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# Development of neuropeptide Y-mediated heart innervation in rats

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ABSTRACT

Neuropeptide Y (NPY) plays a trophic role in the nervous and vascular systems and in cardiac hypertrophy. However, there is no report concerning the expression of NPY and its receptors in the heart during postnatal development. In the current study, immunohistochemistry and Western blot analysis was used to label NPY, and Y1R, Y2R, and Y5R receptors in the heart tissue and intramural cardiac ganglia from rats of different ages (newborn, 10 days old, 20 days old, 30 days old, 60 days old, 1 year old, and 2 years old).
The obtained data suggest age-dependent changes of NPY-mediated heart innervation. The density of NPY-immunoreactive (IR) fibers was the least in newborn animals and increased in the first 20 days of life. In the atria of newborn and 10-day-old rats, NPY-IR fibers were more abundant compared with the ventricles. The vast majority of NPY-IR fibers also contained tyrosine hydroxylase, a key enzyme in catecholamine synthesis.
The expression of Y1R increased between 10 and 20 days of life. Faint Y2R immunoreactivity was observed in the atria and ventricles of 20-day-old and older rats. In contrast, the highest level of the expression of Y5R was found in newborn pups comparing with more adult rats. All intramural ganglionic neurons were also Y1R-IR and Y5R-IR and Y2R-negative in all studied animals.
Thus, the increasing of density of NPY-containing nerve fibers accompanies changes in relation of different subtypes of NPY receptors in the heart during development.



## 1. Introduction

Neuropeptide Y (NPY) is a 36-amino acid peptide, including five tyrosine residues on each molecule and a C terminal amide structure (Tatemoto 1982; Tatemoto et al. 1982). NPY is widely distributed in both the central and peripheral nervous systems and has been functionally related to regulation of blood pressure, circadian rhythms, feeding behavior, anxiety, memory processing, and cognition in the CNS and to vasoconstriction and gastrointestinal tract motility in the PNS (Hodges et al. 2009; Nozdrachev and Masliukov 2011; Kageyama et al. 2012; Masliukov and Nozdrachev 2013).

The various biological effects of NPY and its homologs are mediated by the activation of at least five receptors, known as the Y1R, Y2R, Y4R, Y5R, and Y6R. Among the six NPY receptor subtypes, Y3R subtype has not been cloned, Y4R subtype reacts with pancreatic polypeptide (PP), and Y6R is a nonfunctional receptor in rat and human (Balasubramaniam 1997). Therefore, it seems that Y1R, Y2R, and Y5R are the three major subtypes of NPY receptors that mediate the biological functions of NPY in human and rat. All known NPY receptors belong to the large superfamily of G-protein-coupled, heptahelical receptors (Walther et al. 2011). Actions of NPY on peripheral target-organs are predominantly realized through postsynaptic Y1R, Y5R, and presynaptic Y2R (Balasubramaniam 1997; Michel et al. 1998; Hu et al., 2014).

In the heart, the most prominent source of NPY is postganglionic sympathetic fibers, the majority originating from neurons located in the stellate ganglion (Richardson et al. 2006; Masliukov et al. 2012). In rodents, NPY is also expressed by the parasympathetic neurons of the intrinsic cardiac ganglia (Richardson et al. 2003). Sensory neurons do not produce NPY under physiological conditions (Wakisaka et al. 1991; Valder et al. 2003; Chottová Dvoráková et al. 2008).

NPY can both decrease and increase the contractile response of electrically stimulated rat ventricular cardiomyocytes (Piper et al. 1989; Millar et al. 1991; Allen et al. 2006). The two opposing inotropic effects of NPY in adult rat cardiac myocytes are mediated by different NPY receptor subtypes: positive effects by Y1R and negative effects by Y2R (Protas et al. 2003).

The negative effect, observed in isoproterenol-treated cells, is due primarily to stimulation of the transient outward current ($I_{to}$) and mediated through an inhibitory G protein/adenylate cyclase pathway (Kassis et al. 1987; Piper et al. 1989; Millar et al. 1991). NPY also activates the slow inward L-type Ca2+ current ($Ica_L$) and therefore

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increases the contraction of the cell. NPY may affect heart rate by decreasing $I_f$ (Protas et al. 2003).

NPY may play an important developmental role by promoting growth and/or differentiation of a variety of cells in a receptor-specific manner. Via its Y1R, Y2R, and Y5R, NPY promotes angiogenesis and preadipocyte differentiation (Kuo et al. 2007; Parker and Balasubramaniam 2008; Zhang et al. 2015). NPY has been shown to be a potent, multifunctional angiogenic factor, which stimulates proliferation, migration, and capillary tube formation in endothelial cells. NPY is angiogenic at concentrations below those required for vasoconstriction (Zukowska-Grojec et al. 1998).

NPY is also associated with cardiac hypertrophy. NPY produces hypertrophy by increasing protein synthesis. The effect is age dependent, with a maximum (20% increase of de novo protein synthesis) observed in cells from 16-week-old animals. Experiments using selective NPY agonists and antagonists suggest that the hypertrophic effect of NPY in cardiac myocytes isolated from spontaneously hypertensive rats is mediated by Y5R (Pellieux et al. 2000).

The expression of the spectrum of neurotransmitters in autonomic ganglionic neurons is subject to changes during pre- and postnatal development (Apostolova and Dechant, 2009, Young et al. 2011; Masliukov et al. 2012, 2015). Sympathetic NPY-immunoreactive (IR) nerve fibers show the earliest expression by 16 days of rat gestation in myocardium (Shoba and Tay 2000). However, NPY-IR nerve fiber densities are low at birth. Densities increase during first 2 weeks of postnatal life and then remain relatively stable until adulthood. Later, developing nerve fibers in the atria of young rats express abundant NPY and this fact would suggest that the peptide may play a trophic role in the development of specialized cardiomyocytes (Nyquist-Battie et al. 1994).

The purpose of this study was to investigate the developmental changes of NPY-IR structures (nerve fibers and intramural ganglionic neurons), Y1R, Y2R, and Y5R in the heart of rats of different ages from newborn through senescence using immunohistochemistry and Western blot analysis.

## 2. Materials and methods

### 2.1. Experimental animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Yaroslavl State Medical University and were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 85-23, revised 1996) as well as the relevant Guidelines of the Russian Ministry of Health for scientific experimentation on animals.

Newborn, 10-day-old, 20-day-old, 30-day-old, 2-month-old, 1-year-old, and 2-year-old Wistar rats ($2 \times 7$ groups each containing 5 animals) were used in this work to label NPY and Y1R, Y2R, Y5R in the heart tissue by immunohistochemistry and Western blot analysis.

All animals were kept in acrylic cages with wood shavings in an acclimatized room (12/12 h light/dark cycle; $22 \pm 3$ °C) and were provided with water and pellets ad libitum.

### 2.2. Tissue preparation

All animals were killed with a lethal dose of sodium pentobarbital (Nembutal®, 300 mg/kg, i.p.), after which they were perfused transcardially with 20 ml (newborn and 10 days old), 100 ml (20 and 30 days old), or 500 ml (2 months old and older) of physiological saline and 1 μl heparin followed by a similar volume of fixative composed of 4% paraformaldehyde (PF) in 0.1 M phosphate buffer. After perfusion, the right and left ventricles and the atria were dissected out, rinsed in physiological solution, and immersed in 4% PF for 1–2 h at room temperature. Following fixation, they were washed in three 30-min changes of phosphate-buffered saline (PBS; 0.01 M; pH 7.4), cryoprotected by overnight immersion in 20% buffered (pH 7.4) sucrose solution at 4 °C, and mounted in TissueTek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) on a cryostat chuck and frozen. Twelve-micrometer-thick cross sections were cut with a cryostat, mounted on poly-L-lysine-coated slides, and air-dried for 1 h.

### 2.3. Western blot analysis

Samples from heart tissues were homogenized with a lysis buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, protease inhibitor cocktail (Sigma)). The concentration of the total proteins was determined in all lysed tissues using the Bradford reagent (Fermentas, USA). Each tissue lysate was diluted in sample buffer (Bio-Rad Laboratories Inc., USA) and denatured at 95 °C for 5 min. An equivalent amount of samples were loaded and separated by 10% polyacrylamide gel electrophoresis and transferred to PVDF transfer membranes (AppliChem, Germany). Membranes were blocked with a blocking solution containing 3% non-fat dry milk (AppliChem, Germany) in TBS-T (0.1% Tween 20, 0.2 mM Tris, 137 mM NaCl) for 30 min at room temperature. After washing with TBS-T, membranes were incubated with primary antibodies (rabbit anti-Y1R, 1:1000, Abcam, ab73897, UK; rabbit anti-Y2R, 1:500, Santa Cruz, H-147, USA; rabbit anti-Y5R, 1:10,000, Abcam, ab 133,757, UK; rabbit anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), 1:2500, Abcam, ab9485, UK) diluted in same the blocking solution at 4 °C overnight. Following washing with TBS-T, membranes were incubated with secondary antibodies (goat HRP-conjugated anti-rabbit IgG, Abcam, ab6721) at 1:3000. The immunoblots were detected by chemiluminescence (ECL Prime Western blotting detection reagent, BioRad) with a Syngene G:BOX Chemi XR5E imaging system (Syngene, UK). Chemiluminescent signals were quantified with Gene Tools Gel Analysis software (Syngene, UK), and expressed relative to GAPDH. Protein molecular weight markers were included in each Western blot analysis.

### 2.4. Immunohistochemistry

Serial sections of the heart tissue were processed for the immunohistochemistry. The sections were preincubated for 30 min at room temperature with the blocking buffer containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories, USA) and 0.3% Triton X-100 (Sigma, USA) in PBS to prevent non-specific binding of secondary antibodies. In order to visualize NPY, Y1R, Y2R, Y5R, tyrosine hydroxylase (TH), choline acetyltransferase (ChAT), and single or double immunostaining with antibodies (raised in different host species; see Table 1a)

**Table 1**

a) Primary antisera used for immunohistochemistry

| Primary antisera | Host species | Dilution | Source |
|---|---|---|---|
| NPY | Sheep | 1:500 | Abcam, ab6173 |
| NPY | Rabbit | 1:500 | Abcam, ab43871 |
| PGP9.5 | Rabbit | 1:200 | Abcam, ab10404 |
| TH | Sheep | 1:1000 | Abcam, ab113 |
| ChAT | Goat | 1:50 | Millipore, AB144P |
| Y1R | Rabbit | 1:500 | Abcam, ab73897 |
| Y2R | Rabbit | 1:500 | Santa Cruz, H-147 |
| Y5R | Rabbit | 1:500 | Abcam, ab133757 |

b) Secondary antisera used for immunohistochemistry

| Secondary antisera | Dilution | Source |
|---|---|---|
| Donkey anti-goat IgG FITC | 1:200 | Jackson Immunoresearch |
| Donkey anti-rabbit IgG FITC | 1:200 | Jackson Immunoresearch |
| Donkey anti-rabbit IgG CY3 | 1:200 | Jackson Immunoresearch |
| Donkey anti- sheep IgG FITC | 1:200 | Jackson Immunoresearch |
| Donkey anti-sheep IgG CY3 | 1:200 | Jackson Immunoresearch |

CY3—cyanine 3, FITC—fluorescein isothiocyanate.



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was performed. Antibodies to PGP9.5 were used in to label the neuronal cell bodies in the intramural ganglia.

Subsequently, the sections were incubated in the primary antisera for 24 h at room temperature, rinsed in PBS and further incubated in the corresponding secondary antisera for 2 h at room temperature (see Table 1b). The sections were then rinsed three final times in PBS, mounted on glass slides, allowed to dry overnight and coverslipped using VectaShield (Vector Bioproducts, USA). The control experiments were carried out with the primary antibody replaced with NDS.

### 2.5. Image processing and statistics

The specimens were examined using an Olympus BX43 fluorescence microscope (Tokyo, Japan) fitted with filter sets that allowed separate visualization of FITC or CY3. Images from the fluorescence microscope were recorded using a TCH 5.0 cooled CCD digital camera and ISCapture version 3.6 for Windows imaging software (Tucsen, China). Each image was processed using a sharpen filter and contrast and brightness adjustment only. All photomicrographic plates were made using Adobe Photoshop 6.0 software (Adobe Systems, USA).

To determine the percentage of NPY-IR profiles, we counted the total number of PGP-IR neurons in the measured area and considered them as 100%. NPY-IR neuronal profiles were counted in randomly selected measured areas (1 microscopic field was 0.12 $mm^2$) at 200-fold magnification. To avoid duplicate counts of neurons in the serial sections of the ganglia, only those nerve cell bodies containing a clearly identified nucleus were counted in any given section. Data from 10 measured areas per ganglion per age-group, per animal, were included in this study. Data from individual ganglia in each age-group were meaned yielding group sizes of $n = 5$.

The relative numbers of NPY-IR fibers (minimum length 50 mm) were determined in randomly selected measured areas as was described for NPY-IR neuronal profiles.

Statistical methods include calculation of the mean and standard error of the mean. Differences in means were subjected to one-way ANOVA, followed by Tukey's post-test of multiple comparisons. Differences were considered statistically significant if $p < 0.05$.

## 3. Results

Immunohistochemistry and Western blot analysis was used to label NPY, Y1R, Y2R, and Y5R in the heart tissue and intramural cardiac ganglia from rats of different ages. There were no significant differences in the expression of NPY or its receptors between the left and right atria or left and right ventricles at particular time points. Therefore, the remaining results represent pooled data for both the left and right heart.

### 3.1. Immunohistochemical studies of NPY and its receptors

#### 3.1.1. Development of NPY-IR nerve fibers in the heart

Immunohistochemistry was performed to investigate the pattern of NPY expression in the heart during rat postnatal development. NPY immunoreactivity was observed in the atria and ventricles of all studied animals from birth onwards (Fig. 1). Nerve bundles and individual varicose nerve fibers were NPY-IR. Nerve fiber plexuses, immunoreactive for NPY, were present around blood vessels, and individual fibers were present between cardiomyocytes. The density of NPY-positive fibers was minimal in newborn animals and increased in the first 20 days of life (Table 2). In the atria of newborn and 10-day-old rats, NPY-IR fibers were more abundant compared with the ventricles ($p < 0.05$).

In newborns, only 62% of NPY-IR fibers colocalized TH. In 10-day-old and older rats, all (100%) NPY-IR fibers were also TH-IR. Some of the NPY-IR fibers also contained ChAT in the atria but not in the ventricles of young and old rats.

#### 3.1.2. Development of receptors to NPY in the heart tissue

Immunohistochemistry was performed in order to show the expression and distribution of Y1R, Y2R, and Y5R in the heart. These receptor types were found in the atria and ventricles of newborn and more adult rats.

Y1R immunoreactivity was most distinct on the membrane of cardiomyocytes, with additional granular intracellular labeling. Density of Y1R significantly increased between 10 and 20 days of life (Fig. 2). Faint Y2R immunoreactivity was only observed in the atria and

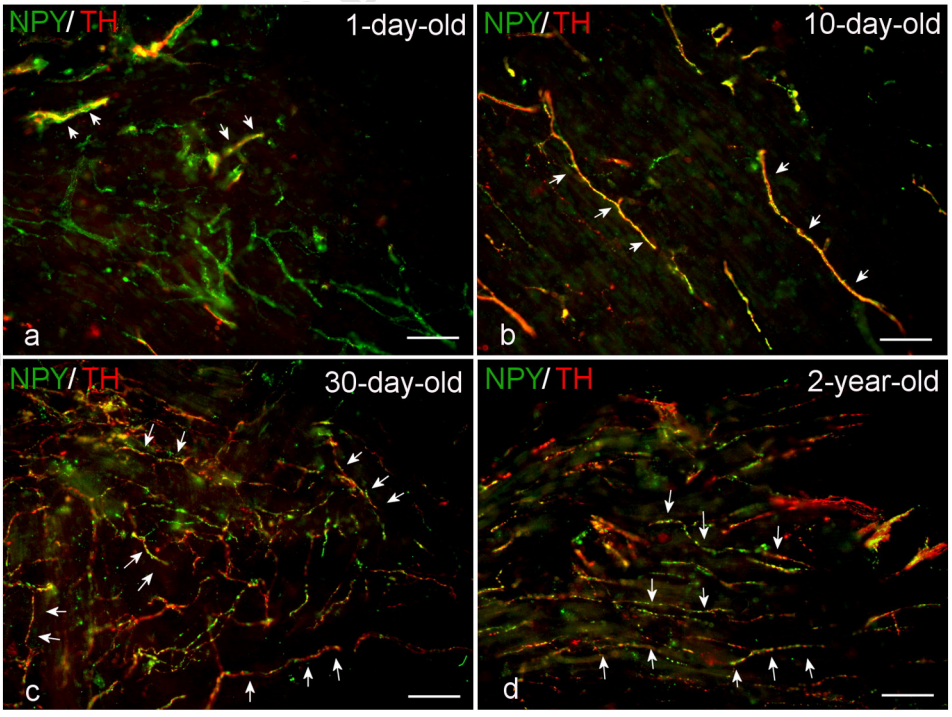


**Fig. 1.** Fluorescence micrographs of NPY (green) and TH (red) immunoreactivity in the left atria of newborn (a), 10-day-old (b), 30-day-old (c), and 2-year-old (d) rats. NPY-IR fibers colocalizing TH are indicated by arrows. Bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
Density of NPY-IR fibers in the rat heart during the development (per mm$^2$, $n = 5$ in each age-group).

| Age | Atria | Ventricles |
|---|---|---|
| Newborn | 78 ± 12,4** | 34 ± 10,2 |
| 10 days old | 368 ± 36,8** | 252 ± 26,4 |
| 20 days old | 526 ± 46,2* | 470 ± 41,2* |
| 1 month old | 502 ± 35,6* | 542 ± 44,6* |
| 2 months old | 494 ± 38,2* | 509 ± 49,4* |
| 1 year old | 518 ± 29,0* | 521 ± 26,4* |
| 2 years old | 487 ± 53,6* | 514 ± 62,4* |

* $p < 0.01$, compared with newborn and 10-day-old rats.
** $p < 0.05$, compared with ventricles.

ventricles of rats after 20 days of life (Fig. 3). Granules of Y2R were located in cardiomyocytes. The density of Y2R-IR granules increased in 30-day-old rats compared with 20-day-old rats and did not change during further development. In contrast, the highest level of the expression of Y5R was found in newborn pups compared with more adult rats (Fig. 4). In newborns, receptors were observed in the coronary vessels, cardiomyocytes and NPY-IR nerve fibers. In 10-day-old and older rats, Y5R were found mainly in NPY-IR nerves.

*3.1.3. Immunohistochemistry of the cardiac intramural ganglia during postnatal development*

NPY-IR neurons were observed in the cardiac intramural ganglia from birth onwards (Fig. 5). In these ganglia, the percentage of NPY-IR profiles significantly increased from the moment of birth (66 ± 5.3%) until 10 days of life, where the number of profiles became 98 ± 0.3% ($p < 0.01$) and did not change during further development. In 10-day-old and older rats, all NPY-IR intramural ganglionic neurons were also ChAT-IR.

In newborn rats, the vast majority (87 ± 4.6%) of NPY-IR neurons were also TH-IR. Further, the expression of TH was downregulated, and in 10-day-old rats, we did not observed TH-IR neurons in the intramural ganglia.

All neurons in the intramural ganglia also had Y1R and Y5R immunoreactivity. No Y2R-IR profiles were observed in the ganglia during the development.

*3.2. Expression of NPY and receptors to NPY by Western blot analysis*

In order to further measure the protein expressions of three NPY receptor subtypes, total protein extracted from atria and ventricles were subjected to Western blot analysis. Fig. 6 showed the data of specific antibodies probed to NPY, Y1R, Y2R, and Y5R, in which the targeted bands were 11, 43, 42, and 52 kDa, respectively, using immunoblot.

NPY was expressed in newborn and 10-day-old rats at a low level (Fig. 7). Its expression significantly increased between 10 and 20 days ($p < 0.05$). The level of NPY expression in the atria was larger compared with ventricles in newborn and 10-day-old rats ($p < 0.05$).

The expression of Y1R, Y2R, and Y5R did not differ in atria compared with ventricles in all studied age-groups ($p > 0.05$). The expression of Y1R was low in newborn and 10-day-old rats and was upregulated in next 10 days of life ($p < 0.05$). Further, we did not find statistically significant differences between 20-day-old and older rats ($p > 0.05$).

The expression of Y2R was not observed in newborn and 10-day-old animals and appeared only in 20-day-old rats. The expression of Y2R increased in 30-day-old rats compared with 20-day-old ($p < 0.05$) and did not change later during development and senescence.

The expression of Y5R was maximal in newborn pups and significantly decreased in the first 10 days ($p < 0.05$). No statistically significant differences were found between 10-day-old and older age-groups ($p > 0.05$).

Among all types of receptors, Y5R had the highest level of expression in newborns. In 20-day-old and older rats, the expression of Y1R prevailed. The ratio of Y1R to Y5R expression was 0.8 in newborns and raised to 3.0–3.1 in 20-day-old and older rats. Y1R/Y2R ratio was 3.0 in 20-day-old rats and 2.0 in animals after 30 days of life. The relation of Y2R/Y5R was 1.4–1.5 in 20-day-old and older rats.

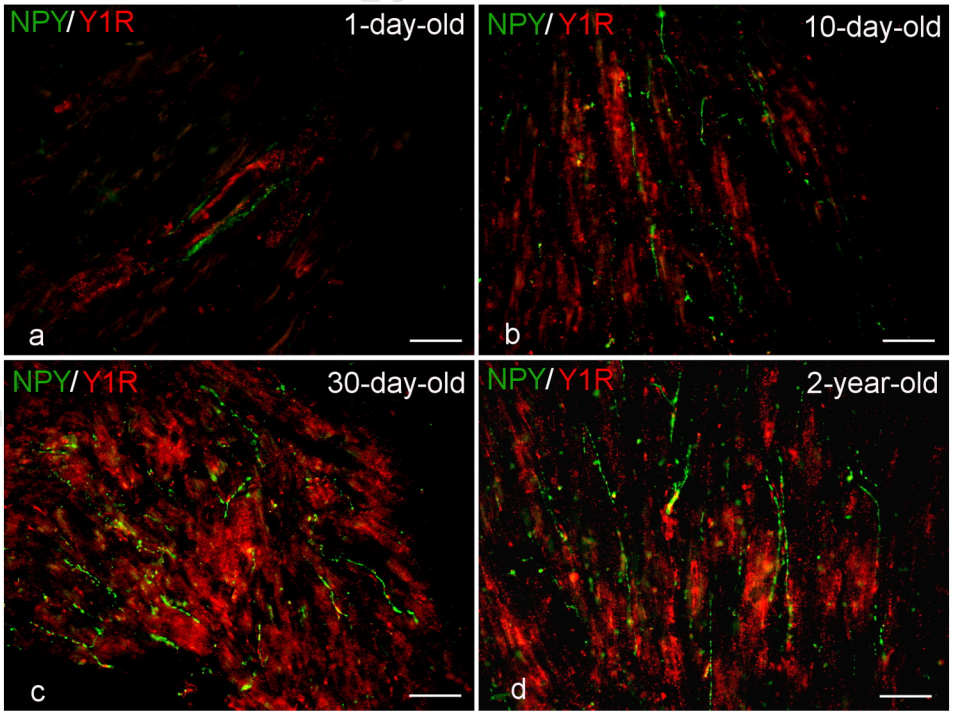


**Fig. 2.** Fluorescence micrographs of NPY (green) and Y1R (red) immunoreactivity in the left atria of newborn (a), 10-day-old (b), 30-day-old (c), and 2-year-old (d) rats. Bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

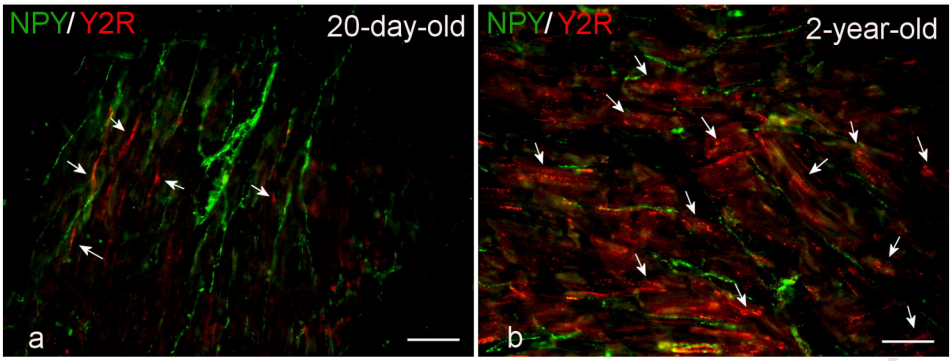


**Fig. 3.** Fluorescence micrographs of NPY (green) and Y2R (red, arrows) immunoreactivity in the left ventricles of 20-day-old (a) and 2-year-old (b) rats. Bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 4. Discussion

The present study shows changes in the expression of NPY, Y1R, Y2R, and Y5R in the rat heart during postnatal development.

The autonomic innervation of the cardiovascular system in rats is immature at birth and reaches adult characteristics between the 5th and 8th week of life (Kuncova and Slavikova, 2000). Biochemical and morphological works confirmed that sympathetic innervation of the newborn rat heart is poor developed at birth (De Champlain et al. 1970; Pappano, 1977). In newborn rats, we observed a small amount of NPY-IR fibers. The density of NPY-positive fibers increases in the first 20 days of rats' life. An increase of NPY-IR sympathetic ganglionic neurons is observed at the same period (Maslyukov et al. 2007, 2010; Masliukov et al. 2012; Masliukov and Nozdrachev 2013).

The majority of NPY-IR fibers are postganglionic sympathetic fibers, originating from the stellate ganglion neurons (Richardson et al. 2006; Masliukov et al. 2012). We also found many NPY-IR fibers colocalizing TH, the enzyme of catecholamine synthesis, from birth onwards. However, there is a delay in co-localization of NPY and TH in sympathetic fibers. In newborn, some NPY-IR fibers are TH-negative. NPY in newborn sympathetic fibers is particularly important due to its possible trophic action. Some studies indicate that NPY plays a critical role as a mediator of the innervation-dependent change in α1-adrenoceptor-dependent chronotropic responsiveness (Sun et al. 1991, 1998) and also promotes growth of cardiomyocytes and capillaries (Pellieux et al. 2000; Movafagh et al. 2006). In the atria, some of the NPY-IR fibers are also cholinergic originating from intramural ganglionic neurons and expressing ChAT.

Previous studies have shown that the right atrium receives the predominant sympathetic innervation to the neonatal rat heart (De Champlain et al. 1970). We did not find statistically significant differences between the densities of NPY-IR fibers in the right and left atria. However, the density of NPY-IR fibers was larger in the atria comparing with the ventricles in young rats.

Horackova et al. (2000) did not find NPY-IR neurons in the intramural ganglia of the heart during the first 2 weeks of postnatal development. However, we observed that NPY immunoreactivity is exist in small number of ganglionic neurons even at birth and their number

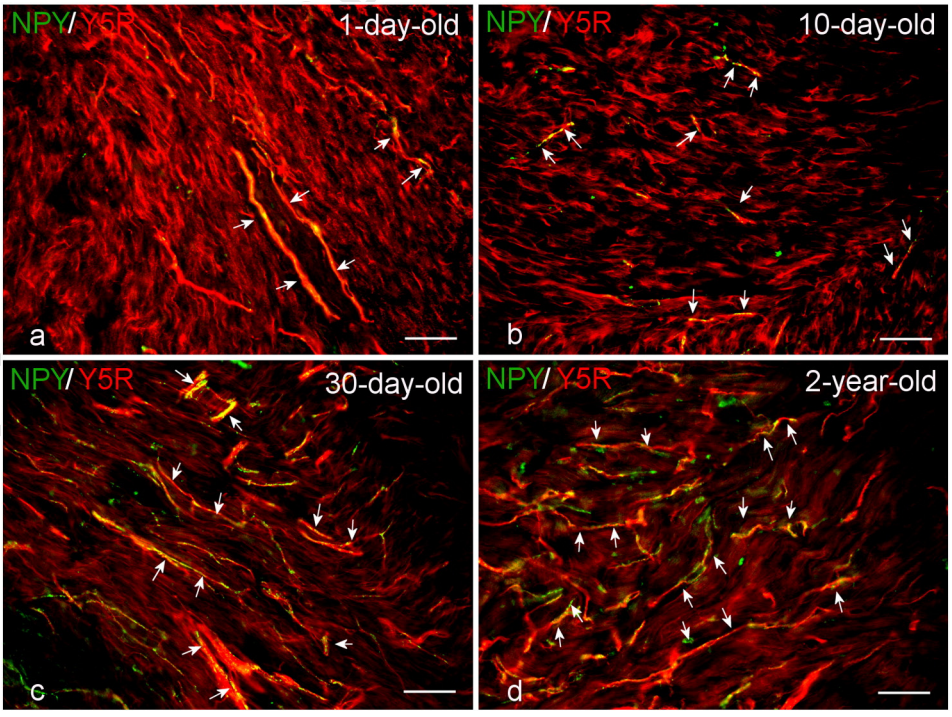


**Fig. 4.** Fluorescence micrographs of NPY (green) and Y5R (red) immunoreactivity in the left atria of newborn (a), 10-day-old (b), 30-day-old (c), and 2-year-old (d) rats. NPY-IR fibers colocalizing Y5R are indicated by arrows. Bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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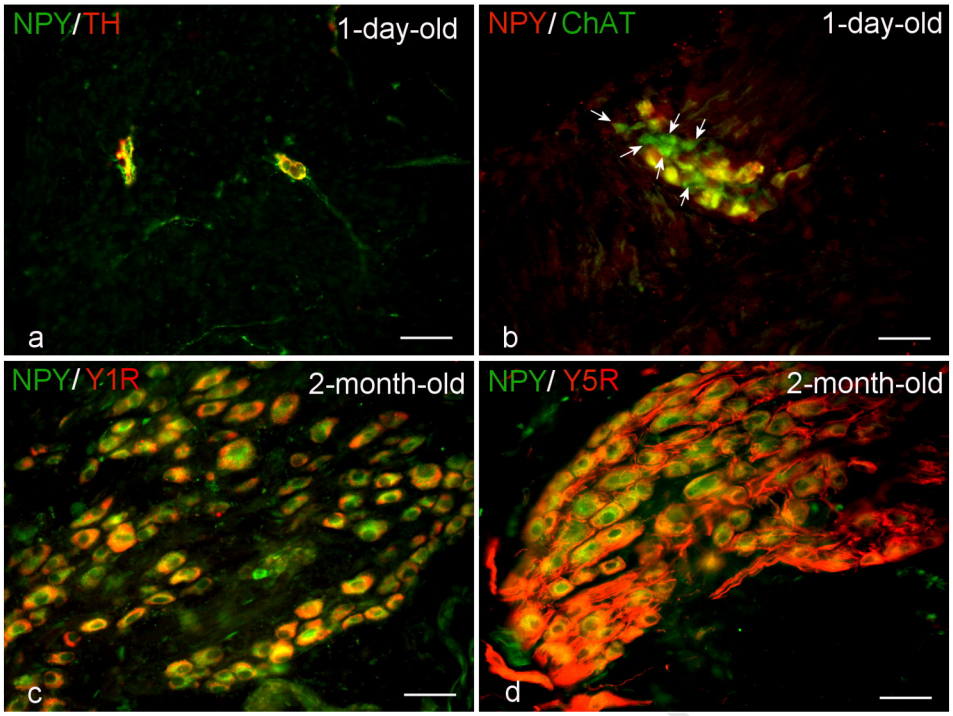


**Fig. 5.** Fluorescence micrographs of NPY-IR neurons (a, c, d—green, b—red) colocalizing TH (red, a), ChAT (green, b), Y1R (red, c), and Y5R (red, d) in intramural ganglia of the right atrium of newborn (a, b) and 2-month-old (c, d) rats. NPY-negative neurons are indicated by arrows. Bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

rapidly increased during first 10 days of life. Some authors also identified NPY-IR neurons in the heart of newborn rats (Nyquist-Battie et al. 1994). Possibly, these disagreements can be caused by the small amount of NPY in the ganglionic neurons of newborn animals and different sensitivity of antibodies used in the above mentioned works.

The percentage of NPY-IR neurons in the intramural ganglia of the heart significantly increased in the first 10 days of the development. Only in newborn animals the vast majority of NPY-IR neurons also expressed TH. Our results are consistent with other studies in the rat (Horackova et al. 1990; Richardson et al. 2003; Slavikova et al., 1993). Q8 Q9

We focused on Y1R, Y2R, and Y5R because they are most abundant in the heart of adult rats (Protas et al. 2003; Masliukov and Nozdrachev 2013). Western blot analysis verified the results we got from immunohistochemistry. A downregulated level of Y5R and a reverse trend in Y1R and Y2R were clearly observed in the heart tissue.

In newborn and 10-day-old rats, only small number of Y1R was found by means of immunohistochemistry and Western blot analysis. However, Y1R was upregulated in the next 10 days, and a high level of Y1R in the heart tissue was observed in 20-day-old and older rats. Previous reports show that in the rat heart, the expression of Y1R is dominates over Y2R (Chottová Dvoráková et al. 2008). We also found lower level of Y2R expression compared with Y1R. Y2R were found at low level only in 20-day-old and older rats.

In the human heart, immunohistochemistry localized both the Y1R and the Y2R subtype on nerve fiber bundles, conducting fibers of the atrioventricular node, and some but not all arterioles and atrial and ventricular cardiomyocytes (Jönsson-Rylander et al. 2003). A positive contractile effect of NPY is mediated by Y1R, whereas a negative contractile effect is mediated by Y2R (McDermott et al., 1997). Q10

We first noticed Y5R-IR in the rat heart during the development by immunohistochemistry and Western blot analysis. We found that Y5R was downregulated in the first 10 days. In young and adult rats, Y5R were located primarily in the NPY-IR nerve fibers. Developmental changes of Y5R were also found in the central nervous system, where Y5R inhibited synaptic excitation in the hippocampal slice only in young rats and their contribution declined with age (Ho et al. 2000).

NPY acts as a trophic factor by promoting growth and/or differentiation of a variety of cells in a receptor-specific manner. The hypertrophic effect of NPY is accompanied by increased activity of cytosolic creatine kinase, protein kinase C, and protein kinase C-dependent activation of mitogen activated protein kinase in adult and neonatal myocytes and activation of phosphoinositol 3-kinase in adults (Protas et al. 2003).

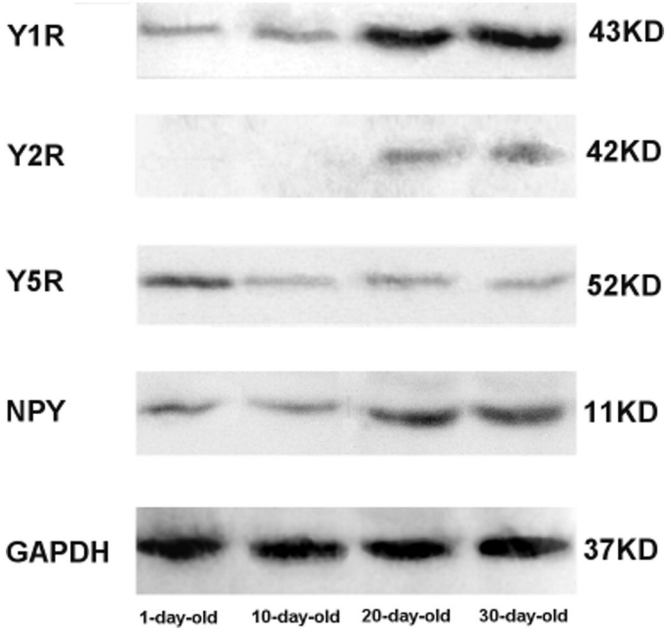


**Fig. 6.** The representative immunoblot bands of NPY, Y1R, Y2R, Y5R and GAPDH in the left atria of newborn, 10-day-old, 20-day-old, and 30-day-old rats by Western blot.

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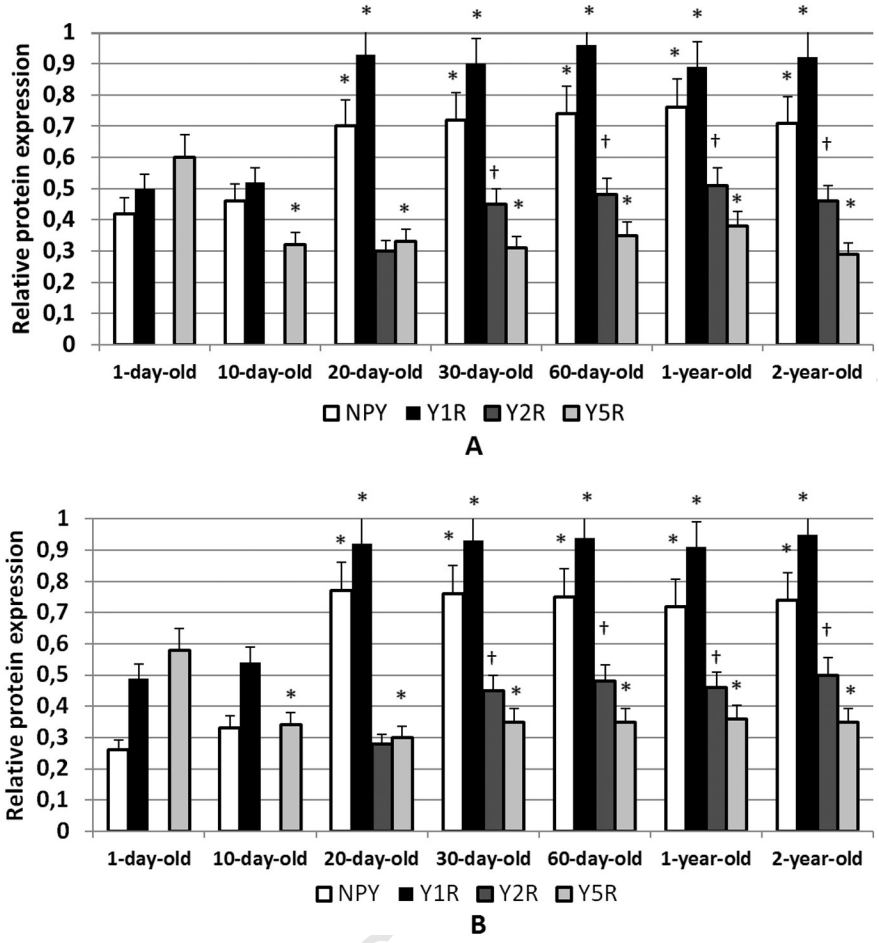


**Fig. 7.** The data analysis of NPY, Y1R, Y2R, and Y5R expression in the atria (A) and ventricles (B) by Western blot. All data represented a relative quantification to internal control GAPDH. $^{*}p < 0.05$ compared to newborns. $^{\dagger}p < 0.05$ compared to 20-day-old rats.

Via its Y1R, Y2R, and Y5R, NPY promotes angiogenesis (Kuo et al. 2007; Parker and Balasubramaniam 2008; Zhang et al. 2015), where the Y5R acts as an enhancer (Movafagh et al. 2006). In rats, the transmural number of capillaries in the left ventricle increases 3-fold from 1 to 5 days and 5-fold from 1 to 11 day of life. At 11 days, the capillary density in the myocardium is comparable to that in adult myocardium (Anversa et al. 1978; Olivetti et al. 1980). By our data, the highest level of expression had Y5R in the first 10 days of age. We could suggest that during early development Y5R have the most important role in stimulating blood vessels growth.

The expression of the Y5R was detected in primary cultured cardiomyocytes of mouse by reverse transcriptase-polymerase chain reaction (Pellieux et al. 2000). The growth stimulating effect of Y5R activation leads to increased protein kinase C activity, cAMP inhibition and to mitogen activated protein kinase phosphorylation and activity (Pellieux et al. 2000; Sheriff et al. 2010). The activation of mitogen-activated protein kinase appears could be important in the intracellular-signaling pathways leading to growth-promoting effects of cardiomyocytes in situations of low sympathetic activity in newborns.

In rats, the cardiac α1-adrenergic chronotropic response changes from stimulatory to inhibitory postnatally (Drugge et al. 1985). In vitro studies indicate that neurally released NPY plays a critical role in the innervation-dependent maturation of the α-adrenergic response (Sun et al. 1991, 1998; Robinson 1996). Sun et al. (1998)) suggested that the Y2R or "peptide YY preferring" is the subtype likely mediating the trophic effect of NPY on α-adrenergic signaling in the neonatal rat ventricles. However, our data indicate that Y2R do not express in the heart of newborn rats. It seems that commercially available NPY peptide receptor agonists may not be adequate tools for assessing the role of specific NPY receptor subtypes in vitro. The limitation of this approach is that peptide fragments bind NPY receptors with low nanomolar affinities and therefore can be classified as "receptor preferring" but are not strictly receptor selective (Guo et al. 2002). NPY 13-36, used in the study of Sun et al. (1998) as Y2R agonist at a concentration of $10^{-7}$ M, may react with Y2R and also with Y5R (Gerald et al. 1996). Therefore, further pharmacological studies with more selective agonists of Y2R and Y5R are required.

Thus, in the heart of newborn rat, NPY may play a trophic function via Y5R promoting growth of cardiomyocytes and myocardial capillaries. This type of receptors may be very important at the period of rapid increasing of the heart weight and establishing of sympathetic innervation.

We did not find differences on the expression of NPY, Y1R, Y2R, and Y5R between young (1- and 2-month-old) and aged (24-month-old) rats. These data confirm our earlier observations about constancy of the number of sympathetic NPY-IR neurons during aging (Masliukov et al. 2012). Therefore, NPY-ergic system of the rat heart regulation is transformed in the first 20 days of life and remains stable throughout the senescence.

## 5. Conclusion

Thus, the increasing of density of NPY-containing nerve fibers accompanies changes in relation of different subtypes of NPY receptors in the heart during development. In the rat heart, Y1R and Y2R expression is upregulated, whereas Y5R expression is downregulated during the development. Further physiological and pharmacological studies using selective agonists and antagonists of NPY receptors are required

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to understand better the function NPY during the development. The information provided here further serves as a basis for future studies investigating the mechanisms of development of autonomic regulation.

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