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Immunochemical Homology of 3 Developmentally Regulated Brain Proteins and Their Developmental Change in Neuronal Distribution

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Proteins S5, S6, and S54 (mol. wts. 95,000, 100,000, and 110,000 Da) appear characteristically at certain developmental stages in the chick brain (Shirao, T. and Obata, K., *J. Neurochem.*, 44 (1985) 1210–1216). In the present study polyclonal and monoclonal antibodies were developed against electrophoretically purified S5 and S6 proteins. Each polyclonal and monoclonal antibody specifically recognized all 3 proteins, S5, S6, and S54, by immunoblot analysis. The tissue specificities of these proteins were examined by immunoblot analysis with these antibodies. Proteins S5 and S6 were found in the neural tissue and in some non-neural tissues of chick embryo. In the adult chicken, however, they were detected neither in neural nor in non-neural tissues with the exception of the spinal ganglion. Protein S54, on the other hand, was found both in late embryonic and adult neural tissues. It was detected neither in embryonic nor in adult non-neural tissues. Immunohistochemical analysis of adult nervous system showed that S54 protein was present only in neurons. Therefore it is concluded that S54 protein is a neuron-specific protein. Developmental changes of localization of these proteins were then examined by immunohistochemistry. In the developing brain, immunostaining was first observed in newly differentiated neurons, later becoming localized in the neuronal processes. In the adult brain, the immunoreactivity was mainly localized in certain types of synaptic regions, but it was also observed in a small population of neuronal somata.

INTRODUCTION

In the course of the development of the nervous system, neurons are generated in the ventricular zone, migrate from there to their permanent location, extend axonal and dendritic processes in the proper direction, and form synapses selectively with their appropriate partners. These events appear to require the temporal expression of certain specific proteins[7,11,31–33] or modulation of certain existing proteins[2,3,9]. For example, Skene and Willard[31] have reported that two polypeptides (GAP-23 and GAP-43) are expressed specifically in association with periods of axonal growth or synaptogenesis in the mammalian central nervous system. They proposed a hypothesis that the neuronal growth state can be defined as an altered program of gene expression.

We have recently analyzed 54 proteins (S1–S54) in the chick optic tectum by two-dimensional electrophoresis and demonstrated that 8 proteins change remarkably in their concentration during development[30]. Proteins S5 and S6 appear temporarily at the developmental stages corresponding to the generation of neurons, and with the growth of neuronal processes, respectively. They are present in all brain regions at certain embryonic stages, but they are not detected in the adult brain[30]. On the other hand, S54 protein appears in parallel with further maturation of the nervous system, and is present in the adult brain[30]. Therefore we have suggested that these proteins may play major roles in brain development. The mol. wts. of S5, S6, and S54 proteins are 95, 100 and 110 kDa, respectively, and their isoelectric points are about 4.5. Proteins S5 and S6 have been purified to homogeneity from chick embryonic brains. There is a high degree of structural homology between S5 and

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S6 proteins[30]. In the present investigation we produced polyclonal and monoclonal antibodies against purified S5 and S6 proteins. With these antibodies we first examined the similarities and differences in antigenicity between S5, S6, and S54 proteins. We then analyzed the tissue specificities of these proteins by immunoblot technique, and also the developmental changes occurring in their distribution within the chicken nervous system using immunohistochemistry.

MATERIALS AND METHODS

Materials

The following materials were purchased from the sources indicated: fluorescein isothiocyanate-conjugated goat anti-mouse IgG and horseradish peroxidase-conjugated goat anti-mouse IgG from Cappel; Vectastain ABC Kit from Vector Laboratories. The other materials used were as described previously[4,30].

Preparation of immunogen proteins and antisera

Each of S5 and S6 proteins was purified from chick embryonic brain as described previously[30]. In brief, the soluble fraction obtained from whole brains of 11-day chick embryos was brought to pH 5.5. The suspension was centrifuged, and then the supernatant was subjected to DEAE-Sepharose column chromatography and ammonium sulfate precipitation. This 20–30% ammonium sulfate fraction highly enriched with S5, S6, and S54 proteins was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli[12]. Each band of S5 or S6 protein was excised from the Coomassie blue-stained gel, homogenized and reapplied separately to sodium dodecyl sulfate-polyacrylamide gel. After re-electrophoresis, S5 or S6 protein was excised from the gel.

The gel slice containing S5 protein (ca. 0.1 mg of protein) was homogenized with a glass homogenizer, and emulsified with Freund's complete adjuvant. On the other hand, S6 protein (0.1 mg of protein) was eluted from the gel slice electrophoretically, and then emulsified with Freund's complete adjuvant. A white rabbit was injected s.c. with S5 protein containing gel homogenate[14,21]. A booster injection containing Freund's incomplete adjuvant was then administered 3 weeks after the initial injection. Another rabbit was injected with eluted S6 protein, and a booster injection was administered 9 weeks after the initial injection. Collection of antisera was started one week after the last injection.

Generation of monoclonal antibodies (MAbs)

Purified S6 protein eluted from the gel was used as an immunogen. The sample (0.1 mg of protein) was emulsified with Freund's complete adjuvant and administered to a female BALB/c mouse i.p. Six months later, intravenous booster injection was given with the same sample (0.1 mg of protein) without adjuvant. After 3.5 days, splenocytes (1.2×10^8 cells) were obtained from the mouse and combined with X63-Ag8-653 myeloma cells (3.6×10^7 cells). These were treated with polyethylene glycol 1000 to achieve fusion[4]. The cells were plated into 192 wells and cultured according to a standard procedure[26]. Their culture supernatants were screened by immunohistochemistry on frozen sections of the optic tectum of 7-day chick embryos or by immunoblot analysis of extract from the optic tectum of an 11-day chick embryo (see below). Cloning was performed by limiting dilution. MAbs were obtained as culture supernatant.

Gel electrophoresis and immunoblot analysis

The procedures used for gel electrophoresis and immunoblot analysis have been described in detail previously[4,30]. In brief, isolated tissues were homogenized in 10 vols. of sample buffer composed of 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, 1 mM EDTA, 40 mM Tris, and 240 mM glycine at pH 8.5. After being boiled in water for 3 min, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the procedure of Laemmli[12]. In some cases isolated tissues were homogenized in 10 vols. of isoelectric focusing lysis buffer containing 9 M urea, 2% Nonidet P-40 (w/v), 2% Ampholine (1.6%, pH range 5–7 and 0.4%, pH range 3.5–10), and 5% 2-mercaptoethanol. Then samples were subjected to two-dimensional gel electrophoresis according to the method of O'Farrell[24] with some modifications as described previously[18]. After the electrophoresis the proteins were transferred to nitrocellulose sheets by electroblotting for 2–12 h at 200 mA (see ref. 25). In some cases the

blots were stained with Amido black for demonstration of protein. Nitrocellulose sheets containing the transferred proteins were incubated in Tris-buffered saline (pH 7.4) containing 5% bovine serum albumin for 60 min and then further incubated with antibodies in the same buffer for 90 min. After washing, the sheets were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG in the above buffer for 60 min. The blots were developed using 0.05% 4-chloro-1-naphthol and 0.01% hydrogen peroxide[8].

Immunohistochemistry

Samples of brain or other tissues were obtained from chick embryos (4–20 days of incubation) and newly hatched chicks (White Leghorn). Young chickens (5 months old) and adult hens (ca. 1.5 years old) were also used. The animals after hatching were anesthetized with ether and perfused with phosphate-buffered saline (pH 7.4) containing 10% formalin. Dissected tissues were fixed with 10% formalin in phosphate-buffered saline for more than 12 h.

Tissue specimens for use as paraffin sections were dehydrated in a graded ethanol and xylene series, embedded in paraffin, and sliced into 3-μm-thick sections. The sections were stained using the avidin–biotin–peroxidase complex method with MAbs as described previously[6,10]. In brief, the deparaffinated sections were incubated with MAbs, with biotinylated anti-mouse IgG, and then with a solution of avidin–biotinylated horseradish peroxidase complex. Peroxidase activity was identified by incubating with a solution of 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in phosphate-buffered saline. In some cases sections were counterstained with hematoxylin.

Tissue specimens to be used for frozen sections were equilibrated with 30% sucrose in phosphate-buffered saline. Frozen sections 12–14 μm in thickness were cut on a cryostat microtome, and indirect immunohistochemistry with fluorescein isothiocyanate-conjugated goat anti-mouse IgG was performed as described previously[4,22,23].

Immunoglobulin class typing

The immunoglobulin class of MAbs was determined histochemically with a mixture of rhodamine-conjugated goat IgG specific to mouse γ-chain and fluorescein-conjugated goat IgG specific to mouse μ-chain. IgG MAbs were stained by rhodamine but not by fluorescein, while the converse was obtained with IgM MAbs[4].

Protein determination

Protein was assayed by the method of Lowry et al.[17].

RESULTS

Characterization of polyclonal and monoclonal antibodies against S5, S6 and S54 proteins

Polyclonal and monoclonal antibodies were produced against S5 protein or S6 protein, as described in Materials and Methods. Each of the resulting antisera (RS5 and RS6) and MAbs (M2A6, M2D8, M2E7, M2F6 and M2H1) was examined for its specificity for the 3 proteins S5, S6, and S54 in terms of the following 3 features. First, these antibodies were used for immunoblot analysis of extract from the optic tectum of an 11-day chick embryo. Each antibody bound specifically to two intense bands (mol. wts. 95 and 100 kDa) and one faint band (110 kDa) in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1A), or to 3 spots in two-dimensional gel electrophoresis (Fig. 1B). The migration patterns of these 3 bands or spots in electrophoresis were identical to those of S5, S6, and S54 proteins[30]. Second, in the chick embryonic optic tectum S5 protein is known to increase from day 4 to day 7, after which it decreases again. On the other hand, S6 protein increases from day 7 to day 9, and decreases after day 18. Protein S54 appears by day 11, and then increases. As shown in Fig. 1C, the developmental changes observed in the amounts of the antigens were consistent with the above-described changes occurring in S5, S6, and S54 proteins. Finally, the antigens were found to be enriched in the 20–30% ammonium sulfate fraction that was rich in S5, S6, and S54 proteins[30]. In contrast to this, β-tubulin recognized by MAb 152H6 (unpublished) and one of the neurofilament proteins recognized by MAb 82E10 (see ref. 4) were not enriched in this fraction (Fig. 1D). A rabbit antiserum RS6 faintly stained some other bands besides those of S5, S6, and S54 proteins (Fig. 1D; lane 10). These were probably breakdown products of S5, S6, and S54 proteins or their aggregates.

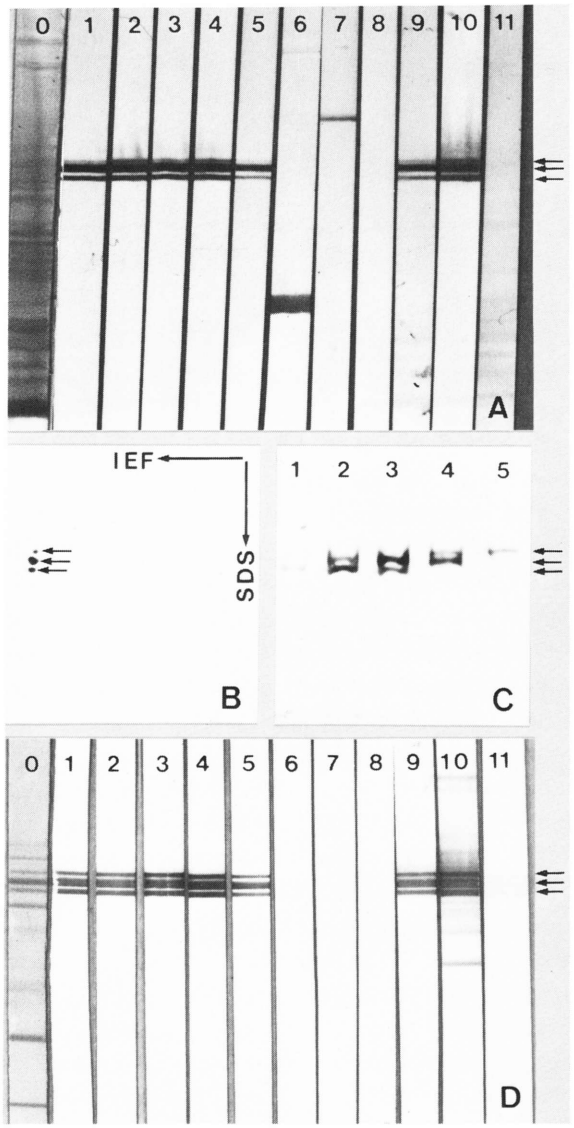

Fig. 1. Immunoblot analysis of antisera and monoclonal antibodies. An extract of tissue homogenate (600 μg of protein) (A) or a partially purified fraction (20 μg of protein) (D) was electrophoresed on sodium dodecyl sulfate-polyacrylamide gel (7%), and electroblotted onto a nitrocellulose sheet. The sheet was cut into 12 strips and each strip (lanes 1–11) except lane 0 was treated for staining with MAbs or antisera as described in Materials and Methods. Lanes in A, D: 1, M2A6; 2, M2D8; 3, M2E7; 4, M2F6; 5, M2H1; 6, β-tubulin-specific 152H6 (unpublished); 7, 82E10 (ref. 4); 8, myeloma culture supernatant; 9, RS5; 10, RS6; 11, rabbit preimmune serum. Proteins in lane 0 were stained with Amido black. B: tissue homogenate was subjected to two-dimensional gel electrophoresis, electroblotted onto a nitrocellulose sheet, and immunostained with MAb M2F6. C: the blots of tissue homogenate at various developmental stages were immunostained with MAb M2F6. Lanes in C: 1, day 4; 2, day 7; 3, day 9; 4, day 16 of incubation; 5, 10 days posthatching. The arrows indicate S5, S6 and S54 proteins.

These observations indicate that each of the polyclonal and monoclonal antibodies obtained in the present study is specific for S5, S6, and S54 proteins in the chicken. Since none of the obtained antibodies could recognize S5, S6, and S54 proteins distinctively, these 3 proteins seem to have very similar antigenicity.

Other properties of the obtained MAbs are summarized in Table I. All MAbs were IgGs. They bound to the antigens on frozen sections of chick embryonic optic tectum. On paraffin-embedded tissues, the immunoreactivity was preserved only for MAbs M2D8, M2E7, and M2F6. Three MAbs cross-reacted with the rat and guinea pig antigens in immunoblot analysis but the remaining two did not. These observations indicate that the epitopes recognized by MAbs M2A6, M2E7, M2F6, and M2H1 are different from one another. Therefore it is concluded that S5, S6, and S54 proteins have at least 4 different epitopes in common.

Distributions of S5, S6, and S54 proteins in non-neural and peripheral nervous tissues

The distribution of S5, S6, and S54 proteins among non-neural and peripheral nervous tissues was examined by immunoblot analysis with MAb M2F6. In an 11-day chick embryo, S5 and S6 proteins were found in the muscle and intestine, but not in the liver (Fig. 2A). In the intestine in particular, S6 protein was present at a level comparable to that in the optic tectum. On the other hand, none of S5, S6, or S54 proteins were detected in the adult non-neural tissues (liver, heart, pectoral muscle, and intestine; Fig. 2B). Several faintly stained bands were observed as seen in Fig. 2B. However, they appeared to be non-specific, because these bands were also stained with myeloma culture supernatant. By immunohistochemistry of the intestine on day 11 of incubation, intense immunostaining was observed in the longitudinal muscular layer. The intestinal epithelium was not stained. On day 11 of incubation pectoral muscle was immunostained faintly. On day 2 of incubation, however, myotome was intensely immunostained. These observations indicate that S5 and S6 proteins are present in developing smooth and skeletal muscle in addition to neuronal cells in the chick embryo.

The labeled antigens were found in the spinal ganglion, but not in the sciatic nerve of the adult chicken.

TABLE I

Monoclonal antibodies specific for S5, S6 and S54 proteins

Antibody no.	*Ig class*	*Immunohistochemistry*		*Immunoblot analysis*		
		Frozen section	*Paraffin section*	*Chicken*	*Rat*	*Guinea pig*
M2A6	G	+	–	+	–	–
M2D8	G	+	+	+	+	+
M2E7	G	+	+	+	–	–
M2F6	G	+	+	+	+	+
M2H1	G	+	–	+	+	+

A characteristic feature of the spinal ganglion of the adult chicken was the presence of S6 protein in addition to S54 protein. On the other hand, S6 protein was detected neither in the optic tectum nor in the cerebellum of the adult chicken[30].

Histochemical examination of the developing chick optic tectum and cerebellum

Immunohistochemistry was performed with antiserum RS6 and MAbs M2A6, M2D8, M2E7, M2F6, and M2H1. Immunostaining patterns of these antibodies were indistinguishable and the results were analogous in paraffin sections stained with the avidin–biotin–peroxidase complex method and in frozen sections with the indirect immunofluorescent method.

In the previous paper[30] the developmental changes of amounts of S5, S6, and S54 proteins were investigated in the chick embryonic optic tectum with two-dimensional gel electrophoresis. Therefore we first examined the developmental changes of immunostaining patterns in the optic tectum. In the optic tectum on day 4 of incubation, immunostaining was observed in the somata and growing processes of differentiated neurons in the marginal zone (the superficial cell-sparse layer; Fig. 3A, B). On day 7 of incubation, intense immunostaining of parallel rows, corresponding to the fasciculated tectobulbar tract[5], was observed (TB in Fig. 3C). The marginal zone were also intensely stained (MZ in Fig. 3C). The ventricular zone (VZ in Fig. 3C), which consists of undifferentiated cells, was not stained. On day 11 of incubation the antigens were widely distributed in the optic tectum except for the ventricular zone. Intense stain-

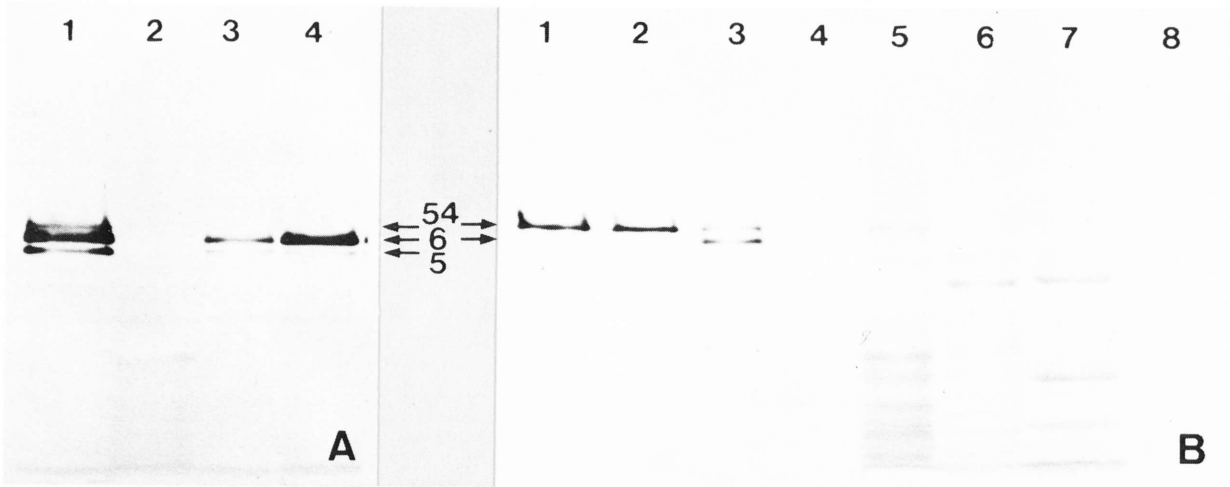

Fig. 2. Tissue specificity of S5, S6, and S54 proteins in an 11-day chick embryo (A) and an adult chicken (B). The blots of various tissue homogenates (0.5 mg wet wt.) were immunostained with MAb M2F6. Lanes in A: 1, optic tectum; 2, liver; 3, pectoral muscle and 4, intestine. Lanes in B: 1, optic tectum; 2, cerebellum; 3, spinal ganglion; 4, sciatic nerve; 5, liver; 6, heart, 7, pectoral muscle and 8, intestine. 5, S5 protein; 6, S6 protein; 54, S54 protein.

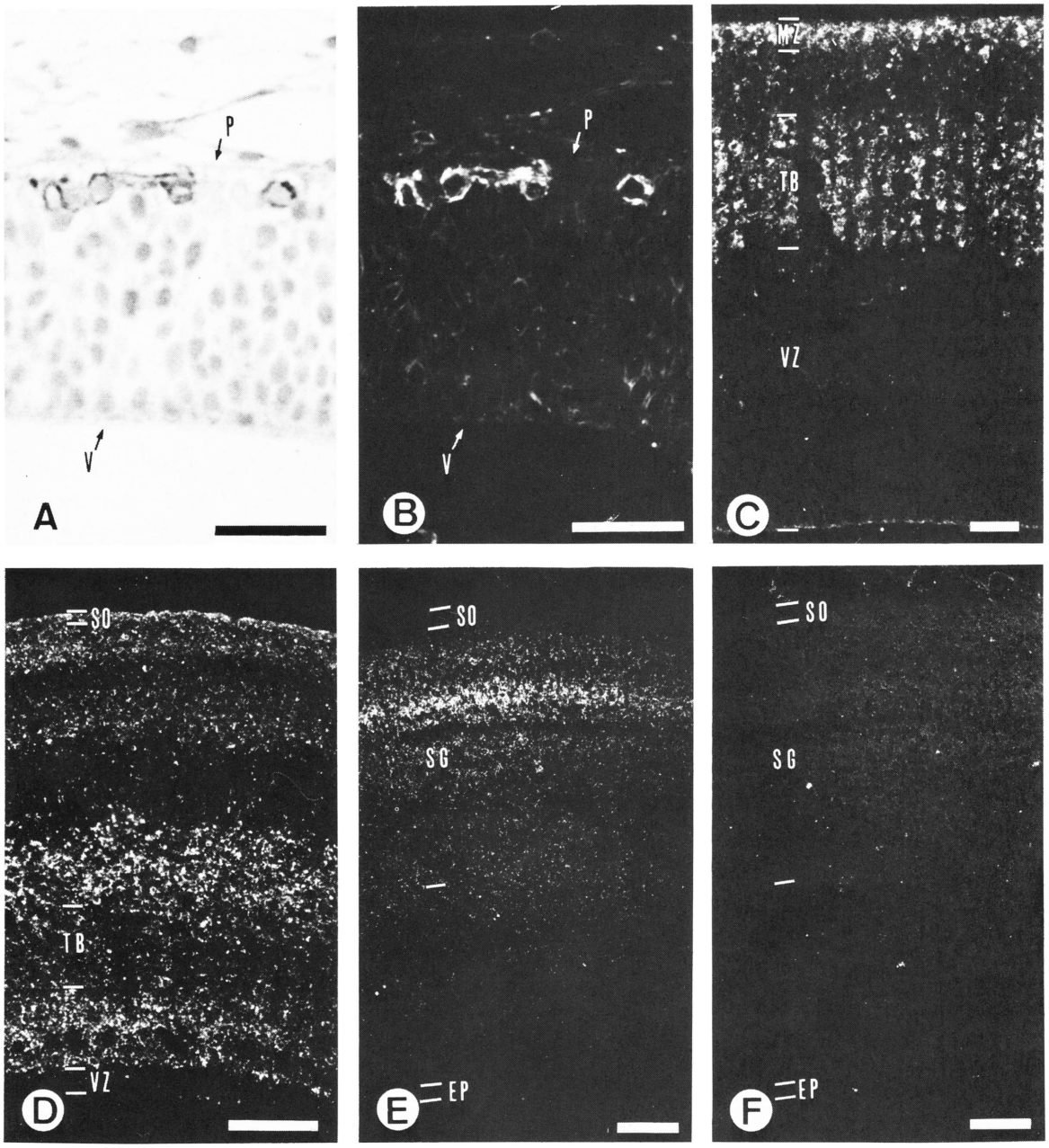

Fig. 3. Light-field (A) and dark-field (B–F) photomicrographs of sagittal sections showing the distribution of antigens in the embryonic optic tectum at different developmental stages. A–E were stained with MAb M2F6. A was counterstained with hematoxylin. F is a control section stained with myeloma culture supernatant. A and B: day 4 of incubation. Differentiated neurons in the superficial layer are stained. Bars = 20 μm. C: day 7 of incubation. Bar = 20 μm. D: day 11 of incubation. Bar = 100 μm. E, F: day 16 of incubation. Bars = 100 μm. P, pial surface; V, ventricular surface; EP, ependyma; MZ, marginal zone; SG, stratum griseum et fibrosum superficiale; SO, stratum opticum; TB, tectobulbar fiber layer; VZ, ventricular zone.

ing was observed in the cellular layer just above the ventricular zone, in the developing stratum griseum centrale[13], in the cell-sparse layers of the developing stratum griseum et fibrosum superficiale, and in the developing stratum opticum (SO in Fig. 3D), which is formed largely by the growing retinal fibers[13]. The immunoreactivity in the layer of the tectobulbar fibers on day 11 of incubation was weaker than that on

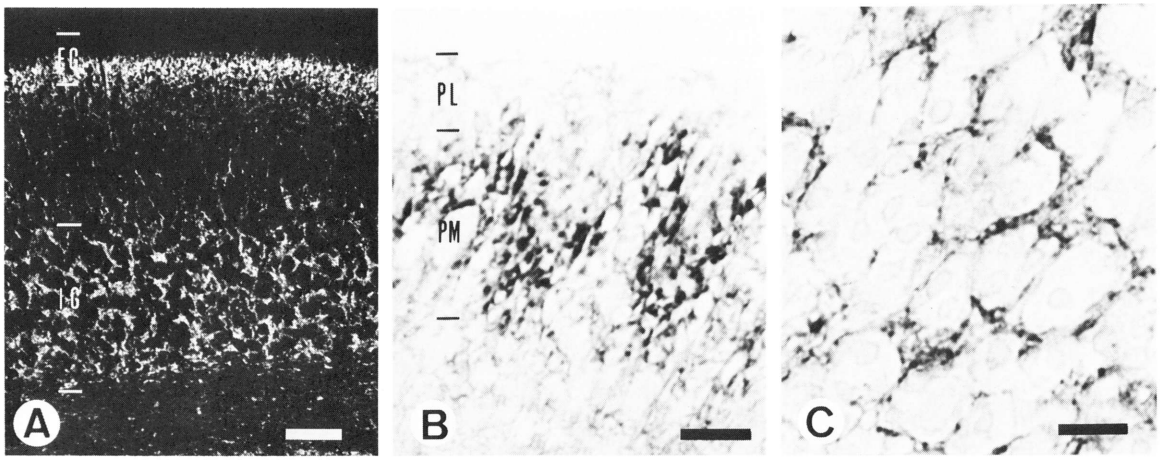

Fig. 4. The developing cerebellum on day 16 of incubation stained with MAb M2F6. Sagittal sections. EG, external granular layer; IG, internal granular layer; PL, proliferative zone and PM, premigratory zone of the external granular layer. A: dark-field photomicrograph. Bar = 40 μm. B, C: light-field photomicrographs. The proliferative zone of the external granular layer is not stained, but the premigratory zone is intensely stained. In the internal granular layer (C) cell somata are not stained, but cell processes are stained. Bars = 10 μm. Hematoxylin counterstain.

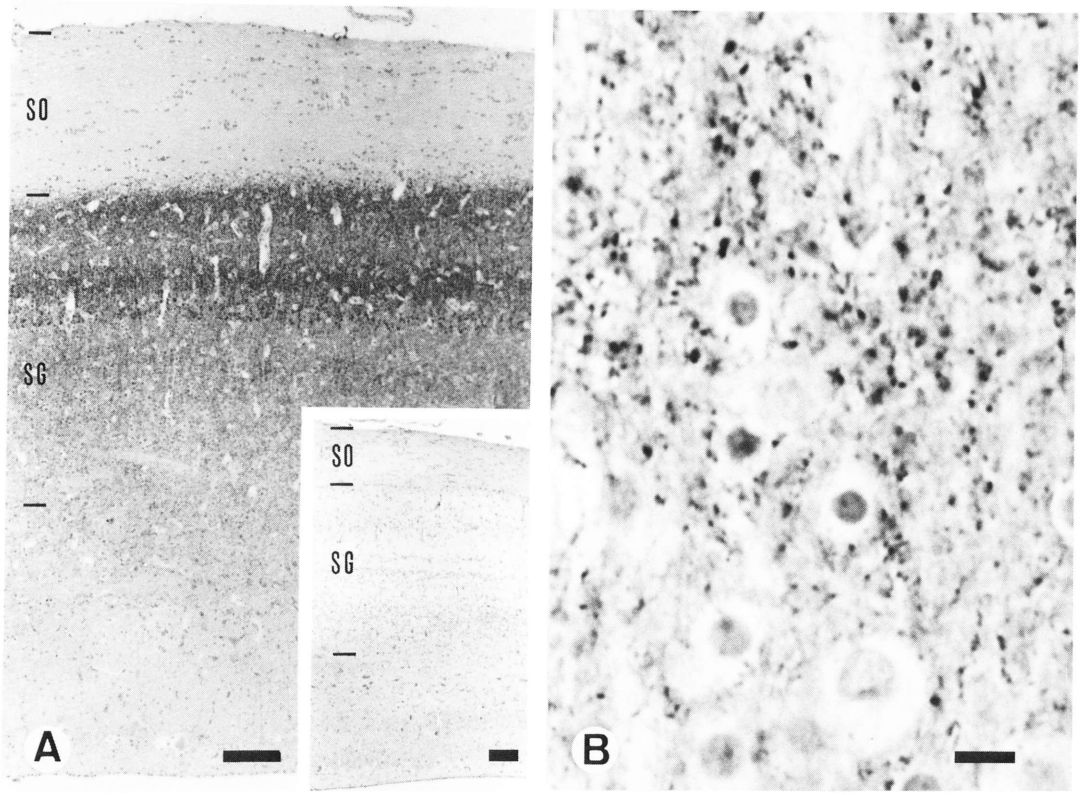

Fig. 5. Light-field photomicrographs of sagittal sections of the adult optic tectum stained with MAb M2F6. A: intense immunostaining is visible in the superficial laminae in the stratum griseum et fibrosum superficiale (SG). There is no immunostaining in the stratum opticum (SO). The inset is a control section stained with myeloma culture supernatant. B: high magnification of the superficial laminae of the stratum griseum et fibrosum superficiale. Immunoreactive dots are not present in the cytoplasm of somata. Bars in A = 100 μm. Bar in B = 5 μm. Hematoxylin counterstain.

day 7 of incubation (Fig. 3; compare TB in C and D). On day 16 of incubation, intense immunostaining was observed in the superficial laminae of the stratum griseum et fibrosum superficiale where retinal fibers ingrow and terminate[13,28] (Fig. 3E). Immunostaining in the layers of retinal fibers (the stratum opticum), and of tectobulbar fibers (the stratum album centrale) was weak. At this developmental stage, immunostaining was hardly observed in the cytoplasm of cell somata.

In order to see whether such developmental changes of the localization of antigens occur in other brain regions, we examined the cerebellum, because the histogenesis of the cerebellum has been investigated in detail[1,19,20,22]. Fig. 4A shows a section of chick cerebellum on day 16 of incubation. Characteristic immunostaining was observed in the external granular layer and in the internal granular layer. The superficial zone of the external granular layer was not stained, but the deeper zone was intensely stained (Fig. 4B; compare PL with PM). The former probably corresponds to Altman's proliferative zone, which contains only undifferentiated cells, and the latter to the premigratory zone, in which post-mitotic cells begin to differentiate[1]. In the internal granular layer, areas of cell processes between the nests of cell somata were intensely stained (Fig. 4C). These neuropil areas consist of the dendrites of granule cells, the axonal knobs of mossy fibers and the cell processes of Golgi cells[19,20]. Most granule cell somata in the internal granular layer were not stained. Weak immunostaining was observed in the molecular layer and white matter.

Histochemical examination of the adult chicken nervous system

Immunoreactivity was widely distributed in the adult optic tectum except for the ependyma and the layer of the retinal fibers (SO in Fig. 5A). Closer examination revealed that the staining consisted of small dots (Fig. 5B). The immunoreactivity was enriched in the neuropil. Although in a young chicken (5 months old) immunostaining was observed in some neuronal somata, in an adult chicken (ca. 1.5 years old) there was no immunostaining in cell somata. In the stratum griseum et fibrosum superficiale

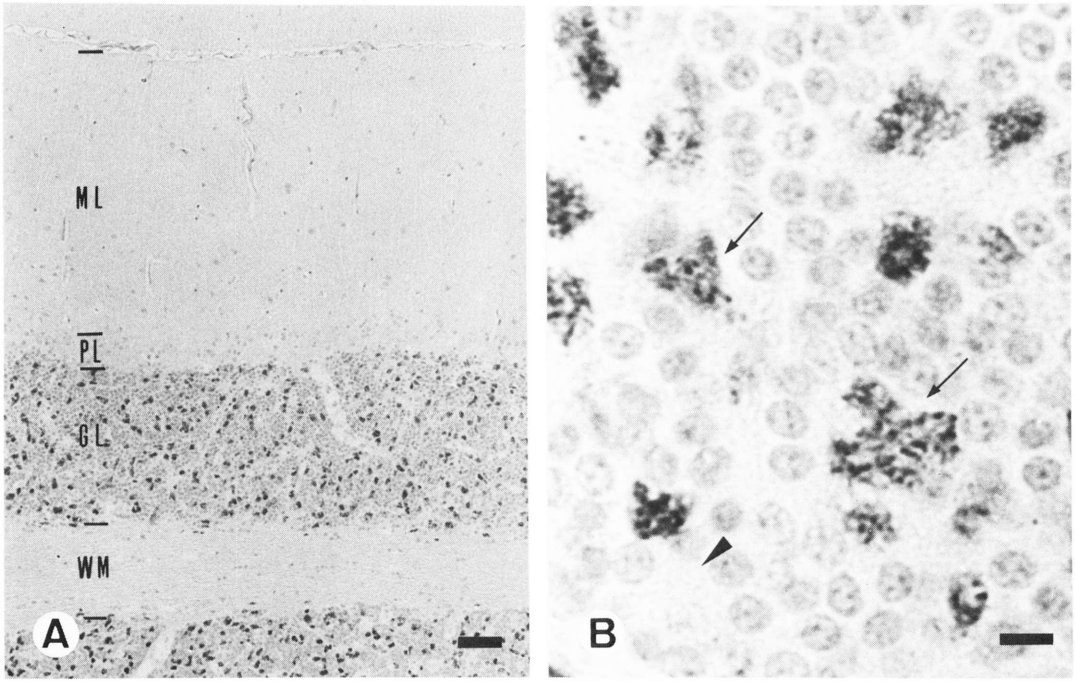

Fig. 6. Light-field photomicrographs of sagittal sections of the adult cerebellum stained with MAb M2F6. A: immunostaining is observed in the granular layer. ML, molecular layer; PL, Purkinje cell layer; GL, granular layer; WM, white matter. B: high magnification of the granular layer illustrated in A. Intensely stained (indicated by arrows) and hardly stained cerebellar glomeruli (indicated by an arrowhead) are visible. Bar in A = 50 μm. Bar in B = 5 μm. Hematoxylin counterstain.

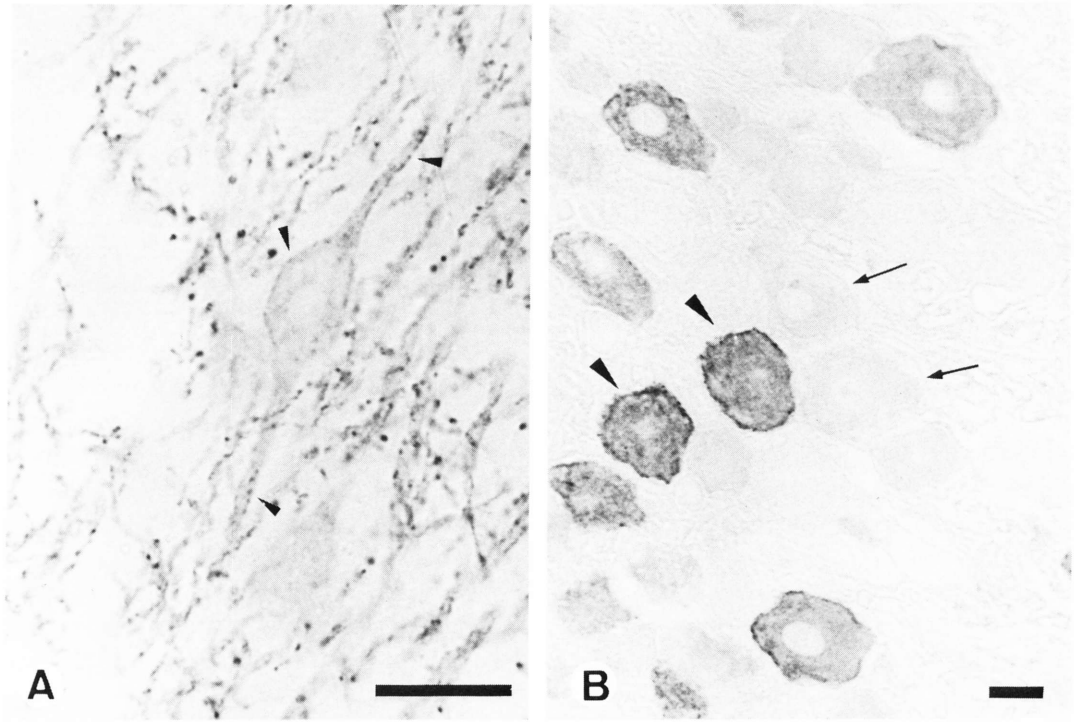

Fig. 7. Light-field photomicrograph of the nucleus isthmi, pars parvocellularis (A) and the spinal ganglion (B) stained with MAb M2F6. A: immunoreactive dots (indicated by arrowheads) surround the neuronal somata and primary dendrites. The cytoplasm of the neuron is also weakly stained. Bar = 20 μm. B: intensely stained (indicated by arrowheads), weakly stained, and hardly stained neuronal somata (indicated by arrows) are visible. No immunostaining is apparent in fibers. Bar = 20 μm.

(SG in Fig. 5A) immunoreactive dots were more numerous and intense in the superficial laminae a–f than in the deeper laminae. The retinal ganglionic fibers terminate on dendrites of tectal neurons in the superficial laminae[13].

Fig. 6 shows the cerebellar cortex stained by MAb M2F6. Intense immunostaining was observed in the granular layer. Closer examination revealed that strong immunoreactivity was present in some but not all of the cerebellar glomeruli, which are complex synaptic arrangements involving mainly mossy fiber terminals and granule cell dendrites[27] (Fig. 6B). Although many synapses are densely packed in the molecular layer, immunostaining was hardly observed in this layer. Purkinje cells and white matter were completely unstained. Thus the strong immunoreactivity seemed to be characteristic in certain neuropils.

Immunoreactive dots were also observed in other brain regions. In addition immunoreactive cells were found in some brain nuclei. Fig. 7A shows a section of the subtectal nucleus isthmi, pars parvocellularis. In addition to the immunoreactive dots surrounding neuronal somata and their dendrites, weak immunostaining was observed in the cytoplasm of some somata. In deep cerebellar nuclei, some neuronal somata were also immunostained (not shown). Furthermore, in the spinal ganglion, immunostaining was present only in neuronal somata (Fig. 7B). Most neurons were intensely or weakly stained, but some were hardly stained. No immunostaining was observed in fibers in the spinal ganglion. These observations indicated that the antigens are also concentrated in some neuronal somata in addition to neuropils.

DISCUSSION

In the previous study[30] it had been concluded that S5 and S6 proteins form a family of structurally related developmentally regulated proteins. The present immunoblot analyses confirmed this finding: polyclonal and monoclonal antibodies developed against purified S5 or S6 protein bound both of these proteins. In addition, all of these antibodies bound

S54 proteins in more developed chicken nervous system. These observations indicate that S5, S6, and S54 proteins make a family of proteins which has a common antigenicity and is developmentally regulated in their expression.

The present immunohistochemical analysis indicated that: (A) in the brain this protein family first accumulates in both the somata and cell processes of newly differentiated neurons. (B) it then becomes localized in neuronal processes. (C) finally it is mainly localized in synaptic regions. In the optic tectum of the 4-day embryo, immunoreactivity appeared in differentiated neurons, but not in undifferentiated or mitotic cells. At the above developmental stage, S5 protein is present in the optic tectum, but S6 and S54 proteins are not[30]. Therefore the labeled antigen first accumulating in newly differentiated neurons is mainly S5 protein. The immunoreactivity in fiber tracts was observed in the early developmental stages, becoming subsequently weaker, and eventually disappearing. The immunohistochemical analysis of the developing cerebellum showed that the labeled antigens were markedly accumulated in the cells in the premigratory zone of the external granular layer, but no such accumulation occurred in the cells in the proliferative zone. Undifferentiated cells are situated in the proliferative zone. When the cells become postmitotic, they are transferred to the premigratory zone, and begin to extend their cell processes[1]. Immunostaining of the growing axons in the molecular layer was not strong. In the cerebellum, therefore, the labeled antigens in newly differentiated neurons accumulate characteristically at the beginning of neurite growth.

In the adult optic tectum and adult cerebellar cortex, the strong immunoreactivity seems to be localized in some synaptic regions. In both two-dimensional electrophoresis[30] and immunoblotting, the adult optic tectum and adult cerebellum contain only S54 protein. Therefore S54 protein seems to be concentrated in synaptic regions. Furthermore, immunoelectron microscopy (Shirao et al., in preparation) showed that in the cerebellar glomeruli immunostaining was observed diffusely in postsynaptic neuronal processes facing the mossy fiber terminals, but not in glial elements. Immunostaining was not observed either in the neuronal somata or in the axons in fiber tracts. In some brain nuclei, however, immunoreactivity was also present in the cytoplasm of some neuronal somata. One possible explanation for these observations is that S54 protein is concentrated in both immunoreactive synaptic regions and neuronal somata. S54 protein should be produced in neuronal somata, carried through axons or dendrites to the synaptic regions, and then concentrated there. In the immunoreactive somata S54 protein may reach the critical concentration detectable by the immunostaining used in this study.

Another possible explanation for the above observations is that S54 protein is concentrated only in synaptic regions, and that another cross-reactive protein(s) (S5 or S6 protein) accumulate in immunoreactive neuronal somata. The amount of this cross-reactive protein is possibly too small to be detected by immunoblot analysis because only a small number of the cells are immunoreactive. The adult spinal ganglion contained some neuronal somata with strong immunoreactivity, and had S6 protein in addition to S54 protein. The differential localization of two different proteins within a cell has been already reported. The cerebellum contains two forms of spectrin (brain and erythrocyte forms). The brain form of spectrin is present in neuronal somata and in their processes, but the erythrocyte form accumulates exclusively in neuronal somata[15,16]. But whether S6 and S54 proteins are differentially localized within a cell cannot be answered without more discriminating specific antibodies.

The previous study[30] showed that in an 11-day chick embryo S5 and S6 proteins are mainly present in the central nervous system, but only in trace amounts in the rest of the embryo. It was suggested that this slight content could be explained by the existence of S5 and S6 proteins in the peripheral nervous system. In the present study immunoblot analysis revealed the existence of S5 and S6 proteins in some non-neural tissues of an 11-day chick embryo. In particular, S6 protein was present in the intestine at levels equivalent to that in the neural tissue. Immunohistochemical analysis also showed that immunoreactivity was present in non-neuronal cells in addition to neurons in the chick embryo. Therefore S5 and S6 proteins seem to be present in some non-neuronal cells. On the other hand, S54 protein was detected neither in the embryonic nor in the adult non-neuronal tissues by immunoblot analysis. Furthermore,

immunohistochemical analysis of adult nervous system showed that S54 protein is present only in neurons. Therefore it is concluded that S54 protein is a neuron-specific protein.

The neural cell-adhesion molecule exists in embryonic and adult forms, and these two forms share a variety of epitopes[29]. It has been suggested that a transition in the carbohydrate portion of the molecule occurs from embryonic to adult forms, and that this transition may act to stabilize the final patterns of interneuronal adhesion[3,9,29]. The orderly and temporal appearances of S5, S6, and S54 proteins during development may be concerned in cell migration, neurite growth, and synaptogenesis. It will be of interest in future investigation to elucidate whether these developmentally regulated forms arise as a result of posttranslational modification of a single protein or not.

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