Morphogenetic Roles of Acetylcholine

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In the adult nervous system, neurotransmitters mediate cellular communication within neuronal circuits. In developing tissues and primitive organisms, neurotransmitters subserve growth regulatory and morphogenetic functions. Accumulated evidence suggests that acetylcholine, (ACh), released from growing axons, regulates growth, differentiation, and plasticity of developing central nervous system neurons. In addition to intrinsic cholinergic neurons, the cerebral cortex and hippocampus receive extensive innervation from cholinergic neurons in the basal forebrain, beginning prenatally and continuing throughout the period of active growth and synaptogenesis. Acute exposure to ethanol in early gestation (which prevents formation of basal forebrain cholinergic neurons) or neonatal lesioning of basal forebrain cholinergic neurons, significantly compromises cortical development and produces persistent impairment of cognitive functions. Neonatal visual deprivation alters developmental expression of muscarinic acetylcholine receptors (mAChR) in visual cortex, whereas local infusion of mAChR antagonists impairs plasticity of visual cortical neurons. These findings raise the possibility that exposure to environmental neurotoxins that affect cholinergic systems may seriously compromise brain development and have long-lasting morphologic, neurochemical, and functional consequences. — *Environ Health Perspect* 107(Suppl 1):65–69 (1999). *http://ehpnet1.niehs.nih.gov/docs/1999/Suppl-1/65-69lauder/abstract.html*

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Classical neurotransmitters, such as acetylcholine (ACh), dopamine (DA), noradrenaline (NA), adrenaline (A), serotonin (5-HT), and γ-aminobutyric acid (GABA), act as developmental signals in all animal species studied. These substances play regulatory roles throughout ontogenesis, including stages prior to development of the nervous system [see reviews by Buznikov et al. (*1*) and Lauder (*2–4*)].

Morphogens are developmental signals that exert specific effects on receptive cells, depending on concentration. Morphogens are present in gradients created by the presence of a "source" and a "sink." Developing cells are affected in specific ways along this concentration gradient [reviewed by Lauder (*2*)]. This concept has traditionally been applied to substances involved in pattern formation and morphogenesis, such as retinoic acid. However, it may also be appropriate to consider neurotransmitters as morphogens when they act as dose-dependent morphogenetic signals in neural and nonneural tissues. Neurotransmitters have these types of actions in primitive organisms and embryos, where they exert their effects using receptors and signal transduction mechanisms similar to those in the adult nervous system (*1–3*). This raises the possibility that the highly specialized roles played by neurotransmitters in synaptic transmission may have evolved from phylogenetically old functions, many of which are recapitulated during development. This may make developing neurotransmitter systems especially vulnerable to environmental neurotoxins, such as pesticides, designed to target receptors for these neurochemicals in lower organisms.

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Abbreviations used: 5-HT, 5-hydroxytryptamine; A, adrenaline; ACh, acetylcholine; AChE, acetylcholinesterase; ChAT, choline acetyltransferase; CNS, central nervous system; DA, dopamine; FAS, fetal alcohol syndrome; GABA, γ-aminobutyric acid; M1, M3, M5, mAChR cholinergic receptor subtypes 1,3,5; mAChR, muscarinic acetylcholine receptor; NA, noradrenaline; nα, non-alpha; nAChR, nicotinic acetylcholine receptor; nBM, nucleus basalis magnocellularis; PI, phosphoinositide; PLC, phospholipase C-β; PNS, peripheral nervous system.

Developmental Roles of Acetylcholine

Acetylcholine is a major excitatory neurotransmitter in the nervous system of vertebrates and invertebrates. Accumulated evidence suggests that ACh also plays a key role in regulation of morphogenetic cell movements, cell proliferation, growth, and differentiation in species as diverse as echinoderms, insects, worms, avians, rodents, and humans.

Cholinergic Regulation of Morphogenetic Cell Movements in Early Embryos

In sea urchin embryos, cell movements occurring during gastrulation and postgastrulation stages appear to be regulated by ACh and biogenic monoamines (*5–10*). Specific antagonists of receptors for these neurotransmitters act as inhibitors of morphogenetic cell movements during specific phases of gastrulation. For example, 5-HT receptor antagonists are effective in blocking cell movements throughout this period, whereas ACh antagonists act only during the final phases of gastrulation (*5*), and do not affect cleavage divisions (*8,9*). The ability of both 5-HT and ACh to affect gastrulating sea urchin embryos suggests that receptors for these neurotransmitters are expressed at the time of gastrulation by cells of the primary gut and mesenchyme (*5,6*). Histochemical evidence has demonstrated the presence of acetylcholinesterase (AChE) during sea urchin gastrulation where it is localized predominantly in the primary gut, which is the site of the most active cell movements (*7,10*).

Similar functions of ACh during gastrulation of vertebrate embryos are suggested by the presence of AChE during gastrulation in the chick embryo (*11,12*). Other morphogenetic roles for ACh during vertebrate embryogenesis are suggested by the early presence of choline acetyltransferase (ChAT), cholinesterases, and muscarinic cholinergic receptors (mAChR) in mesenchyme and developing cartilage of the chick limbud (*13,14*) and myotomes (*15*). In the mouse embryo, ACh may also play a role in palate morphogenesis as suggested by the ability of cholinergic receptor agonists to stimulate elevation of palatal shelves—effects that are blocked by nicotinic cholinergic receptor (nAChR) antagonists, but not by mAChR receptor antagonists (*16*).

Cholinergic Regulation of Cell Proliferation

Neurotransmitters stimulate or inhibit proliferation of nonneuronal cells by

activating receptors coupled to different G-proteins and second messenger pathways [reviewed by Lauder (*3*) and Weiss et al. (*17*)]. Stimulation of proliferation is most often associated with activation of G-proteins negatively coupled to adenylyl cyclase (G_i), or positively coupled to other pertussis toxin-sensitive G-proteins (G_o) or phospholipase C-β (PLC) G_q-coupled pathways. In contrast, activation of neurotransmitter receptors positively coupled to cAMP usually inhibits cell proliferation and causes cell shape changes indicative of differentiation.

Neurotransmitters that promote cell proliferation include 5-HT, adenosine (A), noradrenaline (NA), and ACh. Stimulation of mAChR by carbachol promotes DNA synthesis in cultured astrocytes and in fibroblasts transfected with genes coding for m1, m3, and m5 subunits of the human mAChR. These mitogenic responses are correlated with increased activity of PLC (*18–20*). Recent evidence suggests that activation of G_q-coupled M1, M3, and M5 mAChR receptor subtypes stimulates mitogen-activated protein kinase by PLC-dependent and PLC-independent mechanisms (*21*), and that mAChR activate the *Egr* family of transcription factors (*22*). These findings may shed light on mechanisms underlying mitogenic effects of mAChR activation. Muscarinic receptor-dependent activation of PLC has also been reported in human fetal brain slices (*23*), suggesting that similar mechanisms may be important for development of the human nervous system. Growth-promoting actions of endogenous ACh are suggested by the severe growth defects observed in ChAT-deficient nematode and *Drosophila* mutants (*24*).

Activation of G_q-coupled M1, M3, and M5 mAChRs can also stimulate adenylyl cyclase and inhibit cell proliferation in a number of cell types. These effects occur by mechanisms independent of PLC-mediated phosphoinositide (PI) hydrolysis, possibly involving liberation of βγ subunits from G_q (*25*). Such mechanisms may underlie the inhibitory effects of carbachol on proliferation of fibroblasts transfected with gene sequences encoding M1, M3, and M5 mAChRs (*19,26*). Inhibitory effects of acetylcholine on stem cell proliferation are indicated by the positive effects of antisense oligonucleotides for AChE on proliferation and expansion of progenitor cells in bone marrow cultures (*27*).

Cholinergic Regulation of Cell Survival and Neurite Outgrowth

Acetylcholine has been shown to regulate neurite outgrowth in both snail (*Helisoma*) and mammalian neurons [reviewed by Lipton and Kater (*28*)]. When ACh is directly applied to cultured *Helisoma* neurons, it inhibits neurite outgrowth, but when added together with 5-HT, it prevents inhibition of neurite outgrowth by 5-HT (*29*). Acetylcholine may be a negative influence on neurite outgrowth in mammalian neurons, as nicotinic antagonists enhance process outgrowth in cultured rat retinal ganglion cells, presumably by blocking the inhibitory effects of ACh released from amacrine neurons into the culture medium (*30*). Acetylcholine has also been reported to inhibit neurite outgrowth in rat hippocampus (*31*). However, activation of nAChRs has been reported to promote survival of chicken spinal motoneurons that would otherwise undergo programmed cell death when deprived of trophic factors (*32*). In addition, significant direct roles for AChEs in neurite outgrowth have recently been reported, as discussed by Brimijohn and Koenigsberger (*33*) and Bigbee et al. (*34*).

Development of the Central Cholinergic Nervous System

Central cholinergic neurons detected by ChAT immunoreactivity are concentrated in the mediobasal forebrain, and brainstem (*35*), but are also present throughout the cerebral cortex (*36*) and hippocampus (*37*). Mediobasal cholinergic forebrain neurons project to cortex and hippocampus (*38*), where nAChRs and mAChRs are expressed in both neonatal and adult rat brain (*39–41*).Most neurons expressing these receptors also contain AChE (*42*). Cloning of the nAChR and *in situ* hybridization have revealed multiple subunits that are differentially expressed throughout the brain. Several of these subunits are highly expressed in cortex, hippocampus, and the septal area (*39,43,44*).

Prenatal Ontogeny of Cholinergic Neurons

Ontogeny of cholinergic neurons in the brain and spinal cord of mouse and rat embryos has been studied in detail using ChAT immunocytochemistry to study prenatal development of the cholinergic phenotype (*45,46*) and ChAT immunocytochemistry combined with long survival [^{3}H]thymidine autoradiography to date the time of origin (day of last cell division of progenitors) of cholinergic neurons [reviewed by Semba (*47*)]. From these studies, a detailed picture of prenatal ontogeny of the cholinergic system has been revealed.

In the rat embryo, cholinergic neurons are generated earliest in the spinal cord and brainstem, beginning at embryonic day (E)11, and continue to be generated in these regions through E14, with peak generation at E12 to E13 (*47*). Cholinergic neurons in most other brain regions are generated between E12 to E16, with the exception of the basal ganglia, where cholinergic neurons begin to be generated on E11. Most studies in the rat embryo suggest that the cholinergic phenotype (ChAT immunoreactivity) appears 1 to 2 days following generation of neurons from dividing progenitor cells. However, this view differs from evidence for the sequence of cholinergic phenotype development in the mouse embryo, as discussed below.

In the mouse embryo, Schambra et al. (*45*) demonstrated that cholinergic neurons of the forebrain are generated from distinct germinal zones in the olfactory, lateral, and third ventricles between E14 to E16. In this study, ChAT immunoreactivity revealed mitotic figures in these germinal zones, suggesting that progenitor cells already expressed a cholinergic phenotype prior to the time of last cell division and onset of neuronal differentiation. Because of this early expression, ChAT immunoreactive cells could be followed along their migratory routes to final destinations in specific brain structures. From this analysis, germinal zones giving rise to specific populations of cholinergic neurons could be determined. For example, cholinergic neurons of the basal ganglia were found to originate from germinal zones located in the ventricular epithelium of the ganglionic eminence, adjacent to the lateral ventricle, whereas intrinsic cholinergic neurons of the cerebral cortex and hippocampus, as well as projection neurons in the mediobasal forebrain, were derived from other germinal zones of the lateral ventricles. Cholinergic neurons of the hypothalamic preoptic nucleus and habenula were derived from germinal zones of the third ventricle. By late gestation (E16–E18) cholinergic neurons could be seen migrating within the cortical plate, which gives rise to the six layers of the neocortex. Other cholinergic neurons were found in the CA2 region of the hippocampus, where they were located in a position to form transient targets of granule cell mossy fibers, which do not cross this region

into the more distal hippocampal CA1 region. Further migration of cholinergic neurons into the basal ganglia and cerebral cortex was observed just following birth.

Prenatal Ontogeny of Muscarinic and Nicotinic Receptors

Both mAChR and nAChR cholinergic receptors have been detected by radioligand autoradiography in the prenatal rat brain, where they appear to develop in a caudal-rostral gradient (*48,49*). However, nAChRs appear to be expressed earlier than mAChRs, where they can be detected in spinal cord and brainstem by E12. These receptors appear in more rostral brain regions (mesencephalon, diencephalon) by E14 and can be detected in deep layers of neocortex by E18 (*48*). In the human fetus, nAChRs are highly expressed in the brainstem at midgestation, followed by a decline to low levels prior to birth (*50*).

Muscarinic receptors are not detectable in rat brainstem and spinal cord until E16 and do not reach maximal levels until the end of gestation. Expression of these receptors in more rostral brain regions occurs after E16 and only begins to reach maximal levels just before birth (*49*). In cerebral cortex, mAChR appear to surround the cortical plate at E18, during the period of active migration of cholinergic neurons through this cortical region (*45*), and by E22 are found throughout the developing neocortex (*49*). Accumulating evidence indicates that expression of nAChR may be regulated by signals intrinsic (*51*) and extrinsic to their cortical location (*52,53*). Postnatally, many of these influences may come from cortical cholinergic and noncholinergic afferents, as discussed below.

Acetylcholine and Cortical Plasticity

Although intrinsic cholinergic neurons are born and migrate to the cerebral cortex prenatally, innervation of the cortex by cholinergic afferents (primarily from the mediobasal forebrain nucleus basalis magnocellularis, nBM) does not begin until after birth and is only completed by about 2 months postnatally in rodents. This coincides with the period of most active cortical development and synaptogenesis [reviewed by Bachman et al. (*54*) and Hohmann and Berger-Sweeney (*55*)].

Neonatal Lesions of Forebrain Cholinergic Neurons. Convincing evidence for a developmental role of ACh in postnatal cortical development comes from the work of Hohmann and colleagues (*54–58*) who have shown that neonatal lesions of mouse of nBM cholinergic neurons produce abnormal cortical development, including aberrant lamination, dendritic growth, and positioning of pyramidal cells, as well as altered cortical connectivity. These changes lead to cognitive deficits in adult animals. A recent report that growth and differentiation of cortical neurons is stimulated in co-cultures with nBM cholinergic neurons (*59*) provides further support for the view that forebrain cholinergic neurons are important for normal development of the cerebral cortex.

Many of the effects of neonatal lesioning of mouse forebrain cholinergic neurons appear to be mediated by mAChRs (*55,60–62*), in particular, the M1, M2, and M4 subtypes, which exhibit different patterns of expression in developing cortex. The M1 and M4 subtypes exhibit interesting transient expression patterns in fiber tracts, the cerebral peduncles, and barrel fields, suggesting that they may be involved in axonal growth and cellular rearrangements during barrel field morphogenesis.

Rett Syndrome. Rett syndrome is a developmental disorder that presents in early childhood following a relatively normal infancy. This syndrome is characterized by deceleration of head growth and acquired microcephaly, reduced neuropil development in the cerebral cortex and hippocampus, and decreased numbers of basal forebrain cholinergic neurons. These children exhibit behavioral regression, stereotyped hand movements, seizures, breathing abnormalities, and severe mental retardation. This syndrome shares many similarities with the effects of basal forebrain lesions in neonatal mice. As such, it provides further evidence for the importance of acetylcholine in postnatal brain development and consequent behavior (*63*).

Acetylcholine and Ocular Dominance

During development of the kitten visual cortex, synaptic competition between thalamocortical afferents driven by each eye leads to synaptic rearrangements that are critical for the formation of ocular dominance columns. Deprivation of input from one eye during a critical period of cortical development interferes with these synaptic rearrangements and leads to inability of the affected eye to drive visual cortical cells even after vision has been restored (*64,65*). Ocular dominance plasticity can be blocked by combined lesions of cholinergic and noradrenergic cortical afferents (*66*). This effect can be mimicked by infusion of M1 mAChR antagonists (*67*), suggesting that these receptors are involved in mediating the effects of cholinergic afferents on visual cortical plasticity. Monocular deprivation causes transient changes in developmental regulation of M2, M3, and M4 but not M1 mAChR mRNA expression in rat visual cortex (*68,69*), although with receptor autoradiography, effects on M1 receptors were found (*70*). The latter finding is supported by studies in kitten visual cortex, where M1 mAChRs also appear to be affected by monocular deprivation (*71*). Together, all of these studies suggest that neonatal visual cortical plasticity involves activation of mAChRs. In the chick optic tectum, however, nAChRs may also play a role in wiring of visual pathways. In this species, nα (non-alpha) subunits of the nAChR are transiently expressed in the optic tectum during innervation by optic nerve fibers, whereas in "eyeless" embryos in which both optic vesicles have been removed, this transeint expression does not occur (*72*). This suggests that nAChR may be involved in development of the retinotectal projection in the chick embryo.

Effects of Cholinergic Disruptors on Neural Development

Evidence that acetylcholine plays key roles in neural development suggests that disruptors of cholinergic function could disturb these actions if present during key critical periods. The cerebral cortex may be especially vulnerable to such insults because of the important roles cholinergic afferents play in cerebral morphogenesis and synaptogenesis. Disruptors of cholinergic function that may have significant effects on brain development include alcohol, nicotine, and cholinergic pesticides. Effects of nicotine and cholinergic pesticides are covered in the review by Slotkin (*73*).

Fetal Alcohol Syndrome

Abuse of alcohol in pregnancy has been linked to mental retardation and developmental delays, growth deficiencies, and craniofacial abnormalities in children (fetal alcohol syndrome [FAS]). In an animal model of FAS (*74*), administration of two doses of ethanol (500–600 mg/dl final blood concentration) to pregnant mice on E7 caused similar craniofacial defects in mouse embryos and severely affected development of the forebrain CNS cholinergic system. As might be expected from the effects of neonatal lesions of forebrain cholinergic neurons

discussed above, deleterious effects on synaptic targets of these cholinergic neurons were also observed, including reduced thickness of the cerebral cortex and decreased size of the basal ganglia.

Summary and Conclusions

Prenatal development of the central cholinergic nervous system coupled with the important developmental roles played by acetylcholine in both neural and nonneural tissues should make the vertebrate embryo especially vulnerable to the gestational effects of environmental neurotoxins that target cholinergic receptors or choline esterases. It seems especially important to study the effects of chronic prenatal exposure to cholinergic pesticides on pre- and postnatal brain development as well as behavioral consequences of these exposures.

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