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TH-, NPY-, SP-, and CGRP-immunoreactive nerves in interscapular brown adipose tissue of adult rats acclimated at different temperatures: an immunohistochemical study

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Summary

Interscapular brown adipose tissue (IBAT), a site of nonshivering thermogenesis in mammals, is neurally controlled. The co-existence of sympathetic and peptidergic innervation has been demonstrated in different brown adipose depots. We studied the morphological profile of IBAT innervation and tested by immunohistochemical methods whether cold and warm stimulation are accompanied by modifications in the density of parenchymal noradrenergic nerve fibers. We also studied the immunoreactivity of afferent fibers—which contain calcitonin gene-related peptide (CGRP) and substance P (SP)—in different functional conditions. IBAT was obtained from adult rats (6 weeks old) acclimated at different temperatures (4°, 20°, and 28°C). Tissue activity was evaluated by studying the immunolocalization of uncoupling protein (UCP-1), a specific marker of brown adipose tissue. Noradrenergic and peptidergic innervation were seen to arise from morphologically different nerves. Fibers staining for tyrosine hydroxylase (TH) were thin, unmyelinated hilar nerves, and CGRP- and SP-positive fibers were in thick nerves containing both myelinated and unmyelinated fibers. Under cold stimulation, noradrenergic neurons produce greater amounts of TH, and their axons branch, resulting in increased parenchymal nerve fibers density. Neuropeptide Y (NPY) probably co-localizes with TH in noradrenergic neurons, but only in the perivascular nerve fiber network. The parenchymal distribution of NPY to interlobular arterioles and capillaries suggests that this peptide must have other functions besides that of innervating arteriovenous anastomoses, as hypothesized by other researchers. The different distribution of CGRP and SP suggests the existence of different sensory neuronal populations. The detection of CGRP at the parenchymal level is in line with the hypothesis of a trophic action of this peptide.

Introduction

Interscapular brown adipose tissue (IBAT) is a thermogenic organ that participates in the regulation of energy expenditure. Brown adipocytes are able to uncouple mitochondrial respiration from phosphorylation and provide for energy dispersion as body heat due to the unique presence of UCP-1, a 32 kDa protein of the inner mitochondrial membrane (for a review, see Himms-Hagen & Ricquier, 1998).

The thermogenic activity of brown adipocytes is neurally controlled. IBAT is innervated by several nerves proceeding from the intercostal area and from the lateral peduncles, where they are associated with vascular structures (vascular-nervous peduncles) (Girardier & Seydoux, 1986; Cinti, 1988). These nerves bring to the organ visceral efferent and afferent fibers (Norman *et al.*, 1988; Lever *et al.*, 1989). Some efferent visceral fibers also reach IBAT through a periarterial plexus (Mukherjee *et al.*, 1989).

Efferent visceral neurons are either purely noradrenergic or noradrenergic co-localizing neuropeptide Y (NPY). The former constitute a plexus that makes direct contact with the adipocytes (parenchymal fibers); the latter are associated with the arterial branchings within the parenchyma but do not form parenchymal fibers (Cannon *et al.*, 1986; Mukherjee *et al.*, 1989). Parenchymal fibers are responsible for the functional activation of adipocytes via β-receptors (Jacobsson *et al.*, 1986; Ricquier & Bouillaud, 1986). During cold ex-

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posure, owing to the increase in orthosympathetic activity, the functional activity (thermogenesis) of brown adipocytes intensifies while, simultaneously and unexpectedly, nerve growth factor (NGF) production decreases (Nisoli *et al.,* 1996).

The aim of the present work was to test whether cold and warm stimulation are accompanied by a modification in the density of parenchymal nerve fibers. To this purpose, we performed the immunohistochemical localization of protein gene product 9.5 (PGP 9.5), an enzyme that is considered a highly sensitive marker for neuronal elements, including axonal projections (Thompson *et al.,* 1983; Wilson *et al.,* 1988), and of TH, a specific marker of noradrenergic nerve fibers. We also studied whether the immunoreactivity of afferent fibers (which contain calcitonin gene-related peptide [CGRP] and substance P [SP]; see Himms-Hagen, 1991), whose functional significance is still obscure, undergoes modifications in rats exposed to cold and warm stimulation.

Materials and methods

ANIMALS

Eighteen Sprague–Dawley male rats 6 weeks of age were provided with *ad libitum* food and water and maintained in the following experimental conditions: six control animals were kept at room temperature (20°C), six were cold-acclimated (4°C for 2 weeks), and six warm-acclimated (28°C for 2 weeks).

Animals were anesthetized with 0.36 M chloral hydrate (40 g/kg). Three rats from each experimental group were then perfused intra-aortically with a 3% paraformaldehyde solution in 0.1 M phosphate buffer (PB) at pH 7.4; the remaining nine rats, to be used for transmission microscopic studies, were perfused with a 2% glutaraldehyde–2% paraformaldehyde mixture in 0.1 M PB at pH 7.4. After perfusion, the IBAT, peduncles, and nerves were removed and immediately fixed overnight in the same solution at 4°C.

LIGHT MICROSCOPY

For light microscopy and immunohistochemistry, IBAT samples were cut in two halves along the middle plane of symmetry; the two parts were then rapidly washed with 0.1 M PB at pH 7.4, dehydrated in ethanol, paraffin-embedded with the same orientation, and cut into 3 μm thick sections.

For light microscopy, sections were stained with hematoxylin and eosin.

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Serial sections from IBAT samples prepared for light microscopy were processed for immunolocalization according to the avidin–biotin–peroxidase (ABC) method (Hsu *et al.,* 1981) with the following incubation steps: (1) hydrogen peroxide 0.3% in methanol for 30 min at room temperature (r.t.) to block endogenous peroxidase; (2) two 15 min washes in phosphate-buffered saline (PBS; 0.015 M, pH 7.4); (3) normal goat serum (neuropeptide schedule) and normal rabbit serum (UCP-1 schedule) (Vector Laboratories, Burlington, CA) 1:75 in PBS for 20 min at r.t. to reduce nonspecific background staining; (4) polyclonal rabbit antibodies against tyrosine hydroxylase (TH) (Chemicon, Temecola, CA), neuropeptide Y (NPY), substance P (SP), and calcitonin gene-related peptide (CGRP) (Amersham, Little Chalfont, UK); a polyclonal rabbit antibody against protein gene product 9.5 (PGP 9.5) (Ultraclone, Isle of Wight, UK) and a polyclonal sheep anti-rat uncoupling protein (UCP-1) antibody diluted 1:10,000 in PBS; sections were incubated overnight at high humidity at 4°C; (5) two 15-min washes in PBS at r.t.; (6) biotinylated secondary antibody: goat anti-rabbit IgG (Vector) diluted 1:200 (neuropeptide schedule) and rabbit anti-sheep IgG (Vector), dilution 1:300 (UCP-1 schedule), 30 min at r.t.; (7) two PBS washes of 15 min at r.t.; (8) ABC complex (Vector), 60 min at r.t.; (9) two PBS washes of 15 min at r.t.; and (10) histochemical visualization of peroxidase using 0.075% diaminobenzidine hydrochloride as chromogen (Sigma, St. Louis, MO) and hydrogen peroxide 0.02% in Tris buffer 0.05 M, pH 7.6, for 5 min in a dark room; sections were then rinsed in tap water, counterstained with hemotoxylin, dehydrated, and finally mounted in Eukitt (Kindler GmbH & CO, Freiburg, Germany).

Antibody specificity was tested by in-parallel demonstration of specific antigens in known distributions: NPY in myometrial nerves, CGRP in thyroid C cells, and SP in nerve fibers of the posterior horn of the spinal cord. All positive controls were positive. For each antibody, optimal dilutions were investigated to obtain the maximum specific reaction product and the lowest background. Dilutions were TH, 1:50; NPY, 1:600; CGRP, 1:1500; SP, 1:1500; and PGP 9.5, 1:250.

MORPHOMETRIC ANALYSIS

Serial sections from the IBAT of rats of all experimental groups were processed for immunohistochemistry with specific antibodies. TH- and PGP 9.5-immunoreactive (ir) parenchymal fibers were then counted in transverse sections with a Zeiss light microscope (Phomi 2; final magnification 630x) on 20 random fields for each animal. The overall number of positive fibers was calculated as the mean of the values observed in each animal and was expressed as proportion of the ir fibers found in cold-acclimated rats (the condition of highest expression).

In each vascular and vascular-nervous peduncle and in the subscapular nerve trunk, the diameter of small and large nerves was measured for each animal on semithin sections. Nerve structures were drawn at 25x with a light microscope equipped with a camera lucida. Mean diameter was measured as area equivalent diameter by means of the circles test (Weibel, 1979).

TRANSMISSION ELECTRON MICROSCOPY

Samples of IBAT, of vascular and vascular-nervous peduncles and of nerve bundles were sectioned in fragments of about 1 mm^3 and fixed in a 2% glutaraldehyde/2% paraformaldehyde mixture in 0.1 M PB (pH 7.4) overnight at 4°C. Specimens were post-fixed in 1% OsO4, dehydrated in ethanol, and embedded in an Epon–Araldite mixture. Semithin sections (2 mm) were stained with toluidine blue; thin section were obtained with a Reichert Ultracut E, stained with lead citrate, and examined with a Philips CM 10 transmission electron microscope.

STATISTICAL ANALYSIS

Values are expressed as mean ± standard error. Significance was analyzed by Student's *t*-test.

Results

MORPHOLOGICAL DATA

IBAT innervation is provided by a bundle of nerves that enter the organ bilaterally, coursing along blood vessels at the superficial lateral boundary (vascular-nervous peduncle). These nerves do not reach the deep lateral boundary (vascular peduncle). Another large bundle of nerves of intercostal origin enters IBAT bilaterally from the subscapular region.

Both nerve bundles consisted of 4–5 thick nerves 120.85 ± 7.18 μm in diameter and of numerous thin nerves of an average diameter of 36.81 ± 2.48 μm (from now on designated thick and thin nerve fibers), which by ultrastructural analysis were seen to have different compositions: thick nerves were made up of both myelinated and unmyelinated fibers, and thin nerves exclusively of thin unmyelinated fibers (Fig. 1a and b).

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The immunohistochemical data are summarized in Table 1.

TH immunoreactivity.

TH-positive (noradrenergic) fibers were observed in all three experimental conditions. At the level of the hilar nerves, TH-ir fibers were numerous in thin nerves, whereas thick nerves were faintly reactive (Fig. 2a and b). At the vascular level, positive fibers were observed in the adventitia of large inter- and intralobular vessels, as well as more peripherally around the finer vascular ramifications (Fig. 3a). There were TH-ir fibers associated with capillaries (capillary nerve fibers) or closely apposed to adipocytes (parenchymal nerve fibers) (Fig. 3b).

Substantial differences among the groups were observed in the intensity of TH immunoreactivity (much stronger in cold-than in warm-acclimated subjects) and in its distribution (besides the thin hilar nerves and interlobular vessels, it was mainly observed in the parenchyma). In cold-acclimated animals, several parenchymal fibers were intensely TH positive, whereas in warm-acclimated subjects and in controls these fibers were present only occasionally. TH-ir elements were not observed in the unilocular peripheral parenchyma.

NPY immunoreactivity.

Positivity for NPY was almost exclusively vascular and was distributed to inter- and intralobular arteries, to arterioles, and to the finest capillary ramifications (Fig. 4a and b).

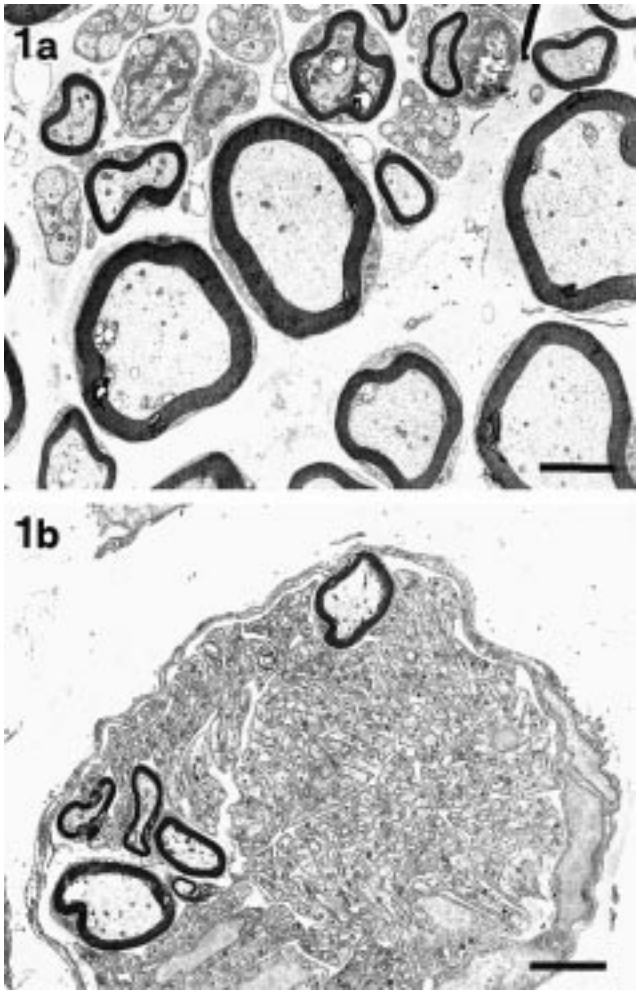

Fig. 1. (a) Adult rat IBAT electronmicrograph of a representative area of a thick nerve. The nerve contains myelinated fibers and clusters of unmyelinated fibers. Bar: 3 lm. (b) Adult rat IBAT electronmicrograph of a thin nerve. The nerve contains predominantly unmyelinated fibers. Bar: 3 lm.

The analysis of nerve immunoreactivity performed in serial sections evidenced NPY labelling only in rare fibers of some thin, mainly TH-positive nerves. In animals exposed to the cold stimulus, NPY-ir fibers were much more evident and were observed especially around thin intralobular arterioles (Fig. 5). Capillary fibers were positive only in this group.

CGRP immunoreactivity.

In hilar nerves, the analysis of serial sections showed that thin TH-positive nerves were also CGRP negative. Conversely, thick nerves were CGRP positive and almost completely TH negative. In these nerves, there were many CGRP- negative fibers, but TH-negative fibers were also observed (Fig. 2c and d). This was especially evident at 4°C and 28°C. There were CGRP-ir

Table 1. Semiquantitative assessment of the distribution of neuropeptide-positive fibers in interscapular adipose tissue (IBAT) of control (20°C), cold-acclimated (4°C), and warm-acclimated (28°C) rats

		Connective tissue					
		Arteries		*Hilar nerves*		*Parenchymal field*	
Peptides	*Environmental temperature*	*Interlobular*	*Intralobular*	*Large*	*Small*	*Capillaries*	*Adipocytes*
	20° C	+++	+++	0	+++	+++	+++
TH	4° C	++++	+++++	+++	+++++	+++++	+++++
	28° C	0	++	0	+++	++	+
	20° C	++	+++	0	+/−	0	0
NPY	4° C	++++	+++++	0	+/−	++	+/−
	28° C	+	+	0	+/−	0	0
	20° C	+	0	+	0	0	+/−
SP	4° C	0	0	+	0	0	0
	28° C	+	0	+	0	0	0
	20° C	+/−	0	+	+	0	0
CGRP	4° C	++	0	++++	++	++	+
	28° C	++	++	++++	++	++	+

Arbitrary evaluation of the density of vascular and parenchymal nerve fibers: O, no fibers; +/−, very rare fibers; +, rare fibers; ++, small number of fibers; +++, moderate number of fibers; ++++, large number of fibers; +++++, very large number of fibers.

fibers also in the adventitia of intralobular vessels (Fig. 3c), where some positive capillary and parenchymal fibers were seen in the two extreme conditions (4°C and 28°C), though not in control rats (Fig. 3d).

SP immunoreactivity.

Rare SP-positive fibers were consistently observed in the thick hilar nerves and around large interlobular vessels in all groups.

UCP-1 immunoreactivity.

Positivity for UCP-1 was observed in all three conditions: strong in cold-acclimated subjects, weaker in controls, and fainter still in warm-acclimated rats (not shown).

UCP-1 specificity was tested on skeletal muscle, liver, and kidney from all animals. All the tissues examined were negative.

Count of PGP 9.5- and TH-ir fibers.

All nerve fibers stained for PGP 9.5, a general marker of axonal projections (Thompson *et al.,* 1983; Wilson *et al.,* 1988), at the level of the thick and thin hilar nerves and of the adventitia of vessels, but also in the parenchyma, in the fine nerve ramifications associated with capillaries (capillary nerve fibers), and in those closely associated with adipocytes (parenchymal nerve fibers). In controls, PGP 9.5-ir fibers were 74% of those present in animals kept at 4°C (the condition of highest expression), and in the warm-acclimated group they were only 47%. TH-ir fibers in controls and warm-acclimated rats were 12% and 6%, respectively, of those observed in cold-acclimated subjects (Fig. 6).

Discussion

The present data demonstrate that the nerve bundles that enter IBAT consist of two types of nerves. We have distinguished them by size into thick and thin nerves. The former contained CGRP- and SP-ir fibers and were both myelinated and unmyelinated; the latter were prevalently TH positive and unmyelinated. Since many fibers of the thick nerves were both CGRP and TH negative, the presence in these nerves of other neuromodulatory substances cannot be excluded.

The perivascular plexus of hilar nerves was TH and NPY positive. These data are in line with previous reports of the presence of TH, CGRP, SP, and NPY in the fibers innervating IBAT (Lever *et al.,* 1988; Norman *et al.,* 1988; Nnodim & Lever, 1988), as well as with the hypothesis of the existence of two TH-ir fiber populations: noradrenergic perivascular fibers exhibiting NPY co-localization and parenchymal, purely noradrenergic fibers (Cannon *et al.,* 1986; Mukherjee *et al.,* 1989).

Cold stimulation is known to result in a functional increase of IBAT (confirmed by the evident increase in UCP-1 expression found in this work), associated with an augmented noradrenaline and TH content (Cottle *et al.,* 1967; Cottle & Cottle, 1969; Young *et al.,* 1982; Kennedy *et al.,* 1997). Our data suggest that this increase is due to the branching of noradrenergic fibers, as demonstrated by the parallel increase in the number of

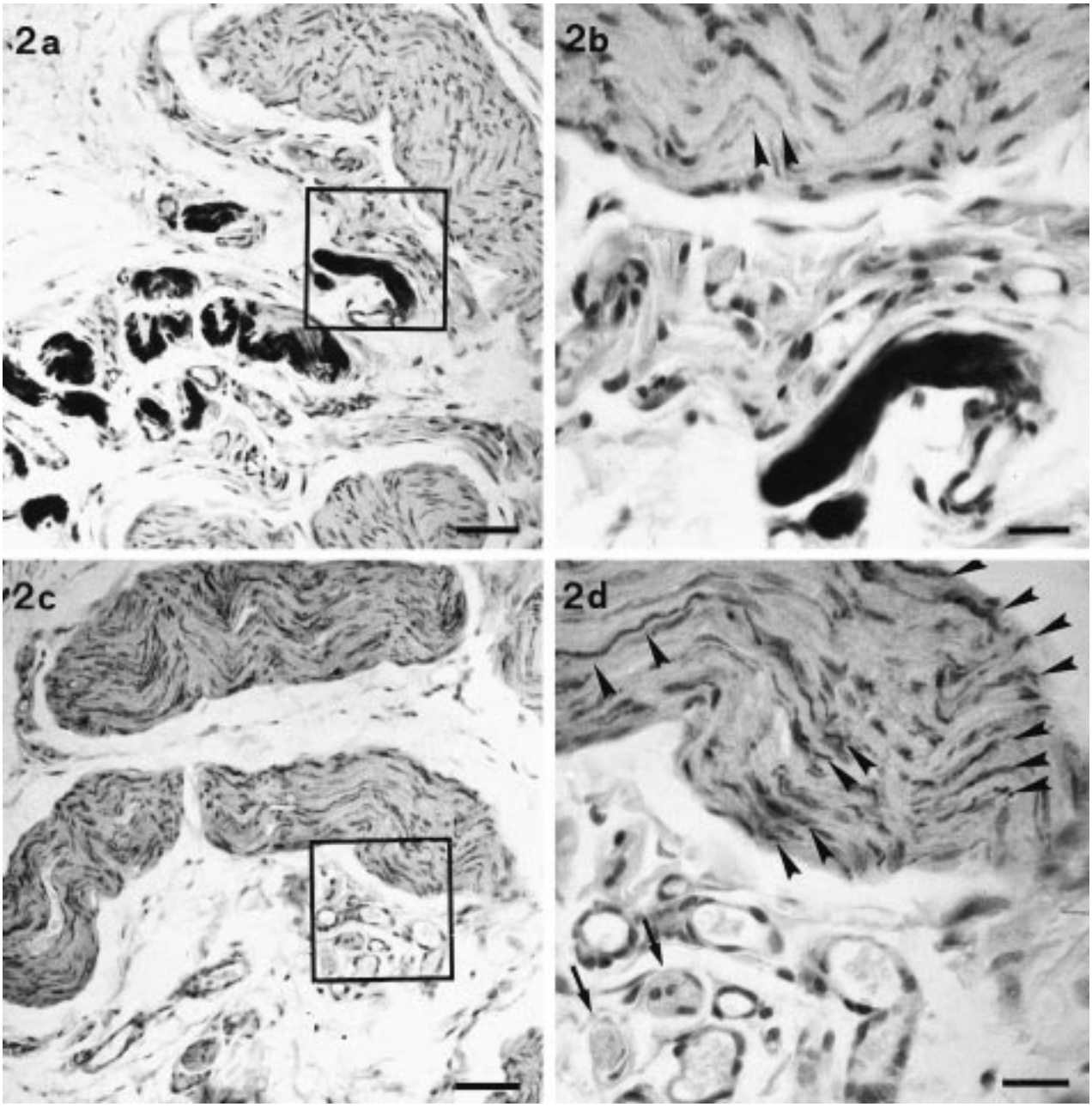

Fig. 2. (a) TH immunoreactivity in the thin and thick nerves making up the subscapular trunk of adult rat IBAT acclimated at 4°C. Thin nerves are intensely positive; thick nerves exhibit rare TH-ir fibers. Bar: 60 μm. (b) Magnification of the framed area in (a). All nerve fibers of a thin nerve are TH positive, while those of the thick nerve are only occasionally and faintly marked (arrowheads). Bar: 23 μm. (c) CGRP immunoreactivity in the thin and thick nerves making up the subscapular trunk in a section serial to the one shown in (a). CGRP-positive fibers are located in the thick nerves; the thin nerves are CGRP negative. Bar: 60 μm. (d) Magnification of the framed area in (c). CGRP immunoreactivity (arrowheads) is prevalently found in thick nerve fibers. Thin nerves are negative (arrows). Bar: 23 μm.

fibers reactive for PGP 9.5 (a general marker for peripheral nerve fibers).

This growth of neuronal projections suggests that brown adipocytes produce one or more neuronal growth factors, but although brown adipocytes produce NGF (Nechad, 1986; Nechad *et al.*, 1994), its production decreases in situations of higher functional stimulation (Nisoli, 1996). The presence of other neurotrophic factors cannot therefore be excluded.

Cold stimulation produced an increase in NPY expression, especially at the level of intralobular arterioles, and gave rise to capillary parenchymal immunoreactivity. Based on functional data, Wood and Stock (1996) advanced the hypothesis that NPY may

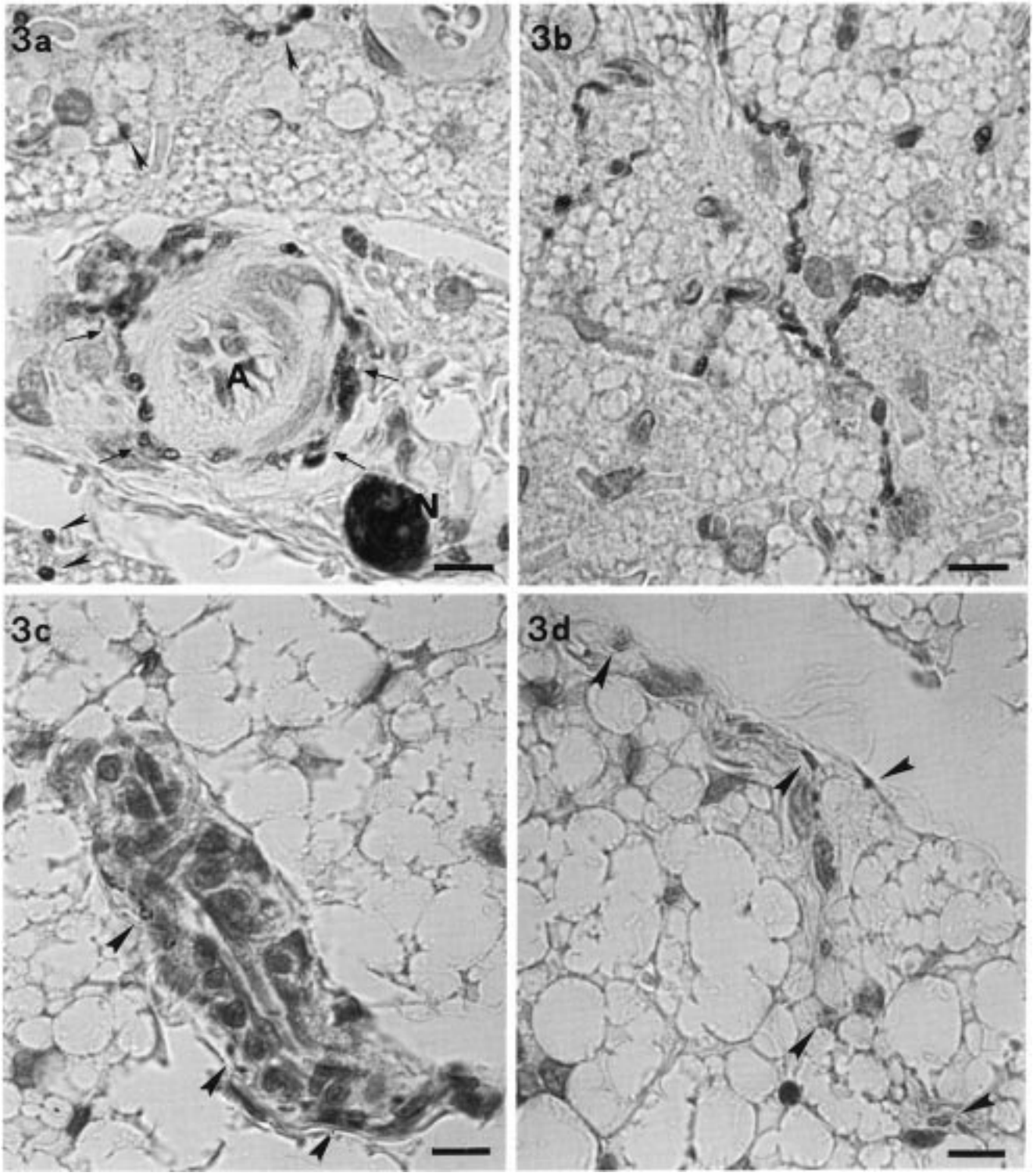

Fig. 3. (a, b) TH immunoreactivity in IBAT of adult rat acclimated at 4°C. Numerous TH-positive nerve fibers can be observed at the interlobular and parenchymal levels (a). A thin, strongly TH-ir nerve (N) is associated with a small arteriole (A) displaying several TH-ir fibers in the adventitia (arrows). TH-positive parenchymal and capillary fibers are also visible (arrowheads). A TH-positive nerve fiber in longitudinal section courses through closely packed multilocular adipocytes (b). Bars: 9 μm. (c, d) CGRP immunoreactivity in IBAT of adult rat acclimated at 28°C. CGRP-ir fibers (arrowheads) can be observed in the adventitia of an intraparenchymal blood vessel (c) and among multilocular adipocytes or in close association with a capillary (arrowheads, d). Bars: 9 μm.

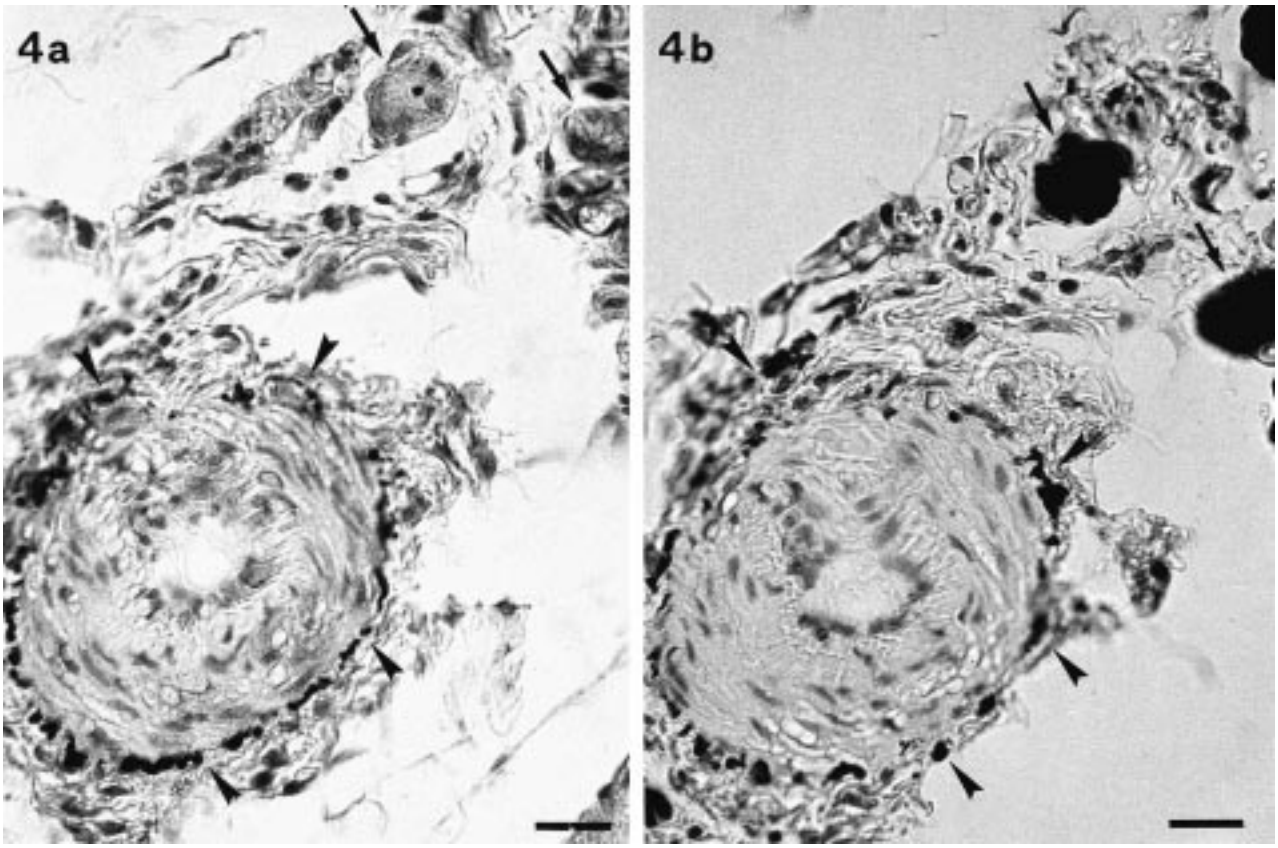

Fig. 4. Serial sections of IBAT of adult rat acclimated at 4°C. NPY- and TH-ir nerve fibers of the perivascular plexus are present in arteriolar adventitia (arrowheads), while the nerves of the vascular-nervous peduncles are TH positive and NPY negative (arrows). Bars: 23 μm.

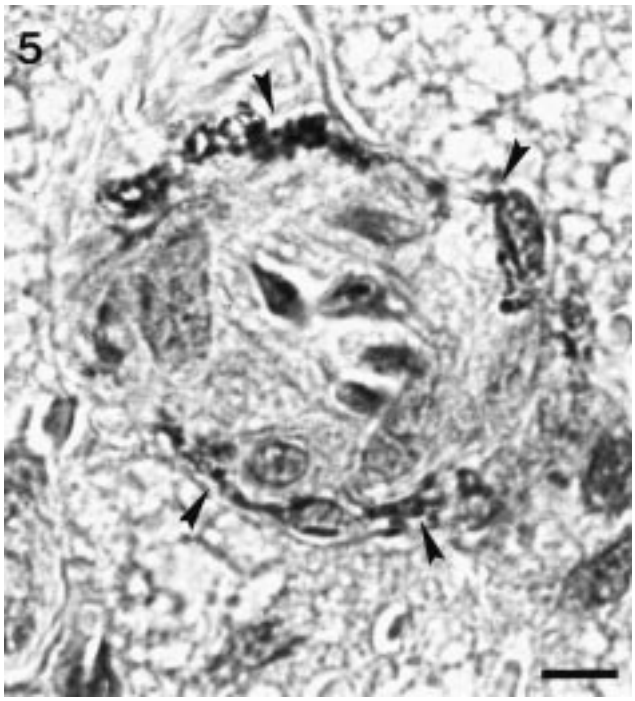

Fig. 5. NPY immunoreactivity in adult rat IBAT acclimated at 4°C. An intralobular arteriole exhibits intensely NPY-ir fibers (arrowheads). Bar: 6 μm.

have a vasoconstrictor role at arteriovenous anasthomoses (AVA). Since the sections prepared for NPY detection did not contain AVA, we can neither nor counter this hypothesis, but the action of NPY during cold exposure is accompanied by a parallel increase in the number of noradrenergic fibers. Moreover, given that the latter are involved in the induction of nitric oxide (vasodilator) secretion by brown adipocytes (Nisoli, 1997) and that NPY has been seen to potentiate the action of noradrenaline (Wahlestedt, 1985), the hypothesis may be advanced that NPY is also implicated in nitric oxide secretion by brown adipocytes. The fibers containing SP and CGRP, two typical sensory-nerve neuropeptides (Himms-Hagen, 1991), had different distributions: interlobular vascular (SP) and intralobular vascular and parenchymal (CGRP). Different populations of neurons thus seem to be responsible for sensory innervation.

In warm- and cold-adapted rats, the immunoreactivity of CGRP-ir fibers increased also at the parenchymal (capillary nerve fibers) level, but the number of these fibers was much lower than that of efferent noradrenergic fibers.

The detection of CGRP-ir fibers in the parenchyma seems to be in line with the finding that their main

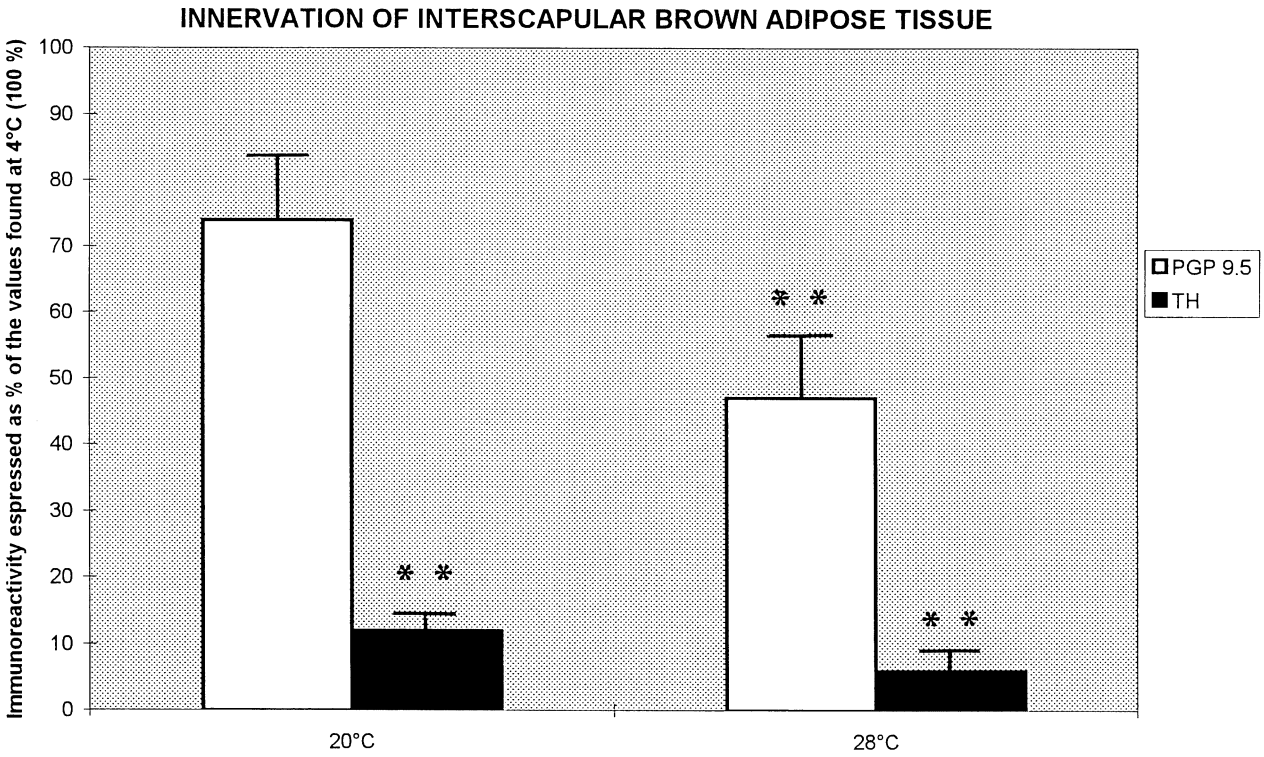

Fig. 6. IBAT innervation. TH- and PGP 9.5-ir parenchymal nerve fibers in control (20°C) and warm-acclimated (28°C) rats, expressed as % of fibers found at 4°C (100%). PGP 9.5 is a general marker for peripheral nerve fibers. The parallel increase in TH- (noradrenergic) and PGP 9.5 (general)-positive fibers indicates a branching of nerve fibers during cold acclimatization. **, $p < 0.01$ (*vs.* cold acclimatization).

activity is to transport the thermal stimulus (Holzer, 1988; Maggi & Meli, 1988). However, other activities of the sensory peptidergic innervation cannot be excluded. The administration of high doses of SP reduces IBAT temperature both in basal and thermogenic conditions, a phenomenon that has been compared to the production of a vasodilator effect on AVA, with reduction in parenchymal flow (Wood & Stock, 1996). However, so far only CGRP has been localized immunohistochemically in AVA (Nnodim *et al.*, 1988). By indicating that CGRP is widely present in the vasculature down to the capillary level, our data suggest that its action is unlikely to be restricted to AVA regulation.

In capsaicin-desensitized rats, which have a long-lasting impairment of a subpopulation of sensory nerve fibers containing CGRP and SP (Buck & Burns, 1986; Maggi & Meli, 1988; Holzer, 1988), IBAT is atrophic, and total proteins and UCP and DNA content are significantly reduced (Himms-Hagen *et al.*, 1988; Himms-Hagen *et al.*, 1990; Cui *et al.*, 1990; Cui & Himms-Hagen, 1992). Furthermore, we have recently demonstrated that CGRP-ir fibers are crucial for the normal proliferation of brown adipocytes in the periovarian white adipose tissue of cold-acclimated rats (Giordano *et al.*, 1998). CGRP could therefore have a trophic sensitive-efferent function, as suggested by other authors (Szolcsanyi, 1984; Maggi & Meli, 1988; Abelli *et at.*, 1993), in line with the proven trophic activity of noradrenaline (Park & Himms-Hagen, 1988).

In conclusion, our data demonstrate the presence in adult rat IBAT of small, almost exclusively noradrenergic unmyelinated nerves and of large nerves constituted by prevalently sensitive unmyelinated and myelinated fibers. During cold acclimatization, noradrenergic neurons increase amounts of TH and their axons branch. NPY co-localizes with TH in noradrenergic neurons, but only in the perivascular nerve fiber network. The parenchymal expression of NPY increases as a consequence of cold stimulation, suggesting that this peptide potentiates noradrenergic activity. The different distributions of CGRP and SP allow the existence of different sensory neuronal populations to by hypothesized. The detection of CGRP at the parenchymal level is in line with a possible trophic action of this peptide.

Acknowledgments

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References

ABELLI, L., GEPPETTI, P. & MAGGI, C.A. (1993) Relative contribution of sympathetic and sensory nerves to thermal nociception and tissue trophism in rats. *Neuroscience* **57,** 739–745.

BUCK, S.H. & BURNS, T.F. (1986) The neuropharmacology of capsaicin: review of some recent observations. *Pharmacological Reviews* **38,** 179–226.

CANNON, B., NEDERGAARD, J., LUNDBERG, S.M., HOKFELT, T., TERENIUS, L. & GOLDSTEIN, M. (1986) "Neuropeptide Tyrosine" (NPY) is co-stored with noradrenaline in vascular but not in parenchymal sympathetic nerves of brown adipose tissue. *Experimental Cell Research* **164,** 546–50.

CINTI, S. (1988) Considerazioni sul ruolo del tessuto adiposo bruno nei mammiferi non ibernanti. *Archivio Italiano di Anatomia e Embriologia* **93 (Suppl),** 7–42.

COTTLE, W.H., NASH, C.W., VERESS, A.T. & FERGUSON, B.A. (1967) Release of noradrenaline from brown fat of cold-acclimated rats. *Life Science* **6,** 2267–71.

COTTLE, M.K.W. & COTTLE, W.H. (1969) Adrenergic fibers in brown fat of cold-acclimated rats. *Journal of Histochemistry and Cytochemistry* **18(2),** 116–9.

CUI, J., ZAROR-BEHRENS, G. & HIMMS-HAGEN, J. (1990) Capsaicin desensitization induces atrophy of brown adipose tissue in rats. *American Journal of Physiology* **259,** R324–32.

CUI, J. & HIMMS-HAGEN, J. (1992) Rapid transient atrophy of brown adipose tissue in capsaicin-desensitized rats. *American Journal of Physiology* **262,** R324–32.

GIORDANO, A., MORRONI, M., CARLE, F., GESUITA, R., MARCHESI, G.F. & CINTI, S. (1998) Sensory nerves affect the recruitment and differentiation of rat periovarian brown adipocytes during cold acclimation. *Journal of Cell Science* **111,** 2587–94.

GIRARDIER, L. & SEYDOUX, J. (1986) Neural control of brown adipose tissue thermogenesis. In: *Brown Adipose Tissue* (edited by TRAYHURN, P. & NICHOLLS, D.G.), pp 122–151. London: E. Arnold.

HIMMS-HAGEN, J. (1989) Role of thermogenesis in regulation of energy balance in relation to obesity. *Canadian Journal of Physiology and Pharmacology* **67,** 394–401.

HIMMS-HAGEN, J., CUI, J. & SIGURDSON, S.L. (1990) Sympathetic and sensory nerves in control of brown adipose tissue: effects of denervation and of capsaicin. *Biochemistry International* **17,** 271–9.

HIMMS-HAGEN, J. (1991) Neural control of brown adipose tissue thermogenesis, hypertrophy and atrophy. *Frontiers in Neuroendocrinology* **12,** 38–93.

HIMMS-HAGEN, J. & RICQUIER, D. (1998) Brown adipose tissue. In: *Handbook of Obesity* (edited by BRAY, G.A., BOUCHARD, C. & JAMES, W.P.T.), pp 415–41. New York: Marcel Dekker.

HOLZER, P. (1988) Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience,* **24,** 739–768.

HSU, S.M., RAINE, L. & FARGER, H. (1981). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabelled antibody (PAP procedures). *Journal of Histochemistry and Cytochemistry* **29,** 577–80.

JACOBSSON, A., NEDERGAARD, J. & CANNON, B. (1986). α and β adrenergic control of thermogenin mRNA expression in brown adipose tissue. *Bioscience Reports* **6,** 621–31.

KENNEDY, D.R., HAMMOND, R.P. & HAMOLSKY, M.W. (1977) Thyroid cold acclimation influences on norepinephrine metabolism in brown fat. *American Journal of Physiology* **232,** E565–69.

LEVER, J.D., MUKHERJEE, S., NORMAN, D., SYMONS, D. & JUNG, R.T. (1988) Neuropeptide and noradrenalin distributions in rat interscapular brown fat and its intact and obstructed nerves of supply. *Journal of the Autonomic Nervous System* **25,** 15–25.

LEVER, J.D., MUKHERJEE, S., NORMAN, D., SYMONS, D., WHEELER, M.H., CONNACHER, A. & JUNG, R.T. (1989) Catecholaminergic and peptidergic nerves in naturally occurring and pheochromocytoma-associated human brown adipose tissue. *Clinical Anatomy* **2,** 157–66.

MAGGI, C.A. & MELI, A. (1988) The sensory-efferent function of capsaicin-sensitive sensory neurons. *General Pharmacology* **19,** 1–43.

MUKHERJEE, S., LEVER, J.D., NORMAN, D., SYMONS, D., SPRIGGS, T.L. & JUNG, R.T. (1989) A comparison of the effects of 6-hydroxydopamine and reserpine on noradrenergic and peptidergic nerves in rat brown adipose tissue. *Journal of Anatomy* **167,** 189–93.

NECHAD, M. (1986) Structure and development of brown adipose tissue. In: *Brown Adipose Tissue* (edited by TRAYHURN, P. & NICHOLLS, D.G.), pp 1–30. London: E Arnold.

NECHAD, M., RUKA, E. & THIBAULD, J. (1994) Production of nerve growth factor by brown fat in culture: relation with the in vivo developmental stage of the tissue. *Comparative Biochemical Physiology and Comparative Physiology* **107,** 381–8.

NISOLI, E., TONELLO, C., BENARESE, M., LIBERINI, P. & CARRUBA, M.O. (1996) Expression of nerve growth factor in brown adipose tissue: implications for thermogenesis and obesity. *Endocrinology* **137,** 495–503.

NISOLI, E., TONELLO, C., BRISCINI, L. & CARRUBA, M.O. (1997) Inducible nitric oxide synthase in rat brown adipocytes: implications for blood flow to brown adipose tissue. *Endocrinology* **138,** 676–82.

NNODIM, J.O. & LEVER, J.D. (1988) Neural and vascular provisions of rat interscapular brown adipose tissue. *American Journal of Anatomy* **182,** 283–293.

NORMAN, D., MUKHERJEE, S., SYMONS, D., JUNG, R.T. & LEVER, J.D. (1998) Neuropeptides in interscapular and perirenal brown adipose tissue in the rat: a pluarality of innervation. *Journal of Neurocytology* **17,** 305–11.

PARK, I.R.A. & HIMMS-HAGEN, J. (1988) Neural influences on trophic changes in brown adipose tissue during cold acclimation. *American Journal of Physiology* **255,** R874–81.

RICQUIER, D. & BOUILLAUD, F. (1986) The brown adi-

pose tissue mitochondrial uncoupling protein. In: *Brown Adipose Tissue* (edited by TRAYHURN, P. & NICHOLLS, D.G.), pp 86–104. London: E. Arnold.

SZOLCSANYI, J. (1984) Capsaicin-sensitive chemoceptive neural system with dual sensory-efferent function. In: *Antidromic Vasodilation and Neurogenic Inflammation* (edited by CHAHL, L.A., SZOLCSANYI, J. & LEMBECK, F.), pp 26–52. Budapest: Akademiai Kiado.

THOMPSON, R.J., DORAN, J.F., JACKSON, P., DHILLON, A.P & RODE, J. (1983) PGP 9.5—a new marker for vertebrate neurons and neuroendocrine cells. *Brain Research* **278,** 224–8.

WAHLESTEDT, C., EDVINSSON, L., EKBLAD, E. & HAKANSON, R. (1985) Neuropeptide Y potentiated noradrenaline-evoked vasoconstriction: mode of action. *Journal of Pharmacology and Experimental Therapeutics* **234,** 735–41.

WEIBEL, E.R. (1979) *Stereological Methods. Practical Methods for Biological Morphometry, Vol. 1,* pp 162–203. London: Academic Press.

WILSON, P.O.G., BARBER, P.C., HAMID, Q.A., POWER, B.F., DHILLON, A.P., RODE, J., DAY, I.N.M., THOMPSON, R.J. & POLAK, J.M. (1988) The immunolocalization of protein gene product 9.5 using rabbit polyclonal and mouse monoclonal antibodies. *British Journal of Experimental Pathology* **69,** 91–104.

WOODS, A.J. & STOCK, M. (1996) Inhibition of brown fat activity during hypothalamic stimulation in the rat. *American Journal of Physiology* **270,** R605–13.

YOUNG, J.B., SAVILLE, E., ROTHWELL, N. & STOCK, M.J. (1982) Effect of diet and cold exposure on norepinephrine turnover in brown adipose tissue of the rat. *Journal of Clinical Investigation* **69,** 1061–71.