Pharmacology, Biochemistry and Behavior 91 (2009) 339–344

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Changes in glutamate decarboxylase enzyme activity and *tau*-protein phosphorylation in the hippocampus of old rats exposed to chronic mild stress: Reversal with the neuronal nitric oxide synthase inhibitor *7-nitroindazole*

Yasser A. El-faramawy [a], Mohamed H. El-banouby [a], Pavel Sergeev [c], Ahmed K. Mortagy [a], Motassem S. Amer [a], Ahmed M. Abdel-tawab [b,*]

[a] *Department of Geriatrics, Faculty of Medicine, Ain Shams University, Cairo, Egypt*
[b] *Department of Pharmacology, Faculty of Medicine, Ain Shams University, Cairo, Egypt*
[c] *Department of Molecular Pharmacology, ETH, Zurich, Switzerland*

ARTICLE INFO

Article history:
Received 9 April 2008
Received in revised form 23 July 2008
Accepted 1 August 2008
Available online 8 August 2008

Keywords:
Glutamate decarboxylase enzyme
tau-protein phosphorylation
Hippocampus
Chronic mild stress
Old rats
7-nitroindazole

ABSTRACT

Effects of chronic stress are not completely understood. They may underlie depression and dementia. This study assessed the association between chronic stress, glutamate levels, *tau*-protein phosphorylation, and nitric-oxide in old rats exposed to chronic mild stress (CMS). Old (>15 months) male Wistar rats were exposed to CMS. Comparison groups included old and young control rats, young CMS-exposed, and old CMS-exposed rats treated with the neuronal nitric-oxide synthase (nNOS) enzyme inhibitor, 7-nitroindazole (20 mg/kg/day i.p.). Hippocampal glutamate levels and glutamate decarboxylase (GAD) activity were determined and *tau* protein phosphorylation was assessed. Age was a significant ($p=0.025$) source of variation in glutamate level [811.71±218.1, 665.9±124.9 μmol/g tissue protein (M±SD) in young and old control rats, respectively]. Old rats exposed to CMS were characterized by an increased risk to develop anhedonia. There was significant ($p=0.035$) decrease in GAD enzyme activity (−60.06%) and increased *tau* protein hyperphosphorylation in old rats exposed to CMS compared to control. Administration of 7-nitroindazole to CMS-exposed old rats significantly ($p=0.002$) increased GAD activity, decreased glutamate levels (7.19±3.19 vs. 763.9±91 μmol/g tissue protein; $p=0.0005$), and decreased phosphorylation of *tau* proteins compared to CMS exposed rats.

1. Introduction

There is evidence suggesting that elderly patients with depression or depressive symptoms have an increased risk of developing Alzheimer Type Dementia (ATD) (Wragg and Jeste, 1989; Wilson et al., 2002; Ownby et al., 2006).

One hallmark of the ATD pathology is the postmortem finding of dysregulated phosphorylation of the microtubule-associated *tau*-protein in the hippocampus of ATD patients, resulting in the formation of the characteristic neurofibrillary tangles (Grundke-Iqbal et al., 1986). Interestingly, although down-stream the deposition of β-amyloid (the other hallmark of ATD), reducing *tau* levels significantly ameliorated the behavioral manifestations of amyloid-induced excitotoxicity in an animal model of Alzheimer, even though there was no decrease in the levels of β-amyloid deposits (Roberson et al., 2007). Several studies reported the association between the hyperphosphorylation of *tau* and stress (Korneyev, 1998) and stress-activated kinases, both in vitro (Goedert et al., 1997; Yoshida et al., 2004) and in intact cells (Buée-Scherrer and Goedert, 2002). Added to that, stress-levels of corticosteroids were reported to enhance *tau* accumulation (Green et al., 2006).

Glutamate-induced excitotoxicity is thought to be part of the pathophysiological mechanisms underlying the development of ATD (Pietrzik and Behl, 2005; Ringheim and Szczepanik, 2006). Glutamate is suggested to induce differential age-related effects that add up to excitotoxicity (Brewer, 1998; Patel and Brewer, 2003; Kannurpatti et al., 2004), and this is associated with increased *tau*-protein phosphorylation (Jäsmä et al., 2006). The hippocampus is considered to be the main site for glutamate-regulated neuroplasticity pertinent to the adaptive response to stress (McEwen, 2001).

Colton et al. (2002) noticed an increased level of nitric oxide (NO) production in mice expressing the apolipoprotein E4 (APOE4), but not the APOE3 protein. Similarly, Keil et al. (2004) reported an enhanced production of NO in cells bearing the Swedish double mutation in the amyloid precursor gene, and they hypothesized that it may mediate the apoptotic effects of β-amyloid in these cells. However, in a previous study, the role played by NO in mediating effects of β-

* Corresponding author. Department of Pharmacology, Faculty of Medicine, Ain Shams University, Postal Code 11566, Abbassia, Cairo, Egypt. Tel.: +20 12 3937457.
E-mail addresses: amtawab@asunet.shams.edu.eg, amtawab@yahoo.com (A.M. Abdel-tawab).

0091-3057/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2008.08.003

amyloid was not shown to be statistically significant (Lahiri et al., 2003). These inconsistent findings, together with the reported anti-depressant-like effect of nitric oxide synthase (NOS) inhibitors (Harkin et al., 1999; Joca and Guimaraes, 2006), justify the search for more insights into the possible role of regulating NO synthesis in depression and ATD.

It is being increasingly emphasized that stress is associated with depression and with the altered hypothalamic-pituitary-adrenal (HPA) axis responsiveness characteristic of depression (Post, 1992; Checkley, 1996; Tafet and Smolovich, 2004; Firk and Markus, 2007), the hippocampus being one major location where this association is expressed, (Sapolsky, 2000; McEwen, 2005). Added to that the studies addressing the perturbation of glutamate metabolism and signaling in depression were thoroughly reviewed by Paul and Skolnick (2003).

In the present study, an animal model of depression addressing the role of chronic unpredictable mild stresses, namely chronic mild stress (Willner et al., 1992), will be used. There is evidence that this model has enough predictive as well as construct validity to demonstrate "antidepressant-reversible depressive like effects in rodents", using the decrease in the preference of the intake of sucrose solution as a marker of anhedonia, a core symptom of depression in humans (Willner, 2005). At the same time, Zhou et al. (2007) reported that mice exposed to CMS over-express hippocampal nNOS gene, and mice lacking this gene were less likely to express CMS-induced changes in the hippocampus.

The objective of the present study was to study the changes in glutamate metabolism and *tau* phosphorylation in the hippocampus of old rats, exposed to CMS, compared to young rats and to test the if these changes are halted by chronically administering the neuronal nitric oxide (nNOS) inhibitor 7-nitroindazole in a dose which was not previously reported to induce learning deficits, (Meyer et al., 1998).

2. Materials and methods

2.1. Animals and design

Male Wistar rats were purchased from the animal facility of the Egyptian Atomic Energy Agency a week before the start of the experiment to acclimatize them to the laboratory conditions: Temperature: ≈25 °C; light/dark cycle: 12 h/12 h, starting at 06:00 am; housing: 1 rat cage; and food (prepared in-house as a standard diet) and water, which were both provided *ad libitum* (unless a restriction was required as part of the study protocol). The European Community guidelines for the use of experimental animals were adhered to throughout the study.

Animals were allocated to the following study groups: young control animals not exposed to CMS ($n=12$), young animals exposed to CMS ($n=12$), old control animals not exposed to CMS ($n=12$), old animals exposed to CMS ($n=12$), old animals treated with 7-nitroindazole while exposed to CMS ($n=5$). The *young* rats were (120–140 g) <4 months old and the *old* rats were (>400 g) >15 months old.

7-nitroindazole (Sigma-Aldrich, Germany) dissolved in Tween 80 and saline was administered intra-peritoneal '*i.p.*' 20 mg/kg/day for 4 weeks to the animals being exposed to the CMS battery. The dose of the 7-nitroindazole was selected after a review of the literature, and specifically according to the findings of Meyer et al. (1998). The other groups were injected with Twin 80 and saline *i.p.* daily throughout the experiment.

The animals were anesthetized using *i.p.* urethane (1 g/kg), decapitated and their hippocampus dissected out under cooling conditions. One side of hippocampus was dedicated for *tau* analysis. The other side was divided into two portions, and randomly allocated for either GAD or neurotransmitter analysis. The portions of the dissected hippocampus taken for the neurotransmitters and GAD enzyme activity estimations were quickly put into an Eppendorf tube containing 1.5 ml of ice-cold 80% ethanol, containing 150 nmol of norvaline (the internal standard). These portions were analyzed after no more than one day of storage at −70 °C, while the portions taken for *tau* protein determinations were stored for 3–6 months at −70 C.

2.2. Chronic Mild Stress (CMS)

The CMS battery was applied according to Willner et al. (1992). It consisted of exposure to the following stressors in a random order: Two 24 h periods of food and water (12 h) deprivation, one of them immediately followed by the sucrose preference test; two periods of overnight illumination; two periods (7 and 17 h) of 45° cage tilt; one 17 h-period of paired housing; one 17 h-period in a soiled cage (moistening dust with 100 ml of water); two periods (5 h) of intermittent white noise [85 dB]; and three periods (6 h) of stroboscopic illumination (60 flashes/min). The rats were trained for one week to consume a palatable, weak (2%) sucrose solution; a bottle of sucrose was put in the cages with a bottle of bland water. The 2% concentration was selected after several experiments and consultation (Willner, *personal communication*, p.willner@swansea.ac.uk). The sucrose preference test (1 h) took place once/week at ≈09:00–10:00 a.m. The baseline ratio of sucrose solution to water consumption in g was determined for two consecutive weeks and averaged, before starting the CMS. The rats were considered anhedonic if their sucrose/water consumption ratio decreased by 50% compared to the baseline ratio.

2.3. Glutamic Acid Decarboxylase (GAD) enzyme activity

GAD enzyme activity was determined according to the method described by Chakraborty et al. (1991). Briefly, one portion of the dissected hippocampus was homogenized separately in a mixture of ice-cold 0.01 M 3-N-morph-olino-propane-sulfonic acid (MOPS) buffer, pH 7.4 and 150 nmol of the internal standard norvaline, to a total volume of 1.5 ml. A mixture of 1 ml of solution containing 4 mM pyridoxal phosphate, 0.6 M tetraethylamine and 2 mM 2-aminoethyl isothiouronium was added to 1 ml of the homogenate. The final suspension was treated with 8 µL/ sample (0.4%) Triton X-100. The mixture was centrifuged at 15,000 g for 5 min in a cooling centrifuge at 4 °C. Two Eppendorf tubes were prepared for each sample; each tube containing 100 µl of a mixture composed of: 200 µl of homogenate, 200 µl of 200 mM KH2PO4, 50 µl of 5 mM glutamic acid, 25 µl 0.2 mM pyridoxal phosphate, and 25 µl of 200 µg/ml gabaculine. The reaction mixture of each area was incubated 37 °C for 0 and 15 min in the Eppendorf tubes. The reaction was stopped by the addition of 50 µl of 100% trichloroacetic acid. The suspension was centrifuged at 10,000 g for 4 min. 500 µl of supernatant was aspirated and processed for derivatization and HPLC assay of GABA.

All chemicals used in this assay were purchased from Sigma-Aldrich (Germany), except the potassium dihydrogen phosphate and HPLC-grade water, which were purchased from (Merck).

2.4. HPLC determinations of glutamate and GABA

The HPLC method with pre-column phenyl-iso-thio-cyanate (PITC) derivatization was applied for the determination of glutamate and GABA levels in the hippocampus tissue homogenates according to the method described by Gunawan et al. (1990). Briefly, after the homogenization was complete, samples were centrifuged in a cooling (4 °C) centrifuge at 15,000 rpm for 10 min. The supernatant was aspirated and transferred to an Eppendorf tube, and 100 µL of the aspirated supernatant were dried in a centrivap under vacuum at 40 °C. The residue was dissolved in 20 µL of ethanol-water-triethylamine (2:2:1) and evaporated to dryness under vacuum. 30 µL of ethanol-water-triethylamine-phenylisothiocyanate (PITC) (7:1:1:1) were added to the residue and allowed to react for 20 min

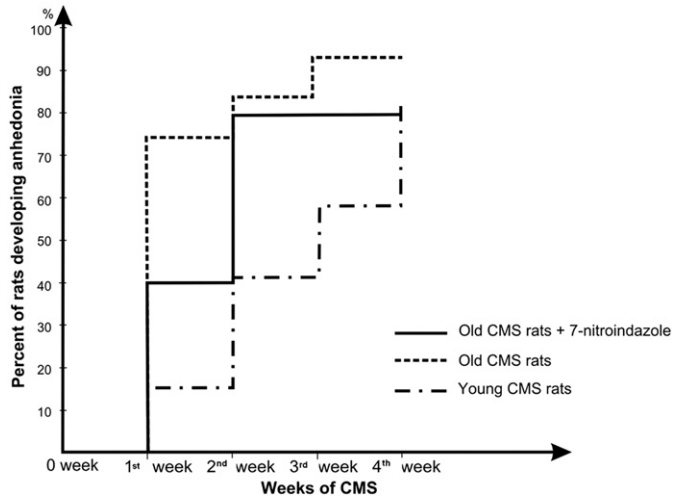

Fig. 1. The effect of CMS on development of anhedonia in old and young rats and the old 7-nitroindazole treated rat groups throughout the 4 weeks of the experiment.

at room temperature to form the PITC derivatives of the amino acids. The excess reagent was then evaporated under vacuum. Dried samples were reconstituted in 50 µl of solvent B. Stock solutions of glutamate and GABA (20 mM each) were prepared in 0.1M hydrochloric acid and stored at −20 °C for no longer than 1 week. The internal standard norvaline (20 mM) was also dissolved in 0.1 M hydrochloric acid and similarly stored at −20 °C for no longer than 3 months. The HPLC system consisted of an HPLC Beckman System Gold 125 dual pump; a Kanauer Injector 166 variable UV detector with a 20 µL loop; and a Phenomenex HPLC column (Lichrosorb RP-18; 5 µm, 250×4.6 mm ID, USA). The mobile phase consisted of solvents A and B: Solvent A: 0.1 M sodium acetate buffer prepared by adding 8.4 g sodium acetate anhydrous to 0.8 L of HPLC water, which was then filtered using 0.22 µm filters. 0.5 ml of triethylamine, 0.7 ml of glacial acetic acid and 5.0 ml acetonitrile were added to this, as was HPLC water, to make up a 1 L mixture. The pH of the mixture was adjusted to 5.8. Solvent B: acetonitrile: water (60: 40, v: v). The solvent A and B mixture was adjusted for the gradient HPLC separation, as shown below. The flow rate was set at 0.6 ml/min, the injected sample volume was 20 µl, and the peaks were detected at 254 nm wave length.

Time in minutes	Solvent A%	Solvent B%
0.0	100	0
2.0	85	15
8.0	85	15
12.0	75	25
20.0	75	25
24.0	55	45
32.0	55	45
37.0	0	100

All chemicals used in this part of the study were purchased from Sigma-Aldrich (Germany), except the acetonitrile and sodium acetate anhydrous, which were purchased from Merck.

2.5 Determination of tau protein

2.5.1. Protein preparation

≅100 µg tissue proteins were dissolved in 50 µl of the 2% SDS sample buffer [40 ml 0.1 M Tris HCL, pH 6.8 (0.5 M); 40 ml 2% SDS10%; 40 ml 20% glycerin; and 0.004 g of 0.002% bromophenol) and 200 ml water (HPLC grade) were added to make a 2-fold sample buffer], 140 µl urea in water 6 M, and 10 µl mercaptoethanol (ME). Sonication was performed for 1 min using an Ultrasonic Homogenizer (Cole-Parmer Instrument Corp., 4710 series, USA) under strict cooling conditions. Centrifugation at 10,000 rpm was performed for 5 min to precipitate the undissolved proteins. The fraction containing the solubilized proteins was incubated at 90 °C for 5 min to denature and reduce the sample for PAGE.

2.5.2. Immunoblot analysis

Equal amounts of protein were separated on 7.5% polyacrylamide gel (Bio-Rad), transferred into polyvinylidene difluoride membranes (Bio-Rad), and incubated in a blocking buffer consisting of PBS, pH 7.4, 1% Tween 20 with 5% nonfat dry milk. The blots were incubated for 30 min at room temperature with monoclonal antibodies anti-*tau* (Sigma-Aldrich, Germany) and anti-phospho-*tau* AT8 (Innogenetics, Germany). After washing with several volumes of PBS supplemented with 0.1% Tween 20 (PBST), the blots were incubated for 30 min in a blocking buffer with peroxidase-conjugated affinity purified F(ab) fragmented Donkey anti-mouse IgG (Jackson Immuno-research Lab., USA) and washed again in PBST. Signals were visualized with the enhanced chemoluminesense technique and band intensities were determined on X-ray film (Amersham Biosciences).

2.6. Quantification of total tissue proteins

Tissue proteins were determined to quantify the neurotransmitter levels in the tissues, to express it in µmol/g tissue protein, and to adjust the volume of samples during protein separation by electrophoresis in the *tau* protein experiments. The quantification of proteins was performed according to the method described by Bradford (1976).

2.7. Statistical analysis

The cumulative risk of developing anhedonia throughout the 4-week exposure to CMS was assessed in terms of *time-subject* units, in which time (in weeks) at which anhedonia happened was censored. The relative risk (RR) for developing anhedonia, considering the time of its occurrence, among the different groups was compared using either the χ^2 statistic or the Fisher's Exact test. Comparisons were made between the means of the two study groups and the statistical significance of the difference, if any, was assessed by the unpaired Student's *t*-test. ANOVA was performed whenever a comparison between the means of more than two groups was made.

3. Results

3.1. Development of anhedonia

From the CMS experiments, old rats were shown to be at a higher risk than young rats of developing anhedonia in response to the CMS battery. The RR of developing anhedonia for the group of old rats was 1.923; CI (1.123–3.293), which was almost double the risk for the young rats. The χ^2 test revealed that this risk is statistically significant (two-tailed $p=0.01$). Interestingly, chronic administration of 7-nitroindazole (n-NOSi) decreased the RR to 0.619, CI (0.308–1.178), which is almost half the risk compared to the untreated old CMS group. However, this decrease was not shown to be statistically significant.

Table 1
The changes in GAD enzyme activity [determined as the GABA concentration (µmol/g tissue protein)] in hippocampus homogenates of old male rats exposed to CMS and treated with *i.p.* 20 mg/kg/day 7-nitroindazole compared to control and CMS groups

	GABA concentration		
	Control ($n=7$)	CMS ($n=10$)	7-nitroindazole ($n=5$)
M.±S.D.	82.05±46.70	32.77±13.77	*253.59±167.91
Kruskal–Wallis statistic	KW=12.239		P=0.002
% change from control level		−60.06%	+209.07%

Table 2
Comparison between the glutamate concentration (μmol/g tissue protein) in hippocampus homogenates of young male rats in the control group and the group exposed to CMS

	Glutamate concentration	
	Young Control (n=11)	Young CMS (n=12)
M.±S.D.	811.71±218.1	*1297.4±864.2
One-tailed Student's t-test with Welch correction	p=0.04	
% change from control level		+59.83%

*Significant at $p<0.05$.

When the development of anhedonia was considered as just a decrease in sucrose/water ratio rather than a 50% decrease, clearly visible differences between the young CMS, old CMS, and 7-nitroindazole treated groups were noticed (Fig. 1).

3.2. GAD enzyme activity

As shown in Table 1 GAD enzyme activity, measured by the GABA concentrations, in the control group of the old rats (82.05±46.70 μmol/g tissue protein, mean±S.D.) was significantly higher (p=0.032) than the GAD enzyme activity in the control group of the young rats (39.62±34.02 μmol/g tissue protein, mean±S.D.). The GAD enzyme activity in the old CMS rats decreased by 60.06% and this decrease was statistically significant (p=0.035).

Administration of 7-nitroindazole increased GAD activity (253.59±167.91 μmol/g tissue protein, mean±S.D.). Application of the Kruskal–Wallis test showed that the difference among the three old comparison groups was statistically significant (p=0.002). Dunn's multiple comparison test showed that the 7-nitroindazole group was the group responsible for this difference. The increase in GAD activity in the 7-nitroindazole-treated group, compared to the control group, was as high as 209.07%.

3.3. Glutamate and GABA levels

The glutamate levels found in the hippocampuses of the young and old rats in the control and CMS groups are shown in Tables 2 and 3. A two-way ANOVA demonstrated that age (young vs. old) is a significant (p=0.025) source of variation in glutamate levels. Given the effect of age, the effect of CMS is barely significant (p=0.053). This could be understood in the light of the tiny, although significant, change in glutamate levels induced by CMS in the group of old animals.

Administration of 7-nitroindazole decreased the mean concentration of glutamate when the control and CMS old groups were compared: [(665.9±124.9; 763.9±91, 7.19±3.19 μmol/g tissue protein, mean±S.D.) control, CMS, and 7-nitroindazole groups respectively]. Application of the Kruskal–Wallis test showed that the difference among the three comparison groups was statistically significant (p=0.0005). Dunn's multiple-comparison test showed that the 7-nitroindazole group was the group responsible for this difference.

Table 3
Concentration of glutamate (μmol/g tissue protein) in hippocampus homogenates of old male rats exposed to CMS and treated with *i.p.* 20 mg/kg/day 7-nitroindazole compared to control and CMS groups

	Glutamate concentration		
	Control (n=10)	CMS (n=10)	7-nitoindazole (n=5)
M.±S.D.	665.9±124.9	763.9±91	*7.19± 3.19
Kruskal–Wallis test	KW=15.319		p=0.0005
% change from control level		+14.74%	−98.92%

*Significant at $p<0.05$.

Table 4
Concentration of GABA (μmol/g tissue protein) in hippocampus homogenates of old male rats exposed to CMS and treated with *i.p.* 20 mg/kg/day 7-nitroindazole compared to control and CMS groups

	GABA concentration		
	Control (n=10)	CMS (n=12)	7-nitroindazole (n=5)
M.±S.D.	25.49±15.05	28.76±10.33	∫16.02±9.92
One-way ANOVA test		p=0.1692	
% change from control level		+12.83%	−37.15%

∫=insignificant at $\alpha>0.05$.

GABA levels did not show significant changes across the study groups (Table 4).

3.4. Changes in tau protein phosphorylation

Aliquots containing equal amounts of protein were analyzed by quantitative immunoblotting using phosphorylation-dependent anti-*tau* antibodies (AT8). Changes in the phosphorylation state of *tau* were reflected by the altered signal intensities of the different *tau* bands. The immunoblots are shown in Fig. 2, with the bands in A and B representing the different rat groups. Each group contained 5 rats, other than the young control (YC) group, which contained 4.

Two major bands, designated in the figure as 1 and 2, were detected as having the AT8 antibodies. Clear evidence of the hyperphosphorylation of *tau* proteins, corresponding to band 1, was seen in the old stressed rats. An increase in the phosphorylation of band 1 was observed in the group of young rats, but not as strongly as in the old rats. This indicates that CMS has a different influence on the phosphorylation of *tau* related to the age of rats.

On the other hand, chronic administration of the nNOS inhibitor 7-nitroindazole was associated with an unambiguous effect on the phosphorylation of *tau* protein. The signal obtained from both of the

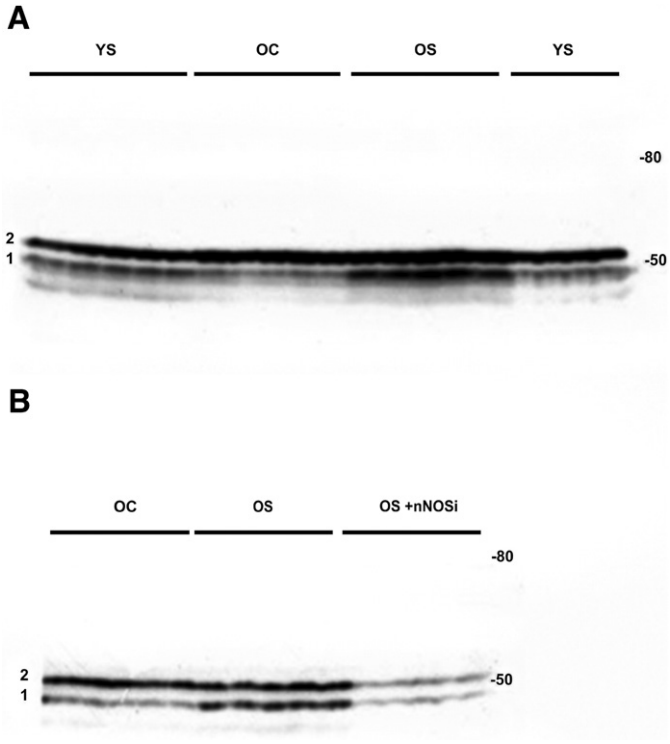

Fig. 2. The proteins isolated from the hippocampus of the following rat groups: YS (young CMS), OC (old control), OS (old CMS) and OS+nNOS (old CMS treated with 7-nitroindazole). The antibodies used were AT8 anti-phospho-*tau* protein.

phosphorylated forms of *tau* was reduced after treating the old rats with 7-nitroindazole. The extent of phosphorylation was even lower than in the old control animals.

These results show that phosphorylation of *tau* at multiple phosphorylation sites takes place in the hippocampus of rats during CMS, and that hyperphosphorylation is related to the age of the rats. nNOS is also suggested to be involved in the processes leading to hyperphosphorylation of *tau* in rats.

4. Discussion

In this study, it was shown that old rats were more likely to develop anhedonia after exposure to the CMS battery compared to younger rats. Chronic administration of the nNOS inhibitor 7-nitroindazole was associated with the alteration of this likelihood, although the alteration was statistically insignificant. Added to that, CMS was associated with a significant decrease in GAD enzyme activity and an increased glutamate level, while chronic administration of 7-nitroindazole was associated with a considerable increase in GAD enzyme activity and a marked decrease in the glutamate level in the hippocampus of CMS exposed rats. CMS exposed old rats showed a greater increase in the phosphorylated *tau*-protein in the hippocampus than young rats, and this increase was markedly reversed by administering 7-nitroindazole.

Studies using the CMS animal models of depression have been widely cited. Specifically, and relevant to the present subject of study, the model has been used to predict vulnerability differences (Dalla et al., 2005), altered GABA levels (Grønli et al., 2007). Also, data on proliferative and structural changes in the dendate gyrus were reported by Jayatissa et al. (2006) and Jayatissa et al. (2008).

Old rats are more vulnerable as regards their response to stress (Anisman and Matheson, 2005). An age-dependent hyper-responsivity to chronic unpredictable stresses was shown to be associated with elevated levels of CRF receptors mRNA (Herman et al., 2001). Interestingly, corticosteroid secretion by the adrenals does not show an age-related decline (Ferrari et al., 2001). HPA axis dysregulation and excitotoxicity are mainstays underlying the pathophysiology of depression and the stress-related animal models of depression (Zarate et al., 2003).

GAD enzyme activity was shown, in the present study, to be significantly decreased in the hippocampus of old rats exposed to CMS. Specifically, GAD enzyme is expressed in the adult brain in two isoforms, namely GAD_{65} and GAD_{67} (Soghomonian and Martin, 1998). The two isoforms were shown to substantially differ in their response to phosphorylation; GAD_{67} is inhibited by phosphorylation and activated by calcineurin-mediated dephosphorylation, while the GAD_{65} isoform is activated by phosphorylation (Hsu et al., 1999). Herman and Larson (2001) reported that exposure to chronic intermittent stress decreased GAD_{65} mRNA levels in the hippocampal-paraventricular hypothalamic nucleus (PVN) relays of old Fischer rats, which was not the case with the young and middle-aged animals. Added to this are the findings of Sommer et al. (2002), who reported a selective inhibition of the phosphatase activity of calcineurin (compared to other phosphatases) in response to reactive oxygen species (ROS) and reactive nitrogen species, reminiscent of stress effects on the aging neuronal cells. Taken together, the effects of chronic stress on GAD isoforms in old rats would lead to a decrease in GAD_{67} re-activation and also a decrease in GAD_{65} expression. This could explain the increased glutamate in response to stress, as shown in the hippocampus homogenates of old rats exposed to CMS compared to the relatively lower levels in the control in the present study.

There was a substantial decrease (more than 98%) in glutamate levels in response to chronic administration of 7-nitroindazole in the present study. It is interesting that Watts et al. (2005) reported a 50% decrease in released glutamate in the hippocampus in response to 90 min local infusion of 1 mM dose of 7-nitroindazole. The decrease in glutamate demonstrated in the present study was more elaborate as it was consistent with the demonstrated increase in GAD activity as well as reflecting the changes in the intra- and extracellular levels in brain homogenates.

Subchronic stress is associated with increased nNOS immunoreactivity in various regions of the hippocampus proper and dentate gyrus of male rats (Echeverry et al., 2004). In a recent review, McEwen (2007) discussed the possible mechanisms that relate stress to hippocampal structural changes, including suppressed neurogenesis, especially in the aging brain. Reasonable evidence suggests that nNOS-derived NO suppresses neurogenesis in the dentate gyrus, and in some way this effect relates to NMDA signaling (Zhu et al., 2006). Actually, activation of nNOS was reported to be coupled to glutamate-induced excitotoxicity (Rameau et al., 2007). These findings are consistent with the results in the present study, where the nNOS inhibitor 7-nitroindazole significantly reversed both the CMS-induced decrease in GAD enzyme activity and the increase in glutamate levels in the hippocampus of old rats. Zhu et al. (2006) demonstrated that 7-nitroindazole significantly reversed the nNOS-derived NO suppressed hippocampal neurogenesis, although, in another study, this enhancement was shown in areas not including the hippocampus (Moreno-Lopez et al., 2004).

The microtubule-associated *tau* protein has considerable influence on the morphology and function of neurons. The phosphorylation of *tau* influences its functioning and subcellular localization, and the sites of its phosphorylation differ, occurring at an increased number of sites in pathological conditions (e.g. ATD) as compared to physiological conditions (Avila et al., 2004). Acute cold water stress was shown to be associated with *tau* hyperphosphorylation, especially in the hippocampus (Okawa et al., 2003). This hyperphosphorylation was reversed when inspected 90 min after the end of the stress, but sustainable *tau* phosphorylation has been shown only after repeated stress (Rissman et al., 2007). There was also, more of a move towards disposition into the insoluble fragments in the repeated stress compared to the acute stress animal groups, as reported by the same authors. In the present study, the phosphorylated *tau* was not shown in the soluble fragments (results not shown). Hyperphosphorylation of *tau* in the CA3 region of the hippocampus was also demonstrated in mice exposed to chronic immobilization stress and in old mice by Jeong et al. (2006).

In the present study, chronic administration of the nNOS inhibitor 7-nitroindazole was associated with an obvious decrease in the CMS-induced increase in phosphorylated *tau*. In ATD patients, nNOS is colocalized with neurofibrillary tangles and plaques in hippocampal cells (Thorns et al., 1998) and increased nNOS reactivity in the frontal cortex (Yew et al., 1999). This was suggested to result from increased nNOS mRNA expression (Galimberti et al., 2005). nNOS immunoreactivity was also colocalized with advanced glycation end products (AGES) and neurofirillary tangles in some of the cortical neurons of the brains of ATD patients (Luth et al., 2005).

This study therefore emphasizes the importance of using wild type animal models in studying the changes in the cytoskeleton-associated *tau* protein phosphorylation. Regulation of nNOS-derived nitric oxide may be one target for pharmacological manipulation of *tau* phosphorylation, when the latter is associated with glutamate excitotoxicity. Given that the present study used only one dose of nNOS inhibitor, it may not suggest a definite cause-effect relationship. But the study clearly addressed, at this particular dose, the association, in CMS-exposed old animals, between glutamate metabolism and *tau* phosphorylation in the hippocampus. It also demonstrated a possible role of nNOS as part of the mechanisms underlying this association. It also demonstrated that these changes were shown at a dose that was previously reported not to induce learning deficit, (Meyer et al., 1998). However, at this dose level, the study could not show a statistically significant behavioral change in the sucrose preference that correlates with these neurobiological findings.

References

Anisman H, Matheson K. Stress, depression, and anhedonia: caveats concerning animal models. Neurosci Biobehav Rev 2005;29:525–46.

Avila J, Lucas JJ, Perez M, Hernandez F. Role of tau protein in both physiological and pathological conditions. Physiol Rev 2004;84:361–84.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

Brewer GJ. Age-related toxicity to lactate, glutamate, and beta-amyloid in cultured adult neurons. Neurobiol Aging 1998;19:561–8.

Buée-Scherrer V, Goedert M. Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases in intact cells. FEBS Lett 2002;515:151–4.

Chakraborty M, Lahiri P, Anderson GM, Chatterjee D. Use of high-performance liquid chromatography for assay of glutamic acid decarboxylase. Its limitation in use for post-mortem brain. J Chromatogr 1991;571:235–40.

Checkley S. The neuroendocrinology of depression and chronic stress. Br Med Bull 1996;52:597–617.

Colton CA, Brown CM, Czapiga M, Vitek MP. Apolipoprotein-E allele-specific regulation of nitric oxide production. Ann N Y Acad Sci 2002;962:212–25.

Dalla C, Antoniou K, Drossopoulou G, Xagoraris M, Kokras N, Sfikakis A, et al. Chronic mild stress impact: are females more vulnerable? Neuroscience 2005;135:703–14.

Echeverry MB, Guimaraes FS, Del Bel EA. Acute and delayed restraint stress-induced changes in nitric oxide producing neurons in limbic regions. Neuroscience 2004;125:981–93.

Ferrari E, Cravello L, Muzzoni B, Casarotti D, Paltro M, Solerte SB, et al. Age-related changes of the hypothalamic-pituitary-adrenal axis: pathophysiological correlates. Eur J Endocrinol 2001;144:319–29.

Firk C, Markus CR. Serotonin by stress interaction: a susceptibility factor for the development of depression? J Psychopharmacol 2007;21(5):538–44.

Galimberti D, Venturelli E, Gatti A, Lovati C, Fenoglio C, Mariani C, et al. Association of neuronal nitric oxide synthase C276T polymorphism with Alzheimer's disease. J Neurol 2005;252:985–6.

Goedert M, Hasegawa M, Jakes R, Lawler S, Cuenda A, Cohen P. Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases. FEBS Lett 1997;409:57–62.

Green KN, Billings LM, Roozendaal B, McGaugh JL, LaFerla FM. Glucocorticoids increase amyloid-beta and tau pathology in a mouse model of Alzheimer's disease. J Neurosci 2006;26:9047–56.

Grønli J, Fiske E, Murison R, Bjorvatn B, Sorensen E, Ursin R, et al. Extracellular levels of serotonin and GABA in the hippocampus after chronic mild stress in rats. A microdialysis study in an animal model of depression. Behav Brain Res 2007;181:42–51.

Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci U S A 1986;83:4913–7.

Gunawan S, Walton NY, Treiman DM. High-performance liquid chromatographic determination of selected amino acids in rat brain by precolumn derivatization with phenylisothiocyanate. J Chromatogr 1990;503:177–87.

Harkin AJ, Bruce KH, Craft B, Paul IA. Nitric oxide synthase inhibitors have antidepressant-like properties in mice. 1. Acute treatments are active in the forced swim test. Eur J Pharmacol 1999;372:207–13.

Herman JP, Larson BR. Differential regulation of forebrain glutamic acid decarboxylase mRNA expression by aging and stress. Brain Res 2001;912:60–6.

Herman JP, Larson BR, Speert DB, Seasholtz AF. Hypothalamo-pituitary-adrenocortical dysregulation in aging F344/Brown-Norway F1 hybrid rats. Neurobiol Aging 2001;22:323–32.

Hsu CC, Thomas C, Chen W, Davis KM, Foos T, Chen JL, et al. Role of synaptic vesicle proton gradient and protein phosphorylation on ATP-mediated activation of membrane-associated brain glutamate decarboxylase. J Biol Chem 1999;274:24366–71.

Jäsmä A, Backstrom A, Gustafsson E, Dehvari N, Hiller G, Cowburn RF, et al. Glutamate treatment and p25 transfection increase Cdk5 mediated tau phosphorylation in SH-SY5Y cells. Biochem Biophys Res Commun 2006;345:324–31.

Jayatissa MN, Bisgaard C, Tingström A, Papp M, Wiborg O. Hippocampal cytogenesis correlates to escitalopram-mediated recovery in a chronic mild stress rat model of depression. Neuropsychopharmacology 2006;31(11):2395–404.

Jayatissa MN, Bisgaard CF, West MJ, Wiborg O. The number of granule cells in rat hippocampus is reduced after chronic mild stress and re-established after chronic escitalopram treatment. Neuropharmacology 2008;54(3):530–41.

Jeong YH, Park CH, Yoo J, Shin KY, Ahn SM, Kim HS, et al. Chronic stress accelerates learning and memory impairments and increases amyloid deposition in APPV717I-CT100 transgenic mice, an Alzheimer's disease model. FASEB J 2006;20:729–31.

Joca SR, Guimaraes FS. Inhibition of neuronal nitric oxide synthase in the rat hippocampus induces antidepressant-like effects. Psychopharmacology (Berl) 2006;185:298–305.

Kannurpatti SS, Sanganahalli BG, Mishra S, Joshi PG, Joshi NB. Glutamate-induced differential mitochondrial response in young and adult rats. Neurochem Int 2004;44:361–9.

Keil U, Bonert A, Marques CA, Scherping I, Weyermann J, Strosznajder JB, et al. Amyloid beta-induced changes in nitric oxide production and mitochondrial activity lead to apoptosis. J Biol Chem 2004;279:50310–20.

Korneyev AY. Stress-induced tau phosphorylation in mouse strains with different brain Erk 1 + 2 immunoreactivity. Neurochem Res 1998;23:1539–43.

Lahiri DK, Chen D, Ge YW, Farlow M, Kotwal G, Kanthasamy A, et al. Does nitric oxide synthase contribute to the pathogenesis of Alzheimer's disease?: effects of beta-amyloid deposition on NOS in transgenic mouse brain with AD pathology. Ann N Y Acad Sci 2003;1010:639–42.

Luth HJ, Ogunlade V, Kuhla B, Kientsch-Engel R, Stahl P, Webster J, et al. Age- and stage-dependent accumulation of advanced glycation end products in intracellular deposits in normal and Alzheimer's disease brains. Cereb Cortex 2005;15:211–20.

McEwen BS. Plasticity of the hippocampus: adaptation to chronic stress and allostatic load. Ann N Y Acad Sci 2001;933:265–77.

McEwen BS. Glucocorticoids, depression, and mood disorders: structural remodeling in the brain. Metabolism 2005;54:20–3.

McEwen BS. Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol Rev 2007;87:873–904.

Meyer RC, Spangler EL, Patel N, London ED, Ingram DK. Impaired learning in rats in a 14-unit T-maze by 7-nitroindazole, a neuronal nitric oxide synthase inhibitor, is attenuated by the nitric oxide donor, molsidomine. Eur J Pharmacol 1998;341:17–22.

Moreno-Lopez B, Romero-Grimaldi C, Noval JA, Murillo-Carretero M, Matarredona ER, Estrada C. Nitric oxide is a physiological inhibitor of neurogenesis in the adult mouse subventricular zone and olfactory bulb. J Neurosci 2004;24:85–95.

Okawa Y, Ishiguro K, Fujita SC. Stress-induced hyperphosphorylation of tau in the mouse brain. FEBS Lett 2003;535:183–9.

Ownby RL, Crocco E, Acevedo A, John V, Loewenstein D. Depression and risk for Alzheimer disease: systematic review, meta-analysis, and metaregression analysis. Arch Gen Psychiatry 2006;63:530–8.

Patel JR, Brewer GJ. Age-related changes in neuronal glucose uptake in response to glutamate and beta-amyloid. J Neurosci Res 2003;72:527–36.

Paul IA, Skolnick P. Glutamate and depression: clinical and preclinical studies. Ann N Y Acad Sci 2003;1003:250–72.

Pietrzik C, Behl C. Concepts for the treatment of Alzheimer's disease: molecular mechanisms and clinical application. Int J Exp Pathol 2005;86:173–85.

Post RM. Transduction of psychosocial stress into the neurobiology of recurrent affective disorder. Am J Psychiatry 1992;149:999–1010.

Rameau GA, Tukey DS, Garcin-Hosfield ED, Titcombe RF, Misra C, Khatri L, et al. Biphasic coupling of neuronal nitric oxide synthase phosphorylation to the NMDA receptor regulates AMPA receptor trafficking and neuronal cell death. J Neurosci 2007;27:3445–55.

Ringheim GE, Szczepanik AM. Brain inflammation, cholesterol, and glutamate as interconnected participants in the pathology of Alzheimer's disease. Curr Pharm Des 2006;12:719–38.

Rissman RA, Lee KF, Vale W, Sawchenko PE. Corticotropin-releasing factor receptors differentially regulate stress-induced tau phosphorylation. J Neurosci 2007;27:6552–62.

Roberson ED, Scearce-Levie K, Palop JJ, Yan F, Cheng IH, Wu T, et al. Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. Science 2007;316:750–4.

Sapolsky RM. The possibility of neurotoxicity in the hippocampus in major depression: a primer on neuron death. Biol Psychiatry 2000;48:755–65.

Soghomonian JJ, Martin DL. Two isoforms of glutamate decarboxylase: why? Trends Pharmacol Sci 1998;19:500–5.

Sommer D, Coleman S, Swanson SA, Stemmer PM. Differential susceptibilities of serine/threonine phosphatases to oxidative and nitrosative stress. Arch Biochem Biophys 2002;404:271–8.

Tafet GE, Smolovich J. Psychoneuroendocrinological studies on chronic stress and depression. Ann N Y Acad Sci 2004;1032:276–8.

Thorns V, Hansen L, Masliah E. nNOS expressing neurons in the entorhinal cortex and hippocampus are affected in patients with Alzheimer's disease. Exp Neurol 1998;150:14–20.

Watts J, Fowler L, Whitton PS, Pearce B. Release of arginine, glutamate and glutamine in the hippocampus of freely moving rats: involvement of nitric oxide. Brain Res Bull 2005;65(6):521–8.

Willner P. Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. Neuropsychobiology 2005;52:90–110.

Willner P, Muscat R, Papp M. Chronic mild stress-induced anhedonia: a realistic animal model of depression. Neurosci Biobehav Rev 1992;16:525–34.

Wilson RS, Barnes LL, Mendes de Leon CF, Aggarwal NT, Schneider JS, Bach J, et al. Depressive symptoms, cognitive decline, and risk of AD in older persons. Neurology 2002;59:364–70.

Wragg RE, Jeste DV. Overview of depression and psychosis in Alzheimer's disease. Am J Psychiatry 1989;146:577–87.

Yew DT, Wong HW, Li WP, Lai HW, Yu WH. Nitric oxide synthase neurons in different areas of normal aged and Alzheimer's brains. Neuroscience 1999;89:675–86.

Yoshida H, Hastie CJ, McLauchlan H, Cohen P, Goedert M. Phosphorylation of microtubule-associated protein tau by isoforms of c-Jun N-terminal kinase (JNK). J Neurochem 2004;90:352–8.

Zarate Jr CA, Du J, Quiroz J, Gray NA, Denicoff KD, Singh J, et al. Regulation of cellular plasticity cascades in the pathophysiology and treatment of mood disorders: role of the glutamatergic system. Ann N Y Acad Sci 2003;1003:273–91.

Zhou QG, Hu Y, Hua Y, Hu M, Luo CX, Han X, et al. Neuronal nitric oxide synthase contributes to chronic stress-induced depression by suppressing hippocampal neurogenesis. J Neurochem. 2007;103(5):1843–54.

Zhu XJ, Hua Y, Jiang J, Zhou QG, Luo CX, Han X, et al. Neuronal nitric oxide synthase-derived nitric oxide inhibits neurogenesis in the adult dentate gyrus by down-regulating cyclic AMP response element binding protein phosphorylation. Neuroscience 2006;141:827–36.