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EFEITOS DO DECANOATO DE NANDROLONA NA HOMEOSTASIA GLUTAMATÉRGICA E NO COMPORTAMENTO AGRESSIVO

Porto Alegre, dezembro de 2014.

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Efeitos do Decanoato de Nandrolona na Homeostasia Glutamatérgica e no Comportamento Agressivo

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas - Bioquímica, como requisito parcial obtenção do título de Doutor em Ciências Biológicas – Bioquímica.

Orientador: Prof. Luis Valmor C. Portela.

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"Os conceitos e princípios fundamentais da ciência são invenções livres do espírito humano"

Albert Einstein

Às duas pessoas mais importantes da minha vida,
meus pais.

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Resumo

Nos últimos anos, houve um aumento significativo no uso abusivo dos Esteróides Anabólicos Andrógenos (EAAs). Um dos efeitos comportamentais mais marcantes da administração crônica de EAAs como o Decanoato de Nandrolona (DN) é a indução do comportamento agressivo exacerbado. Atualmente o sistema glutamatérgico tem sido associado ao comportamento agressivo induzido pelos EAAs, principalmente no que se refere à modulação dos receptores N-Methyl-D-Aspartato NMDA (NMDAr). Nós investigamos os efeitos centrais e periféricos da administração do DN ao longo do tempo (4, 11 e 19 dias consecutivos de administração), e a participação de mecanismos glutamatérgicos. Para isso, camundongos CF-1 tratados com DN foram avaliados em relação ao comportamento agressivo pelo teste do intruso. Além disso, investigamos a captação de glutamato, o imunoconteúdo de GLT-1, os níveis de glutamato no líquido extracelular, e a participação dos NMDAr na manifestação do comportamento agressivo. O fenótipo agressivo foi evidenciado somente no longo tempo de exposição à DN (19 dias). Na mesma janela temporal que os animais apresentaram o fenótipo agressivo houve redução significativa de captação de glutamato em fatias cerebrais de córtex e hipocampo, como também a redução do imunoconteúdo do transportador astrocitário GLT-1 nas mesmas estruturas cerebrais. A administração de antagonistas de NMDAr como MK-801 e memantina antes do teste do intruso diminuiu o comportamento agressivo dos animais tratados cronicamente com DN a níveis iguais aos do grupo controle. Ainda, o comportamento agressivo induzido pela administração crônica de DN diminuiu a remoção do glutamato da fenda sináptica, culminando com o aumento do glutamato extracelular no SNC, o que resultou na hiperexcitabilidade dos NMDAr. Este trabalho enfatiza o papel da comunicação entre astrócitos e neurônios e a relevância da hiperstimulação de NMDAr na manifestação do comportamento agressivo.

Palavras Chaves: Decanoato de Nandrolona, agressividade, receptor NMDA (NMDAr), transportador de glutamato GLT-1, Memantine e MK-801.

Abstract

Nandrolone decanoate (ND), an anabolic androgenic steroid (AAS), induces an aggressive phenotype by mechanisms involving glutamate-induced N-methyl-d-aspartate receptor (NMDAr) hyperexcitability. The astrocytic glutamate transporters remove excessive glutamate surrounding the synapse. However, the impact of supraphysiological doses of ND on glutamate transporters activity remains elusive. We investigated whether ND-induced aggressive behavior is correlated with GLT-1 activity, glutamate levels and abnormal NMDAr responses. Two-month-old untreated male mice (CF1, n=20) were tested for baseline aggressive behavior in the resident-intruder test. Another group of mice (n=188) was injected with ND (15mg/kg) or vehicle for 4, 11 and 19 days (short-, mid- and long-term endpoints, respectively) and was evaluated in the resident-intruder test. Each endpoint was assessed for GLT-1 expression and glutamate uptake activity in the frontoparietal cortex and hippocampal tissues. Only the long-term ND endpoint significantly decreased the latency to first attack and increased the number of attacks, which was associated with decreased GLT-1 expression and glutamate uptake activity in both brain areas. These alterations may affect extracellular glutamate levels and receptor excitability. Resident males were assessed for hippocampal glutamate levels via microdialysis both prior to, and following, the introduction of intruders. Long-term ND mice displayed significant increases in the microdialysate glutamate levels only after exposure to intruders. A single intraperitoneal dose of NMDAr antagonists, memantine or MK-801, shortly before the intruder test, decreased aggressive behavior. In summary, long-term ND-induced aggressive behavior is associated with decreased extracellular glutamate clearance and NMDAr hyperexcitability, emphasizing the role of this receptor in mediating aggression mechanisms.

Keywords: Aggressive behavior, Nandrolone decanoate, Glutamate transporter 1 (GLT-1), N-methyl-D-aspartate receptor (NMDAr), Memantine, MK-801.

Apresentação

Esta Tese é constituída de 5 partes:

Parte I. Introdução e Objetivos

Parte II. Artigo cientifico.

Parte III. Discussão e Conclusão Final.

Parte IV. Referencias bibliográficas

Lista de abreviaturas

5-HT - 5-hidroxitriptamina ou serotonina

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

DN - Decanoato de Nandrolona

EAAs - Esteróides Anabólicos Androgênicos

GABA - Ácido gama-aminobutírico

GAD65 - Glutamato Descarboxilase 65

GLT-1 - Transportador Glial de Glutamato sódio-dependente

HDL - Lipoproteína de alta densidade

LDL - Lipoproteína de baixa densidade

MEM - Memantina

MK-801 - Dizocilpina

MTT - *(3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide thiazolyl blue)*

MWM - Labirinto Aquático de Morris ou *Morris Water Maze*

NMDA - N-metil D-Aspartato

NMDAr - Receptor N-metil D-Aspartato

RA - Receptor Andrógeno

SNC - Sistema Nervoso Central

T - Testosterona

PARTE I.

1. INTRODUÇÃO

1.1 Esteróides Anabólicos Androgênicos (EAAs)

1.1.1 Efeitos fisiológicos dos EAAs

Desde a primeira síntese, isolamento e caracterização dos Esteróides Anabólicos Androgênicos (EAAs), particularmente da testosterona (T), diversos estudos se propuseram a explicar os seus efeitos biológicos (Basaria et al., 2001; Kicman, 2008; Shahidi, 2001). A T exerce seus efeitos androgênicos e anabólicos nos tecidos reprodutivos, como testículos e ovários, e em tecidos não reprodutivos como músculo esquelético, tecido ósseo, rins, fígado e sistema nervoso central (SNC) (Basaria et al., 2001; Kicman, 2008; Shahidi, 2001; Snyder, 1984). A ação androgênica da T permite o desenvolvimento dos tecidos reprodutivos masculinos e das características sexuais secundárias. No que se refere às ações anabólicas, há estímulo da fixação do nitrogênio e o aumento da síntese protéica (Basaria et al., 2001; Evans-Brown et al., 2008; Kicman, 2008; Shahidi, 2001; Snyder, 1984). Destes efeitos surgiu o interesse em estudar o potencial terapêutico da T, e posteriormente levou ao surgimento dos derivados sintéticos chamados de EAAs.

O desenvolvimento dos diferentes EAAs pela indústria farmacêutica visou mimetizar a ação endógena da T, porém com menores efeitos androgênicos e prolongando a sua meia vida útil. Entretanto, apesar dos mais de 100 análogos disponíveis, ainda não foi possível a obtenção de um EAAs desprovido dos efeitos androgênicos devido à farmacodinâmica complexa em relação aos receptores andrógenos (RA) (Basaria et al., 2001; Kicman, 2008; Shahidi, 2001; Snyder, 1984).

Os EAAs, assim como os andrógenos endógenos, são estruturas de quatro anéis com 19 átomos de carbono. Por seu caráter lipofílico, a T e os EAAs atravessam as

membranas biológicas muito facilmente. No ambiente intracelular há formação do complexo hormônio-receptor, através de uma ligação específica, que depende das características químicas de cada esteróide (Goodman et al., 2001). Os andrógenos ligam-se aos receptores de andrógenos (RA) citoplasmáticos, e podem interagir com sítios nucleares afetando a transcrição de genes, ações que caracterizam a via denominada "via clássica" de ação dos esteróides (Rang and Dale, 2007). Existe ainda outra via de sinalização destes hormônios denominada "via rápida", que pode ocorrer independentemente da ligação aos RA. O efeito rápido dos hormônios esteróides consiste basicamente na alteração da fluidez da membrana plasmática através de três distintos mecanismos de ação: (i) independente de ligação ao receptor; (ii) através da ligação a receptores que não RA; e (iii) decorrentes da ligação aos RA. Na via rápida, há alteração nas vias de sinalização envolvendo, por exemplo, proteínas como adenilato ciclase, proteína cinase ativada por mitógeno e fosfatidilinositol 3-cinase (Cato et al., 2002; Falkenstein et al., 2000; Kicman, 2008).

Existem três principais classes de EAAs: 1) Os ésteres do grupo 17-β-hidroxil (propionato e cipionato de testosterona). A esterificação retarda a degradação do composto e prolonga o tempo de ação devido à liberação sistêmica mais lenta (Basaria et al., 2001; Shahidi, 2001). Os ésteres de testosterona podem ser hidrolisados a T livre, e posteriormente reduzidos a 5α-diidrotestosterona, um metabólito com maior atividade biológica, devido a sua maior afinidade pelos RA do que a T. Alternativamente, podem ser aromatizados a estrógenos pela ação da enzima aromatase (Kochakian, 1993; Martini, 1982; Winters, 1990); 2) Os derivados da 19-nortestosterona, quimicamente derivados dos ésteres de T através da adição de uma cadeia carbonada longa no C17. Também sofrem a substituição de um hidrogênio por uma metila no C19. O Decanoato de Nandrolona (DN) pertence a esta classe de EAAs, cujo alongamento da cadeia

carbonada confere atividade androgênica reduzida pela conseqüente redução da afinidade ao RA. O DN pode ser aromatizado a 17β estradiol, embora isso não ocorra de maneira tão eficiente e direta quanto em relação aos ésteres (Basaria et al., 2001; Ryan, 1959; Shahidi, 2001; Winters, 1990); 3) Os compostos alquilados no C17 que incluem a metiltestosterona, oximetolona e estanozolol são menos suscetíveis aos efeitos da metabolização hepática, possibilitando a este grupo ser administrado por via oral. Não há conversão de esteróides 17α- alquilados a diidrotestosterona ou 17β-estradiol, porém outros metabólitos andrógenos e estrógenos ativos podem ser formados (Basaria et al., 2001; Winters, 1990).

1.1.2 Aplicações dos EAAs

A aplicação clínica dos EAAs deve ser analisada conforme a relação entre suas estruturas químicas e as propriedades biológicas, e geralmente é limitada pela seletividade tecidual, farmacocinética e toxicidade sistêmica e central. Estas variáveis tendem a ser moduladas no processo de produção dos análogos sintéticos, os quais buscam a diminuição dos efeitos masculinizantes (Basaria et al., 2001; Kicman, 2008; Snyder, 1984). A indicação mais clara para terapia androgênica é o hipogonadismo masculino, onde o tratamento é capaz de restabelecer os níveis normais da T plasmática, substituindo algumas das funções testiculares conhecidas, exceto a espermatogênese (Goodman et al., 2001). Originalmente, além do tratamento da disfunção hipogonadal, os andrógenos foram aplicados na terapia do retardo da puberdade e na promoção de crescimento corporal (Basaria et al., 2001). Devido aos seus efeitos benéficos na promoção do ganho de massa magra corpórea, os EAAS têm sido empregados com sucesso no tratamento de queimaduras, na recuperação pós-operatória, anemias, politraumatismos, osteopatologias (osteoporose) e doenças com perda de massa

muscular (caquexia) (Basaria et al., 2001; Evans-Brown et al., 2008; Evans, 2004; Shahidi, 2001). Além disso, os EAAs têm sido utilizados como adjuvantes no tratamento de câncer de mama, por causarem uma regressão temporária de tumores sensíveis à ação de hormônios e novamente por apresentarem efeitos benéficos sobre a composição corporal (aumento de massa magra) (Basaria et al., 2001; Kennedy, 1974).

Na hepatite alcoólica, o tratamento com EAAs 17α-alquilados (oximetolona), associado à suplementação nutricional, levou a uma melhora da função hepática com um conseqüente aumento da sobrevivência dos pacientes. Contudo, a administração dos EAAs sem a combinação com suplementação nutricional não causou efeito benéfico, ao contrário, agravaram a toxicidade hepática. Neste sentido, mais estudos são necessários para avaliar a utilização terapêutica dos EAAs nesta patologia (Basaria et al., 2001; Mendenhall et al., 1984; Mendenhall et al., 1995; Snyder, 1984). Nos pacientes com queimaduras severas, o tratamento com EAA diminuiu a degradação protéica, favoreceu o ganho de peso corporal, aumentou a retenção de nitrogênio e diminuiu o período de recuperação (Demling and DeSanti, 1997).

Os avanços e a maior compreensão dos efeitos dos EAAs que impulsionaram a aplicação clínica também levaram à utilização não terapêutica destes compostos. Um dos primeiros relatos de objetivos não terapêuticos ocorreu em 1954, na Áustria. Desde então estas substâncias vêm despertando a atenção de profissionais da área da saúde e pesquisadores devido à sua utilização por atletas profissionais e amadores, com o objetivo de aumentar a massa muscular, melhorar o desempenho físico e a estética corporal (Brooks et al., 1975; Creutzberg and Schols, 1999; Santos et al., 2011). O uso abusivo dos EAAs por atletas de elite se difundiu entre 1950 – 1960 nos países do leste Europeu, coincidindo com o término da Segunda Guerra Mundial e com o crescimento da indústria farmacêutica (Handelsman and Heather, 2008). O uso exacerbado de EAAs

no meio desportivo para melhorias no desempenho físico tornou-se público em 1970, e em 1974, o uso destas substâncias sintéticas foi banido pelo Comitê Internacional de Jogos Olímpicos (Brooks and Reddon, 1996; Schanzer, 1996). De acordo com a Agência Mundial de Anti-doping, estas substâncias químicas são proibidas devido a três critérios: por melhorar o desempenho dos atletas, por representarem um risco a saúde e violarem o espírito esportivo (Handelsman and Heather, 2008).

Estudos demonstram que o benefício da atividade anabólica dos EAAs em indivíduos saudáveis ocorre quando administrados em superdoses (10 a 100 vezes maiores que as doses terapêuticas). Poucas semanas após a suspensão do tratamento, a massa e força muscular decaem, por isso faz-se necessário a aplicação crônica destas substâncias pelos atletas, para manutenção dos efeitos anabólicos desejados (Clark et al., 1995; Grivetti and Applegate, 1997; Lukas, 1996; Sjoqvist et al., 2008). O aumento da massa muscular e da força, diminuição do tempo de recuperação após a sobrecarga de treinamento, aumento de agressividade, diminuição do tempo de recuperação das lesões estão entre os efeitos desejados pelos atletas que utilizam a superdosagem com EAAs (Hough, 1990; Maravelias et al., 2005). Posteriormente, os EAAs deixaram de ser consumidos somente pela elite desportiva e passaram a ser utilizados pela população em geral (Evans-Brown et al., 2008). O aumento do comércio destes fármacos (20-30% ao ano) para fins não terapêuticos se tornou um problema de saúde pública (Evans, 2004), e os EAAs predominantemente utilizados de maneira abusiva são: testosterona, nandrolona e estazonol (McGinnis, 2004). Em resposta ao aumento do consumo destas substâncias, à fabricação clandestina e comércio ilegal, em 1991 os EAAs foram classificados como substâncias de uso controlado do tipo 3 pelo congresso norte americano (Shahidi, 2001; Sjoqvist et al., 2008). No Brasil estes fármacos também são substâncias de consumo controlado (Kicman, 2008; Santos et al., 2011).

1.1.3 Efeitos adversos dos EAAs

A literatura indica que um número consideravelmente importante de atletas aceita os riscos da utilização dos EAAs (Hartgens and Kuipers, 2004). Os efeitos indesejados que decorrem da utilização de tais substâncias se relacionam com a dose, frequência de uso, idade e interações medicamentosas (Bahrke and Yesalis, 2004; Maravelias et al., 2005). Mesmo assim, graves problemas de saúde e sociais associados ao uso abusivo são comuns. A maioria dos usuários dos EAAs apresenta efeitos adversos como acne (40%-54%), atrofia testicular (40%-51%), ginecomastia (10%-34%), estrias cutâneas (34%) e dor no local da injeção (36%) (Bolding et al., 2002; Evans, 1997, 2004). O uso abusivo pode causar dano irreversível em diversos órgãos, entretanto, mesmo em doses clínicas, os EAAs apresentam efeitos adversos como hepatotoxicidade, nefrotoxicidade e doenças cardiovasculares (Bonetti et al., 2008; Luciano et al., 2014; Rockhold, 1993). Os efeitos adversos mais comuns dos EAAs são decorrentes do efeito andrógeno, podendo-se destacar a masculinização em mulheres e crianças e mudanças comportamentais (agressividade). As alterações nos níveis plasmáticos de lipídios podem estar associadas ao surgimento de problemas no sistema cardiovascular (Evans, 2004; Shahidi, 2001; Snyder, 1984).

Diversos estudos demonstram que o tratamento clínico com andrógenos apresenta significantes reduções da lipoproteína de alta densidade (HDL) e concomitante aumento da lipoproteína de baixa densidade (LDL) (Evans, 2004; Glazer, 1991; Kicman, 2008). No estudo de Bonetti et al., 2008 realizado em fisiculturistas, a auto-administração de combinações de classes de EAAs, em doses supra fisiológicas, acarretou na diminuição dos níveis plasmáticos de glicose, insulina, globulina de ligação a hormônio sexual, HDL e, concomitantemente, alterações nos níveis de

linfócito T (glóbulos brancos), diminuição da contagem de espermatozóides e diminuição de índice de fertilidade (Bonetti et al., 2008). Também são relatadas alterações no SNC com importantes implicações morfológicas e comportamentais (Lukas, 1996; Rockhold, 1993; Sjoqvist et al., 2008; Snyder, 1984).

1.2 EAAs e o Sistema Nervoso Central

1.2.1 Efeitos comportamentais dos EAAs

Os efeitos dos EAAs sobre a locomoção espontânea no campo aberto ou câmara de atividade, tanto em ratos como em camundongos machos, demonstram que este parâmetro comportamental não é afetado pelo tratamento (Clark and Henderson, 2003). Em contrapartida, sabe-se muito pouco sobre os efeitos dos EAAs na ansiedade. Neste aspecto, Bitran et al., 1993 demonstrou que altas doses de propionato de T alteram o comportamento de ratos Long–Evans no aparato de labirinto em cruz elevado. Neste estudo, os animais foram testados em 6 e 14 dias de tratamento com propionato de T. Com 6 dias de tratamento, os animais aumentaram a exploração nos braços abertos, isto é, teve um efeito ansiolítico. Porém com 14 dias de tratamento, os animais tratados com o propionato de T não foram diferentes do grupo controle (Bitran et al., 1993). Apesar dos indícios sobre um possível caráter ansiolítico dos EAAs, sua influência sobre a ansiedade é controversa (Bitran et al., 1993; Clark and Henderson, 2003).

Da mesma forma, a ação dos EAAs em parâmetros como aprendizado e memória tem sido pouco explorados. Em estudo feito por Clark et al., 1995, não foram observadas alterações na memória espacial de ratos machos no labirinto aquático de Morris (*Water Maze*) decorrentes da administração de EAAs (Clark et al., 1995). Em contrapartida, o estudo realizado por Magnusson et al., 2009 foi demonstrado que o

tratamento de 14 dias com DN (15mg/kg) em ratos machos *Sprague–Dawley* causou prejuízo na memória espacial no labirinto aquático de Morris (Magnusson et al., 2009). Por outro lado, Smith et al., 1996 testou os efeitos do tratamento crônico (30 dias) com os EAAs sobre a memória espacial no aparato labirinto radial em ratos machos e não encontrou diferenças entre os grupos tratados e controle (Smith et al., 1996). Assim, torna-se evidente a necessidade de considerar que combinações ou uso individual de EAAs, dose, tempo de tratamento e metodologia são variáveis que limitam conclusões mais seguras (Clark and Henderson, 2003).

1.2.2 EAAs e o comportamento agressivo

Em humanos, o uso de EAAs está fortemente associado com comportamento agressivo e violento, todavia cabe ressaltar que a agressividade é uma resposta biológica natural, proeminente no gênero masculino, na busca da satisfação das necessidades básicas (McGinnis, 2004). Dessa forma, a agressividade é um comportamento social complexo que envolve o contexto na obtenção de recursos (Berkowitz, 1993). Estudos neurobiológicos reportam a participação de diversos sistemas de neurotransmissores e regiões cerebrais durante a manifestação do comportamento agressivo em seres humanos e não-humanos (Nelson and Trainor, 2007). De uma perspectiva evolutiva a agressividade é utilizada como mecanismo de sobrevivência na disputa por território, comida e reprodução. De uma perspectiva psiquiátrica a agressividade é motivada pelos sentimentos como: raiva, irritabilidade, frustração, medo e, em alguns casos, prazer (Nelson, 2006).

Estudos em modelos animais têm sido empregados para elucidar o comportamento, neurobiologia e mecanismos moleculares da agressividade, por possuírem fatores sociais relativamente menos complexos. O ato agressivo em seres

não-humanos consiste em combinações de mordidas, luta corporal e perseguição, em seres humanos consiste tanto em forma física e verbal. Todas essas diversidades limitam a comparação direta, entretanto pode fornece similaridades entre as espécies. Charles Darwin relatou que provocação social gera o aumenta a freqüência cardiorrespiratória, e mudanças na expressão facial em seres humanos e não-humanos (Ekman, 2006). Essas observações demonstram que diferenças no estado afetivo entre humanos e não-humanos são mais de caráter quantitativos do que qualitativos. Está cada vez mais evidente que os mecanismos de regulação dos circuitos neurais, envolvendo agressividade em não-humanos, compartilham características funcionais com os circuitos dos humanos (Nelson and Trainor, 2007).

Em modelos experimentais, o comportamento agressivo associado ao uso do EAAs é de caráter contraditório, pois a análise dos resultados é afetada por diversas variáveis, como classe, combinação ou não de classes de EAAs, dose, gênero, tempo de tratamento, idade dos animais e metodologia empregada para avaliar o comportamento agressivo (Clark and Henderson, 2003; McGinnis, 2004). Apesar das contrariedades, o efeito da T na manifestação da agressividade é fundamentado, tendo em vista que a castração diminui o fenótipo agressivo, e a reposição da T restaura este fenótipo em roedores (Nelson, 2006).

1.2.3 Neurobiologia do comportamento agressivo associado aos EAAs

Os andrógenos desempenham um papel crítico no comportamento masculino, embora este seja profundamente influenciado por fatores psíquicos, sociais, somáticos e culturais. Os EAAs podem ter atividade deletéria sobre o SNC de humanos que pode se manifestar por alterações morfológicas, funcionais e comportamentais. O aumento da agressividade, euforia, depressão, transtornos de humor, alterações na libido, e em casos

mais graves, até alucinações (psicose) são freqüentemente relatados em indivíduos que utilizam os EAAs, principalmente em doses elevadas (Cooper et al., 1996; Hall and Chapman, 2005; Hartgens and Kuipers, 2004; Parrott et al., 1994; Su et al., 1993; Uzych, 1992). Os EAAs administrados cronicamente produzem diversas alterações nos sistemas serotoninérgico, dopaminérgico, gabaérgico e glutamatérgico (Carrillo et al., 2011c; Fischer et al., 2007; Grimes et al., 2003; Henderson et al., 2006; Lindqvist et al., 2002; Thiblin et al., 1999), porém a relação entre estas alterações e o fenótipo agressivo ainda não é bem compreendida. Baixos níveis de serotonina estão associados com altos níveis de impulsividade e agressividade (Ferris et al., 1997). Corroborando com isto, a exposição aos EAAs produz uma marcada redução nos níveis de serotonina em áreas específicas do cérebro que se correlacionam com o comportamento agressivo (Grimes and Melloni, 2002). Contrariamente, o aumento da atividade serotoninérgica, diminuição da captação de serotonina, ou agonismo dos receptores 5-$HT1_A$ e 5-$HT1_B$ podem induzir o comportamento agressivo (Chiavegatto et al., 2001).

Algumas evidências indicam que os EAAs podem alterar aspectos morfológicos e neuroquímicos de sinapses glutamatérgicas no hipotálamo, hipocampo e córtex cerebral e, com isso, ter importantes implicações comportamentais (Le Greves et al., 1997; Rossbach et al., 2007). O sistema glutamatérgico é o principal mecanismo de neurotransmissão excitatória do SNC de mamíferos. Ele está envolvido em processos de memória e aprendizado, proliferação e sobrevivência celular. Uma vez liberado na fenda sináptica, o glutamato se liga a seus receptores do tipo ionotrópicos (NMDAr, AMPAr e Kainato) e/ou metabotrópicos (acoplados a proteínas G). A remoção do glutamato da fenda sináptica é feita por transportadores específicos, principalmente astrocitários, que protegem os neurônios da excitotoxicidade decorrente dos elevados níveis de glutamato na fenda sináptica (Danbolt, 2001). A hiperestimulação

glutamatérgica causa aumento do Ca^{2+} intracelular, causando dano neuronal e reatividade glial. Dos 5 transportadores de glutamato em mamíferos clonados, GLAST e GLT-1 (predominantemente localizados em membranas de astrócitos) são os principais responsáveis pela manutenção do tônus glutamatérgico fisiológico (evitando o tônus neurotóxico) (Anderson and Swanson, 2000).

Alguns estudos apontam para uma associação entre o fenótipo agressivo induzido pelos EAAs e o sistema glutamatérgico: a administração de EAAs induziu alterações progressivas em parâmetros glutamatérgicos de hamsters (Carrillo et al., 2011c), e modificações nas projeções neuronais glutamatérgicas do hipotálamo às estruturas adjacentes, foram sugeridas como mecanismos facilitatórios do comportamento agressivo (Carrillo et al., 2011b). Adicionalmente, doses elevadas de DN aumentam a imunorreatividade da enzima glutaminase, que possui um papel fundamental na produção de glutamato em diferentes áreas cerebrais, inclusive naquelas associadas à agressividade. Porém, ainda não está elucidado se esta alteração se reflete necessariamente no aumento de liberação de glutamato pelas vesículas sinápticas (Fischer et al., 2007). Deste modo, é possível inferir que o DN poderia aumentar a síntese e a liberação de glutamato pelo terminal pré-sináptico. Assim, o aumento excessivo de glutamato na fenda sináptica causaria a hiperatividade do NMDAr e o influxo excessivo de cálcio, contribuindo para o fenótipo agressivo. Os mecanismos pelos quais os EAAs causam a agressividade ainda necessitam ser desvendados, principalmente no que se refere aos componentes do sistema glutamatérgico neuronais e gliais.

1.3 Objetivos

1.3.1 Objetivo Geral

Investigar os efeitos do DN em mecanismos cerebrais associados à homeostasia glutamatérgica e comportamento agressivo.

1.3.2 Objetivos Específicos

Avaliar os efeitos a curto, médio e longo prazo da administração do DN em:

a) Parâmetros comportamentais: locomoção espontânea no campo aberto; comportamento agressivo pelo teste do intruso; memória espacial no labirinto aquático de Morris;

b) Parâmetros neuroquímicos: captação de glutamato em fatias de córtex e hipocampo; imunoconteúdo do GLT-1 nessas mesmas áreas; níveis de glutamato no fluido extracelular cerebral obtido através da microdiálise; viabilidade celular através do ensaio de MTT;

c) Parâmetros metabólicos: níveis séricos de lipídeos, uréia, creatinina, albumina, transaminases hepáticas, e teste de tolerância a glicose através de kits comerciais.

PARTE II.

2. CAPÍTULO I

Hormones and Behavior 66 (2014) 383–392

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Regular article

Nandrolone-induced aggressive behavior is associated with alterations in extracellular glutamate homeostasis in mice

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ABSTRACT

Nandrolone decanoate (ND), an anabolic androgenic steroid (AAS), induces an aggressive phenotype by mechanisms involving glutamate-induced N-methyl-D-aspartate receptor (NMDAr) hyperexcitability. The astrocytic glutamate transporters remove excessive glutamate surrounding the synapse. However, the impact of supraphysiological doses of ND on glutamate transporters activity remains elusive. We investigated whether ND-induced aggressive behavior is interconnected with GLT-1 activity, glutamate levels and abnormal NMDAr responses. Two-month-old untreated male mice (CF1, n = 20) were tested for baseline aggressive behavior in the resident–intruder test. Another group of mice (n = 188) was injected with ND (15 mg/kg) or vehicle for 4, 11 and 19 days (short-, mid- and long-term endpoints, respectively) and was evaluated in the resident–intruder test. Each endpoint was assessed for GLT-1 expression and glutamate uptake activity in the frontoparietal cortex and hippocampal tissues. Only the long-term ND endpoint significantly decreased the latency to first attack and increased the number of attacks, which was associated with decreased GLT-1 expression and glutamate uptake activity in both brain areas. These alterations may affect extracellular glutamate levels and receptor excitability. Resident males were assessed for hippocampal glutamate levels via microdialysis both prior to, and following, the introduction of intruders. Long-term ND mice displayed significant increases in the microdialysate glutamate levels only after exposure to intruders. A single intraperitoneal dose of the NMDAr antagonists, memantine or MK-801, shortly before the intruder test decreased aggressive behavior. In summary, long-term ND-induced aggressive behavior is associated with decreased extracellular glutamate clearance and NMDAr hyperexcitability, emphasizing the role of this receptor in mediating aggression mechanisms.

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Introduction

Anabolic androgenic steroids (AAS), such as nandrolone decanoate (ND), are synthetic derivatives of testosterone that were developed to improve anabolic functions with fewer androgenic effects (Jones and Lopez, 2006; Shahidi, 2001). However, humans and rodents submitted to high AAS dose regimens may display exaggerated emotional reactivity and aggressive behavior, which ultimately is associated with glutamatergic hyperexcitability in brain areas such as the hypothalamus, cortex, and hippocampus (Breuer et al., 2001; Carrillo et al., 2009, 2011a; Diano et al., 1997; Kanayama et al., 2010; Le Greves et al., 1997; McGinnis, 2004; Ricci et al., 2007; Robinson et al., 2012; Talih et al., 2007).

The mechanism underlying the AAS-induced aggressive phenotype is dynamic and not restricted to proteins of the synaptic milieu. For instance, select hypothalamic neurons express dramatic increases in phosphate-activated glutaminase, the rate-limiting enzyme in the synthesis of glutamate, in aggressive, adolescent, AAS-treated, male Syrian hamsters (Fischer et al., 2007). Steroids also increase the rate of glutamate and aspartate release, thus increasing the binding probability of glutamate to NMDA or AMPA receptors (Brann and Mahesh, 1995; Ventriglia and Di Maio, 2013). Actually, AAS-induced aggression is mechanistically associated with glutamatergic hyperexcitability. This concept has been supported by studies on genetically modified animals and pharmacological studies addressing glutamate fast-acting ionotropic receptors, i.e. kainate (KAr), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAr), and N-methyl-D-aspartate (NMDAr) (Fischer et al., 2007). To date, studies on knockout mice lacking the AMPAr 1 subunit (GluR1) show reduced aggressive behavior (Vekovischeva et al., 2004), whereas AAS-treated aggressive hamsters

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display a significant increase in the number and density of GluR1-expressing hypothalamic neurons compared to non-aggressive, vehicle-treated controls (Fischer et al., 2007). Moreover, hypothalamic administration of a KAr agonist or L-glutamate stimulates aggressive attacks in rats and cats respectively (Brody et al., 1969; Haller et al., 1998). Although pharmacological antagonism of NMDAr may cause non-specific behavioral effects due to sedation, this receptor has multiple regulatory binding sites that may serve as anti-aggressive targets (Bortolato et al., 2012; Umukoro et al., 2013). Accordingly, the administration of memantine (MEM), a low-affinity uncompetitive NMDAr antagonist, decreases aggression induced by social isolation or morphine withdrawal in rodents (Belozertseva et al., 1999; Sukhotina and Bespalov, 2000). Moreover, knockout mice for monoamino oxidase 'A' exhibited pathological aggressive behavior mediated by the higher expression of NR2A and NR2B subunits of NMDAr in the prefrontal cortex (Bortolato et al., 2012). Remarkably, systemic administration of selective NR2A and NR2B antagonists as well as dizocilpine (MK-801) an uncompetitive high-affinity NMDAr antagonist, countered the enhanced aggression (Bortolato et al., 2012). Collectively, these studies highlight a positive correlation between heightened aggression and increased glutamatergic tonus in a range of animal models.

Because there are no extracellular enzymes that can degrade glutamate, the maintenance of physiological concentrations requires glutamate transporters activity present in both astrocytes and neurons. The high-affinity glutamate transporter 1 (GLT-1) is predominantly expressed in astrocytes and is responsible for more than 90% of glutamate clearance from the synaptic cleft (Lehre and Danbolt, 1998). Further, GLT-1 is highest expressed in the hippocampus and neocortical areas (Ullensvang et al., 1997). In contrast, glutamate transporter 3 (EAAC1) is widely distributed in neurons and even GLT-1 can be found at lower levels in neurons, particularly in axon terminals. Despite neuronal EAAC1 being quantitatively lower when compared to astrocytes, its functional role cannot be neglected. Notably, it can be assumed that astrocytes (via GLT-1) are the main regulators of extracellular glutamate levels (Danbolt, 2001). Although astrocytic glutamate uptake is recognized as an important mechanism to avoid excessive glutamate levels associated with prolonged receptor activation, the impact of supraphysiological doses of ND on glutamate transporters activity remains elusive.

Here, we investigate whether ND-induced aggression is mechanistically interconnected with GLT-1 activity, glutamate levels, and NMDAr response in the brains of male, gonad-intact CF1 mice.

Material and methods

Animals

Two-month old CF1 male mice (total n = 208) weighting 32–38 g were housed in standard polycarbonate cages (cm: 28 × 17.8 × 12.7), and kept in a temperature-controlled room (22 ± 1 °C) with a 12 h light/12 h dark cycle (light on at 7 a.m.). The animals were permitted free access to food and water. To avoid social isolation, both resident and intruder mice were housed at four per cage (Leasure and Decker, 2009). All experimental procedures were performed according to the *NIH Guide for Care and Use of Laboratory Animals* and the *Brazilian Society for Neuroscience and Behavior* (SBNeC). Recommendations for animal

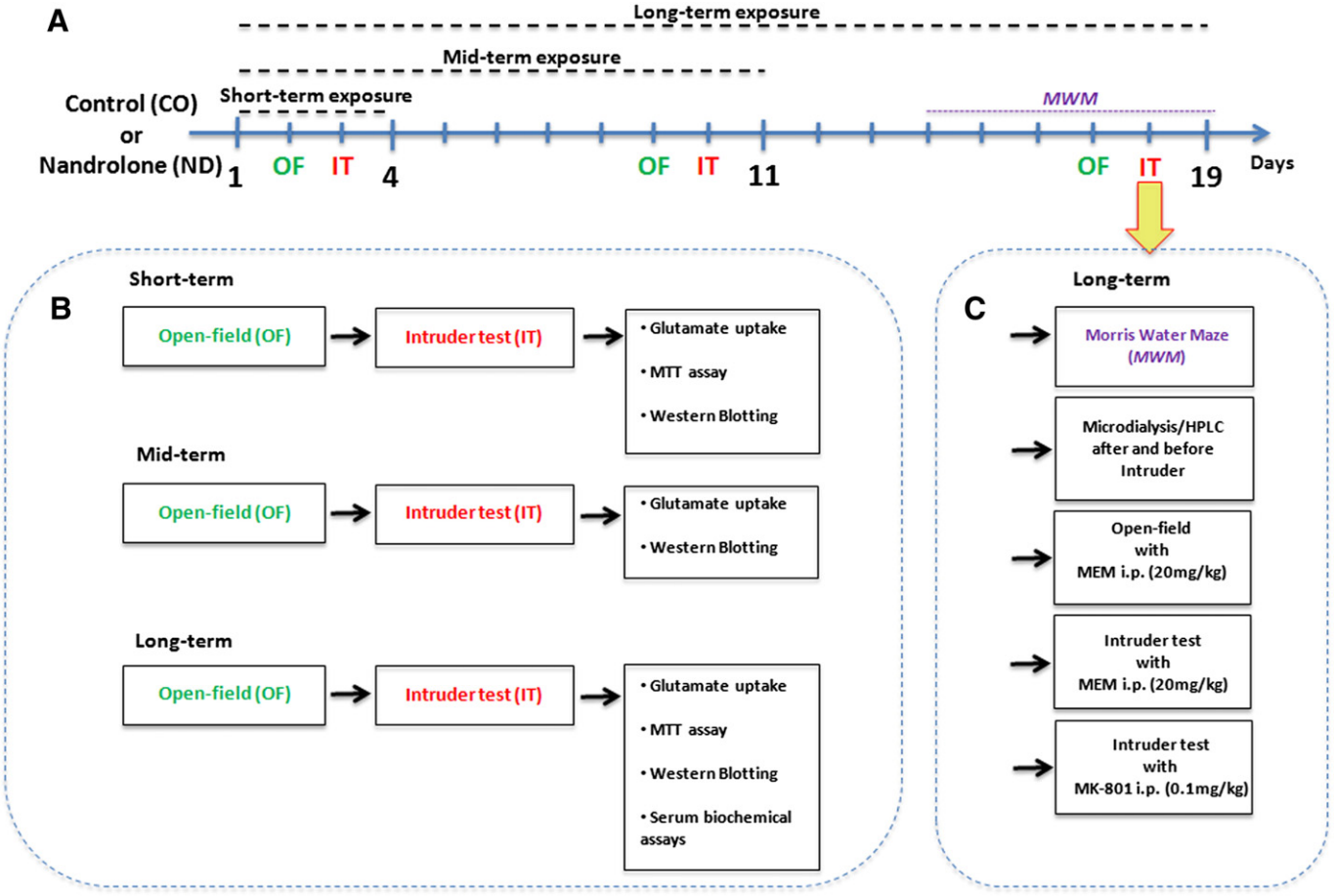

Fig. 1. Schematic experimental design. Behavioral and neurochemical endpoints in control (CO) and nandrolone (ND) groups: (A) The timeline showing each endpoint (short-, mid- and long-term exposure); (B) Flowchart representing behavioral and biochemical outcomes for each endpoint; (C) Boxes with arrows representing five independent experiments conducted after long-term exposure. Abbreviations: high-performance liquid chromatography (HPLC), memantine (MEM), dizocilpine (MK-801), intraperitoneal (i.p.).

care were followed throughout all the experiments in accordance with project approved by the ethical committee from the Federal University of Rio Grande do Sul-UFRGS #26739.

Experimental design and drug administration

All experimental procedures are shown in Fig. 1: (A) The timeline showing each endpoint (short-, mid- and long-term exposure); (B) Flowchart representing behavioral and biochemical outcomes for each endpoint; and (C) Boxes with arrows representing five independent experiments conducted after long-term exposure.

The mice home cages were allocated in one of the following groups; resident or intruder. Resident mice home cages were assigned to treatment groups. Animals received single daily subcutaneous injections of oil-vehicle (CO) or nandrolone decanoate 50 mg (ND; Deca-Durabolin®, Organon; 15 mg/kg/day in a volume of 1 ml/kg, dissolved in corn oil) for 4 days (short-term exposure), 11 days (mid-term exposure) and 19 days (long-term exposure). The ND and CO-treated animals were not housed in the same cage. This strategy avoids ND mice establishing social dominance over CO animals before the intruder test. The dose and treatment regimen were based on previous published works (Le Greves et al., 1997; Rossbach et al., 2007). All the behavioral experiments were conducted during light phase, from 9 a.m. to 3 p.m. (de Almeida et al., 2010) whereas the vehicle and ND were administered from 3 p.m. to 4 p.m. Thus, the last injection was performed on the day prior to the behavioral and neurochemical experiments.

The NMDAr antagonist memantine (MEM; Sigma-Aldrich, M183), and dizocilpine (MK-801; Sigma-Aldrich, M107) were dissolved in saline 0.9%, and were administered intraperitoneally (i.p.) using a single injection volume of 10 ml/kg. The time of peak concentration (60 min) in the rat brain after MEM administration was chosen based on the work of Kotermanski et al. (2013). The dose of MEM (20 mg/kg) was based on the neuroprotective effects achieved in a rat model of glutamatergic hyperexcitability (Zimmer et al., 2012b). The time and dose regimen of MK-801 (0.1 mg/kg) were based on previous published work (Bortolato et al., 2012).

Open-field test

Animals (n = 10 per group/n = 20 per endpoint) were tested for spontaneous and locomotor activity after short, mid and long-term CO or ND administration using a black box apparatus 50 cm × 50 cm × 50 cm. The experiments were conducted in a sound-attenuated room under low-intensity light (12 lx). Animals were placed in the right corner of the arena and locomotor activity was recorded with a video camera for 10 min (Prut and Belzung, 2003). All analyses were performed using a computer-operated tracking system (Any-maze, Stoelting, Woods Dale, IL) (see Supplementary material for detailed description).

Additionally, an independent group of mice were long-term treated with ND or vehicle (CO) and received an i.p. injection of MEM (20 mg/kg) or saline, 60 min before the open-field test (Kotermanski et al., 2013). Animals were assigned to one of the following groups: control + saline (CO + SAL), control + memantine (CO + MEM), ND + saline (ND + SAL) and ND + memantine (ND + MEM); n = 9 per group.

Morris water maze task

We performed the Morris water maze task (MWM) to evaluate spatial memory performance in one independent group of long-term treated animals. The apparatus was a black, circular pool (110 cm diameter) with a water temperature of 21 ± 1 °C. The long-term CO (n = 10) and ND mice (n = 10) were trained daily in a four-trial MWM for five consecutive days; each trial lasted up to 60 s and was followed by 20 s of rest on a hidden black platform. During training, mice learned to escape from the water by finding a hidden, rigid, black platform submerged about 1 cm below the water surface in a fixed location. If the animal failed to find the platform in 60 s, it was manually placed on the platform and allowed to rest for 20 s. Each trial was separated by at least 12 min to avoid hypothermia and facilitate memory acquisition (Muller et al., 2011), see Supplementary Fig. 2.

Resident–intruder test

In this test, aggressive behavior is tested by introducing an intruder into a resident's home cage. As expected, residents are typically more aggressive because they are familiar with the environment and are defending their home territory (Nelson and Trainor, 2007). The protocol of the intruder test used in this study was adapted from a previous work of our group (Kazlauckas et al., 2005). To date, dominant or subordinate animals were not distinguished before the test. If the intruder animal attacks the resident first (~10% of intruders) or showed an exacerbated offensive profile, both animals were excluded from the analysis and were not retested. Thus, all residents attacking the intruder were considered dominant. The test was performed during the light phase based on previous work of Bortolato et al. (2012) and in the proposed concept that light or dark phases produce reliable results in social behavior testing (Yang et al., 2008). As cited above the mice home cages were assigned as resident (ND or vehicle) or intruder.

Two days prior to the intruder test, the home cage sawdust of ND and CO groups was not changed in order for the animals to establish and maintain territoriality within their home cage. Before the test, three resident CF1 mice were removed to an identical and clean cage. One resident mouse remained alone for 2 min habituation prior to introducing the intruder mouse. After testing, the animal number one was moved to the clean cage. For each ND one CO mouse was assessed in the intruder test. The ND and CO animals were tested in their own home cages. The time-delay between each animal test was 15 min, including the first 2 min habituation. Observations of dominant and subordinate status, latency to the first aggressive attack, and the number of attacks of the resident toward the intruder were recorded during a period of 10 min maximum. In this work, an attack was defined as a bout of activity (fight) lasting up to several seconds during which the resident mouse bites the intruder at least once. The behaviors that can be displayed by mice consist of a stationary one-sided attack, frontal attack, chase, wrestle, lunge or boxing inflicted by the resident against the intruder. These components were then recorded as the number of aggressive acts (adapted from Thurmond, 1975).

The intruders did not receive any treatment and were housed in different home cages than were the residents (ND or CO). Residents and intruders were submitted to the resident–intruder test only once. For ethical reasons, if the aggressive behavior put the animals at risk of injuries like blooding or suffering the test was stopped earlier than the maximum test duration of 10 min and both resident and intruder animals were excluded from the analysis (Defensor et al., 2012; Kazlauckas et al., 2005; Simon and Whalen, 1986; Thurmond, 1975).

To characterize the baseline aggressive behavior, three groups of untreated two-month old male mice were subjected to the resident–intruder test for 10 min, with intervals of 48 h (day 1; n = 6, day 2; n = 7 and day 3; n = 7). The aggressive scores of each group did not vary significantly across three independent experiments. The mean latency to first attack was 471.5 ± 42.7 s (S.E.M.), and the mean number of attacks was 2.10 ± 0.65 (S.E.M.).

After that, another group of mice was injected with vehicle (CO) or ND and tested for aggressive behavior at short-, mid- and long-term exposures (n = 10 per group/per endpoint). In addition, another group of long-term ND and CO mice received a single i.p. injection of MEM (20 mg/kg) or saline 60 min before the resident–intruder test (Kotermanski et al., 2013). For this approach, animals were assigned to the following groups: CO + SAL, CO + MEM, ND + SAL and ND + MEM (n = 6 per group, total n = 24).

A separate group of long-term ND and CO mice were i.p. injected with MK-801 (0.1 mg/kg) or saline 30 min before the resident–intruder test (Bortolato et al., 2012). Animals were then assigned to the following groups: CO + SAL, CO + MK-801, ND + SAL and ND + MK-801 (n = 10 per group, total n = 40).

Sodium-dependent glutamate uptake activity

After short-, mid- and long-term vehicle (CO) or ND exposure (n = 6 per group) animals were decapitated and the right hippocampus and frontoparietal cortex were immediately dissected on ice (4 °C). Slices from the hippocampus and frontoparietal cortex (0.4 mm thick) were rapidly prepared using a McIlwain Tissue Chopper, separated in HBSS (in mM: 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 1.11 glucose, pH 7.2) at 4 °C. Hippocampal and frontoparietal cortical slices were pre-incubated with HBSS at 37 °C for 15 min, followed by the addition of 0.33 μCi ml^{-1} L-[^{3}H] glutamate (PerkinElmer®). Incubation was stopped after 5 min for hippocampus and 7 min for frontoparietal cortex with 2 ice-cold washes of 1 ml HBSS. After washing, 0.5 N NaOH was immediately added to the slices and they were stored overnight. Na^+-independent uptake was measured using the above-described protocol with alterations in the temperature (4 °C) and the composition of the medium (N-methyl-D-glucamine instead of NaCl). Results (Na^+-dependent uptake) were measured as the difference between the total uptake and the Na^+-independent uptake. Each incubation was performed in quadruplicate (Thomazi et al., 2004). Incorporated radioactivity was measured using a liquid scintillation counter (Hidex 300 SL).

Cell viability

Mice (n = 5 per group) were decapitated after long-term exposure and the right hippocampus and frontoparietal cortex were immediately dissected on ice (4 °C). Cellular viability was measured in brain slices (0.4 mm thick) by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Oliveira et al., 2002) (for detailed description of MTT assay, see Supplementary material). Active mitochondrial dehydrogenases from living cells are able to reduce MTT to form a purple formazan product. This is feasible method for measuring cell proliferation and neural cytotoxicity in response to drugs.

Western blotting

Mice (n = 6–8 per group) exposed to short-, mid- and long-term ND were decapitated and the left hippocampus and frontoparietal cortex were immediately dissected on ice (4 °C) and stored at −80 °C for homogenate preparations. Hippocampal and frontoparietal cortical homogenates were prepared in PIK buffer (1% NP-40, 150 mM NaCl, 20 mM Tris pH 7.4, 10% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1% phosphatase inhibitor cocktails I and II; Sigma-Aldrich, USA) at 4 °C, and centrifuged. Supernatants were collected and the total protein was measured using the method described by Peterson (1977). For Western blot analysis, 20 μg of protein from hippocampus and frontoparietal cortex homogenate preparations was loaded into each well, separated by electrophoresis on 8% polyacrylamide gel and electrotransferred to PVDF (polyvinyl difluoride, Thermo Scientific Pearce) membranes. Protein bands within each sample lane were compared to standard molecular weight markers (Precision Plus Protein™ Dual Color Standards, Bio-rad), which were used to identify the molecular weight of proteins of interest. Nonspecific binding sites were blocked with Tween-Tris buffered saline (TTBS, 100 mM Tris–HCl, pH 7.5) containing 5% bovine albumin serum (Sigma-Aldrich, USA) for 2 h and then incubated overnight at 4 °C with polyclonal antibody against GLT-1 dissolved in TTBS (1:1000, Alpha Diagnostic International) and monoclonal antibody anti-β-actin (1:3000, Sigma-Aldrich, USA). Membranes were then rinsed 3× for 10 min with TTBS and incubated with secondary antibodies horseradish peroxidase-conjugated secondary antibody (1:3000 dilution, anti-rabbit and 1:3000 dilution anti-mouse, GE Healthcare Life Sciences) for 2 h at room temperature, membranes were then rinsed 4× for 10 min with TTBS and incubated with enhanced chemiluminescent substrate (PerkinElmer®) for 1 min or 2 min at room temperature. The resulting reaction was displayed on autoradiographic film by chemiluminescence (Moreira et al., 2011; Muller et al., 2011). The X-ray films (Kodak X-Omat, Rochester, NY, USA) were scanned and band intensity was analyzed using Image J software (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

Microdialysis

Long-term ND and CO mice (n = 4 per group) were implanted with a guide cannula under anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg) using the following coordinates: anterior = 1.8 mm and lateral = 1.5 mm (both from the bregma), and ventral = 1.5 mm (from the skull) (Paxinos and Franklin, 2001). Animals assigned in each experimental group were randomly chosen from two different cages, and were not distinguished as being dominant or submissive. The mice were allowed to recover for 48 h after implantation of the guide cannula. The location of the dialysis probe was confirmed at the end of the experiment using a vibratome (Leica, Germany) and magnifying lens.

Animals used for microdialysis were allowed to move freely in their cages. A microdialysis probe was slowly inserted into the hippocampus (dentate gyrus-CA3 area) through the guide cannula. The animals were habituated to microdialysis for 1 h (1 μl/min with artificial extracellular fluid aECF; 124 mM NaCl, 3 mM KCl_2, 1 mM $MgSO_4$ $7H_2O$, $NaHCO_3$, 2 mM $CaCl_2$ H_2O, 1 mM glucose, buffered at pH 7.4) using BASi brain microdialysis probes (MD-2200-BR2, USA) (Garrido et al., 2012). To analyze baseline levels of extracellular glutamate, we collected 3 samples at 10 minute increments. Subsequently, individual animals were exposed to an intruder (as previously described) without aECF perfusion, and soon afterward four samples in fractions of 10 min were collected during 40 min to analyze post-test glutamate levels. These animals were used only for microdialysate sampling. Glutamate concentration was analyzed through high-performance liquid chromatography (HPLC).

HPLC procedure

HPLC was performed with cell-free supernatant aliquots to quantify glutamate levels (Schmidt et al., 2009). Briefly, samples were derivatized with *o*-phthalaldehyde and separation was carried out with a reverse phase column (Supelcosil LC-18, 250 mm × 4.6 mm, Supelco) in a Shimadzu Instruments liquid chromatograph (200 μl loop valve injection). The mobile phase flowed at a rate of 1.4 ml/min and column temperature was 24 °C. Buffer composition is A: 0.04 mol/l sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 20% of methanol; and B: 0.01 mol/l sodium dihydrogen phosphate monohydrate buffer, pH 5.5, containing 80% of methanol. The gradient profile was modified according to the content of buffer B in the mobile phase: 0% at 0.00 min, 25% at 13.75 min, 100% at 15.00–20.00 min, and 0% at 20.01–25.00 min. Absorbance was read at 360 nm and 455 nm, excitation and emission respectively, in a Shimadzu fluorescence detector. Samples of 8 μl were used and concentration was expressed in μM.

Biochemical evaluations

After long-term exposure to ND or the vehicle (CO), the animals (n = 10 per group) were sacrificed by decapitation, and then blood was collected and centrifuged (10,000 ×g, 10 min) to obtain serum samples. We evaluated serum biochemical parameters linked with metabolic profile (glucose tolerance test and lipids) and tissue-specific toxicity: kidney (urea, creatinine) and liver (albumin, AST; alanine

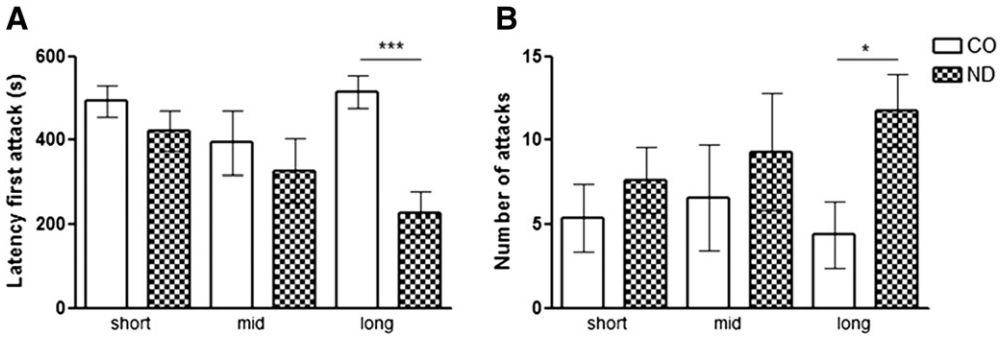

Fig. 2. Resident–intruder test. Long-term ND exposure (15 mg/kg) induced an exacerbated aggressive behavior in CF1 male mice. (A) Latency to first attack ($p < 0.0001$); (B) Number of attacks; ($p = 0.0168$). Data represented mean ± S.E.M. ($n = 10$ per group). *$p = 0.0168$ and ***$p < 0.0001$ indicate significant statistical difference between groups.

aminotransferase and ALT; aspartate aminotransferase) (Muller et al., 2011; Zimmer et al., 2012a) (for detailed description see Supplementary material).

Statistical analysis

Data distribution was analyzed using the Kolmogorov–Smirnov test. The Mann–Whitney *U*-test was used to analyze differences between each ND endpoint (short-, mid-, long-term) and its respective CO group in the resident–intruder test. Differences between groups in neurochemical and biochemical parameters were analyzed using Student's *t* test. Nonparametric comparisons were performed by the Kruskal–Wallis test, with Dunn's post-hoc test comparisons to analyze differences in the resident–intruder test with MEM and MK-801 administration. The differences between groups were considered statistically significant if $p < 0.05$. Results were presented as mean ± standard error of mean (S.E.M.).

The Cohen's *d* was used to estimate the effect size, which is the difference between means divided by standard deviation (S.D.). According to Cohen's *d*, effect size was classified as small ($d = 0.2$), medium ($d = 0.5$) and large ($d \geq 0.8$) (Sullivan and Feinn, 2012).

Results

Nandrolone-induced aggressive behavior

ND exposure did not cause significant decrease in the latency to first attack in short- ($p = 0.199$; Cohen's $d = 0.36$), and mid-term exposures ($p = 0.662$; Cohen's $d = 0.28$) compared to their respective CO groups (Fig. 2A). Also, there was no significant difference in the number of attacks in short- ($p = 0.372$; Cohen's $d = -0.25$), and mid-term exposures ($p = 0.525$; Cohen's $d = -0.26$) compared to their respective CO groups (Fig. 2B). In contrast, long-term administration of ND significantly decreased the latency to first attack (Fig. 2A, $p < 0.0001$; Cohen's $d = 1.41$), and increased the total number of attacks against an intruder compared to the CO group (Fig. 2B, $p = 0.0168$; Cohen's $d = -0.77$). For more detailed pairwise comparisons see Table 1.

Nandrolone decreased glutamate uptake and immunocontent of GLT-1

Long-term ND exposure significantly decreased glutamate uptake in slices of hippocampus (Fig. 3A, $p = 0.03$; Cohen's $d = 1.83$) and frontoparietal cortex (Fig. 3B, $p = 0.004$; Cohen's $d = 1.20$) when compared to the CO group. Moreover, the immunoquantification of the glutamate transporter 1 (GLT-1) by Western blotting revealed a significant decrease in hippocampus (Fig. 3C, $p = 0.03$; Cohen's $d = 1.26$) and frontoparietal cortex (Fig. 3D, $p = 0.0003$; Cohen's $d = 2.02$). For more detailed pairwise comparisons see Tables 2 and 3. The decreased glutamate uptake activity and GLT-1 expression may cause increases in the glutamate levels associated with hyperstimulation of glutamate receptors and cell damage and/or dysfunction. Thus, we performed analyses of cell viability through MTT assay in slices of frontoparietal cortex and hippocampus after short- and long-term ND exposures ($n = 5$ per group). There was no significant difference observed between the ND and CO groups, suggesting no overt signs of decreased neural cells' viability/damage in frontoparietal cortex (short-, $p = 0.577$; Cohen's $d = 0.39$ and long-term, $p = 0.682$; Cohen's $d = 0.61$) and hippocampus (short-, $p = 0.629$; Cohen's $d = -0.29$ and long-term, $p = 0.579$; Cohen's $d = 0.42$) (see Supplementary Table 2).

Short- and mid-term exposures to ND or vehicle (CO) ($n = 6–8$) did not cause significant changes, either in the immunocontent of GLT-1 in hippocampus (short-, $p = 0.785$; Cohen's $d = 0.10$ and mid-, $p = 0.086$; Cohen's $d = 0.81$) and frontoparietal cortex (short-, $p = 0.694$; Cohen's $d = 0.25$ and mid-term, $p = 0.069$; Cohen's $d = 0.88$), or in glutamate-uptake capacity in same brain areas (data not shown).

Long-term ND exposure increased hippocampal extracellular glutamate levels

The glutamate levels in the long-term (ND) group not exposed to intruder (baseline) did not show statistical significant difference compared to the control (CO) group (Fig. 4, $p = 0.06$; Cohen's $d = -1.70$). After exposure to the intruder test, the glutamate levels in the ND group significantly increase compared to CO animals (Fig. 4, $p =$

Table 1
Resident–intruder test after vehicle (CO) or nandrolone (ND) administration (short-, mid-, and long-term endpoints, $n = 10$ per group/per endpoint).

Behavior test	Time regimen	Parameters	Control (CO)	Nandrolone (ND)	Cohen's *d*	Statistical difference	n per group/per endpoint
Intruder test	Short-term exposure	Latency to first attack (s)	493.6 ± 172.0	422.7 ± 221.8	0.36	n.s.	10
	Mid-term exposure		393.7 ± 240.6	327.0 ± 240.8	0.28	n.s.	
	Long-term exposure		515.9 ± 176.9	227.2 ± 233.4	1.41	$p < 0.0001$	
	Short-term exposure	Number of attacks	5.4 ± 9.2	7.6 ± 9.1	−0.25	n.s.	
	Mid-term exposure		6.6 ± 10.0	9.3 ± 11.0	−0.26	n.s.	
	Long-term exposure		4.4 ± 9.2	11.8 ± 10.0	−0.77	$p = 0.0168$	
						n Total	*60*

Values are expressed as mean ± S.D.

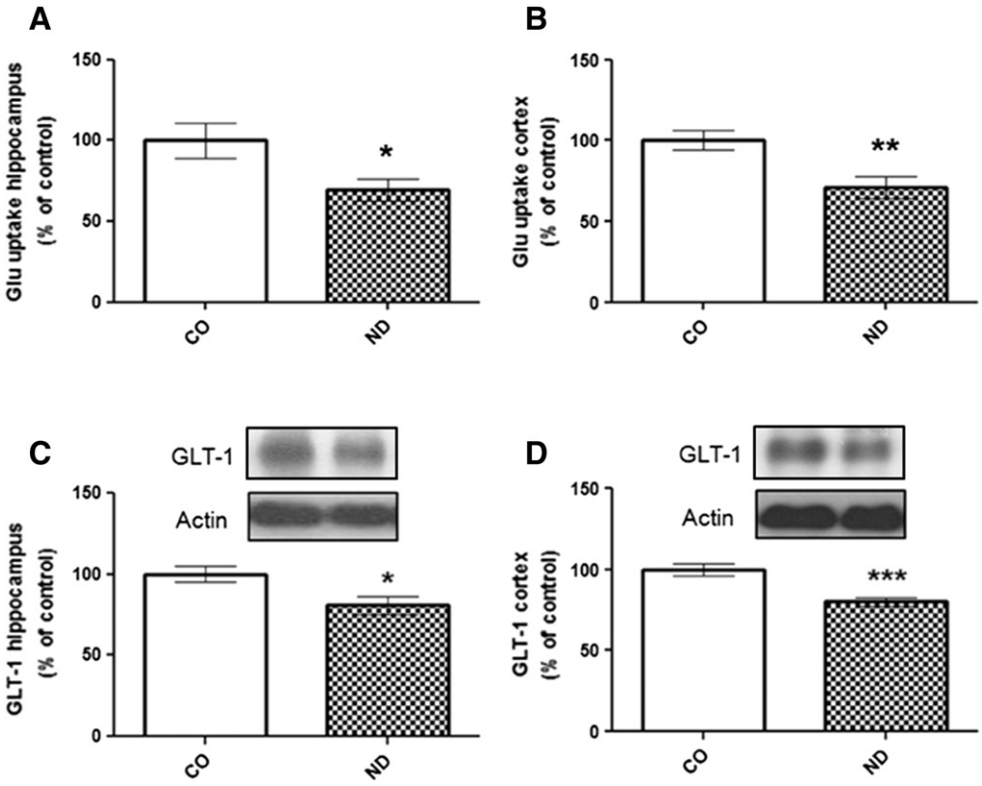

Fig. 3. Glutamate uptake and Western blotting. Nandrolone (ND) decreases glutamate uptake and immunocontent of GLT-1 in hippocampus and frontoparietal cortex. (A) Glutamate uptake in slices of hippocampus, and (B) frontoparietal cortex (n = 6). (C) Immunocontent of GLT-1 in hippocampus and, (D) frontoparietal cortex (n = 6–8). Data represented mean ± S.E.M. *p = 0.03, **p = 0.004 and ***p = 0.0003 indicate significant difference between groups. Short- and mid-term ND exposures were not significantly different from their respective control groups.

Table 2
Glutamate uptake in slices of hippocampus and cortex in long-term groups (CO and ND, n = 6 per group).

Neurochemical assay	Brain structure	Time regimen	Control (CO)	Nandrolone (ND)	Cohen's *d*	Statistical difference	n per group/per endpoint
Glutamate uptake	Hippocampus	Long-term exposure	100.0 ± 11.1	69.56 ± 22.2	1.83	p = 0.03	6
	Cortex	Long-term exposure	100.0 ± 22.7	70.93 ± 25.7	1.20	p = 0.004	
						n Total (hippocampus)	*12*
						n Total (cortex)	*12*

Values are expressed as mean ± S.D.

0.04; Cohen's $d = -3.00$). Extracellular microdialysate fluid was collected and glutamate was measured with HPLC. Data represent mean ± S.E.M. (n = 4 per group). For more detailed comparisons see Table 4.

Memantine and MK-801 decreases aggressive behavior induced by nandrolone

A single MEM administration after long-term ND exposure did not cause significant changes in the locomotor activity on open-field test (see results in the Supplementary Table 3). This indicates an absence of sedative effects, which could be a confounding factor in the resident–intruder.

We additionally investigated the possible connection between increased extracellular glutamate levels and NMDAr hyperexcitability mediating aggressive behavior. Thus, long-term ND + SAL group significantly decreased the latency to first attack and increased the number of attacks compared to CO + SAL group (Fig. 5A, p = 0.022; Cohen's $d = 2.97$ and Fig. 5B, p = 0.0457; Cohen's $d = -2.73$ respectively). The

Table 3
Immunocontent of GLT-1 in homogenates of hippocampus and frontoparietal cortex after vehicle (CO) or nandrolone (ND) administration (Short-, mid- and long-term endpoints).

Neurochemical assay	Brain structure	Time regimen	Control (CO)	Nandrolone (ND)	Cohen's *d*	Statistical difference	n per group
Western blotting	Hippocampus	Short-term exposure	100.0 ± 14.0	98.0 ± 24.4	0.10	n.s.	6
		Mid-term exposure	100.0 ± 18.9	84.1 ± 20.2	0.81	n.s.	
		Long-term exposure	100.0 ± 13.9	80.9 ± 16.5	1.26	0.03	
	Cortex	Short-term exposure	100.0 ± 15.4	96.01 ± 16.3	0.25	n.s.	8
		Mid-term exposure	100.0 ± 15.9	84.98 ± 18.3	0.88	n.s.	
		Long-term exposure	100.0 ± 11.3	80.25 ± 8.3	2.02	p = 0.0003	
						n Total (hippocampus)	*36*
						n Total (cortex)	*48*

Values are expressed as mean ± S.D., n = 6 for hippocampus and n = 8 for cortex per group/per endpoint (short-, mid- and long-term exposures).

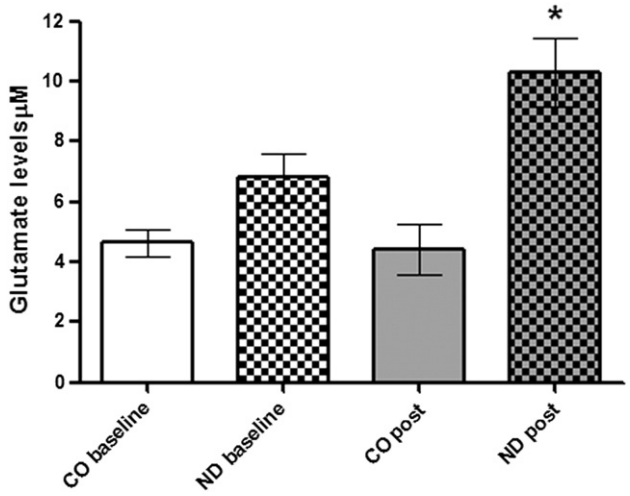

Fig. 4. Glutamate levels in extracellular fluid of hippocampus. The glutamate levels in long-term nandrolone (ND) and vehicle (CO) animals not exposed to intruder (baseline). After exposure to intruder test (post-test), the animals significantly increase the glutamate levels when compared to CO animals ($p = 0.04$). Extracellular microdialysate fluid was collected and glutamate was measured with HPLC. Data represent mean ± S.E.M. ($n = 4$ per group).

administration of MEM significantly increased the latency to first attack and decreased the number of attacks (Fig. 5A, $p = 0.017$; Cohen's $d = -2.99$ and Fig. 5B, $p = 0.003$; Cohen's $d = 3.50$) in ND + MEM compared to the ND + SAL group, respectively. The MEM per se did not significantly affect the latency to first attack or number of attacks in CO + SAL compared to CO + MEM (Figs. 5A and B, $p = 0.999$; Cohen's $d = 0.08$ and $d = -0.04$ respectively).

Additionally, long-term ND + SAL group significantly decreased the latency to first attack and increased the number of attacks compared to CO + SAL group (Fig. 5C, $p = 0.004$; Cohen's $d = 1.57$ and Fig. 5D, $p = 0.024$; Cohen's $d = -1.05$ respectively). The administration of MK-801 significantly increased the latency to first attack and decreased the number of attacks (Fig. 5C, $p = 0.004$; Cohen's $d = -1.32$ and Fig. 5D, $p = 0.002$; Cohen's $d = 1.38$) in ND + MK-801 compared to ND + SAL group, respectively. As expected, the MK-801 per se did not significantly affect the latency to first attack or number of attacks in CO + SAL compared to CO + MK-801 (Figs. 5C and D, $p = 0.999$; Cohen's $d = -0.10$ and $d = 0.22$ respectively). These pharmacological approaches with MEM or MK-801 reinforce the implication of NMDAr hyperexcitability in the mechanism of aggression For more detailed pairwise comparisons see Tables 5 and 6.

Biochemical evaluation

Serum biochemical results are presented in Supplementary Table 1. After long-term exposure, ND significantly increased serum levels of triglycerides ($p = 0.0468$; Cohen's $d = -1.32$) and decreased HDL-cholesterol serum levels ($p = 0.0017$; Cohen's $d = 1.46$) compared to the CO. The capacity of mice in regulating glucose homeostasis (GTT) was not affected by treatments (data not shown). Similarly, there were no significant alterations in serum biochemical markers of liver and kidney damage. The food consumption and body weight gain during short-term to long-term exposure were similar between groups. As expected, testicular weight (in grams) was significantly decreased in ND relative to CO (0.291 g ± 0.04 versus 0.251 g ± 0.04, $p = 0.007$; Cohen's $d = 1.00$).

Discussion

Our results demonstrate that long-term ND-induced aggressive behavior is associated with glutamatergic abnormalities like down-regulation of astrocytic glutamate uptake and increases in the glutamate levels mediating NMDAr hyperexcitability. In accordance to this, the administration of NMDAr antagonists MEM (uncompetitive low-affinity NMDAr antagonist) and MK-801 (uncompetitive high-affinity NMDAr antagonist), shortly before the intruder test reduce the aggressive behavior in long-term ND group.

Short-, mid- and long-term ND exposures did not affect locomotor and exploratory activity on open-field test (see Supplementary Fig. 1) indicating that there was no alteration in the motor components of aggressive responding. In fact, studies evaluating putative effects of AAS on spontaneous locomotion and exploratory profile in rodents have shown no significant changes (see review of Clark and Henderson, 2003). Similarly, the spatial memory performance on MWM in both acquisition and retention phases showed no significant differences between ND and CO groups suggesting normal spatial information processing (see Supplementary Fig. 2). As the hippocampus participates in both memory and aggressive behavior and is very sensitive to repetitive stress, this evaluation allows ruling out the overlap of hippocampal responses associated with long-term ND administration. In contrast to our findings, long-term administration of ND (15 mg/kg) impairs MWM performance in both acquisition and retention phases in rats (Magnusson et al., 2009; Tanehkar et al., 2013). Overall, these behavioral outcomes, associated with the lack of differences on kidney and liver serum biochemical markers (see Supplementary Table 1), strengthen the view that our long-term ND treatment regimen impacts central mechanisms associated with aggression without apparent toxic effects on these organs.

Also, the temporal aggressive profile reported here corroborates published data by Carrillo et al. (2011a), in which an AAS cocktail induced progressive alterations in the glutamatergic parameters associated with increased aggressive scores. Glutamatergic neurotransmission in the hypothalamus is an output aggressive system modulated by gonadal hormones and AAS (Brann and Mahesh, 1995; Brody et al., 1969; Diano et al., 1997; Fischer et al., 2007; Haller, 2013), which also receives many inputs from different brain areas and neurotransmitter systems. This implies that the neurobiology of aggressive behavior involves a complex and intricate collaborative network (Carrillo et al., 2011b; Ferris et al., 2008; Nelson and Trainor, 2007). Accordingly, a neural circuit composed of several regions including the prefrontal cortex, amygdala, hippocampus, hypothalamus, anterior cingulated cortex, and other interconnected structures has been implicated in emotion regulation. Consequently, functional or structural abnormalities in one or more of these regions or in the interconnections among them can increase the susceptibility for impulsive aggression and violence (Nelson and Chiavegatto, 2001). Considering these functional interconnections,

Table 4
Microdialysis/HPLC in long-term groups (CO and ND) at baseline and after exposed an intruder ($n = 4$ per group).

Behavior test	Time regimen	Parameters	Time period	CO	ND	Cohen's *d*	Statistical difference	n per group
Microdialysis/HPLC after and before intruder	Long-term exposure	Glutamate levels (µM)	Baseline	4.63 ± 0.92	6.80 ± 1.64	−1.70	$p = 0.06$	4
			Post-test	4.43 ± 1.65	10.30 ± 2.26	−3.00	$p = 0.04$	
							n Total	*8*

Values are expressed as mean ± S.D.

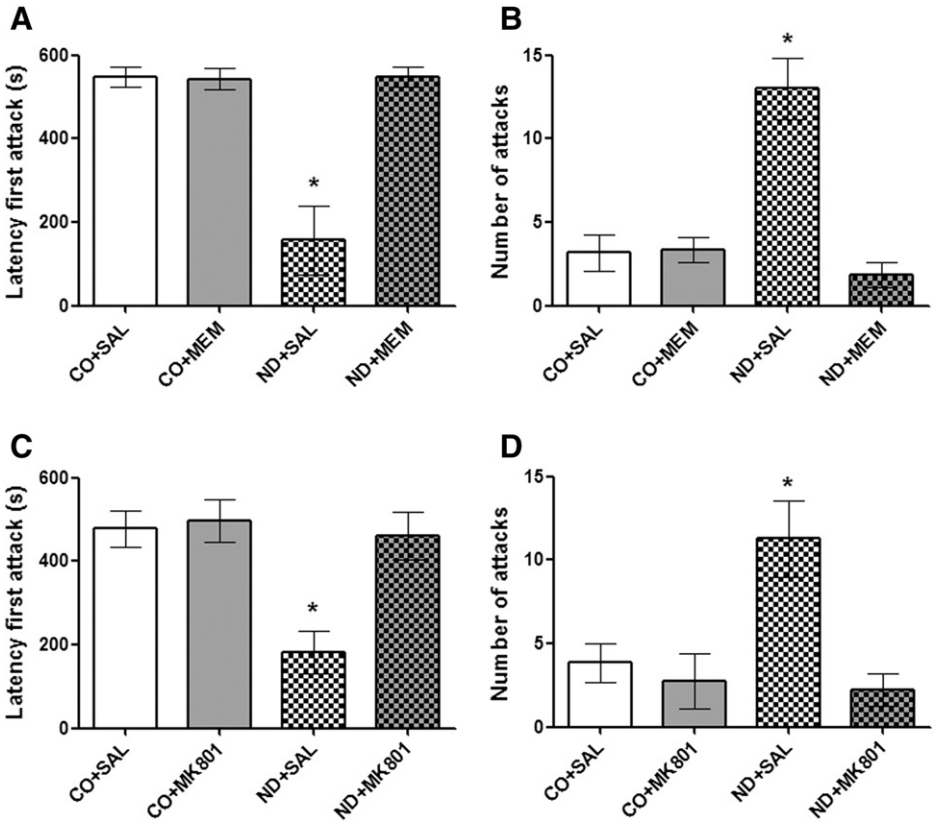

Fig. 5. N-methyl-D-aspartate receptor (NMDAr) antagonists and resident-intruder test. Memantine (20 mg/kg i.p.) or MK-801 (0.1 mg/kg i.p.) administration in long-term ND mice, 60 min and 30 min respectively prior to intruder test, restores the aggressive scores to levels of the CO group: (A) Latency to first attack (CO + SAL versus ND + SAL; $p = 0.0219$ and ND + SAL versus ND + MEM; $p = 0.0168$) and (B) Number of attacks (CO + SAL versus ND + SAL; $p = 0.0457$ and ND + SAL versus ND + MEM; $p = 0.0029$, $n = 6$ per group) after memantine administration; (C) Latency to first attack (CO + SAL versus ND + SAL; $p = 0.0041$ and ND + SAL versus ND + MK-801; $p = 0.0041$); and (D) Number of attacks (CO + SAL versus ND + SAL; $p = 0.0239$, and ND + SAL versus ND + MK-801; $p = 0.0015$, $n = 10$ per group) after MK-801 administration. Data represent mean ± S.E.M. *$p < 0.05$ indicates significant statistical difference between groups.

Table 5
Resident–intruder test in long-term groups (CO and ND) after a single memantine (MEM) administration ($n = 6$ per group).

Behavior test	Time regimen	Parameters	CO/SAL	CO/MEM	ND/SAL	ND/MEM	Cohen's *d*	Statistical difference	n per group
Intruder test with MEM	Long-term exposure	Latency to first attack (s)	548.3 ± 59.6	543.3 ± 60.6			0.08	n.s.	6
					156.7 ± 203.8	547.7 ± 57.4	−2.99	$p = 0.017$	
			548.3 ± 59.6		156.7 ± 203.8		2.97	$p = 0.022$	
				543.3 ± 60.6		547.7 ± 57.4	−0.04	n.s.	
		Number of attacks	3.2 ± 2.6	3.3 ± 1.9			−0.07	n.s.	
					13.0 ± 4.6	1.8 ± 1.8	3.50	$p = 0.003$	
			3.2 ± 2.6		13.0 ± 4.6		−2.73	$p = 0.046$	
				3.3 ± 1.9		1.8 ± 1.8	0.81	n.s.	
								n Total	*24*

Values are expressed as mean ± S.D. Oil vehicle (CO), nandrolone (ND), saline (SAL) and memantine (MEM, 20 mg/kg). The MEM was administered (i.p.), 60 min before the test.

Table 6
Resident–intruder test in long-term groups (CO and ND) after a single MK-801 administration ($n = 10$ per group).

Behavior test	Time regimen	Parameters	CO/SAL	CO/MK-801	ND/SAL	ND/MK-801	Cohen's *d*	Statistical difference	n per group
Intruder test with MK-801	Long-term exposure	Latency to first attack (s)	478.4 ± 164.74	496.3 ± 194.64			−0.10	n.s.	10
					180.8 ± 215.39	460.6 ± 207.53	−1.32	$p = 0.004$	
			478.4 ± 164.74		180.8 ± 215.39		1.57	$p = 0.004$	
				496.3 ± 194.64		460.6 ± 207.53	0.18	n.s.	
		Number of attacks							
			3.86 ± 4.40	2.73 ± 6.39			0.22	n.s.	
					11.28 ± 9.66	2.23 ± 3.54	1.38	$p = 0.002$	
			3.86 ± 4.40		11.28 ± 9.66		−1.05	$p = 0.024$	
				2.73 ± 6.39		2.23 ± 3.54	0.10	n.s.	
								n Total	*40*

Values are expressed as mean ± S.D. Oil vehicle (CO), nandrolone (ND), saline (SAL); dizolcipine (MK-801). The MK-801 (0.1 mg/kg) was administered (i.p.), 30 min before the test.

it seems that the facilitating role attributed to glutamatergic system on aggressive responding is widespread in the brain (Ricci et al., 2007).

Furthermore, long-term ND exposure decreased the immunocontent of GLT-1 and Na^+-dependent glutamate uptake activity in the hippocampus and frontoparietal cortex, acting as contributory factors to the observed increase in hippocampal glutamate levels when animals were exposed to an intruder. These findings support the concept that the peak concentration and rate of clearance of glutamate from the synaptic cleft are important determinants of synaptic function (Clements et al., 1992), and also shed lights for the putative impact of glutamate transporters dysfunction in the mechanisms of aggression. Considering that high glutamate levels are implicated in the genesis of aggression and violent behavior (Munozblanco and Castillo, 1987), and that NMDAr is a candidate modulator of several behavioral phenotypes, we conjectured that ND mediates the aggressive responding through NMDAr.

Therefore, we administrated MEM and MK-801 antagonists to modulate NMDAr activity and aggression in long-term ND exposed mice. Although, the NMDAr antagonists may exhibit a sedative effect (Umukoro et al., 2013), long-term ND mice injected with MEM (20 mg/kg, 60 min before the test) did not show a significantly reduced locomotor profile in the open-field test, implying that MEM might modulate specific mechanisms of aggression without sedative effects. Indeed, the same dose and time regimen of MEM used in the open-field decreased the aggressive outcomes in the long-term ND mice. It seems clear that NMDAr antagonism is the primary mechanism of action of therapeutic relevance for MEM although additional effects at 5-HT3, α-7 nicotinic acetylcholine and dopamine D2 receptors may be supportive for therapeutic effects (Rammes et al., 2008). Thus, the significantly reduced aggressive behavior attained with MEM may also include the participation of these neurotransmitter systems. However, this issue was not addressed by this study. Similarly to MEM, acute injection of MK-801 mitigates the aggressive outcomes in long-term ND exposed mice. It is recognized that MK-801 increases spontaneous locomotion (Bortolato et al., 2012; Tort et al., 2004), albeit doses up to 0.15 mg/kg barely increase locomotor activity in mice (Su et al., 2007) and rats (Ouagazzal et al., 1993). Bortolato et al. (2012) showed that wild-type mice injected with MK-801 (0.1 mg/kg) caused hyperlocomotion and none anti-aggressive effects; but despite this the same dose in our work decreased the aggressive outcomes in long-term ND mice. In contrast to our hypothesis, there are studies showing that NMDAr antagonism with phencyclidine or memantine increases rather than decrease aggressive behavior (Audet et al., 2009; Newman et al., 2012). Taken together, these results corroborate the functional relevance of increased brain glutamate levels and the NMDAr binding sites in the neurobiology of aggression.

Conclusion

In summary, long-term ND-induced aggressive behavior is associated with decreased extracellular glutamate clearance and NMDAr hyperexcitability emphasizing the role of this receptor in mediating mechanisms of aggression.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2014.06.005.

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The authors have no biomedical financial interests to disclose.

Conflict of interest

There are no conflicts of interest with other people or organizations.

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Supplementary Material

Materials and methods

Cell viability

Mice (n=5 per group) were decapitated after long-term exposure and the right hippocampus and frontoparietal cortex were immediately dissected on ice (4°C). Slices (0.4 mm thick) from the respective structures were rapidly prepared using a McIlwain Tissue Chopper, separated in HBSS. Cellular viability was measured in brain slices by MTT assay. Active dehydrogenases from living cells reduce tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to form a purple formazan product, which is quantified spectrophotometrically at a wavelength of 550 nm.

Biochemical evaluations

After long-term exposure to ND or vehicle, animals (n=10 per group) were sacrificed by decapitation, and then blood was collected and centrifuged (10,000 x g, 10 min) to obtain serum samples. We evaluated serum biochemical parameters linked with metabolic profile (glucose tolerance test and lipids) and tissue-specific toxicity: kidney (urea, creatinine) and liver (albumin, AST and ALT). All biochemical determinations were performed using commercial kits (Labtest, MG, Brazil) in a Spectramax M5 (Molecular Devices, USA). For glucose tolerance test (GTT) a glucose solution (2 mg/g i.p.) was injected into mice fasted for 12 h, and blood was collected through a small puncture on the tail at 0, 30, 60, and 120 min. Blood glucose level was measured with glucometer (Accu-Chek Active, Roche Diagnostics, USA), and the area under the curve was used to compare the glucose tolerance among groups. Testicular weight was also measured.

Supplementary Figures

Figure 1

A

Open-field test in long-term groups (CO and ND, *n=10 per group*)

Behavior test	Time regimen	Parameters	Control (CO)	Nandrolone (ND)	Cohen's *d*	Statistical difference	**n** *per group*
Open-field	Long-term exposure	Distance (m)	44.68 ± 12.32	41.69 ± 12.52	0.24	n.s.	10
		Mean speed (m/s)	0.074 ± 0.02	0.069 ± 0.02	0.25	n.s.	
		Time in the center (s)	164.53 ± 46.96	154.41 ± 60.05	0.19	n.s.	
						n TOTAL	***20***

Values are expressed as mean ± S.D.

B

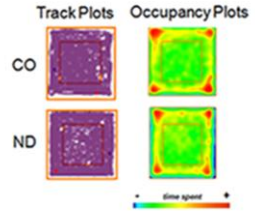
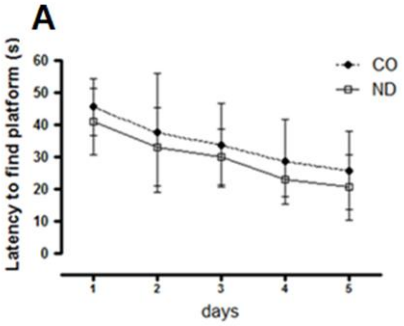
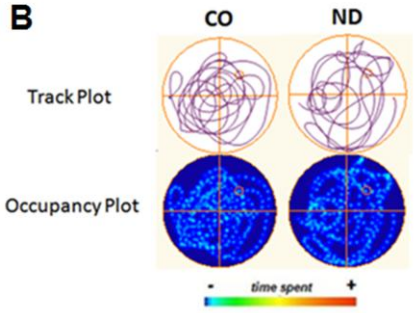


Figure 2





C

Water-Maze (probe session) in long-term groups (CO and ND, *n=10 per group*).

Behavior test	Time regimen	Parameters	Control (CO)	Nandrolone (ND)	Cohen's *d*	Statistical difference	**n** *per group*
Water-Maze (probe session)	Long-term exposure	Distance (m)	13.36 ± 2.34	12.94 ± 1.35	0.23	n.s.	10
		Time (s) in target quadrant	17.10 ± 5.47	14.31 ± 4.31	0.57	n.s.	
		Mean speed (m/s)	0.22 ± 0.04	0.22 ± 0.02	0.00	n.s.	
						n TOTAL	***20***

Values are expressed as mean ± S.D.

Table 1

Serum biochemical evaluations in long-term groups (CO and ND, *n=10 per group*).

Parameters	CO	ND	Cohen's *d*
ALT (IU/L)	7.19 ± 1.99	7.06 ± 3.46	0.05
AST (IU/L)	22.92 ± 15.11	21.87 ± 10.19	0.08
Albumin (g/dL)	3.54 ± 0.23	3.56 ± 0.25	-0.08
Creatinine (mg/dL)	0.26 ± 0.11	0.29 ± 0.19	-0.20
Urea (mg/dL)	42.78 ± 9.2	40.17 ± 7.1	0.32
Triglycerides (mg/dL)	71.2 ± 16.6	109.8 ± 42.3 #	-1.32
Cholesterol (mg/dL)	151.4 ± 49.7	126.0 ± 37.6	0.58
HDL-cholesterol (mg/dL)	36.96 ± 7.15	26.43 ± 7.28 #	1.46

Values are expressed as mean ± S.D.
ALT; Alanine aminotransferase, AST; Aspartate aminotransferase, CO; control and ND; nandrolone.
Significant difference compared with the control ($p < 0.05$).

Table 2

Cell Viability Assay (MTT) in slices of hippocampus and cortex after short- and long-term CO and ND administration (*n=5 per group/per endpoint*).

Neurochemical assay	Brain structure	Time regimen	Control (CO)	Nandrolone (ND)	Cohen's d	Statistical difference	**n** *per group*
Cell Viability Assay (MTT)	Hippocampus	Short-term exposure	100.0 ± 4.97	102.3 ± 9.22	-0.29	n.s.	5
		Long-term exposure	100.0 ± 15.33	90.00 ± 16.79	0.42	n.s.	
	Cortex	Short-term exposure	100.0 ± 17.00	94.00 ± 13.65	0.39	n.s.	
		Long-term exposure	100.0 ± 12.96	93.25 ± 9.29	0.61	n.s.	
						n TOTAL *(hippocampus)*	***20***
						n TOTAL *(cortex)*	***20***

Values are expressed as mean ± S.D. CO; control, ND; nandrolone and MTT; (3-(4,5- 284 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

Table 3

Open-field test in long-term groups (CO and ND) after a single MEM administration (*n=9 per group*).

Behavior test	Time regimen	Parameters	CO/SAL	CO/MEM	ND/SAL	ND/MEM	Cohen's *d*	Statistical difference	**n** *per group*
Open-field with MEM	Long-term exposure	Distance (m)	37.33 ± 9.94	29.71 ± 24.50			0.44	n.s.	9
					40.39 ± 10.45	32.34 ± 18.69	0.55	n.s.	
			37.33 ± 9.94		40.39 ± 10.45		-0.30	n.s.	
				29.71 ± 24.50		32.34 ± 18.69	-0.12	n.s.	
		Mean speed (m/s)	0.062 ± 0.017	0.05 ± 0.04			0.41	n.s.	
					0.07 ± 0.02	0.05 ± 0.03	0.54	n.s.	
			0.062 ± 0.017		0.07 ± 0.02		-0.29	n.s.	
				0.05 ± 0.04		0.05 ± 0.03	-0.11	n.s.	
								n TOTAL	***36***

Values are expressed as mean ± S.D. Oil vehicle (CO), nandrolone (ND), saline (SAL) and memantine (MEM). The MEM (20 mg/Kg) was administered (i.p), 60 min before the test.

PARTE III.

1. DISCUSSÃO

Administrações de curto, médio e longo período com DN não afetaram a atividade locomotora e exploratória no teste de campo aberto em camundongos CF1. Nossos achados corroboram com a literatura, uma vez que não foram observados efeitos dos EAAs sobre a locomoção espontânea no campo aberto, tanto em ratos como em camundongos (Clark and Henderson, 2003). Também não observamos alterações em parâmetros de ansiedade acessados através do labirinto em cruz elevado (dados não apresentados), o que contrapõe o efeito ansiolítico observado em ratos Long–Evans no mesmo aparato (Bitran et al., 1993). A administração de altas doses de propionato de T durante 6 dias de tratamento, aumentou a exploração nos braços abertos, efeito considerado ansiolítico. De uma maneira geral, os poucos estudos que abordaram EAAs e ansiedade apresentam resultados controversos; mais especificamente, o tratamento com DN em ratos apresentou tanto efeitos ansiolíticos como ansiogênicos (Bitran et al., 1993; Clark and Henderson, 2003).

Corroborando com os resultados de Clark et al., 1995, não observamos prejuízos na memória espacial no labirinto aquático de Morris (MWM) diante do tratamento crônico com DN. O desempenho da memória espacial no MWM em nas fases de aquisição e retenção não apresentaram diferenças significativas entre os grupos ND e CO sugerindo o processamento normal de informações espaciais (ver Figura Suplementar 2). Em contraste com os nossos resultados, outros autores relataram que a administração a longo prazo da DN (15 mg/kg) prejudicou o desempenho de ratos no MWM (Magnusson et al., 2009; Tanehkar et al., 2013). Além disso, não observamos efeitos nos parâmetros de toxicidade avaliados através do MTT e de marcadores bioquímicos séricos (ver Tabelas Suplementares 1 e 2), embora os níveis de triglicerídeos tenham aumentado e os de HDL tenham sido reduzidos frente a exposição

crônica ao DN. Observamos também uma redução significativa no peso dos testículos dos animais. Estes resultados são semelhantes aos encontrados em humanos que fazem uso abusivo de EAAs (Bonetti et al., 2008) e em camundongos submetidos à administração crônica de propionato de T (Martinez-Sanchis et al., 2003).

Os andrógenos são reconhecidos como moduladores do comportamento agressivo (Clark and Henderson, 2003; McGinnis, 2004; Nelson, 2006). A neurobiologia do fenótipo agressivo envolve uma rede complexa e intrincada, abrangendo diversas regiões cerebrais e sistemas de neurotransmissão (Carrillo et al., 2011c; Ferris et al., 2008; Nelson and Trainor, 2007). A exposição aos EAAs diminui os níveis de serotonina em regiões cerebrais (Grimes and Melloni, 2002), e diminui os níveis de RNA mensageiro dos receptores 5-HT na amígdala e no córtex pré-frontal. Isto sugere um papel central dos receptores 5-HT_{1B} nas alterações comportamentais observadas diante de altas doses de EAAs (Ambar and Chiavegatto, 2009). No que diz respeito ao sistema dopaminérgico, o antagonista dos receptores D2 haloperidol, é efetivo no tratamento de pacientes agressivos (de Almeida et al., 2005). Neste contexto, os receptores D2 no hipotálamo têm influência direta no comportamento agressivo induzido pelos EAAs (Schwartzer and Melloni, 2010). Associado a isto, sugere-se um potencial mecanismo da estimulação dopaminérgica associada aos EAAs causando o fenótipo agressivo através da diminuição dos efeitos inibitórios do ácido gama-aminobutírico (GABA) (Schwartzer et al., 2009). Estudos demonstram que a exposição crônica aos EAAs altera a função e expressão dos receptores $GABA_A$, bem como a síntese a funcionalidade da enzima glutamato descarboxilase (GAD_{65}) (Grimes et al., 2003; Henderson et al., 2006).

A neurotransmissão glutamatérgica no hipotálamo é modulada pelos hormônios gonodais e EAAs (Brann and Mahesh, 1995; Brody et al., 1969; Diano et al., 1997;

Fischer et al., 2007; Haller, 2013). Nossos resultados demonstram que administração crônica do DN aumenta o fenótipo agressivo por mecanismos associados ao desequilíbrio do sistema glutamatérgico no córtex e no hipocampo de camundongos CF1. Nós observamos que essa alteração comportamental se deve à ruptura da homeostase glutamatérgica, que por sua vez, é resultante da diminuição da atividade/imunoconteúdo do transportador glutamatérgico GLT-1, culminando no aumento dos níveis extracelulares de glutamato. Esse comportamento agressivo foi significativamente atenuado pelo antagonismo dos NMDAr. O perfil agressivo induzido pelo DN aqui relatado se assemelha aos dados publicados por Carrillo et al. (2011a), em que um *cocktail* de EAAs induziu alterações progressivas em parâmetros glutamatérgicos em hamsters (Carrillo et al., 2011a). Modificações nas projeções neuronais glutamatérgicas do hipotálamo às estruturas adjacentes, foram sugeridas como mecanismos facilitatórios do comportamento agressivo induzido por EAAs (Carrillo et al., 2011b). O estudo de Bortolato et al., (2012) demonstrou o envolvimento das subunidades regulatórias NR2A e NR2B do NMDAr no córtex pré-frontal mediando o fenótipo agressivo (Bortolato et al., 2012).

O tratamento crônico com DN aumentou os níveis de glutamato no hipocampo do animal residente após a exposição ao intruso (ver Figura 4), demonstrando a implicação das alterações funcionais no transporte de glutamato. De acordo com o estudo de Takahashi et al., (2011) o fenótipo agressivo resultante da remoção cirúrgica do bulbo olfatório, foi atenuado pela administração de riluzole, com concomitante redução dos níveis de glutamato no córtex pré-frontal (Takahashi et al., 2011). Nós verificamos que a administração de memantina (MEM) resultou na diminuição da agressividade induzida pela administração crônica de DN (ver Figura 5), embora a MEM possa apresentar um efeito sedativo (Umukoro et al., 2013). Nós excluímos este

efeito sedativo no nosso modelo experimental, tendo em vista que os camundongos não apresentaram alterações locomotoras (MEM 20 mg/kg, 60 minutos antes do teste de intruso). O antagonismo do NMDAr é o principal mecanismo de ação de relevância terapêutica da MEM, embora os efeitos adicionais nos receptores de serotonina ($5\text{-}HT_3$), de acetilcolina (α-7 nicotínico) e de dopamina (D_2) também possam influenciar nos efeitos terapêuticos (Rammes et al., 2008). Assim, o comportamento agressivo atenuado pela administração da MEM pode compartilhar mecanismos de ação com estes sistemas de neurotransmissão. Da mesma forma que a MEM, a injeção aguda de MK-801 resultou em diminuição da manifestação agressiva em animais expostos ao DN. Contrapondo nossas evidências, estudos relataram que os antagonistas do NMDAr, como fenciclidina e a MEM, aumentam o comportamento agressivo (Audet et al., 2009; Newman et al., 2012). Em conjunto, nossos resultados ressaltam a relevância funcional do sistema glutamatérgico na neurobiologia da agressividade induzida por EAAs.

2. CONCLUSÃO

O comportamento agressivo induzido pela administração crônica de DN diminui a remoção do glutamato da fenda sináptica, culminando com o aumento nos níveis do glutamato extracelular no SNC, o que resulta na hiperexcitabilidade do NMDAr. O fenótipo agressivo parece não estar associado com mecanismos tóxicos, enfatizando o papel da comunicação entre astrócitos e neurônios na facilitação da manifestação agressiva.

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