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Neurobehavioral teratogenicity of sarin in an avian model

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1. Introduction

Sarin, a potent organophosphate and acetylcholinesterase inhibitor [12], is among the nerve agents most likely to be used on the battlefield or in a terrorist attack, such as that experienced in the Tokyo subway [20]. Adult human and animal studies have demonstrated long lasting neurological abnormalities among survivors of sarin exposure who showed initial, acute signs of toxicity [29,30]. However, there is obviously a much wider circle of individuals who are exposed at lower, nonsymptomatic levels, about which much less is known. In particular, an urban terrorist incident will almost assuredly involve numerous pregnant women and young children who may not display acute toxicity; nevertheless, because of the higher vulnerability of the developing brain to neurotoxic insult, these children may nevertheless manifest long-term deficits in brain development and function. It is therefore critical to establish an animal model for nerve agent neurobehavioral teratogenicity, including elucidation of the synaptic mechanisms by which the nerve gas agents might produce behavioral deficits after developmental exposure.

Although organophosphates as a chemical class, share the ability to inhibit cholinesterase [19], their adverse effects on brain development reflect families of mechanisms that operate below the threshold for cholinesterase inhibition and therefore are not shared equally. These diverse outcomes are especially apparent at low levels of exposure that elicit no initial signs of toxicity [review, 29]. Accordingly, whereas a large body of literature has examined the mechanisms and consequences of neurobehavioral teratogenicity of organophosphate pesticides, this information may not necessarily apply to a shorter-acting but more potent nerve agent like sarin. It is therefore important to note that, to date, there is apparently no published literature on the neurodevelopmental effects of sarin or related nerve gas organophosphates. Here, we present a chick model of sarin neurobehavioral teratogenicity that parallels our previous work with the organophosphate pesticide, chlorpyrifos. Chlorpyrifos evokes widespread disruption of neuronal cell replication and differentiation, culminating in abnormalities of synaptic function.
that involve many neurotransmitter systems and circuits, but most especially those involving acetylcholine and serotonin [29]. Thus, in mammalian models, fetal or neonatal chlorpyrifos exposure leads to cognitive deficits involving impaired presynaptic cholinergic function in critical regions such as the hippocampus, striatum and cerebral cortex [29]. Postsynaptically, the behavioral impairment was closely associated with deficits in receptor-mediated cell signaling cascades, involving both cyclic AMP-dependent pathways and those governed by protein kinase C (PKC).

Notwithstanding these results, mammalian models are always subject to confounds that result from toxicant effects in the mother, the maternal–fetal unit, or maternal–neonatal interactions. These include potential effects of the agent on maternal body temperature or oxygenation [24], maternal stress [38], or the “litter” effect [33], which involves nest-making, pup retrieval littermate competition for teats, and pup vocalizations that trigger suckling and maternal care [2,8,23] none of which are directly operational in humans. Recently, we developed an avian model for studies of developmental neurotoxicity to obviate these confounds [10]. With chicks, we can administer agents directly to the medium surrounding the embryo, eliminating the variables related to maternal physiology in mammalian models. During the early incubation period, the fluid volume of the chick egg is large compared to the embryo, so that the exposure is uniform. Cognitive performance and its neurochemical underpinnings can be tested right after hatching, before the chicks consume food or water, and are thus independent of any potential changes in self-sufficiency. On the other hand, the chick model is limited in that it cannot recapitulate the pharmacokinetics of the maternal–fetal unit, or other aspects of development that are specifically mammalian.

We used the chick model to evaluate the effects of prehatch exposure to chlorpyrifos, which elicited severe deficits in imprinting behavior, a direct parallel to cognitive tests in mammals, involving cholinergic function in a specific region, the left side of the intermedial part of the hyperstriatum ventrale (IMHV), which parallels the function of the mammalian hippocampus [10,32]. Our findings recapitulated our earlier work in the rodent model, demonstrating cognitive impairment, reductions in the presynaptic high-affinity choline transporter and abolition of cholinergic-induced PKC translocation/activation, all of which were present with otherwise subtoxic exposures. In the current study, we performed corresponding work using sarin. As in our study with chlorpyrifos, we administered sarin beginning early in development, parallelizing rodent models with treatment in early gestation, which are known to produce deficits in both the cholinergic synaptic function and related behaviors [29].

We evaluated cholinesterase activity to confirm that the doses of sarin did not lead to persistent cholinesterase inhibition. We used imprinting to evaluate impaired behavioral performance and then evaluated potential, mechanistically-related aspects of cholinergic synaptic function known to be targets for organophosphate-induced developmental neurotoxicity. For presynaptic function, we assayed choline acetyltransferase (ChAT) activity, and binding of hemicholinium-3 (HC3) to the presynaptic high-affinity choline transporter. ChAT, the enzyme that synthesizes acetylcholine, is a constitutive component of cholinergic nerve terminals and thus provides a measure of the development of cholinergic projections [9,22,30]. In contrast, HC3 binding to the choline transporter is responsive to neuronal activity [13,27], so that measurement of both parameters enables the distinction between effects on the development of innervation from those on synaptic activity. These markers have been used previously to characterize effects of chlorpyrifos on cholinergic systems in adult rats [18,17] and to evaluate the immediate and delayed effects of developmental chlorpyrifos exposure in rodents, PC12 [21,30,31] and chicks [32]. Finally, we evaluated the concentration of PKC isoforms related to cholinergic behavioral function in the IMHV [1,10,26].

2. Methods

2.1. Treatments

All animal studies were reviewed and approved by the Duke University Institutional Animal Care and Use Committee, in accordance with all federal, state and local regulations. Fertile Leghorn-line chicken eggs (Gallus gallus domesticus) were purchased from the Department of Animal and Food Science, University of Delaware (Newark, DE), and an injection window was prepared by drilling a hole in the pointed end and sealing it with medical silicon (Dow Corning Type A). Eggs were weighed and, after correcting for shell weight (11%), the concentration of sarin was adjusted to deliver 2.6 or 12 μg/kg in a volume of 3 μl on incubation days 2 and 6. Sarin was dissolved in saline vehicle and control eggs received an equivalent volume of vehicle. Sarin treatment was carried out under a subcontract to the United States Army Medical Research Institute of Chemical Defense (Aberdeen Proving Ground, MD). The eggs were placed in a commercial incubator and a week after the second injection, were transported via ground vehicle to Duke University Medical Center in an incubator. Embryonic survival was monitored via candling and at the end of the incubation period, the hatch rate and physical attributes were noted.

2.2. Imprinting

The apparatus has been described in detail earlier [10]. Briefly, we used a box containing three 20 cm diameter running wheels with the sides covered in black metal, permitting the chicks to see only forward or backward. The imprinting objects were an illuminated red box (15×10×18 cm high) or a blue cylinder with black stripes (13.5 cm diameter×15.5 cm high), located 50 cm from the front, open side of the running wheel, lit from within by a 40 W bulb with holes covered with red or blue filters, and rotated at 30 rpm.

The chicks were hatched in total darkness and handling was done in the dark, aided by a dim green light, which has a minimal effect on imprinting. Each chick was transferred to an individual dark, enclosed wooden chamber warmed to 30 °C, where they were physically and visually isolated from each other. Twenty to 26 h posthatch, the chicks underwent 45 min of “priming,” a 30 min exposure to a light (60 W bulb) followed by 15 min of darkness. Immediately after, they were placed individually on the running wheel for training. The chicks were divided into groups trained for 60 min with either blue or red imprinting objects. The numbers of wheel rotations made by the chick towards the imprinting object were recorded by videotaping and scored by a blinded observer. After training, the chicks were returned to the enclosed chambers for 60 min, after which testing took place. Recorded maternal calls were played continually throughout training but not during testing.

There were four testing sessions in counterbalanced randomized order, each lasting 5 min; in two of the tests, the chick was allowed to run toward the imprinting rotating object and in the other two, toward the control object. The red-light box was used as the imprinting object and the blue-light box served as the control object for chicks trained to follow a red object and vice versa for the chicks trained to follow the blue-light box. The number of wheel rotations completed by the chick toward the imprinting or the novel (control) object was recorded. The imprinting is expressed as a preference score calculated as the number of rotations toward the imprinted light divided by the total number of rotations combined from the test with the imprinted light and the control light. Thus, a perfect score would be 1.0 whereas no imprinting would result in a score of 0.5.

2.3. Neurochemistry

Chicks were decapitated immediately after behavioral testing and the brain was dissected into the cerebral cortex, striatum, cerebellum,
brainstem and left IMHV. Tissues were flash-frozen in liquid nitrogen and stored at −45 °C until assayed. Cholinesterase measurements were made on the cerebellum and the rest of the brain after removal of the IMHV, which represents a minuscule proportion of the total tissue weight (3 mg out of 630 mg). Tissues were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in ice-cold 50 mM Tris (pH 7.4), and aliquots of the homogenate were withdrawn for measurement of total protein and cholinesterase activity. The homogenate was diluted in 0.5% Triton X100, 0.1 M Na2HPO4/KH2PO4 (pH 8) and left on ice for 15 min to allow the Triton X100 to solubilize membrane-associated cholinesterase. Homogenates were sedimented at 40,000 × g for 15 min and aliquots of the supernatant solution were added to final concentrations of 0.5 mM acetylthiocholine iodide and 0.33 mM 5,5′-dithiobis(2-nitrobenzoic acid) in the same buffer without Triton (all reagents from Sigma Chemical Co., St. Louis, MO). Assays were incubated at room temperature for 4, 8, 12, 16 and 20 min, and the enzyme activity was assessed from the linear portion of the time course, reading the absorbance at 415 nm. The assay was standardized using mercaptoethanol as a substrate and calculated relative to total protein.

For PKC determinations, tissues were thawed in 79 volumes of ice-cold 10 mM sodium–potassium phosphate buffer (pH 7.4) and homogenized (Polytron). Assays contained 60 mM sodium phosphate (pH 7.9); 200 mM NaCl, 20 mM choline chloride, 17 mM MgCl2, 1 mM EDTA, 0.2% Triton X-100, 0.12 mM physostigmine, 0.6 mg/ml bovine serum albumin and 50 μM [3H]acetyl-coenzyme A (PerkinElmer Life Sciences, Boston, MA; specific activity 60 mCi/mm, diluted with unlabeled compound to 6.7 mCi/mmol). Samples were preincubated for 15 min on ice, transferred to a 37 °C water bath for 30 min, and the reaction terminated by placing the samples on ice. Labeled acetylcholine was then extracted, counted, and the activity determined relative to total protein.

For measurements of [3H]HC3 binding, the cell membrane fraction was prepared by sedimenting an aliquot of the same tissue homogenate at 40,000 × g for 15 min. The membrane pellet was resuspended (Polytron) in the original volume of sodium–potassium phosphate buffer, resedimented, and the resultant pellet was resuspended using a smooth glass homogenizer fitted with a Teflon pestle, in 10 mM sodium–potassium phosphate buffer (pH 7.4) and 150 mM NaCl. Radioligand binding was evaluated with 2 nM [3H]HC3 (PerkinElmer; specific activity 125 Ci/mmol, with incubation for 20 min at room temperature, followed by rapid vacuum filtration onto glass fiber filters (presoaked for 30 min with 0.1% polyethyleneimine in buffer). The nonspecific component was defined as radioligand binding in the presence of an excess concentration (10 μM) of unlabeled HC3 (Sigma). Binding values were expressed relative to membrane protein.

For PKC determinations, the left IMHV (~3.0 mg) was quickly dissected [10], and frozen in liquid nitrogen. Basal levels of the PKC isoforms γ, δI and α in the cytosolic and membrane fractions of the IMHV were assayed using published protocols modified [10,25,34,40] from earlier techniques [36]. The tissues were homogenized in buffer and then sedimented at 100,000 × g for 1 h at 4 °C, after which the supernatant solution containing the cytosolic fraction was frozen. The membrane pellet was resuspended and solubilized with 0.5% Triton X-100, then sedimented as already described, and the supernatant solution was frozen.

Western blot analysis was carried out by gel electrophoresis (Invitrogen EP048-08, Carlsbad, CA) of 10–15 μg aliquots of cytosolic and membrane protein using specific primary antibodies for each PKC subtype. The PKCγ antibody 36G9 recognizes chick PKCγ [37] and was produced especially for this study (E.A. Van der Zee), whereas cPKCδI and pPKCα were purchased from Santa Cruz Biochemicals (Santa Cruz, CA); IgG HRP conjugated (Bio–Rad) secondary antibody, was then applied exactly as described earlier [25,40]. We did not assess internal standards of structural “housekeeping” proteins (α-tubulin or β-actin) because many neuroteratogens, including the organophosphates, influence neuromorphological development [6] and consequently cause alterations in the expression of these cytoskeleton proteins, unrelated to the neurobehavioral effects linked to specific cell signaling pathways. Instead, to ensure standardization, triplicate samples from all groups were run in proximity to each other on the
same gel, adjusting the sample dilution to make sure that each lane has precisely the same protein load. Since the levels are assessed as changes from the matched control on the same gel, any inherent gel-to-gel differences in hybridization or factors other than the specific concentration of PKCy and PKC\(\alpha\)II are equally represented in both the numerator and denominator and do not contribute to the measured outcome.

2.4. Data analysis

All data are presented as means and standard errors. Treatment effects on neurochemical and behavioral variables were examined by multivariate nested ANOVAs, incorporating factors of treatment and, for neurochemistry, the brain region (a repeated measure, since each animal contributed multiple brain regions), followed, where permitted, by appropriate interaction terms, by lower-order ANOVAs and Fisher’s Protected Least Significant Difference Test to evaluate specific treatment groups that differed significantly from the corresponding control values. To enable convenient comparison of treatment effects across different regions and measures, some data are presented as the percent change from control, with the corresponding control values presented in the figure legends; however, statistical analyses were always conducted on the original data.

For the PKC studies, each gel contained a sample representing one individual from each treatment group; since the samples thus involved multiple gels, the results were evaluated nonparametrically as either an increase or a decrease relative to the paired control sample. We then used \(\chi^2\) analysis to determine if the treatments altered the incidence of increases or decreases from the random value of 50%. Where there was a significant effect on the distribution of PKC values, we then compiled the median increase or decrease as an index of the magnitude of change. Significance for all tests was assumed at \(p<0.05\).

3. Results

Sarin exposure did not decrease the hatch rate nor did it increase the incidence of malformations (data not shown). Despite the absence of overt teratogenesis or impaired viability, the sarin-exposed chicks showed deficits in imprinting performance (Fig. 1). Whereas chicks in the control group showed a preference score above 0.8, those exposed to 2 or 6 \(\mu\)g/kg of sarin showed much lower values, representing nearly a 50% loss of imprinting performance. However, the effect was lost in the group receiving the highest dose of sarin (12 \(\mu\)g/kg).

None of the sarin doses elicited significant cholinesterase inhibition in the hatched chicks (Fig. 2A), nor did they show any deficits in ChAT activity in any of the brain regions (Fig. 2B). In contrast, HC3 binding showed significant reductions at all sarin doses (Fig. 3A); although the effects were smaller in the cerebral cortex than in the other two regions, there was no significant treatment \(\times\) region interaction, indicating the need for caution in interpreting any differences in regional selectivity. However, because HC3 binding is influenced by the density of cholinergic innervation as well as by cholinergic presynaptic activity, we also determined the HC3 binding as the ratio to ChAT, the constitutive marker for cholinergic terminals, so as to give an index of impulse activity per terminal (Fig. 3B). Again, even the lowest dose of sarin elicited a significant reduction, and with this measure, a significant treatment \(\times\) region interaction emerged, reflecting a smaller, nonsignificant effect in the cerebral cortex as compared to the robust, significant reductions seen in the other two regions.

Similar to our previous findings for chlorpyrifos [10], prehatch exposure to sarin reduced the concentration of PKC isoforms in the left IMHV (Fig. 4). Across all sarin doses, PKC subtypes and subcellular fractions, 60% of the samples showed a reduction in PKC, a significant decrease (\(p<0.003\)) from the expected random incidence of 50%; the magnitude of the median decrease was 37%. Although each dose group showed the same trend, i.e. reduction in PKC, the effect was individually significant only in the 6 \(\mu\)g/kg group, in which 70% of the samples showed a lowering of PKC, with a median decrease of 38%. We then examined PKC subtypes and subcellular fractions within that dose group. All three subtypes showed a higher-than-normal incidence of PKC values below the paired control (68% for PKC\(\alpha\), 66% for PKC\(\beta\), 63% for PKC\(\gamma\)). Although the latter was (barely) not individually statistically significant, the incidence was itself not distinguishable from the significant differences seen for the \(\alpha\) and \(\beta\) isoforms, so it is unlikely that there were subtype-selective differences; indeed, the median magnitude of the decrease was also similar for the three isoforms: 42% for \(\alpha\), 34% for \(\beta\), 42% for \(\gamma\). There was, however, a notable selectivity for membrane-associated PKC as opposed to cytosolic PKC. Whereas the incidence of decreases in cytosolic PKC was indistinguishable from random (52%), nearly 80% of the membrane PKC samples were below the corresponding control, a difference that was significant both from random (\(p<0.0005\)) and from the cytosolic proportions (\(p<0.0003\)); the magnitude of the median decrease was 42%. Finally, we examined the three PKC subtypes within the membrane fraction. Each one showed a higher
incidence of decreases from the expected random values: 73% for PKCα and 81% for PKCβ and PKCγ. Although only the latter two were statistically distinguishable from the random 50% value, it must be noted that the successive subdivisions reduced the number of determinations to the point where individual statistical tests become insensitive; the nonsignificant PKCα effect was not distinguishable from the significant effects on the other two isoforms and in fact, the median decrease for the α isoform was actually larger (55% for PKCα, 35% for PKCβ, 43% for PKCγ).

4. Discussion

To our knowledge, the current results represent the first demonstration that developmental exposure to sarin or organophosphate pesticides is apparently subtoxic, producing no dysmorphology or reductions in viability, nevertheless elicits neurobehavioral teratogenicity. Not only did we identify cognitive impairment but we were also able to show the presence of cholinergic and cell signaling deficits. At hatching, we did not detect any residual inhibition of cholinesterase from the sarin treatments on incubation days 2 and 6. Nevertheless, an initial effect on cholinesterase cannot be ruled out, since these enzymes originate early in embryonic development, during the period in which we gave sarin [15]; organophosphates have been shown to evoke cholinesterase inhibition during this period [7]. Indeed, inhibition at the highest dose may account for the nonmonotonic dose–response curve found for impairment of imprinting behavior. In the developing brain, acetylcholine provides trophic input that promotes neuronal survival and differentiation [14], so that if the proper balance is achieved, a small degree of cholinesterase inhibition might actually offset some aspects of the deleterious effects. In keeping with this interpretation, the same nonmonotonic dose–effect relationship for behavioral deficits has been noted in rodent models of developmental exposure to organophosphate pesticides [16,35].

In our earlier work with chlorpyrifos, we found that cognitive impairment was directly associated with deficient cholinergic signaling, characterized by a reduction in HC3 binding and in the ability of cholinergic receptor stimulation to evoke translocation of PKCβ and PKCγ to the membrane [10,29,32]. Similarly, with sarin we found a significant deficit in HC3 binding without a reduction in ChAT, as well as reductions in the concentrations of PKC isoforms, with particular loss in the membrane fraction, evaluated immediately after imprinting, a behavior which depends upon cholinergic activation in the left IMHV. HC3 binding reflects the concentration of high-affinity presynaptic choline transporters, the rate-limiting factor in acetylcholine biosynthesis, which highly reflects presynaptic neuronal activity [27]. Accordingly, our finding a reduction in HC3 binding without any significant decrease in ChAT, a constitutive marker for cholinergic nerve terminals, connotes a deficiency in cholinergic impulse flow rather than a loss of innervation, as confirmed by the decrease in the HC3/ChAT ratio. The magnitude of this effect of sarin seen here corresponds to that seen in rats with fetal or neonatal exposure to chlorpyrifos, and which is known to produce cholinergic-neurotransmitter receptor signaling [4]. In contrast, we found widespread reductions in all subtypes throughout the brain, lasting weeks beyond the exposure period, and selective for the membrane fraction.

There are a number of key differences between chlorpyrifos and sarin that contribute to some of the divergent effects. Chlorpyrifos is metabolically converted to chlorpyrifos oxon, which is the active cholinesterase inhibitor, whereas sarin acts directly, without requiring conversion. Thus, whereas cholinesterase inhibition from chlorpyrifos can occur only after the development of the necessary cytochrome P450 enzymes, sarin can act at any point in development. Second, whereas chlorpyrifos and the other organophosphate pesticides are chemically stable, sarin has a short half-life (hours) in aqueous solutions, so that its duration of action is likely to be much shorter [28]. However, we found effects of sarin at much lower doses than for chlorpyrifos, emphasizing again that sarin is particularly neuroteratogenic despite its short duration of action. Other differences may emerge when mammalian models are examined. For example, sarin may not penetrate the placental barrier as readily as the organophosphate pesticides or may be degraded more rapidly in aqueous solutions, so that its duration of action is likely to be much shorter [28]. However, we found effects of sarin at much lower doses than for chlorpyrifos, emphasizing again that sarin is particularly neuroteratogenic despite its short duration of action. Other differences may emerge when mammalian models are examined. For example, sarin may not penetrate the placental barrier as readily as the organophosphate pesticides or may be degraded more rapidly in aqueous solutions, so that its duration of action is likely to be much shorter [28]. However, we found effects of sarin at much lower doses than for chlorpyrifos, emphasizing again that sarin is particularly neuroteratogenic despite its short duration of action. Other differences may emerge when mammalian models are examined. For example, sarin may not penetrate the placental barrier as readily as the organophosphate pesticides or may be degraded more rapidly inaqueous solutions, so that its duration of action is likely to be much shorter [28]. However, we found effects of sarin at much lower doses than for chlorpyrifos, emphasizing again that sarin is particularly neuroteratogenic despite its short duration of action.
the maternal unit. Clearly, these issues will need to be resolved. However, our results point to potentially greater adverse effects of sarin even if these factors lower the exposure substantially below those seen with the pesticides.

In conclusion, our results provide the first evidence that sarin is a neurobehavioral teratogen at exposures that do not elicit signs of systemic toxicity. As with our earlier work with chlorpyrifos [10,32], sarin exposure produces cognitive impairment in association with deficient cholinergic presynaptic function and postsynaptic signaling mediated through PKC. Finally, the fact that we identified these deficits in an avian model means that the effects of sarin are indeed mediated directly on the developing brain, rather than representing secondary actions that could confound such relationships in a mammalian model. In the event of a terrorist incident involving sarin, there will be a wide circle of low-level exposures that are nonsympotomatic and thus may be likely to be undetected. Our results indicate nevertheless that there may be serious consequences for fetal and neonatal brain development. Further, understanding the defects and their mechanism is an important initial step toward designing therapies that may offset or reverse the sarin-induced neurodevelopmental defects [3,11], an approach we are currently investigating in the chick model [5].

Conflict of interest

The authors declare they have no financial conflicts of interest.

Acknowledgments

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