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Cellular localization of proenkephalin mRNA in rat brain: Gene expression in the caudate-putamen and cerebellar cortex

(opioid peptides/*in situ* hybridization/motor control)

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ABSTRACT **The cellular locations of proenkephalin mRNA have been determined for the caudate-putamen and cerebellar cortex of the rat brain by *in situ* hybridization. In the caudate-putamen, more than half of the neurons express the proenkephalin gene. Morphologically, they are medium-sized cells that may represent projection neurons. In the cerebellar cortex, proenkephalin mRNA is present in a subpopulation of neurons in the granule layer that appear to be Golgi cells—i.e., inhibitory interneurons. The presence of [Met]enkephalin, a pentapeptide derived from proenkephalin, in these two brain areas is consistent with a synthetic role for this mRNA and implicates proenkephalin gene expression in the control of motor function.**

This report describes a portion of the results of a survey in progress of the neuronal circuitry in rat brain that uses proenkephalin-derived peptides for passing chemically coded messages. Using *in situ* hybridization to locate cells that synthesize proenkephalin mRNA, we have identified unambiguously the location of neuronal subpopulations that express the proenkephalin gene in two brain areas responsible for motor control, the caudate-putamen and the cerebellar cortex. The functional nature of this mRNA is attested to by the presence of [Met]enkephalin in these brain regions.

In the caudate-putamen, immunocytochemical studies have suggested that perhaps only as many as 20% of the medium-sized, spiny neurons produce proenkephalin-derived peptides (1). However, results of the present study suggest that an unexpectedly high percentage (>50%) of these neurons have this capacity. In the cerebellar cortex, we found proenkephalin mRNA in Golgi interneurons of the granule layer, resolving a discrepancy regarding the origin of immunoreactive enkephalins in this region (2–5).

MATERIALS AND METHODS

Animals and Tissue Preparation for *in Situ* Hybridization. Adult male rats (Sprague–Dawley, Charles River Breeding Laboratories) were fed and housed under standard conditions. The animals were decapitated, and their brains were removed and frozen onto brass chucks within 2 min by using powdered dry ice. Cryostat sections, 10 μm in diameter and adhering to microscope slides coated with poly(L-lysine) (50 μg/ml), were fixed for 5 min in 3% neutral-buffered paraformaldehyde containing 0.02% diethylpyrocarbonate, rinsed twice in phosphate-buffered saline, dehydrated in an alcohol series, and stored desiccated at −80°C until use.

Preparation of the Rat Radiolabeled Proenkephalin cDNA Probe. The 435-base-pair DNA probe used in these studies is complementary to the mRNA encoding amino acids 56–200 of preproenkephalin (6). The DNA was excised from pRPE-1 by using *Pvu* II restriction endonuclease, purified by agarose gel electrophoresis, and nick-translated by using all four tritiated deoxyribonucleotide triphosphates (specific activity, 25–96 Ci/mmol, Amersham; 1 Ci = 37 GBq) to a specific activity of about 10^7 cpm/μg or nick-translated in the presence of [α-^{32}P]dCTP (specific activity, about 6000 Ci/mmol) and unlabeled dNTPs to a specific activity of 1–5 × 10^8 cpm/μg. The DNase conditions used yielded DNA fragments 75–150 base pairs long.

Cellular Localization of mRNA. The method for localizing autoradiographically radiolabeled DNA hybridized to cellular mRNA (*in situ* hybridization) has been described (7). Briefly, tissue sections were prehybridized for 2 hr at 23°C to decrease nonspecific binding of radiolabeled nucleic acids to tissue. The sections were rinsed in 2× NaCl/Cit (1× NaCl/Cit = 0.15 M sodium chloride/0.015 M sodium citrate), dehydrated in 95% ethanol, and then hybridized for 3 days at 37°C with heat-denatured (95°C for 10 min) and tritiated proenkephalin cDNA (10,000–30,000 cpm/20 μl of hybridization buffer per section) or 100,000 cpm/20 μl per section of proenkephalin [^{32}P]cDNA. We (7) and others (8) have found that, for double-stranded DNA probes of this size, 72-hr hybridizations yielded stronger hybridization signals than did 24-hr hybridizations, presumably because of limited diffusion of these probes through the tissue. The hybridization buffer contained 0.6 M sodium chloride, 10 mM Tris·HCl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 1 mM EDTA, 0.05% yeast total RNA, 0.005% tRNA, 0.05% herring sperm DNA, 0.05% inorganic sodium pyrophosphate, 10 μM unlabeled deoxynucleotides, 10% (wt/vol) dextran sulfate, and 50% (vol/vol) deionized formamide.

After hybridization, the sections were rinsed twice, 10 min each in 2× NaCl/Cit containing 0.05% inorganic sodium pyrophosphate, followed by a 2-day rinse in 0.5× NaCl/Cit containing 0.05% inorganic pyrophosphate with two changes—all at room temperature. As recommended by Haase and coworkers (8), long washes after hybridization with double-stranded DNAs were used to allow time for diffusion of unhybridized probe out of the tissue, lowering the background. Sections were dehydrated in an alcohol series in which 0.3 M ammonium acetate replaced water (8). The sections were dipped in Kodak NTB 2 emulsion, and the autoradiograms were exposed at 4°C for 40 or 46 days. The autoradiograms were developed in Kodak D19 developer and fixed in Kodak Fix; the sections were stained with 1% fast green in 0.4% acetic acid/95% ethanol followed with 0.5% cresyl violet. The sections were observed by using bright-

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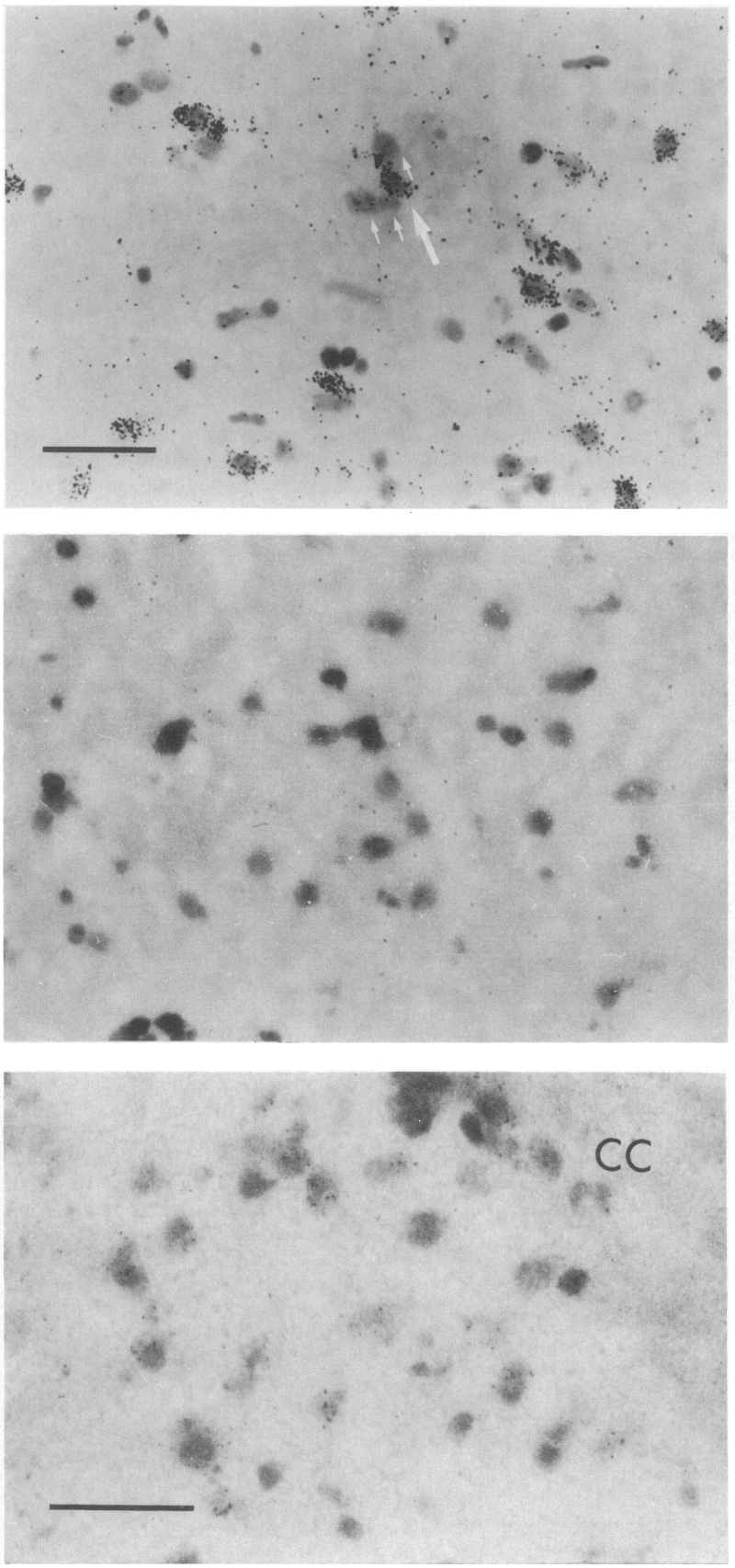

FIG. 1. Bright-field photomicrographs of autoradiograms of three sections of caudate-putamen hybridized with radiolabeled proenkephalin cDNA. (*Top*) The section was probed with 20,000 cpm of tritiated cDNA per 20 μl and was exposed for 46 days. The large white arrow indicates an example of a labeled medium-sized cell, and the small white arrows indicate examples of unlabeled cells. More than half of the cells in this nucleus were labeled. (Bar = 30 μm.) (*Middle*) Pretreatment of the section with RNase before hybridization with 100,000 cpm of [^{32}P]cDNA per 20 μl and exposure for 35 days eliminated the signal. (*Bottom*) Hybridization with a 25-fold excess of unlabeled probe before hybridization with 20,000 cpm/20 μl of tritiated cDNA with an exposure of 45 days greatly reduced the hybridization signal. (Bar for *Middle* and *Bottom* = 30 μm.) CC, corpus collosum.

field and dark-field microscopy and photographed by using Kodak Technical Pan film 2415, ASA 25, with a green filter for bright-field photographs.

Extraction, Separation, and Assay of [Met]Enkephalin. For the detection and quantification of [Met]enkephalin, brains were removed quickly, dissected to obtain caudate-putamen and cerebellum, and then frozen in liquid nitrogen. The frozen tissue, which was stored at −70°C, was homogenized with a Brinkmann Polytron in 10 volumes (wt/vol) of 1 M acetic acid/0.1% 2-mercaptoethanol containing 1 μg of each of the protease inhibitors pepstatin, leupeptin, and phenylmethylsulfonyl fluoride per ml. The homogenate was centrifuged for 45 min at 12,000 rpm in a Sorvall HB-4 rotor at 2°C. The supernatants were fractionated on a Sephadex G-75 column (2.5 × 70 cm) that was equilibrated and eluted with 1 M acetic acid/0.1% 2-mercaptoethanol. Aliquots (0.5 ml) of every three fractions (5 ml each) were pooled, evaporated to dryness, and resuspended in 0.15 ml of 0.2 M *N*-ethylmorpholine acetate buffer (pH 8.0). Ten microliters from each sample was assayed in duplicate with a [Met]enkephalin radioimmunoassay (9). The identity of the predominant peak of [Met]enkephalin immunoreactivity that comigrated with the free pentapeptide in both tissues was confirmed by injecting aliquots onto a reverse-phase HPLC system (5-μm LiChrosphere RP-18 column, 4.6 × 260 mm; Merck) along with ^{125}I-labeled [Met]enkephalin, which served as an internal standard. Peptides were eluted with a 0–20% gradient of 1-propanol in 0.5 M acetic acid, brought to pH 4.0 with pyridine. Fractions were monitored with a fluorescamine detection system (10) and were quantified by using the [Met]enkephalin radioimmunoassay. All of the immunoreactivity migrated in the position of authentic [Met]-enkephalin. The recovery of ^{125}I-labeled [Met]enkephalin averaged 90%. Amounts of [Met]enkephalin reported were not corrected for recovery.

RESULTS

Interpretive Controls for *in Situ* Hybridization. No labeled cells were observed when sections were incubated in hybridization buffer without addition of radiolabeled nucleic acid (not shown). Pretreatment of sections with RNase A (20 μg/ml at 37°C for 30 min) eliminated the signal (compare Fig. 1 *Top* and *Middle*). Hybridization with a 25-fold excess of unlabeled cDNA before addition of radiolabeled cDNA greatly reduced the signal (compare Fig. 1 *Top* and *Bottom*). Hybridization of sections with tritiated proopiomelanocortin cRNA did not yield any signal in the caudate-putamen or cerebellar cortex (not shown).

Cerebellar Cortex. A subpopulation of cells in the granule layer was labeled with clusters of reduced silver grains in the overlying emulsion (Fig. 2). These polygonally shaped cells containing proenkephalin mRNA were found in every granule layer from each lobule of the cerebellar cortex. The relative frequency and location of these cells can be appreciated in a drawing of a representative 10-μm section of the vermis (Fig. 3). The cell bodies of the labeled cells were 10–15 μm in diameter, and they were slightly smaller than the cell bodies of Purkinje cells, which were 16–20 μm along their long axis. The labeled cells were more frequent in the granule layer region nearest to the Purkinje cell body layer, and sometimes they were located nearly adjacent to a Purkinje cell (Fig. 2). The Purkinje-to-labeled-cell ratio was 2:1 in the section of vermis illustrated (Fig. 3).

In addition to granule and Golgi neurons, the granule layer contains a rare, cellular type known as the Lugaro cell. It has been described as having a fusiform-shaped cell body with its processes oriented horizontally and as being located adjacent to the Purkinje cell layer (11, 12). Its relative infrequency and morphological appearance make it unlikely that the labeled

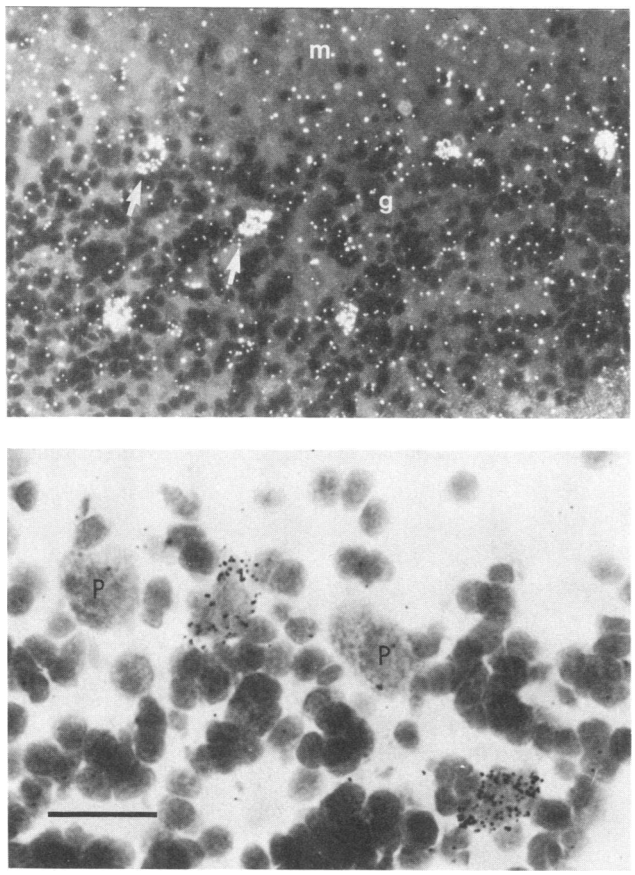

FIG. 2. Dark-field (*Upper*) and bright-field (*Lower*) photomicrographs of an autoradiogram of a 10-μm section of vermis of the rat cerebellar cortex hybridized with tritiated proenkephalin cDNA (20,000 cpm/20 μl) and exposed for 40 days. Labeled cells are located in the granule layer and appear to be Golgi neurons. In *Lower*, the two labeled cells indicated by white arrows in *Upper* appear at higher magnification. [Bar (for *Lower* only) = 15 μm.] g, Granule layer; m, molecular layer; P, Purkinje cell.

cells we observed represent Lugaro cells. Based on cellular location, size, and frequency as criteria (11, 13), it is likely that the labeled cells are Golgi neurons.

Radioimmunoassayable [Met]enkephalin, 60 pmol/g of wet weight, was detected in cerebellar protein extracts. Cerebellar content of this peptide and [Met]enkephalin-Arg^6-Gly^7-Leu^8, an octapeptide also derived from proenkephalin,

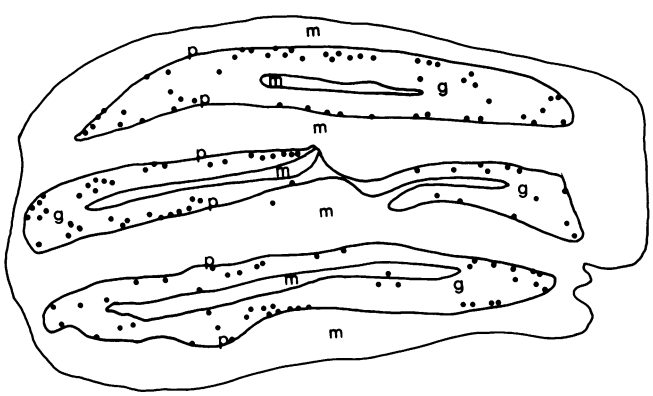

FIG. 3. Drawing of a 10-μm coronal section from the vermis of rat cerebellar cortex showing the location and number of cells labeled with tritiated proenkephalin cDNA in the granule layer. Each dot represents one cell. The Purkinje-to-labeled-cell ratio was about 2:1 in this section. p, Purkinje cell layer; m, molecular layer; g, granule layer.

is relatively low compared to the content of other brain regions (14, 15).

Caudate-Putamen. In the caudate-putamen, >50% of the neurons were labeled (Fig. 1 *Top*). The labeled cell bodies were 10–15 μm in diameter and belong to the medium-sized class of neurons (16). The density of grains per cell was uniform throughout the structure, and the location of labeled cells did not conform to any recognizable striosomal organization (17) as determined by examining coronal sections (Fig. 4). It was consistently observed that the frequency of labeled cells was slightly higher in the ventral aspects of the caudate-putamen (not shown), similar to that described previously in an immunocytochemical study (1).

Radioimmunoassayable [Met]enkephalin, 1.2 nmol/g of wet weight, was measured in the caudate-putamen. These concentrations and those for the octapeptide, [Met]enkephalin-Arg^6-Gly^7-Leu^8, are high in this nucleus relative to other brain regions (14, 15).

DISCUSSION

Cerebellar Cortex. In the cerebellar cortex of the rat, Golgi cells are inhibitory interneurons found nearly exclusively in the granule cell layer (13). Golgi neurons are clearly distinguishable from the much smaller granule cells and are nearly the same size as Purkinje cells. The Golgi cells are more frequent in the regions nearest the Purkinje cell layer, and in the cat, the Purkinje-to-Golgi cell ratio is 3:1 (18). In the present study, the labeled cells clearly have the characteristics of Golgi neurons, and the results suggest that [Met]enkephalin, presumably an inhibitory neuropeptide (19), is released at Golgi synapses on granule cells in this layer.

The results, then, provide definitive proof that opioids are synthesized in a discrete population of cerebellar cortical neurons, confirming and extending previous work showing the existence of proenkephalin mRNA and immunoreactive enkephalins in cerebellar cortex (2–4, 14, 15, 20). Immunocytochemical studies using antisera that cross-reacted with both [Met]- and [Leu]enkephalin suggested that an opioid substance was present in the cerebellar cortex, perhaps in Golgi neurons (2–4). Another study using [Leu]enkephalin antiserum failed to detect immunoreactive enkephalin cells in cerebellar cortex (5). The origin of this negative finding may reside in the 1:4 ratio of [Leu]- to [Met]enkephalin per molecule of precursor.

A high proportion of Golgi neurons are believed to use the inhibitory amino acid neurotransmitter, γ-aminobutyric acid, as a chemical messenger (13). The implication of the large numbers of Golgi neurons that contain proenkephalin mRNA is that some of the same Golgi neurons release both γ-aminobutyric acid and enkephalins, as was suggested by results of a previous immunocytochemical study (4). It should be interesting to determine if all Golgi neurons express the proenkephalin gene and whether conditioning of motor responses (21) alters gene expression in this circuit.

Caudate-Putamen. Our findings and previous reports (13, 14) showing a relatively high content of immunoreactive [Met]enkephalin and other proenkephalin-derived peptides in extracts from the caudate-putamen and terminal fields for this nucleus are consistent with the relatively high abundance of proenkephalin mRNA found previously in RNA extracts of caudate-putamen (14, 20). However, results of immunocytochemical studies have suggested that only a relatively small percentage, perhaps 20%, of the medium-sized, spiny neurons contained immunoreactive enkephalins (1). Results of the present study suggest that, in fact, >50% of the neurons in this nucleus contain proenkephalin mRNA.

The labeled cells were of the medium-size class of neurons (16). Ultrastructural analysis of neurons containing immunoreactive enkephalins revealed spines on the dendritic processes of medium-sized neurons (1). Medium-sized, spiny neurons are believed to be a heavily represented cell type (22) and to contribute substantially to striatal projections (23). Knife-cut studies combined with the localization of immunoreactive enkephalins revealed the existence of a strong striatal-pallidal connection (24). Therefore, it is likely that the medium-sized, proenkephalin mRNA-containing neurons observed in the present study are projection neurons. Some of the medium-sized neurons have been shown to contain both immunoreactive enkephalins and γ-aminobutyric acid-synthesizing enzyme (25).

The medium-sized neurons receive a strong dopaminergic input from the substantia nigra, one of the striatal terminal fields, and many of these cells contain the *d*opamine- and c*A*MP-*r*egulated *p*hospho*p*rotein, DARPP-32, having an apparent molecular weight of 32,000 daltons (26). Dopamine

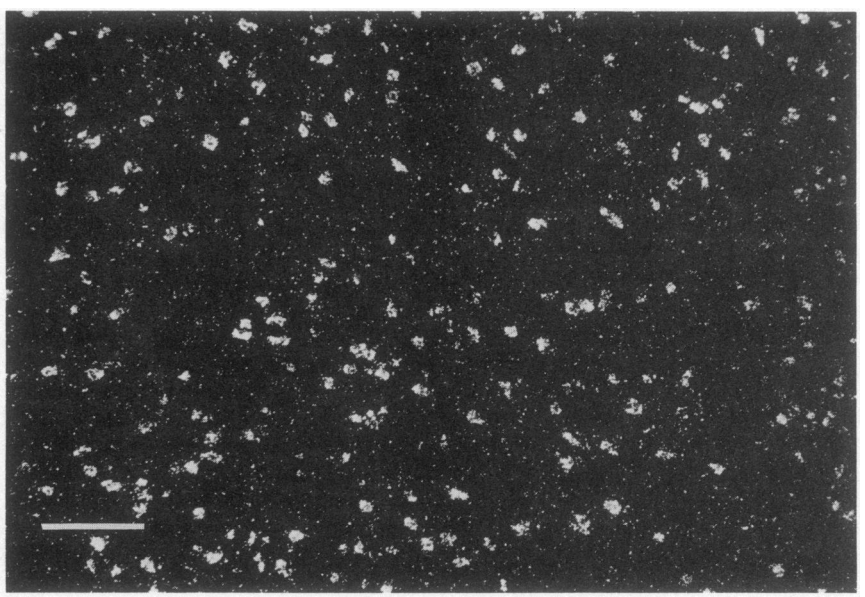

FIG. 4. Dark-field photomicrograph of an autoradiogram of a 10-μm section of caudate-putamen hybridized with tritiated proenkephalin cDNA (20,000 cpm/20 μl) and exposed for 46 days. The labeling intensity was similar for all cells throughout this nucleus. No obvious compartmentalization of the labeled cells was observed in coronal sections. (Bar = 0.1 mm.)

negatively regulates proenkephalin gene expression in the caudate-putamen (ref. 14; G.J.R., unpublished observations). Since as many as 60% of the neurons in the caudate-putamen contain immunoreactive DARPP-32 and since >50% of the neurons in this nucleus contain proenkephalin mRNA, it is of interest to know whether proenkephalin mRNA-containing neurons contain DARPP-32.

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