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Received: 21 July 2022 | Revised: 20 October 2022 | Accepted: 24 October 2022

DOI: 10.1111/and.14633

ORIGINAL ARTICLE

First International Journal of Andrology ANDROLOGIA WILEY

Identification of some calcium binding proteins and neural cell markers in rat testis and epididymis during postnatal development

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Abstract

Calcium-binding proteins (CaBPs) have an essential role in male reproduction by modulating calcium ion metabolism. Although the brain and testis are structurally and functionally different, they show a high degree of transcriptomic and proteomic similarities. The purpose of the present study was to explore some CaBPs (Iba-1, Calbindin, Calretinin and Parvalbumin) and neural cell markers (CNPase, NG2 and Drebrin) expression in rat testis and epididymis during postnatal periods by using immunohistochemistry. Iba-1, calbindin, calretinin, parvalbumin, CNPase, NG2 and drebrin were expressed in the epididymal epithelium in each postnatal period. Iba-1 and calbindin expression showed stage-dependent expression in spermatids. CaBPs and neural cell markers showed a positive reaction in Leydig cells in the postpubertal and mature periods. Sertoli cells, gonocytes, spermatogonium, and peritubular myoid cells showed heterogeneity in the expression of CaBPs and nerve markers throughout postnatal development. Interestingly, CNPase, NG2 and drebrin were positive in spermatocytes, spermatids, and sperm. Expression dynamics of calcium-binding proteins and nerve markers showed differences in germ cells and somatic cells during postnatal development. The expression of these proteins in the testis and epididymis supports that they may have important roles in reproductive physiology.

KEYWORDS
calbindin, calretinin, CNPase, Iba-1, parvalbumin

1 | INTRODUCTION

In the testis, sperm are immotile and incapable of fertilizing the oocytes. The fertilization ability of sperm depends on the successful completion of hyperactivation, capacitation, and the acrosome reaction, where extracellular Ca^{2+} has participated in each stage (Breitbart, 2002; Ho et al., 2002; Publicover et al., 2007). During the capacitation, spermatozoa alter their motility pattern from progressive to hyper-activated motility and undergo a modulated release of acrosomal content in a process referred to as acrosome reaction (Visconti et al., 2002). In these maturational processes, Ca^{2+}-signalling pathways play essential roles (Rahman et al., 2014). Therefore, dysregulation of the Ca(2+) signalling pathway has been likely to promote infertility. To fulfil various cellular activities, Ca^{2+} ions reversibly bind to Ca^{2+}-binding proteins (Hooper & Patel, 2012). The role of calcium in many biological processes is probably mediated by interactions with a variety of calcium-binding proteins (CaBPs). Research has shown that male germ cells, including sperm, express CaBPs. CaBPs have molecular chaperones participated in germ cell development (Ikawa et al., 1998), calmodulin (Yamamoto, 1985), protein phosphatase (Nishio et al., 1992), Ca^{2+} sequestering molecule (Nakamura et al., 1993), membrane fusion of spermatozoa, and acrosomal

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component (Noland et al., 1994). Therefore, the expression of calcium-binding proteins is essential for the functional differentiation of germ cells and the general cellular process during spermatogenesis (Ward & Kopf, 1993).

Despite morphological and structural differences between the human testis and brain, it has become increasingly evident in the last four decades that they show some similar features. In addition, gene expression analyses support the evidence that the testes and brain have highly similar active genes among all human organs (Guo et al., 2003; Guo et al., 2005). In addition, Leydig cells are considered to be involved in the neuroendocrine system in humans (Davidoff et al., 2002). Therefore, Leydig cells express various neuron-specific proteins and peptides, such as synaptophysin, neural cell-adhesion molecule, and Substance-P (Davidoff et al., 1993; Schulze et al., 1987). Moreover, Leydig cells have some glial-cell-specific proteins, including cyclic 2′,3′-nucleotide-3′-phosphodiesterase (CNPase) (Davidoff et al., 2002). Besides Leydig cells, some glial cell- and neuron-specific proteins were also detected in human Sertoli cells, such as CNPase (Davidoff et al., 1999, 2002). Drebrin, one of the actin-binding proteins isolated in the nervous system, has also been reported to be present in the testis (Li et al., 2011; Shirao & Obata, 1985). Besides drebrin, nerve/glial antigen 2 (NG2), which is a type 1-transmembrane protein, is found by various cell types within and outside the nerve tissue (Trotter et al., 2010).

CaBPs modulate ion metabolism and the essential signalling pathways for male germ cell maturation (Shawki et al., 2019). Expression dynamics of calcium-binding proteins in the testis and epididymis during postnatal periods may provide essential knowledge about the physiology of spermatogenesis. Recent studies have reported that the testis and brain have the highest number of similar proteins compared to other tissues (Matos et al., 2021). To the best of our knowledge, no investigations have been conducted on the expression patterns of some calcium binding (Ionized calcium-binding adapter molecule 1 (Iba1), Calbindin, Calretinin, and Parvalbumin) and neural proteins (CNPase, NG2, and Drebrin) in the testis and epididymis throughout postnatal development. The localization and expression dynamics of nerve markers in germ and somatic cells during postnatal development may shed light on reproductive physiology. For these purposes, we studied some calcium-binding proteins (Ionized calcium-binding adapter molecule 1 (Iba1), Calbindin, Calretinin, and Parvalbumin) and neural cell markers (CNPase, NG2, and Drebrin) expression in testis and epididymis during postnatal periods.

2 | MATERIALS AND METHODS

2.1 | Experimental design and animals ethics statement

Experimental studies requiring were performed according to Ankara University guidelines and with the approval of the Ethics Committee of Ankara University, Turkey (2014-18-128). After euthanasia, the tissue samples ($n = 6$) were obtained from healthy neonatal (postnatal day 5, PND5), infantile (postnatal day 20, PND20), postpubertal (postnatal day 50, PND50), and mature (postnatal day 70, PND70) Wistar albino rats as previously described (Ojeda et al., 1980). The testicular and epididymal tissue samples were incubated in Bouin's solution for 18 h. Following fixation, samples were incubated in 70% alcohol for 15 h and dehydrated in graded alcohols. After clearing, tissues were embedded in paraffin.

2.2 | Immunohistochemistry

As described previously, we used the streptavidin-biotin-peroxidase complex method for immunohistochemical labelling (Özbek et al., 2018). In brief, the testicular and epididymal samples were cut into 5-μm thick sections by using a rotatory microtome and mounted to amino-propyl-tri-ethoxy-silane (APES)-covered slides. After the routine deparaffinization and rehydration process, the slides were washed in phosphate-buffered saline (PBS). Then, samples were incubated in 3% H_2O_2 in distilled water. For heat-induced antigen retrieval, sections were treated with the boiled citrate buffer (10 mM, pH 6.0). After washing 5 min in PBS, we encircled the samples using Dakopen. To reduce non-specific binding of the primary antibodies, slides were treated with 10% standard goat serum blocking solution.

TABLE 1 Primary antibodies used for immunohistochemistry

Antibody	Host	Application	Dilution	Catalogue no	Species reactivity
Iba1 pAb	Rabbit	IHC	1/400	FUJIFILM Wako Pure Chemical Corporation, 019–19,741	Mouse, rat and human
Calbindin 1 (CB-955) mAb	Mouse	IHC	1/400	Sigma-Aldrich, C9848	Human, mouse, rabbit, rat, bovine, feline, guinea pig, canine, pig, sheep and goat
Calretinin pAb	Rabbit	IHC	1/400	SWant, 7697	Rat, mouse, human, guinea, pig, monkey and fish, and chicken
Parvalbumin pAb	Rabbit	IHC	1/400	Calbiochem®, PC255L	Mouse, rat and human
NG2 pAb	Rabbit	IHC	1/400	Chemicon, AB5320	Mouse and rat
Drebrin pAb	Rabbit	IHC	1/400	Sigma-Aldrich, D3816	Rat andhuman
CNPase pAb	Rabbit	IHC	1/400	Sigma-Aldrich, C9743	Mouse, rat and human

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TABLE 2 Semiquantitative evaluation of Iba1, Calbindin 1, Calretinin and Parvalbumin staining in rat testis and epididymis during postnatal development

	Tissue	PND5	PND20	PND50	PND70
IBA1	Cell type				
	Testis				
	Spermatogonium	Gonocyte –	–	–	–
	Spermatocyte/spermatid		–	Steps 9–19 spermatid +++	Steps 9–19 spermatid +++
	Sperm		–	++	++
	Sertoli cells	–	–	–	
	Leydig cells			+	+
	Interstitial cells	+++	+++	+	+
	Peritubular myoid cells	–	–	–	–
	Vessel wall muscles	–	–	–	–
	Epididymis				
	Epithelial cell	–	+	I: ++, C: ++, Co: ++, Ca: ++	I: ++, C: ++, Co: ++, Ca: ++
	Interstitial cells	++	++	I: ++, C: ++, Co: ++, Ca: ++	I: ++, C: ++, Co: ++, Ca: ++
	Vessel wall muscles	–	–	I: –, C: –, Co: –, Ca: –	I: –, C: –, Co: –, Ca: –
	Muscle layer in the ductal wall	–	–	I: –, C: –, Co: –, Ca: –	I: –, C: –, Co: –, Ca: –
Calbindin 1	Testis				
	Spermatogonium	Gonocyte ++ +	++	+	+
	Spermatocyte/spermatid		++	Steps 1–9++	Steps 1–9++
	Sperm			–	–
	Sertoli cells	++	++	–	–
	Leydig cells	++	++	+	+
	Interstitial cells	++	++	+	+
	Peritubular myoid cells	+	+	–	–
	Vessel wall muscles	+	+	–	–
	Epididymis				
	Epithelial cell	+++	+++	I: ++, C: ++, Co: ++, Ca: ++	I: ++, C: ++, Co: ++, Ca: ++
	Interstitial cells	++	++	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
	Vessel wall muscles	+	+	I: –, C: –, Co: –, Ca: –	I: –, C: –, Co: –, Ca: –
	Muscle layer in the ductal wall	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
Calretinin	Testis				
	Spermatogonium	Gonocyte +	–	–	–
	Spermatocyte/spermatid		–	++	++
	Sperm			++	++
	Sertoli cells	–	–	–	–
	Leydig cells	++	++	++	++
	Interstitial cells	++	++	++	++
	Peritubular myoid cells	–	–	–	–
	Vessel wall muscles	+	+	+	+
	Epididymis				
	Epithelial cell	++	++	I: +++, C: +++, Co: +++, Ca: +++	I: +++, C: +++, Co: +++, Ca: +++
	Interstitial cells	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +

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TABLE 2 (Continued)

	Tissue	PND5	PND20	PND50	PND70
	Vessel wall muscles	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
	Muscle layer in the ductal wall	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
Parvalbumin	Testis				
	Spermatogonium	Gonocyte +	−	−	−
	Spermatocyte/spermatid		+	+	+
	Sperm			+	+
	Sertoli cells	−	+	−	−
	Leydig cells	+	+	+	+
	Interstitial cells	+	+	+	+
	Peritubular myoid cells	+	+	+	+
	Vessel wall muscles	+	+	+	+
	Epididymis				
	Epithelial cell	++	++	I: ++, C: ++, Co: ++, Ca: ++	I: ++, C: ++, Co: ++, Ca: ++
	Interstitial cells	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
	Vessel wall muscles	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
	Muscle layer in the ductal wall	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +

Abbreviations: −, negative staining; +, weak staining; ++, moderate staining; +++, strong staining; C, caput epididymis; Co, corpus epididymis; Ca, cauda epididymis; I, initial segment.

After serum blocking, the sections were overnight treated with primary antibodies (Table 1). Subsequently, samples were washed with PBS and incubated with secondary antibodies. After rinsing in PBS, the samples were treated with enzyme-conjugated streptavidin. To visualize the immunostaining, DAB (3,3′-diaminobenzidine) was used as the chromogen. Then, sections were counterstained with Gill's haematoxylin and covered in an aqueous mounting medium.

For analysis results of immune reactions, we used Olympus BX51 light microscope with cooled digital colour camera (DP72, Olympus). To confirm the specificity of immunohistochemical procedures, negative and positive control were applied to the sections.

2.3 | Quantification of immunoexpression

In the postnatal 50 and 70 days period, 14 different stages were observed in the seminiferous tubules, and the evaluations were made by considering the cell types in these stages. Moreover, we examined the epididymis by considering the initial segment, caput, corpus, and cauda parts separately. Transverse sections from each animal's testicular and epididymal tissue were first analysed with low magnification (×10) to evaluate the quality of the immunolabelling, histomorphological alterations, and cell labelling patterns during the postnatal period. Since the immunohistochemical scoring algorithms of the markers were not standardized or optimized, in the present study, immunohistochemical staining was semiquantitatively assessed based on a score (density score, IS) of immunoreaction intensity. According to staining intensities, immunostaining in testicles and epididymis is presented as negative (−) (staining cannot be seen even at high magnification—40×—), weak (+) (staining can be seen only at high magnification—40×—), moderate (++) (staining can be seen at low magnification—10×—easily visible) and strong (+++) (staining—10×—strikingly visible at low magnification) as previously described Staining intensity were examined by two independent researchers (M.Ö., F.B.), and the mean values of the data obtained from two researchers were calculated (Liman & Ateş, 2020). In four postnatal periods (PND5, PND20, PND50 and PND70), immunohistochemical reactions were evaluated separately. Semiquantitative evaluation of immunohistochemical staining is presented in Tables 2 and 3.

3 | RESULTS

3.1 | Positive and negative control

During postnatal development, testicular and epididymal tissues were examined for the expression and localization of Iba1, calbindin, calretinin, parvalbumin, CNPase, NG2 and drebrin. For antibody validation, the hippocampus, cerebellum, brain, and retina were used as positive control. For negative control, testicular and epididymal samples were treated with PBS instead of primary antibodies, and the procedures were continued. No immunoreaction was observed as a result of this procedure. Positive and negative control sections are presented in Figure 1.

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TABLE 3 Semiquantitative evaluation of CNPase, NG2, and Drebrin staining in rat testis and epididymis during postnatal development

	Tissue	PND5	PND20	PND50	PND70
CNPase	Cell type				
	Testis				
	Spermatogonium	Gonocyte + +	−	−	−
	Spermatocyte/spermatid		++	++	++
	Sperm		−	+	+
	Sertoli cells	−	−	−	−
	Leydig cells	+	+	+	+
	Interstitial cells	+	+	+	+
	Peritubular myoid cells	−	−	−	−
	Vessel wall muscles	+	+	+	+
	Epididymis				
	Epithelial cell	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
	Interstitial cells	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
	Vessel wall muscles	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
	Muscle layer in the ductal wall	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
NG2	Testis				
	Spermatogonium	Gonocyte −	−	−	−
	Spermatocyte/spermatid		+	+	+
	Sperm			+	+
	Sertoli cells	−	−	−	−
	Leydig cells	+	+	+	+
	Interstitial cells	+	+	+	+
	Peritubular myoid cells	+	+	−	−
	Vessel wall muscles	++	++	++	++
	Epididymis				
	Epithelial cell	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
	Interstitial cells	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
	Vessel wall muscles	++	++	I: ++, C: ++, Co: ++, Ca: ++	I: ++, C: ++, Co: ++, Ca: ++
	Muscle layer in the ductal wall	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +
Drebrin	Testis				
	Spermatogonium	Gonocyte + +	−	−	−
	Spermatocyte/spermatid		++	+++	+++
	Sperm			++	++
	Sertoli cells	+	+	+	+
	Leydig cells	++	++	++	++
	Interstitial cells	++	++	++	++
	Peritubular myoid cells	+	+	+	+
	Vessel wall muscles	+	+	+	+
	Epididymis				
	Epithelial cell	+++	+++	I: +++, C: +++, Co: +++, Ca: + ++	I: +++, C: +++, Co: +++, Ca: + ++
	Interstitial cells	++	++	I: ++, C: ++, Co: ++, Ca: ++	I: ++, C: ++, Co: ++, Ca: ++

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TABLE 3 (Continued)

Tissue	PND5	PND20	PND50	PND70
Vessel wall muscles	++	++	I: ++, C: ++, Co: ++, Ca: ++	I: ++, C: ++, Co: ++, Ca: ++
Muscle layer in the ductal wall	+	+	I: +, C: +, Co: +, Ca: +	I: +, C: +, Co: +, Ca: +

Abbreviations: −, negative staining; +, weak staining; ++, moderate staining; +++, strong staining; C, caput epididymis; Co, corpus epididymis; Ca, cauda epididymis; I, initial segment.

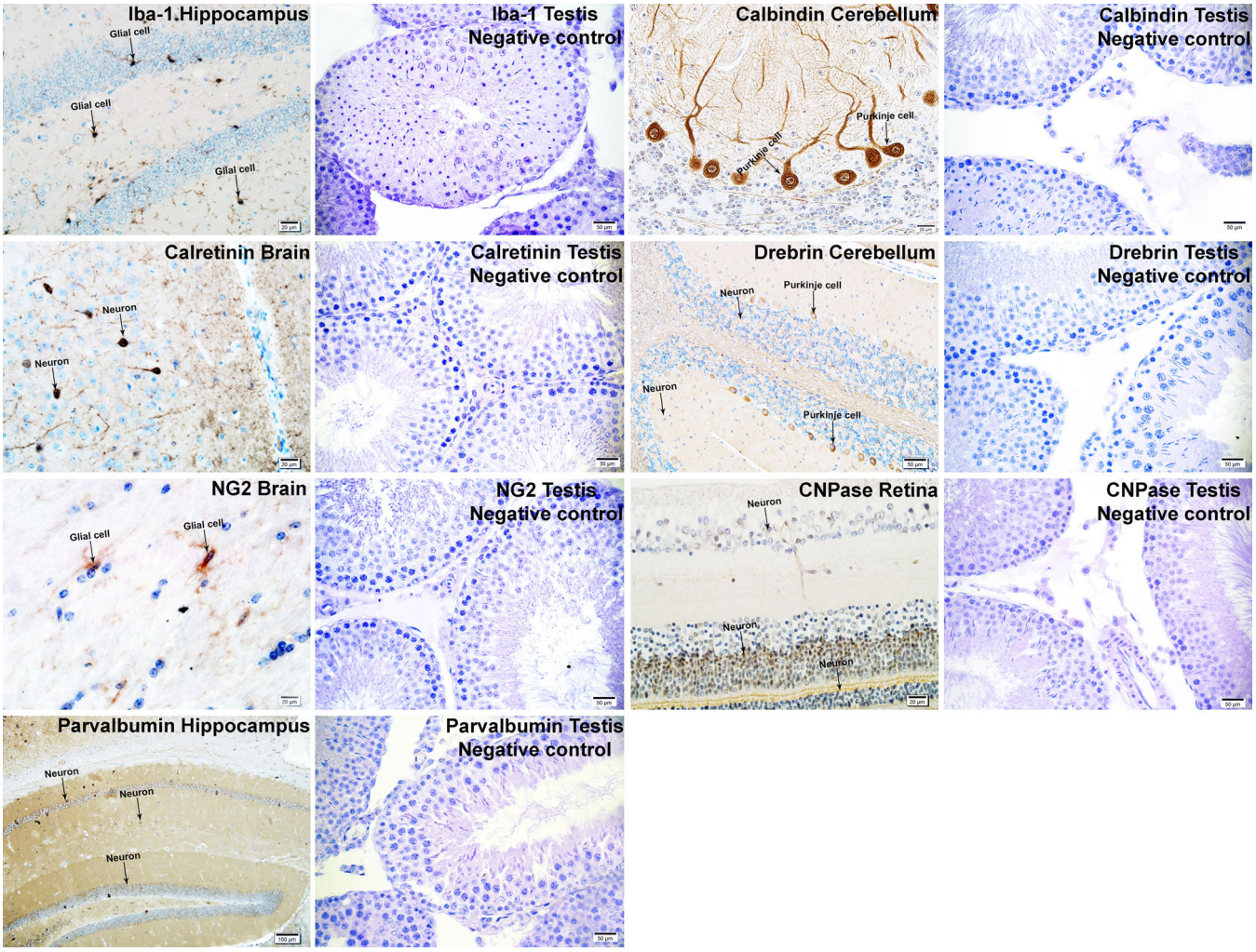


FIGURE 1 Representative figures showing the hippocampus, cerebellum, brain and retina as positive control and testis sections as a negative control for immunostaining. Strept-ABC, DAB. Iba1 staining was observed in glial cells in the hippocampus. Calbindin staining was detected in Purkinje cells in the cerebellum. Neurons were positive for calretinin in the brain. Drebrin immunostaining was observed in neurons and Purkinje cells in the cerebellum. Glial cells were positive for NG2 in the brain. CNPase immunoreaction was detected in neurons in the retina. Neurons were positive for parvalbumin in the hippocampus. No immunoreaction was observed in the testis sections that were incubated without the primary antibody

3.2 | Iba1

In the neonatal period, we observed positive labelling in some interstitial cells in the testis and epididymis. In addition, some epididymal epithelium and interstitial cells were stained with Iba1 in the infantile period. In the postpubertal and mature period, we detected intense immunoreactivity in elongated spermatids (steps 9–19) and weak labelling in Leydig cells. Moreover, some basal cells of the epididymal epithelium and interstitial cells were stained with Iba1 in all epididymal parts (Figure 2).

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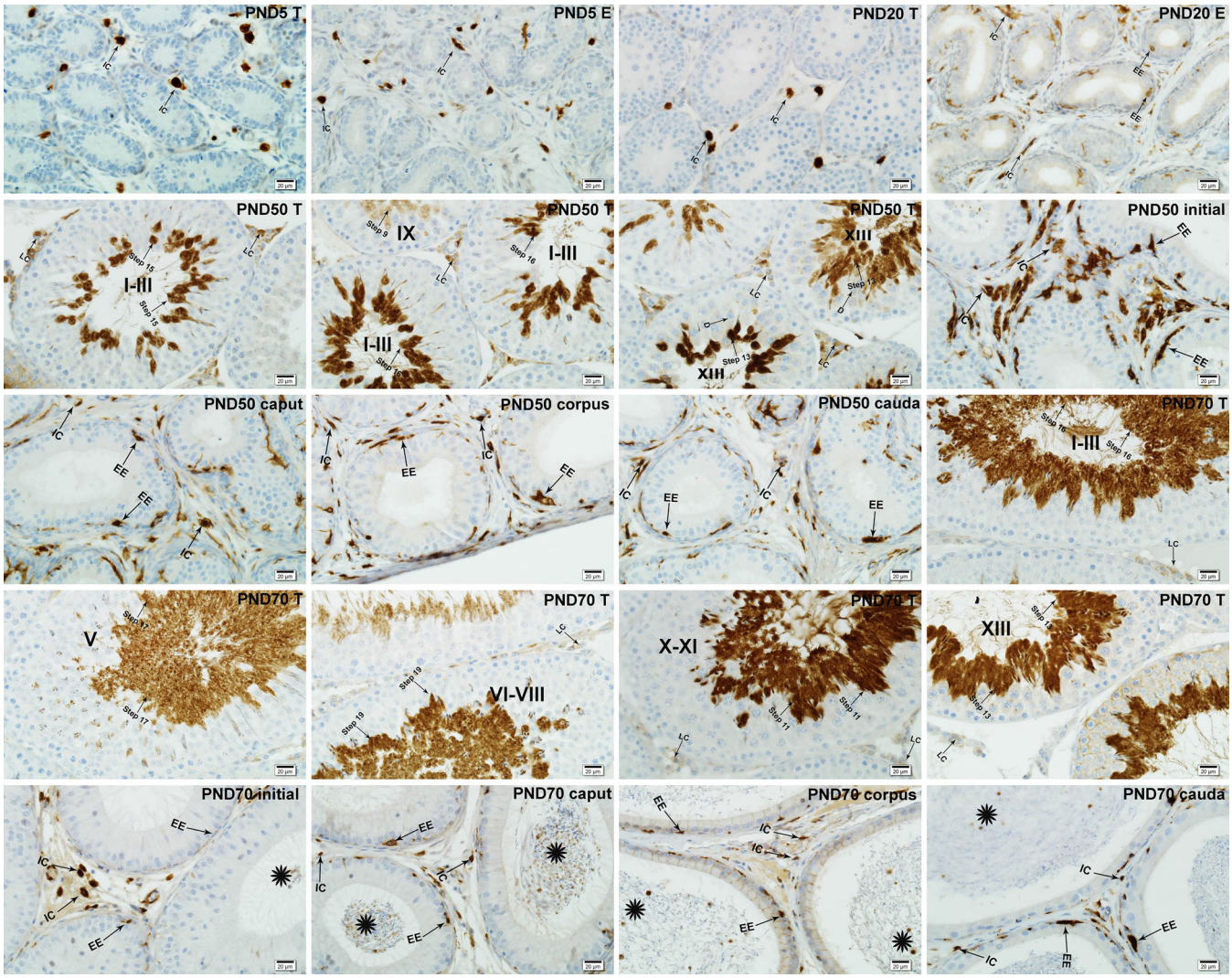


FIGURE 2 Representative immunostaining images showing Iba1 in testis and epididymis on postnatal days 5 (PND5), 20 (PND20), 50 (PND50) and 70 (PND70). Strept-ABC, DAB. Intense staining in interstitial cells (IC) of testis and epididymis on PND5. Moderate staining in IC and weak staining in some epididymal epithelium on PND20. Intense immunoreactivity in elongated spermatids (steps 9–19) in different seminiferous tubule stages (indicated by Roman numerals) and weak labelling in Leydig cells (LC) on PND50 and PND70. Moderate staining in sperm (asterisk) on PND50 and PND70. Moderate staining in some basal cells of the epididymal epithelium (EE) and IC of all epididymis segments including initial segment, caput, corpus, and cauda. D, diplotene spermatocyte

3.3 | Calbindin 1

In the neonatal period, we detected nuclear immunolabelling in gonocytes, progenitor Leydig cells, peritubular myoid cells, and interstitial cells. Furthermore, all epithelial cells of the epididymis, vessel walls, and some interstitial cells showed nuclear immunoreaction in this period. We observed nuclear immunoreaction in spermatogonium, spermatocytes, Sertoli cells, Leydig cells, epididymal epithelium, and some interstitial cells in the infantile period. In the postpubertal and mature period, calbindin immunolabelling was found in spermatogonium, spermatocytes, and round spermatids (Steps 1–9) but not in elongated spermatids (Steps 9–19) and sperm. We observed calbindin-positive (cytoplasmic and nuclear) epithelial cells in all epididymal sections. Epididymal smooth muscle, some interstitial cells, and vessels wall also showed positive immunolabelling in this period (Figure 3).

3.4 | Calretinin

In the neonatal and infantile period, we found immunoreaction in gonocytes, Leydig cells, epididymal epithelium, vessels wall, some interstitial cells, and epididymal smooth muscles. In the postpubertal and mature period, positive immunolabelling was observed in spermatocytes, round and elongated spermatids, sperm, and Leydig cells. Most of the epididymal epithelium, some interstitial cells, and epididymal smooth muscles also showed immunoreaction in all epididymal parts (Figure 4).

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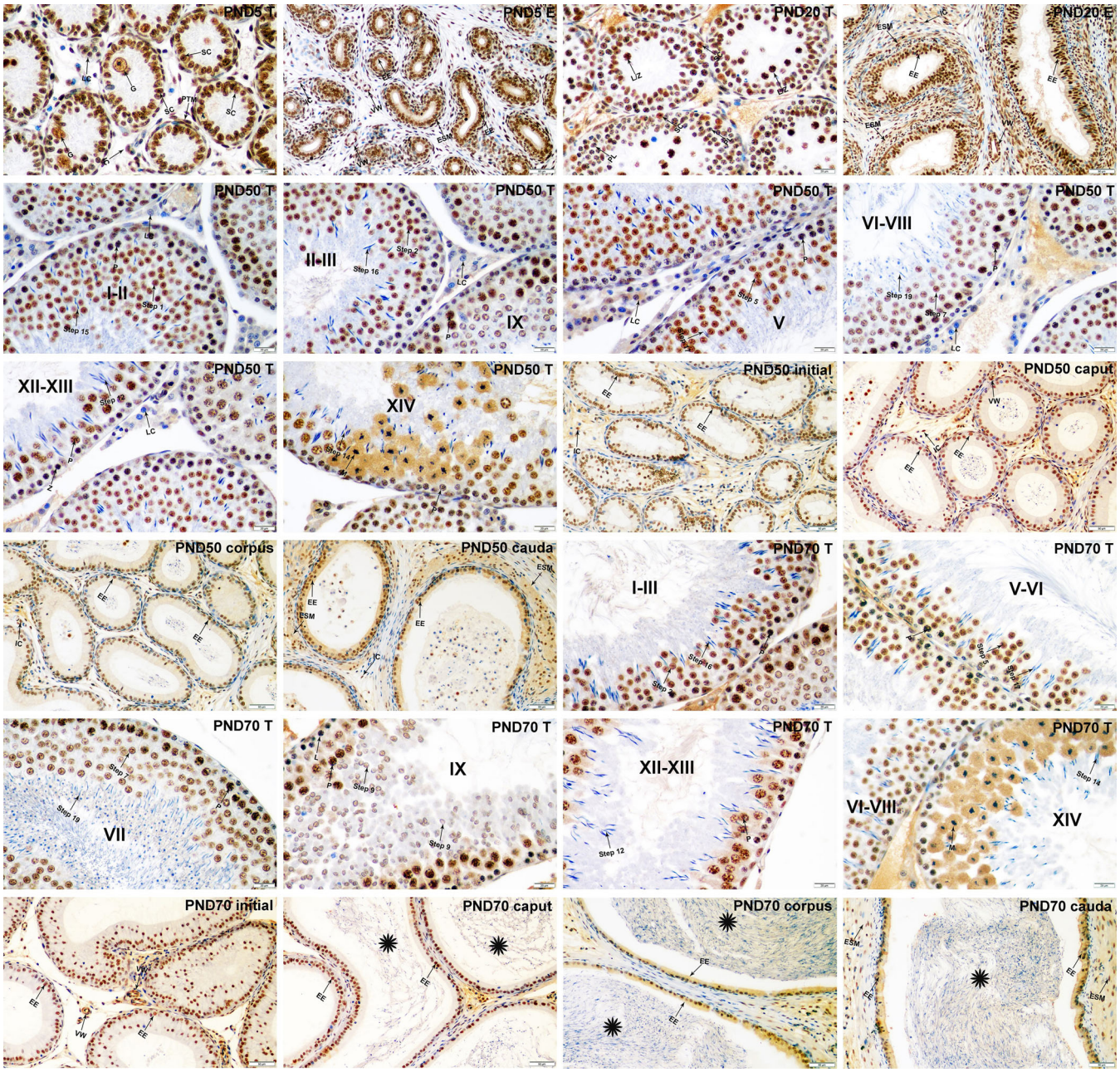


FIGURE 3 Representative immunostaining images showing Calbindin in testis and epididymis on postnatal days 5 (PND5), 20 (PND20), 50 (PND50) and 70 (PND70). Strept-ABC, DAB. On PND5, intense nuclear staining in gonocytes (G), moderate nuclear staining in progenitor Sertoli cells (SC), Leydig cells (LC), and some interstitial cells, weak staining in peritubular myoid cells (PTM). On PND 20, moderate staining in primary spermatocytes (L/Z, leptotene/zygotene; PL, pre-leptotene), Sertoli cells (SC) and weak staining in peritubular myoid cells (PTM). On PND5 and PND20, intense nuclear staining in the epididymal epithelium (EE), weak staining in vessels wall and epididymal smooth muscles (ESM). On PND50 and PND70, weak staining in LC and interstitial cells (IC), epididymal smooth muscles (ESM), moderate staining in round spermatid (steps 1–9), the epididymal epithelium (EE).

3.5 | Parvalbumin

In the neonatal period, an immunoreaction was seen in gonocytes, progenitor Leydig cells, peritubular myoid cells, epididymal epithelial cells, vessels wall, epididymal smooth muscles, and some interstitial cells. We also observed immunolabelling in spermatocytes, Sertoli cells, vessels wall, Leydig cells, peritubular myoid cells, epididymal epithelium, epididymal smooth muscles, and some interstitial cells in the infantile period. In the postpubertal and mature period, a parvalbumin-positive reaction was found in spermatocytes, round and elongated spermatids, sperm, Leydig cells, and vessels wall. Furthermore, we observed immunoreaction in all epididymal epithelium, some interstitial cells, and epididymal smooth muscles (Figure 5).

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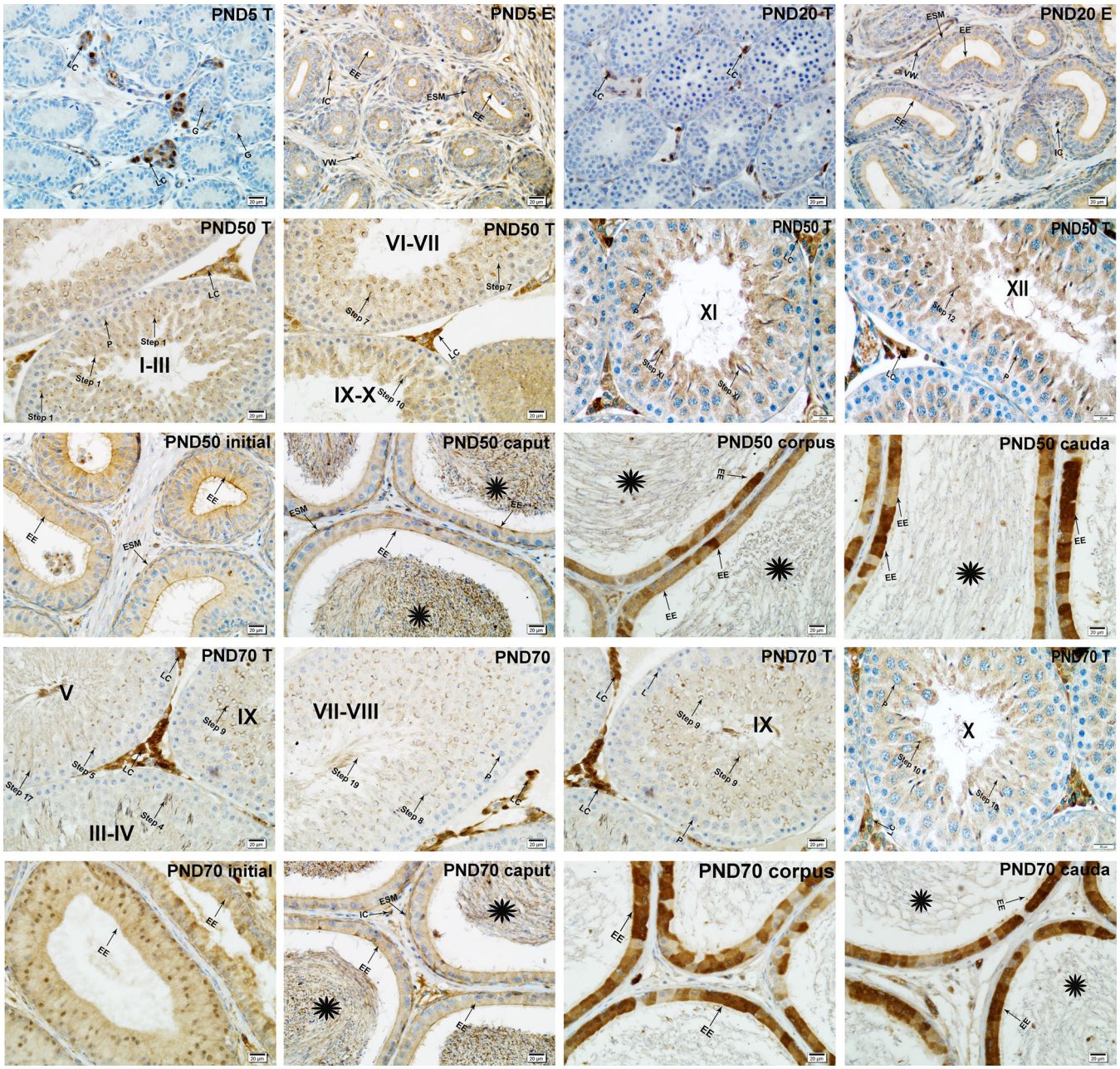


FIGURE 4 Representative immunostaining images showing Calretinin in testis and epididymis on postnatal days 5 (PND5), 20 (PND20), 50 (PND50), and 70 (PND70). Strept-ABC, DAB. On PND5 and PND20, weak staining in gonocytes, vessels wall (VW), interstitial cells (IC) of the epididymis, and epididymal smooth muscles (ESM), moderate immunostaining in Leydig cells (LC), IC of testis and epididymal epithelium (EE). On PND50 and PND70, weak staining in VW, IC of the epididymis, and ESM, moderate immunostaining in primary spermatocytes (P, pachytene), the round (steps 1–9) and elongated spermatids (steps 9–19), sperm, and IC of the testis, intense immunostaining in EE (EE) of all epididymis segments including initial segment, caput, corpus, and cauda

3.6 | CNPase

We observed an immunoreaction in gonocytes, Leydig cells, spermatocytes, Sertoli cells, epididymal epithelium, and vessel walls in the neonatal and infantile periods. In the postpubertal and mature period, positive immunoreaction was present in spermatocytes, round and elongated spermatids, sperm, vessel walls and Leydig cells. In addition, all epididymal epithelium and epididymal smooth muscles showed immunoreaction in all epididymal parts in this period (Figure 6).

3.7 | NG2

In the neonatal and infantile period, we detected intense immunoreaction in Leydig cells, peritubular myoid cells, vessels wall, and epididymal

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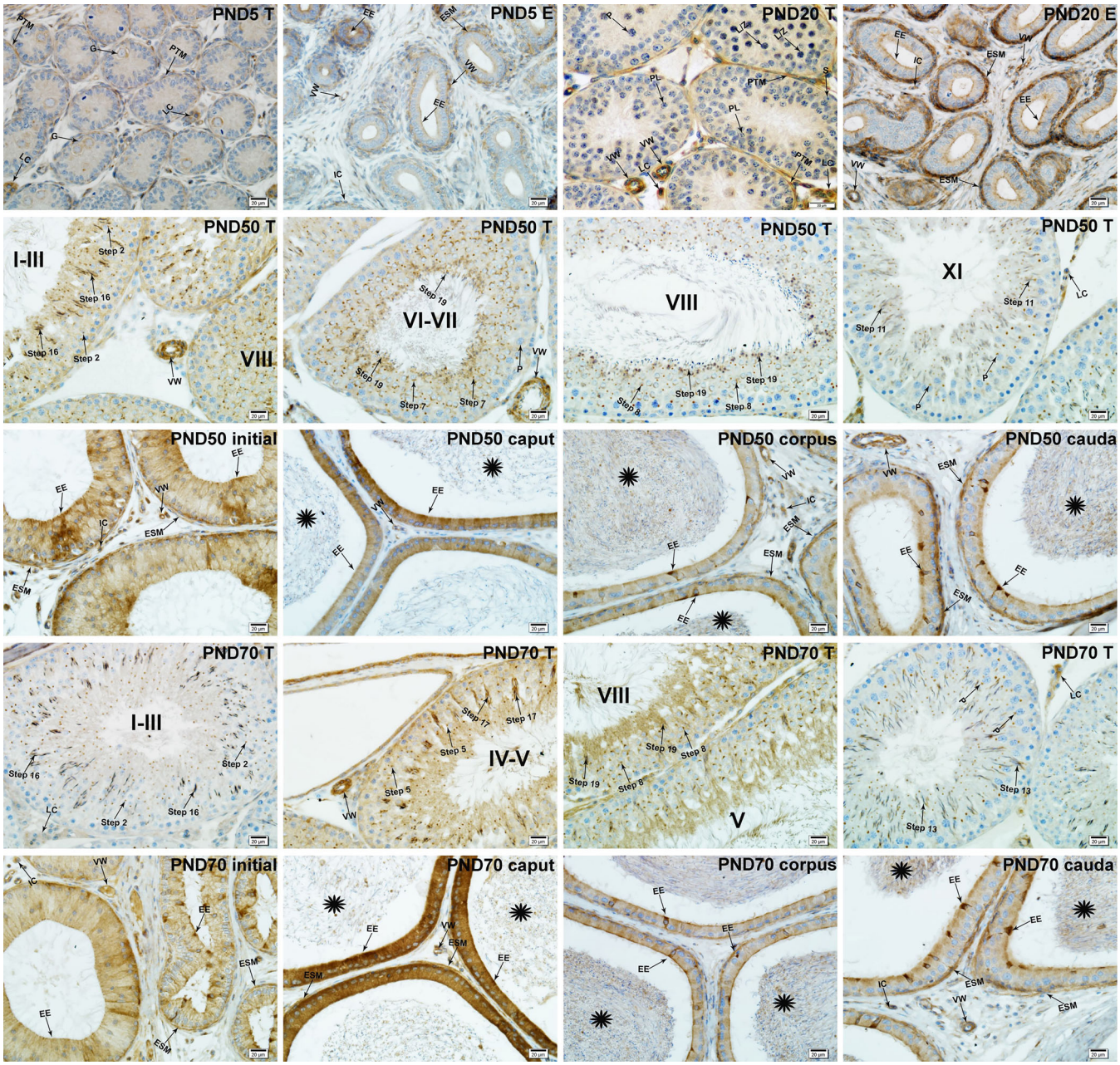


FIGURE 5 Representative immunostaining images showing Parvalbumin testis and epididymis on postnatal days 5 (PND5), 20 (PND20), 50 (PND50) and 70 (PND70). Strept-ABC, DAB. On PND5, PND20, PND50 and PND70, weak immunostaining in gonocytes, spermatocytes (L/Z, leptotene/zygotene; P, pachytene; pre-leptotene, PL), the round (steps 1–9) and elongated spermatids (steps 9–19), sperm, peritubular myoid cells (PTM), Leydig cells (LC), interstitial cells (IC), and vessels wall (VW), moderate immunostaining in the epididymal epithelium (EE)

smooth muscles, but epididymal epithelium showed weak immunolabelling. In the postpubertal and mature period, positive immunoreaction was observed in spermatocytes, round and elongated spermatids, vessel walls, and Leydig cells. We also detected immunolabelling in the vessel wall and all epididymal epithelium in all epididymal parts in this period (Figure 7).

3.8 | Drebrin

In the neonatal period, intense immunoreaction was seen in gonocytes, progenitor Leydig cells, peritubular myoid cells, Sertoli cells, epididymal epithelial cells, vessels wall, epididymal smooth muscles, and some interstitial cells. We also observed intense immunolabelling in spermatocytes, Sertoli cells, vessels wall, Leydig cells, peritubular myoid cells, epididymal epithelium, epididymal smooth muscles, and some interstitial cells in the infantile period. In the postpubertal and mature period, an intense drebrin-positive reaction was present in spermatogonium, spermatocytes, round and elongated spermatids, sperm, Sertoli cells, Leydig cells and vessels wall. Moreover, we observed intense immunoreaction in all epididymal epithelium of the epididymal parts. Some interstitial cells and epididymal smooth muscles also showed immunoreaction in the postpubertal and mature periods (Figure 8).

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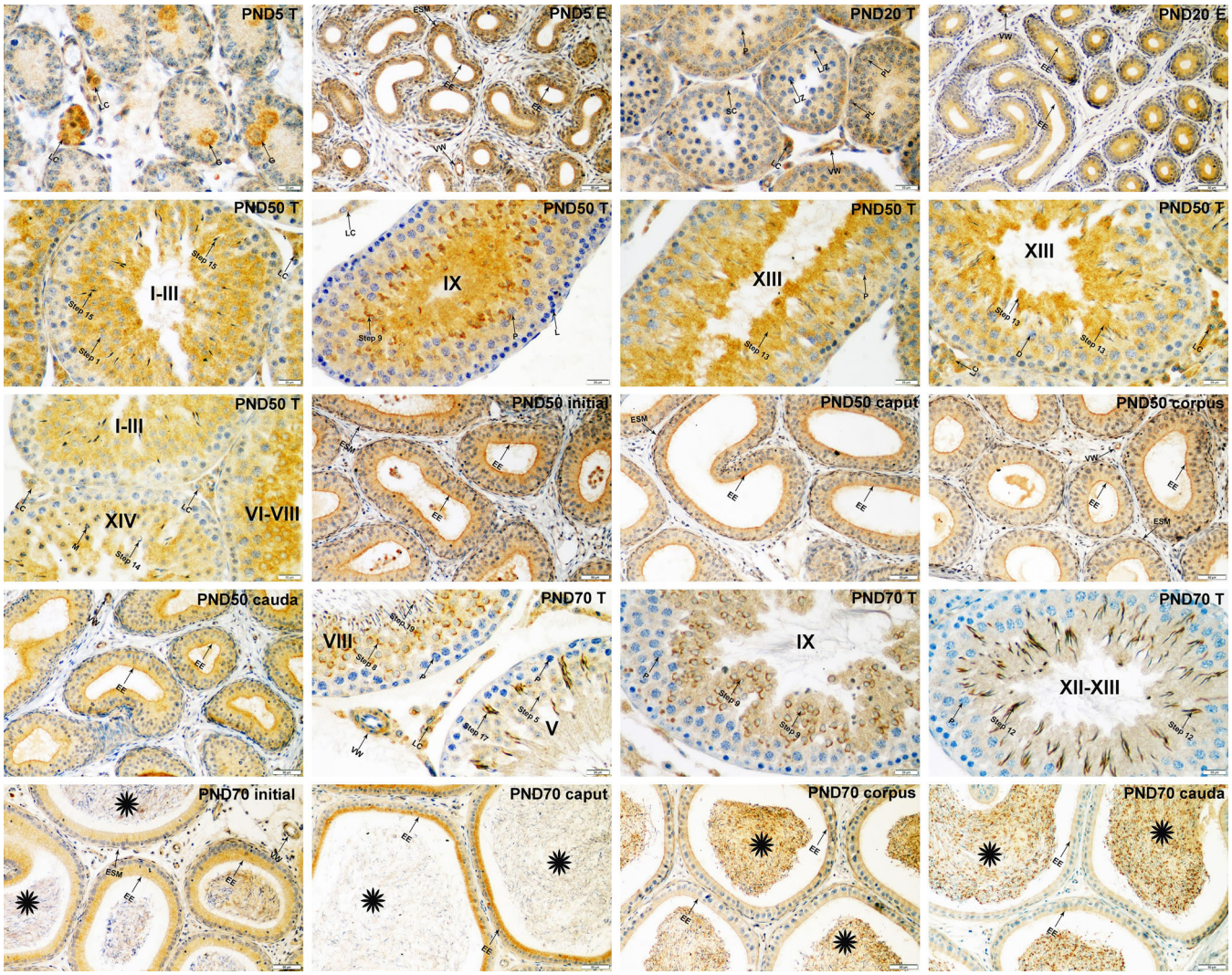


FIGURE 6 Representative immunostaining images showing CNPase testis and epididymis on postnatal days 5 (PND5), 20 (PND20), 50 (PND50) and 70 (PND70). Strept-ABC, DAB. On PND5, PND20, PND50 and PND70, weak immunostaining in Leydig cells (LC), interstitial cells (IC), vessels wall (VW), the epididymal epithelium (EE), epididymal smooth muscles (ESM), and sperm, moderate immunostaining in gonocytes, spermatocytes (L/Z, leptotene/zygotene; P, pachytene; PL, pre-leptotene), the round (steps 1–9) and elongated spermatids (steps 9–19)

4 | DISCUSSION

Here, we report an immunohistochemical analysis of Iba1, calbindin 1, calretinin, parvalbumin, CNPase, drebrin, and NG2 expression in rat testis and epididymis during postnatal development. Not only did their cellular expression show differences, but also showed alteration of expression profile in testis and epididymis throughout postnatal development.

Iba-1 is a calcium-binding protein, which is specifically and constitutively expressed in all microglia. This protein was expressed in both the nucleus and cytoplasm of glial cells. Iba-1 is reported to function in actin-crosslinking and involves phagocytosis and membrane ruffling. Moreover, increased Iba-1 expression is associated with microglial activation (Ohsawa et al., 2004; Sasaki et al., 2001). However, Iba-1 protein is also observed in rat testis, especially in elongated spermatids. In addition, this expression was observed diffusely in stage 10–12 spermatids, more concentrated in stage 18 spermatids, and extracted in the final stages of spermatid (Iida et al., 2001). In this study, Iba-1 expression was observed in elongated spermatids. It is suggested that this situation is regulated in postpubertal and mature period and depends on spermiogenesis. Iba-1 may participate in the rearrangements of the actin filaments during spermiogenesis (Iida et al., 2001). We also observed an immune reaction in epididymal epithelial cells. We speculate that this reaction originates from halo cells located in the epididymal epithelium.

Immunohistochemical studies have shown that calbindin-D28K is localized in spermatocytes and spermatogonia in rat and chick testes. Calbindin-D28K expression also was observed in some Leydig cells (Inpanbutr & Taylor, 1992; Kägi et al., 1988). Furthermore, some studies have reported that calbindin and vitamin D receptor expression is

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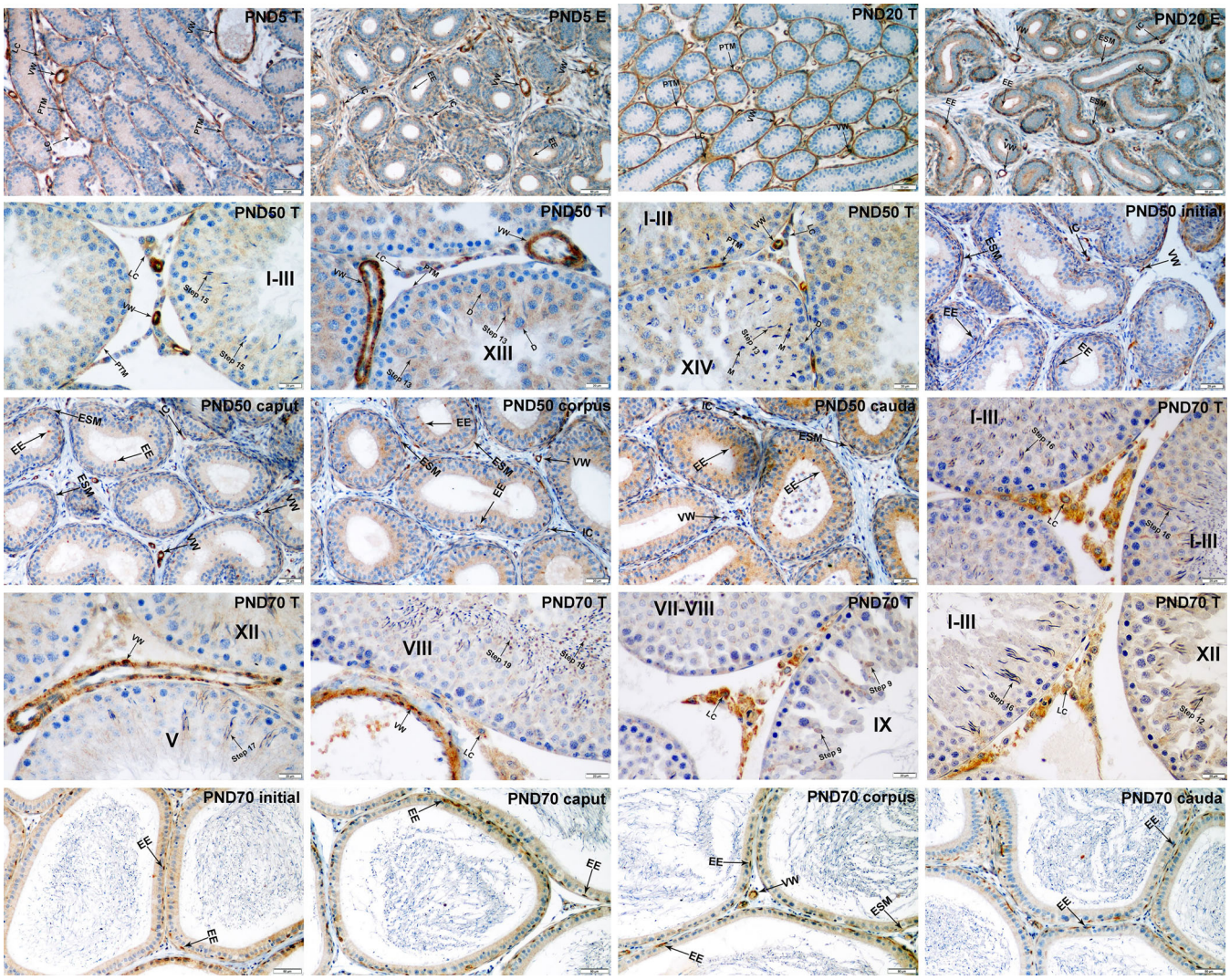


FIGURE 7 Representative immunostaining images showing NG2 testis and epididymis on postnatal days 5 (PND5), 20 (PND20), 50 (PND50), and 70 (PND70). Strept-ABC, DAB. On PND5, PND20, PND50 and PND70, weak immunostaining in Leydig cells (LC), interstitial cells (IC), peritubular myoid cells (PTM), the epididymal epithelium (EE), epididymal smooth muscles (ESM), spermatocytes (D, diplotene; M, Meiotic spermatocytes; P, pachytene), the round (steps 1–9) and elongated spermatids (steps 9–19), moderate immunostaining in vessels wall (VW) of testis and epididymis

positively correlated with testicular maturation (Kägi et al., 1988; Walterst, 2016). Moreover, it has been claimed that calbindin-D28k protects against necrotic and apoptotic cell death by suppressing caspase-3 and buffering calcium (Shum et al., 2022). In androgen-deficient male mice, it was determined that TRPV5 and calbindin-D28K mRNA and protein decreased and it returned to normal levels with testosterone administration (Hsu et al., 2010). In the present study, we observed calbindin expression in germ cells, Leydig cells, Sertoli cells, and some interstitial cells in concordance with previous studies. However, this expression gradually decreased in germ and somatic cells during postnatal development. As previously claimed (Hsu et al., 2010), we suggest that this expression reduction is related to the increased androgenic hormone level after puberty.

As in other systems, Ca^{2+} homeostasis in the epididymis is modulated by a network of signalling pathways and cell–cell interactions. The dysregulation of Ca^{2+}-regulated proteins and Ca^{2+} levels in the epididymis interrupts this network and causes male infertility (Shum et al., 2022). In roosters, calbindin-D28k expression was observed in the cytoplasm of the epithelial cells lining the efferent ductules, rete testis, and epididymal duct. Moreover, it has been reported that positive immunoreaction for calbindin-D28k was detected in principal cells of epididymal epithelium. The researchers also found positive staining in many mononuclear cells in the epididymal connective tissue (Oliveira et al., 2012). In our study, a positive reaction was detected in all epididymal epithelial cells. Parts of the epididymis showed similar reactions in terms of staining properties. However, a decrease was observed in the reaction intensity in epithelial cells as in testis after puberty. This provides evidence suggesting that puberty affects calbindin expression in testis and epididymis.

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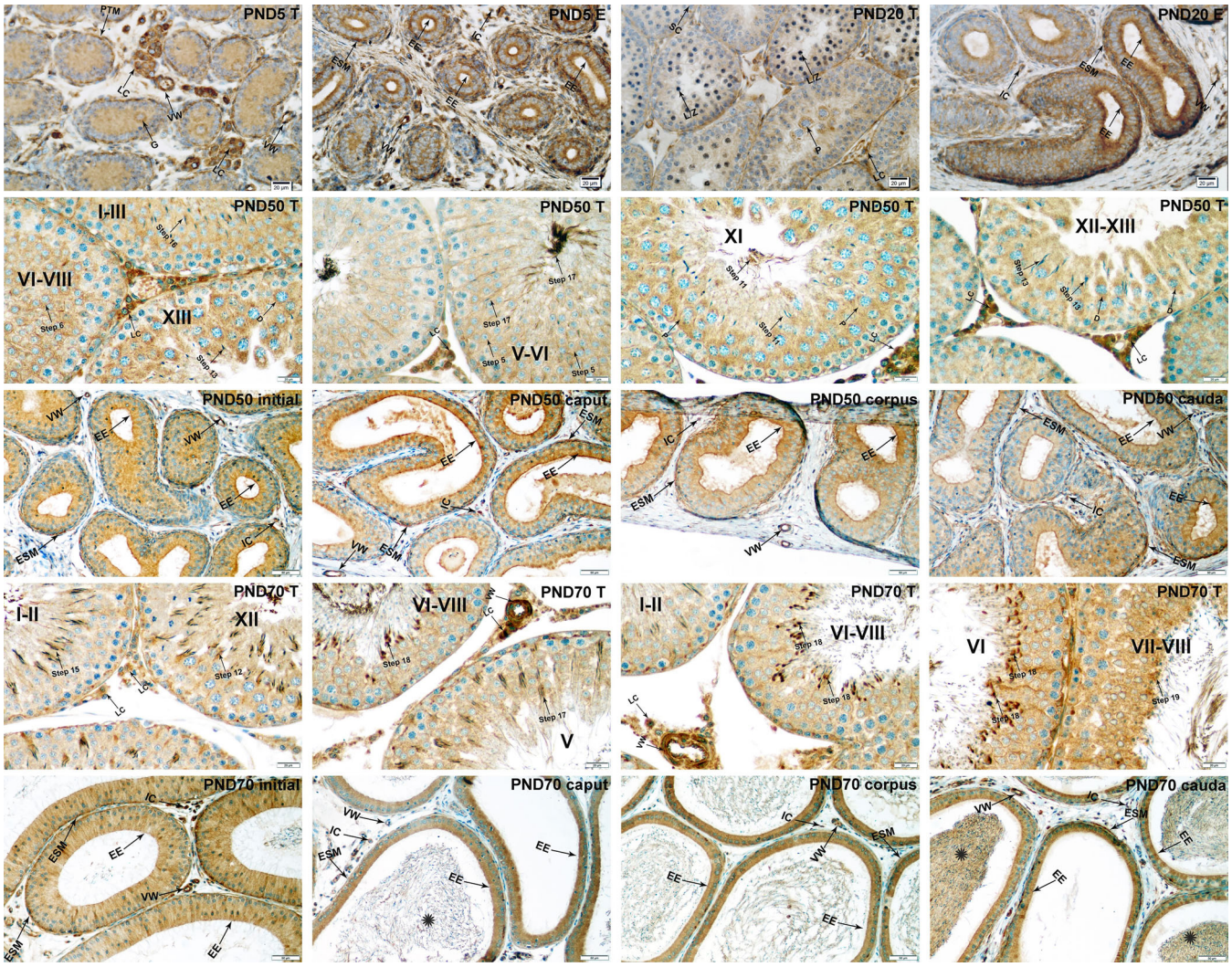


FIGURE 8 Representative immunostaining images showing Drebrin testis and epididymis on postnatal days 5 (PND5), 20 (PND20), 50 (PND50), and 70 (PND70). Strept-ABC, DAB. On PND5 and PND20, weak staining in Sertoli cells (SC), vessels wall (VW) of the testis, peritubular myoid cells (PTM), and epididymal smooth muscles (ESM), moderate staining in gonocytes, spermatocytes (L/Z, leptotene/zygotene; P, pachytene; PL, pre-leptotene), interstitial cells (IC), Leydig cells (LC), and VW of the epididymis, intense staining in the epididymal epithelium (EE). On PND50 and PND70, weak staining in SC, PTM, VW of the testis, and ESM, moderate immunostaining in the round (steps 1–9) and elongated spermatids (steps 9–19), sperm, LC, IC and VW of the epididymis, intense immunostaining in EE OF all epididymis segments including initial segment, caput, corpus and cauda

Calretinin is predominantly present in the nervous system. Moreover, it is expressed in the testis, ovary, and adrenal glands (Afework & Burnstock, 1995; Barinka & Druga, 2010; Hack et al., 2000; Strauss et al., 1994). The significant effect of calretinin is as the buffer of intracellular calcium ions to inhibit the overload of Ca^{2+}. Furthermore, calretinin is able to work as the Ca^{2+} receptor (Schwaller, 2014). Ca^{2+}, as a second messenger in the cytoplasm, is involved in a variety of biological functions, such as cell apoptosis and proliferation, the pass of neurotransmitters in nerve cells, smooth muscle cell contraction, and so forth (Berridge et al., 2000, 2003; Ikura et al., 2002; Walterst, 2016). Moreover, Ca^{2+} participates in the modulation of the production of sex hormones. In humans, calretinin was observed in Leydig cells during adult and fetal life (Altobelli et al., 2017; Augusto et al., 2002). There is a direct correlation between the amount of calretinin in the testis and the number of Leydig cells in the fetal period (Altobelli et al., 2017). Moreover, calretinin is reportedly involved in modulating steroidogenesis by PLC-Ca^{2+}-PKC pathway in Leydig cells (Xu et al., 2018). We observed intense calretinin expression in Leydig cells at all stages of the postnatal period, as in previous studies. Only gonocytes showed calretinin expression in the germ cells during the neonatal periods. In accordance with our findings, Altobelli et al. (2017) observed calretinin expression in human testicular germ cells in the fetal period. The same researcher claimed that no reaction was found in the germ cells of the seminiferous tubules during the neonatal period (Altobelli et al., 2017). We detected calretinin expression in spermatocytes, spermatids, and spermatozoa after puberty. Calretinin expression in spermatids, and spermatozoa may be related to the sperm acrosome

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reaction. Because there is an increase in the intracellular concentration of Ca2+ ([Ca2+]i) in the course of acrosome reaction (Darszon et al., 1999; Sánchez-Cárdenas et al., 2014). Calretinin may be a protein involved in the calcium homeostasis of the acrosome reaction and therefore begins to appear in germ cells after puberty. As stated above, expression of calretinin was also in human epididymal cells in the fetal period as in the present study. Additionally, we detected a gradual increase in calretinin expression in epididymal cells from infantile to mature periods. In addition, calretinin expressions were relatively higher in the corpus and cauda epididymis than in other epididymal parts. Sperm maturation occurs in the unique microenvironment of the epididymal lumen, which is a complex mixture of proteins, ions and other organic molecules created by the specific absorptive and secretory activities of the epididymal epithelium (Guyonnet et al., 2011). Some studies have shown that regucalcin, one of the calcium-binding proteins, is participated in maintaining Ca^{2+} haemostasis in the epididymal fluid. Similarly, calretinin may be participated in maintaining calcium homeostasis of the epididymal fluid.

Parvalbumin is a member of the EF-hand calcium-binding proteins. They have a broad expression in the muscle and brain (Celio, 1990; Schwaller, 2010). Parvalbumin levels are generally strongly modulated throughout the developmental process (Erickson & Lewis, 2002; Reynolds & Beasley, 2001). For example, Kägi et al. (1987) observed strong parvalbumin immunoreaction in fetal Leydig cells of the rat. This expression increased until puberty, then gradually decreased. In this study, parvalbumin expression was observed in Leydig cells in all postnatal periods, but the immunoreaction in Leydig cells at different periods was not significantly different in terms of semiquantitative evaluation. We also observed parvalbumin expression in germ cells (gonocytes, spermatocytes, spermatids, and spermatozoa), consistent with previous studies (Kägi et al., 1988). The expression in spermatids and spermatozoa was intense in the acrosomal region, suggesting that parvalbumin may mediate Ca^{2+}-dependent events during the acrosome formation (U. Kägi et al., 1987). Intracellular Ca^{2+} also modulates muscle contraction. Parvalbumin plays an essential role as a cytosolic Ca^{2+} buffer in skeletal muscle fibres (Butera et al., 2021). In this study, we detected an immunoreaction in the smooth muscles of the vessels, epididymal ducts, and seminiferous tubules in all postnatal periods. Parvalbumin expressed in the smooth muscles may be participated in the regulation of muscle contraction as in skeletal muscles.

CNPase is a membrane-anchored enzyme that hydrolyzes cyclic nucleotides to monophosphates. Most CNPase expression studies have focused on its roles in myelinogenesis and myelin-producing glial cells. However, recent studies have shown the existence of CNPase in non-myelinating cells, such as neuroblastoma and adrenal cells (McFerran & Burgoyne, 1997; Müller et al., 1981). Moreover, some studies detected a remarkable level of CNPase expression and CNPase-like enzyme activity in membranes of cells in the bone marrow, skeletal muscles, heart, spleen, liver, and kidney (Brdiczka et al., 1998; Cao et al., 2007; Halestrap & Brenner, 2003). CNPase enzyme activity and expression were reported to be explicitly localized in the mitochondrial membranes (Dreiling et al., 1981; Galvita et al., 2009; Lee et al., 2005; McFerran & Burgoyne, 1997). Researchers observed the acceleration of calcium release from the mitochondria in CNPase knockdown oligodendrocytes, suggesting that CNPase could be involved in the modulation of mitochondrial Ca^{2+} fluxes and may modulate the mitochondrial permeability transition pore (Azarashvili et al., 2009). To the authors' knowledge, there are few studies on CNPase expression in testis (Davidoff et al., 2002), but none in the epididymis. In testis, CNPase expression was observed in Sertoli cells, Leydig cells, some elongated cells in the intertubular space ("Cocells"), and blood vessels. However, we detected CNPase expression in germ cells, including gonocytes, spermatocytes, spermatid, and sperm. CNPase expression was also found in Leydig cells, some interstitial cells, smooth muscles, and epididymal epithelium. Moreover, no significant difference was observed in the CNPase expression of testis and epididymis throughout postnatal development. Davidoff et al. (2002) claimed that the expression of CNPase in Leydig cells may be related to the similarity of Leydig cells to nerve and glial cells with some features. First, Leydig cells have neuroendocrine properties and express some neuroactive substances. Second, Leydig cells are somewhat similar to multipotential neuroectodermal cells (Davidoff et al., 2002). We considered that CNPase expression in germ cells and epididymal epithelial cells might be associated with calcium metabolism in some vital physiological functions of sperm, such as acrosome reactions, capacitation, and epididymal maturation.

NG2 is a transmembrane protein and is mainly known as a marker of an activated mitogenic cell (Yadavilli et al., 2016). Hence, NG2 is present on many progenitors as well as cancer cells. It is involved in adhesion, angiogenesis, motility, cell proliferation, tumour invasion, and neovascularization. NG2 induces cell proliferation by stimulating integrin on the cell surface and activating growth factor receptor potentiates (Goretzki et al., 1999; Makagiansar et al., 2007). Moreover, it interacts with fibronectin and type VI collagen (Burg et al., 1996). NG2 proteoglycan is found on many progenitor cells, including chondroblasts, keratinocytes and oligodendrocyte progenitors. It is also present in some tumours such as chondrosarcoma, melanoma, osteosarcoma, leukaemia, and glioma (Pluschke et al., 1996; Schwartz et al., 2003; Shoshan et al., 1999). Little is known about NG2 expression in testis and epididymis. In mouse embryos, Combes et al. (2009) observed NG2 expression throughout the gonad in the interstitium and at lower levels in the testis cords. They also detected NG2 expression in the mesonephric tubules in the mesonephros. In rats, Davidoff et al. (2004) observed NG2 immunoreactivity in pericytes, vascular smooth muscle cells, and Leydig cells. In the present study, we observed NG2 expression in Leydig cells and vascular smooth muscles, consistent with previous studies. However, we also detected NG2 immunoreaction in germ cells and epididymal epithelium in each postnatal period. The presence of NG2 in germ cells suggests that NG2 may play a role in the differentiation of male germ cells.

Actin is one of the three major components of the cytoskeleton in eukaryotic cells, which exists either as filamentous actin (F-actin) or globular actin (G-actin). Germ and Sertoli cells also express actin filament (Pellegrin & Mellor, 2007). Drebrin binds to actin microfilaments, changes the helical twist of actin filaments, strengthens their stiffness, and stabilizes their structures (Mikati et al., 2013; Sharma et al., 2011).

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Studies have shown that Drebrin E is expressed in the rat testis and is mainly found in Sertoli cells (Li et al., 2011). Studies have also shown that actin is present in organelle of spermatogenic cells such as the manchette and acroplaxome. The acroplaxome is an actin containing a cytoskeletal plate anchored to the acrosome and the nuclear envelope of the spermatid. The acroplaxome plays an essential role in acrosomal sac transformation by fusing and packaging Golgi-derived proacrosomal vesicles (Kierszenbaum et al., 2003; Kierszenbaum & Tres, 2004). During the seminiferous epithelial cycle in rat testes, Drebrin E displayed a stage-specific localization at the apical ectoplasmic specializations (ES) and the basal ES at the blood–testis barrier (BTB). It is first observed at stage V tubules at the basal ES. However, Drebrin E is downregulated markedly by stages VII and VIII (Li et al., 2011).

This study observed Drebrin expression in Leydig, Sertoli, and germ cells, parallel to the above studies. In the semiquantitative evaluation, we did not observe any difference in Drebrin expression density in Sertoli cells during postnatal development. However, in mature periods, we observed a more intense immune reaction in elongated spermatids (steps 12–19). We suggest that this reaction difference may be due to acrosomal development during the epithelial cycle. The occurrence of the reactions predominantly in the heads of spermatids supports our speculation. We also observed Drebrin expression in epididymal epithelial cells.

Interestingly, Drebrin expression was primarily at the apical ends of epithelial cells during the neonatal and infantile periods. We attributed this to the fact that stereocilia, as is known (Tilney et al., 1992), have actin filaments in their structure and are located on the apical surface of the epithelium. Drebrine may play an essential role in the physiology of stereocilia during these periods.

5 | CONCLUSION

Calcium-binding proteins and nerve markers have a dynamic expression in both epididymis and testis during postnatal development. The presence of calcium-binding proteins in germ and somatic cells in the testis and in the epididymal epithelium suggest that they play an essential role in spermatogenesis. Interestingly, the expression of neuronal markers in testis support that even though the brain's and testis' structural and functional properties are different, they may have some similar properties.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Özbek, M., Beyaz, F., Ergün, E., Ergün, L., Karaca, H., Cabir, A., & Alesawi, Y. A. K. (2022). Identification of some calcium binding proteins and neural cell markers in rat testis and epididymis during postnatal development. *Andrologia*, e14633. https://doi.org/10.1111/and.14633

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