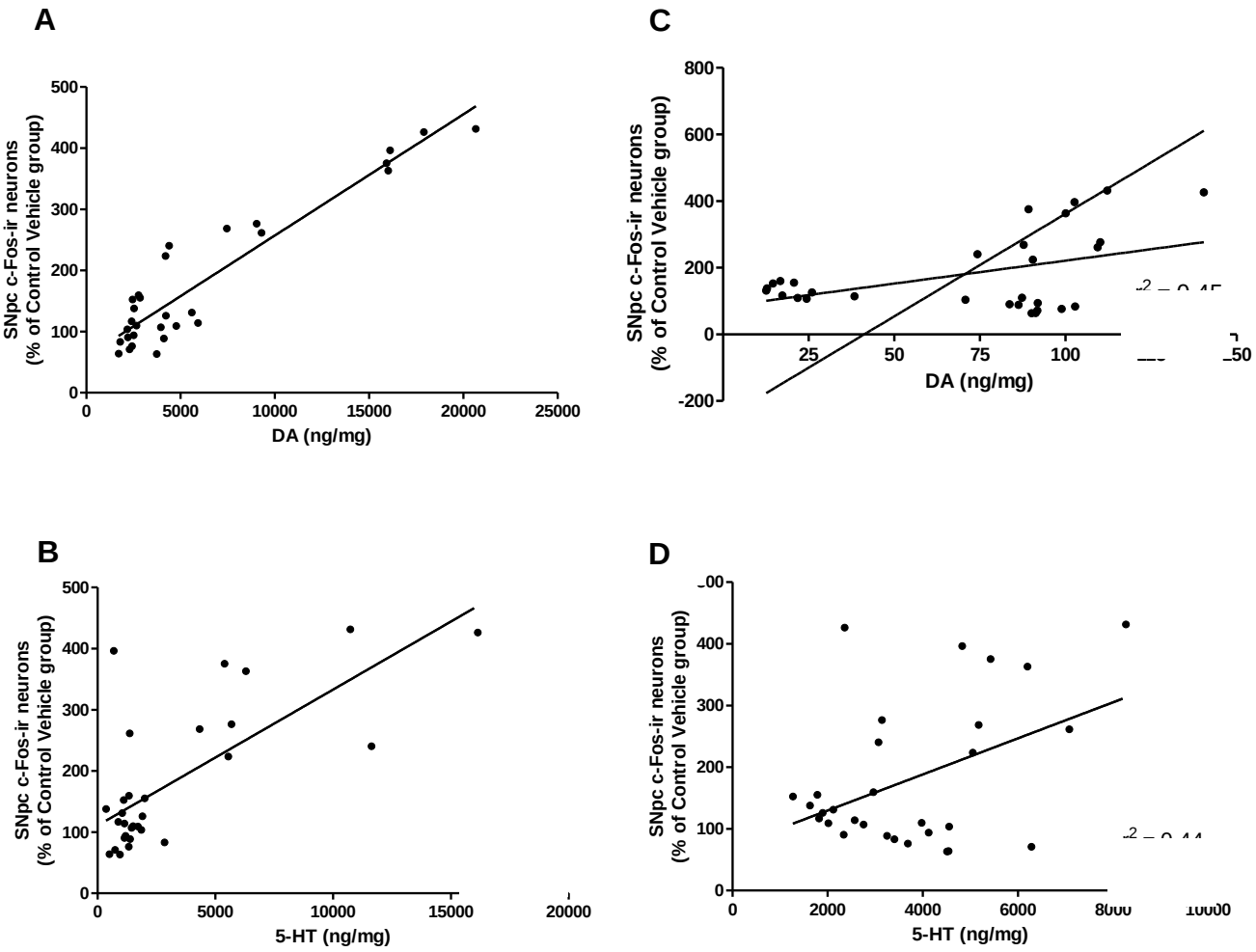

Figure 6: The induction of c-Fos-ir neurons within the SNpc closely correlate with the DA levels in the striatum after REMSD. Pearson's correlation coefficients were calculated considering the following: (A) SNpc c-Fos-ir neurons vs. striatal DA; (B) SNpc c-Fos-ir neurons vs. striatal 5-HT; (C) SNpc c-Fos-ir neurons vs. hippocampal DA; (D) SNpc c-Fos-ir neurons vs. hippocampal 5-HT.

DISCUSSÃO - ARTIGO

In the current study we demonstrated that striatal DA release is strongly enhanced by the selective D2 agonist, piribedil, and this effect was even more boosted by the REMSD. Conversely the blockade of D2 receptors by haloperidol prevented that alteration. A similar profile was obtained for the striatal 5-HT release. However, regarding the DA and 5-HT within the hippocampus, the haloperidol and piribedil groups exhibited comparable levels, although with increased contents also for the haloperidol groups. Additionally, the cognitive task was massively impaired by the D2 blockade associated to the REMSD, but not by the piribedil treatment. This effect was also detected after the REB period. Besides, an increased SNpc c-Fos immunoreactivity was observed for the REMSD and piribedil REMSD groups, indicating that changes in the DA release and in neuronal activity within the nigrostriatal system, promoted by REMSD, are strongly correlated to each other. Therefore, the present data indicate that the D2 receptor is a key player in the SNpc neuronal activation mediated by REMSD, hence, reverberating in neurochemical and cognitive functions associated to DA neurotransmission.

It was reported that the occurrence of a strong relationship between motor impairment and rhythm disorganization in MPTP-treated monkeys (Almirall et al. 2001). Indeed, electrophysiological data showed that the absence of half of the SNpc dopaminergic neurons provoked a major impairment in the sleep-wake parameters, mainly REM sleep (Lima et al. 2007a). Furthermore, normal REM sleep can be suppressed in both normal and DAT-KO mice without affecting motor functions by diminishing dopaminergic tone (Dzirasa et al. 2006). Moreover, it has been demonstrated the existence of changes in the

extracellular levels of DA in the terminal regions of VTA neurons over the course of the sleep-wake cycle (Lena et al. 2005).

The current neurochemical results are in accordance with these previous findings, thus indicating that REMSD was able to promote a remarkable neurochemical imbalance, predominantly of DA neurotransmission within the striatum. Moreover, REMSD demonstrates to increase striatal DA turnover, which is interpreted as a compensatory mechanism associated to DA receptor sensitization (Tufik et al. 1978; Enz et al. 1984; Nunes Junior et al. 1994). In addition, it was observed that piribedil generated a significant increase in the DA content as well as 5-HT. However, such result was not detected for the haloperidol treated groups, suggesting that the activation of D2 receptors could rescue the striatal DA levels depleted by the REMSD. Indeed, the REMSD produced an apparent additive effect on the levels of DA in the striatum that were not observed for the 5-HT. Particularly, striatal 5-HT or 5-HIAA levels were incremented only by the presence of piribedil, despite the REMSD. Concerning the hippocampal neurotransmission, both haloperidol and piribedil produced similar effects on DA and 5-HT levels, i.e., increase in comparison to vehicle groups. Hence, REMSD seems to be overlooked in this context.

Two effects of this neurochemical modulation were of special note: first, haloperidol and piribedil pretreatment affected DA and 5-HT synthesis and degradation in the same general manner, although piribedil's effects were more robust (both in striatum and hippocampus). Second, the treatment with haloperidol and piribedil appeared to attenuate the acute effects of REMSD on the DA turnover increase in the striatum. In fact, it has also been reported that acute administration of apomorphine to haloperidol-pretreated rats causes a potentiated reduction in DA synthesis (Bannon et al. 1980; Reches et al. 1985), which is in accordance to our findings of piribedil/REMSD-induced striatal DA synthesis. Instead, it is also been reported the manifestation of a potentiated quinpirole-induced decline in DA synthesis did not occur after D2 blockade (Der-Ghazarian et al. 2010).

It has been reported that DA neurons exhibit enhanced c-Fos activity in bursts of spikes that are associated with REM sleep (Maloney et al. 2002). Furthermore, a robust increase in the firing of dopaminergic neurons of the VTA has been identified during REM sleep (Dahan et al. 2007). However, few

studies approach this issue focusing specifically on the SNpc neuronal activation-induced by REMSD. Thus, as well as measuring DA synthesis, the ability of haloperidol and piribedil to modulate SNpc neuronal activation was assessed in control and REMSD rats. In the present experimental conditions c-Fos was induced in a substantial portion of the SNpc after REMSD, although, haloperidol appeared to block this activation.

On the contrary, piribedil associated to REMSD generated a synergic induction of c-Fos content within the SNpc. It should be considered that this protein induction requires synaptic receptor activation and increased concentration of intracellular calcium but that increased spike activity does not necessarily induce c-Fos (Luckman et al. 1994). Conversely, c-Fos expression can occur independently of neuronal discharge (Morgan and Curran 1991). However, our findings provided remarkable evidence of the occurrence of a strong positive correlation (r=+0.87) between striatal DA levels and nigral c-Fos activation. That is, the association of D2 activation and REMSD manipulations that produced more predominantly DA increase tend to elicit increases in the nigral c-Fos activation. A somewhat similar, but weaker (r=+0.51), correlation is also observed regarding the striatal 5-HT levels and the nigral c-Fos activation. Complementarily, these correlations (DA x c-Fos and 5-HT x c-Fos) seems to be weaker in the hippocampus, compared to the striatum. Therefore, the notion that dopaminergic neurons purportedly present a static firing rate throughout sleep-wake cycles, a concept that was previously promulgated in the literature, is strongly refuted by these demonstrations and by several others in the literature (Tufik 1981; Asakura et al. 1994; Maloney et al. 2002; Lena et al. 2005; Dzirasa et al. 2006; Santos et al. 2008; Lima et al. 2012; Lima 2013).

Concerning the cognition, the control haloperidol and the REMSD haloperidol groups treated the novel object as familiar, thus failing to recognize the novelty. This effect was even clearly observed considering the discrimination index, which revealed that the impairment of the REMSD haloperidol group was larger compared to the control haloperidol group. Corroborating that, after the REB period only the REMSD haloperidol still showed a significant reduction in this cognitive parameter. In opposite, piribedil treated rats did not manifested such decline, in any period of analysis, even when they were REM sleep deprived. These results suggest that consolidation

or retention of recognition memories processes were severely disrupted by both D2 blockade and REMSD and even more by their combination. In light of the relationship between D2 receptors and REM sleep, it was reported that dopaminergic D2 blockade may produce the reduction or even suppression of REM sleep after a period of REMSD (Lima et al. 2008). In these findings, it is indicated that D2 antagonism (promoted by haloperidol) generated a robust REM sleep suppression, although the administration of a D2 agonist (piribedil) did not produce the inverse (Lima et al. 2008). In view of that, it is conceivable that the memory deficit inflicted by the D2 blockade could be related to the REM sleep suppression, even when the animals were allowed to perform the REB sleep. This proposition seems to be pertinent in relation to the activation of D2 receptor that prevented the potential harmful cognitive effect elicited by the REMSD.

Interestingly, these familiarity-based memory task is correlated to the human episodic-like memory (Morris 2001; Dere et al. 2004) which is impaired in early-stage PD patients (Souchay et al. 2006). Therefore, according to our results it is reasonable to suggest that REMSD could potentiate the memory deficits observed in the early-phase of development of PD. In fact, given the correlation between sleep disturbances and cognitive impairment, it is possible that sleep symptoms in PD patients might be considered as an early marker of dementia (Erro et al. 2012).

It is worth mentioning that to ensure an unbiased detection of the cognitive profile we performed the open-field test concomitant to the memory task in order to discard the motor influence potentially produced by the dopaminergic modulation. Interestingly, the REB was a period that still perpetrated the cognitive decline inflicted by the REMSD, but without the presence of any locomotor influence. This evidence support that our cognitive data from the REMSD groups were not merely a pharmacological product of a motor bias.

In summary, the D2 agonist piribedil and the D2 antagonist haloperidol caused similar changes in hippocampal DA and 5-HT levels, however, in the striatum; DA release was strongly enhanced by piribedil in the REMSD group. In opposite, the blockade of D2 receptors by haloperidol prevented that alteration. To the extent that it was determined, a c-Fos activation characteristic

of REMSD was affected in a synergic manner by piribedil, thus indicating that a strong positive correlation between striatal DA levels and nigral c-Fos activation occurs. Hence, it is suggested that consolidation or retention of recognition memories processes were severely impacted by both D2 blockade and REMSD and even more by their combination. Conversely, the activation of D2 receptor counteracted the memory impairment imposed by the REMSD. The current findings are in accordance with others that provide evidence of robust spectrum of antiparkinsonian actions of piribedil in rodent and in primate models of PD (Millan 2010). Therefore, the present evidence reinforce that the D2 receptor is a key player in the SNpc neuronal activation mediated by REMSD (Lima et al. 2008), as a consequence these changes may have direct impact for cognitive and sleep abnormalities found in patients with PD.

DISCUSSÃO DOS RESULTADOS

No presente estudo foi demonstrado que a liberação de dopamina estriatal é fortemente aumentada pelo agonista seletivo D2, o piribedil, e este efeito foi ainda mais aumentado pelo PSREM. Um perfil semelhante foi obtido para a liberação de 5-HT no estriado. No entanto, em relação a DA e 5-HT no hipocampo, os grupos de haloperidol e piribedil exibiram níveis comparáveis, embora com teores aumentados também para os grupos de haloperidol. Além disso, a tarefa cognitiva foi massivamente prejudicada pelo bloqueio D2 associado ao PSREM, mas não pelo tratamento com o piribedil. Este efeito foi também detectado após o período de rebote. Além disso, um aumento da imunorreatividade da c-Fos na SNPC foi observada para os grupos de PSREM e piribedil PSREM, indicando que as alterações na liberação de DA e na atividade neuronal do sistema nigroestriatal, promovidas por PSREM, estão fortemente correlacionados uns com os outros. Portanto, os dados indicam que o receptor D2 é uma peça chave na ativação neuronal mediada pela privação de sono REM na SNpc.

Dados eletrofisiológicos demonstraram que a ausência de metade dos neurônios dopaminérgicos na SNpc provocou um grande impacto negativo nos parâmetros de sono-vigília, principalmente no sono REM (Lima et al. 2007a). Além disso, demonstrou-se a existência de variações nos níveis extracelulares

de DA nas regiões terminais dos neurônios VTA ao longo do ciclo sono-vigília (Lena et al. 2005).

Os resultados neuroquímicos atuais estão de acordo com estas descobertas anteriores, indicando que a PSREM foi capaz de promover um desequilíbrio neuroquímico notável, predominantemente da neurotransmissão DA no estriado. Além disso, demonstra-se que a PSREM pode aumentar os níveis de DA no estriado, o que é interpretado como um mecanismo compensatório associado á sensibilização do receptor dopaminérgico (Tufik et al, 1978; Enz et al 1984; Nunes Junior et al 1994.). Observou-se também que o piribedil gerou um aumento significativo de DA, bem como de 5-HT. No entanto, este resultado não foi detectado para os grupos tratados com haloperidol, sugerindo que a ativação de receptores D2 pode resgatar os níveis de DA estriatais esgotados pela REMSD. A PSREM produziu um efeito aditivo aparente sobre os níveis de DA no estriado, que não foram observados para a 5-HT. Particularmente, os níveis do estriado de 5-HT ou 5-HIAA foram incrementados apenas pela presença de piribedil, apesar da PSREM. No que diz respeito à neurotransmissão hipocampal, tanto o haloperidol quanto o piribedil produziram efeitos semelhantes na DA e 5-HT. Assim, a PSREM parece ser ignorada neste contexto.

Tem sido relatado que os neurônios DA exibem uma atividade de c-Fos melhorada em explosões de picos que são associados com o sono REM (Maloney et al. 2002). Além disso, um aumento robusto no disparo de neurônios dopaminérgicos da VTA foi identificado durante o sono REM (Dahan et al. 2007). Entretanto, poucos estudos abordam esta questão com foco específico na ativação neuronal na SNpc induzida por PSREM. Assim, bem como medir a síntese de DA, a capacidade de haloperidol e piribedil para modular a ativação neuronal SNpc foi avaliada em ratos controle e PSREM. Nas presentes condições experimentais a c-Fos foi induzida em uma porção substancial do SNpc após PSREM, embora o haloperidol pareceu bloquear esta ativação.

Pelo contrário, piribedil associada a PSREM gera uma indução sinérgica de c-Fos dentro da SNpc. Deve considerar-se que esta indução de proteínas requer

a ativação do receptor sináptico e aumento da concentração de cálcio intracelular, mas que a atividade aumentada não necessariamente induz c-Fos (Luckman et al. 1994). Por outro lado, a expressão de c-Fos pode ocorrer independentemente da descarga neuronal (Morgan e Curran, 1991). No entanto, os nossos resultados forneceram evidências notáveis da ocorrência de uma forte correlação positiva (r = 0,87) entre os níveis de DA estriatais e ativação nigral da c-Fos. Isto é, a associação da ativação D2 e manipulações de PSREM que produziu aumento mais predominantemente DA tendem a provocar o aumento da ativação de c-Fos nigral. Um tanto semelhante, mas mais fraca (r = 0,51) correlação, também se verifica em relação aos níveis estriatais de 5-HT e a ativação de c-Fos nigral. Complementarmente, as correlações (DA x c-fos e de 5-HT x c-Fos) parecem ser mais fracas no hipocampo, em comparação com o estriado. Portanto, a noção de que os neurônios dopaminérgicos supostamente apresentam uma taxa de queima estática ao longo de ciclos de sono-vigília, um conceito que foi anteriormente promulgada na literatura, é fortemente refutada por estas manifestações e por vários outros na literatura (Tufik 1981; Asakura et al. 1994; Maloney et al 2002;. Lena et al 2005;. Dzirasa et al 2006;. Santos et al 2008;. Lima et al 2012;. Lima 2013).

Em relação à cognição, no haloperidol controle e os grupos tratados com haloperidol e PSREM, o animal ficou mais tempo no objeto tido como familiar, deixando de reconhecer a novidade. Este efeito foi claramente observado, mesmo considerando o índice de discriminação, o qual revelou que o comprometimento do grupo haloperidol PSREM foi superior quando comparado com o grupo controle de haloperidol. Corroborando que, após o período de REB apenas o haloperidol PSREM ainda apresentou redução significativa neste parâmetro cognitivo. Já os ratos tratados com piribedil não manifestaram tal declínio, em qualquer período de análise, mesmo quando eram privados do sono REM. Estes resultados sugerem que a consolidação ou a retenção de processos de reconhecimento de memórias foram gravemente perturbada por ambos, bloqueio D2 e REMSD e ainda mais pela combinação destes. Tendo em vista o relacionamento entre os receptores D2 e sono REM, foi relatado que o bloqueio dopaminérgico D2 pode produzir a redução ou mesmo a supressão

do sono REM, após um período de REMSD (Lima et al. 2008). Nestes resultados, é indicado que o antagonista D2 (haloperidol) gera uma supressão do sono REM robusto, embora a administração de um agonista de D2 (piribedil) não produziu o inverso (Lima et al. 2008). Em vista disso, é concebível que o déficit de memória causado pelo bloqueio D2 poderia estar relacionado com a supressão do sono REM, mesmo quando os animais foram deixados livres para o sono REB. Esta proposição parece ser pertinente em relação à ativação do receptor D2, que impediu o efeito cognitivo nocivo provocado pela REMSD.

Curiosamente, estas tarefas de memória com base na familiaridade está correlacionada com a memória episódica do ser humano (Morris 2001;. Dere et al 2004), que é prejudicada em pacientes em estágio inicial DP (Souchay et al 2006.). Portanto, de acordo com nossos resultados, é razoável sugerir que REMSD pode potencializar os déficits de memória observados na fase inicial de desenvolvimento da DP. De fato, tendo em conta a correlação entre os distúrbios do sono e disfunção cognitiva, é possível que os sintomas do sono em pacientes com DP seja considerado como um marcador precoce de demência (Erro et al. 2012).

Vale ressaltar que, para garantir uma detecção imparcial do perfil cognitivo foi realizado concomitantemente o teste de campo aberto para a tarefa de memória, a fim de descartar a influência do comportamento motor potencialmente produzido pela modulação dopaminérgica.

CONCLUSÕES

- Tanto o piribedil quanto o haloperidol causaram mudanças similares nos níveis de dopamina do hipocampo.
- As mudanças nos níveis de 5-HT no hipocampo causadas pelo agonista e antagonista dopaminérgico também foram similares.
- No estriado, o piribedil, juntamente com a REMSD, realçou fortemente a liberação de dopamina.
- Quanto ao haloperidol, houve um bloqueio dos receptores D2, impedido que ocorresse qualquer alteração.
- Nenhum dos grupos de tratamento apresentou mudanças no comportamento motor, avaliado pelo teste de campo aberto.Isso nos mostra que os resultados alcançados não se dão pelo fato de alterações no comportamento motor dos animais.
- Sugere-se que a consolidação ou a retenção de processos de reconhecimento de memórias foram gravemente afetados por ambos, bloqueio D2 e REMSD, e ainda mais pela combinação destes. Sendo razoável sugerir que REMSD pode potencializar os déficits de memória observados na fase inicial de desenvolvimento da DP.
- A tarefa cognitiva foi massivamente prejudicada pelo bloqueio D2 associado ao REMSD, mas não pelo tratamento com o piribedil. Este efeito foi também detectado após o período de rebote.
- Um aumento da imunorreatividade da c-Fos na SNPC foi observada para os grupos de REMSD e piribedil REMSD indicando que o receptor D2 é uma peça chave na ativação neuronal mediada pela privação de sono REM na SNPC.

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ANEXO

ARTIGO 1

Piribedil prevents cognitive, neurochemical and nigral neuronal activation impairments promoted by haloperidol after REM sleep deprivation

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Abstract

Currently, several studies addresses the novel link between sleep and dopaminergic neurotransmission, focusing most closely on the mechanisms by which Parkinson's disease (PD) and sleep may be intertwined. Therefore, variations in the activity of afferents during the sleep cycles, either at the level of DA cell bodies in the ventral tegmental area (VTA) and/or substantia nigra pars compacta (SNpc) or at the level of dopamine (DA) terminals in limbic areas may impact functions such as memory. Accordingly, we performed striatal and hippocampal neurochemical quantifications of DA, serotonin (5-HT) and metabolites of rats intraperitoneally treated with haloperidol (1.5 mg/kg) or piribedil (8 mg/kg) and submitted to REM sleep deprivation (REMSD) and sleep rebound (REB). Also, we evaluated the effects of REMSD on motor and cognitive parameters and SNpc c-Fos neuronal immunoreactivity. The results indicated that DA release was strongly enhanced by piribedil in the REMSD group. In opposite, haloperidol prevented that alteration. A c-Fos activation characteristic of REMSD was affected in a synergic manner by piribedil, indicating a strong positive correlation between striatal DA levels and nigral c-Fos activation. Hence, it is suggested that memories processes were severely impacted by both D2 blockade and REMSD and even more by their combination. Conversely, the activation of D2 receptor counteracted such memory impairment. Therefore, the present evidence reinforce that the D2 receptor is a key player in the SNpc neuronal activation mediated by REMSD,

as a consequence these changes may have direct impact for cognitive and sleep abnormalities found in patients with PD.

Keywords: dopamine, haloperidol, Parkinson´s disease, piribedil, REM sleep deprivation, substantia nigra pars compacta,

Introduction

Currently, several studies addresses the novel link between sleep and dopaminergic neurotransmission, focusing most closely on the mechanisms by which Parkinson's disease (PD) and sleep may be intertwined, whether as predictors or consequences of dopaminergic neurodegeneration (see Lima, 2012). Episodes of excessive daytime somnolence after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) - a well-known dopaminergic neurotoxin that mimics PD - injections in monkeys have been anecdotally reported as a prominent feature of Parkinsonism (Langston et al. 1984; Forno et al. 1986). The first report to provide evidence of sleep disruption by MPTP demonstrated a selective rapid eye movement (REM) sleep suppression that lasted 6-9 days after the last dose of the neurotoxin in cats (Pungor et al. 1990). Besides, it was demonstrated a strong relationship between motor impairment and rhythm disorganization in MPTP-treated monkeys (Almirall et al. 2001).

Moreover, it has been characterized that extracellular levels of dopamine (DA) changes in the terminal regions of ventral tegmental area (VTA) neurons over the course of the sleep-wake cycle (Lena et al. 2005). It was also observed that DA neurons exhibit enhanced c-Fos activity in bursts of spikes that are associated with REM sleep (Maloney et al. 2002). Furthermore, a robust increase in the firing of dopaminergic neurons of the VTA has been identified

during REM sleep (Dahan et al. 2007). Moreover, clinical evidence has illustrated a transient restoration of motor control in PD patients during episodes of REM sleep (De Cock et al. 2007). Therefore, the notion that dopaminergic neurons purportedly present a static firing rate throughout sleep-wake cycles, a concept that was previously promulgated in the literature, is strongly refuted by these demonstrations (Lima 2013).

In light of the relationship between dopaminergic neurotransmission and sleep, it was reported that dopaminergic D2 blockade may produce the reduction or even suppression of REM sleep after a period of REM sleep deprivation (Lima et al. 2008). Furthermore, electrophysiological data indicated that the absence of half of the substantia nigra pars compacta (SNpc) dopaminergic neurons, in rats, provoked a major impairment in the sleep-wake parameters, predominantly in REM sleep (Lima et al. 2007b). In addition, REM sleep could be recovered in the dopaminergic transporter knockout (DAT-KO) mice by selective activation of the D2, but not the D1, suggesting a particular role of this receptor in the regulation of REM sleep (Dzirasa et al. 2006). Such involvement of DA has been previously reported subsequent to sleep deprivation protocols, as being directly involved in the generation of burly dopaminergic D2 supersensitivity (Tufik et al. 1978; Tufik 1981; Nunes et al. 1994; Nunes Junior et al. 1994).

These observations, together with the theory that the pedunculopontine tegmental nucleus (PPT) and laterodorsal tegmental nuclei (LDT) - areas classically associated to REM sleep - are closely connected to the SNpc and VTA and consequently are directly affected by imbalances in DA levels (Lima 2013) could explain the participation of DA in REM sleep. These changes could

result from variations in the activity of afferents during the sleep cycles, either at the level of DA cell bodies in the VTA and/or SNpc or at the level of DA terminals in limbic areas (Lena et al. 2005) also impacting functions such as memory. To test this rationale we performed neurochemical quantifications of DA, 5-HT, as well as its metabolites levels within the striatum and hippocampus of rats treated with haloperidol (selective D2 antagonist) or piribedil (selective D2 agonist) and submitted to REM sleep deprivation (REMSD) and sleep rebound (REB). Then we evaluated the effects of REMSD on motor and cognitive parameters assessed through the open-field and object recognition tests. Lastly, c-Fos neuronal immunoreactivity was quantified within the SNpc was determined in both REMSD and REB paradigms.

Material and 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±2°C) on a 12-h light-dark cycle (lights on at 7:00 AM). The animals had free access to water and food throughout the experiment.

Ethics statement

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 Federal University of Paraná and was approved by the Institutional Ethics Committee (approval ID #555).

REMSD procedure

REMSD was attained by means of the single platform method, in which each sleep deprived animal is placed onto a cylindrical platform, 6.5 cm in diameter and surrounded by water about 1 cm below the platform surface (Lima et al. 2008). At the onset of each REM sleep episode, the animal experiences a loss of muscle tonus and falls into the water, thus being awakened. When platforms of this size are used, REM sleep is completely eliminated (Machado et al. 2004). Throughout the study, the experimental room was maintained at controlled conditions (22±2 ◦C, 12 h light/dark cycle, lights on 7:00 a.m.). The control group was kept in the same room as the REMSD rats during the study. Food and water were provided *ad libitum* by placing chow pellets and water bottles on a grid located on top of the tank.

Experimental design

The animals were distributed randomly in six groups for each experimental evaluation: control vehicle (n=10), control haloperidol (n=10), control piribedil (n=10), REMSD vehicle (n=10), REMSD haloperidol (n=10), REMSD piribedil (n=10). The rats from the sleep deprived groups underwent 24 h of REMSD and subsequently the respective groups received a single intraperitoneal (i.p.) injection of DMSO/saline 0.9% or haloperidol hydrochloride (1.5 mg/kg; Tocris Biosciences Bristol, UK) or piribedil dihydrochloride (8.0 mg/kg; Tocris Biosciences Bristol, UK) and 60 min after started the behavioral

testing, including the open-field and the object recognition tests. At the end of these tests, the rats were allowed to sleep for 24 h (REB period). Afterwards, the groups were re-tested for the same behaviors and immediately decapitated for tissue dissection of striatum and hippocampus for neurochemical purposes or intracardially perfused and the brains were processed for immunohistochemistry to assess c-Fos expression within the SNpc.

Open-field test

The apparatus consists of a circular arena (1 m of diameter) limited by a 40 cm-high wall and illuminated by four 60 W lamps situated 48 cm above the arena floor, providing illumination around 300 lx (Broadhurst 1960). The animals were gently placed in the center of the arena and were allowed to freely explore the area for 5 min. During the experiments, the open-field was video recorded and the measures the locomotion and mean velocity were computed online by an image analyzer system (Smart junior, PanLab, Harvard Apparatus, Spain).

Object recognition test

The apparatus consists of an open box (width x length x height = 80 cm x 80 cm x 50 cm) made of wood and covered with a black opaque plastic film. The illumination on the floor of the box apparatus was around 186 lx. The objects to be discriminated were available in triplicate copies and were made of a biologically neutral material such as glass, plastic or metal. The objects were weighted so that the animals could not move them around in the arena. They are not known to have any ethological significance for the rats and they had never been associated with a reinforce (Ennaceur and Delacour 1988).

The object recognition test consists of two phases, a sample phase (3 min duration) and a choice phase (3 min duration) with 15 min retention interval between the two phases (Ennaceur et al. 2005). In the sample phase two identical objects are exposed in the back corners of the open box, 10 cm away from the sidewall. The rat is placed in the open box facing away from the objects. The total time spent in exploring the two objects was video recorded. After 3 min of exploration, the rat is removed from the open box and returned to its cage. After a delay of 15 min elapsed the rat is reintroduced to the open box and the choice phase is started for a further 3 min. In the choice phase two different objects are exposed in the same locations that were occupied by the previous sample objects. One of the objects is identical to the object seen in the sample phase and the other is a novel object. The frequencies of approaches of each object are recorded.

The exploration is recorded only when the rat touches the object with its nose or that rat's nose is directed toward an object at a distance ≤2 cm. As a measure of discrimination, "discrimination index (DI)" was calculated by dividing the difference in number of explorations between the two objects (object N-object F) by the total amount of exploration for both objects (object N+object F). DI was then multiplied by 100 to express as a percentage.

Quantification of striatal and hippocampal neurotransmitters and metabolites

The striatum and hippocampus of the rats were rapidly dissected and stored at -80°C until the neurochemical quantification. The endogenous concentrations of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA)

noradrenaline (NA) and dihydroxyphenylglycol (DHPG) were assayed by reverse-phase high performance liquid chromatography (HPLC) with electrochemical detection.

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 (25ºC). 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 10,000x*g* for 30 min at 4ºC, 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 μm 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.

c-Fos immunohistochemistry

For the immunohistochemical study of the SNpc c-Fos containing-neurons, rats were deeply anesthetized with ketamine immediately after the PSD and rebound, and were intracardially perfused with saline first, then with 4% of the fixative solution formaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed from the skulls and were immersed for 1 week in that fixative solution at 4ºC. Subsequently, the brains were placed in 30% sucrose solution for 48 h before sectioning. Six 30 µm sections per animal were taken between bregma -4.92 mm and -5.28 mm coordinates (Paxinos and Watson 2005). These sections and coordinates were chosen because of their location in the mid-rostrocaudal part of the SNpc, which contains the highest dopaminergic neuronal density (Petroske et al. 2001; Reksidler et al. 2008).

Tissue sections were incubated with primary antibody anti-c-Fos, raised in rabbits, diluted in PBS containing 0.3% Triton X-100 (1:500; Chemicon, CA, USA) overnight at 4°C. Biotin conjugated secondary antibody incubation (1:200 Vector Laboratories, USA), was performed for 2 h at room temperature. After several washes in PBS, antibody complex was localized using the ABC system (Vectastain ABC Elite kit cat # PK6101, Vector Laboratories, USA) followed by 3,3´-diaminobenzidine reaction with nickel enhancement. The sections were then mounted onto gelatin-coated slides and coverslipped after dehydration in ascending concentrations of ethanol-xylene solutions. Cell counts and neuronal density determination were conducted making use of the software Image-Pro

Express 6 (Media Cybernetics, CA, USA). The mean number of c-Fos-ir neurons in each hemisphere was considered to be representative of the SNpc neuronal cells in each animal. An adjacent series of sections was stained with cresyl violet to serve as a reference for cytoarchitectural purposes. The selected areas were digitized with a digital camera DP71 of a BX51 Olympus microscope (Olympus Optical Co, Japan).

Statistical analysis

Homogeneity of variance was assessed by the Bartlett test and normal distribution of the data by the Kolmogorov-Smirnov test. Differences between groups in the object recognition test were analyzed by two-way analysis of variance (ANOVA) - with treatment as the between-subjects factor and object as the within-subjects factor - followed by the Bonferroni post hoc test. Open-field test, discrimination index, neurochemical and histological findings were analyzed be one-way ANOVA followed by the Newman-Keuls post hoc test. Pearson's correlation coefficients (r^2) were calculated to establish relationships between neurotransmitters concentrations and respective SNpc neuronal activation. Values are expresses as mean ± standard error of mean (SEM). The level of significance was set at $P \leq 0.05$.

Results

Open field test

As can been seen in Fig. 1A the control haloperidol and the REMSD haloperidol groups were equally impaired in comparison to the control vehicle ($P<0.001$) and REMSD vehicle ($P<0.001$) groups, respectively [$F(5.48)=53.96$; $P<0.0001$]. A similar effect was observed comparing the control haloperidol and

the REMSD haloperidol groups to the control piribedil (P<0.001) and REMSD piribedil (P<0.001) groups. However, the REMSD piribedil group exhibited an increased locomotion when compared to the control piribedil (P<0.05) and REMSD vehicle groups (P<0.01). Considering the rebound period (Fig. 1B), the control piribedil group showed a higher locomotion in comparison to the control vehicle (P<0.001) and control haloperidol (P<0.001) groups. In addition, the REB haloperidol (P<0.001) and the REB piribedil (P<0.001) groups presented augmented locomotion when compared to the REB vehicle group. Conversely, the locomotion of the REB piribedil was higher (P<0.01) than the REB haloperidol group [F(5.48)=32.31; P<0.0001].

Fig. 1C shows the comparison between the REMSD groups to the REB groups regarding the locomotion. This demonstrated that the REB vehicle exhibited a decrease (P<0.001) in this parameter when compared to the REMSD vehicle group. In opposite, the REB haloperidol group presented an increment (P<0.001) in comparison to the REMSD haloperidol group [F(5.48)=45.38; P<0.0001]. The analysis of the mean velocity obtained from the groups revealed an absolutely equal statistical effect in the groups tested; therefore, these data are not presented in this article.

Object recognition test

The results present in Fig. 2A unveil that the control vehicle group spent more time exploring the novel object in comparison to the familiar (P<0.001), during the choice phase, as indicated by the object factor [F(1.46)=21.92; P<0.0001]. A similar result was detected for the control piribedil group which explored more often the novel object (P<0.01) in comparison to the familiar.

Conversely, the control haloperidol group demonstrated a similar number of explorations for both objects, indicating impairment in this function. REMSD produced a clear deficit in the object recognition task according since the REMSD vehicle group explored both objects equally. Besides, the novel object was less explored for the REMSD vehicle group when compared to the control vehicle group ($P<0.01$). In addition, the REMSD haloperidol group demonstrated a comparable diminished exploratory capacity, compared to the control haloperidol group according to the treatment factor [$F(5.48)=38.98$; $P<0.0001$]. In contrary, REMSD piribedil group exhibited the same increased exploration to the novel object ($P<0.001$) apparently reversing the damaged produced by the sleep deprivation, demonstrated by the REMSD vehicle group as indicated by the interaction factor [$F(5.48)=11.97$; $P<0.0001$].

Fig. 2B demonstrates the comparison between the control and REB groups after the rebound period. The control vehicle groups demonstrated a preserved capacity of increased exploration of the novel object ($P<0.001$), compared to the familiar as demonstrated by the object factor [$F(1.48)=40.56$; $P<0.0001$]. However, both control haloperidol and control piribedil groups did not show differences in object recognition. Thus, these very groups explored less frequently the novel object ($P<0.001$) and ($P<0.05$), respectively, compared to the control vehicle group. Moreover, the REB vehicle group presented an increased exploration towards the novel object ($P<0.001$) compared to the familiar. Although, the REB haloperidol and REB piribedil groups did not presented differences in the objects exploration parameter. However, the REB piribedil group exhibited an increased number of explorations of both familiar

(P<0.01) and novel objects (P<0.01) in comparison to the REB haloperidol group according to the treatment factor [F(5.48)=19.49; P<0.0001].

As depicted by Fig. 2C the comparison between the REMSD and REB groups indicated a significant effect of the object [F(1.48)=17.52; P=0.0001], treatment [F(5.48)=60.01; P<0.0001] and interaction [F(5.48)=5.22; P=0.0007] factors. The REB vehicle explored more often both, familiar (P<0.001) and objects (P<0.001), compared to the REMSD vehicle group. A similar pattern is observed for the REB haloperidol compared to the REMSD haloperidol (P<0.001; for both objects) and for the REB piribedil compared to the REMSD piribedil group (P<0.001; for both objects).

Lastly, Fig. 2D shows the discrimination index obtained from the number of exploration recorded for each group in the different REMSD and REB paradigms [F(11.94)=11.16; P<0.0001]. Accordingly, the control haloperidol group exhibited a significant reduction in this parameter compared to the control vehicle group (P<0.01). Moreover, the control piribedil group demonstrated an increment in this index, in comparison to the control haloperidol group (P<0.01). As projected by the previous data, REMSD promoted a significant decrease in this index for the REMSD vehicle group when compared to the control vehicle group (P<0.01). Likewise, the REMSD haloperidol group showed a decrease in this parameter compared to the REMSD vehicle group (P<0.01). In opposite, the REMSD piribedil group exhibited a significant increase when compared to the REMSD vehicle (P<0.01) and REMSD haloperidol (P<0.001) groups. Observing the REB period, the results indicated that the discrimination index is increased for the control haloperidol REB group (P<0.001) in comparison to the REMSD haloperidol REB group. However, this parameter was still impaired for

the REMSD haloperidol REB group compared to the control haloperidol REB group ($P<0.05$).

Quantification of striatal and hippocampal neurotransmitters and metabolites

Fig. 3 shows the alterations in the neurotransmission within the striatum. Accordingly, DA levels (Fig. 3A) were reduced in the REMSD group compared to the control vehicle group ($P<0.05$). Moreover, the control haloperidol group also showed a reduction in the striatal DA content compared to the control vehicle group ($P<0.01$). In opposite the control piribedil group presented an interesting increase of this neurotransmitter level compared to the control vehicle ($P<0.001$) and among all the others treated with vehicle or haloperidol ($P<0.001$). Still of note, the REMSD piribedil group demonstrated a significant increase in the DA levels in comparison to the control piribedil group ($P<0.001$), [$F(5.48)=152.7$; $P<0.0001$]. Considering the striatal DOPAC levels Fig. 3B the REMSD control group presented an increase of this metabolite compared to the control haloperidol group ($P<0.05$). In addition, the REMSD piribedil group exhibited a remarkable increase ($P<0.001$) in this metabolite among all the others [$F(5.48)=19.23$; $P<0.0001$]. An analogous profile of alterations was observed regarding the striatal HVA levels [$F(5.48)=7.17$; $P<0.0001$] data not shown. The calculation of the striatal DA turnover (Fig. 3C) indicated that the REMSD vehicle group presented an increase in this parameter ($P<0.05$), compared to the control vehicle group [$F(5.48)=8.34$; $P<0.0001$]. Besides, this increase was also significant ($P<0.01$) compared to the haloperidol and piribedil treated groups.

Regarding the 5-HT levels detected in the striatum (Fig. 3D) the control piribedil group showed an increase in this neurotransmitter ($P<0.05$) in comparison to the vehicle and haloperidol treated groups. In addition, the REMSD piribedil group presented a significant increase ($P<0.001$) in the 5-HT compared to the control piribedil group [$F(5.48)=8.16$; $P<0.0001$]. In addition, the metabolite 5-HIAA (Fig. 3E) presented a significant increase for both control piribedil ($P<0.05$) and REMSD piribedil ($P<0.001$) groups compared to all other groups. However, Fig. 3F shows the absence of statistical differences among the groups, considering the striatal 5-HT turnover, [$F(5.48)=1.63$; $P=0.17$].

Fig. 4 shows the alterations in the neurotransmission within the hippocampus. DA levels (Fig. 4A) have been found to be increased for the haloperidol (control and REMSD) and piribedil (control and REMSD) treated groups in comparison to the control vehicle ($P<0.001$) and REMSD vehicle ($P<0.001$) groups. Nevertheless, the REMSD piribedil group presented a significant increase ($P<0.01$) in the hippocampal DA levels compared to the control haloperidol and REMSD haloperidol groups $F(5.48)=120.2$; $P<0.0001$]. Furthermore, considering the striatal DOPAC content (Fig. 4B), a quite similar effect is observed. The haloperidol (control and REMSD) and piribedil (control and REMSD) treated groups demonstrated significant increases in the DOPAC levels in comparison to the control vehicle ($P<0.001$) and REMSD vehicle ($P<0.001$) groups. Although, the control piribedil group showed higher levels of DOPAC compared to the control haloperidol group ($P<0.01$). Moreover, the REMSD piribedil group exhibited increased levels of this metabolite ($P<0.01$) in comparison to the REMSD haloperidol group $F(5.48)=112.5$; $P<0.0001$]. Again, a similar profile of alterations was observed regarding the hippocampal HVA

levels [F(5.48)=101.1; P<0.0001] data not shown. Regarding the hippocampal DA turnover (Fig. 4C), a massive reduction of this parameter was detected for the haloperidol (control and REMSD) and piribedil (control and REMSD) treated groups in comparison to the control vehicle (P<0.05) and REMSD vehicle (P<0.05) groups [F(5.48)=6.31; P=0.0002].

5-HT levels within the hippocampus (Fig. 4D) demonstrated to be increased for the control piribedil group compared to the REMSD vehicle group (P<0.05). Also, the REMSD piribedil group presented a significant increase (P<0.05) in this parameter compared to the vehicle control and REMSD vehicle groups [F(5.48)=3.71; P=0.0007]. In addition, the 5-HIAA levels (Fig. 4E) were found to be increased for both the haloperidol (control and REMSD) (P<0.001) and piribedil (control and REMSD) (P<0.001) groups [F(5.48)=22.5; P<0.0001]. Finally, regarding the hippocampal 5-HT turnover (Fig. 4F), only the control haloperidol group presented a significant increase in this parameter (P<0.05), compared to the control vehicle group [F(5.48)=2.91; P=0.02].

c-Fos immunohistochemistry

As depicted in Fig. 5B, SNpc neuronal activation demonstrated to be significantly increased in the REMSD vehicle group in comparison to the control vehicle group (P<0.01). In opposite, the control haloperidol group presented a decrement in the nigral c-Fos immunoreactivity within the SNpc when compared to the control vehicle group (P<0.01). Interestingly, the REMSD haloperidol group showed an increment in the neuronal activity in comparison to the control haloperidol group (P<0.001). Of note, the control piribedil and the REMSD piribedil groups showed increased nigral c-Fos immunoreactivity in comparison

to the control vehicle group (P<0.001). Thus, the REMSD piribedil demonstrated a significant increment (P<0.001) in the c-Fos expression compared to the control piribedil group [F(5.48)=247.4; P<0.0001].

Neurotransmitters-induced alterations by REMSD strongly correlate with nigral c-Fos immunoreactivity

Pearson's correlation coefficients revealed a strong positive correlation (r= +0.87; P<0.001) between the striatal DA concentration and the nigral c-Fos immunoreactivity for the groups analyzed (Fig. 6A). Additionally, a moderate positive correlation (r= +0.51; P<0.001) was found between striatal 5-HT and c-Fos immunoreactivity in the SNpc (Fig. 6B). In fact, hippocampal DA also closely correlated (r= +0.45; P=0.01) to the SNpc neuronal activation (Fig. 6C). Furthermore, hippocampal 5-HT levels moderately correlated (r= +0.44; P=0.015) c-Fos neuronal activation within the SNpc (Fig. 6D).

Discussion

In the current study we demonstrated that striatal DA release is strongly enhanced by the selective D2 agonist, piribedil, and this effect was even more boosted by the REMSD. Conversely the blockade of D2 receptors by haloperidol prevented that alteration. A similar profile was obtained for the striatal 5-HT release. However, regarding the DA and 5-HT within the hippocampus, the haloperidol and piribedil groups exhibited comparable levels, although with increased contents also for the haloperidol groups. Additionally, the cognitive task was massively impaired by the D2 blockade associated to the REMSD, but not by the piribedil treatment. This effect was also detected after

the REB period. Besides, an increased SNpc c-Fos immunoreactivity was observed for the REMSD and piribedil REMSD groups, indicating that changes in the DA release and in neuronal activity within the nigrostriatal system, promoted by REMSD, are strongly correlated to each other. Therefore, the present data indicate that the D2 receptor is a key player in the SNpc neuronal activation mediated by REMSD, hence, reverberating in neurochemical and cognitive functions associated to DA neurotransmission.

It was reported that the occurrence of a strong relationship between motor impairment and rhythm disorganization in MPTP-treated monkeys (Almirall et al. 2001). Indeed, electrophysiological data showed that the absence of half of the SNpc dopaminergic neurons provoked a major impairment in the sleep-wake parameters, mainly REM sleep (Lima et al. 2007a). Furthermore, normal REM sleep can be suppressed in both normal and DAT-KO mice without affecting motor functions by diminishing dopaminergic tone (Dzirasa et al. 2006). Moreover, it has been demonstrated the existence of changes in the extracellular levels of DA in the terminal regions of VTA neurons over the course of the sleep-wake cycle (Lena et al. 2005).

The current neurochemical results are in accordance with these previous findings, thus indicating that REMSD was able to promote a remarkable neurochemical imbalance, predominantly of DA neurotransmission within the striatum. Moreover, REMSD demonstrates to increase striatal DA turnover, which is interpreted as a compensatory mechanism associated to DA receptor sensitization (Tufik et al. 1978; Enz et al. 1984; Nunes Junior et al. 1994). In addition, it was observed that piribedil generated a significant increase in the DA content as well as 5-HT. However, such result was not detected for the

haloperidol treated groups, suggesting that the activation of D2 receptors could rescue the striatal DA levels depleted by the REMSD. Indeed, the REMSD produced an apparent additive effect on the levels of DA in the striatum that were not observed for the 5-HT. Particularly, striatal 5-HT or 5-HIAA levels were incremented only by the presence of piribedil, despite the REMSD. Concerning the hippocampal neurotransmission, both haloperidol and piribedil produced similar effects on DA and 5-HT levels, i.e., increase in comparison to vehicle groups. Hence, REMSD seems to be overlooked in this context.

Two effects of this neurochemical modulation were of special note: first, haloperidol and piribedil pretreatment affected DA and 5-HT synthesis and degradation in the same general manner, although piribedil's effects were more robust (both in striatum and hippocampus). Second, the treatment with haloperidol and piribedil appeared to attenuate the acute effects of REMSD on the DA turnover increase in the striatum. In fact, it has also been reported that acute administration of apomorphine to haloperidol-pretreated rats causes a potentiated reduction in DA synthesis (Bannon et al. 1980; Reches et al. 1985), which is in accordance to our findings of piribedil/REMSD-induced striatal DA synthesis. Instead, it is also been reported the manifestation of a potentiated quinpirole-induced decline in DA synthesis did not occur after D2 blockade (Der-Ghazarian et al. 2010).

It has been reported that DA neurons exhibit enhanced c-Fos activity in bursts of spikes that are associated with REM sleep (Maloney et al. 2002). Furthermore, a robust increase in the firing of dopaminergic neurons of the VTA has been identified during REM sleep (Dahan et al. 2007). However, few studies approach this issue focusing specifically on the SNpc neuronal

activation-induced by REMSD. Thus, as well as measuring DA synthesis, the ability of haloperidol and piribedil to modulate SNpc neuronal activation was assessed in control and REMSD rats. In the present experimental conditions c-Fos was induced in a substantial portion of the SNpc after REMSD, although, haloperidol appeared to block this activation.

On the contrary, piribedil associated to REMSD generated a synergic induction of c-Fos content within the SNpc. It should be considered that this protein induction requires synaptic receptor activation and increased concentration of intracellular calcium but that increased spike activity does not necessarily induce c-Fos (Luckman et al. 1994). Conversely, c-Fos expression can occur independently of neuronal discharge (Morgan and Curran 1991). However, our findings provided remarkable evidence of the occurrence of a strong positive correlation (r^2=+0.87) between striatal DA levels and nigral c-Fos activation. That is, the association of D2 activation and REMSD manipulations that produced more predominantly DA increase tend to elicit increases in the nigral c-Fos activation. A somewhat similar, but weaker (r^2=+0.51), correlation is also observed regarding the striatal 5-HT levels and the nigral c-Fos activation. Complementarily, these correlations (DA x c-Fos and 5-HT x c-Fos) seems to be weaker in the hippocampus, compared to the striatum. Therefore, the notion that dopaminergic neurons purportedly present a static firing rate throughout sleep-wake cycles, a concept that was previously promulgated in the literature, is strongly refuted by these demonstrations and by several others in the literature (Tufik 1981; Asakura et al. 1994; Maloney et al. 2002; Lena et al. 2005; Dzirasa et al. 2006; Santos et al. 2008; Lima et al. 2012; Lima 2013).

Concerning the cognition, the control haloperidol and the REMSD haloperidol groups treated the novel object as familiar, thus failing to recognize the novelty. This effect was even clearly observed considering the discrimination index, which revealed that the impairment of the REMSD haloperidol group was larger compared to the control haloperidol group. Corroborating that, after the REB period only the REMSD haloperidol still showed a significant reduction in this cognitive parameter. In opposite, piribedil treated rats did not manifested such decline, in any period of analysis, even when they were REM sleep deprived. These results suggest that consolidation or retention of recognition memories processes were severely disrupted by both D2 blockade and REMSD and even more by their combination. In light of the relationship between D2 receptors and REM sleep, it was reported that dopaminergic D2 blockade may produce the reduction or even suppression of REM sleep after a period of REMSD (Lima et al. 2008). In these findings, it is indicated that D2 antagonism (promoted by haloperidol) generated a robust REM sleep suppression, although the administration of a D2 agonist (piribedil) did not produce the inverse (Lima et al. 2008). In view of that, it is conceivable that the memory deficit inflicted by the D2 blockade could be related to the REM sleep suppression, even when the animals were allowed to perform the REB sleep. This proposition seems to be pertinent in relation to the activation of D2 receptor that prevented the potential harmful cognitive effect elicited by the REMSD.

Interestingly, these familiarity-based memory task is correlated to the human episodic-like memory (Morris 2001; Dere et al. 2004) which is impaired in early-stage PD patients (Souchay et al. 2006). Therefore, according to our

results it is reasonable to suggest that REMSD could potentiate the memory deficits observed in the early-phase of development of PD. In fact, given the correlation between sleep disturbances and cognitive impairment, it is possible that sleep symptoms in PD patients might be considered as an early marker of dementia (Erro et al. 2012).

It is worth mentioning that to ensure an unbiased detection of the cognitive profile we performed the open-field test concomitant to the memory task in order to discard the motor influence potentially produced by the dopaminergic modulation. Interestingly, the REB was a period that still perpetrated the cognitive decline inflicted by the REMSD, but without the presence of any locomotor influence. This evidence support that our cognitive data from the REMSD groups were not merely a pharmacological product of a motor bias.

In summary, the D2 agonist piribedil and the D2 antagonist haloperidol caused similar changes in hippocampal DA and 5-HT levels, however, in the striatum; DA release was strongly enhanced by piribedil in the REMSD group. In opposite, the blockade of D2 receptors by haloperidol prevented that alteration. To the extent that it was determined, a c-Fos activation characteristic of REMSD was affected in a synergic manner by piribedil, thus indicating that a strong positive correlation between striatal DA levels and nigral c-Fos activation occurs. Hence, it is suggested that consolidation or retention of recognition memories processes were severely impacted by both D2 blockade and REMSD and even more by their combination. Conversely, the activation of D2 receptor counteracted the memory impairment imposed by the REMSD. The current findings are in accordance with others that provide evidence of robust spectrum

of antiparkinsonian actions of piribedil in rodent and in primate models of PD (Millan 2010). Therefore, the present evidence reinforce that the D2 receptor is a key player in the SNpc neuronal activation mediated by REMSD (Lima et al. 2008), as a consequence these changes may have direct impact for cognitive and sleep abnormalities found in patients with PD.

Conflict of interests

The authors have declared that no conflict of interests exists.

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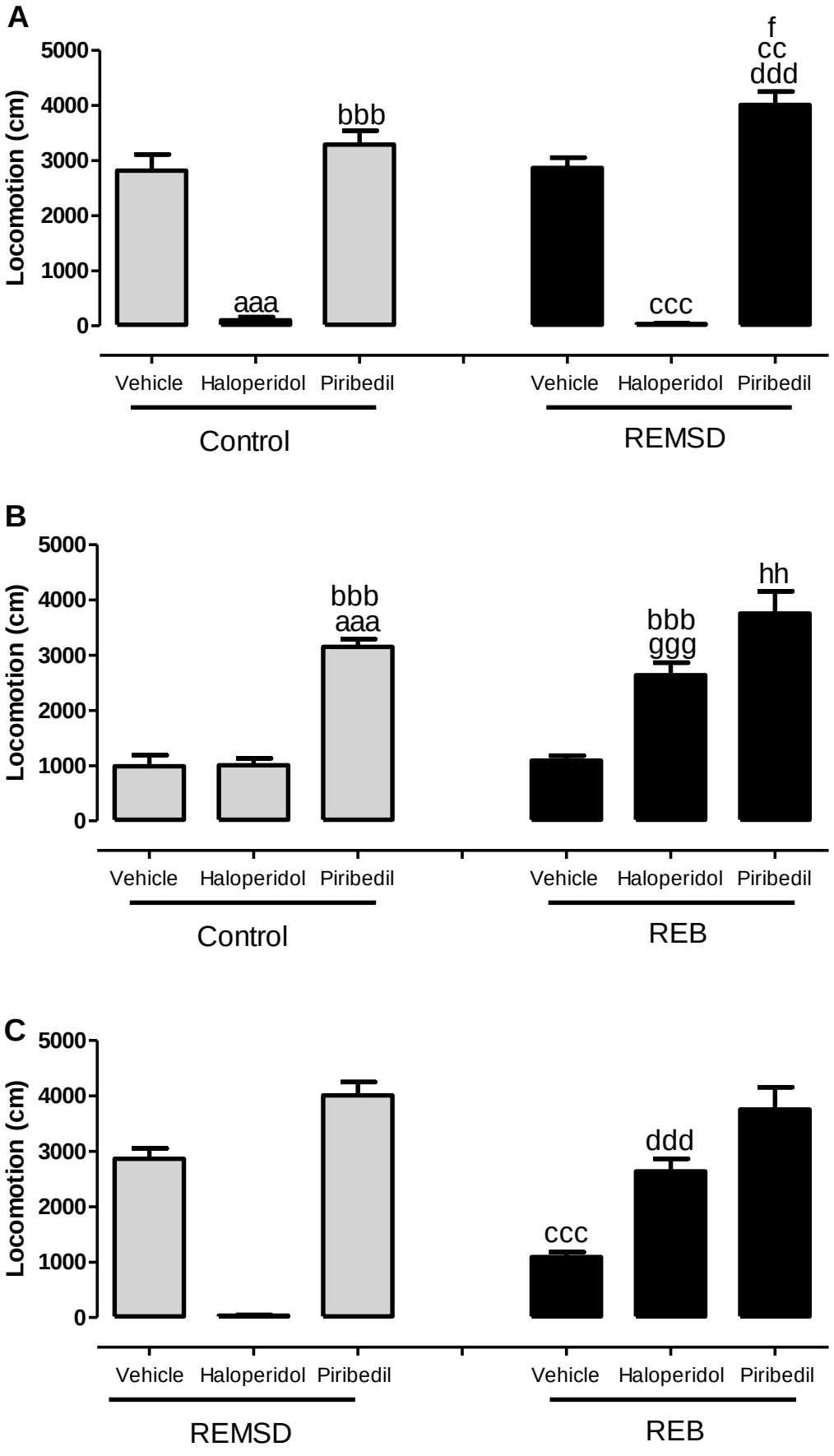
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Figure 1

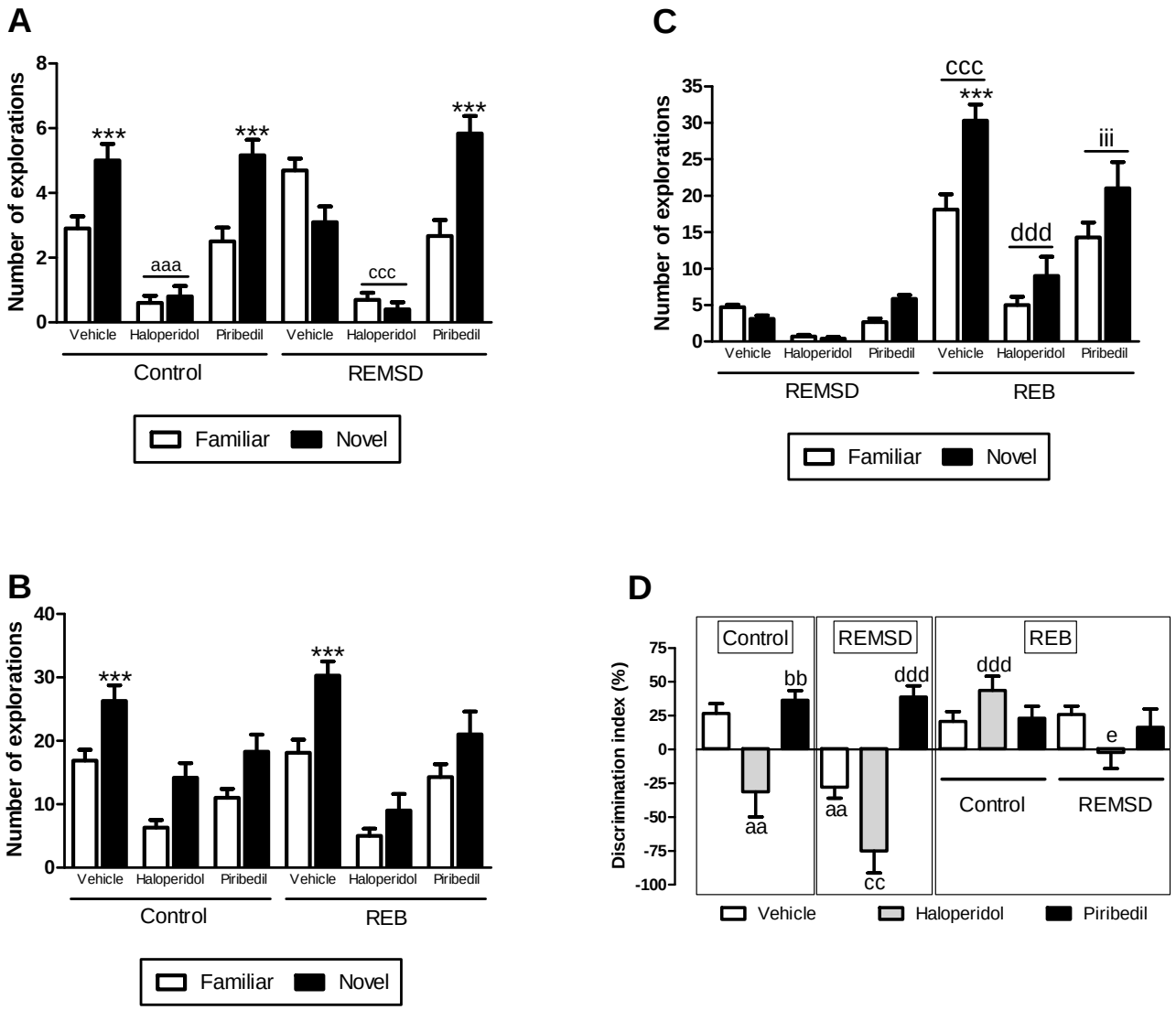
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Figure 2

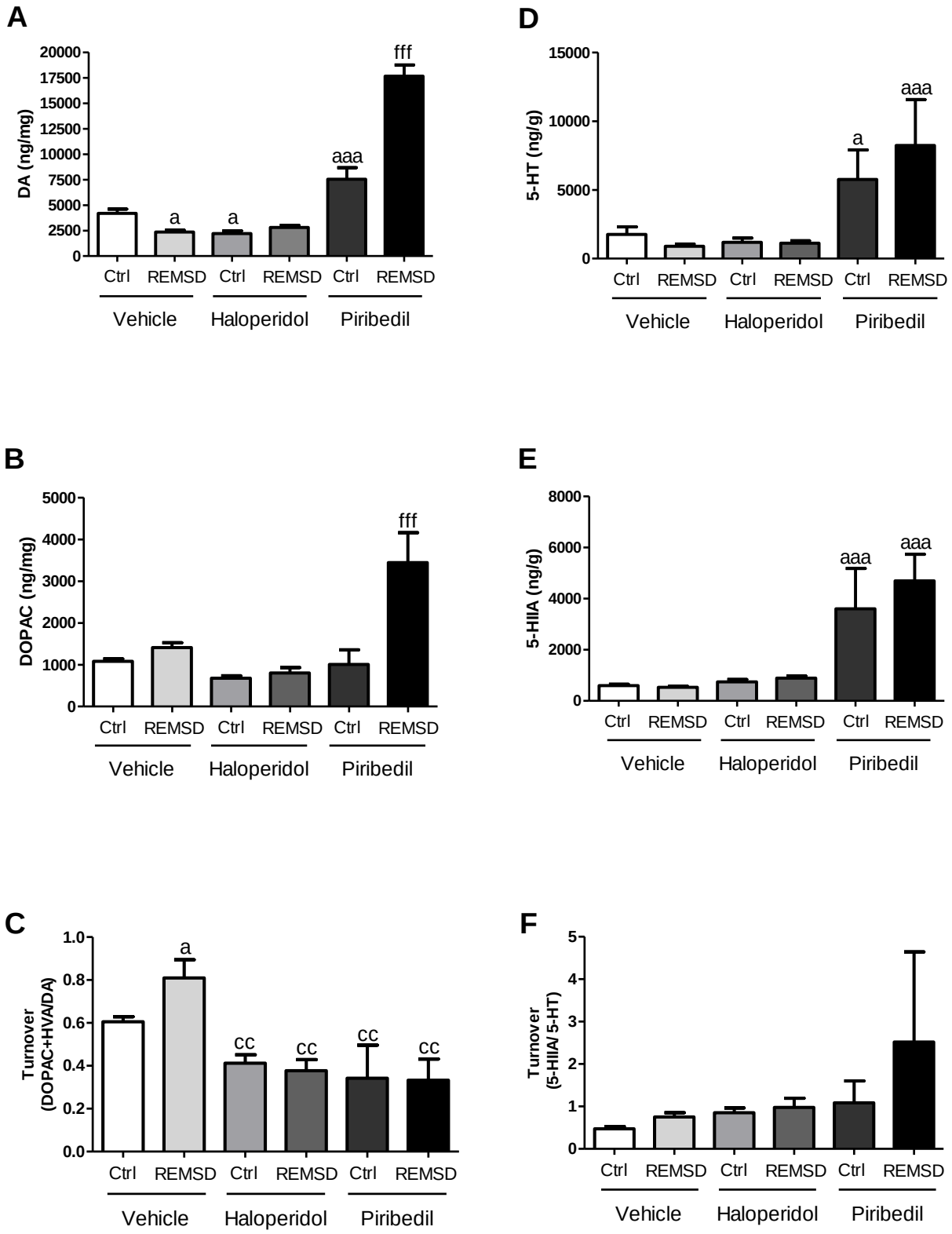
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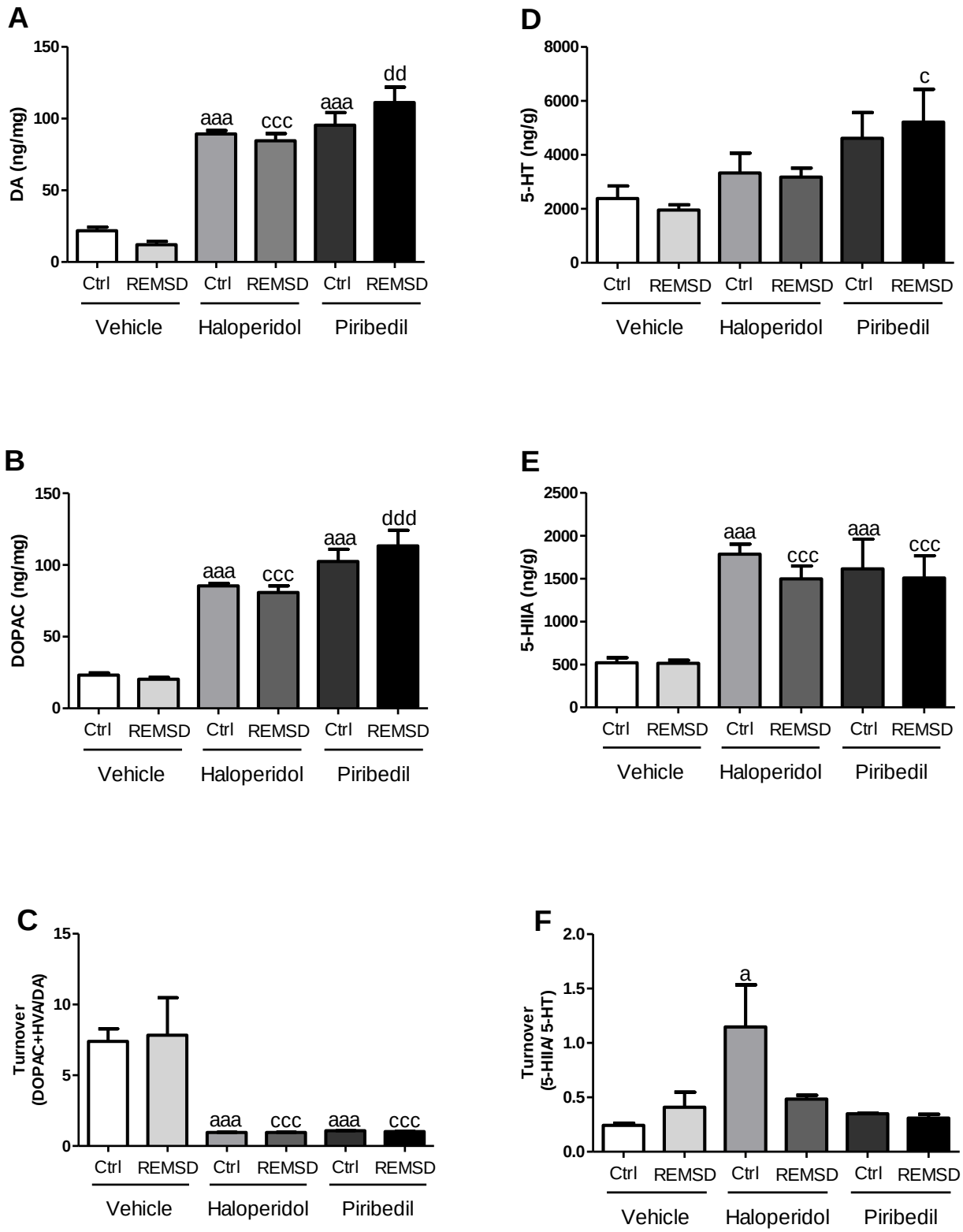
Figure 3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Piribedil

Figure 4

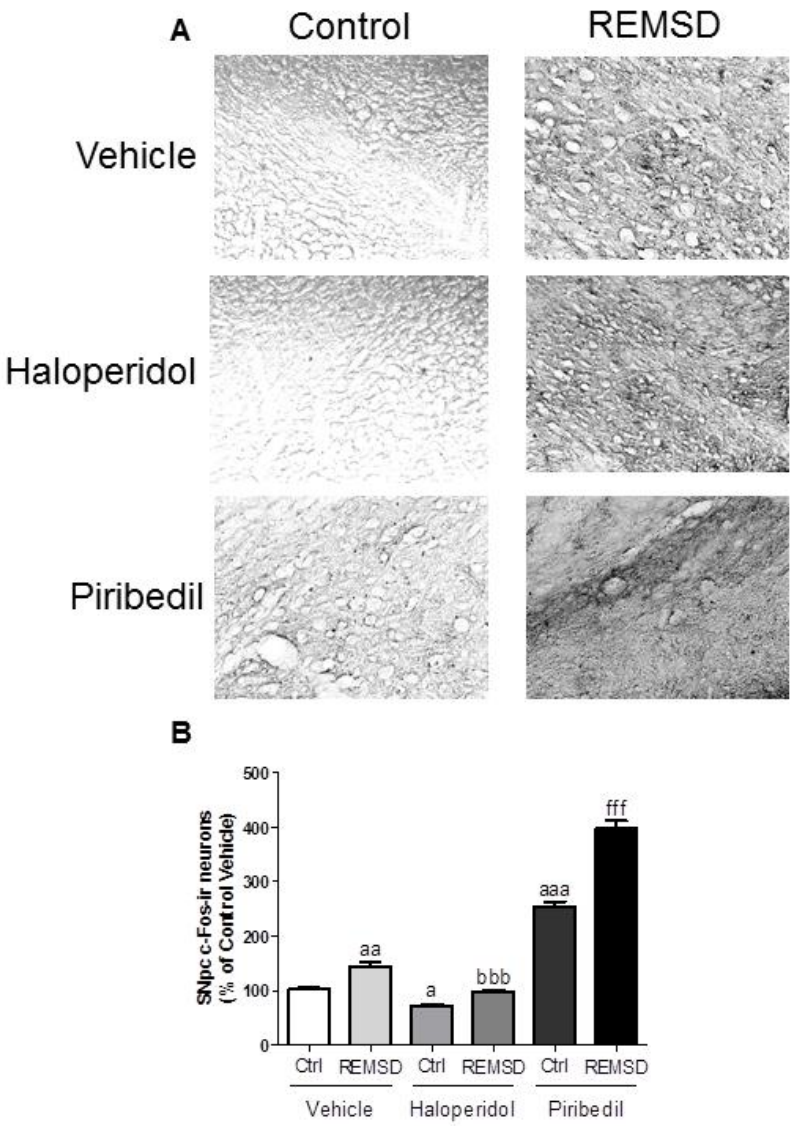
A
Control
REMSD
Vehicle
Haloperidol
Piribedil
B
SNpc c-Fos-ir neurons
(% of Control Vehicle)
500
400
300
200
100
0
aa
a
bbb
aaa
fff
Ctrl
REMSD
Ctrl
REMSD
Ctrl
REMSD
Vehicle
Haloperidol
Piribedil

Figure 5

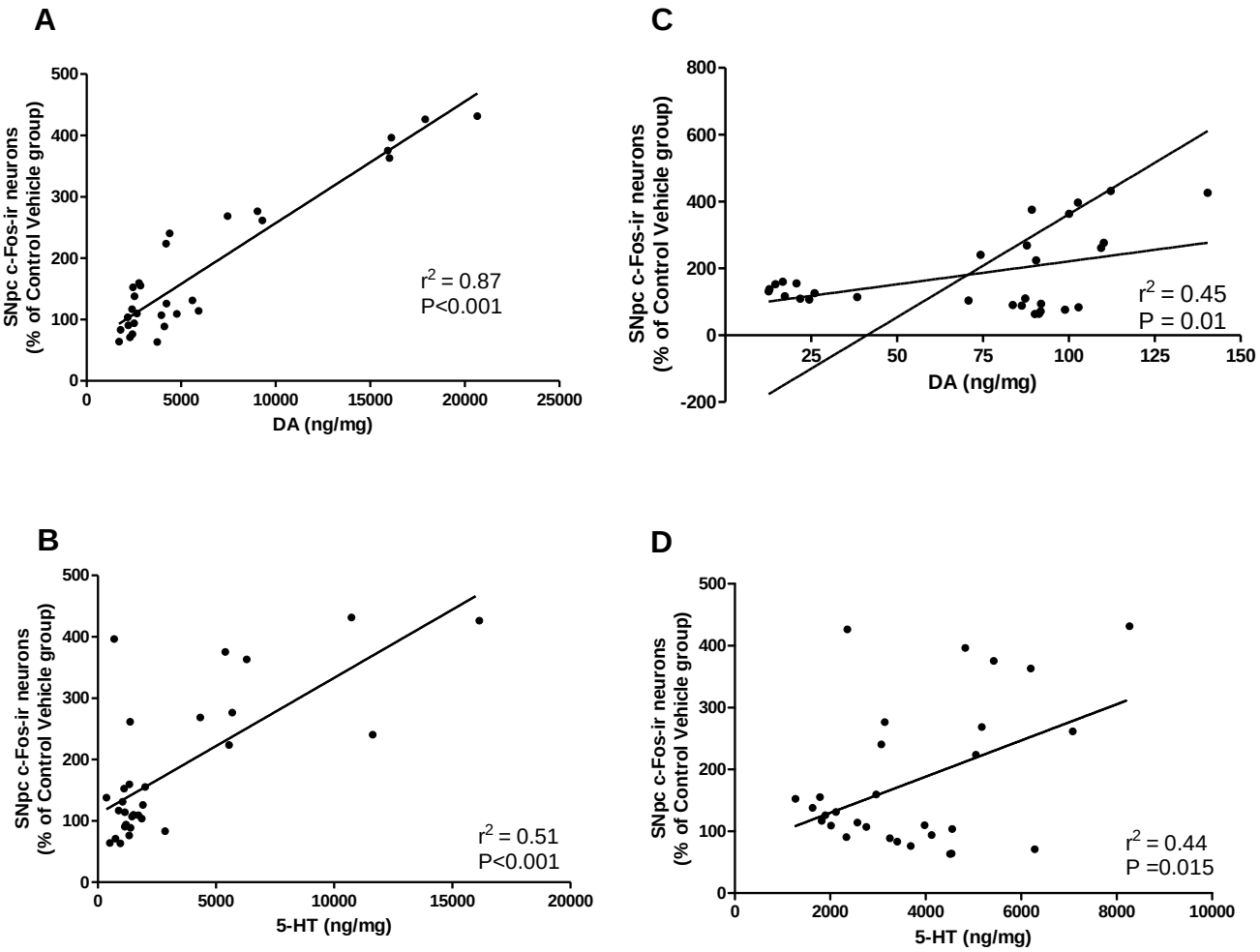
A
SNpc c-Fos-ir neurons
(% of Control Vehicle group)
DA (ng/mg)
r2 = 0.87
P<0.001
C
SNpc c-Fos-ir neurons
(% of Control Vehicle group)
DA (ng/mg)
r2 = 0.45
P = 0.01
B
SNpc c-Fos-ir neurons
(% of Control Vehicle group)
5-HT (ng/mg)
r2 = 0.51
P<0.001
D
SNpc c-Fos-ir neurons
(% of Control Vehicle group)
5-HT (ng/mg)
r2 = 0.44
P =0.015

Figure 6

Figure Legends

Figure 1. Locomotion parameter obtained from the open-field test. (A) Comparison between control and REMSD groups; (B) Comparison between control and REB groups; (C) Comparison between REMSD and REB groups. Values are expressed as mean ± SEM. ^{aaa}P<0.001 vs. control vehicle group; ^{bbb}P<0.001 vs. control haloperidol; ^{ccc}P<0.001 vs. REMSD vehicle; ^{ddd}P<0.001 vs. REMSD haloperidol; ^{f}P<0.05 vs. control piribedil; ^{ggg}P<0.001 vs. REB vehicle; ^{hh}P<0.01 vs. REB haloperidol. One-way ANOVA followed by the Newman-Keuls test.

Figure 2. Cognitive effects elicited by haloperidol and piribedil after REMSD and REB. (A) Number of objects exploration after REMSD; (B) Number of objects exploration after REB; (C) Comparison between REMSD and REB periods; (D) Discrimination index. Values are expressed as mean ± SEM. ^{aaa}P<0.001 vs. control vehicle group; ^{ccc}P<0.001 vs. REMSD vehicle; ^{ddd}P<0.001 vs. REMSD haloperidol; ^{iii}P<0.001 vs. REMSD piribedil ***P<0.001 vs. the familiar object. Panels A, B and C were analyzed by two-way ANOVA followed by the Bonferroni test. Panel C was analyzed by one-way ANOVA followed by the Newman-Keuls test.

Figure 3. Neurochemical examination of the striatal content of DA, 5-HT and metabolites. (A) DA, (B) DOPAC, (C) DA turnover; (D) 5-HT; (E) 5-HIAA; (F) 5-HT turnover. Values are expressed as mean ± SEM. ^{a}P<0.05, ^{aaa}P<0.001 vs. control vehicle group; ^{cc}P<0.01 vs. REMSD vehicle; ^{fff}P<0.001 vs. control piribedil. One-way ANOVA followed by the Newman-Keuls test.

Figure 4. Neurochemical examination of the hippocampal content of DA, 5-HT and metabolites. (A) DA, (B) DOPAC, (C) DA turnover; (D) 5-HT; (E) 5-HIAA; (F) 5-HT turnover. Values are expressed as mean ± SEM. $^{a}P<0.05$, $^{aaa}P<0.001$ vs. control vehicle group; $^{c}P<0.05$; $^{ccc}P<0.001$ vs. REMSD vehicle; $^{dd}P<0.01$; $^{ddd}P<0.001$ vs. REMSD haloperidol. One-way ANOVA followed by the Newman-Keuls test.

Figure 5. Nigral c-Fos activation after REMSD of haloperidol and piribedil groups. (A) Photomicrograph of representative sections of c-Fos immunoreactive (c-Fos-ir) neurons in the SNpc of the groups (magnification 200x). (B) Quantitative estimation of the SNpc c-Fos-ir neurons of the groups. $^{a}P<0.05$, $^{aa}P<0.01$ $^{aaa}P<0.001$ vs. control vehicle group; $^{bbb}P<0.001$ vs. control haloperidol; $^{fff}P<0.001$ vs. control piribedil.

Figure 6: The induction of c-Fos-ir neurons within the SNpc closely correlate with the DA levels in the striatum after REMSD. Pearson's correlation coefficients were calculated considering the following: (A) SNpc c-Fos-ir neurons vs. striatal DA; (B) SNpc c-Fos-ir neurons vs. striatal 5-HT; (C) SNpc c-Fos-ir neurons vs. hippocampal DA; (D) SNpc c-Fos-ir neurons vs. hippocampal 5-HT.

ARTIGO - 2

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Motor and Non-Motor Features of Parkinson's Disease – A Review of Clinical and Experimental Studies

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Abstract: Classically, Parkinson's disease (PD) is considered to be a motor system affliction and its diagnosis is based on the presence of a set of cardinal motor signs (e.g. rigidity, bradykinesia, rest tremor and postural reflex disturbance). However, there is considerable evidence showing that non-motor alterations (e.g. anxiety, depression, sleep, gastrointestinal and cognitive functions) precede the classical motor symptoms seen in PD. The management of these non-motor symptoms remains a challenge. A pattern of regional neurodegeneration that varies considerably depending upon the neuronal population affected may explain the different symptoms. In fact, differential mechanisms of neuronal vulnerability within the substantia nigra pars compacta (SNpc) suggests that factors other than location contribute to the susceptibility of these neurons. In this review we discuss how these factors interact to ultimately target the SNpc. Remarkably, this region consists of approximately 95% of the tyrosine hydroxylase (TH)-immunoreactive neurons in both human and rat brains, and consequently this implicates elevated levels of dopamine metabolites, free radicals and other hazard species in these neurons. An understanding of how these factors promote neuronal death may be useful for the development of novel neuroprotective and/or neurorestorative strategies for PD.

Keywords: Tyrosine hydroxylase, dopamine, animal models, nigrostriatal pathway, neurodegeneration.

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease afflicting about 1% of people over 65 years old and 4-5% of people over 85 years [1, 2]. Major clinical features at presentation include the asymmetric onset of bradykinesia, rigidity, rest tremor and disturbances in balance. These are the result of the degeneration of the neurons of the substantia nigra pars compacta (SNpc) which leads to subsequent reduction of dopaminergic input to the striatum (Fig. **1**). Moreover, there is a degeneration of neurons of selected brain stem nuclei (locus coeruleus, raphe nuclei, dorsal motor nucleus of the vagus), cortical neurons (particularly within the cingulated gyrus and the entorhinal cortex), the nucleus basalis of Meynert and preganglionic sympathetic and parasympathetic neurons [3]. These, apparently, non-motor features progress and come to dominate the later onset of PD producing symptoms that include cognitive decline, depression, gastrointestinal and genitourinary disturbances, as well as sleep abnormalities [4]. Nevertheless, the dopamine (DA)-containing neurons seems to be the key players in PD.

Several mechanisms are implicated in the degeneration of nigrostriatal neurons such as oxidative stress, mitochondrial dysfunction, protein misfolding, disturbances of intracellular calcium and iron homeostasis besides polymorphisms in genes regulating DA metabolism and transport, neuroinflammation and necrosis/apoptosis [5-7]. It has been recently discussed that clinical deficits in PD are strongly associated with the location, rather than mechanism, of brain cell death. The regional selectivity of brain cell loss in different neurodegenerative disorders is reflected in the overlapping motor and non-motor impairments associated to the neurodegeneration [8]. Particularly in PD, this idea is strengthened by the Braak´s hypothesis that describes the existence of six neuropathological stages. Each of these stages is marked by the continual development of distinctive inclusion bodies that present in the form of spindle-like or thread-like and, in part, branching Lewy neurites (LNs) within cellular processes and as granular aggregations and spherical pale bodies and/or Lewy bodies (LBs) in the somata of the involved nerve cells [9].

It is postulated that only a few of the many nerve cell types within the human nervous system are prone to develop the abnormal proteinaceous aggregations. Other neuronal types, even when they are located directly next to involved nerve cells, maintain their morphological and functional integrity. This means that neuronal damage in the brain during PD is not random but obeys certain rules, thereby leaving a distinctive lesional distribution pattern (see Table **1**) [10-12]. The reasons for the marked vulnerability of some neuronal types and the decided resistance of others are still not adequately understood. Although, growing evidence originated from clinical studies, autopsy materials, and *in vitro* and *in vivo* experimental models of PD are available [13-17]. Recent studies discuss that the dopaminergic nature of SNpc neurons, and the subsequent consequences of this neurochemical phenotype for oxidative stress, has long been

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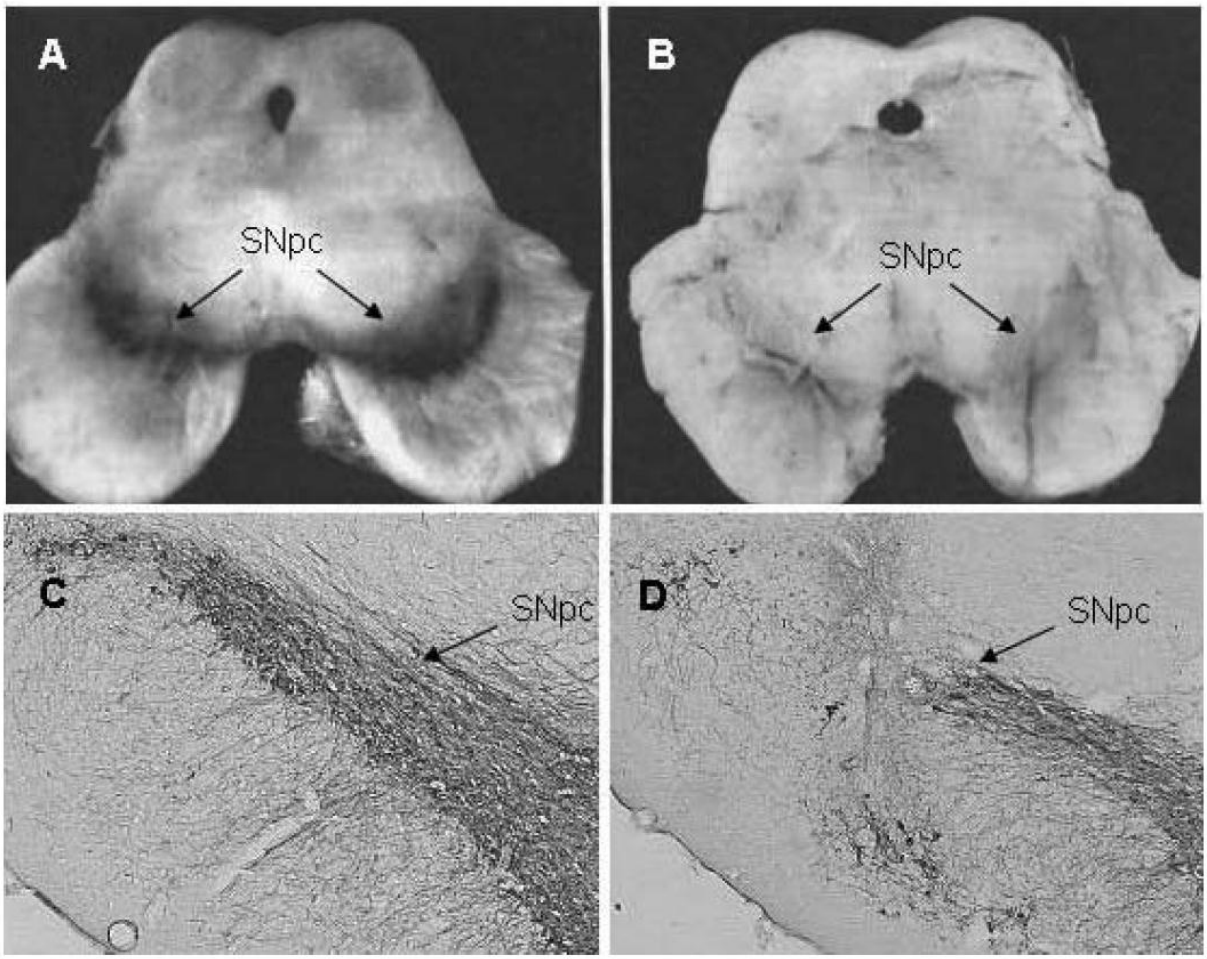

Fig. (1). Massive loss of midbrain dopaminergic neurons in the substantia nigra pars compacta (SNpc). Panels **A** and **B** represent the human postmortem bilateral midbrain of normal and Parkinsonian subjects, respectively. **C** and **D** show the unilateral equivalent of the rat midbrain after vehicle (**C**) or MPTP (**D**) intranigral injection. Arrows indicate the tyrosine hydroxylase immunoreactivity present within the SNpc.

suspected to contribute to their relative vulnerability. The most visually apparent feature of these cells, neuromelanin, is thought to form as a consequence of DA metabolism, and DA levels and/or the rate of DA metabolism within the neuron may vary due to the differential expression of tyrosine hydroxylase (TH) [18].

Modifications in this neuronal function as a result of PD, may contribute to dysfunctional dopaminergic circuitry and thus to the occurrence of non-motor and motor signs and symptoms. This review discusses how these factors interact, lead to mechanisms of selective dopaminergic neurodegeneration and correlate with PD symptoms in both clinical and experimental research. An understanding of these mechanisms will be helpful for the development of novel strategies to improve the survival of targeted neurons in PD.

MOTOR IMPLICATIONS

Regional vulnerability in PD includes specific neurons other than those present within the SNpc. For example, significant neurodegeneration is observed in autonomic ganglia [19]; spinal cord [20]; brainstem [21]; basal forebrain [22]; limbic lobe [23] and the neocortex [24]. It is expected to observe a large variety of motor and non-motor disorders, however, the former is mainly associated to the overwhelming amount of damage of the SNpc neurons, impairing the projections to the caudate/putamen (nigrostriatal pathway) and consequently the DA release [25-27]. The common clinical syndrome associated with PD and other kinds of Parkinsonism includes motor problems related with slowing of movements (bradykinesia) [17, 28, 29], weakness [17, 28], rigidity [17, 28], tremors [30], postural instability [17] and fatigue (Table **1**) [31]. Here, it will be described the relationships of the selective dopaminergic neurodegeneration with some of the major motor implications and their impacts in the functional independence.

Bradykinesia is considered one of the cardinal features observed in PD [28]. This symptom describes the slowness of a voluntary movement, also referring to poverty of spontaneous and associated movements commonly

Table 1. The Clinical-Pathological History of PD

Stage	Neuropathological Findings	Clinical Manifestations
1-2 (pre-clinical)	• LNs and LBs inclusions in the anterior olfactory structures and regions of the medulla oblongata (in the dorsal motor nucleus of the vagus) and pontine tegmentum (including the noradrenergic neurons of the locus coeruleus and the serotonergic neurons of the caudal raphe nuclei)	• Olfactory disturbance • Depression, anxiety • Sleep disturbance • Gastrointestinal dysfunction
3 (early)	• LNs and LBs inclusions in the cholinergic nucleus basalis of Meynert	• Cognitive decline ($^1/_3$ PD patients) • Depression, anxiety
4 (late)	• LNs and LBs inclusions in the temporal mesocortex • Considerable loss of dopaminergic neurons in the SNpc.	• Rest tremor, muscle rigidity and bradykinesia • Postural instability • Depression, anxiety, psychosis • Cognitive decline ($^2/_3$ PD patients)
5-6 (late)	• LNs and LBs inclusions in the neocortex	• Dementia • Psychosis, depression, anxiety • Cognitive decline (90% PD patients)

Abbreviations: Lewy bodies (LBs); Lewy neurites (LNs); Parkinson´s disease (PD); substantia nigra pars compacta (SNpc).

manifested as freezing and the prolonged time it takes to initiate a movement (reaction time) [17, 28, 30, 31]. Central mechanisms of bradykinesia and how they relate to the basal ganglia dysfunction is the core of the motor deficits in PD, however there are additional secondary factors that can potentially contribute to bradykinesia: e.g. muscle weakness, rigidity, tremor, movement variability and slowing of thought [28].

Intrinsic muscle properties could not have changed when the strength of PD patients presented reduction, compared to controls [17, 30-33]. These results showed that weakness was likely due to an inability to activate the muscle voluntarily. In all studies, patients appeared to be readily motivated to produce strong contractions, so that lack of volitional drive was not thought to be a related factor, reinforcing an involvement of the central nervous system pathways related to voluntary muscle recruitment and avoiding an involvement of motivational factors. Other support that indicates that patients lack some part of the normal volitional input to lower motor centers is the existence of a physiological difference between PD and non-PD subjects in the electromyography activity suggesting disorganized voluntary drive to contract muscles in the same way as usual [34]. Rest and action tremor as well as secondary factors could also contribute to the bradykinesia, because they prolong reaction times and slow the initiation of any voluntary movement [17, 28, 29, 31]. All mentioned factors contribute to generate balance-related problems, interfering in many daily activities such as walking or standing up from a chair promoting fear of falling [17].

Recordings before the onset of movements revealed that bradykinesia could be due to slowness in activating the circuitry responsible to perform the voluntary movement. These studies include reaction time tests, electroencephalographic and magnetic encephalographic records, as well as records of the neuronal circuits activity at different times preceding a voluntary action [35, 36]. One reason for that could be explained by results showing that the increase in the threshold level of the motor excitability observed previous the movement or even during electromyography activity in PD patients is reached more slowly than the normal [28]. Pre-movement studies attempted to understand which areas of the motor system are functioning abnormally in bradykinesia and a general finding from these studies is that supplementary motor cortex and nearby areas (midline cortical motor areas) present reduced activity, perhaps coupled with extra activation of the lateral pre-motor area [28].

Decreased activity may be related to difficulties in preparing instructions to move and the increased activation may be an active process of compensation to improve performance that can be observed when external cues are available in the environment or they are given by physical therapists during rehabilitation programs to guide movement of PD patients [28, 37]. Evidence suggests that, although secondary factors such as weakness, rigidity, tremors, postural instability and fatigue may contribute to deficits in the functional independence, the main factor is probably related to the insufficient recruitment of muscle fibers by the descendent pathways during the initiation of movement [28, 29].

Despite a lot of primary and secondary factors that may contribute to clinical motor implications, characteristics of motor recruitment observed in PD can distinguish two features of Parkinsonian bradykinesia: (1) an undervalued muscle force resulted from an underscaling of commands in internally generated movements and (2) an ameliorated performance when external cues are given to guide movement [28]. These features have important therapeutic value and a wide impact in the functional independence of the PD patients.

NON-MOTOR IMPLICATIONS

Remarkably, systematic reviews have indicated non-motor symptoms (e.g. depression, anxiety, cognitive and sleep disturbances) as major factors in determining health-related quality of life, progression of disability, and nursing home placement in PD patients (Table **1**) [38, 39]. Moreover, non-motor features of PD usually do not respond to dopaminergic therapy and probably is the major current

challenge faced in the clinical management [40]. Due to the novelty of this field the predominance of data that have been obtained recently is from animal models of Parkinsonism. In view of that, a selected core of topics regarding the non-motor implications of PD is scrutinized in the following sections.

ANXIETY

Anxiety disorders are the second most common affective disturbance in PD and are present in up to 40% of patients [41]. Anxiety disorders include generalized anxiety disorder, agoraphobia, panic disorder, and social phobia, and frequently co-occur with depression in PD. Although some of these symptoms such as apprehension, fear, or worry are under-recognized and under-treated, others can be improved with available treatments [41]. According to Braak staging of PD pathology, noradrenalin (NA) dysfunction likely occurs prior to significant degeneration of dopamine (DA) neurons [9].

In view of the neurotoxin-related animal models of PD, that has been used extensively to investigate the motor deficits of the nigrostriatal degeneration, also can reveal different features of anxiety and depression. In view of that, it is described that bilateral nigrostriatal damage inflicted by 6-hydroxidopamine (6-OHDA) lesions induced symptoms of depression and anxiety characterized by several behavioral measures (depression: forced swim test, sucrose consumption; anxiety: elevated plus maze) [42]. Furthermore, other studies described the occurrence of depressive-like behaviors promoted by different neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), lipopolysaccharide (LPS) and rotenone [43-45]. Although, so far few studies have investigated the effects of MPTP administration in non-human primates on affect, whereas, MPTP-treated mice show profound increases in immobility in the tail-suspension test [46]. In addition, an increased anxiety-like behavior in open-field and light-dark box in Parkin null mice has been described [47]. However, transgenic mice over-expressing alpha-synuclein A53T exhibited reduced anxiety-like behavior by spending markedly greater amounts of time on the open arms of the plus maze and by a higher proportion of entries to the open arms [48]. In the open field test, these transgenic mice showed a trend toward reduced locomotor habituation and increased thigmotaxis [48]. Moreover, in a novel vesicular monoamine transporter-2 (VMAT-2) deficient mouse model of PD, mice displayed enhanced anxiety and depressive-like behaviors, which became more severe with advancing age [49]. Indeed, the contributions of the studies with animal models do corroborate the premise of different phases of the neurodegeneration in PD, and show the limitations of investigating these phases separately.

Several studies have noted that the onset of affective disorders predates the emergence of motor symptoms [50, 51], since motor symptoms typically do not manifest until about 70% of nigral DA neurons have been lost. In this sense it is observed that affective behaviors may be more sensitive to such depletion. In fact, it is reported an association between decreased binding to DA transporters in the left ventral striatum and depressive and anxious symptoms [52, 53]. DA-depleted rats manifest enhanced learned helplessness behaviors that were only partially alleviated by 3,4-dihydroxyphenylalanine (L-DOPA) [54]. Thus, it is suggested that such inability is associated with a supraphysiological release of DA into the prefrontal cortex and hippocampus [55].

DEPRESSION

Depression has been found to be important determinant of PD patients in Europe [56-59]. In India female gender, presence of depression and low degree of independence had the most detrimental impact in patients with PD [60]. 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 [61, 62].

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 [63]. In addition, the nigral neuronal loss was seven times greater in post-mortem brains of PD patients with depression compared to non-depressed PD patients (Frisina *et al.*, 2009), suggesting that depression may be the result of more severe DA depletion.

There are pathophysiological evidence of serotonin (5-HT) alterations in patients with PD-associated depression [64], and a hypothesis concerning 5-HT has even been proposed for depression in PD [62]. This hypothesis considers a 5-HT-induced DA release in the nucleus accumbens which is down-regulated by 5-HT_{2C} receptors [65]. As a result, reductions in the 5-HT content or increases in the 5-HT_{2C} inhibitory activity could be associated to a decline on dopaminergic neurotransmission in PD patients and subsequent worsening of mood symptoms. However, noradrenergic anti-depressants, such as nortriptyline have recently demonstrated to be more effective than selective 5-HT reuptake inhibitors in PD patients with depression, and may suggest a more prominent role for NA [66].

Upon chronic medication, the loss of efficacy of L-DOPA that commonly leads to increasing L-DOPA dose regimen [67] may reflect a lower capability of 5-HT neurons to promote DA effects of L-DOPA. It is not known whether the lesions of serotoninergic neurons reported in Parkinsonian patients [68-70] result from the pathophysiological process of the disease or the chronic use of L-DOPA. Chronic treatment with an excessive dose of L-DOPA in normal rats has been shown to decrease striatal 5-HT tissue content [71]. Moreover, a single intranigral infusion of L-DOPA could promote a significant lesion in TH-ir neurons associated to decreased levels of striatal DA and motor impairment in the open field [72].

However, the impact of chronic L-DOPA treatment at therapeutically relevant dose in Parkinsonian rats on the integrity of 5-HT neurons is presently unknown although it could lead to aberrant *in vivo* 5-HT and DA releases in the brain. It was recently reported the occurrence of a strong *in vivo* evidence for a deleterious impact of chronic L-DOPA on 5-HT neuron function associated with a heterogeneous

loss of efficacy of L-DOPA to maintain DA transmission in the Parkinsonian brain [73]. This report demonstrated that chronic L-DOPA treatment results in two distinct inhibitory effects on 5-HT transmission. First, chronic L-DOPA treatment homogeneously decreases basal release of 5-HT in the brain *in vivo* and second, chronic L-DOPA treatment heterogeneously affects the ability of L-DOPA to inhibit 5-HT release [73]. Overall, depression in PD patients may exacerbate existing anatomical and functional abnormalities of the 5-HT and DA systems resulting in decompensated neurotransmitter systems that lead to vulnerability to depressive symptoms.

COGNITIVE DEFICITS AND DEMENTIA

Many studies of PD describe elevated incidence of cognitive deficits associated with dementia, ranging from 20% to 80% [74]. This massive variation among studies is probably due to differences in the application of methods for cognitive assessment, dementia definition and data collection [75]. Cognitive dysfunction in patients with PD can be classified as domain-specific cognitive impairments, and dementia, a very common non-motor feature in PD, has important clinical consequences for the patients in terms of excess disability, risk for psychosis, reduced quality of life and increased mortality [75, 76]. TH enzyme and DA transporter (DAT) expressions are classic indicators of dopaminergic neuronal death and subsequent denervation in PD. These proteins are decreased in the striatum of PD patients with or without dementia compared to control patients i.e. 44 and 50%, respectively [77, 78]. On the other hand, neurochemical studies in postmortem inferior frontal gyrus from patients with PD and dementia appears also to sustain greater loss (40%) of both dopaminergic neurons and nerve terminals comparing to PD without dementia (20%) [77]. Significant loss of striatal dopaminergic terminals and DAT have been described either in PD patients with or without dementia, however, a greater loss of these markers is observed within the striatum and inferior frontal gyrus of PD patients with dementia [79].

Clinically, the cognitive decline comprises impairment of executive functions or memory deficits including working memory, long-term, visuospatial and procedural memories as well [75, 80-83]. The impairment of executive functions is the main feature in demented PD patients, including impairment in concept formation and rule finding, problem solving, set elaborating and planning, set shifting and set maintenance.

Several longitudinal studies have confirmed that patients with more severe and advanced Parkinsonism have a higher risk for dementia than those with less advanced PD. Motor symptoms such as rigidity, postural instability and gait disturbance predict more rapid cognitive decline and time to dementia [76, 84, 85]. Many authors considered the age factor as a potential risk to the development of dementia in PD [74-76]. On the other hand it is very difficult to distinguish the importance of age, duration of disease and age at onset of PD as the individual risk factor for dementia in PD, because all these parameters are highly correlated in PD cohorts. The previous existence of mild cognitive impairment in PD patients, mainly detected in tests that evaluated memory and executive function, is determinant in predicting a shorter time to develop dementia [86-89].

SLEEP DISORDERS

The neurobiology of sleep has developed rapidly in the recent years, with remarkable neurophysiological and molecular progress of knowledge, about its mechanisms [90]. An overview of the literature demonstrates that studies concerning a role played by DA in the sleep regulation have becoming more numerous, especially after 1990 [91].

Sleep disturbances and daytime sleepiness are well-known phenomena in PD and were reported in the original description by James Parkinson (insert reference). Sleep disorders have a complex etiology related not only to the underlying neurodegenerative process, but also to the motor and non-motor features of PD and to dopaminergic therapy. A community-based study revealed that nearly two-thirds of PD patients reported sleep disturbances, which is significantly more frequently than observed in patients with diabetes and healthy control subjects [92]. Furthermore, about a third of PD patients rated their overall night-time problems as moderate to severe. Virtually all patients with PD suffer from various sleep disruptions [93]. In a prospective study in PD patients, cabergoline (a D_1 and D_2 agonist) treatment increased arousals, stage shifts, and awakenings, although quantitative electrophysiological measures of sleep were maintained, and subjective measures of sleep quality even improved [94].

The prevention of sleep and enhancement of waking by DA reuptake inhibitors and DA receptors agonists are the basis for their use in the treatment of other conditions such as narcolepsy and somnolence associated with hypodopaminergic states [95]. Moreover, PD patients present several disruptions of sleep, which are deteriorated by the use of dopaminergic D_2 receptor agonists [96-98]. In contrast, treatment with the D_2 antagonist and antipsychotic agent haloperidol attenuates hippocampal theta and gamma oscillations which is characteristic of rapid eye movement (REM) sleep [99]. The basal ganglia have been related to a variety of cognitive functions in addition to motor control. Experimental evidence has suggested a role for these nuclei in attention, time perception and learning and memory, while clinical data has contributed by pointing out the cognitive impairments in neurodegenerative disorders involving the basal ganglia, such as Huntington´s disease and PD. Impaired basal ganglia function has also been correlated with other pathologies such as obsessive-compulsive disorder, attention-deficit disorder, autism and schizophrenia [100].

GASTROINTESTINAL DISTURBANCES

Gastrointestinal symptoms are common in PD and many such as dysphagia, dribbling of saliva and esophageal dysmotility can occur in advanced PD [101]. These symptoms contribute directly to the morbidity and complicate the disease's clinical management [102]. Constipation may precede development of PD [103]. A prospective study followed the bowel habits of 7,000 men for 24 years reported that those with initial constipation (< 1 bowel movement/day) had a threefold risk of developing PD after a mean interval of 10 years from initial constipation

[103]. Large projection cells of the dorsal motor nucleus of the vagal nerve generate long unmyelinated preganglionic fibers that connect the central nervous system with the postganglionic nerve cells of the enteric nervous system [104, 105]. Patients with PD present abnormal electrogastroenterography motility parameters which are similar to the acute stage of vagotomized patients [106]. It is postulated that the participation of dorsal motor nucleus of the vagal nerve is critical in Braak stage 1 [9]. At this stage, cases exhibit no more than a few isolated LB and LN-like inclusions in the dorsal motor nucleus and in the adjacent intermediate reticular zone and few spindle-shaped LN-like inclusions also may appear in the preganlionic axons of the vagal nerve [9, 107]. In addition, it is observed that similar forms of inclusion bodies as those found in the central nervous system occur in select neuronal types within the enteric nervous system [9].

However, a recent report evidence that there is comparable involvement of the dorsal vagal nucleus in multiple system atrophy (a condition associated with impaired control of gastrointestinal function) and different stages of LB disorders although, the relationship of dorsal vagal nucleus involvement and gastrointestinal symptoms is uncertain in PD [108]. A study analyzing eight patients with PD by use of defaecography and anorectal manometrics reported that after apomorphine treatment, defaecographic abnormalities were normalized in one of three patients, and all five individuals who underwent repeated anorectal manometry showed substantial improvements in manometric parameters [109]. The authors concluded that apomorphine can correct anorectal dysfunction in PD and suggested that abnormalities of defaecation and anorectal function occur as a consequence, at least in part, of dopamine deficiency secondary to the pathological changes of PD [110]. Data from a multi-centre, open-label observational study that used intrajejunal L-DOPA/carbidopa gel infusion in patients with non-motor symptoms and with advanced-stage PD demonstrated a significant beneficial effect in the majority of non-motor symptom domains, with paralleling improvement of motor symptoms [111]. According to the authors, this study was the first demonstration that a L-DOPA-based continuous stimulation was beneficial for non-motor symptoms and health-related quality of life of the patients, in addition to the reduction of motor fluctuations and dyskinesias [111].

MECHANISMS OF NEURODEGENERATION IN PARKINSON'S DISEASE - CONTRIBUTION OF THE ANIMAL MODELS

In rodents a profusion of studies based on intracerebral injections of neurotoxins such as 6-OHDA, MPTP, rotenone and LPS (Fig. **1**) have been published [26, 42, 43, 112-116]. The neurotoxicity of 6-OHDA is attributed to its ability to generate reactive oxygen species (ROS) and quinones [117]; however, evidence also suggest that 6-OHDA can act directly by inhibiting the mitochondrial respiratory chain at the level of complex I [118]. In a translational perspective it is important to note that depending on the location of the injection site and dose of neurotoxin, the animal model can present different time-courses of progression and severity of lesion. Middle forebrain bundle or intranigral infusions of 6-OHDA induce an almost complete depletion on neuronal density of tyrosine hydroxilase immunorreative (THir) neurons (80-90% loss) similar to advanced stage of PD [119, 120]. Accompanying this marked degeneration several studies evaluated the efficacy of anti-dyskinetic treatments in unilateral 6-OHDA-lesioned-rats after repeated administration of either L-DOPA or dopamine agonists such as apomorphine [116, 121]. When the focus is to reproduce early stages of PD the 6-OHDA can be administrated into the striatum once the degeneration of the nigrostriatal system is protracted, and induces cell death in a retrograde fashion [122].

Considering a more prolonged time frame (approximately two weeks) to observe the maximal loss of THir neurons [123] and consequently the time for the agent tested to act, the 6-OHDA model is widely employed to investigate neuroprotective treatments [112]. In addition, this model reproduces non-motor symptoms including mild emotional dysfunction, depression, anxiety and cognitive decline that may be of equal or greater significance than that motor symptoms [42, 43, 124].

Many animal species including rats, mice, monkeys and non-human primates are commonly used to reproduce Parkinsonism induced by the MPTP [125-127]. For some researchers it is considered the best experimental model of PD, especially in the context of studies designed to explore molecular mechanisms involved in the dopaminergic neurons degeneration [128]. Once inside the dopaminergic neurons the 1-methyl-4-phenylpyridinium (MPP^+) (metabolite generated from monoamine oxidase-B conversion of MPTP) impairs mitochondrial electron transport by inhibiting of complex I, reducing ATP generation and causing increased production of ROS [129]. The toxin MPTP can be given by several different routes, including systemic (s.c; i.v; i.p; i.m.) or intracarotid artery injection, oral administration and intracerebral sterotaxic injection [130]. Comparatively this toxin reproduces an early phase of PD, since animals that received 6-OHDA intranigral bilaterally exhibited more intense loss of THir neurons and striatal dopamine depletion than MPTP-lesioned animals [113, 120]. Nevertheless, still challenging to reproduce the dopaminergic neurodegeneration chronic and progressively, analogous to patient with DP.

Repeated intranigral MPTP injection in rodent resulted in loss in TH protein expression and THir neurons in the SNpc 24h after the first neurotoxin administration, however during the time frame tested the MPTP effect was not progressive or cumulative [131]. Recently was reported that MPTP-induced a dose-dependent progressive loss in the SNpc THir neurons. These data suggest that an intermittent washout period (10 days) between each increased dose of MPTP is responsible to continued cell death; this finding is important for therapeutic interventions to be applied at any of several stages during progressive neurodegeneration [132]. In addition to these classically known toxins, in recent years the pesticide rotenone has shown promise results as an effective model of PD. Highly lipophilic, it easily crosses the blood brain barrier - without coupling to DAT - and diffuses into neurons where, in a manner similar to MPTP, accumulates within mitochondria and inhibits the complex I [129].

Because rotenone can freely enter all cells there remain critical issues regarding the translatability of this model [133]. Though, different routes of administration such intraperitoneal injection [134], intranigral [135], chronic oral administration [136] and chronic intravenous or subcutaneous administration [137] have been demonstrated success in to mimic the loss of THir neurons and the decrease of striatal DA. On the other hand, are reported consistent disadvantages in consequence of systemic administration of rotenone, such high mortality and systemic organ toxicity [138, 139]. Considering these drawbacks, there are growing interest in to investigate the intracerebral rotenona administration. A single bilateral intranigral injection of rotenone produced an intense reduction in THir neurons and severe depletion in striatal DA and apparently proved an ideal model to promote cognitive deficits that mimics the presyntomatic state of PD [135].

It has been shown that cyclooxygenase-2 (COX-2) induction produced by LPS and by other neurotoxins suggest an increase in microglial activation in the SNpc which, by itself, can be interpreted as a manifestation of damage in the dopaminergic system [113]. Conspicuously, the inflammatory stimuli and ROS imbricates in one activation of NF-κB in microglial cells, oligodendrocytes and neurons to promote the transcription of inflammatory cytokines (IL-1β, IL-6, interferon-γ, TNF-α), apoptosis-promoting factors (p53, Bax), COX-2 and inducible nitric oxide synthase. Considering these findings, it is suggested that intranigral LPS could be considered a neuroinflammatory model of PD in terms of the of COX-2 up-regulation and neurochemical alterations. Otherwise, LPS was surprisingly unable to replicate the motor impairment, which is the strongest characteristic of this disease, despite the TH content depletion achieved after MPTP the intranigral injection [113].

CONCLUSION - TRANSLATIONAL PERSPECTIVE

Despite the focus on translational medicine and an upsurge in interest and funding [13, 14, 140-146], the idea of translated basic research into clinical treatments is not a novelty in PD. Indeed it yielded the Nobel Prize for Arvid Carlsson shared in Physiology and Medicine in 2000 along with Eric Kandel and Paul Greengard [147]. In this review, we have highlighted the varying aspects of both experimental research and clinical non-motor and motor features of PD. Intense efforts have been made toward developing improved PD models to better understand the etiology and pathogenesis of PD, and to identify new drug targets. However, neither neurotoxin induced nor transgenic animal model of PD, perfectly recapitulates all human symptoms. The various above findings provided compelling evidence of the different characteristics of the disease, such as motor deficit, neuroinflammation, oxidative stress and all the plethora of non-motor alterations. We envisage that the road ahead for detecting non-motor signs before the occurrence of motor ones is perhaps the brightest way to provide a window for more effective preventive/restorative treatments for PD.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interests exists.

ABBREVIATIONS

PD	=	Parkinson´s disease
DA	=	Dopamine
LNs	=	Lewy neurites
LBs	=	Lewy bodies
TH	=	Tyrosine hydroxylase
SNpc	=	Substantia nigra pars compacta
NA	=	Noradrenalin
VMAT-2	=	Transporter-2
L-DOPA	=	3,4-dihydroxyphenylalanine
5-HT	=	Serotonin
BDNF	=	Brain derived neurotrophic factor
DAT	=	DA transporter
s.c.	=	Subcutaneous
i.v.	=	Intravenous
i.p.	=	Intraperitoneal
i.m.	=	Intramuscular
6-OHDA	=	6-hydroxydopamine
MPTP	=	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
LPS	=	Lipopolysaccharide
ROS	=	Reactive oxygen species
MFB	=	Middle forebrain bundle
THir	=	Tyrosine hydroxilase immunorreative
COX-2	=	Cyclooxygenase-2

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