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B-50 (GAP-43) in Onuf's nucleus of the adult cat

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The nucleus of Onuf in the sacral spinal cord contains motoneurons that innervate the pelvic floor muscles and possess somatic and autonomic characteristics. We show in this study that in the intact adult cat, the immunocytochemical labelling of the nervous tissue-specific growth-associated protein, B-50 (GAP-43), which persists in Onuf's nucleus, differs markedly from that in the remaining 'purely somatic' motor nuclei of the sacral spinal cord. At the light microscopic level, an intense B-50 (GAP-43) immunoreactivity (B-50-IR) in the neuropil of Onuf's nucleus contrasts with a faint staining in the other spinal motor nuclei. Ultrastructurally, B-50-IR is found in Onuf's nucleus within some unmyelinated small diameter nerve fibres and numerous axon terminals on dendritic and somatic surfaces. Conversely, in all other motor nuclei only a few of these structures are stained. No other cellular profiles show B-50-IR in the tissue examined. According to the proposed functions of B-50 (GAP-43), its persistence in mature spinal axon terminals may indicate a latent capability of functional and structural remodeling, as well as an involvement in long-term enhancement in synaptic transmission. If so, these properties would be considerably more pronounced in Onuf's nucleus as compared to purely somatic motor nuclei.

INTRODUCTION

The neuroanatomist Onufrowicz, who called himself Onuf, described in 1900 a small circular nucleus of neurons located in the ventral horn of the human spinal cord, extending from the caudal S1 to the rostral S3 segments[42]. According to Onuf, motoneurons in this nucleus would innervate striated muscles of the urethral and anal sphincters[42]. More recent studies in the cat, using retrograde tracing techniques, have confirmed this notion[23,27,44]. In the cat, the Onuf's nucleus homologue is located in sacral segments S1–S2, and shows a strict subdivision, with motoneurons innervating the external urethral sphincter located in the ventrolateral portion of the nucleus and those innervating the external anal sphincter located in the dorsomedial portion[1,23,46,55]. Nucleus of Onuf neurons are partially somatic in nature, since they innervate striated muscles and are under voluntary control[22,23]. In addition, they have autonomic properties for the following reasons: (1) cytoarchitectonically they resemble autonomic neurons[43], (2) they receive direct input from the hypothalamus[22,23], (3) they are strongly interrelated with sacral parasympathetic neurons[23,36,43], and (4) in contrast to the purely somatic spinal motoneurons, neurons of Onuf's nucleus are preserved in motoneuron disease[17,29] and affected in Shy–Drager syndrome in which autonomic neurons in the intermediolateral nucleus are predominantly involved[30,51,52].

In the present study, we compare in the adult cat the immunocytochemical distribution pattern of persisting growth-associated protein B-50 in Onuf's nucleus with that in purely somatic spinal motor nuclei. B-50 (otherwise known as GAP-43, GAP-48, F1, neuromodulin, and pp46[4,18,19,28,37,38,48]) has been strongly implicated in axonal outgrowth and synaptogenesis

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during development[4,9,11,13,19–21,24,25,32,34,41,47] and regeneration[3,6,18,26,31,45,48,53,57,58,60,61]. Moreover, the protein is believed to have a crucial role in the regulation of synaptic transmitter release[12,18,19] and long-term potentiation[28,37]. B-50 is commonly referred to as a neuron-specific protein. Recent evidence has shown, however, that B-50 is also expressed by some glial cells in the central nervous system[8,14,59] and by Schwann cells[7,56,62] under certain conditions, raising new questions about the role of this protein. The persistence of B-50 in distinctive regions of the mature nervous system has been linked to the maintainance of neuronal capability for structural remodeling and functional plasticity[2,13,15,33,41]. This capability has been ascribed to the mature Onuf's nucleus[5,54]. Accordingly, we aimed at investigating the precise localization of B-50-containing structures in Onuf's nucleus as compared to exclusively somatic spinal motor nuclei, using light and electron microscopic immunocytochemistry. This approach may provide further insight into the differential functional properties of Onuf's nucleus and purely somatic motor nuclei.

MATERIALS AND METHODS

The subjects of these experiments were one male and one female adult cat, about 3 kg in body weight.

Fixation and immunohistochemical procedures

The cats were deeply anesthetized with pentobarbital and perfused through the heart with 0.9% saline solution until the outflow was clear, followed by 3 l of 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After the perfusion was complete, the lumbosacral spinal cord was fully exposed. The sacral segments were identified using the dorsal root entry zones as landmarks and separately removed. The segments S1 and S2 which contain Onuf's nucleus were examined in both animals. The tissue blocks were postfixed for 24 h in fixative solution as used for perfusion. Part of the tissue was then cryoprotected in graded sucrose (10%, 20%, and 30%) in 0.1% phophate-buffered saline (PBS) for 24–48 h, frozen in liquid nitrogen and sectioned on a cryostat at transverse plane (14 μm) for light microscopy. The rest of the tissue was transferred to 2% paraformaldehyde in 0.1 M phosphate buffer and cut on a vibratome at transverse plane (50 μm) for electron microscopy.

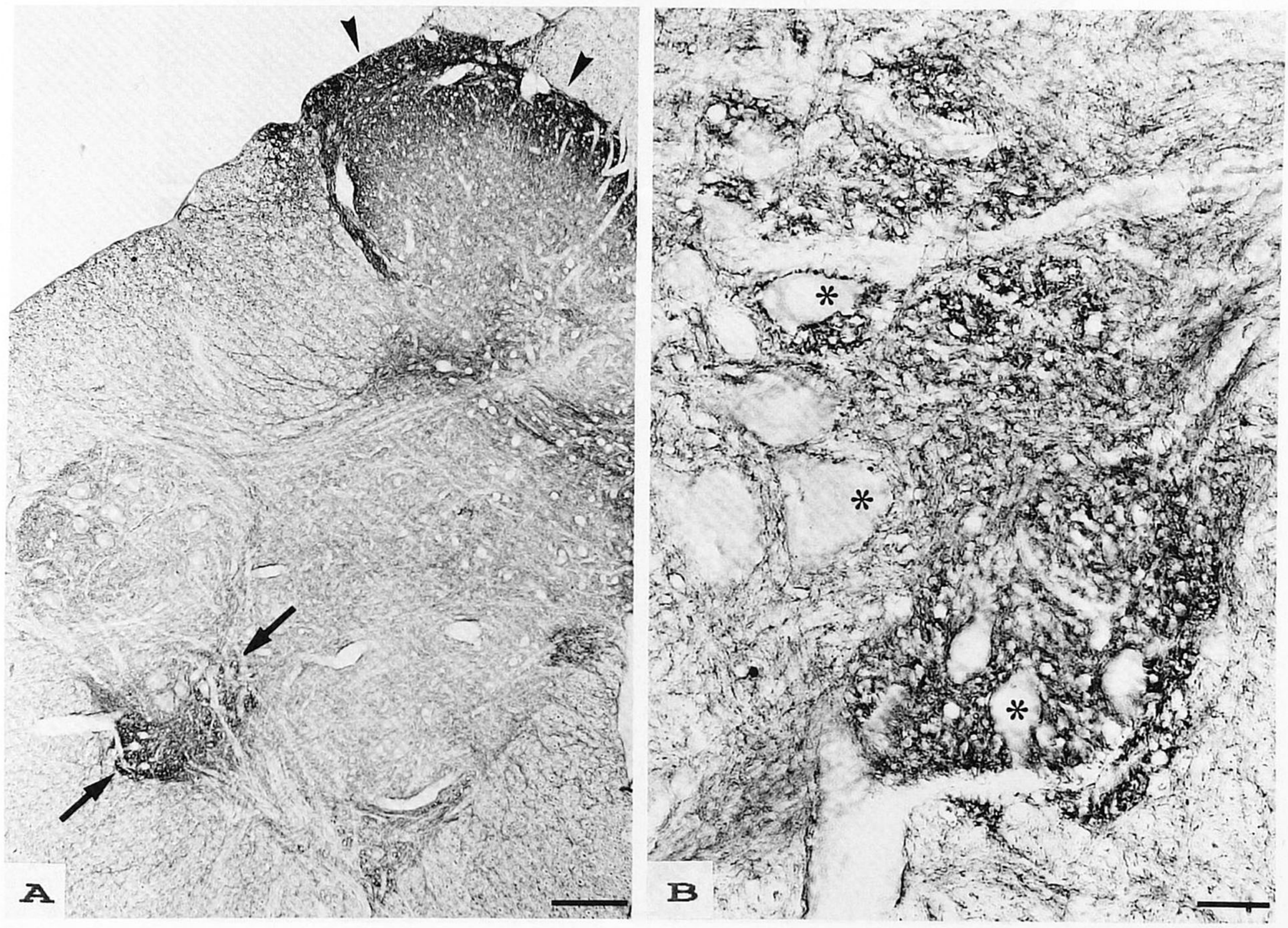

Fig. 1. Light micrograph showing B-50 immunoreactivity in the segment S1 of the adult cat. A: in the ventral horn, a circumscribed intense staining (arrows) is located in Onuf's nucleus, whereas only a faint labelling is detectable in the remaining ventral horn. An intense immunoreactivity is found in superficial laminae of the dorsal horn (arrowheads). Bar = 200 μm. B: higher magnification of Onuf's nucleus. Note that the B-50 labelling is restricted to the neuropil, whereas the neuronal perikarya (asterisks) are unstained. Bar = 40 μm.

Free floating tissue sections were immunohistochemically stained for B-50 using the peroxidase-antiperoxidase method of Sternberger[49]. Prior to immunostaining, sections were incubated in a solution containing 0.3% hydrogen peroxide in PBS for 30 min, to block endogeneous peroxidase activity, followed by three 10-min rinses in PBS. Briefly, sections were then incubated in affinity purified polyclonal rabbit antibodies to rat B-50 (anti-B50 IgGs of rabbit 8920; 1:2,000) for 48 h at 4°C and in secondary antisera (goat-antirabbit IgG, 1:200, Accurate Chemicals) and PAP (1:500, Accurate Chemicals) for 1 h each at room temperature. Primary antisera, secondary antisera, and PAP were diluted in 0.1 M PBS containing 1% normal goat serum (NGS). Tissue sections were rinsed in two changes of 0.1 M PBS (10 min each) and 3% NGS in PBS (30 min) before each antisera or PAP incubation. Following the PAP incubation, tissue sections were rinsed twice in PBS and reacted in 0.05% diaminobenzidine (Sigma) and 0.01% hydrogene peroxide in 0.1 M PB for 10 min. Cryostat sections were mounted on L-polylysine-coated slides, dehydrated in alcohols, cleared in xylene, and coverslipped with Depex. Vibratome sections were postfixed in 2.5% glutaraldehyde for 20 min and in Dalton's chrome-osmium tetroxide solution for 30 min, dehydrated and flat embedded in Araldite between slides coated with Repelcoat (dimethyldichlorsilane). After

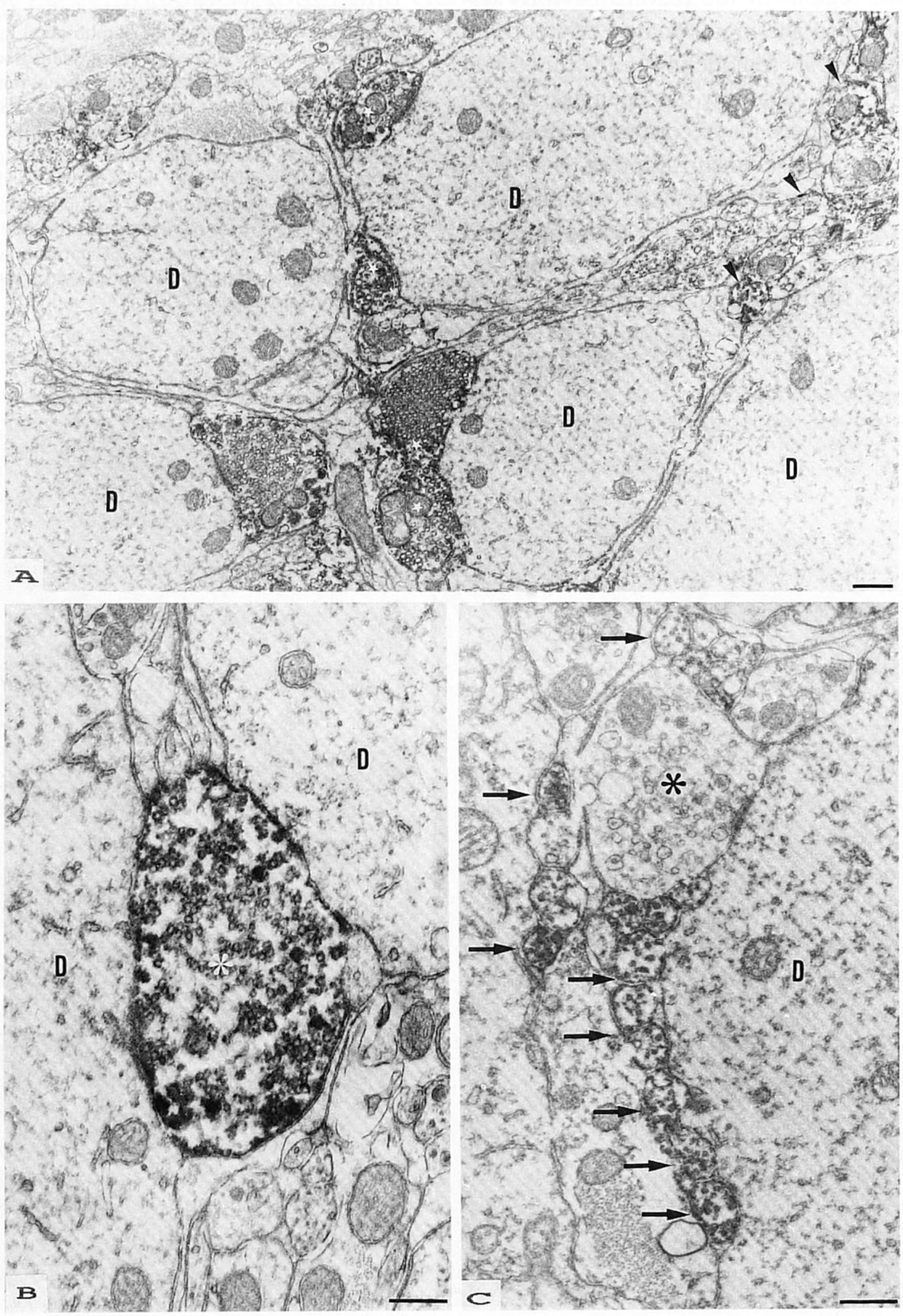

Fig. 2. A: electron microscopic overview showing the distribution of B-50 immunoreactivity in Onuf's nucleus. Labelling is found within numerous axon terminals (white asterisks) on dendrites (D) and within unmyelinated axons (arrowheads). Bar = 0.3 μm. B: B-50-IR in Onuf's nucleus within an axon terminal (white asterisk) in contact with two dendrites (D). Bar = 0.3 μm. C: B-50-IR in Onuf's nucleus within small diameter unmyelinated axons (arrows). An adjacent unlabeled axon terminal (black asterisk) is shown in synaptic contact with a dendrite. Bar = 0.3 μm.

polymerisation, regions of interest were excised from the tissue and re-embedded in Araldite blocks. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined in a Zeiss EM10 electron microscope. Serial ultrathin sections were made from some selected regions in which stained and unstained boutons were found in close proximty.

Control sections for both light and electron microscopic immunohistochemistry were incubated in PBS/NGS or preimmune-rabbit IgG instead of primary antisera, and resulted in no staining of the tissue. The specificity and characteristics of the rabbit polyclonal antibody against B-50 have been reported previously[21,40].

RESULTS

Light microscopy

In this paper, we describe the B-50-immunoreactivity in the ventral horn of segments S1 and S2 (i.e. Onuf's nucleus and surrounding motor nuclei) of the adult cat. The distribution pattern of B-50 immunolabelling in the remaining regions of the mature cat

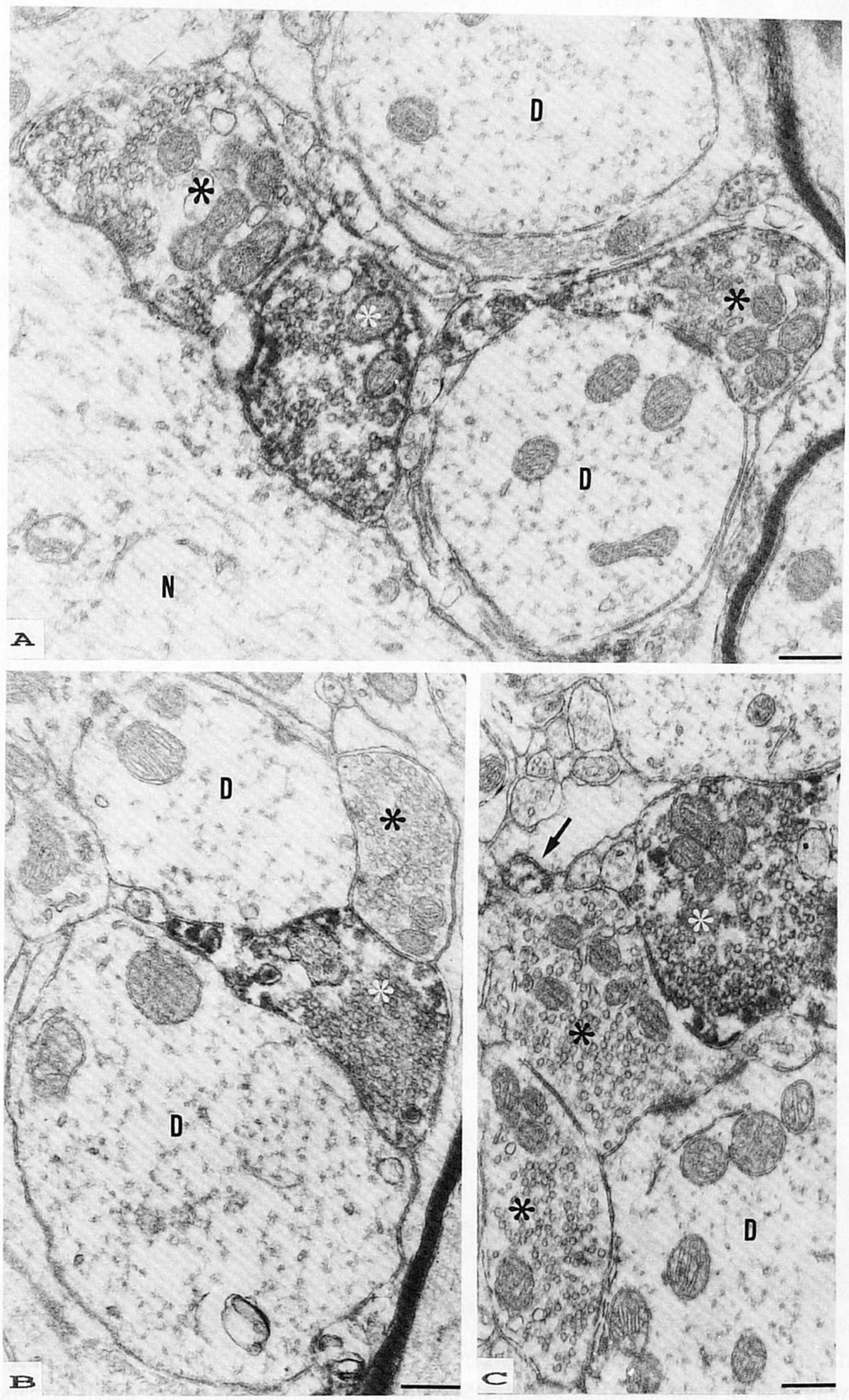

Fig. 3. A: B-50-IR within axon terminals (white asterisk) on the somatic surface of a neuron (N) in Onuf's nucleus. Note that unstained axon terminals (black asterisks) are located in close vicinity on somatic and dendritic (D) surfaces. Bar = 0.3 μm. B and C: B-50-positive boutons (white asterisks) in Onuf nucleus in synaptic contact with dendrites (D). Unstained axon terminals (black asterisks) are detectable in close vicinity. A B-50 labeled unmyelinated axon is shown in C (arrow). Bar = 0.3 μm.

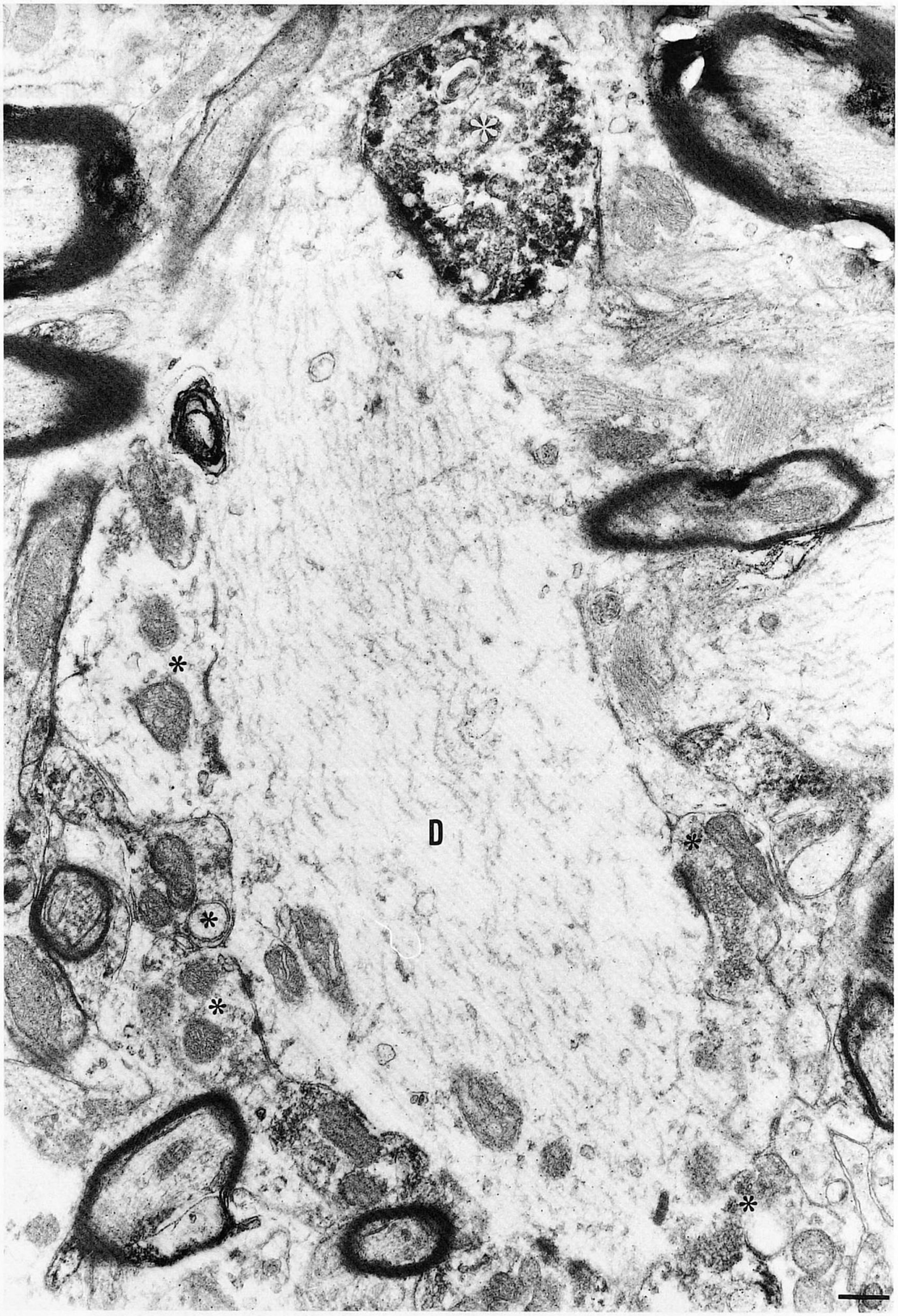

Fig. 4. B-50-IR in the ventral horn (lamina IX) of segment S1 outside Onuf's nucleus where purely somatic motoneurons are located. Immunolabelling is present in only one axon terminal (white asterisk) on a dendrite (D). The remaining boutons on this dendrite are unstained (black asterisks). Bar = 0.3 μm.

spinal cord will be described elsewhere[35]. In the sacral spinal gray, a granular pattern of staining is seen restricted to the neuropil; the neuronal cell bodies are unreactive. In sacral segments 1 and 2, a well-circumscribed intense B-50-staining is detectable in Onuf's nucleus (Fig. 1A–B). This finding clearly contrasts to the rather low levels of labelling in the remaining ventral horn (Fig. 1A) where purely somatic motor nuclei are located. The stained cellular elements cannot be identified at the light microscopic level.

Electron microscopy

In Onuf's nucleus, many axon terminals contacting dendritic and somatic surfaces of neurons display an intense B-50-IR (Figs. 2A–B, 3A–C). Here, the B-50 labelling appears to be predominantly associated with vesicle membranes, but some B-50-IR is also localized at the cytoplasmic side of plasma membranes. However, some boutons in close proximity to the labeled terminals on the same neuronal profile are unstained (Fig. 3 A–C). The complete absence of B-50-IR in such axon terminals is confirmed in serial ultrathin sections of some selected regions. In addition, numerous unmyelinated axons are intensely stained (Fig. 2A,C).

In purely somatic motor nuclei of the spinal cord, only a few axon terminals on the dendritic and somatic surfaces of motoneurons contain B-50-IR (Fig. 4). A conspicuous contrast is noted between the solitary intensely stained axon terminals and the numerous axon terminals without any B-50-label in the closely adjacent neuropil. Occasionally, some unmyelinated axonal profiles are seen in the neuropil of somatic motor nuclei.

Myelinated axons, dendritic and somatic profiles of neurons, as well as glial and vascular cells are devoid of B-50-immunoreactivity in all regions examined. The light and electron microscopic distribution pattern of B-50-IR is identical in the male and the female cat.

DISCUSSION

The neurons of Onuf's nucleus innervate striated pelvic muscles and external sphincters[1,22,23,27,44]. However, whether these neurons are purely somatic or both somatic and autonomic in nature has been the subject of debate for many years[1,23]. A close similarity exists between the structure and function of Onuf's nucleus in animals and humans[23]. Therefore, the present immunocytochemical study allows the further elucidation of particular characteristics of Onuf's nucleus with the advantage of a well-preserved tissue for light and electron microscopic examination.

The growth-associated nervous-tissue specific protein B-50 was first identified as a membrane phosphoprotein in neurons[4,11,18,19,21,34,40,47,53]. B-50 is synthesized at high levels during axonal outgrowth in development or regeneration and then transported to the growth cone where it is associated with both the cytoskeleton and plasma membrane[4,6,18–21,25,26,45,58,60,61]. The location of the protein at the cytoplasmic surface of the plasma membrane in growth cones is consistent with a direct involvement of the protein in membrane addition at the growth cone[19]. In the present study, however, we found B-50-IR mainly associated with vesicle membranes in axon terminals. This apparent organellar location of B-50, which has also been demonstrated with the immunoperoxidase method in the adult rat neostriatum[15], is most likely due to diffusion of the diaminobenzidine reaction product. We also found B-50-IR at the cytoplasmic side of plasma membranes in boutons which is in keeping with previous studies employing immunogold labelling[18,19,21].

B-50 remains detectable in the adult rat brain in areas of continuing synaptic turnover and in some axon tracts, but synthesis normally declines an order of magnitude after synaptogenesis[15,18,19,21,41,47]. We found in Onuf's nucleus B-50-labeled axon terminals in close proximity to unstained boutons. The presence of B-50 may indicate a particular state of readiness for participating in plasticity, or even an ongoing remodeling of presynaptic structures. This notion is supported by previous studies in which a synaptic reorganization has been observed in Onuf's nucleus of adult mammals in response to hormonal alterations and spinal cord injury[5,54].

The idea that B-50 affects the rate of surface membrane addition at the growth cone may explain the location of the protein in particular regions of the intact adult CNS[19]. Here, B-50 has been found in high concentrations within presynaptic terminals in certain regions of the frontal cortex, the basal ganglia, the limbic system, and the hippocampus[2,4,13,15,18]. These are partially regions where memory formation is thought to occur[28,37–39]. In the hippocampus, long-term potentiation (LTP) – a long lasting increase in synaptic strength produced by repeated use – correlates with phosphorylation of B-50 by protein kinase C[16,28] and with neurotransmitter release[28]. Recently, it has been shown that pre- and postsynaptic elements contribute to the generation of LTP[10]. The presynaptic change is an increase in neurotransmitter release[10,18,28], that is, an increase in the rate of exocytosis. This latter occurs in parallel with an increase in the rate of surface membrane addition, and in this respect resembles surface membrane addition at the growth cone[18,19]. These presumptive functions of B-50 are of particular interest in the light of our present finding that an intense B-50

immunolabelling prevails in numerous axon terminals on dendrites and somata of neurons in Onuf's nucleus. The latter are continuously discharging, even during sleep, to ensure tonic innervation of striated sphincter muscles[22]. This could be mediated by a continuous excitation of Onuf's neurons by their presynaptic input which in turn would be associated with a continuous neurotransmitter release. The latter implicates a high efficiency in the synaptic transmission and is, therefore, comparable to the situation in LTP.

In a very recent study, Stewart et al.[50] found that GAP-43 (B-50) is extensively expressed in normal peripheral autonomic nervous tissue of the adult rat. Interestingly, the intensity and ultrastructural distribution pattern of B-50-IR are essentially identical in Onuf's nucleus and in the intermediolateral nucleus of the intact cat[35]. Since in humans both nuclei are affected in Shy-Drager syndrome[30,51,52], autonomic characteristics have been assigned to Onuf's nucleus[23,30]. Our observations on B-50-IR adds to the notion of common functional properties of both nuclei. However, these are not necessarily autonomic properties, since the sacral parasympathetic nucleus in cat shows only low levels of B-50-IR[35].

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