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ScienceDirect
NeuroToxicology 28 (2007) 175–181
NeuroToxicology

Brief communication

Differential effects of methylmercury intoxication in the rat's barrel field as evidenced by NADPH diaphorase histochemistry

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Received 12 January 2006; received in revised form 20 June 2006; accepted 22 June 2006
Available online 4 July 2006

Abstract

In the present study, we investigated the effects of mercury intoxication on the structure of the posteromedial barrel subfield (PMBSF) in the primary somatosensory cortex (SI) of adult rats, as revealed by histochemical reactivity to the enzyme NADPH diaphorase (NADPH-d). Enzymatic reactivity in the neuropil inside barrels was drastically reduced in intoxicated animals, suggesting that the synthesis and/or transport of the nitric oxide synthase enzyme can be altered in acute mercury intoxication. However, the cell bodies and dendrites of barrel neurons, also strongly reactive to the enzyme, were spared from the mercury's deleterious effects.

Keywords: Methylmercury; NADPH diaphorase; Somatosensory cortex; Barrel field; Optical density; Rat

1. Introduction

Mercury is a toxic waste product with many harmful effects in the nervous system (WHO, 1990). In the Amazon basin, for instance, mercury contamination is still a major health concern (Malm, 1998; Nriagu et al., 1992) due to its clandestine use in small-scale gold extraction (Malm, 1998) and the posterior discharge of contaminated rejects directly into rivers. Methylmercury (MeHg) is produced environmentally by biomethylation of the inorganic mercury present in aquatic sediments, leading to subsequent accumulation in the aquatic food chain (Clarkson et al., 2003).

Although the toxic effects of MeHg have long been known (Bakir et al., 1973; Davis et al., 1994; Hunter and Russell, 1954), they began to be more specifically investigated only after a serious contamination incident in Minamata Bay, Japan (Nagashima, 1997). Acute MeHg poisoning is known to cause a wide range of neurological abnormalities in adult humans, including progressive impairment of visual functions, cerebellar ataxia and deficits in motor performance (Davis et al., 1994; Dolbec et al., 2000; WHO, 1990). The pathological effects of MeHg in the brain have also been investigated in experimental animals (Himi et al., 1996; Kobayashi et al., 1998; Nagashima, 1997; O'Kusky, 1985) and are markedly similar to those described in humans (Castoldi et al., 2001; Chang, 1977; Nagashima, 1997).

Two major characteristics of primary cortical areas in the brain are their high metabolic activity and the presence of an intrinsic modular architecture organized in a columnar fashion (Mountcastle, 1997). The primary somatosensory cortex (SI) of some rodents, for instance, displays the conspicuous barrels in layer IV (Welker and Woolsey, 1974; Woolsey and Van der Loos, 1970). The most salient barrels are located at the posteromedial barrel subfield (PMBSF), and are organized in rows that replicate the arrangement of the large facial whiskers (Welker and Woolsey, 1974; Woolsey and Van der Loos, 1970). Besides their striking isomorphic relationship with the whiskers, the barrels also have a very high metabolic rate (Mayhew et al., 2000), which allows them to be revealed by the histochemical reactivity to enzymes such as cytochrome oxidase (Freire et al., 2004; Wallace, 1987), succinic dehydrogenase (Wallace, 1987), and

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0161-813X/$ – see front matter
doi:10.1016/j.neuro.2006.06.007

NADPH diaphorase (NADPH-d) (Franca and Volchan, 1995; Freire et al., 2004, 2005; Pereira et al., 2000).

The NADPH-d histochemistry is based on the presence within some certain neurons of an enzyme that can catalyzes the NADPH-dependent conversion of a soluble tetrazolium salt to an insoluble, visible reaction product, called formazan (see Scherer-Singler et al., 1983 for details).

In addition, this simple and robust technique reveals the localization in the brain of nitric oxide synthases (NOS), the rate-limiting enzymes involved with the production of nitric oxide (NO) in the nervous tissue (Dawson et al., 1991; Hope et al., 1991). NO is a highly diffusible gaseous molecule implicated with several important physiological and pathological roles in the nervous system (Contestabile, 2000). NOS has three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). As a matter of fact, other enzymes located in the brain parenchyma also have diaphorase activity. NOS are recognized as diaphorase enzymes and it has been shown that the use of aldehyde fixatives inhibits their activity while enhancing the staining of the nNOS-positive population (Buwalda et al., 1995). Thus, it is reasonable to suggest that the NADPH-d reactivity observed in the brain reflects specifically the NOS activity. In support to this claim, it has been demonstrated that NOS enzyme itself is widely resistant to aldehyde fixation (Buwalda et al., 1995).

Although several groups have examined the alterations induced by MeHg in the brain of experimental animals (Himi et al., 1996; Kobayashi et al., 1998; Nagashima et al., 1996; Oliveira et al., 1998), no one has yet investigated specifically the actions of MeHg in the somatosensory cortex. Since metabolic rate seems to be a critical factor explaining the tropism of MeHg for the nervous system (WHO, 1990), the rat's PMBSF is an obvious target due to its high oxygen utilization (Mayhew et al., 2000). Besides, the regular, unvarying structure of the rat's PMBSF is another reason we elected this region as a model to investigate both qualitatively and quantitatively the effects of MeHg in the brain.

2. Materials and methods

2.1. Animals, perfusion and histochemical procedures

Adult male Wistar rats ($n = 15$) weighting 230–250 g were used in the present study. The animals were kept in a controlled environment, with temperature around 26 °C, on a 12 h light–dark cycle, and with free access to food and water. All experimental procedures were in strict accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

The animals were divided into two groups. Group I animals ($n = 9$) were orally intoxicated with MeHg chloride, 4 mg/10 ml dissolved in a vehicle (ethyl alcohol 4%), at a rate of 4 mg/kg/day during 7 days (modified from Nagashima et al., 1996). Group II animals ($n = 6$) received only the vehicle. Twenty-four hours after the last MeHg administration, animals were deeply anaesthetized with a mixture of ketamine chloridrate and xylazine chloridrate (1.8 and 0.5 ml/kg, respectively, i.p.) and perfused transcardially with 0.9% heparinized-saline and 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were dissected, flattened between two glass slides, immersed in PB, and cut into 100 μm thick sections in a Vibratome (Pelco International, Series 1000). The flattening of the neocortex allowed us to obtain a complete visualization of the entire barrel field (Welker and Woolsey, 1974; Woolsey and Van der Loos, 1970).

The sections were then collected and washed three times in PB and reacted free-floating in a NADPH-d solution containing 0.6% malic acid, 0.03% nitroblue tetrazolium, 1% dimethylsulfoxide, 0.03% manganese chloride, 0.5% β-NADP and 1.5–3% Triton X-100 in 0.1 M Tris buffer (pH 8.0) (modified from Scherer-Singler et al., 1983). In the present study the incubation time was strictly controlled in both groups. The reaction's development was monitored with an optical microscope, being interrupted after 4 h of incubation by rinsing sections in Tris buffer (pH 8.0). Finally, all sections were dehydrated and coverslipped with Entellan (Merck).

2.2. Measurement of MeHg levels in the nervous tissue

After perfusion, four brains of each group were frozen to allow for the posterior measurement of total Hg content in the region enclosing the barrel field (Zilles and Wree, 1985), by acid digestion of samples, as follows: 0.2 g of each brain was incubated in a solution of 10 ml of nitric acid (HNO_3) and 1 ml of hydrochloric acid (HCl). After, the solution was warmed to 110 °C for 4 h in order to solubilize the tissue. The resultant solution was left to cool down and was fractionated into 200 μl aliquots. Afterwards, the samples were included in a recipient containing stannous chloride, which is used to reduce all Hg forms to the Hg metallic form (vapour). Quantitative analysis of total mercury content was performed by vapour atomic absorption spectrophotometry (CVAFS-2 Mercury Analyzer, Brooks Rand, USA). This method is remarkably more sensitive and has several other advantages compared to other techniques (Liang et al., 1994).

2.3. Qualitative and quantitative analysis

A complete reconstruction of the PMBSF for both groups was made using the software *Neurolucida* (MicroBrightField Inc., USA) (http://www.mbfbioscience.com/neurolucida). For each animal, the barrels were reconstructed from three 100 μm-thick tangential sections through layer IV and superimposed using the blood vessels as landmarks. Since the reactive neuropil forms barrels that span the PMBSF's middle layers vertically across 300 μm (Zilles and Wree, 1985), this procedure allowed for its complete reconstruction (see Freire et al., 2004, 2005 for details).

The area of individual substructures inside the barrel field was measured using the image processing software *Scion Image for Windows*, version Beta 4.0.2 (Scion Corporation, USA) (http://www.scioncorp.com) as follows: the outermost limits for the PMBSF were delineated in order to calculate the total PMBSF area. The area for each barrel was also measured. The septal areas were obtained by the difference

between the total area of the PMBSF and the sum of all barrels' areas.

NADPH-d activity inside PMBSF barrels of control and intoxicated animals was assessed by computer densitometry using digital images captured with a camera attached to the microscope. Average densitometric values were obtained with the help of the software *ImageJ* (http://rsb.info.nih.gov/ij/) from 10 individual barrels in tangential sections. To avoid variations in lighting, which could affect measurements, all images were acquired from one section where the complete PMBSF could be discerned. The measurements were obtained inside a 0.02 mm^2 square window positioned inside each barrel. To minimize the effects of within-group variability, we adopted a normalized scale based on the reactivity of the underlying white matter (averaged over measurements of 10 different sites using the same window). For each animal, the average optical density (OD) for the barrels was designated B, for the underlying white matter W and a contrast index was calculated according to the equation: $C = (B - W)/(B + W)$ (Picanço-Diniz et al., 2004).

The number of type I NADPH-d neurons located in the reconstructed sections of the PMBSF was counted and compared between groups. This approach enabled us to easily evaluate the distribution of the total number of NADPH-d type I neurons across the entire barrel field.

Thirty cells for each group were chosen across the PMBSF and reconstructed using a binocular light microscope (Nikon ADF-DX Optiphot-2, 60x-oil objective), equipped with a motorized stage and connected to a microcomputer running the *Neurolucida* morphometric program. The main criterion to select the cells to be reconstructed was the presence of a fairly complete dendritic arborization (cells whose dendrites seemed to be cut during sectioning were not included in the quantification). Dendritic field and cell body areas were measured (values expressed in μm^2) using the *Scion Image* software. Average values for all measurements were compared between groups using two-tailed Student's *t*-test. The criterion for statistical significance was preset at an alpha level of 0.05. Average values are expressed as mean ± S.E.M.

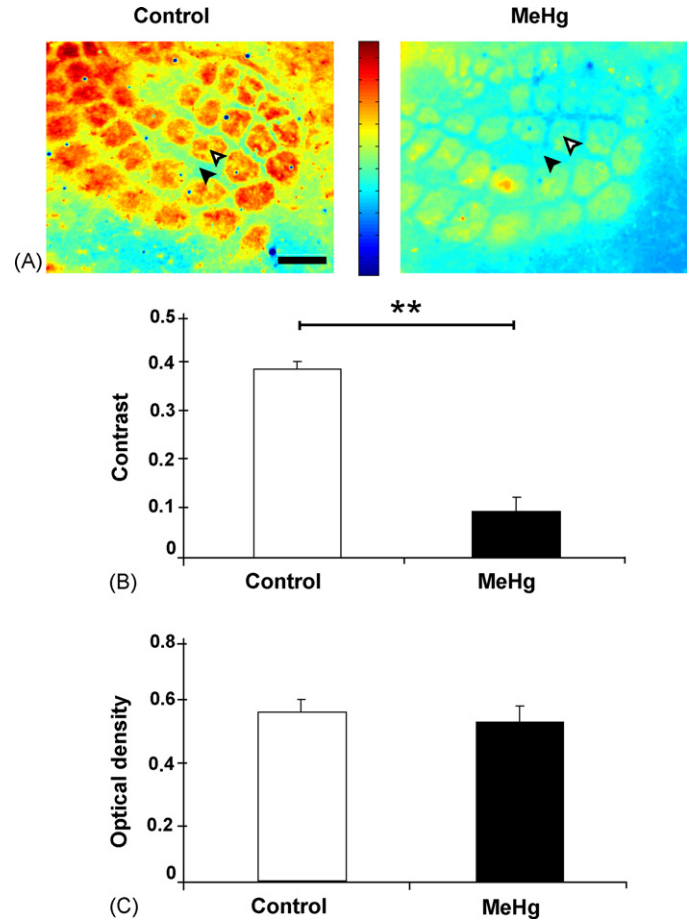

Fig. 1. Densitometric analysis of NADPH-d neuropil reactivity in the PMBSF. (A) Color-coded microphotographs showing the differences in PMBSF enzymatic reactivity, measured by the amount of transmitted light, between representative control (left) and intoxicated animals (right). The red color indicates a higher enzymatic reactivity (control), while green/blue colors indicate a progressively lower reactivity. (B) Average values of the neuropil contrast index ($C = (B - W)/(B + W)$, see text) ($^{**}p < 0.01$, Student's *t*-test). (C) Optical density values of white matter in both groups ($p > 0.05$, Student's *t*-test). Legends: C, contrast index; B, barrel optical density; W, white matter optical density. Arrowheads: white, example of barrels; black, septa. Scale bar in A: 300 μm.

3. Results

3.1. Levels of MeHg in the brain

The total Hg content in the brain of intoxicated animals was 7.15 ± 1.15 μg/g, a value significantly higher than the levels found in control animals (0.06 ± 0.01 μg/g).

Half of the animals intoxicated with MeHg ($n = 5$) had pronounced bristling of the back hair, a hallmark of mercury intoxication. No spasticity or hindlimb paralysis was observed, since these events occur only after more prolonged intoxication (Kobayashi et al., 1998).

3.2. General pattern of NADPH diaphorase reactivity and optical density on the PMBSF

In the control group, as expected, NADPH-d histochemistry revealed the complete pattern of the SI barrel field (Franca and Volchan, 1995; Freire et al., 2004), including the PMBSF (Fig. 1). The NADPH-d histochemistry is unevenly distributed across the PMBSF, being more reactive in the neuropil located inside barrels (Fig. 1, white arrowheads). The septa surrounding barrels are much less reactive (Fig. 1, black arrowheads) (Franca and Volchan, 1995).

The general structure and intrinsic organization of the barrel field was not altered by MeHg intoxication (Fig. 1). However, there was a dramatic decrease in NADPH-d reactivity inside the barrels of intoxicated animals (Fig. 1A), which was confirmed by densitometric analysis (Fig. 1B). The average OD contrast index, normalized to the white matter, was significantly lower for the barrels of intoxicated animals than control ones (intoxicated: 0.111 ± 0.032; control: 0.449 ± 0.017; $p < 0.01$) (Fig. 1B).

In order to establish a comparative analysis of tissues that have not been reacted at the same time and thus have experienced slightly different experimental conditions, we have adopted a normalized relative scale based on a choice of an

internal control measurement in each specimen. Thus, the values of the densitometric measurements in each PMBSF section were normalized for the optical density values measured in the underlying white matter. Since the white matter is similarly non-reactive to NADPH-d in both conditions, as demonstrated by optical density measurements of the white matter in both control and intoxicated groups (intoxicated: 0.569 ± 0.055; control: 0.597 ± 0.047; $p > 0.05$) (Fig. 1C), this procedure allowed the unbiased comparison of different animals and experimental groups.

3.3. PMBSF area and NADPH diaphorase cells' quantification

Measurements of PMBSF area were found to be similar in both groups (intoxicated: 3.36 ± 0.23 mm^2; control: 3.31 ± 0.24 mm^2) and were not different from previously published data (Freire et al., 2004) (Fig. 2A). In addition, the average area occupied by either barrels or septa did not differ when comparing intoxicated (septa: 1.64 ± 0.11 mm^2; barrels: 1.67 ± 0.13 mm^2) and control animals (septa: 1.64 ± 0.13 mm^2; barrels: 1.72 ± 0.12 mm^2) (Fig. 2B).

Even though there is a great diversity in both cell body and dendritic field sizes of NADPH-d neurons in the PMBSF (Franca and Volchan, 1995; Freire et al., 2004) (Fig. 3A), statistical analysis did not reveal any significant differences in these parameters when comparing the two groups (cell body area - intoxicated: 197.7 ± 3.49 μm^2, control: 199.5 ± 4.12 μm^2; $p > 0.05$; dendritic field area - intoxicated: $18.35 \pm 0.68 \times 10^3$ μm^2, control: $19.17 \pm 0.45 \times 10^3$ μm^2; $p > 0.05$) (Fig. 3B and C). These results are similar to those obtained in the visual cortex of the cat (Oliveira et al., 1998), where the morphology of NADPH-d neurons was not affected by mercury intoxication.

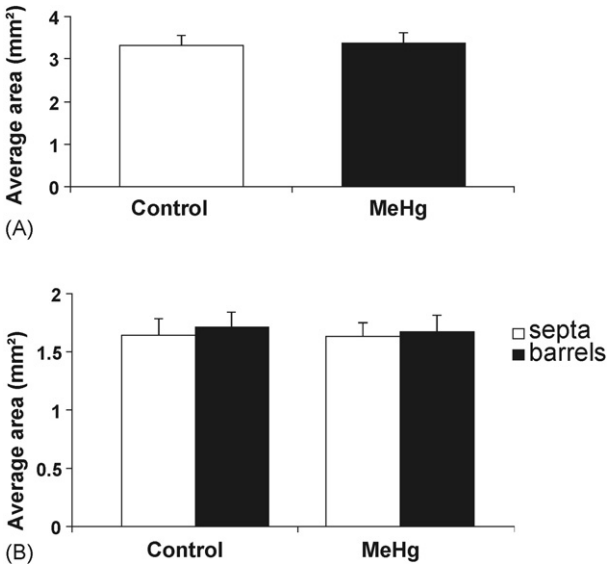

Fig. 2. Effects of MeHg intoxication on areal measurements in the PMBSF. Both total PMBSF area (A) and the areas of individual PMBSF's sub-compartments (barrels and septa) (B) were similar in control and intoxicated animals ($p > 0.05$, Student's *t*-test).

The average number of NADPH-d neurons in the PMBSF also did not differ between the two groups (intoxicated: 68 ± 2.63 neurons; control: 71 ± 3.27 neurons; $p > 0.05$) (Fig. 3D), suggesting that NADPH-d neurons in the rat's PMBSF are spared during MeHg intoxication.

We were also able to identify type II neurons throughout SI, but since NADPH-d histochemistry fails to reveal the dendritic tree of these cells, which are also highly susceptible to tissue fixation (Freire et al., 2004), they were not evaluated in the present study.

4. Discussion

In the present work, we evaluated the effects of MeHg intoxication on the neuronal reactivity of the enzyme NADPH-d in rat's PMBSF. Our main results were two-fold. First, NADPH-d neurons were selectively resistant to the harmful action of the metal, as revealed by quantitative morphometric analysis. Second, the neuropil reactivity was strikingly decreased in the barrel field after acute MeHg intoxication. The significance of these findings will be discussed below.

NADPH-d neurons located in the rat's barrel field did not seem to be damaged by MeHg intoxication. This finding is in agreement with previous studies which suggest a selective resistance of these neurons to a wide range of insults, including traumatic injury, malnourishment, ischemia and neurodegenerative disorders (Boegman and Parent, 1988; Ferrante et al., 1985; Koh et al., 1986; Oliveira et al., 1998; Picanço-Diniz et al., 1998; Thomas and Pearse, 1964). Even though the actual mechanisms behind this resistance are unclear (Ferrante et al., 1985), it is well documented that NO can act as a protective factor following injury to the nervous system (Lipton et al., 1993; Rauhala et al., 2005). For instance, cerebellar Purkinje cells in the mouse, otherwise non-reactive for NADPH-d/NOS, are able to synthesize NO after MeHg intoxication (Himi et al., 1996).

It is known that NADPH-d neurons synthesize GABA in the cerebral cortex (Valtschanoff et al., 1993), and their unusual response to injuries may be a characteristic of inhibitory neurons. Inhibitory neurons play a critical role in regulating cerebral excitability (Somogyi et al., 1998; Tecoma and Choi, 1989), despite their small number (about 20% of the total neuronal population of the cerebral cortex). Alternatively, the relative invulnerability of NADPH-d neurons to injury could reflect an unusual aspect of their metabolism or even a protective action of NO.

Another crucial finding was the decrease in NADPH-d reactivity in the PMBSF neuropil of intoxicated animals. A similar result was previously reported for the cat's visual system (Oliveira et al., 1998). The enzymatic product found within the barrels is diffuse and is probably located inside presynaptic terminals. This hypothesis is supported by findings by Aoki et al. (1993) in layer IV of the monkey's visual cortex, where there is also a high concentration of dispersed NOS, but no obvious cellular profiles. These authors have demonstrated that NOS immunoreactivity is contained in presynaptic terminals (Aoki et al., 1993). The decreased NADPH-d

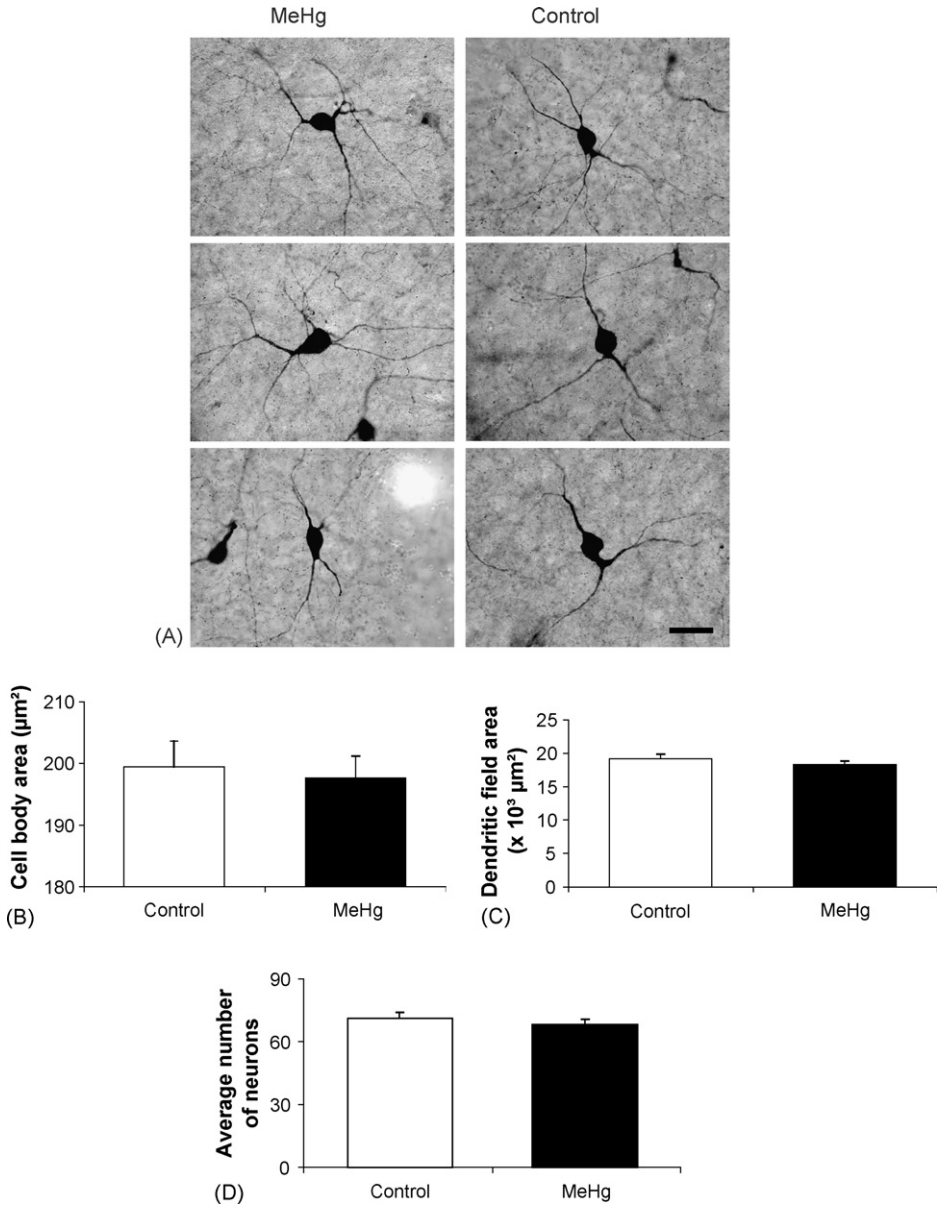

Fig. 3. NADPH diaphorase neurons are spared from MeHg intoxication. (A) A qualitative evaluation did not reveal any significant difference between both groups of cells, what it was confirmed by quantitative assessment of parameters such as cell body (B) and dendritic field (C) area ($p > 0.05$, Student's *t*-test). In addition, the average number of neurons in the PMBSF is not significantly different between both groups (D) ($p > 0.05$, Student's *t*-test). Scale bar in A: 30 μm.

reactivity inside contaminated barrels could result from physical damage to axon terminals by MeHg and/or alterations in the transport of NADPH-d/NOS enzymes from the cell body to axon terminals, as evidenced by MeHg effects on protein synthesis, and transport (Clarkson, 1997; Mottet et al., 1997), and microtubule integrity (WHO, 1990). The weak NADPH-d reactivity observed could also indicate an overall metabolic effect, which could have caused the vasodilatation/vasoconstriction in barrel field or even impaired the neurotransmitter action of NO.

It seems equally conceivable that the decreased neuropil reactivity to NADPH-d may also be related to astrocyte dysfunction. MeHg preferentially accumulates in astrocytes and, by potently and specifically inhibiting glutamate uptake in these cells, it could lead to an harmfully elevated concentration of excitatory amino acids in the extracellular medium (Aschner et al., 2000).

The barrel field morphology was not modified by MeHg action. Probably eventual alterations become apparent only after a more severe or chronic intoxication, since it has been reported that clinical symptoms due to MeHg poisoning in both humans and experimental animals appear only after a more prolonged period of intoxication (Davis et al., 1994; Dolbec et al., 2000; WHO, 1990).

Because of their conspicuous organization and high metabolic activity, the rodent's barrel field constitutes a suitable model for studies of MeHg intoxication. Further investigations are necessary, however, for a better under-

standing of the cellular mechanisms safeguarding NADPH-d neurons against damage by toxic injury.

Acknowledgements

Study supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Programa de Apoio a Núcleos de Excelência (PRONEX-MCT) and Financiadora de Estudos e Projetos (FINEP), Brazil. The authors wish to thank Mr. Joanilson Guimarães for help in some experiments.

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