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Letter to Neuroscience

NATURALLY OCCURRING FREE D-ASPARTATE IS A NUCLEAR COMPONENT OF CELLS IN THE MAMMALIAN HYPOTHALAMO-NEUROHYPOPHYSEAL SYSTEM

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It is generally believed that only L-amino acids have a physiological role in species other than bacteria. Recently, the existence of some D-amino acids, particularly D-aspartate, in various organs of several higher animals has been reported. Here we demonstrate that naturally occurring free D-aspartate is localized subcellularly to the heterochromatin in the nucleoli (but not in either the dendrites or axonal terminals) of magnocellular neurosecretory neurons in the rat hypothalamus, and also of microglia and pericytes in the posterior pituitary. Our results imply that naturally occurring free D-aspartate might have a physiological role in nuclear function in mammals. The findings provide new insight for the biological function of D-stereoisomers of amino acids as well as the organization of the nucleus of at least some eukaryotic cells. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Based on the widely accepted assumption that D-amino acids do not have physiological function in higher species, many D-isomers have been used to investigate various physiological and pathological processes *in vivo*. For example, D-aspartate is widely used as a putatively metabolically inert analogue of L-glutamate to study glutamate transporter systems. However, the existence of some D-amino acids, particularly D-aspartate, in a number of higher animals has recently been demonstrated by several groups (Dunlop et al., 1986; Hashimoto et al., 1992, 1993; Kera et al., 1995; Hamase et al., 1997; Neidle et al., 1990). Additional evidence has also suggested several functions for these D-isomers (Sakai et al., 1997, 1998; D'Aniello et al., 1996; Difiore et al., 1998; Schell et al., 1995). Speculation has therefore arisen that some D-amino acids might be involved in the physiology of eukaryotes.

By using an antiserum highly specific against free D-aspartate, this D-isomer had previously been localized to various regions of the brain and numerous endocrine glands in the rat (Schell et al., 1997). One of these endogenous D-aspartate-positive structures was the hypothalamo-neurohypophyseal system, the anatomical organization of which is relatively well known. Mammalian magnocellular neurosecretory neurons are primarily clustered in the supraoptic nucleus (SON) and the paraventricular nucleus of the hypothalamus. They principally secrete either vasopressin or oxytocin from their nerve terminals in the posterior pituitary into the systemic circulation in response to a variety of different physiological stimuli (Gainer and Wray, 1994). Their dendrites in the hypothalamus, in addition to the classic role of signal reception, contain substantial amounts of neuropeptides and release the oxytocin and vasopressin within the brain (Morris et al., 1998). To begin to elucidate the precise role of naturally occurring D-aspartate, immunogold labeling at the electron microscope level was employed in the present study to localize subcellularly the D-amino acid in the rat SON of the hypothalamus and posterior pituitary.

In the hypothalamic SON, in which the cell bodies and dendrites of magnocellular neurons cluster, naturally

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Abbreviations: SON, supraoptic nucleus.

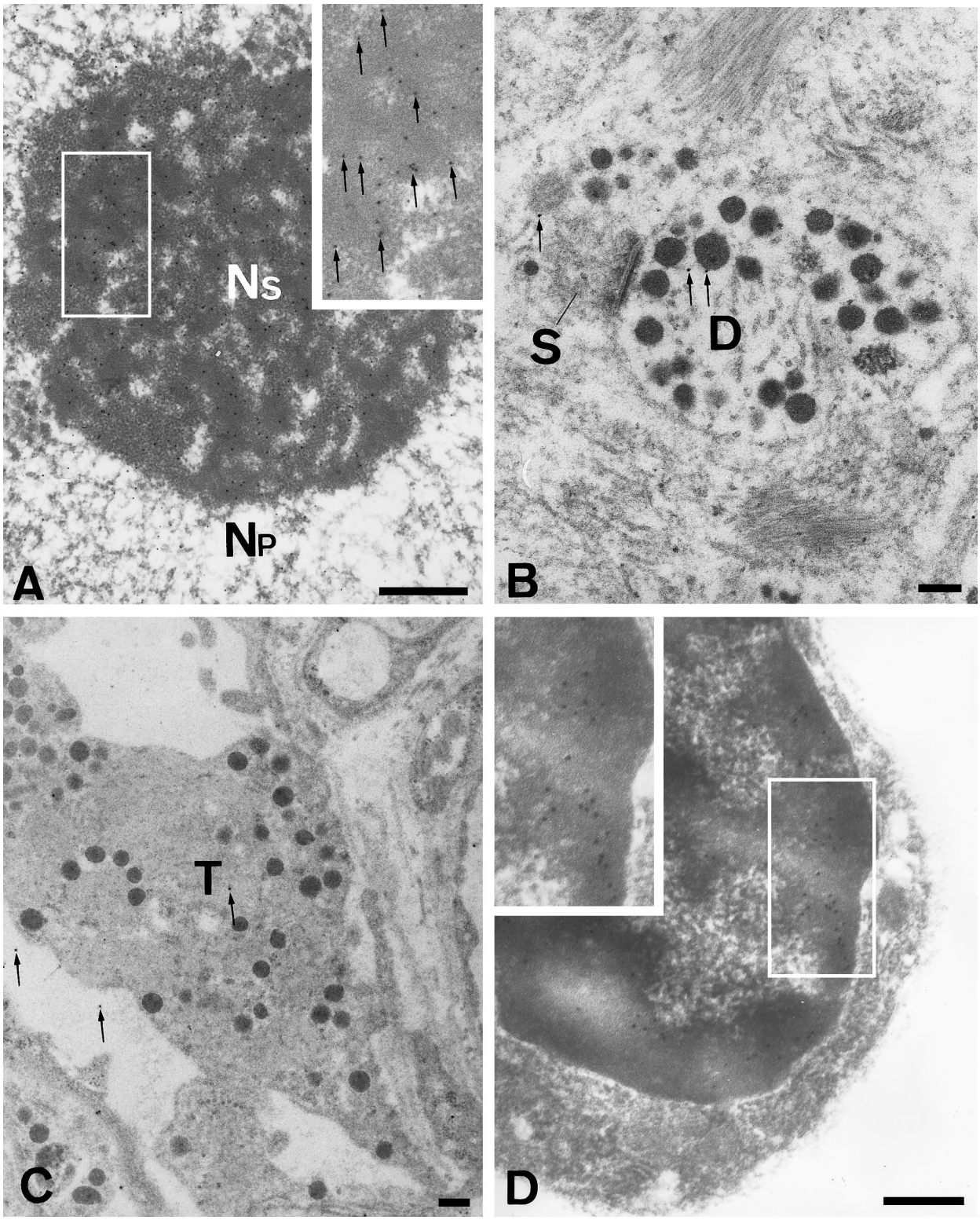

Fig. 1. Electron micrographs of the SON of the hypothalamus and posterior pituitary after immunogold labeling of ultrathin sections for naturally occurring free D-aspartate. Osmium tetroxide fixation could not be used in the processing for electron microscopy because this treatment completely abolished immunoreactivity of D-aspartate (Gundersen et al., 1993). Subcellular structure and particularly membrane structure was therefore not optimally preserved. (A) D-aspartate immunoreactivity was found in the nucleolus (Ns) but not the nucleoplasm (Np) of the magnocellular neurons. The higher magnification insert is printed at low contrast so that the gold particles (arrows) can be distinguished against the electron-dense heterochromatin. (B) No D-aspartate immunoreactivity was found in the cytoplasm or in dendrites (D) of the magnocellular neurons (S, synaptic bouton identifying the profile as a dendrite) or (C) in the magnocellular axonal terminals (T) in the posterior pituitary. In these tissues only scattered non-specific gold particles are present (arrows). (D) Naturally occurring free D-aspartate was also detected in the heterochromatin beneath the nuclear envelope of microglia in the posterior pituitary. The insert is again printed at low contrast to allow the gold particles to be distinguished from the electron-dense heterochromatin. Scale bars = 200 nm.

occurring D-aspartate was selectively localized to the nucleoli of the magnocellular neurons (Fig. 1A). More precisely, the immunolabeling was predominantly associated with the electron-dense structure generally termed heterochromatin (Ross et al., 1995). Few gold particles were found in any other parts of the nucleoli, even in the electron-sparse areas intermingled with the heterochromatin (Fig. 1A). There was no labeling above background in other subcellular structures of the nucleus and soma, including the nucleoplasm and cytoplasm. The D-amino acid was also not found in the dendrites of these neurons (Fig. 1B). No immunogold labeling above background was found in the heterochromatin or nuclei of astrocytes in the supraoptic nucleus, or in cells just outside the supraoptic nucleus, demonstrating that the D-aspartate immunoreactivity observed in the magnocellular neurons is not due to the non-specific binding of immunoglobulins to the materials in the nucleolus.

In the posterior pituitary, where the axon terminals of the magnocellular neurons contact capillaries, no D-aspartate was detectable in the neurosecretory terminals (Fig. 1C), implying that naturally occurring D-aspartate is unlikely to be released as a neurotransmitter or neurohormone. Interestingly, the nuclei of microglial cells in the posterior pituitary were intensely labeled. Here, D-aspartate was immunolocalized to the heterochromatin under the nuclear membrane envelope (Fig. 1D). Again, no gold particles were found in the nucleoplasm or outside the nucleus. A similar localization of the naturally occurring D-aspartate was observed in putative pericytes (not illustrated). Pituicytes, the equivalent of astrocytes in the pituitary, lacked any free D-aspartate immunolabeling, which again demonstrates that the immunogold labeling is specific.

Background gold particles in all specimens examined were negligible (< 2 gold particles/μm^2, compared to an average of 32 ± 7 particles/μm^2 in the nucleoli of magnocellular neurons). Very low numbers of gold particles were observed in all other areas, even within the nucleoplasm of neurons with intensely labeled heterochromatin (Fig. 1A, D). The localization to heterochromatin in some neuronal and non-neuronal cells *in situ* suggests a physiological role of naturally occurring D-aspartate in the function of the nucleus. Because one of its locations is in the nucleoli (predominantly in the heterochromatin) of neurons and these cells no longer divide, D-aspartate is unlikely to be involved in DNA replication. We hypothesize that a likely physiological function of naturally occurring free D-aspartate could be in the control of gene expression. At least two broad mechanisms for the function of free D-aspartate in the nucleus could be proposed: D-aspartate could directly interact with DNA; and/or D-aspartate could act on nuclear proteins to maintain the structure and/or active/inactive state of genes in order to control transcription. Resolution of these possibilities will require substantial further experimentation.

EXPERIMENTAL PROCEDURES

Twelve 2- to 10-month-old female Long–Evans rats were purchased from Charles River (Wilmington, MA, USA), housed under conditions of 14 h of light and 10 h of darkness, and provided with food and water *ad libitum*. All animal care and experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all efforts were made to minimize the number of animals used and their suffering. Individual rats were processed separately. All animals were anaesthetized by overdose of pentobarbital and perfused transcardially with 5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) between 10:00 and 11:00 a.m. The brains and pituitaries were removed and immersed in the same fixative overnight at 4°C. Serial coronal sections, 100 μm thick, across the SON of the hypothalamus and posterior pituitary were obtained from the animals and processed further for electron microscopic visualization and immunogold labeling as previously described, but without osmium tetroxide fixation, because this treatment resulted in complete removal of the immunoreactivity of D-aspartate (Gundersen et al., 1993). The subcellular structures were therefore not optimally preserved. The rabbit anti-D-aspartate antibody used in the immunogold labeling was produced by immunizing the animals with D-aspartate coupled to bovine serum albumin with glutaraldehyde and the colloidal gold technique, and then affinity purified. Its high specificity for the single D-isomer (not L-aspartate, its analogues, or a variety of aspartate-containing peptides) has been established, and preabsorption with free D-aspartate completely abolishes the immunoreactivity (Schell et al., 1997). Technical controls without the primary detection agent (antibody against D-aspartate) were included in all the experiments in the present study. All cell types and subcellular structures were identified by their electron microscopic morphology. Quantitative analyses of the intensity of immunogold labeling were carried out as follows: two ultrathin sections from each sectioned SON (tissue block) were examined and, in each section, the first three magnocellular neuron profiles with transected nucleoli were observed. The numbers of immunogold particles were counted, the radii of the respective nucleoli were measured, and their area calculated. The values for the number of gold particles and the areas of the sectioned nucleoli from any one animal were added together. The number of gold particles per μm^2 of nucleolus was calculated to represent the average intensity of immunoreactivity for that animal. The background immunogold was determined by counting all the gold particles in randomly sampled electron microscopy grids (two from each ultrathin section).

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REFERENCES

D'Aniello, A., Di Cosmo, A.M., Di Cristo, C., Annunziato, L., Fisher, G., 1996. Involvement of D-aspartic acid in the synthesis of testosterone in rat testes. Life Sci. 59, 97–104.

Difiore, M.M., Assisi, L., Botte, V., D'Aniello, A.J., 1998. D-aspartic acid is implicated in the control of testosterone production by the vertebrate gonad. Studies on the female green frog Rana esculenta. J. Endocrinol. 157, 199–207.

Dunlop, D.S., Neidle, A., McHale, D., Dunlop, D.M., Lajtha, A., 1986. The presence of free D-aspartic acid in rodents and man. Biochem. Biophys. Res. Commun. 141, 27–32.

Gainer, H., Wray, S., 1994. Cellular and molecular biology of oxytocin and vasopressin. In: Knobil, E., Neill, J.D. (Eds.), The Physiology of Reproduction. Raven Press, New York, pp. 1099–1129.
Gundersen, V., Danbolt, N.C., Ottersen, O.P., Storm-Mathisen, J., 1993. Demonstration of glutamate/aspartate uptake activity in nerve endings by use of antibodies recognizing exogenous D-aspartate. Neuroscience 57, 97–111.
Hamase, K., Homma, H., Takigawa, Y., Fukushima, T., Santa, T., Imai, K., 1997. Regional distribution and postnatal changes of D-amino acids in rat brain. Biochim. Biophys. Acta 1334, 214–222.
Hashimoto, A., Nishikawa, T., Oka, T., Hayashi, T., Takahashi, K., 1993. Widespread distribution of free D-aspartate in rat periphery. FEBS Lett. 331, 4–8.
Hashimoto, A., Nishikawa, T., Oka, T., Takahashi, K., Hayashi, T., 1992. Determination of free amino acid enantiomers in rat brain and serum by high-performance liquid chromatography after derivatization with N-tert.-butyloxycarbonyl-L-cysteine and o-phthaldialdehyde. J. Chromatogr. 582, 41–48.
Kera, Y., Aoyama, H., Matsumura, H., Hasegawa, A., Nagasaki, H., Yamada, R., 1995. Presence of free D-glutamate and D-aspartate in rat tissues. Biochim. Biophys. Acta 1243, 283–286.
Morris, J.F., Budd, T.C., Epton, M.J., Ma, D., Pow, D.W., Wang, H., 1998. Functions of the perikaryon and dendrites in magnocellular vasopressin-secreting neurons: New insights from ultrastructural studies. Prog. Brain Res. 119, 21–30.
Neidle, A., Dunlop, D.S., 1990. Developmental changes in free D-aspartic acid in the chicken embryo and in the neonatal rat. Life Sci. 46, 1517–1522.
Ross, M.H., Romrell, L.J., Kaye, G., 1995. Histology, 3rd Edn. Williams and Wilkins, Baltimore, MD, pp. 44–49.
Sakai, K., Homma, H., Lee, J.A., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T., Imai, K., 1997. D-aspartic acid localization during postnatal development of rat adrenal gland. Biochem. Biophys. Res. Commun. 235, 433–436.
Sakai, K., Homma, H., Lee, J.A., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T., Imai, K., 1998. Localization of D-aspartic acid in elongate spermatids in rat testis. Arch. Biochem. Biophys. 351, 96–105.
Schell, M.J., Cooper, O.B., Snyder, S.H., 1997. D-aspartate localizations imply neuronal and neuroendocrine roles. Proc. Natl. Acad. Sci. USA 94, 2013–2018.
Schell, M.J., Molliver, M.E., Snyder, S.H., 1995. D-serine, an endogenous synaptic modulator: Localization to astrocytes and glutamate-stimulated release. Proc. Natl. Acad. Sci. USA 92, 3948–3952.

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