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Nestin expression defines both glial and neuronal progenitors in postnatal sympathetic ganglia

Huilin Shi¹, Hongjuan Cui¹, Goleeta Alam¹, William T. Gunning^{1,2}, Andrea Nestor^{2,3}, David Giovannucci^{2,4}, Ming Zhang⁵, and Han-Fei Ding^{1,*}

¹Department of Biochemistry and Cancer Biology, University of Toledo Health Science Campus, Toledo, Ohio 43614 ²Advanced Microscopy and Imaging Center, University of Toledo Health Science Campus, Toledo, Ohio 43614 ³Department of Surgery, University of Toledo Health Science Campus, Toledo, Ohio 43614 ⁴Department of Neurosciences, University of Toledo Health Science Campus, Toledo, Ohio 43614 ⁵Department of Anatomy and Structural Biology, University of Otago, Dunedin, New Zealand

Abstract

Sympathetic ganglia are primarily composed of noradrenergic neurons and satellite glial cells. Although both cell types originate from neural crest cells, the identities of the progenitor populations at intermediate stages of the differentiation process remain to be established. Here we report the identification in vivo of glial and neuronal progenitor cells in postnatal sympathetic ganglia, using mouse superior cervical ganglia as a model system. There are significant levels of cellular proliferation in mouse superior cervical ganglia during the first 18 days after birth. A majority of the proliferating cells express both nestin and brain lipid-binding protein (BLBP). BrdU fate-tracing experiments demonstrate that these nestin and BLBP double positive cells represent a population of glial progenitors for sympathetic satellite cells. The glial differentiation process is characterized by a marked downregulation of nestin and upregulation of S100, with no significant changes in the levels of BLBP expression. We also identify a small number of proliferating cells that express nestin and tyrosine hydroxylase, a key enzyme of catecholamine biosynthesis that defines sympathetic noradrenergic neurons. Together, these results establish nestin as a common marker for sympathetic neuronal and glial progenitor cells and delineate the cellular basis for the generation and maturation of sympathetic satellite cells.

Keywords

noradrenergic neurons; satellite cells; superior cervical ganglia; postnatal sympathetic development

The sympathetic nervous system is composed of sympathetic ganglia and the adrenal medulla, a specialized sympathetic ganglion containing secretory chromaffin cells. Sympathetic ganglia of mammals are organized into two paravertebral chains that span from cervical to sacral regions, with the ganglia being interconnected with pre- and postganglionic sympathetic nerve fibers. Sympathetic ganglia contain two major cell types, neurons and glial cells. Most mammalian sympathetic neurons use noradrenaline as a neurotransmitter and, thus, are called noradrenergic neurons. These neurons are commonly marked by their expression of tyrosine hydroxylase (TH) that catalyzes the rate-limiting step

*Correspondence to: Han-Fei Ding, Department of Biochemistry and Cancer Biology, BHSB 461, University of Toledo Health Science Campus, 3035 Arlington Avenue, Toledo, Ohio 43614. han-fei.ding@utoledo.edu.

in the biosynthesis of catecholamines including dopamine, noradrenaline, and adrenaline. Sympathetic glial cells include Schwann cells and satellite cells. Schwann cells provide myelin to insulate axons of the peripheral nerves whereas satellite cells line the exterior surface of sympathetic neurons. Within a sympathetic ganglion, the majority of glial cells are satellite cells and Schwann cells are generally associated with intra-ganglionic nerve fibers. A common marker for the sympathetic glial cells is S100, an acidic calcium-binding protein (Cocchia and Michetti, 1981).

It is well established that sympathetic neurons and glia are derived from neural crest cells (Anderson, 1989; LaBonne and Bronner-Fraser, 1998; Le Douarin and Dupin, 1993), a transient, highly migratory population of multipotent stem/progenitor cells. The neural crest can be divided into four regions along the anterior–posterior axis: cranial, vagal, trunk, and lumbosacral neural crest. During sympathetic development, neural crest cells, mainly from the trunk region of the neural crest, migrate ventrally and aggregate adjacent to the dorsal aorta to form the primary sympathetic chain. A subpopulation of the cells then undergo dorsal migration to form the paravertebral sympathetic ganglia where they differentiate into sympathetic neurons and glial cells (Francis and Landis, 1999; Kirby and Gilmore, 1976).

The generation of sympathetic neurons (neurogenesis) and glia (gliogenesis), which is best studied in rat superior cervical ganglia (SCG), occur during different periods of sympathetic development. While neurogenesis peaks around embryonic day 14.5 (E14.5) and is essentially completed at the time of birth, gliogenesis begins around E16.5 and continues postnatally (Hall and Landis, 1991; Hall and Landis, 1992; Hendry, 1977). Consistent with the temporal pattern of in vivo sympathetic neurogenesis and gliogenesis, in vitro fate tracing experiments revealed that proliferating cells isolated from the E14.5 rat SCG gave rise predominantly to clones containing only neurons, whereas those from the E17.5 rat SCG generated mostly clones that contained only glial cells (Hall and Landis, 1991). These findings have led to the suggestion that post-migratory neural crest cells commit to a neuronal or glial fate at a very early stage of the sympathetic development (Hall and Landis, 1991). However, the identities of sympathetic neuronal and glial progenitors have not been clearly defined.

In this study, we examined the development of mouse sympathetic ganglia during the first eight weeks after birth, with the goal of identifying molecular markers that define distinct sympathetic progenitor populations. A detailed characterization of the cellular basis for postnatal sympathetic development should facilitate the investigation of genes and signaling pathways that control the developmental process.

MATERIALS AND METHODS

Mice

Male and female C57BL/6J mice 1-day to 8-week old were used in this study. Mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained under Specific Pathogen Free conditions in the animal facility of University of Toledo Health Science Campus. All animal experiments were pre-approved by the Institutional Animal Care and Use Committee.

BrdU injection and SCG collection

The thymidine analog 5-bromo-2'-deoxyuridine (BrdU, Sigma, St. Louis, MO) was dissolved in saline at 10 mg/ml, and mice were injected intraperitoneally with BrdU at 50 mg/kg (body weight) 2 hours prior to being euthanized by CO₂ inhalation on postnatal day 4, or they were injected once daily for 3 days on postnatal day 4, 5 and 6, and sacrificed on day 1, 7, 14, 21, 28, and 56 after the final BrdU injection. C57BL/6J mice without BrdU

injection were also euthanized by CO₂ inhalation on postnatal day 1, 4, 7, 10, 14, 18, 22, 26, 30, 42 and 56. For each time point, at least 3 mice were used. Mouse SCG were collected, fixed in 10% neutral buffered formalin and embedded in paraffin. The paraffin tissue blocks were stored at 4°C until sectioned.

Immunofluorescence

Sections of 4-μm were cut from the SCG paraffin blocks and mounted on 3-aminopropyltriethoxysilane-treated slides (Sigma). After de-paraffinization and rehydration, the sections were treated in a microwave oven at 95 degree for 20 minutes in 10 mM citrate buffer (pH= 6) for antigen retrieval and washed in PBS. Sections were blocked with 10% horse serum for 1 hour and incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: mouse anti-Ki67 (1:100, BD Pharmingen, San Jose, CA), rat anti-BrdU (1:200, Abcam, Cambridge, MA), mouse anti-TH (1:5000, Sigma), rabbit anti-TH (1:1000, Chemicon, Temecula, CA), rabbit anti-S100 (1:200, Dako, Carpinteria, CA), rabbit anti-BLBP (1:2000, Chemicon), and chicken anti-nestin (1:1000, Novus Biologicals, Littleton, CO). For double or triple immunostaining, various combinations of primary antibodies were used. After washing in PBS, the sections were again blocked with 10% goat serum for 1 hour, followed by incubation with secondary antibodies for 2 hours. All secondary antibodies were purchased from Invitrogen (Carlsbad, CA) and used at 1:400 dilutions. The secondary antibodies used were: goat anti-mouse (FITC or Texas-Red), goat anti-rabbit (Alexa fluor 594 or Alexa Fluor 647), goat anti-chicken (Alexa Fluor 488), and goat anti-rat (Alexa Fluor 488). After secondary antibody incubation, the sections were washed in PBS and counterstained with DAPI to stain nuclei. Sections were mounted in Fluorescence Mounting Medium (Dako) and ProLong Gold antifade reagent (Invitrogen), coverslipped, and examined using a TCS SP5 multi-photon laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL) or a Nikon ECLIPSE E800 fluorescence microscope (Nikon Instruments Inc., Melville, NY). Images were processed using Image J (NIH shareware) and Adobe Photoshop CS (Adobe Systems, Mountain View, CA). Figures were assembled and labeled in Canvas 9 (ACD Systems, Miami, FL).

Antibody characterization

The clone B56 anti-Ki67 mouse monoclonal antibody (BD Pharmingen, #550609, Lot #32659) was raised against human Ki67 protein. It has the same antigen specificity as the well-characterized clone MIB 1 anti-Ki67 monoclonal antibody (Key et al., 1993). Both MIB 1 and B56 antibodies recognize a double band of 345 and 395 kD on Western blot. Flow cytometry analysis reveals that the binding of Phycoerytherin-conjugated B56 can be blocked by purified MIB 1 antibody. Immunohistochemistry analysis demonstrates that B56 gives the same staining pattern as MIB 1 on both frozen and paraffin-embedded tissue sections (manufacturer's Technical Data Sheet).

The specificity of the monoclonal rat anti-BrdU antibody (Abcam, ab6326, Lot #163833) has been confirmed by several assays. It reacts with free BrdU, BrdU in single-stranded DNA, or BrdU attached to a carrier protein, but not with thymidine (manufacturer's technical information). In our study, no staining was seen when the antibody was used to stain tissue sections from mice that had not been injected with BrdU.

The mouse monoclonal anti-TH antibody (clone TH-2, Sigma, T1299) was raised against rat TH. It recognizes an epitope present in the N-terminal region (amino acids 9–16) of both rodent and human TH. The rabbit polyclonal anti-TH antibody (Chemicon, AB152, Lot #0603025098) was prepared against denatured TH from rat pheochromocytoma. Both

antibodies detect a single band of ~60 kD on Western blot (manufacturer's technical information), and only stained sympathetic cells with the morphology of neurons.

The rabbit polyclonal anti-S100 antibody (Dako, Z0311, Lot #00015317) was raised against S100 isolated from cow brain. In western blotting of purified recombinant human S100 proteins, the antibody stains S100B strongly, S100A1 weakly, and S100A6 very weakly. No reaction was observed with S100A2, S100A3 and S100A4 (manufacturer's technical information). Also according to the information provided by Dako, Z0311 only labels glial cells in the brain and gives the same staining pattern as a monoclonal anti-S100 antibody previously reported (Hagen et al., 1986). In our study, we found that Z0311 only stained cells with the morphology and distribution of glial cells in adult mouse SCG (Fig. 3).

The rabbit polyclonal anti-BLBP antibody (Chemicon, AB9558, Lot #0603024945) was raised against recombinant BLBP. It stains a single band of ~15 kD on Western blot and only glial cells in immunohistochemistry (manufacturer's technical information). We also found that the antibody only stained cells with the morphology and distribution of glial cells in mouse SCG (Fig. 2).

The chicken anti-nestin antibody (Novus Biologicals, NB100-1604, Lot #0305) was raised against mouse nestin. According to the information provided by the company, the antibody stains neural stem cells of the ventricular zone of the mouse brain by immunofluorescence. In Western blotting, this antibody reacts with recombinant mouse nestin and detects a single band of approximately 250 kD in mouse brain homogenate. No staining was seen in Western blotting of mouse liver homogenate. We also found that the antibody only stained neural crest cells, but not their neuronal progeny cells (unpublished data), which was identical with previous descriptions (Stemple and Anderson, 1992).

Quantification

For sections from each mouse at each time point, a total of 1000 cells (DAPI-positive) were counted from at least 4 randomly selected 400x fields, and the percentages of proliferating cells (Ki67- or BrdU-positive) and progenitor- and lineage-marker expressing cells were determined. All data were presented as mean $\pm$ standard deviation (SD).

RESULTS

Cell proliferation in postnatal mouse SCG

Progenitor cells are generally defined as populations of dividing cells with the capacity to differentiate (Smith, 2006). To identify the progenitor cell populations in postnatal mouse SCG, we first performed Ki67 immunofluorescence staining of SCG sections (Fig. 1A). Ki67 is a nuclear protein specifically expressed in cells undergoing active proliferation (Sawhney and Hall, 1992). From postnatal day 1 (P1) to P4, approximately 20% of ganglionic cells in mouse SCG expressed Ki67. The number of Ki67-expressing cells decreased gradually with time, and by the end of the third postnatal week, only 0.4% of ganglionic cells were stained positively for Ki67 (Fig. 1). Thus, during normal development, sympathetic cell genesis in mice continues at high levels for approximately 2 to 3 weeks after birth. Importantly, the proliferating cells in early postnatal mouse SCG were evenly distributed and no specialized proliferation zones were observed (Fig. 1A). These observations indicate that postnatal development of the sympathetic nervous system is markedly different from that of the central nervous system where continuous neurogenesis persists throughout adulthood in specialized proliferation zones, such as the subventricular zone, the olfactory bulb and the dentate gyrus of the hippocampus (Gross, 2000; Ming and Song, 2005).

Identification of proliferating cells in postnatal mouse SCG

To establish the identities of the Ki67-expressing cells in postnatal mouse SCG, we examined these cells for their expression of lineage markers. For glial markers, we chose S100 and brain lipid-binding protein (BLBP, also called brain fatty acid-binding protein, BFABP). It has been shown previously that sympathetic satellite cells express S100 (Cocchia and Michetti, 1981); BLBP is a marker for Schwann cell precursors and immature Schwann cells of the peripheral nervous system (Jessen and Mirsky, 2005). We found that all satellite cells in mouse SCG expressed high levels of BLBP, and the intensity of its expression did not change significantly from P1 to P30 (Fig. 2). This finding indicates that BLBP is an early marker for sympathetic satellite cells. By contrast, P1 SCG contained no detectable S100-expressing cells, which were first seen in P4 SCG (Fig. 3, A,B). The number of S100-expressing satellite cells increased gradually thereafter, and by P22 all satellite cells were stained positively for S100 (Fig. 3). The expression of S100 was maintained in sympathetic satellite cells throughout adulthood (data not shown). Thus, S100 appears to be a late marker for sympathetic satellite cells.

Double immunofluorescence staining and quantification revealed that between P1 and P18, a period of high levels of postnatal sympathetic cell synthesis, approximately 80% of Ki67-positive cells expressed BLBP (Fig. 2 and 4A), demonstrating that gliogenesis is a predominant event during postnatal sympathetic development. The observation also suggests that early BLBP-expressing satellite cells possess substantial proliferation potential. During the same period, there was a gradual increase in the percentage of Ki67-positive cells that expressed S100, which peaked at the level of ~27% between P14 to P18 (Fig. 3 and 4B). The timing of their appearance suggest that these Ki67 and S100 double positive cells probably represent a subpopulation of immature satellite cells undergoing final rounds of proliferation before differentiation into mature satellite cells. There were a few Ki67-positive cells in P22 and P30 SCG that stained negatively for either BLBP or S100 (Fig. 2–4), and their identity is unknown at the moment.

We also examined Ki67-positive cells for expression of TH, a marker for sympathetic noradrenergic neurons. We found that approximately 5% of Ki67-positive cells expressed TH in mouse SCG of P1, P4, and P7 (Fig. 5), indicating that sympathetic neurogenesis continues at low levels for at least one week after birth. We were not able to detect consistently Ki67 and TH double positive cells in SCG from older mice (data not shown). Together, the data from our immunostaining studies suggest that most proliferating cells in early postnatal mouse SCG are of glial or neuronal lineage.

Differentiation potential of proliferating cells in postnatal mouse SCG

To confirm that the proliferating cells are sympathetic progenitors with the capacity to differentiate into mature glial cells or neurons, we traced the fates of the cells that were proliferating during the first postnatal week. We injected 4-day-old neonatal mice with BrdU intraperitoneally once daily for 3 days to label all cycling cells; the mice were euthanized at various time points after the final BrdU injection. We then performed triple immunofluorescence staining of SCG sections for BrdU, TH, and BLBP or S100 to follow the fates of BrdU-labeled cells. Quantification of BrdU-positive cells at different time points showed a gradual decrease in their numbers, with approximately 50% of them retaining the BrdU label after 8 weeks (Fig. 6A). This result suggests that about half of the progenitors continued to proliferate extensively after BrdU labeling, while the remaining half underwent limited cell divisions and thus retained the BrdU label.

Consistent with our Ki67 studies, we observed that a small number (~5%) of BrdU-positive cells expressed high levels of TH with the morphology of mature neurons in mouse SCG

collected within a week after BrdU labeling (Fig. 6B,C), indicating the presence of neuronal progenitors and continuation of neurogenesis in early postnatal mouse sympathetic ganglia.

Staining for BLBP revealed that approximately 80–85% of BrdU-labeled cells expressed BLBP throughout the time points examined (Fig. 6D–J). Using S100 as a marker for glial differentiation, we found that an average of 15% of the BrdU-labeled cells stained positively for S100 one day after the final round of BrdU injection (Fig. 6D,K). Thereafter, there was a gradual increase in the number of BrdU and S100 double positive cells. The number peaked by the end of 3 weeks after BrdU labeling, with ~80% of BrdU-labeled cells expressing S100 (Fig. 6D,K–P). These findings indicate that most of the ganglionic cells that were proliferating during the first postnatal week are BLBP-expressing satellite cell progenitors, which eventually differentiate into S100-expressing satellite cells. The data from our fate-tracing studies also provide further evidence that the generation of satellite cells is a predominant process of postnatal sympathetic development.

Nestin expression marks both glial and neuronal progenitor cells in postnatal mouse SCG

The postnatal sympathetic glial and neuronal progenitors express the lineage markers BLBP and TH, respectively. However, neither marker is specifically associated with sympathetic progenitor cells, as BLBP expression is maintained in differentiated satellite cells and TH in mature neurons. To understand the molecular mechanisms that control the progenitor cell differentiation during postnatal sympathetic development, it is essential to identify the molecular markers whose expression marks distinct stages of the developmental process. We focused our studies on nestin, a filament protein initially identified as a marker for neural stem cells in the central nervous system (Lendahl et al., 1990). Importantly, it has been shown previously that nestin is expressed in neural crest cells from which all sympathetic neurons and glia originate (Stemple and Anderson, 1992).

Immunofluorescence staining revealed that a large number of cells in P1 and P4 SCG expressed significant levels of nestin, which often formed a thin layer of filamentous structure around nuclei (Fig. 7A,B). Nestin expression was gradually downregulated thereafter and was largely undetectable around P18 (Fig. 7C–F). This temporal pattern of nestin expression closely matches that of Ki67 (Fig. 1). Indeed, double immunofluorescence staining for Ki67 and nestin revealed that approximately 92–93% of Ki67-positive cells in P1 and P4 SCG also expressed nestin (Fig. 7G,H,I). The percentage of Ki67 and nestin double positive cells decreased with time, and by P18 only about 13% of Ki67-positive cells showed detectable levels of nestin expression (Fig. 7I). Given that most of Ki67-positive cells in P1 SCG differentiated into glia or neurons by P18 (Fig. 2–5), the Ki67⁺nestin⁺ cells in P7–P18 SCG probably represent a population of proliferating cells undergoing differentiation or immature glial cells and neurons. Taken together, our data suggest that nestin is a specific marker for sympathetic progenitor cells.

We next determined whether nestin expression is specific for glial or neuronal progenitor cells. We injected mice at P4 with BrdU and euthanized them 2 hours later to minimize the chance of BrdU-labeled cells to undergo extensive differentiation. Triple immunofluorescence staining of SCG sections was performed with antibodies against BrdU, nestin, and BLBP or TH, and the sections were examined with a confocal microscope. Consistent with the results of our Ki67 and nestin double staining experiments, approximately 95% of the BrdU-labeled cells expressed nestin (data not shown). Importantly, almost all of BrdU- and nestin-positive cells that were examined expressed either BLBP (Fig. 7J,K) or TH (Fig. 7L,M). These findings demonstrate that nestin is a marker for both glial and neuronal progenitor cells.

DISCUSSION

In this paper, we report three major findings from our *in vivo* examination of the postnatal development of mouse sympathetic ganglia, using mouse SCG as a model system. First, we show that nestin is a molecular marker for both sympathetic glial and neuronal progenitors. Second, we identify a population of nestin and BLBP double positive cells as the direct precursors of sympathetic satellite cells. Third, our study reveals a temporal expression pattern of glial lineage markers that defines the cellular intermediates in the process of satellite cell differentiation (Fig. 8). These findings define the cellular basis of postnatal sympathetic development, which should facilitate the investigation of the genes and signaling pathways that regulate the developmental process.

Nestin as a marker for both glial and neuronal progenitors in postnatal sympathetic ganglia

Nestin is an intermediate filament originally identified in neural stem cells (Lendahl et al., 1990). In the central nervous system, nestin expression marks the proliferating state of neurogenesis and is rapidly downregulated during differentiation (Dahlstrand et al., 1995). Neural crest cells isolated from rat embryonic trunk neural tubes have also been shown to express nestin (Stemple and Anderson, 1992), which give rise to both neurons and glia of the sympathetic nervous system (Le Douarin and Kalcheim, 1999). Our study reveals that during postnatal sympathetic development, nestin expression levels are at the highest at P1 and gradually decline thereafter, becoming largely undetectable by P18. This temporal expression of nestin correlates closely with the proliferating phase of postnatal sympathetic development. In addition, triple immunofluorescence staining of multiple lineage markers and BrdU-based fate-tracing experiments show that nestin is expressed in both glial-restricted and neuron-restricted progenitor cells. These observations, coupled to the previously reported nestin expression in neural crest cells (Stemple and Anderson, 1992), identify nestin as a molecular link between neural crest cells and sympathetic progenitors. Also importantly, our findings provide nestin as a molecular marker for identifying genes and signaling pathways that promote the differentiation and maturation of sympathetic neurons and satellite cells.

The identities of neuronal and glial progenitors in postnatal sympathetic ganglia

Noradrenergic neurons and satellite glial cells are the two main cell types that constitute sympathetic ganglia. The progenitors for sympathetic neurons have been extensively studied. It is generally accepted that both sympathoadrenal progenitors from the trunk neural crest and sympathoenteric progenitors from the anterior vagal neural crest contribute to the generation of sympathetic neurons (Anderson, 1993; Birren and Anderson, 1990; Carnahan and Patterson, 1991; Durbec et al., 1996; Francis and Landis, 1999). These progenitors are characterized by their nonneuronal morphology and expression of neurofilaments (NF68 and NF160) and c-Ret (Guillemot et al., 1993; Sommer et al., 1995), a protein tyrosine kinase co-receptor for glial cell-derived neurotrophic factor (Plaza-Menacho et al., 2006). It is not known whether these early neuronal progenitors are present in postnatal sympathetic ganglia. Our study reveals that in postnatal mouse SCG, approximately 5% of proliferating cells express nestin and TH. These cells are morphologically indistinguishable from the rest of post-mitotic sympathetic neurons, suggesting that they may represent a population of progenitor cells late in the neurogenic process.

In contrast, very little is known about the identity of the glial progenitors that give rise to sympathetic satellite cells. Using Ki67 to mark cells in an active proliferation state, a property of progenitor cells, we found that the vast majority of Ki67-positive cells in postnatal mouse SCG express BLBP, a nervous system-specific member of the lipid-binding

protein family that was first identified in radial glial cells and immature astrocytes of the central nervous system (Feng et al., 1994; Kurtz et al., 1994). Our BrdU fate-tracing experiments further show that the BLBP-positive proliferating cells eventually differentiate into S100-expressing satellite cells, demonstrating that they function as progenitors of sympathetic satellite cells. However, BLBP is not an exclusive marker of the glial progenitors, as mature satellite cells maintain high levels of BLBP expression. In contrast to BLBP, nestin is expressed at high levels in proliferating glial progenitors but is markedly downregulated in differentiated satellite cells. Also, very few S100-positive satellite cells express detectable levels of nestin (data not shown). Thus, nestin and BLBP expression defines a population of satellite progenitors in mouse postnatal sympathetic ganglia.

Postnatal sympathetic development

The embryonic development of mammalian sympathetic ganglia has been extensively studied, which in rat SCG is characterized by the generation of neurons which peaks around E14.5, followed by a wave of gliogenesis that begins around E16.5 (Hall and Landis, 1991; Hall and Landis, 1992; Hendry, 1977). For postnatal sympathetic development, the predominant event is the proliferation of nonneuronal cells, which, in rat SCG, lasts for at least two weeks after birth (Hendry, 1977). We confirm this observation in postnatal mouse SCG, which shows significant levels of proliferation up to 18 days after birth. We further demonstrate that, based on both morphology and expression of lineage markers, the vast majority of the proliferating cells are glial progenitors that give rise to satellite cells. Thus, the continuing generation and further differentiation of satellite cells characterize the postnatal development of mouse sympathetic ganglia.

During the postnatal gliogenic process in mouse SCG, both glial progenitors and mature satellite cells maintain high levels of BLBP expression, whereas nestin expression is downregulated and S100 expression is upregulated (Fig. 8A). Based upon lineage marker expression profiles and proliferation states, we suggest a model for the glial cell populations that may represent distinct stages of sympathetic gliogenesis (Fig. 8B). The glial progenitors are defined by the expression of both nestin and BLBP, and they possess the capacity to undergo multiple rounds of proliferation. The mature satellite cells are defined by the expression of both BLBP and S100, and they are post-mitotic cells. There is also a population of BLBP-positive glial cells with no or low levels of nestin and S100 expression, which may represent immature satellite cells with limited proliferation potential. The identification of the distinct glial populations and the developmental stages that they represent should help uncover the genes and signaling pathways that control the generation of satellite cells in the sympathetic nervous system.

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Fig. 1. Identification of proliferating cells in postnatal mouse SCG. **A–J:** Immunofluorescence staining for Ki67-positive cells (red) in SCG from P1 to P56. Sections were counterstained with DAPI (blue) to visualize nuclei. There were significant levels of proliferation in SCG during the first 18 days after birth. Scale bar = 100 μ m. **K:** Quantification of Ki67-positive cells in SCG from P1 to P30. At least 1000 cells were counted from each ganglion and 3–4 ganglia were examined for each time point. Data are presented as mean $\pm$ SD.

Fig. 2. BLBP immunofluorescence staining for lineage identification of proliferating cells in postnatal mouse SCG. Sections of SCG from P1 to P30 were stained with antibodies against Ki67 (green) and BLBP (red). All satellite cells stained positively for BLBP. The majority of Ki67-positive cells expressed BLBP (yellow), indicating that the postnatal development of sympathetic ganglia is predominantly a gliogenic process that was essentially completed by P22. Scale bar = 50 μ M.

Fig. 3. S100 immunofluorescence staining for lineage identification of proliferating cells in postnatal mouse SCG. Sections of SCG from P1 to P30 were stained with antibodies against Ki67 (green) and S100 (red). Postnatal sympathetic development in SCG is characterized by a gradual increase in the number of S100-expressing satellite cells, which peaked by P22. In SCG from P7 to P18, a subpopulation of Ki67-positive cells expressed S100 (yellow). Scale bar = 50 μ M.

Fig. 4. Quantification of Ki67-positive cells that express the glial lineage marker BLBP or S100. For each time point, 3–4 ganglia were examined, and Ki67 single positive and Ki67-BLBP (A) or Ki67-S100 (B) double positive cells were counted from at least 4 randomly selected fields (400X). Data are presented as mean $\pm$ SD.

Fig. 5.

TH immunofluorescence staining for lineage identification of proliferating cells in postnatal mouse SCG. Sections of SCG from P1 to P7 were stained with antibodies against Ki67 (green) and TH (red), and counterstained with DAPI (blue) to visualize nuclei. Arrows indicate Ki67 and TH double positive cells, which represented approximately 5% of Ki67-positive cells. The Ki67-positive neurons are morphologically indistinguishable from the rest of post-mitotic neurons. Scale bar = 25 μ M.

Fig. 6.

BrdU fate-tracing study of proliferating cells in postnatal mouse SCG. Mice at P4 were injected with BrdU once daily for 3 days and sacrificed on day (D) 1, 7, 14, 21, 28, 56 after the final round of BrdU injection. Mouse SCG sections were stained with antibodies against BrdU (green), TH (blue), and BLBP or S100 (red). **A:** Quantification of BrdU label-retaining cells in SCG from D1 to D56. For each time point, 3–4 ganglia were examined. BrdU-positive cells were counted from at least 4 randomly selected fields (400X) and the average number of BrdU-positive cells per field from D1 SCG was defined as 100%. Data are presented as mean $\pm$ SD. **B,C:** Confocal immunofluorescence images showing postnatal mouse SCG at D1 (**B**) and D7 (**C**) contained BrdU and TH double positive neurons (arrows), which account for approximately 5% of total BrdU-positive cells. Scale bar = 25 μ m. **D:** Quantification of BrdU-positive cells that express the glial lineage (Lin⁺) marker BLBP or S100 at the indicated time points. For each time point, 3–4 ganglia were examined. BrdU single positive and BrdU-BLBP or BrdU-S100 double positive cells were counted from at least 4 randomly selected fields (400X). Data are presented as mean $\pm$ SD. **E–P:** Confocal immunofluorescence images of SCG sections from mice at different time points (D1–D56) following the final round of BrdU injection. The sections were stained with antibodies to BrdU, TH, and BLBP (**E–J**) or S100 (**K–P**), showing that BrdU-labeled, BLBP single-positive cells in D1 SCG differentiated into satellite cells expressing both BLBP and S100 in a time-dependent manner. Scale bars = 50 μ m.

Fig. 7.

Nestin is a common marker for glial and neuronal progenitors in postnatal mouse SCG. **A–F**: Time-course study of nestin expression in mouse SCG from P1 to P18. Sections were stained with an antibody against nestin (green), counterstained with DAPI (blue), and examined with a confocal microscope. Nestin expression was essentially undetectable by P18. Scale bar = 50 μ m. **G,H**: Confocal immunofluorescence images of SCG sections from mice at P1 (**G**) and P4 (**H**). The sections were stained with antibodies against nestin (green) and Ki67 (red), showing nestin expression in Ki67-positive proliferating cells. Scale bar = 25 μ m. **I**: Quantification of Ki67 and nestin double positive cells in mouse SCG at the indicated time points (P1–P18). For each time point, 3 ganglia were examined. Ki67 single-positive and Ki67-nestin double positive cells were counted from 3 randomly selected fields (400X). Data are presented as mean $\pm$ SD. **J–M**: Confocal immunofluorescence images of SCG sections from mice at P4. The mice were injected with BrdU and sacrificed after 2 hours. **J,K**: Sections were stained with antibodies against BrdU (blue), nestin (green) and BLBP (red), showing nestin expression in BrdU and BLBP double positive glial progenitors

(arrows). Scale bar = 25 μm . **L,M:** Sections were stained with antibodies against BrdU (blue), nestin (green), and TH (red), showing nestin expression in BrdU and TH double positive neuronal progenitors (arrows). Scale bar = 25 μm .

Fig. 8.

A model for the generation and differentiation of satellite cells in postnatal sympathetic ganglia. **A:** A diagram for the temporal expression pattern of molecular markers (nestin, BLBP and S100) during postnatal sympathetic gliogenesis. **B:** A model for the putative glial cell populations that represent distinct stages of satellite cell differentiation (see text for detail).