CHAPTER TWO

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Stereological and Allometric Studies on Neurons and Axo-Dendritic Synapses in Superior Cervical Ganglia

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Abstract

The superior cervical ganglion (SCG) plays an important role in neuropathies including Horner's syndrome, stroke, and epilepsy. While mammalian SCGs seem to share certain organizational features, they display natural differences related to the animal size and side and the complexity and synaptic coverage of their dendritic arborizations. However, apart from the rat SCG, there is little information concerning the number of SCG neurons and synapses, and the nature of relationships between body weight and the numbers and sizes of neurons and synapses remain uncertain. In the recognition of this gap in the literature, in this chapter, we reviewed the current knowledge on

the SCG structure and its remodeling during postnatal development across a plethora of large mammalian species, focusing on exotic rodents and domestic animals. Instrumentally, we present stereology as a state-of-the-art 3D technology to assess the SCG 3D structure unbiasedly and suggest future research directions on this topic.

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1. INTRODUCTION

While mammalian superior cervical ganglion (SCG) neurons and their axo-dendritic synapses seem to share certain organizational features, they display natural differences related to such factors as animal size, animal side, and the complexity and synaptic coverage of their dendritic arborizations (Forehand, 1985; Loesch et al., 2010; Purves and Lichtman, 1985; Purves et al., 1986; Smolen and Beaston-Wimmer, 1986). Larger species possess SCG neurons which exhibit greater convergence, more complex arborizations and a greater proportion of axo-dendritic compared to axo-somatic synapses (Forehand, 1985; Purves and Lichtman, 1985; Purves et al., 1986). However, apart from the rat SCG, there is still little information concerning numbers of SCG neurons and axo-dendritic synapses, and the nature of the relationships between body weight and the numbers and sizes of neurons and synapses remain uncertain.

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Recognizing this gap in the literature, over the past 10 years, we have investigated the quantitative structure of SCG from a broad range of mammals, including both small laboratory and exotic rodents such as rats and capybaras (Loesch et al., 2010; Ribeiro et al., 2004), pacas (Abrahão et al., 2009; Melo, 2009), guinea pigs (Toscano et al., 2009), and preás (Ladd et al., 2012) and also domestic large mammals such as dogs and cats (Fioretto et al., 2007), sheep (Fioretto et al., 2011), and horses (Fioretto et al., 2007; Loesch et al., 2010; Ribeiro et al., 2004). In our own published work, the quantitative assessment of SCG structure—neurons, synapses, neuropil, vessels, and connective tissue among others—was conducted using 3D technology employing the design-based stereology. Au2

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By pursuing our investigations in various mammals, we have found a suitable animal model for studying allometric relationships between SCG volume and neuron number and between SCG volume and synapse number. The model provides a structural foundation, which is of paramount importance for understanding “the aging sympathetic nervous system” and for clinical research, since SCG plays an important role in grave neuropathies including Horner’s syndrome (Bell et al., 2001; Boydell, 1995; Kisch, 1991), stroke (Campbell, 2000; Palmer, 2007; Shaibani et al., 2006), and epilepsy (Kokaia et al., 1994). Au3

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2. STRUCTURAL ORGANIZATION OF SCG

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2.1. Macrostructure

p0020 Due to the SCG's role in some neuropathies, it is useful to know SCG's macrostructure including its dimensions and accurate localization, especially when pursuing operative procedures in the neck of animals, for example in cervical tumors, Horner's syndrome (Boydell, 1995; Panciera et al., 2002), Wobbler's syndrome (Nanai et al., 2006), or during the treatment of guttural pouch mycoses in horses (Freeman, 2006; Millar, 2006).

s0020 2.1.1 Localization

p0025 The cervical part of the autonomic nervous system in mammals consists of a vast array of nerves and ganglia connected to the central nervous system on one side and to the viscera on the other side. The sympathetic cervical chain lies dorsally to the vagus nerve and ventrally to transverse processes of vertebrae and prevertebral muscles. Two paravertebral ganglia are present in the neck: SCG or cranial cervical ganglion and the caudal cervical ganglion or stellate ganglion (when the latter includes between one and three uppermost thoracic sympathetic ganglia). In addition, a small intermediate ganglion is sometimes found, that is, the middle cervical ganglion (Baljet and Drukker, 1979; Gabella, 2004; Hedger and Webber, 1976).

p0030 The SCG of rats, guinea pigs, preás, cutias, pacas, capybaras, sheep, dogs, cats, and horses is located near the base of the skull and deeply into the bifurcation of the common carotid artery, in the origin of the external and internal carotid arteries close to the distal ganglion of the vagus nerve (Abrahão et al., 2009; Coppi and Loesch, 2011; Fioretto et al., 2007, 2011; Gabella, 2004; Hedger and Webber, 1976; Ladd et al., 2012; Loesch et al., 2010; Melo, 2009; Ribeiro, 2006; Ribeiro et al., 2004; Toscano et al., 2009) (Fig. 2.1). In camels, SCG was located on the rostro-lateral aspect of m. longus capitis, ventral to m. sternomastoideus and covered by the mandibular gland. The caudo-dorsal border of the ganglion was parallel to the vagus nerve, while the rostro-ventral margin ran along the carotid sinus branch of the glossopharyngeal nerve. The rostro-dorsal end of the ganglion pointed toward the jugular foramen and the caudo-ventral apex encompassed the rostral extremity of the cervical sympathetic trunk (Sheng et al., 1998). By the same token, sympathetic and parasympathetic nerve structures of young pigs, SCG, and distal vagus ganglion (DVG), respectively, were just close to each other without any connection and located near the base of the

Figure 2.1 Macrostructure of the SCG of an adult dog (A), cat (B), and horse (C). The SCG is located near the distal vagus ganglion (DVG) and related to internal carotid (IC) artery, hypoglossal nerve (H), common carotid artery (CC), and occipital artery (O) in the cat. Authorization will be required once the review process is accomplished. *Image and legend extracted from Fioretto et al. (2007).*

skull and could descend to the first cervical vertebra level (Kabak et al., 2005; Pospieszny and Bruzewicz, 1998).

Topographically speaking, the SCG is located dorso-cranially to the larynx, cranio-medially to the thyroid gland, and ventro-caudally to the tympanic bulla. This ganglion is also very closely adjacent to the hypoglossal nerve. In all species studied, the SCG was white in color and roughly spindle shaped, independently of the animal age (Abrahão et al., 2009; Coppi and Loesch, 2011; Fioretto et al., 2007, 2011; Ladd et al., 2012; Loesch et al., 2010; Melo, 2009; Ribeiro, 2006; Ribeiro et al., 2004; Toscano et al., 2009).

2.1.2 Macromorphometry

The macromorphometric aspects of the SCG—length, width, and thickness—vary greatly according to the animal species and animal age

Figure 2.2 The complete catalog of mammalian species. (A) (from left to right): rats, guinea pigs, preás, cutias, pacas, capybaras and (B) (from left to right): cats, dogs, sheep, horses—from which superior cervical ganglia (SCGs) were harvested and investigated in our laboratory over the past 10 years. Scale bars: 15 mm (A) and 30 mm (B).

investigated, that is, rats, guinea pigs, preás, rabbits, cutias, pacas, capybaras, cats, dogs, sheep, horses, and camels (Abrahão et al., 2009; Coppi and Loesch, 2011; Fioretto et al., 2007, 2011; Kabak, 2007; Ladd et al., 2012; Loesch et al., 2010; Melo, 2009; Najafi and Nejati, 2009; Ribeiro, 2006; Ribeiro et al., 2004; Sheng et al., 1998; Toscano et al., 2009) (Fig. 2.2A and B). Data concerning SCG's dimensions in different animal species and ages have been collated in form of a table (Table 2.1).

2.1.3 Target organs

In mammals, the SCG provides sympathetic innervation to the head and neck as well as to the mandible, submandibular and pineal glands, cephalic blood vessels, choroid plexus, eye, carotid body, salivary, and thyroid glands (Arbab et al., 1986; Bowers, 1984; Cardinali et al., 1981; Gibbins, 1991; Gibbins et al., 1996; Morris et al., 1999; Reuss and Moore, 1989; Uddman, 1989; Voyvodic, 1989). Removal of SCG brings about several neuroendocrine dysfunctions in mammals, including the disruption of water balance in pituitary stalk-sectioned rats and the alteration of the normal

Table 2.1 Mean values of macromorphometric parameters: length (mm), width (mm), thickness (mm) of superior cervical ganglia (SCG) from a plethora of mammalian species: rats, preás, guinea pigs, cutias, pacas, capybaras, cats, dogs, pigs, sheep, horses, and camels—at different time points (newborn, young, adult, and elderly) during postnatal development

Species	Age	Length (mm)	Width (mm)	Thickness (mm)	References
Camels	Adult	17.50	5.00	3.00	Sheng et al. (1998)
Pigs	Young	3.16	1.49	1.11	Kabak (2007)
Sheep	Young	8.52	2.31	2.00	Najafi and Nejati (2009)
		7.10	4.40	2.40	Fioretto et al. (2011)
Rats	Adult	3.3	0.90	–	Ribeiro et al. (2004)
Horses		19.7	7.70	–	
Capybaras		15.9	5.70	–	
	Young	13.27	5.35	–	Ribeiro (2006)
Dogs	Young	3.50	1.80	–	Fioretto et al. (2007)
	Adult	4.40	2.34	–	
Cats	Young	3.30	0.80	–	
	Adult	3.30	1.60	–	
Horses	Young	19.6	5.90	–	
	Adult	26.3	6.40	–	
Guinea pigs	Newborn	2.25	1.11	0.63	Toscano et al. (2009)
	Young	2.70	0.99	0.67	
	Adult	3.70	1.58	0.79	
	Elderly	3.83	1.45	1.05	
Pacas	Newborn	4.50	2.21	1.26	Melo (2009)
	Young	5.37	1.98	1.31	
	Adult	7.36	3.17	1.59	
	Elderly	7.66	2.86	2.20	
Pacas	Newborn	3.74	1.66	1.23	Abrahão et al. (2009)
	Young	5.60	1.34	1.32	
	Adult	7.40	1.69	1.53	
	Elderly	7.70	2.00	2.19	

Table 2.1 Mean values of macromorphometric parameters: length (mm), width (mm), thickness (mm) of superior cervical ganglia (SCG) from a plethora of mammalian species: rats, preás, guinea pigs, cutias, pacas, capybaras, cats, dogs, pigs, sheep, horses, and camels—at different time points (newborn, young, adult, and elderly) during postnatal development—cont'd

Species	Age	Length (mm)	Width (mm)	Thickness (mm)	References
Rats	Adult	3.20	0.8	0.63	Loesch et al. (2010)
Capybaras	Adult	16.5	5.4	4.67	
Horses	Adult	6.80	20.6	5.79	
Preás	Newborn	1.29	0.84	0.82	Ladd et al. (2012)
	Young	2.08	1.03	0.78	
	Adult	2.30	1.06	0.80	
	Elderly	2.08	1.17	0.86	
Cutia	Newborn	2.68	2.16	1.14	Ladd (2007), unpublished data from a Master thesis
	Young	3.11	1.96	1.14	
	Adult	4.55	2.36	1.28	

Data were extracted from the references provided on the rightmost column.

photoperiodic control of reproduction in hamsters, ferrets, voles, rams, and goats (Cardinali et al., 1981).

s0035 **2.2. Microstructure**

p0050 The SCG is the most studied sympathetic ganglia and many of its structural features are reproducible in the other sympathetic ganglia, although important differences are being found with more detailed studies, especially between paravertebral and prevertebral ganglia. In addition to ganglion nerve cells (principal ganglion neurons), sympathetic ganglia contain several other cell types. These include small granular cells (or small intensely fluorescent (SIF) cells), vascular cells (mainly endothelial cells), mast cells, and fibroblasts (in thin septa of connective tissue and in the capsule) (Gabella, 2004; Miolan and Niel, 1996).

s0040 **2.2.1 Ganglion capsule**

p0055 The SCG's capsule (Fig. 2.3) is conspicuous and composed of layers of collagen fibers and flattened fibroblasts. Therefore, each ganglion consists of an

Figure 2.3 Macrostructure of the left superior cervical ganglion (SCG) from an adult preá depicting the conspicuous SCG connective capsule (*) surrounding SCG parenchymal compartments (arrow). Scale bar: 100 μ m.

agglomeration of clusters of neurons separated by nerve fibers, capillaries, and prominent septa of collagen fibers (Fioretto et al., 2007)

The SCG is divided into distinct compartments by capsular septa of connective tissue as reported for mammalian sympathetic ganglia (Fioretto et al., 2007; Gabella et al., 1988; Miolan and Niel, 1996; Ribeiro et al., 2004; Schmidt, 1996; Szurszewski and King, 1989; Szurszewski and Miller, 1994). In addition, Gabella (1976) postulated that the capsule of connective tissue which surrounds the ganglia of the sympathetic trunk is continuous with the epineurium of the associated nerves. Shanthaveerappa and Bourne (1964) described the “perineurial epithelium” of sympathetic ganglia as being continuous with that of the attached nerve trunks. By the same token, Arvidson (1979) and Al-Khafajit et al. (1983) described epineurial and perineurial components of the SCG’s capsule in mice and rats.

The presence of a perineurial layer in the capsule and its continuity with the perineurium of attached nerves is of paramount importance, since the perineurium of peripheral nerve trunks acts as a diffusion barrier, which is not readily penetrated by macromolecules. Nonetheless, the permeability of sympathetic ganglia capsule has yet to be elucidated (Al-Khafajit et al., 1983).

p0070 Fioretto et al. (2011) reported that the sheep SCG is composed of several ganglion units, each one being constituted of clusters of neurons and glial cells. Each ganglion unit is separated by SCG capsular septa of connective tissue. Apart from ganglion neurons and glial cells, other cell types have been found within SCG, such as SIF cells, Schwann cells, fibroblasts, mast cells apart from capillaries harboring endothelial cells. The aforementioned authors have suggested that there are variations in the SCG structure in large mammals when compared to that in small laboratory rodents, such as rats (Ribeiro et al., 2004) and mice (Purves et al., 1986). [Au6]

p0075 With regards to the thickness of SCG capsule during animal maturation (from young to adult specimens), there was a 32.7% increase in that of dogs, a 25.8% increase in that of cats, and a 33.2% increase in that of horses. By performing an interspecies comparison of SCG capsule thickness, a 0.14-fold increase was observed from cats to dogs, a 2.2-fold increase was seen from dogs to horses and, finally a 2.6-fold increase between cats and horses (Fioretto et al., 2007). In adult animals, Ribeiro et al. (2004) reported that SCG capsule thickness ranged from 15 to 30 μm in rats, from 30 to 70 μm in capybaras, and from 60 to 80 μm in horses. Additionally, Ribeiro (2006) described a range of 30–50 μm for SCG capsule thickness in young capybaras, which represents a 28.5% reduction in relation to adult capybaras.

s0045 **2.2.2 SCG neurons**

p0080 SCG neurons are generally spindly shaped—or circular—and readily distinguishable due to their large size, clear nucleus, and the evident nucleolus. Ganglion neuron profiles are normally surrounded by one to two intraganglionic capillaries (in this case they are named intraunit capillaries) as well as by one to three glial cell nuclei (satellite cells) whose processes formed the ganglion neuron glial capsule (Abrahão et al., 2009; Coppi and Loesch, 2011; Fioretto et al., 2007, 2011; Ladd et al., 2012; Loesch et al., 2010; Melo, 2009; Toscano et al., 2009) (Fig. 2.4).

p0085 In horses, the neuron perikarya occupy a larger proportion of the sectional area than in dogs and cats and the neuropil is therefore proportionally less extensive. All neurons are uninucleate and some nuclei were located in the center of the perikaryon, while the majority is eccentric, but none resided at the periphery of the neuron profile. Between 1 and 2 nucleoli were observed in each neuron nucleus (Fioretto et al., 2007; Loesch et al., 2010; Ribeiro et al., 2004).

p0090 In pacas—a South American wild rodent—SCG neuron profiles are circular or, more commonly, ovally shaped. As with guinea pigs and other

Figure 2.4 Light microscopic images of Toluidine Blue-stained physical or optical sections of superior cervical ganglia (SCGs) from a rat (A), guinea pig (B), preá (C), cutia (D), paca (E), capybara (F), cat (G), dog (H), sheep (I), and from a horse (J), depicting details of the ganglion microstructure: uninucleate neurons (black arrowheads) and binucleate neurons (white arrowheads)—the latter is seen only in the SCGs of exotic rodents (B, C, D, E, F). SCG neurons are separated by spaces mainly occupied by the neuropil (*). Scale bars: 75 μ m.

South American wild rodents—preás, cutias, capybaras—ganglion neurons are either uni- or binucleate. In uninucleate neurons, some nuclei are located in the center of the perikaryon, while the majority is eccentric, but none of them are placed at the periphery of the neuron profile. On the contrary, in binucleate neurons, the nuclei occupy the two poles of the cell having a very distinct and defined position within the perikaryon (Abrahão et al., 2009; Melo, 2009).

More recently, Ladd et al. (2012) described the SCG neurons' structure in preás—another wild rodent found in South America. According to the

Figure 2.5 Light microscopic deconvoluted image at a maximal focal plane of a Giemsa-stained, 40- μm -thick, optical section of a left superior cervical ganglion (SCG) from an adult preá depicting three large binucleate neurons (black arrowheads) surrounding one uninucleate neuron (white arrowhead). Note that in binucleate neurons, the nuclei are very distinct and typically occupy the two poles of the cell. SCG uni- and binucleate neurons are separated by spaces mainly occupied by the neuropil (*). Scale bar: 30 μm .

authors, SCG neuron profiles are roughly circular or spindly shaped. SCG neurons are densely distributed and packed in newborn and young specimens. However, in adult and elderly preás, neurons are further apart and separated by spaces mainly occupied by neuropil (neurites and glial processes) and by blood vessels and connective tissue. Regardless of animal ages, nuclei are located either centrally or eccentrically in the perikarya of uninucleate neurons, though they occupy the two cell poles in binucleate neurons (Fig. 2.5).

2.2.2.1 Binucleate neurons

Mammalian sympathetic ganglion neurons are commonly uninucleate, though cells with two nuclei are also found (Dalsgaard and Elfvin, 1982; Filichkina, 1981; Huber, 1899; Macrae et al., 1986; Purves et al., 1986; Smith, 1970) and the frequency in which they appear changes considerably from one species to another and during the postnatal development. In the heart, binucleate cells appear at the stage in the development of the

innervation when the immature neuroblasts seen in early stages are becoming fewer in number and when their migration along nerve bundles passing from the cardiac plexus has almost ceased. On this basis, binucleate nerve cells are found when cell division is occurring and it may represent a stage in a mechanism to increase the number of ganglion cells, by a process of active division, at a time when migration of neuroblasts into the heart has virtually ceased (Smith, 1970).

p0105 One explanation for the occurrence of two nuclei in the SCG neurons of guinea pigs, preás, cutias, pacas, and capybaras is that a mitotic arrest late in the differentiation process can occur. There is no known reason for this, but it has been suggested that it might be due to a response to nerve growth factor (NGF) stimulation of the ganglion cells in their final differentiation to adrenergic cells. It is, however, unclear why only these cells respond in such a manner and no other adrenergic cell populations (Levi-Montalcini and Aloe, 1983; Purves et al., 1988) and why binucleate SCG neurons only occur in rodents—excluding mice and rats—and are not present in even larger mammals such as cats, dogs, sheep, and horses.

p0110 The functional significance of binucleate cells is yet to be clarified. It is possible that the occurrence of the two nuclei is an adaptation to some specific demand imposed on the cells due to protein synthesis. It is unclear whether the binucleate cells' genome is broader, since there is no indication that one of the nuclei has a larger proportion of euchromatin than the other, nor that the nuclei are different from those of the uninucleate cells in their heterochromatin/euchromatin ratio (Ribeiro, 2006; Smith, 1970). These speculations are, however, contradicted by Filichkina (1981), where binucleate SCG neurons in rabbits did not show an increase in the total protein synthesis or axonal protein transport when compared to uninucleate SCG cells.

p0115 The number of uni- versus binucleate neurons was found to vary with the stage of development with about 40% of the cells being binucleate in adult guinea pigs and 50% in the late prenatal stage (60-day-old fetuses) (Forsman et al., 1989). On the contrary, our recent study in preás (Ladd et al., 2012) showed that the number-derived proportion of uninucleate versus binucleate neurons was higher in newborn animals when compared to all remaining age groups, that is, in newborn preás, there were about two uninucleate neurons for each binucleate nerve cell and the proportion decreases and ultimately equates to 1:1 from young to old animals. Mechanistically speaking, we hypothesized that those uninucleate neurons were undergoing cell death or cell repair leading to a ratio equality between uni- and

binucleate neurons during postnatal development: maturation and aging. A second hypothesis would be that uninucleate neurons might be turning into binucleate neurons during postnatal development (Ladd et al., 2012).

- p0120 On the whole, there is still a dearth of knowledge of the origin of binucleate neurons and their functional meaning, since there is no plausible explanation for the large occurrence of binucleate SCG neurons, especially in rabbits and wild rodents as previous publications have demonstrated (Abrahão et al., 2009; Filichkina, 1981; Ladd et al., 2012; Loesch et al., 2010; Melo, 2009; Ribeiro, 2006; Ribeiro et al., 2004; Toscano et al., 2009).

s0055 **2.2.3 Glial cells**

- p0125 Two types of glial cells are found in most peripheral sensory and autonomic ganglia and nerves: (i) Schwann cells, which ensheath axons in peripheral nerves and may produce myelin (Bunge et al., 1986; Webster, 1975) and (ii) satellite cells, which are associated with neuron cell bodies within the peripheral ganglia (Pannese, 1981; Pomeroy and Purves, 1988). In peripheral ganglia, most glia are satellite cells, although Schwann cells may also be found associated with intraganglionic tracts. While Schwann cells in myelinated nerves have been proposed to regulate the ionic environment of the nerve and to provide trophic factors after injury, the role of satellite cells in peripheral ganglia remains less clear (Hall and Landis, 1991; Pannese, 1981) and warrant further systematic investigations (Fig. 2.6).

s0060 **2.2.4 SIF cells**

- p0130 Despite somewhat confused terminology: SIF cells or small granule-containing cells (SGC) or interneurons, these particular cells have been found in all mammalian sympathetic ganglia studied hitherto (Chiba and Williams, 1975; Gabella, 2004).
- p0135 SIF cells in the rat SCG have been studied extensively and, in this species, some or all of them receive preganglionic innervation and at least some of them synapse with principal ganglion cells (Matthews and Raisman, 1969; Williams, 1967; Williams and Palay, 1969; Yokota, 1973). Most rat SIF cells occur in clusters and are in close relation to fenestrated capillaries (Siegrist et al., 1968) (Fig. 2.6).
- p0140 Yokota (1973) performed a three-dimensional analysis of a cluster of four SIF cells within the rat SCG. All four of these cells received afferent synapses but only two of them made efferent somatodendritic synapses with principal ganglion neurons. The other two cells were naked, that is, without Schwann

Figure 2.6 Light microscopic image of a Toluidine Blue-stained 0.5-μm-thick semithin section of a superior cervical ganglion (SCG) from a young capybara. Some details of SCG microstructure are shown: one uninucleate (*) and two binucleate (**) neurons surrounded by three glial cell nuclei (black arrowheads) and separated by a blood vessel (v). It can also be noticed a Schwann cell (white arrowhead), a cluster of three vascular small intensely fluorescent (SIF) cells (arrows) and one axon (A) emerging from the uninucleate neuron (*). Scale bar: 5 μm. *Image extracted from Ribeiro (2006). Authorization will be required once the review process is accomplished. However, the present figure legend has been modified to show additional structures, not shown in Ribeiro (2006).*

cell's sheath over a greater area than those which possessed efferent synapses. However, it should be mentioned that since Yokota's careful study was confined to the immediate neighborhood of these four SIF cells, the possibility that there may have been additional (more distant) efferent synapses cannot be ruled out (Chiba and Williams, 1975).

A direct diffusion mechanism of the secretory products within the rat SCG was suggested by Taxi et al. (1969) and Yokota's findings supported the view that there may be two functional pathways for SIF cells in the SCG. Some SIF cells may operate as interneurons and others through a neurosecretory mechanism. We should not however exclude the possibility that the same cell may harbor these two mechanisms of action (Chiba and Williams, 1975).

Although interneurons are presumed to exist in the SCG of species other than rats, morphological proof for the existence of efferent synapses in these other species has not been presented. In most species studied, it was possible

to distinguish, on the basis of morphological characteristics, two categories of SIF cell in the SCG. Type I SIF cells were those which have long varicose processes extending among ganglionic neurons. Type II SIF cells, on the other hand, are intimately related to subcapsular or stromal blood vessels: an arrangement reminiscent of some endocrine glands. Generally speaking, type I SIF cells are solitary, whereas type II SIF cells are found in clusters (Chiba and Williams, 1975).

- p0155 Rats and guinea pigs break this rule, however, since in both species fairly long varicose fluorescent processes can be observed in the clustered SIF cells. In both these rodents, small clusters of SIF cells are located among ganglion neurons. Therefore, it is speculated that type I and type II SIF cells form intermingled clusters in some species, specifically in the rat and guinea pig SCG. Given the reasons above Chiba and Williams (1975) made no attempt to differentiate between type I and type II SIF cells in either of these species. By the same token, SIF cells were seen placed in two different locations, namely close to neurons or encompassing tight clusters comprising two to five cells in the proximity of blood vessels within the SCG of cats, dogs, and horses (Fioretto et al., 2007).
- p0160 Matthews and Raisman (1969) and Jew (1985) reported the presence of SGC in the SCG, which are distinct to sympathetic neurons and other satellite cells. These SGC were also identified as SIF cells and formed tight clusters associated with small capillaries and, as stated by Miolan and Niel (1996), may play a neuroendocrine role. SIF cells were classified into two types: type I with granular vesicles ranging from 80 to 100 nm and type II with granular vesicles varying from 150 to 300 nm (Gabella, 2004). This author also described that SIF cells presented synapses to preganglionic fibers and few SIF cells had synaptic contacts to ganglion neurons. Furthermore, SIF cells, which had afferent and efferent synapses, were named interneurons (Fioretto et al., 2007).
- p0165 Black et al. (1974) summarized the differences observed between the SCGs of hamsters, guinea pigs, rats, cows, rabbits, cats, and monkeys. There are significant differences in the numbers and anatomic distribution of SIF cells in these species. Whereas the numbers of SIF cells vary with the size of the ganglia, the number of SIF cells per milligram of SCG tissue is very similar in cows, rabbits, cats, and monkeys. By contrast, rodent SCG contains from 20 to 100 times as many SIF cells per milligram of SCG tissue as do nonrodent species.
- p0170 The biologic significance of such relatively huge numbers of SIF cells in the rodent SCG is unknown. Among nonrodents, the percentage of type

I SIF cells varies from a high of 74% in rabbits to a low of 0.48% in cats, though SIF cells classification is ambiguous in those species. On the contrary, all rodents exhibit mixed clusters in which both types of SIF cells are present. Thus, the number of SIF cells per milligram of SCG tissue and the anatomic arrangement of these cells within the SCG differ clearly between rodents and nonrodents (Black et al., 1974).

s0065 **2.2.5 Nerve fibers**

p0175 Rami communicantes are short nerve trunks connecting the ganglia of the sympathetic chain—including SCG—to the spinal nerves. The rami are particularly short in the rat and even when they are multiple and separated into two or more bundles they cannot be distinguished as white and gray rami (Gabella, 2004). Mixed within each of the above-mentioned rami there are preganglionic and postganglionic fibers and, since they have no distinctive structural features to indicate their orientation and nature and their identification can be made only after experiments of selective nerve sections (Gabella, 2004).

p0180 Depending on the level of origin, preganglionic fibers travel some distance up or down the sympathetic chain, forming synaptic contacts with SCG neurons. The length of preganglionic fibers can therefore be considerable. In the upper thoracic chain segment, they are mainly ascending (cranially directed) and in the cervical sympathetic trunk—where SCG is inserted—all the preganglionic fibers are directed cranially. In this trunk, however, there are also caudally directed postganglionic fibers, originating in the SCG, and cranially directed postganglionic fibers, originating in the middle and lower cervical ganglia (Bowers and Zigmond, 1981). In the rat, unlike other species, such as humans and cats, the great majority of preganglionic fibers are unmyelinated. For example, less than 1% of the axons in the cervical sympathetic trunk are myelinated (Brooks-Fournjer and Coggeshall, 1981; Dyck and Hopkins, 1972; Hedger and Webber, 1976).

s0070 **2.2.6 Synapses**

p0185 The chemical organization of the sympathetic nervous system is somewhat more complex. Generally speaking, there are at least three types of ganglion neurons in the paravertebral portion of the sympathetic nervous system: those that contain norepinephrine alone, those that contain norepinephrine along with neuropeptide Y (NPY), and those that contain acetylcholine and vasoactive intestinal peptide. The first type provides innervation to the parenchyma of the target organs, while the second mainly innervates blood

vessels. The third type innervates the sweat glands (Gibbins and Morris, 2006; Smolen, 1988).

p0190 The SCG is the site of synapses between preganglionic sympathetic nerve fibers and postganglionic sympathetic neurons (Guth and Bernstein, 1961; Loesch et al., 2010) (Figs. 2.7 and 2.8). The postnatal biochemical maturation of SCG has been described in rodents (Black et al., 1971a; Thoenen et al., 1972a). Both choline acetyltransferase—which is contained chiefly in the preganglionic terminals (Hebb and Waites, 1956)—and tyrosine hydroxylase (TH)—which is localized to adrenergic neurons—have been described in the SCG (Black et al., 1971b; Smolen and Raisman, 1980). In the SCG of rodents, it has been shown that TH—the rate-limiting enzyme in catecholamine biosynthesis—undergoes a normal postnatal increase in activity (Black et al., 1971a; Thoenen et al., 1972a). In addition, it has been depicted that when the afferent input to SCG neurons is removed at the time of birth, TH activity fails to undergo its normal postnatal increase and remains at neonatal levels (Black and Mytilineou, 1976; Black et al., 1972; Thoenen et al., 1972b).

s0075

3. STEREOLOGICAL ASSESSMENT OF THE SCG DURING POSTNATAL DEVELOPMENT: EFFECTS OF AGING AND ALLOMETRY

s0080

3.1. Ganglion volume

p0195 In order to estimate the volume of the SCG the most accurate and modern method of choice is design-based stereology using Cavalieri's principle (Abrahão et al., 2009; Ladd et al., 2012; Melo, 2009; Ribeiro, 2006). There are several advantages in applying this method: it is possible to estimate the coefficient of error and the estimations are not dependent upon the shape of SCG (Gundersen et al., 1999; Howard and Reed, 2010).

p0200 The basic principle is very straightforward. The organ is cut into slabs of equal thickness beginning with a random start. An estimate of the total volume of the organ can then be calculated by multiplying the slab mean thickness by the sum of the areas of each slab. Then,

$$V_{\text{SCG}} = T \sum A_{\text{SCG}},$$

where T is the between-section distance (in μm) and $\sum A_{\text{SCG}}$ is the sum of the delineated profile areas of the chosen set of SCG sections. Profile areas are estimated from the numbers of randomly positioned test points (at least

Figure 2.7 Transmission electron microscopic images of SCG in rat (a, b), capybara (c, d), and horse (e) immunolabeled for SYP (black immunoprecipitate). (a) Note SYP-positive axon profile (Ax) asymmetrically synapsing on a dendrite profile (D); postsynaptic densities (white arrows) are clearly visible. Note also that the axon profile is rich in small agranular vesicles (av); a few granular vesicles (gv) can also be seen (m mitochondrion). (b) SYP-positive axon profile synapsing with dendritic spine (ds). (c) SYP-positive axon terminal asymmetrically synapsing on a dendrite profile; the axon terminal contains a number of small agranular vesicles. (d) SYP-positive axon profile synapsing on dendritic spine. A few granular vesicles can be seen in addition to small agranular vesicles. (e) Asymmetric axo-dendritic synapse with heavily labeled axonal profile. Bars 0.5 μm . Image and legend extracted from Loesch et al. (2010). Authorization will be required once the review process is accomplished.

Figure 2.8 Confocal immunofluorescence microscopy of SCGs (here, 30 consecutive 1- μ m images were merged as maximal projections) immunolabeled for SYP (light green in original images). (A) Rat, (B) capybara, and (C) horse. In (A–C), SYP-positive labeling (arrows) was located in the neuropil among the ganglion neurons (N). Note that labeling is more densely packed in the rat SCG compared with that in capybara and horse. (D) Immunohistochemical control of horse SCG; no immunolabeling for SYP was observed when the primary antibody was replaced by nonimmune normal goat serum. Bars 50 μ m. Image and legend extracted from Loesch et al. (2010). Authorization will be required once the review process is accomplished.

~ 100 per SCG) hitting the whole reference space and the areal equivalent of a test point (for more details, see Howard and Reed, 2010; Ladd et al., 2012). Ganglion volume is an important parameter which may reflect global alterations in the organ. This parameter is affected by several factors like species-related differences (Loesch et al., 2010; Ribeiro et al., 2004), age (Abrahão

Figure 2.9 Simple histogram (bar chart) showing the superior cervical ganglion (SCG) volume across a plethora of different mammalian species: rats, guinea pigs, préas, cutias, pacas, capybaras, sheep, and horses. Every bar uppermost edge indicates the mean value for each species. Data were extracted from the references identified by specific and corresponding drawing patterns.

et al., 2009; Ladd et al., 2012; Melo, 2009; Toscano et al., 2009) and side (Abrahão et al., 2009) (Fig. 2.9).

In addition, hypertrophy is a widely frequent adaptive mechanism during either SCG postnatal development from medium-sized to large mammals (Abrahão et al., 2009; Ladd et al., 2012; Melo, 2009; Miolan and Niel, 1996; Ribeiro, 2006; Ribeiro et al., 2004; Toscano et al., 2009) or after unilateral ganglionectomy (Fioretto et al., 2011). There has also been noticed an allometric association between SCG volume and body weight (Loesch et al., 2010; Ribeiro et al., 2004).

It should be stressed that the larger proportion of the SCG volume is occupied by the nonneuronal tissue compartment, that is, neuropil, connective tissue, and blood vessels, and that differences in ganglion volume may reflect a variation in the total volume of the aforementioned tissue compartments (Abrahão et al., 2009; Ladd et al., 2012; Toscano et al., 2009).

3.2. Total number of neurons

Cutting-edge 3D technology—employed by using design-based stereology—provides us with a much more accurate and unbiased method for quantifying aspects of the structure of the SCG neurons in healthy and diseased mammals from a plethora of species (Abrahão et al., 2009; Ladd et al., 2012; Melo, 2009; Ribeiro et al., 2004; Toscano et al., 2009). The most powerful stereological tool for estimating the total number of a given particle in 3D is the disector method, described for the first time by Sterio

(1984). The disector probe can be applied either physically or optically (Howard and Reed, 2010).

p0220 Recently, it is considered state-of-the-art to associate the optical (Fig. 2.10) (Ladd et al., 2012) or the physical disector (Abrahão et al., 2009; Melo, 2009) with the fractionator sampling regime (Gundersen, 2002; Howard and Reed, 2010)—which uses several sampling fractions—to assess remodeling-induced changes in the number of SCG neurons during postnatal development (maturation and aging) or as a consequence of both allometric and disease-related adaptive responses.

p0225 In a recent study by Ladd et al. (2012), the total number of SCG neurons in preás was estimated by multiplying the counted number of particles—uninucleate or binucleate neurons—by the reciprocal of sampling fractions:

$$N_{\text{uni- or biSCG}} := \text{ssf}^{-1} \text{hsf}^{-1} \text{asf}^{-1} \Sigma Q^{-}$$

where $N_{\text{uni-orbiSCG}}$ is the total number of uninucleate or binucleate neurons, ssf is the mean section sampling fraction, hsf is the mean height sampling fraction, asf is the mean area sampling fraction, and ΣQ^{-} is the total number of particles (neurons) counted using optical disectors (for more details, revisit Howard and Reed, 2010; Ladd et al., 2012). The several effects exerted by species, maturation (or aging), body weight, and disease on the quantitative structure of SCG may be translated as decrease (Santer, 1991; Toscano et al., 2009), increase (Abrahão et al., 2009; Melo, 2009), or stability (Ladd et al., 2012) in the total neuron number. Figure 2.11 summarizes the differences in the total number of SCG neurons in different species of mammals.

p0230 As reported earlier, a catalogue of publications describing the existence of binucleate neurons—which may imply that some cell division and resulting neurogenesis are still in place—in the SCG of adult wild rodents such as capybaras (Ribeiro et al., 2004), pacas (Abrahão et al., 2009; Melo, 2009), and yellow-tooted cavies (preás) (Ladd et al., 2012) in conjunction with studies depicting substantial increases in the total number of SCG neurons in aged pacas (Abrahão et al., 2009; Melo, 2009) add further fuel and strongly advocate that a postnatal neurogenesis may indeed occur in sympathetic ganglia.

p0235 A robust strategy for tackling neurogenesis in the mammalian SCG is coupling design-based stereology with immunohistochemistry. The latter is attained by using commercially available cell-cycle phase or proliferation markers such as thymidine-analog 5-bromo-2-deoxyuridine (BrdU) and Ki-67, though every marker has advantages and pitfalls and the conclusions they lead to have to be drawn in the overall context of the experiment.

Figure 2.10 Images of successive focal planes throughout a Giemsa-stained optical section of a superior cervical ganglion (SCG) from an adult preá, illustrating the application of the optical disector. The distance between each focal plane is 5 μm . On plane (A) (uppermost surface of the section) a field of view—selected using an unbiased counting frame—is followed along the whole section thickness (planes B, C, D, E, and F) and neurons are sampled and counted as they come into focus on each focal plane. For instance, on plane (C) (10 μm apart from plane A) one uninucleate (U) and one binucleate (B) neuron are sampled. The lowermost focal plane (F) (bottom surface of the section) is 25 μm apart from plane (A) and no particles are sampled on it. Scale bars: 30 μm .

Figure 2.11 Simple histogram (bar chart) showing the total number of superior cervical ganglion (SCG) neurons across a catalogue of different mammalian species: rats, guinea pigs, preás, pacas, cutias, sheep, capybaras, and horses. Every bar uppermost edge indicates the mean value for each species. Data were extracted from the references identified by specific and corresponding drawing patterns.

In light of that, in our recent study (Ladd et al., 2012), the total number of BrdU-positive neurons was stereologically estimated and “always” related to the total number of Giemsa-stained neurons in order to see whether the BrdU subpopulation of neurons would exert an effect on the whole neuronal population, viz, the Giemsa-stained nerve cells. Therefore, by plotting the BrdU chemical-coded subpopulation of SCG neurons against the whole Giemsa-stained SCG neuronal population, we have found that the total number of neurons is surprisingly constant in the SCG of preás during the entire postnatal development, viz, maturation and aging. The aforementioned stereological-plotting approach is novel in the SCG-specialized literature and leads to a more mechanistic explanation for the events underpinning the adaptation and remodeling of SCG neurons during postnatal development.

On the other hand, the increase in neuron number is not always attributable to neurogenesis, but may also be the outcome of late maturation or incomplete differentiation of nerve cells (Ciaronia et al., 2000; Farel, 2003; Miolan and Niel, 1996). In juvenile bullfrogs, dorsal root ganglion (DRG)—a sensory ganglion—houses a population of immature, incompletely differentiated neurons that can be induced to differentiate into a mature form in response to injury (Farel and Boyer, 1999). Indeed, new DRG neurons derive mostly from differentiation rather than from

proliferation of precursor cells (Meeker and Farel, 1997). By the same token, pelvic ganglion neurons more than double between birth and adulthood, probably due to continuous maturation of p75-positive undifferentiated neuronal precursors rather than to cell division (Yan Hui Keast, 2008).

s0090 3.3. Neuron volume

p0250 Cell volume estimated by design-based stereology is considered as a state-of-the-art, assumption-free, and unbiased procedure for estimating neuron size, when compared to 2D or 3D particle shape-dependent methods (Ladd et al., 2012; Ribeiro et al., 2004). Two main stereological methods are currently used for estimating neuron volume: nucleator (Gundersen, 1988) and rotator (Vedel Jensen and Gundersen, 1993). Both methods require isotropic and uniform random or vertical and uniform random sections and the usage of either physical or optical disector (Howard and Reed, 2010).

p0255 The nucleator employs the following formula to estimate the neuron volume ($\bar{v}_N$):

$$\bar{v}_{N\text{mye/sub}} := \Sigma(4p/3)\bar{I}_n^3$$

where $\bar{I}_n^3$ is the mean of all cubed distances from a central point (nucleolus) within the perikaryon to its cell boundaries (Gundersen, 1988).

p0260 The rotator is based upon the Pappus–Guldinus theorem. Technically speaking, the method requires a uniquely defined subspace of the particle, for example, the nucleus or the nucleolus of a cell and the optical disector is used as a probe to sample particles. The following formula is used to estimate neuron volume ($\bar{v}_N$):

$$\bar{v}_N = \pi t \sum_i l_i^2$$

where t is the distance between six parallel half-lines (which constitute a grid) placed perpendicular to the vertical axis of the section and l_i^2 represents the squared distance between cell profile boundary and the vertical axis measured in the focal plane where the nucleolus was identified (Vedel Jensen and Gundersen, 1993). Figure 2.12 collates estimates of SCG neuron volume obtained from quite a few species. Generally speaking, binucleate SCG neurons are twice as big as uninucleate ones.

p0265 Sympathetic neuron hypertrophy seems to be a predominant event during postnatal development in order to compensate for a neuron loss in the same period (Cabello et al., 2002) or as an adaptive mechanism for coupling with

Figure 2.12 Simple histogram (bar chart) showing data on the volume of superior cervical ganglion (SCG) uni- and binucleate neurons across a surfeit of different mammalian species: rats, guinea pigs, preás, cutias, pacas, capybaras, sheep, and horses. Every bar uppermost edge indicates the mean value for each species. Data were extracted from the references identified by specific and corresponding drawing patterns.

aging or with body size increase (Abrahão et al., 2009; Ladd et al., 2012; Melo, 2009; Sanchez, 2008). Along the same lines, neuron volume increased by 217% in dog's caudal mesenteric ganglion—a prevertebral ganglion (Gagliardo et al., 2005) and in the human SCG neurons (Liutkiene et al., 2007) during postnatal development. In contrast, Ribeiro (2006) reported no changes in the volume of SCG neurons in capybaras during postnatal development, whereas Toscano et al. (2009) have recently communicated 27.5% and 40% decreases (atrophy) in the volume of uni- and binucleate SCG neurons in guinea pigs during aging, respectively, which may be attributable to a selective age-related atrophy and neurodegeneration mediated by an increase in the sortilin expression in SCG neurons, compounded with elevated expression of pro-NGF levels in some targets (Al-Shawi et al., 2008). A third hypothesis, which would account for the increase in the size of uninucleate neurons during aging, might be that uninucleate neurons are on the brink of becoming binucleate neurons (which are considerably larger). Given the paradoxical outcome measures reported above, the chances are that alternative neuron-size-regulating mechanisms, apart from hypertrophy, may be acting or coacting in the autonomic nervous system during postnatal development in order to guarantee functional homeostasis, especially in the total amount of neurotransmitters produced (Cabello et al., 2002; Ladd et al., 2012).

s0095 3.4. Synapses

p0270 Estimates using model-based (particle shape-assumption dependent) and design-based stereology (disector method) suggest that the SCG of an adult rat harbors $4\text{--}12 \times 10^6$ synapses (Field and Raisman, 1985; Siklós et al., 1990; Smolen and Raisman, 1980) and $19\text{--}36 \times 10^3$ neurons (Ostberg et al., 1976; Purves et al., 1986; Ribeiro et al., 2004). This gives an average synapse:neuron ratio of about 290:1 and the overwhelming majority of these, 90% or more, are axo-dendritic (Forehand, 1985). Larger species possess SCG neurons which exhibit greater convergence, more complex arborizations and a greater proportion of axo-dendritic compared to axo-somatic synapses (Forehand, 1985; Purves and Lichtman, 1985; Purves et al., 1986). Therefore, in autonomic ganglia—including the SCG—a decision should be made to confine counts to axo-dendritic synapses rather than axo-somatic ones, since the former occur more frequently (Forehand, 1985; Gibbins and Morris, 2006; Loesch et al., 2010; Matthews, 1983).

p0275 Quantitative data on synapses are undoubtedly obtained using transmission electron microscopy because this is the only technique which reveals whether or not a preganglionic bouton makes contact with the surface of a neuron and shows the morphological features conventionally associated with synaptic specializations, that is, clusters of small vesicles directed toward a well-defined density on a presynaptic membrane. However, even this appearance may not imply a functional synapse and the same is true for light microscopy (Gibbins and Morris, 2006). Ideally, a range of chemical markers needs to be localized and correlated with electrophysiological data for the same populations of neurons (Gibbins and Morris, 2006).

p0280 The immunochemical labeling of SYP, a synaptic vesicle membrane glycoprotein, has been used to detect functioning synaptic terminals (Calhoun et al., 1996; Loesch et al., 2010; Mochida et al., 1994; Navone, 1986; Tarsa ^[Au9] and Goda, 2002; Weidenmann and Franke, 1985). SYP is a member of the physin family, which also comprises synaptophysin 1, synaptoporin, pan-tophysin, mitsugumin, and synaptogyrin (Janz et al., 1999; Leube, 1994). Given the variety of potential labels of synapses and their vesicles, it is possible that the synapse labeling achieved in a particular study is subtotal and that the quantitation based upon synaptophysin staining may underestimate total synapse number.

p0285 Loesch et al. (2010) have recently found that rat SCG harbors 10.3 million SYP-labeled axo-dendritic synapses and this estimate is within the range of previously published values for the same species and based upon both

design-based (Siklós et al., 1990) and model-based (Field and Raisman, 1985; Ostberg et al., 1976; Smolen and Raisman, 1980) estimates. The numbers equate to about 430 axo-dendritic synapses per neuron and this estimate is somewhat greater than earlier values of 250–330 per neuron (Forehand, 1985; Ostberg et al., 1976). In the same study, Loesch et al. (2010) reported that the number of SYP-labeled axo-dendritic synapses was found to be 45 million and 56 million in horses and capybaras, respectively. Numbers per neuron were roughly 48-fold larger in capybaras and 25-fold higher in horses (Fig. 2.13).

p0290 Due to the fact that the target tissue of the SCG increases in volume and surface area, it is reasonable to hypothesize that the size and number of ganglion neurons and the number of synapses would vary as a function of the quantity of target tissue innervated by them (Loesch et al., 2010). However, having a heterogeneous population of nerve cells (e.g., secretomotor, pilomotor, vasomotor), SCG neurons, and their synapses may be subject to a variety of structural changes depending upon the organs they target. For instance, neurons innervating the iris have the largest cell bodies and most extensive dendritic arborizations, whereas vasomotor neurons are the smallest. Neurons innervating the middle cerebral artery halt dendritic

f0065 **Figure 2.13** Simple histogram (bar chart) showing data on total number of superior cervical ganglion (SCG) axo-dendritic synapses across three different mammalian species: rats, capybaras, and horses. Every bar uppermost edge indicates the mean value for each species. Data were extracted from Loesch et al. (2010).

growth early in postnatal development, whereas the dendritic complexity of neurons supplying the submandibular gland increases well into adulthood (Andrews, 1996).

s0100

4. CONCLUDING REMARKS AND FUTURE RESEARCH DIRECTIONS

p0295

We have reviewed processes underlying SCG adaptation and remodeling during postnatal development: maturation and aging. Further investigations are warranted—using a plethora of cell-cycle phase markers such as: BrdU, BrdU/PCNA, thymidine, and Ki-67 and combined with cell differentiation (p75) and apoptosis markers (caspase 3)—to shed light on a portfolio of other mechanisms—cell repair, cell division, maturation, differentiation, and death—that we hypothesize to be intertwined and implicated in the qualitative and quantitative structural changes in SCG, its neurons and synapses during postnatal development.

p0300

Another interesting line of investigation would be whether age-related changes preferentially target a neuron population and the interaction between ganglion neurons, their innervation targets, and the signaling neurotrophic factors produced by the latter. This could be achieved by labeling neurons with specific markers, for example, NPY coupled with the usage of neurotracers. Would the differences in the sizes of the innervation targets and in the quality of the signals raised by the target organs explain the changes in SCG, its neurons and synapses during maturation and aging?

p0305

Finally, we hope that the data yielded by previous and forthcoming studies will bridge the gap between basic and translational clinical research, since the role SCG plays is of paramount importance in the understanding and treatment of some important neuropathies including Horner's syndrome, stroke, and epilepsy.

ac0005 ACKNOWLEDGMENT

p0310

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Non-Print Items

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