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The Sea Urchin Embryo, an Invertebrate Model for Mammalian Developmental Neurotoxicity, Reveals Multiple Neurotransmitter Mechanisms for Effects of Chlorpyrifos: Therapeutic Interventions and a Comparison with the Monoamine Depletor, Reserpine

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Abstract

Lower organisms show promise for the screening of neurotoxicants that might target mammalian brain development. Sea urchins use neurotransmitters as embryonic growth regulatory signals, so that adverse effects on neural substrates for mammalian brain development can be studied in this simple organism. We compared the effects of the organophosphate insecticide, chlorpyrifos in sea urchin embryos with those of the monoamine depletor, reserpine, so as to investigate multiple neurotransmitter mechanisms involved in developmental toxicity and to evaluate different therapeutic interventions corresponding to each neurotransmitter system. Whereas reserpine interfered with all stages of embryonic development, the effects of chlorpyrifos did not emerge until the mid-blastula stage. After that point, the effects of the two agents were similar. Treatment with membrane permeable analogs of the monoamine neurotransmitters, serotonin and dopamine, prevented the adverse effects of either chlorpyrifos or reserpine, despite the fact that chlorpyrifos works simultaneously through actions on acetylcholine, monoamines and other neurotransmitter pathways. This suggests that different neurotransmitters, converging on the same downstream signaling events, could work together or in parallel to offset the developmental disruption caused by exposure to disparate agents. We tested this hypothesis by evaluating membrane permeable analogs of acetylcholine and cannabinoids, both of which proved effective against chlorpyrifos- or reserpine-induced teratogenesis. Invertebrate test systems can provide both a screening procedure for

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mammalian neuroteratogenesis and may uncover novel mechanisms underlying developmental vulnerability as well as possible therapeutic approaches to prevent teratogenesis.

Keywords

Acetylcholine; Cannabinoids; Chlorpyrifos; Development; Dopamine; Neurotoxicity; Organophosphate pesticides; Reserpine; Sea urchin; Serotonin

INTRODUCTION

Although developmental neurotoxicity represents one of the major concerns for exposures to environmental contaminants [26,32,75,76], fewer than 10% of all chemicals are ever screened for neurotoxicity [41], whereas a much higher proportion will ultimately prove to be neurotoxic either directly or through indirect mechanisms [9]. In recent years, attention has been focused on pesticides in light of the ubiquitous exposure of human populations [78] but relatively few compounds have actually been tested relative to the total number of pesticides in use [46,78]. Chlorpyrifos and other members of its class, the organophosphate insecticides, provide a critical example of this problem. Because these agents elicit their systemic toxicity through inhibition of acetylcholinesterase, it was originally thought that this mechanism provides a predictive index for the outcomes of developmental exposures, whereas it is now apparent that their ability to elicit developmental neurotoxicity involves multiple mechanisms, many of which operate at doses below the threshold for cholinesterase inhibition [24,29,51,63–65]. These observations indicate that, in order to screen organophosphates for their developmental neurotoxicity potential, developmental mechanisms and outcomes rather than surrogate markers of exposure should be investigated [78]. In that regard, it is critically important that, whereas the developing organism is resistant to the peripheral neuropathies typical of organophosphate poisoning in the adult [33], the developing brain is far more vulnerable at apparently subtoxic exposures [24,29,51,63–65].

Recent proposals for screening techniques focus on cell culture systems or lower organisms as an initial step that would enable subsequent mammalian studies to concentrate on those congeners with the highest potential for developmental neurotoxicity [28,64], an approach endorsed in a report from the Inspector General of the EPA [78] as well as by outside groups [27–29,64]. Because these techniques can focus on actual mechanisms involved in developmental neurotoxicity, they are inherently more focused than a surrogate exposure marker like cholinesterase activity. Indeed, we recently showed how neuronotypic PC12 cells can serve to detect neurotoxicant actions at various stages of neural cell differentiation, with screening capabilities extending to organophosphates, carbamates, organochlorines and heavy metals [67]. Nevertheless, cell culture models share a critical limitation in that they focus on direct neurotoxicant injury to neuronal cells and are problematic for assessment of cell-to-cell interactions that play critical roles in brain assembly and are likely targets for disruption by neurotoxicants. This is especially true for the organophosphates, which are toxic toward glial cells that provide metabolic support for developing neurons and that guide axonal projections to their appropriate targets during brain development [5,34–36,47,53,82,83]. Furthermore, these agents also alter the release of a variety of neurotransmitters that have important trophic functions during brain development, thus disrupting the differentiation and phenotypic fate of their target cells [31,45,77,79].

Although these more complex mechanisms can be elucidated to some extent in whole embryos, brain micromass cultures or mixtures of dissociated cells [47,57,82,83], approaches using lower organisms are also suited for such purposes [16,19,23,44,50,58]. In a number of studies, we evaluated the mechanisms involved in the adverse effects of organophosphates and a wide

variety of related and unrelated environmental contaminants on mammalian brain development using the sea urchin embryo [12,13,16–19,21–23,52,59]. Just as in the mammalian central nervous system, the sea urchin embryo develops the ability to synthesize, store and release neurotransmitters like acetylcholine, serotonin (5HT), dopamine and norepinephrine, and possesses homologous populations of receptors and their downstream signaling cascades, with appearance of all these components over a defined developmental period surrounding cell differentiation and morphological assembly [17,37]. Accordingly, the neuroteratogens that target specific neurotransmitter mechanisms or signaling cascades in the mammalian brain have counterparts in “pre-nervous” morphogenetic anomalies in the developing sea urchin [12,13,16–19,21–23,52,59]. The normal morphologic and biochemical features of sea urchin development are well characterized and visible with routine light microscopy, and the technique has been applied to developmental malformations evoked by a wide variety of embryotoxins, teratogens and neurotoxicants [12,16,18–20,23,38,52]. Each sea urchin produces numerous offspring that develop rapidly and with a specific sequence of morphological events and the adverse effects of toxicants produce a uniform phenotype [18, 19,23,52].

In our previous work with chlorpyrifos and other organophosphates in the sea urchin model, we focused on disruption of acetylcholine neurotransmitter systems as a target for underlying mechanisms and adverse outcomes, as distinct from cholinesterase inhibition per se [15,19, 23,52]. However, organophosphates also disrupt the development of other neurotransmitter systems that subserve trophic functions in the developing mammalian brain, notably 5HT and dopamine [1–4,30,67,68,70,72]. In the current study, we examined the role of these systems in the developmental outcomes evoked by chlorpyrifos exposure in three ways. First, we compared the effects of chlorpyrifos to those of reserpine, a drug that depletes monoamines by inhibiting the vesicular monoamine transporter [61] and as a consequence, disrupts mammalian brain development linked to the trophic effects of monoamine transmitters [6,10, 42,49,74]. Second, we evaluated the ability of different analogs of these neurotransmitters to offset the effects of chlorpyrifos and compared the results to their ability to prevent the adverse effects of reserpine. The analogs all possess arachidonoyl moieties to facilitate lipid solubility as required to penetrate into the embryo, and the biological activities of these agents have been verified in extensive earlier work [7,14,15,18,19]. Finally, we evaluated a broader spectrum of analogs aimed at additional neurotransmitters systems, including acetylcholine and cannabinoids so as to point to future directions for expanding the identified targets for organophosphate effects on brain development. It is important to note that the use of agents to prevent the adverse effects of chlorpyrifos not only provides information about the mechanisms underlying developmental disruption, but also points to the potential development of mechanistically-based interventions to offset neuroteratogenicity.

METHODS

Adult *Lytechinus variegatus* sea urchins were obtained from the Duke University Marine Laboratory (Beaufort, NC) and were transported to the University of North Carolina at Chapel Hill, maintaining a temperature of 2–4° C. They were then kept in circulating and filtered artificial sea water (ASW; 445 mM NaCl, 24.6 mM MgCl₂, 18.1 mM MgSO₄, 9.2 mM KCl, 2.36 mM NaHCO₃, 11.5 mM CaCl₂, pH 7.5) with their normal food (kelp) at 21° C. *Strongylocentrotus droebachiensis*, *Sphaerechinus granularis* and *Dendraster excentricus* sea urchins were collected using scuba and then used immediately for experiments or maintained in natural sea water up to a salinity of 3.3‰ at the appropriate temperature for each species (8–12.5° C for *S. droebachiensis* and *D. excentricus*; 21° C for *S. granularis*). The techniques for maintenance of these species of adult sea urchins and for insemination and handling of embryos and larvae in ASW have been described previously in detail, along with all the landmarks of normal development [20]. The eggs or sperm were obtained by injecting the ripe animals with

0.2–4 ml (depending on the size of the animals) of 0.55 M KCl; some animals were re-injected after about 20 days for an additional harvest. The suspensions of unfertilized eggs (except for *D. excentricus*) were passed through a nylon mesh for elimination of the jelly coat and fertilization of the eggs was carried out using the combined sperm of 2–3 males for each female; for *D. excentricus*, the jelly coat was removed by washing in acidified (pH 5) ASW prior to filtration.

For the testing of neurochemicals, approximately 50–100 embryos or larvae were placed in each 1 ml well of a multiwell cell culture plate. The substances were added at various stages of development from the initial cleavage divisions, or more commonly at mid-blastula 2 or early gastrula 1 stages [19,23] and were then present continuously thereafter. The effects on embryonic and/or larval phenotypes were documented by digital photography using a Leitz Orthoplan or Olympus Universal Research microscope. Imaging began at 2–4 hr after the start of treatment, with image capture occurring at appropriate times to document treatment effects, by an observer blinded to the treatments. Chlorpyrifos (Chem Service, West Chester PA) and reserpine (Sigma Chemical Co., St. Louis MO) were tested as developmental disruptors, with concentrations based on earlier studies showing teratogenic effects in the sea urchin by chlorpyrifos [19,23], or depletion of monoamines and impaired growth in *Tetrahymena* for reserpine [8]. The neurotransmitter analogs used for amelioration of the effects of chlorpyrifos and reserpine were chosen on the basis of their actions on specific receptors and/or their lipophilicity, as required to penetrate the hyaline barrier that impairs the entry of hydrophilic substances into the embryo as well as the membrane barriers within the embryo [52]. For serotonin, we used 5HT HCl (Sigma Chemical Co.), the hydrophilic derivative, N,N,N-trimethyl 5HT (5HTQ, Sigma Chemical Co.) and the lipophilic congener, arachidonoyl serotonin (AA-5HT, synthesized in the laboratory of Dr. V.V. Bezuglov); for dopamine, we tested the lipophilic derivatives, arachidonoyl dopamine (AA-DA, Dr. Bezuglov) and arachidonoyl tyramine (AA-TA, Dr. Bezuglov); for acetylcholine, we evaluated *l*-nicotine bitartrate (Sigma Chemical Co.) and the lipophilic congener, arachidonoyl dimethylaminoethanol (AA-DMAE, Dr. Bezuglov); and for cannabinoid systems, we utilized arachidonoyl vanillylamine (AA-VAN, Dr. Bezuglov) and arachidonoyl ethylene glycol (AA-EG, Dr. Bezuglov). Stock solutions of the hydrophilic agents (5HT, 5HTQ) were prepared in distilled water, whereas the lipophilic agents were dissolved in methanol, ethanol or dimethylsulfoxide [15,19,23,52], with the final vehicle concentration in the ASW medium ranging from 0.1–0.2%. We chose a variety of vehicles so as to enable us to verify that none of the vehicles had any direct actions by themselves, and to assure that the choice of vehicle did not influence the outcome from chlorpyrifos or reserpine exposure or for any of the amelioration treatments. The control groups for each study always contained the same vehicle or mixture of vehicles and in no case did any of the control groups show differences in development from each other or from the established morphological landmarks or temporal relationships for normal sea urchin development [23].

Studies were conducted in all the sea urchin species, with similar results except where indicated. We examined thousands of embryos from each batch of fertilized eggs from each individual female. Because the same phenotype was seen for virtually all embryos at the doses tested, the results are given as representative photomicrographs (magnification 100–200×) from two of the species: *S. granularis* and *S. droebachiensis*. Within an experiment, each individual adult female provided embryos for all treatment groups so that comparisons of treatment effects could be made within the same cohort of embryos. Treatment effects were uniform across cohorts of embryos from all the individuals. The total numbers of females used to obtain the embryos were 25 for *S. granularis*, 23 for *S. droebachiensis*, 18 for *L. variegatus* and 9 for *D. excentricus*. Given that the thousands of embryos in each treatment group had virtually identical phenotypes and that malformed embryos were never seen in the control groups, statistical analyses were restricted to simply assessing the proportions of wells showing malformations

in the treated vs. control groups, regarding the embryos in a given well as a single sample. Using Fisher's Exact Test, all treatment effects described in the Results section were significant at $p < 0.0001$.

RESULTS AND DISCUSSION

In our previous work with chlorpyrifos in the sea urchins, *S. droebachiensis* and *S. purpuratus* embryos and larvae, we provided extensive documentation and images concerning the critical periods of vulnerability and concentration-effect relationships for adverse effects on development [19,23]. These included a scoring scale for the types of defects as well as quantitative analyses across thousands of embryos but since the phenotypic outcomes for any given treatment condition tend to be entirely uniform, the current results are presented as representative photomicrographs. In the current work, we examined several other species and found virtually identical effects of chlorpyrifos with the same threshold (2–5 μM) for all (data not shown). Accordingly, only a few pictures for effects of chlorpyrifos alone will be shown here to enable comparisons with reserpine and with therapeutic interventions. As discussed below, we also found anomalies for reserpine that resembled those of chlorpyrifos in some but not all developmental periods, and which responded similarly to many of the therapeutic agents. Notably, we did find a species difference in the sensitivity to reserpine: the threshold concentration for adverse effects in *S. granularis* (0.25–0.5 μM) was about ten-fold lower than in the other species (2–4 μM).

Chlorpyrifos

Even at high concentrations, addition of chlorpyrifos during the first few cleavage divisions has little or no effect on development up to the beginning of gastrulation [19,23], a result that we replicated in the present study (Figure 1A). With treatment initiated later, at or just before the mid-blastula 2 stage, chlorpyrifos elicited the same malformations we found earlier [19, 23], with all embryos displaying a uniform phenotype characterized by accumulation of pigmented cells in the larval cavity (blastocoele) and their extrusion through the animal pole, forming a “mushroom”-shaped larva (Figure 1B). Also like our previous results [19,23], we again found the greatest sensitivity to chlorpyrifos with exposure commencing at the late blastula stage (30 hr after fertilization), after which the sensitivity declines (data not shown).

Reserpine

In contrast to the limited effects of chlorpyrifos on early cleavage divisions, reserpine appeared to inhibit or, at higher concentrations, completely block cell division, resulting in the formation of abnormal blastulae (Figure 1A); the reserpine-exposed embryos extruded cells into the perivitelline space (a lethal phenotype) and were incapable of further development. Despite this difference in the response to early developmental exposure, when reserpine was introduced at later stages of development we found reserpine-induced malformations similar to those seen for chlorpyrifos [19,23], with the types of malformations shifting with stage of exposure in parallel for both agents. With administration of reserpine during the mid-blastula 2 stage, we found abnormal, pigmented cells, just as was true for chlorpyrifos, but with reserpine, pigmented cells were found throughout the embryo (Figure 1B). Also, like chlorpyrifos, reserpine elicited exogastrulation simultaneously with the appearance of pigmented cells. When reserpine was given later, at the early gastrula 2 stage, we saw the same progression of anomalies emerging by the pluteus stage, ranging from undeveloped arms at low concentrations, to absent arms and eventual block of development at progressively higher concentrations (Figure 2). Again, this mirrors the actions of chlorpyrifos and/or agents that interfere with functioning of acetylcholine systems [19,23,52], raising the possibility of convergent effects of different neurotransmitter systems on the same developmental outcomes; we will see later on how this comes about.

Amelioration by 5HT analogs

Our results showed that, although chlorpyrifos and reserpine differ in their actions at initial stages of development, with treatment begun at or after the mid-blastula stage, they eventually converge on a common set of anomalies. The initial cleavage divisions of the sea urchin embryo are controlled in part by 5HT mechanisms [12,20,60], so the fact that reserpine, but not chlorpyrifos, interferes with the divisions suggests that chlorpyrifos does not have adverse effects on 5HT function at this earliest stage, or at least not to the extent that reserpine does. In the developing mammalian brain, reserpine also interferes with neuronal mitosis but this effect occurs even in brain regions very sparse in 5HT projections [43,49] and can also be demonstrated in regenerating liver and tumors [25,40], raising the possibility that the actions of reserpine on mitotic events are separable from its effects on 5HT and/or other monoamines. Accordingly, to determine if the outcomes seen here in the sea urchin model are indeed related to 5HT depletion, we examined whether they could be prevented by coadministration of 5HT analogs (Figure 3). The lipophilic 5HT analog, AA-5HT, provided complete protection from the effects of reserpine introduced during the early cleavage divisions, whereas the less-lipophilic native neurotransmitter, 5HT provided only partial protection. The hydrophilic analog, 5HTQ, was not effective. Accordingly, the damage resulting from reserpine exposure at the initiation of embryonic development is most likely related to its interference with 5HT mechanisms, and it is then equally clear that chlorpyrifos does not share this particular effect. In turn, this implies that the early stages of effects of chlorpyrifos on 5HT systems in the developing mammalian brain are probably a consequence of disruption of neurodevelopmental mechanisms, rather than by interference with 5HT as a trophic factor that produces the disruption. As shall be seen later, however, this is not necessarily the case throughout development. Finally, the fact that 5HT itself has partial activity effectively rules out the potential artifact that the AA-5HT analog might work because of the arachidonoyl moiety acting as part of the arachidonic acid signaling cascade. In fact, arachidonic acid alone has no such effects (data not shown).

Given the convergence of effects of chlorpyrifos and reserpine on later-stage defects, our next objective was to determine the extent to which 5HT mechanisms participate in these, again using the strategy of prevention by AA-5HT. First, we conducted an extensive bidirectional concentration-response study, varying both the concentrations of reserpine and AA-5HT (Figure 4). By itself, reserpine at concentrations ranging from 2–16 μ M produced profound disruption of development, with the characteristic appearance of pigmented cells, mushroom-shaped larvae, and exogastrulation. As little as 10 μ M AA-5HT provided nearly complete protection against the lowest reserpine concentration, whereas progressively high concentrations of AA-5HT were required as the reserpine exposure was increased. Nevertheless, even at the highest reserpine concentration, 40 μ M AA-5HT was effective. Accordingly, we used this concentration in a parallel test of protection against chlorpyrifos and reserpine and found clear-cut protection against either agent (Figure 5); again, the impermeant analog, 5HTQ was ineffective.

This finding indicates that, when the adverse effects of chlorpyrifos emerge, they too can be reversed by activating 5HT systems, just as is the case for the monoamine depleter, reserpine. Accordingly, there are two distinct phases of developmental sensitivity to chlorpyrifos: little or no effect early in development (initial cleavage divisions) but later emergence of profound effects at the mid-blastula stage. This has its parallel in the effects of chlorpyrifos on the immature mammalian brain, where greatest sensitivity is noted at stages of neuronal development just at the point of differentiation into specific neurotransmitter phenotypes [39, 63,65,69]. Again, this includes prominent effects on 5HT systems in the mammalian brain [3,56,69,73]. However, the reversal of adverse effects by AA-5HT in the sea urchin model suggests that, during this phase, alterations in 5HT function by chlorpyrifos do affect the trophic

functions of this neurotransmitter, since this hydrophobic 5HT analog prevented the adverse morphological outcomes. In that sense, 5HT systems and the downstream events they mediate are a target for disruption by chlorpyrifos exposure, and simultaneously a participating mechanism by which subsequent disruption of other systems occurs.

Amelioration by analogs of other neurotransmitters

The protection of the effects of both chlorpyrifos and reserpine by 5HT analogs findings presents us with a conundrum. Reserpine depletes other monoamines besides 5HT [61], and chlorpyrifos is known to act on a variety of neurotransmitters besides 5HT, including the other monoamines and of course, acetylcholine [62,63,65]. Why, then, should simply replacing the function of 5HT with 5HT itself or AA-5HT reverse the adverse actions of either of these agents? To answer this question, we next pursued a series of parallel studies with analogs of dopamine, acetylcholine and cannabinoids, to see if agents targeting the receptors for these systems would exert protective effects similar to that of AA-5HT (Figure 5). The membrane-permeant dopamine congener, AA-DA provided some protection against reserpine as well as chlorpyrifos, albeit less so for the latter. Surprisingly, however, a similar catecholamine analog based on tyramine (which lacks the meta-hydroxyl group present in dopamine), AA-TA, proved to be extremely effective against the adverse effects of reserpine but not chlorpyrifos. We reinforced this unexpected finding by conducting a dose-response study and found demonstrable protection from reserpine with as little as 2.5 μ M AA-TA (Figure 6, top panel). There are two questions raised by these results. First, why is AA-TA much more effective than AA-DA, and second, why can AA-TA reverse the effects of reserpine but not chlorpyrifos? For the first question, there may be significant pharmacokinetic, metabolic or pharmacodynamic differences between AA-DA and AA-TA. The additional hydroxyl group on dopamine increases its hydrophilicity, which would render it less permeant than the corresponding tyramine analog; similarly, the native compounds have differing affinities for monoamine oxidase, so that AA-DA could be metabolically inactivated to a greater extent than AA-TA. However, given the large disparity in protective actions between AA-DA and AA-TA, a third alternative seems the most likely: pharmacodynamic differences in the mechanisms of action of the two agents. One possibility is that the attachment of the arachidonoyl moiety to dopamine and tyramine could change their relative affinities for dopamine receptors, although it is difficult to conceive why that should create a preferential effect for AA-TA. A second possibility seems more likely: the existence of neurotransmitter receptors for tyramine or its β -hydroxylated downstream metabolic product, octopamine. Receptors for these amines are notable in lower organisms, and it is now clear that, rather than just representing “trace” amines in the mammalian brain, receptors for tyramine and octopamine are present and active there, too [11]. Our findings for AA-TA and AA-DA thus point to a potential developmental role for these simpler amines and their receptors, an area deserving of future investigation.

The second issue, the ability of AA-TA to provide protection from reserpine, but not chlorpyrifos, points toward a further important difference between the two developmental disruptors. Whereas reserpine exerts its effects almost exclusively against monoamine systems, chlorpyrifos affects diverse neurotransmitter systems over and above 5HT or dopamine, most notably acetylcholine [62,63,65]. In our earlier work, we showed that AA-DMAE, which binds to nicotinic acetylcholine receptors and, dependent upon concentration, produces both stimulation and inhibition/desensitization (as do all nicotinic receptor agonists), can protect sea urchin embryos from the adverse effects of chlorpyrifos [19,23]. With the additional species in the current study, we repeated that finding: 40 μ M AA-DMAE prevented the effects of chlorpyrifos added at the mid-blastula stage and furthermore, also protected them from reserpine (Figure 5). The latter effect was entirely surprising, given the specificity of reserpine for monoamine systems, and accordingly, we again pursued this more extensively through a dose-response study (Figure 6, bottom panel), where a concentration of 20 μ M AA-DMAE

provided complete protection against reserpine. We also evaluated nicotine itself, which proved to be partially effective against reserpine, albeit less so than AA-DMAE, and relatively ineffective against chlorpyrifos (Figure 5). In studies with developing neuronotypic cells in culture, nicotine does provide partial protection against the adverse effects of chlorpyrifos on differentiation and oxidative stress [54,55], so the relative insensitivity here may again reside in pharmacokinetic differences such as metabolic or oxidative degradation of nicotine. Alternatively, the concentration of nicotine chosen here may not reside on the appropriate part of its biphasic concentration-response curve to achieve the comparable balance of receptor stimulation and inhibition/desensitization. Again, further studies are warranted to examine this possibility.

In a final set of experiments, we explored whether analogs of cannabinoid receptor agonists could also protect sea urchin embryos from the adverse effects of chlorpyrifos or reserpine given at the mid-blastula stage (Figure 5). Here, we found that AA-VAN provided effective protection from the actions of either chlorpyrifos or reserpine, and another analog, AA-EG was somewhat effective. Recent studies show that cannabinoids can partially protect adult rats from acute organophosphate intoxication, with the presumed mechanism of reducing acetylcholine release so as to lessen the consequences of cholinesterase inhibition [48]. That mechanism seems highly unlikely here, since AA-VAN worked against both chlorpyrifos and reserpine, and further interference with neurotransmitter release or actions would be expected to worsen the outcomes in the sea urchin embryos. Instead, all our results point in a different direction. We found that, regardless of originating mechanism of action, the effects of chlorpyrifos or reserpine could actually be prevented by a variety of agents acting on different transmitter systems: AA-5HT, AA-TA, AA-DMAE, AA-VAN. So, for reserpine, despite the fact that it acts by depleting monoamines, agents like AA-DMAE, nicotine and AA-VAN provided protection. For chlorpyrifos, although it acts on a wide variety of neurotransmitter systems, agents working through any one of the targeted systems can similarly reverse the adverse effects, at least in part. It is thus apparent that all these various activities converge on a shared set of downstream signaling elements that are essential for proper morphological assembly of the embryo. Indeed, for chlorpyrifos, we already know that actions exerted on these common signaling pathways, most prominently adenylyl cyclase/protein kinase A and protein kinase C, provide both a mechanism for disruption of development and a therapeutic target for preventing those effects [80,81]. In the current study, then, profound activation of any one of the inputs to these pathways may serve to offset the adverse effects mediated by other, parallel inputs to those pathways or by disruption of multiple inputs. At the same time, this provides information that may be essential to designing ameliorative therapies for mammalian exposure to organophosphates or other neurodevelopmental toxicants, i.e. regardless of underlying mechanism, certain neuroactive agents that share common signaling cascades, may prove to be effective against a wide variety of disruptors.

Conclusion

There are two essential findings from the current study. First, the adverse effects of chlorpyrifos on 5HT systems in the immature mammalian brain, and similar effects reported for other organophosphates [53,62,63,65–67,69,71,73], likely represent a downstream target of the primary disruption of neural cell differentiation by these agents; but at the same time, because of the neurotrophic role of 5HT, the effects of organophosphates on this transmitter system provide a mechanism by which more widespread developmental perturbations come about. Second, the fact that the effects of agents with disparate originating mechanisms of action can be offset by administering single neuroactive congeners that work on specific neurotransmitter systems points to therapeutic approaches that may be applicable to a wider variety of neurodevelopmental insults. We are currently exploring this possibility in a variety of test systems.

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Abbreviations

5HT	serotonin (5-hydroxytryptamine)
5HTQ	N,N,N,-trimethyl 5HT
AA-5HT	arachidonoyl-5HT
AA-DA	arachidonoyl dopamine
AA-DMAE	arachidonoyl dimethylaminoethanol
AA-EG	arachidonoyl ethylene glycol
AA-TA	arachidonoyl tyramine
AA-VAN	arachidonoyl vanillylamine (arvanil)
ASW	artificial sea water

Figure 1. Effects of chlorpyrifos or reserpine on the early development of *S. droebachiensis*: (A) exposures begun at the 2–4 cell stage (3.7 hr after fertilization) and images obtained at 22.5 hr after fertilization; (B) exposures begun at the mid-blastula stage (22.5 hr after fertilization) and images obtained at 34.7 hr after fertilization.

Figure 2.

Effects of reserpine on the development of *S. granularis* larvae, given at the early gastrula 2 stage (15.75 hr after fertilization); images were obtained at the mid-pluteus 1 stage (45 hr after fertilization). Note the dose-dependent progression of malformations: (0.5 μ M) almost normal development, (1 μ M) underdeveloped first pair of arms and absent second pair of arms, (2 μ M) no arms and hyperproduced chromatophores, (4 μ M) block of development before the end of gastrulation and accumulation of pigmented cells in the blastocoele.

Figure 3.

Protective actions of 5HT derivatives in *S. granularis* embryos. Reserpine was introduced simultaneously with the 5HT analogs at the two-cell stage (2 hr post-fertilization) and images obtained at the early prism stage (26.1 hr post-fertilization). Reserpine alone results in death of the embryos, whereas AA-5HT provides full protection for reserpine. 5HT and 5HTQ, which are progressively less permeable, provide correspondingly less protection.

Figure 4. Protective actions of AA-5HT against the effects of reserpine in *S. granularis* embryos. Reserpine and AA- 5HT were introduced simultaneously at the mid-blastula stage (13.75 hr post-fertilization) and images obtained at the late gastrula stage (24.1 hr post-fertilization). The dose-dependent adverse effects of reserpine and their prevention by AA-5HT are obvious.

Figure 5. Protection of *S. granularis* larvae against chlorpyrifos or reserpine. Agents were given at the mid-blastula 2 stage (15.75 hr after fertilization) and images were obtained at 23.1 hr after fertilization.

Figure 6.

Dose-dependent protection of *S. granularis* larvae against reserpine by AA-TA (top row) and AA-DMAE (bottom row). Agents were given at the mid-blastula 2 stage (13.75 hr after fertilization) and images were obtained at 26.5 hr (top) or 24.2 hr (bottom) after fertilization.