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# Expression of nestin in superior cervical ganglia of rats is influenced by gender and gonadectomy

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ABSTRACT

Neurons and glia arise from neural progenitor cells that express nestin. Although substantial changes in neuronal development were observed during the postnatal period, data concerning dynamics of nestin expression in the superior cervical ganglia (SCG) of rat during that period are lacking. It is known that gonadectomy and steroid hormones influence the development of neurons in the SCG during the postnatal period, but there are no data on how they influence the persistence of nestin expression in the SCG cells.

The dynamics of nestin expression in the SCG in rats of three age groups, as well as the influence of gender and gonadectomy, was investigated.

Three groups of male rats were sacrificed at 2, 3 and 6 months of age. Additional groups of male and female Sprague-Dawley rats were gonadectomized at the age of 2 months. After 30 days, they were sacrificed and SCGs were harvested and processed immunohistochemically. Immunoreactivity for nestin in the SCG was observed in satellite glia, based on their expression of s100. The proportion of neurons that were encircled with nestin-immunoreactive satellite cells (nestin encircled neurons, NEN) decreased between second and third month of age ($p < 0.05$). The proportion of NEN was greater in the NPY+ than in the NPY− subpopulation. The proportion of NEN in the SCG of female rats was significantly higher ($p < 0.05$) than that of both, the male rats and ovariectomised groups. The percentage of these neurons was significantly higher ($p < 0.05$) in orchidectomised, in comparison to male rats.

Results show the existence of nestin-immunoreactive satellite cells in the SCG of adult rats. A substantial decrease of nestin expression in SCG cells of rats, after the onset of sexual maturation, was observed. This decrease showed significant sex-dependence and was dramatically influenced by gonadal activity.



## Introduction

The superior cervical ganglion (SCG) is a sympathetic chain ganglion that supplies postsynaptic innervations to vessels, glands and smooth muscles of the head and neck (Arbab et al., 1986; Grkovic and Anderson, 1997, 1995). Cells of sympathetic ganglia are divided into postsynaptic neurons, satellite cells and Schwann cells. They develop by migration of neural crest cells, starting from the fifth week of embryonal development in humans (Carlson, 2004; Sadler, 2012). Both, neurons and glia arise from neural progenitor cells that express nestin. Nestin is an intermediate filament of class VI, usually used as a marker for the neural crest progenitor cells (Lendahl et al., 1990; Vukojevic et al., 2009, 2010). Nestin is also recognized as a marker for sympathetic neuronal and glial progenitor cells (Shi et al., 2008).

Substantial changes take place in neurochemical specificity and size of the SCG neurons during postnatal development until 6 months of age in rats (Masliukov et al., 2012a,b). Nevertheless, a significant increase in the number of neurons in the trigeminal ganglion of rats was observed between the third and eighth month of age, due to a protracted maturation process (Lagares et al., 2007), raising reasonable presumption that a similar process could be occurring in different ganglia, including the SCG. Moreover, a significant level of proliferation in the mouse SCG until 18th postnatal day was observed, leading to both, neuro- and gliogenesis (Shi et al., 2008).

Gender and sex-steroids could be a significant cause of variations in both central and peripheral nervous systems,

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including sympathetic ganglia (Dey et al., 2013; Fuente-Martin et al., 2013; Filipovic et al., 2014a,b; Habib and Beyer, 2014; Wright and Smolen, 1983a,b, 1985, 1987). A rise in concentration of sex-steroids is the most accentuated change during the post-pubertal period, when the most extensive changes in SCGs of rats were observed (Filipovic et al., 2014a; Masliukov et al., 2012a,b).

Neurons and glial cells express oestrogen and progesterone receptors and do respond to sex-steroids (Habib and Beyer, 2014; Khalaj et al., 2013; Thi et al., 1998; Zoubina and Smith, 2003, 2002). In addition, the influence of sex hormones on neural stem cell activity was observed leading to the conclusion that this contributes to the sexually dimorphic postweaning development in the CNS (He et al., 2013; Nakafuku and Nakamura, 1995).

Although data on the influence of gonadectomy and steroid hormones on development of neurons in superior cervical ganglia during the postnatal period exist (Filipovic et al., 2014a,b; Wright, 1988; Wright and Smolen, 1983a,b, 1985, 1987), there is no study of the influence of gonadectomy on expression of nestin in SCG cells.

Despite the fact that substantial changes in SCGs were observed in rats during the postnatal development, there is no study dealing with dynamics of nestin expression in the SCG of rats during that period, that would reveal persistence and dynamics of the neural crest progenitor cells, as a possible explanation for these changes.

In the present study, we investigated expression of nestin in the SCG of rats during 6 months of age. We observed the persistence of nestin, as a marker of neural crest progenitor cells, in the SCG of rats during the postnatal period. The expression of nestin decreased substantially between the second and third month of age, after the onset of puberty. Taking into consideration that gonadal hormones have significant influence on the development of the nervous system, we hypothesized that the onset of gonadal activity during puberty could influence nestin expression persistence in SCG cells. To explore that possibility, we investigated the influence of gonadectomy on expression of nestin in SCG cells of rats.

## Materials and methods

All experimental procedures were approved by the Ministry of Agriculture (UP/I-322-01/11-01/117, 526-06-1-0255/11-1) and were performed according to the European Union Directive (2010/63/EU).

### *Animal experiments*

A total of 28, Sprague-Dawley rats were used. The animals were raised under controlled conditions (temperature 22 ± 1 °C, under 12/12 light/dark cycle). They were fed ad libitum with standard laboratory chow (4RF21 GLP, Mucedola srl, Settimo Milanese, Italy) and housed individually in plastic cages with sawdust bedding.

At the age of 8 weeks four male rats were anaesthetized with a combination of ketamine (90 mg/kg) and xylazine (10 mg/kg) and perfused transcardially with 0.9% saline followed by 300 mL of Zamboni's fixative (4% paraformaldehyde and 0.20% picric acid in 0.1 M phosphate-buffered saline (PBS) at pH 7.4). Two additional groups of rats ($n = 4$ each) were sacrificed at the age of 3 and 6 months.

To obtain data on influence of gonadectomy on expression of nestin in the SCG, an additional series of experiments was preformed. At the age of 8 weeks, animals were anaesthetized as described previously. Female rats were ovariectomised ($n = 4$), using bilateral dorsal incision or were sham operated ($n = 4$) and used as controls. Male animals ($n = 4$), were orchidectomised through a bilateral incision of the scrotum, or were sham operated ($n = 4$), as described previously (Filipovic et al., 2014a).

Four weeks later, rats were anaesthetized and perfused transcardially, as described previously. Sham operated female animals were sacrificed in proestrus, according to the cytology of the vaginal swab (Gettayacamin et al., 1999). Uteri of the sacrificed animals were weighed in order to confirm success of the ovariectomy.

### *Immunohistochemical procedures*

The superior cervical ganglia were removed and postfixed overnight in Zamboni's fixative.

After washing in PBS, tissues were embedded in paraffin, and sectioned at 5 μm. Sections were placed on glass slides (Histobond + Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). After deparafinisation, immunohistochemical staining was performed.

The following primary antibodies were used: monoclonal mouse anti-rat-nestin (56309, BD Pharmingen, San Jose, CA, USA), at 1:200 dilution; rabbit polyclonal anti-rat-nestin (ab93157, Abcam, Cambridge, UK), 1:200; mouse monoclonal anti s100 antibody (ab4066, Abcam, Cambridge, UK), 1:200 and polyclonal sheep anti-NPY antibody (ab6173, Abcam, Cambridge, UK), 1:1000. For the purpose of counter-staining neurons we used staining with tyrosine hydroxylase (Fig. 3; ab113, Abcam, Cambridge, UK) or rabbit polyclonal PGP 9.5 antibody (Fig. 6; 318A-16, Cell Marque, Rocklin, CA, USA).

Detection was performed using Rhodamine Red TM-conjugated donkey anti-mouse (715-295-151), Rhodamine Red TM-conjugated donkey anti-rabbit (711-295-152), both diluted at 1:300; FITC-conjugated donkey anti-sheep (713-095-147), and FITC-conjugated donkey anti-mouse (715-095-150) secondary antibodies diluted at 1:200 (all Jackson ImmunoResearch Laboratories, Inc., Baltimore, PA, USA). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Slides were rinsed in PBS, mounted, air-dried, and cover slipped (Immu-Mount, Shandon, Pittsburgh, PA, USA). Staining controls included omission of primary antibody from the staining procedure, which resulted in no staining in tissue.

### *Antibodies specificity*

Mouse anti-rat-nestin antibody (56309) recognizes rat nestin (does not cross-react with human nestin). In western blot (WB) analysis, it recognizes nestin as a doublet with an apparent molecular weight of ~200 kDa and recognizes a band of ~198 kDa in WB of lysate from PC-12 cells (manufacturers' data). Rabbit anti-rat-nestin antibody (ab93157) recognizes synthetic peptide within residues 1550–1650 of rat nestin (manufacturers' data). It was used as a marker of progenitor cells during cardiac infarct healing in our previous studies on rats (Agnić et al., 2014a,b). Anti-S100 antibody [4C4.9] (ab4066) is specific for beta subunit of S-100 protein. It recognizes 15,18,21 kDa bands in WB of homogenates of rat and mouse spinal cord and rat brain staining astroglia in sections and culture of the rat brain and spinal cord (manufacturers' data). It was used as a marker of satellite glia in our previous study on the SCG (Filipovic et al., 2014b). Anti-NPY antibody (ab6173) recognizes Neuropeptide Y in the rat brain tissue sections. Specificity of antibody was tested by radioimmuoassay (manufacturers' data). It was used as a marker of vasomotor neurons in our previous studies on the SCG (Filipovic et al., 2014a,b). Anti-Tyrosine Hydroxylase antibody (ab113) reacts with mouse and rat tyrosine hydroxylase. In WB analysis it shows specific immunolabeling of the ~60k tyrosine hydroxylase protein (manufacturers' data). Positive control for TH included staining of rat adrenal medulla (data not shown). Rabbit polyclonal PGP 9.5 antibody recognizes Protein gene product 9.5 (PGP 9.5), a general neuronal marker (manufacturers' data). Positive control for PGP 9.5 included staining of the rat spinal cord and neuronal fibers in the rat skin (data not shown).

### *Imaging and analysis of microphotographs*

A microscope (BX61, Olympus, Tokyo, Japan) with a cooled digital camera (DP71, Olympus, Tokyo, Japan) was used to obtain images (under 40x objective) for further analyses. Details of characteristics of filter sets used are: FITC Ex 460–490 nm, Em 510 nm (U-MWIB2); rhodamine Ex 530–550 nm, Em 590 nm (U-MNG2); DAPI Ex 360–370 nm, Em 420 nm (U-MNU2). Image J software (National Institutes of Health, Bethesda, MD, USA) was used to perform counting of neurons that were encircled with ring of nestin-immunoreactive satellite glia (NEN). Counting was made on a whole section area for each ganglion. Only neurons with visible nuclei and those that had at least 50% of their perimeter encircled with nestin immunolabeling were taken into consideration as positive (Figs. 1, 3 and 6). Percentage of encircled neurons was calculated from the number of total neurons present on a section. Data were shown as mean of percentage of NEN (± standard deviation) for each group of animals ($n = 4$).

### *Statistical analysis*

Statistical software Statistica 2010 (Statsoft, CA, USA) was used for descriptive statistics and Chi-square test. $p < 0.05$ was considered as statistically significant.

## Results

### *Nestin is present in the SCG of adult rats*

The presence of nestin in the SCG of adult rats was examined by immunofluorecence. Results of our study show a presence of nestin immunoreactivity in the SCG of two, three and 6-month-old animals. Immunoreactivity for nestin in the SCG was observed in satellite glia, detected by colocalisation with s100, a marker for glial cells (Fig. 1). Immunoreactivity was present in the cytoplasm