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Localization of neuropeptide Y Y1 receptors in cerebral blood vessels

(arterioles/brain circulation/coexistence/immunohistochemistry/vascular smooth muscle)

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ABSTRACT **The localization of neuropeptide Y (NPY) Y1 receptor (R) -like immunoreactivity (LI) has been studied in cerebral arteries and arterioles of the rat by immunohistochemistry using fluorescence, confocal, and electron microscopy. High levels of Y1-R-LI were observed in smooth muscle cells (SMCs) in the small arterioles of the pial arterial network, especially on the basal surface of the brain, and low levels in the major basal cerebral arteries. The levels of Y1-R-LI varied strongly between adjacent SMCs. Y1-R-LI was associated with small endocytosis vesicles, mainly on the outer surface of the SMCs, but also on their endothelial side and often laterally at the interface between two SMCs. NPY-immunoreactive (Ir) nerve fibers could not be detected in association with the Y1-R-rich small arterioles but only around arteries with low Y1-R levels. A dense network of central NPY-Ir nerve fibers in the superficial layers of the brain was lying close to the strongly Y1-R-Ir small arterioles. The results indicate that NPY has a profound effect on small arterioles of the brain acting on Y1-Rs, both on the peripheral and luminal side of the SMCs. However, the source of the endogenous ligand, NPY, remains unclear. NPY released from central neurons may play a role, in addition to blood-borne NPY.**

The extensive innervation of brain vessels by sympathetic noradrenergic nerves was described by Nielsen and Owman (1; also see ref. 2). Neuropeptide Y (NPY) (3) coexists with noradrenaline (NA) in sympathetic neurons in general (4) and also in NA nerves around brain vessels (5, 6). NPY causes vasoconstriction (7), and potentiates NA-induced vasoconstriction (8), effects that also can be seen on brain vessels (5). NPY causes a reduction in cerebral blood flow after injection into the internal carotid artery (6). In addition, NPY is present in parasympathetic cholinergic neurons and cerebrovascular nerves (9, 10).

Two types of NPY receptors were identified on pharmacological grounds as post- and prejunctional NPY receptors, referred to as Y1 and Y2 receptors (Y1- and Y2-Rs), respectively (11). The Y1-R was first cloned (12–14). Pharmacological and physiological studies have shown that the constricting effect of NPY on blood vessels mainly is mediated by Y1-Rs (15, 16), and this is also true for cerebral vessels (17, 18). The cellular localization of Y1-R binding sites has been observed on vascular smooth muscle cells (SMCs) with autoradiographic ligand binding methodology (19–21), and Y1-R mRNA has been demonstrated in vascular SMCs (13, 14) and with *in situ* hybridization in arterioles and small arteries in testis, ovary, and oviduct (22). Y1-Rs and Y1-R mRNA have also been found in cerebral arteries (17, 18). Recently, the localization of the Y1-R protein has been shown in sensory ganglia, spinal cord, and the brain (23), and in arterial SMCs in the testis (22) with an antiserum raised against the Y1-R.

In the present study we have used an affinity-purified antiserum directed against a C-terminal peptide fragment of the Y1-R to study rat cerebral blood vessels with immunohistochemistry in fluorescence, confocal, and electron microscopes. The localization of Y1-R-like immunoreactivity (LI) and the relationship between the receptor protein and NPY-immunoreactive (Ir) nerve fibers are described.

MATERIALS AND METHODS

Immunofluorescence Studies. Eleven male Sprague–Dawley rats (body weight 200–250 g; B&K, Stockholm) were anesthetized, perfused, and postfixed with 4% paraformaldehyde and 0.2% picric acid in phosphate buffer and cut into 50- or 100-μm-thick sections with a Vibratome. The superficial cerebral arteries on the basal surface were isolated from four rat brains. From three other brains, a slice with the superficial layers was peeled out with a blade. Coronal sections (14 μm thick) were cut from four brains. The tissues were processed according to tyramide signal amplification (TSA)-indirect technology using a commercial kit (TSA-indirect kit; DuPont/NEN) and a rabbit antiserum against a C-terminal fragment (amino acids 355–382) of the Y1 receptor (1:8,000) (H.W. and J.W., unpublished data). Streptavidin fluorescein isothiocyanate-conjugate (1:500) (kit) was used for detection. Using a similar procedure, some of the tissues were incubated in a mixture of rabbit Y1-R antiserum (as above) and a mouse mAb against NPY (1:2,000) (24), whereby lissamine rhodamine-conjugated donkey anti-mouse IgG (1:80; Jackson ImmunoResearch) (1:500; kit) was used to visualize the NPY fibers. The sections were examined in a Nikon Microphot-FX microscope and in a Bio-Rad MRC-600 laser scanning confocal microscope.

Electron Microscopy. Four rats (as above) were perfused and postfixed with 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.02% picric acid in phosphate buffer. Vibratome sections (300 μm-thick) were processed according to the immunoperoxidase method using the Vector ABC kit (Vector Laboratories) and the Y1-R antiserum (1:8,000) (as above). After dehydration, the sections were embedded in Epon 812, cut on an LKB III ultratome and, without counter-staining, examined in a JEOL-1200 electron microscope.

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Abbreviations: Ir, immunoreactive; LI, like immunoreactivity; NPY, neuropeptide tyrosine Y (tyrosine); NPY$^+$, NPY-positive; SMC, smooth muscle cells; Y1-R, type 1 NPY receptor.

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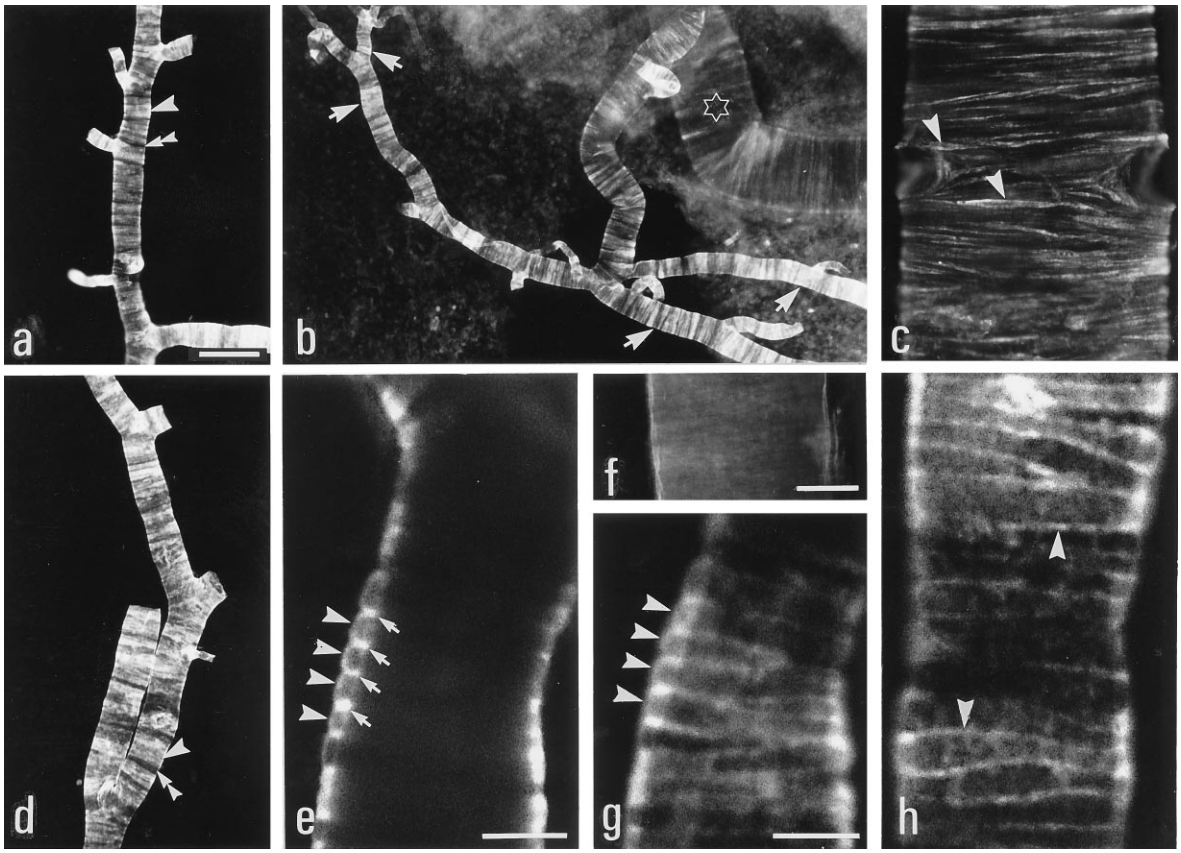

FIG. 1. Immunofluorescence (*a–d* and *f*) and confocal (*e*, *g*, and *h*) micrographs of arterioles and arteries isolated from the basal surface of rat brain. (*a*, *b*, and *d*) Many strong Y1-R$^+$, circularly oriented SMCs (arrowheads) are seen along the small arteries with intermingled Y1-R-negative cells (double arrowheads). The number of Y1-R-Ir SMCs is high and the intensity of labeling is relatively stronger along the small branches of the first branch of the middle cerebral artery (star). (*c*) Weak Y1-R-LI is observed in some circularly oriented SMCs (arrowheads) of the basilar artery. (*f*) Incubation with pre-absorbed antibody completely abolishes immunostaining. (*e*, *g*, and *h*) Confocal micrographs of Y1-R-LI in SMCs in isolated arteriole scanned at different levels. Y1-R-LI is seen on the perivascular side (arrowheads) with the highest intensity along the lateral portion (arrows) of SMCs (*e*). The pattern of labeling extends along the surface of SMCs (arrowheads) (*g*). Y1-R-LI is seen on the plasmalemma of entire SMCs (arrowheads) (*h*). [Bars = 100 μm (*a–d*, *f*) and 5 μm (*e*, *g*, and *h*).]

Controls. The specificity of the antisera was tested by absorption with, respectively, synthetic Y1 receptor peptide and NPY peptide, both at 10^{-6} M.

Quantification. The intensity of immunostaining of Y1-R-LI in the isolated arteries and arterioles was measured on a Macintosh IIx-based imaging system connected to a Nikon Mikrophot-FX microscope using NIH-image software (courtesy W. Rasband, National Institute of Mental Health). An area over the blood vessel of at least 600 μm^2 was included in each measurement. The diameter of the arteries and arterioles was measured at the same time. The intensity values given represent the grey levels of immunolabeled blood vessels minus the grey levels of blood vessels with a corresponding diameter taken from experiments with incubation with control serum. In the histogram the diameters of the blood vessels are plotted against grey levels (fluorescence intensity) (mean of grey level ± SEM) and include major arteries (≈400–300 μm diameter), their primary branches (170–65 μm), and arterioles (≈64–10 μm).

RESULTS

Isolated Basal Cerebral Arteries and Arterioles. Y1-R-LI was observed in SMCs both in superficial cerebral arteries (diameter, 100–400 μm) and arterioles (diameter, below 100 μm) taken from the basal surface of the brain (Fig. 1*a–d*) with a stronger immunostaining in the arterioles, especially the ones with a diameter of 15–30 μm (Figs. 1 *a*, *b*, and *d*; and 2), than in the arteries (Figs. 1 *b* and *c*; and 2). A moderately strong immunoreactivity was seen in the SMCs of the basilar artery (380–400 μm in diameter) (Fig. 1*c*), the artery of the circle of Willis (340–360 μm), the middle cerebral artery (300–320 μm), the anterior and posterior cerebral arteries (320–340 μm in diameter), the corticoamygdaloid artery, and their large primary branches (65–170 μm in diameter). Strongly Y1-R-positive (Y1-R$^+$) and Y1-R-negative cells were seen in the arterioles at irregular intervals (Fig. 1 *a*, *b*, and *d*). In the confocal microscope Y1-R-LI was mainly detected on the

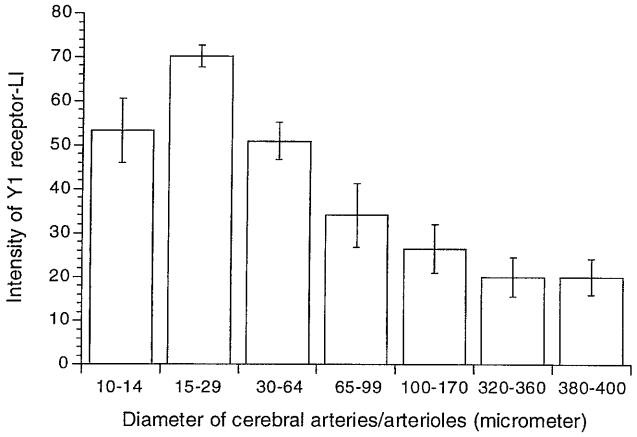

FIG. 2. Histogram plotting the intensity of Y1-R-LI in basal arteries and arterioles against vessel diameter.

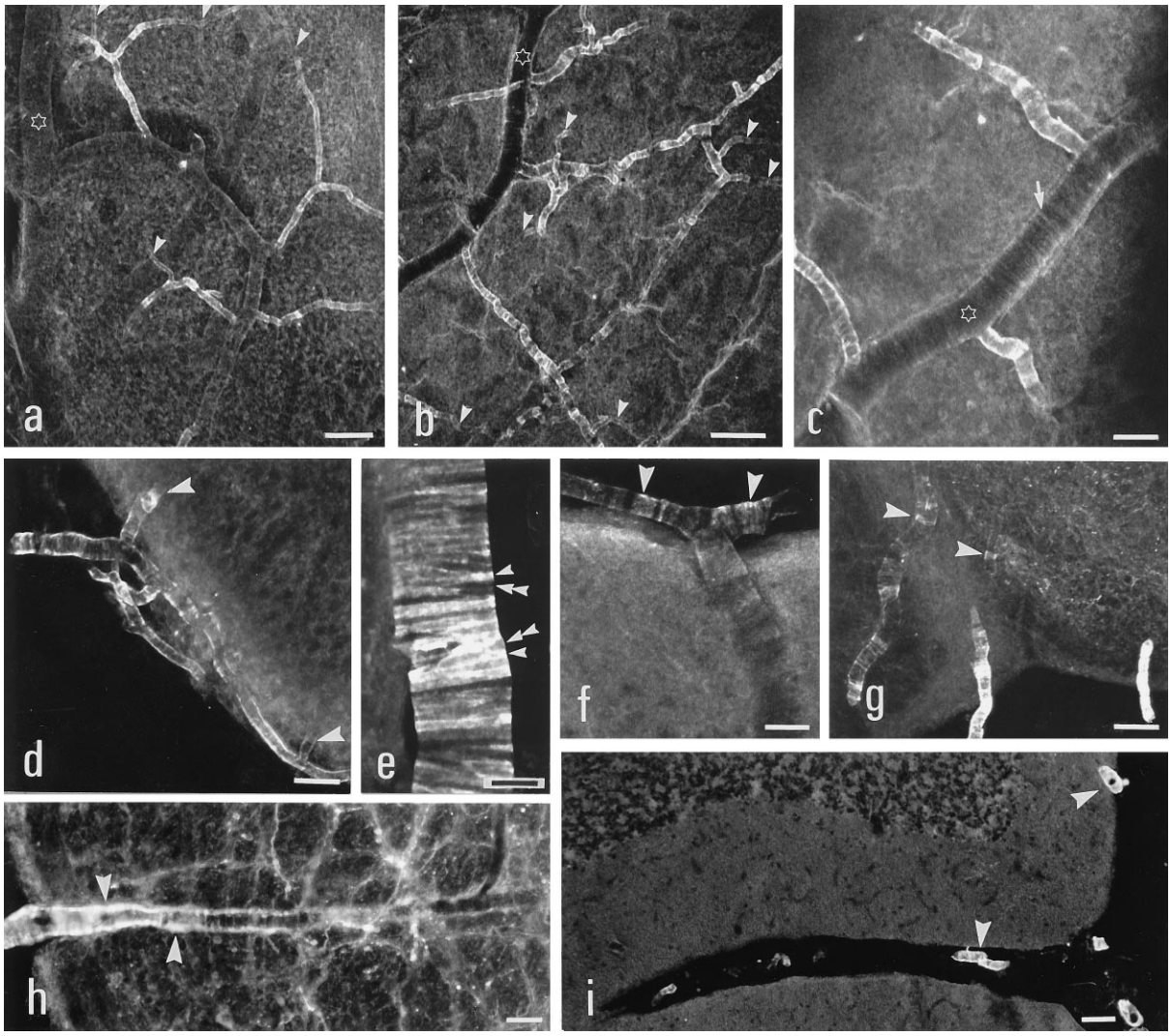

FIG. 3. Confocal (*a–g*) and immunofluorescence (*h* and *i*) micrographs of basal (*a–e*, *g* and *h*), dorsal (*f*), and cerebellar (*i*) vessels. (*a* and *b*) Strong Y1-R-LI is located on the small arterioles given off by second- (stars) or third-order branches of the middle cerebral artery. Y1-R-LI decreases in the smallest arterioles (arrowheads). (*c*) Weak Y1-R-LI in SMCs (arrow) on a second branch artery (stars) of the middle cerebral artery. (*d*) Y1-R-LI disappears when the arteriole has entered hypothalamus (arrowheads). (*e*) Strongly Y1-R-Ir SMCs (arrowheads) with interspersed negative cells (double arrowheads) in an arteriole. (*f*) Weak Y1-R^+ SMCs (arrowheads) in an arteriole on the dorsal cerebral cortex. (*g*) Y1-R-LI is seen within the brain along arterioles (arrowheads) around the interpeduncular nucleus. (*h*) Y1-R-LI (arrowheads) is present on an arteriole at some distance from the medullary surface. (*i*) Small arterioles (arrowheads) on the cerebellar surface are Y1-R^+. [Bars = 100 μm (*a*, and *b*), 50 μm (*d*, *g*, and *i*) and 25 μm (*c*, *e*, *f*, and *h*).]

plasmalemma facing the perivascular space and with a high intensity along the lateral portion of the SMCs (Fig. 1 *e*, *g*, and *h*).

Superficial Arteries and Arterioles *in Situ*. Confocal (Fig. 3 *a–g*) and fluorescence microscopical (Fig. 3 h and i) analysis of brain slices/sections confirmed a strong Y1-R-LI in the arterioles with a small diameter (15–30 μm) on the basal brain surface (Fig. 3*a–e* and *g*), mainly representing second or third order branches of the middle (Fig. 3 *a* and *b*), the anterior and posterior cerebral arteries, the corticoamygdaloid artery, and small branches directly given off by the basal artery. Y1-R-negative SMCs were interspersed with Y1-R^+ ones (Fig. 3*e*). The intensity of immunostaining decreased as the arterioles became smaller (diameter below 10 μm) (Fig. 3 *a–c*). A weak Y1-R-LI was detected along the second-order branches of the arteries (Fig. 3*c*). Y1-R-LI was very weak in the major arteries. On the lateral and dorsal (Fig. 3*f*) surface of the brain, including cerebellum (Fig. 3*i*), the staining intensity was stronger in arterioles (Fig. 3*f*) than in small arteries. In general Y1-R-LI became very weak after the arterioles had entered the superficial layers of the brain (Fig. 3 *d* and *f*), but in several areas, such as most cortical and some diencephalic areas, the interpeduncular nucleus (Fig. 3*g*), medulla oblongata (Fig. 3*h*) and the spinal cord, arterioles with Y1-R^+ SMCs could be followed into the brain. Y1-R^+ vessels were observed in all layers in the olfactory bulb, cerebellum, and the hippocampal formation .

Ultrastructural Localization. In general, the Y1-R^+ SMCs had a higher diffuse electron-density than the negative ones with a prominent, intense, punctuate labeling near the plasmalemma (Fig. 4 *a–c*). Y1-R-LI was highly concentrated at some sites of plasmalemma with small endocytosis vesicles (50–65 nm in diameter) (Fig. 4 *e–i*). A strong labeling for Y1-R-LI was associated with the membrane and sometimes the matrix of vesicles (Fig. 4 *f–i*). These sites were mainly located on the plasmalemma facing the perivascular space (Fig. 4 *d, e, g*, and *i*) but also on the endothelial side (Fig. 4 *a–c*, *e*, and *f*). A particularly intense Y1-R labeling of many endocytotic small vesicles was located in the lateral portion of one or both SMCs, where they interlaced (Fig. 4 *a–c* and *h*). Scattered labeling was occasionally seen in cells peripheral to the SMCs (Fig. 4*a*).

Double-Immunolabeling. In the confocal microscope, a network of NPY-Ir nerve fibers was observed around the

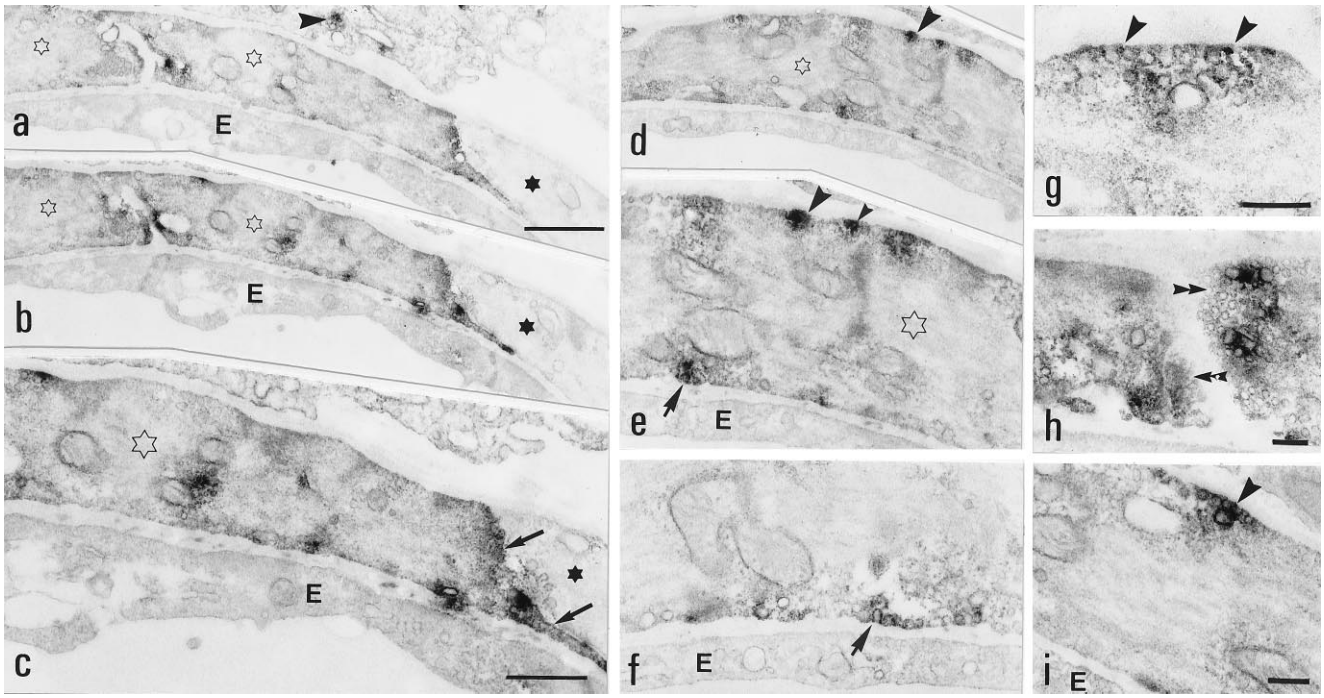

FIG. 4. Electron micrographs of arterioles. (*a–c*) A Y1-R$^+$ (open stars) and a Y1-R-negative (stars) SMC in two adjacent sections. The Y1-R-LI is distributed in the cytoplasm and more intensely at lateral portions (arrows) of the SMC. The scattered labeling (arrowhead) in the cell peripheral to the SMC is unspecific. (*c*) Higher magnification from *b*. (*d–i*) Several intensely Y1-R-Ir sites are seen along the plasmalemma facing the perivascular space (arrowheads), the endothelium (arrows), or laterally toward another SMC (double arrowheads). Note close association with vesicles in many cases. E, endothelium. [Bars = 1 μm (*a*, *b*, and *d*), 500 nm (*c* and *e*), 250 nm (*f* and *i*; *g* and *h*).]

basilar and posterior communicating arteries, the circle of Willis, the anterior cerebral, middle cerebral, posterior cerebral, superior cerebral, and inferior anterior cerebellar (Fig. 5*a*) arteries and their first branches and initial portion of the second branches. No autonomic NPY-Ir nerve fibers were associated with the small arterioles containing Y1-R-LI (Fig. 5 *a* and *b*). However, strongly Y1-R$^+$ arterioles were, in most cases, closely associated with the networks of central NPY-Ir nerve fibers in the superficial layer of the brain (Fig. 5 *c–d*).

Controls. The immunoreactive structures described above were not observed after incubation with control sera (Fig. 1*f*) with the exception of labeling in cells peripheral to SMCs.

DISCUSSION

NPY receptors are involved in the regulation of central blood circulation (2). Here, we provide direct morphological evidence that the Y1-R is a postsynaptic or postjunctional receptor present in many SMCs in arteries and especially arterioles. However, there are distinct variations with regard to size of vessels, and location, and levels of the Y1-Rs. Thus, in general, Y1-R$^+$ vessels are more frequent on the basal brain surface as compared with dorsal and lateral aspects. Moreover, arterioles (diameter, 10–65 μm) have considerably higher receptor concentrations than arteries. The arterioles are mainly third- or fourth-order branches of the major cerebral arteries as well as arterioles directly derived from the basilar artery. Also, along a single arteriole, the Y1-R concentrations are very high in some SMCs, whereas adjacent cells apparently lack the receptor. Often, Y1-R-LI is not detectable after the arterioles have entered into the brain, but in several brain regions Y1-R$^+$ arterioles can be seen deeper in the brain, and for example, in the olfactory bulb strongly labeled arterioles are seen in all cell layers. Y1-R-LI was only occasionally found in arteries within the brain. Y1-R-LI markedly decreased in blood vessels with a diameter below 10 μm, presumably due to a lack of SMCs in the arterial capillaries. These results are in good agreement with studies showing that NPY only has a weak contractile effect on the rat basilar artery *in vitro* (25), whereas arterioles are more sensitive (ref. 26; see also ref. 2).

The Y1-R-LI is associated with small smooth vesicles along the plasmalemma of SMCs, predominantly toward the perivascular space, in agreement with the finding that perivascular application of NPY produces a vasomotor response (27). Y1-R-LI is also associated with small, smooth vesicles along the lateral short ends where two SMCs oppose each other, suggesting that this area plays an important role in the life cycle of the receptor, perhaps as a site of processing and incorporation of the receptor into the membrane or endocytosis. However,Y1-Rs are found also toward the endothelial space, suggesting access of NPY from the luminal side via the periendothelial space. In fact, Allen *et al.* (6) demonstrated that a bolus injection of NPY into the carotid artery produced a profound and long-lasting reduction in cortical blood flow.

The localization of Y1-Rs along invaginations of the plasma membrane and to vesicles is in agreement with findings that other G protein-coupled, seven-transmembrane receptors, such as the β-adrenergic receptor, are associated with non-coated vesicles during the internalization process (28, 29) and with the concept (30) that one of several functions of plasmalemmal caveolae is to process hormonal (transmitter) stimuli, thus representing "message centers" for the cell.

Strongly NPY-Ir nerve fibers were found around the major cerebral arteries as shown in many previous studies (2, 5, 6). Although these arteries only contained low Y1-R levels, NPY released from these autonomic fibers could still directly regulate tonus, in agreement with the general view on the role of NPY in vascular control (15, 16). It is possible that the apparently low levels of Y1-Rs in these arteries are due to insensitivity of our immunohistochemical technique. The SMCs could also express other types of NPY receptors.

More intriguing however, are the abundant Y1-Rs in the SMCs of small arterioles, here shown to lack NPY innervation, because these arterioles may mediate major effects of NPY on

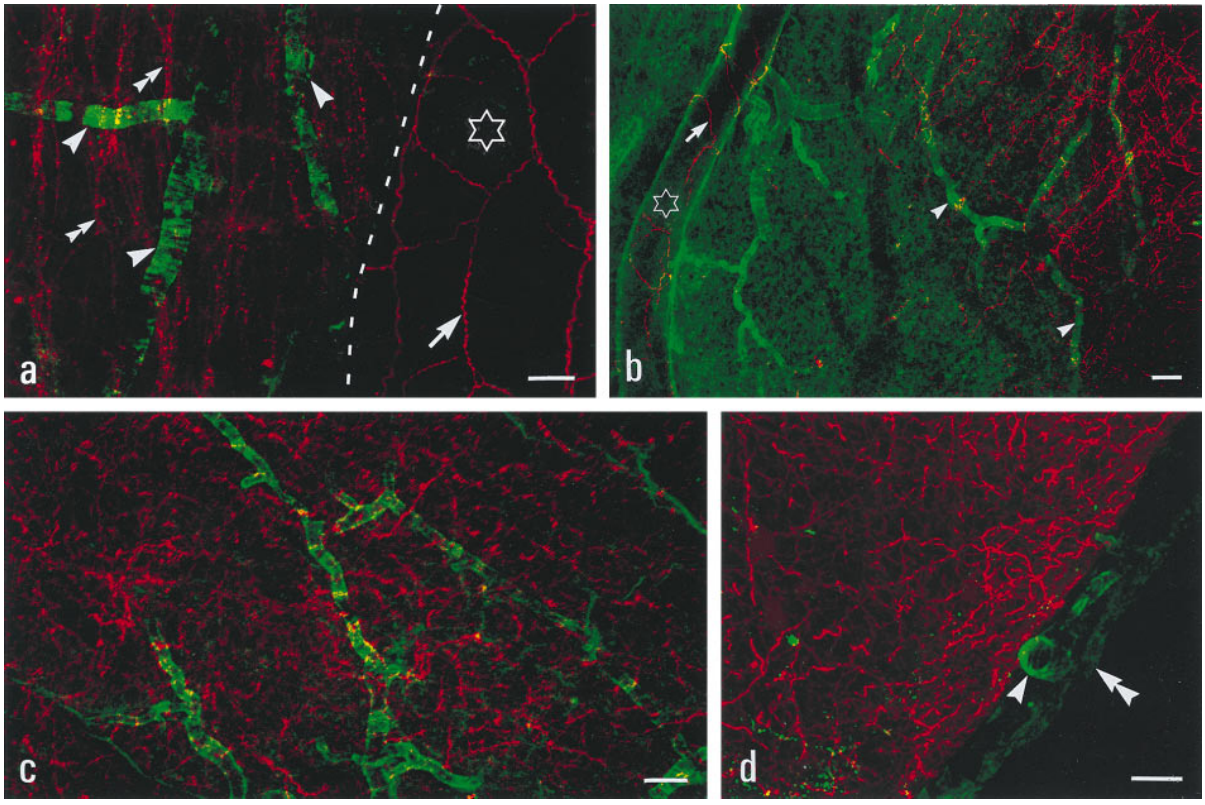

FIG. 5. Confocal micrographs showing Y1-R (green) and NPY (red) -LIs. (*a*) NPY^+ nerve fibers (arrows) but no Y1-R-LI are seen around the inferior anterior cerebral artery (stars). Strong Y1-R-LI around some small arterioles directly derived from the basilar artery, but no NPY^+ nerve fibers are seen along these arterioles. Double arrowheads pointing to central NPY^+ nerve fibers. (*b*) Strongly Y1-R-Ir arterioles lacking NPY^+ nerve fibers (arrowheads). NPY^+ fibers are seen around a first branch (stars) of the middle cerebral artery. (*c*) A dense network of central NPY^+ nerve fibers intermingles with strong $Y1\text{-}R^+$ arterioles (basal cortex). (*d*) A $Y1\text{-}R^+$ superficial arteriole (arrowheads) adjacent to NPY^+ nerve fibers in the basal hypothalamus. The pia mater (double arrowheads) is not labeled. [Bars = 25 μm (*a*, *b*), 50 μm (*c*), and 100 μm (*d*).]

the blood flow in the capillary bed in the brain. An important question, therefore, is the origin of the NPY activating these receptors. Blood-borne NPY could be released from the NPY fibers around larger vessels close to or at some distance from the brain, especially under certain situations, such as physical exercise (31), shock (32), and stress (33). Blood-borne NPY could also originate from the adrenal gland (4) as well as, in the rat, from platelets (34). However, it cannot be excluded that NPY released from terminals of the numerous NPY-containing cortical interneurons (35) can act on arteriolar Y1-Rs, as suggested here by the close association of such central nerve fibers with Y1-R-positive arterioles. Loesch *et al.* (36) have provided evidence that NPY-LI is present in endothelial cells, representing another source of endogenous ligand. Finally, it should be pointed out that NPY may have other functions than controlling blood vessel tone, including trophic actions on SMCs (37, 38) that perhaps could be mediated via Y1-Rs.

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