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SISTEMAS DOPAMINÉRGICOS E AÇÃO ANTIPSICÓTICA: ABORDAGENS EXPERIMENTAIS E TEÓRICAS

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“Fiquei ali parado,
assim pensando,
o que é que o poste tinha para dizer...”
(Nei Lisboa)

“O que um louco pode compreender,
outro louco também pode.”
(Dito popular)

Para minha mãe.

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inúmeras (e interessantes) discussões científicas que tivemos nos últimos anos acerca do tema, muitas vezes realizadas em ambientes outros além dos acadêmicos, onde também é muito bom estar com ele.

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PARTE I

Onde é feita uma introdução e os objetivos são traçados.

PREFÁCIO

Dentro do abrangente campo de pesquisa em neurociências, e mais especificamente dentro da área de neuropsicofarmacologia, a presente tese de doutorado versa sobre os sistemas dopaminérgicos, e, em particular, estuda a ação de fármacos antipsicóticos sobre eles. A tese é dividida em três partes. A primeira delas é destinada a uma breve revisão a cerca dos conhecimentos necessários para o entendimento da segunda parte, onde são apresentados os resultados obtidos através de artigos científicos. Esta segunda parte da tese pode ainda ser dividida em dois conjuntos de resultados, a saber, os obtidos através de procedimentos experimentais realizados dentro do Departamento de Bioquímica da UFRGS, e um segundo conjunto de resultados que foi obtido através de estudos teóricos acerca do tema, entendendo por isso, basicamente, revisão de literatura e inferências secundárias a modelagem matemática. Por fim, na terceira parte da tese os resultados obtidos são um pouco mais explorados e discutidos, desta vez de forma um pouco mais informal do que a exigida pelos padrões atuais adotados na preparação de manuscritos científicos. Espero que esta terceira parte também ajude na integração dos resultados e na geração de perspectivas para futuras pesquisas.

De uma maneira geral, acredito que a descrição de potenciais novos antipsicóticos atípicos de baixo preço comercial, associado ao melhor entendimento do mecanismo de ação que diferencia os antipsicóticos atípicos dos típicos, constituem as duas contribuições mais importantes que esta tese fornece. Dentre as contribuições secundárias, destaco a apresentação de um programa de computador que tem sido utilizado de forma rotineira em nosso departamento, bem como a proposta de um algoritmo para a redução de dose em pacientes tratados com antipsicóticos apresentando sintomas extrapiramidais.

Por fim, acredito que esta tese reflita um pouco da minha trajetória profissional até o momento. Durante o período deste doutoramento, adquiri alguns conhecimentos de matemática e de física que me fizeram ganhar uma nova perspectiva acerca do modo de se fazer ciência. Assim, esta tese, de certa forma, registra o início da transformação de um pesquisador de bancada a teórico.

LISTA DE ABREVIATURAS

AMPA – ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropionato

ATV – área tegmental ventral

CPF – córtex pré-frontal

EPS – sinais e sintomas extrapiramidais

GABA – ácido γ-aminobutírico

5-HT – 5-hidroxitriptamina (serotonina)

[I^{123}]IBZM – iodo-2-hidroxi-6-metoxi-N-[1-etil-2-pirrolidinil metil] benzamida

***K_d* –** constante de dissociação de equilíbrio

k_{off} – constante de taxa de dissociação

MK-801 – (+)-10,11-dihidro-5-metil-5H-dibenzo[a,d]ciclohepteno-5,10 imina

NMDA – N-metil-D-aspartato

PCP – fenciclidina

PET – tomografia por emissão de pósitrons

SNC – sistema nervoso central

SNPc – *pars compacta* da substância negra

SPECT – tomografia computadorizada por emissão de fóton único

RESUMO

(TORT ABL – SISTEMAS DOPAMINÉRGICOS E AÇÃO ANTIPSICÓTICA: ABORDAGENS EXPERIMENTAIS E TEÓRICAS) – Os objetivos da presente tese de doutorado foram os de buscar novos antipsicóticos atípicos de baixo preço comercial e também procurar entender o mecanismo de ação que leva a um perfil antipsicótico atípico. Os resultados da tese são divididos em duas partes, de acordo com sua natureza, em experimentais (primeira parte) e teóricos (segunda parte). Para o desenvolvimento da primeira parte, foi necessária primeiramente a programação de um *software* para medir locomoção em roedores após filmagem com *webcam*. A seguir, foram investigados os efeitos da guanosina, flunarizina e cinarizina em modelos animais de psicose, bem como em outros paradigmas comportamentais. A guanosina foi escolhida para estudo uma vez que tem se mostrado que ela interage com o sistema glutamatérgico – que sabidamente está envolvido na fisiopatologia da esquizofrenia – promovendo a captação astrocitária de glutamato. Já a flunarizina e a cinarizina, dois bloqueadores de canal de cálcio empregados para tratar enxaqueca e vertigem foram escolhidas pelo fato delas produzirem sinais e sintomas extrapiramidais em pacientes idosos, o que posteriormente foi relacionado às suas propriedades como antagonistas moderados dos receptores dopaminérgicos do tipo D2. A guanosina diminuiu o aumento de locomoção induzido por um antagonista NMDA (MK-801), enquanto que não apresentou efeito sobre o aumento de locomoção induzido por anfetamina, de forma que sua utilidade como potencial antipsicótico deve ser ainda melhor estudada. Tanto a flunarizina quanto a cinarizina foram capazes de diminuir o aumento de locomoção induzido por MK-801 e por anfetamina em doses que não causam efeitos catalépticos importantes. Portanto, foi concluído que estes dois compostos apresentam um potencial perfil de antipsicótico atípico, com as vantagens de já estarem disponíveis para uso comercial, boa tolerabilidade e baixo custo quando comparados com os antipsicóticos atípicos disponíveis comercialmente nos dias de hoje. A segunda parte da tese apresenta alguns resultados teóricos matemáticos que podem ser derivados da teoria da lei de ação das massas aplicada ao *binding* de receptores, utilizando também resultados experimentais já conhecidos de PET. Estes resultados apresentam *insights* ao entendimento das diferenças entre os perfis antipsicóticos atípicos e típicos em relação à geração de sinais extrapiramidais. É discutido que fatores culturais e comerciais relacionados à posologia atual empregada no tratamento com antipsicóticos típicos podem ser os responsáveis pelas diferenças de perfis, uma vez que alguns deles são prescritos em doses proporcionalmente maiores em relação à sua afinidade, atingindo assim maiores níveis de bloqueio dopaminérgico no estriado. Uma curta meia-vida plasmática também é apontada como um possível parâmetro importante na geração de um perfil atípico. É mostrado ainda alguns erros de concepção relacionados ao curso temporal da ocupação dopaminérgica que tem sido atualmente cometidos na literatura científica, como o conceito de meia-vida de ocupação de receptores. Como um último resultado teórico, é proposto um algoritmo para a redução de dose em pacientes tratados com antipsicóticos apresentando sinais e sintomas extrapiramidais.

ABSTRACT

(TORT ABL – DOPAMINERGIC SYSTEMS AND ANTIPSYCHOTIC ACTION: EXPERIMENTAL E THEORETICAL APPROACHES) –The aims of this work were the search for new atypical antipsychotics presenting low cost and the understanding of the mechanism of action leading to atypical antipsychotic profile. The results obtained are presented in two distinct parts based on their nature, namely, experimental (first part) or theoretical (second part). For the development of the first part, a webcam based software to measure locomotion of rodents was programmed. After that, it was investigated the effect of guanosine, flunarizine and cinnarizine on animal models of psychosis, as well as in other behavioral tasks. Guanosine was chosen because it has been shown to interact with the glutamatergic system – which is known to be involved in the pathophysiology of schizophrenia – by promoting astrocytic glutamate reuptake. Flunarizine and cinnarizine, two calcium channel blockers commonly used in many countries to treat vertigo and migraine, were chosen because they were shown to induce extrapyramidal signs in elder patients, which was later related to moderate antagonist properties at dopamine D2 receptors. Guanosine was able to reduce a NMDA antagonist (MK-801) induced hyperlocomotion, whereas it had no effect on the hyperlocomotion induced by amphetamine, and it is discussed that its utility as antipsychotic drug should be further evaluated. Both cinnarizine and flunarizine were able to reduce the hyperlocomotion induced by MK-801 and amphetamine at doses that presented no significant cataleptic behavior. It was therefore concluded that these compounds have a potential atypical antipsychotic profile, with the advantage of already approved for commercial use, presenting well tolerability and very low cost when compared to current commercially available atypical antipsychotics. The second part of this thesis presents some theoretical mathematical results that can be derived from the law of mass action theory applied to receptor binding linked with known PET experimental data. These results present insights to the understanding of the differences between typical and atypical profile of antipsychotics regarding the generation of extrapyramidal syndrome. It is argued that cultural and commercial aspects related to the nowadays employed posology of typical antipsychotics can be responsible for the difference seen in profile, once some typical antipsychotics are prescribed in proportionally higher doses in relation to their affinities, leading therefore to higher dopaminergic blockade. A short plasmatic half-life is also pointed as a possible important factor leading to an atipical profile. Moreover, the second part of this thesis also points to some misconception currently being used in the scientific literature regarding the time-course of dopaminergic occupation, such as the concept of receptor occupation half-life. As a last theoretical based result, it is proposed an algorithm for antipsychotic dose reduction in patients presenting extrapyramidal signs and symptoms.

I.1 INTRODUÇÃO

I.1.a SISTEMAS DOPAMINÉRGICOS

A dopamina é um neurotransmissor clássico do tipo catecolaminérgico. Cerca de 80% do total da dopamina no sistema nervoso central (SNC) encontra-se no estriado, enquanto que o restante encontra-se distribuído difusamente pelo córtex e outras regiões cerebrais. Os receptores dopaminérgicos são metabotrópicos acoplados a proteínas G, e podem ser encontrados tanto pré- quanto pós-sinapticamente. Eles são subdivididos em receptores dopaminérgicos do tipo D1 (receptores D1 e D5), que causam ativação da enzima adenilato ciclase, e do tipo D2 (receptores D2, D3 e D4), que inibem esta enzima[1].

Os neurônios dopaminérgicos se encontram organizados em grupos celulares no SNC. Existem quatro principais sistemas dopaminérgicos centrais, sendo que três deles se originam no mesencéfalo. Estes sistemas são também conhecidos como as vias dopaminérgicas:

- Mesolímbica: da área tegmental ventral (ATV) a diversos componentes do sistema límbico, como o núcleo acumbens, o hipocampo e a amígdala; envolvido em processos de emoção, memória e recompensa.
- Mesocortical: da ATV ao neocórtex, em particular ao córtex pré-frontal (CPF); envolvido em funções cognitivas como atenção, motivação, planejamento, e comportamento social.

[1] Para esta seção, nenhuma referência específica foi indicada; estas informações podem ser encontradas em qualquer livro de neurociência básica, como, por exemplo, "*Principles of neural science*" de Eric Kandel.

- Nigroestriatal: da substância negra *pars compacta* (SNPc) ao estriado; envolvido na coordenação sensório-motora e na iniciação do movimento.
- Túbero-infundibular: do núcleo arqueado do hipotálamo à glândula pituitária; envolvido na regulação da liberação de hormônios.

Acredita-se que os sistemas dopaminérgicos mesocortical e mesolímbico estejam diretamente envolvidos na fisiopatologia da esquizofrenia, enquanto que é bem sabido que a doença de Parkinson tem sua etiologia relacionada à morte das células dopaminérgicas da SNPc. Na via túbero-infundibular, a dopamina age como um fator inibidor da secreção de prolactina. Estas informações serão mais discutidas e utilizadas – implícita ou explicitamente – nas seções seguintes desta tese.

I.1.b ESQUIZOFRENIA E AS HIPÓTESES DOPAMINÉRGICA E GLUTAMATÉRGICA

A esquizofrenia é uma doença mental severa, crônica, de alta prevalência, atingindo cerca de 0,5 – 1% da população (Carpenter e Buchanan, 1994; Lewis e Lieberman, 2000). Os sintomas da esquizofrenia tipicamente surgem durante o final da adolescência e o início da adultez, e podem ser classificados como positivos, negativos ou cognitivos (Crow, 1985; Carpenter e Buchanan, 1994; Lewis e Lieberman, 2000). Dentre os sintomas positivos estão incluídas alucinações (tipicamente as auditivas), delírios (tipicamente persecutórios ou de megalomania), e desorganização severa do pensamento e do discurso. Os sintomas negativos constituem um conjunto de déficits em várias dimensões,

como afeto embotado, apatia, anedonia e comportamento anti-social. Já os sintomas cognitivos incluem déficits em capacidades como a atenção e a memória.

A hipótese dopaminérgica clássica da esquizofrenia postula que os sintomas positivos da doença sejam secundários a uma hiperatividade dopaminérgica subcortical ou, mais precisamente, mediados pelos receptores dopaminérgico do tipo D2 da via dopaminérgica mesolímbica (Laruelle et al., 1996; Abi-Dargham et al, 1998, 2000; Abi-Dargham, 2004; Abi-Dargham e Laruelle, 2005). Esta hipótese é sustentada por duas observações principais, a saber: a) agonistas dopaminérgicos do tipo D2 em uso continuado induzem sintomas similares aos positivos esquizofrênicos e, b) toda medicação que é efetiva como antipsicótica necessariamente bloqueia em algum grau os receptores D2 (Abi-Dargham, 2004; Abi-Dargham e Laruelle, 2005). Cabe citar que esta hiperatividade dopaminérgica subcortical tem sido recentemente demonstrada através de trabalhos utilizando o *binding* de [I^{123}]IBZM *in vivo* (Laruelle et al, 1996, 1999; Breier et al, 1997; Abi-Dargham et al, 1998, 2000). De fato, foi verificado que, após a administração de anfetamina, pacientes esquizofrênicos apresentam um aumento subcortical na transmissão dopaminérgica cerca de 2.5 vezes maior do que o encontrado em sujeitos controles (Laruelle et al, 1999). Mais ainda, neste paradigma, os pacientes esquizofrênicos apresentam 40% de chance de desenvolver, ou até piorar, sintomas psicóticos, contra chance nula nos controles (Laruelle et al, 1999).

A revisão da hipótese dopaminérgica para a esquizofrenia atribui também um déficit de atividade da via dopaminérgica mesocortical (Knable e Weinberger,

1997; Davis et al, 2001), ou, em específico, uma hipoatividade do receptor dopaminérgico do tipo D1 no CPF (Abi-Dargham, 2004). Esta hipofunção dopaminérgica estaria relacionada com os sintomas negativos e cognitivos vistos na doença (Knable e Weinberger, 1997; Davis et al, 2001; Goldman-Rakic et al, 2004), e tem sido corroborada por estudos em humanos e em animais mostrando que a depleção dopaminérgica em CPF causa sintomas semelhantes, bem como pela verificação de que os receptores dopaminérgicos D1 encontram-se aumentados em CPF de pacientes esquizofrênicos (Knable e Weinberger, 1997; Davis et al, 2001; Abi-Dargham et al, 2002; Goldman-Rakic et al, 2004).

Em suma, acredita-se atualmente que um déficit de atividade dopaminérgica cortical e um aumento de atividade dopaminérgica subcortical coexistam na doença (Abi-Dargham, 2004; Abi-Dargham e Laruelle, 2005). Esta coexistência é explicada pelo fato das transmissões dopaminérgica dos sistemas mesocortical e mesolímbico serem reguladas por circuitos neuronais complexos que incluem sinapses glutamatérgicas e GABAérgicas, interagindo entre si indiretamente (Abi-Dargham, 2004), como exposto na Figura 1. Tal interação é corroborada empiricamente através de diversos estudos realizados em animais, onde foi mostrado que manipulações que diminuem a atividade dopaminérgica no CPF geram um aumento na atividade das vias dopaminérgicas subcorticais, tanto a espontânea quanto aquela induzida por anfetamina ou apomorfina (Pycock et al, 1980; Davis et al, 1991).

Contudo, diversos trabalhos têm oferecido evidências de que uma disfunção da transmissão glutamatérgica envolvendo os receptores NMDA está associada à esquizofrenia (Bressan e Pilowsky, 2003; Goff e Coyle, 2001; Coyle et

al, 2003; Abi-Dargham e Laruelle, 2005). De fato, é sabido que antagonistas de receptores NMDA, como o PCP e a quetamina, são capazes de induzir tanto os sintomas positivos como os negativos e cognitivos da doença em sujeitos hígidos, bem como em pacientes esquizofrênicos (Krystal et al, 1994; Lahti et al, 1995). Ainda, há evidências sugerindo que a desregulação dopaminérgica encontrada na esquizofrenia pode ser secundária a um déficit na função do receptor glutamatérgico NMDA (Jentsch e Roth 1999). Todas estas informações levaram a formulação da hipótese glutamatérgica da esquizofrenia.

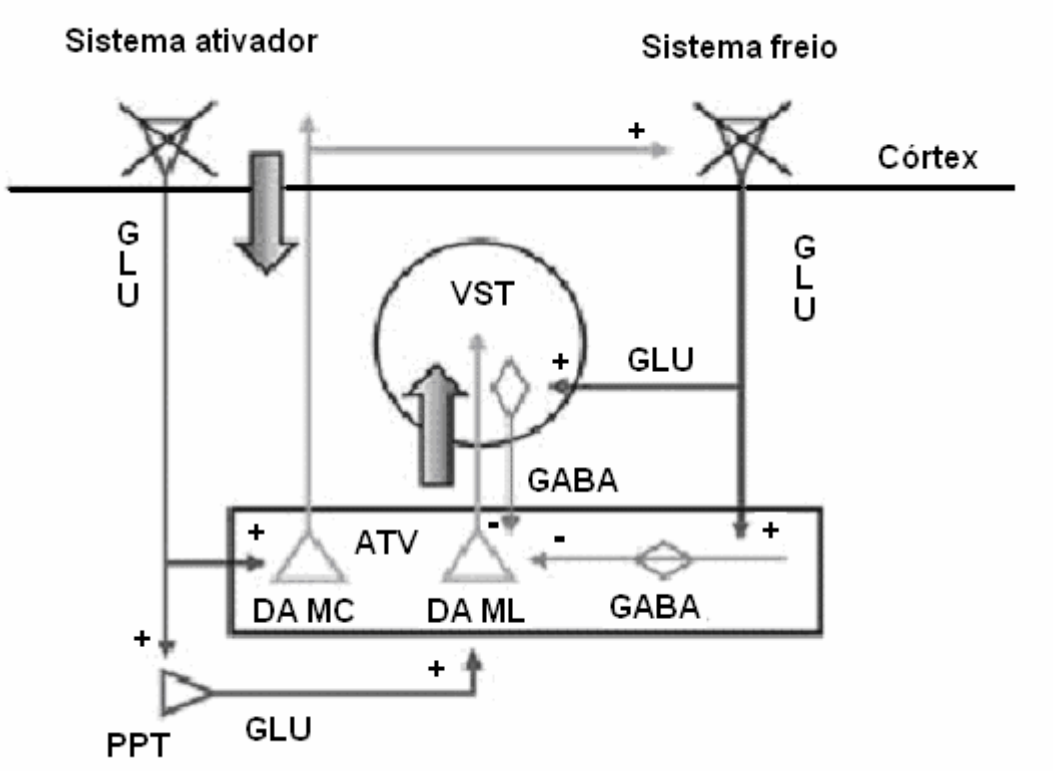

Figura 1. Representação da interação dos sistemas dopaminérgicos mesocortical e mesolímbico via circuitos neuronais incluindo sinapses glutamatérgicas e GABAérgicas. GLU: neurônios glutamatérgicos, GABA: neurônios GABAérgicos, VST: estriado ventral; ATV: área tegmental ventral; DA MC: via dopaminérgica mesocortical; DA ML: via dopaminérgica mesolímbica; PPT: tegumento pedúnculo-pontino (Fonte: Abi-Dargham, 2004).

Entretanto, uma desregulação dopaminérgica também causa alterações na transmissão glutamatérgica, uma vez que aferências glutamatérgicas corticais e projeções dopaminérgicas convergem em sinapses envolvendo neurônios GABAérgicos no estriado (Kotter, 1994; Cepeda e Levine, 1998). De uma maneira geral, mostrou-se que os receptores D1 e D2 apresentam papéis antagônicos em relação à transmissão glutamatérgica via receptor NMDA no estriado (Cepeda e Levine, 1998; Abi-Dargham e Laruelle, 2005). Como representado na Figura 2, a estimulação dos receptores D2 inibe a transmissão glutamatérgica por receptor NMDA, enquanto que a dos receptores D1 a favorecem (Cepeda e Levine, 1998; Abi-Dargham e Laruelle, 2005). Assim, tanto as interações glutamato/dopamina quanto as dopamina/glutamato parecem ser relevantes para a fisiopatologia da doença (Figura 2).

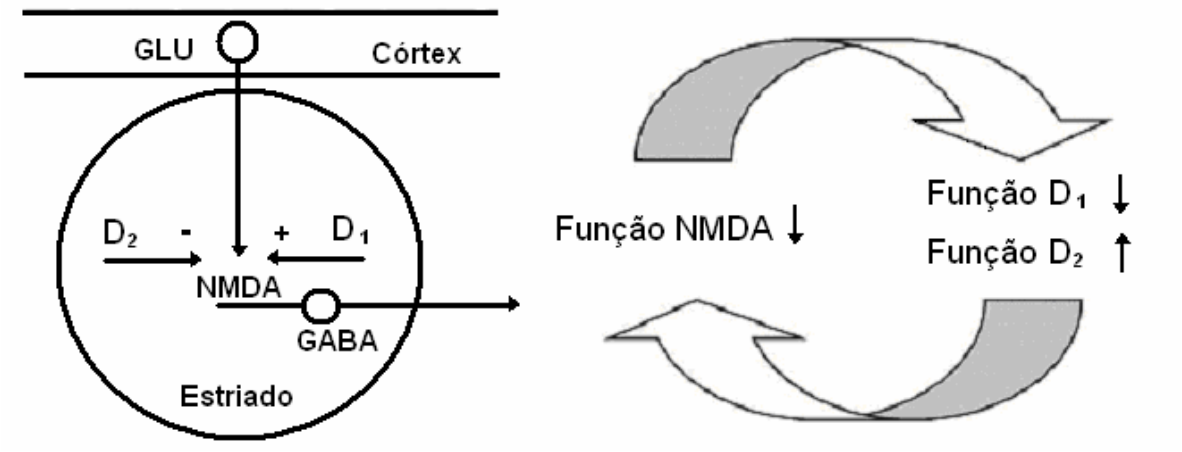

Figura 2. Esquerda: esquema ilustrando que receptores dopaminérgicos D1 e D2 apresentam efeitos opostos na modulação da transmissão glutamatérgica via NMDA em neurônios GABAérgicos do estriado. Direita: representação esquemática dos três desbalanços neuroquímicos principais presentes nos cérebros de pacientes esquizofrênicos, evidenciando suas

intercorrelações através de *loops* de retroalimentação positiva (Fonte: Abi-Dargham e Laruelle, 2005).

Independente da discussão acerca da desregulação dopaminérgica presente na esquizofrenia ser causa ou conseqüência de uma disfunção glutamatérgica, para esta tese, utilizaremos dois referenciais bem consolidados: a) os sintomas positivos da doença estão associados a uma hiperatividade dopaminérgica dos receptores D2 em regiões límbicas, e b) fármacos antagonistas D2 são sabidamente medicações antipsicóticas.

I.1.c ANTIPSICÓTICOS: TÍPICOS E ATÍPICOS

O estudo dos antipsicóticos constitui uma das áreas de pesquisa em psiquiatria que mais recebeu atenção nos últimos anos, sendo também responsável pelo melhor entendimento de muitos aspectos relacionados à fisiopatologia da esquizofrenia. Os primeiros antipsicóticos, clorpromazina e haloperidol, foram introduzidos na década de 50 e sua capacidade de alterar o curso e o prognóstico da doença, reduzindo a severidade e a recorrência dos sintomas, são bem aceitos (Abi-Dargham e Laruelle, 2005). Os antipsicóticos não são específicos para nenhum tipo em particular de psicose; entretanto, alguns (principalmente a clozapina) têm sido apontados como tendo melhor efeito sobre os sintomas negativos (Tuunainen et al, 2002).

Apesar da empolgação inicial causada pelos primeiros antipsicóticos, hoje em dia denominados de antipsicóticos típicos ou de primeira geração, verificou-se que estas drogas causam muitos efeitos colaterais neurológicos importantes,

como acatisia, discinesia tardia, distonia (aguda e crônica), bem como sinais e sintomas extrapiramidais (EPS)[2], como rigidez, bradicinesia, e tremores.

Recentemente, a eficácia de um segundo grupo de drogas, denominadas de antipsicóticos atípicos ou de segunda geração, também foi demonstrada. Os antipsicóticos atípicos, como a clozapina e a quetiapina, são vantajosos em relação aos típicos por não causarem, ou causarem pouco, EPS. Porém, estes fármacos possuem um custo muito elevado em relação ao preço dos antipsicóticos típicos, o que impede seu uso generalizado, especialmente em países de terceiro mundo.

Na presente tese, o conceito de antipsicótico atípico é empregado para denotar uma medicação que trata a psicose gerando pouco ou nenhum EPS. Contudo, cabe citar que há uma outra definição de antipsicótico atípico a qual, além destas propriedades, requer que o fármaco apresente efeito sobre os sintomas negativos da doença.

I.1.d TEORIAS ATUAIS PARA A ATIPICALIDADE

Ainda não se sabe exatamente qual o mecanismo de ação responsável pela diferença clínica vista entre antipsicóticos típicos e atípicos; a determinação de tal mecanismo teria grande importância para o desenvolvimento de novos fármacos atípicos. Atualmente, há na literatura científica diversas teorias para tentar explicar o mecanismo responsável pela atipicalidade. Muitas destas teorias postulam que o perfil atípico é secundário a interações que estes fármacos exerceriam em outros

[2] Existe aqui uma pequena imperfeição na literatura científica específica: às vezes encontramos EPS como significando “extrapyramidal symptoms”, mas, claramente, estão se referindo também aos sinais. Também é comum encontrarmos EPS como significando “extrapyramidal side-effects”.

receptores dopaminérgicos que não o D2 (isto é, em D1, D3, D4), ou mesmo da relação de potência de bloqueio entre eles (por exemplo, D4/D2, D1/D2) (Seeman et al, 1997; Strange, 2001; Tauscher et al, 2004). Outras teorias afirmam que o perfil atípico é secundário à ação dos fármacos em receptores não dopaminérgicos, em especial os 5-HT_{2A} serotoninérgicos, ou mesmo secundário à relação 5-HT_{2A}/D2 (Meltzer et al, 2003). Há também teorias que afirmam que a atipicalidade é secundária a bloqueios dopaminérgicos seletivos no SNC, ou seja, algumas drogas apresentariam maior propensão a bloquear zonas límbicas do que estriatais (Pilowsky et al, 1997; Strange, 2001; Bressan et al, 2003).

Há ainda uma teoria recente que foca exclusivamente no receptor D2, a saber, a hipótese da rápida dissociação (Kapur e Seeman, 2001). Uma vez que os antipsicóticos atípicos possuem em geral menor afinidade a este receptor do que os típicos – principalmente por possuírem alto valor de constante de taxa de dissociação (k_{off}) – é postulado que eles permitem uma certa transmissão dopaminérgica fisiológica por perderem em competição com a dopamina na sinapse (Kapur e Seeman, 2001).

Assim como esta última teoria, a presente tese almejou uma explicação para a geração de um perfil atípico focando exclusivamente no efeito dos antipsicóticos sobre receptor dopaminérgico D2.

I.1.e MODELOS ANIMAIS

O aumento da locomoção induzido em roedores por agentes psicoativos tem sido empregado como um modelo de psicose, uma vez que a capacidade de reversão de tal efeito por fármacos vem sendo validada como possuindo uma boa

previsão de que estes compostos irão apresentar atividade antipsicótica quando administrados em humanos (Ninan e Kulkarni, 1999; O'Neill e Shaw, 1999; Geyer e Ellenbroek, 2003; Kapur e Mamo, 2003). Na presente tese, foram utilizados dois modelos de aumento de locomoção induzidos por psicofármacos em camundongos: (i) a administração sistêmica de anfetamina – um agonista dopaminérgico indireto; (ii) a administração sistêmica de dizocilpina (MK-801) – um antagonista de receptores glutamatérgicos NMDA. O primeiro constitui o modelo de psicose classicamente usado, e é sabido que todo antipsicótico é capaz de reverter o aumento de locomoção secundário à anfetamina (Ellenbroek, 1993; Geyer e Ellenbroek, 2003; Kapur e Mamo, 2003). O segundo modelo vem sendo apontado recentemente como o melhor modelo farmacológico para esquizofrenia (Ninan e Kulkarni, 1999; O'Neill e Shaw, 1999), uma vez que em humanos o PCP e a quetamina (antagonistas NMDA) são capazes de reproduzir tanto os efeitos positivos como os negativos da doença (Goff e Coyle, 2001). De nota, nem todo antipsicótico é capaz de reverter o aumento de locomoção induzido por antagonistas NMDA em doses que não causem diminuição da locomoção espontânea (O'Neill e Shaw, 1999).

A avaliação do grau de efeitos extrapiramidais ocasionados pelos fármacos em estudo foi realizada através da análise do tempo no qual os camundongos, após terem suas patas dianteiras apoiadas em uma barra elevada em relação ao solo, permaneciam em postura de catalepsia.

Através destes dois modelos, um determinado fármaco pode ser considerado com possuindo perfil de antipsicótico atípico se for capaz de reverter o aumento de locomoção induzido por ambas as drogas psicoativas (isto é,

reverter a psicose) em doses que não induzam catalepsia nos animais (isto é, que não causem EPS). Os detalhes técnicos de cada procedimento são expostos nas seções de materiais e métodos dos trabalhos publicados, bem como a descrição de outros paradigmas comportamentais que foram eventualmente utilizados.

I.1.f EFEITOS INDIRETOS DE ANTAGONISTAS NMDA

Recentemente, foi demonstrado que antagonistas NMDA, como a quetamina e o PCP, provocam um aumento da liberação de glutamato, levando a maior estimulação dos receptores AMPA e cainato (Moghaddam et al, 1997). Além disso, antagonistas de receptores não-NMDA inibem o aumento de locomoção e a neurotoxicidade induzidas por antagonistas NMDA (Hauber e Andersen, 1993; Olney e Farber, 1995), e os efeitos bioquímicos (aumento de glutamato extracelular, mas não de dopamina), comportamentais e cognitivos do PCP foram revertidos em ratos por um agonista dos receptores glutamatérgicos metabotrópicos do tipo II/III, que inibe a liberação de glutamato (Moghaddam e Adams, 1998). O paralelo em humanos foi que a lamotrigina, um anticonvulsivante que também inibe a liberação de glutamato, foi capaz de atenuar os efeitos neuropsiquiátricos da quetamina (Anand et al., 2000). Dessa forma, pode-se considerar que grande parte dos efeitos dos antagonistas NMDA seja decorrente dessa liberação de glutamato, levando à hiperatividade dos receptores não-NMDA concomitantemente à hipofunção NMDA.

I.1.g GUANOSINA

Nos últimos anos, vem sendo construído um corpo de evidências caracterizando uma atividade antiglutamatérgica dos nucleotídeos GTP, GDP e GMP e do nucleosídeo guanosina, a qual não é diretamente relacionada à modulação de proteínas-G que estes compostos sabidamente exercem (Baron et al., 1989; Souza e Ramirez, 1991; Ramos et al., 1997; Porciúncula et al, 2002). Em particular, foi mostrado que a guanosina extracelular é capaz de prevenir as convulsões induzidas por compostos que causam hiperestimulação do sistema glutamatérgico (como o ácido quinolínico e a alfa dendrotexina) (Lara et al, 2001; Schmidt et al, 2000, 2005; Vinade et al, 2003), possui efeito neuroprotetor em eventos convulsivos e hipóxicos em animais adultos e jovens (Lara et al, 2001; Frizzo et al, 2002; Soares et al, 2004, Oliveira et al, 2004), e tem se mostrado amnésica em diversos testes de memória, como a esquiva inibitória (Roesler et al, 2000; Vinadé et al, 2003, 2004, 2005). O efeito da guanosina parece ser mediado pela estimulação do transporte do glutamato astrocitário, conforme observado em fatias de cérebro e em cultura de astrócitos (Frizzo et al, 2001, 2002, 2003).

Baseado nesta propriedade antiglutamatérgica da guanosina, e no exposto na seção anterior acerca da dependência glutamatérgica da ação de fármacos antagonistas NMDA, bem como levando em conta o envolvimento da hipofunção NMDA na fisiopatologia da esquizofrenia, esta tese estudou a ação da guanosina frente ao aumento de locomoção induzido por MK-801, bem como frente a outros paradigmas comportamentais.

I.1.h FLUNARIZINA E CINARIZINA

Flunarizina e cinarizina são dois fármacos bloqueadores não seletivos de canal de cálcio (principalmente do tipo T) que têm sido clinicamente utilizados nos últimos anos em países europeus e sul americanos para o tratamento de vertigem e enxaqueca, sendo geralmente bem tolerados pelos pacientes (Todd e Benfield, 1989; Schmidt e Oestreich, 1991; Leone et al, 1991). A maior diferença entre estes dois compostos é a meia-vida de eliminação, cerca de 3 horas para a cinarizina, e de 16-20 dias para a flunarizina (Kariya et al, 1995). Entretanto, alguns relatos clínicos mostraram que ambas as drogas podem agravar sinais extrapiramidais em pacientes com parkinsonismo prévio e, em pacientes idosos, podem mesmo induzir tal sintomatologia (Fernandez et al, 1988; Garcia-Ruiz et al, 1992; Brücke et al, 1995; Daniel e Mauro, 1995). Estudos animais sugerem que tais efeitos colaterais poderem ser devidos a um bloqueio dos receptores dopaminérgicos do tipo D2 no estriado (Kariya et al, 1995). De fato, um estudo com humanos utilizando SPECT evidenciou bloqueio dopaminérgico estriatal moderado em pacientes cronicamente tratados com flunarizina e cinarizina (Brücke et al, 1995).

Com base nisso, estes dois fármacos foram estudados na presente tese para verificar sua potencial utilização como novos antipsicóticos atípicos. Eles apresentariam ainda a vantagem de já estarem disponíveis no mercado, com boa tolerabilidade e baixíssimo custo, especialmente quando comparados aos antipsicóticos atípicos atuais.

I.1.i INDUÇÃO DE SINTOMAS EXTRAPIRAMIDAIS E A JANELA TERAPÊUTICA DE OCUPAÇÃO

Uma vez que os fármacos antipsicóticos são administrados de forma sistêmica, além de sua ação (desejável) no sistema mesolímbico, eles também interagem com os demais sistemas dopaminérgicos. Assim, é sabido que um bloqueio dopaminérgico elevado na via túbero-infundibular é responsável pelo aumento dos níveis de prolactina sérica muitas vezes observados em pacientes esquizofrênicos em tratamento. Da mesma forma, um elevado nível de bloqueio dopaminérgico da via nigroestriatal é responsável por gerar os indesejados EPS.

Em estudos utilizando PET e SPECT para estimar o nível de bloqueio dopaminérgico estriatal em sujeitos tratados com antipsicóticos, verificou-se que este se correlaciona com a melhora clínica dos pacientes, bem como com alguns efeitos colaterais. De fato, é aceito que um bloqueio superior a 65% está associado a um tratamento antipsicótico efetivo na maioria dos pacientes (Kapur et al, 2000a)**.** Por outro lado, o aumento de prolactina sérica ocorre quando mais do que 72% dos receptores dopaminérgicos estão ocupados, enquanto que os EPS tipicamente surgem com um nível de bloqueio superior a 78%[3] (Kapur et al, 2000a).

Assim, observamos que é possível definir uma janela terapêutica de ocupação dopaminérgica que é compatível com eficácia terapêutica sem geração

[3] Note que podemos dizer que um bloqueio dopaminérgico estriatal em um nível superior a 78% causa EPS na maioria dos pacientes. Entretanto, quando nos referimos à eficácia antipsicótica, ou ao aumento de prolactina sérica, devemos ter claro que o nível de bloqueio estriatal é correlacionado a tais efeitos, e não é o causador direto deles, já que o aumento de prolactina é causado pelo bloqueio dopaminérgico na pituitária, enquanto a ação antipsicótica requer um bloqueio em áreas límbicas.

de EPS, a saber, um nível de bloqueio entre 65-78%. Tal informação será utilizada para deduzir alguns resultados apresentados nesta tese.

I.1.j A LEI DE AÇÃO DAS MASSAS

A lei de ação das massas foi inicialmente anunciada em 1864 por dois químicos noruegueses, Cato Maximilian Guldberg e Peter Waage. Infelizmente, eles publicaram seu trabalho em norueguês, ocasionando um atraso de cerca de 15 anos até que o conhecimento chegasse aos químicos franceses e alemães. Com o passar dos anos, a importância da lei de ação das massas foi sendo cada vez mais reconhecida. Por exemplo, o estudo da cinética das reações enzimáticas, incluindo a famosa equação de Michaelis-Menten, é muito baseado em conseqüências da lei da ação das massas (Keener e Sneyd, 1998). Mais ainda, hoje em dia se acredita que ela é uma lei geral da natureza aplicável a muitas circunstâncias além das reações químicas[4]. Atualmente, esta lei é mais conhecida sob o seguinte enunciado: “a velocidade de uma reação química, à temperatura constante, é proporcional ao produto das concentrações molares das substâncias reagentes”. Esquematicamente, freqüentemente encontramos uma reação genérica do tipo

$$\mathbf{A + B \rightarrow C}$$

e a lei de ação das massas nos diz neste caso que (Keener e Sneyd, 1998):

$$\mathbf{dC/dt = k\, A\, B}$$

[4] Por exemplo, a velocidade de formação de novos casais é proporcional ao número de homens e ao número de mulheres numa determinada comunidade.

onde *dC/dt* é a velocidade de formação de *C*, *k* é uma constante de proporcionalidade, enquanto que as letras (*A,B,C*) designam as concentrações das substâncias em questão[5].

Na presente tese, a lei de ação das massas será utilizada para descrever uma reação bilateral (**A + B ↔ C**), onde os reagentes serão um antipsicótico e o receptor dopaminérgico do tipo D2, enquanto que o produto será o complexo unido receptor-antipsicótico. A formulação matemática da lei de ação das massas para este caso específico é apresentada na seção de apêndice do trabalho exposto no capítulo II.2.a, onde é mostrado também como se obtém as equações para as soluções no equilíbrio, que são utilizadas nos capítulos II.2.b e II.2.c. A validade da lei de ação das massas como sendo capaz de descrever o nível de ocupação dopaminérgica central após a administração de um antipsicótico é corroborada através de inúmeros trabalhos já publicados na literatura científica utilizando PET (por exemplo, Suhara et al, 2002; Takano et al, 2004; Talvik et al, 2004; Mamo et al, 2004) e resultados expostos nesta tese fazem uso implícito de tal fato.

[5] Na realidade, uma vez que a igualdade da expressão acima se mantém ao multiplicarmos ela por uma constante qualquer, e lembrando que a concentração é definida como a massa por volume, vemos que esta expressão é igualmente válida para as massas das substâncias em questão.

I.2 OBJETIVOS E ESTRUTURA DA TESE

Os trabalhos realizados na presente tese foram divididos em dois conjuntos: os experimentais e os teóricos. Os objetivos gerais e específicos de cada uma destas partes são expostos a seguir. A importância e o embasamento destes objetivos podem ser encontrados no capítulo anterior de introdução desta tese.

I.2.a TRABALHOS EXPERIMENTAIS

Objetivo geral: investigar potenciais novos antipsicóticos atípicos de baixo custo comercial.

Objetivos específicos:

a) Estudar o efeito da guanosina frente a modelos farmacológicos de esquizofrenia em camundongos.

b) Estudar o efeito da flunarizina frente a modelos farmacológicos de esquizofrenia em camundongos.

c) Estudar o efeito da cinarizina frente a modelos farmacológicos de esquizofrenia em camundongos.

Detalhes técnicos mais precisos sobre a metodologia empregada em cada trabalho podem ser encontrados nos artigos científicos correspondentes.

Estrutura de apresentação dos resultados: os resultados das pesquisas propostas nos itens a, b e c são apresentados, respectivamente, nos capítulos II.1.b, II.1.c e II.1.d. O capítulo II.1.a desta tese descreve um *software* de computador que foi utilizado nestas pesquisas.

I.2.b TRABALHOS TEÓRICOS

Objetivo geral: contribuir para a elucidação do mecanismo de ação que diferencia os antipsicóticos atípicos dos típicos em relação à geração de EPS.

Objetivo específico:

a) Realizar uma revisão de literatura sobre o tema.

b) Utilizar modelos matemáticos junto com as informações obtidas em a) para estudar possíveis mecanismos responsáveis pela geração de um perfil de antipsicótico atípico.

Estrutura de apresentação dos resultados: os principais resultados obtidos são expostos no capítulo II.2.a. O desenvolvimento desta pesquisa gerou ainda outras contribuições teóricas que são expostas nos capítulos II.2.b, II.2.c e II.2.d.

PARTE II

Onde são apresentados os resultados.

II.1 RESULTADOS EXPERIMENTAIS

II.1.a A SIMPLE WEBCAM-BASED APPROACH FOR THE MEASUREMENT OF RODENT LOCOMOTION AND OTHER BEHAVIOURAL PARAMETERS

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A simple webcam-based approach for the measurement of rodent locomotion and other behavioural parameters

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Abstract

We hereby describe a simple and inexpensive approach to evaluate the position and locomotion of rodents in an arena. The system is based on webcam registering of animal behavior with subsequent analysis on customized software. Based on black/white differentiation, it provides rapid evaluation of animal position over a period of time, and can be used in a myriad of behavioural tasks in which locomotion, velocity or place preference are variables of interest. A brief review of the results obtained so far with this system and a discussion of other possible applications in behavioural neuroscience are also included. Such a system can be easily implemented in most laboratories and can significantly reduce the time and costs involved in behavioural analysis, especially in developing countries.

Keywords: locomotion, displacement, rodent, webcam, software

Introduction

Measurement of position and locomotion are central to the study of innumerable features of animal behaviour, including memory, anxiety, spatial orientation and novelty seeking. Moreover, locomotion analysis is also important to screen for neurological effects of various drugs, and pharmacologically induced hyperlocomotion is one of the most widely used models to test compounds for antipsychotic activity (Kapur & Mamo, 2003; Geyer & Ellenbroek, 2003).

Analysis of animal movement has come a long way from the early days of neuroscience. For decades, little more was available to measure locomotion other than a stopwatch, an arena containing a grid pattern on its floor and a hard-labouring postgraduate student to count the number of squares crossed over a given period of time. In fact, this approach was so widely used that it still holds its ground in some recent work, particularly in the developing world (Vianna et al., 2000), due to its extremely low cost in financial terms, if not in time-efficiency.

This method was initially replaced by photocell-based systems which automatically recorded animal crossings (Ericson et al., 1991). These began to be used more than 30 years ago (Weifenbach et al., 1969) and gradually gained acceptance, becoming perhaps the most popular way to evaluate locomotion until recently. These systems, however, were (and still are) relatively expensive and based their estimates of locomotion on indirect discrete measures (number of crossings, as opposed to actual path measurements).

Video analysis of animal movement began to be used in the early 80's (Godden & Graham, 1983) and started to gain wider acceptance about a decade later (Schwarting et al., 1993). However, most of these systems were and still are dependent on specific hardware

(usually commercialized in a package with the software) and brought little cost improvement (Pan et al., 1996). Only recently, with the explosion of cheap "webcam" equipment, has this technology been brought to the point where it can be implemented using standard computer equipment already present on virtually any laboratory in the world. A few studies have been published in recent years describing webcam-based software for locomotion analysis (Lind et al., 2005; Togasaki et al., 2005); surprisingly, however, we have found little data dealing specifically with the application of such software in rodents.

We hereby propose a simple software algorithm for the tracking of rodent position by analyzing webcam-registered images. This algorithm can be easily adapted to other species and special behavioral situations through simple variations in its code. We discuss some of the myriad applications of this inexpensive methodology in the neurosciences, briefly presenting results we have obtained with it (Coitinho et al., 2002; Lourenco da Silva et al., 2003; Dall'Igna et al., 2003, 2004, 2005; Tort et al., 2004, 2005; Dietrich et al., 2004, 2005; da Silva et al., 2005; de Oliveira et al., 2005; Kazlauckas et al., 2005) and proposing other applications to which it can be easily adapted.

Materials and Methods

Our system consists of a program written in Pascal language through a Delphi interface**,** which is able to run on most Microsoft Windows® operational systems (Windows 95/98/2000/XP) and was programmed to export data to Microsoft Excel®. The software, which we have baptized as Mousetracker, was developed to analyze video files of animal experiments previously recorded through a webcam or other electronic video recording apparatus. A current version of the software can be obtained by contact with the authors at mousetracker@gmail.com for no charge. Later, improved versions may be made available commercially in the future, at prices which will still be substantially lower (around 90% cheaper) than hardware/software packages available in the market.

The program has been programmed with a simple, instinctive interface, based on a relatively small amount of buttons and functions (Figure 1). The program occupies little hard drive space (less than 600 KB) and its hardware requirements include no more than a Pentium class or equivalent processor (we routinely use a 1.6 GHz Athlon processor with 512 MB RAM) with a webcam and proper video recording software installed. We usually set the webcam recording software to acquire videos in AVI format at 4 frames/second in 320x240 pixels resolution (yielding an occupation of about 500 KB of hard drive space per minute), but this is not mandatory, although the same configuration should be kept throughout a set of experiments. Microsoft Excel® software is required for exporting of the data.

The software has been initially designed to detect light-coloured animals in a dark arena, and it will be thus described, although it can be easily adapted to do the opposite (as

could be of interest for experiments involving C57BL/6 mice, for example). It works through black/white differentiation, based on a grayscale threshold for animal detection. The program will then consider every pixel lighter than the threshold as being white (i.e., part of the animal), and every pixel darker than the threshold as being black (i.e., part of the arena), as shown in Figure 2. This threshold can be modified by the user, who can visualize the "detected" areas as he varies this parameter until they include the animals, but exclude features of the background. However, we emphasize that the threshold should not be set in a level which includes only small portions of the animals as white, as this can produce fluctuations in animal detection and overestimation of movement (Figure 2).

An ideal arena should be dark and opaque, and the illumination of the room should be diffuse enough not to allow light to reflect on any point of the arena. Note that the arena we have shown in Figure 2 is not ideal, as it contains some reflected light, which nevertheless is below the grayscale threshold level. Moreover, we stress that the arena should also be tested for the onset of light reflection when it is wet, once animal urine can be produced during experiment recordings. Still, if small white areas persist in the background, the program has a routine that allow them to be painted "black" with the aid of the mouse, leading them to be ignored by the system throughout the analysis period.

Once the grayscale threshold is set, the user has to identify the initial position of each animal by using the mouse. The software can analyze as many animals as desirable, providing that each animal remains in a separate part of the arena and the walls dividing them are large enough to prevent animals from approaching each other excessively. Moreover, the separate arenas should be symmetrically distributed around the camera's location to avoid distortion. In measuring locomotion, we have routinely analyzed up to eight mice on each trial.

Besides the grayscale threshold and the initial position of the animals, the user must also inform a value (in pixels) for a parameter called "radius". This variable has this name because, after localizing the animal, the program draws a circle with such a radius around the position of the animal, in a way that the user can be confident that the program is working well (see Figure 1).

The central algorithm for the localization of the position of the animals on each frame is summarized in Figure 3. For each animal, the system will automatically calculate the "center of white" (analogously to the calculation of a mass center) in a square area centered on the initial position of the animal and with a side length equal to twice the selected radius, as exposed in Figure 3A and 3B. This "limited-radius" search for the animal was found to greatly improve processing speed as opposed to calculations performed on the whole arena. After finding the center of white (i.e., the position of the animal), the program proceeds to the next video frame. It can also be set to skip video frames on a multiple of a parameter called "frames", which has a default value of 1. In this new frame, the program will again calculate the center of white on a square of the same area, but this time centered on the position of the animal in the previous frame (Figure 3B and 3C). This procedure is then repeated until the end of the analysis.

The process described above occurs very quickly (around 50-350 miliseconds per frame, depending on the number of animals and radius value, on a 1.6 GHz processor with 512 MB RAM), in a way that the user has the impression of a continuous displacement of the circle containing the animal. Therefore, the conversion of the results is usually faster than the actual length of the video file (i.e. the conversion of a 1-minute-long video including 2 animals with a radius of 10 pixels takes about 14 seconds; analysis of 8 animals at the same time will increase this to 55 seconds). Shorter frame intervals, higher numbers

of animals and greater radius values cause the program to run more slowly. If the radius value is too small, the program can eventually lose track of an animal if its dislocation exceeds the area of the square from one analyzed frame to the next. If this happens, however, the user can easily click on the animal to allow the computer to track it again. It is also possible to pause the analysis and manually modify parameters such as radius and grayscale threshold after the program is running if needed.

By knowing the position of the animals at each point in time, the software can then compute locomotion, speed and acceleration. A button click can also access a feature which traces the animal's path on a white screen (Figure 1). Moreover, for behavioral tasks in which animal position/place preference is a concern (e.g. object recognition, water maze probe tests), the user can select an area of interest in the arena, and the software will also obtain data on time spent within and outside of that area at the same time that it computes locomotion.

Lastly, note that locomotion and other variables can be obtained for different intervals of time within the video file's length. Therefore, after data is obtained, the user selects the time intervals (i.e., blocks of 30 s, 5 min, etc.) for the data to be exported to an Excel$^{@}$ spreadsheet.

Results and Discussion

1. Validation

To confirm the validity of our method in measuring locomotion, we performed 5-minute recordings of 22 animals in a 50x50 cm square arena with our webcam system. The locomotion data obtained for these animals with the software was compared with manually obtained analysis of the number of crossings of each animal after dividing the arena in 5x5 cm squares. Linear correlation of these two variables (automated and manual locomotion measurements) was performed (Figure 4) and yielded a highly significant correlation coefficient (r) of 0,976 ($p<0,001$). Moreover, we believe the minor deviations from the straight line seen in the figure are likely to represent limitations inherent to estimating locomotion by manual counting of crossings, rather than inaccuracies of the software in measuring this parameter. This strong correlation, therefore, makes one comfortable that the software does indeed provide reliable measurements of locomotion.

2. Applications

a) Evaluation of spontaneous locomotor activity

Perhaps the most obvious application for a system designed to measure locomotion is the evaluation of spontaneous locomotion, as shown in figure 5a. By simply exposing an animal to an open field, one can look for motor impairment caused by drugs and other interventions on animal locomotion. Although this is rather nonspecific, as increases or decreases in locomotion can occur due to a variety of causes (including some not related to motor impairment), this can be useful as a screening test. In this setting, we have used our

system to show evidence of motor impairment by chronic, low-dose exposure to methylmercury in drinking water (Dietrich et al., 2005).

b) Drug-induced hyperlocomotion

Models of drug-induced hyperlocomotion have been traditionally used as predictive models to screen for the antipsychotic activity of different compounds. Although the links between the pathophysiology underlying psychiatric illness such as schizophrenia and the increased locomotion induced by compounds such as amphetamine and the N-methyl-D-aspartate (NMDA) receptor blockers phencyclidine (PCP) and dizocilpine (MK-801) are unclear, these animal models have shown good predictive validity for the antipsychotic activity of various drugs (Ninan & Kulkarni, 1999; O'Neill & Shaw, 1999; Geyer & Ellenbroek, 2003)

The use of our software allows us to monitor animals in these models for a reasonably long period of time. Therefore, one can analyze locomotion before and after the injection of a hyperlocomotion-inducing compound and the effect of potential antipsychotic drugs in blunting this response. As can be seen in figure 5 (b and e), both amphetamine and MK-801 produce marked increases in total locomotion which gradually decrease with waning of the drugs. A similar effect, although more subtle, can be observed with caffeine (figure 5d), which also increases dopaminergic tonus in the central nervous system (Cauli & Morelli et al., 2005). Note also the "priming" response of multiple injections of amphetamine, which potentiate the response in subsequent administrations (figure 5c).

In focusing our search for novel antipsychotic compounds, we have found that compounds known to possess some degree of dopamine D2-receptor blockade activity such as flunarizine (Tort et al., 2005) and cinnarizine (Dall'Igna et al., 2005) are able to blunt and/or abolish the hyperlocomotion response induced by both amphetamine and MK-801

(figure 5e). Measurements of these effects can therefore be easily and rapidly performed by our software and can be of use in screening for drugs with potential antipsychotic activity. These results are in accordance with other evidence for antidopaminergic actions of flunarizine (Hori et al., 1998) and have warranted currently ongoing clinical studies involving this drug.

c) Object exploration

By focusing on animal position rather than on locomotion, other behavioural information can be obtained by the use of our system. Measures of exploratory activity, for example, are central to various models used in the evaluation of behavioural features such as anxiety (Belzung & Griebel, 2001) and memory (Rampon, 2000).

Exploration of a novel object or environment is an innate feature of rodent behaviour, but can be inhibited in situations in which anxiety or avoidance-related features are prominent in the animals. Our system has been innovatively used for the evaluation of object exploration as a distinguishing feature of animal temperament, which was used to differentiate mice into two groups with high and low innate exploratory activity, respectively (Figure 6). The system was used to measure time spent in the center of an arena (in which the object to be explored was placed and "high explorer" animals tended to dwell) and in its periphery (in which "low explorers" remained, sometimes throughout the observation period). These two groups of animals were later shown to behave very differently in several other behavioural tasks involving memory, anxiety and aggressiveness (Kazlauckas et al., 2005), a fact which may render the model relevant for the study of personality traits underlying mood disorders.

d) Other potential applications

The position tracking feature described above to measure object exploration can also be used in a myriad of other behavioral tasks. Object recognition memory, for example, can be measured by tracing areas of interest around two objects (only one of which has been previously explored), as mice which remember having explored an object will tend to prefer a novel object instead of the known one in a subsequent session (Rampon et al., 2000). The system can also be used to measure place preference in various anxiety tasks, such as light/dark preference and the elevated plus-maze, in which animals are given a choice between sheltered (e.g. dark chamber or closed arms) and non-sheltered environments (e.g. light chamber or open arms). Finally, although we have not yet used the system for spatial memory tasks, such as the Morris' water maze, the software is able to track the position of animal and can feasibly be used for the acquisition of both quantitative (i.e. time spent in an area) and descriptive (i.e. pathway tracing) information, provided that that adequate contrast between the animal and the pool is achieved.

Conclusion

In summary, we have described a simple, webcam-based software which can be run on an average personal computer and is robust enough to be useful for most behavioural tasks dealing with locomotion and/or position, as discussed above. Although the use of video tracking systems is vital to some of the tasks we have discussed, and therefore widespread among laboratories around the world, it is still done through commercially available hardware/software systems in most laboratories. By offering an alternative which can be implemented at a much lower cost, we hope to help behavioural neuroscience remain one of the few domains of knowledge in which good ideas can still be worth more than large research grants, making opportunities a bit more equal than usual for scientists all over the globe.

Acknowledgements

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Figure legends.

Figure 1.

User interface of the Mousetracker software. Note video display with circled animals (upper left), selected parameters and control buttons (top and lower left), locomotion counts (upper right) and path tracer (lower right).

Figure 2.

Effect of the grayscale threshold on animal detection. (a) shows an actual frame from the video file, (b) is an example of a threshold set too low, which includes lighter areas in the background, (c) represents the adequate threshold, with minimal background interference, and (d) shows an example of a threshold set too high, with inadequate sampling of the animals.

Figure 3.

Scheme of the system's central algorithm: (A) Initial position (X_0,Y_0) of the animal should be informed manually with a mouse click. (B) The software then analyzes the next video frame, searching for the center of white on a square area centered on (X_0,Y_0), which corresponds to the new position of the animal (X_1,Y_1). (C) Similarly, in the next frame, the program searches for the animal's new position (X_2,Y_2) in a square centered on (X_1,Y_1), and so on. Based on the information of the position (X_t,Y_t) of the animal on each time *t*, it is possible to obtain the displacement, velocity, path and time spent in an area of interest, among other variables.

Figure 4.

Correlation between manual recording of number of crossings (y-axis) and automated registering of locomotion (number of "counts", or pixels traveled) by the mousetracker software (x-axis). Points are very close to the straight line, and statistical analysis yields a strong and significant correlation coefficient ($r=0,976$, $p<0,001$).

Figure 5.

Locomotion curves obtained with the system: (a), spontaneous exploration of an open field over a 2 hour interval, with the y-axis ("counts") representing the number of pixels traveled by the animal; decreasing locomotion over time is due to habituation to the environment; (b), hyperlocomotion induced by the administration of amphetamine 5 mg/kg after the habituation period; (c), locomotion over a two-hour period after priming with amphetamine (1 mg/kg daily), evidencing greater response in the seventh administration of the drug than in the first; (d), hyperlocomotion induced by the administration caffeine 30 mg/kg after the habituation period; (e) reversal of MK-801-induced hyperlocomotion by various doses of flunarizine (Tort et al., 2005).

Figure 6.

Separation of high- and low-exploratory phenotypes among a population of CD-1 mice. The graph shows the percentage of time spent in the central portion of an arena with a central object over 5 minutes, as analyzed by the system. Animals were divided as high- (gray dots) and low-exploring (white dots) animals according to their performance in the test at 16 weeks of age, and differences between the two populations (dashes represent

mean values for each group) remained significant when they were retested after at 17 and 52 weeks of age ($^{*}P < 0.05$; $^{**}P < 0.001$) (Kazlauckas et al., 2005).

Figure 1.

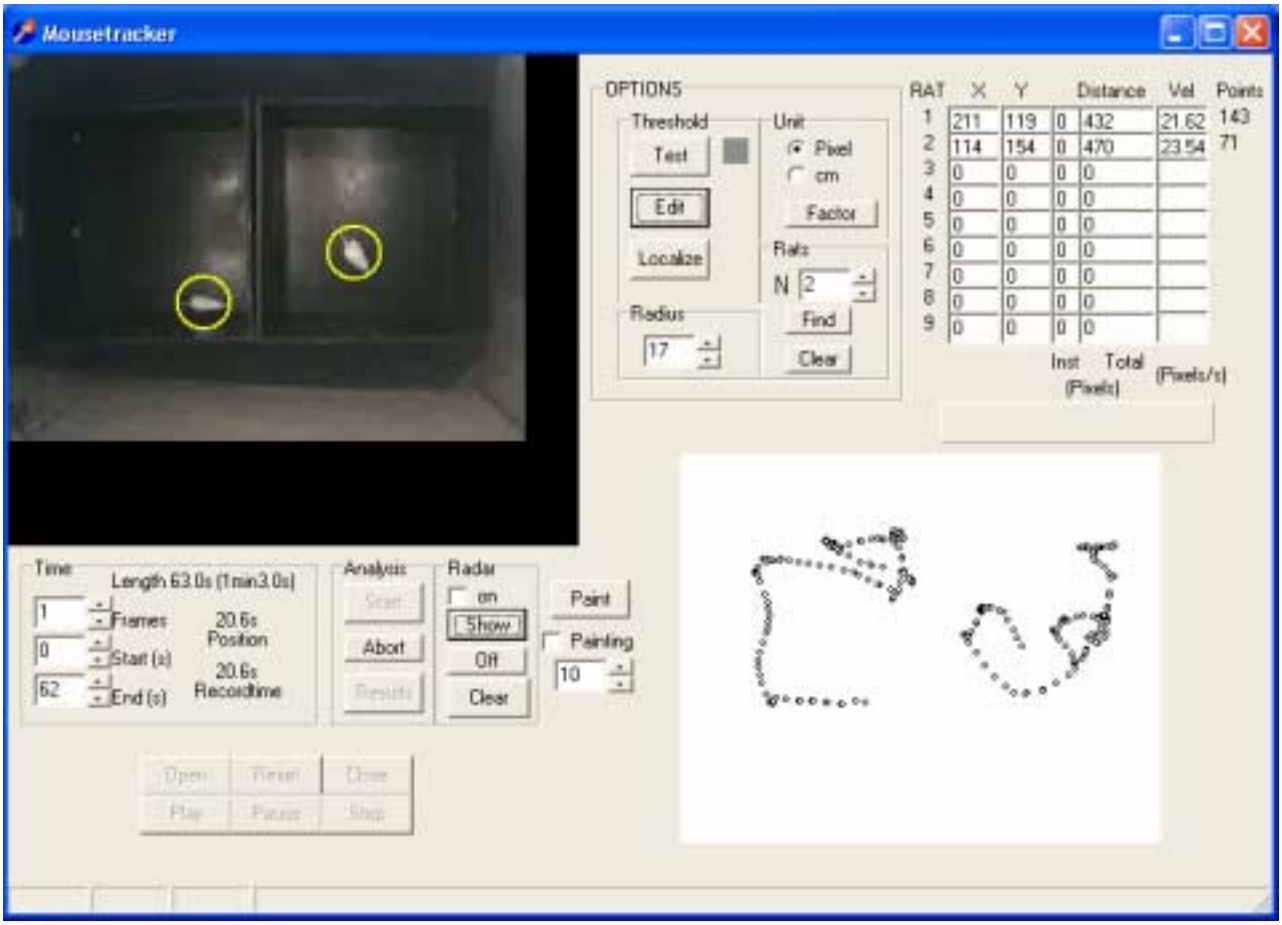
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Figure 2.

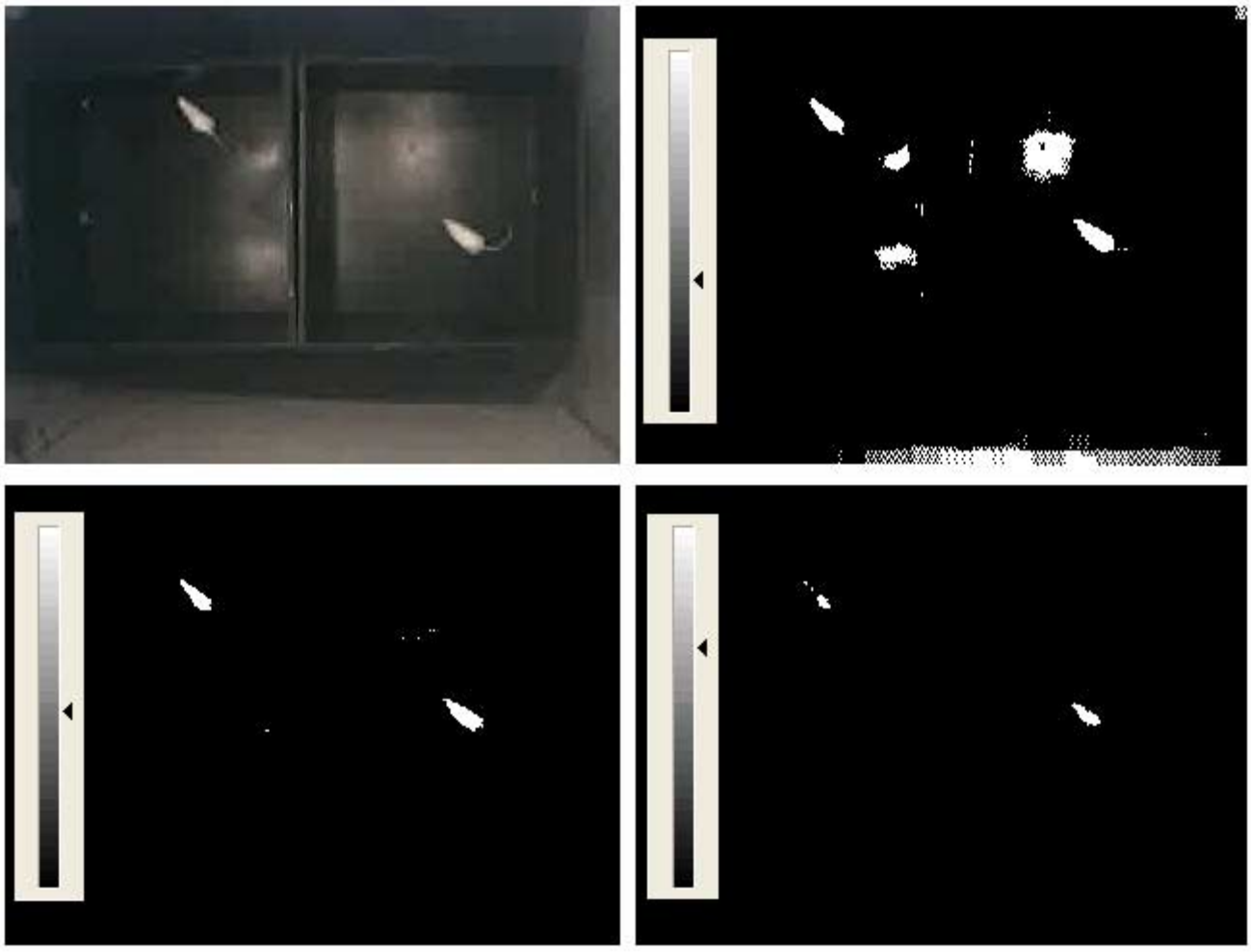

Figure 3

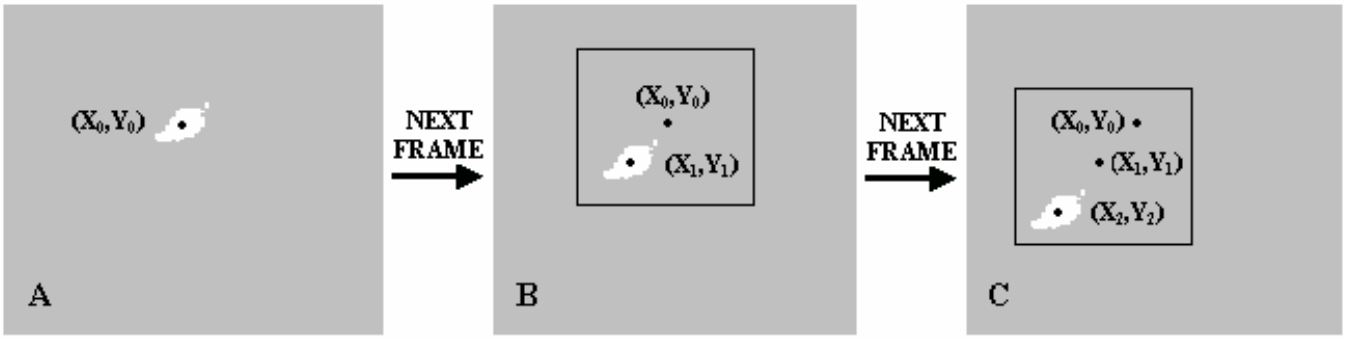

Figure 4

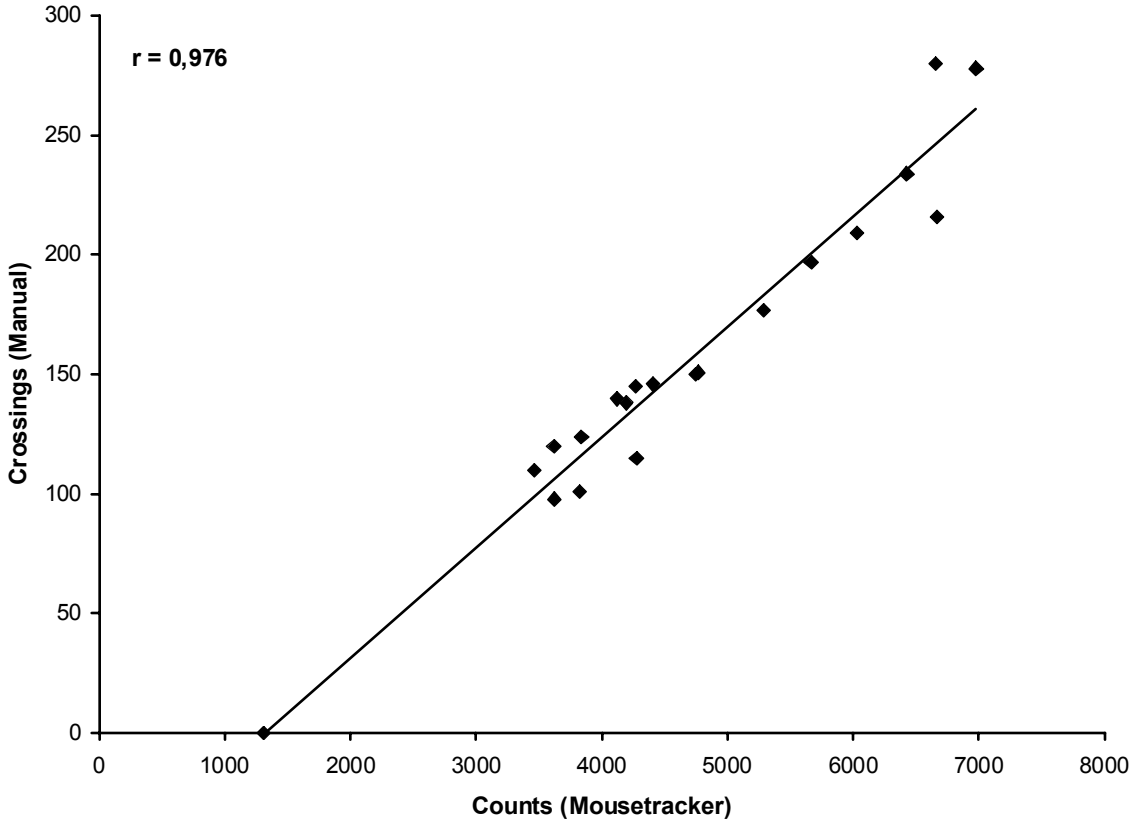
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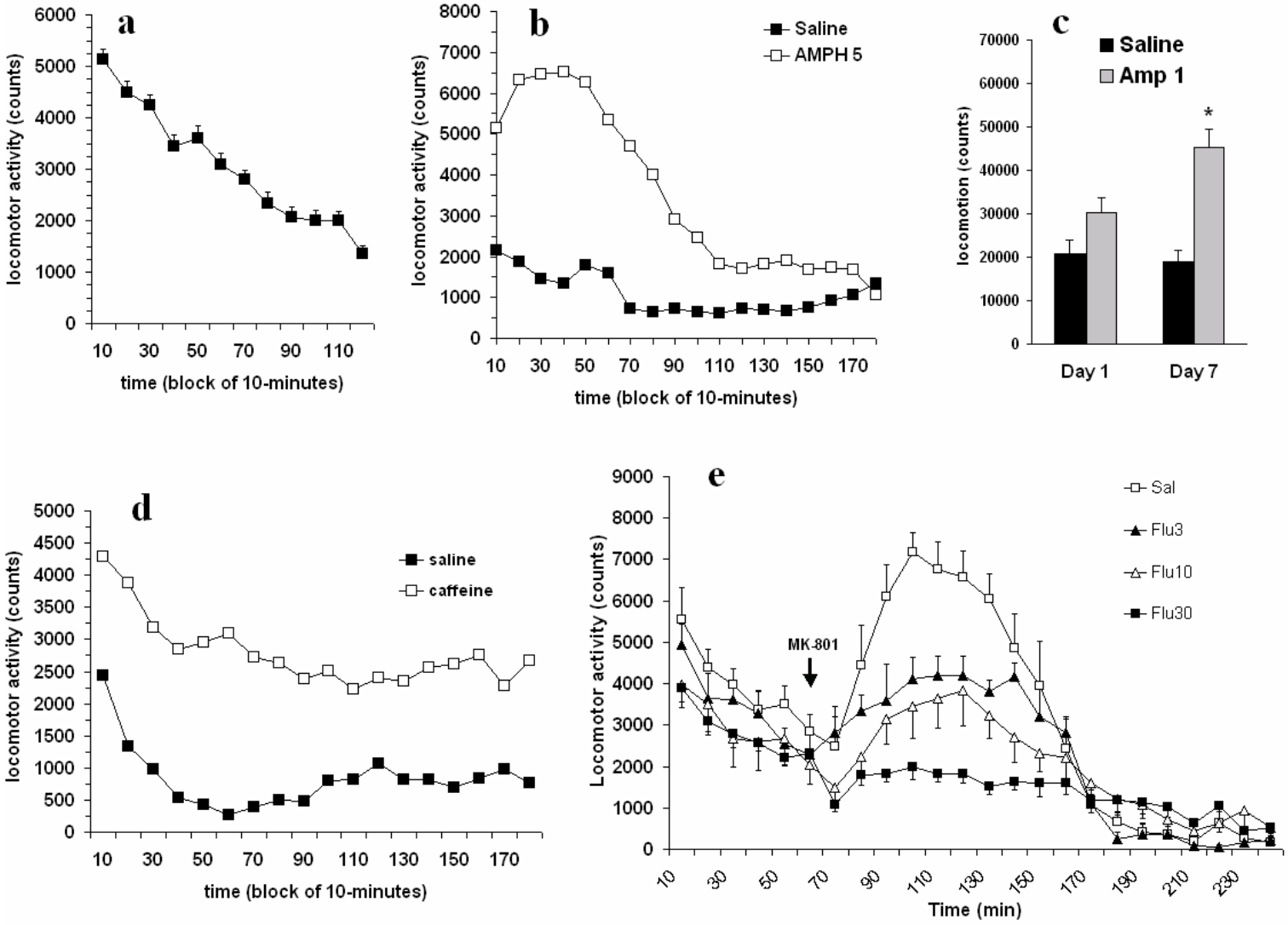
Figure 5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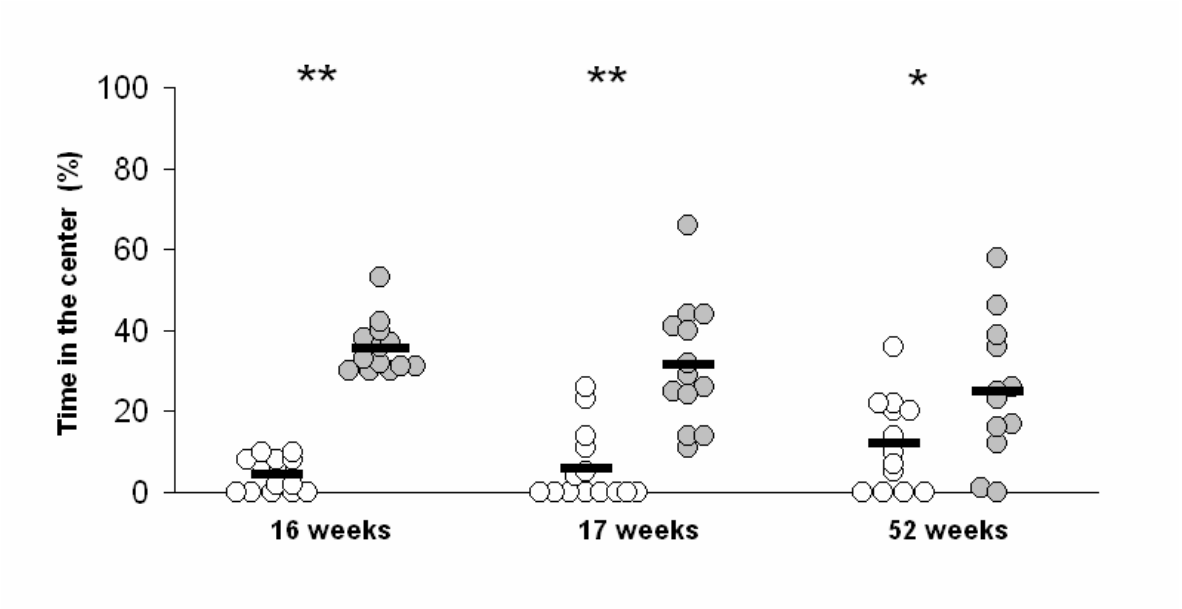
locomotor activity (counts)
time (block of 10-minutes)
b
Saline
AMPH 5
locomotor activity (counts)
time (block of 10-minutes)
c
Saline
Amp 1
*
locomotion (counts)
Day 1
Day 7
d
saline
caffeine
locomotor activity (counts)
time (block of 10-minutes)
e
Sal
Flu3
Flu10
Flu30
MK-801
Locomotor activity (counts)
Time (min)

Figure 6.



II.1.b GUANOSINE SELECTIVELY INHIBITS LOCOMOTOR STIMULATION INDUCED BY THE NMDA ANTAGONIST DIZOCILPINE.

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Research report

Guanosine selectively inhibits locomotor stimulation induced by the NMDA antagonist dizocilpine

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Abstract

Guanosine has been shown to modulate glutamate system by stimulating astrocytic glutamate uptake. Recent evidence suggest that the locomotor effects of NMDA receptor antagonists, an animal model of schizophrenia, is associated with activation of non-NMDA glutamatergic receptors caused by increased glutamate release. The present work was undertaken to evaluate whether guanosine could have influence on the hyperlocomotion induced in mice by dizocilpine (MK-801), a NMDA antagonist. We also evaluated the effect of guanosine on the hyperlocomotion induced by the indirect dopamine agonist amphetamine, and by the non-selective adenosine receptor antagonist caffeine. Guanosine (7.5 mg/kg) produced an attenuation of about 60% on the hyperlocomotion induced by dizocilpine (0.25 mg/kg), whereas it did not affect the hyperlocomotion induced by amphetamine (5 mg/kg) or caffeine (30 mg/kg). Guanosine pre-treatment did not affect total spontaneous locomotion in all experiments. To test neuronal pathway selectivity, we evaluated MK-801 against guanosine in a working memory paradigm (spontaneous alternation task). Guanosine did not reverted the impairment caused by MK-801 in the spontaneous alternation test, and when administered alone also presented an amnesic effect. The results are discussed based on the current hypothesis of locomotor activation induced by the psychoactive drugs studied. Further studies are necessary to evaluate if guanosine could have clinical utility for the treatment of schizophrenia.

Keywords: Guanosine; Amphetamine; Caffeine; MK-801; Locomotion; Schizophrenia

1. Introduction

Extracellular guanine-based purines (GBPs), namely the nucleotides GTP, GDP, and GMP and the nucleoside guanosine, have been shown to exert effects not directly related to the modulation of G-proteins. GBPs (including GMP and guanosine) have been studied in several in vivo and in vitro approaches, producing inhibition of binding of glutamate and analogs [6,23,24], neuroprotective effects to excitotoxic conditions [11,19], anticonvulsant action against seizures induced by glutamatergic agents [17,27,33], as well as an amnesic effect [26,33]. In line with these antiglutmatergic effects, we have recently shown that guanosine stimulates astrocytic glutamate uptake [10,12], which is the main mechanism of glutamate removal from the synaptic cleft [5,9].

In the last years, locomotor stimulation induced in rodents by psychoactive drugs has been used as a model with predictive validity for identification of novel antipsychotics. Among them, glutamate NMDA receptor antagonists, such as phencyclidine (PCP) and dizocilpine, have been regarded as the best pharmacological model for schizophrenia [1,2]. Recent evidence suggest that NMDA receptor antagonism is also associated with glutamatergic activation in non-NMDA receptors induced by increased glutamate release, which appears to be closely related to the behavioral alterations observed [2,3,20,21,31].

Based on such glutamatergic dependence of NMDA antagonists action, the present work was undertaken to evaluate whether guanosine, by its antiglutamatergic properties, could have influence on the locomotor stimulation induced by dizocilpine.

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Since Loeffler et al. [18] have shown that guanosine decreases dopamine synthesis in cultured rat pheochromocytoma PC12 cells, we also evaluated the effect of guanosine on the hyperlocomotion induced by the indirect dopamine agonist amphetamine (Amph), a classical model of psychosis in rodents. Finally, as some in vitro effects of guanosine seemed to be mediated by adenosine release [25], we also investigated the role of adenosine in the mechanism of action of guanosine by studying the hyperlocomotion induced by the non-selective adenosine receptor antagonist caffeine.

2. Material and methods

2.1. Animals

Experiments were performed with male adult albino mice (CF1) purchased from Fundação Estadual de Pesquisa em Saúde (FEPS) and maintained in our own animal facilities under controlled environment (23 ± 2 °C, 12-h light:12-h dark cycle, free access to standard food and water) until 3–4 months old (35–45 g). All behavioral experiments were performed between 10:00 and 14:00 h, in accordance with the Guidelines for Animal Care of our university. Different groups of animals were used in the distinct experiments.

2.2. Locomotor activity assessment

To assess locomotor activity, mice were randomly allocated to individual triangular boxes (50 cm × 30 cm × 30 cm, 50 cm high) with rounded corners, placed on the floor of a soundproof and diffusely illuminated room. Locomotor activities of eight mice were recorded simultaneously by a video-computerized system, with image analysis at four frames per second. The software (programmed by ABL Tort) tracked the animals by distinguishing their white color from the black background of the floor, registering *X* and *Y* horizontal coordinates. The method was set to examine horizontal locomotor activity, ignoring small movements, such as breathing, head and tail actions, and tremors. In all experiments, animals had not been previously habituated to the boxes. The data on locomotor activity is divided in 10 min blocks and presented as a function of time.

2.3. Experimental design

2.3.1. Dizocilpine experiment

Mice were treated with i.p. injection of guanosine at three different doses (0.75, 2.5, and 7.5 mg/kg) or saline and immediately had their locomotor activity recorded for 30 min, followed by i.p. injection of dizocilpine (0.25 mg/kg) and further recording for 3 h. Two control groups consisted of i.p. injection at time 0 of guanosine (7.5 mg/kg) or saline followed by a saline i.p. injection after 30 min.

2.3.2. Amphetamine experiment

Mice were treated with i.p. injection of guanosine at 7.5 mg/kg or saline and immediately had their locomotor activity recorded for 30 min, followed by i.p. injection of amphetamine (5 mg/kg) and further recording for 3 h. Two control groups consisted of i.p. injection at time 0 of guanosine (7.5 mg/kg) or saline followed by a saline i.p. injection after 30 min.

2.3.3. Caffeine experiment

Mice had their spontaneous locomotor activity recorded for 30 min; afterwards they were treated with i.p. injection of guanosine at 7.5 mg/kg or saline and had their locomotor activity recorded for more 30 min, followed by i.p. injection of caffeine (30 mg/kg) and further recording for 2 h. Two control groups consisted of i.p. injection at time 30 min of guanosine (7.5 mg/kg) or saline followed by a saline i.p. injection after 30 min.

2.4. Spontaneous alternation

Spontaneous alternation performance was assessed in the Y-maze. Each arm was 30 cm long, 20 cm high and 6 cm wide, and converged to an equal angle. Each mouse was placed at the end of one arm and allowed to freely move through the maze during 5 min. The series of arm entries was recorded visually. An alternation was defined as entries in all three arms on consecutive occasions. The percentage of alternation was calculated as (total of alternation/total arm entries − 2). Treatments were administered 30 min prior to test, and four groups of mice were studied: saline, guanosine (7.5 mg/kg), dizocilpine (0.25 mg/kg), and guanosine (7.5 mg/kg) + dizocilpine (0.25 mg/kg).

2.5. Drugs

Dizocilpine, amphetamine, guanosine, and caffeine were purchased from Sigma (St. Louis, MO, USA) and were dissolved in distilled water for acute administrations. For all injections, a volume of 10 ml/kg was administered.

2.6. Statistical analysis

The total locomotor activity in each experiment was quantified by calculating the area under the curve (of the function of locomotor activity versus time) obtained after the injection of different treatments. Comparisons of total locomotor activities and of spatial alternation scores among groups were performed with one-way ANOVA, followed by Duncan's post-hoc to determine differences among specific groups. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

Guanosine treatment did not affect total locomotor activity of mice during the habituation period of 30 min after the first injection compared to the saline group (Figs. 1–3). Guanosine at the doses of 0.75 and 2.5 mg/kg did not interfere with the hyperlocomotion induced by dizocilpine (data not shown). However, at the dose of 7.5 mg/kg, guanosine produced a statistically significant attenuation of about 60% (in relation to baseline activity) on the locomotor stimulation induced by dizocilpine (Fig. 1).

Guanosine at 7.5 mg/kg failed to affect the hyperlocomotion induced by amphetamine and caffeine, as shown in Figs. 2 and 3, respectively.

Guanosine at 7.5 mg/kg did not reverted the impairment caused by dizocilpine in the spontaneous alternation task, and, when administered alone, also caused an impairment in the task (Fig. 4).

4. Discussion

The present study demonstrated a selective effect of guanosine in counteracting the locomotor stimulatory effect of the NMDA receptor antagonist dizocilpine without affecting spontaneous locomotor activity, whereas it presented no effect on the locomotor activation induced by the indirect dopamine agonist amphetamine and by the adenosine receptor antagonist caffeine.

In the last years, the antiglutamatergic effects of the GBPs have been intensively studied [3,10–12,17,19,23,24,26,27,33]. We have shown that systemic administration of guanosine and GMP prevent seizures induced by compounds that overstimulate the glutamatergic system (quinolic acid, alpha-dendrotoxin), but not by the GABAergic antagonist picrotoxin [17,27,33]. We also reported that GMP is neuroprotective against intrastriatal quinolinic acid lesion [19], and, in vitro, guanosine protected brain slices exposed to

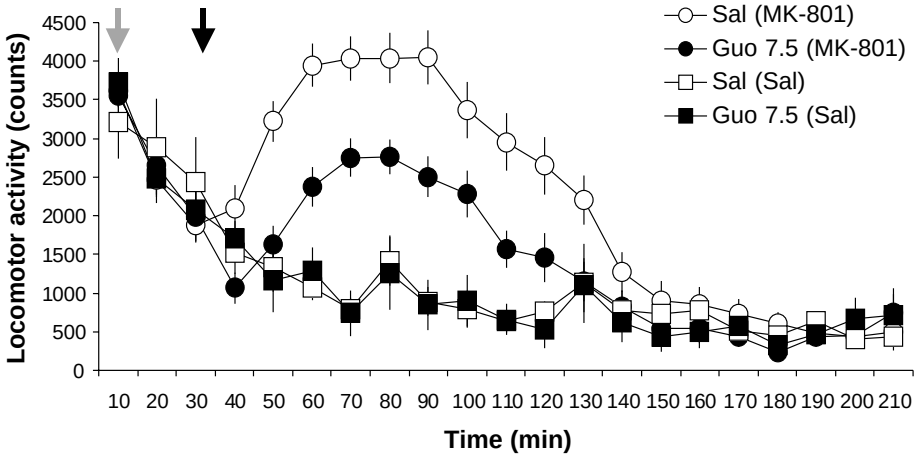

Fig. 1. Locomotor stimulatory effect induced by dizocilpine (MK-801) was inhibited by guanosine pre-treatment ($P < 0.05$). Grey arrow denotes first injection (guanosine 7.5 mg/kg (black symbols) or saline (white symbols)), and black arrow denotes second injection (MK-801 0.25 mg/kg (circle symbols) or saline (square symbols)). $N = 10$ in MK-801 treated groups, and $N = 4$ in control groups. Error bars represent standard error of the mean.

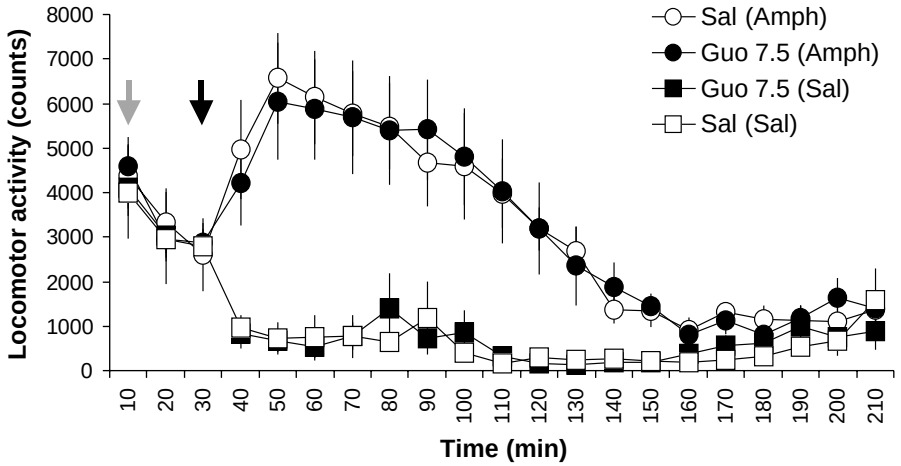

Fig. 2. Locomotor stimulatory effect induced by amphetamine was not affected by guanosine pre-treatment. Grey arrow denotes first injection (guanosine 7.5 mg/kg (black symbols) or saline (white symbols)), and black arrow denotes second injection (amphetamine 5 mg/kg (circle symbols) or saline (square symbols)). $N = 6$ in amphetamine treated groups, and $N = 4$ in control groups. Error bars represent standard error of the mean.

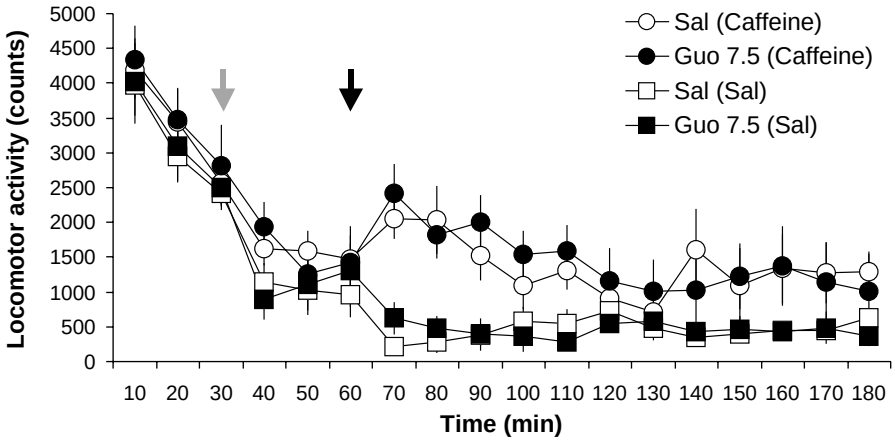

Fig. 3. Locomotor stimulatory effect induced by caffeine was not affected by guanosine pre-treatment. Grey arrow denotes first injection (guanosine 7.5 mg/kg (black symbols) or saline (white symbols)), and black arrow denotes second injection (caffeine 30 mg/kg (circle symbols) or saline (square symbols)). $N = 6$ in caffeine treated groups, and $N = 4$ in control groups. Error bars represent standard error of the mean.

hypoxia/hypoglycemia [29]. Guanosine also impaired inhibitory avoidance performance in rats [26,33], a model that also reveals amnesic effect of classical glutamatergic antagonists [14]. Regarding the mechanism of action of guanosine, a direct antagonistic action on glutamatergic receptors is unlikely, since guanosine is a poor displacer of glutamate ligands [28]. However, we showed that this antiglutamatergic effect could be mediated by astrocytes, as guanosine potently enhanced glutamate uptake in rat astrocytic cultures in a concentration-dependent manner [10,11]. More recently, we showed that the astrocytic glutamate uptake induced by guanine nucleotides depends on their conversion to guanosine [12]. Of note, astrocytic glutamate removal is know to play a major role in maintaining extracellular glutamate concentrations below neurotoxic levels [5,9].

The present results can be explained based on the different neurochemical mechanisms involved in the hyperlocomotion induced by each psychoactive drug studied. Moghaddam and coworkers have characterized the neurochemical and behavioral effects of NMDA antagonists [1–3,20,21,30,31], demonstrating that these compounds promote an increase in the efflux of both glutamate and dopamine in prefrontal cortex (PFC) and nucleus accumbens (NAc) [2,20,21,31], whereas they have only minor effects on striatal dopamine levels [4]. Thus, despite of reducing glutamate neurotransmission at NMDA receptors, dizocilpine promotes an increased stimulation of non-NMDA receptor [2,20,21,31], which may be due to disinhibition of GABAergic or other inhibitory inputs to glutamatergic neurons [31]. This non-NMDA receptor activation could then lead to the subsequent observed increase in dopamine extracelullar level, once it has been shown that AMPA and kainate glutamatergic receptors agonists could promote an increase in dopamine efflux in PFC [15], whereas the AMPA/kainate receptor antagonist LY293558 diminish dopamine levels in PFC [30]. In this context, non-NMDA receptor antagonists as well as inhibitors of glutamate release have been shown to counteract the behavioral and neurochemical effects of NMDA antagonist compounds [7,8,13]. Of functional anatomic importance, besides NMDA antagonists lead to subsequent increase in dopamine efflux in both PFC and NAc (probably via non-NMDA receptors activation), in recent works it was demonstrated that the locomotor activity induced by the NMDA antagonist PCP is closely related to

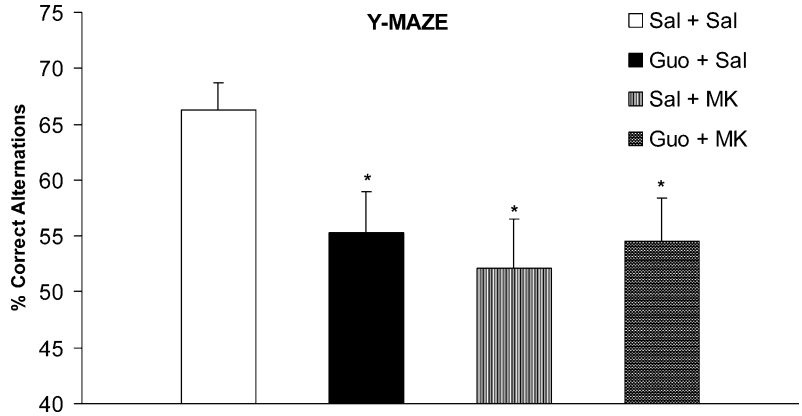

Fig. 4. Spontaneous alternation in the Y-maze. MK-801 (0.25 mg/kg) and guanosine (7.5 mg/kg) caused a significant impairment on the task when each drug was administered alone or combined. $N = 10$ in each group. Error bars represent standard error of the mean.

increased dopaminergic activity in the PFC but not in the NAc [16,21].

Altogether, we can thus hypothesize that the attenuation effect of the hyperlocomotion induced by dizocilpine observed in the present work could be due to an increase of glutamate uptake by astrocytes promoted by guanosine, reducing the neurotransmitter levels at the synaptic cleft, leading to less activation of non-NMDA receptors, with subsequent less increase in the efflux of dopamine in PFC. Guanosine did not affect the hyperlocomotion induced by amphetamine most probably due to the fact that the step of locomotor activation induced by dopamine is posterior in the neuronal circuitry to the action of glutamate in non-NMDA receptors, and hence is not interfered by antiglutamatergic compounds as guanosine. In agreement, as is known, selective D2 receptors blockers counteract the hyperlocomotion induced by NMDA antagonists, although usually at doses that also inhibit spontaneous locomotor activity [22].

We performed a paradigm of working memory with the same drug dosages studied in locomotion experiments to investigate the selectivity of the dizocilpine counter-regulatory effect of guanosine to motor activation pathways. However, in this behavioral task both guanosine and dizocilpine were amnesic when administered alone, which is in line with previous results showing an amnesic effect of both compounds in inhibitory avoidance task [26]. This effect may be related to an inhibition by guanosine of a physiological role of glutamate in learning and memory. Moreover, this cognitive impairment by guanosine may be a drawback in terms of developing new pharmacological treatments increasing guanosine activity, unless distinct receptor types for guanosine, not yet described, mediate the effect on locomotion and cognition.

In a previous work, Loeffler et al. [18] observed that guanosine at high concentration decreases dopamine synthesis in cultured rat pheochromocytoma PC12 cells. However, these results in vitro were not related to our results in vivo, since guanosine presented no effect in the hyperlocomotion induced by amphetamine. Similarly, an in vivo role of adenosine on the effect of guanosine is unlikely, since it failed to inhibit caffeine-induced hyperlocomotion.

Finally, despite of no effect on amphetamine induced hyperlocomotion, the present result point to a potential antipsychotic property of guanosine, once it was shown that NMDA antagonists model of schizophrenia could evaluate compounds that target psychotic symptoms that are not generally treated with typical antipsychotics [3]. Moreover, the neuroprotective and neurotrophic effects of guanosine may also be advantageous for the treatment of schizophrenia, which is associated with inadequate neurodevelopment and increased brain loss after onset of the disorder [32].

5. Conclusions

In conclusion, the present study shows that guanosine selectively counteracts the locomotor activation induced by the NMDA antagonist dizocilpine. This result is in agreement with the known antiglutamatergic effects of guanosine together with the nowadays accepted theory of motor activation induced by NMDA antagonists. Further studies could evaluate if glutamate and dopamine levels in the PFC are indeed inhibited by guanosine administration as well as explore its potential clinical utility for the treatment of schizophrenia.

Acknowledgements

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II.1.c ATYPICAL ANTIPSYCHOTIC PROFILE OF FLUNARIZINE IN ANIMAL MODELS.

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ORIGINAL INVESTIGATION

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Atypical antipsychotic profile of flunarizine in animal models

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Abstract *Rationale:* Flunarizine is known as a calcium channel blocker commonly used in many countries to treat migraine and vertigo. Parkinsonism has been described as one of its side-effects in the elderly, which is in agreement with its recently characterized moderate D_2 receptor antagonism. *Objectives:* To perform a pre-clinical evaluation of flunarizine as a potential antipsychotic. *Methods:* We evaluated the action of orally administered flunarizine in mice against hyperlocomotion induced by amphetamine and dizocilpine (MK-801) as pharmacological models of schizophrenia, induction of catalepsy as a measure for extrapyramidal symptoms and impairment induced by dizocilpine on the delayed alternation task for working memory. *Results:* Flunarizine robustly inhibited hyperlocomotion induced by both amphetamine and dizocilpine at doses that do not reduce spontaneous locomotion (3–30 mg/kg). Mild catalepsy was observed at 30 mg/kg, being more pronounced at 50 mg/kg and 100 mg/kg. Flunarizine (30 mg/kg) improved dizocilpine-induced impairment on the delayed alternation test. *Conclusions:* These results suggest a profile comparable to atypical antipsychotics. The low cost, good tolerability and long half-life (over 2 weeks) of flunarizine are possible advantages for its use as an atypical antipsychotic. These results warrant clinical trials with flunarizine for the treatment of schizophrenia.

Keywords Flunarizine · Amphetamine · Dizocilpine · Locomotion · Antipsychotic · Schizophrenia

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Introduction

Atypical antipsychotics were an important advance in the treatment of schizophrenia and other psychotic disorders (Meltzer et al. 2002). Their main advantages include better tolerability, especially regarding extrapyramidal symptoms, efficacy in a wider range of symptoms (Volavka et al. 2002) and increase in quality of life (Karow and Naber 2002). However, there are concerns about metabolic and cardiovascular side-effects that may affect morbidity and mortality of patients (Meltzer et al. 2002), added to the high cost of treatment, making them inaccessible for many patients, particularly in developing countries. Furthermore, except for injectable depot risperidone, atypical antipsychotics are not yet available in long-acting formulations, which facilitate adhesion to treatment.

Flunarizine, a piperazine derivative with chemical structure similar to the neuroleptic trifluoperazine, is a non-selective T-type, N-type and L-type calcium channel blocker, which has long been used in some countries for the treatment of migraine, vertigo and cerebrovascular disorders (Todd and Benfield 1989; Leone et al. 1991; Schmidt and Oestreich 1991). Flunarizine is usually well tolerated, but clinical reports showed aggravation and inducement of extrapyramidal motor signs secondary to chronic treatment with flunarizine, mainly in elderly patients (Chouza et al. 1986; Brücke et al. 1995). Accordingly, animal studies suggested that this side-effect could be due to moderate striatal D_2 receptor antagonism (Pani et al. 1990; Ambrosio and Stefanini 1991; Kariya et al. 1995; Haraguchi et al. 1998), which was in the low to moderate micromolar range. In humans, this was confirmed by Brücke et al. (1995), who found around 50% of D_2 receptor blockade in a SPECT study in patients chronically treated with flunarizine. Thus, based on its in vivo D_2 receptor occupancy and in vitro affinity, flunarizine can be categorized as a dopamine D_2 receptor antagonist of moderate affinity, in the range between olanzapine and clozapine (Seeman et al. 1997), therefore sharing the main mechanism of atypical antipsychotics, according to Seeman's proposal (Seeman and Tallerico

1998; Kapur and Seeman 2001). Importantly, extrapyramidal symptoms typically appear after at least 6 months of treatment with flunarizine, which can be explained by its long-half life (around 15–20 days) (Kariya et al. 1995), leading to its accumulation due to daily administration up to the point when dopaminergic activity is excessively inhibited. Also, all patients who experienced extrapyramidal symptoms in the literature were older than 55 years, when the physiological dopaminergic tone is decreased (Brücke et al. 1995).

Despite these findings, flunarizine has not been proposed for the treatment of psychotic disorders or adequately tested in pre-clinical studies aiming at its putative antipsychotic actions. However, flunarizine, among other calcium channel blockers, has already been used as a pharmacological tool to study the role of calcium channels in the effects of amphetamine and NMDA receptor antagonists, which are pharmacological models with predictive validity for antipsychotics in pre-clinical studies. It was observed that flunarizine produced a significant inhibitory effect against behaviors induced by the indirect dopaminergic agonist amphetamine in rodents and monkeys (Grebb 1986; Rosenzweig-Lipson and Barrett 1995; Hori et al. 1998) and a borderline inhibitory effect against the NMDA receptor antagonist PCP (Grebb 1986; Hori et al. 1998). Of note, flunarizine also prevented, whereas haloperidol potentiated, the EEG effects of PCP (Popoli et al. 1992; Feinberg and Campbell 1998). Importantly, in all these studies flunarizine has been administered up to 30 min before testing, not taking into account the 2–4 h period to reach peak serum levels (Kariya et al. 1995).

In this study we investigated the profile of flunarizine as an atypical antipsychotic. To this end, we evaluated the effect of orally administered flunarizine on hyperactivity induced by systemic administration of the NMDA receptor antagonist dizocilpine (MK-801) and the indirect dopamine agonist amphetamine as pharmacological models of schizophrenia. The motor side-effects of flunarizine were also evaluated by testing the potency to reduce spontaneous locomotor activity and to induce catalepsy. Finally, cognitive impairment induced by dizocilpine on the delayed alternation task was used as a measure of working memory.

Materials and methods

Animals

Experiments were performed with male adult albino mice (CF1) purchased from Fundação Estadual de Pesquisa em Saúde (FEPS) when 21 days old and maintained in our own animal facilities under controlled environment (23±2°C, 12 h light/dark cycle, lights on at 7:00 a.m. with free access to standard food and water) up to 3–4 months old (35–45 g). All behavioral experiments were in accordance with the Guidelines for Animal Care of our university. Different groups of animals were used in the distinct experiments.

Locomotor activity experiments

Mice were orally treated at 8:00 a.m. with vehicle or flunarizine at different doses (1.0, 3.0, 10.0, 30.0 mg/kg). Three hours later, spontaneous locomotor activity was recorded for 1 h, followed by IP injection with dizocilpine (0.25 mg/kg) or amphetamine (5 mg/kg) and further recording for 3 h. A control group with oral vehicle (water) and IP saline was also included. For all injections (oral and IP), a volume of 10 ml/kg was administered.

To assess locomotor activity, mice were randomly allocated to individual triangular boxes (50 cm×30 cm×30 cm, 50 cm high) with rounded corners, placed on the floor of a soundproof and diffusely illuminated room. Locomotor activity of eight mice was recorded simultaneously by a video-computerized system, with image analysis at four frames per second. The software (programmed by ABL Tort) tracked the animals by distinguishing their white color from the black background of the floor, registering X and Y horizontal coordinates. The method was set to examine horizontal locomotor activity, ignoring small movements, such as breathing, head and tail actions, and tremors. Animals had not been previously habituated to the boxes and were observed for a total of 4 h (1 h habituation, and 3 h after IP injection), with data divided into 10 min blocks.

Catalepsy experiment

Mice were orally treated with flunarizine at different doses (3.0, 10.0, 30.0, 50.0 and 100.0 mg/kg) or vehicle, and had their catalepsy time determined 3 h and 6 h later. Mice treated with haloperidol 1 mg/kg PO were used as positive controls. Catalepsy time was measured after mice forepaws were placed over a horizontal glass bar (0.6 cm diameter), elevated 6 cm from the floor. The time mice maintained both forepaws over the bar and both hindpaws on the ground was recorded with a cut-off time of 180 s, allowing three immediate attempts to replace the animal in cataleptic position within the first 10 s. Mice that kept their paws over the bar, but showed active body or head movements were also not considered as cataleptic. The experimenter was blind to drug treatment.

Delayed alternation task

Delayed alternation performance was assessed in the T-maze task. The starting arm is 60 cm long, each side arm is 30 cm long, and both are 20 cm high and 10 cm wide, and the test was performed in a dimly illuminated room.

Mice were deprived from food until they achieved 80% of their initial weight. Then they were habituated in the T-maze for 4 days, receiving a food reward (Nescau cereal) at the end of the goal arms. In this habituation period, each mouse was placed in the start arm of the maze and permitted to explore it freely for 10 min, with the two open "goal" arms baited.

After these adaptation sessions, mice were trained as follows. In the first trial, food reward was presented in both goal arms. During the next 15 trials, the arm opposite to the one the animal had entered on the previous trial was baited with food reward, except when the animal had gone to the empty arm on the last trial. In this case, the food was left in the same place and the baited side was changed only after the animal had alternated. Sliding doors were used to keep the animal for a 10 s inter-trial interval in the starting arm, and to confine the mouse into the goal arm for 20 s, once it had entered in it. This training continued until the animal reached a criterion of at least 11 correct choices (score) in 15 trials on 3 consecutive days. A maximum of 10 blocks of 15 trials (10 days) was given to each mouse. Animals that failed to reach the criterion in these training sessions were discarded.

In the day after they matched the criterion, they received flunarizine (10 mg/kg or 30 mg/kg) or vehicle PO and after 3 h they were tested (15 trials). This first score was considered as predizocilpine. As soon as this session was over, they received

dizocilpine (0.4 mg/kg IP) and after 30 min they were retested. This second testing session was called post-dizocilpine.

Drugs

Dizocilpine maleate and *d*-amphetamine sulfate were purchased from Sigma (St Louis, Mo., USA) and were dissolved in fresh saline (0.9% NaCl) for acute administrations. Commercially available solutions for oral use of flunarizine (Flunarin, Asta Medica) and haloperidol (Haldol, Janssen) were used.

Statistical analysis

Comparisons of locomotor activities at different time points were analyzed using General Linear Model (GLM) repeated measure (drug treatment versus time) with time as the repeated measure. Duncan's post hoc was used to determine differences among specific groups. Catalepsy time and delayed alternation task performance were analyzed using the Kruskal–Wallis followed by the Mann–Whitney *U*-test due to cut-off time. A value of $P<0.05$ was considered statistically significant.

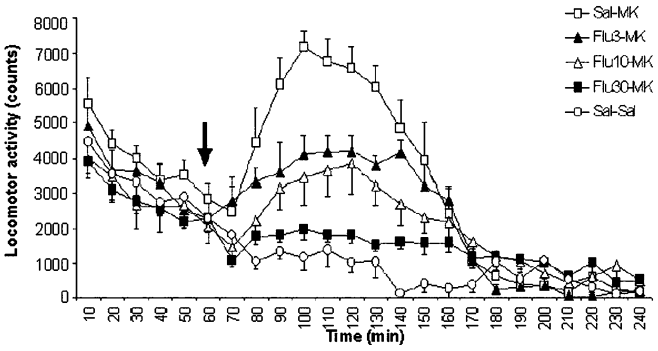

Fig. 2 Flunarizine inhibits hyperlocomotion induced by dizocilpine in mice. Flunarizine was orally administered to male adult albino mice 3 h before spontaneous locomotor recording in a computerized system. After 1-h habituation, mice were injected with 0.25 mg/kg dizocilpine or saline IP and locomotion was recorded for 3 h (n=6 per group). Results shown as mean±SEM. Statistics (two-way ANOVA with time as the repeated measure): no difference between groups at 0–60 min interval; saline > flu3=flu10 > flu30 at 70–240 min interval (P<0.05)

Results

Flunarizine dose-time-dependently inhibited amphetamine-induced hyperlocomotion [$F(85,612)=3.523$; $P<0.001$], with sal=1.0>3.0=10.0=30.0 mg/kg [between groups: $F(5,36)=7.205$; $P<0.001$] (Fig. 1). Against dizocilpine, flunarizine presented a dose-time-dependent inhibition of the hyperlocomotion induced by this NMDA receptor antagonist [$F(68,374)=7.779$; $P<0.001$], with sal >3.0=10.0>30.0 mg/kg [between groups: $F(4,22)=9.008$; $P<0.001$] (Fig. 2).

Regarding motor side-effects, considering the data of the 1 h habituation period in both trials, flunarizine 30 mg/kg presented, if anything, a mild inhibition of spontaneous locomotion (about 18% reduction), which was not statistically different from saline controls (P=0.08). Flunarizine caused catalepsy in a dose-dependent fashion, with no or minimal catalepsy up to 30.0 mg/kg (at 6 h: $Z=-2.747$; P=0.006), whereas the higher doses of 50.0 mg/kg and 100.0 mg/kg produced consistent catalepsy at both 3 h ($Z=-3.724$ for 50.0 mg/kg and −3.832 for 100.0 mg/kg; $P<0.001$) and 6 h ($Z=-3.592$ for 50.0 mg/kg and −3.622 for 100.0 mg/kg; $P<0.001$) after oral injections, but still less than 1 mg/kg haloperidol (at 3 h: $Z=-4.310$ and at 6 h $Z=-4.203$; $P<0.001$) [(Fig. 3)].

In the delayed alternation task, flunarizine 30 mg/kg attenuated the impairment provoked by dizocilpine ($Z=-1.983$; $P<0.05$), while the dose of 10 mg/kg did not achieve statistical significance ($Z=-1.512$; P=0.16) (Fig. 4).

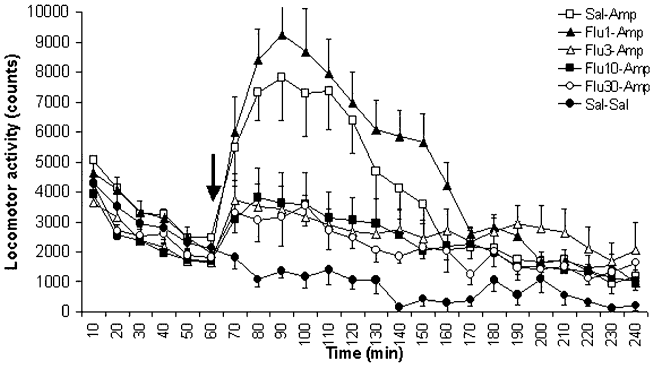

Fig. 1 Flunarizine inhibits hyperlocomotion induced by amphetamine in mice. Flunarizine was orally administered to male adult albino mice 3 h before spontaneous locomotor recording in a computerized system. After 1-h habituation, mice were injected with 5 mg/kg amphetamine or saline IP and locomotion was recorded for 3 h (n=6 per group). Results shown as mean±SEM. Statistics (two-way ANOVA with time as the repeated measure): no difference between groups at 0–60 min interval; sal=flu1 > flu3=flu10=flu30 at 70–240 min interval (P<0.05)

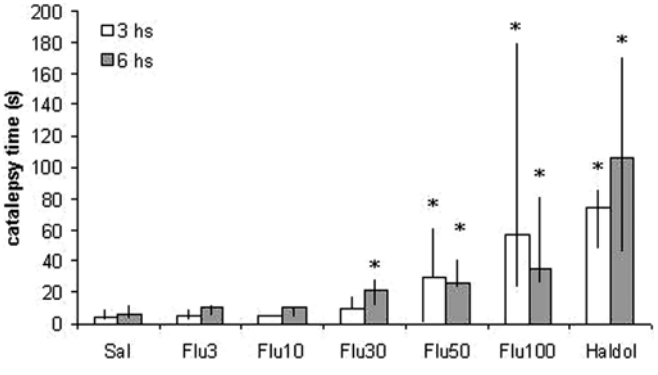

Fig. 3 Effect of flunarizine and haloperidol on catalepsy. Catalepsy time was determined 3 h and 6 h after treatment with vehicle, flunarizine or haloperidol PO. A cut-off time of 180 s was used. n=8 for all groups. Data presented as medians and interquartile range. * Denotes statistically significant (P<0.05) difference from control group

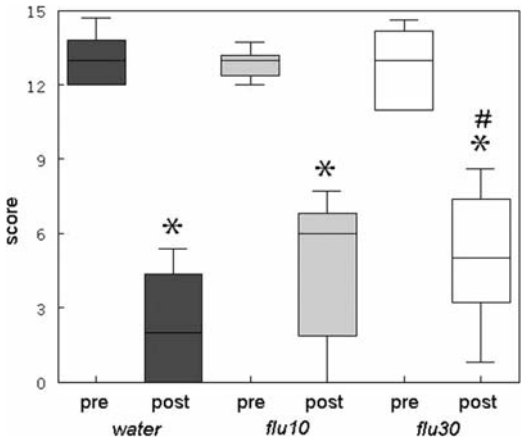

Fig. 4 Flunarizine attenuates dizocilpine induced impairment in the delayed alternation task. In mice previously trained to perform a delayed alternation task, flunarizine (10 mg/kg or 30 mg/kg) or vehicle PO was orally administered to male adult albino mice 3 h before testing 15 trials (*pre*). As soon as this session was over, they received dizocilpine (0.4 mg/kg IP) and after 30 min they were retested (*post*). n=8 for all groups, * denotes statistically significant (P<0.05) difference from its respective pre-test control, # denotes statistically significant (P<0.05) difference from saline post-test group

Discussion

The present work showed that flunarizine potently inhibited hyperlocomotion induced by amphetamine and dizocilpine, two models with predictive validity for antipsychotics, at doses that produced no hypolocomotion and cataleptic behavior, a characteristic suggestive of atypical antipsychotics (Ninan and Kulkarni 1999). Flunarizine also improved dizocilpine-induced impairment in the delayed alternation test at 30 mg/kg, a dose that caused only mild catalepsy. Such profile was observed with a 3-h pretreatment interval, which is more suitable to the pharmacokinetic profile of flunarizine, in contrast with previous studies, which typically administered flunarizine 15–30 min before the experiments (Grebb 1986; Sukhotina et al. 1999).

Among animal models to identify novel compounds with potential antipsychotic action, the indirect dopamine agonist amphetamine has been the most used pharmacological strategy for decades (Ellenbroek 1993). This model has gained further merit after direct evidence of increased dopaminergic activity in a high proportion of schizophrenic patients (for review, see Kapur 2003). Flunarizine potently inhibited amphetamine induced locomotion without a gradual dose response, since 1 mg/kg was ineffective and the doses of 3, 10 and 30 mg/kg were equally effective. Given the complex mechanism of action flunarizine, perhaps this effect may not be ascribed solely to its D_2 receptor antagonist properties. Inhibition of calcium and sodium channels by flunarizine (Holmes et al. 1984; Velly et al. 1987; Pauwels et al. 1991) can inhibit catecholamine release. Also, a possible increase in adenosine (Phillis et al. 1983; Popoli et al. 1990) by flunarizine treatment can also attenuate dopaminergic activity presynaptically by A1 receptors, which inhibit dopamine release, as well as post-synaptically by decreasing D_2 receptor affinity via A_{2a}–D_2 receptor interactions (Lara and Souza 2000). Nevertheless, these combined mechanisms seem not to excessively decrease dopaminergic activity based on its much lower potency to produce significant catalepsy and hypolocomotion, which is at least 1 order of magnitude distant from the effective doses against amphetamine and dizocilpine induced hyperlocomotion. A similar pattern has been observed for olanzapine (Ninan and Kulkarni 1999).

Age (especially >70 years old) was found to be a risk factor for developing extrapyramidal symptoms with flunarizine (Brücke et al. 1995), similarly to antipsychotics. This profile is probably due to the ontogenetic decay of dopaminergic tone (Brücke et al. 1995). To our knowledge, there is no report of extrapyramidal effect of flunarizine in patients younger than 55 years old. Long-term use (usually more than 6 months) was another risk factor, which is not unexpected considering flunarizine's long half-life (more than 2 weeks). This characteristic has been consistently overlooked in clinical practice, since it is normally prescribed at daily intakes. With such long half-life, rats treated daily with flunarizine presented an almost linear accumulation of the drug in plasma and striatum (Kariya et al. 1995), indicating that dose reduction or longer intervals between intakes should be considered to avoid motor side-effects. Apart from this side effect after long-term use, flunarizine is well tolerated even by the elderly.

Glutamate NMDA receptor antagonists, such as phencyclidine and dizocilpine, have also been used as a pharmacological model for schizophrenia, producing both hyperlocomotion and cognitive deficits in rodents (Ninan and Kulkarni 1999). Of note, typical antipsychotics typically inhibit hyperactivity induced by NMDA receptor antagonists at doses that inhibit spontaneous activity per se, contrary to atypical antipsychotics (O'Neill and Shaw 1999). Flunarizine produced a substantial dose-dependent effect in this model without significantly inhibit spontaneous locomotion. Flunarizine was also able to attenuate the cognitive impairment induced by dizocilpine in the delayed alternation test for working memory, which is thought to assess frontal lobe function (Le Marec et al. 2002). These results, therefore, further suggest an atypical profile of flunarizine, which may count with the contribution of other mechanisms of action, such as sodium channel blockade, which may also inhibit the effects of NMDA receptor antagonists (Farber et al. 2002).

Based on its pharmacological profile in clinical practice (regarding tolerability) and in these models, flunarizine has putative antipsychotic action without major motor side-effects, similarly to atypical antipsychotics. Moreover, after target symptoms have improved, flunarizine has the potential to be orally administered weekly or twice a month, which could considerably improve typically poor treatment compliance in psychotic patients (Perkins 2002). The long half-life also may prevent abrupt exacerbation of symptoms in the case of abandoning the treatment.

Conversely, if extrapyramidal side-effects occur, anticholinergic treatment would have to be initiated until plasma levels decrease significantly after dose adjustment. Another notable advantage of flunarizine is its very low cost, which is 10–40 times lower in comparison with atypical antipsychotics. It is also available in liquid formulation, which was used in this study. Taken together, these characteristics may increase treatment compliance with flunarizine in comparison with commercially available antipsychotics. Clinical trials with flunarizine for the treatment of schizophrenia and other psychotic disorders are therefore warranted to confirm its putative profile as a long-acting atypical antipsychotic.

Acknowledgements This work was supported by grants of CNPq and CAPES.

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II.1.d CINNARIZINE HAS AN ATYPICAL ANTIPSYCHOTIC PROFILE IN ANIMAL MODELS OF PSYCHOSIS.

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Cinnarizine has an atypical antipsychotic profile in animal models of psychosis

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Abstract

Cinnarizine, a drug known as a calcium channel blocker, is currently used for the treatment of migraine and vertigo. Induction of extrapyramidal signs by cinnarizine has been reported in the elderly, which is related to its moderate antagonistic properties at dopamine D2 receptors, resembling the mechanism of action of most antipsychotic drugs. Despite this effect, cinnarizine has never been tested as a putative antipsychotic drug. Here we evaluate the potential effect of cinnarizine in two pharmacological models of psychosis, namely amphetamine- and MK-801-induced hyperlocomotion, as well as its ability to induce catalepsy. Cinnarizine significantly counteracted MK-801 (0.25 mg/kg) and amphetamine (5 mg/kg) locomotor effects at doses as low as 20 mg/kg, having no incremental effect at 60 or 180 mg/kg. Regarding side-effects, cinnarizine induced no catalepsy in mice at the effective dose of 20 mg/kg, inducing only mild catalepsy at the doses of 60 and 180 mg/kg. Based on these results and on the antagonist effect of cinnarizine on dopamine D2 receptors, we suggest that it has a potential antipsychotic effect with an atypical profile that should be evaluated clinically.

Keywords

cinnarizine, MK-801, amphetamine, catalepsy, locomotion, schizophrenia, psychosis, mice

Introduction

Diphenylpiperazines compounds, such as cinnarizine and flunarizine, are usually known for their ability to inhibit calcium channels, especially of the T-type, and have been clinically used in some European and South American countries for the treatment of migraine (Rossi *et al.*, 2003) and vertigo (Pianese *et al.*, 2002). Cinnarizine is usually well tolerated by most patients, but case reports showed that its chronic use may exacerbate and even induce extrapyramidal symptoms, especially when administered to the elderly (Daniel and Mauro, 1995). This effect is explained by its low-to-moderate dopamine D2 receptor antagonist effect in the striatum, leading to impairment of nigrostriatal transmission (Brucke *et al.*, 1995). Dopamine D2 receptor antagonism is the main mechanism of action of antipsychotic drugs, which can be divided in two distinct groups: typical (or first generation) and atypical (or second generation) antipsychotics. Typical antipsychotics act mainly on positive symptoms of schizophrenia (hallucinations and delusions), induce more intense extrapyramidal symptoms, tend to induce higher prolactin secretion and act through a potent dopamine D2 receptor blockade (Seeman *et al.*, 1997). In contrast, atypical antipsychotics exert only moderate blockade of dopamine D2 receptor and interfere with other neurotransmitter systems, resulting in broader symptomatic relief (including negative and disorganized symptoms) associated with milder or absent extrapyramidal and prolactin related symptoms (Seeman and Tallerico, 1998; Kapur and Seeman, 2001).

Considering that the affinity of cinnarizine for blocking dopamine D2 receptor is similar to atypical antipsychotics, we hypothesized that cinnarizine would have such a profile in animal models, which could reinforce its putative therapeutic effects for the treatment of psychotic disorders and schizophrenia. In order to test this hypothesis we evaluated the effect of cinnarizine on MK-801 and amphetamine-induced hyperlocomotion, two pharmaco-

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logical models of psychosis and schizophrenia, and tested its ability to induce catalepsy in mice as a model for extrapyramidal side-effects.

Material and methods

Animals

Experiments were performed with male adult albino mice (CF1) purchased from Fundação Estadual de Pesquisa em Saúde (FEPPS) when 21 days old and maintained in our own animal facilities under a controlled environment ($23 \pm 2\,^{\circ}C$, 12 hr light/dark cycle, free access to standard food and water) up to 3–4 months old (35–45 g). All behavioural experiments were in accordance with the Guidelines for Animal Care of our university. Different groups of animals were used in the distinct experiments. All experiments were conducted between 10 AM and 6 PM.

Drugs

MK-801 maleate and d-amphetamine sulphate were purchased from Sigma (St Louis, MO) and dissolved in fresh saline (0.9% NaCl) for acute administrations. Commercially available solutions for oral use of cinnarzine (Stugeron® – Janssen Cilag) and haloperidol (Haldol® – Janssen Cilag) were used. For all injections (oral – by gavage – and i.p.), a volume of 10 mL/kg was administered.

Locomotor activity experiments

Mice were treated orally with water or cinnarizine solution at different doses (6, 20, 60, 180 mg/kg). One hour later, their spontaneous locomotor activity was recorded for 1 h, followed by i.p. injection of either MK-801 (0.25 mg/kg) or amphetamine (5 mg/kg) and further recording for 3 h. A control group with oral water and i.p. saline was also included.

To assess locomotor activity, mice were randomly allocated to individual triangular boxes (50 cm × 30 cm × 30 cm, 50 cm high) with rounded corners, placed on the floor of a soundproof and diffusely illuminated room. Locomotor activity of eight mice was recorded simultaneously by a video-computerized system, with image analysis at four frames per second. The software (programmed by Tort ABL) tracked the animals by distinguishing their white colour from the black background of the floor, registering X and Y horizontal coordinates. The method was set to examine horizontal locomotor activity, ignoring small movements, such as breathing, head and tail actions, and tremors. Animals had not been previously habituated to the boxes and were observed for a total of 4 h (1 h habituation and 3 h after i.p. injection), with data divided in 10 min blocks.

Catalepsy experiment

Mice were orally treated with cinnarizine at different doses (6, 20, 60 and 180 mg/kg) or water, and had their catalepsy time determined 1.5 h and 3 h later. Mice treated with haloperidol (1 mg/kg p.o.) were used as positive controls.

Catalepsy time was measured after mice forepaws were placed over a horizontal glass bar (0.6 cm diameter), elevated 6 cm from the floor. The time mice maintained both forepaws over the bar and both hindpaws on the ground was recorded with a cut-off time of 180 s, allowing three immediate attempts to replace the animal in cataleptic position within the first 10 s. Mice that did not move their paws, but showed active body or head movements were not considered as cataleptic. The experimenter was blind to drug treatment. A control group with oral water treatment was also included.

Statistical analysis

Locomotor activities at different time points and groups were analysed using two-way ANOVA (General Linear Model) with time as the repeated measure. Duncan's post hoc test was used to determine differences among specific groups. Catalepsy time of different groups were analysed using the Kruskal-Wallis followed by the Mann-Whitney *U*-test due to use of a cut-off time. A value of $p < 0.05$ was considered statistically significant.

Results

Cinnarizine did not consistently affect spontaneous locomotor behaviour up to 60 mg/kg, as observed during the habituation period (Fig. 1, Fig. 2). MK-801 (0.25 mg/kg) significantly increased mice locomotor activity during approximately 120 min (Fig. 1). Cinnarizine pre-administration at the doses of 20 and 60 mg/kg, but not 6 mg/kg, significantly counteracted MK-801-induced hyperlocomotion. This effect was time-dose dependent ($F(68,459) = 1.582$; $p < 0.01$). The dose of 180 mg/kg inhibited spontaneous locomotion by around 40% and was not included in the analysis (Fig. 1).

Acute amphetamine (5 mg/kg) treatment induced a significant increment in mice locomotor behaviour (Fig. 2). Cinnarizine pretreatment at the dose of 20 and 60 mg/kg, but not 6 mg/kg, produced a significant time-dose dependent ($F(68,187) = 2{,}087$; $p < 0.001$) attenuation of amphetamine effect (Fig. 2).

Cinnarizine 20, 60 and 180 mg/kg failed to induce cataleptic behaviour in mice 1.5 h after administration. However, the doses of 60 and 180 mg/kg produced significant catalepsy 3 hours after administration compared to saline, but significantly lower than haloperidol (Fig. 3). Haloperidol at the dose of 1 mg/kg induced significant catalepsy, near the ceiling point of 180 s at both time points (Fig. 3).

Discussion

The main finding of this study is that cinnarizine attenuated the psychostimulant effects of two pharmacological models of psychosis, MK-801 and amphetamine. This effect was present even at doses that did not elicit important extra-pyramidal effects in mice

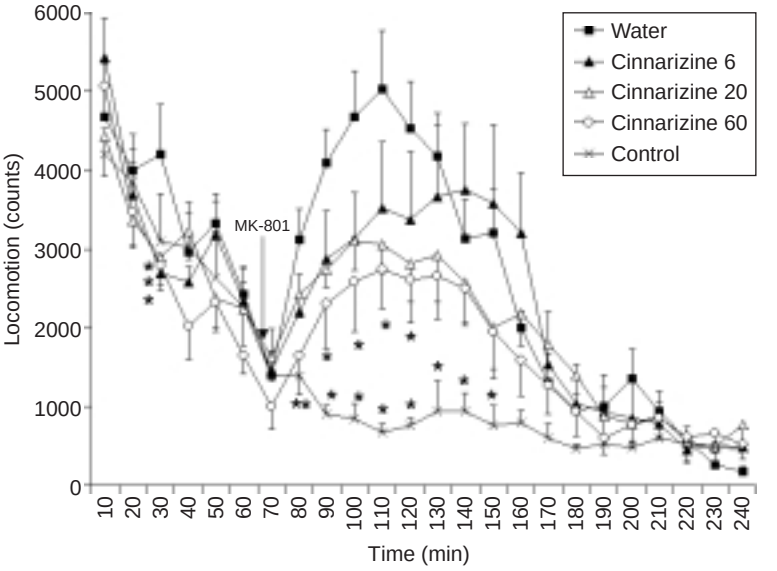

Figure 1 Effect of cinnarizine on MK-801-induced hyperlocomotion. Cinnarizine (6, 20 and 60 mg/kg) or water was administered 1 h before the experiment, and MK-801 (0.25 mg/kg) or saline (control group) was administered after a 1-h habituation period. *denotes significant ($p < 0.05$) difference when compared to water group, which was also injected with MK-801. Data presented as mean ± S.E.M. ($n = 8$–10 animals per group)

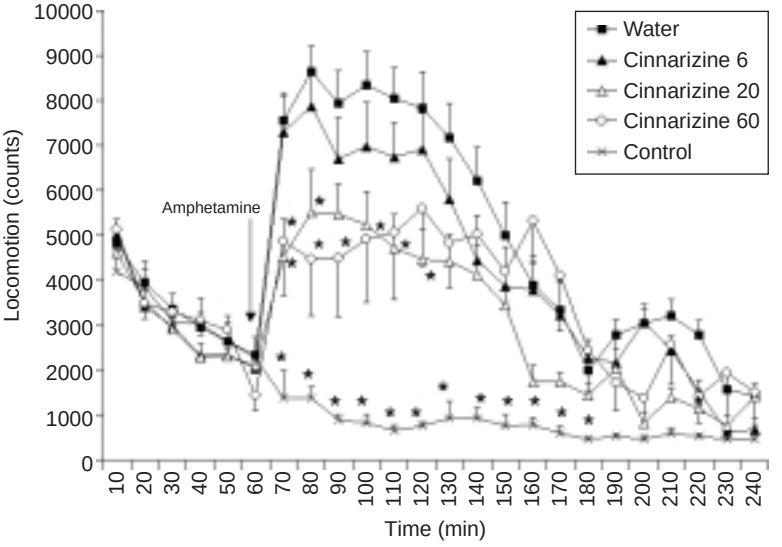

Figure 2 Effect of cinnarizine on amphetamine-induced hyperlocomotion. Cinnarizine (6, 20 and 60 mg/kg) or water was administered 1 h before the experiment, and amphetamine (5 mg/kg) or saline (control group) was administered after a 1-h habituation period. *denotes significant ($p < 0.05$) difference when compared to water group, which also received amphetamine. Data presented as mean ± S.E.M. ($n = 8$–10 animals per group)

and without effects on spontaneous locomotion. These results, allied with existing data, suggest that cinnarizine exerts a behaviourally significant dopamine D2 receptor blockade, similar to atypical antipsychotics in these experimental models (Geyer and Ellenbroek, 2003).

Cinnarizine is clinically used for the treatment of vertigo and migraine, and extra-pyramidal side-effects such as rigidity and tremors that occasionally occur in elderly patients (Daniel and Mauro, 1995). Cinnarizine was also shown to aggravate Parkinson's disease (Fernandez *et al.*, 1988) and even induce parkinsonism in primates (Garcia Ruiz *et al.*, 1992), suggesting a relevant anti-dopaminergic effect. This was demonstrated by Brucke *et al.* (1995), who showed around 40% striatal dopamine D2 receptor occupancy in patients using cinnarizine or flunarizine in a SPECT study, although extrapyramidal side-effects only occurred with higher occupancy rates. In comparison, occupancy level with atypical antipsychotics quetiapine (mean 550 mg/day), clozapine (mean 450 mg/day) and olanzapine (mean 18 mg/y) were 20, 33 and 74%, respectively, with the same radioligand (Tauscher *et al.*, 2002). *In vitro*, using the same radioligand, the affinity (*Ki*) of cinnarizine for dopamine D2 receptor was 13.2 nM, while haloperidol *Ki* is 0.125 or ~100-fold lower (Kariya *et al.*, 1995). In the binding assays by Seeman *et al.* (1997), *Ki* for haloperidol (0.35 nM) is ten-fold lower than that for olanzapine (3.7 nM), but

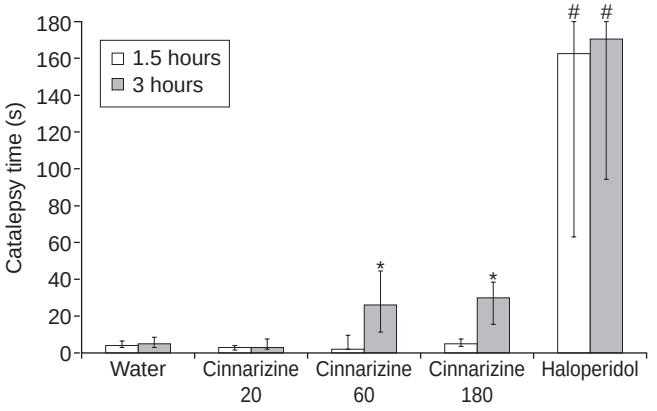

Figure 3 Effect of cinnarizine on catalepsy. Catalepsy time was determined 1.5 h and 3 h after treatment with water, cinnarizine (20, 60 or 180 mg/mg) or haloperidol (1 mg/kg). A cut-off time of 180 s was used. *denotes significant ($p < 0.05$) difference when compared to water group. # denotes significant difference from all other groups. Data presented as medians and interquartile range ($n = 8$–10 animals per group)

between 120- and 220-fold lower than clozapine (40 nM) and quetiapine (78 nM). Thus, based on its D2 receptor occupancy (*in vivo*) and affinity (*in vitro*), cinnarizine can be categorized as a dopamine D2 receptor antagonist of low-to-moderate affinity in between olanzapine and clozapine (Seeman *et al.*, 1997), therefore sharing the main mechanism of atypical antipsychotics, according to Seeman´s proposal (Seeman and Tallerico, 1998; Kapur and Seeman, 2001). Importantly, extrapyramidal symptoms typically appear only in the elderly, who have a decreased dopaminergic tone (Brucke *et al.*, 1995). Furthermore, cinnarizine exerted a fairly potent antagonism of 5-HT2 receptors *in vivo* (*Ki* 0.32 nM) (Okoro, 1999), an action shared with atypical antipsychotics such as olanzapine, risperidone and clozapine (Bymaster *et al.*, 1996).

Amphetamine-induced hyperlocomotion has long been used as a pharmacological model for psychosis in animals (Geyer and Ellenbroek, 2003) and is very useful in pre-clinical research for new antipsychotic drugs as it may mimic the hyperdopaminergic tone present in many schizophrenic patients (Kapur and Mamo, 2003). Cinnarizine was able to significantly counteract amphetamine-induced hyperlocomotion at doses as low as 20 mg/kg, a dose that neither interfered in spontaneous locomotion nor induced cataleptic behaviour in mice. At least part of this effect should result from cinnarizine antagonistic effect on dopamine D2 receptors, although other functions such as inhibition of glutamate (Terrian *et al.*, 1990) and dopamine release (Mena *et al.*, 1995) could be involved. These anti-dopaminergic mechanisms seem not to excessively decrease dopaminergic activity beyond a point to produce significant catalepsy and hypolocomotion, which occurred only in much higher doses than the ones effective against amphetamine induced hyperlocomotion, similarly to olanzapine (Ninan and Kulkarni, 1999). Importantly, catalepsy was not increased despite a three-fold dose increment up to 180 mg/kg, indicating that the effective dose of cinnarizine would be unlikely to induce robust parkinsonism in humans, especially in younger psychiatric patients.

Glutamate NMDA receptor antagonists, such as phencyclidine and dizocilpine, have also been regarded as a pharmacological model for schizophrenia, producing both hyperlocomotion and cognitive deficits in rodents (Ninan and Kulkarni, 1999; Dall'Igna *et al.*, 2003). Of note, typical antipsychotics typically inhibit hyperactivity induced by NMDA receptor antagonists only at doses that inhibit spontaneous activity *per se*, contrary to the more effective and safe atypical antipsychotics with 5-HT2 receptor antagonism (O'Neill and Shaw, 1999; Geyer and Ellenbroek, 2003). Here we have found that the cinnarizine effect on MK-801 hyperlocomotion is also similar to atypical antipsychotic profile, occurring at doses that do not affect spontaneous locomotion or induce catalepsy. NMDA receptor antagonists have been shown to indirectly activate non-NMDA glutamatergic receptors by an increase in glutamate release (Moghaddam *et al.*, 1997). Cinnarizine, mainly due to its ability to block calcium channels, inhibits glutamate release from intact synaptosomes (Terrian *et al.*, 1990), an action that *per se* could possibly prevent behavioural effects of NMDA receptor antagonists both in rodents (Moghaddam and Adams, 1998) and humans (Anand *et al.*, 2000). Another pharmacological action of cinnarizine is the blockade of sodium channels (Velly *et al.*, 1987), which is also a mechanism that prevents NMDA receptor antagonist neurotoxicity (Farber *et al.*, 2002). Also, cinnarizine has been shown to exert neuroprotective (Eichler *et al.*, 1994), antistress (Ossowska *et al.*, 1994) and neurotrophic effects (Tong and Rich, 1997), which are of potential interest for the treatment of schizophrenia and other psychotic disorders.

Based on its pharmacological profile in clinical practice (regarding tolerability) and in these models, cinnarizine has a putative antipsychotic action without major motor side-effects, similarly to atypical antipsychotics. Its mechanisms of action – dopamine D2 and 5-HT2 receptor antagonism associated with inhibition of calcium and sodium channels – could give cinnarizine potential therapeutic advantage over other antipsychotics and is also an interesting pharmacological profile as a mood stabilizer. Clinical trials are necessary to investigate if the effect on animals models and the theoretical advantage of cinnarizine are relevant in the management of patients with psychotic disorders and schizophrenia.

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II.2 RESULTADOS TEÓRICOS

II.2.a THEORETICAL INSIGHTS ON THE MECHANISM OF ACTION OF ATYPICAL ANTIPSYCHOTICS

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Review article

Theoretical insights into the mechanism of action of atypical antipsychotics

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Abstract

The present work discusses some theoretical mathematical results that can be derived from the theory of receptor binding linked with PET experimental data and presents insights to the understanding of the differences between typical and atypical profile of antipsychotics regarding the generation of extrapyramidal syndrome. The first part of the paper discusses the importance of the drug affinity to dopamine D2 receptors (D2R) and of the therapeutic window of drug concentration for antipsychotic action without EPS, whereas the second part discusses the contribution of the plasma half-life in the time-course of D2R occupancy. Together with current experimental data, we concluded that the key factors leading to an atypical profile would be adequate posology, low affinity of the drug to D2R and/or short plasma half-life.

Keywords: D2R; Half-life; Law of mass action; Mathematical model; Schizophrenia; Therapeutic window

Contents

Abbreviations: $[A]$, concentration of antipsychotic; $[A_{0.65}]$, concentration of antipsychotic to block D2R in 65%; $[A_{0.78}]$, concentration of antipsychotic to block D2R in 78%; $[AD_2]$, concentration of blocked dopamine D2 receptor; ΔA, size of the therapeutic window of drug concentration without EPS; $C(t)$, plasma antipsychotic concentration; $[D]$, dopamine concentration; D2R, dopamine D2 receptor; $[D_2]$, concentration of free dopamine D2 receptor; $D_{2\text{Occup}}$, dopamine D2 receptors occupancy; ED50, effective dose 50 value; EPS, extrapyramidal motor side-effects; F, initial fraction of blocked receptors; K_{on}, association rate constant; K_{off}, dissociation rate constant; K_d, equilibrium dissociation constant; PET, positron emission tomography; SPECT, single photon emission computed tomography; $t_{1/2}$, antipsychotic plasma half-life; $T_{1/2}$, time necessary to reach half of receptor occupancy.

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1. Introduction

Antipsychotic therapy was first introduced in the early 1950s, and since then much effort has been employed to understand their mechanism of action, as well as the pathophysiology of psychotic disorders, such as schizophrenia. They are known to be effective in reducing positive schizophrenic symptoms, and some can to a lesser extent also reduce negative symptoms. Clinical doses of typical antipsychotics such as haloperidol are known to produce severe extrapyramidal motor side-effects (EPS), while atypical antipsychotics like clozapine and quetiapine do not.

Since the initial works of Farde et al. (1988, 1990, 1992), the use of positron emission tomography (PET) and of single photon emission computed tomography (SPECT) in psychiatry have provided insights into the understanding of antipsychotic mechanism of action. In the last decade, a body of evidence has been built by several reports indicating that the blockade of dopamine D2 receptors (D2R) is necessary and probably sufficient to achieve antipsychotic effect (Kapur and Remington, 2001; Kapur and Mamo, 2003), corroborating to the hyperactivation of the dopaminergic mesolimbic pathway theory of schizophrenia. However, it has also been consistently observed that excessive D2R blockade in the striatum is related to the generation of EPS (Kapur et al., 2000a). Therefore, the occupancy of striatal D2R receptors became an important objective measure for the development of EPS.

The present work aims to show and discuss some theoretical results that can be derived from the theory of receptor binding linked with PET experimental data and presents insights to the understanding of the differences between typical and atypical profile of antipsychotics regarding generation of EPS. The first part of the paper will discuss the importance of D2R affinity and of the therapeutic window of drug concentration for antipsychotic action without EPS, whereas the second part will discuss the contribution of plasma half-life of an antipsychotic in the time-course of D2R occupancy. Even though they are all straightforward theoretical results, we believe that such an exposition may clarify and review some key aspects related to antipsychotic action.

2. On the therapeutic window of drug concentration: the chief role of affinity

As cited above, it is currently accepted that antipsychotics block D2R in limbic regions, leading to antipsychotic action, whereas excessive D2R blockade in the striatum generates EPS. Using striatum D2R occupation as a marker, a level of 65% blockade is related in most cases to effective antipsychotic action, while EPS typically appear when more than 78% of D2R are blocked (Kapur et al., 2000a). Kapur and Seeman (2000, 2001) and Seeman (2002) have elegantly shown that typical antipsychotics, which commonly induce EPS, bind more tightly to D2R, in contrast to atypical antipsychotics, which present low or moderate affinity for this receptor, mainly because of a higher dissociation rate constant.

The kinetics of the antipsychotic–D2R interaction is said to obey the law of mass action, which can be represented as

$$[A] + [D_2] \overset{K_{on}}{\rightarrow} [AD_2] \overset{K_{off}}{\rightarrow} [A] + [D_2]$$

where $[A]$ stands for the concentration of antipsychotics, $[D_2]$ for free D2R, $[AD_2]$ for blocked D2R by antipsychotics, and K_{on} and K_{off} denote the association and dissociation rate constants, respectively. The affinity of the antipsychotic for the D2R is inversely proportional to its equilibrium dissociation constant (K_d), defined by K_{off}/K_{on}. A straightforward derivation from the law of mass action predicts that the fraction of D2R occupancy (D_{2Occup}) is given by (Appendix):

$$D_{2Occup} = \frac{[A]}{[A] + K_d}. \tag{1.1}$$

This equation shows that larger K_d values are associated with more gradual increases in the fraction of bound D2R with increasing antipsychotic dosage. This is shown in Fig. 1 as plots of D_{2Occup} as a function of $[A]$ for representative antipsychotics. Moreover, by defining the size of the therapeutic window of drug concentration without EPS, denoted by ΔA, as being the magnitude of the dose range between $[A_{0.65}]$ and $[A_{0.78}]$ (the effective antipsychotic concentration that maintains the fraction of blocked D2R between 65% and 78%), we have that ΔA is given by (Appendix):

$$\Delta A = 1.69 K_d. \tag{1.2}$$

Eq. (1.2) shows that ΔA presents a linear relation with K_d, i.e. a K_d ten times higher will produce a therapeutic window ten times wider. Kapur and Seeman (2000, 2001) and Seeman (2002) have reported differences on K_{off}, and therefore on K_d, as much as 1000 times between atypical and typical antipsychotics. Therefore antipsychotics with low affinity for D2R (e.g. clozapine and quetiapine) do not cause EPS clinically, i.e., their extremely wide therapeutic window for drug concentration without EPS makes it difficult to exceed 78% of striatal D2R blockade.

However, by noting that the therapeutic index is the same for all antipsychotics ($[A_{0.78}]/[A_{0.65}] = 1.9$, Appendix), one could argue that it is only a matter of providing a better dosage partition, with more gradual increases of the dose when necessary, and that the therapeutic window of drug concentration without EPS has minimal influence in differing the

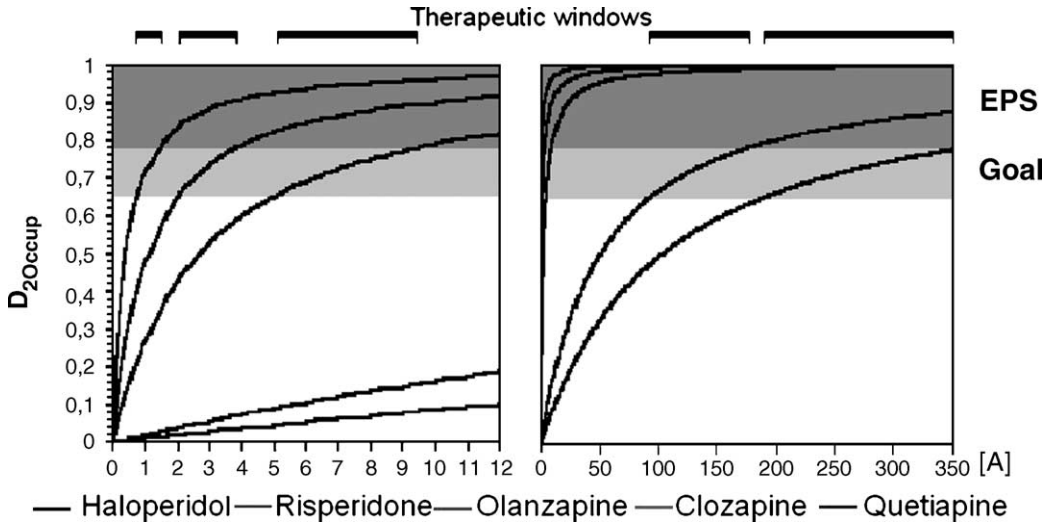

Fig. 1. Plots of D2R occupation (D_{2Occup}) vs. antipsychotic concentration ([A] (nM)) for some commonly used antipsychotics. The size of the therapeutic window without EPS of each antipsychotic is shown on top. Note that the left and right figures are the same, except for the horizontal axis scale. These simulations were performed considering haloperidol, risperidone, olanzapine, clozapine and quetiapine as having K_d values of 0.4, 1.1, 2.7, 51 and 104 nM, respectively (Seeman, 2002). It can be seen the higher the K_d, the wider the therapeutic window without EPS. Note that the [A] within therapeutic windows roughly correspond to the minimal effective dose of antipsychotic in mg/day.

antipsychotics. Indeed, every antipsychotic would have a perfect regime in which major symptoms are controlled without inducing EPS, and the therapeutic window upon rescaling can be as great as wished (i.e. a size of 1 mg/mL is a size of 1.000.000 ng/mL). Commercial and cultural aspects certainly influence this issue at least for some commonly prescribed typical antipsychotics. Accordingly, we can see that commercially available tablets of 25 mg of clozapine would correspond to a lesser change in the level of D2R blockaded than 1 mg haloperidol (Table 1 for some of these relations). Of note, risperidone presents high affinity to D2R, and even so it is sometimes considered as atypical drug. This could be explained by the effort employed in the search of its perfect dosage, compared to the mishandled use of haloperidol. Perphenazine, another typical antipsychotic, had also its adequate dose regime (i.e. control of symptoms without EPS) recently characterized (Talvik et al., 2004), resembling an atypical profile when correctly dosed. In that line, one would conclude that typical and atypical profile differences are strongly related to strategies to finding a perfect dose regime in the clinical setting.

Of clinical relevance, since the fraction between the upper and lower bound of effective dosage ($[A_{0.78}]/[A_{0.65}]$) is 1.9, once EPS is present, the model predicts that antipsychotic dosage could be halved and the level of D2R blockade should remain above 65%, which is compatible with optimal therapeutic effect.

Table 1
Minimum tablet dose of some typical and atypical commonly prescribed antipsychotics and their corresponding dose/size of the therapeutic window of drug concentration without EPS

Antipsychotic	mg	ΔA^* (nM)	mg/ΔA (mg/nM)	% of haloperidol
Haloperidol	1	0.93	1.07	100
Risperidone	1	1.86	0.54	50
Olanzapine	2.5	8.62	0.29	27
Clozapine	25	106.47	0.23	21
Quetiapine	25	206.18	0.12	11

* Calculated through Eq. (1.2). Constants values were obtained from Seeman (2002).

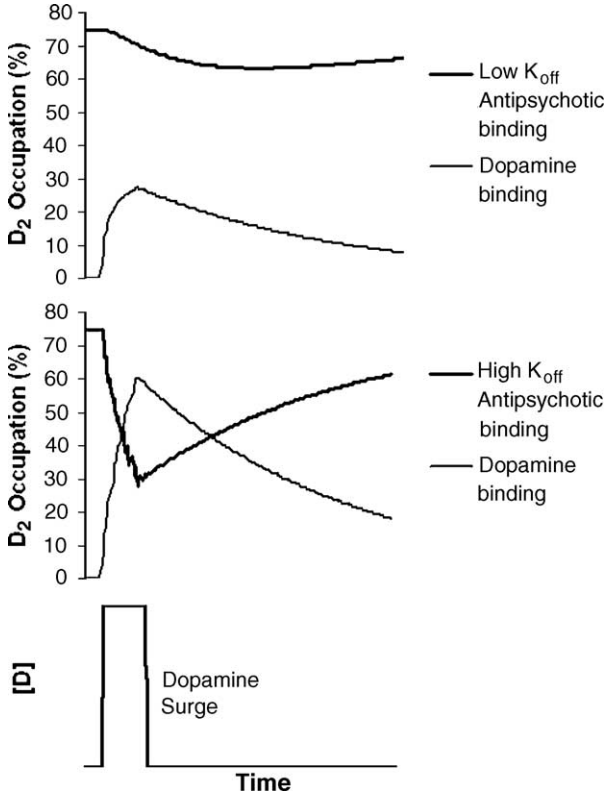

Fig. 2. Simulations of the levels of D2R blockade and dopamine binding during a surge of dopamine after the same level of blockade was obtained with a low and high K_{off} antipsychotic. The symbol [D] stands for the concentration of dopamine. It can be seen that the higher the K_{off}, the higher will be the permissiveness for dopamine binding.

Lastly, we point to another important factor previously observed by Kapur and Seeman (2000, 2001) that could also explain the differences among typical and atypical profile. They have shown that the observed difference in affinity between atypical and typical antipsychotics is mainly due to larger dissociation rate constants (K_{off}) present in atypical drugs, with little variation on the association rate constant (K_{on}) (Kapur and Seeman, 2000, 2001; Seeman, 2002). As can be seen in Fig. 2, drugs with large K_{off} lose when competing against dopamine, allowing an effect of surges of dopamine transmission, whereas drugs with low K_{off} do not allow this transmission. Based on this property, Kapur and Seeman (2001) concluded that atypical antipsychotics would produce less EPS because they would permit at least some degree of phasic striatal physiological dopamine transmission. Of note, losing in competition against surges of dopamine is a property of high K_d rather then high K_{off} per se, once low K_{on} also leads to this same feature.

Until now, we have considered that a striatal blockade between 65% and 78% of D2R is observed when effective antipsychotic action without EPS takes place. Interestingly, it was recently demonstrated that the intermittent blockade of these levels could be as effective as continuous blockade to antipsychotic effect (Kapur et al., 2000b). This will be the subject of discussion of the next section.

3. On the time-course of D2R occupancy: the chief role of plasma half-life

The time-course of the D2R occupancy associated with antipsychotic therapy became an important issue in the treatment of schizophrenia. The presumed notion of the necessity of continuous D2R blockade to achieve control of symptoms was questioned by recent findings showing that transiently high D2R occupancy is sufficient for obtaining and maintaining antipsychotic effect, even in neuroleptic-naïve schizophrenic patients (Kapur et al., 2000b; Tauscher-Wisniewski et al., 2002). Next, we will show that the plasma half-life of an antipsychotic presents a leading role in the time-course of D2R occupancy.

As a first approximation, we can think that plasma levels of an antipsychotic are related to the levels of the available drug concentration at the synaptic cleft. There is also an equation to describe D2R occupancy very related to Eq. (1.1) based on peripheral parameters, which is given by:

$$D_{2Occup}(t) = \frac{100 \times C(t)}{ED50 + C(t)} \tag{2.1}$$

Note the similarity between Eqs. (2.1) and (1.1). With Eq. (2.1), we have only transferred our attention to plasma pharmacokinetics, instead of local synaptic events. In that line, the plasma concentration ($C(t)$) is equivalent to the concentration of the drug [A] at the synaptic cleft, whereas ED50, the drug plasma concentration able to block 50% of D2R, is equivalent to K_d. Moreover, note that from the Eq. (2.1), once we know at a given time the plasma concentration of the drug and the level of receptor occupancy achieved, we can determine the values of ED50, given by:

$$ED50 = \frac{(100 - D_{2Occup})C(t)}{D_{2Occup}} \tag{2.2}$$

And, in most studies, $C(t)$ is fitted as (Tauscher et al., 2002; Takano et al., 2004):

$$C(t) = me^{-bt} \tag{2.3}$$

where m is the plasma maximal concentration at 0 h, b is a constant dependent on the plasma half-life ($t_{1/2}$) of the drug (in fact, $b = \ln 2 / t_{1/2}$), and t is the time after the drug administration.

An important point that is often misleading in PET studies regards the time-course of D2R occupancy, namely the half-life concept. Classically, a function like Eq. (2.3) does have a property of presenting a half-life, which is defined as the time necessary for the plasma concentration to be reduced by 50%. In these cases, $t_{1/2}$ will be always given by $\ln 2/b$, and this result is independent of the initial concentration being handled (Appendix), i.e. the time necessary for risperidone to drop from 6 to 3 ng/mL is the same as to drop from 3 to 1.5 ng/mL and so on (see Table 2). Inadequately, the same definition is also commonly employed to D2R occupancy (Gefvert et al., 1998; Tauscher et al., 2002; Takano et al., 2004). The point is that an equation like Eq. (2.1) does not present the same property, once the time required for the occupancy levels to be halved are dependent on the initial level being handled (Table 2), and the same is valid even if Eq. (2.1) is approximated by a linear polynomial, as is often the case (Gefvert et al., 1998; Tauscher et al., 2002; Takano et al., 2004). Moreover, starting from a D2R occupancy obtained from a given concentration $C(t_0)$, the time necessary to reach half of receptor occupancy ($T_{1/2}$) is given by (Appendix):

$$T_{1/2} = \frac{t_{1/2}}{\ln 2} \ln\left(\frac{C(t_0)}{ED50} + 2\right) \tag{2.4}$$

Therefore, as previously commented, $T_{1/2}$, differently from $t_{1/2}$, is dependent on the concentration of the drug (non-linearly) and will be higher if the concentration is higher and vice versa. Moreover, note also that $T_{1/2}$ is always greater

Table 2
Central and peripheral half-lives of risperidone

Time (h)	$C(t)$ (ng/mL)	D_{2Occup} (%)	$t_{1/2}$ (h)	$T_{1/2}$ (h)
0	45.0	87.6	19	60
19	22.5	77.8	19	47
38	11.2	63.7	19	36
57	5.6	46.8	19	29
60	5.0	43.8	19	28
66	4.0	38.9	19	27
74	3.0	31.8	19	25
76	2.8	30.5	19	24
86	2.0	23.4	19	23

Simulations performed using Eqs. (2.3) and (2.1) for calculations of $C(t)$ and D_{2Occup} respectively. We used $b = 0.036$, $m = 45.0$, ED50 = 6.4 ng/ml (Takano et al., 2004).

than $t_{1/2}$, and, curiously, $T_{1/2}$ approaches $t_{1/2}$ when the initial drug concentration becomes small (i.e., $T_{1/2} \rightarrow t_{1/2}$ if $C(t_0) \rightarrow 0$). Eq. (2.4) nevertheless gives a false impression that $T_{1/2}$ is dependent on the affinity of the drug, once the factor ED50 appears. This is certainly not the case, since antipsychotics with less affinity (high ED50) will also be required at higher concentrations to achieve the same level of D2R blockade. If we define $T_{1/2}$ as being the time necessary for a given fixed fraction (F) of D2R occupancy to drop to half ($F/2$), then we can find $T_{1/2}$ as a function of F (Appendix):

$$T_{1/2}(F) = \frac{t_{1/2}}{\ln 2} \ln\left(\frac{F}{100 - F} + 2\right) \tag{2.5}$$

In the particular case of defining $T_{1/2}$ as being the time for D2R occupancy to drop from 80% to 40% (i.e., $F=80$), we have that $T_{1/2} = (t_{1/2}/\ln 2) \times \ln 6 = 2.6 t_{1/2}$. Eq. (2.5) shows that the higher the initial fraction of blocked receptors ($F = D_{2\mathrm{Occup}}(t_0)$) considered, the higher the $T_{1/2}$ obtained (Fig. 3). As an illustrative example, in the work of Gefvert et al. (1998) they concluded that quetiapine presented lower $T_{1/2}$ for D2R then for 5HT2, but it should be remembered that quetiapine has higher affinity to 5HT2 receptors than to D2R, presenting therefore higher occupancy levels of this receptor than of D2R at the same plasma concentration, which was a confounding factor (Figs. 3 and 4). Moreover, Eq. (2.5) shows that once the initial occupancy fraction F is fixed, the half-life of the receptor occupancy is solely determined by the half-life of the drug plasma concentration, and in particular it is not dependent on its affinity. Which means, once the same

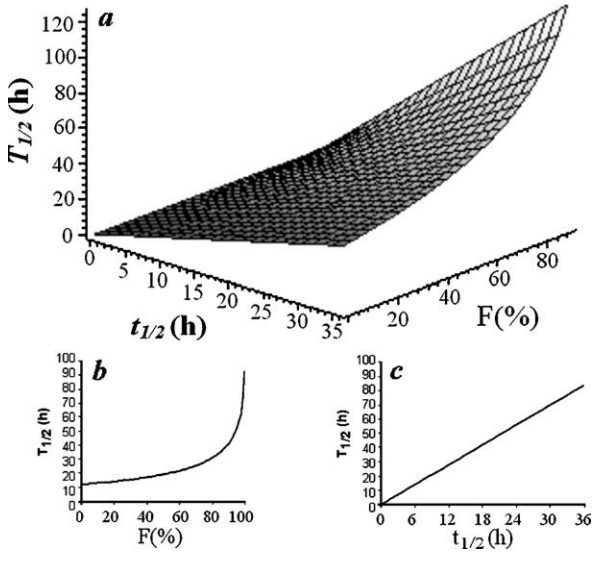

Fig. 3. (a) Plot of the "half-life" of D2R occupancy ($T_{1/2}$) as a function of the initial fraction of D2R receptors blocked (F) and of the plasma half-life of the antipsychotic ($t_{1/2}$); we have plotted $T_{1/2}$ for values of F up to 80% in order to keep clarity (above this, $T_{1/2}$ grows very fast, reaching very high values, as shown in (b)). Note that the higher $t_{1/2}$ or F, the higher is $T_{1/2}$, and this dependence is non-linear on F and linear on $t_{1/2}$, as shown in (b) and (c) respectively. These plots were performed using Eq. (2.5); in (b) we fixed $t_{1/2}=12$ h and in (c) we fixed $F=75\%$.

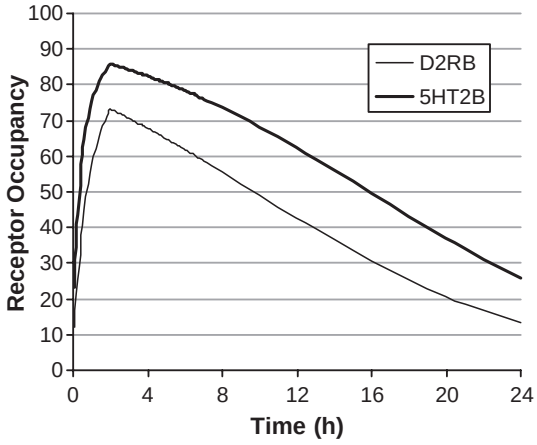

Fig. 4. Simulation of blockade of dopamine D2R and serotonine 5HT2 receptors obtained for quetiapine (continuous lines). We have used Eq. (2.3) for plasma concentrations after drug peak (and a linear polynomial until this) and Eq. (2.1) for receptor occupancy. We considered plasma half-life ($t_{1/2}$) of quetiapine as 5.3 h, ED50 for D2R of 550 ng/mL and for 5HT2 receptors of 247 (since quetiapine present a ratio of affinity between D2R/5HT2 of 1:2.22). Considering the time that the receptor occupancy takes between the peak and half of this value as the half-life of receptor occupancy ($T_{1/2}$), then quetiapine would present higher $T_{1/2}$ for 5HT2 than to D2R. However, if we define $T_{1/2}$ as being the time to decrease a fixed occupancy interval, e.g., the time to drop from 80% to 40%, than $T_{1/2}$ is the same for both receptors.

levels of receptors blockade are achieved with distinct antipsychotics, if they present the same plasma half-life, they will have the same time-course of decrease in occupancy, independently of their affinity (Fig. 4). Of note, as cited above, in a work questioning the need or not of continuous D2R blockade to achieve control of symptoms in schizophrenia, Kapur et al. (2000b) have recently shown only transiently high D2R occupation by quetiapine given once daily. In the same way, the transient blockade observed by Kapur et al. (2000b) was due to the short half-life of quetiapine (5.3 h), and not by its known low affinity to D2R (or large ED50).

4. Discussion

With all these ideas in mind, we can turn back to the discussion of what renders an atypical profile for an antipsychotic. If the transiently high D2R blockade is really proved to be sufficient to achieve control of symptoms, and based on the results presented above, we can thus postulate that an ideal antipsychotic would be the one presenting a short half-life. We can even question the results obtained in the first section of this paper attributing the atypical profile of quetiapine to its wide therapeutic window of drug concentration without EPS in favor of its short half-life. Analogously, the same question could apply for clozapine, which is known for its low D2R occupancy levels (at the time of scan), and presents a 12 h plasma half-life (compared to 24 h for haloperidol, 19 h for risperidone, 24 h for chlorpromazine and 30 h for olanzapine).

Moreover, generally speaking, one can also suppose that it does not matter if small levels above the threshold for EPS are reached after an administration of a short plasma half-life

antipsychotic, meaning that the half-life would be more important than the affinity. In fact, motor side effects would also be transient and bedtime administration would minimize the chance of experiencing EPS.

Based on the concepts above, what one can conclude as being really necessary to a D2R antagonist have an atypical profile? To present a lower affinity and therefore large therapeutic window of drug concentration without EPS? To possess an adequate dose regime? To present high K_{off} and therefore permit the effect of surges of dopamine? To present a short plasma half-life? Most probably, all these factors contribute, and they should be taken into account to the design of new atypical antipsychotics, not to mention other recent strategies, such as partial agonist activity in the case of aripiprazole.

5. Limitations

It is worth pointing that our work is focused on the central role of D2R, which we are assuming to be the key target for antipsychotic therapy. However, it is still a matter of debate to involve or not other neurotransmitters systems in the treatment of psychosis or schizophrenia. Also, different affinities for D2 long and short receptors can play an important role. Moreover, this theory does not address why clozapine can effectively treat refractory patients, which probably involves actions unrelated to D2R blockade. Of note, we have focused on D2R and antipsychotics, but clearly several results are valid for any receptor and ligand, as long as the steady state of occupancy is reached in a time scale shorter (i.e. seconds, minutes) than the time scale of the drug metabolism (i.e. hours, days).

Several factors may also account for the discrepancies between the theoretical results presented here and real data, such as the effects of the metabolites of a given antipsychotic. It is often the case that metabolites of an antipsychotic are also D2R antagonists and present different plasma half-lives. Therefore, when not taking into account the influence of these metabolites, the simulations will underestimate the real level of D2R occupancy.

Another important factor is the pharmacokinetics profile of an antipsychotic in the central nervous system, which in the present model was considered to be the same as in plasma. Other confounding factors are the variations associated to the plasma determinations of antipsychotics and to the measurement of D2R occupancy, since the latter could vary in about ±10% depending on the study (Takano et al., 2004), and also the influence of up-regulation of D2R presented in patients already medicated with antipsychotics (Silvestri et al., 2000). Moreover, it must be considered that the data defining the 65–78% D2R blockade as effective without EPS is based on few studies with a limited number of patients.

Lastly, it is worth pointing that these derivations are based on a mathematical model, which, as every model, is an approximation of the reality and presents limitations. Particularly, this mathematical model is based on derivations from the law of mass action and on the fitting of drug blood concentrations from an equation like Eq. (2.3). The law of mass action presents some assumptions (Appendix) that are known not to be valid in some cases, as well as the fitting of peripheral concentrations by an exponential function is also not always accurate (especially at low or high concentrations). Finally, given the variable levels of D2R blockade that can be achieved with similar doses of antipsychotic in distinct patients, the results presented in this work should be regarded as referring to the average behavior.

6. Conclusion

The present work linked known PET experimental data to the theory of receptor binding and drug pharmacokinetics and could provide some insights to the understanding of the atypical profile presented by some antipsychotics. Although many of the insights presented here are subject to several limitations, we believe that research on this theoretical field together with experimental work will help to improve the models and consequently provide deeper knowledge to the understanding of the mechanism of antipsychotic action.

Acknowledgement

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil.

Appendix A

A.1. Proof of Eq. (1.1)

From the law of mass action applied to receptor binding, we have that the ratio of $[AD_2]$ formation per unit of time ($d[AD_2]/dt$) is given by

$$\frac{d[AD_2]}{dt} = K_{on}[A][D_2] - K_{off}[AD_2] \quad (1)$$

We now create a new variable, called D_{2Occup}, which represents the fraction of blocked D2R from all D2R (free and bound), so D_{2Occup} is given by

$$D_{2Occup} = \frac{[AD_2]}{[D_2] + [AD_2]} \quad (2)$$

By the assumption that the number of D2R, even if up-regulated by antipsychotic treatment, reaches a constant level (i.e., $[D_2]+[AD_2]=C$), and by noting that $1-D_{2Occup}=[D_2]/([D_2]+[AD_2])$, we can use Eq. (2) in Eq. (1) to arrive at the following ordinary differential equation for D_{2Occup}

$$\frac{dD_{2Occup}}{dt} = K_{on}[A]\left(1 - D_{2Occup}\right) - K_{off}D_{2Occup} \quad (3)$$

If we now consider the steady state, where the equilibrium between bound and free D2R is reached (i.e. equal association

and dissociation rates, meaning that $dD_{2Occup}/dt=0$), we have the following equation for D_{2Occup}

$$D_{2Occup} = \frac{K_{on}[A]}{K_{on}[A] + K_{off}} \tag{4}$$

which gives rise to Eq. (1.1).

A.2. Proof of Eq. (1.2)

If we isolate $[A]$ in the Eq. (4), and using the index D_{2Occup} to denote the dependence on D_{2Occup}, we get

$$\left[A_{D_{2Occup}}\right] = \frac{K_{off} D_{2Occup}}{K_{on}\left(1 - D_{2Occup}\right)} \tag{5}$$

We can now define the size of the therapeutic window without EPS, denoted by ΔA, as being the magnitude of the dose range between $[A_{0.65}]$ and $[A_{0.78}]$ (the effective amount of antipsychotic that maintains the fraction of blocked D2R between 65% and 78%), thus ΔA is given by

$$\Delta A = [A_{0.78}] - [A_{0.65}] \tag{6}$$

Upon simple calculation, using Eq. (5) in Eq. (6), we get ΔA as function of K_{off} and K_{on}

$$\Delta A = \frac{K_{off} 0.78}{K_{on}(1 - 0.78)} - \frac{K_{off} 0.65}{K_{on}(1 - 0.65)} = 1.69 \frac{K_{off}}{K_{on}} \tag{7}$$

which is Eq. (1.2).

A.3. Proof of the constant value of the therapeutic index among distinct antipsychotics

Using Eq. (5) and the definition of $[A_{0.65}]$ and $[A_{0.78}]$, we calculate the therapeutic index, which is the fraction between the upper and lower bound of effective dosage without EPS ($[A_{0.78}]/[A_{0.65}]$), getting

$$\frac{[A_{0.78}]}{[A_{0.65}]} = \frac{K_d 0.78}{1 - 0.78} \cdot \frac{1 - 0.65}{K_d 0.65} = 1.9 \tag{8}$$

as stated.

A.4. Proof of the existence of the half-life concept for an exponential function

Suppose that at a given time t_0 we have a certain concentration $C(t_0)$ of drug in the plasma. We are asking how long it takes for the concentration to reach half of this initial value. Mathematically speaking, we are searching a time $t_{1/2}$ such that at time $t_0+t_{1/2}$ we will have

$$C\left(t_0 + t_{1/2}\right) = \frac{C(t_0)}{2} \tag{9}$$

By using Eq. (2.3) in Eq. (9) we get to:

$$me^{-b\left(t_0 + t_{1/2}\right)} = \frac{me^{-bt_0}}{2} \tag{10}$$

After some algebra, Eq. (10) becomes:

$$t_{1/2} = \frac{\ln 2}{b} \tag{10}$$

Hence the plasma half-life of a given antipsychotic is a constant, and therefore independent of the initial level of drug concentration being handled, as stated.

A.5. Proof of Eq. (2.4)

Suppose that at a given time t_0 we have a certain level of D2R blockade given by $D_{2Occup}(t_0)$. We are asking how long it takes for the fraction of blocked receptors to reach half of this initial value. Mathematically speaking, we are searching a time $T_{1/2}$ such that at time $t_0+T_{1/2}$ we will have

$$D_{2Occup}\left(t_0 + T_{1/2}\right) = \frac{D_{2Occup}(t_0)}{2} \tag{12}$$

By using Eq. (2.1) in (12) we get to:

$$\frac{100 \times C\left(t_0 + T_{1/2}\right)}{ED50 + C\left(t_0 + T_{1/2}\right)} = \frac{50 \times C(t_0)}{ED50 + C(t_0)} \tag{13}$$

We now substitute Eq. (2.3) in Eq. (13), arriving at:

$$\frac{100 \times me^{-b\left(t_0 + T_{1/2}\right)}}{ED50 + me^{-b\left(t_0 + T_{1/2}\right)}} = \frac{50 \times me^{-b(t_0)}}{ED50 + me^{-b(t_0)}} \tag{14}$$

With a little algebra, Eq. (14) becomes:

$$T_{1/2} = \frac{1}{b} \ln\left(\frac{me^{-bt_0}}{ED50} + 2\right) \tag{15}$$

which is Eq. (2.4).

A.6. Proof of Eq. (2.5)

We are now fixing a given initial fraction of D2R blocked denoted as F. We ask for the time necessary to reach half of this value ($F/2$). We proceed exactly as the proof above to get Eq. (15). Now we note that Eq. (2.2), with a change of notation, can be rearranged as:

$$\frac{C(t_0)}{ED50} = \frac{F}{(100 - F)} \tag{16}$$

Substituting Eq. (16) into Eq. (2.4), we get the desired result. Hence the time required to reach half of values is dependent on the initial value being handled, and the concept of "half-life" is therefore incorrectly employed in this case.

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II.2.b ON THE SIMULATION OF THE TIME-COURSE OF DOPAMINE D2 RECEPTOR OCCUPANCY FROM THE PHARMACOKINETICS OF ANTIPSYCHOTICS

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On the simulation of the time-course of dopamine D2 receptor occupancy from the pharmacokinetics of antipsychotics

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We have read with great interest the elegant paper by Takano et al. (2004) regarding the estimation of the time-course of dopamine D2 receptor (D2R) occupancy from plasma pharmacokinetics of antipsychotics, with special emphasis on risperidone. It is our view that studies aiming to achieve a correct equation that could predict the level of central D2R blockade based on the measurement of antipsychotic blood levels are valuable in helping clinicians to more objectively control the treatment of patients. We have three comments that could be helpful in this research field.

First, the function of time describing the levels of an antipsychotic in the blood that was used to estimate D2R occupancy in the work of Takano et al. (2004) was:

$$C(t) = m\mathrm{e}^{-bt}, \tag{1}$$

where C is the plasma concentration, m is the estimated plasma maximal concentration at 0 h, b is a constant dependent on the plasma half-life ($t_{½}$) of the drug (in fact, $b = \ln 2/t_{½}$), and t is the time after the drug administration. As can be seen in Figure 2 of Takano et al., the estimated D2R occupancy was in most points smaller than the data obtained from PET scans. One factor contributing to this underestimation could be that the absorption state and particularly the time necessary to reach the peak concentration of the antipsychotic in blood (t_{peak}) were not taken into account. We propose the following modified function of time to describe the drug levels in blood:

$$C(t) = (m/t_{\mathrm{peak}})tH(t_{\mathrm{peak}} - t) + m\mathrm{e}^{-b(t-t_{\mathrm{peak}})}H(t - t_{\mathrm{peak}}), \tag{2}$$

where $H(x)$ is the Heaviside function. Note that equation (2) is essentially equation (1) shifted to the right plus a linear factor until the plasma maximal concentration is reached. Plots of equations (1) and (2) for olanzapine are shown in Figure 1a. Using the same equation for D2R occupancy employed by Takano et al. (2004):

$$\mathrm{D}_{\mathbf{2},\mathrm{occ}} = 100 \times C(t)/(\mathrm{ED}_{\mathbf{50}} + C(t)), \tag{3}$$

where $\mathrm{D}_{\mathbf{2},\mathrm{occ}}$ is the percentage level of D2R occupancy and $\mathrm{ED}_{\mathbf{50}}$ is the apparent in-vivo affinity parameter, we can see in Figure 1b that by using equation (2) instead of equation (1) the levels of D2R blockade become higher after t_{peak}. Although in the work of Takano et al. (2004) this influence was minimal, since risperidone presents a t_{peak} of 2 h, in studies simulating other antipsychotics with higher t_{peak} (such as olanzapine) this factor can have more marked influences on the results.

Secondly, we disagree with the conclusion that the half-life of the D2R occupancy ($T_{½}$) becomes longer as $\mathrm{ED}_{\mathbf{50}}$ becomes smaller and vice versa as suggested by the simulations performed by varying the $\mathrm{ED}_{\mathbf{50}}$ of the drug (shown in Figure 3 of Takano et al.). $\mathrm{ED}_{\mathbf{50}}$ (plasma concentration to block 50% of D2R), similarly to K_{d}, can be regarded as a parameter inverse to the affinity of the antipsychotic to D2R. The maximal plasma concentration (m) of the antipsychotic is also dependent on the affinity to D2R (i.e. the less potent the antipsychotic, the higher its effective dose and plasma concentration) and, therefore, drugs with lower affinity will present greater m and vice versa. Our main point is that the parameter m in the work of Takano et al. (2004) was estimated by the first $\mathrm{ED}_{\mathbf{50}}$ used (6.4 ng/ml) for risperidone, which can be done using equation (3), but then it was held constant to simulations with others values of $\mathrm{ED}_{\mathbf{50}}$. Since m is dependent on $\mathrm{ED}_{\mathbf{50}}$, if m is corrected to each new $\mathrm{ED}_{\mathbf{50}}$, for example by fixing for each $\mathrm{ED}_{\mathbf{50}}$ a value of m able to block 87% of the receptors (as in their work for the first $\mathrm{ED}_{\mathbf{50}}$ given), one can see that the $T_{½}$ does not change. In summary, once the same levels of D2R blockade are achieved with distinct antipsychotics, $T_{½}$ is not dependent on the affinity of the drug. On the other hand, the second set of simulations in the work by Takano et al. (2004) we consider to be legitimate and very illustrative in showing the dependence of $T_{½}$ on the plasma half-life of the drug ($t_{½}$). They have shown that varying $t_{½}$ of

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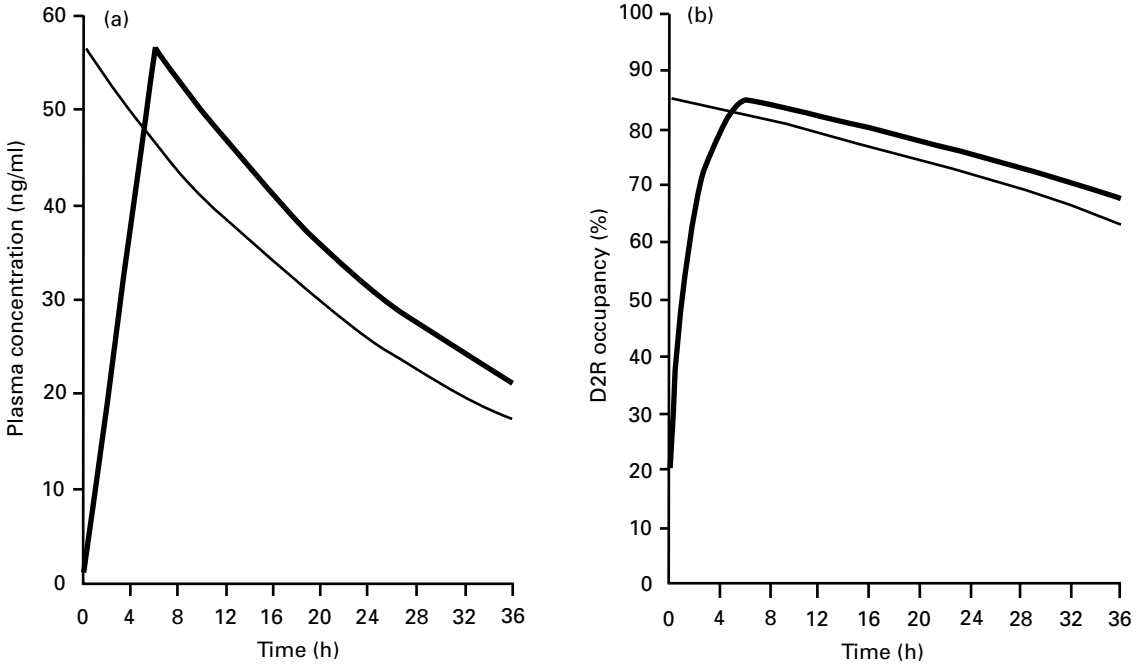

Figure 1. Plasma concentrations of the drug (a) and levels of D2R occupancy (b) as a function of time obtained by two distinct simulations with olanzapine. Simulation B (▬▬) differs from simulation A (——) by considering the time necessary for the plasma concentration of the drug to reach the peak. Note that D2R occupancy levels predicted without taking into account the kinetics of the absorption phase are lower after t_{peak}. For these simulations, olanzapine was considered to have a t_{peak} of 6.1 h (Baldessarini and Tarazi, 2001), an ED_{50} of 10 ng/ml, and a $t_{½}$ of 21 h (Takano et al., 2004).

the drug would produce a change in $T_{½}$ in the same direction. Of note, Kapur et al. (2000) have recently shown only transiently high D2R occupation by quetiapine, in a work questioning whether there is a need of continuous D2R blockade to achieve control of symptoms in schizophrenia. In the same way, the transient blockade observed by Kapur et al. (2000) was due to the short half-life presented by quetiapine (6 h), and not by its known low affinity to D2R (or large ED_{50}).

Thirdly, we would like to comment on the concept of the half-life of D2R occupancy ($T_{½}$). When we are dealing with plasma drug concentrations, an equation like (1) does present a concept of half-life, since the same time necessary to reduce 50% of plasma concentration is independent on the drug level (e.g. since $t_{½}$ of risperidone is 19 h, it will take 19 h to the drug drop from 6 ng/ml to 3 ng/ml and a further 19 h to drop from 3 ng/ml to 1.5 ng/ml and so on). However, the equation describing D2R occupation [equation (3)] does not follow this pattern, since the time necessary to reach half of the levels is dependent on the drug level. Therefore, we question this concept, which should be carefully analysed, especially when data from different works are compared. Moreover, given the dependence on the initial drug level, we propose that $T_{½}$ should be defined for a fixed occupancy interval, e.g. we should consider in the literature $T_{½}$ for receptor occupancy as the time necessary for a decrease of D2R occupancy from 80% to 40%.

We hope these comments will aid future receptor imaging studies to help provide important results that can improve clinical practice.

Acknowledgement

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Statement of Interest

None.

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II.2.c HALF THE DOSE OF ANTIPSYCHOTIC IN CASE OF EXTRAPYRAMIDAL SYMPTOMS

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Letter to the Editors

Half the dose of antipsychotic in case of extrapyramidal symptoms

Keywords: Schizophrenia; Antipsychotic; Dose; D_2; EPS; Occupancy

Dear Editors,

In the last decade, the use of in vivo radioligand neuroimaging in psychiatry has provided important information for the understanding of therapeutic action and extrapyramidal symptoms during antipsychotic therapy (Farde et al., 1992; Nordstrom et al., 1993). In fact, the existence of a therapeutic window for D2R occupancy is currently accepted, since a level above 65% of blockade is related in most cases to antipsychotic efficacy, while extrapyramidal symptoms (EPS) typically appear when more than 78% of D2R are blocked (Farde et al., 1992; Nordstrom et al., 1993; Kapur et al., 2000, Kapur and Mamo, 2003).

The kinetic of the antipsychotic–D2R interaction obeys the law of mass action, which can be represented as

$$[A] + [D_2] \overset{k_{on}}{\rightarrow} [AD_2] \overset{k_{off}}{\rightarrow} [A] + [D_2]$$

where $[A]$ stands for the concentration of antipsychotics, $[D_2]$ for free D2R, $[AD_2]$ for blocked D2R by antipsychotics, and k_{on} and k_{off} denote the association and dissociation rate constants, respectively. The affinity of the antipsychotic for the D2R is inversely proportional to its equilibrium dissociation constant (K_d), defined by k_{off}/k_{on}. A straightforward derivation from the law of mass action predicts that the fraction of D2R occupancy (D_{2Occup}) is given by $D_{2Occup}=100\times[A]/([A]+K_d)$. Moreover, this last result has a peripheral equivalent, which is given by

$$D_{2Occup} = \frac{100 \times C}{C + ED_{50}} \tag{1}$$

where C is the plasmatic concentration of the drug, whereas ED_{50}, the concentration on plasma able to block 50% of D2R, is equivalent to K_d. If we isolate C in Eq. (1), and using the index D_{2Occup} to denote the dependence on D_{2Occup}, we get

$$C_{D_{2Occup}} = \frac{D_{2Occup}}{100 - D_{2Occup}} ED_{50}. \tag{2}$$

Using Eq. (2), we calculate the "therapeutic" index, which is defined as the fraction between the upper and lower bound of effective dosage without EPS, getting

$$\frac{C_{78}}{C_{65}} = \frac{ED_{50}78}{100-78} \cdot \frac{100-65}{ED_{50}65} = 1,9. \tag{3}$$

This last result has two relevant clinical consequences: i) once 65% of D2R blockade is achieved by an antipsychotic, the dosage cannot be doubled in order to keep the D2R blockade inside the therapeutic window of D2R occupation without EPS. ii) on the other hand, we can also conclude that, once EPS is present (meaning $D_{2Occup}>78\%$), the model predicts that antipsychotic dosage could be halved and the level of D2R blockade should remain above 65%, which is compatible with therapeutic effect in most of patients. Such drastic reduction is intuitively too aggressive, because the hyperbolic relationship between dose and occupancy is not taken into account. In Fig. 1 we have plotted a function as Eq. (1), where we can observe the validity of this claim. Note that in cases of parkinsonism with very

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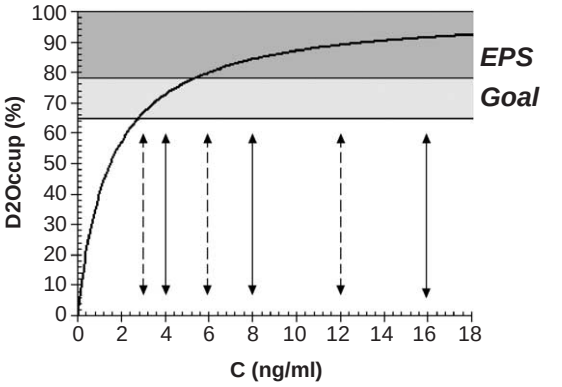

Fig. 1. Plot of the percentage of D2R occupation (D_{2Occup}) obtained using Eq. (1) for different plasmatic concentrations of a fictitious antipsychotic (C). We choose $ED_{50}=1.5$ ng/ml. Note that the plot is qualitatively the same for drugs of any D2R affinity (ED_{50}). D2R occupation above 78% induces EPS and the range of occupation between 65% and 78% is the goal for antipsychotic efficacy without EPS. Any drug concentration responsible for a D2R occupation greater than 78% can be halved and the level of D2R blockade remains above 65%. The arrows illustrate two fictitious examples. The continuous arrows denote a case of a patient which had its drug concentration halved firstly from 16 to 8 ng/ml, which required another halving to 4 ng/ml in order to reach D_{2Occup} value inside the therapeutic window, whereas the dashed arrows represent a similar case for values of 12, 6 and 3 ng/ml.

high D2R occupancy, it is also possible that, even after halving the dosage, the blockade still remains above 78%, which would require further halving of the dosage and so on.

Since in clinical practice a minority of patients should require blockade above 70%, we suggest the following algorithm for changing antipsychotic dosage when EPS appear: halve the dosage and introduce anticholinergic treatment for 3–4 days (the longer the plasmatic half-life, the more days), observing both psychotic and motor symptoms. Then titrate down the anticholinergic treatment and if EPS reappear, halve the antipsychotic dose again, reintroduce anticholinergic treatment, wait a few of days and so on. In case EPS does not recur and clinical effectiveness fails to take place or diminishes, we recommend a slow increase of the medication, since the level of D2R blockade should be inside the putative therapeutic window ($D_{2Occup} \geq 65\%$).

We hope this suggestion can be tested in clinical practice and using in vivo radioligand studies, which can be especially helpful for patients on antipsychotics with high D2R affinity, such as typical antipsychotics and risperidone.

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II.2.d CRITICAL COMMENTS ON THE FAST-OFF D2 THEORY OF ATYPICALITY OF ANTIPSYCHOTIC DRUGS

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Critical comments on the fast-off D2 theory of atypicality of antipsychotic drugs.

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To the Editor:

In a recent letter (1), Seeman presents evidence for the theory that an antipsychotic is atypical (low induction of extrapyramidal signs (EPS) and prolactinemia) when its dissociation rate constant (k_{off}) is high, leading to rapid displacement from D2 receptors (D2R) by dopamine and favoring its physiological effects. Seeman lists *in vitro* (fast dissociation of some, but not all, atypicals from D2R) and brain imaging results (haloperidol, unlike quetiapine and clozapine, still occupies a high percentage of D2R after a 24-hour interval) to support the 'fast-off' hypothesis.

We argue that, although k_{off} values are important, they cannot account alone for atypicality. Various atypical antipsychotics can produce significant EPS at high enough doses (2), while amisulpride produces significant hyperprolactinemia despite dissociating rapidly from D2R, probably due to its high concentrations in the pituitary (3). Therefore, the main factor responsible for induction of EPS is likely to be the net competition for D2R between the antipsychotic and dopamine, which depends not only on affinity but also on synaptic concentration. High-affinity (low k_{off}) drugs at low doses, therefore, may produce as much prolactinemia or EPS as lower affinity drugs at doses high enough to compensate their 'fast-off' property.

Moreover, regarding differences in the time-course of D2R blockade in brain imaging studies, one should note that, once peak levels of blockade are achieved, decay of receptor occupancy is dependent on the drug's half-life, and not on its affinity (4). Even if an antipsychotic dissociates rapidly from D2R, it will bind again if it remains in the synaptic cleft, in a process that will persist until the drug is metabolized or diffuses away. Thus, the transient blockade of D2R by quetiapine and clozapine can be better explained by

their short plasmatic half-lives (5 and 8-12 hours, respectively) when compared to haloperidol (around 24 hours), as well as by higher initial D2R blockade levels with routine doses of the latter; therefore, it does not constitute evidence for the 'fast-off' hypothesis.

To prove Seeman's theory, one would have to show that a high-affinity D2R antagonist produces more extrapyramidal symptoms or prolactinemia than a lower affinity drug in a condition in which both produce the same level of striatal or pituitary D2R blockade. Differences in pharmacokinetics (e.g. half-life) and antagonism of other receptors (e.g. cholinergic and serotonergic) should also be considered. Until this is achieved, we believe the main reason for the paucity of extrapyramidal symptoms observed with most atypical antipsychotics is likely to be the lower level of striatal dopaminergic blockade obtained with routine doses of these drugs.

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PARTE III

Onde são discutidos os resultados e perspectivas.

III.1 DISCUSSÃO DOS RESULTADOS

III.1.a UM PROGRAMA PARA TESTES COMPORTAMENTAIS

O primeiro capítulo de resultados desta tese (II.1.a) descreveu, em linhas gerais, o *software* de computador que foi programado a fim de que muitos dos resultados experimentais apresentados nos capítulos II.1.b, II.1.c e II.1.d pudessem ser obtidos. Como exposto, o programa é baseado em distinguir a cor branca dos animais contra o fundo escuro da arena. Para tanto, é necessário fornecer como uma das variáveis de entrada o limiar de cor. Tudo o que for mais claro do que ele será considerado como sendo a cobaia, e tudo o que for mais escuro será considerado como sendo a arena, conforme mostrado na Figura 2 do capítulo II.1.a. Assim, o programa pode ser empregado em qualquer pesquisa que utilize animais de cor clara[1]. Contudo, observo que através de uma mudança simples no código do programa, é possível também empregá-lo em experiências envolvendo animais escuros filmados em arenas claras, o que poderia ser de interesse para quem trabalha, por exemplo, com camundongos da linhagem C57BL/6.

Baseado neste princípio, é, portanto, importante ter o cuidado de que a arena em que os animais são testados seja escura o suficiente para que esta divisão de cor possa ocorrer. Além disso, é também muito importante que o material constituinte da arena (ie, madeira, acrílico) seja fosco, e que a iluminação do ambiente seja difusa. Infelizmente, estes requisitos foram aprendidos na prática

[1] Dentro do Departamento de Bioquímica da UFRGS, tradicionalmente tem sido utilizado em ratos e camundongos.

por muitos colegas que perderam experimentos inteiros devido a reflexos de luz presentes na arena. Estes reflexos podem alcançar um nível de cor mais claro do que o do limiar, o que faz com que o programa os interprete como sendo parte da cobaia, gerando dados errados[2]. Além disso, ocorreram casos nos quais trabalhamos com arenas inicialmente ideais, mas cuja iluminação fez refletir a urina dos animais que surgiu após o início do experimento. Portanto, é recomendado testar também a existência de possíveis reflexos na arena com a água, ou utilizar algum material para a arena que absorva ou drene a urina.

Ainda assim, para evitar perder gravações inteiras devido a problemas com ruídos de cor clara da arena que podem eventualmente aparecer, ou, mais comumente, devido a interferências vindas da fronteira da arena[3], foi programada uma rotina no *software* que permite "pintar de preto" regiões da arena através do *mouse*. Contudo, é recomendado evitar ao máximo pintar as áreas aonde os animais são analisados, uma vez que, em o animal se localizando em uma área já pintada, o programa não vai reconhecê-lo.

Uma vez diferindo a cobaia de sua arena, o programa faz então o cômputo do centro de branco do animal, de forma análoga a um cálculo de centro de massa. Assim, ele consegue registrar a posição do animal (coordenadas X e Y) num determinado quadro. Após registrar esta informação, ele analisa o próximo

[2] Vale a pena registrar que ocorreram casos onde o problema era justamente o contrário, isto é, a iluminação era tão fraca que os animais não puderam ser diferenciados da arena por aparecerem em tons muito escuros de cinza.

[3] Por exemplo, nos casos da arena consistir de uma caixa negra de paredes finas sobre um chão branco, podem acontecer interferências da região de fronteira quando o animal se aproxima de uma das paredes.

quadro do vídeo[4]. Para um maior ganho de velocidade, a cada quadro analisado, o programa não procura o animal em todos os *pixels* do vídeo, e sim somente dentro da área limitada por um quadrado cujo centro se dá nas coordenadas X e Y obtidas da posição do animal no quadro analisado previamente, e cujo lado vale duas vezes uma variável de entrada denominada raio[5]. Portanto, é extremamente importante que a variável raio seja grande o suficiente para que, no próximo quadro analisado, o animal não tenha se deslocado para além do quadrado definido por ele. Assim, o programa novamente localiza o centro do animal, indo analisar, no próximo quadro, a área do quadrado centrada nestas novas coordenadas X e Y, e assim por diante.

Uma vez sendo capaz de registrar a localização do animal em cada quadro, é possível então o obter o valor do deslocamento empregando-se o teorema de Pitágoras. Ainda, sabendo o espaçamento de tempo entre os quadros, também é possível obter estimativas para valores de velocidade, aceleração, ou outras derivadas de ordem superior que possam vir a interessar.

Existem vários outros dados de interesse em pesquisa comportamental que podem ser obtidos a partir da informação da posição do animal nos diferentes tempos. Entre elas, destaco a possibilidade de registrar toda a trajetória do animal durante o período da análise, bem como a possibilidade de se determinar quanto tempo a cobaia fica em determinadas zonas da arena. Esta última rotina tem

[4] Por isso, é importante, durante a realização das gravações dos vídeos, estabelecer um número de aquisição de quadros por segundo igual em todos os filmes do experimento. Tipicamente, temos utilizado quatro quadros por segundo. Além disso, o programa pode ser instruído a analisar os quadros num múltiplo qualquer, por exemplo, de dois em dois, de três em três.

[5] Esta variável tem este nome, pois, visualmente, o programa circula o animal em cada quadro analisado, a fim de que o usuário possa ter certeza de que ele está convertendo os dados corretamente. Mas, na realidade, o programa analisa os *pixels* num quadrado no qual este círculo que vemos está perfeitamente contido.

aplicações para, por exemplo, arenas de *plus-maze*, de *Y-maze*, de *water-maze*, quantificação do tempo que o animal gasta explorando objetos (*novelty seeking*), entre outras.

Por fim, cito que o programa, além de sua utilização na pesquisa da presente tese, acabou sendo incorporado como uma metodologia de uso rotineiro em diversos outros experimentos realizados por diferentes pesquisadores do departamento. Além disso, algumas variações do *software* para aplicações específicas comentadas acima já foram realizadas. No capítulo de anexo A.1 desta tese, estão listadas as publicações existentes até o momento que utilizaram o programa, ou uma de suas variações, para obtenção de resultados.

III.1.b A GUANOSINA CONTRABALANÇA O AUMENTO DE LOCOMÇÃO INDUZIDO PELO ANTAGONISTA NMDA MK-801

No capítulo II.1.b, foi mostrado o resultado de que a guanosina é capaz de contrabalançar o aumento de locomoção induzido pelo antagonista NMDA MK-801, enquanto que, nas condições desta experiência, não apresentou nenhum efeito sobre o aumento de locomoção induzido por anfetamina e por cafeína.

A hipótese que se levantou para explicar tal resultado foi embasada nos trabalhos do grupo demonstrando que a guanosina estimula a captação de glutamato pelo astrócito (Frizzo et al, 2001, 2002, 2003) – principal mecanismo de remoção do glutamato da fenda sináptica –, junto com a possível dependência glutamatérgica da ação de antagonistas NMDA (Moghaddam et al, 1997; Moghaddam e Adams, 1998).

Embora pareça um pouco paradoxal que antagonistas glutamatérgicos exerçam seus efeitos por um aumento de atividade glutamatérgica, tem sido mostrado que, de fato, muitas das ações dos antagonistas de receptores glutamatérgicos NMDA parecem ser devidas a um aumento da liberação de glutamato em algumas regiões cerebrais, o que resultaria numa maior ativação de receptores não-NMDA (Moghaddam et al, 1997; Moghaddam e Adams, 1998). Uma explicação possível de como isso ocorreria é imaginar que neurônios GABAérgicos não estão sendo mais estimulados por aferências glutamatérgicas via receptor NMDA. Assim, estes neurônios exerceriam uma menor inibição sobre outros neurônios. Em particular, a desinibição de neurônios glutamatérgicos permitiria uma maior liberação de glutamato e subseqüente aumento de sua ação em receptores do tipo AMPA e cainato. Contudo, embora este esquema envolvendo sinapses GABAérgicas fornece uma boa explicação, para o meu conhecimento, ela ainda é especulativa e o verdadeiro mecanismo responsável pelo aumento de glutamato extracelular em algumas regiões cerebrais proporcionado por antagonistas NMDA permanece em estudo.

Neste trabalho, os efeitos do MK-801 e da guanosina também foram investigados na tarefa de alternância espontânea, que é considerado um paradigma para acessar memória de trabalho (Wietrzych et al, 2005). Foi utilizada a mesma dose na qual a guanosina foi efetiva em diminuir o aumento de locomoção induzido por MK-801. Esta parte do trabalho tinha como objetivo mostrar uma certa seletividade de via para o contrabalanço da guanosina perante os efeitos do MK-801 (i.e., sobre as vias responsáveis pela ativação motora). Ou seja, foi uma tentativa de construir um exemplo que mostrasse um efeito do MK-

801 que não era contrabalançado pela guanosina, a fim de ilustrar que nem todos os efeitos de antagonistas NMDA são mediados por ação glutamatérgica. Isto de fato ocorreu, a guanosina não foi capaz de reverter a diminuição de performance causada pelo MK-801 neste paradigma (Figura 4, capítulo II.1.b). Contudo, maiores conclusões não podem ser formuladas a partir destes resultados, uma vez que a guanosina por si só também se mostrou amnésica neste paradigma, resultado este que vai de acordo com resultados prévios obtidos por nosso grupo (Roesler et al, 2000; Vinadé et al, 2003, 2004, 2005).

Embora não tenhamos realizado um teste de catalepsia no presente trabalho ao contrário dos outros trabalhos desta tese que também visaram o *screening* de novos antipsicóticos, é de consenso por parte dos pesquisadores que realizaram pesquisa comportamental utilizando a guanosina que, por inspeção visual, não se nota nenhuma diferença importante de comportamento ou aspecto entre os animais tratados com guanosina e os controles. Em particular, nenhuma postura catatônica foi jamais observada. Um argumento mais objetivo de que a guanosina de fato não causa catalepsia é o de que ela não diminui a locomoção espontânea (Figuras 1,2,3, capítulo II.1.b), o que, como é de se esperar, ocorre com drogas em doses que induzem catalepsia. Ainda, uma vez que a guanosina não interferiu com os efeitos motores da anfetamina, parece bastante improvável que ela possua algum efeito direto sobre o receptor dopaminérgico do tipo D2. De nota, observo aqui que, devido a problemas de solubilidade, a realização de trabalhos empregando doses de guanosina muito maiores do que as utilizadas no presente trabalho é enormemente dificultada.

Uma vez que o uso de antagonistas NMDA tem sido apontado como possivelmente o melhor modelo farmacológico para a esquizofrenia (Ninan e Kulkarni, 1999; O'Neill e Shaw, 1999), um futuro papel para a guanosina como medicação antipsicótica é então questionado. Ainda, conforme discutido acima, parece bastante improvável que a guanosina venha a induzir sintomas de parkinsonismo nos pacientes. Contudo, é observado que sua utilidade potencial como medicação antipsicótica seria visando os sintomas negativos da doença, uma vez que ela não apresentou efeito sobre o aumento de locomoção induzido por anfetamina, que classicamente vem sendo relacionado aos sintomas positivos da doença (Ellenbroek, 1993; Geyer e Ellenbroek, 2003; Kapur e Mamo, 2003). A guanosina apresentaria ainda certas vantagens (como neuroproteção) e desvantagens (como déficits cognitivos) comuns a drogas que interagem com o sistema glutamatérgico, pois, claramente, uma vez que o glutamato constitui o principal neurotransmissor excitatório do SNC, torna-se muito difícil não interferir nos diversos circuitos que ele participa ao se empregar medicações não seletivas a determinados tipos de receptores.

III.1.c FLUNARIZINA E CINARIZINA: DOIS NOVOS ANTIPSICÓTICOS ATÍPICOS?

Nos capítulos II.1.c e II.1.d da presente tese foram apresentados os resultados das investigações envolvendo flunarizina e cinarizina, respectivamente. De uma forma geral, os resultados obtidos nos dois trabalhos são muito semelhantes: ambas as drogas apresentaram perfis de antipsicóticos atípicos nos modelos animais, isto é, foram capazes de reverter o aumento de locomoção

induzido por MK-801 e por anfetamina em doses que não causaram efeitos colaterais extrapiramidais importantes.

Conforme previamente exposto, ambas as medicações foram relatadas como capazes de induzir parkinsonismo, principalmente em pacientes idosos (Fernandez et al, 1988; Garcia-Ruiz et al, 1992; Brücke et al, 1995; Daniel e Mauro, 1995). Como esperado, foi posteriormente demonstrado que estas drogas são de fato antagonistas de receptores D2 dopaminérgicos, apresentando moderada potência (Kariya et al, 1995; Brücke et al, 1995). Em relação ao receptor D2, a cinarizina é menos potente do que a flunarizina, e ambas as drogas são menos potentes do que a olanzapina (Kariya et al, 1995). Este fato por si só já gera a expectativa de que tanto a flunarizina quanto a cinarizina possam vir a ser dois novos antipsicóticos atípicos, uma vez que há teorias para a atipicalidade que postulam que a baixa afinidade ao receptor D2 é o principal fator responsável pelo perfil atípico[6] (Kapur e Seeman, 2001). Além deste antagonismo D2 dopaminérgico, é possível que o fato de serem drogas bloqueadoras de canal de cálcio – e também possivelmente agirem em canal de sódio (Velly et al, 1987; Pauwels et al, 1991) – possa constituir uma vantagem farmacológica extra para estes compostos.

O relato de casos de parkinsonismo induzido por flunarizina e cinarizina está longe de constituir preocupação importante para o emprego destes fármacos na clínica médica como antipsicótico, uma vez que tal sintomatologia foi descrita principalmente em pacientes idosos (Brücke et al, 1995), os quais sabidamente

[6] Contudo, note que esta característica de baixa potência sobre o receptor D2 como sendo realmente necessária para a geração de um perfil atípico é questionada na presente tese; ver capítulos II.2.a e III.1.d.

apresentam menor número de neurônios dopaminérgicos na SNPc e conseqüente diminuição de tônus dopaminérgico nigroestriatal (Brücke et al, 1995). Além disso, tal efeito colateral foi geralmente relatado com flunarizina após meses de tratamento numa posologia inadequada à sua longa meia-vida plasmática, levando à acumulação dos fármacos no corpo. Nestas mesmas condições, é sabido que muitos antipsicóticos atípicos, como a olanzapina, também podem induzir parkinsonismo.

Conforme previamente comentado, estes dois compostos apresentam a vantagem de já existência no mercado, sendo geralmente bem tolerados pela maioria dos pacientes. Além disso, a existência de formulações líquidas também pode vir a ser útil para ajudar na aderência ao tratamento. De nota, uma característica importante que distingue a flunarizina da cinarizina – e também de outros antipsicóticos – é a sua grande meia-vida plasmática, a saber, geralmente reportada como sendo maior do que duas semanas. Esta característica da flunarizina pode também vir a auxiliar bastante na aderência ao tratamento. Por outro lado, como será discutido adiante[7], a curta meia-vida da cinarzina pode também vir a ser uma vantagem perante outros antipsicóticos.

Por fim, ressalvo mais uma vez outra importante vantagem que faz com que a flunarizina e a cinarizina se tornem bastante atrativas para serem testadas na prática clínica: ambas as drogas apresentam preços extremamente baixos quando comparados aos preços atuais dos antipsicóticos de segunda geração. Em alguns casos, o preço destas medicações podem chegar a 10-40 vezes menos. Acredito

[7] Ver capítulos II.2.a e III.1.e.

que este fato é um dos principais responsáveis pelo tom de entusiasmo presente nestes dois trabalhos.

III.1.d ANTIPSICÓTICOS ATÍPICOS vs TÍPICOS: UMA SIMPLES QUESTÃO DE POSOLOGIA?

Na primeira parte do capítulo II.2.a, inicialmente foi revisada a noção corrente na literatura da existência de uma janela terapêutica de ocupação dopaminérgica na qual existe ação efetiva antipsicótica sem a geração dos EPS. Conforme revisado, uma ocupação de receptores D2 dopaminérgicos estriatais entre os níveis de 65-78% parece ser o alvo almejado[8]. O que foi argumentado a seguir é que o conceito de janela terapêutica de ocupação implica na existência de um conceito de janela terapêutica para a concentração dos antipsicóticos. Ainda, como mostrado pela equação 1.2 e na Figura 1 do capítulo II.2.a, o tamanho da janela terapêutica de concentração do fármaco possui uma dependência linear com o K_d, ou seja, se um determinado fármaco possui um K_d cinco vezes maior do que outro, irá, conseqüentemente, apresentar uma janela terapêutica de concentração também cinco vezes maior. Como é sabido, muitos antipsicóticos atípicos apresentam baixa afinidade pelo receptor D2 (alto K_d). Por exemplo, a afinidade da olanzapina é cerca de 10 vezes menor do que a do haloperidol, a da clozapina cerca de 125 vezes menor, e o da quetiapina cerca de 220 vezes menor (Seeman et al, 1997).

[8] Na realidade, a janela de ocupação utilizada nesta tese é conservadora comparada à utilizada pela maioria dos pesquisadores da área, que considera a janela como sendo 60-80%.

Baseado nisso, como discutido no trabalho, poderia se pensar numa primeira instância que esta seria uma explicação possível para a atipicalidade: os antipsicóticos atípicos possuem janela terapêutica de concentração muito maior do que os típicos, fazendo com que seja muito mais fácil acertar na dosagem do que os típicos. Inclusive, este é o próprio título da seção do trabalho, ressaltando a importância da afinidade para o tamanho da janela terapêutica de concentração.

Contudo, numa inspeção mais cuidadosa, definindo uma espécie de "índice terapêutico" como sendo a razão entre a concentração máxima e a mínima da janela, percebemos que ele é o mesmo (=1.9) para todos os antipsicóticos, e, em particular, é independente do K_d. Observo aqui que o trabalho apresentado no capítulo II.2.c sugerindo um algoritmo para redução de dose de antipsicótico em pacientes apresentando EPS é um corolário direto deste resultado. Retomando a discussão, é argumentado a seguir no trabalho que o conceito de tamanho de janela terapêutica de concentração é relativo, uma vez que a escala é arbitrária (por exemplo, 1 mg/mL = 1.000.000 ng/mL). Ou seja, através de um fracionamento de doses adequado, percebemos que a dificuldade de se acertar na janela terapêutica de concentração é a mesma para qualquer antipsicótico, uma vez que o "índice terapêutico" terapêutico é o mesmo. Notamos ainda que, embora as dosagens absolutas dos antipsicóticos atípicos sejam em geral maiores do que as dos antipsicóticos típicos, quando comparados em relação a suas afinidades ao receptor D2, os antipsicóticos atípicos são prescritos em doses mais baixas do que os típicos[9]. Além disso, por questões culturais de posologia, cada vez que se

[9] Por exemplo, uma vez que o K_d da quetiapina é cerca de 220 vezes maior do que o do haloperidol, 10 mg de haloperidol seria equivalente a 2.200 mg de quetiapina. Contudo, estas

aumenta um antipsicótico atípico, a mudança que este aumento ocasiona no nível de ocupação dopaminérgica é geralmente menor do que o aumento causado por um antipsicótico típico. Por exemplo, cada aumento da dosagem de clozapina em 25 mg causa uma mudança no nível de ocupação D2 cerca de 5 vezes menor do que seria causado pelo aumento em 1 mg da dosagem de haloperidol (Tabela 1, capítulo II.2.a).

Ou seja, basicamente está sendo postulado que muito da diferença entre o perfil típico e o perfil atípico em relação à geração de EPS se deve a simples questões de posologia destas medicações. De acordo com esta hipótese, os antipsicóticos de primeira geração induzem mais EPS porque estão sendo prescritos atualmente em doses proporcionalmente maiores em relação a suas afinidades, gerando conseqüentemente um nível de bloqueio dopaminérgico maior do que as doses correntemente empregadas com os antipsicóticos atípicos. Além disso, outro erro cultural de posologia seria o de que, atualmente, o aumento de dose das medicações de primeira geração causa mudanças muito mais bruscas no nível de bloqueio do que as causadas pelo aumento de dose de medicações de segunda geração, que é feito de forma mais gradual em relação às suas afinidades.

Existem alguns fatos que corroboram com esta hipótese. Acredito que o exemplo mais ilustrativo é o caso da risperidona. A risperidona possui afinidade ao receptor dopaminérgico D2 semelhante à do haloperidol (Seeman et al, 1997). Logo que foi lançada no mercado, devido à indução de EPS nos pacientes, o que

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comparações diretas são perigosas para interpretações, uma vez que o K_d é determinado localmente, e as drogas podem diferir bastante quanto à capacidade de penetração no SNC.

fez alguns a considerarem como antipsicótico típico. Contudo, posteriormente, a sua recomendação de dose para o tratamento foi reduzida, o que fez com que ela induzisse muito menos EPS. Desde então, ela vem sendo considerada como um antipsicótico atípico. De nota, um estudo que obteve o mesmo nível de ocupação estriatal D2 dopaminérgica com risperidona e haloperidol mostrou que a chance de induzir EPS era semelhante entre as duas drogas (neste estudo, 42% para a risperidona e 29% para o haloperidol), e concluiu que o bloqueio 5-HT_{2A} não protegia contra EPS[10] (Knable et al, 1997).

Outro exemplo recente é o da perfenazina, que é considerado um antipsicótico típico, mas que foi demonstrado ser igualmente efetivo e não induzir EPS quando adequadamente empregado em doses que causem nível de bloqueio dentro da janela de ocupação (Talvik et al, 2004).

Com relação ao haloperidol, uma meta-análise publicada em 2002 mostrou que o tratamento com doses de 3 –7.5 mg/dia é tão efetivo quanto o de doses maiores (7.5 –15 mg/dia) induzindo menos EPS e apontou para a necessidade de se estudar mais o efeito de doses mais baixas (1.5 – 3 mg/dia) (Waraich et al, 2000). Curiosamente, em 1996, já havia sido mostrado, num estudo avaliando 7 pacientes por PET, que 2 mg/dia de haloperidol causa um bloqueio D2 estriatal entre 53-74% (Kapur et al, 1996). Ainda, neste estudo, cinco pacientes melhoraram substancialmente, enquanto que nenhum apresentou EPS (Kapur et al, 1996). Mais recentemente, em um ensaio randomizado duplo cego em 40 pacientes tratados para o primeiro episódio de psicose, foi mostrado que esta

[10] Risperidona possui alta afinidade ao receptor 5-HT_{2A}.

mesma dosagem (2 mg/dia) de haloperidol é tão efetiva quanto 8 mg/dia, com a vantagem de melhor tolerabilidade (Oosthuizen et al, 2004).

De nota, foi mostrado que o excessivo bloqueio estriatal dopaminérgico é correlacionado com os sintomas depressivos presentes em pacientes esquizofrênicos (Bressan et al, 2002). Ou seja, é possível que os antipsicóticos típicos sejam menos eficazes em tratar os sintomas negativos da doença em relação aos atípicos justamente por ocasionarem um maior bloqueio dopaminérgico.

Cabe citar que por essa conjectura que está sendo apresentada, todo antipsicótico atualmente considerado como atípico também pode causar EPS, bastando, para isso, doses que bloqueiem mais do que 78% dos receptores dopaminérgicos D2 estriatais. Particularmente, acredito que não é correta a noção de que a clozapina e a quetiapina não podem atingir tais níveis de bloqueio. O baixo nível de bloqueio dopaminérgico comumente observados por estas drogas pode ser explicado pelas doses em que eles estão sendo prescritos, bem como o fato de possuírem curta meia-vida[11]. De fato, o emprego de injeção de clozapina em *bolus* em macacos pode causar um nível de bloqueio de 83%, e o curso temporal desta ocupação foi reportado como sendo rápido, durando algumas horas (Suhara et al, 2002). Interessantemente, foi mostrado que a clozapina perde o seu perfil atípico quando a sua afinidade ao receptor D2 é aumentada através de um estudo utilizando a isoclozapina, um isômero da droga que apresenta afinidade 10 vezes maior ao receptor D2, e afinidades semelhantes a outros receptores (5-HT1A, 5-HT2, D1, D4, M1) (Kapur et al, 2002).

[11] Ver discussão III.1.e.

Enfim, o que foi postulado nesta parte do trabalho é que o principal mecanismo de ação que diferencia antipsicóticos típicos dos atípicos pode vir a ser, na realidade, uma simples questão de posologia, ou seja, da existência de regimes de dose adequados nos quais os sintomas possam ser controlados sem a geração de EPS. De acordo, é também postulado que tal regime é em tese possível de ser encontrado para todos os antipsicóticos, mesmo os atualmente considerados como típicos.

III.1.e PODE UMA CURTA MEIA-VIDA EXPLICAR O PERFIL ATÍPICO DE ALGUNS ANTIPSICÓTICOS?

Conforme discutido na seção anterior, na primeira parte do capítulo II.2.a, se trabalhou com a idéia de uma janela terapêutica para a ocupação dopaminérgica, e se atribuiu o perfil típico dos antipsicóticos basicamente como sendo secundário ao seu emprego atual em doses muito elevadas, fazendo com que o bloqueio dopaminérgico atinja níveis acima da janela. Por fim, levantou-se a hipótese de que bastaria considerar melhores regimes de dose para conseguir acertar os níveis de ocupação na janela terapêutica.

Uma idéia intuitiva que estava sendo acreditada era a da necessidade de manter um nível de bloqueio dopaminérgico dentro da janela terapêutica. Interessantemente, trabalhos recentes com a quetiapina mostraram que um bloqueio dopaminérgico transiente nos níveis da janela pode ser igualmente eficaz para o tratamento antipsicótico efetivo, colocando em cheque a necessidade do bloqueio dopaminérgico contínuo para a ação antipsicótica (Kapur et al., 2000b; Tauscher-Wisniewski et al., 2002).

Baseado nestes resultados preliminares, a segunda parte do capítulo II.2.a é voltada inicialmente a mostrar que, após atingir um nível inicial de bloqueio dopaminérgico[12], o parâmetro fundamental que governa o curso temporal do decaimento da ocupação dos receptores é a meia-vida de eliminação do fármaco. Embora este resultado seja intuitivo para muitos pesquisadores, ainda há outros que acreditam que este decaimento seja governado por parâmetros de ligação, como a afinidade, ou mesmo somente o k_{off}. De fato, trata-se de uma grande coincidência que a quetiapina ao mesmo tempo possua um alto valor de k_{off} e uma curta meia-vida, quando comparada aos demais antipsicóticos. E o mesmo parece ocorrer também com a clozapina. De nota, até o momento, há um relato preliminar indicando que a clozapina pode chegar a atingir 80% de bloqueio D2 e depois decair rapidamente (Jones et al, 2000). Uma maneira intuitiva de entender porque que os parâmetros de afinidade sobre o bloqueio não influenciariam no curso temporal do decaimento é imaginar que, mesmo que a droga se desligue rápido de seu receptor (isto é, apresente alto valor de k_{off}), ela ficará disponível na fenda sináptica para se ligar novamente, pelo menos até que seja metabolizada ou difundida, que são processos que estão relacionados com a meia-vida do fármaco.

Ainda, como exposto no capítulo II.2.a, muitos pesquisadores da área chegaram a criar um conceito de meia-vida de ocupação do receptor por parte do antipsicótico (Gefvert et al., 1998; Tauscher et al., 2002; Takano et al., 2004). Este conceito é mal definido, uma vez que o tempo do decaimento da ligação é dependente do nível de bloqueio inicial sendo considerado, como exposto na

[12] "Nível de bloqueio inicial" está sendo utilizado para se referir ao nível de bloqueio atingido no pico de concentração da droga, que constitui o valor máximo de bloqueio a partir do qual começa o decaimento.

equação 2.5 e na Figura 3 do capítulo II.2.a. De fato, o trabalho exposto no capítulo II.2.b, entre outros comentários, chama a atenção para isso. No capítulo de anexo A.2 desta tese, foram colocadas as respostas que este comentário gerou, bem como o trabalho a partir do qual este comentário é baseado[13].

Assim, uma vez se assumindo que o curso temporal de decaimento da ocupação é devido à meia-vida do fármaco, temos como corolário que este parâmetro constitui o principal responsável pela capacidade de provocar bloqueios transientes. A característica de uma curta meia-vida, junto com doses adequadas que não atinjam níveis de bloqueio inicial muito elevado, poderia explicar a ausência de geração de EPS em antipsicóticos como quetiapina e clozapina. Ainda, esta característica pode também explicar recaídas rápidas dos sintomas com a suspensão do tratamento.

Se o bloqueio dopaminérgico transiente for realmente provado como sendo efetivo para o tratamento antipsicótico, a procura de novos antipsicóticos atípicos constituiria da descoberta de antagonistas dopaminérgicos D2 de curta meia-vida.

III.1.f CONSIDERAÇÕES FINAIS

A presente tese apresentou alguns resultados experimentais obtidos das investigações de potenciais novos antipsicóticos atípicos em modelos animais de esquizofrenia, bem como apresentou também alguns *insights* teóricos que podem auxiliar no entendimento do mecanismo de ação responsável pela maior geração de EPS por parte dos antipsicóticos típicos.

[13] Note que a figura apresentada junto com o comentário de Olsson e Farde neste capítulo A.2 de anexo constitui uma das seções da Figura 3 do capítulo II.2.a.

Pode-se perceber um certo antagonismo entre os argumentos apresentados nas discussões da parte experimental com relação aos da parte teórica. De acordo, nas discussões da parte experimental, os conceitos clássicos de divisão de antipsicóticos em atípicos e típicos são utilizados para sugerir que tanto a flunarizina quanto a cinarizina podem vir a ser dois novos fármacos de segunda geração. Por outro lado, em um certo momento, a segunda parte desta tese chega mesmo a sugerir que tal divisão de classificação de antipsicóticos não existe, e que tudo se resumiria a questões culturais envolvidas nas posologias empregadas atualmente com estas medicações. Então, por que a busca de novos antipsicóticos atípicos, tão almejada na primeira parte desta tese, se a própria sugere que é suficiente utilizarmos os antipsicóticos de primeira geração em doses melhores empregadas? Além disso, por que a flunarizina é promovida por ter uma meia-vida longa, se é apontada a potencial importância de uma meia-vida curta para a geração do perfil atípico?

Uma das explicações para este antagonismo é de ordem mais prática, e se deve simplesmente ao fato de que os trabalhos experimentais foram realizados previamente aos trabalhos teóricos. Assim, no período de realização e escrita dos trabalhos experimentais, grande parte dos resultados revisados de literatura e dos conseqüentes *insights* expostos na parte teórica da tese não eram conhecidos. Desta forma, estes trabalhos foram escritos empregando o conceito de divisão clássica de antipsicóticos em atípicos e típicos, uma vez que eram os conceitos utilizados na época.

Uma outra explicação, talvez mesmo a mais correta dentro de uma postura científica, é baseada no fato de que os resultados apresentados na parte teórica

desta tese são altamente especulativos. De maneira que até a sua corroboração ou refutação, não podem ser adotados como verdades. Assim, a parte experimental da tese foi escrita de acordo com os conhecimentos considerados consolidados pela literatura científica, enquanto que a parte teórica se permitiu ousar um pouco mais. De fato, a divisão de antipsicóticos em atípicos e típicos é ainda empregada rotineiramente[14], e pode mesmo ser que permaneça por bastante tempo ainda.

Pelo que foi discutido na tese, percebemos que mais do que um mecanismo de ação em isolado, o perfil atípico é provavelmente secundário a uma série de fatores, que podem mesmo agir em conjunto em algumas drogas[15]. Os que esta tese destacou são os de atingir os níveis de bloqueio dentro da janela terapêutica, bem como a capacidade de alguns fármacos em causar bloqueios intermitentes. Ainda, este último não implica que drogas com meia-vida longa não possam ser bons antipsicóticos. De fato, uma vez utilizados em doses que não ultrapassem o limiar para a geração de EPS, fármacos de longa meia-vida são potencialmente vantajosos na aderência ao tratamento.

Por fim, observo que os resultados apresentados nestes trabalhos se fundamentam numa importância central do receptor D2, a qual, embora também atribuída por outros grupos (Kapur e Remington, 2001; Kapur e Mamo, 2003), gera muita controvérsia e está longe de constituir consenso por parte dos pesquisadores da área. Ainda, é importante ressaltar que foi discutido o possível

[14] Aparentemente, os termos antipsicóticos de primeira e segunda geração têm sido mais empregados nos últimos anos.
[15] Cabe citar que um fator não mencionado nesta tese, mas que certamente é importante na menor indução de EPS, é a atividade anticolinérgica presente em algumas drogas.

mecanismo de ação que diferencia um antipsicótico atípico do típico em relação à geração de EPS, não sendo considerado o mecanismo de ação sobre sintomas negativos e cognitivos.

III.2 CONCLUSÕES

A partir dos resultados expostos nesta tese, as seguintes conclusões podem ser formuladas:

Resultados experimentais[16]

a) A guanosina contrabalança o aumento de locomoção induzido em camundongos por MK-801, enquanto que não apresenta efeito sobre o aumento de locomoção induzido por anfetamina e por cafeína. A guanosina não reverte a piora de performance induzida por MK-801 no teste de alternância espontânea em camundongos, e por si só também causa uma piora de performance neste paradigma. O papel da guanosina como potencial antipsicótico deve ser mais bem estudado.

b) A flunarizina contrabalança o aumento de locomoção induzido em camundongos por MK-801 e por anfetamina em doses que não causam efeitos catalépticos importantes. A flunarizina atenua a piora de performance induzida em camundongos por MK-801 no paradigma do labirinto em T. Estes resultados sugerem um potencial perfil de antipsicótico atípico para a flunarizina e fundamentam seu teste em humanos.

c) A cinarizina contrabalança o aumento de locomoção induzido em camundongos por MK-801 e por anfetamina em doses que não causam efeitos catalépticos importantes. Estes resultados sugerem um potencial perfil de antipsicótico atípico para a cinarizina e fundamentam seu teste em humanos.

[16] Embora não citado explicitamente em cada item, estas conclusões são aplicadas apenas às condições experimentais (doses, via de administração das drogas, etc) empregadas nesta tese.

Resultados teóricos

a) Questões culturais de posologia poderiam explicar a maior indução de EPS por antipsicóticos típicos, devendo ser mais bem investigadas.

b) O curso temporal da ocupação de receptores após um nível de bloqueio inicial obtido pela administração de um fármaco é dependente da meia-vida de eliminação da droga, e não é dependente da sua afinidade ao receptor.

c) O conceito de meia-vida de ocupação do receptor não é bem fundamentado do ponto de vista teórico.

d) Uma curta meia-vida de eliminação do fármaco poderia explicar o perfil atípico por parte de alguns antipsicóticos, devendo ser mais bem investigada.

e) A estratégia de reduzir à metade a dose de medicação antipsicótica em pacientes apresentando EPS é bem fundamentada teoricamente.

f) Estudos teóricos são capazes de gerar *insights* importantes para a pesquisa clínica em neuropsicofarmacologia.

III.3 PERSPECTIVAS

Em relação ao *software* descrito nesta tese, as perspectivas que surgem a partir deste trabalho inicial são as de fazer adaptações no algoritmo já existente do programa, a fim de criar versões que possuam utilidades para aplicações a outros testes comportamentais além da quantificação da locomoção[17]. Uma outra possibilidade de trabalho futuro é o de disponibilizar o programa através da rede mundial de computadores. Assim, pesquisadores de diferentes centros poderiam fazer as filmagens dos experimentos em seus laboratórios, e a conversão dos vídeos para a obtenção dos dados seria efetuada pela internet.

Com relação aos resultados experimentais obtidos usando guanosina, flunarizina, e cinarizina, as perspectivas que naturalmente surgem são as de testar estes compostos na prática clínica, a fim de verificar suas potenciais ações como antipsicóticos. É possível que a guanosina venha a apresentar efeitos clínicos semelhantes aos encontrados com o uso da lamotrigina, um anticonvulsivante inibidor da liberação de glutamato. Contudo, a ausência de estudos prévios empregando a guanosina em humanos torna necessária primeiramente a realização de uma série de testes para verificar sua segurança. Ou seja, neste momento, o uso da guanosina na prática clínica constitui uma perspectiva ainda bastante distante.

Por outro lado, tanto a flunarizina quanto a cinarizina já vêm sendo utilizadas clinicamente há anos, e são consideradas drogas seguras e bem toleradas pela maioria dos pacientes, de maneira que a perspectiva de testá-las

[17] De fato, uma primeira versão modificada do programa já existe. Esta é capaz de quantificar o tempo que o animal gasta ao redor de um objeto colocado no centro da arena, e vem sendo empregada em experiências que avaliam a busca de novidade.

na prática clínica é bem mais próxima. De fato, a avaliação de um destes compostos como potencial medicação antipsicótica já está em andamento. A saber, o Prof. Dr. Diogo Lara está atualmente coordenando um ensaio clínico randomizado duplo cego que visa inicialmente estudar o efeito da flunarizina como antipsicótico e compará-lo ao efeito do haloperidol. Este projeto recebeu verba da fundação Stanley para ser realizado, e possui perspectivas de resultados para o ano de 2006.

Analogamente, em relação aos resultados teóricos, as perspectivas que surgem a partir desta tese são as de testar na prática clínica as hipóteses levantadas. Entre elas, fica a expectativa de virem a testar o algoritmo sugerido para a redução de dose de antipsicótico em pacientes apresentando síndrome extrapiramidal.

A conjectura de que o perfil atípico é simplesmente secundário aos níveis de bloqueio dopaminérgico dentro da janela terapêutica de ocupação, e de que a posologia comumente empregada para os antipsicóticos de primeira geração é inadequada, pode ser testada através de estudos que avaliem antipsicóticos típicos empregados em doses mais baixas do que as usadas atualmente. Além disso, nestes estudos, o aumento de doses quando necessário deverá ser realizado de forma mais gradual do que a empregada hoje em dia.

Fica também a expectativa de virem a estudar a outra conjectura levantada nesta tese atribuindo importância à meia-vida plasmática do antipsicótico na geração do perfil atípico. Para tanto, é necessário encontrar um novo antagonista dopaminérgico D2 apresentando uma meia-vida plasmática curta e testá-lo na prática clínica, usando uma dose que produza níveis iniciais de bloqueio

dopaminérgico semelhantes aos que a clozapina ou a quetiapina causam. Interessantemente, chamo a atenção que a presente tese nos mostrou um fármaco com estas características: a cinarizina, que possui meia-vida de 3 horas.

Além disso, um trabalho teórico a ser realizado num futuro próximo é o de simular o curso temporal de ocupação dopaminérgica secundário aos tratamentos com clozapina e quetiapina, a fim de verificar como ocorre a intermitência do bloqueio dopaminérgico em cada caso. Isto pode vir a gerar informações importantes para a busca de novas drogas, caso o bloqueio transiente seja realmente demonstrado como sendo efetivo para o tratamento antipsicótico.

Um outro trabalho teórico a ser desenvolvido é o de avaliar o erro que existe entre a aproximação de equilíbrio e a real solução analítica da equação da lei de ação das massas, a fim de corroborar ou não o seu emprego freqüente nos modelos de ocupação dopaminérgica central.

Por fim, como uma perspectiva mais ampla e mais de longo prazo, fica o plano de se implantar uma linha de pesquisa dentro da UFRGS que vise fazer estudos teóricos em neurociências, tanto para dar andamento a estas primeiras pesquisas em neuropsicofarmacologia, bem como para iniciar pesquisas em outras áreas. Em 2006 tenho previsto a realização de um pós-doutorado no exterior junto a um grupo de pesquisa em neurociência teórica, onde espero adquirir conhecimentos que ajudem a implementar tal campo de pesquisa quando do meu regresso.

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ANEXOS

A.1 PUBLICAÇÕES RELACIONADAS AO CAPÍTULO II.1.a.

1. Dall'Igna OP, Da Silva AL, Dietrich MO, Hoffmann A, de Oliveira RV, Souza DO, Lara DR. Chronic treatment with caffeine blunts the hyperlocomotor but not cognitive effects of the N-methyl-D-aspartate receptor antagonist MK-801 in mice. Psychopharmacology (Berl). 2003;166:258-63.

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A.2 PUBLICAÇÕES RELACIONADAS AO CAPÍTULO II.2.b

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LETTER TO THE EDITOR

Half-life of receptor occupancy – a meaningless concept

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The interesting paper of Takano et al. (2004) deals with the estimation of brain receptor occupancy from plasma drug concentration values. The letter from Tort et al. (2005) correctly points out that the concept of receptor occupancy half-life is dependent on the initial starting values of receptor occupancy and not on affinity.

The concept of receptor occupancy half-life has been suggested to represent the time for receptor occupancy to reach half of its initial value. The concept has been used in recent efforts to understand differences between time-courses of drug–receptor interaction in brain and drug concentration in plasma. In one study, dopamine D2 receptor occupancy half-life of quetiapine has been compared with its plasma elimination half-life and also with its serotonin 5-HT2 receptor occupancy half-life (Gefvert et al., 1998). In another study, a linear approximation of the time-course for receptor occupancy was applied (Tauscher et al., 2002).

The comments of Tort et al. emphasize the importance of the initial receptor occupancy and stresses that the half-life of receptor occupancy is not a constant but rather a function of the occupancy at time t_0. Interestingly, assuming half initial fractional occupancy ($O_0/2$) at time $T_{\frac{1}{2}}$, equation (3) in Takano et al. (2004) can be solved for $T_{\frac{1}{2}}$ and expressed as:

$$T_{\frac{1}{2}} = -\frac{1}{b}\ln\left(\frac{1-O_0}{2-O_0}\right). \quad (1)$$

Observe that $T_{\frac{1}{2}}$ is independent of affinity (K_D) and only dependent on initial occupancy (O_0) and the plasma concentration elimination constant (b). In Figure 1, $T_{\frac{1}{2}}$ is plotted vs. receptor occupancy at time t_0 for $b = 0.036\ h^{-1}$, the value chosen by Takano et al. (2004). The figure clearly shows that the receptor occupancy half-life is dependent on the initial occupancy value.

A concern is that the concept of receptor occupancy half-life is not explicitly defined in the literature and can easily be confused with the unimolecular dissociation rate constant, k_{off}. We also suggest that an operational definition based on a starting occupancy of 80%, as suggested by Tort et al., has to be avoided. The concept of receptor occupancy half-life is not defined by theory, as is the case with the half-life of a drug in plasma. This is because the time-course of drug–receptor interaction is described by a hyperbolic curve whereas the time-course for plasma drug concentration is described by a mono-exponential curve. It is unclear to us how the operationally defined concept can advance understanding in clinical psychopharmacology. On the contrary, it may fuel the misconception that there is such thing as a constant receptor occupancy half-life that could be compared with, for example, the half-life of drug concentration in plasma. Such a concept would thus run the risk of increasing the confusion on the matter rather than providing clarity. We, therefore, argue that the concept is meaningless and should be dropped altogether.

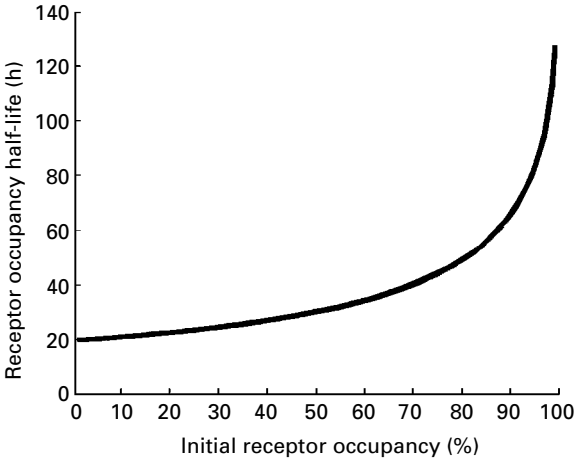

Figure 1. Simulated receptor occupancy half-life as a function of drug plasma concentration elimination constant ($b = 0.036\ h^{-1}$) and initial receptor occupancy (O_0). Note that receptor occupancy half-life is close to the half-life for drug concentration in plasma when initial receptor occupancy is small.

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Statement of Interest

None.

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Gefvert O, Bergstrom M, Langstrom B, Lundberg T, Lindstrom L, Yates R (1998). Time course of central nervous dopamine-D2 and 5-HT2 receptor blockade and plasma drug concentrations after discontinuation of quetiapine (Seroquel) in patients with schizophrenia. *Psychopharmacology* (*Berlin*) *135*, 119–126.

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LETTER TO THE EDITOR

The necessary parameters for estimating the time-course of receptor occupancy

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The dissociation of the kinetics between plasma and the brain has been reported for antipsychotic drugs such as risperidone and olanzapine (Tauscher et al., 2002), and the kinetic profile of antipsychotics at receptor sites has been viewed as an important profile for antipsychotic actions and dosing schedule (Tauscher et al., 2002). Our recent report in this Journal (Takano et al., 2004) showed that the time-course of receptor occupancy could be estimated using plasma pharmacokinetics and drug-affinity parameters.

Tort et al. (2005) and Olsson and Farde (2005) presented interesting views based on our report (Takano et al., 2004), but there seem to be some misunderstandings. Tort et al. used equation (2) in their letter and simulated a curve that is different from the curve simulated using equation (2): $C = me^{-bt}$ in our report. Importantly, 'm' in equation (2) is a value derived from the measured plasma concentration data, whereas Tort et al. seem to assume that 'm' in our equation (2) is related to the ED_{50} value, which is a parameter for in-vivo affinity. However, ED_{50} is independent of the plasma concentration parameter m.

Tort et al. (2005) and Olsson and Farde (2005) pointed out that the value for 'the half-life of receptor occupancy' is dependent on the initial receptor occupancy (O_0) and not on the affinity of the drug. Although we used 'the half-life of receptor occupancy' as an index for the time-course of receptor occupancy, the proposed concept of half-life is not the same as that used for the plasma concentration. As we mentioned in our report, the time- course of receptor occupancy is not an exponential or linear function (Takano et al., 2004). The index 'the half-life of the receptor occupancy' is dependent on the initial occupancy value, but it needs to be pointed out that the initial occupancy value itself is a derivative of the ED_{50} value as can be seen from equation (3) in our report: $D_{2,occu} = 100 \times me^{-bt}/(ED_{50} + me^{-bt})$ (Takano et al., 2004)

$$O_0 = 100 \times m/(ED_{50} + m), \quad (4)$$

where O_0 is the receptor occupancy at time 0, which was defined as the initial occupancy by Olsson and Farde (2005).

As seen from equation (3), the time-course of receptor occupancy is determined by four parameters (ED_{50}, m, b and t). As suggested in our simulation study, the half-life of receptor occupancy ($R_o T_{\frac{1}{2}}$) can be operationally defined as the time required to reach half of the initial receptor occupancy value. From equation (3), the half-life of receptor occupancy ($R_o T_{\frac{1}{2}}$) can be expressed as follows:

$$R_o T_{\frac{1}{2}} = -\frac{1}{b} \times \ln(1/(m/ED_{50} + 1)), \quad (5)$$

From equation (5), three parameters (ED_{50}, m and b), namely, in-vivo affinity and plasma-concentration data, are keys in calculations of the half-life of receptor occupancy. Using equation (4), equation (5) can also be expressed as

$$R_o T_{\frac{1}{2}} = -\frac{1}{b} \times \ln((100 - O_0)/(200 - O_0)). \quad (6)$$

Although our definition [equation (5)] and the definition of Olsson and Farde (2005) [equation (6)] look different, these two definitions are essentially the same.

In conclusion, the time-course of receptor occupancy as described using initial occupancy and the magnitude of change (see Figure 3 in Takano et al., 2004) is dependent on in-vivo affinity, plasma-concentration kinetics and time (ED_{50}, m, b and t). The time-course of receptor occupancy is a consequence of a complex series of conditions. The suggested index 'half-life of receptor occupancy' is one aspect of the concept. It is the entire time-course of receptor occupancy that serves as the fundamental data in investigations of drug dynamics in the brain.

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Statement of Interest

None.

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ARTICLE

Estimation of the time-course of dopamine D_2 receptor occupancy in living human brain from plasma pharmacokinetics of antipsychotics

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Abstract

Although the kinetic profile of antipsychotics at dopamine D_2 receptor sites has been suggested to be important for antipsychotic action and dosing schedule, the kinetic profiles of the respective antipsychotic drugs in the brain have not yet been clearly defined. We aimed to estimate the time-course of dopamine D_2 receptor occupancy from plasma pharmacokinetics and the apparent in-vivo affinity parameter (ED_{50}; concentration required to induce 50% occupancy). Dopamine D_2 receptor occupancies and plasma concentrations of risperidone were measured in five patients with schizophrenia using positron emission tomography with [^{11}C]FLB 457. Measured dopamine D_2 occupancies were compared with those estimated from plasma kinetics and in-vivo ED_{50}. The time-course of dopamine D_2 receptor occupancy was simulated with altered plasma kinetics or apparent in-vivo affinity parameters of the drug. Mean half-life of dopamine D_2 receptor occupancy of risperidone was 80.2 h while that of the plasma concentration was 17.8 h. Dopamine D_2 receptor occupancy estimated from plasma pharmacokinetics and in-vivo ED_{50} was within 1 S.D. of the mean measured occupancy. When the ED_{50} value was changed to one-tenth and 10-fold, the simulated half-life of receptor occupancy changed to 117.6 h and 27.3 h respectively. Using plasma pharmacokinetics and in-vivo ED_{50}, the time-course of receptor occupancy could be calculated. Simulation of drug kinetics at receptors would provide useful information for the evaluation of antipsychotics.

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Key words: Antipsychotics, dopamine D_2 receptor, occupancy, pharmacokinetics, positron emission tomography.

Introduction

The application of positron emission tomography (PET) and single photon emission computed tomography (SPECT) to the receptor-imaging field has made it possible to measure the dopamine D_2 receptor occupancy with antipsychotic drugs (Bench et al., 1993; Bigliani et al., 1999; Farde et al., 1988, 1990). The clinical effect of antipsychotic drugs has been reported to be associated with a striatal dopamine D_2 receptor occupancy level higher than 70% (Kapur et al., 2000; Nordström et al., 1993).

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Relatively rapid kinetics of dopamine D_2 receptor occupancy with transient high occupancy were shown in some antipsychotics such as quetiapine (Kapur et al., 2000), and the kinetic profile at receptors has been suggested to represent an important profile of antipsychotic action (Kapur et al., 2000; Seeman and Tallerico, 1999; Suhara et al., 2002a). Plasma concentrations of antipsychotics have been relied upon as objective indicators of drugs in vivo, and plasma pharmacokinetics have been used for determining rational dosage regimens. On the other hand, significant dissociation of antipsychotic kinetics between plasma and the brain has been reported, and the conventional approach of relying on plasma elimination half-lives for dosing schedules of antipsychotics has been questioned (Tauscher et al., 2002). Although the kinetic profile at the dopamine D_2 receptor site has

been increasingly focused upon, those of the individual antipsychotic drugs still need to be clarified, and they can be expected to be of great value for dosing schedules in clinical situations as well as for drug developments. In addition, cortical regions have been suggested to be the important sites for antipsychotic action, especially for the so-called atypical antipsychotics (Lidow et al., 1998; Pilowsky et al., 1997).

We aimed to estimate the time-course of dopamine D_2 receptor occupancy by risperidone by a combination of the values of the present plasma pharmacokinetics and the in-vivo ED_{50} value (concentration required to induce 50% occupancy) calculated from our previous data; and to simulate the time-course of dopamine D_2 receptor occupancy induced by antipsychotics with different pharmacological profiles and changing pharmacokinetic and apparent in-vivo affinity parameters (ED_{50}).

Methods

Patients

Five male patients (age range 24–45 yr; mean ± S.D., 35.2 ± 9.6 yr) meeting the DSM-IV criteria for schizophrenia participated in this study. The patients were recruited from the outpatient units of Tokyo Medical and Dental University affiliated psychiatric hospitals in the Tokyo and Chiba prefectures in Japan. They had received risperidone for more than 7 months without other medication. Four were maintained on 4 mg and one on 6 mg risperidone once every night.

After description of the study, written informed consent was obtained from all patients. This study was approved by the Ethics and Radiation Safety Committee of the National Institute of Radiological Sciences, Chiba, Japan.

Radioligand

The precursors for the synthesis of [^{11}C]FLB 457 were kindly supplied by Astra Arcus (Sodertalje, Sweden). [^{11}C]FLB 457 was synthesized by *O*-methylation of the corresponding precursors with [^{11}C]methyliodide with high specific radioactivity, which was obtained by a reduction of [^{11}C]CO_2 with $LiAlH_4$ in an inert atmosphere with specially designed equipment (Halldin et al., 1995; Suzuki et al., 1999). The radiochemical purities were more than 95%.

PET procedure

Dynamic scans were performed for 80 min using ECAT EXACT HR+ (CTI-Siemens, Knoxville, TN, USA) immediately after the bolus injection of 155.0–238.7 (mean ± S.D., 207.7 ± 28.5) MBq of [^{11}C]FLB 457 with high specific radioactivities (139.3–394.8 GBq/μmol; mean ± S.D., 237.6 ± 69.5 GBq/μmol).

MRIs were acquired on Gyroscan NT (Phillips Medical Systems, Best, The Netherlands) (1.5 T) to obtain T1-weighted images of the brain.

On the day before the first PET scan (day 0), the patients stopped taking risperidone that night. On the day of the first PET scan (day 1), the patients took breakfast around 07:00 hours and took their usual daily dose of risperidone orally at 10:00 hours. The first PET scan was performed at 15:00 hours (5 h post-risperidone). The second PET scan was performed at 10:00 hours on the next day (day 2) (24 h post-risperidone). The third PET scan was performed at 15:00 hours on the following day (day 3) (53 h post-risperidone). Blood samples were taken to measure the concentrations of risperidone and 9-OH-risperidone just before and after each PET scan.

Data analysis

All emission scans were reconstructed with a Hanning filter cut-off frequency of 0.4. The temporal cortex, the area for calculating ED_{50} in the previous study (Yasuno et al., 2001), was chosen for the region of interest (ROI). Following the previous study (Yasuno et al., 2001), circular ROIs were set at 8 mm diameter to cover 7 slices for the cerebellum and 10–11 slices for the temporal cortex on the PET images of summated activity for 80 min with reference to the individual MR images and Brain Atlas. The average values of right and left ROIs were used to increase the signal–noise ratio for the calculations. Quantification was performed using a three-parameter simplified reference tissue model (Lammertsma, 1996). The cerebellum was used as the reference tissue because of its negligible density of dopamine D_2 receptors (Suhara et al., 1999). This model allows the estimation of binding potential (BP), which was defined as the ratio of receptor density (B_{max}) to dissociation constant (K_d).

The occupancy of risperidone at the dopamine D_2 receptor was estimated using the following equation:

$$Occ = (BP_{baseline} - BP_{drug}) \times 100 / BP_{baseline},$$

where Occ is the receptor occupancy, $BP_{baseline}$ is the BP in the drug-free state, and BP_{drug} is the BP of the patient on the drug. In this study, because we could not perform PET scans in a drug-free state for four of the patients, the age-corrected mean BP in the temporal cortex ($BP = -0.0245 \times age + 2.474$) of 11 drug-naive patients with schizophrenia (age range 19–40 yr;

Table 1. Characteristics of the patients, the time-course of dopamine D_2 receptor occupancy, and the half-life of plasma concentration and dopamine D_2 receptor occupancy of risperidone (Ris.)

Patient no.	Age (yr)	Ris. (mg/d)	D_2 receptor occupancy (%)			Half-life (h)	
			5 h	24 h	53 h	Plasma concentration	D_2 receptor occupancy
1	24	4	81.1	55.6	36.5	11.5	45.5
2	39	4	69.1	62.6	41.1	16.9	62.0
3	42	4	86.2	71.7	57.2	17.7	73.8
4	45	4	87.7	82.6	70.9	23.7	127.3
5	26	6	85.6	77.3	62.8	19.3	92.2
Mean (±S.D.)	35.2 (±9.6)	4.4 (±0.9)	82.0 (±7.6)	70.0 (±10.9)	53.7 (±14.5)	17.8 (±4.4)	80.2 (±31.4)

mean ± S.D., 28.1 ± 7.9 yr) reported in our previous study (Suhara et al., 2002b), was used as $BP_{baseline}$. Individual $BP_{baseline}$ was used for one patient with 4 mg risperidone (patient 2 in Table 1), who had been drug-free for 15 months at the time of the baseline PET scan. Dopamine D_2 receptor occupancies at three time-points were fitted to a linear regression, that can be described by

$$y = o + a \times t,$$

where o is the estimated maximal receptor occupancy at 0 h (Tauscher et al., 2002). Time to reach half of the estimated maximal receptor occupancy was defined as $T_{\frac{1}{2}}$ of receptor occupancy. The R^2 values of linear regression analysis ranged from 0.95 to 0.99.

Plasma concentration of risperidone

The plasma concentrations of risperidone and its active metabolite, 9-OH-risperidone, were determined by HPLC, and their sum was used as the plasma concentration of risperidone because they both have a similar pharmacological profile (Dollery, 1999). The range of the observation time (5–53 h) was considered to equal the elimination phase of the drug, since the plasma concentration of risperidone was reported to reach a peak within 2 h of its oral administration (Dollery, 1999), and oral absorption of risperidone was reportedly not significantly affected by food (Dollery, 1999). The time-course of the plasma concentration was fitted to one-exponential function (Gefvert et al., 1998; Tauscher et al., 2002). The time required to reach half of the plasma concentration of risperidone was defined as $T_{\frac{1}{2}}$ of plasma concentration. The R^2 values of the exponential fitting ranged from 0.95 to 0.99.

Simulation study

A simulation study was performed to estimate the time-course of dopamine D_2 receptor occupancy from the plasma pharmacokinetics and in-vivo ED_{50} value.

The relationship between dopamine D_2 receptor occupancy and plasma concentration of antipsychotics was expressed by the following equation (Fitzgerald et al., 2000; Kapur and Remington, 1996):

$$D_{2,occ} = 100 \times D/(ED_{50} + D), \quad (1)$$

where $D_{2,occ}$ is dopamine D_2 receptor occupancy, D is the concentration of the drug in proximity to the dopamine D_2 receptor, and ED_{50} is the concentration required to induce 50% occupancy.

The decrease in plasma concentration was expressed by the following equation (Gefvert et al., 1998; Tauscher et al., 2002):

$$C = m e^{-bt}, \quad (2)$$

where C is the plasma concentration, m is the estimated maximal plasma concentration at 0 h, b is a constant, and t is the time after the drug administration.

Plasma concentration was used as a functional surrogate of D (Fitzgerald et al., 2000; Kapur and Remington, 1996). Therefore $C = D$.

Combining equations (1) and (2),

$$D_{2,occu} = 100 \times m e^{-bt}/(ED_{50} + m e^{-bt}), \quad (3)$$

The ED_{50} value in the temporal cortex calculated from our previous data was 6.4 ng/ml (Yasuno et al., 2001).

The plasma concentrations of the five patients were averaged at each time-point and the mean plasma

concentration was fitted to one-exponential function and used in this simulation:

$$\text{plasma concentration (ng/ml)} = 45.0 \times e^{-0.036t} \quad (R^2 = 0.969)$$

($T_{\frac{1}{2}}$ of the mean plasma concentration = 19.3 h).

Dopamine D_2 receptor occupancy derived from this equation with mean plasma concentration was compared to the mean of occupancy calculated from the present consecutive PET data. In addition, dopamine D_2 receptor occupancy estimated from individual plasma data was also compared to measured occupancy from individual PET data by repeated-measures ANOVA.

The time-course of dopamine D_2 receptor occupancy was simulated by varying the ED_{50} value of risperidone from one-tenth to 10-fold (0.1, 0.2, 0.5, 2, 5, 10) of 6.4 ng/ml with fixed plasma kinetics (plasma concentration = $45.0 \times e^{-0.036t}$). The time-course of dopamine D_2 receptor occupancy was also simulated by varying $T_{\frac{1}{2}}$ of the plasma concentration from one-tenth to 5-fold (0.1, 0.2, 0.5, 2, 5) of $T_{\frac{1}{2}}$ of the mean plasma concentration of five patients with fixed maximal plasma concentration (45.0 ng/ml) and ED_{50} value (6.4 ng/ml). The time required to reach half of the receptor occupancy from simulated time 0 was defined as simulated $T_{\frac{1}{2}}$ of receptor occupancy.

Results

The half-life ($T_{\frac{1}{2}}$) of dopamine D_2 receptor occupancy of the five patients varied from 45.5 to 127.3 h, with a mean value of 80.2 ± 31.4 h (Table 1). The $T_{\frac{1}{2}}$ of the plasma concentration of risperidone was 11.5–23.7 h, with a mean value of 17.8 ± 4.4 h (Table 1). The patient with the shortest $T_{\frac{1}{2}}$ of dopamine D_2 receptor occupancy had the shortest $T_{\frac{1}{2}}$ of plasma concentration, and the patient with the longest $T_{\frac{1}{2}}$ of dopamine D_2 receptor occupancy had the longest $T_{\frac{1}{2}}$ of plasma concentration (Table 1). Figure 1 shows the time-courses of dopamine D_2 receptor occupancy and plasma concentration of patient 3 with the nearest $T_{\frac{1}{2}}$ to the mean value.

The estimated dopamine D_2 receptor occupancies from the mean plasma pharmacokinetics of the five patients (plasma concentration (ng/ml) = $45.0 \times e^{-0.036t}$) and the in-vivo ED_{50} value (6.4 ng/ml) were 85.4% at 5 h, 74.6% at 24 h and 50.5% at 53 h, which were within 1 S.D., respectively, of the mean dopamine D_2 receptor occupancies of the five patients (82.0 ± 7.6% at 5 h, 70.0 ± 10.9% at 24 h and 53.7 ± 14.5% at 53 h).

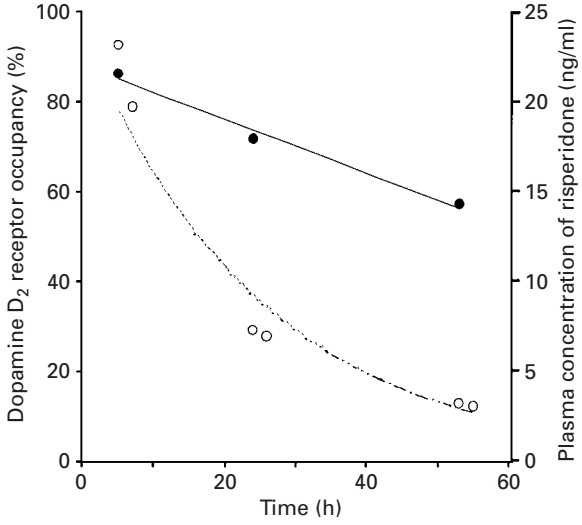

Figure 1. Time-course of dopamine D_2 receptor occupancy in the temporal cortex (●) and the plasma concentrations (○) after taking 4 mg risperidone (patient 3). The sum of the plasma concentrations of risperidone and 9-OH-risperidone was used as the plasma concentration of risperidone. The $T_{\frac{1}{2}}$ of plasma concentration (17.7 h) was shorter than that of dopamine D_2 receptor occupancy (73.8 h).

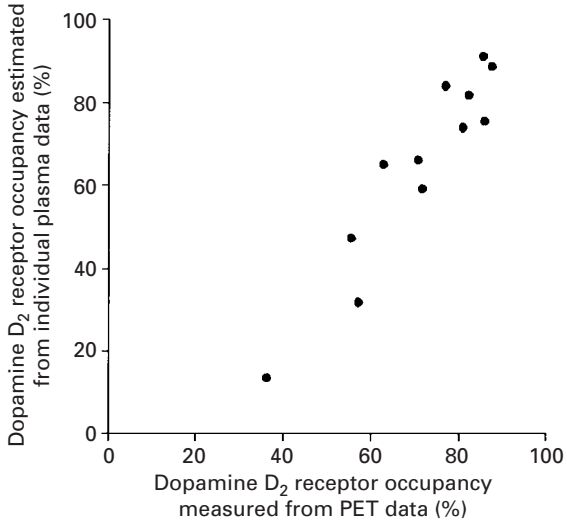

Figure 2. The relationship between the dopamine D_2 receptor occupancy measured from PET data and the dopamine D_2 receptor occupancy estimated from individual plasma data.

Figure 2 shows the relationship between the estimated occupancies from individual plasma data and the individual measured dopamine D_2 receptor occupancies. The estimated occupancies were not significantly different from the measured occupancies ($p > 0.05$).

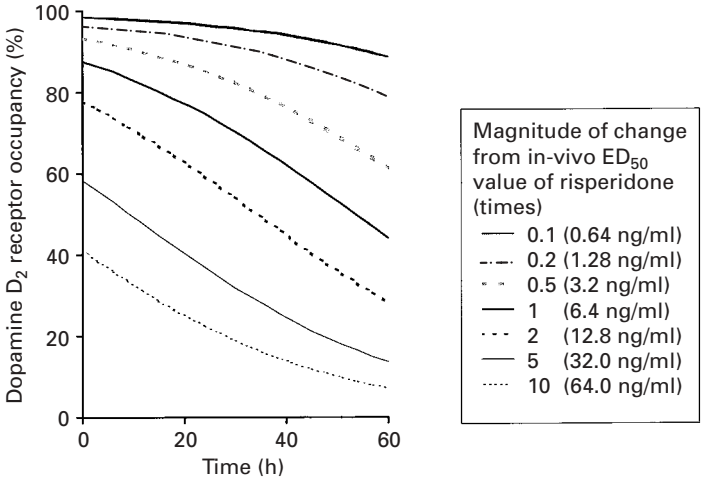

Figure 3. Effect of ED_{50} value change on the time-course of simulated dopamine D_2 receptor occupancy. Values are the magnitudes of change from the ED_{50} value of risperidone (6.4 ng/ml). Estimated dopamine D_2 receptor occupancy increased and the slope of the curves became gentler as the ED_{50} value became smaller.

In the simulation study, when the ED_{50} value was changed to one-tenth (0.64 ng/ml) with fixed plasma pharmacokinetics [plasma concentration (ng/ml) = $45.0 \times e^{-0.036t}$], the simulated dopamine D_2 receptor occupancy was 98.6% at 0 h and 91.0% at 53 h, and the simulated $T_{\frac{1}{2}}$ of dopamine D_2 receptor lengthened to 117.6 h (Figure 3). When ED_{50} was increased to 10-fold (64 ng/ml) with fixed plasma pharmacokinetics, the simulated dopamine D_2 receptor occupancy became 41.3% at 0 h, and 9.3% at 53 h, and the simulated $T_{\frac{1}{2}}$ of dopamine D_2 receptor occupancy shortened to 27.3 h (Figure 3). The simulated $T_{\frac{1}{2}}$ of the time-course of dopamine D_2 receptor occupancy became longer as ED_{50} became smaller, and vice versa.

When the $T_{\frac{1}{2}}$ of the plasma concentration was changed to one-tenth (1.9 h) with fixed ED_{50} (6.4 ng/ml) and the estimated maximal plasma concentration, the simulated dopamine D_2 receptor occupancy was 87.6% at 0 h and 0% at 53 h, and the simulated $T_{\frac{1}{2}}$ of dopamine D_2 receptor occupancy shortened to 6.0 h. When the $T_{\frac{1}{2}}$ of the plasma concentration was changed to 5-fold (96.3 h), the simulated dopamine D_2 receptor occupancy became 87.6% at 0 h and 82.7% at 53 h, and the $T_{\frac{1}{2}}$ of dopamine D_2 receptor occupancy lengthened to 302.2 h.

Discussion

In this study, we demonstrated that the time-course of dopamine D_2 receptor occupancy by various antipsychotics could be estimated from the combination of the plasma pharmacokinetics data and the apparent in-vivo affinity parameter (ED_{50}). The estimated time-course of dopamine D_2 receptor occupancy from the mean pharmacokinetics data and the in-vivo ED_{50} value fitted well with the data from the consecutive PET scans of our patients. Since consecutive PET scans for each antipsychotic drug are not readily performed in routine clinical situations, this estimation of the time-course of dopamine D_2 receptor occupancy from plasma pharmacokinetics, with separately measured apparent in-vivo affinity parameter, would be of great value in the clinical setting in terms of both dosing schedule, and drug development and evaluation. For example, the in-vivo ED_{50} value of striatal D_2 receptor occupancy by haloperidol was reported to be approx. 0.51 ng/ml using [^{11}C]raclopride (Kapur et al., 1997). Although data concerning the time-course of dopamine D_2 receptor occupancy by haloperidol is limited, striatal D_2 receptor occupancy in one volunteer was reported to be 92% at 3 h and 76% at 27 h after single oral administration of 7.5 mg (Nordström et al., 1992). From the reported data (Nordström et al., 1992), the $T_{\frac{1}{2}}$ of the plasma concentration of haloperidol was estimated to be approx. 13 h. Using our equation, the simulated dopamine D_2 receptor occupancy by the oral administration of 7.5 mg haloperidol would be 95% at 3 h and 84% at 27 h. Quetiapine, an antipsychotic drug with low affinity for dopamine D_2 receptor, was reported to show 64% occupancy at 2 h after 450 mg of oral administration, and almost no occupancy on striatal D_2 receptor at 24 h (Gefvert et al., 2001; Kapur et al., 2000). The in-vivo ED_{50} value of quetiapine has not been investigated thoroughly, but it was estimated

Table 2. Reported (Rep.) time-course of dopamine D_2 receptor occupancy and estimated (Est.) occupancy values

Drug	Dose (mg)	in-vivo ED_{50} of drug (ng/ml)	Plasma $T_{\frac{1}{2}}$ of drug (h)	Dopamine D_2 receptor occupancy (%)			
					2–3 h	1 day	2 days
Haloperidol	7.5[a]	0.51[b]	13[a]	Rep.	92[a]	76[a]	–
				Est.	95	84	–
Quetiapine	450[c]	330–770[d]	3[c]	Rep.	64[c]	0[c]	–
				Est.	69–83	1.3–1.5	–
Risperidone	3[e]	6.87[f]	19.5[e]	Rep.	–	72 ± 9[e]	47 ± 16[e]
				Est.	–	65	45
Olanzapine	15[e]	10[g]	20.9[e]	Rep.	83[e]*	78[e]	61[e]
				Est.	86*	77	60

– Indicates that the data was not available.

[a] Data from Nordström et al. (1992); [b] data from Kapur et al. (1997); [c] data from Gefvert et al. (1998); [d] data from Kapur et al. (2000); [e] data from Tauscher et al. (2002); [f] data from Nyberg et al. (1999); [g] data from Kapur et al. (1999).

* Data at 6 h after drug administration.

to be in the range of 330–770 ng/ml (Kapur et al., 2000), and the $T_{\frac{1}{2}}$ of plasma concentration was estimated to be approx. 3 h (Gefvert et al., 2001). The simulated dopamine D_2 receptor occupancy was calculated to be 69–83% at 2 h and 1.3–1.5% at 24 h. Thus, the transient high occupancy reported for the clinical dose of quetiapine can be simulated with its affinity parameter and pharmacokinetics data. Using the reported plasma concentration data from a discontinuation experiment with 3 mg risperidone (Tauscher et al., 2002), the $T_{\frac{1}{2}}$ of mean plasma concentration of risperidone was estimated to be 19.5 h. The striatal D_2 receptor occupancy can be simulated with the reported in-vivo ED_{50} value (6.87 ng/ml) (Nyberg et al., 1999). The simulated value was 65% at 24 h and 45% at 48 h, which was within 1 S.D. of the reported values from the PET measurements (72 ± 9% at 24 h and 47 ± 16 at 48 h) (Tauscher et al., 2002). Although variations in plasma data and in-vivo ED_{50} values can result in deviations in the results of the estimation, the estimated values seemed to be consistent with the reported clinical results (Table 2).

The present results indicated that dopamine D_2 receptor occupancy by risperidone remained high even after the plasma concentration had decreased. This was consistent with a recent report that the kinetics of dopamine D_2 receptor occupancy in the brain and the plasma pharmacokinetics of antipsychotics are dissociated (Tauscher et al., 2002). The dissociation of plasma pharmacokinetics and receptor occupancy was also shown in a dopamine D_1 receptor occupancy study with the dopamine D_1 receptor antagonist NNC 756 (Karlsson et al., 1995). Our simulation method would be useful for investigating the pharmacodynamics of various drugs with specific binding. In this study we used the ED_{50} value measured in vivo because a disparity between in-vivo and in-vitro dissociation rates under different conditions has been reported; environmental factors such as temperature and incubation time can affect the in-vitro data of receptor binding (Kapur et al., 2001; Kessler et al., 1993). Thus, although direct comparative affinity parameters for FLB 457 between in vitro and in vivo were not available, the in-vivo ED_{50} value would be more reliable for estimating the time-course of receptor occupancy in the living human brain.

Several confounding factors must be noted in the present study. First, dopamine D_2 receptor occupancy was calculated using age-corrected mean BP values of other drug-naive patients with schizophrenia as baseline. The absence of the patients' own baseline values introduced a potential error. The coefficient of variance of dopamine D_2 binding potential in the temporal cortex was reported as approx. 15% in schizophrenia using [^{11}C]FLB 457 (Suhara et al., 2002b). If the BP value at baseline were changed by 15%, the occupancy in this study would change from –11% to +8.3%. Secondly, we measured only three time-points to evaluate the time-course of receptor occupancy and six time-points for the plasma pharmacokinetics, and linear regression was used to estimate the half-life of dopamine D_2 receptor occupancy. Obviously, the use of more time-points would be additionally favourable for a more precise fitting. But as shown in Figure 3, as the time-course of dopamine D_2 receptor occupancy was not an exponential or a linear function, the

meaning of the $T_{\frac{1}{2}}$ of receptor occupancy was not equal to that of plasma. Thirdly, in our study we started to measure occupancy and plasma concentration 5 h after oral administration. Although this could simplify the estimation to the elimination phase, estimation of the total kinetics including the absorption phase is needed for more detailed results. Fourthly, in the simulation study we assumed that the same mathematical model could be applied regardless of the degree of in-vivo affinity. However, discussions concerning the effect of the in-vivo environment such as endogenous transmitters on the binding of drugs with different characteristics have been reported (Farde et al., 1990; Seeman and Tallerico, 1999). Fifthly, [^{11}C]FLB 457 has high affinity for both dopamine D_2 and dopamine D_3 receptors in vitro (Halldin et al., 1995). Although distinct anatomical localization has been reported for dopamine D_3 receptors (Hall et al., 1996), further study will be necessary to determine the contribution of dopamine D_3 receptor binding in the human temporal cortex.

In conclusion, the time-course of dopamine D_2 receptor occupancy by risperidone can be estimated from plasma pharmacokinetics and the in-vivo ED_{50} value, and this estimated relationship would be applicable to other antipsychotics. Moreover, the estimation of drug kinetics at the receptor site would undoubtedly provide information useful for the evaluation of new antipsychotics.

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Statement of Interest

None.

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A.3 FORMAÇÃO E PRODUÇÃO CIENTÍFICA DURANTE O PERÍODO DO DOUTORAMENTO (2003-2005):

Formação Acadêmica:

- Graduação no curso de Bacharelado em Física.
  Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.
  Período: 2003-2005*.

- Mestrado em Matemática.
  Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.
  Título: Aplicações de Processos Estocásticos à Biomedicina.
  Período: 2003-2005.
  Orientador: Artur Oscar Lopes.

Lista de artigos científicos publicados internacionalmente em revistas indexadas com índice de impacto conhecido:

1. Dall'Igna OP, **Tort AB**, Souza DO, Lara DR. Cinnarizine has an atypical antipsychotic profile in animal models of psychosis. J Psychopharmacol. 2005;19(4):342-6.

2. Dietrich MO, **Tort AB**, Schaf DV, Farina M, Goncalves CA, Souza DO, Portela LV. Increase in serum S100B protein level after a swimming race. Can J Appl Physiol. 2003;28(5):710-6.

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3. Monte TL, Rieder CR, **Tort AB**, Rockenback I, Pereira ML, Silveira I, Ferro A, Sequeiros J, Jardim LB. Use of fluoxetine for treatment of Machado-Joseph disease: an open-label study. Acta Neurol Scand. 2003;107(3):207-10.

4. de Oliveira RV, Dall'Igna OP, **Tort AB**, Schuh JF, Neto PF, Santos Gomes MW, Souza DO, Lara DR. Effect of subchronic caffeine treatment on MK-801-induced changes in locomotion, cognition and ataxia in mice. Behav Pharmacol. 2005;16(2):79-84.

5. Portela LV, **Tort AB**, Walz R, Bianchin M, Trevisol-Bittencourt PC, Wille PR, Cardoso RC, Ishida MM, vonWangenheim A, Grisard EC, Steindel M, Goncalves CA, Souza DO. Interictal serum S100B levels in chronic neurocysticercosis and idiopathic epilepsy. Acta Neurol Scand. 2003;108(6):424-7.

6. Schaf DV, **Tort AB**, Fricke D, Schestatsky P, Portela LV, Souza DO, Rieder CR. S100B and NSE serum levels in patients with Parkinson's disease. Parkinsonism Relat Disord. 2005;11(1):39-43.

7. Schenatto CB, Xavier RM, Bredemeier M, Portela LV, **Tort AB**, Silva TL, Souza DO, Brenol JC. Elevated serum S100B protein levels in neuropsychiatric lupus. Ann Rheum Dis, *in press*.

8. Schmidt AP, **Tort AB**, Amaral OB, Schmidt AP, Walz R, Vettorazzi-Stuckzynski J, Martins-Costa SH, Ramos JG, Souza DO, Portela LV. Serum S100B in pregnancy-related hypertensive disorders: a case-control study. Clin Chem. 2004;50(2):435-8.

9. **Tort AB**, Dietrich MO, Goncalves CA, Souza DO, Portela LV. Influence of anticoagulants on the measurement of S100B protein in blood. Clin Biochem. 2003;36(7):519-22.

10. **Tort AB**, Goncalves CA, Souza DO, Giugliani R, Portela LV. S100B protein and amniotic fluid. Clin Chim Acta. 2003;335(1-2):165-6;

11. **Tort AB**, Mantese CE, dos Anjos GM, Dietrich MO, Dall'Igna OP, Souza DO, Lara DR. Guanosine selectively inhibits locomotor stimulation induced by the NMDA antagonist dizocilpine. Behav Brain Res. 2004;154(2):417-22.

12. **Tort AB**, Portela LV, da Purificacao Tavares M, Goncalves CA, Netto C, Giugliani R, Souza DO. Specificity and sensitivity of S100B levels in amniotic fluid for Down syndrome diagnosis. Life Sci. 2004;76(4):379-84.

13. **Tort AB**, Souza DO, Lara DR. Half the dose of antipsychotic in case of extrapyramidal symptoms. Schizophr Res. 2005;78(2-3):347-9

14. **Tort AB**, Portela LV, Rockenbach IC, Monte TL, Pereira ML, Souza DO, Rieder CR, Jardim LB. S100B and NSE serum concentrations in Machado Joseph disease. Clin Chim Acta. 2005;351(1-2):143-8.

15. **Tort AB**, Souza DO, Lara DR. On the simulation of the time-course of dopamine D2 receptor occupancy from the pharmacokinetics of antipsychotics. Int J Neuropsychopharmacol. 2005;8(1):137-9.

16. **Tort AB**, Dall'Igna OP, de Oliveira RV, Mantese CE, Fett P, Gomes MW, Schuh J, Souza DO, Lara DR. Atypical antipsychotic profile of flunarizine in animal models. Psychopharmacology (Berl). 2005;177(3):344-8.

17. **Tort AB**, Souza DO, Lara DR. Theoretical insights on the mechanism of action of atypical antipsychotics. Prog Neuropsychopharmacol Biol Psychiatry, *in press*.

18. **Tort AB**, Neto WP, Amaral OB, Kazlauckas V, Souza DO, Lara DR. A simple webcam-based approach for the measurement of rodent locomotion and other behavioural parameters. J Neurosci Methods, *in press.*

19. **Tort AB**, Amaral OB, Souza DO, Lara DR. Critical comments on the fast-off D2 theory of aytipicality of antipsychotic drugs. Am J Psychiatry, *in press*.