Published in final edited form as:
Neurosci Lett. 2008 January 31; 431(2): 167–172. doi:10.1016/j.neulet.2007.11.036.

Ultrastructural localization of extranuclear progestin receptors relative to C1 neurons in the rostral ventrolateral medulla☆

Teresa A. Milner[a,b,*], **Katherine L. Mitterling**[a,b], **Costantino Iadecola**[a], and **Elizabeth M. Waters**[b]

a *Division of Neurobiology, Department of Neurology and Neuroscience, Weill Cornell Medical College, 411 East 69th Street, New York, NY 10021, United States*

b *Harold and Margaret Milliken Hatch Laboratory of Neuroendocrinology, The Rockefeller University, 1230 York Avenue, New York, NY 10021, United States*

Abstract

To better understand the role of progestins in the C1 area of the rostral ventrolateral medulla (RVLM), immunocytochemical localization of progestin receptors (PRs) was combined with tyrosine hydroxylase (TH) in single sections of RVLM from proestrus rat brains prepared for light and electron microscopy. By light microscopy, PR-immunoreactivity (-ir) was detected in a few nuclei that were interspersed between TH-labeled perikarya and dendrites. Electron microscopy revealed that PR-ir was in several extranuclear locations. The majority of PR-labeling was in non-TH immunoreactive axons (51 ± 9%) near the plasma membrane. Additional dual labeling studies revealed that PR-immunoreactive axons could give rise to terminals containing the GABAergic marker GAD65. PR-ir also was found in non-neuronal processes (29 ± 9%), some resembling astrocytes. Occasionally, PR-ir was in non-TH-labeled terminals (10 ± 3%) affiliated with clusters of small synaptic vesicles, or in patches contained in the cytoplasm of dendrites (10 ± 1%). These findings suggest that progestins can primarily modulate neurons in the C1 area of the RVLM by presynaptic mechanisms involving GABAergic transmission. Moreover, they suggest that PR activation may contribute to progestin's effects on arterial blood pressure during pregnancy as well as to sex differences in central cardiovascular regulation.

Keywords

Baroreceptor; Electron microscopy; Bulbospinal neurons; Presynaptic profiles; GABA; Glia

Ovarian hormones, especially estrogens and progestins, regulate arterial blood pressure (ABP) and cardiovascular tone. Within the CNS, estrogens are known to act within the well-established medullary sites that regulate sympathetic outflow (for review see [32]). In particular, local injections of 17β-estradiol into the rostroventrolateral medulla (RVLM), which contains sympathoexcitatory bulbospinal neurons and is critical for tonic (resting) and reflex control of systemic arterial pressure [1,7], decreases sympathetic tone [30,31]. Our recent study revealed that estrogens modulate calcium currents in C1 catecholaminergic bulbospinal neurons in the RVLM and that estrogen receptor (ER) alpha and beta have unique distributions relative to C1 neurons [39].

☆Grant support: NIH grants HL18974, DA08259 (TAM), NS07080 and T32 DK07313 (E.M.W.).

*Corresponding author at: Division of Neurobiology, Department of Neurology and Neuroscience, Weill Cornell Medical College, 411 East 69th Street, New York, NY 10021, United States. Tel.: +1 212 570 2900; fax: +1 212 988 3672. *E-mail address:* tmilner@med.cornell.edu (T.A. Milner).

The role of progestins in the RVLM has been studied largely in the context of pregnancy, where elevated progestins have been associated with decreased ABP likely due to increased GABAergic inhibition of the RVLM [11,17]. Other studies have reported that many genomic and non-genomic effects of progestins are attributable to progestin metabolites; however, a role for progestin activation of progestin receptors (PRs) remains [26,29]. PR expression in many brain regions can be directly regulated by estrogens [9,27]. We have shown that few nuclear PRs in non-catecholaminergic cells are found in the RVLM (unpublished observations). However, whether extranuclear PRs are also present in the RVLM has not been studied.

To better understand the potential role of progestins in modulating neurons in the C1 area of the RVLM, the present study examined the relationship of PR-immunoreactivity (-ir) relative to tyrosine hydroxylase (TH) in the RVLM of female rats by light and electron microscopy (EM). To further characterize the cellular identity of PR in presynaptic profiles, some sections were processed for PR and a GABAergic marker.

Female (N = 8; 275–325 g) adult Sprague–Dawley rats (Charles River Laboratories (Wilmington, MA) or Taconic Laboratories (Chatham, NY)) were used. All rats were housed with 12:12 h light/dark cycles. Estrous cycle stage was determined using vaginal smear cytology [37], and females were assessed for two full cycles. The proestrus (high estrogen) stage was examined since high levels of gonadal steroids are known to produce maximal expression of nuclear PRs in other brainstem regions [10]. Estrous stages were confirmed by uterine weights and by determining levels of estrogens and progestins from blood samples collected during the perfusion procedure using radioimmunoassay. Uterine weights were approximately 21% heavier in proestrus rats (0.8 ± 0.07 g) compared to diestrus rats (0.63 ± 0.04 g). On average the blood levels for estrogens were 10 pg/ml higher at proestrus than diestrus. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee.

PR-labeling was examined using a well-characterized antiserum. A rabbit polyclonal antiserum to a peptide corresponding to amino acids 533–547 (the N-terminal region present in the A and B isoforms) of the human PR was purchased from Dako (Carpinteria, CA). Specificity has been demonstrated by sucrose density gradients and absence of labeling either in absorption controls by immunoprecipitation methods [36] or in thymus and uterus of PR knock-out mice [16,35]. As an additional control, the PR antibody was preadsorbed with 150 mg/ml of the cognate peptide overnight at 4^{o}C before incubating the tissue. After preadsorption of the PR antibody (diluted 1:500) in acrolein/paraformaldehye fixed tissue, no nuclear immunoreactivity was detected in the arcuate nucleus by light microscopy (not shown) and no extranuclear immunoreactivity was detected in a 6050-μm^2 field sampled from the hippocampal CA1 region or the RVLM (colabeled with TH) by EM.

Catecholaminergic labeling was identified using a monoclonal mouse antibody to TH (Incstar (Stillwater, MN)). This antibody has been extensively characterized and localized in fixed rat brain [24].

GABAergic labeling was identified using a monoclonal antibody (MAB 351) to glutamic acid decarboxylase (GAD) 65 (Chemicon (Temecula, CA)). This antibody has been tested previously for specificity [6].

Rats were deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and perfused through the ascending aorta with solutions of (1) 10–15 ml saline (0.9%) containing 1000 units of heparin; (2) 50 ml of 3.75% acrolein (Polysciences, Washington, PA) and 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4); (3) 200 ml of 2% paraformaldehyde

in PB. During the perfusion, aortic blood was collected for radioimmunoassays of circulating hormone levels. Each brain was removed from the skull, cut into 5 mm coronal blocks and post-fixed with 2% paraformaldehyde in PB for 30 min. Sections (40 μm thick) through the RVLM were cut on a Vibratome (Leica, Wien, Austria) and collected into PB. Sections were stored in cryoprotectant (30% sucrose, 30% ethylene glycol in PB) until immunocytochemical processing.

Free-floating sections were dual-labeled for PR-immunoperoxidase and TH- (or GAD-) immunogold using methods described previously [39]. Sections were treated with 1% sodium borohydride in PB for 30 min, incubated in 0.5% bovine serum albumin (BSA) in 0.1 M Tris-saline (TS; pH 7.6) for 30 min and rinsed in TS. Sections were placed in PR antiserum (1:1000) in Triton X 100 (0.25% for light microscopy; 0.025% for EM) and 0.1% BSA in TS for 1 day at room temperature followed by an additional 4–5 days at 4 °C. One day prior to processing, TH antiserum (1:2000) or GAD antiserum (1:1000) was added to the primary antibody diluent.

To visualize PR-labeling, the sections were incubated in (1) biotinylated goat anti-rabbit IgG in 0.1% BSA (1:400, Vector Labs, Burlingame, CA), 30 min; (2) avidin–biotin complex (ABC), 30 min; (3) diaminobenzidine (Aldrich, Milwaukee, WI) and H_2O_2, 6–8 min. All incubations were separated by TS washes. TH- (or GAD-) labeling was visualized using silver-enhanced immunogold. For this, sections were rinsed in TS and incubated in donkey anti-mouse IgG conjugated to 1 nm gold particles (1:50, Electron Microscopy Sciences, Fort Washington, PA) in 0.08% BSA and 0.01% gelatin in phosphate-buffered saline (PBS; pH 7.4) for 2 h at room temperature. Sections were rinsed in PBS, post-fixed in 2% glutaraldehyde in PBS for 10 min, and rinsed in PBS followed by 0.2 M sodium citrate buffer (pH 7.4). The conjugated gold particles were enhanced by treatment with silver solution (EMS) for 5–7 min.

Sections for light microscopy were mounted on gelatin-coated slides, dehydrated through alcohols and xylene and coverslipped with DPX (Aldrich, Milwaukee, WI). Sections for EM were post-fixed for 1 h in 2% osmium tetroxide, dehydrated through alcohols and propylene oxide, and embedded between two sheets of plastic in EMbed 812 (EMS). Ultrathin sections (70 nm thick) were collected from the RVLM (Fig. 1; approximate levels 61–63 of Swanson [33]) as previously described [24]. Sections were counter-stained with uranyl acetate and Reynold's lead citrate.

Light microscopic sections were photographed with a Nikon E800 light microscope using a Micropublisher digital camera (Q-imaging, Barnaby, British Columbia). Electron microscopic preparations were analyzed on a FEI Tecnai Biotwin transmission electron microscope and a digital camera system (Advanced Microscopy Techniques, software version 3.2). Levels, sharpness, brightness and contrast in figures were adjusted in Adobe Photoshop 7.0 and assembled in Quark Xpress 6.1 using an Apple Power Macintosh G5 computer. The subcellular location of PR-labeling relative to TH-labeled profiles was examined by EM from one RVLM block from five rats each. From these rats, one block (approximately 1.2×10^3 μm^2 per block) from each of the three rats with the highest estrogen levels as determined from the radioimmunoassays were selected for semiquantitative analysis. Profiles were sampled near (within 1.5 μm) the plastic–tissue interface to ensure equivalent antibody penetration [18]. In each field, all profiles containing PR-ir were photographed, and categorized as catecholaminergic (TH-containing), non-catecholaminergic, or glial. To correct for differences in areas sampled in each block, the percentage of each type of profile was determined per block. To determine the relative distribution of PR immunoreactive profiles, the mean percentage and standard error was determined by averaging the percentages from one block from each of three rats. PR-labeled profiles were classified according to the nomenclature of Peters et al. [28]. Dendrite profiles contained regular microtubule arrays and usually were postsynaptic to axon terminal profiles. Unmyelinated axon profiles had a diameter less than 0.2 μm, had a few small

synaptic vesicles and lacked synaptic junctions in the plane of section. Terminal profiles had minimal diameters greater than 0.2 μm, contained numerous small synaptic vesicles, sometimes contained large dense-core vesicles and often contacted other neuronal profiles. Glial profiles tended to conform to the boundaries of surrounding profiles and/or lacked microtubules and, if astrocytic, were identified by the presence of glial filaments.

Since previous studies showed that nuclear PR-ir in the rat brainstem was highest at proestrus [10], tissue from this stage was chosen for the analysis. Consistent with our previous observations using two fluorescent markers (unpublished), PR-ir was detected in a few nuclei within the RVLM (Fig. 1). Usually one or two PR-labeled nuclei were detected per section and almost all were in the anterior portion of the RVLM and intermingled with TH-labeled perikarya and dendrites (not shown). No PR-labeled nuclei were found in neurons with TH-ir.

The electron microscopic analysis was focused on the anterior portion of the RVLM since it contains the bulk of C1 and non-C1 sympathoexcitatory bulbospinal neurons [4,25]. The morphological characteristics of catecholaminergic profiles in the C1 area of the RVLM have been described in detail previously [23,39]. PR immunoperoxidase labeling in RVLM tissue prepared for EM was primarily detected at extranuclear sites. In an experiment identifying PR-labeling with the immunogold–silver method, the subcellular localization was similar but less prominent due to the reduced sensitivity of this method for small amounts of antigen (not shown).

Semiquantitative analysis revealed that axonal profiles with PR-labeling were most numerous (51 ± 9% of 91 processes from three blocks from three separate rats). The majority (40 of 42) of PR-labeled axons were small (<0.2 μm in diameter), unmyelinated and lacked TH-ir (Fig. 2A). Rarely (2 of 42), PR-ir was detected in non-TH-labeled myelinated axons (not shown).

Extranuclear neuronal PR-labeling also was detected in axon terminals (9.7 ± 3% out of three blocks). Terminals with PR-ir (n = 9) had an average diameter of 0.95 ± 0.05 μm, contained numerous small synaptic vesicles and 1–3 dense-core vesicles (Fig. 2B). PR-ir in terminals was found in clusters affiliated with the plasma membrane (Fig. 2B). Of the PR-labeled terminals observed, one formed a symmetric contact on the shaft of a TH-labeled dendrite (not shown) and two formed symmetric synapses or closely apposed unlabeled dendritic shafts (Fig. 2B).

The observation that some terminals with PR-labeling formed symmetric synapses suggested that these terminals contained the inhibitory transmitter GABA. Thus, to further characterize the phenotype of PR-labeled axons, sections through the RVLM were dually labeled with PR and the GABAergic marker, GAD65. This marker was selected since our previous studies [21] showed that presynaptic GABAergic profiles are prominent in the RVLM and that they form numerous contacts with bulbospinal neurons. In fortuitous planes of section, PR-ir was detected in axons with GAD65-ir (Fig. 2C). Moreover, PR-ir was detected in preterminal axons of GAD65-immunoreactive terminals (Fig. 2D). Terminals with GAD65-ir were large (about 1.3 μm in diameter), contained numerous small synaptic vesicles and an occasional dense-core vesicle (Fig. 2D).

Extranuclear neuronal PR-labeling also was detected in dendrites (9.7 ± 1% out of three blocks). PR-labeled dendrites (n = 9) had an average diameter of 0.88 ± 0.09 μm and lacked TH-ir (Fig. 3A). Within dendrites, PR-ir was found in clusters in the cytoplasm and not affiliated with the plasma membrane.

In addition to neuronal profiles, extranuclear PR-ir was detected in glial profiles (29 ± 9% out of three blocks). Glial profiles were interspersed in the neuropil and occasionally contained

glial filaments identifying them as astrocytes (Fig. 3B). Sometimes, PR-labeled astrocytic profiles formed gap junctions with unlabeled glial profiles (Fig. 3B).

The present study demonstrates that in addition to nuclei, PR-ir is found at extranuclear sites in the RVLM C1 area. Most PR-ir is in presynaptic profiles, some of which are GABAergic, while the remainder is in non-catecholaminergic dendrites and glia.

The demonstration of PR in nuclei in the RVLM of the female Sprague–Dawley rat is consistent with previous reports describing PR nuclei in ventral brainstem regions [10,15]. However, in contrast to other catecholaminergic brain stem regions (e.g., nucleus of the solitary tract and A1 area), C1 adrenergic neurons in the RVLM did not contain detectable levels of nuclear PR. Whether the little nuclear PR that was observed is contained in non-C1 bulbospinal neurons or intrinsic RVLM cells remains to be determined.

The subcellular localization of PRs in the RVLM is similar to the distribution of extranuclear ERα and ERβ [39]. However, PRs were relatively less abundant and were allocated to fewer and different cellular compartments. In particular, unlike PRs, extranuclear ERs were detected in C1 neuronal perikarya and dendrites. Moreover, ERα-ir was prominent in large terminals containing numerous dense-core vesicles similar to afferents from the nucleus of the solitary tract and hypothalamus. PR-containing terminals more closely resembled ERβ-containing terminals in size but, unlike ERβ-labeled terminals, contained a few dense-core vesicles suggesting that they had different cellular origins.

PR-ir was primarily in unmyelinated axons as well as a few myelinated axons and axon terminals. Within axons and axon terminals, PR-ir was usually associated with the plasma membrane. Although likely underestimated due to their different subcellular locations, at least some of the PR-labeled axons contained GAD65-ir. These findings suggest that progestins may rapidly modulate neurotransmitter release, particularly GABA. In support, progestins are modulators of $GABA_A$ receptors as well as glutamatergic NMDA receptors and others [3]. Moreover, activation of PRs in GABAergic axons may affect transduction of electrical signals to the terminal [38].

Although the source of presynaptic PR-containing profiles is unknown, the presence of dense-core vesicles suggests that they colocalize peptides [34]. At least a portion of PR-labeled terminals are GABAergic which arise in part from the caudal ventrolateral medulla as well as the nucleus of the solitary tract [2,5,12]. Previous studies have shown that GABAergic terminals form synapses on and tonically inhibit bulbospinal neurons in the RVLM [7,21]. Thus, these findings suggest that progestins could directly modulate baroreceptor function by acting directly on PRs in presynaptic GABA inputs.

The demonstration that PR-ir is in dendritic and glial profiles is consistent with our other reports examining the cellular distribution of extranuclear gonadal steroid receptors in the RVLM as well as the hippocampal formation [20,22,39]. Within dendritic profiles, PR-ir was primarily detected in the cytoplasm, distal from the plasma membrane suggesting that PRs are primarily in transit and/or stored for future use. Since PR-containing dendrites lacked detectable TH-ir they probably do not arise from C1 neurons. However, whether they arise from non-C1 bulbospinal neurons or interneurons remains to be determined.

The finding that PR-ir is in glial cells is consistent with previous studies demonstrating PR protein in glial cells *in vitro* [14]. The presence of PRs on glial cells could be important in controlling ionic balance and synaptic transmission [13,19]. Moreover, since some PR-ir was found on astrocytic profiles that formed gap junctions, activation of PRs could be involved in calcium signaling between adjacent cells [8].

In conclusion, these findings suggest that progestins can primarily modulate neurons in the C1 area of the RVLM by presynaptic mechanisms involving GABAergic transmission. The presence of extranuclear PRs in the RVLM suggests another mechanism through which progestins may regulate ABP during pregnancy and may contribute to sex differences in central cardiovascular regulation in this critical sympathoexcitatory region.

Acknowledgements

We thank Dr. Annelyn Torres-Reveron, Ms. Nora Tabori and Mr. Scott Herrick for technical assistance.

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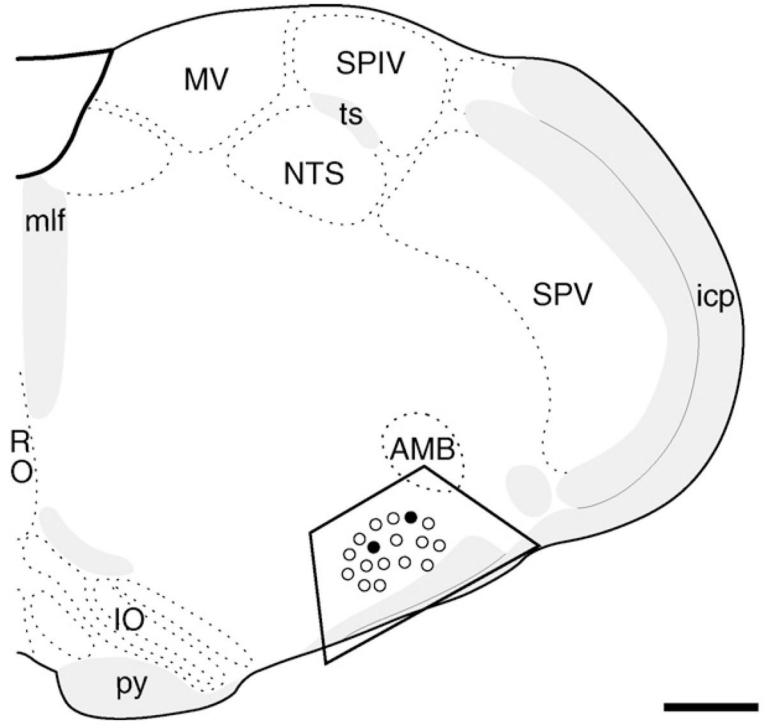

Fig. 1.

Nuclei with PR-ir are intermixed with TH-labeled neurons in the RVLM. Schematic drawing through the medulla showing the level of the RVLM (trapezoid) analyzed light and EM (adapted from Swanson plate 61 [33], about 1.2 mm caudal to bregma) and the relative distribution of PR-labeled (solid circles) and TH-labeled (open circles) nuclei. Abbreviations: AMB, nucleus ambiguus; icp, inferior cerebellar peduncle; IO, inferior olivary complex; mlf, medial longitudinal fascicle; MV, medial vestibular nucleus; NTS, nucleus of the solitary tract; py, pyramidal tract; RO, nucleus raphe obscurus; SPV, spinal nucleus of trigeminal; ts, tractus solitarius; XII, hypoglossal nucleus. Bar, 1 mm.

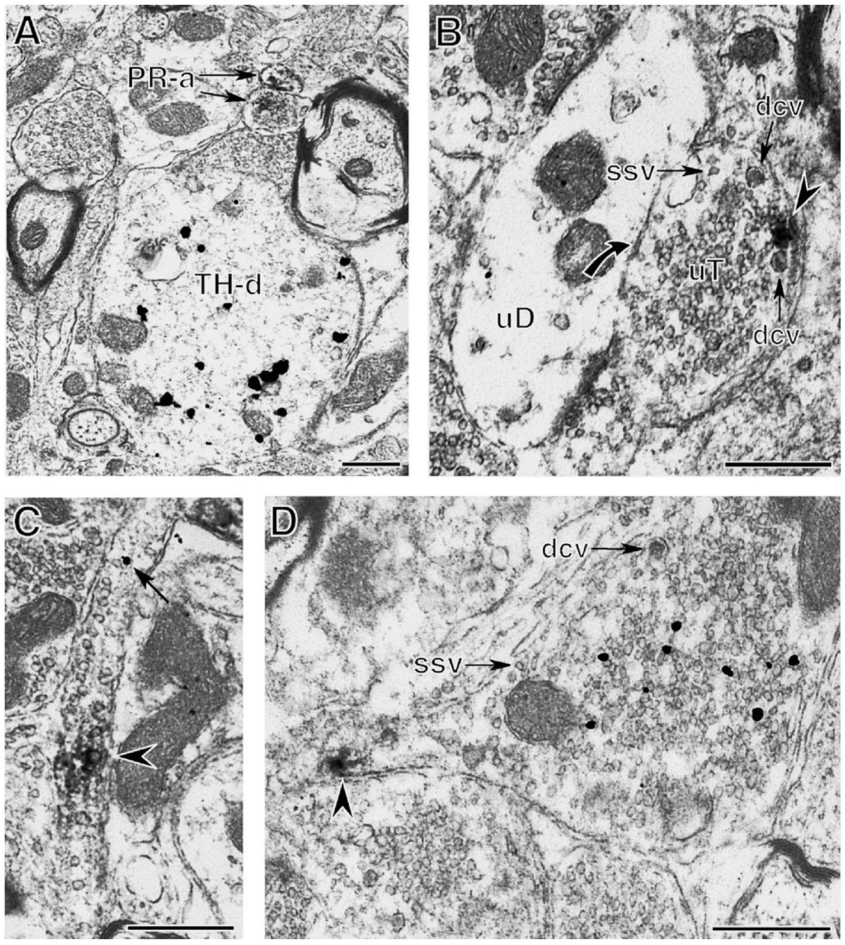

Fig. 2.

Extranuclear PR-ir is found in presynaptic profiles. (A) Two axons with PR-ir (PR-a) are near a TH-immunoreactive dendrite (TH-d). (B) A cluster of PR-ir (arrowhead) is near the plasma membrane of an unlabeled terminal (uT) that contains small synaptic vesicles (ssv) and dense-core vesicles (dcv). The PR-labeled terminal contacts (curved arrow) an unlabeled dendrite (uD). (C) A patch of PR-ir (arrowhead) and immunogold for GAD65 (arrow) are in a longitudinal section of an axon. (D) PR-ir (arrowhead) is found in a preterminal axon of a terminal with GAD65-ir (black particles). The GAD65-labeled terminal has numerous small synaptic vesicles (ssv) and a dense core vesicle (dcv). Bar, 500 nm.

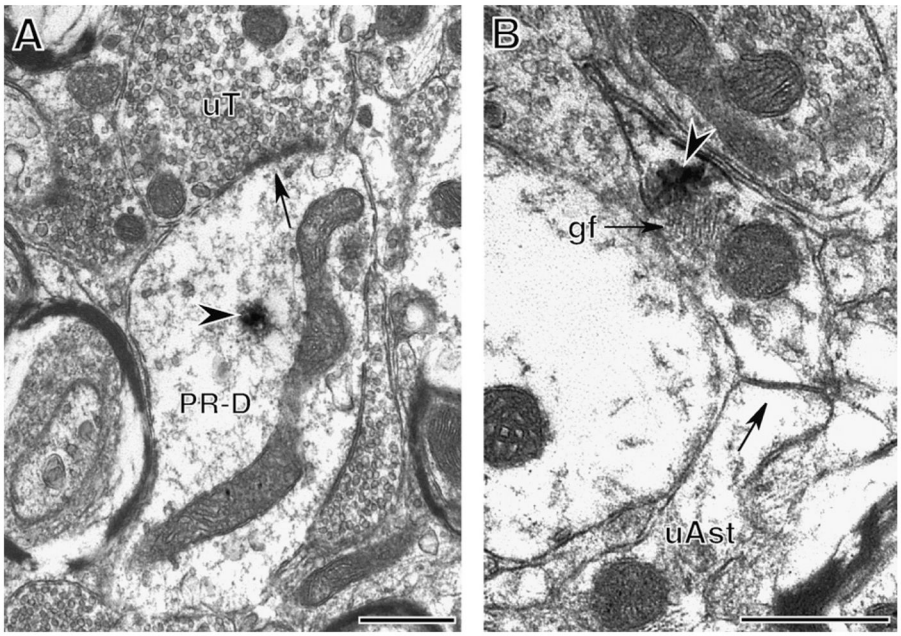

Fig. 3.

PR-ir is found in dendrites and glia. (A) A cluster of PR-ir (arrowhead) is in the cytoplasm of a dendrite (PR-D). An unlabeled terminal (uT) contacts (arrow) the PR-containing dendrite. (B) PR-ir (arrowhead) is in an astrocytic process identified by glial filaments (gf) that forms a gap junction (arrow) with an unlabeled astrocytic profile (uAst). Bar, 500 nm.