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Regulation of molecular components of the synapse in the developing and adult rat superior cervical ganglion

(trans-synaptic regulation/postsynaptic/calmodulin)

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ABSTRACT **Rat superior cervical sympathetic ganglion was used to begin studying the regulation of molecular components of the synapse. Ganglionic postsynaptic densities (PSDs) exhibited a thin, disc-shaped profile electron microscopically, comparable to that described for brain. Moreover, the presumptive ganglionic PSD protein (PSDp) was phosphorylated in the presence of Ca^{2+} and calmodulin, bound ^{125}I-labeled calmodulin, and exhibited a M_r of 51,000, all characteristic of the major PSD protein of brain. These initial studies indicated that ganglionic PSDp and the major PSD protein of brain are comparable, allowing us to study synaptic regulation in the well-defined superior cervical sympathetic ganglion. To obtain enough quantities of ganglionic PSDp, we used synaptic membrane fractions. During postnatal development, calmodulin binding to the ganglionic PSDp increased 411-fold per ganglion from birth to 60 days, whereas synaptic membrane protein increased only 4.5-fold. Consequently, different synaptic components apparently develop differently. Moreover, denervation of the superior cervical sympathetic ganglion in adult rats caused an 85% decrease in ganglionic PSDp–calmodulin binding, but denervation caused no change in synaptic membrane protein 2 weeks postoperatively. Our observations suggest that presynaptic innervation selectively regulates specific molecular components of the postsynaptic membrane structure.**

A central challenge in neurobiology concerns the mechanisms that govern moment-to-moment communication in the nervous system, subserving functions such as coordination and movement, and the mechanisms that underly long-term functions, including learning and memory. The synapse, the communicative junction between neurons, is acknowledged to play a pivotal role in these distinctively neural processes. Indeed, the primacy of synaptic organization and function in learning and memory has been appreciated for decades (1). Remarkably, however, little is known about the factors regulating synaptic structure.

In contrast, extensive study indicates that presynaptic impulse activity regulates multiple events in the postsynaptic neuron through trans-synaptic mechanisms. For example, postsynaptic tyrosine hydroxylase and dopamine-β-hydroxylase, catecholaminergic synthetic enzymes, are induced in the adult rat sympathetic superior cervical ganglion (SCG) by trans-synaptic stimulation (2–4). Moreover, normal biochemical development of postsynaptic neurons in the SCG is dependent on presynaptic regulation (5, 6). Currently, whether the presynaptic innervation similarly regulates the structure of the specialized postsynaptic membrane is unclear.

Our examination of trans-synaptic regulation of synaptic molecular structure began by focusing on the postsynaptic density (PSD), a proteinaceous, disc-shaped structure attached to the postsynaptic membrane at chemical synapses (7–10). Extensive evidence indicates that the PSD plays a critical role in synaptic communication (11–15). Therefore we explored the regulation of specific PSD molecular components by presynaptic innervation in the rat SCG. Although the peripheral sympathetic system offers an accessible model that can be conveniently manipulated *in vivo*, most studies characterizing the PSD have used the cerebral cortex, a rich source of postsynaptic membranes. The cortical PSD is composed of a predominant protein of M_r 51,000 (8, 9), termed the major PSD protein (mPSDp). The mPSDp molecule binds calmodulin in a Ca^{2+}-dependent manner (14) and appears to be a PSD kinase subunit that autophosphorylates (11–13). These observations were particularly interesting because postsynaptic Ca^{2+} influx has been invoked as a step in memory formation (16, 17) and because Ca^{2+}/calmodulin-mediated phosphorylation of the mPSDp may alter molecular conformation leading to changes in PSD size and shape—with altered synapse function (15).

In this study we used the SCG as a model system to examine the role of presynaptic innervation on postsynaptic membrane structure. The presumptive SCG PSD protein (M_r 51,000) was used to monitor postsynaptic structure at the molecular level. Because of the extremely limited amounts of available ganglionic PSD fractions, we used a synaptic membrane (SM) fraction as the source of ganglionic PSDp instead of the PSD fraction itself. Previous work has suggested that the protein serves as a postsynaptic marker in the isolated synaptic membranes, synaptic junctions, or PSD (18). In summary, our results indicate that calmodulin binding of the PSDp of the SCG is regulated by presynaptic innervation.

MATERIALS AND METHODS

Materials. Calmodulin was obtained from Boehringer Mannheim. Na-^{125}I and [γ-^{32}P]ATP were purchased from New England Nuclear. Methyl-4-azidocalmodulin was a product of Pierce.

Animals, Surgical Procedures, and Ganglion Dissection. Adult Sprague–Dawley rats of either sex were housed in a temperature-controlled environment and exposed to a fixed light–dark cycle as described (19). Rats were anesthetized with 4% fluothane, and denervation of the SCG was performed as described (19). Animals were sacrificed by exposure to CO_2 vapor. Ganglia were isolated, stored at −80°C, and used within 1 day.

Subcellular Fractionation. Synaptosomal and SM fractions were prepared from the frozen pooled SCGs according to procedures of Carlin *et al* (10). PSD fractions were prepared as described (20).

Binding of ^{125}I-Labeled Calmodulin or ^{125}I-Labeled Methyl-4-Azidocalmodulin to Ganglionic PSDp in SM Fractions. Two

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Abbreviations: PSD, postsynaptic density; PSDp, PSD protein; mPSDp, major PSD protein of brain; SCG, superior cervical ganglion; SM, synaptic membrane.

approaches were used to study binding of iodinated calmodulin to the ganglionic PSDp in SM fractions. (*i*) ^{125}I-labeled calmodulin was bound to ganglionic PSDp in SM fractions separated by NaDodSO$_4$/PAGE according to procedures of Carlin *et al.* (14). In this method, iodinated calmodulin that bound to the ganglionic PSDp and renatured in the NaDodSO$_4$/gel was used to estimate molecular size of the ganglionic PSDp. (*ii*) Azido-^{125}I-calmodulin was bound to the ganglionic PSDp in SM fractions by affinity photolabeling, and the resulting radiolabeled conjugates were identified by subsequent NaDodSO$_4$/PAGE (21). The cross-linked complex of the ganglionic PSDp and azido-^{125}I-calmodulin has a M_r of 69,000 (M_r of calmodulin is 18,000). The second method estimates the amount of iodinated calmodulin bound to the native physiological form of the ganglionic PSDp and was used to define ganglionic PSDp during development and after denervation. In both approaches, the quantity of ganglionic PSDp was estimated densitometrically and was expressed as arbitrary standardized units.

NaDodSO$_4$/PAGE was performed according to the method of Wu *et al.* (20). Protein concentration was determined as described (22). Iodination of calmodulin was performed as previously indicated (14). Azido-^{125}I-calmodulin was prepared using the procedure of Andreasen *et al.* (21).

Electron Microscopy. Electron micrographs were used to identify structures in the isolated PSD fractions as described (9).

RESULTS

PSD Fractions from the SCG. To begin studying the regulation of postsynaptic membrane structures, we used the accessible, conveniently manipulated SCG (2–5, 19, 23). We isolated the PSD fraction from ganglia using a procedure identical to that employed for the preparation of the PSD fraction from the brain (20) to ensure that comparable entities were being examined. Indeed, the electron micrographic profile of the ganglionic PSD fraction was similar (but thinner) to that of cerebral cortex (10), as predicted. Multiple characteristic crescent-shaped PSD profiles were present in osmium tetroxide-stained sections (Fig. 1). The thin PSD profiles seen resemble those of the cerebellum (10), suggesting that the corresponding major cortical PSDp in SCG may

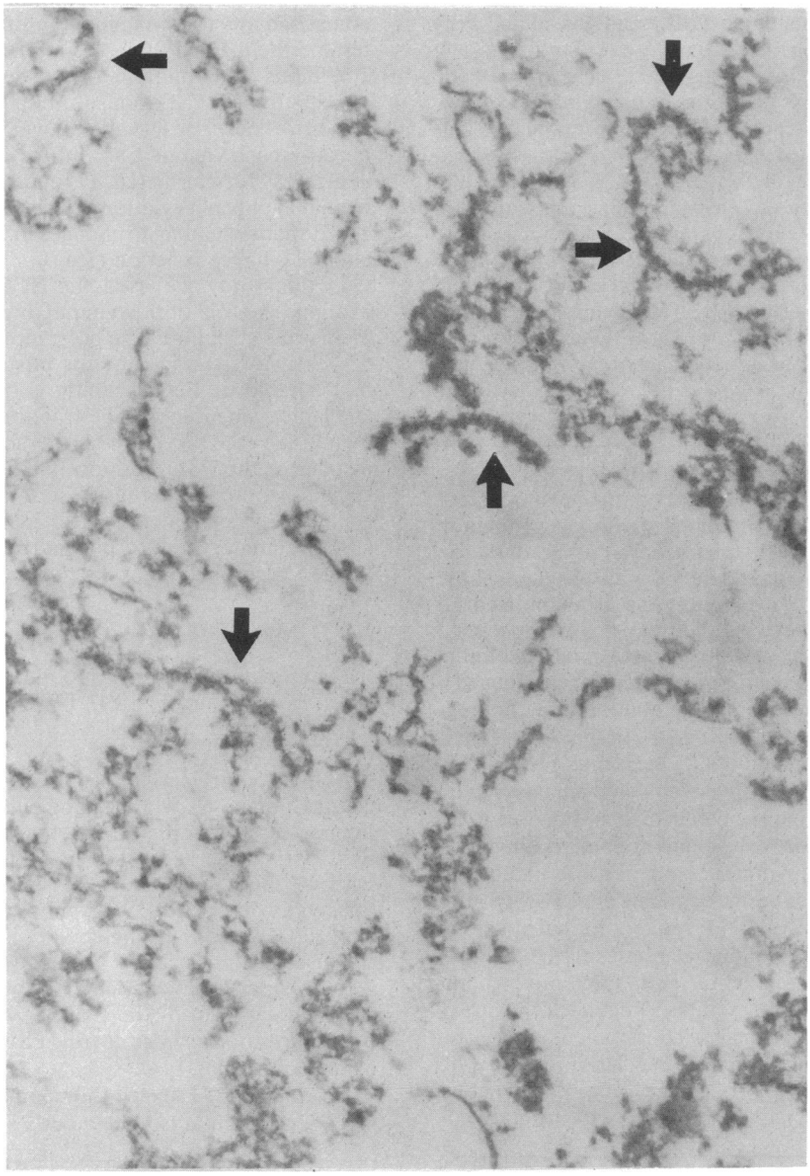

FIG. 1. Electron micrograph of the PSD fraction isolated from the adult rat SCG. The fraction was stained with osmium tetroxide. Arrows, PSDs. Note the thin, crescent-shaped profiles characteristic of PSDs. (×23,140.)

be a minor component of the cerebellar fraction protein (10). Fragmented material seen in the ganglion preparation (Fig. 1) probably reflects the thin, delicate nature of PSDs.

In summary, PSDs from the peripheral ganglion look similar, if not identical, to brain PSDs, allowing the study of regulation in the less complex periphery. However, only miniscule PSD fractions could be obtained from 100 adult ganglia, permitting the morphological studies already discussed but precluding the use of this preparation for routine investigation of biochemical regulation. Consequently, we used the SM fraction to investigate regulation in the SCG.

Characterization of the Ganglionic PSDp in SM Fractions. To determine whether the presumptive PSDp present in the SM fractions of the SCG was the same as that derived from PSD fractions, two distinct biochemical properties were characterized. We defined Ca^{2+}/calmodulin-dependent phosphorylation and calmodulin binding, two well-delineated characteristics of the mPSDp isolated from the brain.

Ca^{2+}/calmodulin-dependent phosphorylation. To determine whether the ganglionic PSDp from the SCG could be autophosphorylated in the presence of Ca^{2+} and calmodulin as in brain (11–13), the ganglionic SM fraction was examined. In fact, the ganglionic PSDp was autophosphorylated under appropriate conditions (Fig. 2), suggesting that the M_r 51,000 protein in the ganglionic SM fraction represents a protein kinase subunit similar to that of brain.

Binding of ^{125}I-labeled calmodulin. To determine whether the presumptive PSDp in the SCG binds calmodulin with high affinity in the presence of Ca^{2+} as in brain (14), the ganglionic SM fraction was examined. In fact, the ganglionic PSDp bound calmodulin as expected (Fig. 3). Moreover, PSDp from the ganglia exhibited the same M_r 51,000 as that of brain, suggesting that central and peripheral proteins share common structural and functional features.

It should be noted that of the three polypeptides—51,000, 60,000, and 27,000—that bound calmodulin in the presence of Ca^{2+} (Fig. 3), the 51,000 and the 60,000 M_r polypeptides were phosphorylated in the presence of Ca^{2+} and calmodulin (Fig. 2). These results strongly suggest that the 51,000 and 60,000 M_r polypeptides are protein kinase subunits that autophosphorylate, reproducing observations made in brain. Moreover, our studies of phosphorylation and calmodulin binding

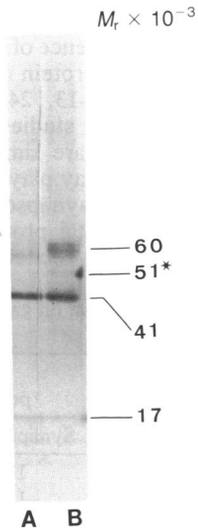

FIG. 2. Ca^{2+}/calmodulin-dependent phosphorylation of ganglionic PSDp. The SM fraction was isolated from adult rat SCG, and the fractions (40 μg each) were phosphorylated in the absence (lane A) or presence (lane B) of Ca^{2+}/calmodulin (see *Materials and Methods*). The autoradiogram of the phosphorylated SM proteins is shown; position of the ganglionic PSDp is indicated by the asterisk.

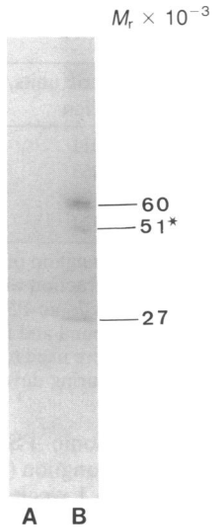

FIG. 3. Binding of ^{125}I-labeled calmodulin to ganglionic PSDp. The SM fraction was obtained from adult rat SCG, and binding of iodinated calmodulin to the ganglionic PSD in the fractions (40 μg each) was done without (lane A) or with (lane B) Ca^{2+} (see *Materials and Methods*). Position of the ganglionic PSDp (51,000) in the autoradiogram is indicated by the asterisk.

indicate that the ganglionic PSD kinase consists of more 60,000 than 51,000 subunits, similar to that in cerebellar PSDs (10, 14). Indeed, the Ca^{2+}/calmodulin-dependent protein kinase purified from rat cerebellum has a subunit ratio of 1:4 (51,000:60,000) (24), in contrast to the forebrain ratio of 3:1 (51,000:60,000) (24). However, in the studies described below, the smaller 51,000 subunit was measured because the 60,000 region may contain other calmodulin binding proteins, such as calcineurin (25).

Developmental Profile of the Ganglionic PSDp. During postnatal development the number of synapses in the SCG increases dramatically (5, 26–28). To ascertain whether the ganglionic PSDp similarly increases and whether, consequently, that protein may serve as a valid marker for synapse number, rats of different ages were examined. SM fractions were isolated and azido-^{125}I-calmodulin was covalently linked to the ganglionic PSDp; binding was then measured (see *Materials and Methods*). The developmental profiles of total SM proteins and of the ganglionic PSDp were defined (Tables 1 and 2 and Fig. 4). Initially, we defined the development of the protein per unit of SM. Between postnatal days 1 and 60, the ganglionic PSDp increased 90-fold per unit of SM (Fig. 4). Rise in the ganglionic PSDp appeared progressive over this period because the protein increased 14-fold by day 7 and 70-fold by day 21. Moreover, the increase was highly specific for the ganglionic PSDp because total SM protein increased only 4.5-fold from days 1–60.

Table 1. Subcellular fractions isolated from rat SCG of varying ages

Age, days	Wet wt, μg	Protein per ganglion, μg		
		Homogenates	Synaptosomes	SM
1	263	29	2.3	0.7
7	725	68	6.4	1.6
21	1,896	101	9.9	2.8
60	2,150	115	11.3	3.2

Ganglia were removed from rats aged 1–60 days postnatally and were processed for subcellular fractionation. Eighty 1-day-old ganglia and 40 for each older age were used. Wet weight is expressed per ganglion.

Table 2. Developmental increases in the ganglionic PSDp per ganglion

Age, days	Ganglia/ fraction	Densitometric units/ ganglion	Ganglionic PSDp/ ganglion
1	57	0.0011	1
7	24	0.0363	33
21	14	0.3128	284
60	13	0.4524	411

The amount of SM protein per ganglion of varying ages and those of ganglionic PSDp per unit of SM fraction were obtained from Table 1 and Fig. 4. The quantity of ganglionic PSDp is expressed as the amount of iodinated calmodulin bound and is presented in arbitrary densitometric units. These units were used to calculate the increases in ganglionic PSDp per ganglion during development.

Development of the ganglionic PSDp was even more dramatic when expressed per ganglion (Table 2). The protein increased 33-fold from birth to 1 week of age, 284-fold by 3 weeks, and 411-fold per ganglion by 60 days.

Effects of Denervation on the Adult Ganglionic PSDp. Previous studies have indicated that preganglionic denervation (decentralization) eliminates virtually all synapses in the adult SCG (27–30) and elicits a decrease in postsynaptic soluble enzymes that synthesize transmitters (31). It was, consequently, of interest to determine whether presynaptic innervation regulates components of the postsynaptic membrane specialization. Ganglia were unilaterally denervated in adult rats, and effects on the ganglionic PSDp were defined. Contralateral intact ganglia and ganglia from unoperated animals served as controls. Denervation evoked a dramatic decrease in the binding of calmodulin to the ganglionic PSDp compared with controls. Calmodulin binding to the ganglionic PSDp decreased to 15% of control in denervated ganglia 2 weeks after surgery (Fig. 5), although total SM protein was not significantly altered (Table 3). Similarly, after 1 and 3 weeks binding was 26% and 30% of control, respectively, whereas SM protein was unaffected (Fig. 5 and Table 3).

In addition to the effects of denervation on calmodulin binding to the ganglionic PSDp, binding to a protein of M_r 60,000 was also reduced. The M_r 60,000 protein may represent another subunit of the Ca^{2+}/calmodulin-dependent protein kinase, as has been reported for brain (11–13, 24).

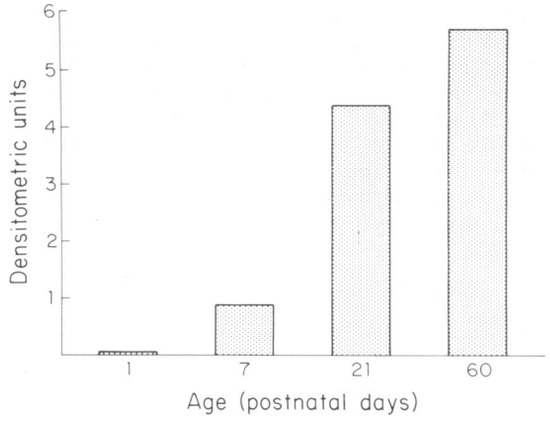

FIG. 4. Developmental increases in ganglionic PSDp per SM fraction. Ganglia were isolated from rats of varying ages and processed for subcellular fractionation. The SM fractions (40 μg each) were incubated with azido-^{125}I-calmodulin, and the amount of iodinated calmodulin bound to the ganglionic PSDp was determined (see *Materials and Methods*). Forty animals were used to obtain fractions from 1-day-old rats, and 20 animals were used for each older age. The results are expressed as arbitrary densitometric units.

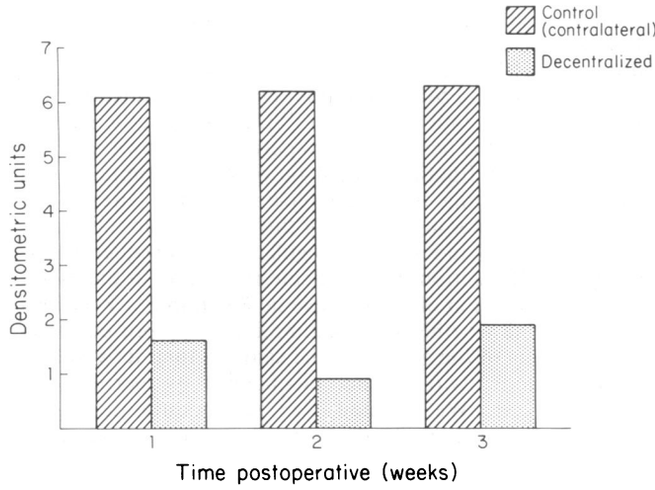

FIG. 5. Effects of decentralization on the binding of azido-^{125}I-calmodulin to ganglionic PSDp in adult SM fractions. Rats aged 3 mo were subjected to unilateral ganglion decentralization, and the denervated and contralateral control ganglia were isolated at 1, 2, and 3 weeks postoperatively. SM fractions were prepared, and binding of azido-^{125}I-calmodulin to the ganglionic PSDp in the SM fractions (40 μg each) was performed (see *Materials and Methods*). Results are expressed as arbitrary densitometric units. A total of 32 animals each was used for each time point.

DISCUSSION

Our results indicate that the easily manipulated SCG is a convenient model for studying regulation of specific synaptic components at the molecular level. The PSD fraction contains characteristic disc-shaped PSD profiles, detected by traditional electron microscopic methods that have been applied successfully in the study of brain (9, 10). The morphological similarity of ganglion profiles to that described for brain suggests that PSDs in the periphery and brain are comparable, a contention supported by a number of biochemical studies.

As in brain, a PSDp of M_r 51,000 is detectable in SMs of the SCG. Moreover, the ganglion protein binds calmodulin in a Ca^{2+}-dependent manner, reproducing properties of the brain mPSDp (14). Finally, the ganglionic PSDp can undergo autophosphorylation in the presence of Ca^{2+} and calmodulin, suggesting that the peripheral protein may be a subunit of a PSD kinase, as in brain (11–13, 24). In aggregate, the morphological and biochemical studies suggest that ganglionic PSDp and brain mPSDp are similar, if not identical. Consequently, the molecules may play analogous functional roles at peripheral and central synapses.

We defined the developmental profile of the ganglionic PSDp to begin examining two related questions. Can the

Table 3. Effects of decentralization on ganglion synaptic fractions

Postoperative time, wk	Protein per ganglion, μg	
	Synaptosomes	SM
1 (control)	11.9	3.4
1 (decentralized)	11.1	3.2
2 (control)	12.7	3.4
2 (decentralized)	11.3	3.1
3 (control)	12.9	3.5
3 (decentralized)	11.6	3.4

Adult rats aged 3 mo were unilaterally decentralized. The decentralized and contralateral control ganglia were isolated after varying periods of time. Thirty-two ganglia were used for each group.

ganglionic PSDp serve as a marker for synapses, thus allowing quantitation biochemically? How are the molecular components of the synapse assembled during development? Previous work, using electron microscopic approaches, indicated a dramatic increase in SCG synapse numbers in the rat postnatally (26–28). However, these morphological approaches are cumbersome and time consuming, involve a number of assumptions, and require the application of correction factors, yielding relative but not absolute values (32, 33).

Biochemical indices of synaptogenesis may yield more precise estimates and new insights. We quantitated synaptic development using several different approaches. In the first, we calculated the increase in the ganglionic M_r 51,000 protein per unit of SM. From birth to 60 days, total SM protein increased ≈4.5-fold per ganglion, while the ganglionic PSDp increased 90-fold per unit of SM. It is apparent, consequently, that the SM and ganglionic PSDp develop differentially and that different molecular components of the synapse are expressed in a differential fashion.

This contention is supported by our observations of the development of ganglionic PSDp per ganglion. From birth to 60 days ganglionic PSDp increased over 400-fold. Because previous work has indicated that morphological synapse numbers increase only 10-fold during a comparable period of time (26–28), it may be concluded that different molecular components of the synaptic complex do, in fact, develop differentially.

We tentatively conclude that the presumptive PSDp may serve as one measure of synapse numbers but that different components of the synapse develop differently. These observations are consistent with previous work on the neuromuscular junction indicating that synaptogenesis is a complex multi-step process and not a simple all-or-none phenomenon (34–37).

In turn, these considerations raise interesting questions about the factors that regulate different components of the synapse throughout life, the subject of other experiments in the present study. Abundant evidence indicates that trans-synaptic influences regulate a variety of postsynaptic transmitter molecules in sympathetic neurons (2–6). It was logical to ask whether presynaptic innervation might also regulate molecular components of the structural synapse in the postsynaptic sympathetic membrane. In fact, our experiments indicate that denervation of the SCG results in an 85% decrease in the ganglionic PSDp, whereas total synaptic membrane protein is unchanged. Further, we found no alteration in the $NaDodSO_4$/gel profiles of SM fractions (data not shown). Consequently, total mass of the PSD apparently remains relatively unaltered, while binding of calmodulin to the ganglionic PSDp markedly decreases. Our observations suggest that denervation alters ganglionic PSDp in the synapse.

The marked decrease in calmodulin binding to the ganglionic PSDp consequent to denervation may be mediated by a number of mechanisms. Most simply, denervation may have caused a loss of the M_r 51,000 protein in synaptic membranes, either through dissociation or degradation. Alternatively, decentralization may somehow have caused the saturation of ganglionic PSDp by endogenous calmodulin or altered conformation of the ganglionic PSDp, thus changing binding properties. Regardless of specific mechanism, however, it is clear that the presynaptic innervation selectively regulates particular synaptic processes or molecules. We are now in a position to identify specific functionally important molecules in the postsynaptic membrane and density that are regulated by presynaptic influences.

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