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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.

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

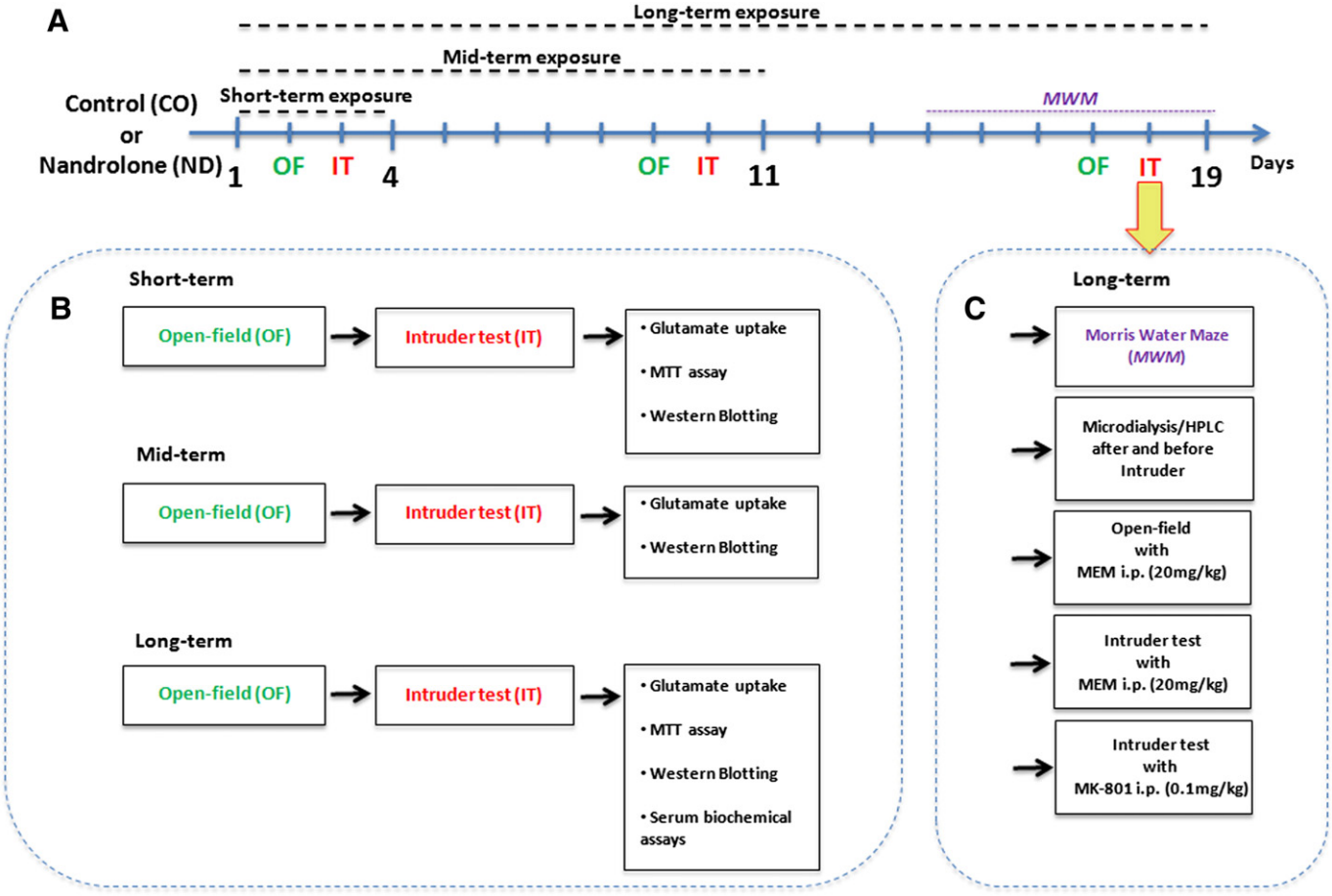

Fig. 1. Schematic experimental design. Behavioral and neurochemical endpoints in control (CO) and nandrolone (ND) groups: (A) The timeline showing each endpoint (short-, mid- and long-term exposure); (B) Flowchart representing behavioral and biochemical outcomes for each endpoint; (C) Boxes with arrows 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

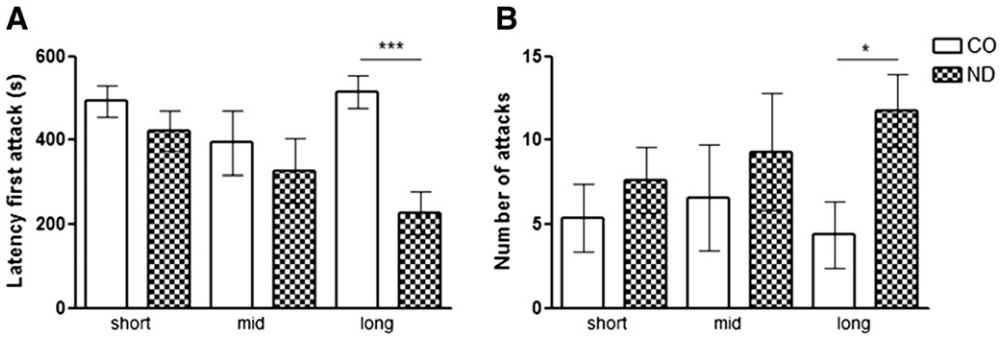

Fig. 2. Resident–intruder test. Long-term ND exposure (15 mg/kg) induced an exacerbated aggressive behavior in CF1 male mice. (A) Latency to first attack ($p < 0.0001$); (B) Number of attacks; ($p = 0.0168$). Data represented mean ± S.E.M. ($n = 10$ per group). *$p = 0.0168$ and ***$p < 0.0001$ indicate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

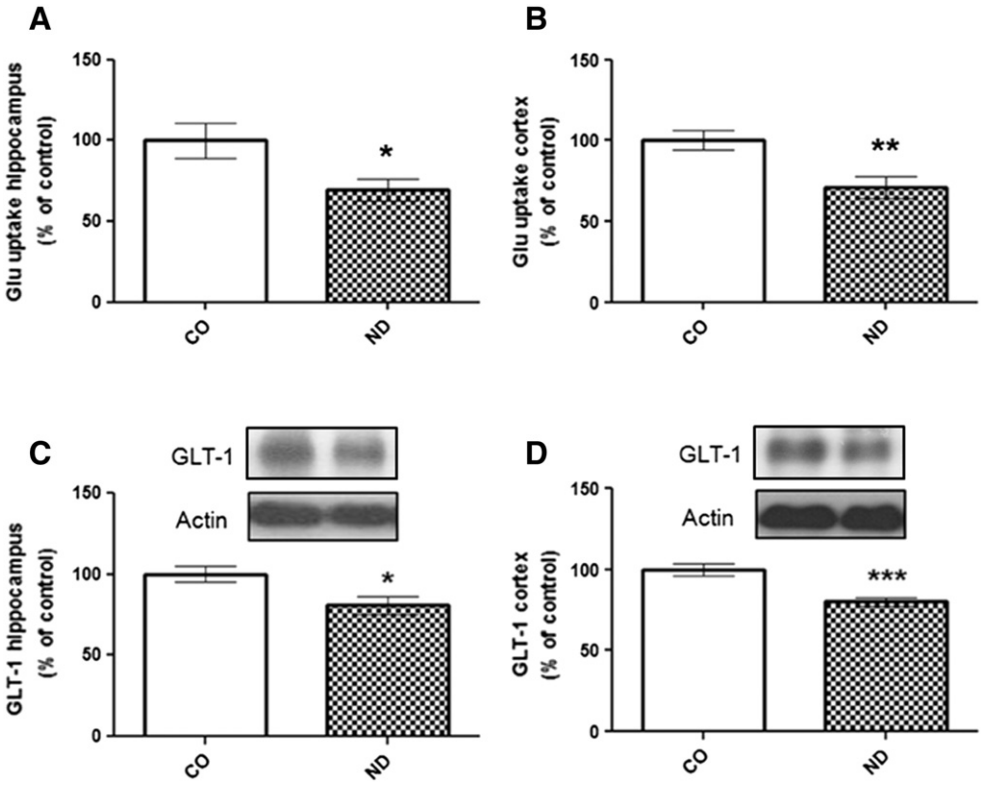

Fig. 3. Glutamate uptake and Western blotting. Nandrolone (ND) decreases glutamate uptake and immunocontent of GLT-1 in hippocampus and frontoparietal cortex. (A) Glutamate uptake in slices of hippocampus, and (B) frontoparietal cortex (n = 6). (C) Immunocontent of GLT-1 in hippocampus and, (D) frontoparietal 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).

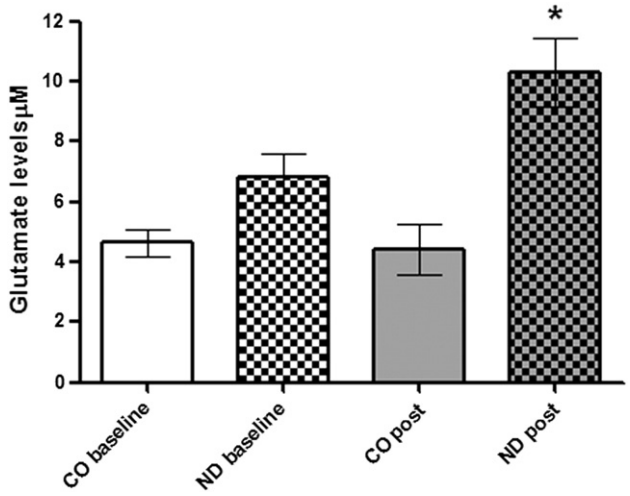

Fig. 4. Glutamate levels in extracellular fluid of hippocampus. The glutamate levels in long-term nandrolone (ND) and vehicle (CO) animals not exposed to intruder (baseline). After exposure to intruder test (post-test), the animals significantly increase the glutamate levels when compared to CO animals ($p = 0.04$). 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.

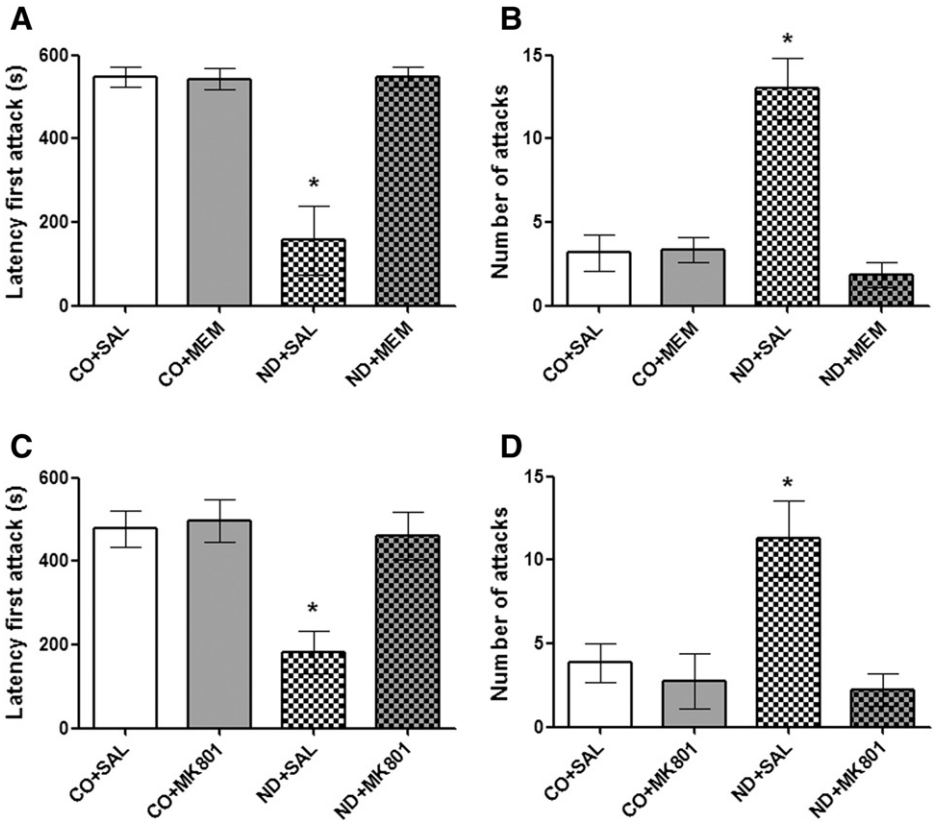

Fig. 5. N-methyl-D-aspartate receptor (NMDAr) antagonists and resident-intruder test. Memantine (20 mg/kg i.p.) or MK-801 (0.1 mg/kg i.p.) administration in long-term ND mice, 60 min and 30 min respectively prior to intruder test, restores the aggressive scores to levels of the CO group: (A) Latency 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.

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Conflict of interest

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

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