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ScienceDirect
Neurochemistry International 52 (2008) 432–437
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

# Changes in regional long-term oxidative metabolism induced by partial serotonergic denervation and chronic variable stress in rat brain

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Received 2 March 2007; received in revised form 5 August 2007; accepted 7 August 2007
Available online 11 August 2007

**Abstract**

Stressful experiences and genetic predisposition have both independent and interactive contributions to the development of depression. The serotonergic system is involved in the development of depression, and administration of neurotoxins that specifically compromise its function leads to symptoms of affective disorders. In order to find out which brain regions are most affected by stress, partial serotonergic denervation and their combination, chronic variable stress (CVS) was applied for 3 week. Serotonergic denervation was elicited by parachloroampetamine (PCA, 2 mg/kg), and cytochrome oxidase histochemistry was used to characterize the long-term levels of neuronal oxidative energy metabolism. PCA pretreatment blocked the increase in oxidative activity in chronically stressed rats in medial preoptic area, cortical and medial amygdala. PCA raised oxidative activity compared to control animals in substantia nigra and ventrolateral division of laterodorsal thalamus. CVS reduced the oxidative activity induced by PCA in suprachiasmatic hypothalamus, anteroventral thalamus, hippocampal CA3 region and cortical amygdala. In the dorsal part of the anterior olfactory nucleus chronic stress blocked the decrease in oxidative activity evoked by PCA. Conclusively, partial serotonergic denervation with PCA and chronic variable stress both had independent effects on long-term energy metabolism in several rat brain structures, tending to increase it. However, partial serotonergic denervation by parachloroampetamine and chronic variable stress had in many brain regions an interactive effect on energy metabolism, each factor reducing the effect of the other, which could reflect the weakening of adaptive mechanisms.


*Keywords:* Chronic variable stress; Parachloroamphetamine; Serotonin; Oxidative energy metabolism; Cytochrome oxidase histochemistry; Depression; Animal models

## 1. Introduction

Depression is a disorder arising from strong negative environmental stressors (Brown, 1998; Pine et al., 2002; van Praag, 2005) and involves dysfunction in the monoamine systems (Chaouloff, 2000; Harro and Oreland, 2001). Stress-induced pattern of changes in hormonal and neuropeptide feedback systems is an important tool to adapt to changes in environment, but prolonged periods of stress can often have counter-adaptive effects on the behaviour (Akil, 2005; Korte et al., 2005; Leonard, 2005). In an attempt to mimic excessive human day-to-day stress, several animal models have been developed. Methods using multiple stressors – chronic variable stress or chronic mild stress – rely on the ability of a sequence of relatively mild stressors to produce behavioural changes that are reversible by antidepressant treatment (Katz, 1982; Willner, 2005).

Many key proteins of the serotonergic system in different brain regions have been implicated in depression both in animal models and in human disorder. A few diverse examples of implications of serotonin in depression are: changes in serotonin and its metabolite levels/turnover or release following chronic stress in different brain regions in animals (Mangiavacchi et al., 2001; Gamaro et al., 2003; Bekris et al., 2005); changes in serotonin receptor sensitivity in response to chronic stress (Leonard, 2005); therapeutic effect of antidepressant treatment by a cascade of events starting with increasing the amount of serotonin in the synapse and resulting in altered (auto)receptor sensitivity (Pineyro and Blier, 1999; Elhwuegi, 2004); depressogenic properties of acute dietary tryptophan (serotonin precursor) depletion (Bell et al., 2005); depressogenic effect of monoamine depletion by reserpine as an rodent

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doi:10.1016/j.neuint.2007.08.003

model of depression and in people taking reserpine as hypertension treatment (O'Neil and Moore, 2003); more frequent occurrence of less functional allelic variations of tryptophan hydroxylase 1 and 2 (enzymes synthesizing serotonin) genes among people with major depression and neurotic/anxious personality (Nash et al., 2005; Zhang et al., 2005). Conclusively, it is clear that the serotonergic system is malfunctioning in affective disorders and in animal models of depression, but there can be multiple simultaneous molecular mechanisms in a number of brain circuits that contribute to the depressive state.

One possible approach to elicit long-lasting serotonergic dysfunction is to apply neurochemically specific neurotoxins. Parachloroamphetamine (PCA) and other substituted amphetamines are potent neurotoxins with a multiphase, time dependent impact on neuronal functioning. The acute effect of PCA is a massive, dose dependent release of serotonin, resulting in a transient depletion followed within days by the onset of degeneration of axon terminals, especially in neocortex, striatum and thalamus, leaving the preterminal axons and cell bodies intact. PCA may have a region specific effect on serotonergic axon terminals depending on the origin, type and density of serotonergic fibres terminating in a given region—fine axons arising from the dorsal raphe nucleus being more vulnerable than "beaded" axons originating in median raphe (Mamounas and Molliver, 1988; Wilson et al., 1993). The abovementioned studies use destruction of the serotonergic system as a tool to study morphology and morphological changes, but such a near-total lesion is of limited value from behavioural viewpoint. It has been suggested that partial lesions with small toxin doses can be utilized to add ecological validity to animal experiments (Datla and Curzon, 1996). Our group has previously shown that small doses of PCA (2 mg/kg) that cause a restricted reduction of serotonin in frontal cortex, cerebral cortex, hippocampus, hypothalamus and cerebellum can induce behavioural alterations bearing similarities to the negative impact of chronic stress—increased anxiety and impulsivity (Harro et al., 2001; Harro, 2002; Haidkind et al., 2004). The effect of such small doses of PCA on regional serotonin levels 1 month after administration is relatively similar in brain regions receiving serotonergic input mainly from dorsal vs. median raphe (Harro et al., 2001).

A possibility to monitor long-term changes in brain in response to certain stimulation is to assess the changes in oxidative phosphorylation. Cytochrome c oxidase (CO, EC 1.9.3.1), an inner mitochondrial membrane protein, is the terminal complex of electron transport chain that catalyses the oxidation of the mobile electron carrier cytochrome c and reduction of oxygen to water (Brunori et al., 2005). In the processes of electron transport a proton gradient is generated, which is used to drive the oxidative phosphorylation of ADP to ATP by F1/Fo ATPase (Oster et al., 2000). The activity of neurons is almost entirely dependent on oxidative metabolism and changes in CO expression in mitochondria can be used as a marker of long-term metabolic activity. Strong excitatory input leading to high frequency of charging is associated with elevated $Na^+/K^+$ ATPase activity, which is proposed to be the main energy-consumer in neurons (Wong-Riley et al., 1998). CO histochemistry is a method that combines good anatomical resolution with a functional outcome—long-term metabolic activity of a given brain region.

It has been suggested by epidemiological research that the genetic makeup of an individual strongly interacts with stressful life situations (Kendler et al., 1995). Results demonstrating that lifetime stress interacts with the serotonin transporter promoter region polymorphism in causing depression have confirmed the notion of genetic susceptibility on molecular level (Caspi et al., 2003; Kendler et al., 2005; Mandelli et al., 2006). The natural variability of the capacity of serotonin system causes chronic stress to induce a mild to severe depression-like state in a number of rats, but some animals remain unaffected. Partial denervation of the serotonergic system with PCA allows us to render the animals more susceptible to environmental stressors and to control for the vulnerability/protective properties of a certain neurotransmitter system.

The aim of this study was to explore the impact of partial serotonergic denervation with parachloroamphetamine and chronic variable stress (CVS) on rat brain regional long-term oxidative metabolism, to reveal which brain regions are most affected by these factors.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats ($n = 29$, weighing 260–332 g at the beginning of the experiment, from Scanbur BK AB, Sweden) were housed four per cage in standard polypropylene cages in a light controlled room (12-h light/dark cycle; lights on at 8:30 a.m.) maintained at 22 °C. Food and water were available ad libitum. Animals were submitted to CVS at 3 months and sacrificed at 4 months of age, 4 days after the last stressful procedure. All experiments were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Ethics Committee of the University of Tartu. All efforts were made to minimize the number of animals used and their suffering.

### 2.2. PCA administration

Administration of the neurotoxin was carried out 1 week before the CVS regimen. PCA (Sigma) in the dose of 2 mg/kg (expressed as for hydrochloride) was dissolved in distilled water and injected in a volume of 1 ml/kg intraperitoneally. Control animals received a vehicle injection. In our previous experiments with a similar design, we have always observed a consistent 15–30% decrease of 5-HT and 5-HIAA levels with administration of 2 mg/kg of PCA (Harro et al., 2001; Haidkind et al., 2004; unpublished data).

### 2.3. Chronic variable stress

Rats belonging to the stress group were submitted to the CVS procedure as previously described (Harro et al., 2001) in a separate room during 21 days. Various stressors of different duration were applied one by one everyday, each one thrice altogether. The stressors, in the order of presentation, included: (1) movement restriction in a small cage (11 cm × 16 cm × 7 cm for 2 h), (2) cage tilt at 45° (for 24 h), (3) tail pinch with a clothes-pin placed 1 cm distal from the base of tail (5 min), (4) cold (4°) water and wet bedding (initially, 400 ml of water was poured on a rat, and the sawdust bedding was kept wet for the following 22 h), (5) forced swimming (5 min at room temperature), (6) strong illumination (900 lx) during the predicted dark phase (for 12 h) and (7) stroboscopic light (for 14 h, 10 Hz).

### 2.4. CO histochemistry and image analysis

Unanesthesised rats were decapitated, brains removed and immediately frozen on dry ice. Brains were stored at −80 °C until coronally sectioned (thickness 40 μm) in a cryostat microtome at −20 °C, slides with sectioned tissue were kept refrigerated at −80 °C until stained. The staining procedure used is based on the protocol described by Gonzalez-Lima and Cada (1998) with minor modifications. The 0.1 M $Na_2HPO_4/NaH_2PO_4$ buffer solution adjusted to pH of 7.4 was used. Automatic agitation was used with all the steps in the protocol. First the refrigerated sections were fixed for 5 min in 0.125% glutaraldehyde (v/v) solution in cold buffer (4 °C). Next the specimens were washed with four changes (5 min each) of 10% sucrose in the buffer solution at room temperature. To enhance staining intensity, the sections were pre-incubated, for 10 min with 0.0275% cobalt chloride (w/v) and 0.5% dimethyl sulfoxide (DMSO, v/v) in 0.05 M Tris buffer with 10% sucrose (w/v) adjusted to pH to 7.4 with approximately 0.1% HCl (v/v). The metal ions included in the previous step were removed by a 5 min wash with the buffer solution. Thereafter the sections were stained for one hour at room temperature in an incubation solution consisting of 0.05% DAB (3,3′-diaminobenzidine tetrahydrochloride, AppliChem), 0.0075% cytochrome c (Sigma, prepared using TCA), 5% sucrose, 0.002% catalase (Sigma) and 0.25% DMSO (v/v) in sodium phosphate buffer. To avoid non-specific auto-oxidation the reaction was conducted in dark. Finally, the reaction was stopped by introducing the slides for 30 min to 3.5% formalin (v/v) and 10% sucrose in phosphate buffer. The sections were dehydrated in ethanol, cleared in xylene and coverslipped. Regions of interest (ROIs) to be compared in data analysis were stained in the same incubation medium.

Stained and coverslipped sections were digitized and saved in a non-compressed format. Image analysis was conducted using the Image J 1.34 s freeware on the blue channel (resulting from a RGB split) of the background-subtracted image. Eighty-nine ROIs were detected from the stained images with the help of Paxinos and Watson (1986) rat brain atlas. Grayscale values were transformed to optical density values (OD) with the help of Kodak grayscale tablet with known grayscale and OD values. OD of any given ROI was sampled from three consecutive slices in one brain and averaged. The OD value was sampled randomly from right or left hemisphere of different animals, on the three consecutive slices of the brain the same hemisphere was sampled. ROIs were selected with a freehand selection tool covering the whole brain region, leaving out defected areas. Distances from bregma for the ROIs with significant group differences were: anterior olfactory nucleus, dorsal division +4.2 mm, medial preoptic area −0.3 mm, suprachiasmatic hypothalamus −1.3 mm, anteroventral thalamus −1.8 and −2.12 mm averaged, ventrolateral division of laterodorsal thalamus −1.8 and −2.12 mm averaged, anterior paraventricular nucleus −2.12 mm, hippocampal CA3 region −2.12 mm, cortical amygdala −1.8 and −2.12 and −2.3 mm averaged, medial amygdala −2.12 mm and substantia nigra −4.8 mm.

### 2.5. Data analysis

The obtained OD values were transformed to standard scores (T scores). All brain areas were treated as independent and a 2 × 2 (stress/no stress, PCA/vehicle) ANOVA with Fisher LSD post hoc test used for group comparisons. In case of the dorsal part of the anterior olfactory bulb, one animal with the oxidative activity value exceeding the mean by two standard deviations was excluded from analysis.

## 3. Results

PCA had a significant main effect on CO activity in hippocampal CA3 region ($F(1, 28) = 5.1$; $p < 0.05$), anteroventral thalamus ($F(1, 26) = 6.8$; $p < 0.05$), substantia nigra

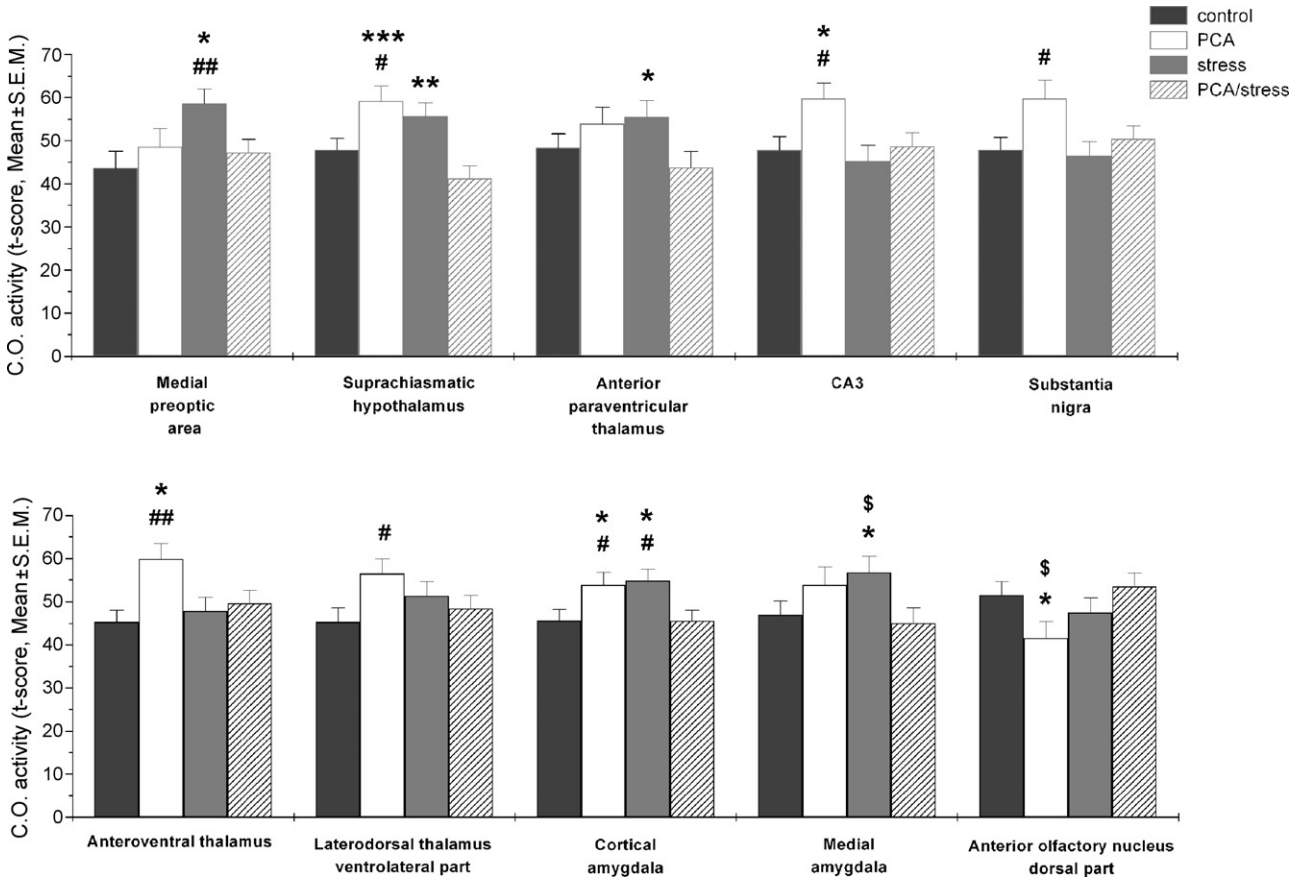


Fig. 1. OD values (T score, M ± S.E.M.) of cytochrome oxidase stained brain slices of chronic variable stress and parachloroamphetamine (PCA) treated rats. #vs. control, $p < 0.05$, ##vs. control, $p < 0.01$, $vs. control, $p = 0.06$, *vs. PCA/stress; $p < 0.05$, **vs. PCA/stress; $p < 0.01$, ***vs. PCA/stress, $p < 0.001$. Fisher LSD post hoc test.

($F(1, 27) = 5.3$; $p < 0.05$) ventral tegmental area ($F(1, 27) = 4.6$; $p < 0.05$), granular retrosplenial cortex ($F(1, 27) = 4.5$; $p < 0.05$) and cerebellar vermis ($F(1, 27) = 6.8$; $p < 0.05$). In all instances PCA caused an increase in oxidative activity.

There was a CVS main effect of borderline significance only in hippocampal CA3 ($F(1, 28) = 4.0$; $p = 0.057$), with stress decreasing oxidative activity. However, interaction between the serotonergic lesioning and chronic stress was the most prevalent finding in this study (Fig. 1). Regions of interaction include: medial preoptic area ($F(1, 24) = 4.9$; $p < 0.05$), cortical amygdala ($F(1, 29) = 9.9$; $p < 0.005$) and medial amygdala ($F(1, 26) = 6.3$; $p < 0.05$), where PCA blocked the stress induced increase in oxidative activity; suprachiasmatic hypothalamus ($F(1, 26) = 17.4$; $p < 0.0005$), ventrolateral division of laterodorsal thalamus ($F(1, 28) = 4.5$; $p < 0.05$), anteroventral thalamus, with borderline significance ($F(1, 26) = 4.1$; $p = 0.054$) and cortical amygdala, where CVS reduced the oxidative activity induced by PCA treatment; dorsal division of the anterior olfactory bulb ($F(1, 28) = 5.6$; $p < 0.05$), where stress blocked the decrease in oxidative activity evoked by PCA. In anterior paraventricular nucleus ($F(1, 27) = 5.7$; $p < 0.05$) stress and PCA combined yielded in a reduction of metabolic activity compared to stress group.

Dorsal raphe nucleus was the only region with a large synergistic increase in oxidative activity in response to CVS and PCA combined (control 48.2 vs. PCA/stress 56.5), but this effect was only seen at arithmetical level, the results of ANOVA were non-significant. Ventral tegmental area and retrosplenial cortex showed no significant post hoc comparisons, in case of cerebellar vermis the differences appeared only between the PCA treated and chronically stressed animals (data not presented).

## 4. Discussion

With the current work we have tried to improve the understanding of depression-related regional metabolic changes in the brain by using a stress-diathesis approach, combining the vulnerability caused by serotonin deficiency with chronic variable stress.

Partial serotonergic denervation by PCA significantly increased oxidative activity in a number of brain areas, such as suprachiasmatic nucleus, CA3, substantia nigra, anteroventral and laterodorsal ventrolateral thalamus and cortical amygdala. It should also be mentioned that in many other brain areas PCA treatment tended to increase oxidative metabolism, and it cannot be excluded that the small number of observations led to type 2 error in some cases. Only in the dorsal part of anterior olfactory bulb the effect of partial serotonergic lesion was to decrease CO activity. Altogether it appears that the long-term effect of PCA is to release the serotonergic target areas from inhibition, as revealed by increased oxidative activity in several brain regions. In several parts of the rodent thalamus administration of serotonin or its agonists have been found to cause inhibition of target neurons firing rate and dorsal raphe lesions resulted in enhanced excitation in target areas (Blasiak et al., 2006; Grasso et al., 2006). The excitation/inhibition evoked by serotonin seems, however, to be dependent on serotonin receptor types predominantly expressed in a given region (Di Mauro et al., 2003; Panksepp, 1998; Stanford et al., 2005). The regional alterations in energy metabolism could of course be secondary to serotonergic denervation and occur not in brain regions where the serotonergic changes are most prominent, but downstream.

In all brain regions where chronic stress had an impact on its own, the oxidative activity was increased. All these regions – medial preoptic area, cortical and medial amygdala – have been implicated in the regulation of hypothalamic-pituitary-adrenal axis through paraventricular hypothalamus (Herman and Cullinan, 1997; Dayas et al., 1999; Herman et al., 2003; Trneckova et al., 2006). From our data it could be concluded that although acute and chronic stress vary in several neurophysiological aspects, some regions controlling the HPA axis seem to be universally active in both circumstances.

In some cases it appeared that in animals with serotonergic dysfunction the regional energy metabolism was not reactive to stress. In medial preoptic area, cortical and medial amygdala PCA treatment inhibited the potential increase in oxidative metabolism induced by stress. Repeated social defeat induces a c-fos expression in both median and dorsal raphe nuclei (Chung et al., 1999) and there is ample evidence of the involvement of dorsal raphe in chronic stress (Leonard, 2005). As reaction to stress is adaptive in essence, it can be suggested that the activation of serotonergic nuclei can be of benefit to the animal (Panksepp, 1998). If the serotonergic system is defective, these adaptive reactions can be compromised, leaving the animal more vulnerable to stress. There is evidence that rats with defective serotonin system have altered adaptive behavioural responses—animals pretreated with the neurotoxin 5,7-dihydroxytryptamine did not display the typical freezing behaviour in a social defeat situation (Chung et al., 1999). The reactivity of medial amygdala to chronic stress in an interactive manner with the condition of the serotonergic system has been previously shown using social defeat paradigm, where c-fos expression in that region was more pronounced in stressed animals with serotonergic lesions (Chung et al., 1999). On the other hand, there were several brain regions where stress had an impact only on PCA treated animals, decreasing the oxidative activity to control levels. These regions include suprachiasmatic hypothalamus, anteroventral thalamus, hippocampal CA3 and cortical amygdala. Though stress alone had no effect on oxidative activity of these regions, it did block the increase in metabolic activity in serotonin deficient rats. As we proposed, serotonergic lesions could release the target areas from inhibition, resulting in elevated metabolic activity. In line with this suggestion, the activation of dorsal raphe by CRF during stress could increase its output and decrease metabolic activity in target areas.

Chronic stress or depression can alter the daily rhythms in rodents, for example chronic mild stress exerts disturbances of the diurnal and circadian rhythms of the locomotor activity and circadian body temperature changes in the rats (Ushijima et al.,

2006; Gorka et al., 1996). The relationship of circadian activity rhythms and chronic stress was further indirectly confirmed by this study. Stress had an impact on oxidative activity of suprachiasmatic nucleus (SCN), the internal pacemaker of the organism, but only in PCA pretreated animals. On the other hand, it is possible that instead non-photic stimulation (stressors and everyday handling) the photic stimuli (stroboscope and light during the habitual dark phase) have affected the function of SCN (Mistlberger, 2006). There is also evidence that rodent brain lesions caused by PCA or another serotonergic neurotoxin, 3,4-methylenedioxymethamphetamine (MDMA), modulate circadian rhythm changes (Penev et al., 1995; Morin and Allen, 2006) and that non-photic stimulation e.g. sleep deprivation by handling, which can be regarded as a stressor to an animal, increases serotonin release in SCN (Grossman et al., 2000). Our results suggest that from a long-term metabolic perspective, stressors could have more marked effect on suprachiasmatic function in animals with serotonergic deficit.

Anteroventral thalamus, the region in our study where stress reversed the effect of PCA, but had no effect on its own, has usually been linked to allocentric spatial learning and memory processes (van Groen et al., 2002). It is known, that chronic stress has a strong impact on spatial memory formation in Y-maze and Morris water maze (Kleen et al., 2006; Song et al., 2006). It could be hypothesized that the adverse impact of chronic stress on memory can partially be mediated by alterations in the anteroventral thalamus, at least among a specific subgroup of animals with inferior capacity of serotonergic system. The impact of PCA and stress on another region of the brain extensively associated with memory, hippocampal CA3, is identical to that of anteroventral thalamus—chronic stress having an impact only on the background of serotonergic dysfunction.

Chronic stress had an effect on dorsal division of the anterior olfactory bulb (AOD) CO activity, though again only in animals with serotonergic dysfunction which had their metabolic activity reduced. AOD is a part of the olfactory system with numerous connections to the limbic system, including the cortical amygdala (Song and Leonard, 2005). This system (olfactory bulbs and amygdaloid complex) has previously been described as a possible part of chronic stress neural substrate, having long-lasting c-fos activation in response to chronic stressors (Matsuda et al., 1996).

In conclusion, partial serotonergic denervation with parachloroamphetamine and chronic variable stress both had independent effects on cytochrome oxidase activity, an indicator of long-term energy metabolism, in several rat brain structures. Both manipulations tended to increase energy metabolism. The main finding of the study is, however, that partial serotonergic denervation by parachloroampetamine and chronic variable stress had an interactive impact on energy metabolism, the combination of manipulations resulting in oxidative energy metabolism comparable to control animals. The functional and behavioural significance of this interaction is yet to be established, but could be related to a reduction in the adaptive capacity of the brain.

## Acknowledgements

This work was supported by the Estonian Ministry of Education and Science project 0182643 and the EU Framework 6 Integrated Project NEWMOOD (LSHM-CT-2004-503474).

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