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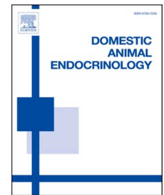

Seasonal variation of NGF in seminal plasma and expression of NGF and its cognate receptors NTRK1 and p75NTR in the sex organs of rams

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ABSTRACT

Nerve growth factor (NGF) has long been known as the main ovulation-inducing factor in induced ovulation species, however, recent studies suggested the NGF role also in those with spontaneous ovulation. The first aim of this study was to evaluate the presence and gene expression of NGF and its cognate receptors, high-affinity neurotrophic tyrosine kinase 1 receptor (NTRK1) and low-affinity p75 nerve growth factor receptor (p75NTR), in the ram genital tract. Moreover, the annual trend of NGF seminal plasma values was investigated to evaluate the possible relationship between the NGF production variations and the ram reproductive seasonality. The presence and expression of the NGF/receptors system was evaluated in the testis, epididymis, vas deferens ampullae, seminal vesicles, prostate, and bulbourethral glands through immunohistochemistry and real-time PCR (qPCR), respectively. Genital tract samples were collected from 5 adult rams, regularly slaughtered at a local abattoir. Semen was collected during the whole year weekly, from 5 different adult rams, reared in a breeding facility, with an artificial vagina. NGF seminal plasma values were assessed through the ELISA method. NGF, NTRK1 and p75NTR immunoreactivity was detected in all male organs examined. NGF-positive immunostaining was observed in the spermatozoa of the germinal epithelium, in the epididymis and the cells of the secretory epithelium of annexed glands, NTRK1 receptor showed a localization pattern like that of NGF, whereas p75NTR immunopositivity was localized in the nerve fibers and ganglia. NGF gene transcript was highest ($p < 0.01$) in the seminal vesicles and lowest ($p < 0.01$) in the testis than in the other tissues. NTRK1 gene transcript was highest ($p < 0.01$) in the seminal vesicles and lowest ($p < 0.05$) in all the other tissues examined. Gene expression of p75NTR was highest ($p < 0.01$) in the seminal vesicles and lowest ($p < 0.01$) in the testis and bulbourethral glands. NGF seminal plasma concentration was greater from January to May ($p < 0.01$) than in the other months. This study highlighted that the NGF system was expressed in the tissues of all the different genital tracts examined, confirming the role of NGF in ram reproduction. Sheep are short-day breeders, with an anestrus that corresponds to the highest seminal plasma NGF levels, thus suggesting the intriguing idea that this factor could participate in an inhibitory mechanism of male reproductive activity, activated during the female anestrus.

1. Introduction

The first member of the neurotrophin family to be identified was the nerve growth factor (NGF) [1], which was followed by brain-derived growth factor (BNDF), as well as additional neurotrophins (NT3, NT4). According to Thoenen and Barde [2] and Snider [3], they are

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crucial for the survival, differentiation, development, and synaptic plasticity of both central and peripheral neurons. The biological effects of NGF are mediated by two classes of receptors, the high-affinity specific 140-kDa neurotrophic tyrosine kinase receptor 1 (NTRK1) and the low-affinity 75-kDa nerve growth factor receptor (p75NTR) belonging to the tumour necrosis factor (TNF) receptor family [4–7].

Although NGF was first identified in neural tissue, it is expressed in a wide variety of non-neuronal cell types, such as thyroid, adrenal, salivary gland [8,9], immune cells [10], vascular endothelial and muscular smooth cells [11], and lung cells [12,13].

Numerous investigations have demonstrated that NGF affects both male and female reproductive function and this topic is covered in interesting and recent reviews [14–20]. In particular, Adams et al. [21] highlighted that NGF, an ovulation-inducing factor (OIF), is a ubiquitous seminal plasma protein involved in the regulation of hormonal and endocrinal events, so controlling reproduction in induced and spontaneous ovulatory species [21].

Bogle et al. [22] analyzed the presence and localization of NGF in male reproductive organs such as testicles, epididymis, ductus deferens, ampulla, coagulating gland, and penis and evaluated the abundance of NGF in the ejaculate of rats, cattle, bison, elks, llamas, and white-tailed deer. In this study [22], NGF concentration in camelid seminal plasma was 10 times higher than in bovine seminal plasma, whereas radioimmunoassay did not identify NGF in horse or porcine ejaculates.

NGF and its receptors are expressed in many male reproductive tract organs of different species such as humans, camelids, and rabbits [21, 23–25], as well as in hamster ejaculated sperm [26].

Squillacioti et al. [27] found that castration increased the expression of NGF and NTRK1 receptors, indicating that these molecules in the accessory sex glands were regulated by testicular steroids.

Further investigation revealed that NGF was also found in the seminal fluid of several livestock species, including alpacas, llamas, rabbits, and camels [28–31]. Additionally, it is well demonstrated that NGF stimulates ovulation in alpacas and llamas [29,32].

Research has demonstrated that NGF affects sperm motility and acrosome reaction in a dose- and time-dependent way [26].

The domestic sheep is a polyestrous species with an average cycle length of 16.5 days. Since its short photoperiod reproductive activity, sheep prefer to breed in the autumn and give birth in the spring [33].

Unlike the female, the ram is less sensitive to seasonal changes. Essentially, the ram’s mating behavior is almost always the same, so that sperm production happens all year round [33].

However, authors report that the best semen yielded is recorded in the reproductive season [34–36]. Additionally, several studies found that sperm quality is impacted by monthly fluctuations in terms of viability, morphology, total and progressive motility, and acrosome integrity [37–39]. The percentage of viable sperm was surprisingly greater in July than it was in November [40,41].

All of this could be influenced by factors produced in the reproductive tract.

To our knowledge, there are no studies about the presence and localization of NGF in the reproductive tract of the ram. Nonetheless, it is quite likely that the ovulatory response may be influenced by the potential OIF presence in the seminal plasma of this spontaneously ovulating species. Additionally, NGF likely influences sperm functions in rams as well. Thus, the primary goal of this investigation was to assess the expression of NGF and its two receptors (NTRK1 and p75NTR) in the sex organs of male rams, whereas, the second goal was to evaluate the annual trend of NGF seminal plasma levels.

The results of this investigation will be used to bolster functional and comparative hints with earlier discoveries about the expression of those compounds in different species.

2. Materials and methods

2.1. Animal and experimental research design

The experiments foreseen in this study were carried out in two different groups of animals. The first group allowed immunohistochemistry and molecular biology studies; the animals came from Italian farms. The second study made it possible to evaluate the concentration of NGF in seminal plasma via ELISA; the animals coming from Spanish farms (Fig. 1).

2.2. Sampling for qPCR and immunohistochemistry

The experimental study was designed and conducted following approval from the Ethical Committee of the University of Perugia (protocol no. 98657 dated May 23, 2023).

Five sexually mature Sarda rams (aged 5–7 years) were utilized in the experimental protocol. The animals involved in this study were slaughtered at the municipal slaughterhouse in Viterbo, following the Council Regulation (EC) no. 1099/2009, ensuring the protection of animals at the time of slaughter, according to Law no. 333/98 (Council Directive 93/119/EC of 22 December 1993). Reproductive organs — testis (T), epididymis (E), vas deferens ampulla (DA), seminal vesicles (SV), prostate (P), and bulbourethral glands (BU) — were sampled for the assessment of NGF, NTRK1, and p75NTR gene expressions via RT-qPCR. The tissues T, E, DA, SV, P and BU were meticulously removed, washed with RNase-free phosphate-buffered saline (PBS), and subsequently frozen at −20 °C until further use. A pilot experiment underscores the requirement for a more specific and sensitive qPCR method for the NTRK1 gene, given its very low expression. It involved the utilization of TaqMan probes and pre-qPCR techniques to enhance the precision and sensitivity of the analysis. The FFPE specimen adjacent to the that employed for RNA extraction was used for immunohistochemistry (IHC) identification of NGF, NTRK1, and p75NTR proteins. Samples for morphology procedures were quickly dipped in 10 % neutral-buffered formalin solution in PBS (0.1M, pH 7.4), left for 36 h, and then processed for histological evaluation until the paraffin wax embedding [42].

2.3. Sampling for ELISA

The experiment was carried out using ejaculates collected from 5 healthy and reproductively mature fertile Rasa Aragonesa rams (aged 48–54 months, 72–89 kg of body weight and genetically heterogeneous). The studies were conducted with the approval of the Ethics Committee for Animal Experiments of the University of Zaragoza (Project License PD28/20, dated September 18, 2020). The experiment was carried out at the farm in Zaragoza, Spain (41°43′26.4″N 0°48′29.9″W/41.724011, -0.808316).

All rams were maintained under uniform nutritional conditions and housed under the same environmental conditions (outdoor access) in isolated enclosures to avoid herd conditioning effects on semen quality (e.g., ewe effect).

2.4. Preparation and fractionation of seminal plasma and ELISA

Two ejaculates were collected twice weekly using a prewarmed artificial vagina and pooled from five rams per day all year-round. Pooled semen was centrifuged for 10 min at 12000 × g at 4 °C. The supernatant was collected and centrifuged again under the same conditions (12000 × g, 10 min, 4 °C) to remove remaining spermatozoa and cell debris. After filtering through a 0.22 mm Millipore membrane (Millipore Ibérica, Madrid, Spain), the recovered seminal plasma was stored in Eppendorf tubes in a −20 °C freezer until use. In the seminal plasma, the NGF protein concentration was evaluated using the ELISA method (Fig. 1).

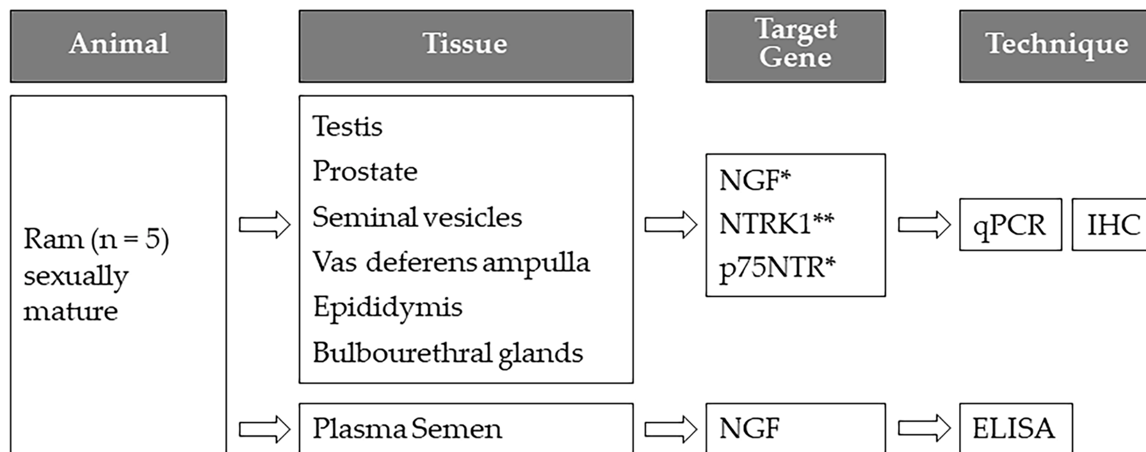

Fig. 1. Experimental Flowchart. The figure shows the experimental design for profiling NGF, NTRK1, and p75NTR gene by qPCR and protein expressions by IHC. * RT-qPCR was carried out using primers and Sybr green DNA intercalating dye. ** RT-qPCR was performed using TaqMan probes.

Seminal plasma NGF concentrations were evaluated using the NGF DuoSet® ELISA Immunoassay kit (DY256, R&D System, Milan, Italy), according to the manufacturer's instructions. This kit has already been used for the detection of NGF in other species (Maranesi 2018 BOR; Maranesi 2020 Animals; Gajardo, 2023; Berland, 2016). Briefly, the plates were pre-coated with the specific Capture Antibody overnight at room temperature. After washing, 100 µl of sample (diluted 1:2 in Reagent Diluent) or NGF standard were added to the plate and incubated 2 h at room temperature. After washing, the specific Detection Antibody and the Streptavidin- Horseradish Peroxidase (HRP) were consecutively added to all wells and incubated at room temperature. Then the Substrate Solution was added to the wells and the plate incubated for 20 min to develop the colour. After adding the stop solution, the optical density of each well was immediately determined using a microplate reader (Biotrak Plate Reader, Amersham Pharmacia Biotech, Cambridge UK) set to 450–570 nm. Assay sensitivity was 12.8 pg/mL, intra- and inter-assay coefficients of variation were 6.8 % and 9.2 %.

2.5. Total RNA purification, and reverse transcription for NGF, NTRK1 and p75NTR genes

Total RNA was purified from ram tissues using the total RNA purification kit (Norgen Biotek Corp., Ontario, Canada). RNA quality and quantity were assessed using NanoDrop™ 2000/2000c (Waltham, Massachusetts, U.S.) and Qubit RNA Assay Life Technologies (Monza, Monza Brianza, Italy).

For reverse transcription, 200 ng of total RNA was reverse transcribed using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Waltham, Massachusetts, U.S.) in a final volume of 20 µL following the manufacturer's guidelines. A negative reverse transcriptase control was included in all RT-qPCR experiments to monitor gDNA contamination.

2.6. Pre-amplification of NTRK1 gene

A preamplification reaction was conducted before qPCR amplification, using 100 ng of cDNA, 12.5 µL of TaqMan™ PreAmp Master Mix (Applied Biosystems, Foster City, CA), 1 µL of each TaqMan probe (Table 1), and water to a final volume of 25 µL.

The preamplification conditions include 10 min at 95 °C (enzyme activation), followed by 13 cycles of 15 s at 95 °C (denaturation) and 4 min at 60 °C (anneal/extend), with a final step of 99 °C for 10 min (enzyme inactivation).

Table 1
TaqMan probes. The table displays the probes utilized in the NTRK1 study. The HPRT1 (hypoxanthine phosphoribosyltransferase 1) and the TBP (TATA-box binding protein) probes are potential endogenous controls; the NTRK1 probe is the target gene. All selected probes are designed for *Ovis aries* sequences (reported in the TaqMan ID column) and bind two exons (Exon Boundary column).

Gene Symbol	TaqMan ID	Reference Sequence	Exon Boundary	Amplicon bp
HPRT1	Oa04825272_gH	XM_015105023.2	7 – 8	52
TBP	Oa04818075_m1	XM_015097549.2	4 – 5	66
NTRK1	Oa04767849_g1	XM_027976575.1	14 – 15	59

2.7. qPCR amplification of NGF, NTRK1 and p75NTR genes

The qPCR amplification was executed using 1,5 µL of cDNA (relative to NGF or p75NTR gene), 1 µL of primer sense and 1 µL of primer reverse (Table 2), and 10 µL of Fast Sybr™ Green Master Mix (Applied Biosystems, Foster City, CA) in a final volume of 20 µL. Following previous studies, a stable reference gene was identified [43].

NTRK1 qPCR amplification was performed using 1 µL of pre-amplified NTRK1, with 1 µL of TaqMan probe (see Table 1) and 10 µL of TaqMan™ Fast Advanced Master Mix (Applied Biosystems, Foster City, CA), in a final volume of 20 µL.

The following qPCR conditions were the same for NGF, NTRK1, and p75NTR. QPCR reactions were amplified under the following conditions: 30 s at 95°, followed by 38 cycles at 95° for 15 s and 60° for 30 s. RT-controls were included to monitor potential genomic DNA contamination. QPCR amplification efficiency was calculated using StepOne Software v2.3 (Applied Biosystems, Foster City, CA). Three technical replicates were performed for each biological sample, and the average Cq value (quantification cycle according to MIQE guidelines) was calculated [44]. Target gene expression was then reported as normalized values according to the Livak 2ˆ-ΔCq method [45].

2.8. Immunohistochemistry of NGF, NTRK1 and p75NTR

Immunohistochemistry was performed on the samples of all tested animals as follows [46]. Formalin-fixed, paraffin-embedded (FFPE) sections of approximately 5 microns were cut and placed on poly-L-lysine coated glass slides followed by deparaffinization in xylene and hydration in ethanol until distilled water.

Sections were microwaved for three 5 min. cycles at 750 W in citrate buffer (pH 6.0) for antigen retrieval and treated for 10 min. with a 3 % hydrogen peroxide solution to block endogenous peroxidase. All subsequent steps were carried out in a moist chamber at room temperature

Table 2
Gene expression primers. The table indicates the primers used in the NGF and p75NTR investigation. The ACTB primers (Sense, Reverse) were used to amplify the endogenous control, whereas the NGF and p75NTR primers were utilized to amplify the target genes. This is followed by the amplicon length (bp) column and the reference sequence column.

Gene Symbol	Forward	Reverse	bp	Sequence
NGF	CAACATCACTGTGGACCCCA	GCCTCGAAGTCCAGATCCTG	117	XM_004002369.5
P75NTR	ACTGTGAACTTGGGGCACAA	TTCAACCCCGTTACCAGCTC	125	XM_027974687.2
ACTB	CCTTAGCAACCATGCTGTGA	AAGCTGGTGCAGGTAGAGGA	130	U39357.2

(RT). Non-specific background and binding were avoided with a 30 min. use of a species-specific normal serum diluted 1:10.

For the immunohistochemical reaction, sections were incubated overnight at RT with a primary antibody (Table 3).

The dilution used for each primary antibody was found to be the best at maintaining signal intensity and reducing background among other dilutions tested in the range of 1:50–1:1000. On the second day, sections were incubated with a 1:200 diluted biotin-conjugated secondary antibody (Table 3). The site of immunological reaction was detected trough an avidin-biotin complex (VectastainElite ABC Kit, Vector Laboratories, Burlingame, CA, USA) and revealed trough the chromogen diaminobenzidine (DAB Substrate Kit, Vector Laboratories, Burlingame, CA, USA). The slides were counterstained with Mayer's hematoxylin and placed with Eukitt medium (Sigma-Aldrich, Alcobendas, Spain) for light microscopy.

Negative control sections were incubated with normal IgG (Novus Biological, Littleton, CO, USA; Table 3) in place of the primary antibody. Gray squirrel ovarian sections were used as a positive control of the reaction [48]. The sections were washed with PBS between all incubation steps, except after normal serum. Sections were observed under a photomicroscope (Nikon Eclipse E800, Nikon Corp., Tokyo, Japan) connected to a digital camera (Nikon Dxm 1200 digital camera).

2.9. Statistical analysis

QPCR output data (Cq) were normalized using the 2ˆ-ΔCq method. The 2ˆ-ΔCq gene expression results were obtained from the normalization of target gene Cq with the selected endogenous control. To compare gene expression 2ˆ-ΔCq among the different reproductive tissues, One-Way ANOVA followed by Tukey's test was used, and the significance level was set at $p \leq 0.05$. Statistical analysis was performed using GraphPad Prism 9 (GraphPad, San Diego, CA, USA).

3. Results

3.1. RNA quality and quantity valuation

The 260/280 and 260/230 RNA absorbance ratios were 1.9 and 2.1, respectively, indicating high RNA purity in each sample. Although the total RNA amount did not significantly differ between samples, minimal variations in total RNA content were adjusted during RT using a fixed RNA input.

Table 3
Antisera characteristics. The table shows the antisera name, the species in which the antibody is raised, the working solution, and the antibody commercialized.

Antisera	Species	Dilution	Commercialized
NGF Recombinant monoclonal	Rabbit	1:100	MA5-32067, Invitrogen, Thermo Fisher Scientific, MA, USA
Anti- NTRK1	Rabbit	1:100/ 1:500	Clary et al., 1994 [47]
Anti-p75 NGF Receptor	Rabbit	1:100	ab52987, Abcam Cambridge, UK
Anti-rabbit biotin-conjugated	Goat	1:200	BA-1000-1.5, Vector Laboratories, CA, USA
Rabbit IgG	Rabbit	1:100	I-1000-5, Vector Laboratories, CA, USA

3.2. Expression level of NGF and p75NTR genes

All primers generated an amplicon with a single peak in the melting analysis confirming the dimer absence. The normalized NGF gene expression (2ˆ - Cq target gene - Cq ACTB) value assessed by qPCR and primers in reproductive tissues revealed a statistically significant NGF upregulation ($p < 0.001$) in SV tissue compared with other male reproductive tissues: BU, P, T, DA, and E. In contrast, the expression of NGF was significantly downregulated in T tissue than all the other tissues ($p < 0.01$; Fig. 2).

The normalized expression value of the p75NTR gene assessed by qPCR and primers pointed out a statistically significant p75NTR upregulation ($p < 0.001$) in SV tissue compared with other male reproductive tissues: BU, P, T, DA, and E. In contrast, the expression of p75NTR was significantly downregulated in T tissue than in P, DA, SV, and E tissues ($p < 0.01$; Fig. 3).

3.3. Expression profiling of NTRK1 gene

The qPCR data were normalized to the most stable endogenous control (EC) selected from two candidates, TBP and HPRT1. The optimal EC was identified by using Delta Ct, BestKeeper, NormFinder, and GeNorm mathematical approaches as described in Guelfi et al. [49]. The RefFinder tools ranked the EC from most stable as TBT to least stable as HPRT1 based on the results of the four algorithms. Consequently, NTRK1 gene expression levels were normalized as follows: 2ˆ - ΔCq = 2ˆ - (Cq target gene - Cq TBP) (Table 4).

The NTRK1 gene evaluated by qPCR and TaqMan probes was expressed in all reproductive tissues examined: T, P, DA, SV, BU, and E, with Cq values ranging between 20 and 30 cycles. The gene expression level of NTRK1 in the SV was significantly upregulated from the expression level in BU, P, T, DA, and E ($p \leq 0.05$). In contrast, no statistically significant differences were observed between NTRK1 expression levels in BU, P, T, DA, and E tissues (Fig. 4).

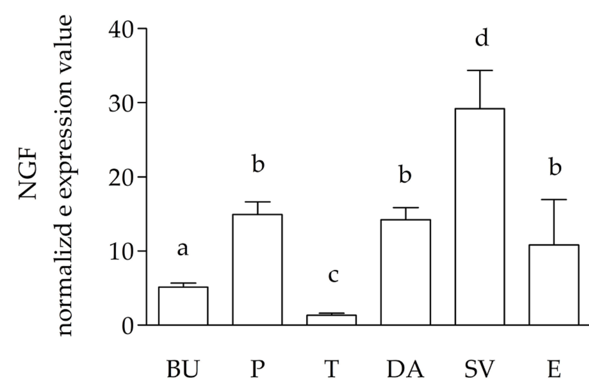

Fig. 2. NGF gene normalized expression values. In SV tissue, normalized NGF expression was significantly upregulated ($p < 0.001$) compared with BU, P, T, DA, and E. In T tissue, NGF expression was significantly downregulated from all other tissues ($p < 0.01$). NGF expression in BU was significantly different versus P, SV ($p < 0.001$), and T, DA, E ($p < 0.01$). The expression of NGF between P, DA, and E shows no significant differences ($p > 0.05$). Each bar represents the mean ± SEM of 5 samples. Different letters indicate a statistically significant difference ($p \leq 0.05$).

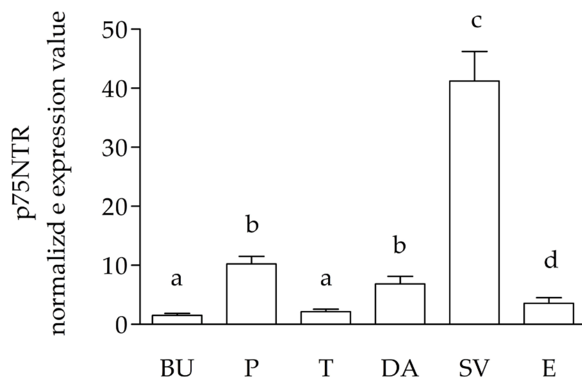

Fig. 3. p75NTR gene normalized expression values. P75NTR expression in BU was significantly downregulated than P, SV ($p < 0.001$), as the same vs DA, and E ($p < 0.01$), while BU vs T showed no differences. P75NTR expression in DA tissue is significantly upregulated versus BU, T, E ($p < 0.01$). In SV tissue, normalized p75NTR was significantly upregulated ($p < 0.001$) compared with BU, P, T, DA, and E. In E tissue p75NTR showed a significantly different expression than all the other tissues ($p < 0.01$). P, DA, and E show no significant differences compared with each other. Each bar represents the mean ± SEM of 5 samples. Different letters indicate a statistically significant difference ($p \leq 0.05$).

Table 4
Endogenous control ranking order. The resulting data obtained from the four methods, Delta Ct, BestKeeper, NormFinder, and GeNorm, were evaluated using the RefFinder algorithm, which provides a comprehensive ranking by ordering the candidate ECs from most stable (first column) to least stable (second column). RefFinder results are highlighted.

Algorithm	EC rank	
Delta Ct	TBP	HPRT1
BestKeeper	HPRT1	TBP
NormFinder	TBP	HPRT1
GeNorm	TBP	HPRT1
RefFinder comprehensive ranking	**TBP**	**HPRT1**

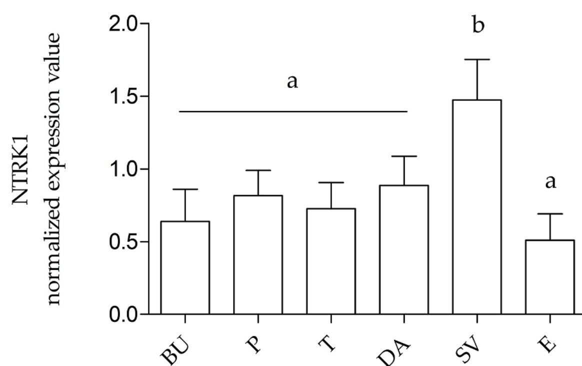

Fig. 4. NTRK1 normalized expression values. The figure shows the NTRK1 normalized gene expression level in BU, P, T, DA, SV, and E tissues. NTRK1 in SV was significantly upregulated ($p \leq 0.05$) versus NTRK1 in BU, P, T, DA, and E tissues. Each bar represents the mean ± SEM of 5 samples. Different letters indicate a statistically significant difference ($p \leq 0.05$).

3.4. NGF protein levels in seminal plasma

The levels of NGF protein were assessed in seminal plasma once a month in all months of the year using the ELISA procedure. Within the 12 months of the year, two clusters of months with significantly different NGF levels ($p < 0.001$) were identified based on ELISA results. NGF levels were 52.00 ± 2.50 pg/mL (mean ± SEM) for the first cluster from January to May while NGF levels were 38.43 ± 1.81 pg/mL (mean ± SEM) for the second cluster from June to December (Fig. 5).

3.5. Immunohistochemical localization of NGF, NTRK1 and p75NTR in the ram reproductive tissues

The immunohistochemical investigation demonstrated the presence of the neurotrophin NGF and the receptors NTRK1 and p75NTR on all examined organs of the ram reproductive system (Table 5).

NGF was mainly observed in the cytoplasmic component of the lining and glandular epithelial cells, with small differences depending on the organ examined (Fig. 6). NGF immunostaining was also observed in nerve fibres and peripheral ganglia, lining epithelium of the vas deferens and urethra, skeletal muscle of the urethra wall (Fig. 7). Nerve fibres and peripheral ganglia positivity was used as a positive control of the antibody, inside the section [50]. In the testis, immunopositivity for NGF was observed in the spermatids, starting from the Golgi phase up to the final maturation phase of spermiogenesis (Fig. 6a, b); indeed, in the different convoluted seminiferous tubules, the immunostaining was visible in the cytoplasmic component of the spermatids with a different localization depending on the stage of maturation: from the perinuclear region in the Golgi phase to the adluminal position in the acrosome phase up to the localization only in the tail in the maturation phase. At the level of the epididymis, NGF staining was localized in the epithelium lining the organ (Fig. 6c). In the ampulla of the vas deferens (Fig. 6d), in the seminal vesicles (Fig. 6e), and in the disseminated component of the prostate (Fig. 6f) positivity for NGF was localized in the glandular epithelium. Finally, in the bulbo-urethral gland NGF was observed in the apical region of the epithelial cells lining the ducts (Fig. 6g) and in the glandular adenomeres (Fig. 6h) where the appearance of the staining varied contextually with the histological characteristics of the cells. In total, secretion-rich pyramidal cells, the immunostaining was localized in the cell basal region near the nucleus, while in the cylindrical and smaller resting state cells the staining was distributed throughout the cytoplasm.

As well as in the epithelial component, NGF immunostaining was also observed in the peripheral ganglia and nerve fibres (Fig. 7a), in the skeletal muscle of the urethra wall (Fig. 7b), in the lining epithelium of the vas deferens (Fig. 7c) and of the urethra, (Fig. 7d). Nerve fibres and peripheral ganglia positivity were used as a positive control of the antibody, inside the section [50]. No staining was evidenced in the negative control sections incubated with normal IgG (data not shown).

The NTRK1 receptor was observed by immunohistochemistry in all examined organs of the ram reproductive system with a localization pattern similar to the NGF molecule one and, in addition, in the lining epithelium of the urethra. In the testis, NTRK1 receptor immunostaining was observed in the cytoplasmic component of spermatids during the different phases of the spermiogenesis (Fig. 8a): in the perinuclear region during the Golgi phase; in the adluminal position of the cytoplasm

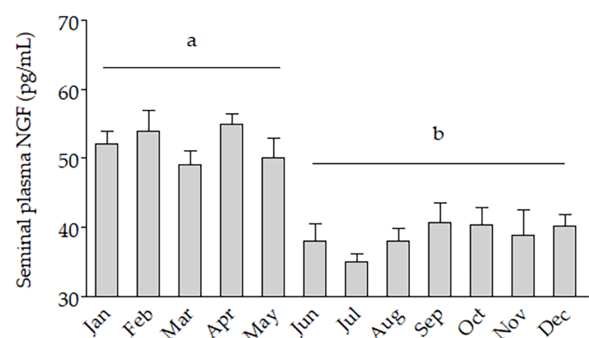

Fig. 5. NGF levels in seminal plasma. Each bar represents the mean ± SEM of 5 samples. Different letters indicate a statistically significant difference ($p \leq 0.01$).

Table 5
NGF, NTRK1 and p75NTF immunopositivity in in the organs of the ram reproductive system. The intensity of the staining was graded in arbitrary units as follows: negative (-), low (+), moderate (++) and intense (+++) [51]. T: Testis; E: epididymis; DA: vas deferens ampulla; SV: seminal vesicles; P: prostate; BU: bulbourethral glands; EC: epithelial cells; NF: nerve fibers.

		T	E	DA	SV	P	BU
NGF		++	+++	+++	++	++	+++
NTRK1		+	-/+	+++	+++	++	++
P75NTR	EC	-	-	-	-	-	-
	NF	+++	+++	+++	+++	+++	+++

during the acrosome phase and in the tail during the maturation phase. In the other organs, immunostaining was localized in the cytoplasmic component of the lining and glandular epithelial cells. In the epididymis, a quite weak NTRK1 receptor immunostaining was localized in the epithelium lining the organ (Fig. 8b). The glandular epithelium of the ampulla of the vas deferens (Fig. 8c) and especially of the seminal vesicles (Fig. 8d) showed the stronger immunostaining. NTRK1 receptor was observed in the glandular epithelium of the disseminated lobules of the prostate (Fig. 8e). Finally, in the bulbourethral gland NTRK1 receptor was observed in the apical region of the epithelial cells lining the ducts and in the glandular adenomeres (Fig. 8f): in tall, secretion-rich pyramidal cells, the immunostaining was localized in the cell basal region near the nucleus, while in the cylindrical and smaller resting state cells the staining was distributed throughout the cytoplasm. No staining was evidenced in the negative control sections incubated with normal IgG (data not shown).

The p75NTR receptor was observed in all organs of the reproductive system of the ram. The immunostaining was localized at the level of the nervous tissue, i.e. in the nerve fibers and the ganglia, while the epithelial components of the organs such as the lining epithelia and glandular epithelia were negative for this receptor (Fig. 9). In the testis and epididymis, positivity was observed in thin nerve fibers located in the lamina propria of both organs, beneath the epithelium and in the perivascular regions (Fig. 9a, b). In the ampulla of the vas deferens (Fig. 9c), in the seminal vesicles (Fig. 9d), in the prostate gland (Fig. 9e), and in the bulbourethral gland (Fig. 9f) positivity was observed in the larger nerve bundles and the ganglia located in the adventitia of the organs as well as in the thinner nerve branches infiltrating in the connective septa and the lamina propria reaching the glandular epithelium and parenchyma of the organs. No staining was evidenced in the negative control sections incubated with normal IgG (data not shown).

4. Discussion

This is the first study that details the differential gene and protein expression for NGF and its cognate receptors, NTRK1 and p75NTR, in the ram male sex organs. NGF immunoendocrine communication can be defined in the sex organs of rams as autocrine and paracrine signaling. We describe the NGF signal as autocrine because the cell can produce NGF mRNA, NGF protein, and NGF receptors to be self-regulating. NGF

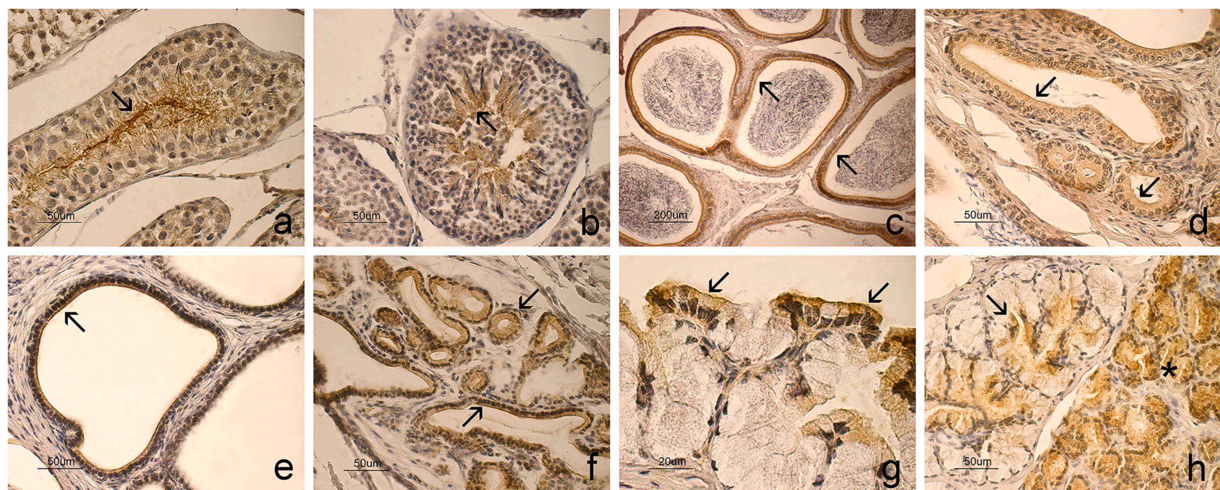

Fig. 6. Immunopositivity for NGF in the organs of the ram reproductive system. NGF-positive spermatids are observed in different phases of spermiogenesis such as the maturation phase where the immunostaining is localized in the tails (a, arrow) and the acrosome phase where the staining is in the cytoplasmic adluminal region (b, arrow). NGF-positivity is observed in the lining epithelium of the epididymis (c, arrows) and the glandular epithelium of the ampulla of the vas deferens (d, arrows), of the seminal vesicles (e, arrows) and the prostate (f, arrows). In the bulbourethral gland, the staining is localized in the supranuclear cytoplasmic region of the cells of the epithelium lining the ducts (g, arrows) and in the secreting adenomeres (h) where positivity is localized in the basal region of the active foamy cells (arrow) and the whole cytoplasm of resting cells (asterisk). Sections are counterstained with hematoxylin.

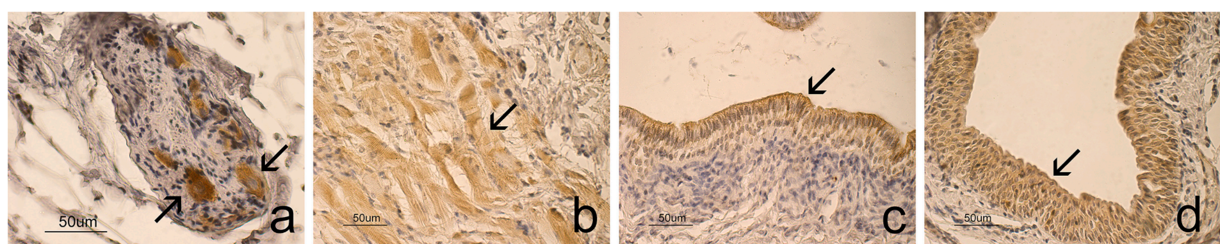

Fig. 7. Immunopositivity for NGF in some structures of ram genital tract. A peripheral ganglion (a) where positivity is localized in the neuron cytoplasm (arrows); NGF-positive skeletal muscle fibres (arrow) in the urethra wall (b); lining epithelium (arrows) of vas deferens (c) and urethra (g). Sections are counterstained with hematoxylin.

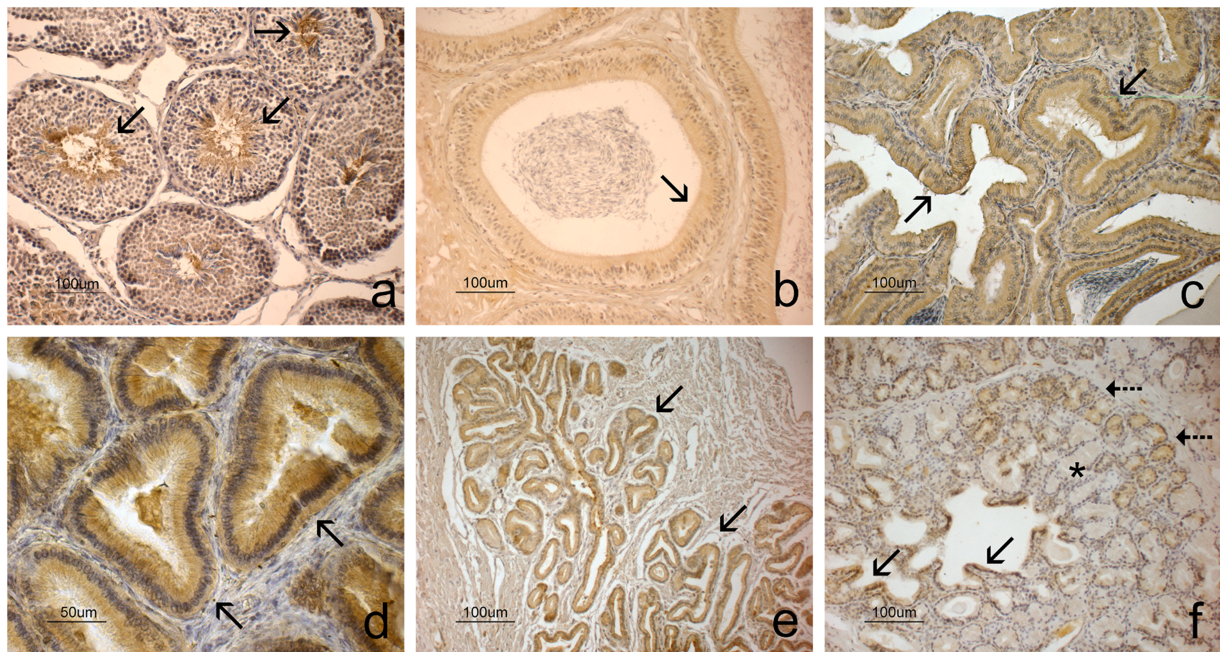

Fig. 8. Immunopositivity for NTRK1 receptor in the organs of the ram reproductive system. NTRK1 receptor positivity is observed in the tails and the cytoplasmic adluminal region (arrows) of the spermatids at different spermiogenesis phases inside convoluted seminiferous tubules (a). A quite weak positivity is observed in the lining epithelium of the epididymis (b, arrow). A strong positivity is observed in the glandular epithelium of the ampulla of the vas deferens (c, arrows), the seminal vesicles (d, arrows) and a prostate lobule (e, arrows). In the bulbourethral gland, the staining is localized in the supranuclear cytoplasmic region of the cells of the epithelium lining the ducts (f, arrows) and in the secreting adenomeres (h), where positivity is localized in the basal region of the active foamy cells (asterisk) and the whole cytoplasm of resting cells (dotted arrows). Sections are counterstained with hematoxylin.

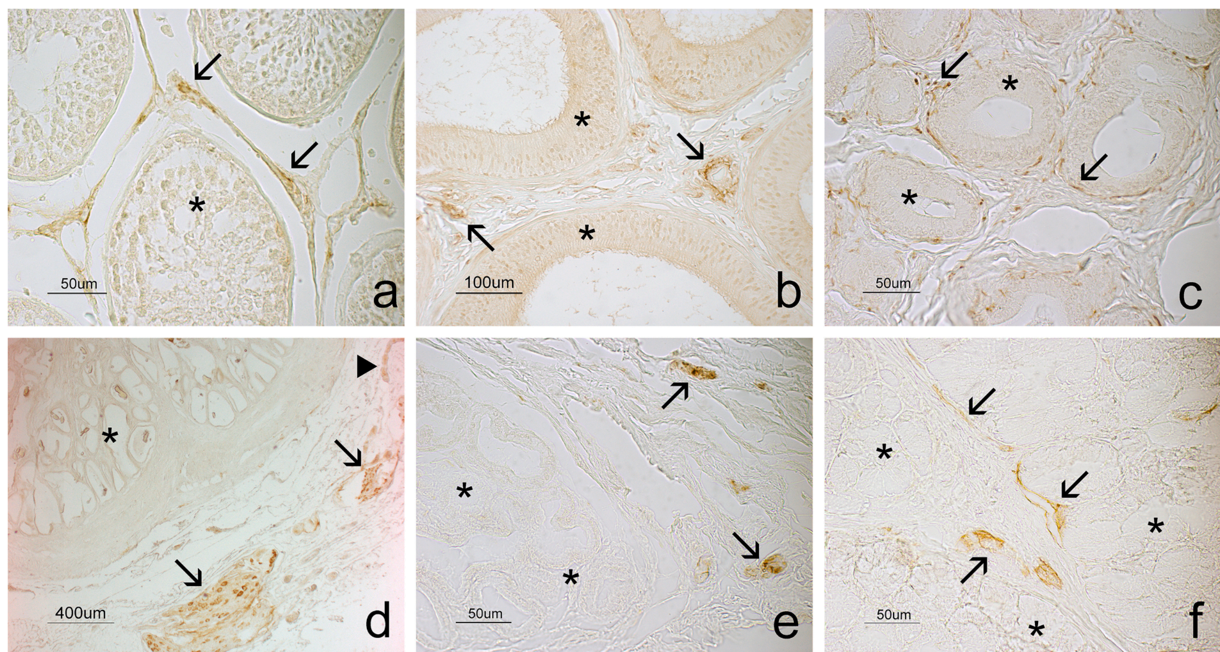

Fig. 9. . Immunopositivity of p75NTR in the organs of the ram reproductive system. Immunostaining is shown in the nerve fibers located in the lamina propria and in the perivascular areas of the testis (a, arrows), epididymis (b, arrows), ampulla of the vas deferens (c, arrows); in a ganglion (arrows) and a nerve bundle (arrowhead) in the adventitia of the seminal vesicles (d); in nerve fibres (arrow) localized in the lamina propria of urethra next to a prostate lobule (asterisks, e); in nerve fibres (arrows) infiltrating in the connective septa separating adenomeres (asterisks) of the bulbourethral glands (f). Asterisks in the images point out negative epithelium of the testis (a), epididymis (b), vas deferens ampulla (c), seminal vesicles (d), prostate (e), bulbourethral glands (f). These sections were not counterstained.

communication is defined as paracrine because the NGF protein produced by one cell can be exported and bind the NGF receptors of neighboring cells. In our research, it is confirmed that sex ram tissues synthesize NGF mRNA and NTRK1 and p75NTR NGF receptors.

Immunohistochemistry revealed, albeit with different levels of staining, positive signals for NGF, NTRK1, and p75NTR in several cells and structures including the germ cells of the gonad, the lining epithelium of the epididymis, glandular epithelium of the vas deferens

ampulla, seminal vesicles, prostate glands and bulbourethral glands as well as the nerve fibres and peripheral ganglia annexed to the analysed organs.

Through RT-qPCR analysis, all sex organs under investigation expressed NGF, NTRK1, and p75NTR mRNAs, albeit in varying relative amounts. There are many intriguing concerns regarding the possible roles of this neurotrophin in the regulation of reproductive function, given the vast distribution and variable expression pattern of each NGF system component inside particular cellular types of ram male sex organs.

The immunohistochemical investigation found NGF in all organs analyzed in this study in agreement with the literature on other species [25,26]. The positivity of the molecule was observed in the cytoplasm of the lining epithelial and glandular epithelial cells about the different analised organs [25,26,52].

At the testis level, NGF was localized in the spermatids, starting from the Golgi phase up to the final maturation phase of spermiogenesis. This localization has been already described in other animal species: in the spermatozoa collected from the rete testis and different regions of the epididymis, such as the head and tail, in the golden hamster [26], in the spermatozoa of the sperm ejaculated in the llama [53], in bull ejaculate [54], and human sperm [55]. It has been previously demonstrated that NGF stimulates important sperm functions such as apoptosis, motility and the acrosome reaction and in the case of the last two activities the regulation occurs in a time-dependent and dose-dependent manner [26]. The regulation of spermatogenesis by NGF occurs through an autocrine and paracrine mechanism [56–59], while a role for NGF in the maturation of spermatids was hypothesized, based on the presence of NGF during the different phases of spermiogenesis [26]. In this study, the NGF localization in the spermatids together with the NTRK1 receptor, strongly supports the hypothesis that also in rams NGF may play an important role in the maturation of these germinal cells as well as in sperm motility and acrosome reaction through an autocrine and paracrine mechanism.

It has been suggested that NGF has an autocrine and/or paracrine role during testicular development and spermatogenesis since NGF and NTRK1 have been selectively identified in the germinal and endocrine cells of the male gonads [58,60]. Using an NTRK1-KO mouse model, direct evidence was produced that the NGF/NTRK1 system negatively affects testicular development [61].

All these findings from other mammalian species support the idea that the NGF system pathway is critical also for the development of the ram's testis.

The columnar secretory epithelial cells of the ram prostate gland exhibited a strong positive response to NGF and NTRK1. The existence of NGF and its receptor in these cell types suggests that this neurotrophin regulates the growth and differentiation of these cells, through intricate autocrine and/or paracrine processes. Additionally, as shown in several other species, including humans, in rabbit the prostate is the primary source of this neurotrophin for the seminal plasma, as confirmed by the extensive dispersion of NGF and its mRNA [62]. NGF has also been shown to have a particular mitogenic effect in rat prostate tissue [63].

These findings suggest that the ovulation-inducing component NGF is less abundant in the seminal plasma of ram (38–52 pg/ml) than in llama (1.2 mg/dl [22]), rabbit (ranging from 1894 pg/ml to 151 μg/ml [25,64]), human (763 pg/ml [65], 820 pg/ml [54]), bulls (0.73 to 7.19 μg/mL [66], 1860 pg/mL [67], 20.5 μg/mL [68]), but more abundant than horses, and pigs, where the NGF concentration resulted to be lower than 10 pg/ml [22,30]. Although at lower concentrations, it cannot be excluded that seminal plasma NGF may have significant effects in ewes, although it could be a different effect to that highlighted in camelids; in the latter, a high concentration of seminal plasma NGF is recorded and it is well established that NGF can induce ovulation in 100 % of llamas [69–71].

Interestingly, in the seminal plasma examined, NGF appears at reduced concentrations during the reproductive activity of this animal species which, being a negative photoperiod, in our latitude, begins in late summer and ends in the autumn season, raising the intriguing possibility that this component may be involved in a mechanism that inhibits male reproductive activity and is triggered during the female anestrus. In contrast, Zhang et al., in the testis of wild ground squirel via immunohistochemistry, western blot and RT-PCR, demonstrated that the expressions of NGF and NTRK1 are higher in the breeding season than in the non-breeding season [72].

Even melatonin, a fundamental molecule for the reproductive activity of this animal species, undergoes important fluctuations in the seminal plasma, with lower concentrations during the non-reproductive season [73,74].

The NGF/NTRK1 system may have a function in the cell biology of these tissues in both healthy and pathological circumstances, as evidenced by its presence in benign prostatic hyperplasia, prostatic cancer cells, and normal prostatic tissue [62,75].

The localization of NGF in the epididymis and accessory glands has been observed in the lining epithelia and the glandular epithelia with results that are sometimes consistent and sometimes contrasting with the numerous data present in the literature about the different animal species [22,25,26,52]. In rabbit, NGF has been highlighted in the seminal vesicles and prostate [25,52]; in rat, NGF has been described in the epididymis, vas deferens, prostate and coagulation glands [76]. In ruminants, NGF positivity was observed in numerous male glands partially related to the abundant distribution observed in the ram in our study. Bogle et al. [22] described its immunopositivity in the epididymis and ampulla of cattle, bison, and deer; in the prostate of llamas, cattle and deer; in the seminal vesicles of cattle and bison; in the bulbo-urethral glands of llamas and deer. Therefore, the literature suggests that NGF is a common protein in the male accessory glands among various animal species and its abundance in camelids, bovids and cervids has an important role in the ovulation mechanisms of both spontaneous and induced species [22].

Similar to the distribution of NGF, the receptor NTRK1 was observed in all the analysed organs of the ram. In particular, this receptor showed a similar localization pattern of NGF attesting that the NGF system plays a role likely related to the local regulation of each organ function, by a paracrine and/or autocrine mechanism. NTRK1 was already observed in the male reproductive organs of other species as in the spermatids of the rat [76]; in the prostate, bulbourethral glands and epididymis of sexual mature male rabbit [25,52] in the prostate, bulbourethral glands, and epididymis of the llama [22,53]. Some authors also suggested an influence of the NGF system on the concentration and sperm motility in the epididymis [54].

The tissue abundance of NGF mRNAs was, in order, seminal vesicles, prostate, vas deferens ampulla, epididymis, bulbourethral gland, and testis, in agreement with IHC findings, where NGF was higher in the seminal vesicle gland than in the other genital tract tissues examined. Remarkably, NTRK1 and p75NTR mRNA levels showed a comparable distribution pattern, with strong expression in the seminal vesicles. These small variations in the NGF system could simply reflect morphological variations in the quantity and kind of positive cells, or they could be related to the distinct embryological origins of the sex organs.

The large quantity of NGF found in the seminal vesicles is in agreement with the study of Hofman and Hunsicker [77], which reported that the bull seminal vesicle was very rich in NGF (up to 0.68 mg/ml) and probably the main source of this factor in the seminal plasma of this ruminant. In addition, NGF is present in the seminal vesicle at amounts similar to those in the mouse submaxillary gland, which is considered the primary tissue source of NGF in this rodent [78].

According to Harper et al. [79] and Squillacioti et al. [27], the prostate and seminal vesicles are the main locations of NGF expression in male guinea pigs and rats. Additionally, the caudal area of the epididymis appears to express more NGF than the other segments [26].

The quantity of NGF mRNA and NTRK1 in accessory glands may suggest that NGF could influence the ejaculated sperm behavior. NGF

and NTRK1 were co-expressed in the seminal vesicle epithelial glandular cells as well as bulbourethral glands and prostate. It suggests that NGF could regulate cell development and function of these organs, through paracrine and/or autocrine mechanisms. A considerable amount of the fluid that eventually turns into semen is secreted by the seminal vesicles, so suggesting that the relative NGF abundance in the glands of this organ may play a key role in its accumulation within the seminal plasma. Similarly, it can be hypothesized that the prostate and the bulbourethral glands may also contribute to the secretion of NGF in the seminal plasma, although to a lesser extent than the seminal vesicles. Our immunohistochemical analysis highlighted the presence of the p75NTR receptor on all the ram samples examined, i.e., gonads, epididymis and glandular tissues responsible for the production of seminiferous plasma. In all organs, p75NTR was observed in the nerve fibers in contrast with some data present in the literature relating to the reproductive system. Indeed, in the rabbit p75NTR was observed in the Leydig cells of the testis, the stromal cells and the glandular epithelium of the prostate, and the stromal cells of the seminal vesicles [25]. In the Japanese monkey, p75NTR has been observed in the epithelium and smooth muscle of the epididymis, prostate and seminal vesicles [80]. In the rat, at the level of germ cells, spermatids, Leydig cells, Sertoli cells, epididymis, vas deferens and accessory glands [76]. However, numerous studies carried out on non-reproductive organs described p75NTR at the level of nerve fibers: it has been identified in sympathetic neurons and small-diameter peripheral sensory neurons that mediate nociception both during development and in adulthood [81,82]; in bone and articular cartilage, p75NTR was located in nerve fibers near NGF-positive blood vessels [83]; in the bladder, this receptor is in the nerve fibers reaching the urothelium and in the nerve bundles of the submucosa [84]. p75NTR is in the peripheral portion of the nerve trunks and fibers reaching the skin [85].

Ultimately, this research validates that the NGF/cognate receptors system is extensively expressed throughout several cell types in the sex organs of ram. This expression implies a role for NGF in spermatogenesis and testicular development. This study evidenced that this system is expressed in the reproductive tissues of all the different genital tracts examined, confirming the role of NGF in ram reproduction. Sheep are short-day breeders, with an anestrus that corresponds to the highest seminal plasma NGF levels, thus suggesting the intriguing idea that this factor could participate in an inhibition mechanism of male reproductive activity, arising during the female anestrus.

The gene and protein expression studies described in this article that evaluated the NGF system in the ram reproductive tract were conducted during the non-breeding season. In future research this system will be examined, from a comparative perspective, in the breeding and non-breeding seasons.

A deeper knowledge of these complex cellular mechanisms could be useful to improve the reproductive performance of farm animals and humans too.

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CRediT authorship contribution statement

Francesca Mercati: Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Gabriella Guelfi:** Writing – review & editing, Supervision, Methodology, Investigation. **Martì José Ignacio Martì:** Methodology, Investigation. **Cecilia Dall'Aglio:** Methodology, Investigation. **Lucía Calleja:** Writing – review & editing, Methodology, Investigation. **Domenico Caivano:** Methodology, Investigation, Conceptualization. **Maria Luisa Marenzoni:** Methodology, Investigation, Conceptualization. **Camilla Capaccia:** Writing – review & editing, Methodology, Investigation. **Polina Anipchenko:** Writing – review & editing, Methodology, Investigation. **Francesco Alessandro Palermo:** Methodology, Investigation. **Paolo Cocci:** Methodology, Investigation. **Mario Rende:** Methodology, Investigation. **Massimo Zerani:** Methodology, Investigation. **Margherita Maranesi:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

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