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Reversal of chlorpyrifos neurobehavioral teratogenicity in mice by nicotine administration and neural stem cell transplantation

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Abstract

Identifying the mechanisms underlying the adverse effects of developmental neurotoxicants enables the design of therapies that can potentially reverse neurobehavioral deficits in adulthood. We administered chlorpyrifos (CPF), a model organophosphate pesticide to pregnant mice and identified visuospatial deficits in adult offspring using performance in the Morris Maze. We then evaluated two strategies to reverse the effects, nicotine administration and transplantation of neural stem cells. Daily administration of nicotine prior to behavioral testing did not alter maze performance by itself, but completely reversed the deficits evoked by prenatal CPF exposure. Similarly, control animals grafted with neural stem cells in adolescence did not show any alterations in behavioral performance as adults, but the grafts completely reversed the effects of prenatal CPF treatment. This study thus provides a model for the development and application of both pharmacologic and cell-based therapies to offset the effects of neurobehavioral teratogens.

Keywords

Chlorpyrifos; Mice; Morris maze testing; Neural stem cell therapy; Neurobehavioral teratogenicity; Nicotine therapy; Prenatal exposure

INTRODUCTION

Over recent decades, there has been an alarming rise in the incidence of neurodevelopmental disorders, coincident with increased exposures to neurotoxic chemicals in the environment, resulting in what has been termed a “silent pandemic” [10]. Although most toxicologic studies focus on the mechanisms underlying the effects of individual toxicants, it is obviously critical to design therapies that may be used to offset the consequences of such exposures, which are likely to remain problematic, given the thousands of new chemicals released each year, few of

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which are ever tested for developmental neurotoxicity. In earlier work, we showed how nicotine treatment in adulthood could offset the adverse effects of developmental neurotoxicants that target septohippocampal cholinergic projections, achieving restoration of synaptic and behavioral function through a pharmacologic intervention that offsets the deficiencies in cholinergic function [2]. In addition to its direct cholinergic actions, nicotine causes release of many other neurotransmitters, thus enabling it to reverse deficits involving other transmitters, such as norepinephrine [1,31], dopamine [26], GABA [39] and glutamate [11]. Accordingly, nicotine therapy has been applied successfully in humans as well as in animal models for the reversal of cognitive dysfunction associated with surgical lesions or neurodegenerative disorders [23], and can be administered conveniently either by transdermal patches in humans [12] or by subcutaneously-implanted osmotic minipumps in animals [29,30].

Nevertheless, there are clear liabilities of continuous pharmacologic replacement of deficient cholinergic synaptic function with nicotine, including loss of effect due to desensitization, cardiovascular complications, and addiction liability. Consequently, it would be valuable to design ameliorative therapies that might actually achieve permanent reversal of synaptic and neurobehavioral deficits. To that end, considerable attention is now being paid to neural stem cells, which have the potential to replace or rescue damaged neural circuits and/or induce trophic processes that foster endogenous repair mechanisms [6,21]. We recently demonstrated the successful use of this strategy in reversing neurobehavioral deficits evoked by prenatal heroin exposure [5,14]. Here, we provide a comparative proof-of-principle of the pharmacologic vs. stem-cell approach to amelioration of neurobehavioral teratogenicity of a defined environmental neurotoxicant, the organophosphate pesticide, chlorpyrifos (CPF). Organophosphates represent nearly 50% of all insecticide use worldwide [7] and are undergoing increased restriction specifically because of their propensity to elicit developmental neurotoxicity. Even low prenatal exposures to CPF compromise cognitive function in children [22]; in animal models, CPF disrupts synaptic development and function, involving not only hippocampal cholinergic systems but a wide variety of regions and circuits [28].

In the present study, we exposed mice to CPF prenatally at doses devoid of adverse effects on viability or morphogenesis, and evaluated cognitive deficits in adulthood. We then demonstrated equivalent reversal of the deficits by nicotine therapy in adulthood, and by administration of neural stem cells in adolescence.

METHODS

CPF administration

The study employed heterogeneous stock (HS/Ibg) mice, since this strain is especially fertile even under exposure to various insults [18]. Four females and one male were housed in each mating cage and maintained under standard conditions of 24° C and a 12-hr light - dark cycle. Females were checked daily at 08:00. Those that had mated, as evidenced by the existence of a vaginal plug, were then housed with other pregnant females (date was termed as gestational day 1, GD1). Chlorpyrifos administration paradigms were based on our previous work with other teratogens [36,37]. In order to identify the maximum dose that allows normal pregnancy without fetal resorption or postnatal mortality, the pregnant mice were divided into several groups, each receiving either 1, 3, 5, 10 or 20 mg/kg/day CPF dissolved in DMSO vehicle, subcutaneously, on gestational days (GD) 9-18, the period in which most brain structures develop [25]. Controls received DMSO injections on the same schedule.

Beginning on GD18, each female was housed individually. Fostering of the offspring of CPF-exposed and control animals to controls was applied in approximately half of the litters, and took place within 24 h of birth. As there was no significant difference between fostered and

non-fostered treated pups, the results from both groups were pooled for presentation. The pups were weaned at postnatal day (PN) 25, segregated by sex and housed in groups of five. Only a one male and one female pup from each litter were used in each experimental group in order to prevent bias resulting from litter effects. Approximately equal numbers of males and females were used and sex effects were taken into account in the analysis.

Monitoring general development of mothers and offspring

Maternal factors, as well as offspring development, were monitored. Of the daily pre-injection maternal body weights, GD9, 12, 15 and 18 are presented to show representative trends (Fig. 1A) and on PN1, 7 and 14; maternal caretaking, measured by pup retrieval, was assessed on PN3 and 7. In the offspring, we monitored the sex ratio, litter size litter survival, and the development of body weight from birth to PN50. We also noted the ages of development of the righting and startle responses, appearance of fur, and eye and ear opening. To evaluate maternal caretaking, 6 pups were placed in their cage (17.5×28.0 cm) at the maximum possible distance from each other; we recorded the amount of time needed for the mother to retrieve the pups and return them to the nest. To evaluate the righting response, animals were placed on their backs and we measured the ability to right themselves within 20 sec. To evaluate the startle response, we produced a sudden sound of approximately 100 dB immediately above the litter.

Morris water maze evaluation

The Morris water maze was conducted in an adaptation of the apparatus originally developed for rats [19], with testing occurring on PN75. The test was performed in a round tank 87 cm in diameter, containing water (24°C) rendered opaque by adding powdered milk. A platform, $8 \times 10 \times 9$ cm high, was placed 1 cm below the surface of the water. Mice were given two blocks of four trials on each day for four consecutive days [20]. On all trial days, each mouse was given 60 sec to swim, find the platform and climb onto it. The mouse was then left on the platform for 20 sec until placed in the water for another trial. Mice that failed to find the platform within 60 sec were removed from the water and placed on the platform for 20 sec until the next trial. A computerized video system was used to monitor and analyze the behavior in the maze. Performance was evaluated as the mean latency across the eight trials on a given day.

To control for possible non-hippocampal and/or activity-related factors, two control tests followed the Morris place test: a. Visible platform test (neural stem cells transplantation) – the mice were given four trials in which the platform was raised above water level; mice that required more than 40 sec to reach the visible platform on any trial were excluded from further analysis [33]. b. Swimming speed (nicotine therapy) – the platform was removed and swimming speed over the missing platform and in the rest of the Morris maze was monitored in four 60 sec trials.

The Morris test established the 3 mg/kg/day dose of CPF as eliciting the greatest behavioral impairment, and the reversal studies therefore employed this dose.

Nicotine therapy

To assess the ability of nicotine to reverse the Morris Maze performance deficits, animals received an intraperitoneal injection of either 0.33 or 1 mg/kg of (-)-nicotine- hydrogen - (+) - tartrate (Sigma, Rehovot) or saline vehicle 20 min before each of the four daily Morris test sessions [40]. These doses are equivalent to 0.1 and 0.3 mg/kg of nicotine free base, respectively.

Neural stem cells therapy

To assess the therapeutic effect of neural stem cells, on PN35, animals received either transplantation of neural stem cells or DMEM control medium, and were tested for maze performance on PN75.

Derivation of neural precursors and growth of neurospheres—We use standard procedures [3,8] as described by in our previous publications [5,14]. Neural stem cells were derived from newborn (PN1) HS/Ibg mice. Briefly, cerebral cortices were minced, dissociated by 30 min incubation with Trypsin and mechanical dissociation into single cells using a 5 ml pipette. The pellet was suspended in serum-free F12/DMEM medium, and then incubated at 37°C and 5% CO₂ for up to 7 days. The cells were supplemented daily with growth factors; 10 ng/ml basic fibroblast growth factor-2 and 20 ng/ml epidermal growth factor (both from Peprotech Asia, Israel). The viability of the cells was monitored throughout the incubation using Trypan Blue. Under these conditions, most cells died and approximately 0.2% of cells proliferated into clusters of small round cells that grew into floating spheres. After three days of incubation, the cells were sedimented at 800 rpm for 8 min and were resuspended in half of the original volume and incubated for three more days. As already described, these spheres consisted mainly of PSA-NCAM+, nestin+, and NG2(−) cells that generated GFAP+ astrocytes, GalC+ oligodendrocytes and few neurofilament+ neurons upon differentiation [4, 8].

On transplantation day, 5-7 days old spheres were dissociated by trituration with 200 µl Accutase (Sigma), followed by 10 min incubation at 37°C in 5% CO₂ to dissociate the spheres into single cells while minimizing cell death [34]. After being washed with 5 ml phosphate-buffered saline, cells were incubated with 1mM CM-Dil cell tracker (Invitrogen, Jerusalem, Israel), for 5 min at 37°C, and then for 15 min at 4°C [16]. The cells were triturated and washed with buffer and counted.

Sphere transplantation—Animals were anesthetized with an intraperitoneal injection of 80 mg/kg pentobarbital and placed in a stereotaxic apparatus. We then injected approximately 50,000 cells in a volume of 5µl using a 10µl Hamilton syringe, delivered into each hippocampus at the following coordinates: 1.8 mm posterior to bregma, ±1.5 mm lateral from the midline and 1.7 mm below calvarium [32,38]. DMEM media was chosen as the control solution. Because Dil-labeled cells that die might transfer the cell tracker to neighboring host cells [15,27], we ran two additional control groups: 1) animals that were transplanted with CM-Dil-labeled, adult frontal cortex-derived cells certain to die in the host brain and, 2) animals that were transplanted with CM-Dil-labeled adult subventricular zone-derived neural stem cells, destined to live.

Evaluation of the transplanted cells in the host brain—The fate of the transplanted cells was evaluated in sample groups of offspring. Twenty-four hours after behavioral testing, the mice were anesthetized with an overdose of pentobarbital and perfused transcardially with 4% paraformaldehyde (Gadot, Netanya, Israel). The brains were then removed and postfixed overnight at 4°C in 4% paraformaldehyde, cryoprotected in 30% sucrose for an additional 24 hours and finally deep frozen in liquid nitrogen and stored in -70°C. The entire hippocampal region was sliced into coronal 10µm frozen sections, using cryostat (Leica, Wetzlar, Germany). Every sixth section was mounted. Dil-positive cells were identified with fluorescent microscopy (Olympus, NY).

Statistical analysis

Data are presented as mean and standard errors, with differences between treatments established by multivariate ANOVAs incorporating all variables (prenatal treatment, postnatal

treatment, sex, day of testing for the Maze performance), followed by Tukey test for post hoc analysis; where variance was heterogeneous, data were log-transformed for the statistical analyses. The global ANOVA did not identify any significant interaction of sex with treatment and accordingly, results from males and females are shown combined, although sex was retained as a factor for the statistical evaluations. Significance for all tests was assumed at the level $p < 0.05$.

RESULTS

Dams given all but the highest dose of CPF showed normal body weight gain during pregnancy and nursing (Fig. 1); their maternal behavior as assessed by pup retrieval test was also normal (data not shown). At birth, CPF did not affect litter size or sex ratio (data not shown). Dams given 20 mg/kg/day, all died before delivery. CPF-exposed offspring did not show any significant changes in body weight development from newborn to young adult (Fig. 2A), nor were there significant differences in standard developmental landmarks (Fig. 2B).

In adulthood (PN75) animals exposed to CPF prenatally showed significant deficits in Morris Maze performance, with a peak effect at 3 mg/kg CPF (Fig 3). For example, on the third testing day, the 3 mg/kg CPF offspring took 84% longer than control to reach the platform ($p < 0.002$). Consequently, the 3 mg/kg dose was employed in the subsequent reversal studies.

By themselves, nicotine injections (DMSO+nicotine) had no effect on Morris Maze performance but nicotine at either the low or high dose reversed the deficits evoked by prenatal CPF exposure (Fig. 4A). The differences in latency did not reflect alterations in swimming ability (Fig. 4B).

In the immunocytochemical studies designed to evaluate the fate of the transplanted cells (Fig 5), Dil-labeled neural precursors were identified in the brain of the grafted animals, mainly in the regions near the lateral ventricles and the cerebral cortex. Fig. 5A shows Dil-labeled transplanted cells near the lateral ventricle. No Dil-labeled cells could be identified in any of the groups that were not grafted with these labeled cells. In the study designed to address the concern of non-specific Dil staining, no Dil-labeled cells were found in the control group transplanted with Dil-labeled adult cortical cells (Fig. 5B) suggesting that the dead adult cells did not “spill” Dil to be uptaken by neighboring cells. On the other hand, Dil-labeled neural stem cells derived from the *adult* subventricular zone migrated extensively and could be seen in great numbers throughout the brain, shown here along the corpus callosum (Fig. 5C) suggesting that Dil labels both young and adult cells, specifically, directly and that concern about inadvertent labeling due to spillage and subsequent uptake, is not relevant in the present investigation.

In the behavioral studies, mice exposed prenatally to CPF and receiving the sham transplantation procedure (CPF+sham) again displayed impaired Morris water maze performance (Fig. 6). By itself, transplantation of neural stem cells (DMSO+NSC) had no effect, but transplantation reversed the deficits evoked by prenatal CPF exposure (CPF+NSC vs. CPF+sham, $p < 0.0001$). The visible platform test showed no difference between groups (data not shown), thus excluding the contribution of motor, visual and other non-hippocampal factors to the prenatal CPF-induced deficits or their reversal by transplantation.

Discussion

Our results indicate that neural stem cells given in adolescence can completely reverse neurobehavioral deficits evoked by prenatal CPF exposure, equivalent to the ameliorating effects of nicotine administration delivered in adulthood. The demonstrable effect of the stem cell therapy on behavioral performance, weeks after delivery of cells, strongly suggest that a

permanent reversal of CPF-induced deficits. These findings thus provide a proof-of-principle of the ability of this therapy to achieve a lasting amelioration of neurodevelopmental damage.

Although the majority of studies of CPF-induced developmental neurotoxicity have been conducted in rats, establishing a parallel model in mice has clear importance not only for the ability to isolate and use neural stem cells and related tools, but also for the design of future work on gene-environment interactions. The effects of neonatal CPF treatment on behavioral endpoints in mice were reported recently [24] and the present study extends the mouse model to include prenatal exposure regimens. In particular, we were able to identify the threshold for maternal toxicity and to show that neurobehavioral deficits are evoked at much lower doses that do not compromise maternal, fetal or neonatal viability, and that are not dysmorphicogenic. Parallel to the earlier findings in the rat, we found a nonmonotonic dose-effect curve on behavior [17]; this is likely due to the positive trophic effects of cholinergic hyperstimulation, resulting from cholinesterase inhibition at higher doses, as discussed earlier [17]. Similarly, although neonatal CPF exposure in rats and mice produce sex-selective neurobehavioral deficits [24,28], exposures begun in midgestation target both sexes equivalently, as found here in mice and previously in rats [13].

Based on the known targeting of hippocampal cholinergic pathways by developmental exposure to CPF [28] and the role of these projections in Morris Maze performance, the ability of nicotine to reverse the deficits was not unexpected; indeed, we showed earlier that the effects of prenatal phenobarbital exposure, which target these same projections, are likewise reversed by nicotine therapy in adulthood [2]. By implication, the lasting repair wrought by the neural stem cells is likely to involve the same circuits. However, stem cell effects are not limited to cholinergic projections, nor to the hippocampus. Indeed, the development of procedures for the production of neural stem cells in various laboratories, including ours [5,14], has led to therapeutic application in animal models of a wide spectrum of brain defects, including Parkinson's disease [6,35] and cognitive disorders [5,14,21]. The rationale behind the use of neural stem cells for the reversal of neurobehavioral birth defects, is that the cells will differentiate in a region-specific manner [9], dictated in part by the specific sites at which deficits are present [4]. More recently, stem cell therapies were shown to induce the formation of endogenous neural precursors in the host brain [5]. These findings suggest that stem cell therapies will be able to reverse multiple types of neurodevelopmental deficits; this is particularly important for toxicants like CPF, that target a wide variety of neurotransmitter systems and neural circuits. Future work should thus concentrate on whether the stem cell approach can reverse, for example, CPF-induced serotonergic deficits that contribute to impaired emotional and appetitive behaviors [28].

In conclusion, we were able to reverse CPF neurobehavioral teratogenicity through two different strategies, one involving nicotine therapy in adulthood, and the other, neural stem cell treatment delivered in adolescence. Although nicotine therapy is more facile for clinical application, it requires lifelong treatment with an agent known to be involved in cardiovascular complications, carcinogenesis and addiction; further, its actions are directed primarily toward cholinergic function and only secondarily to the actions of other neurotransmitters, thus limiting its potential applicability. Neural stem cell therapy, on the other hand, represents a one-time treatment that produces potentially permanent reversal of neurobehavioral deficits, but has substantial procedural limitations that place it much further from immediate clinical application. Recent developments, including adult-derived stem cells, techniques to minimize immune rejection, and delivery via peripheral injection, are all likely to contribute to further advances for this approach.

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Abbreviations

ACh	acetylcholine
ANOVA	analysis of variance
CPF	chlorpyrifos
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
GalC	galactosylceramidase
GD	gestational day
GFAP	glial fibrillary acidic protein
HS	heterogeneous stock
i.p.	intraperitoneal
NG2	proteoglycan NG2
NSC	neural stem cells
PSA-NCAM	poly-sialated neural cell adhesion molecule
PKC	protein kinase C
PN	postnatal day
SC	subcutaneous

Fig 1.
 Effect of CPF treatment (1,3,5, 10 or 20 mg/kg/day) on maternal body weight.
 GD = Gestational Day, PN = Postnatal Day.
 Dams given 20 mg/kg/day died by GD19. No maternal death occurred in any of the lower doses.

Fig. 2.

Effects of prenatal CPF exposure (1,3,5 or 10 mg/kg/day) on general parameters of pup development: body weight (A), and neurological and morphological developmental landmarks (B).

Data represent means and standard errors.

None of the differences were statistically significant.

Abbreviation: PN = postnatal day.

Morris Maze - Dose response

Fig 3.

Dose response (1,3,5 or 10 mg/kg/day) for the effect of prenatal CPF exposure on Morris-Maze performance, determined as the latency to reach and climb on the platform. ANOVA indicates significant deficits caused by CPF ($p < 0.003$ overall, individually significant at 1 and 3 mg/kg). Sample sizes were: Control – 34 animals; 1 mg – 22; 3 mg – 18; 5 mg – 14; 10 mg – 21.

Fig. 4.

A. Effects of prenatal CPF exposure (3 mg/kg/day) and subsequent nicotine therapy on Morris Maze performance: (A) latency to reach and climb on the platform; (B) Swimming speed.

DMSO – Sham, control animals treated with DMSO prenatally and treated with control solution before Morris maze testing (N=21).

DMSO – Nicotine, control animals treated with DMSO prenatally and treated with nicotine before Morris maze testing (N=25; N_{low}=10, N_{high}=15).

CPF – Sham, animals exposed prenatally to CPF and treated with control solution before Morris maze testing (N=21).

CPF – Nicotine (low dose), animals exposed to prenatally to CPF and treated with 0.33 mg/kg nicotine before Morris maze testing (N=13).

CPF – Nicotine (high dose), animals exposed to prenatally to CPF and treated with 1.00 mg/kg nicotine before Morris maze testing (N=15).

For clarity, the scores of the two different DMSO-nicotine dose groups are shown combined, although the separate doses were retained for the statistical analysis. Mice exposed prenatally to CPF (CPF+sham) required more time than controls (DMSO+sham) to reach the platform ($p < 0.002$). Nicotine injections by themselves (DMSO+nicotine) had no effect on latency but reversed the deficits evoked by prenatal CPF exposure (CPF+nicotine) ($p < 0.0001$ for comparison of the two doses of nicotine to CPF+sham). There were no significant differences in swimming speed.

Fig 5.

A. Dil labeled cells in the area near the lateral ventricles (red). B. Control for Dil specificity: Brain tissue of a mouse transplanted with adult cortical cells, pre-labeled with Dil. No Dil marked cells can be visualized. C. Control for Dil specificity: Transplanted neural stem cells derived from the adult subventricular zone are visualized in the host brain along the corpus callosum (red).

Fig. 6.

Effects of prenatal CPF exposure (3 mg/kg/day) and subsequent transplantation of neural stem cells on Morris Maze performance. Mice exposed prenatally to CPF (CPF+sham) required more time than controls (Control+sham) to reach the platform ($p < 0.004$). By itself, grafting of neural stem cells (Control+NSC) had no effect on performance but neural stem cells reversed the deficits evoked by prenatal CPF exposure ($p < 0.0001$ comparing CPF+NSC to CPF+sham). Sample sizes were: DMSO+sham – 10 animals; DMSO+NSC – 9; CPF+Sham – 16; CPF+NSC – 1