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Immunohistochemical Expression of Synaptophysin in the Adult and Developing Cervical and Lumbar Enlargements of the Spinal Cord of Rabbit.

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ABSTRACT

Background: The principle findings of synaptophysin immunoreactivity (SynpIR) during the ontogeny of rabbit spinal cord are: At E14, SynpIR precedes in the entire marginal layer especially at the entrance zone of dorsal root and motor neurite outgrowth emerged from the basal plate. At E21, SynpIR is expressed in the motoneurons of ventral and lateral horns of mantle layer growing into the ventrolateral columns of marginal layer. **Methods:** We found intensely stained thick tracts and diffuse axons among proliferating neuroblasts of mantle layer. The peripheral parts of ventral horns were occupied with closely packed multipolar neurons from which long dendrites departed toward the surface of marginal layer. **Results:** At E28, pronounced SynpIR presented in the ventral grey horn while the white matter was faintly stained., meanwhile the dorsal horn was more cellular than ventral and lateral horns. Few intensively SynpIR fibers cross the dorsal and ventral commissures. In adult, profuse SynpIR appeared in the entire grey matter, and stained dendrites departed from neurons in the lateral laminae into the adjacent funiculi as finger-like projections. These projections did not reach the surface, so that the outer one-third to one-fourth of the funiculi contained little or no SynpIR. In the periphery of ventral horns, we found large multipolar neurons with faintly stained cytoplasm. The white matter and the neuroepithelial cells surrounding the central canal were almost unstained. **Conclusion:** Synaptophysinis a reliable marker for fiber outgrowth and synapse formation in therabbit spinal cord, and its differential expression levels is specific and almost completed before birth.

Keywords: *Synaptophysin, Development, Spinal cord, Rabbit, IHC.*

INTRODUCTION

The spinal cord is part of the central nervous system that functions as a channel for outgoing (efferent) neural motor transmission, and incoming (afferent) sensory information, and also serves as the site of neural reflex circuits and central pattern generators. It is built in a basic three-zone pattern, which is maintained during development: the grey matter layer where cell bodies reside is called the mantle zone, the white matter forms the marginal zone and the ependymal layer, which is the main site of proliferation, and forms the lining of the central canal of the spinal cord. Gradually, the mantle layer becomes a butterfly-shaped structure.The grey matter consists of neurons, their dendrites, and the supportive cells called neuroglia. It also contains a meshwork of neural tissues such as axonal, dendritic, andglial processes that are packed very tightly together and that fill the interneural spaces. The white matter is devoid of neuronal cell bodies and consists primarily of myelinatedaxons, some unmyelinated axons, supportive neuroglial oligodendrocytes, and blood vessels. The myelin sheaths around the axons impart a white color to this region.

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The development of spinal cord has been the subject of intensive neuroanatomical and morphological research mainly neurulation, neuroblast migration and axonal pathfinding.[1,2] Following their proliferation in the ependymal layer, the neuroblasts migrate towards the periphery to form the mantle layer, the presumptive grey matter. The direction of migration of the neurons is orthogonal to the ventricular surface and most likely closely associated with the radially orientated glial processes. In the mantle layer, the postmitotic neurons aggregate in anlagen and start to differentiate developing the outgrowth of their axon and dendrites. The axons extended into the peripheral marginal layer of the presumptive white matter making contact through small synaptic vesicles. Synaptophysin; the first synaptic vesicle protein cloned and characterized[3]is widely accepted as the most reliable synaptic vesicle marker.[4-6] In the spinal cord of rat,[7] demonstrated strong synaptophysinimmunoreactivity in the dorsal and ventrolateral parts of the marginal layer from embryonic day 14 (E14) throughout the prenatal period. They added this staining pattern most likely indicates transient functional synaptic contacts because, in the adult rat spinal cord, the corresponding region, the white matter, exhibited only faint synaptophysin immunoreactivity. In the mantle layer of the embryonic rat spinal cord, which corresponds to the grey matter of the adult spinal cord, strong synaptophysinimmunoreactivefibers were observed prior to the formation of functional synapses. The latter are most likely permanent, since synaptophysinimmunoreactivityin the adult spinal cord is mainly confined to the grey matter. In support, synaptophysin immunoreactivity is no longer present in the cytoplasmic compartments of adult neurons,[8-9] indicating that both the biosynthesis and the sorting of synaptic vesicle proteins are specifically regulated during neuronal development.In addition, variances in gliogenesis, myelination and vasculature of the fetal spinal cord of mouse and rabbit.[10-11] He showed that the most rapid glial proliferation in the white matter coincides with the beginning of a rapid increase in vascularity in both white and grey matter at E16 and E20 in fetal mouse and rabbit spinal cord, respectively. He added that myelinated axons appear in the ventral white matter at E24, and myelination become well established in all ventral, lateral and dorsal tracts of the white matter at E30 in rabbit yet in the mouse the onset of myelination at El8 up to P5 when myelination is fairly well established. Whether this phenomenon is similar in the spinal cord of rabbit or not is not known as knowledge about the distribution of synaptic vesicles are very little and investigations using synaptic markers are not available either. In the present study, we used synaptophysin immunocytochemistry as a marker of timing of the appearance of synaptic vesicles at various gestational ages in spinal cord of rabbit and comparing them with the adult, and to determine whether it followed a similar pattern to that of the rat or not. We conclude that synaptophysin is a reliable marker for the synapses and neuronal outgrowth and the pattern of its distribution during the development of the spinal cord in rabbit is specific.

MATERIALS ANDMETHODS

Animals preparation and tissue collection

Adult pregnant female rabbits (20-22 weeks old) were purchased from the farm of Alexandria University Faculty of Agriculture (Alexandria, Egypt) and used for this investigation. The day after mating was designated as the embryonic day 1 (E1). Adult materials were enucleated fromthe pregnant rabbitsanesthetized by chloroform, surgicallysacrificed and thenperfused transcardially with 0.1 M phosphate buffered saline (PBS, pH 7.4) for 1 minute followed by 4% paraformaldehyde (PFA) in 0.1 M PBS for 10 minutes using peristaltic pump. The embryos were collected at the 14th, 21st and 28th day of gestation (E14, E21, and E28, respectively).Three to five specimens were used for each stageand fixed in PFAin 0.1 M PBS (pH 7.4)at 4°Cfor 3–4 days at 4°C, dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin.Whole El4embryos were sectioned while blocks of tissue containing the cervical and lumbar enlargements of the spinal cord of E21 and E28 embryos as well as the adult were dissected out and removed and the dorsal surface of spinal canal opened to expose the cord during postfixation.

Synaptophysin Immunohistochemistry

For avidin-biotin complex (ABC) method, the tissue sections were deparaffinized in xylene, dehydrated with in a graded series of ethanol, and then processed for antigen retrieval using EDTA buffer (pH 8) in a boiling water bath for 20 minutes. The sections were then treated with 3% H2O2 for 30 min to block endogenous peroxidase activity, and then in normal horse serum as blocking reagent to prevent nonspecific reaction for 1 hour, each at room temperature. The sections were incubated over night at 4□ with 1:500 dilution of mouse monoclonal synaptophysin. Following rinsing in PBS several times, they were incubated with biotinylated anti-mouse (1:5000)followed by PBS rinse and subsequently with ABC complex (Vector, Burlingame, CA) for 90 min each at room temperature. Horseradish peroxidase (HRP) activity was detected by incubating slides with 0.1 M Tris-HCl buffer (pH 7.5) containing 0.04% diaminobenzidine (DAB) and 0.03% H2O2. The immunostained sections were counterstained with Mayer's hematoxylin, dehydrated with a graded series of ethanol, cleared in xylene, cover slipped with

Permount (Fisher Scientific, Fairlawn, NJ). All photographic images were captured on CCD camera mounted on microscope and stored on optical disks for offline investigations. Images were edited by Adobe Photoshop CC (Adobe Systems, San Jose, CA). Contrast was adjusted as needed for all figures. All animal experiments were reviewed and approved by the Damanhour University research committee prior to the experiment.

RESULTS

Synaptophysin immunoreactivity in the adult rabbit spinal cord

In all investigated segments, the spinal cord sections showed thinner dorsal horns compared to the ventral horns, and they were extended up to reach the dorsal border of the spinal cord whereas the ventral horns were wider and shorter and they did not extend to reach the ventral border of the spinal cord. Synaptophysinimmunoreactivity (SynpIR) presentedlittle variationsbetween the segments of the cervical [Figure 1A] and lumbar(Fig. 1B) enlargements. We could not detect any SynpIRin the neuroepithelial cellsof the ependymal layer, whichis a single-cell layer surrounding the central canal that tends to be oval and greatly constricted. Profuse SynpIR consisting of punctate granulesappeared in the entire dorsal, lateral and ventral horns of the grey matter specially along theaxons of dorsal spinal roots at their entrance into the dorsolateral fissures. On the other hand, the dorsal, lateral and ventral funiculi of the white matter, whichdid not exhibit stainghaveabout 3:1 relative proportion to the grey matter at the two spinal cord enlargements. The right and left masses of white matter are separated by deep dorsal and ventral fissures, respectively. The medial laminae of the right and left dorsal horns are smooth thus the dorsal funiculi are entirely devoid of immunoreactivity [Figure 1].

However, SynpIRdendrites from neurons in the medial laminae of the ventral horns departed into the ventral funiculus on each side of the ventral median septum. Bundles of SynpIR nerve fibers, likely commissural fibers, extended throughnarrow dorsal and ventral grey commissuresabove and below the central canal; thus, the grey matter takes the characteristic butterfly shape [Figure 1, 2A]. Intensive SynpIRdendrites from neurons in the lateral laminae of the entire grey matter extended into the dorsal, ventral and lateral funiculi as finger-like projections. These projections appeared to diminish in size as they extended peripherally and, in general, did not reach the surface of the spinal cord, so that the outer one-third to one-fourth of the funiculi contained little or noSynpIR [Figure 1, 2A-B].

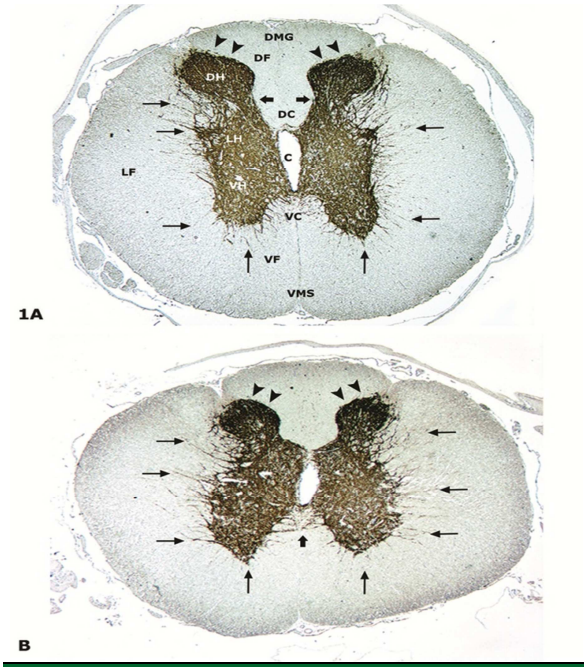

Figure 1: Synaptophysin immunoreactivity in the spinal cord of adult rabbit. Synaptophysinimmunoreactivity (SynpIR)in the adult transverse sections of the spinal cord of rabbit presented little variations between the segments of the cervical enlargement (A) and lumbar enlargement(B). The ependymal layer surrounded the central canal (C) was immunonegative. SynpIR was mainly confined to the gray matter, particularly in the laminae (double head arrows) of the dorsal horn (DH). Intensive SynpIR in dendrites (arrows) from neurons as finger-like projections. Scale bars A-B=2x

In the periphery of ventral horns, wefoundlargemultipolar neuronswith prominent nucleoli and faintlysynaptophysin stained cytoplasm [Figure 2B-D]. Other fusiform middle sized and small unipolar neurons were found in the peripheral parts of the dorsal horn [Figure 2C] and the central part of the ventral horns. Long SynpIRdendrites extended from the large neurons within the white matter. Glial cells and astrocytes were distributednearor around the neurons allover the grey matter.

Synaptophysin immunoreactivityin the early phase of spinal cord development

At E14, after the closure of the neural tube,the neuroepithelium of the primitive spinal cord consists of three distinct layersenclosing a vertically elongated central canal [Figure 3]. The ependymal layer was formed of a thick dorsal and a thin ventral half, both of which meet at the roof and floor plates of the tube. Their proliferating cells migratedorthogonally towards the periphery forming dorsally a small alar plate and ventrally alarge basal plate, together form the mantle layer.The roof plate consisted only of the ependymal layer [Figure 3A1], while the floor plate was formed

from both the ependymal and marginal layers [Figure 3A2]. ExtensiveSynpIR was demonstrated in the entire marginal layer, and the intensity of staining was comparable in the segments of the cervical enlargement than in the lumbosacral segments [Figure 3A, 3B]. SubstantialSynpIR occurred in the axons of the sensory neurons within the entrance zone of the dorsal root and the motor neurite outgrowth that emerged from the differentiating neuroblasts of the basal plate [Figure 3B1, 3B2]. However, faint staining was found in the part of the marginal layer below the floor plate [Figure 3A2]. On the other hand, theproliferating cells within the entire ependymal layer and the dorsal root ganglia as well as roof and floor plates were constantly unstained. Slightstaining was found along the fine axons of the neurons of the mantle layer especially the basal plate [Figure 3B2]. Apart from neuroblasts in the alar and basal plates, unstained glioblastswith prominent nuclei were alsogenerated, and develop into microglial cells, astrocytes, or oligodendrocytes. We could not observe any cells in the marginal layer in all sections studied.

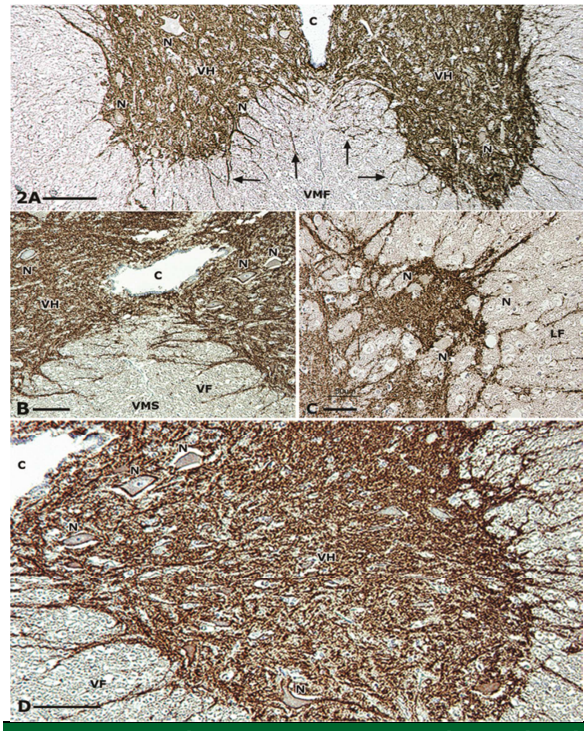

Figure 2: Synaptophysin immunoreactivity in the spinal cord of adult rabbit.(A)Higher magnifications of the ventral horn (VH) of spinal cord transverse sections showed intensive SynpIR in long dendrites (arrows). (B-D) there was a large multipolar neuron with faintly synaptophysin stained cytoplasm. (C) Other fusiform middle sized and small unipolar neurons were found in the peripheral parts of the dorsal horn. Scale bars in A=200µm, B& D = 100µm, C=50µm.

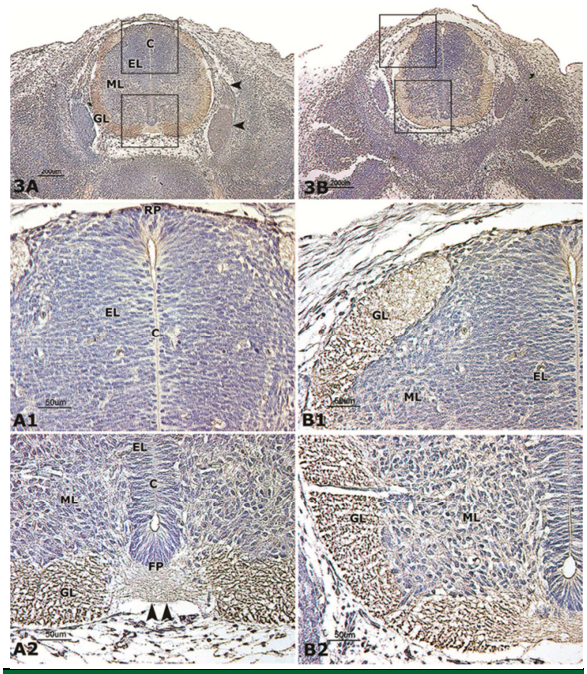

Figure 3: Synaptophysin immunoreactivity in the spinal cord of the embryonic day 14.(A, B) Illustrated intensiveSynpIRin the E14 transverse sections of the spinal cord of rabbit in the entire marginal layer (GL). (A1) The roof plate (RP) consisted only of the ependymal layer (EL), while the floor plate (FP) was formed from both the ependymal and marginal layers in which faint staining (double arrow heads) was found in the part of the marginal layer below the floor plate (A2). Slight staining was found along the fine axons of the neurons of the mantle layer (ML) especially the basal plate (B2). On the other hand, the ependymal layer and the dorsal root ganglia (arrow heads) as well as roof and floor plates were constantly unstained.Scale bars in A&B = 200µm, A1, A2, B1&B2 = 50µm.

At E21, the central canal became oval to elongated in the segments of the cervical and lumbar enlargements, respectively, and surrounded byone flattened cell layer of the ependymal layer [Figure 4A-4B]. The entire marginal layer, presumptive white matterexhibited intensive SynpIR in both thoracic and lumbosacral segments, and thedorsal, ventral and lateral columns could be clearly distinguished from the adjacent dorsal, lateral and ventral grey horns. The intensity of staining was strong within the entrance zone of the primary afferent nervesinto the dorsal horn [Figure 4A1], and the outgrowing nerve fibers of the motoneurons of the ventral and lateral horns of the mantle layer into the ventrolateral columns of the marginal layer [Figure 4B1]. Intensely stained thick tracts of nerve fibers coursed dorsoventrally in the central parts of the mantle layer as well as axons were diffuse among the proliferating neuroblasts [Figure 4A2]. The size and form of the cells was found to be largely variable in the different laminae of the dorsal and intermediate grey

horns [Figure 4A1-2, 4B1]. Theperipheral parts of the ventral horn were occupied with closely packed large and medium-sized multipolar neurons [Figure 4B2]. Long dendrites of the large neurons of the ventral and lateral horns departed toward the surface of the marginal layer.Positively stained neurons were observed in the dorsal root ganglion (arrowheads) [Figure 4B].

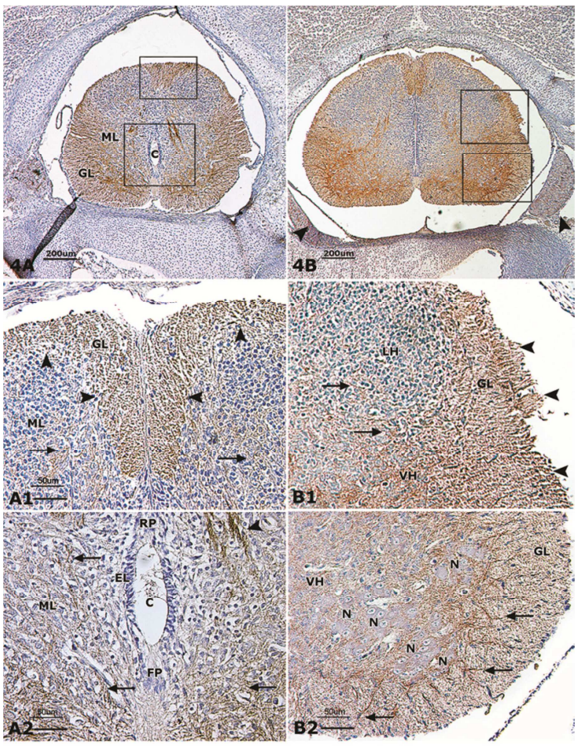

Figure 4: Synaptophysin immunoreactivity in the spinal cord of the embryonic day 21.(A, B) Extensive SynpIRin the E21 transverse sections of the spinal cord of rabbit was distributed in presumptive white matter in both thoracic and lumbosacral segments. (B)Positively stained neurons were observed in the dorsal root ganglion (arrow heads). (A1) strong staining intensity was detected within the entrance zone of the primary afferent nervesinto the dorsal horn (arrow heads). (B1) stained outgrowing nerve fibers (arrows). (A2) Intensely stained thick tracts of nerve fibers (arrow heads) as well as axons were detected among the proliferating neuroblasts (arrows). (B2) immunoreactive long dendrites of the large neurons of the ventral horn. Scale bars in A&B = 200µm, A1, A2, B1&B2 = 50µm.

SynpIR in the late phase of spinal cord development:
At E28, the definite structures of the spinal cord were clearly differentiated, and an explosive proliferation of cells undergo during this stage as thesize of the grey matter was relatively four times that of the white matter [Figure 5A, 5B]. Thecentral canal was oval and greatly compressed and lined by a single cuboidal ependymal layer. The dorsal and ventral commissures became clear and the dorsal median septum and the ventral sulcus were deeper. The white matter was faintly stained and could be clearly distinguished from the deeply stained grey matter.SynpIR was more pronounced in the ventral horn, which was relatively larger in size than the dorsal horn. Nevertheless, the dorsal horn was more cellular than the ventral and lateral horns [Figure 5A1-A2]. Extensively stained outgrowing motor neuron dendrites departed from the ventral and lateral horns into the adjacent medial, ventral and lateral parts of the white matter. In the dorsal and ventral commissures, few intensively SynpIR nerve fibers were found crossing from the dorsal and ventral horns, respectively [Figure 5A2, 5B1].

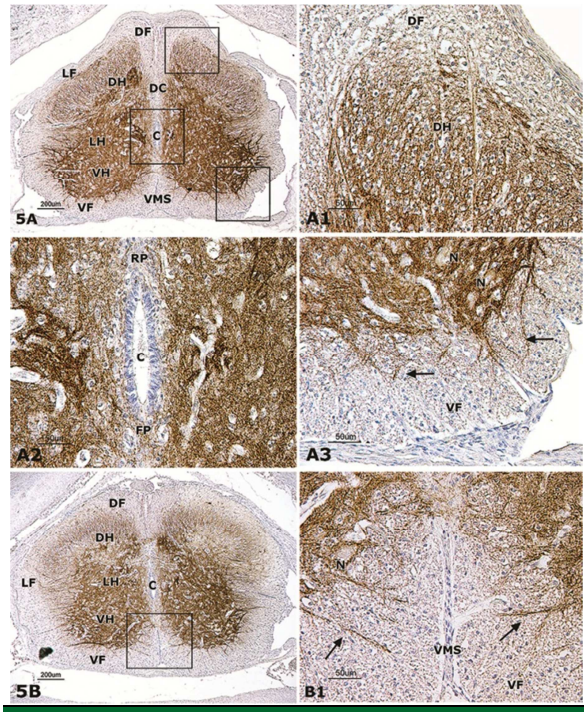

Figure 5: Synaptophysin immunoreactivity in the spinal cord of the embryonic day 28.A, B.SynpIR inthe grey matter was more extensive compared to the faintly stained white matter. The immunoreactivity was pronounced in the ventral horn(A1). The dorsal horn was more cellular than the ventral and lateral horns (A1-A2). Extensively stained outgrowing motor neuron dendrites were detected (A3, B1). In other hand the central canal was unstained (A2).Scale bars in A&B = 200µm, A1, A2, B1&B2 = 50µm.

DISCUSSION

Studies on the developmental expression of synaptophysin in the central nervous system revealed that it is present not only in mature terminals but also in outgrowing axons,[7-9,12] in cytoplasmic bridge

connecting retinal photoreceptor cell bodies with the rod component,[13] in dendrites of the continuously regenerating olfactory receptor neurons and, in dendrites of developing hippocampal pyramidal neurons.[8,9] While synaptic vesicles in outgrowing axons could serve as a reservoir for subsequent synaptogenesis, synaptophysin is improbable to contribute to synaptic organelles in developing dendrites. In the present study, the pattern of synaptophysin immunocytochemical staining exhibited in the adult rabbit spinal cord and during prenatal development consists of punctate granules, suggestiveof a localization to nerve terminals. It was not restricted to synapses but also the migrated neurons and the outgrowing sensory and motor nerve fibers into and out the grey matter.In contrast, the proliferating cells within the ependymal layer or the neuroepithelium surrounding the central canal as well as dorsal root ganglion were constantly unstained as mentioned previously.[7]

Spatial and temporal expression pattern of synaptophysin

In the present study, slight SynpIR was demonstrated at E14only in the marginal layer, namely in the sensory neurons within the entrance zone of the dorsal root and the motor neurons that emerged from the neuroblasts of the basal plate. At E21, the white matter exhibited SynpIR specially in the dorsal column, where the primary afferent nerves projected into the dorsal horn and the ventrolateral columns in addition to the outgrowing nerve fibers of the motor neurons from the ventral and lateral horns. These are extensive network of dendritic branches of the motor neurons and axons of the contralaterally and ipsilaterally projecting interneurons. Similar findings were found in the rat.[7] At E28, the SynpIR become entirely restricted to the grey matter, which has not previously reported. In addition, SynpIR outgrowing motor dendrites extended into the adjacent medial, ventral and lateral parts of the white matter as well as few nerve fibers crossing the dorsal and ventral commissures. Our results show the reduction of SynpIR in the marginal layer, white matter, which contrasts the findings only in adult rat.[7]

Synaptophysin expression in the spinal grey and white matter

SynpIR is principally a marker of maturity as it reflects synapse formation, but it also marks immaturity, such as when it is found in the embryonic white matter. The significance of SynpIR of embryonic white matter is uncertain, but it might represent axonal transport of partial synaptophysin proteins recognized by the anti-synaptophysin antibody.[14] The data derived from the present study reveal that synaptic sites in the spinal white matter of rabbit are organized into outgrowing motor neuron dendrites departed from the ventral horn into the adjacent medial, ventral and lateral parts of the white matter. These data correlate with the patterns of dendritic extensions visualized through neuronal labeling in cat and human.[15,17] In mammals, the peripheral one-fourth to one-third of the lateral and ventral funiculi lack these dendritic extensions. Inthe cat, the ipsilaterally projecting dendrites extended approximately two-thirds of the thickness of the white matter before ending in fine varicosities.[18] This pattern is again revealed in the rat when using an antibody to label a membrane component of vesicles within the presynaptic elements or axons.[5] In contrast, dendrites of motor neurons in frogs and turtles extend to the full thickness of the lateral and ventral funiculi.[19-21]

The relatively thicker and wider ventral horn than the dorsal one of the grey matter may be due to its coordination of motor neurons (Gregory and José, 2008).[22] As mentioned in rat,[7] we found the development of motor neurons in the ventral and lateral horns of the rabbit spinal cordto precede those in the dorsal horn.The synaptophysin-positive ventrolateral parts of the marginal layer consist of an extensive network of dendritic branches of the motor neurons and axons of the contralaterally and ipsilaterally projecting interneurons.[23] Axodendritic synapses have also been observed in rat and mouse at a similar developmental stage within the ventrolateral marginal layer.[17,24] However, functional axodendritic synapses present in the ventrolateral marginal layer do not persist because the intersegmental reflexes gradually disappear during further pre- and postnatal development.[25] Our results show that this process is paralleled with the reduction of SynpIR in the marginal layer in later stages of the prenatal period and during the first week after birth during the formation of the adult white matter.[7]

Prenatal pattern of SynpIR in the rabbit spinal cord support transient synaptic contacts

In the present study, SynpIR in adult spinal cord of rabbit was confined to the grey matter, while the white matter is largely unstained, which is similar to other mammals.[7,26-28] In the embryonic period, we observed profuse SynpIR only within the entire marginal layer of the spinal cord at E14, then it also appeared in the developing ventral and lateral grey horns at E21. SynpIR attained the definite pattern as in the adult at E28. This contrast the findings in the rat spinal cord,[7] where SynpIR is reduced in later stages of the prenatal period and during the first week after birth during the formation of the adult white matter. The difference in the synaptophysin expression between embryonic and adult animal may be due to breakdown of synaptic contacts in the marginal layer and their formation in the mantle layer during development of the white and grey matter.[7] A similar reorganization has been observed in the cerebral cortex, in which immunostaining for

another synaptic vesicle antigen was found to be transiently present in the early area of the white matter in the adult.[29] In summary, the pattern of synaptophysin immunoreactivity in the rabbit spinal cord show peculiar spatiotemporal pattern specially during development. Further studies are planned to examine correlation between thesedifferences with myelination and gliogenesis.

CONCLUSION

We conclude that synaptophysin is a reliable marker for fiber outgrowth in addition to synapse formation in the rabbit spinal cord, and the differential expression levels and patterns of synaptophysin is specific and almost completed in the prenatal stage.

Acknowledgments

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Abbreviations

AP, alar plate; BP, basal plate; C, central canal; DC, dorsal commissure; DF, dorsal funiculus;DH, dorsal horn;DMG, dorsal median groove; EL, Ependymal layer; FP, floor plate;GL, Marginal layer;LF, lateral funiculus; LH, lateral horn; ML, mantle layer; N, neuron; RP, roof plate; VC, ventral commissure; VF, ventral funiculus; VH, ventral horn;VMS, ventral median septum.

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