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COMMENTARY

NEUROTRANSMITTER METABOLISM IN CELL CULTURE

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Cell culture techniques have provided a new dimension in the study of the nervous system. Selected neural tissues can be grown in isolated environments, allowing manipulation of the types of cells grown together and the components of the medium. The inherent capabilities of individual cells, as well as their responsiveness to external stimuli, can be examined. Cell culture studies are proving particularly useful in analyzing the effects of genetic, developmental and pharmacologic factors on neurotransmitter metabolism.

Much of the work to date has been directed toward substantiating that cultured neural and glial cells continue to function *in vitro* as *in vivo*. When their behavior is similar, these cultures serve as models to study molecular mechanisms of neural functioning. When behavior is different, one must consider whether artifacts are being introduced by the conditions of culture. Differences, however, can give insight into the pluripotentiality of these cells and control mechanisms operant *in vivo*. We will comment here primarily on studies and ideas which illustrate unique experimental approaches available through the use of cell culture.

Cell sources

Methods for the culture of neural and glial cells are continually being expanded and refined (for reviews, see Refs. 1 and 2). Embryonic tissues can be dissected from localized areas of the nervous system and adapt more easily than adult tissues to culture. This source often yields "immature" cells which may or may not continue through their normal development *in vitro*. These primary cultures contain glia and "fibroblastic" cells, as well as different types of neurons. Various stratagems can be employed to inhibit overgrowth of the non-neuronal cells.

Continuous cells lines obtained from tumors of the nervous system proliferate rapidly in culture and can be grown as homogeneous, clonal populations derived from single cells. Sources of these transformed cells include tumors arising spontaneously and those induced by exposure to viruses or mutagens. All these oncogenic lines are aberrant with respect to their aneuploid chromosome complement, inexact cell of origin, and unbridled capacity for division; still they express many properties of normal neurons and glia (for reviews, see Refs. 3–5).

Somatic cell hybridization techniques offer another rich source of cell lines for culture. As will be discussed below, neural properties continue to be expressed in hybrids formed from both embryonic and transformed neural cells. Hopefully, in the future, continuously dividing neural cell lines will be obtained from animals carrying inherited lesions of neural metabolism and from specific areas of the nervous systems. Non-neuronal cell types, which possess functional proteins in common with nerve cells, may also prove useful in studies of neurotransmitter metabolism.

Properties of neurotransmission expressed in culture

Various aspects of neurotransmitter metabolism have been examined in culture, including uptake, storage, synthesis, degradation, release and membrane reception. We will consider each of these aspects for several putative transmitters.

Acetylcholine. Choline is thought to be taken up by a specific transport system into neurons which synthesize acetylcholine. Similar transport mechanisms are observed for other transmitters and are characterized by their high substrate affinity, dependence on Na^+ and energy, and inhibition by specific drugs, in thise case hemicholinium[6, 7]. Choline is taken up into cultured neuroblastoma, glia and "fibroblastic" cells by a high affinity system [8], which has none of the other properties of specific transport. Even in neuroblastoma lines with high levels of choline acetyltransferase (CAT, EC 2.3.1.6) activity, transport is only partially energy and Na^+ dependent, not inhibited by hemicholinium and not linked to acetylcholine synthesis [9]. Further studies are needed to determine if specific uptake can occur in association with synapse formation.

Cultured embryonic neurons from many areas of the nervous system show high levels of CAT activity [10–13] and clusters of electron-lucent vesicles (500–700 Å in diameter) [13, 14], presumed to store acetylcholine. Cholinergic neurons are able to form electrophysiologically functional synapses with skeletal myotubules in culture (for review, see Ref. 2), demonstrating that mechanisms are intact for storage and release of acetylcholine and uptake of choline. Continuous lines of mouse neuroblastoma [15, 16] and rat neural [17], but not glial or "fibroblastic" [18] cells also have CAT activity and clusters of small, clear vesicles [19]. Neuroblastoma line NS-20, with high CAT activity [15], stores substantial amounts of acetylcholine [17]; and neuroblastoma × glioma hybrid line NG108-15 (also called 108CC15) can form functional, nicotinic synapses with primary skeletal muscle in culture [20].

Acetylcholinesterase (AChE, EC 3.1.1.7) activity is present at higher levels in primary and continuous neural and muscle cells [14–16, 21] than in glial and "fibroblastic" cells [18]. Both nicotinic and muscar-

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inic acetylcholine receptors appear on a number of different cell types in culture. Nicotinic receptors have been demonstrated on primary and transformed skeletal myotubules [22, 23], peripheral neurons, and neuroblastoma cells [24] by using *d*-tubocurine to block acetylcholine-induced membrane depolarizations and by binding of α-bungarotoxin. Muscarinic receptors have been described on heart muscle [25], neuroblastoma [26–28] and "fibroblastic" cells [29] by using atropine to block membrane hyperpolarizations and formation of cyclic 3′:5′-guanosine monophosphate (C-GMP) induced by acetylcholine and by binding of [^{3}H]quinuclidinyl benzilate.*

Biogenic amines. Membrane uptake of the amine precursors, phenylalanine, tyrosine and tryptophan, is similar in a number of different types of cultured cells, including glioma, "fibroblastic" and neuroblastoma lines, irrespective of their abilities to synthesize catecholamines or serotonin [8].† The high affinity, saturable component of this transport is relatively independent of Na^+ and energy. By contrast, a specific high affinity uptake system for norepinephrine has been demonstrated in cultured neurons from sympathetic ganglia [30] and in one neuroblastoma clone which is able to synthesize this transmitter [31]. This uptake occurs in the absence of adrenergic synapses, indicating it is not restricted to synaptic terminals.

Many cultured cells, including fibroblasts [32] will take up sufficient quantities of catecholamines from the medium to show paraformaldehyde-induced histofluorescence. However, by using low catecholamine concentrations and specific inhibitors, one can distinguish uptake and storage specific to adrenergic neurons [33, 34]. Storage of dopamine and norepinephrine in neuroblastoma cells [35–37] and primary sympathetic neurons [30] is sensitive to reserpine and associated with a particulate subcellular fraction, presumably the dense core vesicles observed in these cells. Two puzzling findings suggest the need for further examination of catecholamine storage in cultured cells. First, the dense core vesicles observed in murine neuroblastoma cells are larger (1000–3000 Å in diameter) than those in adrenergic neurons *in vivo* (400–1000 Å). Second, cultured neurons from several areas of the brain store very little of the catecholamines which they synthesize [38]. However, in cultured primary sympathetic neurons, catecholamine storage is functionally intact. These cells release catecholamines upon exposure to depolarizing stimuli, such as veratridine and high K^+ concentrations [30, 39], and release is blocked by decreased Ca^{2+} or increased Mg^{2+} concentrations, suggesting mediation by vesicular exocytosis.

Enzymes needed in catecholamine and serotonin synthesis have been demonstrated in a number of primary and continuous neural cultures. Tyrosine hydroxylase (TH, EC 1.14.16.2), tryptophan hydroxylase (EC 1.14.16.4) and dopamine β-hydroxylase (DBH, EC 1.14.17.1) activities have been described in cultures of brain [21, 38], sympathetic ganglia [30, 40] and certain cloned neural lines from mouse [15, 16, 41–43] and rat [17] tumors. Some neuroblastoma clones with little or no TH activity [15] still have DBH activity [44], making them candidates for octopamine synthesis. L-Aromatic amino acid decarboxylase activity (EC 4.1.1.28) is expressed variably in culture—it appears at lower than expected levels in brain aggregates [21] and has been undetectable in homogenates of neuroblastoma cells [45], even though these cells can synthesize catecholamines and serotonin [46]. Phenylethanolamine *N*-methyltransferase activity (EC 2.1.1.28) has not been reported in cultured cells, but at least one line of murine neuroblastoma cells does contain epinephrine [46]. Monoamine oxidase (MAO, EC 1.4.3.4), aldehyde reductase (EC 1.1.1.1) and catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) activities have been demonstrated in primary fibroblasts [32, 44] and nerve [47] cultures, as well as continuous neuroblastoma and glioma lines [48–50]. Monoamine oxidase activity in the latter two cell types is exclusively of the A form [51], which is thought to predominate in intraneuronal metabolism.

In contrast to the relative ease of cholinergic synapse formation in culture, no electrophysiologically functional adrenergic synapses have been demonstrated in cultured cells, although such synapses do appear to form by morphologic criteria [1]. Receptors for catecholamines have been demonstrated on a number of cells types. Norepinephrine and isoproterenol interact with β-adrenergic receptors to increase intracellular levels of cyclic adenosine 3′:5′-monophosphate (cAMP) in cultured "fibroblastic" [52], glioma [53] and lymphosarcoma cells [54]. Electrophysiologic studies also suggest the presence of these receptors on atrial cells [25], Purkinje neurons [55] and other brain neurons [56] in culture. Interestingly, β-adrenergic receptors have not been described on murine neuroblastoma cells, presumably because of the sympathetic origin of these cells. Both norepinephrine [57] and dopamine [29, 58] can produce changes in membrane potentials of neuroblastoma cells and hybrids derived from them, but the nature of these transmitter–receptor interactions has not been well characterized.

Other transmitters. Neural and glial cells from both primary and continuous cell cultures show high affinity uptake of the putative transmitters glycine, taurine, glutamic acid and γ-aminobutyric acid (GABA) [8, 59–63].† In many of these studies, transport has been shown to be largely energy and Na^+ dependent. Such findings make it difficult to believe that specific transport mechanisms exist only in neurons which use these substances as transmitters. Storage of GABA appears to be a Ca^{2+}-dependent process in some rat neural lines [61], and cultured primary neurons and glioma cells can accumulate taurine and GABA at concentrations >100-fold above that in the medium [59, 62].

It is difficult to distinguish changes in amino acid metabolism which correlate directly with the role of these compounds as transmitters. Schubert *et al.* [64] have observed higher levels of GABA and β-alanine in free amino acid pools of neural and glial lines, as compared to muscle and "fibroblastic" lines. Glutamic acid decarboxylase activity (EC 4.1.1.15), inhibited by amino-oxyacetic acid, appears in glioma and

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neuroblastoma, but not "fibrolastic" lines [18, 59, 65], and in primary cultures from central [21], but not peripheral [66] nervous tissue. Together these studies suggest an active role for glial cells in neurotransmitter metabolism.

As predicted from studies *in vivo*, glycine hyperpolarizes and glutamate depolarizes neurons from the central nervous system [24]. Glutamate has no effect on cultures of superior cervical ganglia, dorsal root ganglia or skeletal muscle, and can hyperpolarize atrial muscle cells [24, 25]. GABA can depolarize neurons from the superior cervical and dorsal root ganglia [25], but hyperpolarizes neurons from the central nervous system [24, 56, 67].

Regulation of neurotransmitter synthesis

Culture systems offer a means of studying regulation of neurotransmitter metabolism at the cellular level independently of trans-synaptic and intercellular modulations. Most studies to date have examined regulatory influences on catecholamine metabolism. In both noradrenergic neuroblastoma cells and sympathetic neurons, extracellular tyrosine concentrations can affect uptake of tyrosine and TH activity. Short-term (30 sec) uptake of tyrosine in neuroblastoma cells is doubled by a 2-hr preincubation with 10^{-3} M or 10^{-4} M tyrosine [68]. Increasing levels of tyrosine, up to 6×10^{-5} M, increase synthesis of catecholamines [40] and the amount of TH, as measured by specific antibody titration [69]. This latter concentration of tyrosine correlates with the maximum capacity for cellular uptake* [68] and the range of serum levels for this amino acid *in vivo*, indicating that tyrosine is not normally rate-limiting for catecholamine synthesis.

In different culture systems, various co-factors may be rate-limiting for catecholamine synthesis. Addition of biopterin, necessary for TH activity, appears to stimulate catecholamine accumulation in organ cultures [70], but not dissociated cell cultures [40] of sympathetic ganglia. Certain cell types, including neuroblastoma cells [71], synthesize their own biopterin. The low levels of L-aromatic amino acid decarboxylase activity seen in neuroblastoma cells [45] and brain aggregates [21] suggest a need to examine the content and stability of pyridoxine in culture medium. Addition of ascorbic acid, the presumed co-factor for DBH, increases norepinephrine formation in some primary cultures [40], but not in others [38]. As would be expected from studies *in vivo*, drugs which block TH activity, such as α-methyl-*p*-tyrosine [70], or prevent access of dopamine to DBH, such as reserpine [30, 35, 70], depress catecholamine synthesis, while MAO inhibitors increase levels of catecholamines [72].

A number of studies have focused on the ability of depolarization or hormones to alter catecholamine metabolism in adrenergic neurons. Most of these studies have used short-term (48 hr) explant cultures of superior cervical ganglia. Many of the agents used, high K^+ concentrations, dibutyryl cAMP (dBC-AMP) and nerve growth factor (NGF) also affect the survival and/or differentiation of nerve cells making it difficult to assess their specific effects on neurotransmitter enzymes. High K^+ (54 mM) or dBC-AMP (1–5 mM) can increase TH [72–74], DBH [73, 75] and MAO [73] activities in explant cultures by about 50 per cent over control levels. Two-fold increases in TH activity [76] and long-term (20 hr) uptake of tyrosine [50] are also seen in noradrenergic neuroblastoma clones treated with 1 mM dBC-AMP. The slow rates of increase and their inhibition by cyclohexamide suggest the need for new protein synthesis. Nerve growth factor also stimulates TH [72] and MAO [77] activities in cultures of sympathetic ganglia; at optimal NGF concentrations, the addition of adrenocorticotrophic hormone or dBC-AMP can further increase MAO activity [78]. The effects of K^+ and NGF [79] may be mediated through transitory increases in cellular cAMP levels. Both synthesis and degradation of catecholamines may be increased by such treatments. It would be interesting to know if increases in MAO activity occur in both neuronal and non-neuronal cells in these cultures.

In continuous neuroblastoma lines, agents which depress cell division frequently increase the expression of differentiated properties. Such agents include dBC-AMP, Na butyrate, aminopterin, 5-bromodeoxyuridine and low serum levels. Differentiated properties include neurite extension and electrical excitability, as well at CAT, AChE, TH and DBH activities [3]. It is not clear how such agents "induce" differentiation and whether the process bears any relation to regulatory mechanisms operating *in vivo*. Treatment of glioma or neuroblastoma cells with neuraminidase (EC 3.2.1.18) causes a 2- to 3-fold rise in acetylcholinesterase and butyrylcholinesterase activities through an increase in the V_{max} of these enzymes [80]. This finding suggests regulation of enzyme activities through the structural arrangement of macromolecules within membranes.

Developmental insights

Formation of cholinergic synapses in culture has allowed analysis of the roles of acetylcholine and cholinergic receptors in this process. Synapses between spinal cord neurons and skeletal myotubules form in the presence of curare, which blocks the interaction of acetylcholine with these receptors, and xylocaine, which blocks electrical activity [81]. Further, areas of high sensitivity to acetylcholine form on myotubules without innervation [82]. It appears that developmental processes involving movement of neurites toward end-organ muscles, adhesion of neurite endings to muscle membranes and formation of synapses need not be mediated through the interaction of transmitters with their receptors.

Primary embryonic neurons grown *in vitro* may follow a developmental sequence parallel to their course *in vivo*. Noradrenergic neurons from sympathetic ganglia show an increasing capacity to synthesize catecholamines over several weeks *in vitro* [66]. Superior cervical ganglia co-cultured with iris tissue have increased DBH activity and high affinity uptake of norepinephrine, as compared to ganglia grown alone [83]. In spinal cord [14] and brain [21] cultures, increases in CAT and AChE activity appear to accompany the formation of cholinergic synapses. Skeletal muscle myotubules can "induce" CAT acti-

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vity in spinal cord neurons without notably affecting AChE activity [12]. This process does not result from increased survival of neurons, nor does it depend on synaptic contact between neurons and myotubules. Rather, "trophic" factors, secreted by muscle cells into the medium, appear to be responsible; the factor(s) are stable to heating at 58° and relatively large in size (>50,000 daltons).* Dissociated sympathetic ganglia neurons can also form electrophysiologically functional cholinergic synapses with each other and with skeletal myotubules *in vitro* [84]. Choline acetyltransferase activity is increased dramatically in these cultures by exposure to a number of non-neuronal cell types, including fibroblasts, muscle cells and glioma cells, or medium conditioned by them, through factor(s) similar to those described above [13, 30]. Such "trophic" factors may resemble NGF and may represent a class of molecules which plays a critical role in neuronal development.

The demonstration of cholinergic neurons within populations of cultured sympathetic ganglia cells [13] raises interesting questions about the capacity of neurons to synthesize more than one transmitter. Ultrastructural studies have repeatedly demonstrated more than one class of synaptic vesicles in various neuronal populations. Thus, a few dense core granules, similar to those associated with catecholamine storage, are seen intermingled among small, clear vesicles, presumed to contain acetylcholine. Although about 5 per cent of the neurons in the superior cervical ganglia *in vivo* may be cholinergic [85], under certain conditions of culture more than 50 per cent are able to form cholinergic synapses [13]. This phenomenon of apparent pluripotentiality for transmitter synthesis has also been observed with continuous neural lines. Simultaneous presence of CAT and TH activities appears in several clonal neuroblastoma lines [15, 86]; and a rat neural clone, and subclones obtained from it, synthesize detectable amounts of acetylcholine, dopamine and GABA [17]. Studies on transmitter metabolism in single cells are needed to resolve this issue, but it appears that developmentally immature neurons may be able to "program" their transmitter synthesis in several alternate ways.

Drug actions

Culture studies have proven especially useful in analysis of receptor functioning. Responses to drugs and neurotransmitters have been examined: (1) by monitoring changes in membrane potentials, either through electrophysiologic recordings of single cells [24] or by uptake of $^{22}Na^{+}$ in cell populations [87], (2) by measuring changes in intracellular cyclic nucleotide levels, and (3) by receptor-specific binding of isotopically labeled drugs.

Morphine receptors have been demonstrated on hybrid line NG108-15 by the pharmacologically specific binding of labeled morphine analogues and the ability of morphine to depress both resting cAMP levels and those induced by prostaglandin E_1 [88, 89], while increasing C-GMP levels [90]. An interesting model of "tolerance" and "addiction" has developed from these studies. Cells exposed to morphine for several days show a compensatory increase in adenylate cyclase activity (EC 4.6.1.1) necessary to maintain normal intracellular levels of cAMP in the presence of this drug. When morphine is removed, adenylate cyclase activity is unchecked and remains abnormally high for a short period of time, resulting in increased levels of cAMP which could disrupt neuronal functioning.

Desensitization of β-adrenergic and cholinergic receptors has also been observed in cultured cells. Brief exposures of "fibroblastic" or glioma cells to isoproterenol, a β-adrenergic agonist, result in transitory rises in cellular levels of cAMP [52, 91]. With longer exposures (2 hr) the cells become refractory to stimulation by isoproterenol and recovery requires 9–24 hr of culture in the absence of this drug. In studies with frog red blood cells, it appears that β-adrenergic receptors, as measured by alprenolol binding, actually decrease in number during desensitization, and recovery involves new receptor synthesis and/or membrane insertion [92]. Desensitization of nicotinic receptors on cultured myotubules can also be measured by a decrease in the rate of $^{22}Na^{+}$ uptake and occurs after only a 2-min exposure to carbamylcholine [87].

Other studies point to the molecular mechanisms of action of several drugs. 6-Hydroxydopamine is selectively toxic to developing adrenergic neurons *in vivo*. Cultured neuroblastoma cells show 2- to 3-fold greater uptake of this dopamine analogue and sensitivity to its lethal effects than "fibroblastic" cells [93]. 6-Hydroxydopamine taken up into either cell type becomes covalently linked to proteins forming high molecular weight complexes [94]. Linkage is presumed to be random and to inactivate many enzymes. Another drug, diphenylhydantoin, used as an anticonvulsant *in vivo*, has been found to block repetitive action potentials generated in neuroblastoma cells in response to low Ca^{2+} concentrations [95]. In other studies with neuroblastoma cells, C-AMP levels are increased by exposure to adenosine [96], adding further credibility to the role of purines as neurotransmitters [97].

Genetic approaches

One of the main advantages in using continuously dividing neural cells lines is the ability to select mutants or epigenetic variants which show altered neurotransmitter metabolism. Simantov and Sachs [98] have used antibody prepared against AChE as a cytotoxic factor to select against cells with this enzyme. Lines with low AChE activity obtained in this manner showed no change in CAT activity. These workers have also selected neuroblastoma lines with increased resistance to the growth inhibitory influences of dBC-AMP or high temperature (40°). These new lines show alterations in "induction" of AChE activity and in cAMP binding protein [99].

Increased intracellular levels of cAMP can kill lymphosarcoma cells. Bourne *et al.* [54] selected variant lines able to grow in the presence of isoproterenol, normally a stimulator of adenylate cyclase activity. These lines showed normal resting levels of cAMP and phosphodiesterase activity (EC 3.1.4.17), but did not respond to a number of drugs normally able to

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increase cAMP levels. They concluded that these variants contain an altered adenylate cyclase, in contrast to another variant line which seems to lack the cytoplasmic receptor for cAMP. Presumably one could also isolate lines with defective β-adrenergic receptors by this technique.

As with other continuous cells lines, neuroblastoma and glioma lines lacking hypoxanthine phosphoribosyltransferase (HPRT, EC 2.4.2.8) activity can be selected by their resistance to 6-thioguanine. Such lines offer a model for the Lesch–Nyhan syndrome in humans in which low HPRT activity, important in purine salvage, results in neuronal dysfunction. One neuroblastoma line lacking HPRT activity was found to be comparable to its parental clone in morphology, electrical excitability and AChE activity [100]. Other noradrenergic neuroblastoma lines lacking HPRT activity, however, have sharply reduced MAO activity, with no changes in TH, DBH or COMT activities [44]. This latter finding suggests an as yet undefined link between the regulation of purine and catecholamine metabolism.

Since both tyrosine and tryptophan hydroxylases can convert phenylalanine to tyrosine, neuroblastoma lines with these enzyme activities can be selected by their ability to grow on medium lacking tyrosine, normally an essential amino acid [101]. This technique has made it possible to obtain adrenergic and serotonergic clones from mixed populations of neuroblastoma cells.

Another advantage of continuous lines is their ability to form hybrids. Neuroblastoma cells fused to L-cells frequently form hybrids which continue to express differentiated neural properties [102]. In some cases, fusion produces hybrid lines which show higher levels of neuronal activity than either parent. Thus, fusion of neuroblastoma and glioma cells has produced hybrids NG108-5 and NG108-15 which show heightened ability to synthesize neurotransmitters [103, 104], increased frequency of morphine receptors [105] and the ability to form synapses [20]. Hybridization techniques can also be used to confer normal neural properties on continuously dividing lines. One hybrid line formed by fusion of a murine neuroblastoma cell with an embryonic, murine sympathetic neuron has TH activity and electrophysiologic properties similar to normal parental cell [106].

Neurotransmitter metabolism can also be examined in mutant cells obtained from animals or humans possessing inherited neurologic abnormalities. Messer, in Sidman's laboratory, has examined cerebellar cultures from several mouse mutants, with altered cerebellar development *in vivo*; so far such cultures appear to be similar to control in cell survival and GABA metabolism [107]. In the case of human diseases, it is more difficult to examine neural dysfunction in culture, as one cannot obtain viable nerve populations from patients. Perhaps one can use other, more easily obtainable cell types which bear functional properties in common with nerve cells. Thus, human fibroblasts cultures show MAO and COMT activities* [44] and continuous human fibroblastic line VA-2 possesses both β-adrenergic and prostaglandins receptors [53].

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