Acta Neuropathol (1996) 91 : 337–342 © Springer-Verlag 1996

REGULAR PAPER

Keisuke Iwanaga · Mitsunori Yamada
Koichi Wakabayashi · Fusahiro Ikuta
Hitoshi Takahashi

A newly discovered age-related synaptic change in the human locus ceruleus: morphometric and ultrastructural studies

Received: 23 June 1995 / Revised: 13 October 1995 / Accepted: 13 November 1995

Abstract We have recently found that in the human locus ceruleus (LC) some pigmented neurons contain granules in their cytoplasm that are immunoreactive (IR) for the 38-kDa synaptic vesicle-specific protein (SVP). These represent synaptic terminals enveloped in the somatic cytoplasm. In the present study we analyzed LC pigmented neurons morphometrically in 48 autopsied individuals, whose ages at death ranged from 5 to 94 years, and also examined LC pigmented neurons ultrastructurally in 4 of these individuals. The number and incidence of LC pigmented neurons containing SVP-IR intracytoplasmic granules became significantly higher with age. The mean somatic area of the neurons was significantly higher than that of neurons without SVP-IR intracytoplasmic granules. Ultrastructurally, the synaptic terminals, which contained many round or flattened clear vesicles and sometimes dense-cored vesicles, were found to be enveloped by the somatic cytoplasm of some pigmented neurons and occasionally formed synaptic contacts with the cytoplasm. These enveloped synaptic terminals showed no apparent degenerative features. Our results strongly suggest that the enveloping of synaptic terminals by the somatic cytoplasm of human LC pigmented neurons is a phenomenon associated with the aging brain, and that this phenomenon may be related to intrinsic adaptive mechanisms of the LC pigmented neurons to certain environmental changes associated with aging.

Key words Human · Locus ceruleus · Pigmented neuron · Synaptic terminal · Aging

K. Iwanaga (✉) · M. Yamada · K. Wakabayashi · F. Ikuta[1]
H. Takahashi
Department of Pathology, Brain Research Institute,
Niigata University, 1 Asahimachi, Niigata 951, Japan
Tel.: 81-25-223-6161, ext. 5159; Fax: 81-25-223-7503

Present address:
[1] Niigata Neurosurgical Hospital and Brain Research Center,
Kurosaki, Nishikanbara, Niigata 950-11, Japan

Introduction

The presence of synaptic terminals enveloped in somatic cytoplasm is a newly discovered, characteristic feature of pigmented neurons in the human locus ceruleus (LC); such synaptic terminals can be demonstrated as granules immunoreactive (IR) for the 38-kDa synaptic vesicle-specific protein (SVP) [9]. The biological significance of this phenomenon is, however, still unknown. Therefore, as an initial step towards a better understanding of this phenomenon, we performed a morphometric investigation of LC pigmented neurons containing SVP-IR intracytoplasmic granules in a large number of autopsied individuals of various ages. We also examined the ultrastructural profiles of synaptic terminals enveloped in the somatic cytoplasm of LC pigmented neurons in some of these individuals.

Materials and methods

We selected 48 autopsied individuals after carefully reviewing their clinical and pathological records and histological sections containing the bilateral LC. Individuals diagnosed as having brain tumors or neurodegenerative diseases, other than sporadic amyotrophic lateral sclerosis [11], were excluded, as were those who obviously had fewer LC neurons than expected for their ages, with or without evidence of neurodegeneration. The ages of the individuals examined ranged from 5 to 94 years (average = 48.6 years), and the interval between death and removal of the brain ranged from 1.5 to 17 h (average = 5.5 h).

Each brain stem was fixed in formalin, cut transversely at the level of entry of the trigeminal nerve, and embedded in paraffin. Three sections, 4 μm thick, were taken from each specimen at intervals of 40 μm to avoid re-counting the same neurons. These sections were immunostained with a mouse monoclonal antibody against SVP, which is synonymous with synaptophysin [12], using the avidin-biotin-peroxidase complex method and counterstained with methyl green. Details of the procedure have been described previously [23].

Histomorphometric measurements were carried out using a semiautomatic image analysis system (System Supply, Nagano, Japan), consisting of an NEC PC-98 computer linked to a photographic microscope and image analysis system. A high-resolution video camera mounted on the microscope displayed the image of

the specimen on a video monitor. The movement of a pen on a graphics tablet traced the image of the specimen on the video screen. Thus, the region of interest was outlined and the area enclosed by the tracing was calculated automatically by the computer.

Somatic areas of nucleolated pigmented neurons, both with and without SVP-IR intracytoplasmic granules, in the three sections from each specimen were measured using the system, with a square ocular grid covering an area of 0.0256 mm^2 at a magnification of × 767. To avoid gaps or overlaps between fields while counting, the LC sections were moved systematically up and down and from side to side by means of a superimposed squared graticule, whose single fields covered an area corresponding to that of the whole ocular grid. Student's *t*-test was applied for evaluation of the differences between two means. Pearson's product moment method was used to evaluate the relationships between age and cell number or ratio.

For ultrastructural examination, specimens that were fixed in buffered 4% paraformaldehyde and which contained LC from 4 of the 48 individuals (3 men aged 63, 67 and 71 years, and 1 woman aged 66 years) were washed with phosphate-buffered saline, post-fixed with 1% OsO_4, dehydrated in a graded ethanol series and embedded in Epon. After light microscope examination of 1-µm sections stained with toluidine blue, ultrathin sections were cut, stained with uranyl acetate and lead citrate and examined with a Hitachi H-7100 electron microscope at 75 kV.

Results

Immunocytochemistry revealed that in all cases the somatic cytoplasm of some of the LC pigmented neurons contained single or multiple clusters of SVP-IR coarse granules (Fig. 1).

The results of the morphometric analysis are shown in Fig. 2a–c. There was no correlation between the total number of LC pigmented neurons counted and the age of the subject, and a high degree of individual variation was observed (Fig. 2a). On the other hand, the total number of LC pigmented neurons containing SVP-IR intracytoplasmic granules was found to increase significantly with age (n = 48; r = 0.48; P < 0.001) (Fig. 2b). The proportion of LC pigmented neurons which contained SVP-IR intracytoplasmic granules was also found to increase significantly with age (n = 48; r = 0.55; P < 0.001) (Fig. 2c).

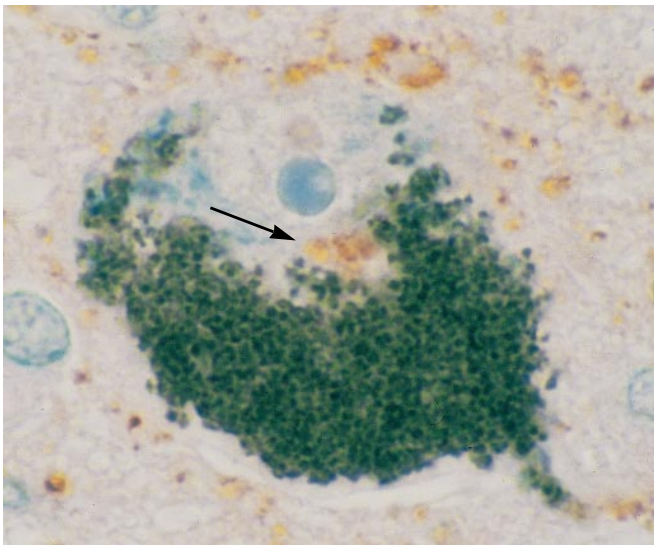

Fig. 1 Clusters of synaptic vesicle-specific protein (SVP)-immunoreactive (IR) granules (*arrow*) in a nucleolated pigmented neurons of locus ceruleus from a 72-year-old woman. × 1460

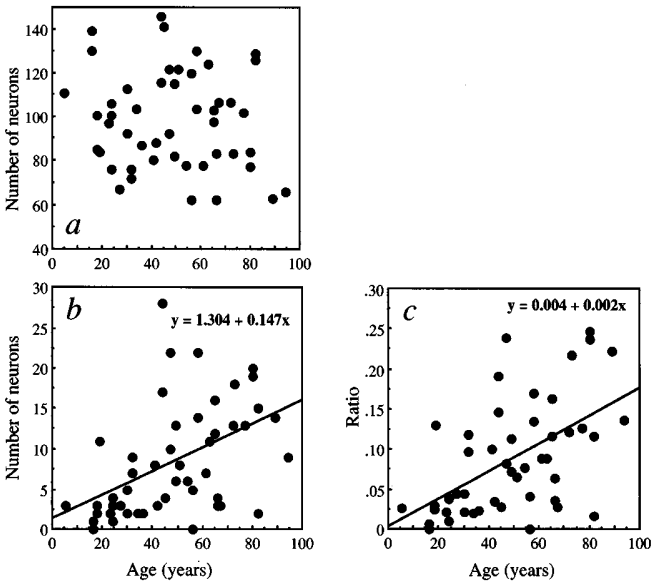

Fig. 2 a–c Morphometrical analysis. **a** Number of nucleolated locus ceruleus (LC) pigmented neurons relative to age (n = 48). **b** Number of nucleolated LC pigmented neurons containing SVP-IR intracytoplasmic granules relative to age (n = 48; r = 0.48; P < 0.001). **c** Ratio of nucleolated LC pigmented neurons containing SVP-IR intracytoplasmic granules to the total cell count of LC pigmented neurons relative to age (n = 48; r = 0.55; P < 0.001)

Measurements of the somatic area of nucleolated pigmented neurons, with and without SVP-IR intracytoplasmic granules, were carried out. The means and standard deviations are shown in Table 1. The results of the Student's *t*-test are also shown. The mean number of neurons without SVP-IR intracytoplasmic granules in the 40- to 59-year age group was significantly higher than that for the 20- to 39-year age group (P < 0.001). No significant differences were found between other adjacent age groups. In each age group, the mean number of neurons containing SVP-IR intracytoplasmic granules was significantly higher than that of neurons without the granules (P < 0.001).

Although the ultrastructural study was performed on tissues which were in a somewhat poor state of preservation, the findings appeared to be sufficiently consistent for the following limited observations to be made. The somatic cytoplasm of LC pigmented neurons was often seen to surround synaptic terminals, which contained many small round or flattened clear vesicles, sometimes with a number of dense-cored vesicles (Figs. 3, 4). Occasionally, asymmetrical synaptic junctions with prominent postsynaptic densities and widened synaptic clefts were evident (Fig. 4) [6]. No convincing symmetrical synaptic junctions could be detected. On rare occasions, dendrite-like profiles without postsynaptic densities, and glial proces-

Table 1 Mean (± SD) somatic areas of nucleolated pigmented neurons in the LC (*SVP* synaptic vesicle-specific protein, *LC* locus ceruleus)

Age (years)	Neurons – SVP[a]		Neurons + SVP[b]	
	No. cells measured	Mean somatic area ± SD (μm²)	No. cells measured	Mean somatic area ± SD (μm²)
≤ 19	583	463 ± 174	18	723 ± 229*
20–39	923	448 ± 153	39	615 ± 182*
40–59	1626	543 ± 170	159	671 ± 149*
60–79	841	557 ± 151	101	683 ± 152*
80 ≤	442	565 ± 142	53	660 ± 165*

[a, b] LC pigmented neurons without[a] or with[b] SVP-immunoreactive intracytoplasmic granules
*$P < 0.001$ (compared to neurons – SVP in each age group)

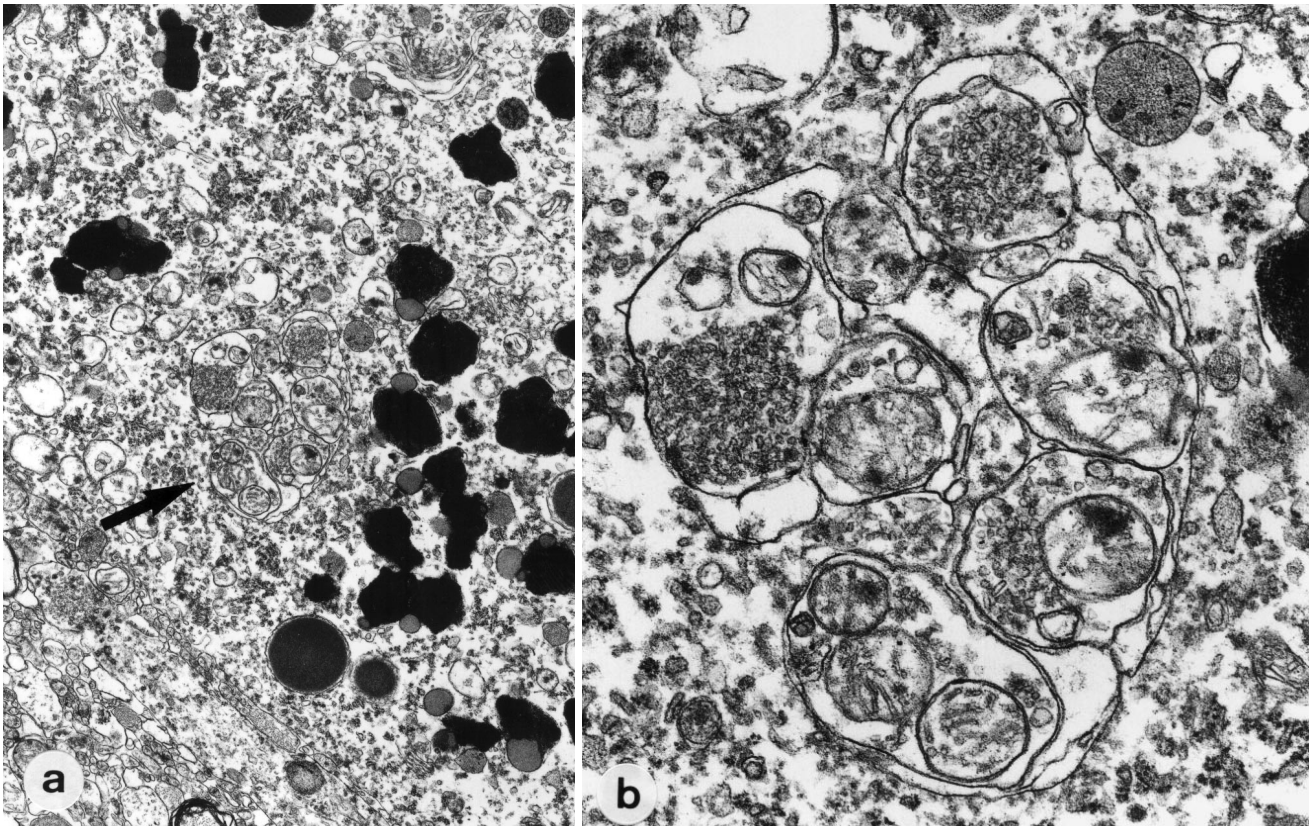

Fig. 3 **a** Electron micrograph of a LC pigmented neuron. **b** Higher magnification of the area indicated by the *arrow* in **a**. A cluster of several synaptic terminals containing many round or flattened clear vesicles is evident within the cytoplasm; tissue from a 67-year-old man. **a** x8750; **b** × 30 000

ses containing bundles of intermediate filaments were also surrounded by the somatic cytoplasm, together with the synaptic terminals. It was also noted that there were sometimes depressions in the somata of LC pigmented neurons in which many axon-like profiles were gathered. Moreover, a few LC pigmented neurons were observed to surround the cytoplasm (or at least the proximal portions of dendrites) (Fig. 5). No synaptic terminals were found to be enveloped in the dendrite-like profiles inside or outside the somatic cytoplasm of LC pigmented neurons.

Discussion

The mouse monoclonal antibody against SVP that we used recognizes synaptic vesicles in the synaptic terminals [12]. In the present study, we used 4-μm sections to detect synaptic terminals within the somatic cytoplasm, rather than on the surface of cell membranes. In all the cases examined, some of the LC pigmented neurons showed single or multiple clusters of SVP-IR coarse granules within their somatic cytoplasm.

We counted the total number of LC pigmented neurons with prominent nucleoli at the level of the upper pons, in fundamentally the same way as in previous studies by Vijayashankar and Brody [22], Tomlinson et al. [21] and Mann et al. [11]. A high degree of individual variation was observed in the numbers of LC pigmented neurons counted. Contrary to the results of previous studies which

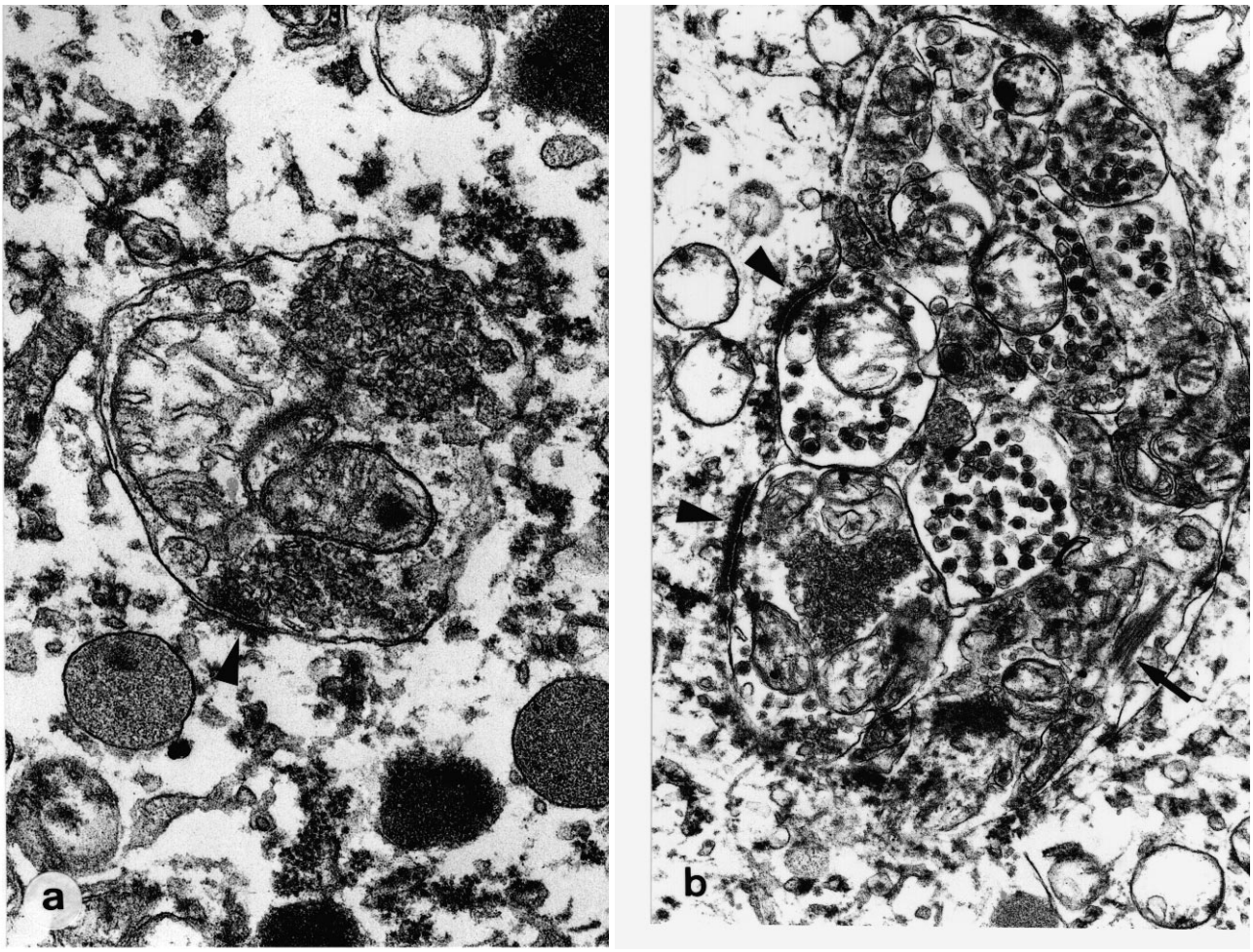

Fig. 4 a, b Synaptic terminals enveloped in the somatic cytoplasm of LC pigmented neurons. **a** A single synaptic terminal containing many round or flattened clear vesicles and showing an asymmetrical synapse (*arrowhead*); tissue from a 67-year-old man. **b** A cluster of synaptic terminals containing clear vesicles and dense-cored vesicles is evident. A glial process with intermediate filaments (*arrow*) is also evident. Accumulations of dense material (postsynaptic density) are evident on the cytoplasmic surface of a pigmented neuron (*arrowheads*); tissue from a 63-year-old man. **a** × 40 000; **b** × 22 400

suggested that LC pigmented neurons decrease in number in older persons [11, 21, 22], there was no significant correlation between the number of pigmented neurons and the age of the subject, although numbers appeared to increase to a peak at around 50–60 years of age, declining thereafter. However, there was a significant correlation between the number of LC pigmented neurons containing SVP-IR intracytoplasmic granules and the individual's age, and between the proportion of LC pigmented neurons which contained SVP-IR intracytoplasmic granules and the subject's age, indicating a substantial, age-related increase in the numbers of these neurons.

A few studies have been carried out on the size of human LC neurons. Mann et al. [10] reported that the cell bodies gradually increased in size until around middle age, and that they subsequently shrank, with large numbers of atrophied cells apparent by late middle and old age. That study was based on a conventional light microscope examination. German et al. [7]and Chan-Palay et al. [3], on the other hand, demonstrated morphometrically that the cell bodies gradually decreased in size with increasing age. These studies dealt with only a small number of cases. In the present study of 48 cases, no age-related decrease in the size of the LC pigmented neurons was confirmed. Moreover, it was demonstrated that for each age group, those pigmented neurons that contained SVP-IR intracytoplasmic granules were significantly larger in size than those without such granules. There is no doubt that the increases in size were partially reflected by the number of synaptic terminals within the somatic cytoplasm. However, even when this was taken into account, it appeared that there was no relationship between the enveloping of synaptic terminals and neuronal shrinkage.

Ultrastructurally, it was particularly interesting that the LC pigmented neurons looked healthy and that the synaptic terminals enveloped in somatic cytoplasm did not apparently show degeneration. Normally, when synaptic terminals are detached from their parent cells, they eventu-

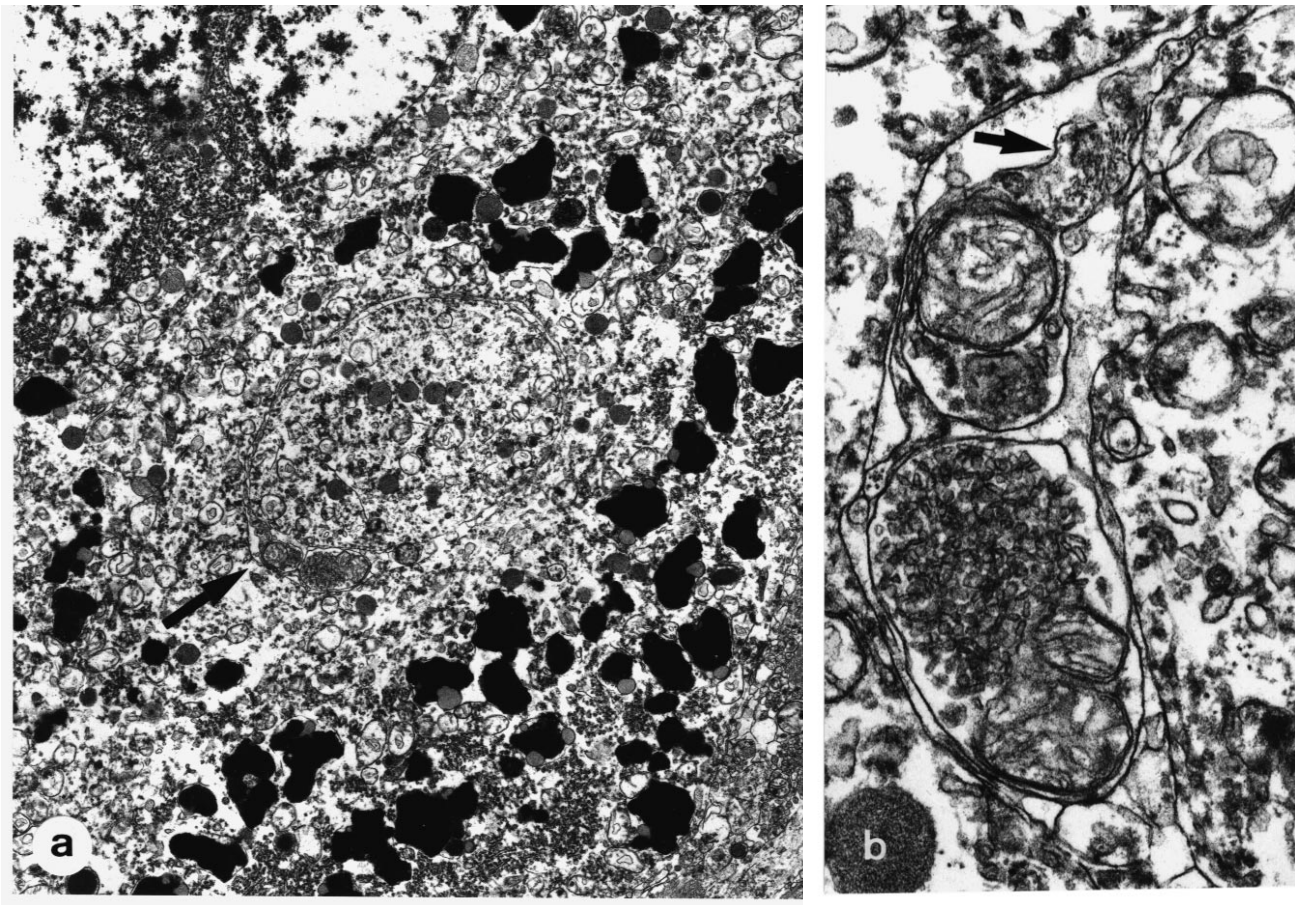

Fig. 5 **a** Electron micrograph of a LC pigmented neuron showing an entrapped, round cytoplasmic island within the cytoplasm. **b** Higher magnification of the area indicated by the *arrow* in **a**. A synaptic terminal containing many flattened clear vesicles and a glial process with intermediate filaments (*arrow*) are also evident; tissue from a 67-year-old man. **a** × 7000; **b** × 37 500

ally become electron dense [14, 17]. It is unlikely that such a phenomenon occurred in the present synaptic terminals; these synaptic terminals were still connected to their axons. We consider that the contours of neurons become increasingly irregular with age so that the synaptic terminals, and sometimes other structures such as dendrites and glial processes, become engulfed by these neurons.

In ultrastructural observations of cat and rat LC, the soma of the neurons was found to be surrounded by glial sheaths, and the major inputs were found to occur on the distal dendrites of the LC neurons [8, 18]. Although few in number, inputs to the soma and proximal dendrites were found to occur on a limited number of somatic and dendritic appendages that pierced the glial sheaths [8, 18]. Furthermore, it was recently demonstrated that the most important inputs to the soma may mainly originate from two cell groups [1], both located in the rostral medulla: the nucleus paragigantocellularis lateralis and the nucleus prepositus hypoglossi, which are the main nuclei projecting, respectively, adrenergic and gamma aminobutyric acid (GABA)-ergic afferents to the LC [2, 4, 5, 15, 16]. Ultrastructurally, adrenergic fibers contain dense-cored vesicles in the terminals, and GABAergic synaptic terminals are reported to contain pleomorphic synaptic vesicles and to show asymmetrical or symmetrical synaptic junctions [14]. Our ultrastructural study confirmed the presence of these two types of synaptic terminals, suggesting that there was no selectivity with regard to those synaptic terminals enveloped in the somatic cytoplasm of LC pigmented neurons.

In conclusion, our present findings strongly suggest that the enveloping of synaptic terminals in the somatic cytoplasm of human LC pigmented neurons is a phenomenon associated with the aging brain. Based on the morphometric and ultrastructural findings discussed above, we consider that the phenomenon is not related to the neurodegenerative process, but may reflect certain intrinsic mechanisms within the LC pigmented neurons themselves. It is difficult to say what purpose or role, if any, it may have in the aging process. However, it is appealing to speculate that the phenomenon represents an essential morphological alteration for LC pigmented neurons to survive and maintain the axo-somatic neurotransmission against certain external environmental changes as the brain ages.

Recently, we examined specimens of the substantia nigra (SN) from 14 of the individuals investigated in this study, with ages ranging from 19 to 77 years, and con-

firmed that similar SVP-IR intracytoplasmic granules can also occur in the pigmented neurons in the SN (data not shown). Ultrastructural study of the SN of one individual revealed synaptic terminals enveloped in the somatic cytoplasm of pigmented neurons (data not shown). So far, we have encountered no SVP-IR intracytoplasmic granules in neurons outside the two pigmented nuclei, the LC and SN.

Synaptic terminals enveloped in somatic cytoplasm were first noticed in LC pigmented neurons containing Lewy bodies, a hallmark of Parkinson's disease [19]. It is known that the LC and SN are involved in Parkinson's disease [13], Alzheimer's disease [20] and other neurodegenerative disorders [13], which generally develop in the elderly. Whether, and if so, how, this phenomenon of synaptic terminals participates in the progression of such disorders is another issue of interest.

Acknowledgements We wish to express our appreciation to Dr. K. Obata, Laboratory of Neurochemistry, National Institute for Physiological Sciences, Okazaki, for providing the anti-SVP antibody; Dr. K. Endoh, Department of Hygiene, Niigata University School of Medicine, Niigata, for his helpful cooperation with the statistical analysis and Ms. A. Ito, Department of Orthopedic Surgery, Niigata University School of Medicine, for statistical advice. We also thank Mr. S. Egawa, Mr. T. Hasegawa, Ms. Y. Ota and Ms. C. Tanda for their technical assistance, and Ms. M. Machida for help in preparing the manuscript. This work was supported in part by research grants for CNS Degenerative Diseases, Aging and Health, and Nervous and Mental Disorders (6A-2) from the Ministry of Health and Welfare, Japan.

References

1. Aston-Jones G, Ennis M, Pieribone VA, Nickell WT, Shipley MT (1986) The brain nucleus locus coeruleus: restricted afferent control of a broad efferent network. Science 234: 734–737
2. Aston-Jones G, Shipley MT, Chouvet G, Ennis M, Bockstaele E van, Pieribone V, Shiekhattar R, Akaoka H, Drolet G, Astier B, Charléty P, Valentino RJ, Williams JT (1991) Afferent regulation of locus coeruleus neurons: anatomy, physiology and pharmacology. Prog Brain Res 88: 47–75
3. Chan-Palay V, Asan E (1989) Quantitation of catecholamine neurons in the locus coeruleus in human brains of normal young and older adults and in depression. J Comp Neurol 287: 357–372
4. Ennis M, Aston-Jones G (1989) GABA-mediated inhibition of locus coeruleus from the dorsomedial rostral medulla. J Neurosci 9: 2973–2981
5. Ennis M, Aston-Jones G (1989) Potent inhibitory input to locus coeruleus from the nucleus prepositus hypoglossi. Brain Res Bull 22: 793–803
6. Forno LS, Norville RL (1981) Synaptic morphology in the human locus ceruleus. Acta Neuropathol (Berl) 53: 7–14
7. German DC, Walker BS, Manaye K, Smith WK, Woodward DJ, North AJ (1988) The human locus coeruleus: computer reconstruction of cellular distribution. J Neurosci 8: 1776–1788
8. Groves PM, Wilson CJ (1980) Fine structure of rat locus coeruleus. J Comp Neurol 193: 841–852
9. Iwanaga K, Takahashi H, Yamada M, Ikuta F (1995) Synaptic terminals wrapped in the somatic cytoplasm of pigmented neurons in the human locus ceruleus. Neurosci Lett 188: 147–150
10. Mann DMA, Yates PO (1974) Lipoprotein pigments: their relationship to ageing in the human nervous system. II. The melanin content of pigmented nerve cells. Brain 97: 489–498
11. Mann DMA, Yates PO, Hawkes J (1983) The pathology of the human locus ceruleus. Clin Neuropathol 2: 1–7
12. Obata K, Kojima N, Nishiye H, Inoue H, Shirao T, Fujita SC, Uchizono K (1987) Four synaptic vesicle-specific proteins: identification by monoclonal antibodies and distribution in the nervous tissue and the adrenal medulla. Brain Res 404: 169–179
13. Oppenheimer DR, Esiri MM (1992) Diseases of the basal ganglia, cerebellum and motor neurons. In: Adams JH, Duchen LW (eds) Greenfield's neuropathology, 5th edn. Arnold, London, pp 988–1045
14. Peters A, Palay SL, Webster HD (1991) Synapses. In: Peters A, Palay SL, Webster HD (eds) The fine structure of the nervous system. Neurons and their supporting cells, 3rd edn. Oxford University Press, New York, pp 138–211
15. Pieribone VA, Aston-Jones G (1991) Adrenergic innervation of the rat nucleus locus coeruleus arises predominantly from the C1 adrenergic cell group in the rostral medulla. Neuroscience 41: 525–542
16. Pieribone VA, Aston-Jones G, Bohn MC (1988) Adrenergic and non-adrenergic neurons in the C1 and C3 areas project to locus coeruleus: a fluorescent double labeling study. Neurosci Lett 85: 297–303
17. Raisman G, Matthews MR (1972) Degeneration and regeneration of synapses. In: Bourne GH (ed) The structure and function of nervous tissue, vol 4. Academic Press, New York, pp 61–104
18. Ramon-Moliner E (1974) The locus coeruleus of cat. III. Light and electron microscopic studies. Cell Tissue Res 149: 205–221
19. Takahashi H, Iwanaga K, Egawa S, Ikuta F (1994) Ultrastructural relationship between Lewy bodies and pale bodies studied in locus ceruleus neurons of a non-parkinsonian patient. Neuropathology 14: 73–80
20. Tomlinson BE (1992) Ageing and the dementias. In: Adams JH, Duchen LW (eds) Greenfield's neuropathology, 5th edn. Arnold, London, pp 1284–1410
21. Tomlinson BE, Irving D, Blessed G (1981) Cell loss in the locus coeruleus in senile dementia of Alzheimer type. J Neurol Sci 49: 419–428
22. Vijayashankar N, Brody H (1978) A quantitative study of the pigmented neurons in the nuclei locus coeruleus and subcoeruleus in man as related to aging. J Neuropathol Exp Neurol 38: 490–497
23. Wakabayashi K, Takahashi H, Obata K, Ikuta F (1992) Immunocytochemical localization of synaptic vesicle-specific protein in Lewy body-containing neurons in Parkinson's disease. Neurosci Lett 138: 237–240