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COMMENTARY

Muscarinic Cholinergic Receptor Signal Transduction as a Potential Target for the Developmental Neurotoxicity of Ethanol

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ABSTRACT. Central nervous system dysfunctions (most notably mental retardation and microcephaly) are among the most significant effects of *in utero* exposure to ethanol. Ethanol has been shown to cause alterations of both neuronal and glial cells, including cell loss, and changes in their migration and maturation. Here, we propose that one of the potential targets for the developmental neurotoxicity of ethanol may be represented by the signal transduction systems activated by cholinergic muscarinic receptors. Ethanol has been shown to inhibit second messenger systems activated by various G-protein-coupled receptors, including certain subtypes of muscarinic receptors. Although the roles of muscarinic receptors in brain development have not been fully elucidated, two potentially relevant effects have been discovered in the past few years. By activating muscarinic receptors coupled to phospholipid metabolism, acetylcholine can induce proliferation of glial cells, and act as a trophic factor in developing neurons by preventing apoptotic cell death. Ethanol has been shown to inhibit both actions of acetylcholine *in vitro*. These effects of ethanol may lead to a decreased number of glial cells and to a loss of neurons, which have been observed following *in vivo* alcohol exposure. In turn, these may be the basis of microencephaly and cognitive disturbances in children diagnosed with Fetal Alcohol Syndrome. BIOCHEM PHARMACOL **57**;7:721–726, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. ethanol; muscarinic receptors; glial cells; neurons

Exposure to ethanol during pregnancy is detrimental to human fetal development. Children exposed to ethanol while *in utero* may present a syndrome (Fetal Alcohol Syndrome or FAS) that is characterized by particular facial features, growth deficiency, and severe CNS dysfunctions [1]. CNS abnormalities, most notably microcephaly and mental retardation, are of the most concern as they appear to be long-lasting, if not irreversible [2–4]. The clinical and social consequences of FAS are highlighted by its increasing incidence [5], which makes FAS one of the leading causes of mental retardation in the general population [6].

A large number of studies have been conducted in laboratory animals to gain an understanding of the characteristics and mechanisms of alcohol teratogenicity, and their results have been summarized in several books and reviews [7–9]. Most studies have been carried out in rodents, where exposure to ethanol may occur during pregnancy (to mimic exposure during the first two trimesters of pregnancy in humans) or postnatally (to mimic human exposure during the third trimester of pregnancy). As the development of the nervous system differs across species, and since, within one species, different brain regions and cell types develop at different times, the timing of exposure to ethanol is extremely important for specific adverse effects to be manifest. For example, microencephaly, which is also seen in 80% of FAS children, can be produced in rats almost exclusively when ethanol is given postnatally, during the brain growth spurt [10].

In addition to microencephaly, several studies have examined the specific effects of developmental ethanol exposure on neurons and glial cells. Selective losses of certain neurons, for example hippocampal pyramidal cells or cerebellar Purkinje and granule cells, have been found [11–13]. Alterations in glial cell development, migration, and number also have been reported [14–17].

Many research efforts have addressed the biochemical and molecular mechanisms involved in the developmental neurotoxicity of ethanol. Ethanol has been shown to affect the development of neurotransmitter systems, second messenger pathways, ion channels, hormones, and cell adhesion molecules [18–20].

In this commentary, it is proposed that a potentially relevant target for ethanol may be represented by the signal transduction pathways activated by the neurotransmitter acetylcholine. Evidence is presented indicating that ethanol disrupts second messenger systems stimulated by certain subtypes of cholinergic muscarinic receptors during brain development, and that this leads, at least *in vitro*, to

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inhibition of muscarinic receptor-induced glial cell proliferation and neuroprotection.

CELL SIGNALING THROUGH MUSCARINIC RECEPTORS

Muscarinic receptors are one of the two classes of receptors (the other being the nicotinic receptors) that bind the neurotransmitter acetylcholine. Five subtypes of muscarinic receptors have been cloned, and their coupling to second messenger systems has been characterized [21, 22].

The m_2 and m_4 receptors are coupled to a pertussis toxin-sensitive G-protein that inhibits adenylate cyclase, leading to inhibition of cyclic AMP formation, while the other three subtypes (m_1, m_3, and m_5) are coupled through pertussis toxin-insensitive G-proteins to PLC* (particularly the β1 isozyme) [23, 24]. Activation of PLC causes hydrolysis of membrane PIs with the formation of IP_3 and DAG. IP_3 causes mobilization of intracellularly stored calcium by acting on specific receptors in the endoplasmic reticulum, while DAG activates several PKC isozymes [25, 26], which can phosphorylate a variety of substrates including receptors, enzymes, and ion channels. The m_1, m_3, and m_5 subtypes also activate other significant intracellular pathways. PLD hydrolyzes PC to phosphatidic acid, which, in turn, can be converted to DAG by a phosphatidate-phosphohydrolase [27]. This pathway may be of importance, since PC constitutes more than 40% of membrane phospholipids, and PC-derived choline appears to be the major source of choline for the synthesis of acetylcholine. Other pathways include activation of phospholipase A_2, which causes the release of arachidonic acid [28], and the production of nitric oxide, which, in turn, leads to the activation of guanylate cyclase and an increase in cyclic GMP levels [29]. Receptor subtypes of both groups can also activate the Ras/Raf/MAP kinase pathway in a pertussis toxin-sensitive (e.g. m_2) or -insensitive (e.g. m_1 and m_3) fashion [30, 31].

MUSCARINIC RECEPTORS IN THE DEVELOPING BRAIN

A number of studies have examined the ontogenesis of muscarinic receptors and associated signal transduction pathways in the CNS, and the topic was reviewed recently [32]. Receptor binding studies have indicated that in rat brain muscarinic receptors can be detected at 15 days of gestation [33], and that their density is low at birth and increases with age, reaching adult levels at about 28 days of age [33, 34]. Studies with receptor subtype-specific antibodies also have shown a gradual postnatal increase in the rat brain [35], while at the mRNA levels m_1 and m_3 receptors increase with age, but m_2 and m_4 mRNA levels do not change significantly over time [36].

Muscarinic receptor-mediated inhibition of cyclic AMP accumulation in rat cerebral cortex has been detected only in postnatal week 3, suggesting that m_2 and m_4 receptors are uncoupled from G_i at earlier ages [37]. Several investigators have shown that muscarinic receptor-stimulated PI metabolism is higher in brains from neonatal rats than in adults [34, 38, 39]. In certain brain regions (e.g. cerebral cortex and hippocampus), the effect of muscarinic agonists appears to peak around postnatal days 6–10, whereas in others (e.g. cerebellum) it decreases from birth [34, 40]. The mechanisms underlying such enhanced stimulation of PI metabolism in immature rats are not fully understood, and the prevalent hypotheses are that it is due to a more efficient coupling at early ages [38], and/or to a lack of inhibitory feedback by PKC or other kinases [41]. Enhanced PI metabolism by muscarinic receptors in neonatal mice [42] and human fetuses [43] also has been reported. Activation of PLD by muscarinic agonists is also enhanced in the neonatal rat brain; indeed, such a biochemical response could be detected in cerebral cortical slices from 7-day-old rats but not in adults [44].

The development of other parameters of the cholinergic system has also been investigated. Activity of choline-acetyltransferase (ChAT) in rat brain increases slowly, as only 19% of adult ChAT is present after 1 week [45]. On the other hand, choline levels, which are considered to be the rate-limiting factor in the biosynthesis of acetylcholine, are actually higher in the neonatal than in the adult rat brain [45]. This fact, together with the low activity of acetylcholinesterase in the neonatal rat brain, may explain why acetylcholine levels are high (40–87% of adult levels) early postnatally [33]. This latter observation is of importance, as sufficient endogenous acetylcholine is present in the neonatal rat brain to activate muscarinic receptors, thus making it plausible that certain effects observed *in vitro* may also occur physiologically *in vivo* (see Ref. 46 for a recent review on non-neuronal effects of acetylcholine).

POSSIBLE ROLES OF MUSCARINIC RECEPTORS IN BRAIN DEVELOPMENT

There is only limited information on the role of the cholinergic system, and muscarinic receptors in particular, in brain development [24, 47]. Most findings indicate that muscarinic receptor formation precedes the development of presynaptic markers, suggesting that muscarinic receptors may have an effect on synaptogenesis. The previously discussed findings of an enhanced phospholipid metabolism in the developing brain upon stimulation of muscarinic receptors have added strength to the hypothesis that this system may have a fundamental role in the regulation of synaptogenesis, neurocytomorophogenesis, and glial cell proliferation [47]. Neonatal lesions of the basal forebrain cholinergic neurons result in abnormal cortical development, whose main features include a delay in the develop-

* *Abbreviations:* PLC, phospholipase C; PLD, phospholipase D; PI, phosphoinositide; DAG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate; PC, phosphatidylcholine; PKC, protein kinase C; IGF-I, insulin-like growth factor-I; NMDA, *N*-methyl-D-aspartate; and MAP, mitogen-activated protein.

mental expression of critical neuronal components of the neocortex [48]. Muscarinic receptors are particularly enriched in nerve growth cones from neonatal rats, and their activation leads to phosphorylation of GAP43, a PKC substrate, which is believed to play a central role in neurite elongation [49]. Two additional effects of muscarinic receptors that have been investigated recently are their ability to induce proliferation of glial cells and to exert a neuroprotective action in neurons [50, 51].

A number of studies have shown that astrocytes and other glial cells possess functional muscarinic receptors [52–54]. However, the role that such receptors may have in the regulation of glial cell physiology is not well understood. An important observation was made some years ago by Ashkenazi *et al.* [55], who showed that activation of muscarinic receptors coupled to phospholipid metabolism, but not those preferentially coupled to inhibition of adenylate cyclase, causes proliferation of rat cortical astrocytes. This finding was subsequently confirmed and expanded by other investigators [50, 56, 57]. Rat cortical astrocytes were found to express mRNA for m_3 and m_2 muscarinic receptors, and proliferation induced by muscarinic agonists appears to be due primarily to stimulation of the m_3 subtype [50]. By activating the same receptor subtype, muscarinic agonists can also induce proliferation of human astrocytoma cells [50]; in addition, carbachol was also found to be mitogenic in oligodendrocyte progenitor cells [58]. The mechanisms by which muscarinic and other G-protein-coupled receptors induce mitogenic signalling are under investigation, but have not been fully elucidated [31, 59, 60]. Hydrolysis of membrane phospholipids (PI, PC) and activation of PKC isozymes, immediate-early genes, and MAP kinases appear to be involved in a rather complex array of pathways leading to the regulation of gene expression.

Acetylcholine also has been found to have a trophic effect in neurons by activating muscarinic receptors. In cerebellar Purkinje cells *in vitro*, acetylcholine analogues increase cell survival induced by nerve growth factor, although they do not have any trophic effect when present alone [51]. The effect of muscarinic agonists is inhibited by atropine and is ascribed to activation of m_1 and/or m_3 receptors [51]. Additional evidence for a role of acetylcholine in cerebellar development is provided by results obtained in cerebellar granule cells. When these cells are maintained in the presence of physiological concentrations of K^+ (nondepolarizing conditions), they undergo apoptosis. By activating muscarinic m_3 receptors, acetylcholine has been shown to inhibit apoptotic cell death [61]. The antiapoptotic effect of muscarinic agonists was direct, and did not involve the release of neurotrophic factors from granule or glial cells. The lowering of $[K^+]_e$ from 25 to 5 mM results in a rapid and dramatic lowering of $[Ca^{2+}]_i$. However, differently from NMDA, which also has an anti-apoptotic effect in these neurons, carbachol has only a small transient effect on $[Ca^{2+}]_i$, suggesting that the ability of muscarinic agonists to block apoptosis is not due to maintenance of elevated $[Ca^{2+}]_i$ [61]. This protective effect of muscarinic agonists in cerebellar granule cells was confirmed recently.* A similar anti-apoptotic effect of acetylcholine has also been observed in PC12 cells stably expressing m_1 muscarinic receptors, when these cells undergo apoptosis upon removal of growth factors [62]. In these cells, the effect of muscarinic agonists does not appear to be associated with alterations of cyclic AMP or Ca^{2+} levels or with activation of PKC [62].

ETHANOL AND MUSCARINIC RECEPTOR SIGNAL TRANSDUCTION IN THE DEVELOPING BRAIN

During the past decade, numerous studies have investigated the hypothesis that muscarinic receptor-stimulated second messenger systems, particularly the hydrolysis of membrane phospholipids, may represent a relevant target for the developmental neurotoxicity of ethanol. Initial studies indicated that administration of alcohol to rats during the brain growth spurt (postnatal days 4–10) causes microencephaly, as well as inhibition of muscarinic receptor-stimulated PI metabolism in brain slices [63]. This biochemical effect of ethanol was seen only in certain brain areas, such as cerebral cortex, hippocampus, and cerebellum, which are known targets for the developmental neurotoxicity of ethanol, and was unique to muscarinic receptors, as other metabotropic receptors were unaffected [63, 64]. Furthermore, ethanol was effective at inhibiting muscarinic receptor-stimulated PI metabolism only in neonatal rats, both *in vivo* and *in vitro*, while adult animals were not affected [63–65]. Inhibition of PI metabolism did not appear to be mediated by the production of acetaldehyde [66], and was seen also in mouse brain tissue [42]. The precise molecular target for the action of ethanol on this system remains elusive, although one or more G-proteins coupling muscarinic receptors to PLC have been suggested [67]. Additional time–course and dose–response studies *in vivo* showed that microencephaly and inhibition of muscarinic receptor-induced PI metabolism are highly correlated, i.e. they are both either present or absent following developmental administration of ethanol [68, 69].

Although these observations showed strong correlations, they do not provide causality. Recent *in vitro* findings, however, are offering some insights on the relationship between inhibition of the effects of acetylcholine and the developmental neurotoxicity of ethanol. Several studies have indicated that ethanol can inhibit the proliferation of astrocytes; this effect, however, is seen at rather high alcohol concentrations (100 mM and above; reviewed in Ref. 70). On the other hand, ethanol was shown to inhibit the mitogenic effect of acetylcholine in rat cortical astrocytes and human astrocytoma cells at lower concentrations

*Castoldi AF, Barni S, Randine G, Costa LG and Manzo L, Ethanol selectively interferes with the tropic action of NMDA and carbachol on cultured cerebellar granule cells undergoing apoptosis. *Dev Brain Res* **111:**279–289, 1998.

(10–50 mM) [57]. These concentrations are comparable to those found in the blood of moderate to heavy drinkers. Proliferation induced by other mitogens (e.g. IGF-I and prolactin) is also inhibited by these low concentrations of ethanol, but that induced by platelet-derived growth factor is not [71, 72]. Ethanol has been shown to inhibit acetylcholine-induced increases in IP_3, Ca^{2+} levels, and phosphatidic acid in glial cells [44, 73]; however, the exact intracellular pathways involved in its inhibitory effect on glial cell proliferation have not been defined. Of particular interest is that the presence of ethanol may not be necessary for the mitogenic action of various agonists (e.g. acetycholine and prolactin) to be manifest (72; Fu C, Guizzetti M and Costa LG, unpublished observations). Thus, ethanol, in addition to directly inhibiting signal transduction processes activated by muscarinic receptors, may also cause still unidentified alterations in astrocytes, which, in turn, lead to a reduction in the effects of certain mitogens. Although the mechanisms of ethanol-induced glial cell proliferation have yet to be clarified, one could speculate that this action of alcohol may contribute to the microencephaly seen in individuals with FAS [9, 57].

Ethanol also has been shown to inhibit the anti-apoptotic action of various trophic factors, such as acetylcholine, NMDA, and IGF-I, in various cellular systems. Ethanol promoted apoptosis in cerebellar granule cells by inhibiting the trophic effect of NMDA [74, 75]. In this case, inhibition of trophism by ethanol was ascribed to its ability to block the initial response to NMDA, i.e. the increase in $[Ca^{2+}]_i$ [75]. In BALB/c3T3 cells, IGF-I markedly protected cells from tumor necrosis factor-induced apoptosis. In this model, ethanol inhibited the anti-apoptotic effect of IGF-I at concentrations of 5–25 mM [76]. Similar results were also observed in cerebellar granule cells (unpublished data reported in Ref. 76], and in both instances the effect may be ascribed to an action at the IGF-I receptor level and/or to downstream effects [71, 76]. Recently, ethanol was found to inhibit the anti-apoptotic effect of carbachol in cerebellar granule cells.* In this study it was also observed that alcohol-elicited inhibition of trophism did not require the concomitant presence of ethanol and carbachol in order to occur; however, the molecular mechanisms involved in this inhibitory action of ethanol are not known. Altogether, these findings raise the interesting hypothesis that ethanol may enhance neuronal death during brain development, by inhibiting the trophic action of various endogenous factors, including acetylcholine. Indeed, excessive neuronal loss is one of the main consequences of developmental ethanol exposure.

* Castoldi, AF, Barni S, Randine G, Costa LG and Manzo L, Ethanol selectively interferes with the trophic action of NMDA and carbachol on cultured cerebellar granule cells undergoing apoptosis. *Dev Brain Res* **111:**279–289, 1998.

CONCLUSIONS

Despite a large number of studies, the cellular and molecular mechanisms involved in the developmental neurotoxicity of ethanol have not been fully elucidated. Several *in vivo* and *in vitro* studies indicating that ethanol can disrupt muscarinic receptor signal transduction have prompted the testing of the hypothesis that this neurotransmitter system may represent a target for alcohol's developmental neurotoxicity. The identification of the mitogenic effect of acetylcholine in glial cells and of its trophic/anti-apoptotic action in neurons, both associated with activation of muscarinic receptors, suggested that these two cellular processes may be affected by ethanol, and initial results confirm this hypothesis. Inhibition of these effects of acetylcholine by ethanol may be responsible, at least in part, for the microencephaly, neuronal death, and associated behavioral disturbances seen following developmental ethanol exposure. Although mechanisms involved in the developmental effects of ethanol are likely to be multiple and complex, this hypothesis, while by no means inclusive, addresses two major, irreversible consequences of developmental ethanol exposure, and adds new insights on the action of acetylcholine and muscarinic receptors in brain development.

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