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Expression of PTHrP and PTH/PTHrP receptor 1 in the superior cervical ganglia of rats

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ABSTRACT

PTHrP and its receptor PTHR1 are found in the CNS and peripheral nervous system. The presence of PTHrP mRNA has been detected in the superior cervical ganglion (SCG), but there are no data on the cellular distribution of PTHrP and PTHR1 in the SCG. Although it is known that ovarian activity and reproductive status influence sympathetic activity, and the PTHrP/PTHR1 system is influenced by estrogens in different tissues, it is not known whether these factors have a similar effect on expression of PTHrP and PTHR1 in the nervous system. Hence, we investigated the presence and distribution of PTHrP and PTHR1 in neurons and glia of the SCG of rats, as well as the influence of ovariectomy on their expression, by using immunohistochemistry. PTHrP and PTHR1 immunoreactivity was observed in cytoplasm as well as in nuclei of almost all neurons in the SCG. In male rats, intensity of PTHrP fluorescence was significantly higher in cytoplasm of NPY–, in comparison to NPY+ neurons ($p < 0.05$). In female rats, 2 months post-ovariectomy, significantly lower intensity of PTHrP fluorescence in cytoplasm of the SCG neurons was observed in comparison to sham operated animals ($p < 0.05$). In addition to neurons, PTHrP and PTHR1 immunoreactivity was observed in most of the glia and was not influenced by ovariectomy. Results show the expression of PTHrP and its receptor, PTHR1, in the majority of neurons and glial cells in the SCG of rats. Expression of PTHrP, but not PTHR1 in the cytoplasm of SCG neurons is influenced by ovarian activity.

1. Introduction

PTHrP is a peptide identical to parathyroid hormone (PTH) in its first 13 amino-acid sequence (Thiede, 1994). Its presence was found in a broad spectrum of normal tissues, including the central nervous system (Weir et al., 1990; Wysolmerski, 2012). PTHrP has different functions, acting normally as an autocrine/paracrine factor, with exception of pregnancy and lactation, when it acts also as a humoral factor (Wysolmerski, 2012). Moreover, in cells and body fluids, PTHrP is normally subjected to posttranslational changes and proteolytic cleavage resulting in occurrence of different peptides with distinctive roles (Orloff et al., 1994). Its amino-terminal peptide (1–37) acts through PTHrP/PTHR1, a G-protein coupled receptor, which is also a common receptor to PTH (Abou-Samra et al., 1992; Wysolmerski, 2012). PTHR1 acts through different signaling pathways and induces the entry of extracellular calcium by regulation of calcium channels (Swarthout et al., 2002).

The presence of PTHrP and PTHR1 in glia of sensory – dorsal root ganglia (DRG) – was observed previously in proliferating Schwann cells (Macica et al., 2006) and their role in nerve regeneration was postulated. Also, a presence of PTHrP and PTHR1 was detected in various parts of the CNS (Swarthout et al., 2002; Weaver et al., 1995; Weir et al., 1990; Wysolmerski, 2012) including cerebral cortex, hippocampus, cerebellar cortex and hypothalamus (Holt et al., 1996; Weir et al., 1990; Yamamoto et al., 2002). Until now, the expression of PTHrP was not investigated in sympathetic neurons, although the presence of PTHrP mRNA in the superior cervical ganglion (SCG) of rats was proved previously by using DNA microarray technology and RT-PCR, along with up-regulation of PTHrP mRNA following axotomy (Boeshore et al., 2004). Nevertheless, the above study did not specify whether PTHrP was expressed in neurons or glia of the SCG, and if expressed in neurons, in which neuronal subpopulations.

It is known that the activity of the sympathetic nervous system varies with the ovarian cycle and pregnancy (Anglin and Brooks, 2003; Greenwood et al., 2001; Kaur et al., 2007; Minson et al., 2000). Ovarian hormones influence the expression of neuropeptides in central, as well as in tissues of the peripheral nervous system (Puri et al., 2005, 2006; Roman et al., 2006; Williams et al., 2011).

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Sympathetic neurons widely express estrogen receptors and are sensitive to the influence of estrogens (Zoubina and Smith, 2002). Influence of ovarian hormones on the level of sympathetic activity is thought to be related to the lower incidence of cardiovascular disease in young women, in comparison to men and postmenopausal women (Birkhaeuser, 2005; Burt et al., 1995). The SCG is a paravertebral chain ganglion that supplies sympathetic innervations to structures of the head and neck (Arbab et al., 1986; Grkovic and Anderson, 1995, 1997). We have recently demonstrated that gender and gonadectomy have a significant influence on density, size of neurons and distribution of populations of neurons in SCG of sexually mature rats (Filipovic et al., 2014). Furthermore, it is known that expression of PTHrP and PTHR1 can be influenced by ovariectomy and estrogens in different tissues (Cros et al., 1998; Paspaliaris et al., 1995) but there are no data linking their expression in neurons and glia with gonadal activity. Also there is some evidence that the PTHrP/PTHR1 system could regulate release of catecholamine from PC12 cells (a cell line derived from a pheochromocytoma of the rat adrenal medulla) (Brines and Broadus, 1999), leaving the possibility for a similar role in sympathetic neurons and potential regulation of overall sympathetic activity.

Since there are no data on the cellular distribution of PTHrP and PTHR1 in the SCG, we investigated their presence and patterns of distribution in neurons and glia of the SCG of rats. In addition, we wanted to find out whether the expression of PTHrP and PTHR1 in neurons of the SCG is linked to a distinct neuronal sub-population, related to the presence/absence of NPY. There is no study on the influence of ovariectomy and/or ovarian hormones on expression of PTHrP and its receptor in neurons of the SCG. Hence, we seek to find out if ovariectomy (and consequent lack of ovarian hormones) influences their expression in neurons of the SCG of rats.

2. Material and methods

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 Communities Council Directive of 24 November 1986 (86/609/EEC). A total of 4 male and 10 female Sprague–Dawley rats were used. The animals were raised under controlled conditions (temperature 22 ± 1 °C, under 12/12 light/dark cycle). They were housed individually in plastic cages with sawdust bedding and fed *ad libitum* with standard laboratory chow (4RF21 GLP, Mucedola srl, Settimo Milanese, Italy).

At the age of 12 weeks male rats were anesthetized 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).

At the age of 8 weeks, female animals were anesthetized as described previously and were ovariectomized through a bilateral dorsal incision, or sham operated ($N = 5$ for each group).

Eight weeks later, rats were again anesthetized and perfused. Sham operated female animals were sacrificed in proestrus, according to the cytology of the vaginal swab as described previously (Filipovic et al., 2014). The superior cervical ganglia were harvested and postfixed in Zamboni's fixative.

Tissues were washed in PBS, embedded in paraffin and sectioned in 5 μm thick sections. Sections were placed on glass slides. After deparafinization, immunohistochemical and immunofluorescence staining was performed.

The following primary antibodies were used: polyclonal rabbit anti-PTHLP (Ab-2)(34–53) antibody (PC09, Calbiochem, Merck KGa, Darmstadt, Germany; at dilution of 1:100); a polyclonal rabbit anti-PTH-PTHrP-R antibody (sc-20749, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, UK; 1:100); a monoclonal mouse [4C4.9] anti-S100 Astrocyte Marker antibody (ab4066, Abcam, Cambridge, UK; 1:200); and a polyclonal sheep anti-Neuropeptide Y (ab6173, Abcam, Cambridge, UK; at dilution of 1:1000). Secondary antibodies that were used were: Rhodamine Red TM-conjugated donkey anti-rabbit (1:300) and FITC-conjugated donkey anti-sheep and donkey anti-mouse secondary antibodies (both 1:200) (711-295-152; 713-095-147 and 715-095-150, respectively, all Jackson ImmunoResearch Laboratories, Inc., Baltimore, PA, USA). The sections were washed in PBS and nuclei were stained with DAPI. After rinsing in PBS, all slides were air-dried and cover slipped (Immu-Mount, Shandon, Pittsburgh, PA, USA). Alternatively, after incubation with primary PTHrP- and PTHR1-antibodies and washing, sections were incubated with secondary goat-anti-rabbit-biotinylated IgG (sc-2040; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, UK; 1:100). Detection was performed by using streptavidine–peroxidase system (K0690, Dakocytomation, Carpinteria, CA, USA) and diaminobenzidine (DAB) reaction. Nuclei were counterstained with hematoxyline. Staining controls in both cases – immunohistochemistry and immunofluorescence – included omission of primary antibody from the staining procedure, which resulted in no staining in tissue. Positive control included staining of paraffin sections of rat tissues: skin and liver (PTHrP and PTHR1), adrenal medulla (PTHrP), cardiac muscle (PTHR1) and dorsal root ganglia (PTHrP and PTHR1; please see a supplementary material).

A microscope (BX61, Olympus, Tokyo, Japan) with a cooled digital camera (DP71, Olympus, Tokyo, Japan) was used to obtain images (under 40× objective). Images were analyzed by using Image J software (National Institutes of Health, Bethesda, MD, USA). Only neurons with visible nuclei were taken into consideration for analysis. The intensity of fluorescence in cytoplasm of neurons was measured on two microphotographs of visual fields obtained by using a 40× objective, by manual outlining of somata using freehand selection function, as described previously (Filipovic et al., 2013). Number of PTHrP and PTHR1 immunoreactive and non-immunoreactive glial cells was determined on 10 square fields size 100 × 100 μm.

Statistical software GraphPad Instat 3 (McIntosh, CA, USA) was used for Student's *t*-test, after the Kolmogorov–Smirnov test of normality. Chi-square test was used for proportions of immunoreactive glia. Statistical significance was set at $p < 0.05$.

3. Results

The results of the present study are shown in Figs. 1–5. PTHrP immunoreactivity was observed in cytoplasm as well as in nuclei of almost all neurons in the SCG. In male rats, intensity of PTHrP fluorescence was significantly higher in cytoplasm of NPY negative somata, in comparison to NPY+ neurons ($p < 0.05$; Fig. 1). Similarly, immunoreactivity for PTHR1 was also observed in all neurons of the SCG, in cytoplasm, as well as in nuclei, but there was no difference in intensity of PTHR1 fluorescence between NPY– and NPY+ populations of SCG neurons in male rats ($p > 0.05$; Fig. 2).

In female rats, 2 months post-ovariectomy, significantly lower intensity of PTHrP fluorescence in cytoplasm of both, NPY–, as well as the NPY+ neurons of SCG, was observed in comparison to sham operated animals ($p < 0.05$; Fig. 3). In contrast, we did not observe a difference in intensity of PTHR1 in cytoplasm of NPY– or NPY+ SCG neurons 2 months post ovariectomy ($p > 0.05$; Fig. 4). A significant difference in the intensity of PTHrP or PTHR1 fluorescence between the two populations of NPY– and NPY+ neurons of the SCG was not observed neither in ovariectomized nor in sham operated female animals.

In addition to neurons, PTHrP immunoreactivity was observed in glia (Fig. 5). 60.47 ± 12.11% of glial cells were PTHrP immunoreactive in male rats, 87.57 ± 2.54% of glial cells were PTHrP immunoreactive in female sham operated rats and 87.86 ± 3.32% were PTHrP+ in ovariectomized animals.