Gene expression and immunolocalization of low-affinity neurotrophin receptor (p75) in rabbit male reproductive tract during sexual maturation

Ana Sanchez-Rodriguez, Maria Arias-Alvarez, Pilar G. Rebollar, Jose M. Bautista, Pedro L. Lorenzo, Rosa M. Garcia-Garcia

Contents

This study reports the gene expression and immunolocalization of the low-affinity neurotrophin receptor, p75, in accessory glands (prostate and bulbourethral glands) and epididymis (caput and cauda) of male rabbits during sexual maturation. We showed that p75 was expressed in all tissues studied with similar mRNA levels during this period. However, it was differentially immunolocalized in bulbourethral glands and stereocilia of epididymis. These findings may be related with some remodeling processes in the accessory glands during sexual maturation, which could be associated with sperm maturation. The interaction of p75 with neurotrophins in rabbit male reproductive tract suggests the possible implication of this system in sexual maturation in rabbits.

KEY WORDS

gene expression, immunolocalization, neurotrophin receptor, p75, rabbit male, sexual maturity

1 | INTRODUCTION

Low-affinity receptor, p75 is a member of the death-promoting tumor necrosis factor family. It mediates the biological function of the neurotrophin beta Nerve Growth Factor (bNGF) and also is able to bind to others neurotrophins known (neurotrophins 3 and 4/5 and brain-derived neurotrophic factor, BDNF), mediating apoptotic and prosurvival signals in the nervous system (Wehrman et al., 2007).

The sexual maturation period is a crucial stage where some important changes occur in the reproductive tract, determining male long-term seminal quality and productivity of rabbit in the farm. We have demonstrated the expression and localization of the neurotrophin bNGF and its high-affinity receptor, TrkA, in rabbit male tract during this period (Sanchez-Rodriguez, Arias-Alvarez, Rebollar, Lorenzo, & Garcia-Garcia, 2018; Sanchez-Rodriguez, Lorenzo, Arias-Alvarez, Rebollar, & Garcia-Garcia, 2017). Thus, NGF/TrkA system

seems to be implicated in the sexual development of the male, but there are no studies about p75. Hence, in this study, we investigated the gene expression and immunolocalization of p75 in accessory glands (prostate and bulbourethral glands) and in epididymis (caput and cauda) of rabbit males at two time-points in the critical period of sexual maturation.

2 | MATERIAL AND METHODS

Eight New Zealand x California male rabbits (*Oryctolagus cuniculus*) were reared on the experimental farm at the Agrarian Production Department (Polytechnic University Spain), in individual cages under ambient controlled conditions (16HL:8HD, 20–25°C of temperature and a relative humidity of 60%–75%) and with free access to food and water. All experimental procedures were approved by the Animal

Ethics Committee of the Community of Madrid (Ref. PROEX 302/15) and in compliance with the Spanish guidelines for the care and use of animals in research (BOE, 2013) and conform to Directive 2010/63/EU.

Rabbit males were trained with an artificial vagina since 20 weeks old, in order to be sexually active and to standardize seminal parameters. Animals were slaughtered by cervical dislocation at weeks 22 and 37 of age ($n = 4$, respectively), and tissues were recovered for PCR studies and fixed for immunohistochemistry analysis.

For PCR studies, total RNA was extracted (Trizol, Life Technologies), purified (PureLink® RNA MiniKit, Thermo Fisher) and treated with DNase (Thermo Fisher). The reverse transcription (RT) was carried out using Superscript™ First-Strand Synthesis System for RT-PCR kit (Life Technologies). Primers for *p75* and *GAPDH* were designed according to GenBank database (Table 1), and an end-point PCR and electrophoresis in 1.5% agarose gel was performed. Thereafter, 100 ng of cDNA were relatively quantified using SYBR Green (Biorad) and an annealing temperature of 62°C. Non-template controls and RT negative samples were run in duplicate as negative controls. Quantification was normalized to *GAPDH* and relativized to the expression in the prostate, as reference tissue (Maranesi et al., 2015). The relative gene expression of *p75* was evaluated using the formula $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001).

Immunohistochemistry was performed as previously described (Arias-Alvarez et al., 2010) with minor modifications. Mouse monoclonal anti-p75 primary antibody (5 µg/ml, ab157295, Abcam) was incubated overnight at 37°C in moist chamber. Secondary antibody (1:200) was added for 30 min at room temperature [biotinylated anti-mouse IgG (Abcam)]. Slides were incubated with the ABC Vector Elite kit and then with peroxidase red chromogen (Vector Laboratories). For negative control samples, PBS was used instead of the primary antibody. Melanoma slides (ab5101, Abcam) were used as positive control tissue. Ten images of each tissue of every male were taken using a light microscope (Leica F550) equipped with a DCF400 camera and evaluated. The intensity of the signal was considered by the same blinded observer as mild +, moderated ++ or strong +++.

The gene expression of *p75* was analysed by one-way ANOVA, with weeks, tissues and the interaction between them as fixed effects (SAS Software version 9.4). Differences were considered significant when p -value < 0.05.

3 | RESULTS

The electrophoresis of cDNA transcripts showed bands of the predicted size of the target *p75* (117 bp, Figure 1a). *p75* mRNA expression assessed by RT-qPCR was similar between tissues and

FIGURE 1 (a): Electrophoresis of *p75* mRNA transcripts of prostate, bulbourethral glands and caput and cauda of epididymis (lane 1–4) obtained by end-point PCR. Lane 5: Negative control. MWM (molecular weight markers): DNA ladder 100–1000 bp. (b): Relative expression of *p75* in prostate, bulbourethral glands, caput and cauda of epididymis assessed by RT-qPCR. Data are represented as means $\pm$ SE. No differences were shown between tissues nor ages studied ($p < 0.05$)

ages (Figure 1b), showing no statistical differences ($p > 0.05$). Furthermore, no interaction between them was found.

Immunolocalization of *p75* was heterogeneous in all tissues. In prostate, some epithelial cells were stained in both ages (+/-), whereas lumen and interstitial tissue had not signal (Figure 2a, d). Epithelial cells of bulbourethral glands were homogeneously and strongly stained (+++) in younger males, whereas the staining was moderated (++) and non-homogeneous in older ones (Figure 2f, j). Interstitial tissue had not signal for *p75*. In epididymis, some stereocilia were stained (+) in caput in both ages (Figure 2k, o) and the interstitial tissue of cauda had a mild signal (+) in younger males (Figure 2p, s). The rest of the structures in epididymis were unstained. Negative control showed no staining.

4 | DISCUSSION

This study reports the expression and immunolocalization of *p75* in rabbit male genital tract during the sexual maturation period. It was

TABLE 1 Primers for *p75* and *GAPDH* for RT-qPCR

Gene	Nucleotide sequence 5'- 3'	Product size (bp)	NCBI reference sequence
<i>p75</i>	F:TCGCCTTCAAGAGGTGGAACA	117	XM_008271321.2
	R:CAGAGATGCCGCTGTCACTA		
<i>GAPDH</i>	F:GTCGGAGTGAACGGATTGG	53	NM_001082253.1
	R:AAAGCAGCCCTGGTGACCA

FIGURE 2 Immunolocalization of p75 in prostate (a, d), bulbourethral glands (f, j), caput (k, o) and cauda of epididymis (p, r) in 22-week old (left panel) and 37 week old (right panel) rabbit males. Negative controls are b, g, m and q for 22-week and c, h, n and r for 37-week old rabbits. Abbreviations: E, epithelial cells; I, interstitial tissue; L, lumen; St, stereocilia. Scale bar is 50 μ m

expressed in a constant manner during this period but differentially immunolocalized in the different organs studied.

All tissues had similar gene expression during sexual maturation. Expression of p75 in prostate has been previously described in adult rabbits (Maranesi et al., 2015) but this is the first time that gene expression of p75 of bulbourethral glands and epididymis in rabbits was determined. In addition, gene expression of the receptor during the sexual maturation process was not previously defined either.

We showed that p75 was immunolocalized only in some tissues of rabbit male tract with different distribution between ages. The prostatic tissue had scarce staining in epithelial cells, similar to results published elsewhere in adult rabbits (Maranesi et al., 2015). To the best of our knowledge, the immunolocalization of p75 in epithelial cells of bulbourethral glands has not been described before. However, in our study, p75 seems to be located mainly in bulbourethral gland which is related to bNGF localization as we have previously demonstrated (Sanchez-Rodriguez et al., 2018). Also, staining signal was higher at younger stages. This could be associated to the remodeling process that is necessary to occur for achieving the sexual maturity around this age according to the role of p75 in remodeling system by apoptosis (Wehrman et al., 2007).

Another interesting finding in this study was the localization of p75 in stereocilia of the epididymis, which, to the best of our

knowledge, was not previously described. Stereocilia are cell elongations that absorb excess of fluid from the lumen of the epididymis to assure the proper sperm concentration, so it could be hypothesized that the interaction of p75 with neurotrophins may be involved in this function. It is remarkable that p75 was localized only in caput but not in cauda of epididymis. Sperm cells go through epididymis and they experiment a change in the motility pattern from caput to cauda, which depends on some factors produced by the epididymis epithelium (Ayer-LeLievre, Olson, Ebendal, Hallbook, & Persson, 1988). In this sense, the interaction of p75 with some neurotrophins could contribute in giving the sperm the proper motility during its transit through epididymis, though further studies are needed to confirm this hypothesis.

In conclusion, this study confirms that male reproductive tract can produce p75, being the protein localized specially in bulbourethral glands and stereocilia of epididymis. These original findings reaffirm the possible contribution of this receptor in the physiological processes of rabbit male development during the sexual maturation.

ACKNOWLEDGEMENTS

This study was financed by the project AGL2015-65572-C2 Grant. Ana Sanchez-Rodriguez was funded by Predoctoral Contract UCM Santander. Authors wish to thank P Abad for its help in PCR techniques and M Rodriguez and B Velasco for their invaluable help in the animal facilities.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

PGR, PLL, RMGG and MAA conceived the study and raised funds. ASR, RMGG and JMB designed the experiments of part of them. ASR, MAA, PGR and RMGG collected the animal samples. ASR and RMG performed the experimental procedures and analysed the data. All authors drafted, revised and approved the manuscript.

REFERENCES

- Arias-Alvarez, M., Garcia-Garcia, R. M., Torres-Rovira, L., Gonzalez-Bulnes, A., Rebollar, P. G., & Lorenzo, P. L. (2010). Influence of leptin on in vitro maturation and steroidogenic secretion of cumulus-oocyte complexes through JAK2/STAT3 and MEK 1/2 pathways in the rabbit model. *Reproduction*, 139, 523–532. <https://doi.org/10.1530/REP-09-0309>
- Ayer-LeLievre, C., Olson, L., Ebendal, T., Hallbook, F., & Persson, H. (1988). Nerve growth factor mRNA and protein in the testis and epididymis of mouse and rat. *Proceedings of the National Academy of Sciences of the United States of America*, 85, 2628–2632. <https://doi.org/10.1073/pnas.85.8.2628>
- BOE: Boletín Oficial del Estado. Real Decreto 53/2013, por el que se establecen las normas básicas aplicables para la protección de los

animales utilizados en experimentación y otros fines científicos, incluyendo la docencia. (2013) BOE, 34, 11370–11421.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. *Methods*, 25, 402–408. <https://doi.org/10.1006/meth.2001.1262>

Maranesi, M., Zerani, M., Leonardi, L., Pistilli, A., Arruda-Alencar, J., Stabile, A., ... Parillo, F. (2015). Gene expression and localization of NGF and its cognate receptors NTRK1 and NGFR in the sex organs of male rabbits. *Reproduction in Domestic Animals*, 50, 918–925. <https://doi.org/10.1111/rda.12609>

Sanchez-Rodriguez, A., Arias-Alvarez, M., Rebollar, P. G., Lorenzo, P. G., & Garcia-Garcia, R. M. (2018). Immunolocalization of β -nerve growth factor (NGF) in male reproductive tract and NGF levels in serum and seminal plasma at puberty and adulthood in rabbit. *Reproduction Fertility and Development*, 30, 214.

Sanchez-Rodriguez, A., Lorenzo, P. L., Arias-Alvarez, M., Rebollar, P. G., & Garcia-Garcia, R. M. (2017). Immunolocalization of nerve growth

factor high-affinity receptor (TrkA) in rabbit male tract. *Reproduction in Domestic Animal*, 52(Suppl. 4), 97–98.

Wehrman, T., He, X., Raab, B., Dukipatti, A., Blau, H., & Garcia, K. C. (2007). Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors. *Neuron*, 53, 25–38. <https://doi.org/10.1016/j.neuron.2006.09.034>

How to cite this article: Sanchez-Rodriguez A, Arias-Alvarez M, Rebollar PG, Bautista JM, Lorenzo PL, Garcia-Garcia RM. Gene expression and immunolocalization of low-affinity neurotrophin receptor (p75) in rabbit male reproductive tract during sexual maturation. *Reprod Dom Anim*. 2018;53(Suppl. 2): 62–65. <https://doi.org/10.1111/rda.13257>