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DYNORPHIN IN PRO-OPIOMELANOCORTIN NEURONS OF THE HYPOTHALAMIC ARCUATE NUCLEUS

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Abstract—Considerable evidence suggests that dynorphin participates in the regulation of energy balance. In this study, we have used immunohistochemistry to investigate in detail the cellular localization of pro-dynorphin (DYN) immunoreactive cell bodies in the mediobasal hypothalamus with special reference to neurons producing orexigenic or anorexigenic transmitters. In colchicine-treated rats, DYN immunoreactivity was demonstrated in many cell bodies of the arcuate nucleus (Arc). Double-labeling revealed that DYN immunoreactivity was present in approximately 30% of pro-opiomelanocortin (POMC) neurons in the ventrolateral Arc as shown by presence of α-melanocyte-stimulating hormone (α-MSH) and cocaine- and amphetamine-regulated transcript (CART). In contrast, DYN immunoreactivity was not demonstrated in agouti-related peptide (AgRP)- or neuropeptide Y (NPY) –containing neurons in the ventromedial aspect of the Arc. Dynorphin immunoreactivity was also colocalized with the vesicular acetylcholine (ACh) transporter (VAChT; a marker for cholinergic neurons) in the cell soma of Arc POMC neurons. Brainstem POMC neurons in the commissural part of the solitary tract nucleus (NTS) were devoid of DYN immunoreactivity, whereas DYN immunoreactivity was detected in a few NPY-containing NTS neurons and cholinergic DMX neurons. Our results showing presence of DYN together with α-MSH in a subpopulation of hypothalamic POMC neurons further point to the neurochemical heterogeneity of hypothalamic POMC neurons. The results suggest a role for DYN in control of energy balance by mediating the effect of peripheral hormones such as leptin and insulin.

Key words: body weight, food intake, arcuate nucleus, hypothalamus, melanocortin, opioid peptide.

The dynorphins are a family of opioid peptides derived from the *prodynorphin* gene (Civelli et al., 1985). Prodynorphin is the precursor of leucine-enkephalin, dynorphin A and B, and α-neoendorphin (Civelli et al., 1985; Day et al., 1998). Prodynorphin-derived peptides play a role in a large number of physiological functions, including e.g. reproduction, cardiovascular regulation, fluid homeostasis, thermoregulation and modulation of the hypothalamic–pituitary axis (Smith and Gallo, 1997; Naqvi et al., 1998; Zhang and Gallo, 2003; Goodman et al., 2004; see Bodnar and Klein, 2005). Extensive research has suggested that the central opioid system also is involved in the regulation of appetite and body weight (see Bodnar, 2004; Levine and Billington, 2004). Among the endogenous opioids, dynorphin, which binds to and activates the kappa receptor, has a role in the regulation of food intake. Intracerebral administration of dynorphin and kappa receptor agonists increases food intake (Morley and Levine, 1983; Silva et al., 2002). Fasting is associated with a significant increase in the expression pre-prodynorphin mRNA in areas of rat hypothalamus involved in energy homeostasis (Berman et al., 1997; Hervé and Fellmann, 1997). Furthermore, the obese and leptin receptor–deficient Zucker rat exhibits elevated central levels of dynorphin (Roane et al., 1988). Dynorphin expression has been detected within leptin-responsive neurons in the hypothalamic dorsomedial and arcuate nuclei (Elias et al., 2000). Moreover, the obesity syndrome of leptin-deficient *ob*/*ob* mice is associated with a fivefold increase in dynorphin peptide levels in the dorsomedial hypothalamus (Khawaja et al., 1991). Recently, it has also been reported that a targeted mutation of the mouse *dynorphin* gene reduces fat mass and increases weight loss during fasting (Sainsbury et al., 2007).

Since dynorphin is implicated in the regulation of food intake and energy balance, it is of interest that dynorphin is expressed in many neurons of the brainstem and hypothalamus (Nakao et al., 1981; Khachaturian et al., 1982), in particular the hypothalamic arcuate nucleus (Foradori et al., 2005). This nucleus, located in a region with a weak blood–brain barrier (BBB), has been shown to play important roles in the regulation of body weight and energy balance (see Morton et al., 2006; Meister, 2007). The arcuate nucleus contain two populations of neurons that exert opposite effect on feeding; neurons located in the ventromedial part of the nucleus produce the orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP) and promote feeding, whereas neurons located mainly in the ventrolateral part of the arcuate nucleus produce the anorexigenic peptides α-melanocyte-stimulating hormone (α-MSH), derived from the pro-opiomelanocortin (POMC) precursor, and cocaine- and amphetamine-regulated transcript (CART), which both inhibit food intake (see Cone, 2005; Morton et al., 2006; Meister, 2007).

In the present study, we have employed double- and triple-labeling immunohistochemistry to investigate in detail the distribution and colocalization of dynorphin-containing neurons within the rat mediobasal hypothalamus and brainstem. Our data show that dynorphin immunoreactivity is present in a large number of hypothalamic, but not brainstem, POMC neurons.

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Abbreviations: AgRP, agouti-related peptide; CART, cocaine- and amphetamine-regulated transcript; DMX, dorsal motor nucleus of the vagus nerve; NPY, neuropeptide Y; NTS, nucleus tractus solitarius; POMC, pro-opiomelanocortin; VAChT, vesicular acetylcholine transporter; α-MSH, α-melanocyte stimulating hormone.

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EXPERIMENTAL PROCEDURES

Male Sprague–Dawley rats of 150–200 g body weight (Scanbur-BK, Stockholm, Sweden) were used. All experiments were performed in accordance with guidelines from the Swedish National Board for Laboratory Animals, which conform to international guidelines on the ethical use of animals and were approved by the local ethical committee. Efforts were made to minimize the number of animals used and their suffering. Rats were anesthetized with a combination of ketamine (75 mg/kg)+medetomidin (1 mg/kg) i.p. and were treated with an injection (20 μl) of colchicine (6 mg/ml in 0.9% NaCl; Sigma-Aldrich, St. Louis, MO, USA) into the lateral ventricle 24 h before the animals were killed. Colchicine is known to arrest axonal transport, thereby increasing levels of transmitters, enzymes and peptides/proteins in the cell soma. Animals were anesthetized with sodium pentobarbital (40 mg/kg; i.p.) and perfused via the ascending aorta with 50 ml of Ca^{2+}-free Tyrode's solution (37 °C) followed by 50 ml of formalin-picric acid fixative (37 °C) (4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer, pH 6.9). Perfusions were thereafter continued for 6 min with ice-cold fixative as above. The brains were rapidly dissected out, post-fixed in the same fixative for 90 min and rinsed for at least 24 h in 0.1 M phosphate buffer (pH 7.4) containing 10% sucrose, 0.02% bacitracin (Sigma) and 0.01% sodium azide (Merck, Darmstadt, Germany). The brains were frozen and sections were cut (14 μm) in a cryostat (Microm HM 560, Walldorf, Germany). Sections were incubated overnight at 4 °C with primary antisera as follows: guinea-pig antiserum to pro-dynorphin (diluted 1:4000; Neuromics, Inc., Minneapolis, MN, USA), sheep antiserum to α-MSH (diluted 1:15,000; Chemicon International, Temecula, CA, USA), rabbit antiserum to CART (diluted 1:2,000; Phoenix Pharmaceuticals, Inc., Belmont, CA, USA), rabbit antiserum to AgRP (diluted 1:1500; Phoenix Pharmaceuticals), mouse monoclonal antibodies to NPY (diluted 1:400; Sigma-Aldrich) and rabbit antiserum to vesicular acetylcholine transporter (VAChT) (diluted 1:1000; Sigma-Aldrich). After rinsing with PBS, the sections were incubated with the following secondary antibodies: donkey anti–guinea pig–Cy3, donkey anti-rabbit-Cy5, donkey anti-rabbit-Cy3, donkey anti-goat-Cy2, donkey anti-goat-Cy5 and donkey anti-mouse-Cy2 (all diluted 1:250; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The sections were mounted in *N*,*N*-p-xylenebis (pyridinium bromide) (DPX; Fluka, Buchs, Switzerland) in order to prevent fading of immunofluorescence. Images were collected by confocal microscopy (BioRad confocal scanning system Radiance Plus, Hemel Hempstead, UK). The proportion of α-MSH/dynorphin immunoreactive cell bodies was quantified by counting the number of

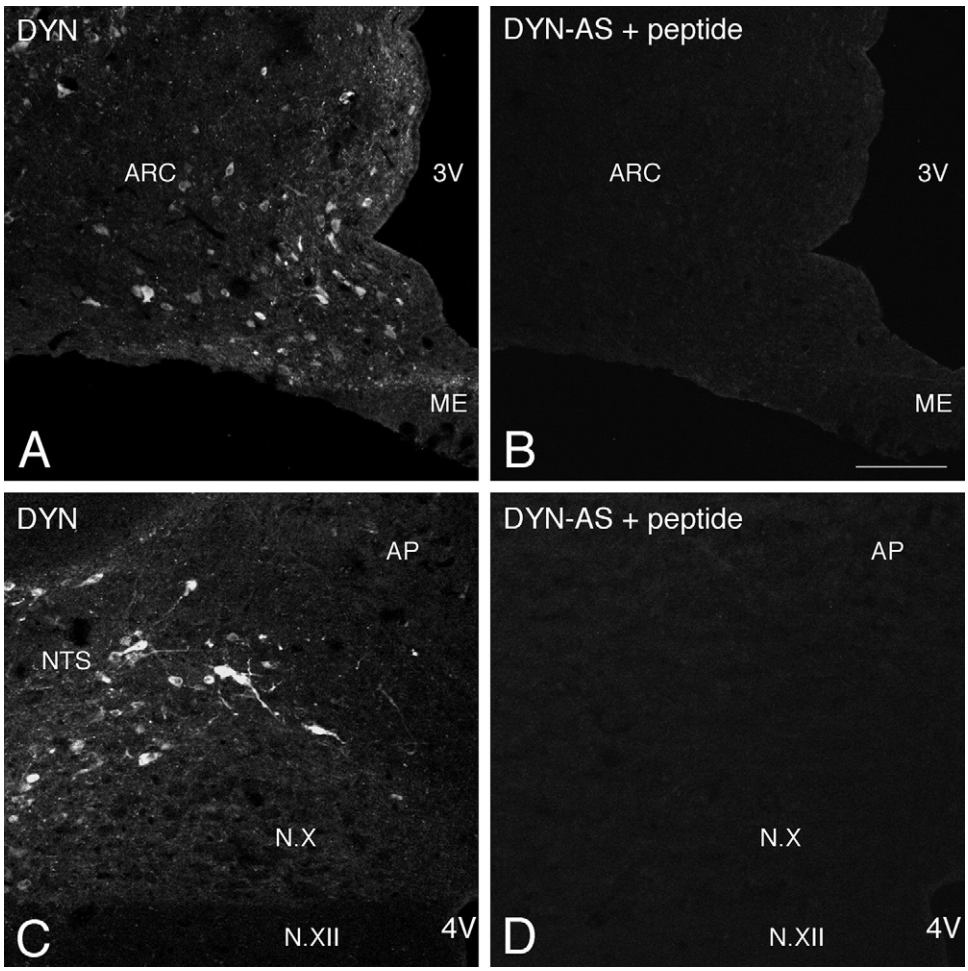

Fig. 1. (A–D) Immunofluorescence of adjacent sections of the arcuate nucleus (ARC) (A, B) and brainstem nucleus tractus solitarius (NTS) (C,D) after incubation with antiserum to pro-dynorphin (DYN) (A, C) and pro-DYN antiserum preabsorbed with pro-DYN blocking peptide at a concentration of 10^{-5}M (B, D). DYN-immunoreactive cell bodies are observed mainly in the ventrolateral part of ARC (A) and in the commissural part of the NTS (C). Preabsorption with pro-DYN blocking peptide results in a total disappearance of all immunoreactivity (B, D). AP=area postrema; ME=median eminence; N.X=nucleus of the vagus nerve; N.XII=nucleus of the hypoglossal nerve; 3V=third ventricle; 4V=fourth ventricle. Scale bar=100 μm.