

Effects of pentoxifylline, 7-nitroindazole, and imipramine on tumor necrosis factor- α and indoleamine 2,3-dioxygenase enzyme activity in the hippocampus and frontal cortex of chronic mild-stress-exposed rats

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Objectives: This study aimed to investigate the role of tumor necrosis factor (TNF)- α and the neuronal nitric oxide synthase enzyme in dysregulation of indoleamine 2,3-dioxygenase (IDO) enzyme, and hence serotonin availability in chronic mild stress (CMS), an animal model of depression.

Methods: Rats were divided into five groups: two control and CMS-exposed for 6 weeks, and another three groups exposed to CMS and administered pentoxifylline 50 mg/kg/day intraperitoneally, 7-nitroindazole 40 mg/kg/day subcutaneously, or imipramine 20 mg/kg/day intraperitoneally for the previous 3 CMS weeks. Rats were assessed for neurochemical and immunohistochemical abnormalities.

Results: Pentoxifylline-, 7-nitroindazole-, and imipramine-treated rats showed amelioration of CMS-induced behavioral deficits that was accompanied by significant reduction in kynurenine/serotonin molar ratio and nitrates/nitrites in frontal cortex and hippocampus. In the pentoxifylline and 7-nitroindazole groups, serum TNF- α was reduced relative to the CMS group (18.54 ± 0.85 and 19.16 ± 1.54 vs 26.20 ± 1.83 pg/mL, respectively; $P < 0.05$). Exposure to CMS increased TNF- α and IDO immunohistochemical staining scores in both hippocampus and midbrain raphe nuclei. 7-Nitroindazole and pentoxifylline significantly ($P < 0.05$) reduced TNF- α immunostaining in hippocampus and raphe nuclei, with significant ($P < 0.01$) reduction of IDO immunostaining in raphe nuclei. Likewise, imipramine reduced TNF- α immunostaining ($P < 0.05$) in hippocampus.

Conclusion: Neuronal nitric oxide synthase and TNF- α may play a concerted role in modulating IDO enzyme activity in CMS-exposed rats and provide additional evidence for possible alternative approaches to switch the neurobiological processes in depression.

Keywords: chronic mild stress, TNF- α , kynurenine, serotonin, nNOS, immunohistochemistry

Introduction

Several studies suggest that the link between stress and depression might involve disturbances in tryptophan metabolism.¹⁻³ Tryptophan undergoes two major metabolic pathways: the serotonin-synthesis pathway and the kynurenine pathway. A shift in the balance of tryptophan metabolism from the serotonin towards the kynurenine pathway might be involved in the pathophysiology of depression. Indeed, kynurenine metabolites contribute to several neurobiological changes associated with depressive illness.⁴

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Not infrequently, clinical reports have revealed an increase in plasma kynurenine/tryptophan ratio, which is associated with increased activity of the cellular immune activation. Disturbed metabolism of tryptophan affects biosynthesis of neurotransmitter 5-hydroxytryptamine (serotonin), and it appears to be associated with an increased susceptibility for depression.⁵ Indoleamine 2,3-dioxygenase (IDO) is predominantly expressed intracellularly as constitutive or inducible forms in blood monocytes and tissue macrophages, including microglial cells within the brain parenchyma.⁶ Actually, IDO degrades tryptophan along the kynurenine pathway, so that fluctuations in its enzymatic activity can alter brain tryptophan metabolism. Enhancement of the kynurenine pathway deprives the neuronal tissue microenvironment from tryptophan and induces subsequent reduction in serotonin synthesis, and this may suggest a strong link between the kynurenine pathway, serotonin synthesis, and depression.^{4,7}

The IDO enzyme is induced by proinflammatory cytokines, mainly interferon (IFN)- γ and tumor necrosis factor (TNF)- α .^{8,9} In chronic inflammatory conditions, IDO is activated, and the degree of activation matches the intensity of depressive symptoms, as in patients suffering from malignancies and those chronically treated with IFN- α .¹⁰ Recently, O'Connor et al¹¹ reported that peripheral administration of lipopolysaccharide (LPS) activates IDO and culminates in a distinct depressive-like behavioral syndrome. This was measured by increased duration of immobility in both the forced-swim and tail-suspension tests. Blockade of IDO activation, either indirectly with the anti-inflammatory tetracycline derivative minocycline¹² or directly with the IDO antagonist 1-methyltryptophan,¹¹ attenuated LPS-induced expression of proinflammatory cytokines (TNF- α , IL-1 β), prevented the development of depressive-like behavior, and normalized the kynurenine/tryptophan ratio in the plasma and brain of LPS-treated mice. Thus, IDO might be considered a critical molecular mediator of inflammation-induced depressive-like behavior, probably through the catabolism of tryptophan along the kynurenine pathway.

On the other hand, speculations were raised on the role of neuronal nitric oxide (nNOS) in modulating depressive-like symptoms.¹³ Yildiz et al¹⁴ reported that nNOS inhibitors produce an antidepressant-like effect in the forced-swimming test in mice and rats. Furthermore, increased expression of TNF- α and nNOS has been noted in brain areas of depressed patients.^{13,15} Like nNOS inhibition, deletion of either TNF- α receptor subtypes (especially, type 2 TNF- α receptor) was reported to induce antidepressant-like effects in experimental animals.¹⁶

Currently used antidepressants, which target monoamines, only produce “remission” in 30% of patients. Part of the problem is the fact that the pathophysiology of depression has not been fully elucidated, and treatments are based more on empirical data rather than on consistent pathophysiological reasoning.^{17,18} Chronic mild stress (CMS), a validated animal model of depression,¹⁹ has been reported to result in long-lasting changes of neurochemical, neuroimmune, and neuroendocrinological variables that resemble the depressive state,^{20,21} hence CMS provides an interesting model to explore the pathophysiology of depression. Accordingly, the present study aims to examine the hypothesis that exposure to CMS might induce alterations in the balance between the two major tryptophan metabolic pathways – kynurenine/serotonin – in the frontal cortex and hippocampus of male Wistar rats. Special interest is directed towards using pharmacological tools for nNOS and TNF- α inhibitors (7-nitroindazole and pentoxifylline, respectively) to investigate their antidepressant-like effects and their modulation of the balance between serotonin and kynurenine in the frontal cortex and hippocampus, compared to the prototype antidepressant imipramine.

Materials and methods

Animals

Adult male Wistar rats weighing 225–275 g were purchased from the National Research Centre, Giza, Egypt. Rats were acclimatized as follows: 12-hour light–dark cycle, light on at 5 am, temperature $\approx 25^{\circ}\text{C}$, and 50%–60% relative humidity. Experimental procedures were approved by the ethical committee of the National Research Centre, Giza, Egypt.

Drugs and chemicals

Pentoxifylline and imipramine hydrochloride were dissolved in saline. 7-Nitroindazole was dissolved in dimethyl sulfoxide, and volume was adjusted with saline. Kynurenine, L-tryptophan, serotonin, 5-hydroxyindoleacetic acid (5-HIAA), and 3-nitro-L-tyrosine were all purchased from Sigma-Aldrich (St Louis, MO, USA).

Study design

Male Wistar rats were divided into five groups ($n = 10/\text{group}$): control group, CMS group (exposed to CMS for 6 weeks and administered saline intraperitoneally for the last 3 CMS weeks), and the other three groups were exposed to CMS for 6 weeks and administered imipramine 20 mg/kg/day intraperitoneally,²² 7-nitroindazole 40 mg/kg/day

subcutaneously,²³ or pentoxifylline 50 mg/kg/day intraperitoneally for the last 3 CMS weeks.²⁴

Chronic mild stress

The CMS battery consisted of exposure to a variety of mild unpredictable stressors: water and/or food deprivation, restricted food access (ie, two to three chow pellets) after food deprivation, empty water bottles after water deprivation, cage tilting, 24-hour lighting, pairing, stroboscopic light, intermittent white noise (85 dB), and cold temperature ($\approx 10^{\circ}\text{C}$). Stressors were repeated each week for a total of 6 weeks, modified after Bekris et al.²⁵ A sucrose preference test (SPT; behavioral test) was used to assess the development of anhedonia. Control animals were kept in a separate room (Table 1).

Sucrose preference test

The SPT was done as a behavioral test at the start of dark cycle, ie, at 5 pm.²⁶ After a 23-hour period of food and water deprivation, animals were presented simultaneously with two preweighed bottles: one with 2% sucrose and the other with plain water for a period of 30 minutes. Two baseline tests were carried out during the training period before the start of the CMS protocol. Then the SPT was conducted weekly in all groups. Sucrose preference was measured by calculating the proportion of sucrose consumption out of total consumption of liquid.²⁵

Table 1 Chronic mild stress (CMS) exposure protocol

Day	Duration/start	Stressor
Saturday	6 hours (start at 9 am)	Pairing
	18 hours (start at 3 pm)	Stroboscopic light
Sunday	8 hours (start at 9 am)	Cage tilt (cold exposure [10°C] for 30 minutes)
	24 hours (start at 5 am)	FWD
Monday	At 5 pm (30 minutes)	SPT
	15 hours (start at 5.30 pm)	Termination of FD and continue WD
Tuesday	2 hours (start at 9 am)	Empty bottles
	4 hours (start at 9 am)	Noise
Wednesday	(Start at 11 am)	Termination of WD
	21 hours (start at 1 pm)	Stroboscopic light
Thursday	6 hours (start at 10 am)	Cage tilt
	17 hours (start at 4 pm)	FWD and pairing
Friday	2 hours (start at 9 am)	Restricted access to food
	4 hours (start at 9 am)	Noise
Saturday	(Start at 11 am)	Termination of FWD (cold exposure for 30 minutes)
	(Start at 1 pm)	Overnight illumination
Sunday	24 hours	Reversal of dark/light cycle

Abbreviations: FWD, food and water deprivation; SPT, sucrose preference test; FD, food deprivation; WD, water deprivation.

Body weight

Body weight was measured weekly for each rat throughout the study.

High-performance liquid chromatography

The rat brain was dissected on ice, with the rat-brain atlas of Paxinos and Watson²⁷ as a guide. Kynurenine determinations in brain tissue homogenates were performed using a Luna high-performance liquid chromatography (HPLC) column (5 μm C18 [2] 150 \times 4.60 mm; Phenomenex, Torrance, CA, USA), according to the method described by Wang and Tang,²⁸ with modifications suggested by Bellac et al.²⁹ Briefly, the mobile phase for ultraviolet detection consisted of 15 mmol/L sodium acetate/acetic acid solution containing 2.7% (v/v) acetonitrile, pH 3.6. The flow rate was set at 1.3 mL/minute. The injected sample volume was 100 μL . Peaks were detected at 365 nm wavelength. 3-Nitro-L-tyrosine was used as an internal standard.

Determinations of tryptophan, serotonin, and 5-HIAA levels in the brain-tissue homogenates were performed according to the method described by Bellac et al and Mefford.^{29,30} Briefly, the mobile phase for the electrochemical detector HP 1049A (Hewlett Packard, Palo Alto, CA, USA) consisted of acetonitrile, 100 mM acetic acid, 100 mM ammonium acetate (10:90) v/v, and 50 mg/L ethylenediaminetetraacetic acid, pH 5.1.³¹ 3-Methoxy-4-hydroxyphenethyl alcohol was used as an internal standard. Measurements were done at an electrode potential of 0.850 mV. The limit of detection for all assayed compounds was 0.05–0.10 pmol. All chemicals were purchased from Sigma-Aldrich.

Nitrate/nitrite assay

Nitric oxide metabolite (nitrates/nitrites) concentrations in frontal cortex and hippocampus homogenates were measured by nitrate/nitrite colorimetric assay kit (23479; Fluka, Buchs, Switzerland), applying the Griess assay. The procedure followed the manufacturer's instructions. Absorbance was measured at 540 nm with a microplate reader. NO metabolites were expressed as nmol/mg tissue.

TNF- α in serum

TNF- α protein was determined using enzyme-linked immunosorbent assay (ELISA) by adding 50 μL of standard and serum samples to a 96-well plate of a commercially available rat TNF- α ELISA kit (RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions.

Immunohistochemical study

Coronal sections of the brain at the hippocampal and raphe nuclei levels were fixed in formalin (10%) and embedded in paraffin blocks. Sequential 4 μ m sections were cut on charged slides. The sections were incubated with the primary antibodies (rabbit anti-IDO polyclonal antibody [EB06950; Everest Biotech, Upper Heyford, UK]) at a concentration 1:50, and the next section with primary monoclonal TNF- α antibody (J1D9; Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 1:50. The immunohistochemical technique was performed by applying the supersensitive avidin-biotin detection kit (Biogenex, CA, USA) and following the technique of Hsu and Raine.³² The immunostained sections were blindly examined qualitatively and semiquantitatively. The semiquantitative immunohistochemical evaluation was done as follows: it was first graded as negative = (0), mild (1+), moderate (2+), or strong (3+) according to the intensity of the staining as well as the percentage of the stained area, then the numerical evaluation was further analyzed statistically.

IDO was topographically expressed as a diffuse intracytoplasmic, while TNF- α was expressed as a diffuse intra- and extracellular staining. A semiquantitative score was calculated based on the staining intensity. The sections were examined for raphe nuclei in the brain stem, CA1 and CA3 of the hippocampus proper, granular, molecular, and polymorphic layers of dentate gyrus, and the alveus, which represent the projecting myelinated axons of the hippocampal pyramidal neurons in the cerebral hemispheres.

Statistical analysis

The results are expressed as means \pm standard error of the mean. A nonparametric Kruskal–Wallis test, *t*-test, and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test were performed for statistical comparisons between experimental groups. Repeated-measures ANOVAs were used for analysis in sucrose preference, followed by Bonferroni's post hoc test. Nonparametric Kruskal–Wallis test followed by post hoc Dunn's test were utilized for analysis of immunohistochemical staining score.

All statistical analyses were performed using the software package SPSS for Windows, version 15.0 (IBM, Armonk, NY, USA). *P*-values were considered significant at <0.05 .

Results

Sucrose preference test

Figure 1A shows the effect of tested drugs on the percentage change in SP from the third week (pretreatment

week). The repeated-measures ANOVA revealed significant within-subject (week) effects ($F[3,27] = 2.97$, $P = 0.049$) and between-subject (treatment) effects ($F[4,29] = 5.531$, $P = 0.002$) and a significant interaction between the factors week and treatment on percentage change of sucrose preference from the third week ($F[12,87] = 2.67$, $P = 0.004$). CMS induced significant reduction in SP in the fifth and sixth weeks ($P < 0.05$ and $P < 0.01$, respectively) in comparison to control rats. Treatment with imipramine, 7-nitroindazole, and pentoxifylline significantly ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively) increased SP at the end of week 6 compared to the untreated CMS group. Interestingly, pentoxifylline significantly ($P < 0.05$) increased SP in week 4 and week 5 ($P < 0.05$ and $P < 0.01$, respectively), while 7-nitroindazole significantly ($P < 0.05$) increased SP in week 5, denoting early reversal of anhedonia by both drugs compared to the delayed reversal with imipramine.

Body weight

Figure 1B shows the effect of tested drugs on the percentage change of body weight from the third week. The repeated-measures ANOVA revealed significant effects of the within-subjects factor, week ($F[3,29] = 7.04$, $P = 0.004$), the between-subjects factor, treatment ($F[4,31] = 4.31$, $P = 0.023$), and a significant interaction between the factors week and treatment on percentage change of body weight from the third week ($F[12,93] = 6.2$, $P = 0.034$). An overall decrease in body weight was observed in the CMS group in the fourth, fifth, and sixth weeks compared to the control group ($P < 0.05$, 0.01 and 0.001, respectively). Treatment with 7-nitroindazole and pentoxifylline significantly reduced the CMS-induced weight loss at the fifth and sixth weeks.

Neurochemical changes

Tryptophan and serotonin concentrations by HPLC in frontal cortex and hippocampus

CMS reduced tryptophan concentrations in frontal cortex and hippocampal homogenates, with no statistical significance, as demonstrated in Table 2; imipramine significantly ($P < 0.01$) reversed this effect in the frontal cortex. However, the increase (21%) was statistically insignificant in the hippocampus (122.1 ± 3.539 vs 100.9 ± 10.61 pmol/mg tissue) in comparison to the CMS-untreated group. Neither 7-nitroindazole nor pentoxifylline induced significant changes.

In rats exposed to CMS, serotonin concentrations in frontal cortex homogenates were decreased by 23% (1.374 ± 0.1174 vs 1.794 ± 0.215 pmol/mg tissue), with no statistical significance, as shown in Table 3. However, CMS induced a

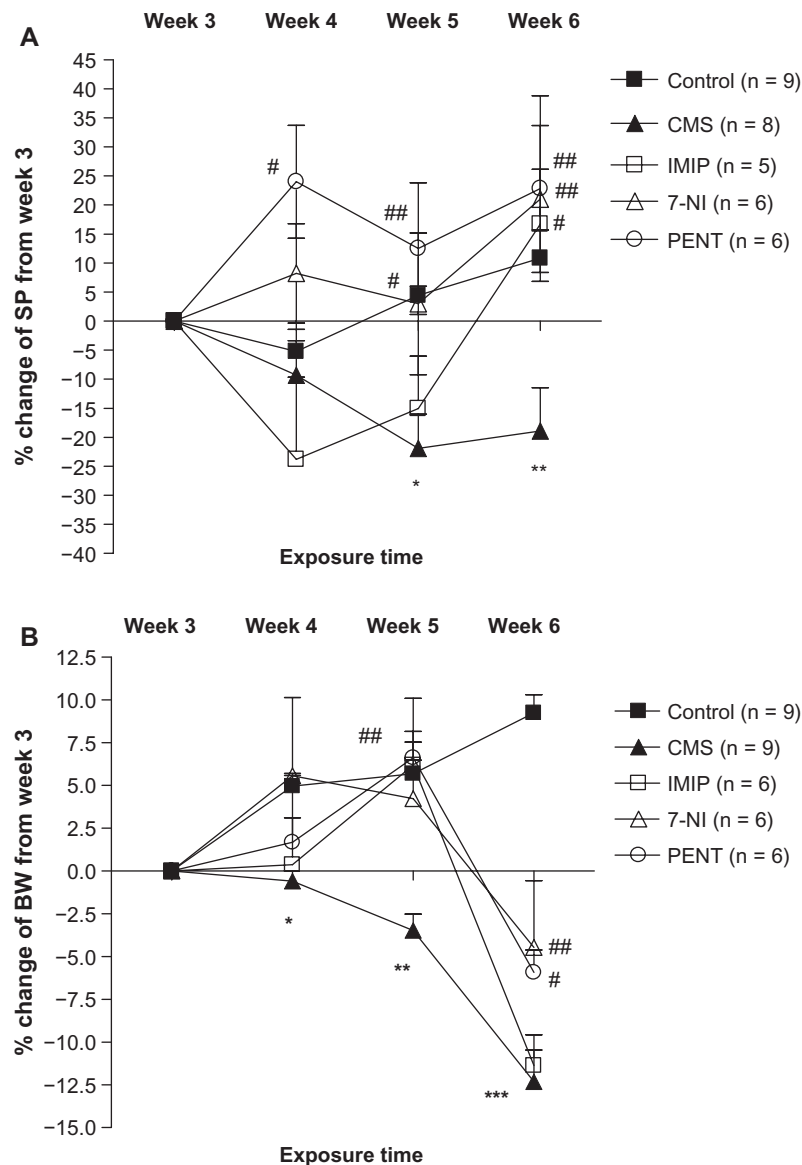


Figure 1 Effects of chronic treatment with imipramine (IMIP), 7-nitroindazole (7-NI), and pentoxifylline (PENT) on weekly percentage changes of (A) sucrose preference (SP) and (B) body weight (BW) from the pretreatment week (week 3) in male Wistar rats exposed to chronic mild stress (CMS).

Notes: Data are presented as means \pm standard error of mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs control group; # $P < 0.05$; ## $P < 0.01$.

significant (1.365 ± 0.116 vs 2.445 ± 0.466 pmol/mg tissue, $P < 0.001$) decrease in serotonin concentrations in hippocampus homogenates compared to the control group. The tested drugs did not induce any significant effect on serotonin concentrations in the frontal cortex or hippocampus.

5-HIAA concentrations by HPLC in frontal cortex and hippocampus

CMS decreased mean concentration of 5-HIAA in the frontal cortex (16.7%) (8.580 ± 1.031 vs 10.30 ± 1.226 pmol/mg tissue) and hippocampus (17.4%) (10.94 ± 1.49 vs 13.25 ± 0.637 pmol/mg tissue) homogenates compared to control,

with no statistical significance. Imipramine induced a significant ($P < 0.001$) increase of 5-HIAA mean concentrations in the frontal cortex and hippocampus compared to the CMS group. 7-Nitroindazole increased 5-HIAA concentrations in the hippocampus by 43.05% (15.65 ± 1.43 vs 10.94 ± 1.49 pmol/mg tissue) compared to the CMS group; however, this effect was statistically insignificant (Figure 2).

Kynurenine concentrations by HPLC and kynurenine/serotonin ratio in frontal cortex and hippocampus

Exposure to CMS induced a significant ($P < 0.01$) increase of kynurenine mean concentration in the frontal cortex

Table 2 Tryptophan concentration in the frontal cortex and hippocampus (pg/mg tissue)

	Control	CMS	IMIP	7-NI	PENT
Frontal cortex					
Mean	85.6	74.95	104.5	72.46	75.13
SD	17.90	17.97	9.554	6.249	5.262
SE	8.007	8.038	4.273	2.794	2.353
t ^a (P)	1.03 (0.3)	–	–3.2 (0.012)*	0.29 (0.8)	–0.2 (0.98)
Hippocampus					
Mean	118.7	100.9	122.1	85.49	82.40
SD	15.00	23.73	7.914	4.547	3.467
SE	6.706	10.61	3.539	2.034	1.551
t ^a (P)	1.4 (0.2)	–	–1.9 (0.09)	1.43 (0.19)	1.73 (0.12)

Notes: ^aIndependent sample t-test (vs CMS); *significant.

Abbreviations: CMS, chronic mild stress; IMIP, imipramine; 7-NI, 7-nitroindazole; PENT, pentoxifylline; SD, standard deviation; SE standard error.

and in hippocampus homogenates compared to control. Treatment with imipramine, 7-nitroindazole, and pentoxifylline significantly decreased kynurenine mean concentration in frontal cortex homogenates ($P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively). In the hippocampus, imipramine, 7-nitroindazole, and pentoxifylline significantly decreased kynurenine mean concentrations ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively) compared to the CMS group (Figure 3A and B).

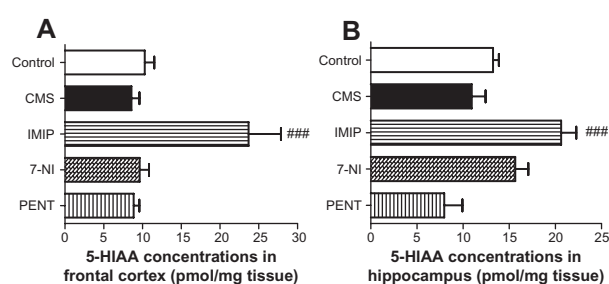
As demonstrated in Figure 3C and D, CMS induced a significant increase of kynurenine/serotonin molar ratios in the frontal cortex and hippocampus ($P < 0.01$ and $P < 0.05$, respectively) compared to the control group. Imipramine, 7-nitroindazole, and pentoxifylline treatment significantly reduced kynurenine/serotonin mean ratio in the frontal cortex ($P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively)

Table 3 Serotonin molar concentration in the frontal cortex and hippocampus (pmol/mg tissue)

	Control	CMS	IMIP	7-NI	PENT
Frontal cortex					
Mean	1.794	1.374	1.434	1.477	1.424
SD	0.4731	0.2626	0.1450	0.09849	0.1717
SE	0.2116	0.1174	0.06486	0.04405	0.07679
t ^a (P)	1.7 (0.12)	–	–0.45 (0.7)	–0.82 (0.43)	–0.36 (0.73)
Hippocampus					
Mean	2.445	1.365	1.540	1.540	1.524
SD	0.4661	0.1125	0.09716	0.1162	0.4463
SE	0.2084	0.05029	0.04345	0.05197	0.1996
t ^a (P)	5.04 (0.001)*	–	–2.6 (0.03)*	–2.4 (0.4)*	–0.77 (0.46)

Notes: ^aIndependent sample t-test (vs CMS); *significant.

Abbreviations: CMS, chronic mild stress; IMIP, imipramine; 7-NI, 7-nitroindazole; PENT, pentoxifylline; SD, standard deviation; SE standard error.

**Figure 2** Effects of chronic mild stress (CMS) and treatments with imipramine (IMIP), 7-nitroindazole (7-NI), and pentoxifylline (PENT) on changes of 5-hydroxyindoleacetic acid (5-HIAA) concentrations (pmol/mg tissue) in frontal cortex (A) and hippocampus (B) homogenates.

Notes: Data are presented as means \pm standard error of mean ($n = 5/\text{group}$). #### $P < 0.001$ vs CMS group by one-way analysis of variance (A $F[4,20] = 9.399$, $P = 0.0002$; B $F[4,20] = 10.35$, $P = 0.0001$) followed by Tukey's post hoc test.

compared to the CMS group. A significant reduction in kynurenine/serotonin mean ratio in hippocampus homogenates was demonstrated for 7-nitroindazole and pentoxifylline ($P < 0.01$ and $P < 0.05$, respectively) compared to the CMS group. Imipramine induced a statistically insignificant reduction (6.70 ± 1.267 vs 10.48 ± 2.22) in the kynurenine/serotonin mean.

Ratio in hippocampus homogenates compared to CMS group

Nitric oxide metabolites in frontal cortex and hippocampus

Figure 4 demonstrates that CMS-exposed rats exhibited a significant ($P < 0.001$) increase in nitric oxide metabolite concentrations in frontal cortex and hippocampus homogenates in comparison to nonexposed control rats.

Treatment with imipramine significantly ($P < 0.001$) decreased nitric oxide metabolite concentrations in the frontal cortex, but was not statistically significant in the hippocampus (569.6 ± 64.15 vs 610.9 ± 42.19 nmol/g tissue). 7-Nitroindazole and pentoxifylline significantly decreased nitric oxide metabolites in the frontal cortex and hippocampus.

Serum TNF- α analysis

Figure 5 shows that CMS induced a significant ($P < 0.05$) increase in TNF- α mean serum concentration compared to control. Treatment with imipramine reduced mean serum TNF- α by 10%, with no statistical significance (23.38 ± 3.860 vs 26.20 ± 1.831 pg/mL) compared to the untreated CMS group. Treatments with 7-nitroindazole and pentoxifylline showed significant ($P < 0.05$) reduction of CMS-induced increase in mean serum TNF- α concentration.

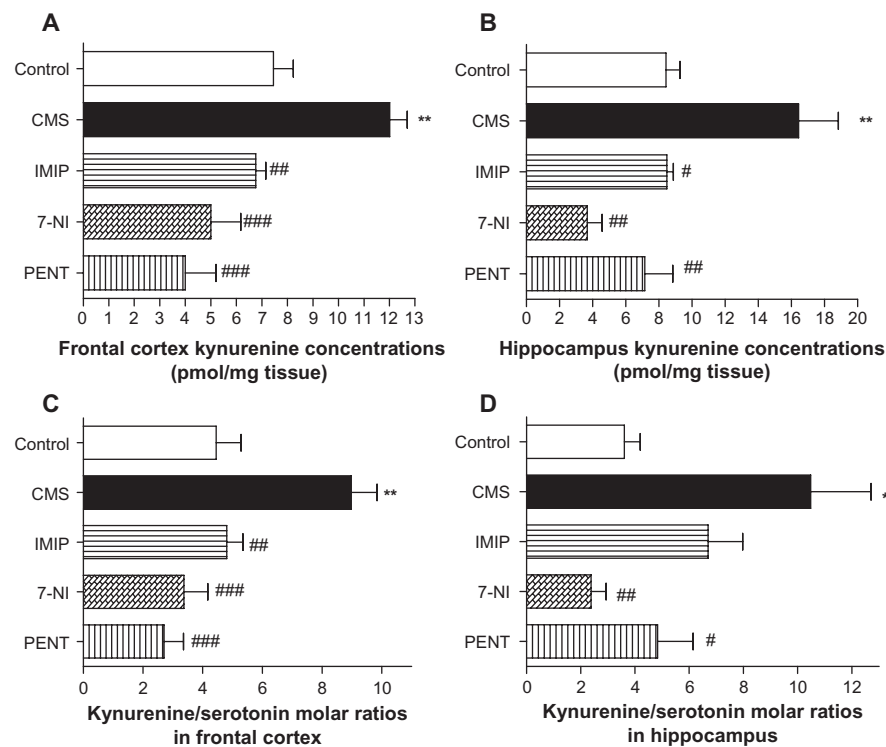


Figure 3 Effects of chronic mild stress (CMS) and treatments with imipramine (IMIP), 7-nitroindazole (7-NI), and pentoxifylline (PENT) on changes of kynurenine concentrations (pmol/mg tissue) and kynurenine/serotonin ratio in frontal cortex (A and C) and hippocampus (B and D) homogenates.

Notes: Data are presented as means \pm standard error of mean ($n = 5$ /group). * $P < 0.05$ vs control group; ** $P < 0.01$ vs control group; # $P < 0.05$; ### $P < 0.01$; #### $P < 0.001$ vs CMS group by one way ANOVA (A $F[4,20] = 11.82$, $P < 0.0001$; B $F[4,20] = 10.56$, $P < 0.0001$; C $F[4,20] = 10.72$, $P < 0.0001$; D $F[4,20] = 5.615$, $P = 0.0034$) followed by Tukey's post hoc test.

Immunohistochemical examination

Qualitative assessment

In the control group, immunohistochemical staining of the hippocampus demonstrated negative IDO and mild positive TNF- α staining, as shown in CA1, CA3, and the dentate gyrus. CMS-exposed rats exhibited strong positive IDO staining in the alveus, although this was negative in the

hippocampus proper, and strong positive TNF- α staining in CA1, CA3, dentate gyrus, and alveus. In pentoxifylline- and 7-nitroindazole-treated rats, there was negative IDO staining and mild TNF- α staining in CA1, CA3, and the dentate gyrus. Imipramine-treated rats demonstrated negative

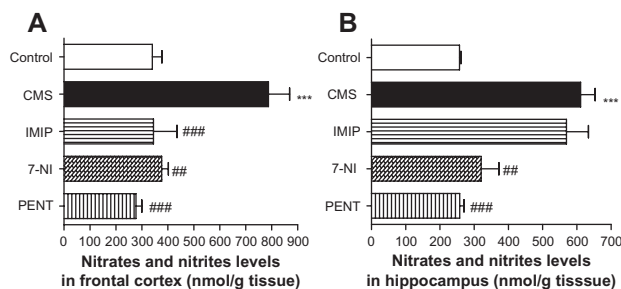


Figure 4 Effects of chronic mild stress (CMS) and treatments with imipramine (IMIP), 7-nitroindazole (7-NI), and pentoxifylline (PENT) on changes of nitric oxide metabolites (nitrates/nitrites, nmol/g tissue) in frontal cortex (A) and hippocampus (B) homogenates.

Notes: Data are presented as means \pm standard error of mean ($n = 5$ /group). *** $P < 0.001$ vs control group; ## $P < 0.01$; ### $P < 0.001$ vs CMS group by one-way analysis of variance (A $F[4,20] = 12.22$, $P < 0.0001$; B $F[4,20] = 17.19$, $P < 0.0001$), followed by Tukey's post hoc test.

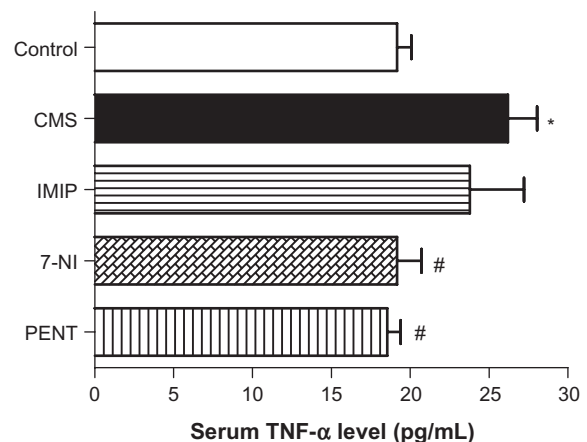


Figure 5 Effects of chronic mild stress (CMS) and treatments with imipramine (IMIP), 7-nitroindazole (7-NI), and pentoxifylline (PENT) on serum tumor necrosis factor (TNF- α level (pg/mL).

Notes: Data are presented as means \pm standard error of mean ($n = 6$ /group). * $P < 0.05$ vs control group; # $P < 0.05$ vs CMS group by one-way analysis of variance ($F[4,25] = 3.072$, $P = 0.0346$), followed by Tukey's post hoc test.

IDO and moderate positive TNF- α staining in CA1, CA3, and the dentate gyrus. As regards midbrain raphe nuclei, immunohistochemical staining demonstrated negative IDO and mild positive TNF- α staining in the control, pentoxifylline, and 7-nitroindazole groups, while the imipramine group showed negative IDO and moderate positive TNF- α . In CMS the mid brain raphe nuclei demonstrated strong positivity with IDO and TNF- α staining which are approximately equal in their staining degree. Consecutive sections showed topographic proximity between IDO and TNF- α staining (Figure 6).

Semiquantitative assessment

Table 4 shows that exposure to CMS induced significant increase in TNF- α immunohistochemical staining score in both the hippocampus and raphe nuclei compared to control group ($P < 0.05$ and $P < 0.01$, respectively). At the same time, the CMS group showed increase in IDO immunohistochemical staining in both the hippocampus and raphe nuclei, which was significant only in raphe nuclei ($P < 0.001$) compared to the control group. Both 7-nitroindazole and pentoxifylline significantly ($P < 0.05$)

reduced TNF- α immunostaining in the hippocampus and raphe nuclei, and this effect was accompanied by reduction of IDO staining in both areas. Nevertheless, this effect was only significant ($P < 0.01$) in raphe nuclei. Likewise, imipramine reduced TNF- α and IDO immunostaining in the hippocampus and raphe nuclei; however, its effects were statistically insignificant, except in hippocampus TNF- α immunostaining ($P < 0.05$).

Discussion

In this study, rats exposed to CMS developed depressive-like behaviors. This was evidenced by anhedonia in SPT, which was associated with neurochemical changes in the serum, hippocampal and frontal cortical molar concentrations of serotonin, kynurenine, NO metabolites and TNF- α , in addition to changes occur in their immunostaining.

The neurochemical changes induced by CMS in this work have been reported individually by other investigators. Indeed, the CMS-induced depressive-like behavior that was associated with increased hippocampal TNF- α level³³ and nNOS expression.³⁴ At the same time, a meta-analysis

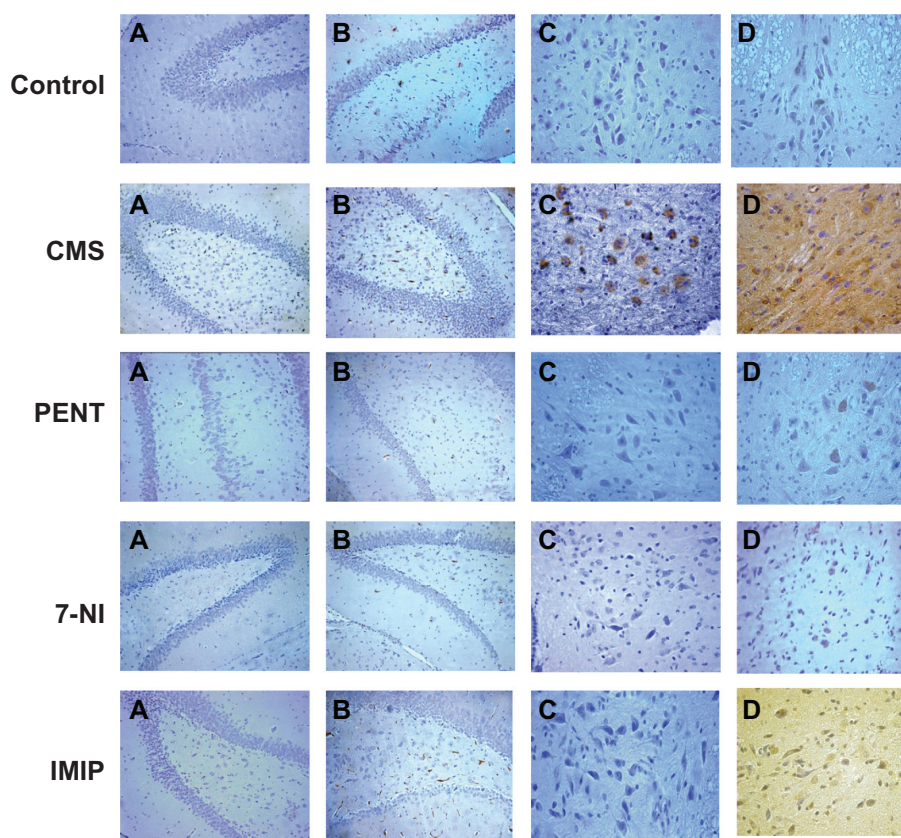


Figure 6 (A–D) Effects of chronic mild stress (CMS) and treatments with imipramine (IMIP), 7-nitroindazole (7-NI), and pentoxifylline (PENT) on indoleamine 2,3-dioxygenase (IDO) and tumor necrosis factor (TNF)- α immunohistochemical staining of hippocampus and raphe nuclei in comparison to control group ($n = 5/\text{group}$). (A) hippocampus proper stained with IDO $\times 200$; (B) hippocampus proper stained with TNF- α $\times 200$; (C) raphe nuclei stained with IDO $\times 400$; (D) raphe nuclei stained with TNF- α $\times 400$.

Table 4 Effects of chronic mild stress (CMS) and treatments with imipramine (IMIP), 7-nitroindazole (7-NI), and pentoxifylline (PENT) on indoleamine 2,3-dioxygenase (IDO) and tumor necrosis factor (TNF)- α immunohistochemical staining semiquantitative score of hippocampus and raphe nuclei in comparison to control group

	Immunohistochemical staining score			
	Hippocampal IDO staining	Hippocampal TNF- α staining	Raphe nuclei IDO staining	Raphe nuclei TNF- α staining
Control	0.0 \pm 0.0	0.71 \pm 0.18	0.0 \pm 0.0	0.71 \pm 0.18
CMS	0.41 \pm 0.20	2.57 \pm 0.20*	2.43 \pm 0.20***	2.57 \pm 0.20**
IMIP	0.24 \pm 0.21	0.70 \pm 0.22 [#]	0.40 \pm 0.24	2.00 \pm 0.32
7-NI	0.23 \pm 0.22	0.56 \pm 0.23 [#]	0.23 \pm 0.20 ^{##}	0.83 \pm 0.20 [#]
PENT	0.20 \pm 0.20	0.60 \pm 0.24 [#]	0.20 \pm 0.22 ^{##}	0.80 \pm 0.21 [#]
Kruskal–Wallis	$P = 0.4350$	$P = 0.0013$	$P = 0.0003$	$P = 0.0003$
(KW) test	KW = 3.791	KW = 17.95	KW = 21.43	KW = 21.30

Notes: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs control group; [#] $P < 0.05$; ^{##} $P < 0.01$ vs CMS group by nonparametric Kruskal–Wallis test, followed by Dunn's post hoc test. Data are presented as means \pm standard error of mean ($n = 5/\text{group}$).

reported significantly higher concentrations of TNF- α in depressed subjects,³⁵ and a postmortem examination of hippocampi of patients with major depression showed more expression of nNOS.¹³ Ito et al³⁶ reported an increase in plasma kynurenine in healthy subjects exposed to physical stress, while Miura et al¹ suggested that proinflammatory cytokines stimulated the IDO enzyme under stress, promoting the kynurenine pathway.

Chronic treatment with pentoxifylline or 7-nitroindazole displayed antidepressant-like activity (reversed anhedonia in SPT) that was comparable to imipramine effect. Moreover, early reversal of CMS-induced anhedonia was observed with pentoxifylline and 7-nitroindazole suggesting a faster antidepressant onset compared to imipramine. Concurrently, all tested drugs reduced kynurenine, the kynurenine/serotonin ratio, and NO metabolites in the frontal cortex and hippocampus, besides reduction in serum TNF- α level and immunohistochemical staining of TNF- α and IDO in the hippocampus and raphe nuclei. Moreover, imipramine and 7-nitroindazole increased hippocampal 5-HIAA concentrations, denoting an increase in extracellular serotonin level, yet the effect of the later was statistically insignificant.

The behavioral findings (anhedonia) in the present study agree with Bah et al,³⁷ who detected that pentoxifylline administration significantly reversed the depressive-like behavior seen after myocardial infarction in rats. Likewise, the behavioral effect of 7-nitroindazole was previously investigated by Yildiz et al¹⁴ and Joca and Guimarães,³⁸ who reported that nNOS inhibitors produced an antidepressant-like activity in the forced-swimming test. Additionally, Zhou et al³⁹ reported that 7-nitroindazole significantly reversed the nNOS-derived NO suppression of hippocampal neurogenesis in depression. Interestingly, Ulak et al⁴⁰ reported that nNOS inhibition potentiated the behavioral

effects of some antidepressants – imipramine, citalopram, and fluoxetine – in rats; however, the exact mechanism was not clear.

One mechanism was suggested by Zhou et al,³⁴ who reported that CMS induced hippocampal nNOS overexpression via activating mineralocorticoid receptors. In turn, hippocampal nNOS-derived NO significantly downregulated local glucocorticoid receptor expression, and consequently nNOS inhibition reversed the CMS-induced depressive behaviors via preserving hippocampal glucocorticoid receptors.

Another possible mechanism of 7-nitroindazole antidepressant effect can be explained by the reported increase in extracellular levels of serotonin⁴¹ and decrease in tyrosine hydroxylase expression in the hippocampus of rat treated with 7-nitroindazole.⁴² Indeed, NO has a bimodal effect in IDO function. High micromolar concentrations of the NO donors decreased IDO activity, while low micromolar concentrations increased IDO activity in IFN-stimulated monocytic cells.⁴³ The iNOS isoform generates much larger quantities of NO (nanomolar range) than constitutive NOS, like nNOS isoforms (picomolar range).⁴⁴ In CMS, the iNOS activity may be attenuated by high glucocorticoid level in the hippocampus,⁴⁵ while nNOS activity increases,³⁴ resulting in low NO concentrations that stimulate IDO activity, leading to a reduction in hippocampus serotonin and an elevation in kynurenine concentrations. This in turn might explain the reduction in kynurenine concentrations and kynurenine/serotonin ratio with nNOS inhibition in the present study.

The findings with pentoxifylline are suggestive of an involvement of TNF- α in the pathophysiology of depression and are supportive of the “inflammatory hypothesis of depression.”⁴⁶ A possible mechanism for the involvement

of TNF- α in depression is thought to be through its ability to affect tryptophan metabolism. Many lines of evidence suggest that the underlying link between stress and depression might involve a shift of tryptophan metabolism from serotonin towards the kynurenine pathway¹ that ultimately produces quinolinic acid, which has neurotoxic properties contributing to several neurobiological changes associated with depressive illness.⁴

TNF- α is reported to induce the activity of the IDO enzyme. At the same time, an association between serum TNF- α and brain kynurenine concentrations was reported by O'Connor et al,¹¹ who demonstrated that induction of the IDO enzyme was associated with increased central TNF- α , and elevated kynurenine was shown to induce degenerative changes in the hippocampus, which might be involved in depression.^{2,3} Indeed, Zhu et al⁴⁷ have shown that treatment with IL-1 or TNF- α is associated with increased activity of serotonin transporters and decrease in its synaptic availability. Immune activation has also been shown to decrease tryptophan availability.^{10,48,49} Targeting the kynurenine pathway by pentoxifylline could potentially reduce TNF- α , with loss of its inducing effect on the IDO enzyme consequently reducing conversion of tryptophan to kynurenine, with its prodegenerative effects on hippocampus tissue.

In the present study, immunoreactivities of both TNF- α and IDO were found to be in close proximity, providing a topographic possibility for TNF- α to exert its proposed effects on IDO. In the immunohistochemistry assays, IDO was negative and TNF- α was mildly positive in control brain sections (both the hippocampal and raphe sections), however, it was strongly positive for TNF- α in the hippocampus of rats exposed to CMS. In the raphe, strong positive reactions were found for both TNF- α and IDO. Raphe regions are the areas of projecting serotonin fibers to the hippocampus. Most probably, the expression of IDO at neurons, microglia, and astrocytes at the raphe regions may compete or deprive the serotonin neurons from their tryptophan resources, by shifting the tryptophan towards the kynurenine pathway.^{7,50}

Increased TNF- α expression in the hippocampus, and specifically at the dentate gyrus, in rats exposed to CMS, as shown in the present study, may point to a role for cytokines in the neurobiology of depression, other than compromising the serotonin pathway. TNF- α may affect neurogenesis in the hippocampus⁵¹ and compromise the expression of BDNF;⁵² both conditions are reportedly associated with depression.⁵³

Interestingly, nNOS inhibition is associated with decreased TNF- α expression in mice exposed to inflammatory pain,⁵⁴

and local application of both nNOS inhibitor and TNF- α antiserum induced neuroprotection and improved functional outcome following spinal cord injury.⁵⁵ The tendency of pentoxifylline to reduce NO metabolites was also noted in other studies. Schwartz et al⁵⁶ demonstrated that the production of NO by glial cells induced by *Streptococcus pneumoniae* was inhibited by pentoxifylline. Furthermore, Beshay et al⁵⁷ reported that pentoxifylline suppressed NO production of LPS/IFN gamma-stimulated macrophages. These studies, as well as the findings of the present study, highlight the relevance of the role of nNOS and TNF- α in neurodegeneration.

In the present work, the antidepressant effects of imipramine were associated with marked elevation of 5-HIAA and reduction in kynurenine, the kynurenine/serotonin ratio, and NO metabolites in the frontal cortex and hippocampus. Imipramine did not significantly decrease serum TNF- α level; however, it could significantly reduce the TNF- α immunostaining in the hippocampus proper. These findings are in agreement with recent studies by Abbas et al and Hsu et al,^{58,59} who demonstrated that imipramine treatment increased 5-HIAA in the hippocampus of forced-swim-stressed rats and mice, respectively. Furthermore, Kubera et al⁶⁰ showed that chronic treatment with imipramine reduced immune activation in rats subjected to CMS. Additionally, imipramine inhibited the production of TNF- α in whole human blood⁶¹ and microglia and astrocyte cultures.⁶²

The early reversal of CMS-induced anhedonia by 7-nitroindazole and pentoxifylline in the present work may be explained by their direct inhibition of nNOS and TNF- α . In contrast, imipramine may affect TNF- α and nNOS later in the process, through the long-term effects on serotonin. Recently, Zhang et al⁶³ reported that the 5-HT1 AR-selective agonist 8-OH-DPAT and fluoxetine downregulated hippocampal nNOS expression. The limitations of this study were that direct enzyme activities of NO (inducible and neuronal) and IDO in the frontal cortex and hippocampus were not measured. Furthermore, TNF- α concentrations were measured in serum, not in the studied brain regions.

In conclusion, the findings of this study add further evidence that disturbance in brain monoaminergic function is not the sole mechanism underlying depression. It highlights the role played by the immune system and TNF- α in the pathophysiology of depression. It also elucidates the intricate relationship between TNF- α , nNOS, and tryptophan metabolism (with its two limbs: kynurenine and serotonin) and their association with depression, which might be relevant to speculating about management strategies for depression.

Finally, pentoxifylline and nNOS antagonists may prove to be a useful adjunct to other antidepressants.

Disclosure

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