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Coexistence of Acetylcholinesterase and Somatostatin-Immunoreactivity in Neurons Cultured from Rat Cerebrum

Abstract. *Cultures derived from rat cerebral hemispheres were sequentially stained for acetylcholinesterase activity and for either somatostatin-like immunoreactivity or cholecystokinin-like immunoreactivity. Somatostatin-like immunoreactivity was found to coexist with acetylcholinesterase activity in individual neurons of several morphological subtypes, but cholecystokinin-like immunoreactivity and acetylcholinesterase activity were never seen in the same neurons. These findings suggest a specific anatomical association, perhaps even an overlap, of the cholinergic and somatostatinergic systems in the mammalian cerebrum, and indicate that the combined deficiencies of somatostatin and cholinergic markers in Alzheimer's dementia and senile dementia of the Alzheimer type may be of pathophysiological importance.*

Numerous studies have demonstrated that there are relatively selective and profound decreases in cholinergic markers such as choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) in cerebral cortex and hippocampal formation in Alzheimer's disease and senile dementia of the Alzheimer type (AD/SDAT) (1-4). More recently, studies in several laboratories have shown that there is also a significant decrease in the assayable levels of somatostatin (SOM) in AD/SDAT (5, 6). Levels of six other neuropeptides, including vasoactive intestinal polypeptide (7, 8), arginine vasopressin (9), cholecystokinin (CCK) (8), and thyrotropin-releasing hormone, luteinizing hormone-releasing hormone, and substance P (10) have been reported as being unchanged in AD/SDAT, and an additional study reported only small decreases in substance P levels as compared with the larger decreases in SOM (11). These data therefore suggest that the decrease in SOM, like that of the cholinergic markers, is relatively selective. However, to date there has been no evidence to suggest possible neuronal associations of the cholinergic and somatostatinergic systems in the brain, and at least three recent studies have suggested that the two systems do not overlap (12-14).

Cultures of dispersed cells derived from cerebrum of fetal rats have proved to be useful for analysis of physiological, biochemical, and pharmacological prop-

erties of cortical neurons (15-18). All of these studies have emphasized the similarities of neurons in culture with those of comparable neurons in situ. Neurons in these cultures have been characterized by numerous techniques, including both silver and tetanus toxoid staining and by extensive intracellular electrophysiological recordings (15), and more recently by morphological classification after intracellular dye injections (19), such that individual cells can be readily identified as neurons on the basis of their phase-contrast appearance in either the living or fixed state.

Immunohistochemical staining has delineated a neuronal localization of several neuropeptides including SOM (20) and cholecystokinin octapeptide (CCK-8) (21), and the cultures have subsequently been used to study the neuronal biosynthesis and release of SOM (22, 23) and to analyze some of the complexities of the neuronal response to SOM (24). A previous study has also demonstrated the feasibility of histochemical staining for AChE in these cultures (25). In the present study we utilized a sequential staining technique for AChE activity and then for either SOM-like immunoreactivity (SOM-LI) or CCK-8-like immunoreactivity (CCK-8-LI) to determine whether one or both of these peptides might coexist with AChE in the cultured cerebral neurons. Our findings of the frequent coexistence of SOM-LI and AChE in individual neurons suggest a relationship

between the cholinergic and somatostatinergic systems in the mammalian cerebrum.

Dissociated cell cultures were prepared from cerebral hemispheres of 15- to 16-day-old rat embryos (15, 16). Cultures were grown on collagen and polylysine-covered glass cover slips in minimum essential medium and 5 percent rat serum in a 5 percent CO₂ atmosphere at 36°C. Cultures were inhibited with cytosine arabinoside for 24 hours when background cells reached confluency. Studies were performed on mature neurons in culture 4 to 8 weeks after plating. To enhance staining for both AChE and SOM, cultures were first incubated with colchicine (100 nM) for 24 hours immediately prior to fixation. Cultures were then rinsed in a HEPES buffer (NaCl, 140 mM; HEPES, 20 mM; glucose, 10 mM; MgSO₄, 1.5 mM; K₂HPO₄, 1.5 mM; CaCl₂, 1.8 mM; brought to pH 7.4 with tris), fixed at room temperature in 4 percent paraformaldehyde for 30 minutes followed by 5 percent dimethyl sulfoxide for 30 minutes.

AChE histochemistry was performed by a method modified from Koelle (26). Cover slips were incubated overnight in a mixture of 4 mM acetylthiocholine iodide (Sigma), 10 mM glycine, and 2 mM copper sulfate in 50 mM sodium acetate (pH 5.0) at 4°C. Ethopropazine (0.2 mM) (27) was included in this mixture to inhibit nonspecific cholinesterases. The stain was developed in 40 mM sodium sulfide (pH 7.8) for 150 seconds followed by an intensification in a freshly prepared 1 percent silver nitrate solution for 2 minutes at room temperature.

For immunohistochemical staining for SOM-LI and CCK-LI, cover slips were first incubated for 1 hour at 36°C under a drop (40 µl) of a 1:200 dilution of a rabbit polyclonal antibody raised against either bovine serum albumin-conjugated cyclic SOM-14 (28) or against hemocyanin-*p,p'*-difluoro-*m,m'*-dinitro-diphenyl sulfone-conjugated CCK-8 (anti-CCK-8) (Immunonuclear Corporation). The second incubation was with a 1:30 dilution of an FITC-conjugated goat antiserum to rabbit immunoglobulin (Cappel Laboratories) for 1 hour at 37°C. Rinsing was accomplished using HEPES buffer to which 0.2 percent Triton-X had been added. For the CCK-8 staining only, the cover slips were incubated with nonimmune goat serum 1:1 for 30 minutes prior to each immunoglobulin incubation. Cover slips were mounted in buffered glycerol (pH 9.0) and examined using a Zeiss Universal microscope equipped for epifluorescence, bright field, and phase

Table 1. Number of neurons staining for SOM-LI and AChE by morphological subtype as determined by phase-contrast microscopy.

Morphology	Neurons examined first for SOM-LI			Neurons examined first for AChE		
	Neurons positive for SOM-LI	Neurons also positive for AChE	Per-cent	Neurons positive for AChE	Neurons also positive for SOM-LI	Per-cent
Monopolar	21	5	24	40	3	8
Bipolar	33	8	24	99	16	16
Multipolar	37	9	24	137	20	15
Pyramidal	24	7	29	70	13	19
Indeterminate	25	2	8	38	7	16
Total	140	31	21.1	391	59	15.1

contrast. All specific staining was eliminated by the substitution of normal rabbit serum for the first antiserum. Specific staining for SOM-LI was eliminated by the preincubation of antiserum to SOM with synthetic SOM-14, but not by incubation with luteinizing hormone-releasing hormone, arginine vasopressin, or adrenocorticotropin. Specific staining for CCK-8-LI was eliminated by the preincubation of the antiserum to CCK-8 with synthetic CCK-8 (sulfated), but not by incubation with SOM-14, vasoactive intestinal polypeptide, substance P, Leu-enkephalin, or thyrotropin-releasing hormone.

Specific staining for SOM-LI was seen in 2 to 5 percent of all neurons, while 4 to 8 percent of neurons stained positively for AChE. These values are consistent

with the findings of previous studies (20, 25). In the analysis of the AChE staining, only those neurons with definite reaction product seen throughout the nerve cell body were considered positive for AChE and cells with only light or scattered staining were counted as negative for AChE.

With sequential staining for SOM-LI and AChE, three populations of neurons could be positively identified: those staining for AChE only, those staining for SOM-LI only, and those neurons in which these two markers coexist (Fig. 1). Both AChE and SOM-LI were found only in cells which appeared to be neurons by phase-contrast examination. Neurons staining for both markers included most morphologies identifiable by phase-contrast appearance, including

monopolar, bipolar, multipolar or stellate, and pyramidal.

The incidence of costaining was analyzed quantitatively in two ways (Table 1). In one series, the culture was initially surveyed for SOM-LI under fluorescence microscopy, and each neuron staining positively was then examined under bright field to determine whether the neuron was also stained for AChE. In this analysis, out of 140 neurons staining for SOM-LI, a total of 31 neurons (22.1 percent) also stained positively for AChE. In the other series, the culture was surveyed under bright field for AChE staining, and each neuron staining positively for AChE was then examined under fluorescence for SOM-LI staining. In this second analysis, out of 391 neurons staining positively for AChE, a total of 59 neurons (15.1 percent) costained for SOM-LI. In some cases, staining for SOM-LI was observed in the proximal processes but not in the cell bodies of neurons that were heavily stained by the AChE reaction product. Therefore, it is likely that the coexistence of SOM-LI and AChE was underestimated because of occasional obscuration of the fluorescence by a very dense AChE reaction product as well as by our elimination from analysis of neurons staining lightly for AChE.

With sequential staining for AChE and CCK-8-LI, no costaining was observed during examinations of more than 200 neurons staining positively for either AChE or CCK-8-LI.

Although somatostatin has been reported to be contained in and released from cholinergic nerves in the heart of the toad (29), two anatomical studies in the mammalian central nervous system have not found any relationship between neurons containing AChE and those containing SOM (12, 13). In another study, lesions of the nucleus basalis or transection of the fornix in the rat caused a marked decrease in ChAT activity, but did not decrease the SOM content of the cortex or of the hippocampal formation (14). This latter result raises the possibility that the decrease in SOM seen in AD/SDAT is due to a loss of SOM neurons intrinsic to the cortex. It is now increasingly likely, from data derived with both lesioning studies (30) and immunocytochemical studies of ChAT (31, 32), that there exists an intrinsic cholinergic neuronal system in cerebral cortex and in hippocampus. That somatostatinergic neurons might interact with this intrinsic cholinergic system, or that SOM might coexist with acetylcholine in neurons of this system is compatible with our present findings.

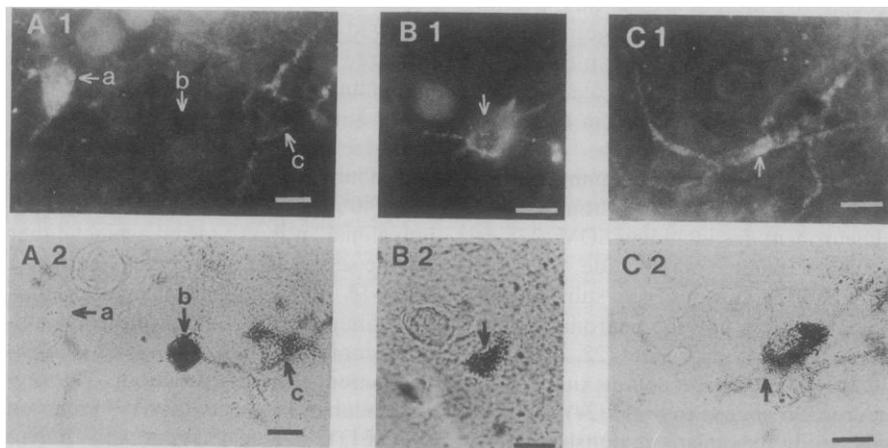


Fig. 1. Fluorescence photomicrographs (top photograph of each set) showing staining for SOM-like immunoreactivity (SOM-LI) and the corresponding bright-field micrographs (bottom of each set) showing staining for acetylcholinesterase activity (AChE). (A1) and (A2) demonstrate three populations of neurons definable by these two methods of staining. Neuron *a* stains positively for SOM-LI, but there is no staining for AChE. Neuron *b* stains darkly for AChE, but there is no staining for SOM-LI. Neuron *c* stains positively for AChE and also stains for SOM-LI, which can be seen in the fluorescence photomicrograph to be particularly prominent in the periphery of the perikaryal cytoplasm and out in the neuronal processes. (B1) and (B2) show a multipolar or stellate neuron which has SOM-LI staining in the cell body and out several processes and prominent staining for AChE in the neuron's cell body. (C1) and (C2) show a large bipolar neuron with SOM-LI staining predominantly in its processes and staining for AChE in the perikaryon and, to a lesser extent, out the neuronal processes. In (B) and (C) arrows point to an identical location on the same neuron in both the fluorescent and the bright-field photomicrographs. In each case the calibration bar represents 20 μ m.

In some areas of rat cerebrum AChE can be used to identify cholinergic neurons; in most areas its presence is not sufficient to identify a neuron as capable of releasing acetylcholine at its axon terminals (33). AChE may therefore be present in some noncholinergic neurons, and ChAT is a more specific marker for cholinergic neurons. Nevertheless, the presence of AChE as a neuronal marker should be of value in itself. For example, AChE in the absence of ChAT could signify a neuron postsynaptic to a cholinergic neuron. In this case, the coexistence of AChE and SOM could indicate that cortical somatostatinergic neurons are innervated by cholinergic neurons, with the deficiencies seen in AD/SDAT reflecting loss of both the presynaptic and postsynaptic elements. Acetylcholine has been shown to stimulate the neuronal release of SOM in similar cultures of rat cerebral cortex (23). Although muscarinic cholinergic receptors are not reduced in the cortex of patients with AD (34, 35), those receptors present on the relatively small population of somatostatinergic neurons could represent only a small fraction of the total number of muscarinic receptors.

In summary, we have demonstrated the coexistence of AChE and SOM-LI in neurons derived from mammalian cerebral hemispheres. As far as we are aware, these findings constitute the first neuroanatomical association of a cholinergic marker and SOM within mammalian central nervous system neurons. The absence of a demonstrable coexistence of AChE and CCK-8-LI suggests some specificity for the relationship between AChE and SOM-LI. The most immediate importance of these findings is in relation to the known diminution of both cholinergic innervation and of SOM levels of brains of persons affected by AD/SDAT. It is not known if SOM and AChE also coexist in cerebral neurons *in vivo*, or if AChE in these neurons represents a cholinergic neuron or a neuron receiving cholinergic input. The implications of the coexistence of AChE and SOM-LI in the same cerebral neurons are that these systems may be both anatomically and physiologically linked, and that this association may be important in understanding the pathophysiology of processes such as AD/SDAT and the functioning of the normal brain.

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Gene Product of *v-fgr onc*: Hybrid Protein Containing a Portion of Actin and a Tyrosine-Specific Protein Kinase

Abstract. *The nucleotide sequence of the region of Gardner-Rasheed feline sarcoma virus (GR-FeSV) encoding its primary translation product, p70^{gag-fgr}, has been determined. From the nucleotide sequence, the amino acid sequence of this transforming protein was deduced. Computer analysis indicates that a portion of P70^{gag-fgr} has extensive amino acid sequence homology with actin, a eukaryotic cytoskeletal protein. A second region of P70^{gag-fgr} is closely related to the tyrosine-specific kinase gene family. Thus, the v-fgr oncogene appears to have arisen as a result of recombinational events involving two distinct cellular genes, one coding for a structural protein and the other for a protein kinase.*

Gardner-Rasheed feline sarcoma virus (GR-FeSV) is a replication-defective, acute transforming retrovirus that was isolated from a cat fibrosarcoma (1). This virus arose by recombination of the non-defective helper feline leukemia virus (FeLV) and cellular sequences present within the normal cat genome (2). Analysis of a DNA clone containing the proviral genome of GR-FeSV revealed the presence of 1.7 kilobase pairs (kbp) of cell-derived sequences that are flanked by a partial *gag* gene at the 5' end and partial *env* gene at the 3' end (2). These cell-derived sequences, termed *v-fgr*, appear to code for the oncogenic potential of the viral genome. Cells transformed by GR-FeSV express a 70,000-dalton protein, which is recognized by antibodies to the helper virus structural protein p15 but not by antibodies to other helper virus proteins (2, 3). Thus the primary

translational product of GR-FeSV, called P70^{gag-fgr}, is a hybrid molecule, containing at least a portion of FeLV p15 as well as FeLV-unrelated component coded by *v-fgr* sequences (2, 3). The hybrid P70^{gag-fgr} has a closely associated kinase activity with specificity for tyrosine residues (3). In an effort to understand the mechanism of action of the *v-fgr* oncogene and its relationship to other cell-derived genes, we have undertaken primary DNA sequence analysis of the region of GR-FeSV encoding P70^{gag-fgr} (1). The nucleotide sequence of the entire region coding for the P70^{gag-fgr}, as determined by the procedures of Maxam and Gilbert (4), is shown in Fig. 1.

The v-fgr nucleotide sequence. Since the *v-fgr onc* gene product contains *gag*-gene determinants, we compared the sequence in Fig. 1 with that of the Snyder-Theilen feline sarcoma virus *gag*-gene