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Distribution of neurotensin/neuromedin N mRNA in rat forebrain: Unexpected abundance in hippocampus and subiculum

(*in situ* hybridization/CA1/precursor)

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ABSTRACT **We have used *in situ* hybridization to determine the regional distribution of mRNA encoding the neurotensin/neuromedin N (NT/N) precursor in the forebrain of the adult male rat. Cells containing NT/N mRNA are widely distributed in the forebrain. These areas include the septum, bed nucleus of the stria terminalis, preoptic area, hypothalamus, amygdala, accumbens nucleus, caudate–putamen, and piriform and retrosplenial cortex. In general, the regional distribution of NT/N mRNA corresponds to the previously determined distribution of neurotensin-immunoreactive cell bodies; however, several notable exceptions were observed. The most striking difference occurs specifically in the CA1 region of the hippocampus, where intense labeling is associated with the pyramidal cell layer despite the reported absence of neurotensin-immunoreactive cells in this region. Analysis of microdissected tissue by S1 nuclease protection assay confirmed the abundance of authentic NT/N mRNA in CA1. A second major discrepancy between NT/N mRNA abundance and neurotensin-immunoreactivity occurs in the intensely labeled subiculum, a region that contains only scattered neurotensin-immunoreactive cells in the adult. These results suggest that, in specific regions of the forebrain, NT/N precursor is processed to yield products other than neurotensin. In addition, these results provide an anatomical basis for studying the physiological regulation of NT/N mRNA levels in the forebrain.**

Neurotensin (NT), a tridecapeptide with widespread distribution in the central nervous system and peripheral tissues of mammals, is one of a family of structurally related peptides with members manifesting diverse biological activities (1). The structure of the rat gene encoding NT has recently been determined (2), and the general features of the predicted NT precursor for the rat are the same as those of the predicted precursors for the dog (3) and cow (2). In each species, a precursor protein of 169–170 amino acid residues encodes both NT and neuromedin N, a peptide similar in structure to NT (4). A comparison of the sequences of the complete neurotensin/neuromedin N (NT/N) precursor from rat, dog, and cow reveals a high degree of conservation. This finding is consistent with the idea that the precursor may be processed to yield biologically active peptides that are as yet unknown.

There is increasing evidence that NT functions as a neuroregulatory substance in the mammalian central nervous system (1, 5). Effects produced by NT administration in the central nervous system include hypothermia, hypotension, and antinociception. NT has also been implicated in the central regulation of anterior pituitary function (6, 7). NT-immunoreactive cell bodies are most numerous in the forebrain, with widespread distribution in limbic regions such as the septum, preoptic area, hypothalamus, and amygdala (8). Other forebrain regions, such as the dorsal subiculum, contain many NT-immunoreactive cell bodies during early stages of development, but the number of neurons with detectable NT-like immunoreactivity decreases dramatically by adulthood (9).

In the present study we have used *in situ* hybridization histochemistry to identify cells in the forebrain of the adult male rat that synthesize mRNA encoding the NT/N precursor. Unexpectedly, NT/N mRNA is abundant in the CA1 (field 1 of Ammon's horn) region of hippocampus and throughout the subiculum. Otherwise, the regional distribution of such cells corresponds well with the previously determined distribution of NT-immunoreactive cells.

MATERIALS AND METHODS

Animals and Tissue Preparation. Adult male Sprague–Dawley rats (Zivic–Miller) were housed under controlled light conditions (lights on 0700–2100) with food and water available ad libitum. At ≈80 days of age, rats were sacrificed by decapitation (1700–1800 hr), and coronal brain sections were prepared from brains that had been frozen in cold isopentane. Sections (20 μm) from four brains were thaw-mounted on gelatin-coated slides and stored at −70°C for *in situ* hybridization histochemistry. Sections (300 μm) from three brains were thaw-mounted on slides and stored at −70°C for microdissection. A 17-gauge stainless steel needle was used to sample the CA1 region of hippocampus bilaterally from several consecutive sections. For comparison, the central amygdaloid nucleus was removed with a 14-gauge needle in a similar manner. Samples were stored at −70°C until assayed.

Synthesis of Probe and Control RNAs. Template plasmid (prNT4) was constructed by ligating a 336-base-pair (bp) *Eco*RV/*Bgl* II fragment (nucleotides 626–961) of the rat NT/N gene (2) into *Bam*HI/*Sma* I-digested pGEM4 (Promega). Labeled antisense RNA probe (7×10^8 cpm/μg) was transcribed from *Eco*RI-linearized template with T7 RNA polymerase (Boehringer Mannheim) and [α-^{35}S]CTP (1250 Ci/mmol; 1 Ci = 37 GBq; Amersham) as described (10). Unlabeled antisense RNA was synthesized similarly. Labeled sense RNA was transcribed from a *Hin*dIII-linearized template by using SP6 RNA polymerase (Boehringer Mannheim). Unincorporated nucleotides were removed by chromatography over Sephadex G-50, and the probe size was reduced by alkaline hydrolysis (11).

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Abbreviations: NT, neurotensin; NT/N, neurotensin/neuromedin N; CA1, field 1 of Ammon's horn.

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***In Situ* Hybridization.** Slide-mounted brain sections were warmed to room temperature, treated sequentially with 4% (wt/vol) paraformaldehyde, 0.25% acetic anhydride (in 0.1 M triethanolamine, pH 8.0), a graded alcohol series, and chloroform and then air dried. Probe (2 pmol/ml) was applied in 45 μl of hybridization buffer [50% (vol/vol) formamide/10% (wt/vol) dextran sulfate/0.3 M NaCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA/0.2% bovine serum albumin/0.2% Ficoll/0.2% polyvinylpyrrolidone/yeast tRNA at 0.5 mg/ml/10 mM dithiothreitol], and slides were incubated at 45°C in a moist chamber overnight. To remove unhybridized probe, slides were washed for 15 min at room temperature with 2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0), followed by RNase A digestion as described (11). Additional washes were performed in 2× SSC for 1 hr at room temperature, 0.1× SSC for 30 min at 62°C, and 0.1× SSC briefly at room temperature. Sections were dehydrated in a graded alcohol series in which water was replaced by 0.6 M ammonium acetate and air dried.

To assess specificity of labeling, some sections were treated as described except for the substitution of labeled sense probe for labeled antisense probe. In addition, other sections were treated with a mixture of labeled antisense probe and excess (250×) unlabeled antisense RNA.

Autoradiography. Slides were apposed to Hyperfilm-βmax (Amersham) for 44 hr, and the film was developed in D19 solution (Kodak). Selected slides were subsequently dipped in NTB2 emulsion (Kodak, diluted 1:1 with 0.6 M ammonium acetate), exposed for 7 days at 5°C, developed in D19 solution diluted 1:1 with water, and counterstained with cresyl violet acetate.

Labeling was considered specific if it was (*i*) bilaterally symmetrical, (*ii*) consistent from section to section and from brain to brain, and (*iii*) absent in sections treated with labeled sense probe or a mixture of labeled antisense probe and excess unlabeled antisense RNA.

S1 Nuclease Protection Assay. S1 nuclease protection assays were done as described (12). To isolate a full-length NT/N cDNA for use as probe, a rat brain cDNA library was constructed in λgt11 and screened as described (3). The

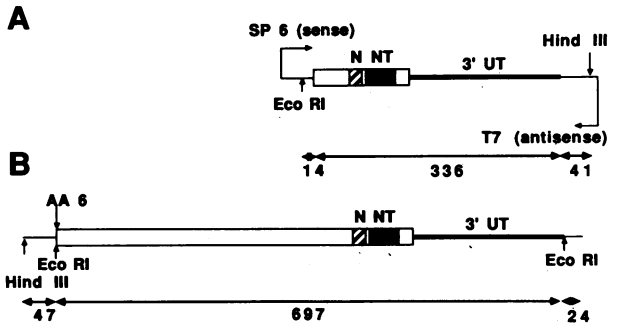

FIG. 1. Schematic diagram of templates used for RNA and DNA probe synthesis. Plasmid sequences (—), 3′ untranslated sequences (▬), and coding regions (□) are shown. The positions of the neuromedin N (▨) and NT (■) coding domains are indicated. The sizes (nucleotides) of various probe regions (delineated by double-headed arrows) are shown. (*A*) RNA probes were synthesized from a portion of exon 4 of the rat NT/N gene subcloned into pGEM4 as template. (*B*) DNA probes were synthesized from a rat brain NT/N cDNA (containing coding sequences from amino acids 6–169 and the proximal 3′ untranslated region) cloned into vector M13mp11.

*Eco*RI insert of a recombinant (rbNT/N-1), encompassing nearly the entire coding region (amino acids 6–169), was subcloned into vector M13mp11 and used to synthesize ^{32}P-labeled, single-stranded probe. RNA was prepared from brain punches by the lithium chloride/urea procedure as described (13). Pulverized skeletal muscle (50 mg) was added before extraction to serve as a carrier. No NT/N mRNA was detected in RNA prepared from skeletal muscle alone (data not shown).

RESULTS

NT/N mRNA in Hippocampus and Subiculum. The distribution of NT/N mRNA in the rat forebrain was determined by *in situ* hybridization with a ^{35}S-labeled RNA probe encompassing the NT and neuromedin N coding domains and part of the 3′ untranslated region (Fig. 1*A*). There was intense

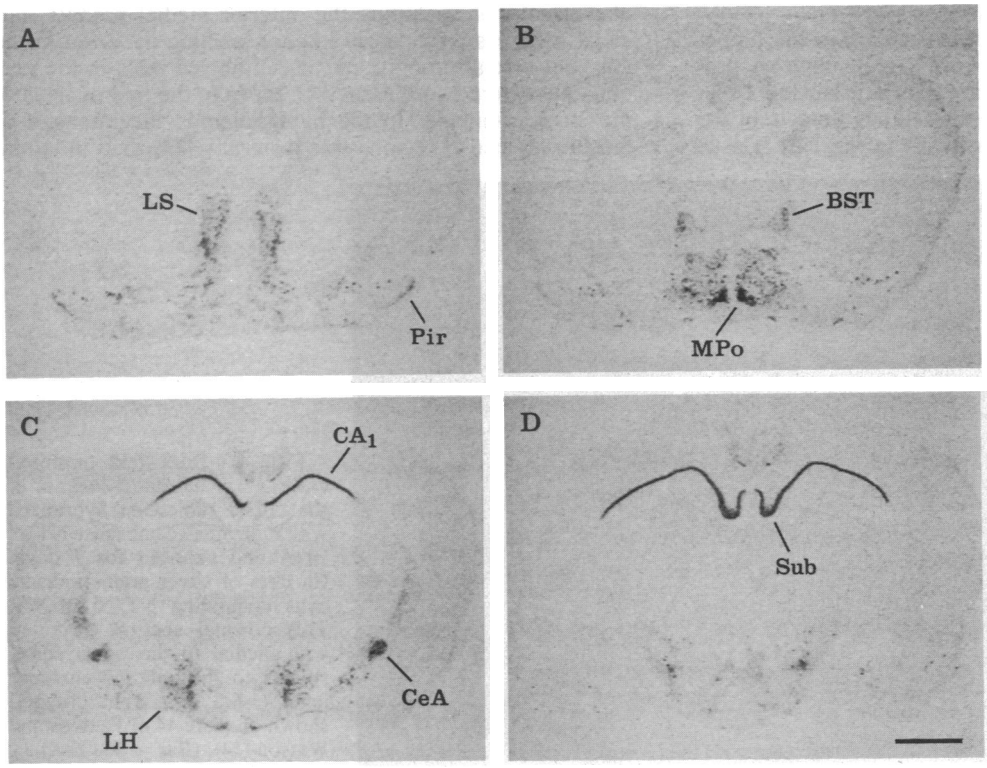

FIG. 2. Distribution of NT/N mRNA in coronal sections of rat forebrain determined by *in situ* hybridization with a ^{35}S-labeled antisense RNA probe (see Fig. 1*A*). Sections were apposed to Hyperfilm-βmax film for 44 hr. Autoradiograms were imaged with a Panasonic CCD black-and-white video camera and Sony video printer. Forebrain regions were identified according to Paxinos and Watson (14). Approximate anterior–posterior level of sections relative to bregma were 0.0 mm (*A*), −0.3 mm (*B*), −3.0 mm (*C*), and −3.8 mm (*D*). BST, bed nucleus of the stria terminalis; CA1, field 1 of Ammon's horn; CeA, central amygdaloid nucleus; LH, lateral hypothalamus; LS, lateral septum; MPo, medial preoptic nucleus; Pir, piriform cortex; Sub, dorsal subiculum. (Bar = 2 mm.)

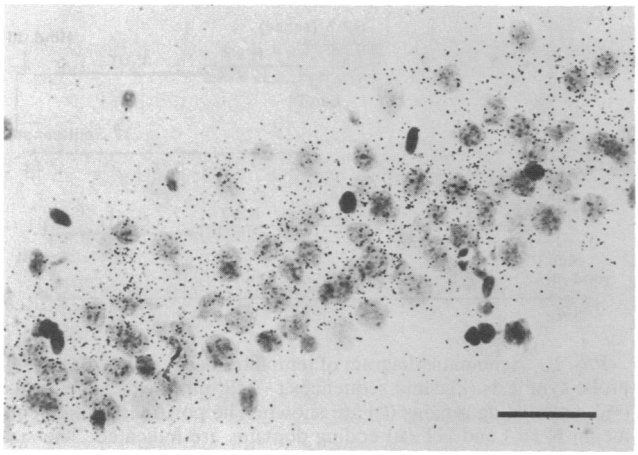

FIG. 3. Bright-field photomicrograph of an autoradiogram of the CA1 pyramidal cell layer hybridized with radiolabeled antisense NT/N RNA and exposed for 7 days. (Bar = 30 μm.)

labeling of the CA1 region of hippocampus (Fig. 2 *C* and *D*) that was unexpected in view of the reported absence of NT-immunoreactive cell bodies in this region (8, 9, 15–17). This labeling occurred at all septotemporal levels of the hippocampus. Examination of CA1 at higher magnification showed labeling to be distributed evenly over the pyramidal cell layer, not concentrated over a subset of the cell bodies (Fig. 3). There was an abrupt decrease in labeling at the approximate region of transition from CA1 to CA3. Neither CA3, CA4, nor dentate gyrus displayed appreciable specific labeling.

Like CA1, the subiculum was labeled along its entire septotemporal extent. Many intensely labeled cells were seen in the dorsal subiculum (Fig. 2*D*; and Fig. 4), despite previous reports of low numbers of NT-immunoreactive cell bodies in the adult (9, 15, 16). Labeling in the ventral subiculum was unexpected because NT-immunoreactive cell bodies have not been reported in that region of the rat at any stage of development (8, 9, 15–17). Labeling was restricted to the subiculum proper, as neither the presubiculum nor the parasubiculum exhibited labeled cells.

To determine whether the intense labeling in the hippocampus indicates authentic NT/N mRNA, S1 nuclease protection assays were done with a uniformly labeled DNA probe encompassing nearly the entire coding domain of the NT/N precursor (depicted schematically in Fig. 1*B*). Tissue was obtained by microdissection, and RNA isolated from CA1 (n = 3; Fig. 5, lanes 1–3) was compared with RNA from samples of the central amygdaloid nucleus (n = 3; Fig. 5, lanes 3–6). The central amygdala was chosen for comparison because it contains many NT-immunoreactive neurons (8, 18) and because it exhibited dense labeling after *in situ* hybridization with the RNA probe (Fig. 2*C*). In all cases, only a single, full-length protection product (Fig. 5, lanes 1–6, arrow) and residual undigested probe were seen. In addition, the levels of NT/N mRNA in samples of CA1 were comparable to those seen in samples of the central amygdaloid nucleus (note that CA1 samples were an estimated 2.7-fold larger than central amygdala samples). These results demonstrate that it is authentic NT/N mRNA that is abundant in the CA1 region of hippocampus even though NT-immunoreactive cell bodies have not been detected in this region.

Distribution of NT/N mRNA in Other Forebrain Regions. Labeled cells were widely distributed in the forebrain, and labeling was particularly heavy in limbic regions (Fig. 2). Beginning rostrally, labeling was present in anterior portions of piriform cortex. The medial portion of the accumbens nucleus exhibited hybridization signal that was continuous caudally with signal in the ventral pallidum and bed nucleus of the stria terminalis. Small numbers of intensely labeled cells were observed in the endopiriform region. The caudate–putamen contained labeled cells concentrated dorsomedially and ventrally at rostral levels and spread throughout the nucleus at caudal levels. Labeling was consistently seen in cerebral cortex just medial to the cingulum; this signal was faint in anterior cingulate cortex but increased in intensity in retrosplenial cortex.

Moderate-to-dense labeling occurred in a rostrocaudal continuum from the septum through the preoptic area and hypothalamus (Fig. 2*A–C*). In the septal region, hybridization signal was intense in rostral portions of the lateral septum, in the diagonal band of Broca, and in the lateral subdivision of the bed nucleus of the stria terminalis. Labeled cells accompanied the medial forebrain bundle from the region of the diagonal band of Broca through the lateral preoptic area and lateral hypothalamus. In the medial preoptic region, only light labeling was observed in periventricular structures, including the anterior medial preoptic nucleus, whereas particularly intense labeling occurred in the adjacent medial preoptic nucleus. Labeled cells in the preoptic area were continuous with those in the bed nucleus of the stria terminalis. In the hypothalamus the intensity of labeling in medial regions was generally less than in lateral

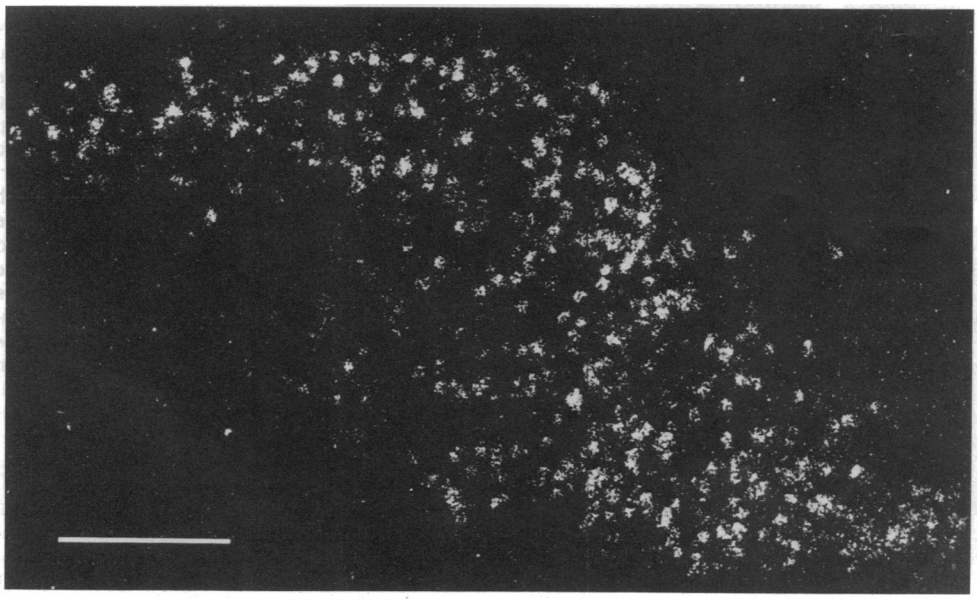

FIG. 4. Dark-field photomicrograph of an autoradiogram of the dorsal subiculum hybridized with radiolabeled antisense NT/N RNA and exposed for 7 days. Clusters of silver grains indicate cells containing NT/N mRNA. This coronal section (oriented with medial to the right) corresponds to the anterior–posterior level (−6.8 mm from bregma) shown on plate 44 of Paxinos and Watson (14). (Bar = 300 μm.)

ones. There was little, if any, signal associated with the paraventricular nucleus or the periventricular region, which was surprising because NT-immunoreactive cell bodies have been seen consistently in these areas (8, 18). Labeled cells were observed in the arcuate nucleus, dorsomedial nucleus, and perifornical region, whereas the ventromedial nucleus was devoid of any labeling. In the mammillary region light-to-moderate labeling was present in the ventral premammillary nucleus and the medial mammillary nucleus.

In the amygdala, labeling was generally restricted to medial structures (Fig. 2*C*). Labeled cells were seen in the anterior cortical, basomedial, medial, and central nuclei of the amygdala; the densest labeling was associated with the central nucleus. A continuum of labeled cells extended medially from the rostral amygdaloid complex across the sublenticular substantia innominata to the lateral hypothalamus and bed nucleus of the stria terminalis. Labeling was also present in the amygdalohippocampal transition area.

DISCUSSION

The distribution of NT in the rat forebrain has been extensively mapped with immunocytochemical techniques (8, 9, 15–23). DNA sequence analysis of cDNA and genomic clones has revealed that NT is synthesized as part of a larger precursor protein that also contains a coding domain for the NT-like peptide neuromedin N (2, 3). To determine whether the distribution of mRNA encoding this precursor corresponds to the previously reported distribution of NT-immunoreactive cell bodies, *in situ* hybridization histochemistry was performed on sections of adult rat forebrain by using an RNA probe derived from the rat NT/N gene. In general, the regional distribution of NT/N mRNA corresponds well with that of NT immunoreactivity. However, NT/N mRNA is abundant in the CA1 region of the hippocampus, a region where NT-immunoreactive neurons have not been detected (8, 9, 15–17), even with the use of colchicine to enhance detection. Similar discrepancies between neuropeptide mRNA abundance and neuropeptide immunostaining have been already noted (24). Our results obtained by S1 nuclease protection assay confirm that high levels of authentic NT/N mRNA exist in CA1. These results effectively rule out the possibility that cells in CA1 use an alternative splicing mechanism (25) to generate one or more mRNAs that lack the NT coding domain.

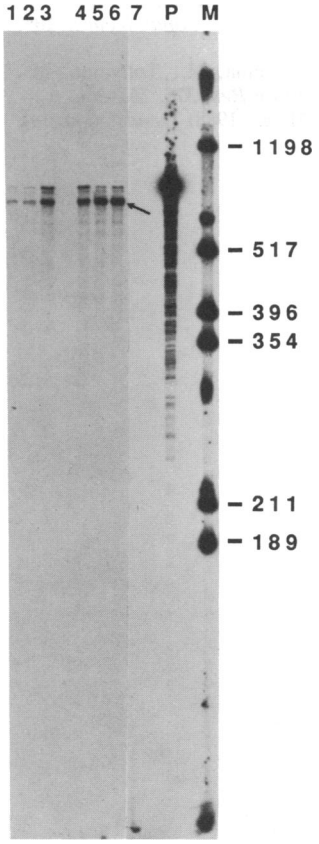

FIG. 5. S1 nuclease protection analysis of RNA from the CA1 region of hippocampus. Individual punches of CA1 and the central amygdaloid nucleus were obtained from three adult rat brains, and RNA was prepared by extraction with 3 M lithium chloride/6 M urea. A portion (40%) of the RNA recovered from each punch (lanes 1–3, central amygdala from rats 1–3; lanes 4–6, CA1 from rats 1–3), or 20 μg of yeast RNA (lane 7) was hybridized with a ^{32}P-labeled antisense DNA probe, followed by digestion with 50 units of S1 nuclease. The probe (lane P) encompasses nearly all of the NT/N precursor coding region and the proximal portion of the 3′ untranslated region (see Fig. 1*B*). Products of the reactions were analyzed on 5% polyacrylamide/7 M urea sequencing gel and visualized by autoradiography for 12 hr with an intensifying screen. Sizes of ^{32}P end-labeled *Hin*fI restriction fragments of pSP65 (Promega Biotech) are indicated (lane M). Position of the full-length protection product is indicated by an arrow.

The absence of NT-immunoreactive cells in the hippocampus could be the result of posttranslational compartmentalization of NT. In adult rats the detection of NT-immunoreactive cell bodies in many brain regions requires colchicine, which blocks axonal transport. Without colchicine NT is presumably transported rapidly out of cell bodies to axonal projection sites, and, thus, the cell bodies contain insufficient NT to be detected immunocytochemically. However, rapid axonal transport of NT cannot readily account for the absence of NT-immunoreactive cell bodies in the CA1 region, because the subiculum, which receives a major projection from CA1 (26), contains relatively low levels of NT (27).

Posttranslational regulation could occur at the level of processing of the NT/N precursor protein to yield processed, bioactive peptides. Tissue-specific processing of the proopiomelanocorticotropin precursor protein yields different sets of peptides in the anterior and intermediate lobes of the pituitary (28). By analogy, the NT/N precursor protein might be differentially processed to yield distinct sets of peptides in different brain regions. An obvious candidate for one product of differential processing is neuromedin N. To our knowledge, the distribution of neuromedin N-immunoreactive cells in mammalian tissues has not been determined. In other vertebrates, however, gastrointestinal cells expressing NT, neuromedin N, or both peptides, have been detected (29). In addition, peptides other than NT and neuromedin N could be generated from the NT/N precursor (2, 3).

Although differential protein processing seems the most likely explanation for the discrepancy between NT/N mRNA abundance and NT-immunostaining, we cannot rule out the possibility of translational regulation. For example, NT/N mRNA may be sequestered in the nuclei of CA1 pyramidal cells and remain untranslated. Another possibility is that NT/N mRNA is transported to the neuronal cytoplasm but translated inefficiently. Alternatively, there may be rapid degradation of the NT/N precursor protein after translation. Immunocytochemical studies with antisera that specifically detect the NT/N precursor, neuromedin N, and other potential processing products should aid experimental testing of these possibilities.

The abundance of NT/N mRNA in the subiculum was also unexpected. Efferents of the dorsal and ventral subiculum of the rat project differentially, but with some overlap, to the entorhinal and presubicular cortex, lateral septum, basal forebrain, and hypothalamus (26, 30). To our knowledge, there have been no reports of NT-immunoreactive cell bodies in the ventral subiculum. In the dorsal subiculum of the rat, the number of cell bodies with detectable NT-like immunoreactivity reaches a maximum during the first postnatal week (9) and then decreases markedly by adulthood, so that only scattered cells are visible (9, 15, 16). There are indications of a similar reduction in NT-immunoreactive cells during development of the human subiculum (31). Parallel changes occur in the density of NT-immunoreactive fibers in the mammillary body, and a NT-immunoreactive pathway from subiculum to mammillary body has been identified in the neonatal rat (17) and human neonate (31). A NT-immunoreactive pathway from dorsal subiculum to anterior cingulate cortex in the adult rat has also been reported (16). The abundance of NT/N mRNA in the dorsal subiculum of the adult rat indicates that the developmental reduction in NT-immunoreactive neurons seen in this region is the result of

posttranscriptional regulation. This fact raises the interesting possibility of a developmental change in processing of the NT/N precursor protein. Recent evidence suggests that transient perinatal expression of neurotransmitters in the developing neocortex is important for the establishment of proper neuronal connectivity (32).

Not all regions previously noted to contain NT-immunoreactive cell bodies were found to possess NT/N mRNA. Labeling was conspicuously absent from the hypothalamic paraventricular nucleus and the adjacent periventricular region, areas previously reported to contain NT-immunoreactive cell bodies (8, 18). One possible explanation for these results is that the content of NT/N mRNA in cells of these regions may be below the limits of detectability by *in situ* hybridization. Alternatively, previously reported NT immunostaining in these regions may not represent authentic NT. An analogous situation has been described for the thyroid-releasing hormone precursor and its mRNA in certain regions of the rat brain (24).

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