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ORIGINAL ARTICLE

A comparison of tabun-inhibited rat brain acetylcholinesterase reactivation by three oximes (HI-6, obidoxime, and K048) *in vivo* detected by biochemical and histochemical techniques

Jiri Bajgar[1], Petr Hajek[2], Jana Karasova Zdarova[1], Jiri Kassa[1], Antonin Paseka[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 Defense, Trebesska, Hradec Kralove, Czech Republic, [2]Department of Anatomy, Medical Faculty of Charles University, Simkova, Hradec Kralove, Czech Republic, [3]Center of Advanced Studies, Faculty of Military Health Sciences, University of Defense, Trebesska, Hradec Kralove, Czech Republic, and [4]Department of Engineering Mechanics, Faculty of Mechanical Engineering, Technical University of Liberec, Halkova, Liberec, Czech Republic

Abstract

Tabun belongs to the most toxic nerve agents. Its mechanism of action is based on acetylcholinesterase (AChE) inhibition at the peripheral and central nervous systems. Therapeutic countermeasures comprise administration of atropine with cholinesterase reactivators able to reactivate the inhibited enzyme. Reactivation of AChE is determined mostly biochemically without specification of different brain structures. Histochemical determination allows a fine search for different structures but is performed mostly without quantitative evaluation. In rats intoxicated with tabun and treated with a combination of atropine and HI-6, obidoxime, or new oxime K048, AChE activities in different brain structures were determined using biochemical and quantitative histochemical methods. Inhibition of AChE following untreated tabun intoxication was different in the various brain structures, having the highest degree in the frontal cortex and reticular formation and lowest in the basal ganglia and substantia nigra. Treatment resulted in an increase of AChE activity detected by both methods. The highest increase was observed in the frontal cortex. This reactivation was increased in the order HI-6 < K048 < obidoxime; however, this order was not uniform for all brain parts studied. A correlation between AChE activity detected by histochemical and biochemical methods was demonstrated. The results suggest that for the mechanism of action of the nerve agent tabun, reactivation in various parts of the brain is not of the same physiological importance. AChE activity in the pontomedullar area and frontal cortex seems to be the most important for the therapeutic effect of the reactivators. HI-6 was not a good reactivator for the treatment of tabun intoxication.

Keywords: *Acetylcholinesterase; tabun; rat; brain parts; reactivators*

Introduction

The basic mechanism of action of organophosphates (OPs)/nerve agents is considered to be acetylcholinesterase (AChE, EC 3.1.1.7) inhibition and subsequent accumulation of neuromediator acetylcholine at the cholinergic synapses, either peripheral or central, leading to cholinergic hyperstimulation and development of the symptoms of poisoning, followed by metabolic dysbalance and, without effective treatment, leading to death. Tabun (*O*-ethyl *N*,*N*-dimethyl phosphoramidocyanidate; GA) is a nerve agent acting at both peripheral and central levels but with prevailing central effects[1–3].

Address for Correspondence: Jiri Bajgar, Department of Toxicology, Faculty of Military Health Sciences, University of Defense, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic. E-mail: bajgar@pmfhk.cz

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The treatment of nerve agent poisoning consists of the administration of parasympatholytics, cholinesterase reactivators (oximes), and anticonvulsants. Atropine is considered as the most common parasympatholytic drug. Among anticonvulsants, diazepam is frequently used. The choice of oximes is broad (e.g. pralidoxime, obidoxime, HI-6, and others) but not so simple, and depends very much on the type of OP[1,4].

There is a general opinion that obidoxime is a good reactivator for OP insecticide poisoning[1,5]. For nerve agents, HI-6 seems to be a relatively good reactivator; however, in the case of tabun intoxication it is not effective[1,6,7]. Therefore, some attempts to synthesize new reactivators with the aim of making them universal or more effective, especially against tabun-inhibited AChE, have been made. After their synthesis, the reactivation efficacy was tested *in vitro* followed by *in vivo* studies[4,6]. In some cases, however, the *in vivo* results did not correspond to the *in vitro* observations. This discrepancy could be caused by different inhibition/reactivation of AChE originating from various sources (human brain, rat erythrocytes, brain, etc.) or by different penetration of the agent to the target organs. It is generally accepted that reactivation on the periphery is without doubt, but with one exception: after nerve agent intoxication followed by monoisonitrosoacetone (MINA) treatment, reactivation of peripheral AChE was not observed, but AChE in the brain (central compartment) was reactivated[8].

K048 (1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)butane dibromide) is one of the newly synthesized reactivators having some reactivation efficacy against tabun-inhibited AChE *in vitro* and comparable therapeutic efficacy with obidoxime *in vivo*[9–14]. On the other hand, HI-6 is less effective than obidoxime. We compared the reactivation effects of these three reactivators (Figure 1) *in vivo* on AChE activity in different parts of the brain in tabun-intoxicated rats.

Materials and methods

Chemicals

Tabun was obtained from the Military Technical Institute of Protection (Brno, Czech Republic). It was of minimal 95% purity and was stored in glass ampoules (1 mL). Solutions of the agents for experiments were prepared before use. All oximes (obidoxime, HI-6, K048) were synthesized at the Department of Toxicology of the Faculty of Military Health Sciences (Hradec Kralove, Czech Republic) Their purities were analyzed using the high performance liquid chromatography (HPLC) technique. All other chemicals of analytical purity were obtained commercially and used without further purification. All substances were administered intramuscularly (i.m.) at a volume of 1 mL/kg body weight.

Animals

Female Wistar rats (Velaz, Prague), weighing 200–220 g, were used in this study. The animals were divided into groups of 10 animals each (six for biochemical and four for histochemical examination). Housing of the rats was realized in the Central Vivarium of the Faculty of Military Health Sciences under veterinary control (light cycle 12h/12h, standard laboratory diet and water *ad libitum*). All the experiments were performed with the permission and under the supervision of the Ethics Committee of the Faculty of Military Health Sciences, Hradec Kralove (permission No 153/06) according to §17 of Czech law No 207/2004, permission of responsible person No 0001/94 – M 699.

Intoxication and treatment

Control group

The animals were injected with saline i.m. and 1 min later they were injected once again with saline i.m. (1.0 mL/kg). Decapitation and brain removal (sampling) was realized 30 min after the last saline injection.

Tabun group

The animals were injected with tabun (i.m.) at a dose of $1 \times LD_{50}$, i.e. 200 µg/kg, and 1 min later they were injected with saline. Then, 31 min after the intoxication, the animals were decapitated and the brains were removed.

Treated groups

HI-6 group The animals were injected with tabun (i.m.) at a dose of $1 \times LD_{50}$, i.e. 200 µg/kg; 1 min later, the animals were injected with one injection (i.m.) of atropine (21 mg/kg) and HI-6 dichloride. Then, 31 min after the intoxication, the animals were decapitated and the brains without perfusion were removed and used for histochemical or biochemical examination.

Obidoxime group The animals were injected with tabun (i.m.) at a dose of $1 \times LD_{50}$, i.e. 200 µg/kg; 1 min later, the animals were injected with one injection (i.m.) of atropine (21 mg/kg)

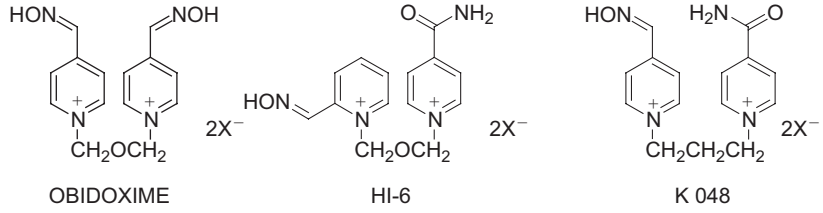

Figure 1. Structural formulae of reactivators used.

and diobidoxime chloride. Then, 31 min after the intoxication, the animals were decapitated and the brains without perfusion were removed and used for histochemical or biochemical examination.

K048 group The animals were injected with tabun (i.m.) at a dose of 1 × LD_{50}, i.e. 200 µg/kg; 1 min later, the animals were injected with one injection (i.m.) of atropine (21 mg/kg) and K048 chloride. Then, 31 min after the intoxication, the animals were decapitated and the brains without perfusion were removed and used for histochemical or biochemical examination.

The doses of all oximes (obidoxime, HI-6, and K048) were equal to 5% of their LD_{50}, i.e. corresponding to human therapeutic doses.

Six animals per group were used for biochemical examination. For histochemical examination, four animals per group were used.

Histochemical determination of AChE

The removed brains were rapidly frozen and cut into series of 20 µm sections using a cryostat. For neuroanatomical mapping according to a rat brain atlas[15], AChE detection[16] was used. The Karnovski–Roots method is based on the hydrolysis of artificial substrate acetylthiocholine (the same as in biochemical examination) and detection of the released reaction product (thiocholine). For digital microphotography, an Olympus BX51 light microscope equipped with CCD (charge coupled device) was used.

Quantitative evaluation was done using 3D Doctor software[17,18]. The image was transposed to a gray scale with density distribution (expressed in pixels) ranging from 0 to 255. A lower pixel number indicates high activity, a higher number shows inhibition. As the density is on a linear scale, the difference (255 – determined density) gives information about 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 the biochemical determination of AChE: nucleus ruber (NR) – section at level 5.8 mm dorsally from bregma; frontal cortex (FC) – level of 2.2 mm rostrally to bregma, containing areas F1, F2, F3; dorsal septum (DS) – level of bregma, including lateral septal nucleus, 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) – 2.8 mm dorsally from bregma including mediodorsal nuclei, intermediodorsal, paraventricular, paracentral, central medial, and centrolateral nuclei; pontomedullar area (PM) – section at level of 13 mm dorsally to bregma, we evaluated only nucleus gigantocellularis as representative of AChE activity, its size, and function. The distances from the bregma and terminology of nuclei are quoted from reference 15.

Biochemical determination of AChE

The brains were frozen and the parts (NR, FC, DS, HTh, Hipp, Th, PM) were prepared. After thawing, the tissue was homogenized (1:10, distilled water, Ultra-Turrax homogenizer) and homogenates were used for enzymatic analysis. The concentration of wet-weight tissue was 2 mg per cuvette (2 mL). AChE activity was determined using the method of Ellman *et al.*[19] as described elsewhere[20]. Acetylthiocholine iodide (0.5 mM) was used as substrate (Tris-HCl buffer, pH 7.6) and 5,5′-dithiobis-2-nitrobenzoic acid (0.5 mM) as chromogen. A Uvikon 752 spectrophotometer was used for determination of the absorbance at 412 nm. The activity is expressed as µmol of substrate hydrolyzed/60 min.kg wet-weight tissue or as % of control value. In the control group, distinguishing between AChE and butyrylcholinesterase activity was performed, and the butyrylcholinesterase activity was found to be negligible (less than 5%).

Statistical evaluation

Enzyme activities determined by the biochemical method are expressed as mean ± SD or % of control values and statistical differences were tested by *t*-test. Histochemical results are expressed as mean only. Transformation of curves and their equations and correlation coefficients were evaluated by the least-squares method using relevant PC programs.

Results

Mortality

Following tabun poisoning, all animals survived for 30 min after tabun injection. However, three animals were clearly *ante finem*. Salivation, tremor, and convulsions were observed in all animals. After tabun intoxication and treatment with atropine and reactivators, fine tremor was observed 10–30 min after the administration only, and all animals survived.

Biochemical

Data dealing with normal AChE activity in the brain areas are presented in Table 1. AChE activity varied from high (PM, Th, Hipp) to low (NR, FC) values. The administration of tabun caused different inhibitions of AChE in all areas studied. Percent inhibition (lower activity) was highest in FC and Hipp, containing about 30–40% of the control AChE activity. Lower inhibition of AChE was observed in the remaining brain structures and in NR. The highest AChE activity increase was observed following obidoxime therapy. This increase, detected by the biochemical method, was statistically significant in all brain structures studied except Th (obidoxime). AChE activity in three structures (FC, Hipp, PM) was significantly increased following treatment with K048. The increase of AChE activity following treatment with HI-6 was statistically significant only in Hipp and DS (Table 2). Percent expression of AChE activity is shown in Table 3.

Histochemical

The histochemical results of AChE distribution in the brain structures showed a picture similar to that of biochemical examination (Table 1). The most sensitive areas to tabun were FC and Hipp (about 35–40% of control values); other high AChE activity following untreated tabun intoxication was observed in NR. Histochemical images of three areas, the relatively sensitive FC, PM, and NR are shown in Figures 2–4. The quantitative evaluation following intoxication with tabun and treatment with atropine and reactivator is also shown in Figures 2–4, where it can be seen that AChE inhibition in all parts had a similar tendency, as observed by biochemical examination. Quantitative evaluation of AChE activity is shown in Table 2 and percent changes following treatment using different oximes are given in Table 3. Quantitative histochemical evaluation showed that the activities were shifted to lower densities, and pixel frequencies were different (Figures 2–4).

Table 1. Control values of AChE activity in different brain areas detected by biochemical and histochemical methods.

	Control (ukat/g)	
Brain area	Histochemical	Biochemical
FC	184.1 + 15.0	253.0 + 13.3
Hipp	220.2 + 16.3	301.6 + 15.3
HTh	201.4 + 17.5	262.0 + 14.3
NR	190.0 + 15.2	251.3 + 12.6
PM	222.6 + 18.2	385.0 + 13.3
DS	200.0 + 17.8	251.2 + 12.3
Th	239.0 + 16.9	325.3 + 14.8

Correlation between results obtained using histochemical and biochemical methods

The comparison of percentage residual AChE activities after the administration of tabun, atropine, and reactivator in seven brain regions (Figure 5) shows a good correlation between results obtained by histochemical and biochemical methods of AChE detection.

Discussion

The main cholinergic pathways in the rat brain are represented by different structures, i.e. the septal nuclei, thalamus, cortex, and hippocampus[21]. AChE activity in these structures is very different, varying from high to low activity[20,22]. It appears from our results that AChE activity in these structures is influenced differently following tabun intoxication and its treatment. Non-uniform AChE inhibition in different brain structures following untreated intoxication with nerve agents in rats and guinea-pigs[3,20,21,23,24] has been demonstrated.

K048 was developed with the aim of obtaining a universal reactivator, but later on, it was tested as a prospective reactivator for tabun and OP pesticide poisoning[11–13]; it showed better antidotal efficacy than that of HI-6, and AChE reactivation in the brain *in vivo* (whole brain homogenate) was higher in comparison with HI-6[13]. Simultaneously, the antidotal efficiency of K048 against tabun poisoning was comparable with trimedoxime[9]. Prospective antidotal efficacy of K048 against paraoxon[25] and methyl-paraoxon[26] was also shown. However, its potency to reactivate AChE in the brain inhibited by tabun *in vivo* as demonstrated in our results was not so unambiguous. In their excellent review, Lorke *et al.*[27]

Table 2. AChE activities in the brain parts of intoxicated and treated animals.

	Tabun		Tabun + HI-6		Tabun + obidoxime		Tabun + K048	
Brain area	Histochemical	Biochemical	Histochemical	Biochemical	Histochemical	Biochemical	Histochemical	Biochemical
FC	66.1 ± 7.5	81.1 ± 9.5	61.9 ± 10.4	75.9 ± 6.8	88.7 ± 8.1*	130.0 ± 10.2*	100.1 ± 4.8*	167.3 ± 10.1*
Hipp	92.7 ± 7.1	117.0 ± 10.2	123.6 ± 6.1*	189.1 ± 15.5*	135.0 ± 5.9*	199.6 ± 14.5*	99.0 ± 5.5	150.5 ± 9.7*
HTh	103.7 ± 7.2	155.3 ± 12.3	138.1 ± 5.7*	185.4 ± 15.6	162.7 ± 7.7*	219.7 ± 19.8*	104.9 ± 7.7	117.9 ± 8.9
NR	133.0 ± 6.0	187.1 ± 14.5	153.7 ± 8.1*	191.9 ± 18.8	180.0 ± 4.2*	240.5 ± 19.9*	144.8 ± 8.3*	200.7 ± 11.2
PM	118.0 ± 6.3	151.1 ± 15.7	154.0 ± 5.8	238.5 ± 20.1	173.9 ± 5.9*	312.5 ± 21.5*	130.6 ± 4.1*	271.8 ± 18.8*
DS	95.0 ± 6.4	125.5 ± 10.1	124.6 ± 4.7*	169.6 ± 10.4*	130.6 ± 8.9*	173.2 ± 12.6*	109.0 ± 4.8	145.6 ± 10.4
Th	117.3 ± 5.6	189.3 ± 15.4	150.0 ± 5.6*	217.7 ± 19.1	118.9 ± 6.1	195.1 ± 15.6	102.6 ± 6.9	146.3 ± 12.3

Note. *$p < 0.05$.

Table 3. Percent values of AChE activity in brain parts following intoxication with tabun and its treatment.

	Tabun (%)		Tabun + HI-6 (%)		Tabun + obidoxime (%)		Tabun + K048 (%)	
Brain area	Histochemical	Biochemical	Histochemical	Biochemical	Histochemical	Biochemical	Histochemical	Biochemical
FC	35.9	32.1	33.6	30.0	48.2	51.4	54,4	66.1
Hipp	42.1	38.8	56.1	62.7	61.5	66.2	45.0	49.9
HTh	51.5	59.3	68.6	70.8	80.8	83.9	52.1	45.0
NR	70.0	74.5	80.9	76.3	94.7	95.7	76.2	79.9
PM	53.0	39.2	69.2	61.9	78.1	81.2	58.7	60.2
DS	47.5	50.0	62.3	67.6	65.3	68.9	54.5	58.0
Th	49.1	58.2	62.8	66.9	49.7	60.0	42.9	45.0

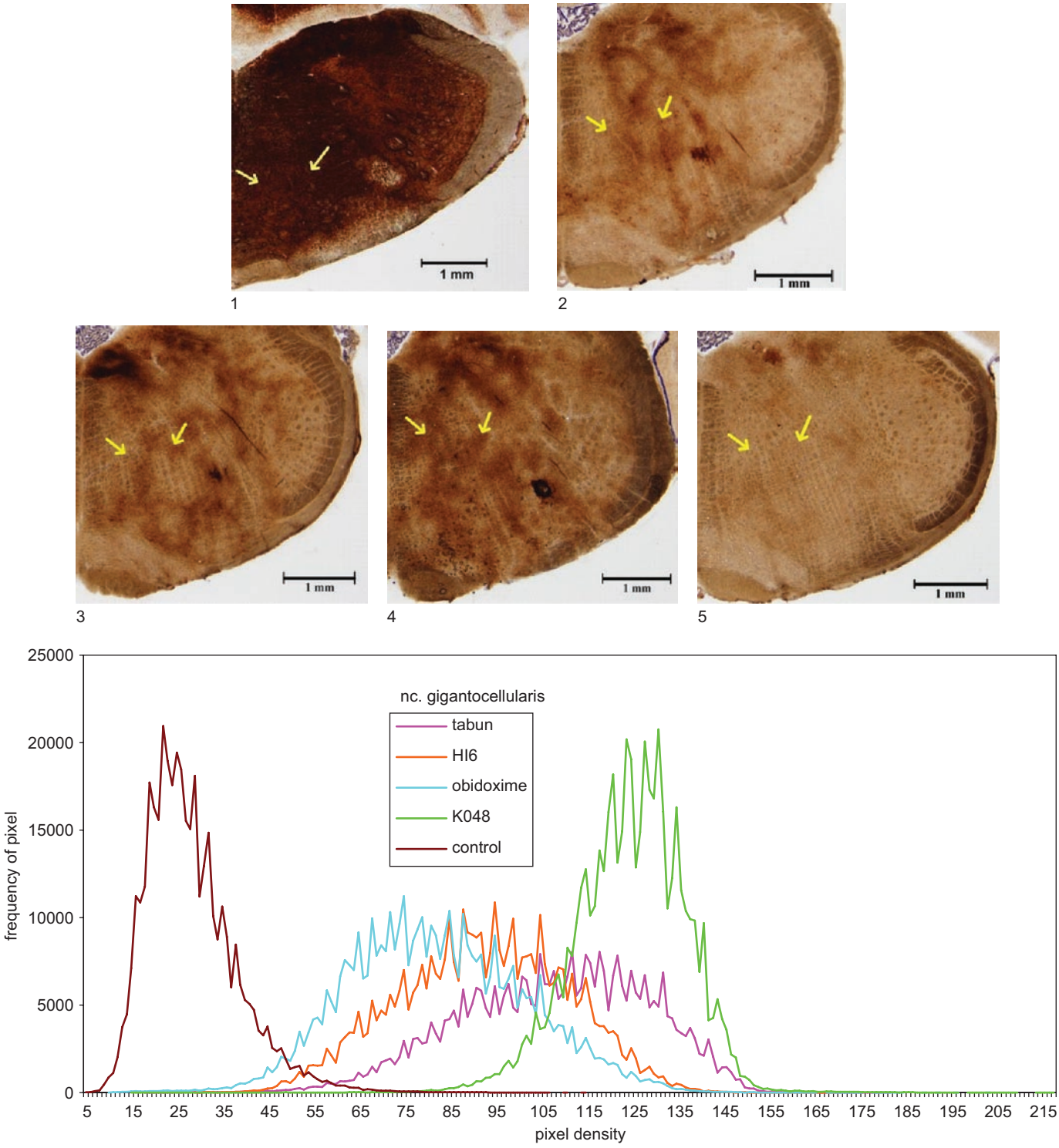

Figure 2. Top: Microphotography of 20 μm sections of rat brain (reticular formation, ncl. gigantocellularis, position marked by arrows) following different treatments: 1, control (not intoxicated); 2, tabun (intoxicated); intoxicated with tabun and treated: 3, HI-6; 4, obidoxime; 5, K048. Magnification: ×40. Staining: AChE. Bottom: Quantitative evaluation of histochemical data: density curves of microphotographs.

demonstrated that the entry of oximes into the brain is a currently hotly debated question, and that only part of the oximes present in the bloodstream are able to penetrate the blood–brain barrier.

Our results showed that different AChE reactivations could be observed in various parts of the brain following treated tabun poisoning; K048 was more effective than HI-6. However, when comparing reactivation effects in the brain parts, obidoxime was the most effective. When the reactivation efficacy *in vivo* is tested using whole brain homogenate as an enzyme source, the results obtained may be a mix of activities of the structures involved.

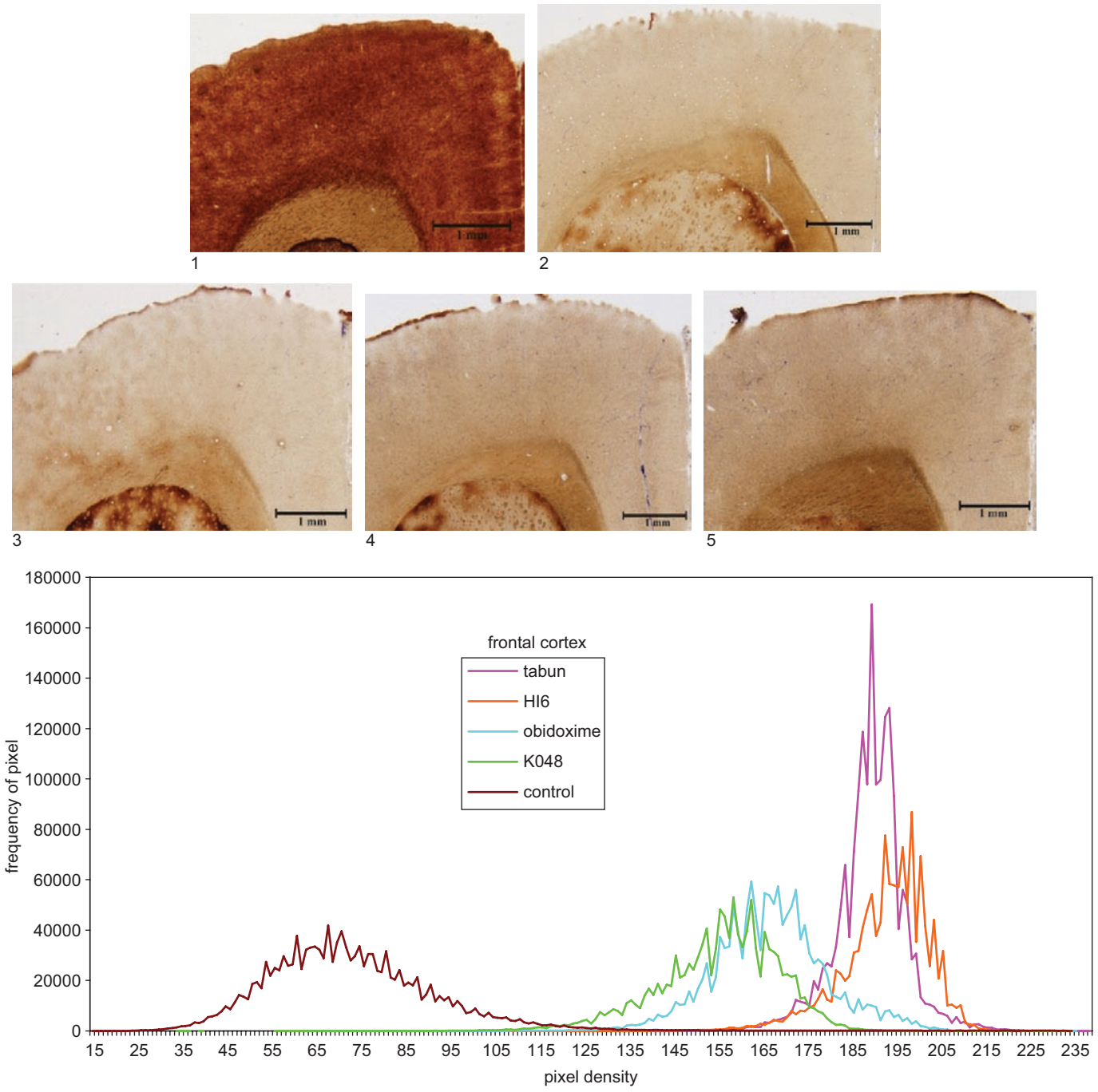

Figure 3. Top: Microphotography of 20 μm sections of rat brain (frontal cortex) following different treatments: 1, control (not intoxicated); 2, tabun (intoxicated); intoxicated with tabun and treated: 3, HI-6; 4, obidoxime; 5, K048. Magnification: ×40. Staining: AChE. Bottom: Quantitative evaluation of histochemical data: density curves of microphotographs.

AChE reactivation in some structures can be functionally more important than that in other areas. These areas can be identified as the frontal cortex, pontomedullar area, and some parts of the limbic system. The importance of AChE inhibition/reactivation in these structures seems to shed more light on the pathogenesis of OP/nerve agent intoxication as well as contributing to the understanding of neurotoxicology in general.

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***Declaration of interest*:** The authors report no conflict of interest.

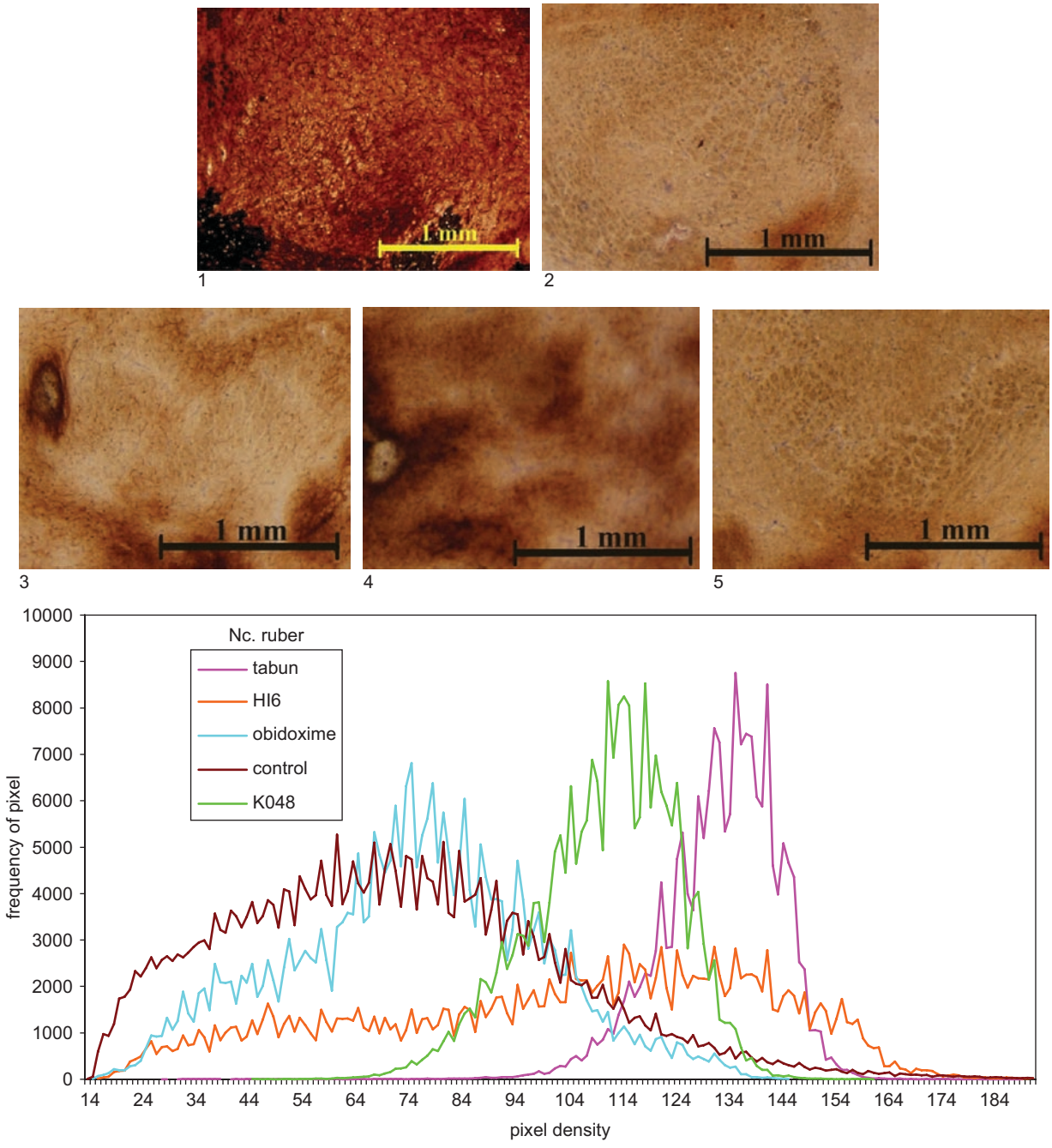

Figure 4. Top: Microphotography of 20 μm sections of rat brain (nucleus ruber) following different treatments: 1, control (not intoxicated); 2, tabun (intoxicated); intoxicated with tabun and treated: 3, HI-6; 4, obidoxime; 5, K048. Magnification: ×40. Staining: AChE. Bottom: Quantitative evaluation of histochemical data: density curves of microphotographs.

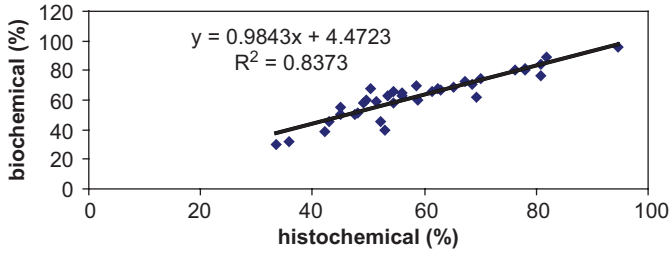

Figure 5. Comparison of results obtained by histochemical and biochemical methods (percent values) following administration of saline, tabun, tabun and atropine with obidoxime, HI-6, or K048.

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