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BRAIN RESEARCH

Short Communication

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

Yumi Takemoto[a,*], *Reiji Semba*[b]

[a]*Department of Neurophysiology, Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan*
[b]*Institute for Developmental Research, Aichi Human Service Center, Japan*

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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.

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 (Felix and Künzle, 1976; Fremeau et al., 1992; Giacobini, 1983; Gogos et al., 1999; Mulder and Snyder, 1974; Renick et al., 1999; Snyder et al., 1973; Yoneda and Roberts, 1982). 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 (Yoneda and Roberts, 1982) and released as radiolabeled L-proline by a potassium stimulation of neurons in brain and spinal cord slices (Mulder and Snyder, 1974). L-proline produces electrophysiological actions in the spinal cord and brain (Felix and Künzle, 1976). Then, a proline transporter cloned from a rat forebrain c-DNA library is widely distributed in the rat brain (Fremeau et al., 1992) and in synaptic vesicles (Renick et al., 1999). 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 (Gogos et al., 1999). In addition, we have demonstrated cardiovascular responses to exogenous injection of L-proline in the brain of the conscious rat (Takemoto, 1990, 1995, 1999). 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 (Takemoto et al., 2004).

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

* *Corresponding author*. Fax: +81 82 257 5128.
E-mail address: yumitake@hiroshima-u.ac.jp (Y. Takemoto).

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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. (1987). 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 (1986). The specificity of the purified (eluted) antibodies was confirmed by a dot immunobinding assay (Hawkes et al., 1982; Wako et al., 1995). Briefly, diluted solutions of amino acid–rabbit serum albumin conjugates prepared by the method of Aoki et al. (1987) 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 (Biomeda 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 (Nishio et al., 1985). The sizes of the aromatic rings in molecular structures of proline and pipecolate are quite similar (Fig. 1). Without pipecolate–glutaraldehyde conjugate absorption, the

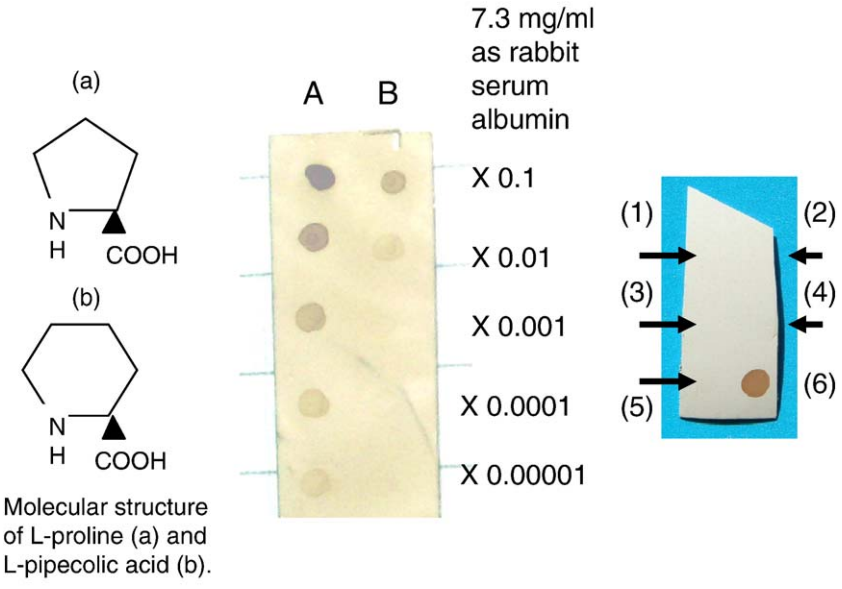

Fig. 1 – Dot immunobinding assay showing high specificity of purified antibodies against L-proline antigen. Brown DAB spots on a nitrocellulose membrane at left were obtained by reacting diluted antigens (A: L-proline–glutaraldehyde–rabbit serum albumin; B: L-pipecolic acid–glutaraldehyde–rabbit serum 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).