Regional Differences of Serotonin-Mediated Synaptic Plasticity in the Chicken Spinal Cord with Development and Aging

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SUMMARY

Previous studies in our laboratory /3,17/ have demonstrated that serotonin (5-HT) appears to have a trophic-like effect in enhancing synapse formation and maintenance in both the developing and the adult central nervous system. In the present study, we focused on age-related changes in the density of the axosomatic and axodendritic synapses and the number of 5-HT-positive fibers in the chicken spinal cord, with special reference to differences between the ventral (laminae VII and IX) and the dorsal (lamina I) horn. At 1 week posthatching (P1W), a transient overproduction of synapses and 5-HT-immunoreactive fibers occurred in lamina IX; all parameters had returned to their initial levels by 1 month post-hatching (P1M). The density of synapses further decreased by about 40% between P6M and P2Y (2 years posthatching). Although the magnitude of the transient increase in lamina VII was less than that in lamina IX, the changing pattern of the synapses and the 5-HT-positive fibers was similar in both regions. In the ventral horn, thin 5-HT-positive fibers were most prominent at P1W and then decreased with development; thin 5-HT-positive fibers were still found at P6M but had almost disappeared by P2Y. By contrast, at P2Y the density of the synapses and the 5-HT-positive fibers in the dorsal horn was even higher than that of younger animals. Reduction of 5-HT levels in P2Y-old chickens by p-chlorophenylalanine (pCPA) administration decreased the synaptic density in lamina I but not in lamina IX. The results of this study demonstrate that 5-HT-mediated synaptic plasticity is markedly different in the ventral and dorsal horns of the aged chicken. In the ventral horn, synaptic plasticity reached a maximum at about P1W, remained stable in the young-adult period, and then finally disappeared in the aged chicken. Conversely, the results suggest that in the dorsal horn, 5-HT fibers continue to mediate the trophic influence on synaptic plasticity even in the old chicken.

KEY WORDS

synaptic plasticity, 5-HT , dorsal horn, ventral horn

INTRODUCTION

Chubakov and colleagues /4/ first demonstrated using *in vitro* preparations that serotonin (5-HT) has a trophic-like role in facilitating the formation of chemical synapses. Subsequent *in vivo* studies in our laboratory /17,3/ have shown that 5-HT enhances synapse formation and maintenance in both the developing and the mature central nervous system (CNS). The synaptic density decreases in a dose-dependent fashion in the presence of 5-HT depletors with different pharmacokinetics; at the maximum dose, almost 70% of the synapses disappear within one week after the decrease in the 5-HT level.

To exclude the possibility that synaptic loss occurs by nonspecific side effects of 5-HT depletors, Niitsu *et al* /16/ identified the 5-HT

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receptor subtype mediating the facilitator role of 5-HT. In the chicken embryo, the number of synapses in the ventral horn of the spinal cord could be artificially manipulated by administering a 5-HT2A antagonist or agonist or both.

Because 5-HT fibers are distributed diffusely in diverse regions of the CNS, the putative role of 5-HT in regulating the number of synapses is important for understanding not only brain function but also the background mechanism for synaptic plasticity. In our previous line of studies /3,17/, we needed more information to understand the regulatory mechanism of 5-HT-mediated synaptic plasticity. Hence, we undertook the present study to clarify the following points. (1) We attempted to analyze in detail the changes in synaptic density with development and aging. (2) We expected to ascertain whether 5-HT-mediated synaptic plasticity is maintained in the aged chicken. As a special interest was directed toward the differences between the motor and sensory systems, we analyzed data from both the anterior horn (laminae VII and IX) and dorsal horn (lamina I).

METHODS

Animals

Over 100 White Leghorn chickens were used in this study. The chickens were examined at posthatching (P) days P0D, P1W (1 week), P2W, P1M (1 month), P2M, P6M, and P2Y (2 years).

Tissue preparation

Chickens were deeply anesthetized with an overdose of chloropent and then perfused from the ascending aorta with a small amount of saline followed by a fixative. A fixative composed of 0.1 M phosphate buffer containing 4% paraformaldehyde and 0.15% picric acid was used for immunohistochemical studies. For electron microscope studies, the fixative buffer contained 2.5% glutaraldehyde and 2% paraformaldehyde. Immediately after the perfusion fixation, lumbosacral spinal-cord segment 3 was removed and immersed in the appropriate fixative for 12 hr at 4°C. For the immuno-histochemistry studies, 40-μm-thick transverse sections of the spinal cord were cut on a freezing microtome. Free-floating sections were reacted with an antibody against 5-HT, using the ABC method /23/. That immunohistochemical staining tends to vary among animals or even within single sections is widely believed. To ensure staining consistency, immunohistochemistry preparations were carefully processed in our laboratory. Perfect perfusion fixation and constant agitation of the sections during the staining process are necessary. For quantitative analyses, we selected several of the best-stained sections.

For electron microscope studies, spinal segments were transversely cut to a thickness of about 1 mm, osmificated, and then embedded in Epon. Semithin plastic sections, 2–4 μm thick, were cut with a glass knife and stained with toluidine blue. The sections were re-embedded in Epon after observation under a light microscope to ascertain tissue orientation. Except for the regions to be examined, the re-embedded sections were trimmed.

Pharmacological experiment

To decrease the amount of 5-HT in the spinal cord of P2Y-old chickens, four injections of p-chlorophenylalanine (pCPA) (800 mg/kg body weight) were given intraperitoneally over a period of 7 days (days 1,2,4, and 6). Control animals received saline injections on the same days. The chickens were perfused with the fixative for electron-microscope studies. We compared the data on P2Y-yr-old chickens with data on P1W-old and P6M-old chickens that were examined in a previous study /17/.

Quantitative analyses

Four animals (either control or experimental) were used for each stage. For each animal, electron micrographs were taken of each axosomatic and axodentritic synapse, from the dorsolateral part of the lateral motor column, at a primary magnification of × 5000. From each animal, 10–20 photographs of both types of synapses were taken from two re-embedded, semi-thin sections. The negatives were enlarged on printing papers, and the final magnification reached × 9000. Synapses were identified as profiles having aggregates of synaptic

Table 1
5-HT-immunoreactive varicosities and synaptic densities in the chicken spinal cord

Lamina	Age	5-HT Immunoreactivity[a]	Synaptic Density[a]	
			Axosomatic[b]	Axodendritic[c]
I	POD	71.72±08.14 (42	0.07±0.07 (54)	28.17±07.42 (36
	P1W	181.94±16.29 (36	0.13±0.09 (50)	50.49±12.69 (45
	P2W	110.80±10.04 (48	0.11±0.09 (40)	36.55±07.68 (40
	P1M	82.50±12.34 (40	0.11±0.07 (48)	38.57±06.76 (44
	P2M	75.00±08.05 (36	0.10±0.14 (50)	41.91±08.29 (42
	P6M	81.40±05.04 (45	0.18±0.11 (42)	40.88±10.03 (32
	P2Y	117.40±07.64 (62	0.16±0.09 (60)	44.75±05.60 (60
VII	POD	55.00±09.97 (62	0.21±0.12 (108	17.74±03.71 (64
	P1W	134.00±08.65 (40	0.36±0.13 (101	24.21±04.44 (64
	P2W	84.00±10.42 (64	0.26±0.11 (78)	15.91±08.70 (44
	P1M	58.60±09.16 (64	0.23±0.11 (68)	16.94±05.01 (48
	P2M	73.00±05.69 (48	0.19±0.11 (56)	15.57±04.19 (24
	P6M	55.00±11.24 (52	0.24±0.11 (60)	14.34±04.38 (38
	P2Y	52.00±09.61 (44	0.20±0.13 (66)	15.56±03.55 (34
IX	POD	96.00±13.02 (60)	0.28±0.12 (118	23.59±05.53 (54
	P1W	334.00±40.43 (100	0.51±0.15 (102	36.47±09.33 (52
	P2W	154.00±14.45 (64)	0.33±0.14 (100	25.06±04.92 (52
	P1M	100.00±13.99 (64)	0.28±0.14 (64)	18.10±03.77 (42
	P2M	92.00±10.49 (48)	0.24±0.16 (52)	18.58±03.90 (24
	P6M	102.00±15.22 (52)	0.30±0.07 (60)	18.19±04.33 (48
	P2Y	81.00±17.84 (64)	0.22±0.13 (67)	12.64±03.54 (36

[a]Means ± standard deviation (no. in parentheses indicates no. micrographs counted). [b]No. synapses per 1μm somatic membrane. [c]No. synapses in 200 μm^2. P=posthatching, D=day, M=month, Y=year

vesicles and a membrane specialization that was characteristic of an active zone. Axosomatic synapses were located on the somatic profiles. The density of axosomatic synapses was expressed as the number of synapses per 1 μm of somatic membrane. Axodendritic synapses were counted in the area of 200 μm^2 in the neuropil. The synaptic density was examined in laminae I, VII, and IX. The data of the synaptic density on POD (lamina IX), P1W (laminae I, VII, IX), and P6M (lamina IX) gathered in a previous study /17/ were compared with the new data from the present study. The results are presented in Table 1.

Fibers positive for 5-HT consisted of varicosities and thread-like parts interconnecting the varicosities. Although we could identify the varicosities in light-microscopic observations (see Figure 1), we did not always recognize the fine, thread-like parts. Therefore, we carried out quantitative analyses on the density of the 5-HT fibers by counting the number of varicosities in a unit area (1000 μm^2), using a light microscope equipped with an oil-immersion objective lens (× 100) and a drawing tube. We counted the number of varicosities from five regions of each lamina; the mean represents the value of one animal. We used ANOVA for statistical comparison between each pair.

RESULTS

Structural changes of 5-HT-positive fibers

Between P6M and P2Y, the number of thin 5-HT-immunoreactive fibers in lamina IX (Figure 1, small arrows) that were not counted in the quantitative study decreased by P2Y. When compared with their appearance at P6M, the thick varicose fibers surrounding the somal profiles of

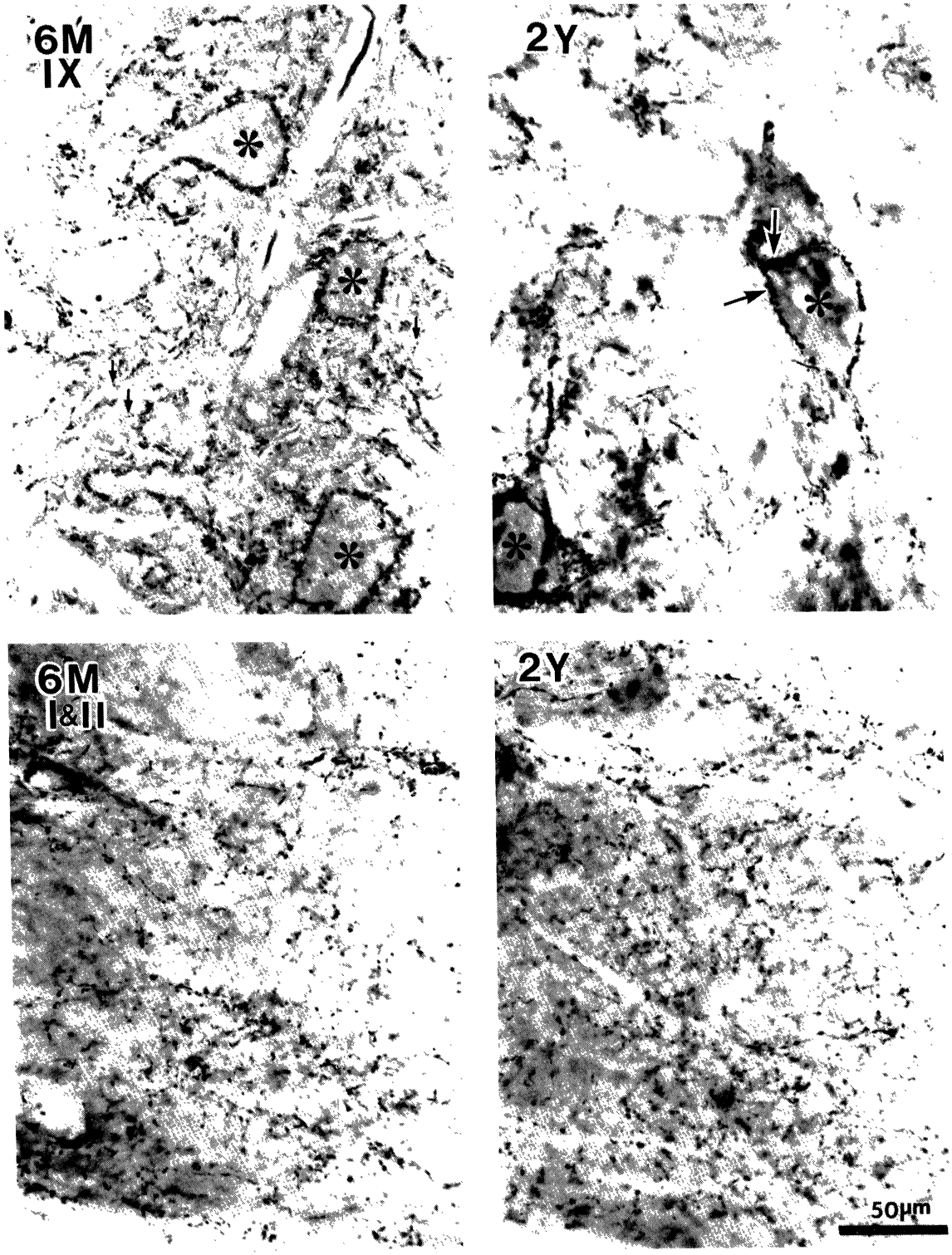

Fig. 1: Photomicrograph of 5-HT-positive profiles in lamina IX (upper) and laminae I and VII (lower) at P6M-old (left column) and P2Y-old (right column) chickens. * indicates motoneuron soma. Section thickness = 40 μm (ABC method). Large arrows indicate thick 5-HT-immunoreactive varicose fibers; small arrows indicate thin 5-HT-immunoreactive fibers.

large-size neurons (Fig. 1, large arrows) appeared to be enlarged or swollen at P2Y. In laminae I and II, neither the ratio of thin fibers *versus* thick varicosities nor general appearance of 5-HT immunoreactive fibers changed between P6M and P2Y.

Table 1 shows the chronological changes in number of 5-HT-positive varicosities counted in the immunohistochemical preparations. The results are presented in graphic form in Figure 2. In all three laminae studied here, the density of varicosities reached a maximum value at P1W and then decreased to near-initial values around P1M. The density of 5-HT varicosities in lamina I, but not those in laminae VII and IX, showed a moderate increase at P2Y.

Synaptic density at different posthatching ages

The chronological changes in the density of 5-HT immunoreactive varicosities and synapses in the chicken spinal cord are presented in Table 1 and shown in graphic form in Figure 2. The sharp rise in the density of both the axodendritic and axosomatic synapses seen in all three laminae was most characteristic of lamina IX. After the first peak, the synaptic density in lamina IX generally decreased with development and aging, except for a small transient increase in the density of the axosomatic synapses at P6M that did not exceed the initial level. The pattern of changing synaptic-density in lamina VII was similar to that of lamina IX. After a moderate increase at P1W, the density of the axosomatic synapses in lamina I did not decrease much at P2W. Rather than decreasing further, the density of both synapses in lamina I were higher at P2Y than at P2W.

Synaptic density in pCPA-treated chickens

The effect of pCPA on eliminating 5-HT differs greatly among different species and among different regions of the CNS. In a previous study /17/, we obtained the maximum effect in the chicken spinal cord by injecting 800 mg pCPA/kg body weight. Data from P2Y-old chickens in the present study were compared with those from P1W-old and P6M-old chickens studied in /17/. Table 2 shows that relative to untreated control animals, the

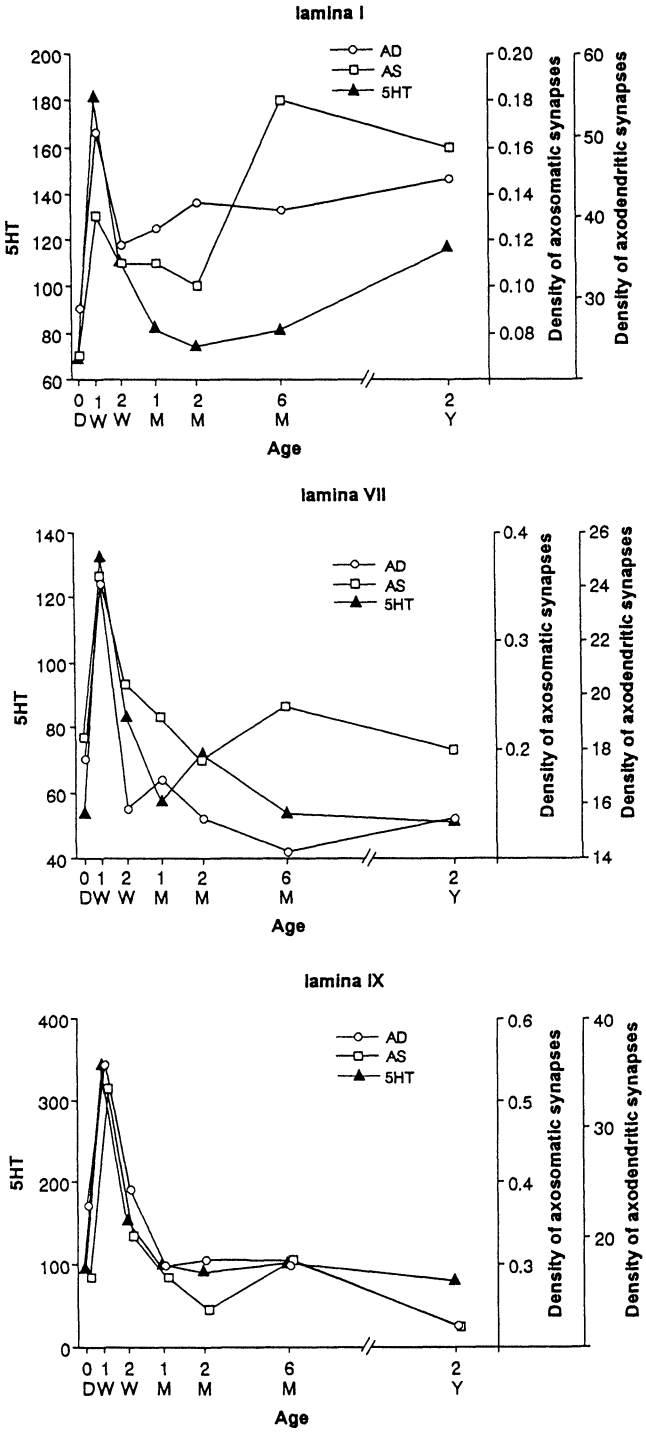

Fig. 2: Changes in 5-HT-immunoreactive varicosities and synaptic density with development and aging. AD=axodendritic, AS=axosomatic, D=day, Y=year, W=week, M=month

Table 2
Synaptic densities in pCPA-treated chickens and untreated control animals[a]

Lamina	Age	Axosomatic[b]			Axodendritic[c]		
		Untreated	pCPA	P	Untreated	pCPA	P
I (SP)	P1W	0.13±0.09 (49)	0.09±0.08 (47)	0.0001 31%	50.49±12.69 (45)	35.84±12.88 (45)	0.0001 29%
	P2Y	0.16±0.09 (60)	0.12±0.08 (41)	0.0279 25%	44.75± 5.60 (60)	38.86± 6.38 (32)	0.0005 14%
IX (SP)	P1W	0.51±0.15 (102)	0.16±0.06 (91)	0.0001 69%	36.47± 9.33 (51)	14.04±4.81 (48)	0.0001 61%
	P2Y	0.22±0.13 (67)	0.22±0.13 (101)	0.9599 0%	12.64±3.54 (36)	13.10±3.54 (41)	0.5734 ±1%

[a]Means ± standard deviation (No. in parentheses indicates no. electron micrographs counted).
[b]No. synapses per 1μm somatic membrane. [c]No. synapses in 200 μm^2.
P=posthatching, D=day, M=month, Y=year, SP=spinal cord, P=posthatching, 1W= one week, 2Y= two years

axosomatic and the axodendritic synaptic densities did not change in lamina IX of P2Y-old, pCPA-treated chickens (see 'Methods'). In lamina I, however, the density of the axosomatic synapses in pCPA-treated chickens decreased by 25% ($p = <0.05$) and the density of the axodendritic synapses decreased by 14% ($p = <0.01$) relative to untreated animals.

DISCUSSION

Changes in 5-HT varicosities and synaptic density with development and aging

The results of the present study have demonstrated differences between the dorsal and ventral horns in both the magnitude and duration of 5-HT-mediated synaptic plasticity. In lamina IX, the synaptic density transiently increased at P1W. Although the magnitude of the transient increase in lamina VII was less than that in lamina IX, the changing pattern of both synapses and of 5-HT-positive fibers was similar to that in lamina IX. Overproduction of synapses was especially prominent in lamina IX. Although synaptic overproduction has long been considered a structural background for generating plasticity in the critical period of development /2,7,8,21/, a substance for regulating the number of synapses in the brain was identified only recently, when a study in our laboratory demonstrated that 5-HT facilitates synaptic overproduction /17/.

Overexpression of 5-HT (monoamines) during development has been found in regions of the CNS other than the chicken spinal cord /10,18/. Using ultrastructural markers of the monoamine 5-hydroxydopamine, Kristt /12/ demonstrated that 20% to 30% of all synapses are monoaminergic in the somatosensory cortex of rodents in the first postnatal week and that the ratio decreases thereafter. Aberrant 5-HT fibers appeared around postnatal day 10 in the barrel fields of the cerebral cortex of rodents (5/6). A transient increase of 5-HT fibers in the spinal cord of rats has also been reported /19/. During the critical period of development, the transient overexpression of 5-HT shown in various regions of the CNS may also have a trophic-like role in producing synaptic over-production.

The synaptic-density pattern seen in lamina I was different from those of laminae VII and IX. When compared with the ventral horn at P1W, the magnitude of synaptic overproduction in lamina I was less prominent. Moreover, the synaptic density in lamina I did not decrease further after P2W as it did in the ventral horn. At P2Y the synaptic density in lamina IX decreased to about 50% of that seen at P6M, whereas in lamina I, the synaptic density of P6M-old and P2Y-old chickens showed very little change. After pCPA injections, the patterns of changes in synaptic density in the dorsal *versus* the ventral horn were different as well.

Because the increase-decrease pattern in the density of synapses at the critical period of development is conspicuous, much attention has been paid to the synaptic overproduction as one mechanism generating plasticity in the developing brain. Although synaptic density does not change for a long time (weeks or months), 5-HT-facilitated

synapse formation is assumed for synaptic turnover or remodeling in the adult CNS.

The results of our recent study /16/ demonstrated that synapse formation is mediated by 5-HT2 receptors The 5-HT2 receptors in the neocortex decrease gradually with aging /9,15/. The brain shows an enormous loss of 5-HT2 receptors in Alzheimer's disease /1,20,22/. In such situations, synaptic loss may occur in the affected brain because the ability of synaptic recruitment is lost. Therefore, we would like to emphasize that synaptic plasticity mediated by 5-HT is an important issue not only in the developing but also in the adult and the aging brain.

Thin *versus* thick 5-HT-positive fibers

The thin 5-HT-positive fibers in lamina IX of P1W-old chickens were most frequently located in the neuropil, where synapse formation is accelerated to the maximum. Although their numbers decreased after P1W, thin fibers were still seen in P6M-old chickens. By contrast, thin 5-HT-positive fibers were not found in lamina IX of P2Y-old chickens, who appeared to have lost synaptic plasticity. Such findings may suggest an involvement of thin, 5-HT-positive fibers in the trophic effects for synapse formation.

Most of the enlarged or swollen varicose 5-HT fibers were found around somal profiles of motoneurons in lamina IX of P2Y-old-chickens. Steinbusch and colleagues /24/ demonstrated similar changes in the 5-HT fibers of aged (28-month-old) rats. Such changes in aged chickens and rats may represent degenerating fibers because they appear structurally similar to the changes seen in 5,7-dihydroxytryptamine-induced denervation /25,26/.

Several groups /11,13,14,27/ have reported different properties for each type of 5-HT fiber. In the neocortex of the rat /11/ and the monkey /27/, the two types of 5-HT fibers have dissimilar regional and laminar patterns of distribution. When compared with thick fibers innervating specific regions or laminar structures, fine 5-HT fibers are distributed more diffusely. These two fiber types exhibit differential vulnerability to neurotoxic amphetamine derivatives. Thin fibers degenerate more easily, whereas thick fibers are consistently spared /14/. Another difference between the two types of fibers is that thin and thick fibers arise from different cells of origin: the dorsal and median raphe nuclei, respectively /11,13/. The collective information from all these studies suggests the presence of morphologically and functionally different classes of 5-HT fibers in the brain. Further study will be necessary to clarify whether a specific type of 5-HT fiber mediates the nontransmitter role in facilitating synapse formation.

In conclusion, this study has demonstrated marked differences between 5-HT-mediated synaptic plasticity in the ventral and dorsal horn in the aged chicken. In the ventral horn, 5-HT-mediated synaptic plasticity appeared to reach a maximum at about P1W, remained stable in the young-adult period, and finally disappeared in the aged chicken. By contrast, the results suggested that even in the old chicken, 5-HT fibers in the dorsal horn mediate the trophic influence for synaptic plasticity.

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