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Spatiotemporal expression patterns of natriuretic peptides in rat testis and epididymis during postnatal development

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Abstract

Natriuretic peptide (NP) family is composed of atrial, brain and C-type NP (NPPA, NPPB and NPPC). Here, we aimed to investigate NP expression in testis and epididymis during postnatal development. NPPA expression was observed in gonocytes at prepubertal period but in only spermatocytes in pachytene and leptotene/zygotene stage at pubertal period. In prepubertal and pubertal periods, we detected NPPB expression in only Leydig cells. However, NPPC expression was detected in all of the gonocytes and Sertoli cells, spermatocytes and some interstitial cells in prepubertal and pubertal periods. In postpubertal and mature periods, NPPA and NPPB staining were detected in Leydig cells, elongated and round spermatids but not in spermatogonia and spermatocytes. However, we observed NPPC expression in all cells of the seminiferous tubules and Leydig cells in the postpubertal and mature periods. Epididymal epithelium showed intense NPPC expression during postnatal period but weak NPPA and NPPB expression in prepubertal and pubertal periods. The expression of three NPs in the testis significantly increased after puberty. In conclusion, puberty had a significant effect on NP expression in testis. Unlike NPPA and NPPB, expression of NPPC in all cells of the seminiferous tubule suggests that NPPC is effective in each step of spermatogenesis.

KEYWORDS

atrial natriuretic peptide, brain natriuretic peptide, C-type natriuretic peptide, epididymis, rat, testis

1 | INTRODUCTION

The postnatal testicular development includes a time period in which both germ and somatic cells undergo proliferation and differentiation that will result in the first stage of spermatogenesis and establish a framework for the future constant sperm production (Honaramooz et al., 2002; Oatley, de Avila, Reeves, & McLean, 2004). During the prepubertal period, both the germ and somatic cells undergo the developmental process. In the early postnatal period, gonocytes pass through a number of developmental stages and become turned into spermatogonia. During this period, the somatic cells are composed of immature proliferative Sertoli and Leydig cells, and become differentiated to perform their supporting roles during spermatogenesis (Herrera-Alarcón, Villagómez-Amezcua, González-Padilla, & Jiménez-Severiano, 2007; Honaramooz et al., 2002; Lunstra, Wise, & Ford, 2003). The maintenance of normal testis physiology is dependent upon hormones acting through paracrine and endocrine pathways both in vitro and in vivo. Testosterone and follicle-stimulating hormone (FSH) are the main hormonal regulators of spermatogenesis (Sofikitis et al., 2008).

Studies have shown that natriuretic peptides (NPs) are involved in the regulation of steroidogenesis in the testis (El-Gehani,

Tena-Sempere, Ruskoaho, & Huhtaniemi, 2001). NPs are composed of three structurally closely related but functionally distinct molecules: atrial natriuretic peptide (NPPA), brain natriuretic peptide (NPPB) and C-type natriuretic peptide (NPPC) (Levin, Gardner, & Samson, 1998; Öztop, Cinar, & Turk, 2018). NPs perform their physiological functions by binding to two different membrane-bound guanylyl cyclase-coupled receptors (NPR-A and NPR-B) and activating cyclic guanosine monophosphate (cGMP) signalling cascades. NPR-A is activated by NPPA and NPPB, while NPPC activates NPR-B. Furthermore, all three peptides bind the natriuretic peptide clearance receptor (NPR-C) that clears themselves from the circulation (Pandey, 2005; Potter, Abbey-Hosch, & Dickey, 2005). NPPA and NPPB are synthesised and secreted predominantly by the atrium and ventricle, respectively (Maack, Nikonova, Friedman, & Cohen, 1996; Stein & Levin, 1998). In contrast, NPPC is expressed in many different tissues, where it can perform locally as an autocrine/paracrine regulator by binding to NPR-B (Komatsu et al., 1991; Nielsen, Gøtze, Jensen, & Rehfeld, 2008; Totsune et al., 1994). NPPA and NPPB are released into the circulation in response to myocardial stretch (Kinnunen, Vuolteenaho, & Ruskoaho, 1993). NPPB produces physiological effects on target organs in a manner similar to those of NPPA. While NPPA is almost exclusively of the atrial origin, a considerable amount of NPPB is produced by cardiac ventricle due to its larger mass (Hosoda et al., 1991). Although NPPA mRNA expression was not observed in testis (El-Gehani et al., 2001), protein expression was determined in both foetal and adult testis (Mourdjeva, Russinova, Kyurkchiev, & Kehayov, 2001). In contrast, NPPB and NPPC mRNAs were detected by Southern hybridisation and real-time polymerase chain reaction (RT-PCR) in the testes during prenatal and neonatal periods (El-Gehani et al., 2001). NPPA is localised in round and elongated spermatids in seminiferous tubules during adulthood. Furthermore, researchers have reported that NPPA was expressed in foetal, progenitor and immature Leydig cell populations with a varied immunoreactive density (Mourdjeva et al., 2001). Collin, Lissbrant, and Bergh (1997) also reported that NPPB is localised to Leydig cells in rats.

The epididymis is a highly convoluted duct composed of structurally four different regions: initial segment, caput epididymis, corpus epididymis and cauda epididymis (Domeniconi, Souza, Xu, Washington, & Hinton, 2016). The epididymis supports male fertility by ensuring spermatozoa maturation (Cornwall, 2009). In the epididymis, the spermatozoa experience different luminal contents allowing the spermatozoa to gain progressive motility and the capability to fertilise an ovum (Robaire, Hinton, & Orgebin-Crist, 2006). Nielsen et al. (2008) reported that NPPC is expressed in the epididymal epithelium. Since region-specific differences exist in the cellular activities of the epididymal epithelium, each segment seems to harbour unique and common regulators that are involved in gene and protein expression and signalling events (Domeniconi et al., 2016). In this respect, the expression of NPPA, NPPB and NPPC in the epididymis has not been studied in a region-dependent manner.

Researchers have reported that NPs play crucial roles in mammals, including blood volume, blood pressure, bone growth, fat metabolism and steroidogenesis in testes (Gnessi, Fabbri, & Spera, 1997; Potter et al., 2005). Furthermore, Kong et al. (2017) reported that NPPC regulates the oocyte attraction of the spermatozoon in the oviduct. In addition, proteomic studies in cattle spermatozoa have shown that NPPC is significantly higher in bulls with high fertility (Viana et al., 2018). Although many studies are available on the expression of NPs in the testis (Collin Lissbrant, & Bergh, 1997; El-Gehani et al., 2001; Pereira, Costa, Rosa-e-Silva, Vieira, & dos Reis, 2008), data on their expression in the epididymis are scarce. In postnatal development, no fully comprehensive study has been conducted that investigates together the quantitative changes of NPs in testis and epididymis. We reason that changes in expression of NPs in the postnatal period would contribute substantially to the field of reproductive physiology. For this purpose, we examined the spatiotemporal expression patterns of NPs in the testis and epididymis during postnatal development.

2 | MATERIALS AND METHODS

2.1 | Experimental design and animal ethics statement

All the experimental protocols were approved by Department of the Experimental Animal Ethics Committee, Ankara University, Turkey (2014-18-128). Testicular and epididymal tissue samples harvested from Wistar albino rats were used in this study. We designed four groups as prepubertal (postnatal day 5), pubertal (postnatal day 20), postpubertal (postnatal day 50) and mature (postnatal day 70). Each group consisted of six animals. The rats were housed at room temperature (RT) in a 12-hr light/12-hr dark cycle, and they had free access to standard diet and water. All rats were euthanised, and tissue samples were collected for immunohistochemical investigation and Western blotting. For Western blotting, the samples were stored at −80°C. For immunohistochemistry, the samples were fixed in Bouin's solution at room temperature (RT). After fixation, tissues were washed in 70% alcohol for overnight and passed through graded alcohols (80%, 96% and 100%). Then, the samples were cleared in benzene and methyl benzoate solutions and embedded in paraplast.

2.2 | Immunohistochemistry (IHC)

We performed immunohistochemical staining, as described previously (Özbek et al., 2018). Briefly, we cut 5-μm thick sections from the paraplast-embedding tissue by using rotatory microtome (RM2125RT; Leica). Then, the samples were transferred on poly-L-lysine-coated slides. The sections were dewaxed with xylene and rehydrated in a series of alcohol. Then, we washed the sections in phosphate-buffered saline (PBS) and incubated the slides in 3% H_2O_2 in distilled water for 20 min. After washing with PBS, we applied the citrate buffer (10 mM, pH 6.0) for antigen retrieval. The slides in citrate buffer were boiled in for 20 min in a microwave oven. After washing with PBS, the sections were encircled using DakoPen and incubated in 10% normal goat serum blocking solution (Ultra V Block®; Thermo Fisher Scientific) for 10 min. Then, the slides were overnight incubated with primary antibodies (Table 1). After rinsing in PBS, the samples were treated with secondary antibody (biotinylated goat

TABLE 1 Primary antibodies used in the immunohistochemical staining and Western blotting

Primary antibodies	Immunogen	Dilution		Code	Reactivity	Company
		IHC	WB			
Rabbit polyclonal NPPA	Amino acids 1–153 representing full-length ANP of human origin	1:100	1:1,000	sc-20158	Human, rat, mouse	Santa Cruz Biotechnology
Rabbit polyclonal NPPB	Amino acids 27–134 mapping at the C-terminus of BNP of human origin	1:100	1:1,000	sc-20159	Human	Santa Cruz Biotechnology
Rabbit polyclonal NPPC	Amino acids 1–115 of CNP of human origin	1:100	1:1,000	sc-20952	Human, rat, mouse	Santa Cruz Biotechnology
Rabbit polyclonal GAPDH	Amino acids 1–335 representing full-length GAPDH of human origin		1:1,000	sc-25778	Human, rat, mouse	Santa Cruz Biotechnology

anti-mouse and rabbit IgG; Thermo Fisher Scientific) for 30 min at RM. After washing in PBS, the slides were incubated with enzyme-conjugated streptavidin (streptavidin–horseradish peroxidase; Thermo Fisher Scientific) for 30 min. We used 3-amino-9-ethylcarbazole (AEC Chromogen & Substrate System; Thermo Fisher Scientific) as chromogen for visualising the immunostaining and counterstained slides with Gill's haematoxylin.

Immunohistochemical staining was evaluated under the Olympus BX51 light microscope with an integrated digital camera (DP74; Olympus). We acquired images from sections by using a digital camera with CellSens software. All the immunostaining procedures were performed in a humidity chamber. We performed positive and negative control to confirm the specificity of the immunohistochemical reaction. For the negative control, the primary antibody was replaced with PBS. At the same time, we used nonspecific rabbit serum (sc-2027; Santa Cruz Biotechnology) instead of primary antibodies for isotype control. Anatolian ground squirrel atria were used for positive controls, as reported in our previous study (Öztop et al., 2019). The same procedure was performed for all the immunostaining processing including the negative and positive controls.

2.3 | Western blot (WB) analysis

2.3.1 | Protein extraction

In brief, snap-frozen testicular and epididymal tissue segment samples collected from each rat were homogenised in cell lysis buffer (PROPREPTM Protein Extraction Solution) containing protease inhibitor cocktail (Cell Signaling Technology) by using an ultrasonic homogeniser. After homogenisation and sonication, the lysates were centrifuged at 13,000 × *g* for 25 min at 4°C, and the supernatant was transferred to new tubes for final collection after centrifugation at 10,000 × *g* for 15 min. The total protein concentrations were detected by using Quick Start Bradford Protein Assay (Bio-Rad Laboratories).

2.3.2 | Western blot

The proteins (25 μg, each) were subjected to 10% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 5% skimmed milk for 2 hr at RT and was incubated with primary antibodies (Table 1) overnight at 4°C. After rinsing, the membranes were treated with goat polyclonal anti-rabbit (sc-2004; dilution 1:1,000; Santa Cruz Biotechnology) horseradish peroxidase conjugate for 2 hr at RT, and washed with 10 mM Tris–HCl pH 7.4, 150 mM NaCl and 0.1% Tween-20 (TBST). The protein in the membranes was visualised by chemiluminescence using Clarity™ Western ECL Substrate. We used GAPDH as the internal control, and the signal intensities were quantified using Image Studio™ Lite (LICOR).

2.4 | Statistical analysis

GraphPad Prism 5 (GraphPad software) was applied to evaluate data. All the data were expressed as mean ± *SEM*. Two groups (testis and epididymis) were compared with a two-tailed Student's *t* test. Multiple groups (comparison between 5th, 20th, 50th and 70th days, and comparison between testis and epididymal regions (caput [CT], corpus [CS], cauda [CA]) were compared using one-way factorial analysis of variance (ANOVA), with Tukey's multiple comparison test. $p < 0.05$ was considered as significant.

3 | RESULTS

3.1 | Immunohistochemistry

The Anatolian ground squirrel atria were used as a positive control in order to determine the specificity of the antibodies. As seen in our previous study (Öztop et al., 2019), NPPA, NPPB and NPPC stainings were observed in cardiomyocytes (Figure 1). For the negative control, nonimmune serum was used instead of the primary antibody and the other immunohistochemical procedures were applied in the same way. We did not observe any nonspecific reaction in the negative control slides (Figure 1).

3.1.1 | Postnatal day 5

We observed weak NPPA staining in gonocytes in the testes and some interstitial cells of the epididymis. However, NPPB staining was detected in only Leydig cells in the testis and in some interstitial cells of the epididymis. On the other hand, we found intense NPPC immunoreaction in gonocytes, Sertoli cells, interstitial cells

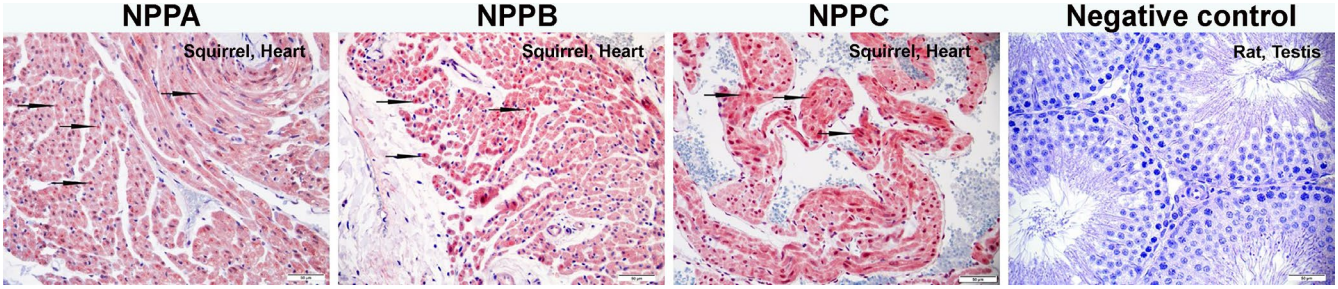

FIGURE 1 Representative figures showing squirrel atria sections as positive control and rat testis sections as negative control for immunostaining. Strept-ABC, AEC, paraffin. NPPA, NPPB and NPPC stainings were observed in the atrial cardiomyocytes. No immunoreaction was detected in the rat testis sections that were incubated without the primary antibody. Scale bars: 50 μm

and the blood vessel walls. Epithelium and smooth muscle cell of the epididymis were also positive for NPPC (Figure 2).

3.1.2 | Postnatal day 20

We found perinuclear NPPA immunoreaction in only spermatocytes in pachytene and leptotene/zygotene stage in testis. In addition, weak NPPA staining was observed in the epididymal epithelium. NPPB immunoreaction was detected in only some Leydig cells in the testis and in the epididymal epithelium. On the other hand, NPPC staining was observed in almost all the cells of seminiferous tubules and Leydig cells. Epididymal epithelium and peritubular smooth muscle cells were also positive for NPPC (Figure 2).

3.1.3 | Postnatal days 50 and 70

In both periods, we found NPPA and NPPB immunoreaction in elongated and round spermatids, but did not detect any reaction in spermatogonia and spermatocytes. While some Leydig cells showed weak NPPA staining, intense NPPB immunoreaction was observed in most of the Leydig cells. On the other hand, we observed NPPC expression in all the cells of the seminiferous tubules and in Leydig cells (Figure 3). We did not detect any NPPA and NPPB staining in the epididymis, whereas NPPC staining was observed intensely in smooth muscle cells and all the epithelial cells of the entire epididymal regions. In addition, we observed NPPC-positive cells in the lumen of caput, corpus and cauda epididymis (Figures 4 and 5).

3.2 | Western blotting findings

In the rat testis and epididymis during postnatal development, the levels of NPPA, NPPB and NPPC expression were detected using Western blot analysis. Our results confirmed the immunohistochemical data. The comparison of these natriuretic peptides' expression in the developing testis and epididymis is given in Figures 6–8 .

3.2.1 | Atrial natriuretic peptide

We found that the expression of NPPA in the testis increased from the prepubertal to the postpubertal period. However, the NPPA increase in the testis was not statistically significant between postpubertal and mature periods. In the epididymis, we found a low level of NPPA expression in the prepubertal and pubertal periods, but we did not observe any NPPA expression in the postpubertal and mature periods (Figure 6).

3.2.2 | Brain natriuretic peptide

We detected that NPPB expression tended to increase in both testis and epididymis from the prepubertal to the pubertal period. NPPB expression significantly increased in the testis after the pubertal period, but NPPB expression was not observed in the epididymis during postpubertal and mature periods (Figure 7).

3.2.3 | C-type natriuretic peptide

From the prepubertal to the mature period, we observed that the NPPC expression continuously increased in the testis ($p < 0.05$). However, NPPC expression tended to increase in the epididymis. In the postpubertal and mature periods, NPPC expression was higher in the testis compared to the epididymal regions. In addition, in the postpubertal periods, NPPC expression was higher in the caput region compared to the other regions of the epididymis ($p < 0.05$) (Figure 8).

4 | DISCUSSION

Here, we examined the spatiotemporal expression profiles of NPs in rat testis and epididymis during postnatal development. The expression of NPs in testis and epididymis varied depending on the postnatal developmental period.

All three NPs, especially NPPA and NPPB, induce testosterone secretion from Leydig cells (Foresta et al., 1991; Khurana & Pandey, 1993; Mazzocchi, Malendowicz, Rebuffat, Kasprzak, & Nussdorfer, 1990). Research on adult mouse Leydig cells showed that NPs stimulate steroidogenesis by affecting both the Δ^4 and Δ^5 signalling pathways (Khurana & Pandey, 1993). In humans, the acute NPPA injection failed to enhance peripheral LH and testosterone levels, but NPPA importantly increased testosterone concentrations

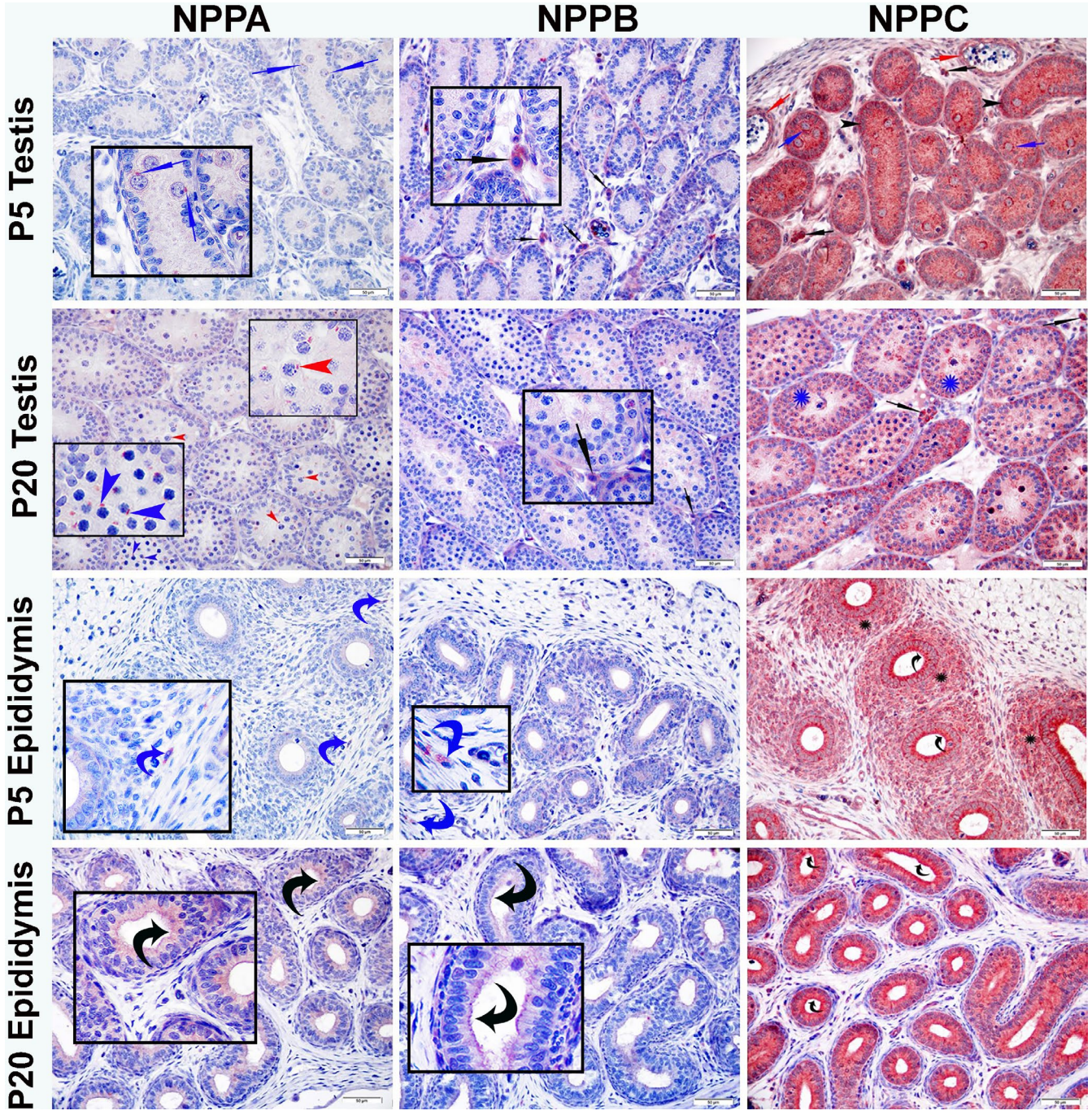

FIGURE 2 Representative immunohistochemistry images showing NPPA, NPPB and NPPC in testis and epididymis on postnatal days 5 (P5) and 20 (P20). Strept-ABC, AEC, paraffin. In postnatal days 5, NPPA expression was observed in gonocytes (blue arrows) and some interstitial cells in the epididymis (blue curved arrows). NPPA staining was detected in only spermatocytes in pachytene (blue arrowheads) and leptotene/zygotene stage (red arrowheads), while NPPB immunoreaction was observed only in some Leydig cells (black arrows). In both periods, NPPC immunoreaction was determined in all the cells (blue asterisk) of seminiferous tubules (blue arrows, gonocytes; black arrowheads; Sertoli cells), Leydig cells (black arrows), vessel wall (red arrows), epithelium (black curve arrowheads) and smooth muscle cells (black asterisks) of ductus epididymis. Scale bars: 50 μm

in vena spermatica, which in turn produces a direct effect on the metabolism of Leydig cells (Foresta et al., 1991). In rats, the long-term administration of NPPA increased both basal and hCG-induced testosterone levels in the blood, and led to hypertrophy of Leydig cells (Mazzocchi et al., 1990). The literature available exists conflicting results regarding NPPA's localisation in the testis. In rats, some researchers observed NPPA immunoreaction in the acrosome of round and elongated spermatids (Pandey & Orgebin-Crist, 1991; Vollmar, Friedrich, & Schulz, 1990), while others detected NPPA immunoreaction in the nuclei of round spermatids and spermatocytes (Collin, Lissbrant, & Bergh, 1997). However, Pereira et al. (2008) and Bakalska, Mourdjeva, Russinova, Kyurkchiev, and Kehayov (1999)

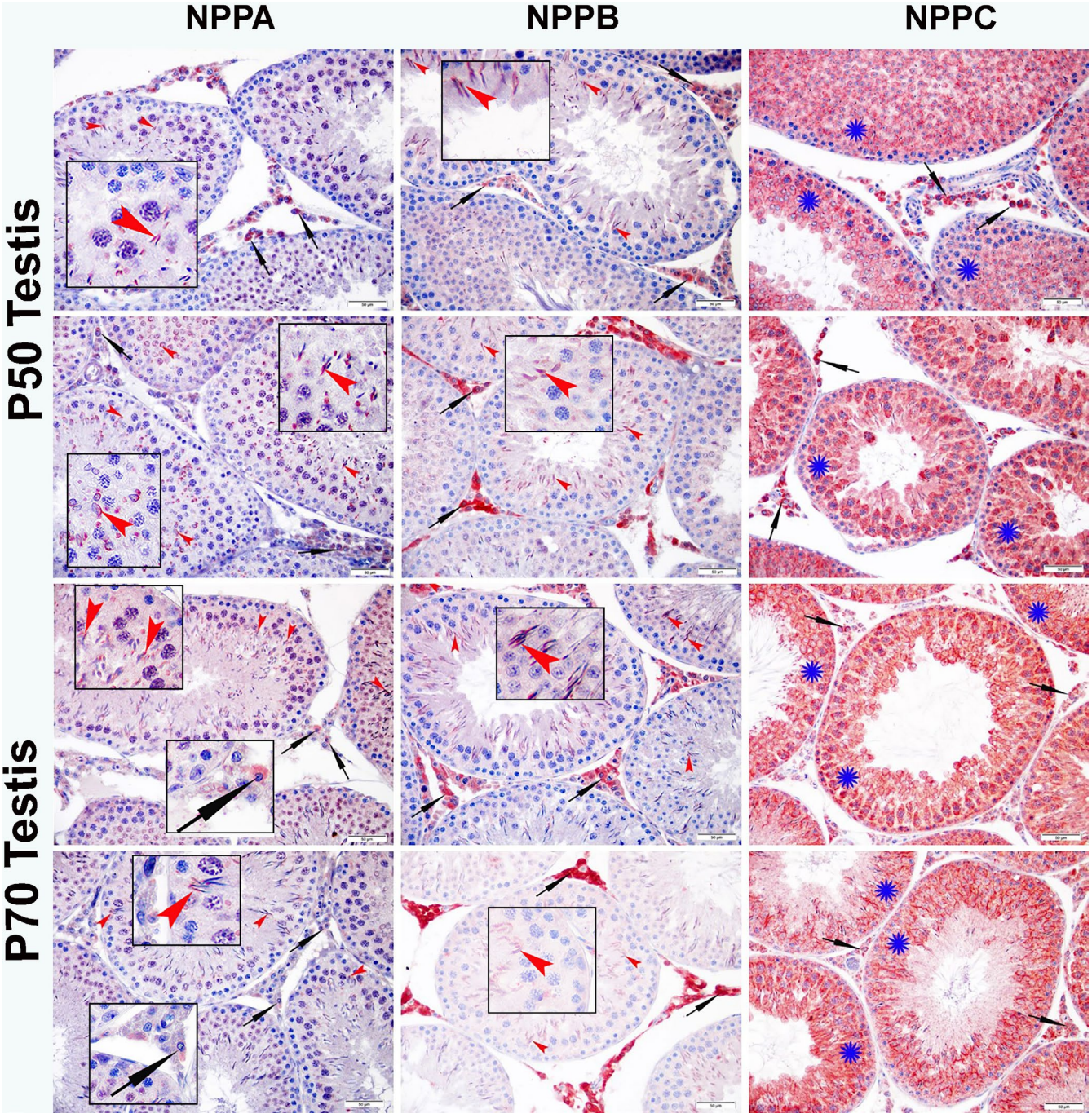

FIGURE 3 Representative immunohistochemistry images showing NPPA, NPPB and NPPC in testis and epididymis on postnatal days 50 (P50) and 70 (P70). Strept-ABC, AEC, paraffin. In both periods, NPPA and NPPB immunoreaction were observed in elongated and round spermatids (red arrowheads). Leydig cells (black arrows) showed weak NPPA staining but intense NPPB staining. NPPC immunoreaction was detected in all the cells of the seminiferous tubule (blue asterisks) and some interstitial cells including Leydig cells (black arrows). Scale bars: 50 μm

reported the intense NPPA staining in only Leydig cells in rat testes. In the present study, we observed NPPA immunoreaction in the gonocytes (postnatal 5th day), pachytene and leptotene/zygotene spermatocytes (postnatal 20th day), round and elongated spermatids (postnatal 50th and 70th), suggesting that NPPA is necessary for the development of the spermatogenic cell series. These differences may result from fixation errors and the specificity of the antibodies used in immunohistochemistry. With the experience we have gained from our previous studies (Özbek et al., 2018), we received more reliable results by fixing testis in Bouin's solution. Interestingly, NPPA expression was not observed in spermatogonium and spermatozoon. Spermatogonium might not express either NPPA or NPR-A before the differentiation step. In addition, NPPA is expressed in spermatids, but not in spermatozoon. As evidenced from NPPC (Xia, Mruk, & Cheng, 2007), this difference in the expression may be due to the fact that NPPA helps the spermatids to pass into the adluminal

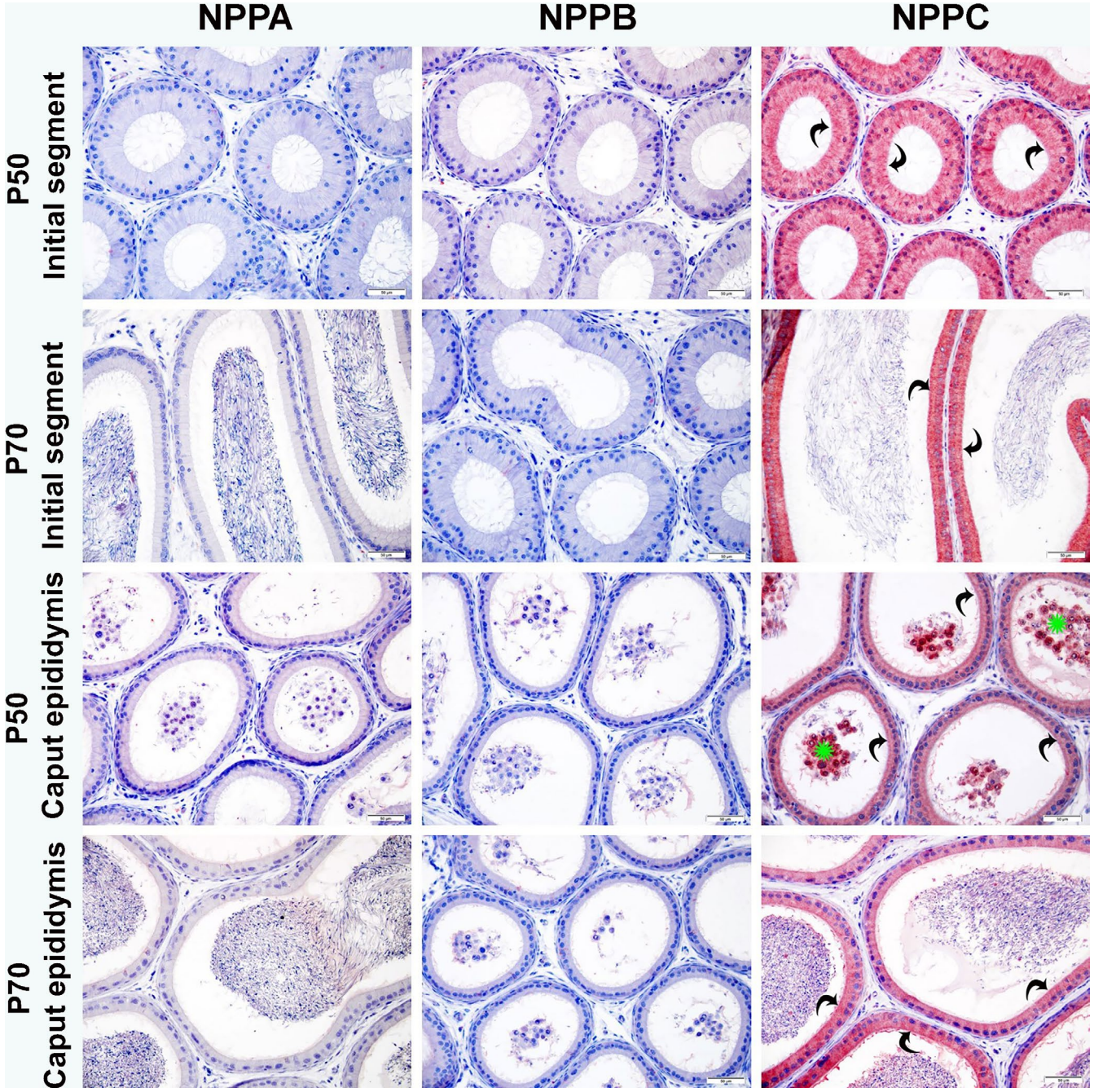

FIGURE 4 Representative immunohistochemistry images showing NPPA, NPPB and NPPC in initial segment and caput epididymis on postnatal days 50 (P50) and 70 (P70). Strept-ABC, AEC, paraffin. NPPA and NPPB stainings were not observed in epididymis including initial and caput segment, while NPPC staining was observed intensely in all the epithelial cells (black curved arrows) of ductus epididymis including initial and caput segment. Moreover, NPPC staining was detected in some cells (green asterisk) in epididymal lumen. Scale bars: 50 μm

compartment. In the prepubertal and pubertal periods, we did not detect any NPPA reaction in immature Leydig cells. However, NPPB expression was detected in only Leydig cells (postnatal 5th day and 20th day) in testis. We observed a significant increase in NPPA and NPPB expression after puberty. Interestingly, NPPA immune reaction was detected in Leydig cells in postpubertal and mature periods. After puberty, the increase in NPPA and NPPB expression is consistent with the finding that NP increases testosterone level, as reported by previous researchers (Foresta et al., 1991; Khurana & Pandey, 1993; Mazzocchi et al., 1990). Although we observed NPPA and NPPB expression in the epididymis in the prepubertal and pubertal periods, we did not detect NPPA and NPPB expression in the epididymis in the postpubertal and mature periods. We could not explain the reason for this difference through the present findings. Therefore, it needs further studies which would examine their expression patterns using animals that lack NPPA and NPPB genes.

As is known, NP exerts their biological effects by binding to NPR-A and NPR-B (Pandey, 2005; Potter et al., 2005). In the

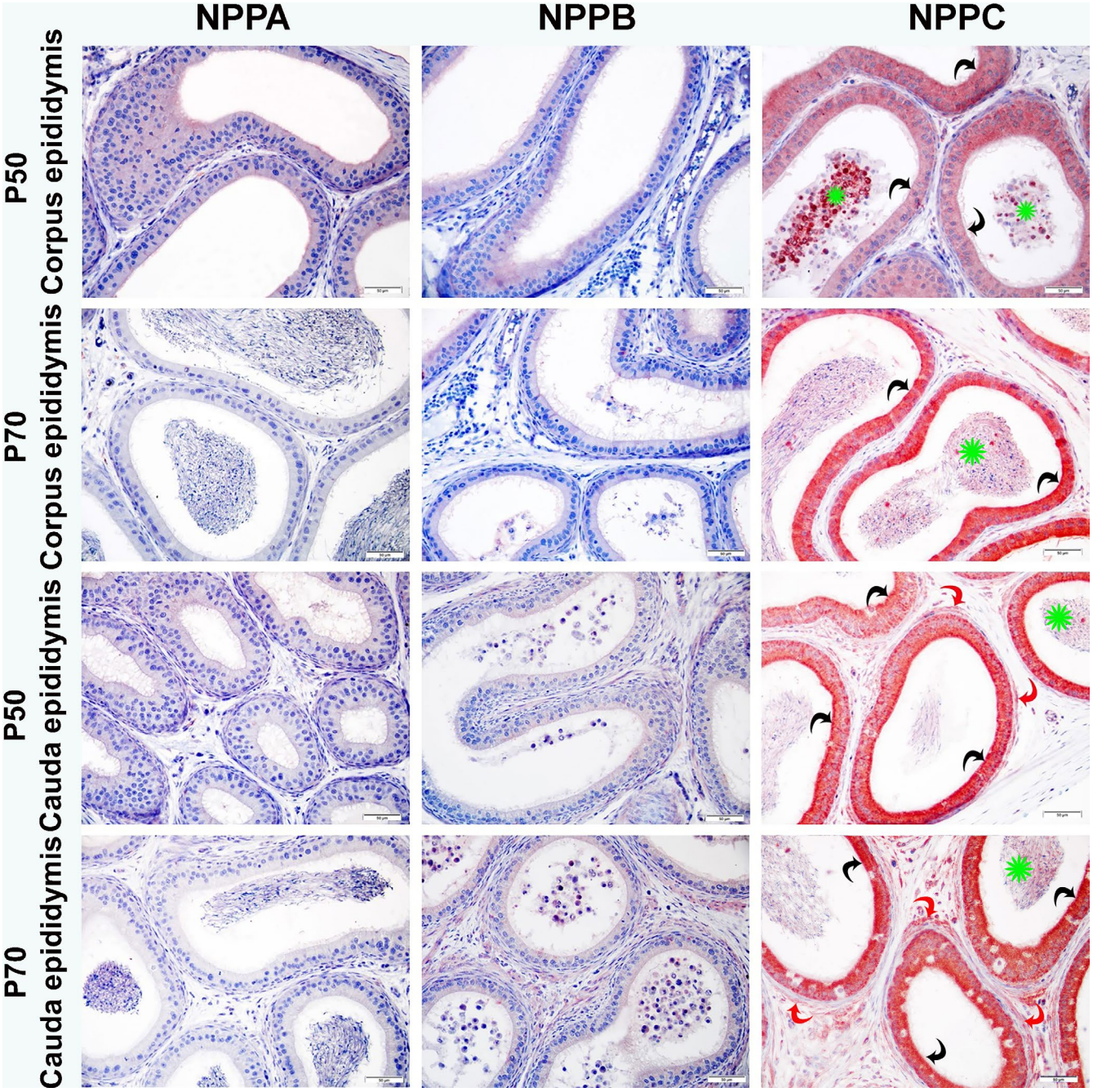

FIGURE 5 Representative immunohistochemistry images showing NPPA, NPPB and NPPC in corpus and cauda epididymis on postnatal days 50 (P50) and 70 (P70). Strept-ABC, AEC, paraffin. NPPA and NPPB stainings were not observed in corpus and cauda epididymis, while NPPC staining was observed intensely in all the epithelial cells (black curved arrows) of epididymis including corpus and cauda segment. Moreover, NPPC staining was detected in some cells (green asterisk) of epididymal lumen in corpus and cauda epididymis. In cauda epididymis, NPPC staining was also observed in smooth muscles (red curved arrows). Scale bars: 50 μm

literature, studies on the expression of these receptors during the postnatal development of the testis are scarce. Müller et al. (2004) reported that seminiferous tubules represented major NPR-A expression site in adult rat testis. Moreover, the NPR-A expression was low in the testis until puberty. The same study also showed that NPR-C expression was observed predominantly in the microvasculature surrounding the seminiferous tubules. Furthermore, NPR-C represented the predominant NPR in testis before the onset of puberty. Interestingly, the ratio of NPR-C/NPR-A dramatically changed in testis during postnatal development (Müller et al., 2004). Data from Müller et al. (2004) and the present study have shown that the puberty has a significant effect on the expression profile of both NPR and NP in testis.

C-type natriuretic peptide, which is produced by cardiomyocytes and known to regulate vascular permeability and constriction/dilation, is also synthesised locally by Sertoli cells in the testis

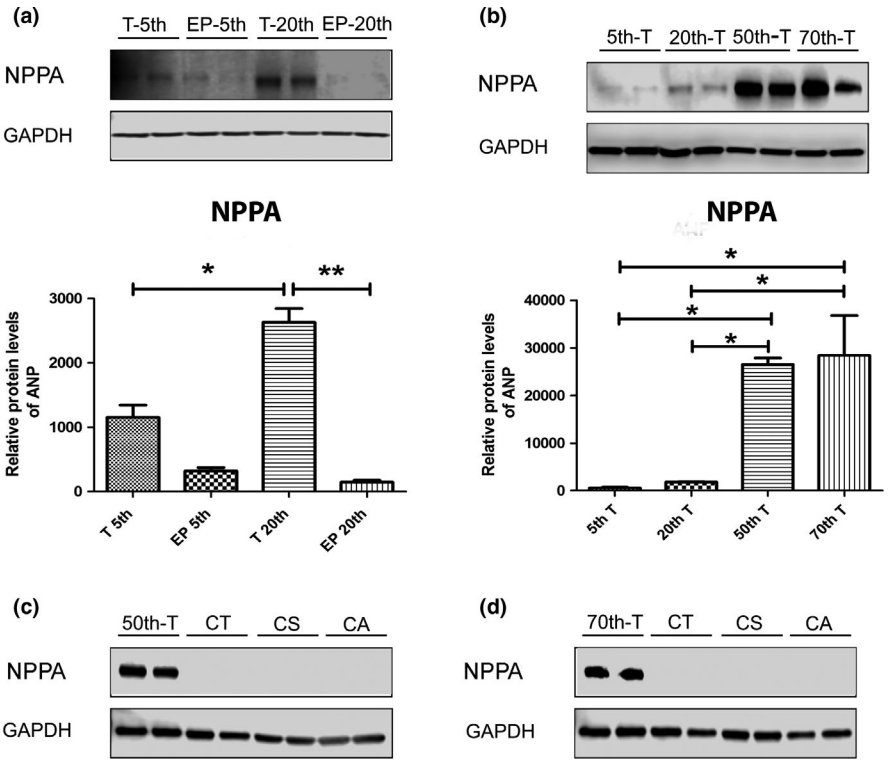

FIGURE 6 Western blot analyses of expression levels of NPPA in rat testis and epididymis. Tissue samples were harvested from rats on postnatal days 5 (5th), 20 (20th), 50 (50th) and 70 (70th). Lysates from testis (T) and epididymis (E) including caput (CT), corpus (CS) and cauda (CA). Each lane in graph indicates one rat and two rats per group. Relative band intensity of NPPA was quantified by densitometry and normalised to GAPDH expression. Data were normalised by dividing arbitrary units of NPPA-GAPDH. The results were presented as means ± *SEM* in arbitrary densitometric units ($n = 6$, three replicates). $^{*}p < 0.05$; $^{**}p < 0.01$

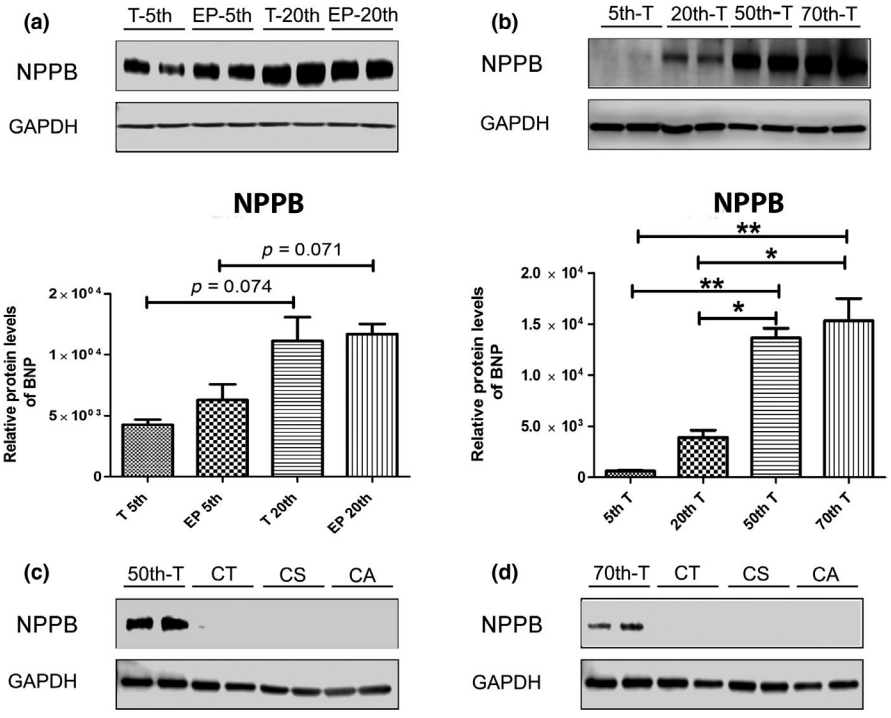

FIGURE 7 Western blot analyses of expression levels of NPPB in rat testis and epididymis. Tissue samples were harvested from rats on postnatal days 5 (5th), 20 (20th), 50 (50th) and 70 (70th). Lysates from testis (T) and epididymis (E) including caput (CT), corpus (CS) and cauda (CA). Each lane in graph indicates one rat and two rats per group. Relative band intensity of NPPB was quantified by densitometry and normalised to GAPDH expression. Data were normalised by dividing arbitrary units of NPPA-GAPDH. The results were presented as means ± *SEM* in arbitrary densitometric units ($n = 6$, three replicates). $^{*}p < 0.05$; $^{**}p < 0.01$

and serves as an autocrine factor (Sellitti, Koles, & Mendonça, 2011). Nielsen et al. (2008) reported that NPPC expression was the highest in male reproductive tissues, and its mRNA in the epididymis and seminal vesicles was 125-fold higher than that in other tissues. Furthermore, NPPC protein levels in porcine seminal plasma and seminal vesicle fluid were 2,000- and 100,000-fold greater than those found in the porcine brain, respectively (Chrisman, Schulz, Potter, & Garbers, 1993). In the present study, NPPC expression was observed in all the cells of the seminiferous tubule and Leydig cells in all the examined periods. We observed a significant increase in NPPC expression in the testes after puberty. We consider that this sudden increase after puberty might be due to the increase in testosterone production, as mentioned in previous studies (Foresta et al., 1991; Khurana & Pandey, 1993;

Mazzocchi et al., 1990). In addition, the NPPC expression in all the spermatogenic cells during postnatal period suggests that this protein is necessary for the development of spermatogenic cells.

The epididymal epithelium is composed of several types of cells, including basal, principal, apical, clear, halo and narrow cells (Cornwall, 2009). The epididymis is also major expression site of NPR-B and NPR-A (Mewe, Bauer, Müller, & Middendorff, 2006; Müller, Mukhopadhyay, Davidoff, & Middendorff, 2011). NPPC binds to epididymal membrane preparations, which in turn results in a considerable amount of cyclic GMP (cGMP). cGMP-sensitive cyclic nucleotide-gated ion channels (CNG) have been found in mammalian spermatozoa (Weyand et al., 1994; Wiesner et al., 1998), controlling the Ca^{2+} entry into spermatozoa (Cisneros-Mejorado, Hernández-Soberanis, Islas-Carbajal, & Sanchez, 2014; Wiesner et al., 1998). NPPC increased cGMP levels and induced Ca^{2+} influx via CNG channels. The elevation of Ca^{2+} also increases the swimming speed of spermatozoa along the chemoattractant gradient (Böhmer et al., 2005; Eisenbach & Giojalas, 2006; Ralt et al., 1994). Kong et al. (2017) reported that the mouse oviductal epithelium in ampulla expresses high levels of NPPC in the occurrence of ovulated oocyte–cumulus complexes, and the spermatozoa express NPR-B on the midpiece of the flagellum. NPPC secreted by oviductal ampulla attracts spermatozoa towards oocytes. They also showed that spermatozoa from NPR-B mutant mice were not attracted by NPPC, preventing fertilisation in vivo. Furthermore, the proteomic analysis showed that NPPC is more abundant in the seminal plasma of high-fertility bulls than that in low-fertility bulls (Viana et al., 2018). In our study, we observed NPPC immunoreactivity in all the epithelial cells of the epididymis and smooth muscle cells. The expression of this peptide in the epididymis was found to be increased from caput to cauda. However, whether this peptide is produced from other parts of the testis or from epithelial cells of the epididymis needs further clarification. Because NPR-B is expressed in epididymal epithelial cells (Nielsen et al., 2008), the immunohistochemical reaction may be describing the NPR-B/NPPC complex. However, researchers reported that proCNP-derived peptides were produced by the seminal vesicles, prostate gland and epithelial cells of the epididymis (Nielsen et al., 2008). Considering previous researchers,

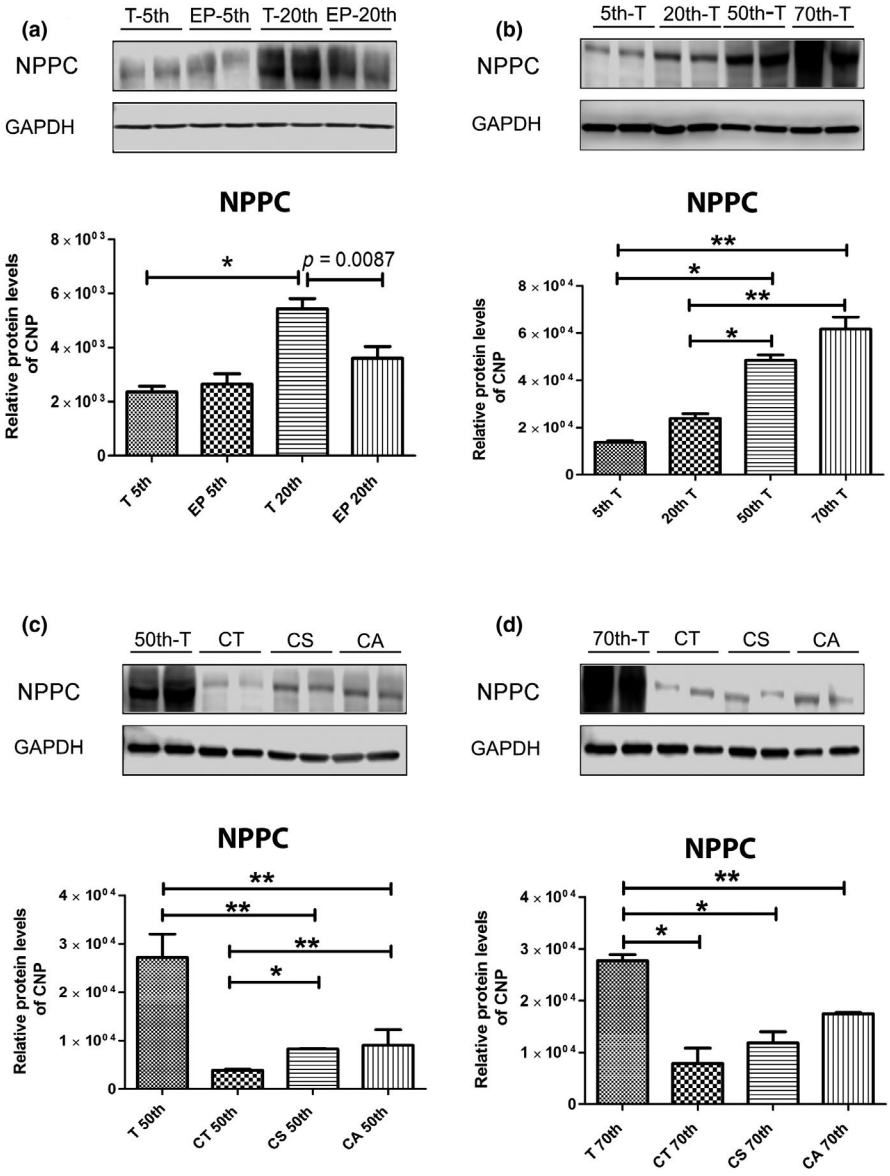

FIGURE 8 Western blot analyses of expression levels of NPPC in rat testis and epididymis. Tissue samples were harvested from rats on postnatal days 5 (5th), 20 (20th), 50 (50th) and 70 (70th). Lysates from testis (T) and epididymis (E) including caput (CT), corpus (CS) and cauda (CA). Each lane in graph indicates one rat and two rats per group. Relative band intensity of NPPC was quantified by densitometry and normalised to GAPDH expression. Data were normalised by dividing arbitrary units of NPPA-GAPDH. The results were presented as means ± *SEM* in arbitrary densitometric units ($n = 6$, three replicates). $^{*}p < 0.05$; $^{**}p < 0.01$

the most likely implication is that NPPC increases the motility of spermatozoa by binding to NPR-B in spermatozoa in the epididymis, thus regulating the reproductive function.

It is generally accepted that the transport of spermatozoon within the epididymis is dependent upon peritubular smooth muscle contraction, ciliated cells and luminal fluid (Elfgen, Mietens, Mewe, Hau, & Middendorff, 2018). In all regions of the epididymis, there are peritubular smooth muscle cells around the basement membrane. The thickness of this smooth muscle layer increases from caput to cauda epididymis. Research on rat and bovine showed that NPR-A and NPR-B were expressed in peritubular smooth muscle cells of the epididymis (Mewe et al., 2006; Müller et al., 2011; Thong et al., 2014). In bovine, NPPA and NPPC have been reported to cause the relaxation of the epididymal duct by binding to NPR-A and NPR-B respectively (Mewe et al., 2006). However, NPPA, but not NPPC, has significant relaxing effects on peritubular smooth muscle cells in rats (Mietens et al., 2014). In our study, the only NPPC expression was shown in the smooth muscle cells of the epididymis in all the examined periods, consistent with previous researchers.

In conclusion, this study shows differences in testicular and epididymal expression of NPs during the postnatal period. Unlike NPPA and NPPB, expression of NPPC in all the cells of the seminiferous tubule suggests that NPPC is involved in each step of spermatogenesis. Puberty had a significant effect on the expression of all the three NPs and led to a significant increase in their expression in testis. After puberty, only NPPC expression was observed in the epithelial and smooth muscle cells of epididymis. In the postpubertal period, NPPC expression increased from caput to cauda epididymis. We suggest that NPPC increases the motility of spermatozoa in epididymis via cGMP.

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