Distribution of Amylin-Immunoreactive Neurons in the Monkey Hypothalamus and their Relationships with the Histaminergic System*

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Summary. Amylin (AMY) is a 37 amino acid peptide of pancreatic origin that has been localized in peripheral and central nervous structures. Both peripheral and central injection of the peptide causes various effects, including anorectic behavior in rats. Prompted by previous reports showing that the anorectic effect of AMY is mediated by histamine release, we immunohistochemically investigated possible relationships between these two systems at the light microscopical level. Monkey (Macaca fuscata japonica) hypothalamus specimens were submitted to immunohistochemical double staining procedures using AMY and histidine decarboxylase (HDC) antisera. AMY-immunoreactive neurons were found widely distributed in several nuclei of the monkey hypothalamus including the supraoptic, paraventricular, perifornical, periventricular, ventromedial, arcuate, and tuberomammillary nuclei. We detected AMY-immunoreactive nerve fibers throughout the hypothalamus, the median eminence and hypothalamus-neurohypophysial tract. Although AMY- and HDC-immunoreactive neuronal cell bodies occupied distinct hypothalamic zones, many HDC-immunoreactive cell bodies and dendrites, particularly those in the periventricular, arcuate, and rostral tuberomammillary regions, were surrounded by numerous AMY-immunoreactive nerve fiber varicosities. These findings demonstrate for the first time the presence of a discrete number of AMY-immunoreactive neurons in the monkey hypothalamus and add morphological support to the experimental data demonstrating that AMY probably exerts its influence on food intake via the histaminergic system.

Amylin (AMY), also named islet amyloid polypeptide (IAPP), is a 37 amino acid peptide first isolated from the amyloid deposits of human insulinomas and from pancreatic islets of non-insulin dependent (Type 2) diabetic patients (Westermark et al., 1986; Cooper et al., 1987). It belongs to a peptide superfamily, along with calcitonin, calcitonin gene-related peptide (CGRP) and adrenomedullin (references: Wimalawansa, 1997). AMY shares a 46% structural homology with CGRP, about 23% with adrenomedullin, and less than 20% with calcitonins. Although the gene for AMY in mammals is located on chromosome 12 and the gene for CGRP on chromosome 11, both peptides probably originate from a duplication of an ancestral gene. AMY is mainly produced by pancreatic B cells (Westermark et al., 1987; Johnson et al., 1988; Mulder et al., 1993) where it is co-stored in the same secretory vesicles as insulin (Fehmann et al., 1989; Lukinius et al., 1989; Kahn et al., 1990; Moore and Cooper, 1991; Stridberg et al., 1993). It is also produced by various endocrine/paracrine cells of the gut mucosa (Toshimori et al., 1990; Ohtsuka et al., 1993; Mulder et al., 1994, 1997; D’Este et al., 1996; Tingstedt et al., 1999). Because previous studies reported little AMY mRNA in the brain, it was usually indicated as an exclusively peripheral peptide that acted centrally because of its ability to cross the blood brain barrier (Banks et al., 1995). In later studies, however, a broad expression of AMY mRNA was found in the chicken brain (Fan et al., 1994) and rat sensory neurons (Ferrier et al., 1989; Nicholl et al., 1992; Mulder et al., 1995), AMY-immunoreactive nerve cell bodies and fibers were also described in the

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Fig. 1 a–d. Schematic drawings of four coronal sections of the monkey hypothalamus at different cranio-caudal levels (insets) indicating the main distribution patterns of AMY-immunoreactive nerve cell bodies (asterisks) and fibers (dashed lines). ch Optic chiasma, in infundibulum, ot optic tract, tm tuberomammillary area.

Fig. 2. AMY-immunoreactivities in monkey hypothalamus. a. Supraoptic nucleus containing numerous nerve cell bodies intensely immunostained for AMY. OT optic tract. b. Higher magnification of the boxed area in a. c. AMY-immunoreactive nerve cell bodies aligned in the retrochiasmatic portion of supraoptic nucleus. ch optic chiasma. d. Medium and small size AMY-immunoreactive neurons occupy the central portion of the paraventricular hypothalamic nucleus. e. Numerous AMY-immunoreactive nerve fibers grouped in the hypophysial stalk. f. AMY-immunoreactive nerve cell bodies and fibers in the ventromedial hypothalamic nucleus. Scale bar: a, d, f=200 μm; b=50 μm; c, e=65 μm
Fig. 2. Legend on the opposite page.
rat brain (SKOFITSCH et al., 1995) and by us in the rat brainstem (D’ESTE et al., 2000a) and, as a preliminary report, in some nuclei of the monkey hypothalamus (D’ESTE et al., 2000b). From a functional viewpoint, AMY is involved in several regulatory functions, including glucose homeostasis, the calcium/bone metabolism, memory modulation, and food intake (review: WIMALAWANSA, 1997). When injected either intraperitoneally (BALASUBRAMANIAM et al., 1991; LUTZ et al., 1994; ASARIAN et al., 1998) or by an intrahypothalamic route (CHANGE et al., 1991) or intracerebroventricularly (LUTZ et al., 1998b) in rats, AMY induces anorectic behavior. This effect is mediated by central histamine release (LUTZ et al., 1996) and unaffected by the lesion or interruption of peripheral inputs (LUTZ et al., 1995; 1998a, c).

The aim of the present study was to complete our early preliminary study on the presence of AMY-immunoreactive neurons in the monkey hypothalamus, by investigating their distribution patterns and — by double immunostaining procedures — their possible relationships with the histaminergic neurons.

MATERIALS AND METHODS

Animals

The brains of six adult male monkeys (Macaca fascata japonica) were used. The animals were housed in the animal house of the Shiga University of Medical Sciences (Otsu, Japan) under optimal environmental conditions with a supply of food and water ad libitum. The animals were treated according to the directions of the local ethical committee. Under deep anesthesia with pentobarbital, the animals were perfused via the ascending aorta with 3 liters of 0.1 M phosphate buffered saline, pH 7.4 (PBS), containing heparin, followed by 5 liters of a cold fixative mixture containing 4% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4 (PAF), containing 35% glutaraldehyde. Each brain was coronally sliced into several specimens that were postfixed in the same cold PAF mixture without glutaraldehyde for a further 48–76 h. The slices corresponding to the hypothalamic areas were frozen and cut by a cryostat into 20-μm thick sections that were stored as free-floating sections at 4°C in 15% sucrose in PB till use.

Immunohistochemistry

To improve tissue permeability to proteins, free-floating sections were preincubated in PBS containing 0.3% Triton X-100 (PBST) for at least 4 days at 4°C. To block the endogenous peroxidase activity, sections were incubated for 30 min at room temperature in PBST containing 0.1% sodium azide and 0.5% H2O2. To minimize non-specific binding, the sections were then incubated for 30 min at room temperature in 2% normal goat serum in PBST containing 1% bovine serum albumin (BSA, Sigma, St Louis, USA) (PBST/BSA). The sections were then incubated for 3 days at 4°C with a polyclonal antiserum raised in a rabbit against the carboxy amided terminal tripeptide (25–37) of human AMY (AMY 10F, produced and characterized by S.J. WIMALAWANSA) diluted 1:40,000 in PBST/BSA. This antiserum to AMY is devoid of any cross-reactivity to CGRP, adrenomedullin, calcitonin, or other known biologically active peptides. Sections were then incubated for 2 h at room temperature with a biotinylated goat anti-rabbit IgG (diluted 1:1,000; Vector Laboratories, Burlingame, CA) and for 1 h at room temperature with streptavidin-peroxidase complex (diluted 1:2,000, ABC Elite, Vector). After each step, sections were rinsed three times for 10 min with PBST. The peroxidase activity was visualized by reaction for 3 min at room temperature with a solution containing 0.04% 3-3′diaminobenzidine tetrahydrochloride (DAB, Fluka, Buchs, Switzerland), 0.4% nickel ammonium sulfate, and 0.003% H2O2 in 0.05 M Tris-HCl buffer, pH 7.6, giving a dark blue granular precipitate. The stained sections were mounted on glass slides, dehydrated, cleared and coverslipped with Permount (Fisher Chemicals, Fair Lawn, New Jersey, USA).

To demonstrate the histaminergic neurons, a polyclonal antiserum — raised in a guinea pig — to histidine decarboxylase (HDC, Eurolab, Malmö, Sweden), diluted 1:10,000, was used either on adjacent sections or on the same sections previously immunostained for AMY. In these sections, a biotinylated donkey anti-guinea pig IgG (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA), diluted 1:1,000, was used as a secondary antiserum, and

Fig. 3. Amy-immunoreactivities in monkey hypothalamus. a–c and e. Sections at various levels of the hypothalamic periventricular area; it contains several nerve cell bodies that vary in size and shape, and fibers. b. A higher magnification of a. The asterisks in a and b label same area. v. Third ventricle. d. AMY-immunoreactive nerve cell bodies and fibers in the arcuate nucleus (the ependymal wall is at the bottom of the figure). f. An immunopositive magnocellular neuron in the perifornical portion of the paraventricular nucleus. The arrows follow its positive axon projecting to the wall of a blood vessel. Scale bar: a, c, e=200 μm; b=30 μm; d=80 μm; f=65 μm.
DAB alone, producing a brown precipitate, or the Novared kit (Vector), producing a brilliant red precipitate, were used in the double immunostaining procedures.

Sections were then observed with a photomicroscope (Olympus AX70 Provis, Olympus Italia, Milan, Italy) equipped with a cooled CCD color digital camera (Spot RT 220-3; Diagnostic Instruments Inc., Sterling Heights, USA) that allowed direct acquisition of desired images in a computer, to then assemble and label them with the aid of a software program (Adobe Photoshop 5.0 LE).

Although the correct use of good histamine antisera necessitates the perfusion of animals with carbodiimide (Panula et al., 1988, 1990), in preliminary trials this procedure proved unsuitable for AMY 10F antisemur activity. It also hindered any meaningful comparison of the two immunohistochemical activities. Instead of labeling histaminergic neurons with a histamine antiserum, we therefore used the HDC antiserum. The results matched those of preliminary trials using the histamine antiserum in carbodiimide-perfused samples, thus giving reliable information on the presence and distribution patterns of histaminergic neurons.

Controls included the omission of primary antibodies or their substitution with either PBST/BSA or normal rabbit (or guinea pig) serum or with the primary antiserum preincubated, overnight at 4°C, with an excess of synthetic AMY (15–25 μg/ml, Peninsula, St Helens, UK). All control sections were devoid of immunostaining. In contrast, preincubating the AMY-antiserum with up to 100 μg/ml of any other peptides, including CGRP, adrenomedullin and calcitonin (Peninsula), did not affect the immunoreaction.

RESULTS

AMY-immunoreactive nerve cell bodies were found in discrete areas of the monkey hypothalamus (Fig. 1). Numerous magnocellular neurons within all the subnuclear subdivisions of the supraoptic nucleus displayed AMY-immunoreactivity (Fig. 2 a–c), as did numerous medium size neurons in the paraventricular nucleus (Fig. 2 d, f) and in the perifornical (Fig. 3 f) and neighboring accessory nuclei. Positive medium and small size neurons were also shown in the hypothalamic periventricular (Figs. 3 a–c, e, 4), ventromedial (Fig. 2 f), arcuate (Fig. 3 d) and tuberomammillary nuclei (Fig. 4). Numerous immunopositive nerve fibers were shown throughout the hypothalamus, mainly along the hypothalamo-neurohypophysial tract (Figs. 2 e, 4 e), the deep layer of the median eminence, and the tuberomammillary region. Many AMY-immunoreactive nerve fibers seemed to reach blood vessel walls (Fig. 4 a, e). In sections double immunostained for AMY and HDC, hardly any AMY-immunoreactive neurons colocalized HDC, but often they layed in close contact with HDC-immunoreactive neurons (Fig. 4). Several AMY-immunoreactive nerve fiber varicosities reached the surface of numerous HDC-immunoreactive cell bodies and dendrites in the periventricular and rostral tuberomammillary regions (Fig. 4 f, g). In these regions, small to medium size HDC-immunoreactive cell bodies were surrounded by a more or less dense network of AMY-immunoreactive nerve fibers (Fig. 4).

DISCUSSION

Using a polyclonal antiserum raised against the COOH-terminal tridecapeptide of human AMY, we have been able to provide new evidence for a discrete number of AMY-immunoreactive neurons in the monkey hypothalamus. The complete lack of immunostaining under control conditions, in particular after a preabsorption test with synthetic AMY, and the unaffected immunostaining after the preabsorption test with all the other peptides, indicated the specificity of our results. The AMY-immunoreactive neurons were demonstrated to be distributed: a) within the hypothalamic magnocellular nuclei and the hypothalamo-hypophysial system; and b) in some parvocellular regions close to the lumen of the third ventricle, i.e., the periventricular nucleus, the arcuate nucleus, the ventromedial nucleus and the rostral tuberomammillary region, where the AMY-immunoreactive nerve fibers often lay close to blood vessel walls.

All these hypothalamic nuclei are involved in the regulation of a variety of visceral activities. Numerous experiments in mammals have already demonstrated AMY’s biological activities, including glucose homeostasis, the calcium/bone metabolism, memory modulation, and food intake. Our immunohistochemical results agree well with the reported anorectic behavior induced by AMY (Balasubramaniam et al., 1991; Chance et al., 1991; Lutz et al., 1994, 1998b; Asarian et al., 1998). They also support experimental evidence attributing this effect to histamine release (Lutz et al., 1996). Many hypothalamic sites we detected AMY-immunoreactive neurons — including the ventromedial, arcuate, periventricular and tuberomammillary nuclei — are closely involved in regulating food intake. In these
Fig. 4 a–e. Monkey hypothalamus double immunostained for AMY (DAB/Nickel = dark blue) and HDC (DAB alone = yellow-brown). Several examples of AMY-immunoreactive cell bodies (arrows) and fibers in rostral tuberomammillary (a), periventricular (b–d) and infundibular (e) regions lying close to HDC-immunoreactive neurons (arrowheads). f–g. Rostral tuberomammillary region double immunostained for AMY (DAB/nickel = dark blue) and HDC (Novared = red). Arrows follow some AMY-immunoreactive fibres contacting several HDC-immunoreactive elements (arrowheads). v Blood vessels. Scale bar: a–f = 25 μm; g = 10 μm.
hypothalamic regions, the most distinctive finding was the intimate contact between numerous AMY-immunoreactive nerve fibers and HDC-immunoreactive neurons.

In conclusion, ample evidence from pharmacological and neuroanatomical experiments shows that several central peptides are directly or indirectly involved in the regulation of food intake, among them cholecystokinin, bombesins, glucagon, neuropeptide Y, corticotrophic releasing hormone, melanocortins, orexins, insulin, and leptin (review in Woods et al., 1998). The present immunohistochemical results strongly suggest that this list should also include AMY. Our findings add morphological support to the experimental data demonstrating that AMY probably exerts its influence on food intake via the histaminergic system.

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