

Immunohistochemical evidence for the localization of neurons containing the putative transmitter, L-proline, in rat brain

Takemoto Yumi* and Semba Reiji**

*Department of Neurophysiology, Graduate School of Biomedical Sciences, Hiroshima University, Japan, **Institute for Developmental Research, Aichi Human Service Center, Japan

Corresponding author: Takemoto Yumi, Kasumi 1-2-3, Minami-ku, Hiroshima, 734-8551 Japan. Fax: +81-82-257-5128. E-mail: yumitake@hiroshima-u.ac.jp

Number of figures: 4 figures, number of pages: 11 pages

Abstract

We examined whether there are the neurotransmitter candidate amino acid L-proline containing neurons localized in the rat brain. Antibodies against L-proline conjugated with rabbit serum albumin were raised in a rabbit and purified with affinity chromatography. Strong L-proline-like immunoreactivity was confined to several groups of neurons in the arcuate nucleus (n) and supraoptic n in the hypothalamus and area postrema. The brainstem had markedly stained fibers in the medial longitudinal fasciculus and localized neuronal cell body labeling in the red n, mesencephalic trigeminal n, lateral reticular n, raphe obscurus n, solitary n, compact ambiguus n, motor trigeminal n, and n of trapezoid body. Our findings are consistent with the hypothesis that L-proline may function as a neurotransmitter or neuromodulator in the brain.

Classification terms

Theme D: Neurotransmitters, modulators, transporters, and receptors.

Topic: other neurotransmitters

Key words: L-proline, immunohistochemistry, putative neurotransmitter, brainstem, hypothalamus, rats

There is a long history of evidence suggesting that the non-essential amino acid L-proline may be a neuronal modulator or transmitter in the central nervous system [2-5, 7, 9, 11, 19]. Namely, Several criteria of neurotransmitter fit L-proline. Namely, this amino acid is biosynthesized by the enzyme, pyrroline 5-carboxylate reductase (EC1.5.1.2) in the rodent brain [19], and released as radiolabeled L-proline by a potassium stimulation of neurons in brain and spinal cord slices [7]. L-Proline produces electrophysiological actions in the spinal cord and brain [2]. Then, a proline transporter cloned from a rat forebrain c-DNA library is widely distributed in the rat brain [3] and in synaptic vesicles [9]. A research using genetically modified mice that have increased plasma concentration due to lack of catabolic enzyme, proline dehydrogenase, indicates modulated function of sensorimotor gating [5]. In addition, we have demonstrated cardiovascular responses to exogenous injection of L-proline in the brain of the conscious rat [12-14]. However, evidence for localized distribution of L-proline itself in the brain is lacking. Therefore, the present study employed immunohistochemistry with newly produced L-proline-specific antibodies to investigate whether L-proline is localized within a particular structure in the rat brain. A brief report of this work has appeared in abstract form [18].

All protocols and surgical procedures used in this study were performed in accordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan and the Guideline for Animal Experiment in Hiroshima university and the Committee of Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University.

The antigen of L-proline (0.1 mmol) conjugated with rabbit serum albumin (12 mg) was produced using the glutaraldehyde method of Aoki et al. [1]. Antiserum was raised in

a rabbit, by intradermal injections of the L-proline-conjugated antigen into the back. The antiserum was first absorbed with the structurally similar amino acid pipecolic acid (Fig. 1) bound to glutaraldehyde, and further pre-absorbed with L-proline bound Sepharose 6B affinity chromatography customized according to the method of Saito and Tanaka [10]. The specificity of the purified (eluted) antibodies was confirmed by a dot immunobinding assay [6, 20]. Briefly, diluted solutions of amino acid-rabbit serum albumin conjugates prepared by the method of Aoki et al. [1] were spotted on a nitrocellulose membrane. The membrane was incubated with purified antibodies (20 ng/ml), then with anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase. Labeling was visualized by reaction with 3-3'-diaminobenzidine tetrahydrochloride (DAB). The dot immunobinding assay detected the conjugate of L-proline at a concentration of 1000 times lower than for L-pipecolate conjugate (Fig. 1). The antibodies selectively detected L-proline antigen, but not that of D-proline, L-glutamate, L-aspartate, gamma-aminobutyric acid (GABA), or glycine (Fig. 1). Thus the purified antibody was highly specific for L-proline.

Brain sections were prepared from 7 male Wistar rats (over 10 weeks old) anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The abdominal aorta was blocked with a hemostat secured below the level of the thoracic aorta and the upper body of the rat was perfused intracardially with physiological saline (20 - 40 ml) followed by the fixative solution containing 4% paraformaldehyde, 0.2% picric acid, 1% glutaraldehyde, 2% sucrose, and 0.9% NaCl in 0.1 M acetate buffer pH 6 (500 ml). Blocks from the removed brain were further post-fixed for 30 - 60 min. Fifty-micron thick sections were collected with a vibratome microslicer (DTK-1500) and further chemically reduced with 0.5 % dimethyl amine borane for 1-2 h.

Immunohistochemistry was performed after the floating slices were treated with

0.3 % triton X-100 - phosphate buffered saline (TPBS) for 20 min and blocked with 1 % bovine serum albumin - TPBS for 10 min. The slices were incubated with purified proline antibody solution (20 - 50 ng/ml TPBS) overnight, and subsequently with biotinylated goat anti-rabbit IgG antiserum for 1 h. The reaction was amplified with a 1-h avidin D-horseradish peroxidase incubation, and labeling was visualized with DAB reaction in the presence of 0.006 % hydrogen peroxide and 100 mM imidazole. Slices were mounted on glass slides, covered with Crystal/Mount (Biomedica Corp.) and examined with a DP12 or DP70 Image Analysis System (Olympus light microscope with mounted digital camera).

The brain contains the lysine metabolite L-pipecolic acid at one tenth to one fiftieth of the quantity of L-proline [8]. The sizes of the aromatic rings in molecular structures of proline and pipecolate are quite similar (Fig. 1). Without pipecolate-glutaraldehyde conjugate absorption, the antibodies showed substantial cross reactivity with pipecolate. Incorporating the pre-absorption step with the pipecolate conjugate enabled us to obtain antibodies that are highly specific to L-proline: L-proline antigen could be detected at a concentration 1000 times lower than L-pipecolate antigen (Fig. 1).

Immunohistochemistry with the pre-absorbed L-proline specific antibody clearly revealed positively labeled cell bodies and fibers in a group of neurons in the brainstem and hypothalamus. Figure 2 shows positively labeled neurons in the magnocellular red nucleus and mesencephalic trigeminal nucleus in a mesencephalic frontal section. The greatest neuronal cell body and/or fiber labeling was observed in the arcuate (Fig. 3) and supraoptic nuclei of the hypothalamus, the area postrema and the medial longitudinal fasciculus in the medulla (Fig. 4). We observed several regions of neuronal cell body labeling in the brainstem, including lateral reticular nucleus, raphe obscurus nucleus, solitary nucleus, compact ambiguus nucleus, motor trigeminal nucleus, and nucleus of

trapezoid body.

The present results indicate that L-proline-like immunoreactivity is localized in a group of fibers and neuronal cell bodies in the rat brainstem and hypothalamus. We expected to see terminal-like bead structures along the immunoreactive fibers (axons) close to the ventral and dorsal medulla, as these sites produce cardiovascular responses to exogenous proline [15-17]. However, we found the potential bead-structures only in the hypothalamus. The proline transporter by Fremeau Jr. et al. was found to be distributed widely in the brain [3] and at synaptic vesicles of neuronal terminals in the rat forebrain [9]. However, this transporter has no characteristics of classic vesicular transporters: no amino acid sequence homology and a sodium gradient energy driving force to transport transmitters distinct from a proton gradient energy driving force of classic vesicular transporters [9]. Fremeau Jr. et al. [3] have obtained the cloned transporter, based on the conserved amino acid sequences from the sodium-dependent norepinephrine and gamma-aminobutyric acid transporters in the rat forebrain cDNA. The methodological origin of amino acid sequence appears to give a limitation for a vesicular transporter. The other type of classic proline transporters which has the vesicular transporter amino acid homology with the proton gradient driving force may exist specifically in neurons of the brainstem or hypothalamus. If so, they would provide a powerful tool for finding general proline containing terminals.

Positive immunoreactivity was observed in areas corresponding to the amygdala, piriform cortex, and hippocampus. However observation of these regions at a higher magnification revealed staining only in the outer parts of the cytoplasm, but not of fibers, as well as strong background staining. The positive reactivity in cortical and allocortical regions was not confined to neurons. Thus although there is L-proline-like material in

neurons and their surrounding areas of the higher brain (e.g. amygdala, piriform cortex, and hippocampus), the role of L-proline as a neurotransmitter in these regions remains unclear.

Genetically modified mice, which produce increased plasma proline concentration due to proline catabolic deficiency, produce a higher content of proline in the hypothalamus, hippocampus, amygdala, and cortex [5], suggesting that these regions may have L-proline containing neurons or terminals. As shown in Fig. 3, we observed clearly stained neuronal cell bodies and fibers in the hypothalamus. The other higher brain regions did not give clear and selective neuronal labeling. The underlying reason for this discrepancy remains to be determined. For example, it is possible that L-proline may function as a diffuse neuromodulator in the higher brain but as a traditional neurotransmitter in the lower brain regions and hypothalamus. Alternatively, the high L-proline content observed in the higher brain regions may not be related to neuronal signaling, but rather an artifact associated with cross reactivity (i.e. high concentrations of pipercolic acid) or metabolism (general increase in brain L-proline content for protein synthesis in genetically modified mice [5]). Additionally, some methodological reasons such as types of fixation may result in the discrepancy.

It may be suggested that the proline antibodies might be detecting constituent L-proline residues within all proteins. However, the dot immunobinding assay using amino acid (D-proline, L-glutamate, L-aspartate, GABA, or glycine) -rabbit serum albumin conjugates did not show staining on the nitrocellulose membrane. This finding demonstrates that reactivity of the antibody with L-proline residues within proteins is quite low or negligible.

In conclusion, by developing a selective L-proline antibody and employing it in

immunohistochemistry experiments, we demonstrated that L-proline appears to be localized within several groups of neurons. Our findings provide support for the hypothesis that L-proline may function as a neurotransmitter or a neuromodulator in the brain.

References

- [1] E. Aoki, R. Semba, K. Kato and S. Kashiwamata, Purification of specific antibody against aspartate and immunocytochemical localization of aspartergic neurons in the rat brain, *Neurosci.* 21 (1987) 755-765.
- [2] D. Felix and H. Künzle, The role of proline in nervous transmission, *Adv. Biochem. Psychopharmacol.* 15 (1976) 165-173.
- [3] R. T. Fremeau Jr, M. G. Caron and R. D. Blakely, Molecular cloning and expression of a high affinity L-proline transporter expressed in putative glutamatergic pathways of rat brain. *Neuron* 8 (1992) 915-926.
- [4] E. Giacobini, Imino acids of the brain. In: Laitha (Ed.), *Handbook of Neurochemistry* 2nd ed. Metabolism in the Nervous System, Vol. 3, Plenum, New York, 1983, pp. 583-605.
- [5] J. A. Gogos, M. Santha, Z. Takacs, K. D. Beck, V. Luine, L. R. Luca, J. V. Nadler and M. Karayiorgou, The gene encoding proline dehydrogenase modulates sensorimotor gating in mice, *Nature Genetics* 21 (1999) 434-439.
- [6] R. Hawkes, E. Niday and J. Gordon, A dot-immunobinding assay for monoclonal and other antibodies, *Anal. Biochem.* 119 (1982) 142-147.
- [7] A. H. Mulder and S. H. Snyder, Potassium-induced release of amino acids from cerebral cortex and spinal cord slices of the rat, *Brain Res.* 76 (1974) 297-308.
- [8] H. Nishio, Y. Yamada, E. Giacobini and T. Segawa, Distribution of pipercolic acid and proline in the developing rat brain and peripheral organs, *Int. J. Devl. Neurosci.* 3 (1985) 379-384.
- [9] S. E. Renick, D. T. Kleven, J. Chan, K. Stenius, T. A. Milner, V. M. Pickel and R. T. Fremeau Jr, The mammalian brain high-affinity L-proline transporter is enriched

preferentially in synaptic vesicles in a subpopulation of excitatory nerve terminals in rat forebrain, *J. Neurosci.* 19 (1999) 21-33.

[10] N. Saito and C. Tanaka, Immunohistochemical demonstration of GABA containing neurons in the guinea pig ileum using purified GABA antiserum, *Brain Res.* 376 (1986) 78-84.

[11] S. H. Snyder, A. B. Young, J. P. Bennett and A. H. Mulder, Synaptic biochemistry of amino acids, *Fed. Proc.* 32 (1973) 2039-2047.

[12] Y. Takemoto, Amino acids with central pressor effect in conscious rats, *Jpn. J. Physiol.* 40 (1990) 561-565.

[13] Y. Takemoto, Regional hemodynamic changes and vasopressin release induced by intracisternal injection of L-proline in the conscious rat, *Jpn. J. Physiol.* 45 (1995) 743-758.

[14] Y. Takemoto, Kynurenic acid inhibits circulatory responses to intracisternally injected L-proline in conscious rats, *Neurosci. Lett.* 261 (1999) 121-123.

[15] Y. Takemoto, Depressor and bradycardic actions of L-proline injected into the nucleus tractus solitarii of anesthetized rats, *Jpn. J. Physiol.* 51 (2001) 687-692.

[16] Y. Takemoto, L-Proline microinjected into the rat ventrolateral medulla induces a depressor response distinct from L-glutamate, *Jpn. J. Physiol.* 54 (2004) 339-345.

[17] Y. Takemoto, Depressor responses to L-proline microinjected into the rat ventrolateral medulla are mediated by ionotropic excitatory amino acid receptors, *Auton. Neurosci.* 120 (2005) 108-112.

[18] Y. Takemoto, H. Kawano and R. Semba, Localization of L-proline in a group of neurons in the rat brain, *Jpn. J. Physiol.* 54 (2004) s212.

[19] S. Yoneda and E. Roberts, A new synaptosomal biosynthetic pathway of proline from ornithine and its negative feedback inhibition by proline, *Brain Res.* 239 (1982) 479-488.

[20] K. Wako, N. Ma, T. Shiroyama and R. Semba, Glial uptake of intracerebroventricularly injected D-serine in the rat brain: an immunocytochemical study, *Neurosci. Lett.* 185 (1995) 171-174.

Figure legends

Fig. 1. Dot-immunobinding assay showing high specificity of purified antibodies against L-proline antigen. Brown DAB spots on a nitrocellulose membrane at left were obtained by reacting diluted antigens (A: L-proline-glutaraldehyde-rabbit serum albumin; B: L-pipecolic acid-glutaraldehyde-rabbit serum albumin) with the purified primary rabbit anti-proline antibody (20 ng/ml) and secondary goat anti-rabbit IgG conjugated with horseradish peroxidase. The right photograph shows no reactivity of the purified anti-proline antibody (0.2 µg/ml) with either glutaraldehyde-rabbit serum albumin conjugate (20 µg/ml) of L-aspartic acid (1), L-glutamic acid (2), gamma-aminobutyric acid (3), glycine (4), or D-proline (5). A brown spot at (6) indicates positive reaction with L-proline-glutaraldehyde-rabbit serum albumin (20 µg/ml).

Fig. 2. A: Localized distribution of anti-L-proline- immunoreactive neurons in the magnocellular red nucleus (RMC) and mesencephalic trigeminal nucleus (Me5) in a frontal section. No neuron-specific labeling was apparent at higher magnification in the area with background staining. B and C: Magnified positively stained neuronal cell bodies and fibers in the RMC and Me5 of the frontal section A. There was stronger staining in the axons than in somata (B). Note that background staining around labeled cells is weak in C. Scale bars in B and C = 50 µm.

Fig. 3. Neuronal cell bodies and fibers in the arcuate nucleus of the hypothalamus in a sagittal section with strong L-proline-immunoreactivity. Several immunoreactive bead-like structures associated with the stained fibers such as marked by dotted ellipses, suggestive of axon terminals, were visible. Scale bar = 50 µm.

Fig. 4. Fibers in the medulla corresponding to the medial longitudinal fasciculus in a frontal section were strongly labeled by immunohistochemistry with the anti-L-proline

antibody. Scale bar = 50 μm .

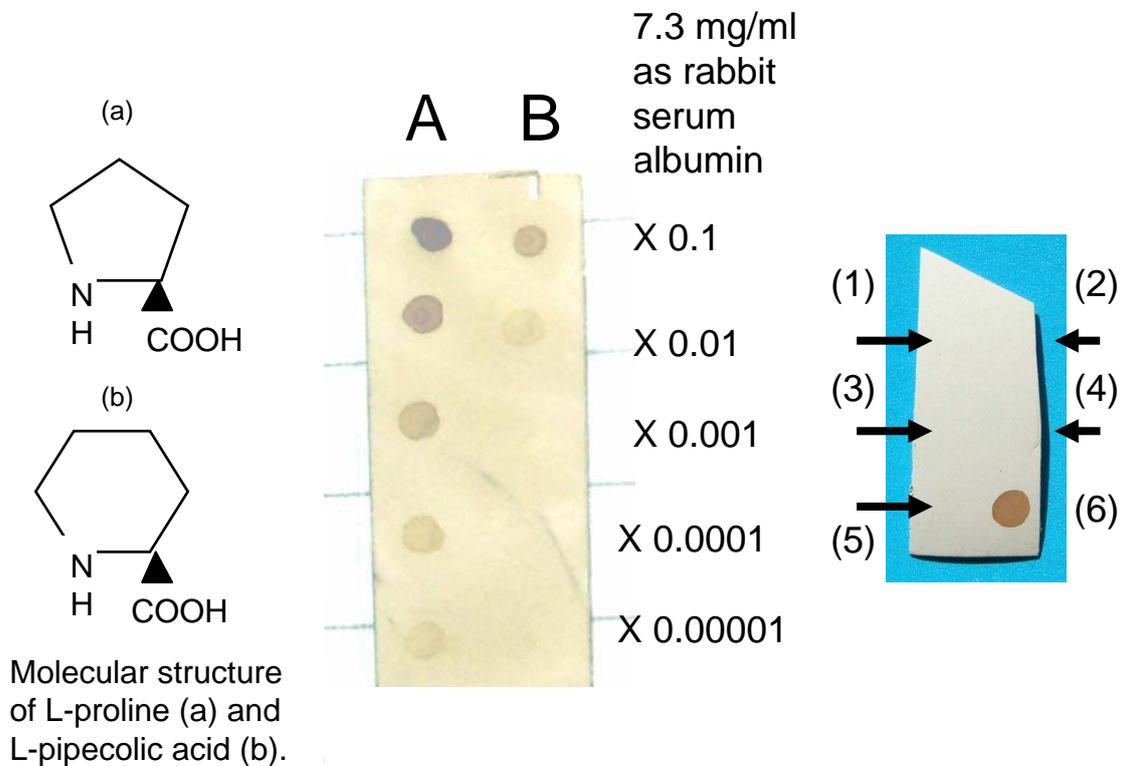
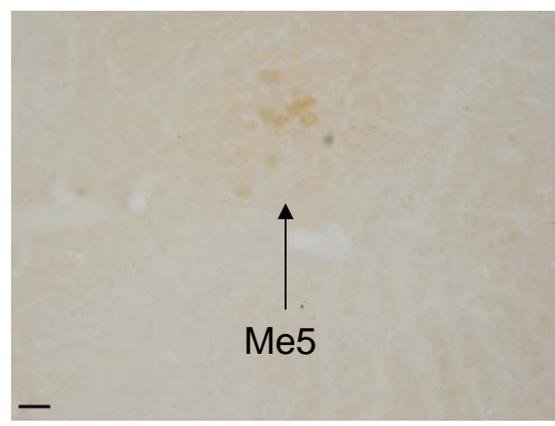
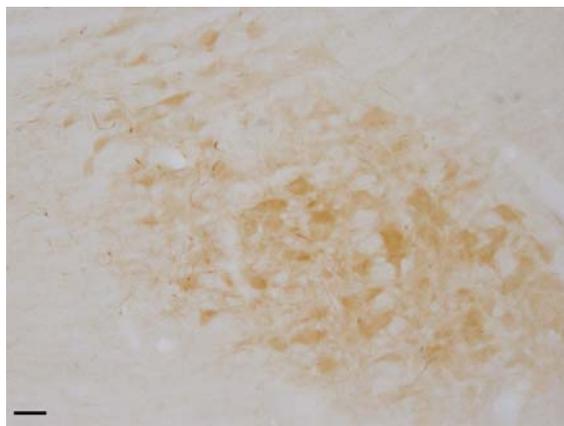
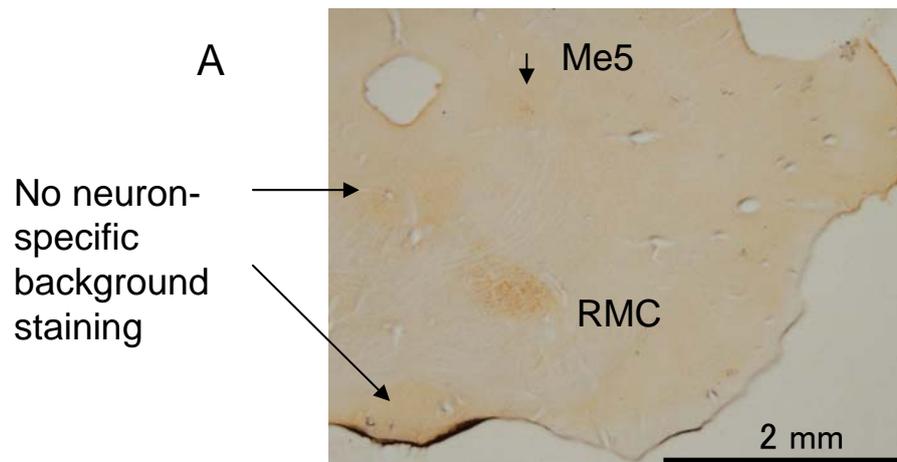


Fig. 1. Dot-immunobinding assay showing high specificity of purified antibodies against L-proline antigen. Brown DAB spots on a nitrocellulose membrane at left were obtained by reacting diluted antigens (A: L-proline-glutaraldehyde-rabbit serum albumin; B: L-pipecolic acid-glutaraldehyde-rabbit serum albumin) with the purified primary rabbit anti-proline antibody (20 ng/ml) and secondary goat anti-rabbit IgG conjugated with horseradish peroxidase. The right photograph shows no reactivity of the purified anti-proline antibody (0.2 $\mu\text{g/ml}$) with either glutaraldehyde-rabbit serum albumin conjugate (20 $\mu\text{g/ml}$) of L-aspartic acid (1), L-glutamic acid (2), gamma-aminobutyric acid (3), glycine (4), or D-proline (5). A brown spot at (6) indicates positive reaction with L-proline-glutaraldehyde-rabbit serum albumin (20 $\mu\text{g/ml}$).



B

C

Fig. 2. A: Localized distribution of anti-L-proline-immunoreactive neurons in the magnocellular red nucleus (RMC) and mesencephalic trigeminal nucleus (Me5) in a frontal section. No neuron-specific labeling was apparent at higher magnification in the area with background staining. B and C: Magnified positively stained neuronal cell bodies and fibers in the RMC and Me5 of the frontal section A. There was stronger staining in the axons than in somata (B). Note that background staining around labeled cells is weak in C. Scale bars in B and C = 50 μm.

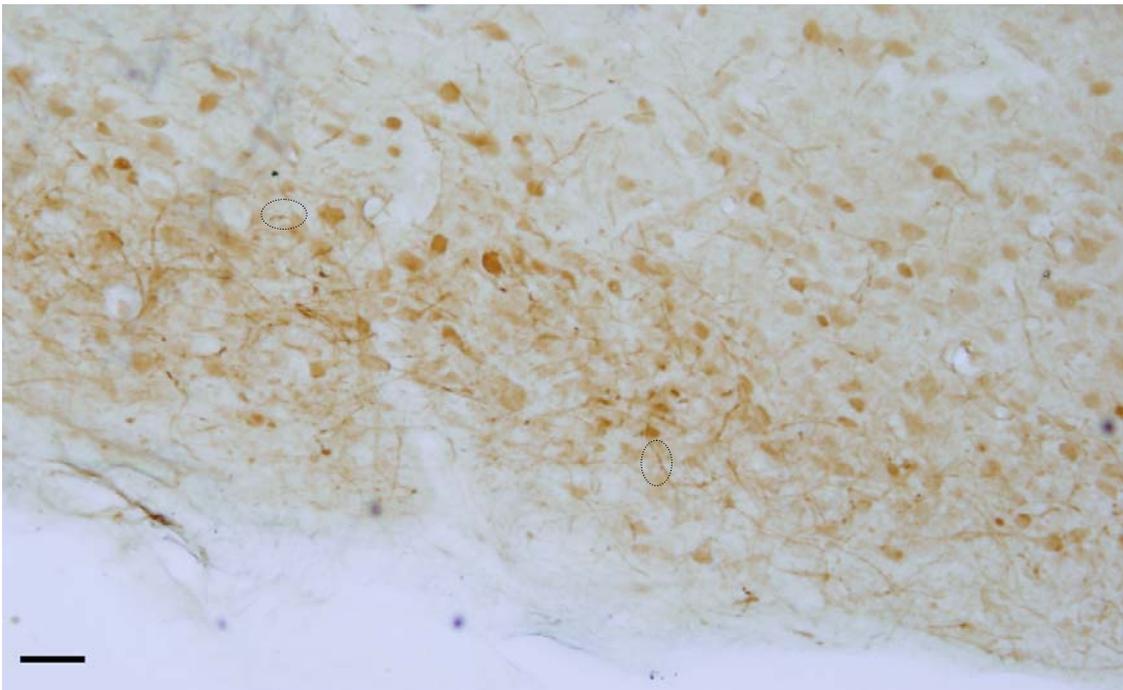


Fig. 3. Neuronal cell bodies and fibers in the arcuate nucleus of the hypothalamus in a sagittal section with strong L-proline-immunoreactivity. Several immunoreactive bead-like structures associated with the stained fibers such as marked by dotted ellipses, suggestive of axon terminals, were visible. Scale bar = 50 μ m.

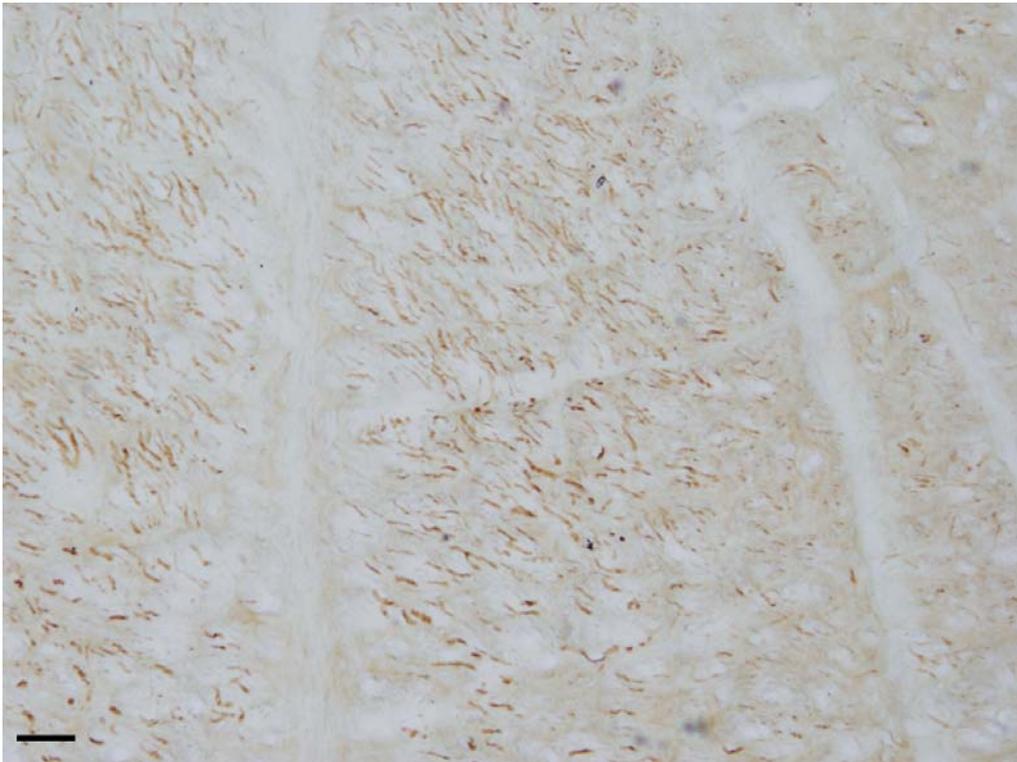


Fig. 4. Fibers in the medulla corresponding to the medial longitudinal fasciculus in a frontal section were strongly labeled by immunohistochemistry with the anti-L-proline antibody. Scale bar = 50 μ m.