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A chick model for the mechanisms of mustard gas neurobehavioral teratogenicity

Uri Wormser^a, Michal Izrael^b, Eddy A. Van der Zee^c, Berta Brodsky^a, Joseph Yanai^{b,d,*}

^aDepartment of Pharmacology, School of Pharmacy, Faculty of Medicine, Institute of Life Sciences, The Hebrew University, Jerusalem, Israel

^bThe Ross Laboratory for Studies in Neural Birth Defects, Department of Anatomy and Cell Biology, The Hebrew University-Hadassah Medical School, Box 12272, 91120 Jerusalem, Israel

^cDepartment of Animal Physiology, University of Groningen, PO Box 14 9750 AA Haren, The Netherlands

^dDepartment of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA

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Abstract

The chemical warfare blistering agent, sulfur mustard (SM), is a powerful mutagen and carcinogen. Due to its similarity to the related chemotherapy agents nitrogen mustard (mechlorethamine), it is expected to act as a developmental neurotoxicant. The present study was designed to establish a chick model for the mechanisms of SM on neurobehavioral teratogenicity, free of confounds related to mammalian maternal effects. Chicken eggs were injected with SM at a dose range of 0.0017–17.0 µg/kg of egg, which is below the threshold for dysmorphology, on incubation days (ID) 2 and 7, and then tests were conducted posthatching. Exposure to SM elicited significant deficits in the intermedial part of the hyperstriatum ventrale (IMHV)-related imprinting behavior. Parallel decreases were found in the level of membrane PKC γ in the IMHV, while eliciting no net change in cytosolic PKC γ . The chick, thus, provides a suitable model for the rapid evaluation of SM behavioral teratogenicity and elucidation of the mechanisms underlying behavioral anomalies. The results obtained, using a model that controls for confounding maternal effects, may be replicated in the mammalian model and provide the groundwork for studies designed to offset or reverse the SM-induced neurobehavioral defects in both avian and mammals.

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Keywords: Chick; IMHV; Imprinting; Mustard gas; PKC γ

1. Introduction

Sulfur mustard (SM), also termed mustard gas, is a chemical weapon employed in various conflicts during the 20th century [20,35] and functions as a powerful alkylator and highly cytotoxic blisterogen in both humans and

animals [8,20,35]. Skin exposed to SM develops erythema within 30 min to several hours after exposure followed by edema, vesicle and blister formation, ulceration, necrosis, and desquamation [26,35]. Due to its powerful alkylating and mutagenic activities, SM like other mustards, such as, nitrogen mustard, may possess teratogenic effects. In the event of a SM attack during war or a terrorist incident there could be numerous pregnant women who survive the SM exposure, however, the transplacentally exposed fetus may bear long-term consequences. Since neurobehavioral birth defects may result from low levels of prenatal exposure, below the threshold for dysmorphology [15] even children who appear normal may display long-term neurobehavioral birth defects. SM is expected to perturb neurobehavioral development due to its cytotoxic and

Abbreviations: IMHV, intermedial part of the hyperstriatum ventrale; PKC, protein kinase C; SM, sulfur mustard or mustard gas.

* Corresponding author. The Ross Laboratory for Studies in Neural Birth Defects, Department of Anatomy and Cell Biology, The Hebrew University-Hadassah Medical School, Box 12272, 91120 Jerusalem, Israel. Tel.: +972 2 675 8439; fax: +972 2 675 8443.

E-mail address: yanai@md.huji.ac.il (J. Yanai).

mutagenic properties, which is particularly potent in the developing cells and adversely affects rapidly proliferating cells. Because of the specific cytotoxicity of SM, nitrogen mustard was widely employed in cancer chemotherapy [24]. Findings of various studies suggest that SM affects neurotransmission systems. It is relevant to the present study that SM targets both, the cholinergic system [11] and the signaling protein, PKC [27].

Despite the fact that neuroteratogens affect a variety of regions and innervations, making the ascertaining of the mechanism difficult, a typical finding is cognitive impairment related to specific regions: hippocampus-related behaviors in rodents and the analogous IMHV-related imprinting and their associated cholinergic inputs [16,30,31,43]. Pinpointing the synaptic components that are affected by the neuroteratogen may offset or reverse the defects. Towards this end, we studied prenatal exposure of mice to teratogens that act directly or indirectly on hippocampal function [25,30,42,43], and identified a defect in the signaling protein, PKC γ [25,43]. Initial studies replicated these finding in the chick IMHV-imprinting model [16]. Ascertaining the locus and mechanism underlying the behavioral deficits in mice enabled us to reverse in the mouse both the synaptic and cognitive dysfunction through therapies targeting septohippocampal cholinergic pathways [31,42]. Consequently, it appears pertinent to apply this model on SM neurobehavioral teratogenicity. However, the rodent model suffers from ineluctable methodological confounds stemming from drug effects on the maternal-fetal unit, maternal physiology or mother-offspring interactions [28]. To mitigate these confounds, it is beneficial to ascertain the major effects on chicks and then apply these findings to a mammal (rodent) model. The advantages of the chick are numerous, making it almost an ideal model: drugs can be administered in defined doses without consideration of maternal-fetal or maternal-neonatal interactions, maternal toxicity or pregnancy stage-related pharmacokinetic changes. Parallel to the hippocampus and its role in cognitive behaviors in mammals, avian species possess the left intermedial part of the hyperstriatum ventrale (IMHV), which is responsible for imprinting behavior [3,5], the tendency of the chicks to follow the first object they encounter; normally the mother [18], although it can be assessed with artificial objects [3]. Corresponding to the rodent hippocampal behaviors—cholinergic innervation model, imprinting involves the cholinergic innervation [33]. The IMHV which contains various neurotransmitter innervations has a particularly high concentration of muscarinic cholinergic receptors [6], and the release of acetylcholine in this region elicits the imprinting stimulus [33]. Most pertinent to the present study, in the chick, the IMHV PKC γ appear to play a pivotal role in the mechanism of imprinting behavior; similar to mammalian hippocampal function [1,34]. Indeed, we have recently established a chick model for the neurobehavioral teratogenicity of nicotine heroin and

chlorpyrifos, all agents that act directly or indirectly on cholinergic innervation. The exposed chicks showed extensive defects in their imprinting behavior paralleled alterations in PKC isoforms [16].

The current study was designed to establish a chick model for SM neurobehavioral teratogenicity similar to the one recently established for nicotine, chlorpyrifos and heroin [16]. The key questions are whether prehatch exposure to SM can affect behavioral development and whether the behavioral defects may be related to defined synaptic alterations. Consequently, chick embryos were exposed to SM prehatch and after hatching, then they were tested for the IMHV related filial imprinting and concomitant alterations in PKC γ , which may have a mechanistic role in imprinting.

2. Materials and methods

2.1. Precautionary steps

The work with SM was conducted according to the precautionary procedures of the Ministry of Labor and Welfare, Section of Labor Inspection, and the Department of Safety of The Hebrew University. All stages of experiments were performed in a continuously operated fume hood with an air flow of 125 ft/min. Investigators wore lab coats, three layers of gloves including one with long sleeves to cover the arm, protective glasses, and masks. All contaminated glass and disposables were neutralized in a solution containing 1:1 1N NaOH:ethanol. The eggs were kept in the hood 3 days after exposure.

2.2. Teratogen treatment

Fertile heterogeneous stock eggs (60 ± 3 g) of the Cobb I chicken broiler strain (*Gallus gallus domesticus*) were obtained from a commercial source and placed in an incubator. To administer the SM, a hole was drilled in the chorioallantois end (pointed end) of the shell, at least 24 h before the first injection, and was covered with an adhesive silicone glue cap (Medical Type A, Dow Corning, Midland, MI). Sulfur mustard, dissolved in ethanol, was then administered on incubation days (ID) 2 and 7 (after 24 and 144 h of incubation, respectively), the period of time in which most of the brain structures, especially the IMHV develop [12,21]. The doses employed were: 0.0017, 0.005, 0.017, 0.05, 0.17, 0.5, 1.7, 5.0, and 17.0 $\mu\text{l}/\text{kg}$ of egg; control eggs received the equivalent volume (50 $\mu\text{l}/\text{kg}$ of egg) of vehicle. We conducted preliminary studies on the effects of the ethanol vehicle as compared to untreated eggs, so as to ensure that it did not elicit developmental toxicity. We were careful to use more than one order of magnitude less ethanol than that required for teratogenic effects in previous studies on ethanol teratogenicity in the chicks [23].

Furthermore, in the present study, animals from the control group receiving ethanol did not show any neurobehavioral differences compared to the un-injected (intact) control chicks.

The eggs were incubated (Model 1202 Incubator, G.Q.F. Manufacturing Savannah, GA) at 37.5 °C with 50–60% humidity and were candled on incubation days 5 and 15. Fourteen to twenty-four hours posthatch, the chicks were trained to follow an imprinting object and were tested for imprinting performance. Afterwards, the left IMHV was removed and taken for Western Blot analysis of basal level of PKC γ .

2.3. Induction and testing of imprinting

As modified [16] from earlier descriptions by McCabe et al. [19], the imprinting apparatus contained three 20-cm diameter running wheels with the sides covered in black PVC, permitting the chicks to see only forward or backward. The imprinting objects were an illuminated red box or a blue cylinder (both 15×10×18 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. Imprinting training and testing were both assessed with this apparatus.

The imprinting procedure was modified from the one developed by McCabe et al. [19]. 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 [17]. Each chick was tagged and then transferred to an individual dark, enclosed wooden chamber warmed to 30 °C, where they were physically and visually isolated from each other. Fourteen to twenty-four hours posthatch, the chicks underwent 45 min of “priming,” 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 or away from the imprinting object were recorded by a self-made computerized system. 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 rotating 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, and the running away (backward)

from the objects was recorded by the apparatus. The imprinting is expressed as preference scores where:

Preference score

$$= \frac{\text{Running toward the training light}}{\text{Running toward the training light} + \text{Running toward a novel light}}$$

Running from the light is deducted from the score for running toward the light. The preference score is a measure of the strength of learning; assessing the selective preference that arises from the experience of the training object. The expected range of the preference score is 0.0–1.0, where 0.5 indicates no imprinting.

2.3.1. Locomotor activity

Because locomotor activity influences the number of wheel rotations, we also assessed locomotor activity of the different experimental groups, as the number of rotations of the wheel made by the chick during training (forward and backward). The locomotor activity during imprinting testing (both training and novel lights) is expressed as the total number of rotations of the wheel (forward and backward) made by the chick.

2.4. Quantitative assessment of the PKC γ isoform

After behavioral testing, the brain was removed and the left IMHV (2.5–3.0 mg) was quickly dissected according to the procedure described by Horn et al. [13], and frozen in liquid nitrogen. Basal levels of the PKC γ in the cytosolic and membrane fractions of the IMHV were assayed using published protocols modified [25,30,43] from earlier techniques [7]. Briefly, the IMHV tissues 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 digested with 0.5% Triton X-100 (Sigma), then sedimented as already described, and the supernatant solution was frozen.

Western blot analysis was carried out by gel electrophoresis of 10–15 μ g aliquots of cytosolic and membrane protein using specific primary antibodies for each PKC subtype, the PKC γ antibody 36G9, recognizes chick PKC γ [34], was specially produced for this study (E.A. Van der Zee), and IgG HRP conjugated (Bio-Rad) secondary antibody, exactly as described earlier [25,43]. We did not assess internal standards of structural “housekeeping” proteins (α -tubulin or β -actin) because many neuroteratogens influence neuromorphological development [10] and consequently cause alterations in the expression of these cytoskeleton proteins, unrelated to the neurobehavioral effects linked to specific cell signaling pathways.

2.5. Data analysis

Data are presented as means and standard errors, with differences between treatments established by multivariate

Table 1

Lack of effect of pre-hatch exposure to SM on the activity level (wheel rotations) during training (left) and during testing (right)

Treatment (<i>n</i>)	Activity during training	Activity during tests
Control (22)	133±22	27±4
Mustard (33)	120±20	33±5

There were no statistically significant differences between control and mustard-exposed groups.

Data represent means and standard errors obtained from the same chicks as in Fig. 1.

ANOVA, and followed by the least significant difference test for post hoc comparisons between groups. χ^2 test was employed for the non-parametric data. Significance for all tests was assumed at the levels of $p < 0.05$. For convenience, some results are presented as the percent change from control values; however, statistical tests were always performed on the original data.

3. Results

Chicks exposed to SM in the present dose range appeared normal, devoid of visible congenital malformations. The rate of the common avian leg deformities in (spread legs), which represents a sensitive indication for dysmorphology, also did not differ from control level. As is shown in Table 1, their general locomotor (number of rotations) activity during imprinting training and imprinting testing was similar to that of control, which excludes potential confounding effects on activity in the imprinting evaluations.

Imprinting preference score in the control group was 0.72 (Fig. 1), well above the “no preference” score of 0.5 ($p < 0.001$). Pre-hatch exposure to SM decreased the imprinting score to 0.59% ($p < 0.05$).

Pre-hatch exposure to SM reduced membrane PKC γ in the IMHV by 22% ($p < 0.05$), while eliciting no net change in cytosolic PKC γ (Fig. 2).

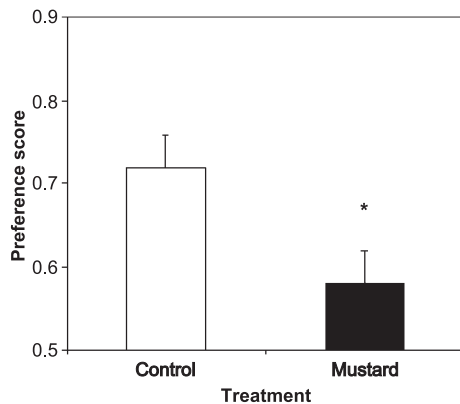


Fig. 1. Preference ratio in control chicks and chicks with pre-hatch exposure to SM. Data represent means±SEM obtained from 22 control and 33 mustard-exposed chicks. * $p < 0.05$ for the difference between the groups (ANOVA).

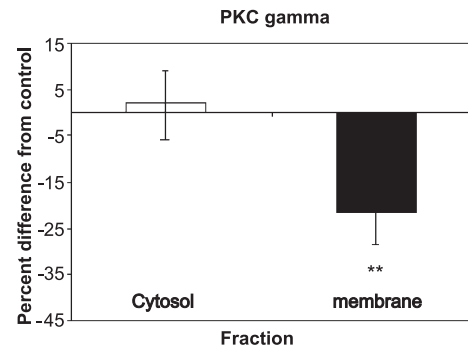


Fig. 2. Effect of pre-hatch exposure to SM on basal membrane and cytosolic levels of the PKC γ isoform. Data represent means±SEM obtained from 22 control and 33 mustard-exposed chicks. ** $p < 0.01$ for the difference from zero change.

Dose response could not be demonstrated for neither the preference ratio nor for membrane PKC γ (data not shown).

4. Discussion

Pre-hatch exposure to subtoxic doses of SM induces marked deficits in the IMHV-related imprinting behavior and concomitant alterations in membrane PKC γ isoform, suggesting that beyond its role as a blistering agent and cytotoxin; SM acts as a developmental neurotoxicant. Our results are in line with the known teratogenicity of mustard-related, chemotherapeutic agents [2,9] which are potent mutagenic compounds due to their DNA alkylating activity.

Mustard gas is used in chemical warfare [20,35] due to its reputation as a blistering agent, however, the mustard compound family is also known as a cytotoxic agent; effecting developing cells [8,20,35]. Because of this capability, nitrogen mustard was used for chemotherapy [24]. Indeed, chemotherapeutic agents often exert neuro-behavioral teratogenicity via their cytotoxic action [22]. It is not surprising that SM alters PKC, since in addition to its known role as a blistering agent, SM targets both, the cholinergic system [11] and the signaling protein, PKC [27]. The regulation of PKC activation by acetylcholine was demonstrated in our previous studies [4,16].

Just as in the mammalian hippocampus, the chick provides a brain region and innervation-specific model for neurobehavioral teratogenicity converging on signaling systems that regulate cognitive function. The essential difference is that in the chick, the perturbations occur in a well controlled environment, free of maternal confounds. In the chick, filial imprinting behavior, the tendency of the chicks to follow the first object they encounter, which is normally the mother [18], can also be assessed with artificial objects [3]. Furthermore, avian species, unlike rodents, recognize color and thus a variety of discrimination tasks based on the imprinting model can be carried out easily. Imprinting depends on the integrity of a specific structure, the left side of the IMHV [19] and the reliance of imprinting

on this structure recapitulates the dependence of learning and memory in the mammalian hippocampus [13,14], as established by biochemical, molecular, morphological, electrophysiological and lesioning assessments [5,19].

Furthermore, the relationship between synaptic function and behavioral endpoints is augmented by the fact that only the left IMHV stores the required imprinting information, whereas the right IMHV acts only as a temporary or “buffer” storage site [5,19].

In addition to the avoidance of maternal confounds, the avian model enables true comparisons to be made of the relative impact of neuroteratogens on brain development as compared to somatic growth, whereas mammalian models are highly dependent on potential adverse effects on maternal nutritional status, uteroplacental function or endocrine changes elicited in the mother. As the teratogen is delivered directly into the yolk sac, a strict relationship between insult and effect can be drawn in the absence of variables of maternal pharmacokinetics, which differ radically among species and with the stage of pregnancy. Moreover, rodents do not provide optimal maternal care to the defective neonates, which fail to provide the proper cues, and require an extended period of postnatal development before cognitive function can be assessed. On the other hand, the chick is entirely self-sufficient, and as shown here, can be tested for cognitive function shortly after hatching. Unlike the rodent, where the “litter effect” needs to be taken into account [29] each chick is a separate subject, so that large numbers of animals can be assessed for high-throughput screening. However, the chick model is insufficient for evaluation of neuroteratogens that require prior metabolic activation by the mother, which are excluded from the fetus by the placenta, or that share other attributes that are unique to mammals and not avian species. Accordingly, avian studies in concert with the mammalian (rodent) model, as carried out in our laboratory on various teratogens [16,30,43], are complimentary and provide an almost ideal control. Therefore, replicating the present study on SM in a rodent model represents the next obvious step.

Because of the homologies between the rodent and chick model in regional specificity, neurotransmitter pathways and cognitive outcomes after exposure to neuroteratogens, avian species are likely to provide valuable information about synaptic mechanisms that underlie behavioral deficits.

Beyond the biologic implications of our findings, there are important methodological considerations that will influence future studies. PKC is translocated from the cytosol to the membrane, where it is being activated, and therefore, the present demonstration of a decrease in membrane level of PKC γ provides an indirect indication for functional impairment. Evaluation of PKC isoforms by Western blot analysis provides an assessment only of the total number of molecules without telling us about their *function*. This is particularly true for PKC γ [16,25,43], where a phospho-specific antibody is not yet available. Even if the antibodies were available, these would be unable

to characterize the specific neurotransmitter-receptor-mediated component of translocation/activation as distinct from the more general pool of enzyme, as was shown for cholinergic innervation in our previous studies with mouse and chick models [16,43]. Accordingly, the study of agonist-induced translocation/activation of PKC isoforms after prehatch exposure to SM, using our recently established procedure, will provide valuable information regarding the identification of the specific neurotransmitter innervation that is mechanistically related to the SM-induced changes in the PKC.

The lack of dose response suggests, in the case of SM, that there is a threshold phenomenon in which defects occur after administering a very low dose, and the larger doses do not exacerbate the damage greatly. An extensive study using a large sample size is required to resolve the issue.

Reversal of neurobehavioral birth defects is increasingly feasible. We were able to reverse neurobehavioral teratogenicity in the mouse model by manipulating the regulating pathways [41], neural grafting [31] and nicotine therapy [4]. Similar models for the reversal of neuroteratogenicity in rodents were demonstrated, for example, fetal alcohol syndrome [32]. Consequently, the present findings may be applied in a model for offsetting or reversing the SM-induced behavioral birth defects. Although the time from hatching to the expression of imprinting is short, it is expected that reversal of the defects in our model can be done, since reversal of prenatally induced behavioral defects by acute therapy was recently demonstrated [44].

Previous studies have shown that topical iodine preparations are potent protectants against SM-induced skin lesions. We demonstrated that post-exposure treatment with iodine significantly reduce the skin damage caused by alkylating agents [37,38,40] and heat stimuli [36,39]. Although iodine is known for its cytotoxic and antiseptic properties, low doses might elicit protection against noxious stimuli. We may raise the hypothesis that the cytoprotective effect of iodine can also be applied to chemical- and irradiation-induced teratogenicity. Future studies with the egg and mammalian models will confirm this hypothesis.

In conclusion, the present study establishes a model for ascertaining the behavioral teratogenicity of SM and for the elucidation of the synaptic mechanisms that connect specific neural defects to adverse behavioral outcomes. Our identifying the specific cellular defects that represent the downstream common pathways mediating cognitive impairment after neuroteratogen exposure will facilitate future research on the development of interventions that enable reversal of neuroteratogenicity.

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