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COEXISTENCE OF GLUTAMIC ACID DECARBOXYLASE- AND SOMATOSTATIN-LIKE IMMUNOREACTIVITY IN NEURONS OF THE FELINE NUCLEUS RETICULARIS THALAMI[1]

WOLFGANG H. OERTEL,*,[2] ANN M. GRAYBIEL,‡ ENRICO MUGNAINI,§ ROBERT P. ELDE, ‖ DONALD E. SCHMECHEL,¶ AND IRWIN J. KOPIN*

** Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20205; ‡Department of Psychology and Brain Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; §Department of Biobehavioral Sciences, University of Connecticut, Storrs, Connecticut 06268; ‖ Department of Anatomy, University of Minnesota, Minneapolis, Minnesota 55455; and ¶Division of Neurology, Duke University Medical Center, Durham, North Carolina 27710*

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Abstract

The presence of somatostatin-like (SOM) immunoreactivity within GABAergic neurons of the nucleus reticularis thalami (NRT) was demonstrated by immunocytochemical methods in the cat using a two-color, double immunoperoxidase method with antisera raised in different species. GABAergic neurons were identified by means of a sheep antiserum to glutamic acid decarboxylase, the biosynthetic enzyme for γ-aminobutyric acid (GABA). *SOM* immunoreactivity was visualized with a rabbit antiserum to synthetic somatostatin. Vibratome sections of perfusion-fixed tissue were processed according to the pre-embedding unlabeled peroxidase antiperoxidase (PAP) method for light and electron microscopy. Single sections through the NRT were first processed for glutamic acid decarboxylase-like (GAD) or SOM immunoreactivity. With either antiserum, most neurons of the NRT were immunoreactive. The intracellular sites recognized by the two antisera had only partially overlapping distributions. SOM immunoreactivity appeared largely restricted to perinuclear structures which were identified by electron microscopy as the Golgi apparatus and multivesicular bodies. *GAD* immunoreactivity also appeared in the Golgi apparatus but was broadly dispersed throughout the cytoplasm of the cell body and dendrites. Intensely GAD-immunoreactive dots in the neuropil of the NRT were shown by electron microscopy to be immunoreactive boutons. Coexistence of the SOM and GAD antigens was demonstrated in sections sequentially incubated with rabbit anti-somatostatin, swine anti-rabbit IgG, rabbit PAP, and 4-chloro-1-naphthol (blue color), followed by sheep anti-rat glutamic acid decarboxylase, donkey anti-goat IgG, goat PAP, and 3,3′-diaminobenzidine (brown color). Many neurons contained blue-black perinuclear skeins (SOM and GAD immunoreactivity) and brown reaction product in the cytoplasm (GAD immunoreactivity) and were contacted by dark brown punctate profiles (GAD immunoreactivity). Control experiments showed that a weak cross-reactivity of the two linking antisera did not impair the simultaneous visualization method.

This study provides direct evidence for the existence, within GABAergic neurons of the cat NRT, of a substance immunocytochemically indistinguishable from somatostatin. The data suggest that neurons of the feline NRT compromise a "SOM-positive subtype" of GABAergic neurons.

Immunohistochemical evidence strongly suggests that neuropeptides coexist with classical and nonclassical neurotransmitters in endocrine cells and in neurons of the central, peripheral, autonomic, and enteric nervous systems. The production of low molecular weight polypeptides by amine-producing endocrine and enteric cells was

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[2] To whom correspondence should be sent at his current address: Neurologische Klinik (Leiter: Prof. Dr. A. Struppler), Technische Universitaet Muenchen, Moehlstrasse 28, D-8000 Muenchen 80, Federal Republic of Germany.

first studied systematically by Pearse (1968, 1969), who formalized the concept of cellular co-production of amines and polypeptides in his characterization of the APUD (amine and precursor uptake and decarboxylation) series. Since this pioneering work, a number of examples of co-localization of biogenic amines and neuropeptides have been studied in central and peripheral neurons (Hökfelt et al., 1977, 1978a, 1980a, b, c, d; Chan-Palay et al., 1978, 1981; Schultzberg et al., 1980). In addition, neuropeptides have been demonstrated in presumed cholinergic neurons (Lundberg et al., 1979; Fex and Altschuler, 1981; Erichsen et al., 1982) and in neurons that contain other neuropeptides (Watson et al., 1978; Schultzberg et al., 1980; Hunt et al., 1981; Erichsen et al., 1982. For review, see Osborne, 1981, and Potter et al., 1981.).

A striking absence in the list of neurotransmitters coexisting with neuropeptides has been γ-aminobutyric acid (GABA),[3] a major inhibitory neurotransmitter in the brain. Only recently, Chan-Palay et al. (1981) and Nilaver et al. (1982) have reported light microscopic evidence for the presence of motilin-like immunoreactivity in some GABAergic cerebellar Purkinje cells. In the present study we applied two-color immunohistochemical methods to examine the possibility that the neuropeptide, somatostatin (Elde and Parsons, 1975), coexists with a specific endogenous marker for GABAergic neurons, namely, glutamic acid decarboxylase, the biosynthetic enzyme for GABA (McLaughlin et al., 1974; Saito et al., 1974; Barber and Saito, 1976; Wu, 1976; Ribak et al., 1976; Roberts, 1979; Oertel et al., 1981a). We chose to study the thalamic reticular nucleus (NRT) because this nucleus has been shown to contain glutamic acid decarboxylase-like (GAD)-immunoreactive neurons in the rat (Houser et al., 1980) and somatostatin-like (SOM)-immunoreactive neurons in the cat (Graybiel et al., 1981) and monkey (Graybiel and Elde, 1983). This work was summarized at the Conference of Cytochemical Methods in Neuroanatomy (June 10 to 12, 1981, Bethesda, MD) and at the 11th Annual Meeting of the American Society for Neuroscience (Oertel et al., 1981c).

Materials and Methods

Observations were made on the brains of 10 young domestic cats (1000 to 2000 gm) and 10 adult Osborn-Mendal and Sprague-Dawley rats (150 to 200 gm). Two rats received an intraventricular injection of 50 μg of colchicine dissolved in 10 μl of saline 36 hr prior to perfusion; in three rats 10 to 25 μg of colchicine in 1 to 2.5 μl of saline was injected stereotaxically in the thalamic relay nuclei 30 hr prior to perfusion. Animals were perfused under deep sodium pentobarbital anesthesia with 4% formaldehyde in 0.12 M sodium phosphate buffer (pH 7.4). Coronal and sagittal (20- to 25-μm) sections through the thalamus were cut on a Vibratome and collected in phosphate-buffered saline (PBS; pH 7.4). For the immunohistochemical localization of glutamic acid decarboxylase, we employed sheep antiserum S3 (second or fourth bleed), and for somatostatin, rabbit antisera R176C and R176D. These antisera have been characterized elsewhere (Elde and Parsons, 1975; Oertel et al., 1981b) and seem to be specific for their respective antigens.

Single-antigen immunohistochemistry was carried out on floating sections according to the pre-embedding unlabeled peroxidase-antiperoxidase (PAP) method of Sternberger (1979). The two-color double PAP technique represented a simple sequential staining procedure for the two antigens (Fig. 6). As linking antisera we used swine anti-rabbit IgG and donkey anti-goat IgG for localizing SOM and GAD immunoreactivity, respectively. Normal donkey serum was employed for blocking of unspecific binding sites.

GAD immunocytochemistry. Sections from rat and cat brains were stained for GAD immunoreactivity at the light and electron microscopic level. All solutions were prepared in PBS and the sera were diluted in 1% normal donkey serum, 0.01 M monohydrochloric lysine, and 0.2% Triton X-100 dissolved in PBS. For light microscopic investigations the sections were pretreated with 3% hydrogen peroxide (H_2O_2)/10% methanol at room temperature (RT) for 5 min, preincubated in 10% normal donkey serum containing 0.1 M monohydrochloric lysine and 0.2% Triton X-100 for 1 hr at RT, and incubated for 16 hr at RT in sheep glutamic acid decarboxylase antiserum S3 diluted 1:2000. Control sections were incubated with preimmune serum S3 diluted 1:1800 (16 hr at RT). All sections were then exposed to donkey anti-goat IgG antiserum (1:50; Miles Laboratories, Elkhard, IN) for 30 min at RT, rinsed, and incubated for 30 min at RT in goat PAP complex (Sternberger-Meyer, Garretsville, MD) diluted 1:70 to 1:100. After being washed in Tris-buffered saline (0.05 M Tris, 0.9% NaCl, pH 7.6; four to five times, 5 min each), the sections were carried through the 3,3′-diaminobenzidine (DAB) procedure of Graham and Karnovsky (1966) or the 4-chloro-1-naphthol procedure of Dacheux and Dubois (1976). Some DAB-treated sections were counterstained with toluidine blue or cresyl violet. Sections were mounted from PBS onto subbed slides and coverslipped with Permount or a glycerol/PBS (3:1, v/v) mixture for bright-field photography.

For electron microscopic immunocytochemistry, 50-μm thick sections were reacted according to the pre-embedding method, as detailed above, with minor modifications: the H_2O_2/methanol pretreatment was omitted and subsequent steps were performed in the absence of Triton X-100. Following DAB incubation, the sections were fixed with 1% OsO_4, treated with uranyl acetate, dehydrated, and embedded for electron microscopic analysis as detailed elsewhere (Oertel et al., 1981a). Semithin sections, lightly counterstained with toluidine blue, were used to determine the localization of immunoreactive neurons.

SOM immunocytochemistry. Sections were treated with primary incubations in rabbit somatostatin antiserum R176C or R176D (1:500 to 1:700) for 48 hr at 4°C and for 5 hr at RT, and with control incubations in antiserum R176C (1:500 to 1:700) absorbed for 30 min prior to the incubation at RT with 10 μM somatostatin 16.4 μg/ml). Sections were then sequentially exposed to the immunoglobulin fraction of swine anti-rabbit IgG

[3] Abbreviations: DAB, 3,3′-diaminobenzidine; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase-like; NRT, nucleus reticularis thalami; PAP, peroxidase-antiperoxidase; PBS, phosphate-buffered saline; RT, room temperature; SOM, somatostatin-like.

antiserum (Dako Ltd., Copenhagen, Denmark) (diluted 1:35 to 1:50) for 30 min at RT, to rabbit PAP complex (Sternberger-Meyer) (1:100) for 30 min at RT, and to the chromogen solution of choice.

Some sections from the cat brains were prepared for electron microscopy as described above. This was not done for the rat because hardly any SOM immunoreactivity was evident in the NRT of normal and colchicine-treated rats at the light microscopic level (see Hökfelt et al., 1978b; Finley et al., 1981).

Sequential immunocytochemistry. To demonstrate the presence of the GAD- and SOM-immunoreactive antigens in the same cell, sequential staining of sections with the two respective antisera was carried out by means of a two-color double immunoperoxidase method without elution of the first antibody linkage (modified from Mason and Sammons, 1978; see Fig. 6). For this sequential staining, SOM immunoreactivity was localized as a blue reaction product using 4-chloro-1-naphthol as chromogen, and after five washes in PBS, GAD immunoreactivity was visualized as a brown color in the same section following all but the initial H_2O_2/methanol pretreatment step of the GAD staining protocol and using DAB as chromogen. The appearance of blue color was interpreted as evidence for SOM immunoreactivity, and the appearance of brown color was evidence for GAD immunoreactivity. The presence of black (blue-brown) cytoplasmic zones in cells stained by a diffuse brown reaction product was taken as an indication of the coexistence of both antigens, as described below, under "Results."

Finally, in some experiments SOM immunoreactivity was visualized by the indirect immunofluorescence method of Coons (1958; see also Hökfelt et al., 1980d). After fluorescence microphotography, the sections were incubated for GAD immunocytochemistry with DAB as chromogen. The areas of interest were rephotographed and the distributions of SOM immunoreactivity (green color) and GAD immunoreactivity (brown color) were compared.

Controls. Controls for the single-antigen immunocytochemistry included (*a*) for GAD immunoreactivity, the replacement of the primary antiserum S3 with preimmune serum, and (*b*) for SOM immunoreactivity, absorption of glutamic acid decarboxylase antiserum S3 with synthetic somatostatin (10 μM) for 30 min at RT was performed to test the cross-reactivity of this serum to somatostatin. The corresponding control, absorption of antiserum to somatostatin with glutamic acid decarboxylase protein, was not carried out due to the lack of pure glutamic acid decarboxylase (the antiserum S3 was raised against a glutamic acid decarboxylase·antibody complex).

It must be emphasized that the immunological controls do not fully guard against false-positive results, inasmuch as the primary antisera used may bind to as yet unidentified compounds sharing some antigenic determinants with glutamic acid decarboxylase and somatostatin.

In the control experiments for the sequential double-immunoperoxidase method without elution of the first antibody sequence, one or both of the primary antisera were replaced by the control serum (preimmune serum for GAD immunoreactivity and absorbed antiserum R176D for SOM immunoreactivity). Furthermore, each of the six different constituents of the two linkages was omitted one at a time. To test for cross-reactivity of the two linking antisera, two procedures were followed. First, staining for GAD immunoreactivity was repeated by substituting the primary, secondary, or tertiary antiserum with the primary, secondary, or tertiary antiserum of the sequence for SOM immunocytochemistry and vice versa. Second, sections were incubated according to the sequence: H_2O_2/methanol, 10% normal donkey serum, rabbit anti-somatostatin, swine anti-rabbit IgG, donkey anti-goat IgG, goat PAP, and DAB. Finally, the chromogen sequence was tested by comparing localization of GAD/SOM and SOM/GAD immunoreactivity with either 4-chloro-1-naphthol or DAB being the first chromogen.

Results

GAD immunocytochemistry showed, in agreement with Houser et al. (1980), that virtually all neurons of the rodent NRT are GAD-immunoreactive. Likewise, the neurons of the feline NRT are GAD-positive. SOM immunocytochemistry confirmed that neurons of the feline NRT (Graybiel et al., 1981; Graybiel and Elde, 1983), but not those of the rodent NRT (Hökfelt et al., 1978b; Finley et al., 1981), contain SOM immunoreactivity. Therefore, the study on the coexistence of the two antigens was carried out in the NRT of the cat. Localization of SOM

Figure 1. Light micrograph of a sagittal section through the rat NRT, incubated in sheep preimmune serum S3 (1:1800), donkey anti-goat IgG, goat PAP complex, and DAB. No specific staining is observed. Lightfield × 38.

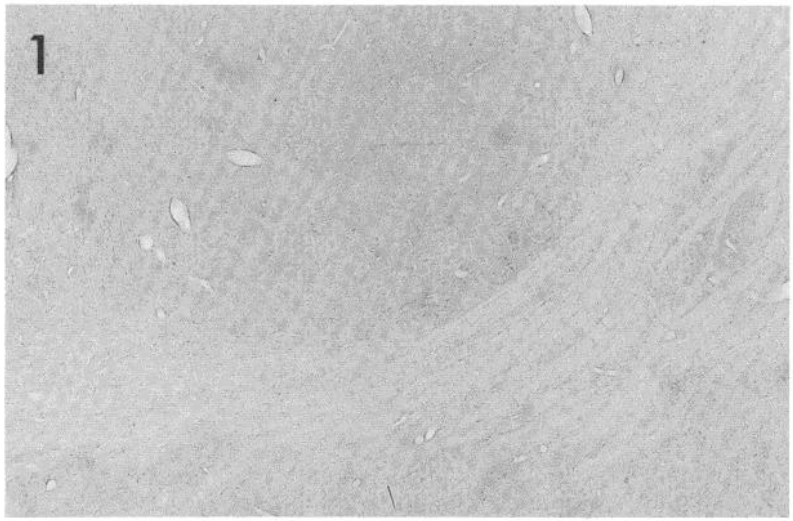
Figure 2. Light micrograph of a sagittal section through the rat NRT, incubated in sheep antiserum S3 to glutamic acid decarboxylase (second bleed) (1:2000), donkey anti-goat IgG, goat PAP complex, and DAB. The NRT forms a shell of GAD-immunoreactive neurons around the thalamic nuclei. Lightfield × 45.

Figure 3. Light micrograph of the border between the NRT and the zona incerta in a sagittal rat brain section, incubated with rabbit anti-somatostatin (1:600), swine anti-rabbit IgG, rabbit PAP complex, and DAB. The NRT and the zona incerta are devoid of SOM-immunoreactive neurons. At the border of both structures, close to the capsula interna, a small group of SOM-positive neurons is visible. Lightfield × 120.

Figures 4 and 5. Light micrographs of the feline NRT in the frontal plane. Notice the common landmarks. Glial profiles in the capsula interna are faintly visible due to osmication and mounting in Permount. Lightfield × 54.

Figure 4. Tissue was incubated as indicated in the legend of Figure 2. The vast majority of neurons, including their proximal dendrites and punctate profiles in the neuropil, are GAD-immunoreactive. In the adjacent thalamic nuclei scattered GAD-positive somata and numerous GAD-positive punctate profiles are revealed.

Figure 5. Tissue was incubated as indicated in the legend of Figure 3. The vast majority of neurons contain SOM-immunoreactive skeins. *No SOM-immunoreactive punctate profiles are seen in the neuropil of the NRT.*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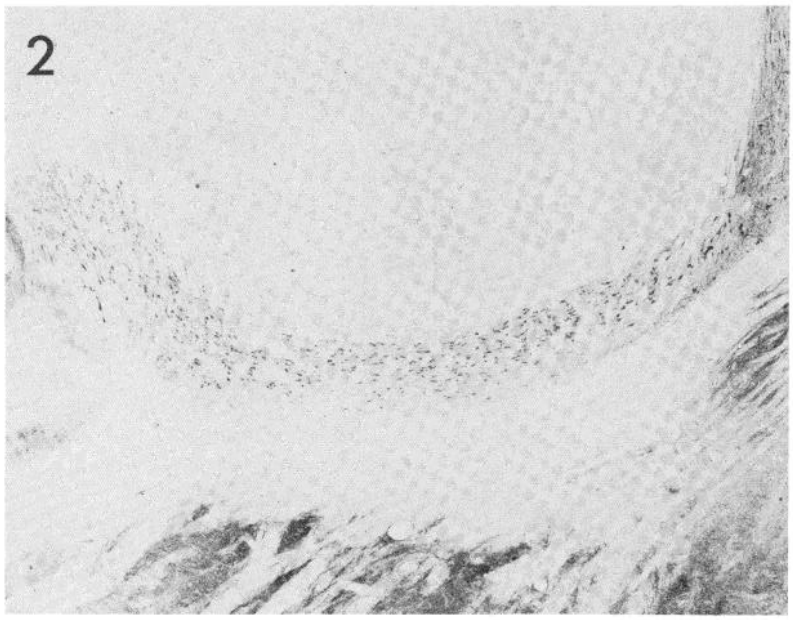
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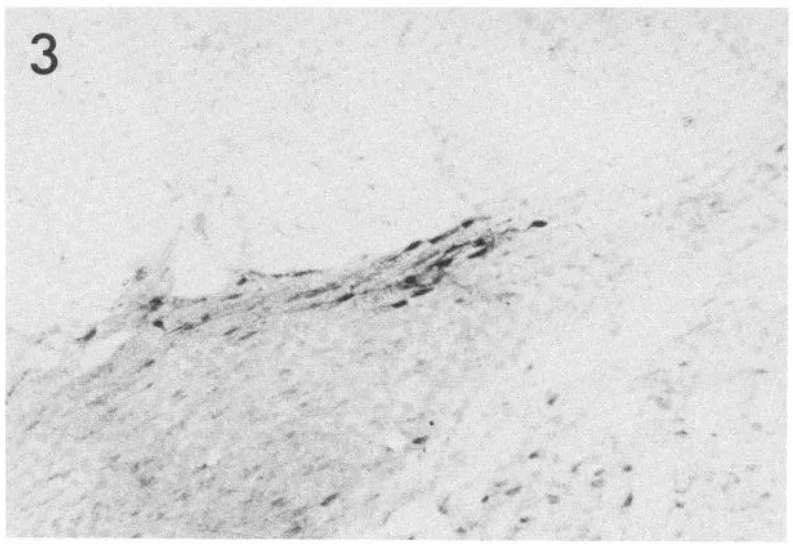
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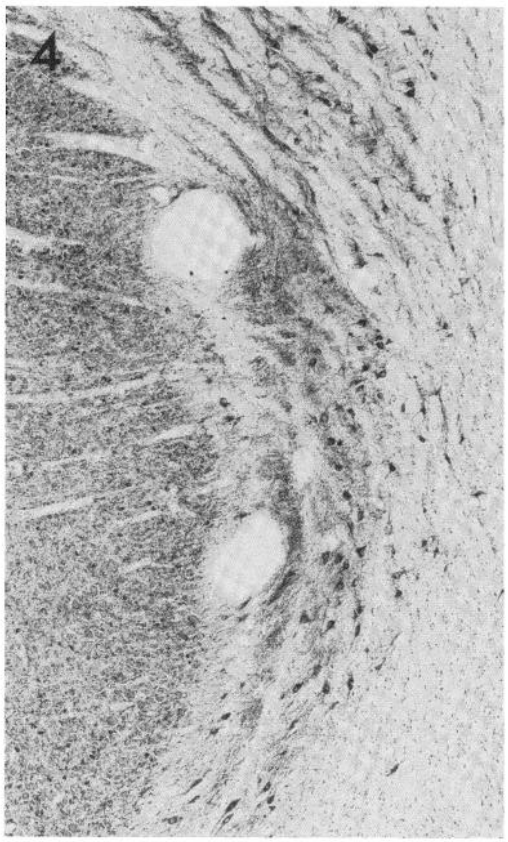
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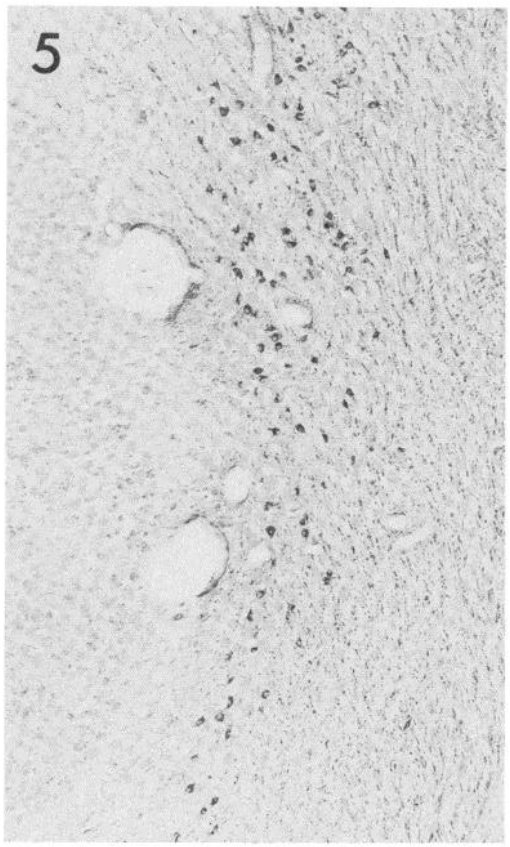
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immunoreactivity, using swine anti-rabbit IgG antiserum, and of GAD immunoreactivity, using donkey anti-goat IgG antiserum, proved to be comparable to the standard methods in which sheep anti-rabbit IgG and rabbit anti-sheep IgG antisera are employed for SOM and GAD immunoreactivity, respectively.

GAD immunocytochemistry. In both the cat and the rat, the cytoplasm of the vast majority of NRT neurons showed GAD immunoreactivity (Figs. 2 and 4) throughout the cell body and dendrites, and zones of heightened immunoreactivity appeared in the perinuclear cytoplasm (Fig. 9). In both species, numerous intensely brown punctate structures were seen in the neuropil of the NRT, in contact with immunoreactive dendrites and cell bodies (Fig. 13). In thalamic nuclei medial to the external medullary lamina, GAD-positive neurons were numerous in the cat but sparse in the rat (Figs. 2 and 4). Clustered and solitary small GAD-positive punctate structures, presumably axon terminals, were common in these nuclei in both species.

Absorption of sheep antiserum S3 to glutamic acid decarboxylase with synthetic somatostatin did not change the distribution of GAD immunoreactivity. With preimmune serum, no specific staining was obtained (Fig. 1). All other controls were negative.

In ultrathin sections of the cat NRT, GAD-positive immunoprecipitate was observed throughout the perikaryal cytoplasm and dendrites (Fig. 13) of NRT neurons. The Golgi apparatus was marked by more immunoprecipitate than was the rest of the cytoplasm, and boutons were very intensely immunoreactive (Fig. 13, *inset*; Table I).

SOM immunocytochemistry. In the cat, SOM immunoreactivity was present in virtually all of the neurons of the NRT (Fig. 5) and appeared mainly in skeins in the perinuclear cytoplasm as described in the accompanying report (Graybiel and Elde, 1983). Some of the proximal dendrites also contained skeins of reaction product (Fig. 10). SOM-immunoreactive punctate profiles only rarely appeared in the neuropil. At the electron microscopic level, SOM immunoreactivity was localized in the Golgi apparatus, which appeared heavily labeled (Fig. 12; Johansson, 1978), and also in multivesicular bodies (Table I).

In normal rats, neurons of the NRT were devoid of SOM immunoreactivity (Fig. 3). Even after colchicine pretreatment, only isolated SOM-immunoreactive cells were observed in the NRT itself. However, just ventral to the region where the NRT and the zona incerta merge, a group of 5 to 15 neurons with a homogeneous cytoplasmic staining was seen (Fig. 3), as has been described also by Hökfelt et al. (1978b) and Finley et al. (1981). Absorption of rabbit somatostatin antiserum with synthetic somatostatin abolished all specific staining. All other controls were negative.

Sequential two-color staining for SOM and GAD immunoreactivity. The results of the control tests indicated that, though slight cross-reactivity of the linking swine anti-rabbit and donkey anti-goat antisera did occur, simultaneous localization of GAD and SOM immunoreactivity was possible without elution of the first antibody linkage or inactivation of the first PAP complex. The main reason for this was the differential distribution of SOM and GAD immunoreactivity described above: the presence of intense immunoreactivity in perinuclear skeins and multivesicular bodies was the hallmark of the SOM-immunoreactive antigen, whereas diffuse immunoreactivity in the perikaryal and dendritic cytoplasm and intense immunoreactivity of puncta (interpreted as boutons) were present in sections processed for GAD immunocytochemistry (Table I).

In detail, the "single control" sequence of rabbit anti-somatostatin antiserum, swine anti-rabbit IgG, rabbit PAP, 4-chloro-1-naphthol, preimmune serum (control), donkey anti-goat IgG, goat PAP, and DAB yielded blue SOM immunoreactivity in the typical perinuclear arrangement on a light brown general background. With absorbed rabbit anti-somatostatin antiserum and sheep antiserum to glutamic acid decarboxylase as primary antisera in the two complete sequences, brown GAD immunoreactivity was revealed as cytoplasmic staining with less distinct perinuclear skeins, and as puncta. "Double control" incubations with absorbed rabbit anti-somatostatin antiserum (control), swine anti-rabbit IgG, rabbit PAP, 4-chloro-1-naphthol, followed by preimmune serum (control), donkey anti-goat IgG, goat PAP, and DAB resulted in a homogeneous gray background staining of the neurons in the NRT (Fig. 7). Finally, the test sequence for the two linking antisera (rabbit anti-somatostatin antiserum, swine anti-rabbit IgG, donkey anti-goat IgG, goat PAP, and DAB) consistently gave weak brown staining for SOM immunoreactivity (Fig. 8). Thus,

TABLE I
Staining for single antigen[a]

Primary Antiserum[b]	Microscopy	Cytoplasm	Golgi Apparatus	Multivesicular Bodies	Proximal Dendrites	Boutons
Sheep preimmune serum S3	LM	---[c]	---	---	---	---
Sheep antiserum S3 to glutamic acid decarboxylase	LM EM	++	+++	---	++	+++++
Absorbed rabbit anti-somatostatin R176D	LM	---	---	---	---	---
Rabbit anti-somatostatin R176D	LM EM	---	+++++	++	---	---

[a] Rating of immunoreactivity at the light (LM) and electron microscopic (EM) level.
[b] For immunocytochemical details, see "Materials and Methods."
[c] ---, background.

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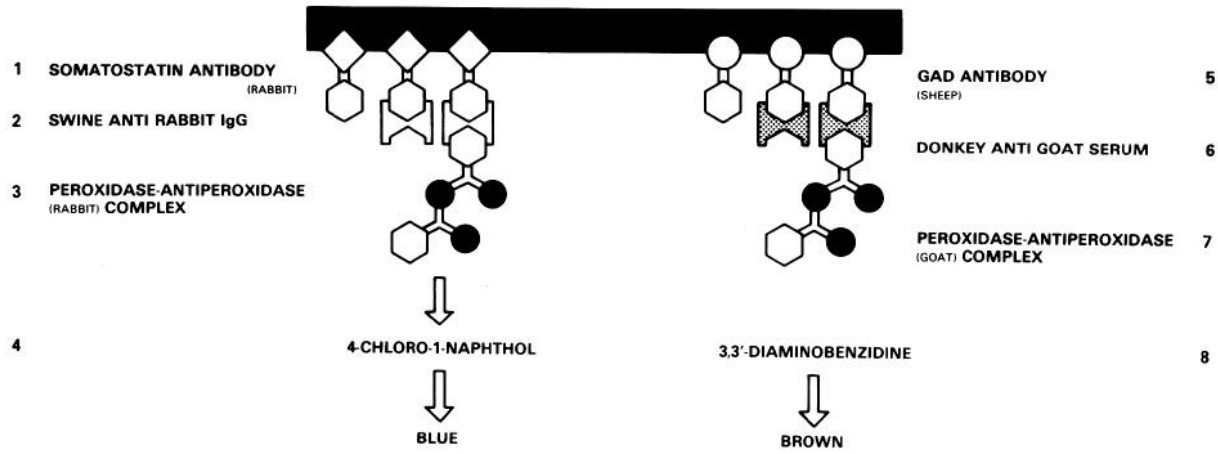

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STEPS 1, 3-8
BACKGROUND

8

STEPS 1,2, 6-8
CROSS-REACTIVITY
OF LINKING ANTISERA

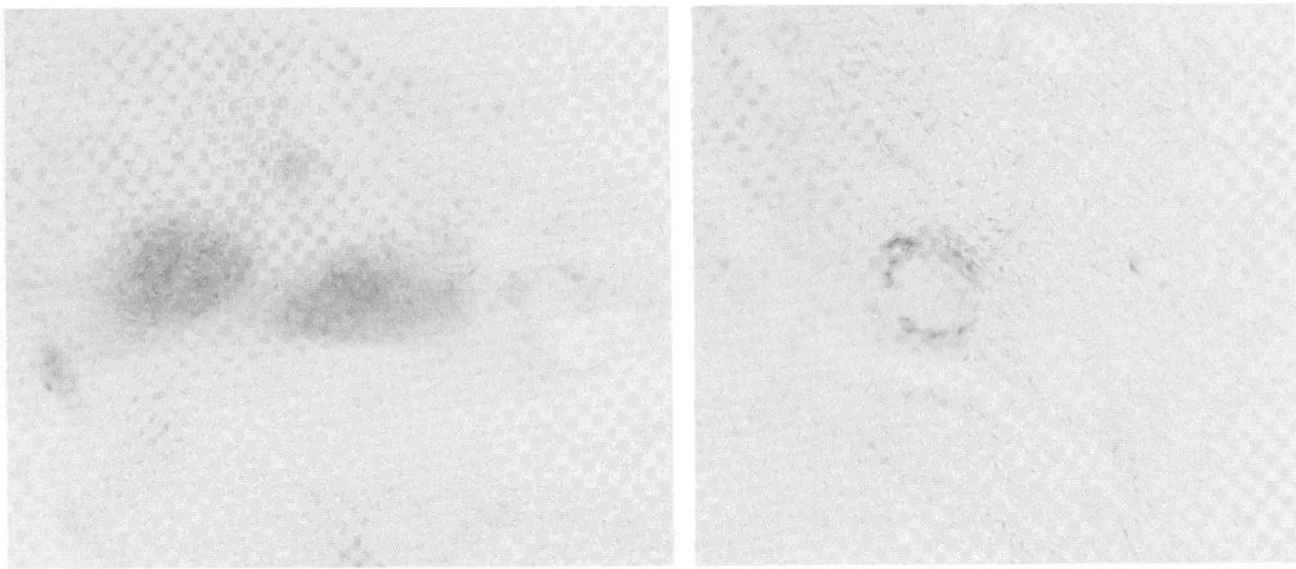
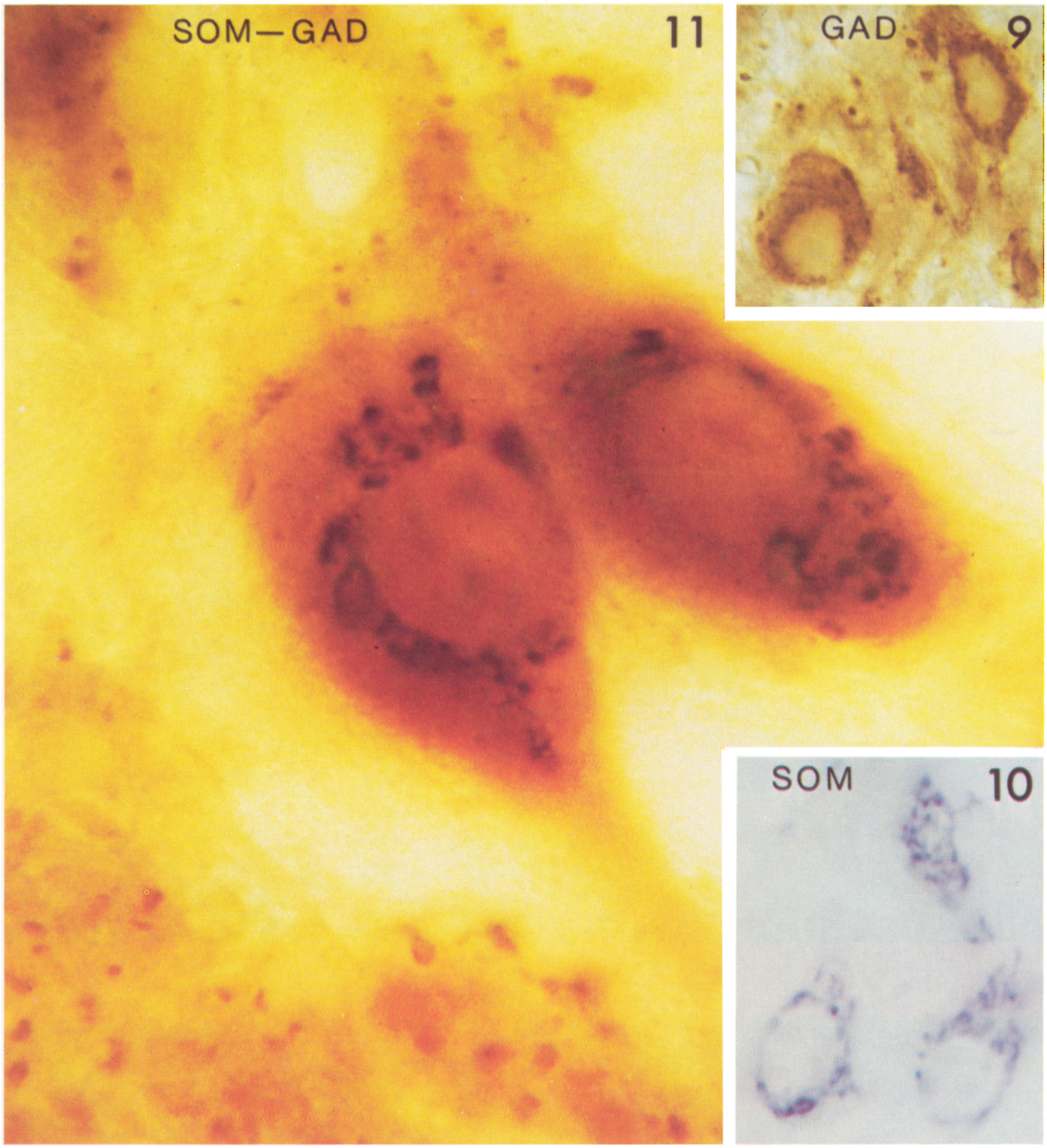


Figure 6. Scheme of the double PAP method.

Figures 7 and 8. Examples of controls for the two-color immunoperoxidase method: light micrographs of neurons in the feline NRT. Lightfield × 860.

Figure 7. Following incubation in rabbit anti-somatostatin, rabbit PAP complex, 4-cholor-1-naphthol, sheep preimmune serum S3, donkey anti-goat IgG, goat PAP complex, and DAB, demonstrating the background level obtained in various controls. Background in the sequence depicted in Figure 6 is much lower.

Figure 8. Following incubation in rabbit anti-somatostatin, swine anti-rabbit IgG, donkey anti-goat IgG, goat PAP complex, and DAB, demonstrating light cross-reactivity of the two linking antisera.

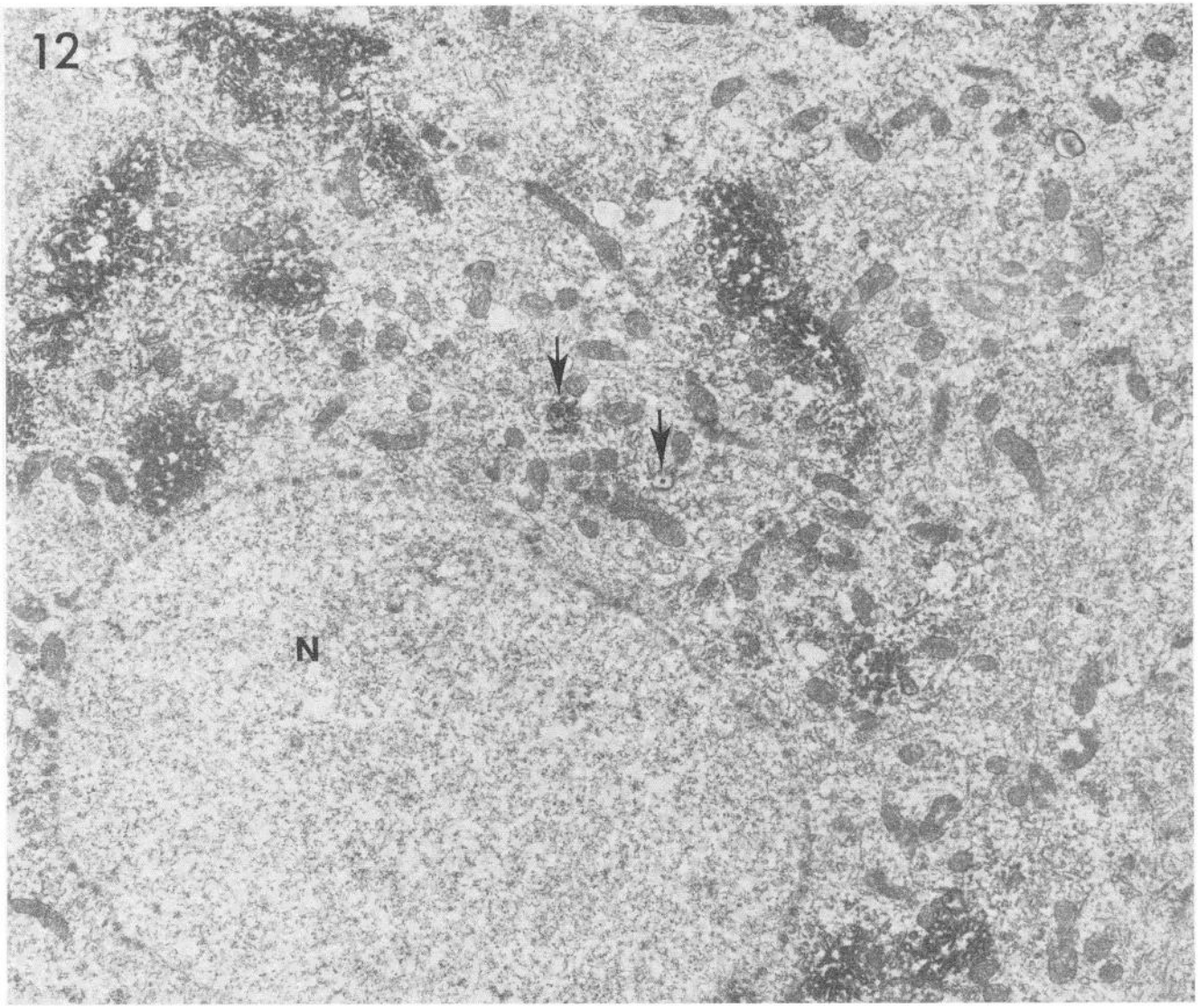


Figures 9 to 11. Color micrographs of the feline NRT in the frontal plane. Lightfield × 500.

Figure 9. Neurons from a section incubated in the sequence of sheep anti-glutamic acid decarboxylase, donkey anti-goat IgG, goat PAP complex, and DAB, demonstrating weak GAD immunoreactivity in the cell body, medium immunoreactivity in the perinuclear region (Golgi apparatus), and intense immunoreactivity in puncta near the GAD-positive cell.

Figure 10. Neurons from a section incubated in the sequence of rabbit anti-somatostatin, swine anti-rabbit IgG, rabbit PAP complex, and 4-chloro-1-naphthol, demonstrating skeins of SOM immunoreactivity (Golgi apparatus).

Figure 11. Neurons from sections incubated in the double-staining sequence as depicted in Figure 6. Note the *black* (blue-brown) *skeins* (SOM immunoreactivity) surrounding the unstained nucleus in a homogeneously stained cytoplasm (GAD immunoreactivity). Intensely stained punctate profiles are present in the vicinity of the neuron and in the neuropil (GAD immunoreactivity).



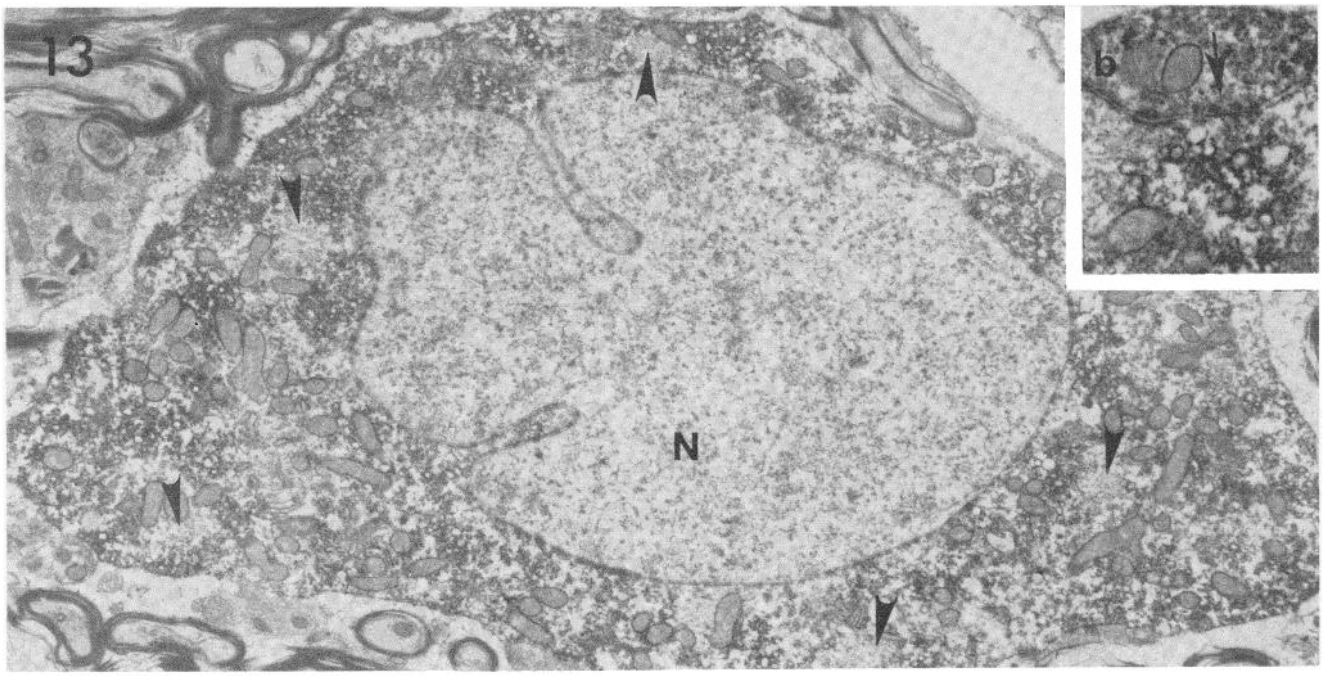


Figure 12. Electron micrograph of a neuron of the NRT in the cat showing that SOM immunoreactivity is localized at the Golgi apparatus and at multivesicular bodies (*arrows*). *N* indicates the cell nucleus. Magnification × 12,400.

Figure 13. Neuron of the cat's NRT with GAD immunoreactivity present throughout the cytoplasm. Bundles of neurofilaments (*arrowheads*) are free from immunoprecipitate. *N* indicates the cell nucleus. Magnification × 7,900. *Inset*, A GAD-positive bouton (*b*) forms a symmetric synaptic junction (*arrow*) with the cell body of a GAD-positive NRT neuron. Magnification × 16,350.

the two linking antisera cross-reacted slightly. Adding an incubation with preimmune serum S3 between the two linking antisera gave a slightly higher light brownish background.

When the full sequential incubation sequence was followed employig rabbit anti-somatostatin with 4-chloro-1-naphthol (blue) and sheep anti-glutamic acid decarboxylase with DAB (brown) (see Fig. 6 for method scheme), brown somata and proximal dendrites were observed which contained blue-black perinuclear skeins. Around these neurons were dark brown punctate profiles (Fig. 11; compare to Figs. 9 and 10). When the chromogens were reversed, so that DAB was used to visualize SOM immunoreactivity and 4-chloro-1-naphthol was used as chromogen for GAD immunoreactivity (as second antigen), brown perinuclear skeins were seen in homogeneously blue stained cells surrounded by blue punctate profiles. A defect of this sequence was that, due to a light grayish-bluish background, the cytoplasmic stain for GAD immunoreactivity was sometimes difficult to define.

Discussion

This study demonstrates that GAD and SOM immunoreactivity coexist in the neurons of the NRT of the cat. By contrast, in the rat, only GAD immunoreactivity appeared in neurons of the nucleus reticularis.

Methodological aspects. The successful demonstration of the coexistence of GAD- and SOM- immunoreactive antigens depended primarily on two factors. First, the anti-glutamic acid decarboxylase and anti-somatostatin antisera was raised in different species. Second, there was only a partial overlap in the distribution of the antigens detected by the two antisera. In practice, the second circumstance proved crucial for the demonstration, because the two linking antisera (swine anti-rabbit and donkey anti-goat) cross-reacted lightly. This was unexpected (see Mason and Sammons, 1978), but the weak cross-reaction was no obstacle in this particular study, given the restricted distribution of the SOM-immunoreactive antigen and the diffuse distribution of the antigen detected by the antiserum to glutamic acid decarboxylase. We thus could rely on simultaneous visualization with bright-field microscopy by a double PAP protocol using chromogens of different colors (Sternberger and Joseph, 1979). We thereby avoided problems associated with elution of the first antibody linkage (Tramu et al., 1978; Nilaver et al., 1979; Hökfelt et al., 1980d) or inactivation of the first PAP complex. Of the various possible combinations, the most consistent results were obtained when the antigen with the more discrete distribution (SOM immunoreactivity) was demonstrated first, using a blue color, and the more abundant antigen (GAD immunoreactivity) was stained subsequently with the brown chromogen.

The blue color of the SOM-immunoreactive skeins visualized the 4-chloro-1-naphthol sections was turned into black by an overlay of brown dye, as a consequence of the second sequence for GAD immunoreactivity with the chromagen DAB. This appeared to be due to colocalization of SOM and GAD immunoreactivity in the Golgi apparatus, but an overlay due to remaining peroxidatic activity of the first antibody linkage was not excluded. Both the anti-somatostatin and the anti-glutamic acid decarboxylase antisera gave apparently specific immunolocalization, and the black profiles could be distinguished from the brown GAD-immunoreactive cytoplasm. Nevertheless, as both SOM and GAD immunoreactivity were found to be localized in the Golgi apparatus, controls were carried out to determine whether the demonstrated coexistence was due to an artifact.

Absorption of antiserum S3 to glutamic acid decarboxylase with somatostatin did not change the distribution of GAD-immunoreactive profiles. Thus, the antiserum S3 apparently did not detect SOM-immunoreactive profiles. In agreement with Graybiel et al. (1981) and Graybiel and Elde (1983), absorption of somatostatin antiserum with synthetic somatostatin abolished SOM immunoreactivity. Furthermore, the somatostatin antiserum did not detect the same antigens as the antiserum S3 to glutamic acid decarboxylase because (*a*) the SOM immunoreactivity it localized in individual NRT cells was far more restricted in its distribution than that detected by antiserum S3, and (*b*) in the experiments on the NRT of normal and colchicine-pretreated rats, virtually all neurons contained GAD immunoreactivity, but not SOM immunoreactivity.

As discussed in the accompanying paper (Graybiel and Elde, 1983), it remains to be shown by biochemical techniques to what form of somatostatin, somatostatin precursor, or SOM antigenic determinant the demonstrated SOM immunoreactivity in the feline NRT corresponds.

Functional aspects. This study presents direct evidence for the coexistence of GAD immunoreactivity, the marker for GABAergic neurons, and a substance immunocytochemically indistinguishable from the neuropeptide, somatostatin. It remains to be determined why the NRT contains GAD-immunoreactive, but not SOM-immunoreactive boutons, despite the fact that axon terminals in the neuropil of the NRT partly stem from intrinsic axon collaterals of NRT neurons (Scheibel and Scheibel, 1966).

In view of the function of GABA as a major inhibitory neurotransmitter, it is noteworthy that somatostatin has also been reported to have mainly inhibitory effects both centrally and in the periphery. Somatostatin was first identified as the factor in hypothalamic extracts capable of inhibiting the release of growth hormone from cells of the anterior pituitary (Brazeau et al., 1973). It has been shown to have inhibitory actions on the release of other hormones (including glucagon and insulin from the pancreas; Curry and Bennett, 1974; Siler et al., 1974; Fujimoto and Ensinck, 1976). With few exceptions (see Dodd and Kelly, 1978), somatostatin has been reported to depress neuronal activity in the central and peripheral nervous systems and in the myenteric plexus (see Reichlin, 1980, for review). Electrophysiological studies (Florey, 1981) in the NRT of the cat should help to decide whether somatostatin, in addition to GABA, could play a role in producing the inhibitory signals for which this nucleus is noted (Lamarre et al., 1971; Schlag and Waszak, 1971).

The lack of SOM immunoreactivity in the rat NRT and in Purkinje cells of the cerebellum of the rat and cat

(unpublished observation) strongly suggests that SOM immunoreactivity is neither linked to GAD immunoreactivity in the general sense, nor necessarily linked in specific brain regions in all species. SOM immunoreactivity coexists with norepinephrine in neurons of the prevertebral ganglion (Hökfelt et al., 1977) and with cholecystokinin-like immunoreactivity in colonic neurons of the submucosal plexus of the guinea pig (Schultzberg et al., 1980). Although GAD and SOM immunoreactivity are present in the rat pancreas, GAD immunoreactivity has been demonstrated in the insulin-containing beta cells (Robbins et al., 1981), and not in the SOM-positive D cells. We conclude that the presence of SOM-immunoreactive material in the feline NRT conspicuously separates this GABAergic nucleus from at least some other groups of GABAergic cells, and may suggest the existence of a "SOM-positive subtype" of GABAergic neurons.

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