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Evidence for Transsynaptic Regulation of Neuronal Cell Surface Heparan Sulfate Proteoglycan in Developing Rat Superior Cervical Ganglion

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The effect of neonatal deafferentation on the expression of a neuronal cell surface heparan sulfate proteoglycan (HeS-PG) was investigated in the developing rat superior cervical ganglion. Two monoclonal antibodies, one directed against the core protein of HeS-PG, and one to a determinant associated with a heparan sulfate side-chain, were used to monitor postnatal increases of HeS-PG by radioimmunoassay. Following neonatal deafferentation by section of the cervical sympathetic trunk, total protein per ganglion was slightly reduced at survival times of 7, 14, and 30 days. Expression of the core protein determinant on HeS-PG was not altered in deafferented ganglia. In contrast, levels of side-chain determinant were significantly reduced at 14 and 30 days. These results suggest that processing of HeS-PG side-chains by principal ganglionic neurons is partially regulated by transsynaptic influences during development. Transsynaptic regulation of neuronal development may be a more general process than was believed previously, with effects not limited to molecules associated with synaptic development.

Key words: radioimmunoassay, superior cervical ganglion, heparan sulfate, transsynaptic regulation

The rat superior cervical ganglion (SCG) has been used as a model system for studies of normal development and of the role of innervation in the regulation of development. Biochemical maturation of ganglionic neurons has been carried out by assay of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis [1,2]. Estimates of the progress of synaptogenesis by cholinergic preganglionic neurons have been carried out by assay of choline acetyltransferase (CAT) [1,3,4]. Morphological assessment of principal ganglionic neurons and of developing synapses has been carried out by electron microscopic analysis [1,5,6]. These studies have shown that the SCG completes its development postnatally, with increases in TH levels [1], and increases in ganglionic cell size. Recognizable synapses are rare in the SCG in neonatal animals, with synaptogenesis occurring most rapidly during the first two postnatal weeks [1,3,6].

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Because of the ease of manipulation of its presynaptic input, the SCG has been used as a model in which to study transsynaptic effects of presynaptic input. Section of the cervical sympathetic trunk (CST) in neonatal rats affects both the pattern of neuronal maturation and that of synaptogenesis [1–3,6–8]. Isolation of the SCG from developing presynaptic input at birth results in reduction in the postnatal increase in TH levels and reduces cell division and growth [1–3]. The development of postsynaptic specializations associated with synaptogenesis also does not occur [6]. Since denervation can be mimicked by treatment with ganglionic blockers such as chlorisondamine and pempidine [4], it has been hypothesized that presynaptic input exerts its developmental effects via direct synaptic activity. However, additional trophic influences may contribute to postsynaptic changes associated with denervation [9].

If reinnervation is permitted, up to 50% of normal levels of synapses can be recovered by 60 days [8]. However, TH levels do not recover [10]. In an apparent paradoxical result, assay by high-performance liquid chromatography (HPLC) of catecholamine levels present in both deafferented and reinnervated ganglia do not differ from those measured in control ganglia [10]. Thus, despite persisting reductions in TH levels, catecholamine pools remain at control levels.

Recently, monoclonal antibodies have been used to monitor the development of a neuronal cell surface heparan sulfate proteoglycan (HeS-PG) in the SCG [11]. Antibodies directed against two different determinants on HeS-PG, one associated with the core protein and one with a heparan sulfate side chain, were used to assess neuronal maturation. Quantitation of antigen levels by radioimmunoassay (RIA) indicated that these antigens increased significantly during postnatal development, with the most dramatic increases occurring during the second postnatal week. No significant redistribution of antigen associated with development was observed. These antigens were of interest because of the association of HeS-PG with a factor that induces neurite outgrowth in vitro [12,13]. The antigen can be used as a marker for neuronal surface to monitor neurite outgrowth and increases in cell diameter. Alterations in expression may be associated with the regulation of neurite outgrowth.

Because HeS-PG may represent a molecule associated with more general characteristics of development than neurotransmitter synthetic enzymes, we were interested in examining whether the expression of determinants on both core protein and side chains is regulated by presynaptic input. If the levels of these molecules are also reduced by neonatal deafferentation, it may be possible to conclude that transsynaptic regulation of development is a factor not limited to the control of molecules directly associated with synaptic transmission. A preliminary report of a portion of this work has been published [14].

METHODS

Reagents

Monoclonal antibodies directed against HeS-PG were obtained from Drs. L.F. Reichardt and W.D. Matthew. Monoclonal antibody PG 3 recognizes an antigenic determinant associated with a heparan sulfate side chain, and PG 22 recognizes the core protein or an N-linked carbohydrate associated with it. These antibodies were used either diluted directly from culture supernatants or from ammonium sulfate precipitations of ascites fluid. Previous studies have indicated that no differences in

the properties of antibodies derived from these sources can be observed [11]. ^{125}I-goat-anti-mouse-IgG (Fab′ fragment) or ^{125}I-sheep-anti-mouse-IgG (whole molecule) were obtained from Amersham.

Experimental Animals and Surgical Procedures

Sprague-Dawley rats were used throughout for these experiments. For neonatal section of the CST, published procedures were followed [6,8]. Litters of pups aged 1–4 days were removed from their mothers. The sex of each pup was determined and equal numbers of males and females were selected for experimental and control groups. Since unilateral deafferentation of the SCG can cause compensatory changes in the contralateral ganglion (Smolen, personal communication), all surgery was carrried out bilaterally. Control rats were littermates of experimental animals in all cases. Experimental animals were anesthesized with methoxyfluorane in air. The skin over the throat was opened and the carotid artery was visualized. The SCG was located beneath the carotid artery and the CST severed at the base of the ganglion. A 2–3-mm section of the CST was removed to prevent reinnervation. Section of the CST was carried out bilaterally. The skin was sealed with collodion dissolved in acetone. Pups were kept warm until wide-awake and were returned as a group, along with control animals, to their mother. Mortality was less than 20% for 7-day survival periods, with somewhat poorer survival rates with longer survival periods.

Surgery on adult female rats was carried out using chloral hydrate (35 mg/kg, 7% solution) as anesthetic. Bilateral section of the CST was carried out in a similar fashion. The fascia surrounding the salivary glands and the overlying skin were sutured separately using 4-0 silk. Rats were given intramuscular injections of 100,000 units of Bicillin to reduce postsurgical respiratory infections. Mortality was less than 5% using prophylactic doses of antibiotic.

Quantitation of Antigen

Assay of antigen levels was carried out according to previously developed methods [11]. Ganglia from experimental animals were pooled to provide sufficient tissue for assay. Control ganglia were treated similarly. Desheathed ganglia were homogenized in 5 mM Tris-HCl, pH 8.1, in the presence of a protease inhibitor, phenylmethylsulfonylfluoride. Protein levels were determined by Amido-Schwarz assay. The total protein per ganglion was determined by correcting this value for total volume of homogenate and the number of ganglia pooled. Twelve dilutions of whole tissue homogenate were prepared (from 1:1 to 1:10,000) and aliquots were incubated with equal volumes of antibody at limiting dilution. After 18–24 hr, bound antibody was precipitated by centrifugation at 100,000g in an Airfuge. The amount of antibody remaining in the supernatant was determined by a standard solid-phase RIA [15], using a crude preparation of rat brain synaptosomes as the antigen. 50% inhibition points (I-50) were determined graphically, and standardized to 4 mg/ml initial concentration of protein. These values were further corrected for variations in protein content per ganglion, to permit comparison between experimental and control groups at each age examined, according to I-50 (corrected) = I-50 × [total protein (control ganglia)/ total protein (experimental ganglia)]. Control results were standardized to a value of 1.0; results from experimental groups were calculated as antigen levels relative to control levels (relative specific activity, RSA). All homogenates were assayed at least twice.

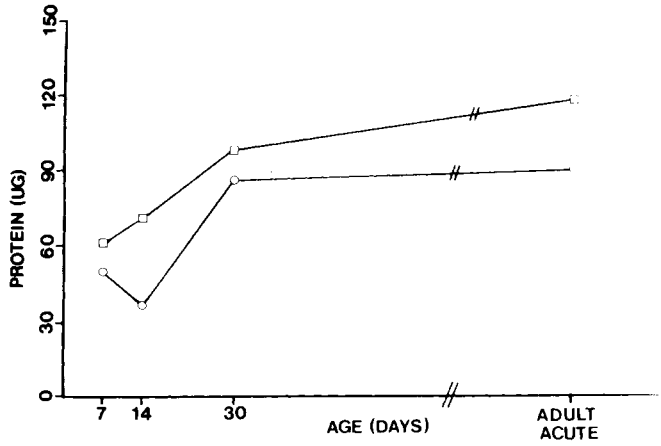

Fig. 1. Protein content of control and deafferented ganglia. Values determined as described in the text from homogenates of pooled SCGs. Open squares = control SCGs; open circles = deafferented SCGs.

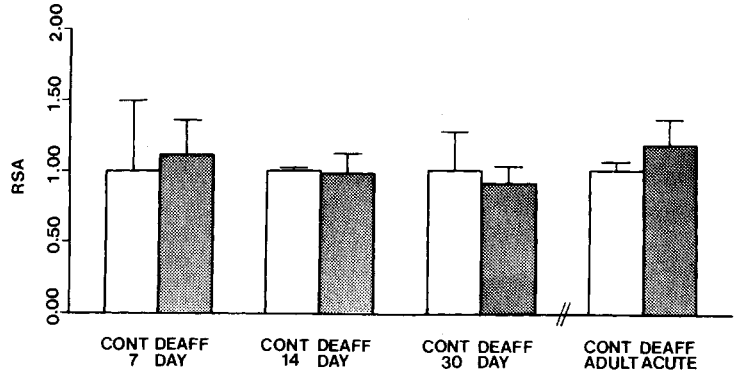

Fig. 2. Radioimmunoassay of levels of core-protein determinant recognized by PG 22. Control values for each age (in days) have been standardized to an average value of 1.0. Values for deafferented groups are presented as fractions of control levels (RSA = I-50 deaff/ I-50 control for each age group). Bars = one standard error of the mean. See text for discussion.

RESULTS

Protein assays of pooled experimental and control ganglia were carried out using tissue from 7-, 14-, and 30-day-old animals. In all cases, protein content of deafferented ganglia was lower than that of control groups (Fig. 1). The difference in ganglionic protein content was greater in younger animals than in 30-day groups, where a 12% reduction in total protein per ganglion was observed. After bilateral section of the CST and 7-day survival in adult rats, a 26% reduction in ganglionic protein content was observed, indicating loss of protein associated with the degeneration of presynaptic terminals (Fig. 1).

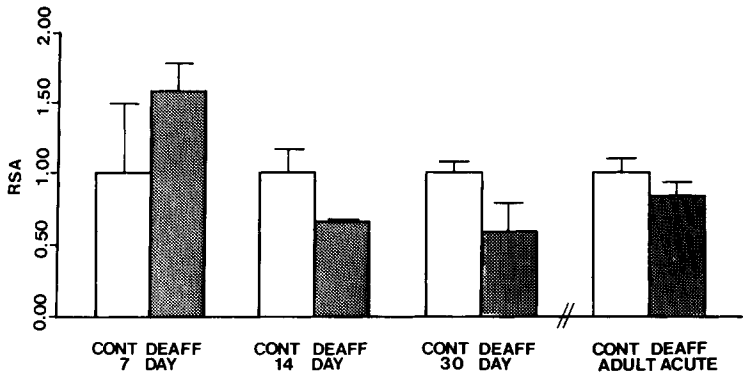

Fig. 3. Radioimmunoassay of levels of heparan sulfate side-chain determinant recognized by PG 3. Data presented as in Figure 2. See text for details.

RIA of levels of antigenic determinants recognized by PG 22 and PG 3 at 7, 14, and 30 days are shown in Figures 2 and 3. No significant differences in antigen levels recognized by PG 22 were observed at any time point following neonatal section of the CST, after correction for reduction in protein content per ganglion in experimental groups. In addition, no changes in levels of antigen recognized by PG 22 were observed following acute section of the CST in adult animals. In contrast, expression of the determinant recognized by PG 3 was significantly reduced at 14 and 30 days after birth (pooled, t-test, $P < 0.01$). Levels were increased over control levels at 7 days, but this difference was not significant. A small reduction in antigen level was also observed after acute section of the CST in adult rats, but this reduction was not statistically significant. Despite the reduction in levels of the side-chain determinant recognized by PG 3, absolute levels of the determinant increased during postnatal development of deafferented ganglia.

Immunocytochemical staining of experimental and control ganglia using either PG 3 or PG 22 followed by horseradish peroxidase-conjugated goat-anti-mouse antibody yielded no marked differences in staining pattern or staining intensity in 14 or 30 day SCG (data not shown).

DISCUSSION

The results of RIA of antigens associated with HeS-PG provide evidence that presynaptic innervation plays a role in the regulation of expression of molecules other than enzymes associated with neurotransmitter synthesis. This finding is important because it demonstrates that molecules that undergo significant changes associated with more general features of neuronal maturation [cf 11] can be influenced by innervation of the cell during critical postnatal periods, possibly mediated by activity [cf 4]. Innervation may play a central role in controlling the maturation of neurons, as has been demonstrated to be the case not only for development and maintenance of the neuromuscular junction but also for muscle tissue [cf 16].

Reductions in total protein per ganglion following neonatal deafferentation are consistent with previous studies [2,10]. SCGs deprived of preganglionic innervation from birth contain less protein at all ages examined, with the greatest reduction from control levels at 14 days. Much of the protein normally contributed by presynaptic axons and terminals is at least partially replaced by other sources, such as glia and connective tissue.

Studies of antigens associated with the HeS-PG indicate that the expression of a core protein antigen recognized by PG 22 does not appear to be regulated by presynaptic input. After correction for reduction in ganglion size, the same relative amount of antigen is synthesized in ganglia which have been deprived for presynaptic input from birth. In contrast, the pattern of synthesis of a heparan sulfate side-chain determinant, recognized by PG 3, is altered. At 7 days, more side-chain antigen is present in denervated SCGs. The variability in results was high for assay of 7-day tissue; further assays are required to determine whether this finding is significant. By 14 days, however, and persisting at 30 days, a 30–40% decrease in the level of side-chain antigen is observed. This result must be considered in view of earlier studies of normal development of these two antigenic determinants [11]. In these earlier studies, the relative amount of antigen recognized by PG 3 was less at birth than that of PG 22. In addition, immunocytochemical staining using PG 3 could not be detected until 7–10 days after birth, while staining using PG 22 was present at birth. These results suggested that postnatal processing of the side-chain determinant bound by PG 3 was required.

The results of the current study are compatible with this hypothesis, and further suggest that synthesis of the side-chain determinant may be partially regulated by presynaptic input. The absence of alterations in antigen levels following section of the CST in adult rats is consistent with reports of little, if any, immediate effect of deafferentation on ganglionic neurons in adult animals [cf 17].

Postnatal alterations in the metabolism of heparan sulfate proteoglycans occur in the rat brain [18], particularly during the first 2 weeks, and are proposed to be involved in postnatal neuronal maturation processes involving synaptogenesis. An embryonic-to-adult conversion of carbohydrate side-chains occurs in a neuronal cell adhesion molecule (N-CAM), in which loss of sialic acid residues enhances cell adhesion [19]. This alteration may be important in the modelling of the developing nervous system by regulating cell-cell interactions. In the ciliary ganglion, the ability of cultured neurons to respond to a neurite outgrowth factor is affected by their developmental stage; cells lose their responsiveness to a conditioned-medium factor after contact with target neurons is made [20].

The HeS-PG has been reported to be part of a complex that is a potent inducer of neurite outgrowth in vitro [12]. A mutant of the PC-12 cell line lacking cell surface HeS-PG also fails to secrete the neurite outgrowth factor. Our current observation that neonatal deafferentation appears to reduce levels of a side-chain determinant on HeS-PG may reflect a developmentally significant conversion of the molecule. However, the antibodies employed in the present study do not block the in vitro effect. Recently, antibodies have been generated to the neurite outgrowth factor that are capable of blocking its in vitro function [21]. It remains to be determined whether these antibodies exert a similar effect in vivo, and whether presynaptic input contributes to the regulation of expression of these antigenic determinants. Further investi-

gation of HeS-PG is required to determine whether alterations in the molecule play a role in control of neurite outgrowth in vivo.

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REFERENCES

1. Black IB, Hendry IA, Iversen LL: Brain Res 34:229, 1971.
2. Black IB: Annu Rev Neurosci 1:183, 1978.
3. Black IB, Hendry IA, Iversen LL: J Neurochem 19:1367, 1972.
4. Black IB, Geen SC: Brain Res 63:291, 1973.
5. Eranko L: Brain Res 46:159, 1972.
6. Smolen AJ, Raisman G: Brain Res 181:315, 1980.
7. Black IB, Coughlin MD, Couchard P: Soc Neurosci Symp 4:184, 1979.
8. Smolen AJ: Dev Brain Res 1:49, 1981.
9. Hendry IA, Hill CE: Brain Res 200:201, 1980.
10. Smolen AJ, Lindley T, Wright LL: Soc Neurosci Abstr 9:935, 1983.
11. Greif KF, Reichardt LF: J Neurosci 2:843, 1982.
12. Matthew WD, Lander AD, Greenspan RJ, Reichardt LF: Soc Neurosci Abstr 8:299, 1982.
13. Lander AD, Fujii DK, Gospodarowicz D, Reichardt LF: J Cell Biol 94:574, 1982.
14. Greif KF, Trenchard HI: J Cell Biochem (Suppl) 8B:116, 1984.
15. Klinman NR: J Exp Med 136:241, 1972.
16. Jacobsen M: "Developmental Neurobiology." New York: Plenum, 1978.
17. Gabella G: "Structure of the Autonomic Nervous System." London: Chapman and Hall, 1976.
18. Margolis R, Margolis RK, Chang LB, Preti C: Biochemistry 14:85, 1975.
19. Edelman GM: Science 219:450, 1983.
20. Collins F, Lee MR: J Neurosci 2:424, 1982.
21. Matthew WD, Patterson PH: Soc Neurosci Abstr 9:206, 1983.