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Chronic Mild Stress Alters Kynurenine Pathways Changing the Glutamate Neurotransmission in Frontal Cortex of Rats

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Abstract

Immune stimulation might be involved in the pathophysiology of major depressive disorder (MDD). This stimulation induces indoleamine 2,3-dioxygenase (IDO), an enzyme that reduces the tryptophan bioavailability to synthesize serotonin. IDO products, kynurenine metabolites, exert neurotoxic/neuroprotective actions through glutamate receptors. Thus, we study elements of these pathways linked to kynurenine metabolite activity examining whether antidepressants (ADs) can modulate them. Male Wistar rats were exposed to chronic mild stress (CMS), and some of them were treated with ADs. The expression of elements of the IDO pathway, including kynurenine metabolites, and their possible modulation by ADs was studied in the frontal cortex (FC). CMS increased IDO expression in FC compared to control group, and ADs restored the IDO expression levels to control values. CMS-induced IDO expression led to increased levels of the excitotoxic quinolinic acid (QUINA) compared to control, and ADs prevented the rise in such levels. Neither CMS nor ADs changed significantly the antiexcitotoxic kynurenic acid (KYNA) levels. The QUINA/KYNA ratio, calculated as excitotoxicity risk indicator, increased after CMS and ADs prevented this increase. CMS lowered excitatory amino acid transporter (EAAT)-1 and EAAT-4 expression, and some ADs restored their expression levels. Furthermore, CMS decreased *N*-methyl-D-aspartate receptor (NMDAR)-2A and 2B protein expression, and ADs mitigated this decrease. Our research examines the link between CMS-induced pro-inflammatory cytokines and the kynurenine pathway; it shows that CMS alters the kynurenine pathway in rat FC. Importantly, it also reveals the ability of classic ADs to prevent potentially harmful situations related to the brain scenario caused by CMS.

Keywords Chronic mild stress · Antidepressants · Indoleamine 2,3-dioxygenase · Kynurenine pathways · Glutamate neurotransmission · Frontal cortex

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Introduction

Among psychiatric diseases, major depressive disorder (MDD) has one of the highest impacts on public health. In fact, over 350 million people suffer from the disease all around the world, and it is one of the main causes of morbidity [1]. There are estimations indicating that MDD impact is going to soar in the near future, becoming one of the leading causes of incapacity measured in disability-adjusted life years (DALYs) [2]. Furthermore, MDD represents not only severe personal costs to the sufferers but also a vast economic burden of direct and indirect costs [3].

The mechanism of action of most of the available drugs for the treatment of patients with MDD is based on restoring the monoaminergic deficit in the synaptic cleft, either blocking reuptake transporters of monoamines (e.g., tricyclic antidepressants and selective reuptake inhibitors) or inhibiting the

enzymes (i.e., monoamine oxidase inhibitors, MAOIs) that degrade them (reviewed in [4]),

Nearly one third of patients with MDD are treatment-resistant (reviewed in [5]), and therefore, more research to improve the available treatments is warranted. New theories have been proposed suggesting that immune activation and subsequent inflammation could play a role in the pathophysiology of the disease (reviewed in [6]). Importantly, the inflammatory hypothesis of depression is not inconsistent with the monoaminergic hypothesis, and actually, both might be complementary through several intersections. One of those intersections could be the activation of the enzyme indoleamine 2,3-dioxygenase (IDO). IDO can be induced by pro-inflammatory cytokines that have been found to be upregulated in patients with MDD [7, 8]. Once activated, IDO catalyzes the conversion of tryptophan into kynurenine, reducing the bioavailability of this amino acid to synthesize serotonin (5-HT).

Some of the kynurenine metabolites as quinolinic (QUINA) and kynurenic (KYNA) acids could exert neurotoxic and neuroprotective actions, respectively, as agonists/antagonists of *N*-methyl-D-aspartate (NMDA) glutamate receptors (reviewed in [9]). NMDA receptors (NMDAR) represent one of the most promising targets in depression research especially, since their blockade could mediate the effects of rapid-acting antidepressants like ketamine [10]. NMDAR subunits 2A and 2B play a leading role in most studies because of their high expression in the mammalian forebrain [11, 12]. It is generally believed that sustained stimulation of NMDAR leads to excitotoxicity and oxidative stress, processes related to depression both in animal models and humans [13, 14]. In this sense, excitatory amino acid transporters (EAATs) responsible for glutamate reuptake in the synaptic cleft stand as the main direct scavenger mechanism to this signaling (reviewed in [15]). However, regulation of glutamate neurotransmission is fine and complex and the roles of interacting components and their full involvement in depressive-like behaviors are still unknown (reviewed in [16]).

Thus, we hypothesized that exposure to a well-characterized multidimensional animal model of depressive behavior, chronic mild stress (CMS) [17], affects kynurenine pathways and can influence glutamatergic neurotransmission. The aim of this study was to evaluate the impact of CMS on the possible activation of the IDO pathway and kynurenine metabolites. In addition, we aimed to study some elements of glutamatergic neurotransmission linked to the activity of kynurenine metabolites and to examine whether classic antidepressants can modulate the expression of the pathways and parameters explored.

The understanding of antidepressant actions on generic molecular targets as well as their potential differing mechanisms may support the development of new treatment strategies that optimize the specific properties of each individual drug [18]. Thus, another main aim of this study was to research the molecular effects of commonly used antidepressants on these pathways.

Materials and Methods

Animals

Male outbred Wistar Hannover rats (HsdRccHan:Wist, from Envigo, Spain) initially weighing 200–225 g were housed one per cage. Animals were maintained under standard conditions of temperature and humidity and a 12-h light-dark cycle (lights on at 08:00 h) with free access to food and water; they were handled daily for the change of cage and bedding for 14 days before the beginning of the stress protocol. All experimental procedures adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense and Madrid Regional Government following European legislation (2010/63/EU). Animal studies are reported in compliance with the ARRIVE guidelines [19] and all efforts were made to minimize animal suffering and to reduce the number of animals used.

Experimental Groups

Animals were randomly assigned to the following groups (n = 6–8 in each group): (1) a control group (animals were handled for few seconds once at 10:00) (CT); (2) a control group with intraperitoneal injection (i.p.) of vehicle (sterile saline) injected daily for 7 days (CT + Veh), (3) a CMS group; and (4) a CMS group injected (i.p.) with vehicle (CMS + Veh) group (days 14–21).

For experiments involving the i.p. injection of antidepressants (days 14–21), three additional experimental groups were employed: (5) a CMS group injected with desipramine (CMS + Desip); (6) a CMS group injected with escitalopram (CMS + Escit); and (7) a CMS group injected with duloxetine (CMS + Dulox).

The vehicle-injected groups did not differ from the same experimental groups without injection in any of the parameters analyzed, and their values have been merged in all the figures.

Chronic Mild Stress (CMS) Protocol

The CMS protocol used was a modification of the one proposed by Willner [20]. Our group has previously used this 21-day CMS protocol in this same strain of rats, and we have shown that it induces depressive-like behavior when analyzed by means of the modified forced swim test, sucrose test, splash test, and elevated plus maze test [21, 22]. The protocol consists of a series of different stressors that were changed daily (two stressors/day) for a period of 21 days. The stressors included the following: (a) food deprivation, (b) water deprivation, (c) cage tilting, (d) soiled cage, (e) grouped housing after a period of water deprivation, (f), stroboscopic illumination (150 flashes/min), and (g) intermittent illumination every 2 h.

Chemical and Pharmacological Tools

Unless otherwise stated, the chemicals employed were from Sigma-Aldrich (Spain). Antidepressant drugs (Sigma-Aldrich, Spain) employed were the tricyclic antidepressant (TCA), desipramine hydrochloride (D3900) (20 mg/kg, i.p.), the selective serotonin-reuptake inhibitor (SSRI), escitalopram oxalate (E4786) (15 mg/kg, i.p.), and the serotonin-norepinephrine reuptake inhibitor (SNRI) (S)-duloxetine hydrochloride (SML0474) (15 mg/kg, i.p.). Doses employed have shown antidepressant activity in rodents in previously published studies using similar protocols. The drugs employed are neither new nor experimental drugs; these are well-established antidepressants, widely used in preclinical studies and in the treatment of patients. Accordingly, and in order to reduce the number and the suffering of animals following the 3Rs principles, we did not perform behavioral tests on these rats. The result that these tests would yield, that the CMS protocol induces a depressive-like behavior and that treatment with canonical antidepressants (i.e., TCAs, SSRIs, and SNRIs) reverses the behavior induced by the stress protocol, has been extensively published with these drugs and doses in this model [23, 24]. Yet more, experiments previously performed in our laboratory employing the same CMS and antidepressant treatment protocols have shown that rats exposed to this CMS protocol present depressive-like behavior, and antidepressants reverse the behavior induced by the CMS protocol. Thus, we can affirm that the CMS and the treatments employed in this study have reduced variability. For all the reasons mentioned above, we do believe that is not necessary to perform behavioral test at this point of the study, which will unnecessarily increase the number of animals employed.

In order to explore the therapeutic effects of the drugs, we choose a protocol close to clinical reality: vehicle (sterile saline) or antidepressants administered during the last 7 days of the CMS protocol.

Tissue Samples

To avoid variations in corticosterone levels caused by circadian rhythm, biological samples were always obtained at the same time of day, namely between 15:00 and 16:00. Samples from CMS-exposed animals were taken the next day after day 21 of stress.

After terminal anesthesia using sodium pentobarbital (320 mg/kg, i.p., Vetoquinol®, Spain; CN: 570681.8), the brain was removed from the skull, and frontal cortical (FC) areas were excised from the brain and frozen at − 80 °C until assayed. Samples were taken from the same coronal cut and their size was very similar among them (65.52 ± 0.43 mg of tissue).

Homogenization of the Samples

A widely utilized method that provides a high purity cytosolic fraction, practically without nuclear contamination, was employed [25]. Briefly, the tissue (frontal cortex, FC) was homogenized in 300-mL buffer (10-mmol/L *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (pH 7.9); 1-mmol/L EDTA, 1-mmol/L EGTA, 10-mmol/L KCl, 1-mmol/L dithiothreitol, 0.5-mmol/L phenylmethylsulfonyl fluoride, 0.1-mg m/L aprotinin, 1-mg/mL leupeptin, 1-mg/mL Na-p-tosyll-lysinechloromethylketone, 5-mmol/L NaF, 1-mmol/L $NaVO_4$, 0.5-mol/L sucrose, and 10-mmol/L Na_2MoO_4). After 15 min, Nonidet P-40® (Roche, Mannheim, Germany) was added to reach a 0.5% concentration. The tubes were gently vortexed for 15 s, and nuclei were collected by centrifugation at 5000 *g* for 5 min. Supernatants were considered as the cytosolic fraction. All steps of the fractionation were carried out at 4 °C.

Western Blot Analysis

To determine the expression levels of the IDO-1, EAAT1–4, and NMDAR 2A and 2B, cytosolic extracts from FC samples were used.

After adjusting protein levels in the homogenates and mixing them with *Laemmli* sample buffer (Bio-Rad, USA), 20 mL (1 mg/mL) was loaded and the proteins size-separated in 10% SDS-polyacrylamide gel electrophoresis (90 V).

After the gel electrophoresis, the membranes were blocked in 30-mL Tris-buffered saline containing 0.1% Tween 20 and 5% skim milk/BSA, then the membranes were incubated with specific primary antibodies against IDO-1 (sc-25809, Santa Cruz Biotechnology, USA, SCB, 1:1000), EAAT1 (sc-515839, SCB, 1:750), EAAT2 (sc-15317, SCB, 1:2000), EAAT3 (sc-25658, SCB, 1:1000), EAAT4 (sc-293344, SCB, 1:750), NMDAR2A (4205, Cell Signaling, 1:750), and NMDAR2B (4207, Cell Signaling, 1:1000). After washing with a TBS-Tween solution, the membranes were incubated with the respective horseradish peroxidase-conjugated secondary antibodies for 90 min at room temperature and revealed by ECL™ kit following the manufacturer's instructions (Amersham Ibérica, RTN2236; Spain).

Blots were imaged using an Odyssey® Fc System (Li-COR Biosciences) and quantified by densitometry (NIH ImageJ® software). All densitometries are expressed in arbitrary units of optical density (O.D.). Several exposition times were analyzed to ensure the linearity of the band intensities. The loading controls (blots shown in the respective figures) were β-actin (Sigma A5441).

Table 1 Primers used for PCR analysis; forward and reverse nucleotides sequences for each target protein

Protein	Forward	Reverse
KAT-1	TCACACCTCGCACCAAGATC	TGGCACAGATTAGCCACCAG
KAT-2	AGGTTCCTCACTGCAACGAG	AGGAGCCAGGGAGATGATGT
KAT-3	TGGCCTGAAACCCATCATCC	GCGGATACAGGAATGGCTGA
Haao	GGCACATCCCTAAGCCTGTT	GCCACAGCCACACATCTACA
NMDAR2A	GGGTTCTGCATCGACATCCT	CACTTCCGAACGCTCCTCAT
NMDAR2B	AAGCCTGGCATGGTCTTCTC	CTGAGGGGAGCCGTTTACTC

Protein Assay

Protein levels were measured using the Bradford method based on the principle of protein-dye binding.

PCR Analysis

Primer oligonucleotides for PCR were designed with the Primer3 tool [26]. Target specificity was checked by in silico PCR using the USCS GenomeBrowser [27] and Blast (NCBI) for cDNA and gDNA; only intron-spanning primer pairs with no unintended targets were selected (Table 1).

PCR analyses were carried out homogenizing FC in 600 mL of TRIZOL® reagent (Invitrogen, Life Technologies, USA) in the TissueLyser LT (QUIAGEN®, Venlo, Netherlands); the frequency used was 50 oscillations per second for 5 min at 4 °C.

Total cytoplasmic RNA was prepared from samples following TRIZOL® datasheet; aliquots were converted to cDNA using random hexamer primers. Semi-quantitative changes in mRNA levels were estimated by real time-PCR (RT-PCR) using the following cycling conditions: 35 cycles of denaturation at 95 °C for 10 s, annealing at 58–63 °C for 15 s depending on the specific set of primers, and extension at 72 °C for 20 s. Reactions were carried out in the presence of SYBR green (Quantimix Easy Master Mix Biotools, B&Mlabs 10607-4154) carried out in a 20-L reaction in a Rotor-Gene (Corbett Research, Australia). Relative mRNA concentrations were calculated from the take-off point of reactions using included software, and tubulin levels used to normalize data.

Kynurenine (Kyn), Tryptophan (Trp), Kynurenic Acid (KYNA), Quinolinic Acid (QUINA), and Glutamate (Glu) Levels

Commercially available ELISA kits were used to measure kynurenine and tryptophan (ImmuSmol, France), kynurenic and quinolinic acids (Cloud-Clone Corp, USA), and glutamate levels (Labor Diagnostika, Germany) in the FC tissue homogenate following the manufacturers' instructions.

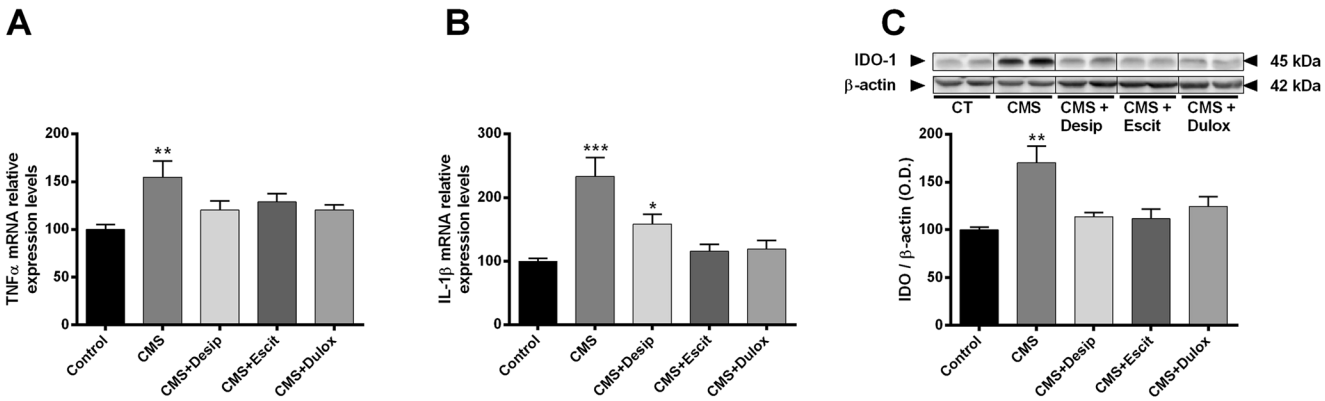

Fig. 1 Effects of CMS and antidepressant treatments on the indoleamine 2,3-dioxygenase (IDO-1) pathways in the FC. CMS induced an increase of TNF-α and IL-1β in the FC (**a**, **b**). Antidepressants did not revert the CMS-induced upregulation of TNF-α (**a**) and escitalopram and duloxetine trend to decrease IL-1β levels (**b**). CMS increased the protein expression levels of IDO-1 (**c**). The three antidepressants restored the IDO-1 protein expression levels compared to the control group (**c**). The vehicle-injected groups did not differ from the same experimental groups without injection, and their values have been merged in the figure. In the Western blots, the densitometric data of the bands of interest were normalized by 훽-actin. In the C group panel blots were cropped (black lines) to improve clarity and conciseness of the presentation. Data are means ± SEM of 6–8 rats per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control; One-way ANOVA following Tukey post hoc test. The values of IL-1β and IDO-1 protein expression (**b**, **c**) did not follow a Gaussian distribution; thus, a nonparametric ANOVA with a Kruskal-Wallis test followed by a Dunn's post hoc test was performed

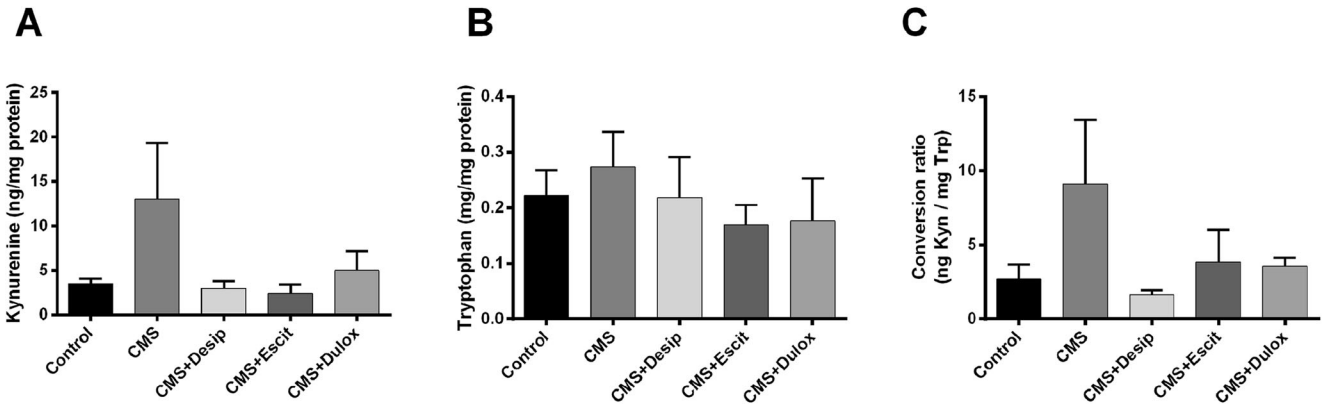

Fig. 2 CMS trended to impact tryptophan (Trp) conversion to kynurenine (Kyn) and antidepressant effects in the FC. The CMS group showed a tendency to increase kynurenine levels (**a**). There were no changes in the amount of tryptophan between the different groups (**b**). The profile described for **a** persisted in the conversion ratio of tryptophan into kynurenine (**c**). The vehicle-injected groups did not differ from the same experimental groups without injection, and their values have been merged in the figure. Data are means ± SEM of six to eight rats per group. One-way ANOVA followed by the Tukey post hoc test

Statistical Analysis

Data are expressed as mean ± SEM of three or more independent experimental replications, and a one-way ANOVA with a Tukey's post hoc test was employed for comparisons between groups. Data were analyzed using the Brown-Forsythe test to assess Gaussian distribution. In those cases, in which the data did not follow a Gaussian distribution, a nonparametric ANOVA with a Kruskal-Wallis test followed by a Dunn's post hoc test was performed. The Grubb's test/ESD method (extreme studentized deviate) was performed with a significance level set at 0.05 for the detection of outliers. In all cases, a p value < 0.05 was considered statistically significant.

Results

Effects of CMS and Antidepressant Treatments on Proinflammatory Cytokines and on the Indoleamine 2,3-Dioxygenase (IDO) Pathways in the FC

CMS exposure for 21 days induced an increase in the levels of the proinflammatory cytokines TNF-α and IL-1β in the FC (Fig. 1a, b). Antidepressants did not revert the CMS upregulation in TNF-α (Fig. 1a). IL-1β levels trend to decrease with escitalopram and duloxetine treatments (Fig. 1b).

CMS exposure increased protein expression levels of IDO-1 compared to the control groups, and antidepressant treatments showed that IDO-1 protein expression levels close to control values (Fig. 1c).

Effects of CMS and Antidepressant Treatments on Tryptophan Conversion to Kynurenine in the FC

IDO-1 can convert tryptophan (Trp) into kynurenine (Kyn). There was a tendency for Kyn levels to increase in the CMS group (Fig. 2a). The amount of tryptophan did not change among the different groups (Fig. 2b). Furthermore, the previously indicated tendency persisted in the CMS group when the Kyn/Trp ratio was analyzed (Fig. 2c).

Effects of CMS and Antidepressant Treatments on the Kynurenine Pathway in the FC

The two principal metabolites of the kynurenine pathway are quinolinic acid (QUINA) and kynurenic acid (KYNA). QUINA levels were increased after CMS exposure when compared to the control group, and the antidepressant treatments reverted the rise in such levels (Fig. 3a).

The enzyme 3-hydroxyanthranilate 3,4-dioxygenase (Haao), which catalyzes the penultimate step of the pathway to QUINA (the last step is a nonenzymatic reaction), is increased in the CMS group compared to control but not in the antidepressant-treated animals (Fig. 3b).

KYNA levels showed a tendency towards increase in the CMS group when compared to the control group (Fig. 3c). Antidepressant treatments did not have any effect on KYNA levels (Fig. 3c). The mRNA levels of some of the KYNA synthesis enzymes, the kynurenine aminotransferases (KAT-2 and KAT-3), were also increased after CMS exposure. Antidepressant treatments did not modify the mRNA kynurenine aminotransferase levels induced by CMS and in particular, the CMS + desipramine group

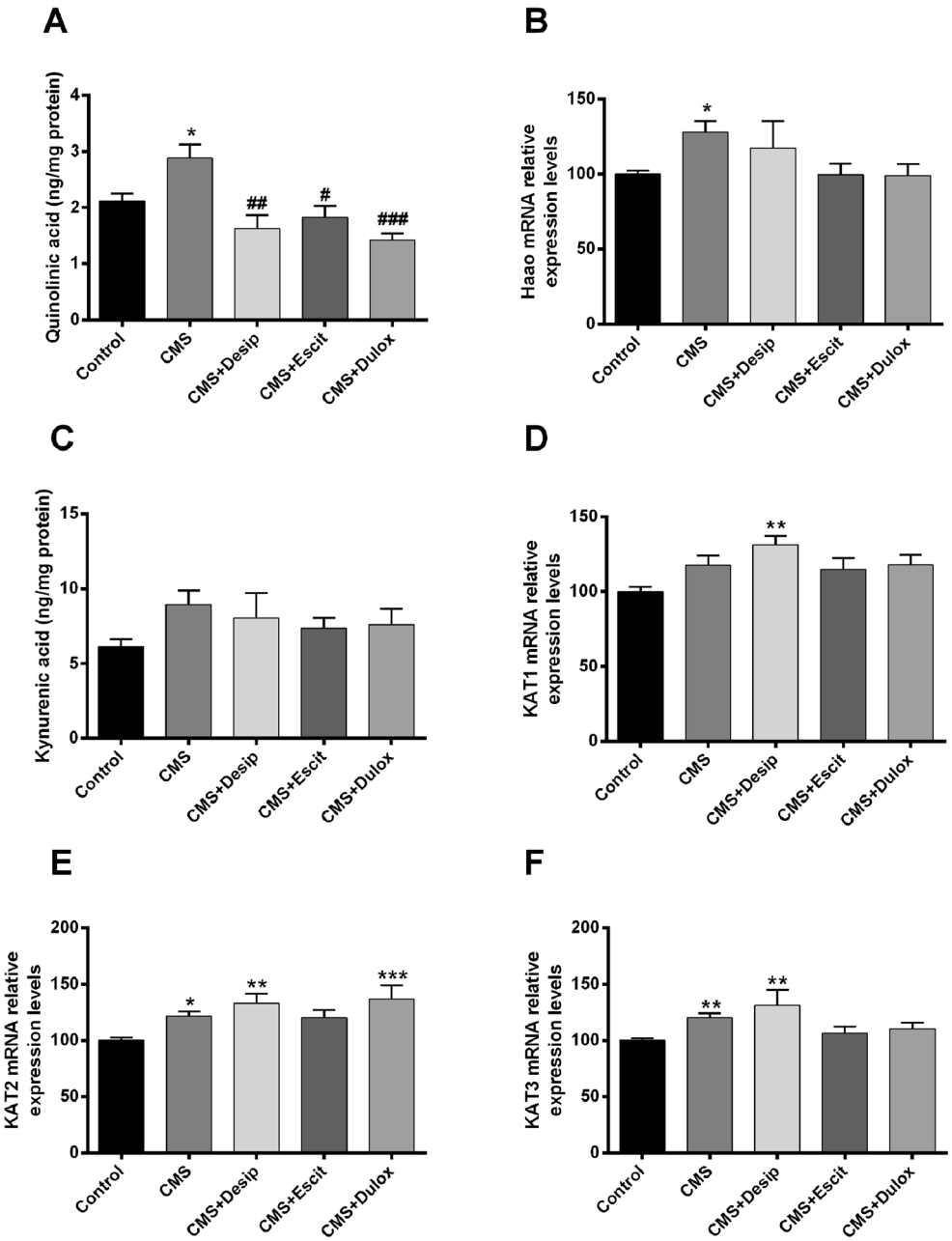

Fig. 3 Effects of CMS and antidepressant treatments on quinolinic acid (QUINA) levels, kynurenic acid (KYNA) levels, and on the ratio between QUINA and KYNA levels in the FC. QUINA levels were upregulated after CMS and the antidepressants restored them to control levels (**a**). The 3-hydroxyanthranilate 3,4-dioxygenase (Haao) levels were increased in the CMS group but not in the treatment groups (**b**). KYNA levels showed a tendency towards increase in the CMS group when compared to the control group (**c**). Antidepressants did not have any effect on KYNA levels (**c**). The mRNA levels of KAT-2 and KAT-3 were increased after CMS exposure (**e**, **f**). The CMS + desipramine group showed statistically increased mRNA levels of the three kynurenine aminotransferases when compared to the control group (**d**, **e**, **f**), and the CMS + duloxetine group showed increased mRNA KAT-2 levels (**e**). The vehicle-injected groups did not differ from the same experimental groups without injection, and their values have been merged in the figure. Data are means ± SEM of 6–8 rats per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. CMS. One-way ANOVA followed by the Tukey post hoc test

showed increased mRNA levels of the three kynurenine aminotransferases when compared to the control group (Fig. 3d, e, f). In the case of KAT-2, duloxetine-treated rats also presented a significant increase (Fig. 3e).

To study the overall equilibrium pathways between pro- and antiexcitotoxic consequences, the ratio between QUINA and KYNA levels, a plausible excitotoxicity risk indicator [28], was calculated. QUINA/KYNA ratio showed a clear trend to increase in the CMS group when compared to the control group (Fig. 4). Treatment with antidepressants prevented from this increase.

Effects of CMS and Antidepressant Treatments on Glutamate Levels, Excitatory Amino Acid Transporter (EAATs) Expression, and mRNA and Protein Expression Levels of N-Methyl-D-Aspartate Receptors (NMDARs) in the FC

Regarding the potential actions of kynurenine metabolites on NMDAR, some of the glutamatergic neurotransmission main elements were studied.

CMS exposure did not modify the FC glutamate levels, but the three antidepressants trend to decrease them (Fig. 5).

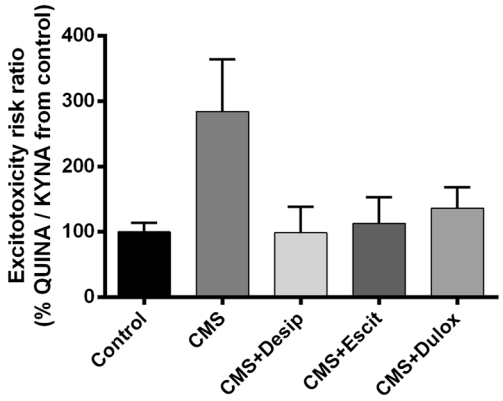

Fig. 4 Effects of CMS and antidepressant treatments on the excitotoxicity risk ratio quinolinic acid (QUINA)/kynurenic acid (KYNA) in the FC. CMS seemed to induce an increased percentage in the QUINA/KYNA ratio when compared to the control group. Antidepressant treated groups showed a QUINA/KYNA ratio similar to the control group. The vehicle-injected groups did not differ from the same experimental groups without injection, and their values have been merged in the figure. Data are means ± SEM of six to eight rats per group. The values did not follow a Gaussian distribution; thus, a nonparametric ANOVA with a Kruskal-Wallis test followed by a Dunn's post hoc test was performed

The CMS protocol lowered the expression of EAAT-1 and trend to decrease EAAT-4 (Fig. 6a, d), but it did not affect the expression of EAAT-2 and EAAT-3. Escitalopram and duloxetine treatments restored EAAT-1 (Fig. 6a) and duloxetine increased EAAT-4 in the FC (Fig. 6d).

No changes evoked by CMS in the expression of NMDAR2A and NMDAR2B mRNA were detected (Fig. 7a, c). Desipramine upregulated NMDAR2A mRNA, and all the antidepressant treatments increased NMDAR2B mRNA levels (Fig. 7a, c). A decrease in NMDAR2A and NMDAR2B protein expression was observed after CMS which was mitigated by the treatments (Fig. 7b, d).

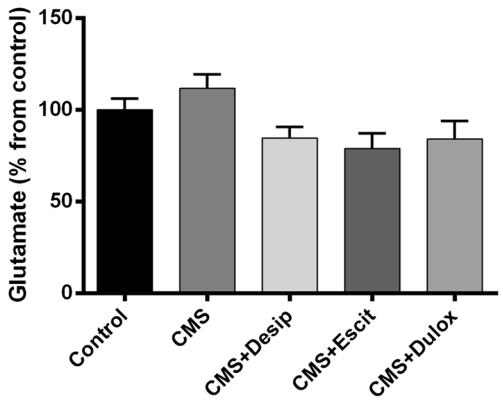

Fig. 5 Effects of CMS and antidepressant treatments on the overall glutamate levels in the FC. No differences were found in the glutamate levels between the control and CMS groups. The antidepressants trended to decrease glutamate under CMS exposure. The vehicle-injected groups did not differ from the same experimental groups without injection, and their values have been merged in the figure. Data are means ± SEM of six to eight rats per group. One-way ANOVA followed by the Tukey post hoc test

Discussion

Our results showed that an experimental model causing depressive-like behavior (i.e., CMS) induced the expression of the enzyme IDO, activating the kynurenine pathways and led to an increase of quinolinic acid, a potentially excitotoxic molecule (reviewed in [9]) in the FC. Furthermore, classical antidepressant treatments seemed to prevent the activation of kynurenine pathways in the FC caused by CMS. These effects could potentially protect the FC from excitotoxicity and they could mitigate the decrease in the synthesis of 5-HT associated with IDO activation.

Accumulating evidence indicates that NMDAR plays an important role in the neurobiology and treatment of MDD (reviewed in [29]). Here, CMS exposure caused changes in glutamatergic neurotransmission, decreasing not only the re-uptake transporters EAAT-1 (present mainly in astrocytes) but also the expression of NMDAR2A and NMDAR2B subunits. Besides, antidepressant treatments were able to restore glutamate receptor levels and trended to reduce the glutamate in the FC, although the impact of glutamate signaling through the NMDAR and its relation to depressive-like behaviors needs to be addressed in the future.

CMS induced an increase of proinflammatory mediators with the capacity to activate IDO, like TNF-α and IL-1β [7, 30–33]. Antidepressant treatments trended to decrease the upregulation of IL-1β and, consequently, its expression profile was inversely correlated with the IDO expression profile. Most studies support the ability of the employed antidepressants to reduce the TNF-α levels in experimental depression models; however, our study did not show antidepressant effects on mRNA TNF-α levels. A feasible explanation for our results could be the statistical power achieved considering the size of the groups with antidepressant treatments.

The activity of IDO diverts the tryptophan available for the serotonin synthesis to the production of kynurenine, potentially linking the inflammatory and monoaminergic hypotheses of depression [34]. Our results did not find a drop in the amount of tryptophan, but exposed a trend towards the increase in kynurenine levels after CMS that persisted in the conversion ratio of tryptophan into kynurenine. Importantly, the antidepressant-treated groups showed values close to the control group.

As already mentioned, IDO has been associated with depressive-like behaviors not only because of the deviation of tryptophan from serotonin synthesis but also for the intrinsic ability of kynurenine metabolites (i.e., QUINA and KYNA) to affect other systems and their actions on the *N*-methyl-D-aspartate (NMDA) receptor (reviewed in [9]).

Our data show an upregulation of QUINA caused by CMS and a return to control levels after antidepressant treatments. In addition, Haao, an enzyme that catalyzes the last enzymatic

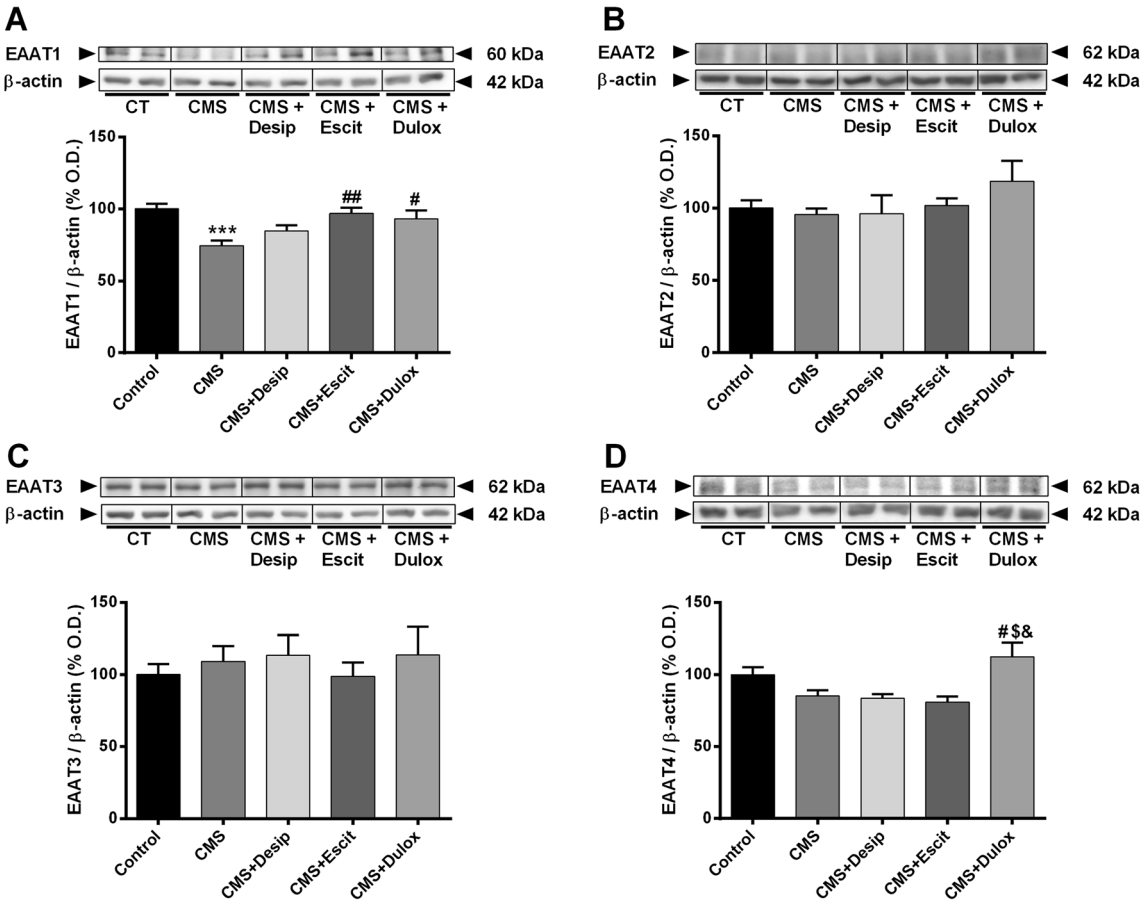

Fig. 6 Effects of CMS and antidepressant treatments on the excitatory amino acid transporters (EAATs) in the FC. CMS decreased expression of EAAT-1 (**a**) and trended to decrease EAAT-4 (**a**, **d**) but did not affect EAAT-2 and EAAT-3 (**b**, **c**). Escitalopram and duloxetine restored EAAT-1 (**a**) and duloxetine upregulated EAAT-4 (**d**). The vehicle-injected groups did not differ from the same experimental groups without injection, and their values have been merged in the figure. The densitometric data of the bands of interest were normalized by β-actin. Blots were cropped (black lines) for improving the clarity and conciseness of the presentation. Data are means ± SEM of six to eight rats per group; *** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$ vs. CMS; $ $p < 0.05$ vs. CMS + desipramine; & $p < 0.05$ vs. CMS + escitalopram. One-way ANOVA followed by the Tukey post hoc test

step of the pathway to quinolinic acid, was increased in CMS but not in antidepressant-treated groups.

KYNA levels and their synthesis enzymes (KATs) showed a tendency to increase only in the CMS group. Antidepressant-treated groups overexpressed KAT-2, and desipramine-treated rats showed increased mRNA levels of the three KATs.

Moreover, antidepressants restrain the induction of the kynurenine pathways, especially QUINA production following CMS. Given our data, we decided to study the QUINA/KYNA ratio [28] as a possible excitotoxicity risk indicator. This ratio shown a trend to increase after CMS exposure and antidepressant treatments seemed to return it to control levels, in the same way as with the kynurenine/tryptophan ratio.

We can infer that the kynurenine pathways are upregulated after CMS and that antidepressant treatments block their activation as well as the potential subsequent damage. Excitotoxicity represents a harmful situation for the nervous tissue, and it is closely related to oxidative stress. We have previously demonstrated, with the same CMS and antidepressant treatment protocols, a CMS-induced increase in the oxidative/nitrosative stress in the FC of rats resulting in higher levels of lipid peroxidation markers, such as malondialdehyde (MDA) and 4-hydroxinonenal (4-HNE) [14]. Importantly, the same antidepressant treatments employed in this study were able to restore the MDA and 4-HNE to control levels in rats exposed to the same CMS protocol [14].

Thus, to further explore the excitotoxicity scenario, we studied some of the main elements of glutamatergic transmission in our CMS and antidepressant-treatment protocols. The glutamate levels in the FC remained unaffected after the CMS protocol and the three antidepressants did not change significantly the glutamate levels. There are previous studies showing that glutamate levels were increased after chronic stress exposure, although these studies employed different CMS protocols from the one utilized here (reviewed in [17]). Moreover, tissue glutamate concentrations are difficult to relate to glutamatergic neurotransmission, since most glutamate

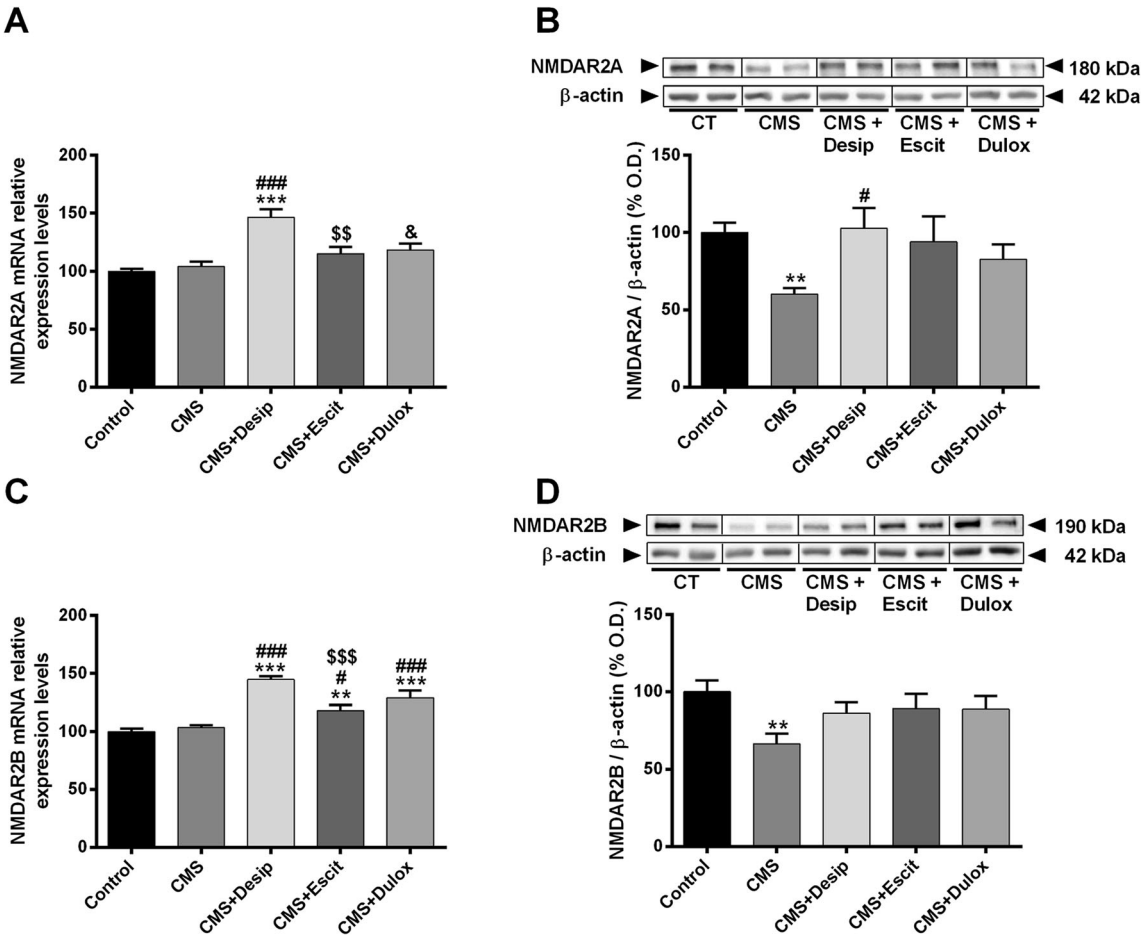

Fig. 7 Effects of CMS and antidepressant treatments on *N*-methyl-D-aspartate receptors (NMDARs) in the FC. CMS did not exert actions on NMDAR2A and NMDAR2B mRNA (**a**, **c**). Desipramine upregulated NMDAR2A mRNA (**a**) and the three antidepressants increased NMDAR2B mRNA (**c**). CMS decreased the protein expression of NMDAR2A and NMDAR2B (**b**, **d**). The antidepressants did not show the decrease induced by CMS (**b**, **d**). The vehicle-injected groups did not differ from the same experimental groups without injection and their values have been merged in the figure. The densitometric data of the bands of interest were normalized by β-actin. Blots were cropped (black lines) for improving the clarity and conciseness of the presentation. Data are means ± SEM of six to eight rats per group; ** $p < 0.01$, *** $p < 0.001$ vs. control; # $p < 0.05$, ### $p < 0.001$ vs. CMS; $$ $p < 0.01$, $$$ $p < 0.001$ vs. CMS + desipramine; & $p < 0.05$ vs. CMS + escitalopram. One-way ANOVA followed by the Tukey post hoc test

is of metabolic origin and unrelated to the neurotransmitter pool.

Taking this into account, we study the EAATs responsible for glutamate reuptake with a vital role in keeping glutamate levels within the brain. CMS exposure reduced glial EAAT-1 protein expression and antidepressants prevented this decrease. Specifically, escitalopram and duloxetine increased EAAT-1 expression levels. Obviously, further research is warranted to fully understand the escitalopram and duloxetine effects on the EAAT-1 expression, considering that desipramine, a TCA and consequently less selective than the other ADs employed, has no effect on it. Glial EAAT-2 is responsible for 90% of glutamate reuptake in the forebrain, and there are studies showing its downregulation after stress protocols [17, 35]. However, the effects of stress and depression on the glial EAAT-1 [36, 37] and neuronal EAAT-4 [38, 39] have been also described in the FC. Taken together and bearing in mind the difficulties when analyzing tissue glutamate levels, our results seem consistent with the ability of antidepressants to reduce glutamate neurotransmission in the FC [40–42].

Several studies have reported results of elevated glutamate content and a trend for reduced glutamine/glutamate ratios in the plasma of patients with MDD compared to healthy individuals. Moreover, there are studies presenting demonstrations that antidepressants may reduce plasma glutamate levels in those patients (reviewed in [43]), and a study employing *postmortem* analysis of depressed individuals has shown upsurges in FC levels of glutamate [44].

The NMDAR expression results are challenging to interpret. CMS did not have any effect on mRNA of the NMDAR2A and NMDAR2B subunits, but it caused a decrease in these protein levels. These findings are consistent with a previous study employing CMS [45] and with *postmortem* studies [46] showing the same protein expression

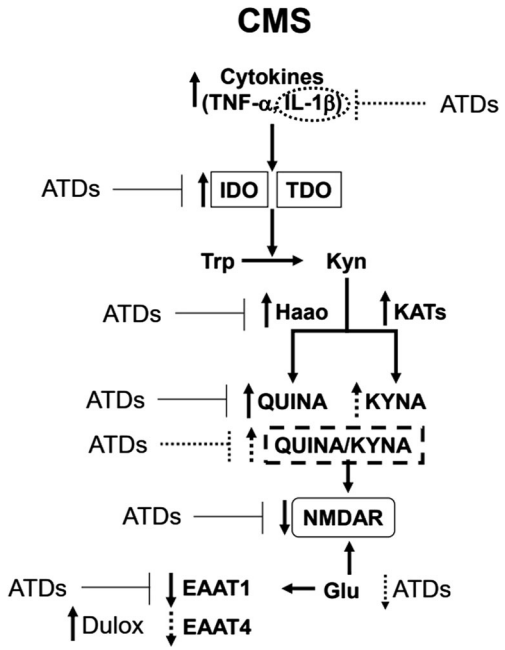

Fig. 8 Schematic overview of the pathways affected in the frontal cortex (FC) after CMS exposure. Dotted lines indicate tendencies

reduction for both subunits in the FC of patients with MDD. Interestingly, the three antidepressant treatments upregulated NMDAR2B mRNA levels (desipramine also increased the subunit 2A mRNA) and mitigated the falling off in expression of both subunit proteins. NMDAR2A has been associated with synaptic plasticity, a phenomenon necessary for memory and the proper working of cognitive functions, and antidepressants have demonstrated positive actions over synaptic plasticity [47]. In contrast, the excitotoxic risk is more related to the NMDAR2B subunit, but its increase has also been proposed as an antidepressant mechanism through a synaptic strength enhancement [48].

NMDAR protein expression decrease seems counterintuitive considering the excitotoxic scenario, but recent studies suggest that the action of rapid-antidepressants could be more related to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) than to the antagonism of NMDAR, suggesting partial agonism of these receptors as key for exerting antidepressant properties [49, 50]. In fact, CMS induces a decrease in AMPA receptor GluR1 expression in the FC [51].

Keeping in mind these considerations and the importance of balance in the glutamatergic neurotransmission, the antidepressant treatments could be mitigating the changes caused by CMS exposure. A theoretical possibility is that antidepressants reduce overall glutamate levels and increase EAATs to reduce the excitotoxicity risk, but they restore the decrease in the NMDARs to maintain a basal tone that allows for plasticity. Although far from the objectives of this research, these are areas worth studying in the future, considering that there is still very little research on the effects of CMS on glutamatergic signaling in the brain.

In summary, our data indicate that exposure to CMS induced IDO, activating the kynurenine pathways in the FC, leading to the increase of quinolinic acid and, therefore, this could result in an increased NMDA-mediated excitotoxicity risk, measured through the QUIN/KYNA ratio. However, the decrease in NMDAR protein expression in the CMS group requires a future exhaustive analysis of kynurenine metabolite implications over this receptor.

Antidepressant treatments reversed the CMS-induced kynurenine pathway activation that could theoretically protect the FC from the excitotoxicity, and it could prevent the decrease in the synthesis of 5-HT caused by the IDO activation and the subsequent decrease of tryptophan bioavailability for serotonin synthesis. Moreover, treatments appeared to control the glutamate acting on the synaptic cleft to achieve a healthy neurotransmission.

For a schematic overview of the pathways being affected in the FC after CMS, see Fig. 8.

New perspectives on molecular targets for mood disorders are necessary to broaden the knowledge of their pathophysiology and to design treatments that are more effective. Our research provides new insights focusing on the inflammatory activation of kynurenine pathways, the effects on the glutamate excitotoxicity risk and the ability of classic antidepressants to prevent potentially harmful situations related to this imbalance. Further exploring this approach may result in interesting advances in stress-related pathologies and psychiatric diseases.

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Compliance with Ethical Standards

Conflict of Interest The authors provide full disclosure of any and all biomedical financial interests.

The authors declare that there are not conflicts of interest.

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