

Full Research Paper

Inhibition of Acetylcholinesterase in Different Structures of the Rat Brain Following Soman Intoxication Pretreated with Huperzine A

Jiri Bajgar^{1,*}, **Petr Hajek**^{2,†}, **Jana Karasova**^{1,‡}, **Dasa Slizova**², **Otakar Krs**², **Kamil Kuca**^{1,3}, **Daniel Jun**^{1,3}, **Josef Fusek**¹ and **Lukas Capek**⁴

1 Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic; †E-mail: karasova@pmfhk.cz

2 Department of Anatomy, Medical Faculty of Charles University, Hradec Kralove, Czech Republic; †E-mail: hajekp@lfhk.cuni.cz

3 Center of Advanced Studies, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic

4 Department of Engineering Mechanics, Faculty of Mechanical Engineering, Technical University of Liberec, Liberec, Czech Republic

* Author to whom correspondence should be addressed; E-mail: bajgar@pmfhk.cz;

Tel: +420 973 251 507; Fax: +420 495 518 094;

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Abstract: Acetylcholinesterase (AChE) activities in different brain parts were determined quantitatively in rats treated with huperzine A, soman, and huperzine A followed by soman, using histochemical and biochemical methods. Following soman intoxication (1.2 x LD₅₀, i.m.), AChE activity was decreased to 30-80% of control values depending on the brain structure. The most sensitive area was the frontal cortex and the most relatively resistant was ncl. ruber. Huperzine A treatment only caused a change in AChE activity varying from 70 to 100 % of control values. In rats pretreated with huperzine A and intoxicated with soman, AChE activity was significantly higher than that observed after soman. In these animals, survival of rats pretreated with huperzine was observed while the mortality of untreated animals was near to 80 %. The results suggest that huperzine A is good candidate for further study for clinical use as a prophylactic drug against nerve agent poisoning.

Keywords: acetylcholinesterase, rat brain parts, soman, Huperzine A, pretreatment

1. Introduction

Nerve agents belong to the most toxic organophosphorus cholinesterase inhibitors. They can be used as chemical warfare agents and, they can be (and have been) misused by terrorists. The countermeasures against their effect (i.e. prophylaxis and treatment) are of high importance [1-4]. Protection against the inhibition of acetylcholinesterase (AChE, EC 3.1.1.7) using reversible cholinesterase inhibitors is the most common approach to pharmacological prophylaxis. The administration of present antidotes or scavengers such as cholinesterase preparations to prevent the effects of OP is also possible [1,5,6]. As for as protection against cholinesterase inhibition, pyridostigmine seems to be a most common prophylactic drug. However, its low central effects and relative high toxicity are disadvantages of its use. Therefore a search for new prophylactics was performed.

Huperzine A is reversible and selective AChE inhibitor compared to BuChE and is a more potent inhibitor of AChE than pyridostigmine. The review of Jiang *et al.* [7] represents a comprehensive documentation of the progress in the studies on huperzine A covering the period 1999-2002. It appears to be a selective inhibitor of red cell AchE, while pyridostigmine or physostigmine additionally inhibit plasmatic butyrylcholinesterase (BuChE, EC 3.1.1.8) in guinea-pigs [8,9]. Since pyridostigmine does not penetrate into the brain, it does not afford protection against seizures and subsequent neuropathology induced by an OP agent such as soman. Besides, huperzine has also been successfully tested for pretreatment of OP poisoning [10,11].

Huperzine A was tested for its prophylactic potential [12-17] and by means of a dynamically working *in vitro* model using AChE from immobilized human erythrocytes. The model predicts that inhibitors with a faster dissociation constant such as huperzine may be superior in case of very effective poisons such as soman [18]. However, there is a lack of information dealing with selective effect of huperzine especially on the AChE in the brain structures. It is known that the brain is well-organized and complex organ containing different levels of neuromediators and relevant enzymes in various structures of the brain. AChE activity varies a minimum 14-fold between striatum and cerebellum [19]. In prophylactic studies, AChE activity was determined in the whole brain homogenate. Some our results showed differential AChE inhibition in various brain regions, ranging from 15 to 86% following intoxication with soman [20]. Thus, the activity determined in the whole brain homogenate can be considered as a "mean" of the activities in different brain structures. It is difficult to correlate biochemical results which are not defined structurally as those in histochemical studies. However, this approach (comparison of these results) was used [20] and showed that it is possible and it would be interesting approach allowing more detailed explanation of action of nerve agents as well as their therapeutic influencing. Therefore (on the basis of above mentioned studies), prophylactic effects of huperzine A against soman using determination of AChE activity (histochemical and biochemical) in selected rat brain structures was studied.

2. Results and Discussion

2.1 Mortality

Following soman treatment, five from six animals died within 30 min after soman poisoning. Typical symptoms of poisoning – salivation, disturbed ventilation and convulsions – were observed in all animals in this group. After administration of huperzine A, fine tremor was observed occurring 10-30 min post administration, as well as in a group of animals pretreated with huperzine A and intoxicated with soman. All animals in these two groups showed practically unchanged ventilation and all animals survived.

2.2 Biochemical

The data on AChE activity in the brain areas are presented in Table 1. AChE activity varied from high (F.ret, Th, Hipp) to low (FC, NR) values.

Table 1. Histochemical (H) and biochemical (B) results of AChE activities following Huperzine A and soman treatment.

Group	Area	CONTROL		SOMAN		HUPERZINE		HUPERZINE+SOMAN	
		H	B	H	B	H	B	H	B
FC	a	181.1	253.0±13.3	53.4	70.1±10.5	125.6	190.2±12.1	83.8	121.4±12.4
	%	100	100	29.V	27.7 ^c	69.3	75.2 ^{a,d}	46.3	48.0 ^{b,c,d}
Hipp	a	221.45	301.6±15.3	89.5	135.5±14.7	165.9	241.3±14.6	112.5	158.2±13.8
	%	100	100	40.4	44.9	74.9	80.0 ^{a,d}	50.8	52.5 ^{b,d}
HTh	a	203.4	262.0±14.3	66.8	92.7±13.8	184.2	249.1±14.2	154.7	209.6±14.3
	%	100	100	32.8	35.4 ^c	90.6	95.0 ^d	76.0	80.0 ^{b,c,d}
NR	a	187.9	251.3±15.3	141.05	198.3±14.3	198.35	261.4±13.9	176.4	246.0±14.1
	%	100	100	75.1	78.9 ^c	105.6	104.0	93.9	97.9 ^c
F.ret	a	226.9	385.0±13.3	63.7	101.5±12.3	176.5	269.5±12.9	147.4	261.8±13.5
	%	100	100	28.1	26.4 ^c	77.8	70.0 ^a	65.0	68.0 ^{b,c}
DSep	a	192.7	251.2±12.3	57.7	89.6±10.8	152.3	213.5±13.0	154.7	205.8±13.1
	%	100	100	29.IX	35.7 ^c	79.0	85.0 ^a	80.3	81.9 ^{b,c}
Th	a	223.9	325.3±14.0	74.1	114.8±13.7	214.1	322.0±13.5	172.6	253.2±14.2
	%	100	100	33.1	35.3 ^c	95.6	99.0 ^d	77.1	77.8 ^{b,c,d}

For biochemical examinations, the results are means with their standard errors (n=6) (AChE activity in $\mu\text{mol}/60 \text{ min}/\text{kg}$) and percent activity expression (%).

For histochemical examinations, the results are means (AChE activity in pixel density, n=3) and percent activity expression (%).

BIOCHEMICAL RESULTS: All control values are significantly different from group soman (all parts) ($p < 0.05$). The letters indicate significant difference for other experimental groups: (NR – nucleus ruber from amygdala, FC – frontal cortex, DSep – dorsal septum, HTh – hypothalamus, Hipp – hippocampus, Th – thalamus, F.ret – pontomedullar area, mostly reticular formation containing nucleus gigantocellularis and others).

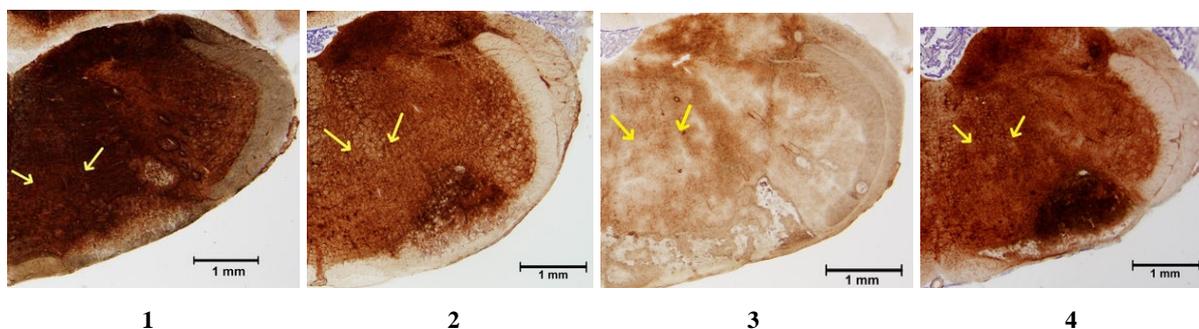
Administration of soman caused strong inhibition of AChE in all areas studied. Percentual inhibition was highest in F.ret and FC, which had about 30 % of the control activity. For huperzine treatment, the highest AChE inhibition was observed in F.ret and FC areas (about 70-75 % of controls). Lower inhibition of AChE observed in the rest brain structures and in NR, AChE inhibition was not detected.

Following pretreatment with huperzine and soman intoxication (HUPERZINE+SOMAN group), AChE activity in all brain parts examined was lower in comparison with huperzine treatment only (HUPERZINE group). However, the activities were higher in all brain parts compared with soman intoxication only (SOMAN group), and they were statistically significant for NR, F.ret, and DSep, respectively.

2.3 Histochemical

The histochemical results showed a similar picture to that of the biochemical examination (Table 1). The area most sensitive to soman was FC and Dsep (about 30 % of control values), and the most resistant was NR (75 %). The other soman treated brain regions had about 30-40 % of the control values. Histochemical inhibition values following administration of huperzine only matched most closely in the FC. The structures most resistant to huperzine seems to be NR. Histochemical pictures of three areas, the relatively sensitive FC, and F.ret and Hipp are shown in Figures 1-3. The quantitative evaluation following intoxication with soman pretreated with huperzine is also shown also in Figures 1-3, where it can be seen that AChE inhibition in all parts has had relatively same tendency as observed for biochemical examinations. According to biochemical results, AChE inhibitions following huperzine administration (HUPERZINE group) and soman intoxication pretreated with huperzine (HUPERZINE+SOMAN group) are very similar. However, after quantitative histochemical evaluation, the activities are shifted to lower densities and frequency of pixels is different.

Figure 1. Top: Microphotography of 20 μm sections of the rat brain (reticular formation, ncl. gigantocellularis, position is marked by arrow) following huperzine, soman and huperzine followed by soman administration. 1 - control, 2 - Huperzine A, 3 – soman, 4 – Huperzine A + soman. Magnification: 40x, Staining: AChE [22].



Bottom: Quantitative evaluation of histochemical data: Density curves of microphotographs.

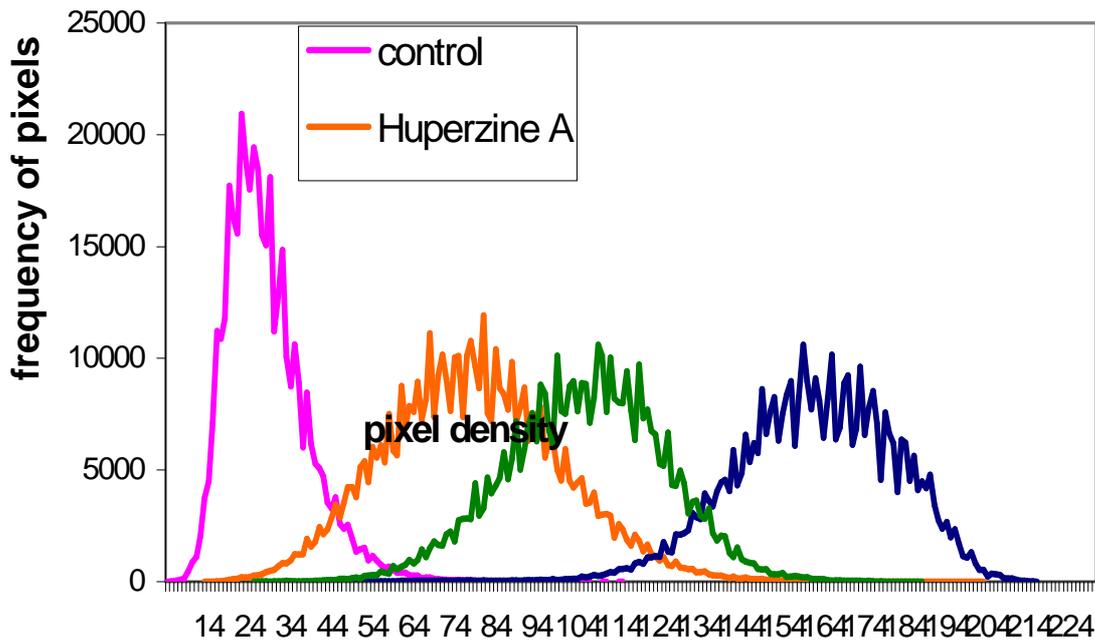
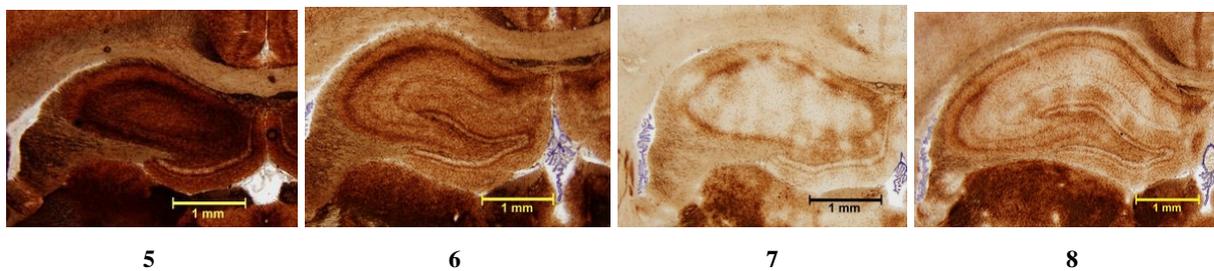


Figure 2. Top: Microphotography of 20 μm sections of the rat brain (hippocampus) following huperzine, soman and huperzine followed by soman administration. 5 - control, 6 - Huperzine A, 7 - soman, 8 - Huperzine A + soman. Magnification: 40x, Staining: AChE [22].



Bottom: Quantitative evaluation of histochemical data: Density curves of microphotographs

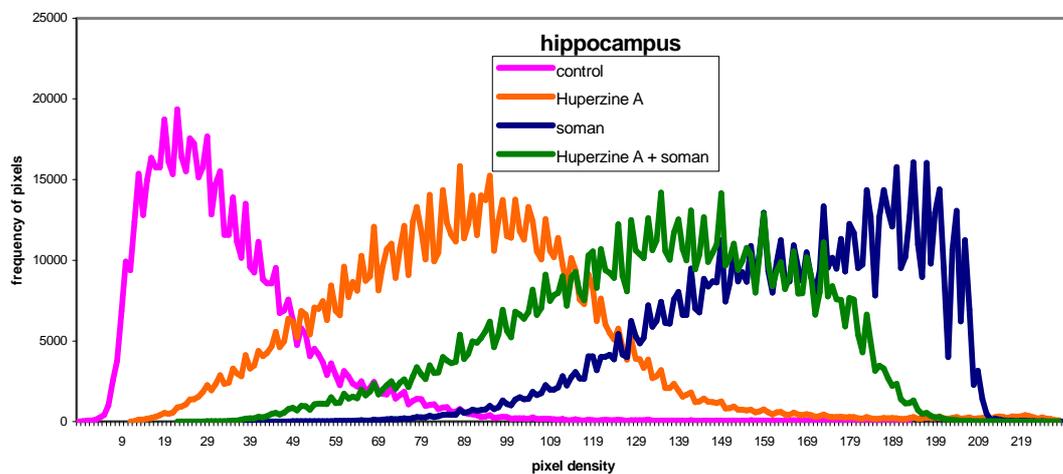
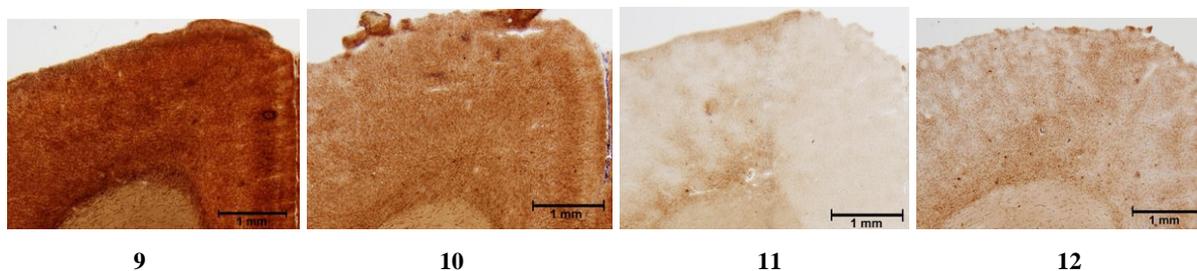
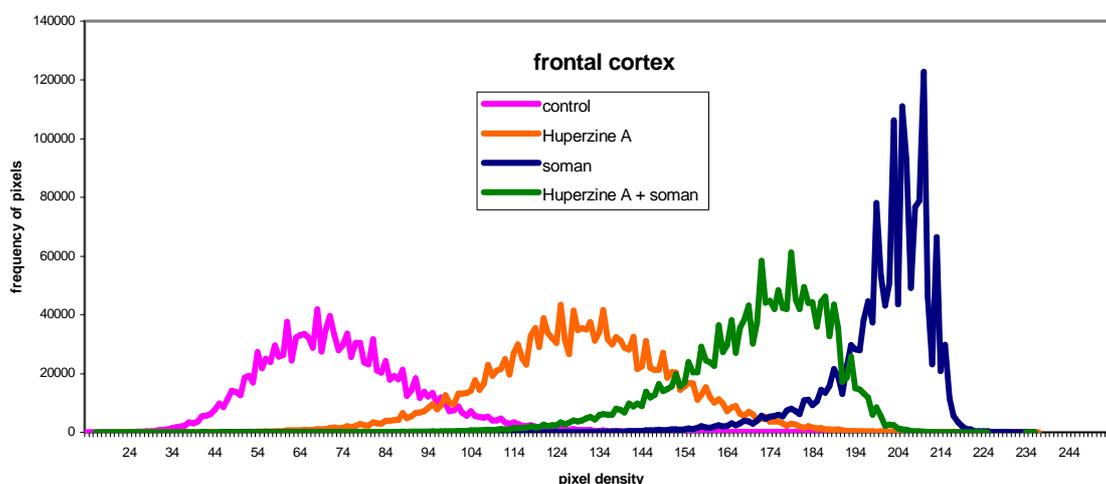


Figure 3. Top: Microphotography of 20 μm sections of the rat brain (frontal cortex) following huperzine, soman and huperzine followed by soman administration. 9 – control, 10 - Huperzine A, 11 – soman, 12 – Huperzine A + soman. Magnification: 40x, Staining: AChE [22].



Bottom: Quantitative evaluation of histochemical data: Density curves of microphotographs.



Correlation between results obtained using histochemical and biochemical methods

The comparison of percentage of residual AChE activity after administration of soman, huperzine and huperzine followed by soman in 7 brain regions (Figure 4) shows a good correlation between results obtained by histochemical and biochemical methods of AChE detection.

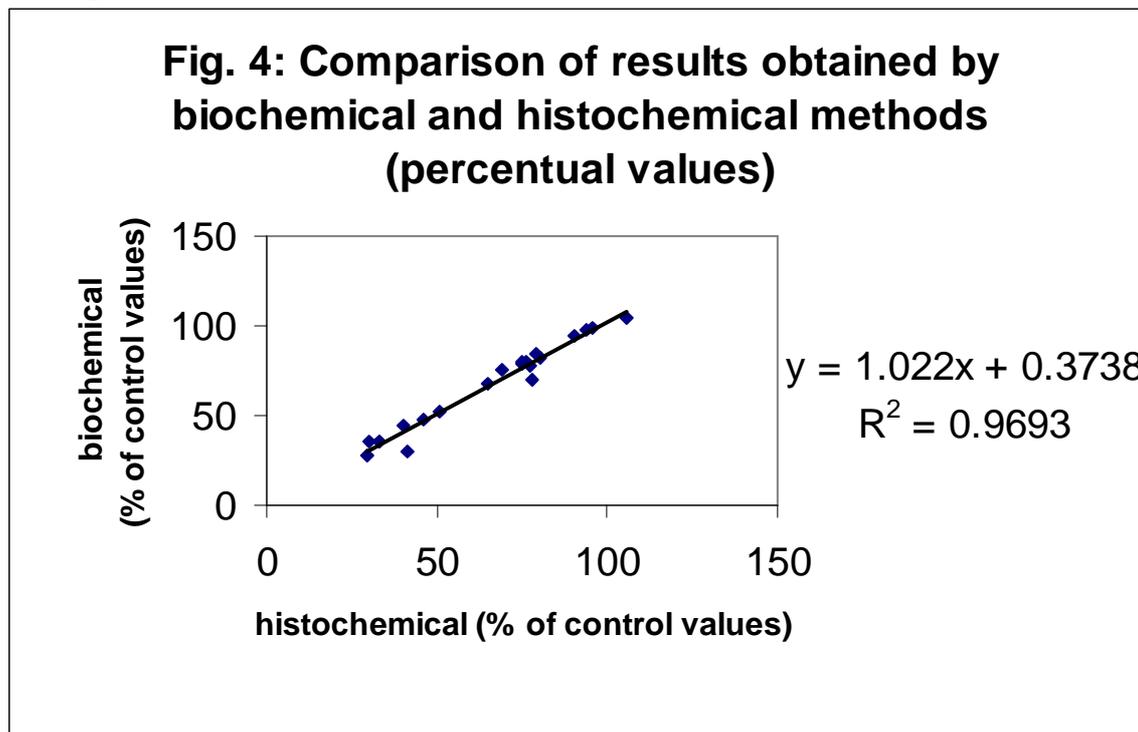
Discussion

Huperzine A is considered and studied with respect to prophylaxis in vitro and in vivo [11,14,16]. In general, reversible inhibitors including huperzine protect organism from the acute toxicity of lethal doses of the nerve agents such as sarin or soman [1,5,10,11,13] as it was also demonstrated in our experiments.

At the light of its particular inhibitory affects on the brain AChE activity, huperzine, compared to the other drugs, seems to be the optimal candidate to be used as pretreatment against OP poisoning [8,9] as demonstrated in guinea-pigs. In preventing the lethality of nerve agents, huperzine was effective compared with other reversible cholinesterase inhibitors (Tacrine, donepezil, rivastigmine,

etc.) [17]. The protective effects against soman toxicity of huperzine were demonstrated also in primates [10].

Figure 4. Comparison of results obtained by histochemical and biochemical methods (percentual values) following administration of soman, huperzine and soman pretreated with huperzine.



The results of Grunwald *et al.* [12] suggest that the protection of mice from soman poisoning by huperzine was achieved by temporary sequestering the active site region of the physiologically important AChE. In this connection, it is of interest that the main cholinergic pathways in the brain cholinergic system are represented i.a by septal nuclei, thalamus, cortex and hippocampus [26]. Some of these structures were studied in our paper and their influencing either by soman or huperzine was different. The not-uniform AChE inhibition in the different brain structures following untreated intoxication with nerve agents in rats and guinea-pigs [27,28] was demonstrated. This non-uniform inhibition suggests that some brain structures more sensitive to huperzine or OP than others can be functionally important for toxic/prophylactic action. The pontomedullar area seems to be the important structure because of its role in the respiratory control through the regulatory centers for respiration [29] in this brain part. Respiration is under cholinergic innervation [29,30]. The depression of central respiratory control centers in the pontomedullar area is considered as a primary event leading to death [31-33]. The highest AChE inhibition in this structure either by soman or huperzine *in vivo* supports a hypothesis that AChE inhibition here could be a cause of an interruption of cholinergic pathways controlling ventilation function. This not-uniformity can be explained by fine differences of huperzine action not only on the enzyme (AChE) activity but also by its different action on a structural level. Nucleus gigantocellularis contained in pontomedullar area and AChE here could be a key target for a survival or death of animals intoxicated with soman. On the other hand, AChE activity in NR was relatively unchanged in groups pretreated with huperzine only and huperzine followed by soman

intoxication. This resistancy supports an idea about different penetration of drugs/poisons into various brain areas.

These results suggest that huperzine is an important drug employed not only in the treatment of Alzheimer disease [26] but it is good candidate for further study for clinical use as prophylaxis against nerve agents. Its generally known effect – protection of AChE against its inhibition by soman – would have different importance for various brain structures. Our results underline the importance of pontomedullar brain area in this connection.

3. Experimental Section

3.1 Animals

Female Wistar rats (VELAZ Prague), weighing 200-220 g, were used in this study. The animals were divided into groups of three (histochemical) and six (biochemical) animals each. Rats were housed in the Central Vivarium of the Faculty of Military Health Sciences under veterinary control. All the experiments were performed under permission and supervision of the Ethics Committee of the Faculty of Military Health Sciences, Hradec Kralove (permission No 153/06) according to § 17 of the Czech law No 207/2004, permission of responsible person 0001/94 – M 699.

3.2 Chemicals

Soman was obtained from the Military Technical Institute of Protection (Brno, Czech Republic). It was of minimally 99% purity and stored in glass ampullas (1 mL). The solutions of the agents for experiments were prepared before use. Huperzine A was obtained from Sigma Aldrich GmbH (Division of Czech Republic) as well as other chemicals of analytical purity.

3.3 Intoxication and pretreatment

CONTROL GROUP: The animals were injected with saline i.p. and 30 min later, they were injected once again with saline i.m. (0.1 mL/kg). The decapitation and brain removal (sampling) was realized 30 min after the last saline injection.

SOMAN GROUP: The animals were injected with saline i.p. and 30 min later, soman was administered i.m. in a dose of 1.2 x LD₅₀, i.e. 84.0 µg/kg; 30 min after the injection/at the time of death, the animals were decapitated, the brain was removed and used for histochemical or biochemical examinations.

HUPERZINE GROUP: The animals were injected i.p. with Huperzine A (500 µg/kg) and 30 min later, they were injected with saline i.m. The dose of Huperzine A as well as the route of administration was used on the base of previously published results [12]. The sampling was performed 30 min after the injection of saline.

HUPERZINE+SOMAN GROUP: the animals were pretreated with Huperzine as it is described in the previous group. 30 min later, the animals were intoxicated with soman and decapitated 30 min after the soman injection.

3.4 Histochemical determination of AChE

Removed brains were rapidly frozen and cut into a series of 20 μm sections in a cryostat. For neuroanatomical mapping according to the rat brain atlas [21], AChE detection [22] was used. The Karnovsky-Roots method is based on hydrolysis of artificial substrate acetylthiocholine (the same as in biochemical examination) and detection of the released reaction product (thiocholine). For digital microphotography, Olympus BX51 light microscope equipped with CCD was used.

Quantitative evaluation was made using software 3D Doctor [23,24]. The picture was transposed to a gray scale with density distribution (expressed in pixels) ranging from 0 to 255. Lower pixel number indicates high activity, higher number shows inhibition. Because the density is of linear scale, the difference (255 - determined density) gives us information on the AChE activity. This pixel density was compared in control and intoxicated animals for each structure examined in absolute and relative (%) values.

We selected the following sections and groups of nuclei to compare quantitative histochemistry with biochemical determination of AChE: ncl. ruber (NR) – section at the level 5,8 dorsally from bregma; frontal cortex (FC) – the level of 2.2 mm rostrally to bregma, containing areas F1, F2, F3; dorsal septum (DS) – the level of bregma, including lateral septal ncl – dorsal and intermediate parts; hypothalamus (HTh), 2.8 mm dorsally to bregma, including ventromedial and dorsomedial hypothalamic nuclei, perifornical, arcuate, periventricular and supraoptic nuclei, tuber cinereum, dorsal and lateral hypothalamic areas; hippocampus (Hipp), 3.5 mm dorsally to bregma; medial thalamus (Th) 2,8mm dorsally from bregma including mediodorsal nuclei, intermediodorsal, paraventricular, paracentral, central medial and centrolateral nuclei; ponto-medullar area (F.ret, section at the level of 13 mm dorsally to bregma, we evaluated only ncl. gigantocellularis as representative by AChE activity, its size, and function. The distance from bregma and terminology of nuclei are quoted from Paxinos and Watson [21].

3.5 Biochemical determination of AChE

The brain parts (NR, FC, DS, HTh, Hipp, Th, F.ret) were prepared and frozen. After thawing, tissue was homogenized (1:10, distilled water, Ultra Turrax homogenizer) and homogenates were used for enzymatic analysis. AChE activity was determined using the method of Ellman *et al.* [25]. Acetylthiocholine was used as substrate (Tris-HCl buffer pH 7.6). UVIKON 752 spectrophotometer was used for determination of absorbancy at 412 nm. The activity was expressed as μmol of substrate hydrolyzed/(60 min.kg) wet weight tissue or as % of control values.

3.6 Statistical evaluation

Enzyme activities were expressed as a mean \pm SD or % of control values (means only) and statistical differences were tested by t-test. Transformation of the curves and their equations and correlation coefficients were evaluated by the least square method using relevant PC programmes.

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