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ORIGINAL ARTICLE

M.E. Pirker · S. Montedonico · U. Rolle · H. Austvoll
P. Puri

Regional differences in nitrergic neuronal density in the developing porcine urinary bladder

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Abstract Nitric oxide (NO) is involved in normal bladder physiology by regulating local arteriolar tone and smooth muscle relaxation and modulating the production of extracellular matrix proteins in vitro. Little information is available regarding the nitrergic innervation of the bladder during development. In this study we investigated the changes in density and morphology of the intramural nitrergic neurons of the porcine urinary bladder during development using whole-mount preparation. Bladder specimens were obtained from porcine foetuses of gestational age 60 days ($n = 5$) and 90 days ($n = 5$) and from newborn piglets ($n = 5$) after perfusion fixation. Bladders were divided into base, body, and dome. Whole-mount preparation using NADPH-diaphorase (NADPH-d) histochemistry was used to visualize nitrergic innervation of the urinary bladders and to measure density of NADPH-positive ganglia (including single neurons), number of NADPH-d positive neurons per ganglion, and size of individual neurons. One-way ANOVA and chi-square tests were used for statistical analysis with a p-value < 0.05 considered statistically significant. NADPH-d positive ganglia were numerous in the muscular layer of all three age groups. At E60, ganglion density was significantly higher in the body (mean 880/cm^2) than in the dome (397/cm^2) or the base (676/cm^2). The ganglion density significantly decreased with age. The number of NADPH-d positive neurons per ganglion increased significantly between E90 and birth ($p < 0.01$). A marked increase in the size of individual neurons over time was also seen ($p < 0.001$), predominantly due to an increase in cytoplasm. Our data on whole-mount preparations demonstrate that significant maturation in nitrergic neuronal density and morphology occurs in the porcine urinary bladder, at least until birth.

Keywords Urinary bladder · Nitrergic innervation · Neuronal density · Whole-mount preparation

M. E. Pirker · S. Montedonico · U. Rolle · P. Puri (✉)
Children's Research Centre,
Our Lady's Hospital for Sick Children and University College Dublin, Dublin, Ireland
E-mail: prem.puri@ucd.ie
Tel.: +353-1-4096420
Fax: +353-1-4550201

H. Austvoll
Institute of Experimental Clinical Research,
University of Aarhus, Denmark

Introduction

Neurophysiology of the urinary bladder is an important topic in paediatric urology; dealing with neurogenic disorders of bladder function, enuresis, and urinary incontinence is part of our daily routine. The foetal bladder is under neural control by the 3rd trimester [1]. At this stage, the bladder shows normal nonphasic contractions as well as immature phasic contractions [2]. Bladder function subsequently undergoes maturation during the first years of life until sufficient bladder capacity and voluntary control of micturition are achieved. Bladder maturation is at least in part caused by a maturation of the neuronal pathways in the urinary bladder [3, 4]. Little information is available regarding the morphological aspects of the developmental changes of bladder's intramural neuronal plexus.

Nitric oxide (NO) is an important nonadrenergic noncholinergic (NANC) inhibitory neurotransmitter. Nitrergic nerve fibres and neurons are present in the urinary bladder of various species, including rat, cat, guinea pig, pig, and human [5–10]. NO is involved in detrusor and sphincter muscle relaxation in various species, including rat, pig, and human [11–13]; regulation of local arteriolar tone [14, 15]; and production of extracellular matrix proteins [16–18].

Whole-mount preparation is an elegant technique that shows the neuronal network in full and, therefore, is far superior to standard tissue sections for investigating neuronal networks. Although well-known for the analysis of developmental changes in the intramural

neuronal plexus of the bowel [20, 21], this technique has not been used yet to study developmental changes in neurons of the urinary bladder. In this study we investigated the changes in density and morphology of the intramural nitrergic ganglia of the porcine urinary bladder during development using whole-mount preparation.

Materials and methods

Tissue sampling and whole-mount preparation

Bladder specimens were obtained from porcine foetuses of gestational age 60 days (E60, $n = 5$) and 90 days (E90, $n = 5$) and from newborn piglets (newborn, $n = 5$). The animals were provided by the Institute of Experimental Clinical Research, Skejby Sygehus, University of Aarhus, Denmark. The study was approved by the Danish authorities of animal protection, permission number 200 601–068. Bladders were removed after perfusion of the whole animal with 4% paraformaldehyde in phosphate-buffered saline (PBS) and placed in the fixative for 48 h at 4°C. Afterwards, bladders were transferred into sterile containers filled with PBS and stored at 4°C until further use.

All bladders were divided into three tissue pieces: cranial pole (''dome''), middle portion (''body''), and caudal pole including the two ureteral and the urethral openings (''base'')[19]. For this dissection, bladders were pinned flat onto a polystyrene block (Fig. 1). The border between base and body was determined as a horizontal line 5 mm cranial to the ureterovesical junctions. The border between body and dome was an arbitrary horizontal line at the point where the cylindrically shaped body started to taper off to form the dome.

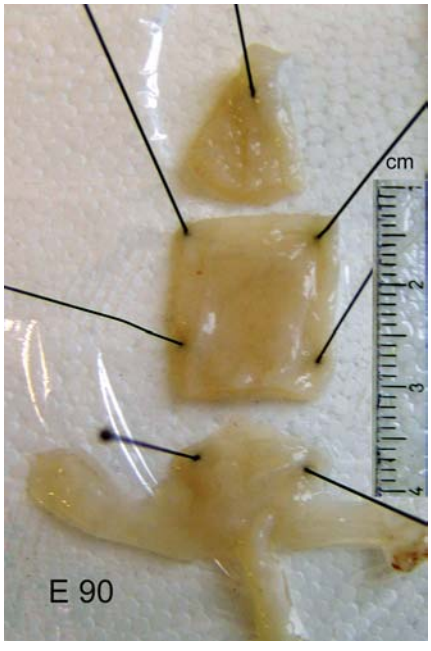

Fig. 1 Dissection of an E90 bladder pinned on a polystyrene block. From cranial to caudal: dome–body–base. Both ureters and the urethra are still attached to the base

After this dissection, the bladder pieces were cut open along the anterior midline, and the cranial borders of the body pieces were marked with fine stitches to allow for correct orientation during mounting and microscopic assessment. The mucosa with some adjacent submucosal tissue was then stripped off from the muscular layers using a fine pair of forceps. The thin serosal layer and all remaining connective tissue on the outer surface of the bladder wall was then carefully removed to ensure that only truly intramural ganglia were assessed. In the E60 and E90 stages, the whole base, body, and dome were used, whereas in the bladders of newborns, squares of 70–100 mm^2 were cut out of the left frontal part of the base, body, and dome and used for staining and analysis because the whole specimens in this age group were unsuitably large for mounting on glass slides.

Staining procedure

For NADPH-diaphorase (NADPH-d) histochemistry, the whole-mount preparations and removed mucosal and serosal layers were incubated in 1 mg/ml β-NADPH (Sigma), 0.25 mg/ml nitro blue tetrazolium, and 0.3% Triton-X in 0.05 mol/l Tris-HCl buffer (pH7.6) at 37°C for 2 h and then left in the staining solution overnight at room temperature. Incubation was performed using a free-floating technique on a 12-well cell culture dish. After the desired staining intensity was achieved, specimens were washed in PBS for 15 min and then mounted on Polysine slides (BDH) using Glycergel mounting medium (Dakocytomation). Gentle pressure was applied manually to the cover glass during mounting to make the preparations as flat as possible for the microscopic assessment.

Microscopy and morphometry

Three bladders of gestational age 60 days, three bladders of gestational age 90 days, and four bladders of newborn piglets were used for morphometric analysis. The remaining bladders were used for preliminary experiments to optimise the whole-mount preparation and staining procedure. Specimens were investigated using a Leica DMLB light microscope equipped with a Leica DC 300F digital camera system attached to a personal computer.

Density of NADPH-d positive ganglia was measured by drawing a numbered grid on the coverslip and then counting the ganglia in each square of the grid under the light microscope at ×200 magnification. Ganglia overlapping the left and lower lines of the square were also counted, and those overlapping the right and upper lines were excluded from the respective square. Especially in the specimens of newborn piglets, frequent focus adjustment was used to ensure that all ganglia in the

relatively thick specimen were counted. To simplify the procedure, single NADPH-d positive cells in the bladder wall were also counted as ganglia. After counting, the area of each numbered square was determined by photographing the grid with a digital camera and measuring the area under each square using image analysis software (Image J, version 1.30). Density of ganglia was then calculated by dividing the number of ganglia by the corresponding area measurement.

The number of NADPH-d positive neurons per ganglion was determined by assessing 30 adjacent ganglia in the base, body, and dome of each specimen, resulting in an overall number of 270 ganglia analysed for the E60 and E90 age groups and 360 ganglia analysed in the neonatal specimens. The evaluation of adjacent ganglia was chosen instead of a random selection of ganglia to eliminate a possible bias of choosing only larger, smaller, or easier-to-count ganglia. Counts were performed by two different investigators (PM and MS) to control for inter-rater variability.

The size of NADPH-d positive neurons was determined by photographing a minimum of 10 single neurons and/or ganglia with clearly delimitated individual neurons of the body region of each specimen, using ×400 magnification. The border of each neuron and its nucleus were marked by hand, and the total area of the neuron and the nucleus was measured using Image-J image analysis software. The area of the cytoplasm was calculated by subtracting the area of the nucleus from the area of the whole neuronal body. A total of 35 neurons per age-stage were measured and the results averaged for each stage.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare ganglion densities and neuron sizes between the different age groups, with the Student–Newman–Keuls test being used for pairwise comparisons. The number of NADPH-positive neurons per ganglion was compared by grouping ganglia into three groups (one to two neurons, three to 10 neurons, and more than 10 neurons) and then assessing the distribution of these ganglion groups among the different age groups using the chi-square test for statistical analysis. All statistical tests were performed using a commercially available software package. A p-value < 0.05 was considered statistically significant. All numerical data are expressed as mean $\pm$ standard deviation unless otherwise indicated.

Results

Architecture and morphology of the NADPH-d positive intramural innervation

Numerous NADPH-d positive ganglia containing one to 30 neurons were found in the muscular layer of the urinary bladder in all three age groups. Ganglia were more abundant near the serosal layer than near the submucosa and were negligible in the mucosal/submucosal lamina that had been stripped off. The removed serosa and connective tissue contained numerous very large ganglia, especially around the ureterovesical junctions (Fig. 2). These large ganglia were not included in the analysis of intramural ganglia because of their extramural location and significantly larger size. Intramural ganglia did not form a uniform neuronal network as seen in the intestinal nerve plexus but were located along the dividing bundles of NADPH-d positive nerve fibres. These nerves entered the bladder as large nerve trunks with the major vessels at the ureterovesical junction. Additionally, ganglia along different larger nerve bundles were linked by small interconnecting nerve bundles. Finally, numerous single NADPH-positive nerve fibres starting from the intramural ganglia were found to run parallel to the smooth muscle bundles of the bladder wall, presumably forming the functional nerve endings. Staining of NADPH-d positive neurons within each ganglion was not uniform. About one-third of the neurons showed very strong NADPH-d positive staining, whereas the remaining neurons were moderately or weakly stained. The shape of individual NADPH-d positive neurons was relatively uniform among all age groups and bladder regions. They were clubbed with a larger ''head'' containing the nucleus and a tapering ''tail'' terminating into the axon. The head had one or more dendrites. The number of dendrites per neuron seemed to increase with age.

Density of NADPH-d positive ganglia in different bladder regions and age groups

No significant difference was found in the total number of intramural ganglia (including single neurons) in the whole bladder of the E60 ($4{,}944 \pm 790$) and E90 age groups

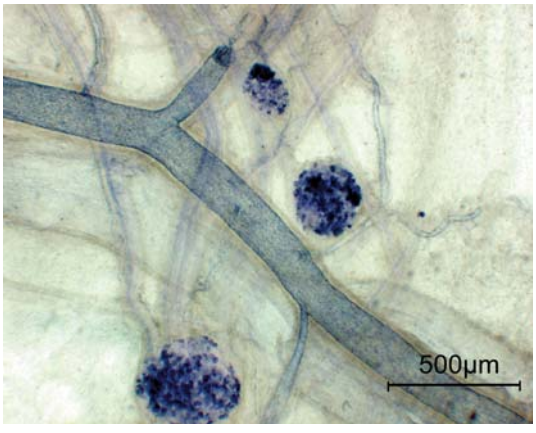

Fig. 2 Photomicrograph of the ureterovesical ganglion complex stained for NADPH-d. The connective tissue at the ureterovesical junction contains large ganglia with many NADPH-d positive neurons. Note that some areas of the ganglia are not stained, representing NADPH-d negative neurons. These ganglia are not located within the bladder wall itself and therefore were not included in the following analysis. (Original magnification ×40)

(5,581 ± 367). Although NADPH-d positive ganglia were abundant in all regions of the bladder, the regional distribution was variable. In the E60 bladders, density was highest in the body (880 ± 108cm^2) followed by the base (676 ± 131 cm^2) and the dome (397 ± 45 cm^2). Intramural ganglion density decreased significantly with age ($p < 0.001$). The overall density of NADPH-d positive ganglia was 720/cm^2 (± 100) in the E60 bladder, 435/cm^2 (± 75) in the E90 bladder, and 175/cm^2 (± 66) in the newborn bladder (Fig. 3), but the extent of reduction was not uniform in different bladder regions (Fig. 4). The body region showed the most significant decrease in ganglion density, while the reduction was less marked in the base and comparably small in the dome. As a result, the overall distribution of ganglion densities among the different bladder regions changed considerably with bladder maturation. In the newborn animals, the density of NADPH-d positive ganglia was highest in the base (200 ± 77 cm^2), followed by the body (183 ± 64 cm^2) and the dome (149 ± 68 cm^2).

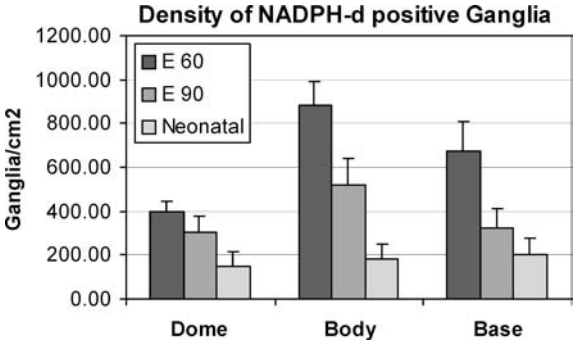

Fig. 4 Reduction in density of NADPH-d positive ganglia during development in different bladder regions

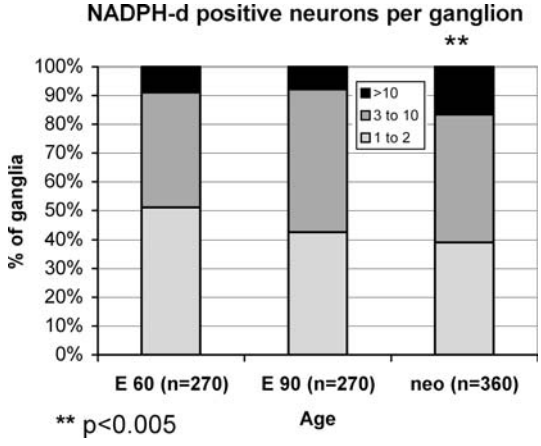

Fig. 5 Significant increase in the number of NADPH-d positive neurons per ganglion during development ($p < 0.001$, chi square). The increase was significant between E90 and neonatal but not between E60 and E90. Note the relatively higher percentage of middle-sized ganglia in the E90 specimen

Increase in number of NADPH-d positive neurons per ganglion with age

An overall significant increase in the number of NADPH-d positive neurons per ganglion with age was found ($p < 0.001$). The proportion of ganglia containing more than 10 NADPH-d positive neurons increased from 9% in the E60 bladder to 17% in the newborn bladder, whereas the proportion of ganglia with only one or two NADPH-d positive neurons decreased from 51% in the E60 to 39% in the newborn bladders. In the pairwise analysis, the number of NADPH-d positive neurons per ganglion increased significantly between E90 and neonatal bladders but was not significantly different between E60 and E90 bladders (Fig. 5). The increase in the number of NADPH-d positive neurons per ganglion with age was also analysed for different regions of the bladder. The increase was most marked in the base ($p < 0.01$), less in the body ($p = 0.03$), and not significant in the dome (Fig. 6).

Increase in size of NADPH-d positive neurons with age

A marked increase in the size of NADPH-d positive neurons was seen with increasing age ($p < 0.001$). The mean area of one photographed neuron was 164 μm^2 (± 41) in the E60, 245 μm^2 (± 103) in the E90, and

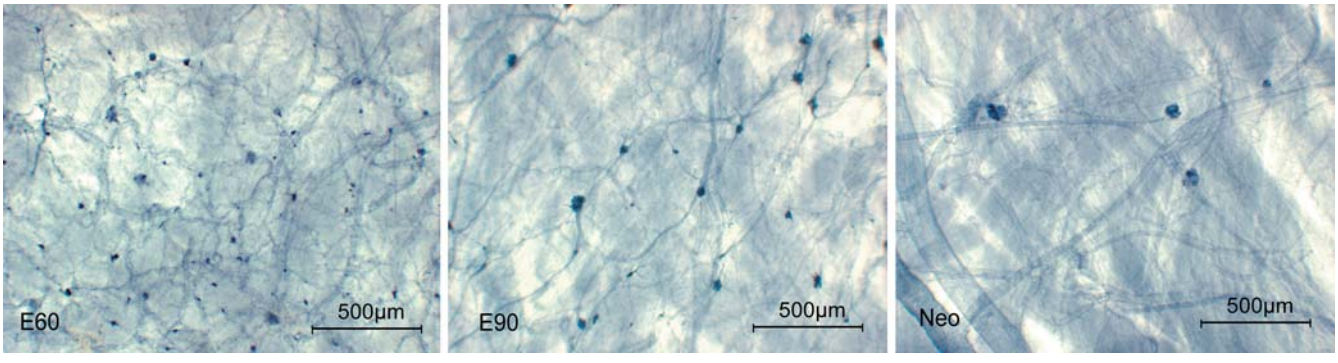

Fig. 3 Decrease in density of intramural NADPH-d positive ganglia during development. All photographs are taken from the body region and shown in identical magnification. Mean density was 720 ganglia/cm^2 in the E60 (**a**), 435/cm^2 in the E90 (**b**), and 175/cm^2 in the neonatal bladder (**c**). (Original magnification ×40)

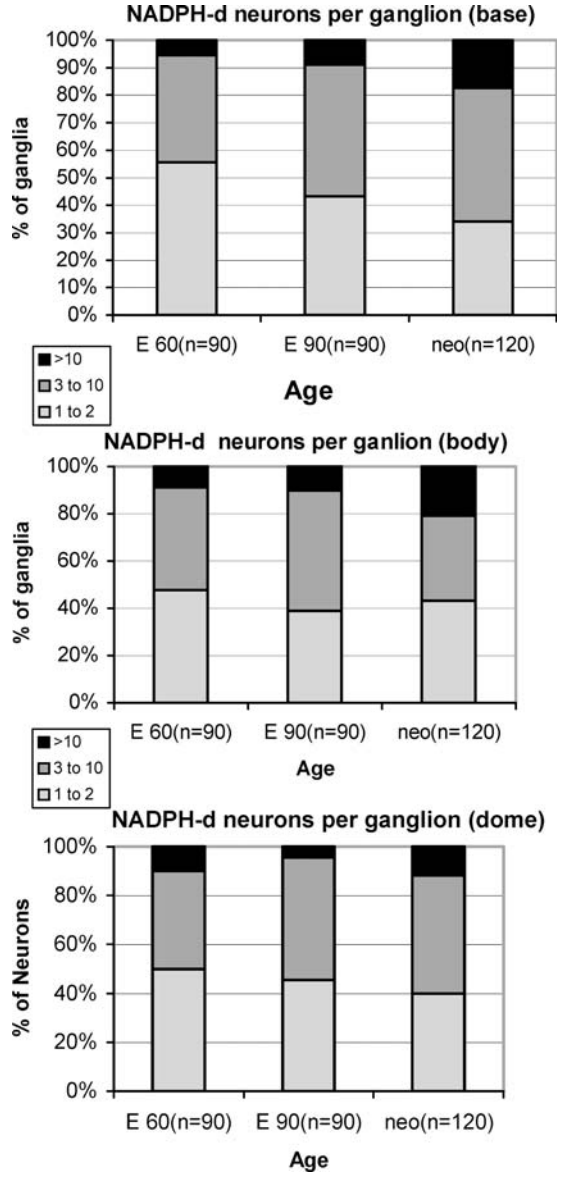

Fig. 6 Increase in the number of NADPH-d positive neurons during development analysed for different bladder regions. The increase was statistically most significant in the base ($p < 0.01$), less in the body ($p = 0.03$), and not significant in the dome

454 μm^2 (± 132) in the neonatal bladders (Fig.7). When analysed separately for the nucleus and cytoplasm, the increase was found to be predominantly caused by an increase in cytoplasm, resulting in a cytoplasm area/nucleus area ratio of 1.38 (± 0.37) in the E60, 1.97 (± 0.66) in the E90, and 3.15 (± 0.93, $p = 0.01$) in the neonatal bladders (Fig. 8).

Discussion

Intramural nitrergic neurons in the porcine urinary bladder during development

Our study was able to demonstrate that a large number of NADPH-d positive ganglia are present in the foetal porcine urinary bladder and that this intrinsic nitrergic neuronal system undergoes significant morphological changes during the second half of gestation.

We found three significant changes in the morphology of the intramural nitrergic ganglia during development: a regionally different decrease in ganglion density, an increase in the number of NADPH-d positive neurons per ganglion, and a considerable increase in the size of individual NADPH-d positive neurons.

A decrease in the density of NADPH-d positive neurons was described in a very similar way for the enteric nervous system by Van Ginneken et al. and Wester et al. [20, 21]. The density of NADPH-d positive neurons in pig duodenum decreased by approximately 90% between the second half of gestation and 6–8 weeks postnatally [21]. This striking similarity indicates that the decrease in neuron density represents a universal pattern of (nitrergic) neuronal plexus maturation and ageing rather than a unique phenomenon of the urinary bladder.

The decrease in ganglion density could be caused by various factors. One possible explanation is the marked increase in the bulk of detrusor muscle fibres and the increase in bladder volume [22] during foetal development. If the total number of ganglia remains quite constant, as indicated by our total ganglion counts in the E60 and E90 specimens, the increase in bladder size and muscle volume can lead to a relative decrease in the density of the nitrergic ganglia. Besides muscular growth, the possibilities of apoptosis of nitrergic neurons and fusion of neurons to form fewer but larger ganglia also have to be considered. The theory of fusion of single neurons or smaller ganglia is also supported by our finding of a significant increase in the number of NADPH-d positive neurons per ganglion. However, the extent of this increase can only explain the marked decrease in ganglion density to a certain extent. On the other hand, it may well reflect a functional maturation of the intrinsic nitrergic neuronal network. It is imaginable that single neurons, as seen quite frequently in the E60 bladder, migrate along the interconnecting nerve fibres while their axons are growing, fusing with other neurons or small ganglia on their way to form larger ganglia. Such a migration of neurons would contribute to the formation of a complex interconnected network.

The striking finding in this study was the significant regional differences in the decrease of ganglion density. The decrease was most marked in the body region, less in the base, and least in the dome. Regional differences in detrusor smooth muscle growth during development could be a possible reason for these regional differences in the decrease of ganglion density. The decrease was most marked in the body region, where the maximum of detrusor growth would be expected. When looking at the decrease of ganglion density in the bladder base, it is interesting that it was also marked between E60 and E90 but much less between E90 and neonatal bladders, whereas the situation is the reverse in the dome. This may suggest that muscular bladder growth slows down first in the trigonal region.

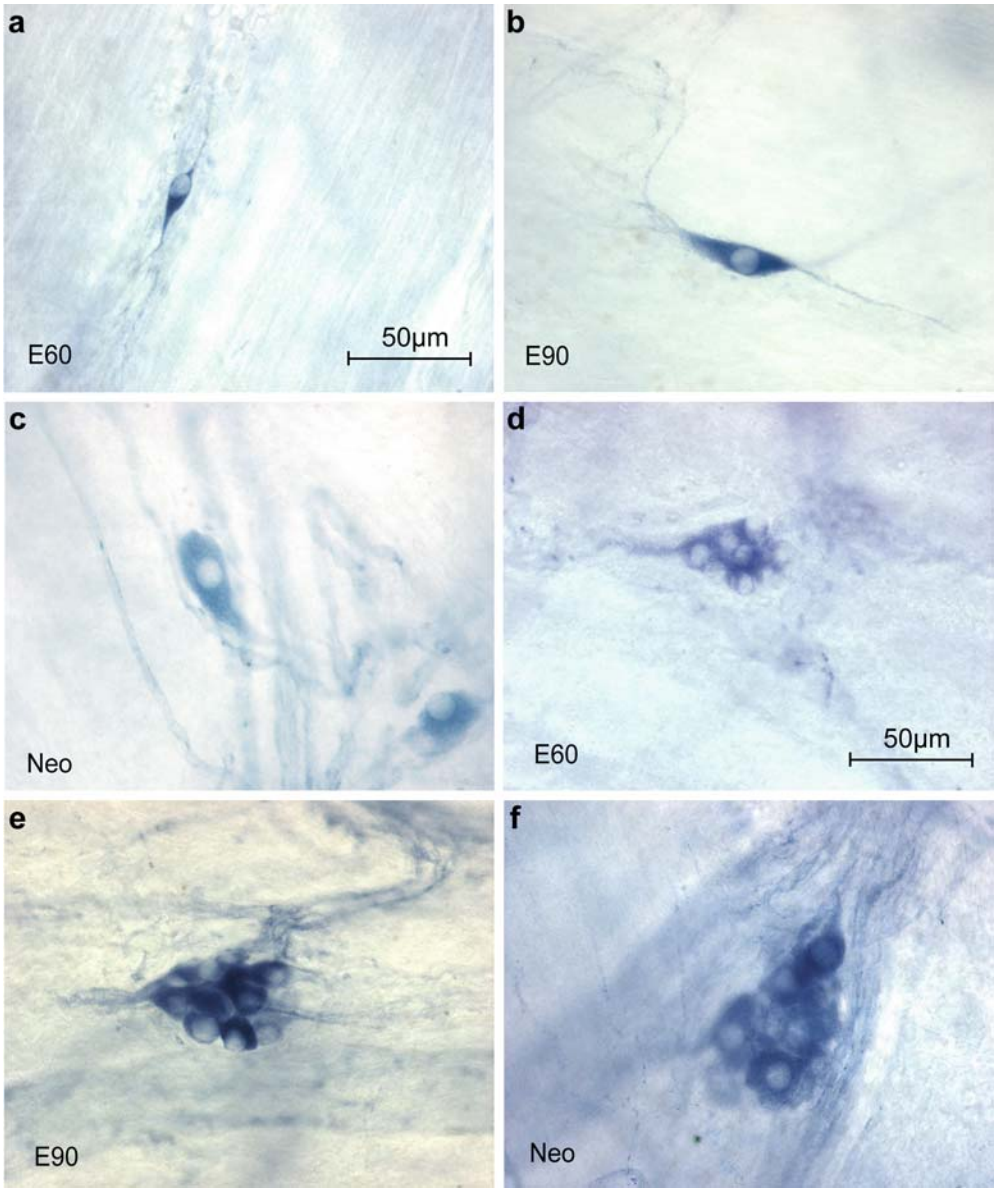

Fig. 7 Increase in neuron size during development. All pictures were taken at ×400 and are shown in identical magnification. The increase in size of individual neurons is mainly caused by an increase in cytoplasm (**a**–**c**). **d**–**f** The ganglia from different age groups with a comparable number of neurons. The increase in size of the individual neurons also causes a marked increase in the total size of the ganglion

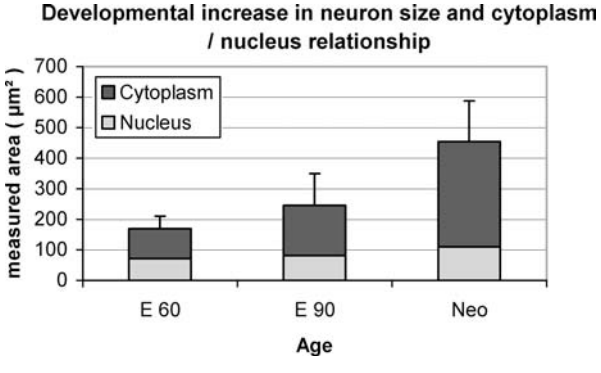

Fig. 8 The increase in neuron size during development was predominantly due to an increase in cytoplasm, while the size of the nucleus remained quite constant.

The regional differences in the decrease of ganglion density led to a marked developmental change in the topical distribution of nitrergic ganglia in our bladder preparations. While the ganglion density was highest in the body region in the E60 specimen, ganglia were most concentrated in the bladder base in the newborn animals. Zhou et al. studied the distribution of NADPH-d positive neurons in adult guinea pigs and found that they were more numerous and organized in larger ganglia in the bladder base than in the body [19]. This could indicate that the relative increase in the density of NADPH-d positive ganglia in the bladder base found in our study may reflect a development towards an ''adult'' pattern of intramural innervation. It would also be in agreement with the results of a functional study in pigs that demonstrated NANC-mediated relaxation (including the NO pathway) predominantly in the urethral and trigonal smooth muscle [11].

Interestingly, our findings of the increase in the number of nitrergic neurons per ganglion also fit into the theory of a developmental ''concentration'' of nitrergic intramural neurons in the bladder base. The increase in the NADPH-d positive neurons per ganglion was statistically most marked in the bladder base, less in the body, and not significant in the dome. This finding is in agreement with another study on the adult guinea pig, which showed that larger NADPH-d ganglia were located predominantly in the bladder base whereas smaller NADPH-d ganglia dominated in the body and the dome [19].

We found a striking increase in the size of nitrergic neurons in the bladders during development. The increase in the size of NADPH-d positive neurons with age has also been described by other authors for different species and different regions of the body [23, 24]. This also indicates that it is a rather universal characteristic of nitrergic neuron maturation. The significant increase in the cytoplasm/nucleus ratio suggests an increase in the functional activity of these neurons, which may be required for the growth and transmitting function of the axons and dendrites.

Technical considerations

The use of NADPH-d histochemistry to demonstrate truly nitrergic innervation is sometimes questioned in the literature today. Although some authors have reported that a number of NADPH-d positive neurons were not immunoreactive for nitric oxide synthase (NOS) in the guinea pig urinary bladder [19], a number of studies have demonstrated a one-to-one correlation of NADPH-d staining and NOS expression [8, 25, 26]. NADPH-d immunohistochemistry was chosen for demonstration of nitrergic innervation in this study for various reasons: It was shown to be almost identical with NOS immunoreactivity in the neuronal structures of the lower urinary tract [27], it leads to easily reproducible results, and it causes far fewer problems in terms of tissue penetration of reagents.

The latter fact becomes very important with the use of whole-mount preparations, especially in the urinary bladder. Whole-mount preparation of the neuronal network within the urinary bladder wall differs in some important points from the preparations known from the enteric plexus. The first major difference is that the bowel musculature is organised in two distinct layers, namely the circular and longitudinal muscle layers. These two layers are easily distinguishable and can be separated under a dissection microscope without destroying the interposed myenteric plexus. Additionally, the intramural nervous system is organised into three different layers: the myenteric plexus and the inner and outer submucous plexuses. Although interconnected, each individual plexus macroscopically appears as a rather two-dimensional network. Therefore, preparation of these plexuses results in very thin tissue preparations suitable for most histochemical and immunohistochemical staining methods. The detrusor and trigonal muscle, on the other hand, has a very complex architecture, and different muscle layers are not readily distinguishable. In concordance with this, the intramural neuronal network spreads through the whole muscular bladder wall, forming a three-dimensional network.

Therefore, for assessment of morphological changes, it is necessary to prepare the complete muscular layer as a whole. These preparations reached a thickness of approximately 0.7 mm (before mounting) in the newborn animals. Because of this thickness, preliminary experiments showed that the applicability of immunohistochemical staining methods on these preparations was rather limited in the newborn animals, and a microscopic investigation of bladders of older animals was not possible using this species.

Conclusions

By using whole-mount preparation on the foetal and neonatal porcine urinary bladder, we could show that significant morphological changes of the intramural nitrergic neuronal system of the urinary bladder occur at least until birth, especially the ''concentration'' of nitrergic neurons in the bladder base with maturation. Further studies of the prenatal and postnatal maturation of the urinary bladder neuronal plexus may enable us in the future to better understand the physiological neuroanatomical changes underlying the maturation of bladder function in children. On this basis, new diagnostic modalities can be developed to help us better discern normal from abnormal bladder function, especially in young children.

References

1. Massad CA, Kogan BA (1997) Fetal urodynamics. In: O'Donnell B, Koff SA (eds) Pediatric urology. Butterworth-Heinemann, Oxford, pp 152–154
2. Kogan BA, Iwamoto HS (1989) Lower urinary tract function in the sheep fetus: studies of autonomic control and pharmacologic responses of the fetal bladder. J Urol 141:1019–1024
3. Bellinger MF (1987) Myelomeningocele and neuropathic bladder. In: Grayhack JT, Howards SS, Duckett JW (eds) Adult and pediatric urology. Year Book Medical Publishers, Chicago, pp 1836–1862
4. de Groat WC (2002) Plasticity of bladder reflex pathways during postnatal development. Physiol Behav 77:689–692
5. Dixon JS, Jen PY, Gosling JA (2000) The distribution of vesicular acetylcholine transporter in the human male genitourinary organs and its co-localization with neuropeptide Y and nitric oxide synthase. Neurourol Urodyn 19:185–194
6. Vizzard MA, Erdman SL, de Groat WC (1993) Localization of NADPH diaphorase in bladder afferent and postganglionic efferent neurons of the rat. J Auton Nerv Syst 44:85–90
7. Keast JR, Kawatani M (1994) Extensive distribution of NADPH diaphorase activity in the nerve supply of the cat lower urinary tract. J Auton Nerv Syst 50:161–169

8. Saffrey MJ, Hassall CJ, Moules EW, Burnstock G (1994) NADPH diaphorase and nitric oxide synthase are expressed by the majority of intramural neurons in the neonatal guinea pig urinary bladder. J Anat 185(Pt 3):487–495
9. Persson K, Alm P, Johansson K, Larsson B, Andersson KE (1995) Co-existence of nitrergic, peptidergic and acetylcholine esterase-positive nerves in the pig lower urinary tract. J Auton Nerv Syst 52:225–236
10. Smet PJ, Edyvane KA, Jonavicius J, Marshall VR (1994) Distribution of NADPH-diaphorase-positive nerves supplying the human urinary bladder. J Auton Nerv Syst 47:109–113
11. Persson K, Andersson KE (1992) Nitric oxide and relaxation of pig lower urinary tract. Br J Pharmacol 106:416–422
12. Ehren I, Iversen H, Jansson O, Adolfsson J, Wiklund NP (1994) Localization of nitric oxide synthase activity in the human lower urinary tract and its correlation with neuroeffector responses. Urology 44:683–687
13. Andersson KE, Persson K (1994) Nitric oxide synthase and nitric oxide-mediated effects in lower urinary tract smooth muscles. World J Urol 12:274–280
14. Vallance P, Collier J, Moncada S (1989) Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. Lancet 2:997–1000
15. Kawabata M, Han WH, Ise T, Kobayashi K, Takabatake T (1996) Role of endogenous endothelin and nitric oxide in tubuloglomerular feedback. Kidney Int Suppl 55:S135–S137
16. Trachtman H, Futterweit S, Singhal P (1995) Nitric oxide modulates the synthesis of extracellular matrix proteins in cultured rat mesangial cells. Biochem Biophys Res Commun 207:120–125
17. Kim NN, Villegas S, Summerour SR, Villarreal FJ (1999) Regulation of cardiac fibroblast extracellular matrix production by bradykinin and nitric oxide. J Mol Cell Cardiol 31:457–466
18. Cai L, Suboc P, Hogue DA, Fei DT, Filvaroff EH (2002) Interleukin 17 induced nitric oxide suppresses matrix synthesis and protects cartilage from matrix breakdown. J Rheumatol 29:1725–1736
19. Zhou Y, Tan CK, Ling EA (1997) Distribution of NADPH-diaphorase and nitric oxide synthase-containing neurons in the intramural ganglia of guinea pig urinary bladder. J Anat 190(Pt 1):135–145
20. Wester T, O'Briain DS, Puri P (1999) Notable postnatal alterations in the myenteric plexus of normal human bowel. Gut 44:666–674
21. Van Ginneken C, Van Meir F, Sommereyns G, Sys S, Weyns A (1998) Nitric oxide synthase expression in enteric neurons during development in the pig duodenum. Anat Embryol (Berl) 198:399–408
22. Nicolaides KH, Rosen D, Rabinowitz R, Campbell S (1988) Urine production and bladder function in fetuses with open spina bifida. Fetal Ther 3:135–140
23. Phillips RJ, Kieffer EJ, Powley TL (2003) Aging of the myenteric plexus: neuronal loss is specific to cholinergic neurons. Auton Neurosci 106:69–83
24. Tay D, Diao YC, Xiao YM, So KF (2002) Postnatal development of nicotinamide adenine dinucleotide phosphate-diaphorase-positive neurons in the retina of the golden hamster. J Comp Neurol 446:342–348
25. Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH (1991) Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc Natl Acad Sci USA 88:7797–7801
26. Young HM, Furness JB, Shuttleworth CW, Bredt DS, Snyder SH (1992) Co-localization of nitric oxide synthase immunoreactivity and NADPH diaphorase staining in neurons of the guinea-pig intestine. Histochemistry 97:375–378
27. Persson K, Alm P, Johansson K, Larsson B, Andersson KE (1993) Nitric oxide synthase in pig lower urinary tract: immunohistochemistry, NADPH diaphorase histochemistry and functional effects. Br J Pharmacol 110:521–530