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Immunohistochemical distribution of dynorphin B in rat brain: Relation to dynorphin A and α-neo-endorphin systems

(opioid peptides/pro-dynorphin/immunofluorescence/colocalization)

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ABSTRACT **A specific antiserum was prepared against dynorphin B, an endogenous opioid peptide contained in a recently isolated 4,000-dalton dynorphin. The antiserum did not crossreact with dynorphin A, α-neo-endorphin, β-neo-endorphin, dynorphin-(1–8), or [Leu]enkephalin. In immunohistochemical staining experiments on frozen sections through rat brains from normal and colchicine-treated animals, the antiserum labeled the same neuronal fiber systems previously described as containing both dynorphin A and α-neo-endorphin immunoreactive material. The α-neo-endorphin/dynorphin A immunoreactive perikarya in the hypothalamic magnocellular nuclei also were labeled by the dynorphin B antiserum. In addition, the dynorphin B antiserum revealed groups of immunoreactive neuronal cell bodies in several other hypothalamic and extrahypothalamic areas, including brainstem, midbrain, central nucleus of amygdala, and in the dorsomedial, lateral, and anterior nuclei of hypothalamus. These perikarya had not been detected in previous studies that used dynorphin A and α-neo-endorphin antisera. The findings are in agreement with recent studies demonstrating a common precursor for dynorphin A, dynorphin B, and α-neo-endorphin. The apparently wider distribution of dynorphin B immunoreactive cell bodies compared to α-neo-endorphin/dynorphin A immunoreactive perikarya may be a reflection of differential processing of the precursor in different brain regions.**

Dynorphin is an extraordinarily potent endogenous opioid peptide that has been isolated from porcine pituitary (1, 2) and gut (3). Porcine pituitary extracts also contain a 4,000-dalton, 32-residue dynorphin (dynorphin-32) that consists of the original dynorphin heptadecapeptide (now called dynorphin A) attached to the aminoterminus of a [Leu]enkephalin containing tridecapeptide (4). This tridecapeptide also has been isolated as such from bovine pituitary extracts (5). Within dynorphin-32 the two peptides are separated by a Lys-Arg dipeptide sequence, indicating a precursor role of dynorphin-32 (4). The [Leu]enkephalin-containing tridecapeptide derived from dynorphin-32 was named dynorphin B because of its structural and pharmacological similarities to dynorphin A (4). Recent studies have shown that dynorphin B is present in large quantities in brain and neurointermediate pituitary of rat (unpublished data). We now provide immunohistochemical evidence that dynorphin B is present in the same neuronal fibers and perikarya that have previously been described as containing α-neo-endorphin and dynorphin A immunoreactive material (6). This finding is in agreement with recent evidence obtained by recombinant DNA methodology that in pig hypothalamus dynorphin A, dynorphin B, and α-neo-endorphin are part of a larger common precursor (pro-dynorphin) (7). In addition to the α-neo-endorphin/dynorphin A immunoreactive neuronal perikarya in the supraoptic and paraventricular nuclei of hypothalamus, the dynorphin B antibodies revealed several other groups of neuronal cell bodies that had not been detected in our previous study that used dynorphin A or α-neo-endorphin antibodies.

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MATERIALS AND METHODS

Peptides and Antisera. Antisera were used that had been raised against synthetic dynorphin B. Dynorphin B was obtained from Avram Goldstein (Addiction Research Foundation, Palo Alto, CA) and from Peninsula Laboratories (San Carlos, CA). All other peptides used in this study were from Peninsula Laboratories. All peptides with the exception of dynorphin B-29, the 29-residue putative peptide at the COOH terminus of pro-dynorphin (7), were shown to yield a single peak on reversed-phase HPLC when using a system as described (8). The synthetic dynorphin B-29 was only 87% pure as judged by this criterion. The dynorphin B antiserum (R3-2) was raised against a carbodiimide-treated peptide-thyroglobulin mixture (9). In a radioimmunoassay with ^{125}I-labeled dynorphin B as a tracer, the antiserum did not crossreact with [Leu]enkephalin, [Met]enkephalin, dynorphin A, α-neo-endorphin, β-neo-endorphin, or dynorphin-(1–8) (up to 1 μM). In some experiments a dynorphin B antiserum (13s) raised against a glutaraldehyde-linked peptide-thyroglobulin complex was used (10). This antiserum was a gift from Avram Goldstein. Its properties in tissue radioimmunoassay have been described (11). Antiserum 13s produced a similar immunostaining as R3-2. The descriptions of immunoreactive neuronal elements (Table 1; Figs. 1 and 2) were taken from sections incubated with antiserum R3-2. Antisera to dynorphin A and to α-neo-endorphin were used to compare the distribution of these two peptides with the distribution of the dynorphin B-like immunostaining. The characterization and specificities of these antisera have been described (6, 10).

Immunohistochemistry. Serial sections through brains from six normal and six colchicine-treated male rats (Sprague–Dawley; 150–250 g) were stained by immunofluorescence for dynorphin B, dynorphin A, and α-neo-endorphin. For colchicine treatment the animals received an intraventricular injection of 50 μg of the drug in 25 μl of water 48 hr prior to perfusion. The animals were perfused with either 4% freshly depolymerized paraformaldehyde in 0.13 M isoosmotic sodium phosphate buffer or with Zamboni's fixative (12) supplemented with 0.02% glutaraldehyde (13) as previously described (6). The perfused brains were removed, blocked, fixed at 4°C for 2 hr, and soaked overnight at 4°C in 5% sucrose in 0.01 M sodium phosphate buffer containing 0.15 M NaCl (pH 7.4). After freezing the brains in liquid dichlorodifluoromethane, coronal sections (13 μm) were cut in a cryostat and processed for immunofluorescence by using primary antiserum at a final dilution of 1:400 and fluorescein-labeled sheep anti-rabbit IgG (Arnel, New York) at a final dilution of 1:40. The immunohistochemical staining protocol

has been described in detail elsewhere (6, 14). After staining, the sections were examined and photographed in a Leitz Orthoplan microscope equipped with an epi-fluorescence attachment. Immunofluorescent staining was correlated with coronal sections from the stereotaxic atlases of Koenig and Klippel (15) and Pellegrino *et al.* (16).

For immunostaining experiments in which two or more antigens were to be visualized in the same neuronal cell bodies, 4-μm thick frozen sections were cut in a Bright OTF cryostat (Hacker Instruments, Fairfield, NJ) equipped with a model 5030 precision microtome. Adjacent serial sections were then processed for immunohistochemistry by using the different primary antisera to dynorphin A, dynorphin B, and α-neo-endorphin.

The specificity of the immunofluorescent staining was tested by incubating adjacent serial sections either with primary antiserum alone or with primary antiserum to which 10–100 μM synthetic peptide was added immediately prior to incubation of the section. The following peptides were tested in the blocking controls: dynorphin A, dynorphin B, α-neo-endorphin, dynorphin-(1–8), [Leu]enkephalin, and dynorphin B-29.

RESULTS

Specificity Controls. The specificity of the immunostaining was tested by blocking controls. The immunohistochemical specificities of the dynorphin A and α-neo-endorphin antisera used in this study have been reported in detail elsewhere (6, 17). In addition, these two antisera were tested for crossreactivity with dynorphin B: neither in radioimmunoassay (up to 1 μM)

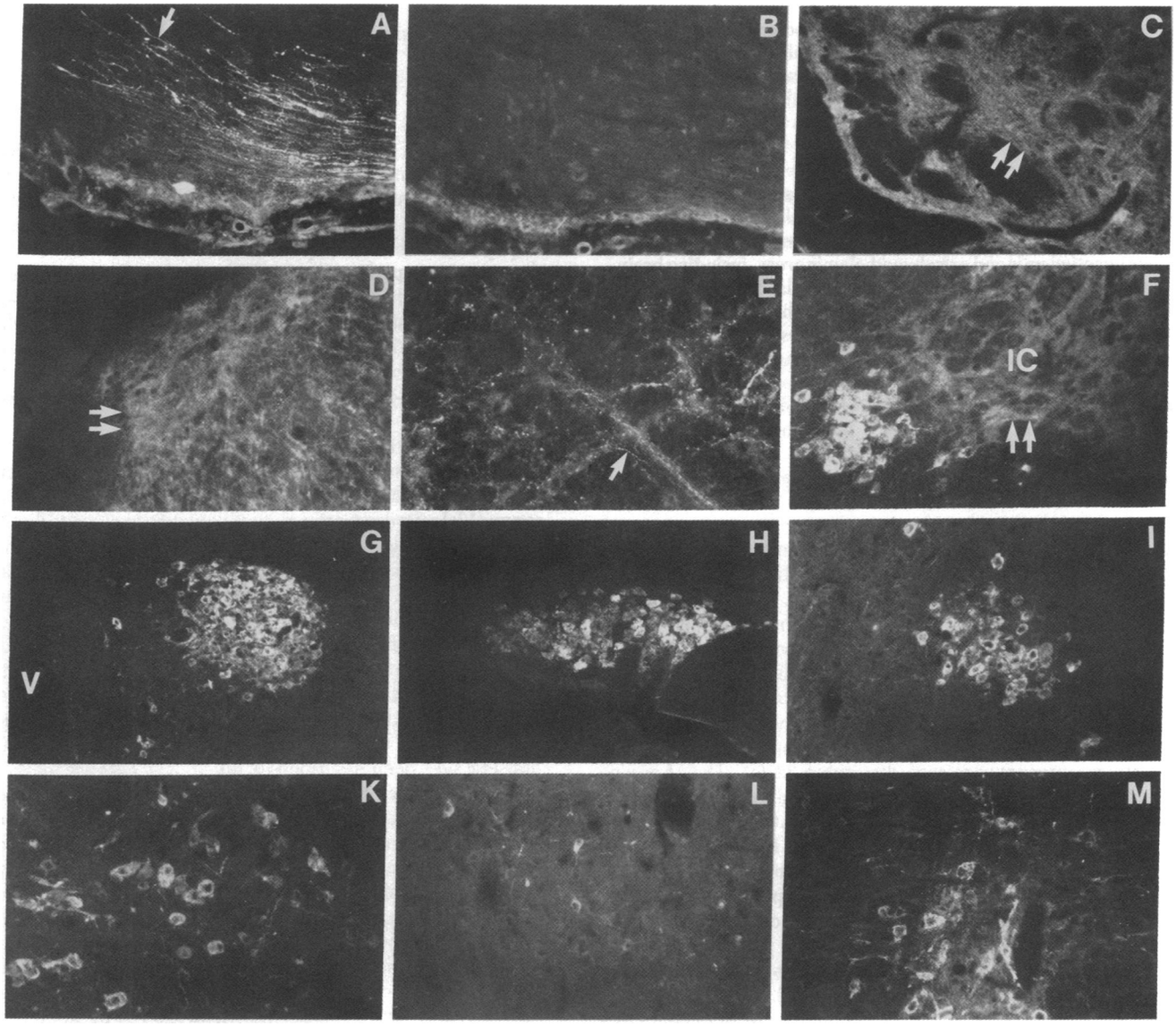

FIG. 1. Photomicrographs of dynorphin B immunoreactive neuronal elements in rat brain. Micrographs were taken from brain sections that had been incubated with antiserum R3-2. (*A* and *B*) Specificity of the dynorphin B-like immunostaining in beaded axons of the ventral hypothalamus. The section in *A* was incubated with dynorphin B antiserum in the presence of 100 μM synthetic dynorphin A and that in *B* was incubated with the same antiserum in the presence of 10 μM dynorphin B. Only dynorphin B blocked the immunofluorescence. (*C–M*) Typical dynorphin B immunostaining in neuronal cell bodies, thin beaded axons (single arrows), and thick fiber and terminal patterns (double arrows) in various brain regions: substantia nigra (*C*), ventral pallidum (*D*), solitary tract (*E*), internal capsule (entopeduncular nucleus region) and lateral hypothalamic nucleus (*F*), paraventricular nucleus (*G*), supraoptic nucleus (*H*), anterior hypothalamic nucleus (*I*), dorsomedial nucleus (*K*), reticular formation dorsolateral of substantia nigra (*L*), and nucleus raphe pallidus of the medulla oblongata (*M*). IC, internal capsule (entopeduncular nucleus region); V, third ventricle.

nor in immunohistochemistry (up to 50 μM) did dynorphin B crossreact with the dynorphin A and α-neo-endorphin antisera. All dynorphin B-like immunostaining reported below was blocked by 10 μM dynorphin B (Fig. 1*B*) but not by 100 μM dynorphin A (Fig. 1*A*), α-neo-endorphin, dynorphin-(1–8), or [Leu]enkephalin. The dynorphin B-like immunostaining was blocked also by 50 μM of a partially purified preparation of synthetic dynorphin B-29, indicating that in immunohistochemistry the dynorphin B antiserum was capable of recognizing COOH-terminal extended dynorphin B peptides.

Distribution of Neuronal Fibers and Terminals. Dynorphin B antisera, when applied to sections through rat brain, produced intense immunostaining in neuronal fiber and terminal patterns that resembled the ones previously described as containing immunoreactive dynorphin A and α-neo-endorphin (6). In adjacent serial sections the distributions of fibers and terminals that were reactive to either dynorphin B or dynorphin A and α-neo-endorphin antibodies appeared virtually identical. However, the fiber staining produced by the dynorphin B antisera often seemed to be more intense with less background staining than the dynorphin A/α-neo-endorphin immunofluorescence. As for dynorphin A/α-neo-endorphin, the densest dynorphin B fiber and terminal systems were seen in substantia nigra, internal capsule at the level of the entopeduncular nucleus, hypothalamus, ventral pallidum (region of the rostral parts of the median forebrain bundle), and the mossy fibers of the hippocampal formation (18) (Fig. 1 *A–D* and *F*). However, immunoreactive fibers and terminals also were seen in many other brain regions throughout the neuroaxis (Fig. 1*E*; Table 1). There were no significant differences in fiber distribution and staining intensities between normal and colchicine-treated animals.

Table 1. Distribution of the major dynorphin B immunoreactive neuronal elements in rat brain

Brain region	Perikarya*	Fibers and terminals†
Medulla/pons		
Nucleus of the solitary tract		++
Solitary tract		++
Parabrachial nucleus		+
Raphe pallidus nucleus	+	++
Mesencephalon		
Substantia nigra (reticular part)		+++
Peripeduncular nucleus region	+	+
Central gray		+
Diencephalon		
Stria terminalis		++
Hypothalamus		
Lateral nucleus	+	+
Dorsomedial nucleus	+	+
Supraoptic nucleus	+	+
Retrochiasmatic supraoptic nucleus	+	+
Paraventricular nucleus	+	+
Periventricular nucleus	+	+
Anterior nucleus	+	+
Telencephalon		
Hippocampal formation (mossy fibers)		+++
Entopeduncular nucleus/ internal capsule		+++
Globus pallidus		+
Caudate/putamen		+
Ventral pallidum		+++
Bed nucleus of the stria terminalis		++
Central nucleus of amygdala	+	+
Lateral septal nucleus		+
Accumbens nucleus		++
Cortex		+
Olfactory tubercle		+

* Presence of cell bodies is indicated by +.
† Sparse, moderate, and dense immunostaining is indicated by +, ++, and +++, respectively.

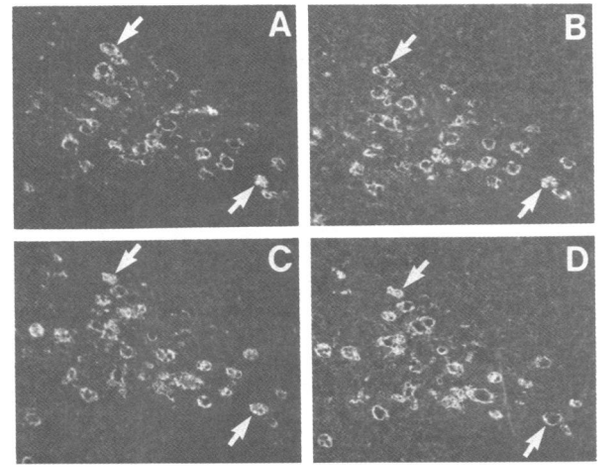

FIG. 2. Demonstration of colocalization of dynorphin B, dynorphin A, and α-neo-endorphin immunoreactivity in cell bodies of the paraventricular nucleus. Adjacent serial thin sections (4 μm; in rostral to caudal order from *A* to *D*) were incubated with dynorphin B antiserum R3-2 (*A* and *C*), dynorphin A antiserum (*B*), and α-neo-endorphin antiserum (*D*). It can be seen clearly that the same neuronal cell bodies (arrows) contain immunoreactivity to all three antisera.

Distribution of Neuronal Cell Bodies. Most dynorphin B immunoreactive neuronal perikarya were seen only in brains from colchicine-treated animals. In normal animals only perikarya in the supraoptic and paraventricular nuclei showed a faint immunofluorescence upon incubation with dynorphin B antisera. In colchicine-treated animals numerous intensely stained, dynorphin B immunoreactive cell bodies were seen in the supraoptic, retrochiasmatic supraoptic, and paraventricular nuclei of hypothalamus (Fig. 1 *G* and *H*). When 4-μm serial sections through the same neurons in these nuclei were stained alternately for dynorphin A, dynorphin B, and α-neo-endorphin, it became obvious that the same cell bodies were labeled by the three different antibodies (Fig. 2 *A–D*), indicating that immunoreactivities for all three opioids are localized in the same hypothalamic magnocellular neurons. These same perikarya also contain vasopressin immunoreactive material (19).

In addition to the magnocellular neurons, the dynorphin B antibodies detected several groups of neuronal perikarya that either were not visualized or were barely visualized by antisera to α-neo-endorphin or dynorphin A. These included cell bodies in the dorsomedial (Fig. 1*K*), periventricular, lateral (Fig. 1*F*), and anterior (Fig. 1*I*) nuclei of hypothalamus. A few cell bodies in the central nucleus of the amygdala and in the region of the peripeduncular nucleus dorsolateral to the substantia nigra (Fig. 1*L*) also were immunostained by dynorphin B antisera. A group of dynorphin B immunoreactive cells was observed in the nucleus raphe pallidus between the superficially lying pyramidal tracts (Fig. 1*M*).

DISCUSSION

We have demonstrated by double-antibody immunofluorescence the distribution in brain of dynorphin B immunoreactive nerve fibers, terminals, and perikarya. The immunoreactive fiber and terminal patterns that were labeled by the dynorphin

B antiserum were identical to the ones that had been demonstrated by dynorphin A and α-neo-endorphin antisera (6). Moreover, we have demonstrated directly on serial thin sections through the hypothalamic magnocellular nuclei that the three peptides are colocalized within the same cell bodies in these nuclei (Fig. 2 *A–D*). These results are in excellent agreement with the recent demonstration by Kakidani *et al.* that in pig hypothalamus dynorphin A, dynorphin B, and α-neo-endorphin are all part of a larger common precursor (7).

However, while demonstrating essentially the same fiber systems as dynorphin A/α-neo-endorphin antisera, the dynorphin B antiserum revealed several groups of neuronal perikarya that were not detected in our previous study (6). However, it is likely that these additional dynorphin B immunoreactive perikarya synthesize dynorphin A and α-neo-endorphin, because all three substances are derived from a common precursor. The apparent difficulties in visualizing dynorphin A and α-neo-endorphin immunoreactivity in neuronal cell bodies other than magnocellular neurons may be due, in part, to the relatively high background staining of these antisera compared to dynorphin B antiserum. Another factor may be that in brain α-neo-endorphin and dynorphin A are subject to complex proteolytic processing pathways. For example, dynorphin A in most brain regions is converted extensively into dynorphin-(1–8) (8). α-Neo-endorphin can be converted into β-neo-endorphin (20) and an even further processing of dynorphins and neo-endorphins into [Leu]enkephalin cannot be excluded (6, 21). Moreover, the extent to which the processing occurs differs among brain regions and therefore, the amounts of the various products of dynorphin A and α-neo-endorphin are extremely variable across brain areas (8, 20).

In view of these complex processing mechanisms, it can be expected that the extent to which neurons that synthesize pro-dynorphin (7) are visualized depends largely on which region of the molecule toward which the antibodies are directed. Indeed, recent studies that utilized antisera directed toward a COOH-terminal segment of dynorphin A showed the presence of dynorphin A immunoreactive cell bodies in numerous hypothalamic and extrahypothalamic locations that seem to be similar to the ones reported here (22, *, †), whereas, in earlier studies that used antisera directed toward the middle region of dynorphin A, the cell body staining was restricted to the magnocellular neurons of hypothalamus (6, 23). Therefore, we can expect that as more antibodies to the various processing products of pro-dynorphin become available, more cell populations synthesizing the precursor and its different products will be detected.

The precise molecular nature of the intraneuronal dynorphin B immunoreactive material that we have visualized must be subject to some speculation. Within the precursor molecule, dynorphin B has a COOH-terminal extension of 16 amino acids, creating a peptide of 29 amino acids (dynorphin B-29) with a single arginine in position 14. Because the dynorphin B-like immunostaining is blocked by both dynorphin B and dynorphin B-29, it is possible that the reactivity demonstrated by the dynorphin B antiserum may be due, in part, to dynorphin B-29 or even to larger precursor molecules. However, gel permeation and HPLC experiments on rat brain and pituitary extracts have shown that the majority of the dynorphin B immunoreactivity in these tissues is dynorphin B rather than dynorphin B-29 or larger precursors (unpublished data). Thus, it seems that besides α-neo-endorphin and dynorphin A we have histochemically visualized a third opioid peptide within a neuronal network that was previously called the α-neo-endorphin/dynorphin neuronal system (6). Biochemical evidence suggests that the system also contains dynorphin-(1–8) and β-neo-endorphin (8, 20). Therefore, it may be more appropriate to use the term neo-endorphin/dynorphin neuronal system.

All five opioid peptides thus far demonstrated in this system can interact with the κ-opioid receptor, with dynorphin A having the highest selectivity (24–26), and therefore the system seems to be geared primarily toward the production of ligands to this important subclass of the multiple opioid receptor system. Future studies must now address the question whether the pro-dynorphin precursor also can give rise to the δ ligand [Leu]enkephalin, a processing step that is not precluded by the primary structure of the precursor (7). Immunohistochemical studies using highly specific antisera to [Leu]enkephalin have shown that many of the neo-endorphin/dynorphin-immunoreactive neuronal elements described here (e.g., fibers in substantia nigra and entopeduncular nucleus) do not contain [Leu]enkephalin-like immunoreactivity, whereas in other regions (e.g., ventral pallidum) the [Leu]enkephalin antisera produce an immunostaining identical to that produced by dynorphin A, dynorphin B, and α-neo-endorphin antisera (unpublished data). Detailed immunohistochemical studies comparing the distribution of [Leu]enkephalin immunoreactivity with pro-dynorphin-related immunoreactivity may provide helpful information if (and in which brain regions) pro-dynorphin could serve as a precursor to [Leu]enkephalin.

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