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Publication Date

2018

DOI

10.1016/b978-0-12-809405-1.00016-x

Peer reviewed

**DEVELOPMENTAL TOXICITY WITHIN THE CENTRAL CHOLINERGIC NERVOUS
SYSTEM [6490 Words]**

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Abstract

Neurons that use acetylcholine (ACh) in the developing brain make up an extremely important network that helps guide other systems during critical time periods of cell proliferation and differentiation. This important function and the variety of molecular targets for developmental neurotoxicants makes the central cholinergic system vulnerable to neurodevelopmental abnormalities that can result in functional deficits later in life. The alteration of brain development by toxicants such as anticholinesterase pesticides, lead (Pb), nicotine, and others is supported by many studies that demonstrate concomitant changes in a variety of cholinergic targets, which provide clues to potential underlying mechanisms for their neurodevelopmental toxicity. This chapter discusses the role of the cholinergic system in brain development, special vulnerabilities related to brain region and developmental time periods, and human, animal, and mechanistic data on the neurodevelopmental toxicity of pesticides and other environmental chemicals.

Key Words

Cholinergic

Neurodevelopment

Central Nervous System

Anticholinesterase

Pesticide

Neurotoxicity

Introduction

Acetylcholine (ACh) is among the oldest neurotransmitters identified, and its role in the adult and developing nervous system has been studied extensively for nearly a century. ACh is also among the oldest classical neurotransmitters from a phylogenetic perspective, and is found throughout the nervous system including some of the more primitive brain structures. Neuronal diversity and cytoarchitecture within the developing central nervous system (CNS) is achieved by complex cellular proliferation and differentiation guided by specific neurochemical cues. The early appearance of ACh and associated metabolic, receptor, and transport proteins suggests it also may serve a basic functional role in cell growth and differentiation. Likewise, the early differentiation of cholinergic neurons may serve as a template for the subsequent development of other cell types. The appearance of ACh and its receptors on non-nervous tissue also suggests a fundamental role for it in cell function and development.

Development and maintenance of the cholinergic system is extremely important in a number of neurological diseases and disorders. For example, the autoimmune disease myasthenia gravis causes changes in the steady-state levels and activity of skeletal muscle nicotinic ACh receptors, and degeneration of striatal cholinergic interneurons is a hallmark for Huntington's disease. In patients with Alzheimer's disease, the cholinergic system is a primary target ¹, and choline acetyltransferase (ChAT) polymorphisms in humans are a risk factor for the disease ². The cholinergic system is also targeted by many neurotoxicants, most notably the organophosphorus (OP) and carbamate insecticides that inhibit acetylcholinesterase (AChE). The developing brain is particularly vulnerable to neurotoxicants that have an impact on the cholinergic system because of the early appearance of ACh and cholinergic receptors, and their

fundamental role in cell proliferation and differentiation. A more detailed description of the developing cholinergic system can be found elsewhere ^{3,4}.

The developing nervous system is more susceptible than the mature nervous system to the neurotoxic effects of OP pesticides such as chlorpyrifos (CPF) ⁵⁻⁸. Perinatal exposure to CPF causes cognitive and behavioral deficits in experimental animal models ⁹⁻¹¹ and other neurodevelopmental effects as evidenced from both animal and human data ^{12,13}. These findings together with evidence of developmental neurotoxicity for other anti-AChE pesticides suggest that the developing central cholinergic system may be especially vulnerable to perturbation by environmental chemicals.

I. The Cholinergic System in CNS Development

The role of the cholinergic system in neurodevelopment has been studied extensively ¹⁴ and is a logical target for neurodevelopmental toxicants. Four major morphologic groups of CNS cell types have been suggested ¹⁵: (1) very large motor (25-45 μm), (2) large forebrain (18-25 μm), (3) medium (14-20 μm), and (4) small (8-16 μm). Alpha motor neurons in the brainstem and spinal cord are among the largest neurons, up to 500 \AA in diameter, whereas gamma motor neurons are among the smallest ⁴. Neurotransmitter receptors found on cholinergic neurons are activated by muscarine or nicotine, and hence are classified as muscarinic or nicotinic receptors, respectively. These structurally unrelated molecules belong to different super-families of genes and differ in their location, specificity for agonists and antagonists, and cellular responses mediated by their activation. Muscarinic receptors are the predominant cholinergic receptor in the CNS and they are abundant in smooth muscle, heart, and exocrine glands.

Muscarinic receptors are activated by ACh and blocked by atropine and other antagonists. Activation by ACh of muscarinic receptors is the first step in a G-protein-coupled signal transduction pathway that ultimately leads to cellular responses such as the modulation of K^+ , Ca^{2+} , or Cl^- channel permeability, the regulation of cell growth, and the switching on of transcriptional factors in the nucleus^{16,17}. These ACh receptors are important in many neurologic disorders, models of neuronal plasticity such as long-term potentiation (LTP), and the neurotoxicity of compounds such as OP insecticides. There are several subtypes. The M1 subtype predominates in the cortex and hippocampus, M2 in the cerebellum and thalamus, M3 at moderate density throughout, M4 in the striatum, and the M5 at very low density in the hippocampus and brainstem¹⁸.

The other type of cholinergic receptor is nicotinic. Nicotinic receptor-ion channel complexes are found in the peripheral nervous system and the CNS. There are several subtypes depending on various combinations of subunits that make up a pentameric channel complex. Two of the major types of receptor complexes in the CNS are described based on their electrophysiological properties¹⁹ and abundance²⁰. The first type is composed of $\alpha 4$ and $\beta 2$ subunits and is most abundant in the CNS, found primarily in the thalamus, midbrain, and brainstem. They bind nicotine with high affinity and exhibit slow-inactivating or type II currents. The second type is homomeric and composed of $\alpha 7$ subunits. This subtype has widespread distribution in the CNS and at neuromuscular junctions and has fast-inactivating type 1A currents associated with high permeability to Ca^{2+} . Nicotinic receptors have been studied extensively and their ligands have been reviewed elsewhere²¹.

Cholinergic receptors are expressed early in CNS development. Muscarinic receptors appear during the postnatal period in rats and are involvement in cell differentiation and process

extension. The general pattern of the ontogeny of muscarinic subtypes includes a rapid postnatal increase of m1 and m3 subtypes on the postsynaptic membrane, and presynaptic m2 and m4-receptors increasing at a somewhat slower rate³. Muscarinic receptors are important in the formation and elimination of synapse in the developing brain²², as well as many other aspects of brain development^{23,24}. Nicotinic cholinergic receptors appear very early as well. In fact, nicotinic mRNA appears at the time of the last mitosis of progenitor neuroepithelial cells, which is the earliest among all the receptors studied²⁵. Nicotinic antagonists have been shown to regulate the growth and growth factors of neurons during CNS development²⁶⁻²⁹, and there is concern for the effect of nicotine from cigarettes on neurodevelopment³⁰⁻³².

Cholinergic neurons in the CNS are generally of two types: interneurons or projection neurons. These neurons are widely dispersed throughout the brain, but five primary cholinergic systems have been identified³³: (1) neostriatum, (2) cerebral cortex and hippocampus, (3) magnocellular basal nucleus, (4) pontomesencephalic tegmentum, and (5) cranial nerve motor nuclei and nerves of the spinal cord. Intrinsic cholinergic neurons are found in the cerebral cortex, striatum, hippocampus, nucleus accumbens, and other areas where they exist primarily as interneurons or in proximity with non-neuronal tissue. The cellular morphology of cholinergic neurons is also diverse and varies from region to region within the brain. Cholinergic interneurons are often associated with the dopaminergic system, as is the case in the striatum. Some of these intrinsic neurons can form local circuits in the same regions that receive extrinsic cholinergic input. For example, in the hippocampus, cholinergic interneurons are present along with major innervation from the medial septum and the magnocellular basal nucleus. Cholinergic projection neurons arising from the basal forebrain and projecting to the cerebral cortex are extensive and play a primary regulatory role in this structure.

Other cholinergic neurons project to the diagonal band, prefrontal cortex, and thalamus. ChAT activity is the highest in the striatum, interpeduncular nucleus, and habenula, suggesting that these structures are major sites of cholinergic neurotransmission. Although the density of certain cholinergic neuronal groups may vary among species, the general organization of the cholinergic system is highly conserved. It may be predicted that during development, major regions of the brain containing cholinergic neurons are more vulnerable to anticholinergic agents. It is also clear that several regions of the brain containing cholinergic neurons would be predicted to be important for critical developmental milestones. For example, cholinergic neurons found within the basal forebrain facilitate the relay of information from the brainstem to the cerebral cortex³⁴, and hence the normal development of this system is critical for normal brain function. It should be noted that any assertion of overall differences in brain region toxicities may be biased by the brain regions studied the most versus those regions not studied due to lack of relevance to the particular study or limited accessibility for detailed analysis.

II. Vulnerable Time Periods of Developmental Neurotoxicity

There are critical times in nervous system development. For example, studies of the development of birdsong and human language have indicated that learning is much more effective earlier in life³⁵. Overall, anticholinesterase pesticides such as CPF have been shown to have effects on the nervous system throughout neurodevelopment (see <http://endocrinedisruption.org/prenatal-origins-of-endocrine-disruption/critical-windows-of-development/timeline-test/view-the-timeline>). For those studies showing brain regional differences in toxicity, this usually depends on the timing of exposure. For example, early

exposure (post-natal days 1-4) to CPF caused cell loss in the brainstem, whereas the damage shifts to the forebrain if the exposure is during post-natal days (PN) 11-14 ³⁶. Likewise CPF-dependent changes in cholinergic markers such as ChAT and hemicholinium-3 (HC-3) are highly dependent on brain region and timing of the exposure during development ³⁷.

One study found that mice exposed to CPF during different prenatal periods responded differently in changes in dopamine levels measured at PN14 and PN60, with more changes in the cerebral cortex than in the hippocampus ³⁸. Other studies have shown that neurobehavioral responses to CPF exposure during development differ depending on the timing of exposure and other variables such as sex ¹⁰. Evidence has also been presented that exposure to neurodevelopmental toxicants during the neonatal period of rapid brain development (“brain growth spurt”) confers sensitivity to, and potentiates the effects of, pesticide exposure during adult life ³⁹. These and other studies suggest that the effects of OPs are different depending on the timing of exposure during development; however, there is no clear evidence that there are time periods of neurodevelopment that are not susceptible to OP toxicity.

III. Functional Effects of Developmental Exposure to Anticholinesterases

The toxicity of anticholinesterase compounds have been studied extensively in occupational ⁴⁰ and non-occupational settings ⁴¹. Acute exposures to sufficient levels of these agents are usually lethal due to respiratory failure. Sublethal effects can include recurring seizures and neuropathies. The development of tolerance to OPs has been well characterized and the mechanism involves the down-regulation of cholinergic receptors after repeated sublethal exposures ⁴²⁻⁴⁴. There has been concern for the effects of chronic low-level exposure in humans, especially as humans may be exposed to low levels of these compounds in the home, in food,

and in occupational environments. Chronic exposure has the potential to cause brain pathology such as necrosis and apoptosis⁴⁵, and the clinical significance of these effects is at least suggested by epidemiological and animal studies. Many studies have suggested that low level exposure can alter cognitive processes that underlie memory⁴⁶⁻⁴⁸. This includes studies of the survivors of exposures to OP nerve agent chemical weapons during the Gulf War and Tokyo subway terrorist attacks^{49,50}. Many people exposed to these compounds complain of memory loss even in the absence of overt toxicity or significant cholinesterase inhibition. A poor correlation between cholinesterase inhibition and toxicity of many important OP insecticides has prompted research on alternative mechanisms of their actions.

Neuropsychological symptoms may be associated with acute exposures to OPs, or with exposure to these compounds during neurodevelopment that result in altered mental capacity later in life. The neurodevelopmental toxicity of pesticides that target cholinergic neurons, such as the OPs, has been suspected for many years, and the hazard identification and reduction of risk has been the focus of many studies^{47,51-54}. Children are especially vulnerable, whether exposure occurs during development and results in long-lasting effects, or if the effects are transient associated with the exposure during a specific time period⁵⁴. Developmental effects may occur in utero since it was found that proximity to OP and carbamate pesticide use during pregnancy was associated with cognitive deficits in 10 year old children⁴¹. Inner-city minority children exposed to higher levels of CPF scored 6.5 points lower on the Bayley Psychomotor Development Index and 3.3 points lower on the Bayley Mental Development Index at 3 years of age than children exposed to lower levels⁵⁵. Children in this cohort also exhibited deficits in working memory and IQ at 7 years of age⁵⁶. It should be noted that not all studies have found an association between OP developmental exposures and cognitive deficits⁵⁷. These and other

studies indicate there may be a link between pesticides that target the cholinergic system and neurodevelopmental effects in humans ⁵⁸⁻⁶⁰, but a causal relationship between these effects and selective, specific alterations of the development of cholinergic neurons in the CNS is difficult to establish. Given the evidence that OPs may have toxicities other than those related to AChE inhibition and their general effects on overall fetal growth ⁶¹, it is likely that developmental exposures to OPs also result in a more generalized effect on neurodevelopment across several neurotransmitter systems.

Animal studies support the evidence in humans that anticholinesterase pesticides alter neurodevelopment. Spyker and Avery ⁶² provided some of the earliest evidence that prenatal exposure to OPs causes behavioral effects in rat pups. Potential effects on the developing CNS were subsequently confirmed by animal studies that reported effects such as anencephaly, cerebellar hypoplasia, stunted growth of various brain structures, altered cholinergic and glutamatergic neurochemistry, and regional histopathology in the brains of neonatal mammals ⁶³⁻⁶⁵. Following early observation of histopathological effects from developmental exposure to OPs, the emphasis on mechanistic (discussed below) and functional behavioral studies emerged to ascertain clinical relevance of developmental effects. Deficits in spatial memory were observed in PN40-45 day guinea pigs after they were exposed to CPF prenatally ⁶⁶. Interestingly, the behavioral effects in this study were associated with morphological changes in the striatum and amygdala. Brain morphological changes were also seen in children exposed prenatally to CPF ⁵³.

The insecticide methyl parathion altered locomotor activity, operant behavior, and brain AChE in rat pups when administered to dams at 1.0 mg/kg on gestational day (GD) 6-20 ⁶⁷. Similarly, daily injections of 1.3 or 1.9 mg/ kg parathion in rat pups during PN5-20 did not cause

neuromuscular effects, but reduced the number of muscarinic receptors and AChE activity with a concomitant reduction in performance in tests of spatial memory ⁶⁸. This study was later repeated by and Pope ⁶⁹, using a slightly lower dose of parathion during PN5-20, a critical time period for development of the rat hippocampus. Morphologic as well as biochemical endpoints assessed in this study revealed significant reductions of AChE activity (73%) and muscarinic receptor binding (36%) at PN12, and cytopathologic lesions in the hippocampus confined to the dentate, CA4 and CA3 subfields.

Many other studies have demonstrated the developmental toxicity of OPs and have been reviewed elsewhere ^{12,51,70}. Chanda and Pope ⁶ injected CPF to dams daily from GD12-19 and found reductions in muscarinic and nicotinic receptor binding, AChE activity, and righting reflex and cliff avoidance behaviors in neonatal pups. It was observed that repeated exposures to low doses caused more neurotoxicity than was observed in their earlier studies using a single high dose. Mice exposed to a single oral dose of CPF (0.1, 1.0 or 5 mg/kg body weight) or carbaryl (0.5, 5.0 or 20.0 mg/kg body weight) on PN10 exhibited developmental neurotoxic effects that were not attributed to concurrent AChE inhibition, but rather neurodevelopment toxicity ⁷¹. Similar effects have been observed where developmental exposure cause later behavioral effects in rats were with CPF ⁹. In this study, exposure to CPF before weaning did not cause signs of overt cholinergic intoxication or impaired growth, nor did the exposures cause significant inhibition of regional brain AChE activity 24 h after the last injection.

The vulnerability of the developing nervous system and toxicities observed later in life likely are responsible for some of the age-related differences in OP toxicity. However, some differences may be related to other factors since dissociations between brain AChE inhibition and the magnitude, as well as recovery of, motor activity changes after carbamate exposure have

been demonstrated in rats ⁷². The effects of OPs on the developing nervous system also varies with the type of OP pesticide. In a zebra fish model of developmental exposure, CPF and Malathion had opposite effects on behavior and major differences in brain morphological effects ⁷³.

IV. Cholinergic mechanisms of developmental neurotoxicity

The functional properties of the vertebrate nervous system are determined by the pattern of synaptic connections formed during development. Key neurodevelopmental events, including neurogenesis and gliogenesis, cell migration, axonal and dendritic outgrowth, the formation and pruning of synapses, and programmed cell death, are critical determinants of synaptic connectivity. Thus, the concomitant and coordinated ontogeny of these events in a temporally- and regionally-dependent manner is required for normal neurodevelopment, and perturbations of either the timing or extent of any of these events can alter patterns of neuronal connectivity, potentially resulting in long-term changes in neural function ⁷⁴⁻⁷⁶.

Various components of the cholinergic system, and in particular ACh, AChE and cholinergic receptors, play important biological roles in regulating these neurodevelopmental events in the cholinergic nervous system (Figure 1)^{14,77}. In the developing rat brain, ChAT and AChE, which are required for ACh synthesis, are first detected in cholinergic cell bodies in the medial septum, diagonal band and magnocellular preoptic region; which precedes the development of enzymatic activity days later in terminal fields in the hippocampus and frontal cortex⁷⁸. Experimental evidence indicates that cholinergic neurons can synthesize and release ACh from their axonal growth cones prior to target contact or synapse formation^{79,80}, and developing cholinergic neurons have also been observed to secrete ACh along their axon and at

the soma^{81,82}. It is thought that this secreted ACh acts as a trophic factor to regulate the development of neural circuitry (Figure 1)^{82,83}. This hypothesis derives from experimental evidence that ACh inhibits *in vitro* neurite outgrowth in spinal motor neurons⁸⁴ and axonal projections in thalamic explants⁸³ via cholinergic receptor-dependent mechanisms, and ACh influences axonal pathfinding by modulating the rate and direction of axonal growth²⁹. In the developing retina, ACh released by starburst amacrine cells causes local Ca²⁺ release in the developing dendrites of adjacent retinal ganglion cells, thereby preventing these processes from retracting⁸⁵.

The level of cholinergic neurotransmission throughout prenatal and postnatal development exerts significant influence on not only axonal outgrowth and pathfinding, but also the formation and refinement of neural networks in the developing brain,^{14,77,86} which includes defining the ratio of excitatory to inhibitory neurotransmission⁸⁶, and the rate of neurogenesis in both the developing and adult brain⁷⁷. Developmental neurotoxicants that alter ACh levels can interfere with these neurodevelopmental processes, as exemplified by aflatoxin, a mycotoxin found in contaminated human and animal cereal-based foodstuffs. One of the most prevalent aflatoxins, aflatoxin B1, and its metabolites, aflatoxin M1 and aflatoxicol, readily cross the placenta and are transferred in milk. Preclinical studies in a rat model have shown that developmental exposures to sublethal concentrations of aflatoxin B1 in the maternal diet impairs locomotor coordination and causes learning deficits in exposed offspring⁸⁷⁻⁸⁹. These behavioral changes have been linked to decreased hippocampal neurogenesis secondary to suppression of cholinergic signals on hilar GABAergic interneurons and release of BDNF from granule cells⁸⁹. Similarly, decreased cholinergic signaling caused by developmental exposure to lead (Pb) can

trigger cholinergic neuronal cell death in the medial septal area of the brain, resulting in long-lasting behavioral deficits⁹⁰⁻⁹².

ACh receptors exert regulatory influence on multiple neurodevelopmental events¹⁴, and reduced cholinergic innervation during development results in reduced cortical thickness and dendritic abnormalities in the adult brain⁷⁷. Nicotinic receptors play a critical role in promoting cell division, differentiation and synaptogenesis, and in regulating neurotransmitter release, with much of this activity attributed to $\alpha 7$ nicotinic receptors^{14,77}. Spontaneous nicotinic excitation in the rodent brain during a brief developmental window in the first postnatal weeks is required for the developmental shift of GABA receptors from depolarizing to hyperpolarizing⁷⁷.

Relative to nicotinic receptors, much less is known about the role of muscarinic receptors in the developing brain, although studies of muscarinic receptor agonists and antagonists indicate that these receptors contribute to effects of ACh on cell proliferation, differentiation and survival in the developing brain¹⁴. Muscarinic receptors appear to play a predominant role in mediating the effects of ACh on neurite outgrowth. The axon inhibitory effects of ACh in thalamic explant cultures from E14 mice are blocked by both nicotinic and muscarinic receptor antagonists, but the latter are more potent⁸³. Conversely, M1 muscarinic receptor agonists stimulate axonal outgrowth in primary cultures of rat hippocampal pyramidal neurons via activation of protein kinase C (PKC) and extracellular signal-related kinase (ERK)1/2⁹³. M3 muscarinic receptor-dependent mechanisms mediate the neurite-stimulating influence of astrocytes. Co-culture of rat hippocampal neurons with astrocytes pre-treated with the cholinergic agonist carbachol enhances neurite outgrowth, presumably via secretion of permissive factors⁹⁴.

Cholinergic receptor activation also plays an important role in regulating gene expression, which is critical to normal brain development. Signaling via nicotinic or muscarinic

receptors can increase phosphorylation of the transcription factor cAMP response element binding protein (CREB), which activates gene transcription¹⁴. CREB regulates the transcription of a large number of genes⁹⁵, and CREB-responsive genes are involved in multiple aspects of neurodevelopment, including neurogenesis, cell survival, axonal and dendritic growth and plasticity, and synaptogenesis⁹⁶⁻¹⁰⁰. We previously demonstrated that CPF activates CREB, evidenced as a significant increase in phosphorylated CREB (pCREB) in cultured primary rat cortical and hippocampal neurons, but not astrocytes (Figure 3)¹⁰¹. This activity was observed in neuronal cell cultures exposed for 1 h to the parent compound CPF or its metabolites, chlorpyrifos oxon (CPF-oxon) and trichloropyridinol (TCPy) with estimated EC₅₀ values of 60 pM, < 30 fM and < 30 pM, respectively. These concentrations are well below the IC₅₀ values for AChE inhibition by CPF and CPF-oxon. This, coupled with the fact that TCPy has negligible effect on the enzymatic activity of AChE, strongly suggests that the canonical mechanism of OP neurotoxicity, AChE inhibition, does not mediate CREB activation by CPF or its metabolites. How these compounds activate CREB has yet to be determined, but likely does not include direct phosphorylation of CREB since TCPy does not contain a phosphate group, nor does it involve muscarinic receptors since atropine did not block the effect¹⁰¹. Possibilities that have yet to be tested in this model system include stimulation of presynaptic nicotinic receptors, activation of signaling molecules upstream of CREB, or inactivation of phospholipases¹⁰¹. Also unknown is the biological significance of OP-induced CREB activation, even whether increased pCREB is deleterious or neuroprotective. CREB activation enhances dendritic growth¹⁰², and CPF, CPFO and TCPy have been shown to promote dendritic arborization in cultured primary rat neurons¹⁰³. Whether these two effects are causally related has yet to be shown, but it suggests the intriguing

hypothesis that CPF alters neuronal connectivity in the developing brain via activation of CREB (Figure 3).

AChE appears quite early in the embryonic development of the CNS. Its mRNA is expressed by progenitor cells in the ventricular zone and in undifferentiated cells in the cortical plate¹⁴. Its expression prior to the formation of cholinergic synapses provided the first clue that AChE has biological roles in the developing nervous system in addition to its canonical function in regulating cholinergic neurotransmission by enzymatic hydrolysis of synaptic ACh. It is now accepted that AChE functions to regulate cell adhesion, neurite outgrowth, synaptogenesis and neuronal apoptosis¹⁰⁴⁻¹⁰⁷.

Neurite outgrowth was the first non-classical activity of AChE to be unequivocally identified¹⁰⁸. In early postnatal rats, upregulation of AChE activity in layer IV of the primary cerebral cortical sensory areas coincides spatially and temporally with transient AChE expression of ingrowing thalamocortical axons¹⁰⁹⁻¹¹¹, and thalamic lesions eliminate AChE activity in the developing cortex¹¹². Functional studies confirmed that AChE functions as a morphogen to promote axonal growth. Cultured chick sympathetic, rat sensory, rat spinal motor neurons and dopaminergic neurons in organotypic cultures from rat midbrain respond with increased neurite outgrowth when plated on substrates pre-coated with AChE or grown in culture medium supplemented with exogenous AChE¹¹³⁻¹¹⁶, and selective pharmacological inhibitors of AChE and function-blocking AChE antibodies inhibited neurite outgrowth in cultured neural cells^{108,113-115,117-119}. The importance of AChE to axon tract formation *in vivo* was demonstrated in studies using the AChE inhibitor BW284C51, which blocks non-catalytic sites in the AChE molecule, to reduce the thickness of the post-optic commissure, the major longitudinal axon tract of the *Xenopus* brain¹²⁰. Genetic manipulation of AChE expression corroborated pharmacological

studies: (1) antisense suppression of AChE decreased neurite outgrowth in PC12 cells, an effect that was rescued by heterologous expression of the synaptic form of human AChE¹²¹; and (2) in neuroblastoma cells, transfection with sense AChE increased neurite outgrowth, which was blocked by AChE antibodies; whereas, transfection with antisense AChE decreased neurite outgrowth¹²².

Interesting, CPF and CPF-oxon, but not TCPy, have been shown to decrease axonal growth in cultured primary rat sensory neurons at concentrations that did not inhibit the enzymatic activity of AChE or cause cytotoxicity¹²³. The axon inhibitory activity of these OPs required AChE. Compared to sensory neurons derived from *AChE*^{+/+} mice, sensory neurons derived from *AChE*^{-/-} mice had shorter axons in the absence of OPs, and their length was not inhibited by CPF or CPF-oxon (Figure 4)¹²³. Transfection of *AChE*^{-/-} DRG neurons with cDNA encoding full-length *AChE* restored the wildtype axonal phenotype and response to the axon inhibitory effects of OPs. These data indicate that inhibition of axonal growth by OPs requires AChE, but the mechanism involves inhibition of the morphogenic rather than enzymatic activity of AChE. These findings suggest a novel mechanism for explaining not only the functional deficits observed in children and animals following developmental exposure to OPs, but also the increased vulnerability of the developing nervous system to OPs.

VI. Other Cholinergic Developmental Neurotoxicants

A. Other Insecticides

Pyrethroids insecticides, a class of synthetic insecticides modeled after the naturally occurring pyrethrins isolated from the *Chrysanthemum* genus of plants, are widely used in

agriculture and for domestic purposes. There are two structural subgroups of this insecticide class based on different intoxication syndromes: Type I pyrethroids produce a tremor syndrome, while Type II pyrethroids produce choreoathetosis. Their canonical mechanism of action is to delay inactivation of voltage-gated sodium channels, leading to repetitive firing of action potentials¹²⁴. The cholinergic system has been implicated in the developmental neurotoxicity of pyrethroids. Specifically, gestational or lactational exposures to pyrethroids altered muscarinic and nicotinic receptor binding in neonatal mice^{125,126} and rats¹²⁷, with the magnitude and direction of these changes depended on the brain region (cortex or hippocampus) and the pyrethroid. Pyrethroid-associated changes in muscarinic receptor expression have been associated with altered AChE activity and with a delay in the functional maturation of the cholinergic system in neonatal rat following gestational or lactational exposure to pyrethroids¹²⁷.

Studies of allethrin similarly demonstrated that prenatal, postnatal and perinatal exposures of rat pups significantly decreased muscarinic receptor binding and inhibited AChE activity in the hippocampus, and showed that these neurochemical changes accompanied deficits in performance in hippocampal-dependent learning and memory tasks¹²⁸. More recent studies with the new generation type II pyrethroid, lambda-cyhalothrin, demonstrated that a 4 week exposure of weanling rats decreased muscarinic receptors in the frontal cortex, hippocampus and cerebellum and decreased ChAT expression and AChE activity in the hippocampus¹²⁹. As observed with allethrin, lambda-cyhalothrin effects on cholinergic signaling were associated with learning deficits. Collectively, these studies suggest that cholinergic dysfunction, particularly in the hippocampus, contributes, at least in part, to the cognitive deficits associated with developmental exposure to pyrethroids.

The neonicotinoids (neonics) are synthetic insecticides developed to selectively target the cholinergic system. These are a commercially important class of insecticides whose use has grown dramatically in recent years^{130,131}. Their insecticidal activity is attributed to the activation of postsynaptic nicotinic receptors in insects¹³². While ligand binding assays confirm that neonics have a higher affinity for insect vs. mammalian nicotinic receptors¹³³, it was recently reported that neonics increase Ca²⁺ oscillations in primary cerebellar neurons via nicotinic receptor-dependent mechanisms with higher potency than would be predicted based on ligand binding studies¹³⁴. Such findings, coupled with the prevalent use of neonics and evidence of human exposures¹³⁵, have prompted several comprehensive reviews of the literature on neonic developmental neurotoxicity. The first, which reviewed *in vitro*, *in vivo* and epidemiology studies in the peer-reviewed literature concluded that no common effects were identified among the neonicotinoids that were consistent with developmental neurotoxicity, or with the neurodevelopmental effects associated with nicotine¹³³. In contrast, a more recent systematic review of the human literature reported an association between chronic neonic exposure and an adverse human health effect¹³⁵. Three of these studies focused on developmental outcomes, including congenital heart defects (CHDs)¹³⁶, neural tube defects (NTDs)¹³⁷, and autism spectrum disorder (ASD)¹³⁸. The authors of the systematic review¹³⁵ concluded that while the studies conducted to date were limited in number, and there were methodological concerns regarding the exposure measures, the findings were suggestive of developmental neurotoxicity associated with exposure to neonics. Interestingly, several cholinergic abnormalities have been reported in autism¹³⁹. Ligand binding and western blotting studies of postmortem brain tissue have reported regional differences in the expression of specific nicotinic receptor subunits with reduced expression of $\alpha 3$, $\alpha 4$ and $\beta 2$ in the parietal and frontal cortex, cerebellum and thalamus, but

increased expression of $\alpha 7$ in the cerebellum in the autistic brain relative to controls¹⁴⁰⁻¹⁴².

Collectively, these observations suggest the possibility that individuals with heritable increases in $\alpha 7$ expression may be uniquely sensitive to the developmental neurotoxicity of neonics.

B. Nicotine

While there has been a significant decline in cigarette sales over the last decades, the use of other tobacco products including electronic cigarettes and electronic nicotine delivery systems has skyrocketed. The 2016 Surgeon General's Report indicated that e-cigarettes are a 2.5 billion dollar business that poses a significant public health issue for our nation's young people. While these newer products contain fewer chemicals than traditional cigarette smoke, they still have a large nicotine content. Nicotine has been associated with negative physiological, cognitive and psychiatric outcomes in children who were exposed during gestation¹⁴³. Nicotine has also been shown to alter brain development when administered prenatally in rodent models, although the neurodevelopmental outcome varies depending on the developmental stage at the time of nicotine exposure¹⁴³⁻¹⁴⁵.

Nicotine readily crosses the fetal-placenta barrier but is slowly cleared from the placenta, resulting in significantly higher nicotine levels in the amniotic fluid compared to the maternal plasma¹⁴⁶. Prenatal exposure to nicotine results in inappropriate nicotinic receptor activation and has been shown to promote a pro-apoptotic environment, resulting in a reduction in the total number of cells within the fetal brain^{147,148}. Detailed morphological analysis has revealed abnormalities in cell size and density within specific brain regions, such as the hippocampus and cortex of rats developmentally exposed to nicotine^{145,148,149}. Many of these morphological changes occur independent of changes in fetal somatic cell growth, and thus can occur without any

obvious changes in birth weight or other birth defects^{145,150}. Human brains continue to develop throughout adolescence, and maturation of cholinergic systems occurs during the periadolescent period^{151,152}. Studies indicate that adolescent rats are more susceptible than adults to nicotine due to a profound upregulation of nicotinic receptor expression, a finding of critical importance for today's society because of the increasing prevalence of adolescent nicotine exposure¹⁴³.

At the molecular level, even minute amounts of nicotine have been shown to activate apoptosis genes and to reduce DNA synthesis, and the temporal and regional selectivity of these effects coincides with the ontogeny of nicotinic receptors and the cholinergic growth spurt during the early postnatal period in rodents¹⁵³⁻¹⁵⁵. In later stages of development corresponding to the third trimester in humans, nicotine exposure has been reported to change cholinergic receptor composition and overall cholinergic transmission. Both nicotinic and muscarinic receptor receptors are altered by prenatal nicotine exposure, resulting in aberrant cholinergic transmission in the brain¹⁴³. The profile of cholinergic receptor subtypes varies as a function of developmental stage, so nicotine-induced effects on cholinergic transmission may fluctuate between hypo and hyper-cholinergic activity depending on the timing of nicotine exposure¹⁴³. In addition to changes in cholinergic receptor composition, prenatal nicotine exposure also decreases presynaptic choline transporter proteins, reduces ChAT activity and alters membrane potential, all of which have a significant impact on cholinergic transmission in the developing brain^{143,147,156}. These changes in cholinergic signaling can ultimately alter their target structures, including neurons in the ventral tegmental area (DA-VTA), the nucleus accumbens and laterodorsal tegmentum^{143,157-160}. Many of these regions are critical neuroanatomical substrates for behaviors such as addiction and reward, as well as cognitive arousal, suggesting that prenatal nicotine exposure can adversely impact global circuitry in the entire brain^{161,162}.

C. Lead (Pb)

A number of metals, including essential metals, are well-documented developmental neurotoxicants¹⁶³, and there is evidence that at least some disrupt cholinergic signaling in the developing nervous system^{164,165}. However, cholinergic mechanisms of developmental neurotoxicity are perhaps best documented for the non-essential metal lead (Pb). While rigorous regulations have resulted in a significant decrease in Pb exposure and a concurrent drop in children's blood Pb levels over the past few decades, Pb remains a significant global public health concern¹⁶⁶. Developmental Pb exposures are associated with decreased IQ, aggression, motor impairments, and deficits in hearing and vision¹⁶⁶. Pb-induced changes in neurobehavioral function persist long after blood Pb levels have returned to normal, and these changes are thought to reflect perturbations in dendritic development, synaptogenesis, and myelin and fiber tract formation, all of which may be secondary to effects of Pb on mechanisms of neurotransmission and energy metabolism¹⁶⁶.

Several studies have implicated the cholinergic septohippocampal pathway in Pb developmental toxicity. Developmental exposure to Pb in the drinking water was observed to cause 30-40% reductions in ChAT activity in the septum and hippocampus of 7- and 28-day-old rats with blood Pb levels of 20 µg/dL¹⁶⁷, and the developmental plasticity of the cholinergic system was compromised in the hippocampus of rats developmentally exposed to Pb¹⁶⁸. The effects of Pb on the development of the septohippocampal pathway may persist into adulthood, resulting in alterations of cholinergic systems similar to those observed after fimbria-fornix

transection⁹⁰. Pb targets additional components that modulate ACh levels: *in vitro* and *in vivo* Pb exposure has been shown to inhibit Na⁺-dependent high-affinity choline uptake and reduce the number of uptake sites^{169,170}.

Several laboratories have shown that chronic *in vivo* exposure to Pb during development causes region-specific decreases in the number of muscarinic receptors in rat brain^{167,171-174}.

Whether Pb targets specific muscarinic receptor subtypes could not be determined from these studies. This is a critical issue given evidence that the ontogeny of muscarinic receptors varies among the different subtypes¹⁷⁵, and that certain subtypes may be more involved than others in the behavioral effects of Pb¹⁷⁶. Early *in vitro* studies demonstrated a direct interaction between Pb and muscarinic receptors, but only at higher Pb concentrations¹⁷⁷. Subsequent ligand binding studies using tissues derived from rats exposed developmentally to lower concentrations of lead observed no effect of Pb on muscarinic receptor binding^{171,172}. Pb alters signal transduction pathways linked to muscarinic receptors, including adenylyl cyclase activity¹⁷⁸, phosphoinositide turnover^{179,180}, and PKC¹⁸¹, but these effects have not been causally linked to Pb-induced changes in muscarinic receptor function.

Nicotinic receptors are heavily involved in learning and memory processes¹⁸², and the nicotinic receptor is one of the most sensitive molecular targets of Pb in neurons. In neuroblastoma cells, 1 nM to 3 μ M Pb reduced nicotinic ion conductance by 26-90%¹⁸³. The mechanism by which Pb inhibits nicotinic receptor function has been characterized in cultured hippocampal neurons as noncompetitive, voltage-independent, and primarily acting on the fast-desensitizing nicotinic current associated with $\alpha 7$ subunit-bearing receptors¹⁸⁴. Pb was a less potent inhibitor of the slowly desensitizing currents associated with other nicotinic receptor subtypes. The $\alpha 7$ receptor subtype is believed to be a presynaptic receptor that is extremely

permeable to Ca^{2+} and functions to modulate synaptic plasticity^{185,186}. These characteristics of Ca^{2+} permeability, involvement in plasticity, and sensitivity to Pb, are strikingly similar to the NMDA glutamate receptor, suggesting that the inhibitory action of Pb at presynaptic nicotinic receptors may contribute to the impairment of behavioral processes believed to be mediated by postsynaptic cholinergic and glutamatergic receptors.¹⁶⁶ In an attempt to investigate the subtype-specific effects of Pb on nicotinic receptors, $\alpha 3/\beta 2$ and $\alpha 3/\beta 4$ subtypes were expressed in *Xenopus oocytes* and Pb was found to potentiate and block ion conductance to varying degrees depending on the receptor subtype¹⁸⁷. While these studies suggest that nicotinic receptors may be involved in Pb-induced learning impairment, the behavioral consequences of changes in nicotinic receptor density and function have yet to be determined.

Pb has also been shown to alter cholinergic neurotransmission via indirect mechanisms. Pb competes with Ca^{2+} binding sites on vesicular docking proteins in order to block the evoked release of ACh from presynaptic neurons⁹⁰⁻⁹². Pb also binds to and inhibits AChE, and *in vivo* studies have shown that developmental Pb exposures decreases AChE activity in a dose-dependent manner in multiple brain regions of the perinatal rats, including the cortex, cerebellum and hippocampus.^{188,189} However, the susceptibility of these different brain regions to the AChE inhibitory effects of Pb, varies according to regional differences in maturation at the time of Pb exposure^{188,190}.

D. Endocrine disruptors

It is widely posited that endocrine-disrupting chemicals (EDCs) alter neurodevelopment by interfering with hormone-mediated processes that influence neurodevelopment^{191,192}. Developing cholinergic neurons are sensitive to thyroid hormone,^{193,194} which regulates the ontogenetic expression of muscarinic receptors in the cerebellum and of ChAT in multiple brain regions¹⁹⁴⁻¹⁹⁷. Neonatal thyroid hormone deficiency transiently decreased expression of cerebellar muscarinic receptors, and markedly delayed developmental expression of ChAT in the hippocampus, cerebral cortex, and cerebellum. Subsequent administration of thyroid hormone restored ChAT to control levels in the hippocampus and cerebellum but not in the forebrain, where ChAT levels remained persistently depressed¹⁹⁶. These data suggest that neonatal thyroid hormone deficiency selectively causes persistent reduction of ChAT in cortical cholinergic neurons.

An example of an EDC that has raised concerns regarding its potential to cause adverse neurodevelopmental outcomes is bisphenol-A (BPA). BPA is a synthetic compound used to produce polycarbonate plastics and epoxy resins, and it is widely found in canned goods, plastics and household dust. BPA is typically considered a weak agonist of the nuclear estrogen receptor¹⁹⁸, but in humans and in rodent models, exposure to BPA during pregnancy has been shown to decrease maternal and offspring thyroid parameters¹⁹⁹⁻²⁰². Exposure of mice to BPA in the maternal diet throughout gestation and lactation also significantly decreased ChAT immunoreactivity in the hippocampus coincident with memory deficits²⁰³. But whether the effects of developmental BPA exposure on thyroid hormone levels are causally linked to altered cholinergic function in the developing brain has not been determined.

Conclusions

The cholinergic system within the CNS plays a critical role during brain development. Cholinergic neurons and their receptors form an early scaffold for other neurotransmitter systems and serve a variety of functions in the adult brain. There are critical time periods during brain development that might be more susceptible to cholinergic neurotoxicants, and certain brain areas may be affected more than others. The alteration of brain development by toxicants such as anticholinesterase pesticides, lead (Pb) and nicotine is supported by many studies that demonstrate concomitant changes in a variety of cholinergic targets (Table 1) which provide clues to potential underlying mechanisms for these effects. During development, as is true in the adult brain, evidence is building for anticholinesterase toxicities unrelated to their effect on the catalytic function of AChE, and toxicity at exposure levels not expected to inhibit this activity.

Table 1. Known cholinergic targets for developmental neurotoxicants

Cholinergic Target	Anticholinesterase Insecticides	Pyrethroids	Neonicotinoids	Nicotine	Lead (Pb)	Endocrine Disruptors
AChE	✓	✓			✓	
ChAT	✓	✓		✓	✓	✓
ACh Release					✓	
Muscarinic Receptor	✓	✓		✓	✓	
Nicotinic Receptor	✓	✓	✓		✓	
Behavioral Deficits	✓	✓			✓	

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Figure 1. Schematic representing cellular events in neurodevelopment influenced by the cholinergic system. Acetylcholine (ACh) has direct (red triangle) and indirect (blue cross) effects on specific cellular events in neurodevelopment. Indirect effects of ACh are mediated by signaling through nicotinic and muscarinic receptors (nAChR and mAChR, respectively, blue cross). Acetylcholinesterase (AChE) also has direct (green asterisk) and indirect effects (via enzymatic modulation of ACh levels). While there are no data demonstrating a direct effect of the presynaptic high-affinity choline transporter (CHT), the choline acetyltransferase (ChAT) or the vesicular acetylcholine transporter (VAChT) on neurodevelopment, these components of the cholinergic signaling system likely influence cellular events of neurodevelopment indirectly via modulation of cholinergic neurotransmission.

Figure 2. Schematic illustrating cellular and molecular mechanisms by which organophosphorus anticholinesterases may interfere with the development of neuronal connectivity.

Figure 3. Chlorpyrifos and its metabolites increase cellular levels of pCREB in primary cortical neurons. (A) Representative immunoblots of lysates from cultured rat cortical neurons exposed to chlorpyrifos or its metabolites, chlorpyrifos-oxon or trichloropyridinol, for 1 h probed with antibodies that specifically recognize pCREB, α -tubulin and total CREB. (B) Densitometric analyses of immunoblots. Densitometric values for pCREB were normalized to total CREB and α -tubulin and expressed as a percentage of control values (cortical cultures exposed to vehicle).

Adapted from Schuh *et al.*, 2002¹⁰¹.

Figure 5. Expression of wildtype AChE restores wildtype phenotype to *AChE*^{-/-} sensory neurons. Representative fluorescence micrographs of *AChE*^{-/-} sensory neurons transfected with pGFP alone (A) or co-transfected with pGFP and pAChE (B). Neurons were treated with vehicle or chlorpyrifos (CPF, 0.1 μM) for 24 h starting the day after transfection. Transfected neurons were identified by GFP expression (green fluorescence) and axons delineated by reacting with antibody specific for phosphorylated neurofilaments (red fluorescence). Adapted from Yang *et al.*, 2008¹²³.