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Sarin (nerve agent GB)-induced differential expression of mRNA coding for the acetylcholinesterase gene in the rat central nervous system

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Abstract

We carried out a time–course study on the effects of a single intramuscular (i.m.) dose ($0.5\times$ LD_{50}) of sarin (*O*-isopropyl methylphosphonofluoridate), also known as nerve agent GB, on the mRNA expression of acetylcholinesterase (AChE) in the brain of male Sprague–Dawley rats. Sarin inactivates the enzyme AChE which is responsible for the breakdown of the neurotransmitter acetylcholine (ACh), leading to its accumulation at ACh receptors and overstimulation of the cholinergic system. Rats were treated with 50 μg/kg of sarin ($0.5\times$ LD_{50}) in 1 mL saline/kg and terminated at the following time points: 1 and 2 hr and 1, 3, and 7 days post-treatment. Control rats were treated with normal saline. Total RNA was extracted, and northern blots were hybridized with cDNA probes for AChE and 28S RNA (control). Poly-A RNA from both treated and control cortex was used for reverse transcription–polymerase chain reaction (RT–PCR)-based verification of the data from the northern blots. The results obtained indicate that a single (i.m.) dose of sarin ($0.5\times$ LD_{50}) produced differential induction and persistence of AChE mRNA levels in different regions of the brain. Immediate induction of AChE transcripts was noted in the brainstem ($126 \pm 6\%$), cortex ($149 \pm 4\%$), midbrain ($153 \pm 5\%$), and cerebellum ($234 \pm 2\%$) at 1 hr. The AChE expression level, however, increased over time and remained elevated after a decline at 1 day in the previously shown more susceptible brainstem. The transcript levels remained elevated at a later time point (3 days) in the midbrain, after a dramatic decline at day 1 ($110 \pm 2\%$). In the cortex, transcript levels came down to control values by day 1. The cerebellum also showed a decline of the elevated levels observed at 2 hr ($275 \pm 2\%$) to control values by day 1. RT–PCR analysis of the AChE transcript at 30 min in the cortex showed an induction to $213 \pm 3\%$ of the control level, confirming the expression pattern obtained by the northern blot data. The immediate induction followed by the complex pattern of the AChE mRNA time–course in the CNS may indicate that the activation of both cholinergic-related and unrelated functions of the gene plays an important role in the pathological manifestations of sarin-induced neurotoxicity.

Keywords: Sarin; Acetylcholinesterase; mRNA expression; Central nervous system; Acute exposure

1. Introduction

Sarin (*O*-isopropyl methylphosphonofluoridate; also known as nerve agent GB), an organophosphorus ester that was developed as a warfare agent [1], is suspected of being one of several chemicals to which several thousand Gulf War veterans were possibly exposed [2]. We have shown that sarin-induced neurotoxicity involves multiple mechanisms [3–5]. Cholinergic inhibition plays an immediate, central, and persistent role in the development of sarin-induced neurotoxicity, even though there may be convergence and/or cross-talk between several signaling pathways [3,5,6].

Hydrolysis of the neurotransmitter ACh is the principal step that terminates the intercellular communication pathway defined as cholinergic signaling. This pathway includes neuromuscular and interneuronal transmission, operates through a variety of receptor subtypes, and activates different intracellular responses in multiple tissues and cell types. AChE is the enzyme that performs this essential step in humans and other species.

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Abbreviations: AChE, acetylcholinesterase; ACh, acetylcholine; ChE, cholinesterase; APS, ammonium persulfate; BuChE, butyrylcholinesterase; DEPC, diethylpyrocarbonate; DFP, diisopropylphosphorofluoridate; IEG, immediate early genes; GFAP, glial fibrillary acedic protein; ChAT, choline acetyl transferase; VAChT, vesicle acetylcholine transporter; p-CREB, phosphorylated cyclic AMP-response element binding protein; PKA, protein kinase A; PSL, photo-stimulated luminescence; TEMED, tetramethylethylenediamine; TBE, Tris borate EDTA buffer.

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AChE degrades the ACh neurotransmitter rapidly [7,8]. This enzyme is also present in non-neuronal cells [9] and in neurons assumed to be non-cholinergic, such as dopaminergic neurons in the substantia nigra [10]. The wide range of cellular distribution of AChE, from meninges to various apparently non-cholinergic glia and neuronal cells, suggests additional important roles for this enzyme in the nervous system. For example, cell types that are known to receive cholinergic input yet contain no AChE include spiny neurons in the striatum [10]. In contrast, cholinoceptive cells that can be excited by ionophoretic ACh (for example, Purkinje cells in the cerebellum) exist, but appear to lack AChE and do not receive cholinergic input in their mature state [11]. Furthermore, identification of newer roles for cholinergic signaling such as hormonal control of hydration levels [12] adds complexity to the AChE function. A statistically significant correlation between body weight loss and plasma cholinesterase levels of sarin-dosed animals has been shown recently, thus establishing a link between sarin, AChE, and weight loss [13]. This above-mentioned study also confirmed the established fact that the satiety center controlling the hypothalamus and CNS may thus be affected by sarin treatment via cholinergic pathways. Similarly newer functions of AChE such as neurite outgrowth [14] and neuronal differentiation [15] make it a key gene whose expression may have a diverse and important role in the recovery or degeneration of different cell types in the CNS after a neurotoxic insult. Thus, studying the mRNA expression pattern of the *AChE* gene in the brain of sarin-treated rats will give more insight into its presumed central role.

2. Materials and methods

2.1. Materials

Sarin (GB) was obtained from the U.S. Army Medical Research and Material Command. Radioactive [α-^{32}P]dATP (3000 Ci/mmol) was purchased from New England Nuclear. Duralon-UV membranes were purchased from Stratagene, and the Random Primer labeling system was obtained from Invitrogen. Other chemicals used were purchased from standard sources.

2.2. Animal treatment

Young adult male Sprague–Dawley rats, weighing approximately 250 g, were purchased from Charles River. The rats were kept in a temperature-controlled room at 21–23° with a 12-hr light–dark cycle, and were provided with Purina rat chow and tap water *ad lib*. Animal treatment with sarin was carried out in specially designated treatment rooms at Duke University. All animal treatments and procedures were approved by the Duke University Institutional Animal Care and Use Committee and carried out according to the recommended guidelines provided by the U.S. Army. We used the minimum number of animals necessary to produce reliable scientific data. Groups of five rats were treated with a single intramuscular injection of sarin (50 μg/kg) in normal saline (1 mL/kg) into the thigh muscle, for each time point. The control group containing five rats was injected with 1 mL/kg of saline as described above. The rats were examined daily for any clinical signs and were weighed two times a week. At the termination of the experiment, the animals were anesthetized with 100 μg/kg of ketamine/xylazine and then were dissected; the brain was removed and washed thoroughly with ice-cold DEPC-treated water to remove traces of blood. The rats were killed at 1 and 2 hr as well as 1, 3, and 7 days post-sarin treatment. The different brain regions, i.e. cortex, cerebellum, brainstem, and midbrain, were quickly dissected at the end of each time point and frozen in liquid nitrogen. The tissues were kept at −70° until used for total RNA extraction.

2.3. Preparation of cDNA probes

Rat AChE cDNA was a gift from Dr. C. Legay of the Laboratoire de Neurobiologie Cellular et Moleculaire, CNRS URA 1875. A 28S RNA cDNA probe was purchased from the American Type Culture Collection. The clones were amplified, and cDNA was purified according to standard methods [16]. The cDNA insert for AChE hybridization was prepared by *Hin*dIII and *Xba*I digestion, while *Eco*RI digestion was used for the preparation of the 28S RNA insert from its vector. The inserts were gel purified using standard protocols [16].

2.4. Total and poly-A RNA extraction

Total RNA was purified from the cortex, cerebellum, brainstem, and midbrain of control and sarin-treated rats using a TRIzol kit from Invitrogen: Life Technologies. Poly-A RNA was prepared from the cortex of both control and treated rats, following a modification of the method of Chomczynski and Sacchi [17]. Briefly, a denaturing solution (250 g guanidine thiocyanate dissolved in 293 mL of double-distilled water and 17.6 mL of sodium chloride, pH 7, along with 26.4 mL of 10% sarcosyl, heated up together to 65° and added with 360 μL of β-mercaptoethanol) was used to homogenize the tissue and then was extracted with phenol and chloroform and purified using oligo-(dt) columns. The purity and quantity of the RNA were determined by A_{260}/A_{280} ratios and then A_{260}, respectively, using a UV-1601 Shimadzu Spectrophotometer (Shimadzu Corp.).

2.5. Reverse transcription (RT)

Using a first strand cDNA synthesis kit (Invitrogen: Life Technologies), 1–2 μg of RNA was reverse transcribed by AMV reverse transcriptase. Reverse transcription was primed with random hexamers or oligo-(dt) in a volume

of 15 or 33 μL. Polymerase chain reactions were performed with 2–4 μL of the resulting cDNA solution.

2.6. Polymerase chain reaction (PCR)

Oligonucleotide primers for the PCR experiments were made in an automated DNA synthesizer in the DNA core facility at Duke University. With the aid of a Seqweb primer designer program of the Shared Bioinformatics Program of Duke University, primers for AChE and cyclophilin were designed. The forward primer used for AChE amplification was 5′-GAC TGC CTT TAT CTT AAT GTG-3′, and the reverse primer was 5′-CGG CTG ATG AGA GAT TCA TTG-3′. The primers for the hybrid product consisting of cyclophilin with the flanking AChE sequences at both ends were as follows: forward primer, 5′-TCT CCT TCT TTG CTC AGC GAC TTA AAC CCC ACC GTG TTC TTC-3′; and reverse primer, 5′-GTT CCC GTC ACA GGT CTG AGC AAT GCC CGC AAG TCA AAG A-3′. DNA was amplified in a Perkin Elmer-Cetus thermocycler (9700 version). Reaction tubes contained, in a final volume of 50 μL, 5 μL of cDNA, 0.5 U of AmpliTaq DNA polymerase, 200 μM dNTPs, and 8 pmol of primers in PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3). Radioactive dCTP was used for PCR reactions. Each PCR cycle consisted of: denaturation temperature, 94°; annealing temperature, 55°; extension temperature, 72°. The PCR products (16 μL of DNA) were separated using 5% polyacrylamide gels (consisting of polyacrylamide, TEMED, TBE, and APS), electroblotted overnight, and exposed to X-ray films. For determining the ideal cycle number for the quantitative analysis, PCR samples were taken out at different cycle numbers such as 18, 21, 24, 30, 35, and 40, run on 1% agarose gels, quantified, and plotted to decide the linearity of the amplification. PCR reactions gave a maximum yield in a linear range at 30 cycles, and hence all other experiments were done for 30 cycles. A total of five animals were used for controls as well as for sarin treatment.

2.7. Data analysis

Each autoradiogram of the gel was scanned into Photoshop 4.0 using a UMAX Astra 2400S Scanner. The bands were quantified using an IP-Lab gel (Molecular Dynamics, Inc.) and the results expressed as a percent of the controls. The value obtained for AChE was divided by that of cyclophilin (internal control). Statistical significance was calculated using Student's *t*-test. A P value <0.05 was considered significant. The radioactive bands were left in the bioimaging system until they were saturated.

2.8. DNA sequencing

Amplified DNA from the PCR reaction was cloned using a One-shot PCR cloning kit (Invitrogen: Life Technologies), and the cloned products were sequenced using the Duke University Core Sequencing Facility. Sequence identity was verified using several software programs provided by the Shared Bioinformatics Program of Duke University.

2.9. Northern blot hybridization

Twenty micrograms of total RNA from each tissue was used for agarose gel electrophoresis and transferred to Duralon nylon membranes. The membranes were hybridized with ^{32}P-labeled cDNA probes, and the latter were labeled by the Random Primer labeling system using [α-^{32}P]dATP as the radioactive nucleotide. The blots were exposed to phosphorimaging plates, and radioactivity in the bands was quantified with the Imagequant System (Molecular Dynamics, Inc.). The radioactivity (PSL values) of the mRNA bands from each tissue for each time point (1 hr to 7 days) was calculated as the percent of the value obtained for the same tissue from control rats. The radioactivity of the mRNA bands from control tissues for any probe and tissue was designated as 100%. The radioactive bands were left in the bioimaging system until they were saturated.

2.10. Statistical analysis

The hybridized blot was exposed to phosphorimaging plates at least two times, and the radioactivity of the bands was quantified and normalized as described above. The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. A P value <0.05 was considered significant.

3. Results

3.1. Clinical signs

All of the animals were observed for the development of clinical signs of sarin toxicity. Those treated with $0.5\times$ LD_{50} were inactive for 4 hr after the treatment. There were no observable changes in their clinical condition thereafter.

3.2. mRNA expression of AChE in the cortex of sarin-treated rats

In the cortex, a single i.m. dose of sarin (50 μg/kg) produced a significant increase in AChE transcript level to $149 \pm 4\%$ at 1 hr and remained more or less at the same level ($147 \pm 3\%$) at 2 hr. This level, however was down-regulated to $110 \pm 2\%$ after 1 day, and stayed at the same level for 3 days ($110 \pm 3\%$) and 7 days ($100 \pm 2\%$) (Figs. 1 and 2A). The expression of 28S RNA was used as a control to reveal any variation in AChE expression due to purity of RNA or error in estimation and loading. The same northern blot was hybridized to both of the probes, and the blot was stripped after hybridization with each probe. The 28S RNA

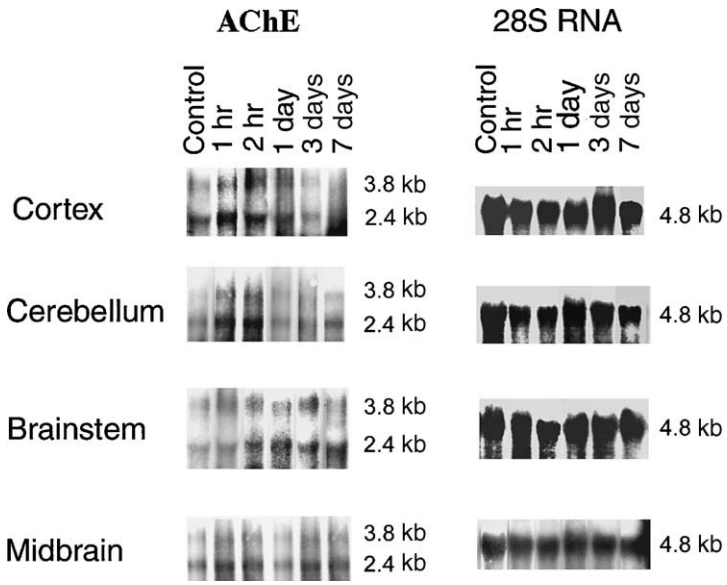

Fig. 1. Northern blots showing the expression of AChE and 28S RNA in the CNS of sarin-treated rats. Rats were treated with a single dose of sarin ($0.5\times LD_{50}$) and killed at 1 and 2 hr and at 1, 3, and 7 days. Total RNA (20 μg) from cortex, cerebellum, brainstem, and midbrain was used as described in Section 2. ^{32}P-Labeled probes prepared from cDNA inserts of rat AChE and 28S RNA were used. Two major bands of 3.8 and 2.4 kb were used for AChE transcript quantitation. Hybridizing bands were quantified using the Imagequant system. The radioactivity (PSL values) of the mRNA bands was calculated as a percentage of the value for the same tissue from the control rats. The radioactive bands were left in the bio-imaging system until they reached saturation. The blots were stripped off of one probe and used for the next one. This figure shows only the relevant portion of representative autoradiograms, although five rats were used in each treatment group. There was a distinct differential expression pattern of transcripts in different regions of the CNS.

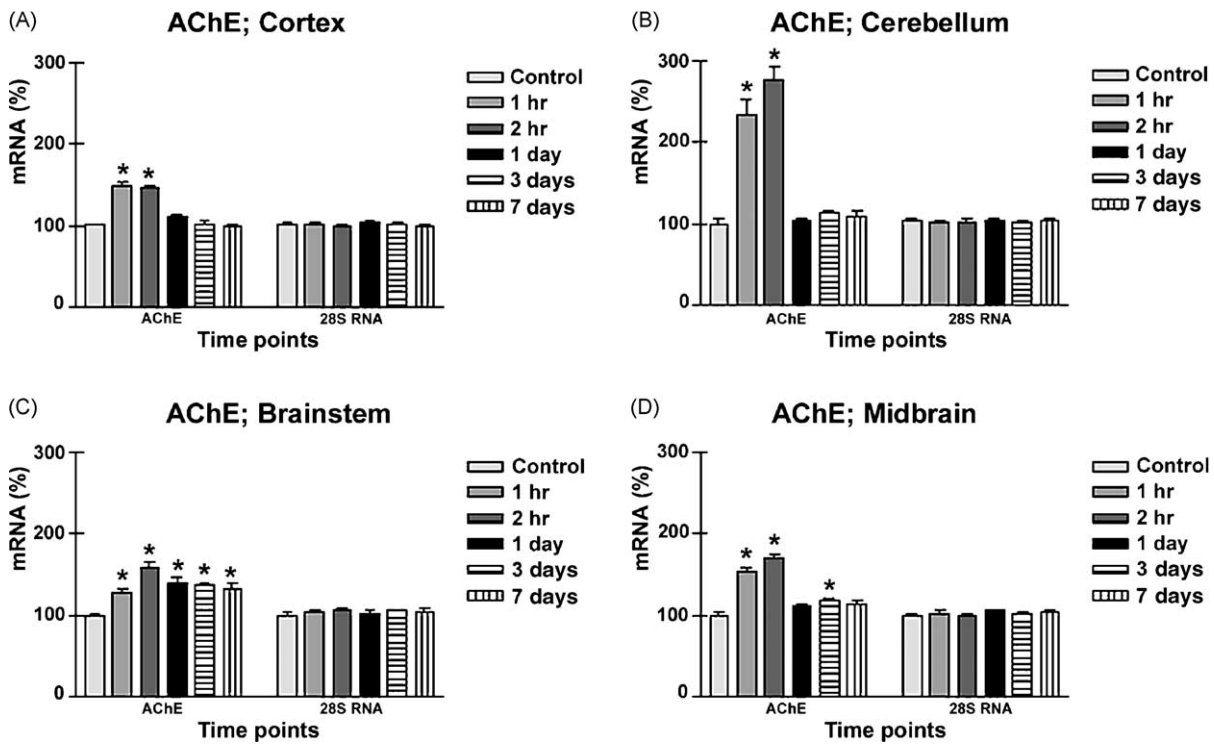

Fig. 2. Expression of AChE and 28S RNA in different regions of the CNS. Rats were treated with a single dose of sarin (50 μg/kg/mL) and killed at 1 and 2 hr as well as 1, 3, and 7 days post-treatment. Total RNA was purified from the cortex, cerebellum, brainstem, and midbrain. Twenty micrograms was used for the northern blotting using standard techniques, and hybridization was carried out as described in Section 2. Values for the AChE and 28S RNA cDNA probes represent the means ± SEM of the percent of control values from untreated rats. The radioactivity of the bands was measured in PSL units by exposing northern blots to phosphorimage plates, followed by quantification with the Fujix Bio-imaging system. Data from control animals were taken as 100%. Five rats were used in each group (control group as well as treated). A significant difference ($P < 0.05$) from the control is marked with an asterisk. There was a statistically significant increase in AChE mRNA levels at 1 hr for all of the tissues, although the induced levels were small in the brainstem and highest in the cerebellum. Both the cortex and the cerebellum continued to show induced levels at 2 hr before reaching control values by day 1 and stayed there for 3 and 7 days. In contrast, brainstem and midbrain persistently showed induced levels to varying degrees at later time points.

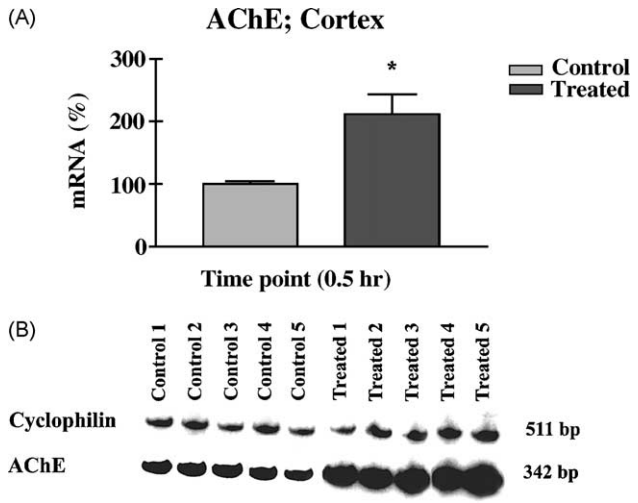

Fig. 3. RT–PCR analysis of the AChE transcripts in the cortex of sarin-treated rats. (A) Poly-A RNA was prepared from the cortex of control and sarin-treated rats, and cDNA was synthesized. RT–PCR reactions were carried out for 30 cycles using the conditions mentioned in Section 2. The products were run in 5% acrylamide gels, electroblotted overnight, and exposed to X-ray films; the bands were scanned into Photoshop version 4.0. The bands were quantified using an IP-Lab Gel. The experiments were done in a group of five control and five treated animals. Data are presented as a percent of the controls. Values are the means ± SEM of five animals. Statistical analysis using an unpaired Student's *t*-test indicated a significant increase (213 ± 30%) in transcript levels at 30 days in the cortex of sarin-treated rats ($P < 0.05$). (B) Gels showing the top hybrid band (consisting of cyclophilin flanked by AChE sequence) and the lower AChE specific band in the control and treated animals. The radioactive bands were left in the bio-imaging system until they reached saturation.

did not show any significant change in expression at any time point after sarin treatment. Alteration in the expression of AChE was real and could not be ascribed to the above-mentioned errors, since the time–course profile was different for both genes. The mRNA expression data of AChE were normalized to control 28S RNA in all the tissues (Figs. 1 and 2).

3.3. RT–PCR of the AChE transcript in the cortex

A single dose of sarin (0.5× LD_{50}) at 30 min post-treatment resulted in a statistically significant induction of AChE transcripts in the cortex to 213 ± 3% of the control level (Fig. 3A and B).

3.4. mRNA expression of AChE in the cerebellum of sarin-treated rats

The AChE transcript levels showed early dramatic induction in the cerebellum at 1 hr (234 ± 2%) that remained elevated until 2 hr (275 ± 2%). These elevated levels were the highest at all of the time points of all of the tissues studied. The transcript levels for AChE declined and were the same at 1 (104 ± 3%), 3 (112 ± 4%), and 7 days (109 ± 6%) post-treatment. There was no alteration in 28S RNA levels at any time point in the cerebellum of sarin-treated rats (Figs. 1 and 2B).

3.5. mRNA expression of AChE in the brainstem of sarin-treated rats

The immediate induction levels of AChE in the brainstem (126 ± 6%) were further increased to 157 ± 8% at 2 hr. Although there was a decline to 139 ± 7% at day 1, the transcript levels remained more or less the same at 3 (137 ± 3%) and 7 days (132 ± 7%). There was no alteration in 28S RNA levels at any time point in the brainstem of sarin-treated rats (Figs. 1 and 2C).

3.6. mRNA expression of AChE in the midbrain of sarin-treated rats

There was a significant increase in AChE transcript levels (153 ± 5%) at 1 hr, which remained high at 2 hr (160 ± 5%) in the midbrain. The transcript levels eventually came down to near control values at day 1 (110 ± 2%). There was a statistically significant increase at day 3 (118 ± 3%), although the level was back to near control values (113 ± 5%) at day 7. There was no alteration in 28S RNA levels at any time point in the midbrain of sarin-treated rats as compared to the controls (Figs. 1 and 2D).

4. Discussion

AChE gene expression is an important part of the cholinergic neuronal systems for the maintenance of CNS homeostasis. Many components of cholinergic neural systems have not been investigated at the level of the gene, and the nature of regulatory mechanisms that foster coordinated expression of cholinergic macromolecules remains relatively unexplored. Hence, our current study provides the preliminary evidence that *AChE* gene expression is differentially modulated in different regions of the brain after sarin treatment, thus confirming the complexity of cholinergic regulation.

We have shown earlier that DFP, a structural analog of sarin, induces some of the IEGs such as c-*fos* [18] and c-*jun* [19], as well as GFAP and vimentin [20]. Our recent studies on the effect of sarin on the mRNA expression of astroglial markers such as GFAP and vimentin suggest that the immediate induction of these genes could be due to the presence of binding sites for IEGs such as c-*fos* and c-*jun*. Similarly, the presence of c-Fos binding sites in the promoters of key cholinergic genes, such as the genes encoding AChE, ChAT, and the VAChT, indicate that elevated c-Fos levels may activate regulatory pathways, leading to long-term changes in the expression of proteins mediating brain cholinergic transmission [21]. These acute cholinergic stimulations promote selective bi-directional changes in the expression of the genes regulating ACh metabolism. Exposure of adult mice to the organophosphorous insecticide fenthion resulted in AChE overproduction [22], which has been reported to cause retinal degeneration [23].

It is interesting to note that in the present study, the previously shown, more susceptible tissue, the brainstem [3], exhibited low levels of immediate induction of *AChE* gene expression, when compared to other CNS regions, followed by persistent levels later on. The inhibition of AChE activity was shown to be maximal at 3 hr in the brainstem at 0.5× LD_{50}, reaching control levels at 24 hr. *AChE* gene expression can be correlated to the highest inhibition of AChE and ChAT at early time points, its recovery to control levels at 24 hr [3], as well as to the increased enzyme activity at a similar dose at a later time point (15 days) [24]. It is also interesting to note that a similar dose of sarin caused a differential induction of the astroglial marker genes GFAP and vimentin [6] and the neuronal marker α-tubulin [5]. In all of these scenarios, it was the brainstem that showed an absence of induction at early time points, followed by late induction. Taken together, there is a direct inhibitory effect of sarin on the brainstem, which not only severely inhibits the cholinergic pathways, but also activates proteases that may lead to synapse loss [25] in an activity-dependent manner. This could result in a significant amount of cell death or cell injury. In a similar way, immediate induction and persistence of induced levels of AChE mRNA in midbrain can be correlated with initial inhibition of AChE enzyme levels [3], followed by an increase of enzyme activity at 15 days [24]. This modified level of transcripts can also be explained by the combined effect of direct inhibition of the cholinergic pathway coupled with the activation of other apoptotic or necrotic pathways.

In spite of a high amount of cholinergic pockets, the cortex showed only a lower level of induction at 1 hr that persisted until 2 hr and eventually came down to control levels at 1 day. On the other hand, a higher level of induction (212%) at 30 min indicates the efficiency of the feedback mechanisms in bringing down the levels later on. AChE enzyme activity remains inhibited at early time points [3] as well as later time points (until 15 days) [24]. As mentioned earlier, this efficient feedback system at the transcriptional level in the cortex, as suggested by Kaufer *et al.* [21], may be modulating the AChE mRNA expression levels.

The most interesting aspect of AChE expression in sarin-induced changes in the brain is that the cerebellum showed the maximum induction of AChE immediately and its levels persisted for 2 hr before returning to basal levels. It is possible that AChE overexpression in the cerebellum may be the classic scenario, where AChE may be switching into non-catalytic functions such as neurite extension [26], interneuronal interactions [27], and neuronal apoptosis [28]. There have been reports of transient expression of AChE in developing embryos [22]. Recently, we have shown that rats treated with 0.5× LD_{50} sarin exhibit decreased plasma BuChE, an increased blood–brain barrier (BBB) permeability in the midbrain and the brainstem, and degeneration of Purkinje neurons in the cerebellum [29]. Earlier we demonstrated that the cerebellum showed immediate induction of IEGs more readily than other CNS regions in DFP-treated hens [19]. We have also shown that DFP differentially modulates the levels of PKA [30] and pCREB [31] in the CNS of the hen. Choi *et al.* [32] showed that PKA mediates the expression of AChE via the cAMP-responsive element. Taken together, it is conceivable that the PKA/pCREB/IEG pathway acts on several proteins including AChE, which may be playing the key role in the pathophysiology of long-term clinical development. Downstream pathways may be of both degenerative and regenerative types, as mentioned above.

At this point it is difficult to speculate whether the stability of the AChE transcript or the rate of transcription plays an important role in the differential induction of AChE mRNA, although the stability of the transcript has been reported to have a crucial role [8,33]. In addition, neurons express two pools of AChE: active and inactive [34]. How these enzyme pools are regulated during the post-translation process is not known. Moreover, Kaufer *et al.* [35] suggested that in addition to the effects of ACh, an autologous feedback response could regulate transcriptional elevation from the *AChE* gene through AChE–anti-ChE complexes acting on signaling intracellular pathways. Complexes of an extracellular protein like AChE could affect intracellular transcriptional signals through their similarity to complexes of neuronal proteins with extracellular domains resembling AChE in their sequence and presumed folding properties [36]. It is also shown that in addition to its catalytic capacity, AChE interacts with target proteins on the neuronal plasma membrane, which can transduce intracellular signals. Such interactions might explain the intensity of the anti-ChE responses, as they add upon the signals induced by the elevated ACh levels.

In summary, treatment with a single i.m. dose of 0.5× LD_{50} sarin resulted in differential induction of AChE mRNA in different regions of the brain. The immediate induction followed by the complex time–course pattern in the AChE mRNA levels of the brain may indicate that the activation of both cholinergic-related and unrelated functions of the gene plays an important role in the long-term pathological manifestations of sarin-induced neurotoxicity. Sarin-induced *AChE* gene expression may explain, at the best in part, our recent findings that acute sarin exposure caused neuronal death of specific regions in the rat brain [37].

Acknowledgments

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