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EXPRESSION OF β-NGF AND HIGH AFFINITY NGF RECEPTOR (TrKA) IN LLAMA (*Lama glama*) MALE REPRODUCTIVE TRACT AND SPERMATOZOA

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Short title:

β-NGF and TrKA expression in llama male

Abstract

β-Nerve Growth Factor (β-NGF) is a seminal plasma element, responsible for inducing ovulation in camelids. The main organ of β-NGF production remains nondescript. The aims of this study were: (1) characterize gene expression and protein localization of β-NGF and its main receptor TrKA in the llama male reproductive tract, and (2) determine whether the seminal β-NGF interacts with ejaculated sperm by localizing β-NGF and TrKA in epididymal, ejaculated, and acrosome reacted (AR) sperms and, additionally, by identifying β-NGF presence in sperm-adsorbed proteins (SAP). Both β-NGF and TrkA transcripts are widely expressed along the male reproductive tract, with a higher expression level of β-NGF at prostate ($p<0.05$). β-NGF immunolabeling was only positive for prostate, whereas TrKA label was present in epithelial and muscular cells of testis, prostate, bulbourethral glands and epididymis. Using an

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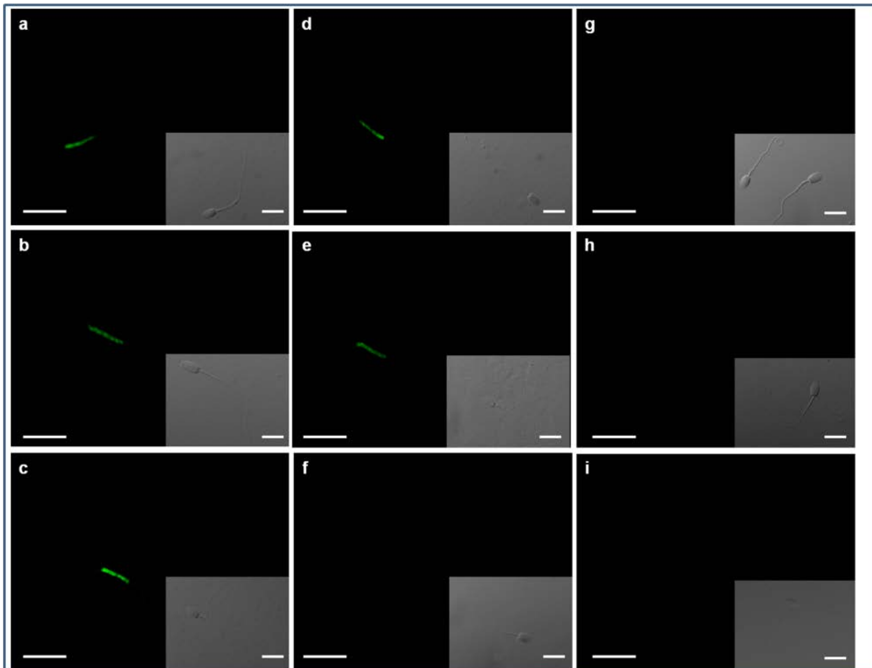
immunofluorescent technique, β-NGF was co-localized with TrKA in the middle piece of ejaculated and AR sperm. However, only TrKA was observed in epididymal sperm indicating that β-NGF could have a seminal origin. This was also confirmed by identification of four β-NGF isoforms in SAP. This study extends the knowledge about the participation of β-NGF/TrkA in llama reproduction, providing evidence that may have roles in the regulation of sperm physiology.

Graphical Abstract

Both β-NGF and TrkA transcripts are widely expressed along the male reproductive tract, with a higher expression level at prostate. TrKA but no β-NGF was observed in epididymal sperm, whereas β-NGF and TrkA were present in ejaculated sperm (acrosome-reacted and non-acrosome-reacted). Four β-NGF isoforms were observed in sperm adsorbed proteins.



Keywords: Spermatozoa, seminal plasma, β-nerve growth factor, South American Camelids

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Abbreviations:

SAP, sperm-adsorbed protein; EE, electroejaculation; OIF, ovulation-inducing factor; SP, seminal plasma; AR, acrosome reacted.

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1. INTRODUCTION

Nerve growth factor-beta (β-NGF) is a polypeptide growth factor that regulates survival, growth, and differentiation of specific peripheral and central neurons via its high-affinity receptor, tyrosine kinase receptor A (TrKA), and low-affinity receptor, p75 neurotrophin receptor (p75NTR) (Dechant & Barde, 1997; Schecterson & Bothwell, 2010). Although initially discovered in the neural tissue, β-NGF was subsequently found to be expressed in a large array of non-neuronal cell types (Ceccanti et al., 2013; Ehrhard, Erb, Graumann, & Otten, 1993; Ricci et al., 2007; Yamamoto, Sobue, Yamamoto, & Mitsuma, 1996).

Several studies have shown that β-NGF also influences the reproductive function of both males and females (Bao et al., 2014; Li & Zhou, 2013). In this regard, expressions of β-NGF and its high and low affinity receptors, TrKA and p75NTR, have been detected in different organs of the male reproductive tract in a large variety of species (Li et al., 2005; MacGrogan, Despres, Romand, & Dicou, 1991; Shikata et al., 1984; Wang, Dong, Chen, Hei, & Dong, 2011). The prostate and seminal vesicles

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are the major sites of β-NGF expression in the bull (Harper & Thoenen, 1980; Hofmann & Unsicker, 1982), the male guinea pig (Harper et al., 1979), the rat (Squillacioti, De Luca, Paino, Langella, & Mirabella, 2009) and the rabbit (Maranesi et al., 2015). Both β-NGF and its corresponding receptors (TrKA and p75NTR) have been implicated in autocrine and paracrine regulation of spermatogenesis (Mutter, Middendorff, & Davidoff, 1999; Parvinen et al., 1992; Persson et al., 1990; Seidl, Buchberger, & Erck, 1996) and also in testis morphogenesis (Russo et al., 1999). Moreover, β-NGF might play a role in regard to sperm function. Presence of β-NGF and its receptor TrKA have been reported in hamster epididymal sperm (Jin, Tanaka, Watanabe, Matsuda, & Taya, 2010) and in ejaculated bull and human sperm (Li et al., 2010; Li, Zheng, Wang, & Zhou, 2010); supporting evidence that β-NGF modulates certain sperm functions such as apoptosis, sperm motility and acrosome reaction in a time- and dose-dependent manner (Jin, Tanaka, Watanabe, Matsuda, & Taya, 2010).

The β-NGF has also been described in the seminal fluid of several livestock species (Adams, Adams, Singh, & Baerwald, 2012; Druart et al., 2013).

In camelids, β-NGF was pointed as the major ovulation-inducing factor (OIF) component of seminal plasma (SP) capable of inducing ovulation in llamas (Ratto, Delbaere, Leduc, Pierson, & Adams, 2011), alpacas (Kershaw-Young, Druart, Vaughan, & Maxwell, 2012) and dromedary (Fatnassi et al., 2017). Additionally, a recent study in the rabbit, other reflex ovulator species, also postulated the β-NGF as the main ovulation inducing factor of the seminal plasma (constituting 1.5% of the total proteins). The β-NGF may induce ovulation via endocrine and neural

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mechanisms (Maranesi et al., 2018). Similar findings were evidenced in urethrostomized llamas (Berland et al., 2016).

A recent study by immunohistochemistry indicates that the primary seminal source of β-NGF would be the prostate in llamas (Bogle et al., 2018), however there is not information regarding to the gene expression of this factor. Similarly, it is also unknown if this factor is capable of influencing the sperm physiology in these species, considering that concentration of β-NGF is particularly high in the SP of camelids, up to 15–45% of total protein (Fatnassi et al., 2017; Tanco, Ratto, Lazzarotto, & Adams, 2011).

The present study has, therefore, been undertaken to: (1) determinate the presence and abundance of *β-NGF* and *TrKA* transcripts and protein localization in the male reproductive tract in llamas, (2) determine whether the β-NGF of SP interacts with ejaculated sperm by localizing β-NGF and TrKA in epididymal, ejaculated non-reacted, and ejaculated acrosome reacted sperm and, additionally, by identifying its presence in sperm-adsorbed proteins (SAP).

2. RESULTS

Experiment 1

mRNA relative abundance of β-NGF and TrKA in llama male reproductive tract

Transcripts for *β-NGF* and *TrKA* were detected in testis, epididymis (head, body, and tail), bulbourethral gland and prostate, but with distinct abundance (Figure 1). *β-NGF* relative abundance was greater in the prostate than in the testis, body, and tail of

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epididymis and bulbourethral gland ($p < .05$). No statistical differences were found for epididymis head expression. While, mRNA relative abundance of *TrKA* was at steady state amounts between testicles, prostate, bulbourethral glands and epididymis.

Immunolocalization of* β-NGF *and* TrKA *in the reproductive tract of llama males

The immunopositive reaction of TrKA was detected along the reproductive tract, whereas only the prostate showed positive immunostaining for β-NGF (Figure 2). No immunostaining was in control sections.

In testicles, clear immunostaining of TrKA was observed in seminiferous tubules: Sertoli cells, spermatocytes, and spermatids (Figure 3a). A strong reaction was also noted in the Leydig cells. No immunoreaction was observed in the tunica albuginea.

In the caput of the epididymis, positive staining for TrKA was restricted to the epithelial cells, being with a strong mark in the stereocilia. All epithelial cell types and smooth muscles of the corpus and cauda epididymis were strongly positive for TrKA (Figure 3b-d).

Within the tubuloalveolar bulbourethral glands, only the apical epithelium of the ducts exhibited a regular TrKA immuno-staining, whereas the mark was irregularly distributed in the acini and smooth muscle (Figure 3e).

In the prostate, positive labeling for TrKA was detected in the apical secretory epithelium, whereas mild mark was observed in the smooth muscles underlying gland cells (Figure 3f).

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Strong immuno-staining of β-NGF was localized in the whole cytoplasm of epithelial cells of prostate glandular epithelium having accumulation of the reaction products on the supra-nuclear portion. No mark was detected at stroma level (Figure 2f).

Experiment 2

Immunolocalization of β-NGF and TrKA in epididymal sperm, ejaculated non-acrosome reacted and ejaculated acrosome reacted sperm

TrKA protein was present in the middle piece of epididymal and in ejaculated sperm, both acrosome reacted (AR) and non-AR (Figure 4 a-c).

β-NGF was mainly localized in the middle piece of both AR and non-AR ejaculated sperm, whereas no label was detected in epididymal sperm (Figure 4 d-e).

Experiment 3

Identification of β-NGF in llama SAP

β-NGF western analysis of SAP revealed four β-NGF species of 13, 17, 23 and 35 kDa (Figure 5). Among them, the 13, 23 and 35 kDa protein bands identity was confirmed by mass spectrometry, showing sequence homology with the *Camelus ferus* Beta-nerve growth factor precursor sequence registered in the database of the National Centre for Biotechnology (Accession Number EPY74508.1) (Table 1). The identity of the 17 kDa band was undetermined due to the absence of a significant score.

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3. DISCUSSION

To the best of our knowledge, this is the first report that describes the differential expression of mRNA and proteins of β-NGF and its high affinity receptor TrKA, in the reproductive tract of male llamas. β-NGF, as a component of the seminal plasma, has an important role in camelids reproduction, being responsible for inducing ovulation (Ratto, Delbaere, Leduc, Pierson, & Adams, 2011). In other species, both prostate and vesicular glands are the major sites of β-NGF expression (Hofmann & Unsicker, 1982; Maranesi et al., 2015; Squillacioti, De Luca, Paino, Langella, & Mirabella, 2009). It is known that in camelids vesicular glands are absent (Tibary & Anouassi, 1997). In the present study, the noticeable greater amounts of *β-NGF* transcripts and protein found in the prostate would indicate that, in the llama, seminal β-NGF has a prostatic origin.

A recent report, based on immuno-histochemical analysis, also pointed the prostate as the main source of β-NGF production in llamas (Bogle et al., 2018). Authors found β-NGF positive signal along the male reproductive tract, whereas in the present study was only found in the prostate. This could be explained by the different primary antibody working dilutions used in these two studies (1:400 vs 1:1,500). Our results also differ from reports in other mammals in which positive β-NGF immunostaining was observed not only in prostate but also in testicles, epididymis and other accessory glands (e.g. rabbit [Maranesi et al., 2015], golden hamster [Jin, Tanaka, Watanabe, Matsuda, & Taya, 2010], and rat [Li et al., 2005]). On the other hand, *β-NGF* gene expression was found along the male reproductive tract. Even though it is advised to use the lesser primary antibody concentration, it should be considered the possibility

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that immunohistochemistry resulted less sensitive than PCR, therefore presence of low amounts of β-NGF in testis, epididymis and bulbourethral glands cannot be discarded.

The presence of β-NGF specific receptor, TrKA indicates that the organs studied are capable of responding to β-NGF. Genetic expression of both, *β-NGF* and *TrKA*, would indicate a role in the reproductive functions. In the gonads, it has been postulated that β- NGF exerts an autocrine and/or paracrine function during testicular development and spermatogenesis (Koeva, Davidoff, & Popova, 1999; Persson et al., 1990). The co-expression of β-NGF and its receptor TrKA, within the same apical epithelium and muscular cells of prostate also supports a possible regulatory function on cell development.

In order to increase our knowledge about the functions of β-NGF described in camelids seminal plasma, we study its presence in spermatozoa. We revealed that β-NGF receptor TrKA, but no β-NGF was expressed at epididymal sperm cells, whereas ejaculated sperm were immune-positive for both, β-NGF and TrKA. This observation led us to presume that β-NGF is provided by seminal plasma to the spermatozoa. Supporting this hypothesis, β-NGF was detected abundantly in SAP fraction, easily removed by 0.5 M KCl. This technique is consistent with descriptions for SAP in llama (Zampini, Sequeira, Argañaraz, & Apichela, 2017) and other livestock (e.g., bulls [Einspanier et al., 1994; Manjunath, Bergeron, Lefebvre, & Fan, 2007], rams [Bergeron, Villemure, Lazure, & Manjunath, 2005; Pérez-Pé, Cebrián-Pérez, & Muino-Blanco, 2001], and boars [Garénaux et al., 2015]).

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Western blot revealed four β-NGF isoforms in SAP, being more abundant the 13 kDa mature form. The 16.5 kDa mature form described by Seidah et al. (1996) could be coincident with the 17 kDa form here described. The 35 kDa precursor has been described in human prostate, as well as the partially processed 22-23 kDa form of pro-NGF (Delsite, & Djakiew, 1999).

In the present study, β-NGF localization was restricted to the middle piece of llama ejaculated sperm. By contrast, in human sperm β-NGF was located at the middle piece and the neck (Li et al., 2010), in bovine above the equatorial region of the sperm head and tail (Li, Zheng, Wang, & Zhou, 2010) and in golden hamster the tail and neck (Jin, Tanaka, Watanabe, Matsuda, & Taya, 2010). Indeed, contrary to the observations described by those authors, we found that TrKA co-localize with β-NGF in llama sperm. Taken together, the results of the present study suggest a species-specific localization of β-NGF and its receptor in the mammalian spermatozoa that could be related to different functional response.

According to our results, the consistent expression of β-NGF in the sperm middle pieces can have an impact on mitochondria function. In this regard, it has been recently demonstrated that β-NGF stimulates mitochondrial activity and biogenesis in TM4 Sertoli cells line (Jiang et al., 2018).

In the sperm cell, presence of β-NGF in the middle piece could also be related to sperm motility. Studies performed on ejaculated sperm (e.g. bovine [Li et al., 2010], human [Shi et al., 2012]) and sperm from epididymis (golden hamster [Jin, Tanaka, Watanabe, Matsuda, & Taya, 2010]), demonstrate that sperm motility increases in a

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time- and dose-dependent manner when β-NGF is added to the sperm culture medium. It is worthy to distinguish that, in camelids, ejaculated sperm lack of progressive motility, having only oscillatory movement (Giuliano et al., 2008); whereas sperm collected form epididymis display forward progressive motility (Morton, K. M., Bathgate, R., Evans, G., & Maxwell, W. C. , 2007). Moreover, when seminal plasma is removed or diluted, the progressive motility increases (Kershaw-Young & Maxwell., 2011; Fumuso et al., 2018). These findings could indicate that one or several factors present in seminal plasma modulate sperm motility. More in-depth studies are needed to determine if β-NGF is one of them. β-NGF was still present in acrosome reacted sperm, suggesting that it may be necessary for the fertilization process. Presence of β-NGF in llama uterine and oviductal fluid is still unknown, but it can be expected to reinforce and control its action during sperm transit along the female reproductive tract.

In conclusion, our findings confirm that the β-NGF/ TrKA system is present in testicles, prostate, bulbourethral glands and epididymis. The prostate is the main source of seminal β-NGF in llamas.

For the first time, presence of β-NGF and its main receptor were described in the sperm of camelids being co-localized in the middle piece.

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4. MATERIALS AND METHODS

4.1 Experiment 1. Presence of β-NGF and TrKA in the reproductive tract of llama males

4.1.a Animals

Three male llamas between 3 and 4 years old from a local abattoir (Bella Vista, Tucumán, Argentina) localized at 27°01′58″S latitude and 65°18′25″W longitude on the sea level were used during the winter of 2016.

4.1.b Reproductive organs

The reproductive organs were obtained immediately after slaughtering, in accordance with protocols approved by local institutional animal care. The testis, epididymis (head, body, and tail), bulbourethral glands and prostate were dissected into small pieces (less than 0.5cm thick). The tissues were fixed with 10% formaldehyde in PBS (pH 7.4) for histological and immunohistochemical analysis or stored in RNAlater solution (Ambion, Austin, TX, USA) at −70°C until RNA isolation, according to the manufacturer's instructions, for gene expression analysis.

A section of the epididymis was reserved for sperm collection, as described below.

4.1.c mRNA relative abundance of β-NGF **and** *TrKA*

Fifty mg of testis, epididymis (head, body, and tail), bulbourethral gland and prostate samples, stored in RNAlater solution were used to isolate mRNA using the Genelute Direct mRNA Miniprep kit (Sigma Co., St. Louis, MO, USA), according to the

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manufacturer's instructions. Reverse transcription was performed using Moloney murine leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI) and oligo $(dT)_{15}$ primer. The reaction mixture (25 µl) consisted of 5.5 µl of mRNA, 50 mMTris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5mM of each dNTP, 25 pmol of oligo $(dT)_{15}$, 200 units of reverse transcriptase, and RNase-free water. Reactions were performed by incubating the mixture in a thermal cycler at 42°C for 90 min, followed by enzyme inactivation at 94°C for 5 min.

PCR amplification of testis, epididymis (head, body, and tail), bulbourethral gland and prostate cDNA samples (n = 3) were carried out in a final volume of 10 µl containing 0.5 µl of cDNA, 2 µl of 5X Green GoTaq Reaction Buffer (pH 8.5), 0.2mM of each dNTP, 2.5 units of GoTaq DNA polymerase (Promega), and 1 µM of each primer pair. Primer sequence for *NGF* and *TrkA* transcripts are displayed in table 2.

The optimized cycling conditions for *β-NGF* were as follows: denaturation at 94°C for 5 min; 35 cycles of 94°C for 15 s, 58°C for 25 s, and 72°C for 20 s; followed by a final step at 72°C for 5 min. The amplification of *TrKA* was under the following condition: 94°C for 5 min; 40 cycles of 94°C for 15 s, 60°C for 25 s, and 72°C for 20 s with a final extension at 72°C for 5 min. PCR products were separated on 1.5% agarose gels containing SYBR Safe (Invitrogen, Carlsbad, CA).

For semi-quantitative expression analysis, gel images were captured with an Optio M 90 Pentax digital camera, and the optical densities of PCR products were quantified using ImageJ 1.42q software (NIH, Bethesda, MD). The relative abundance of the *β*-

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NGF and *TrKA* transcripts were normalized against that of ACTB (reference gene, Table 2).

4.1.d Histology and Immunohistochemistry

Testis, epididymis (head, body and tail), bulbourethral gland and prostate tissues fixed with 10% formaldehyde were dehydrated in a graded series ethanol, cleared in chloroform and embedded in paraffin blocks. Serial sections (5 µm) were mounted on positively-charged slides (HDA microscope slides, Cat. No. HDAS001A,Yancheng Huida Medical Instruments Co., Ltd., China).

After deparaffinization with xylene and rehydration, the tissue sections were incubated at 37°C for 20 min with Proteinase K solution (10 µg/µl in TE Buffer, pH 8.0) (Sigma) for antigen retrieval, followed by incubation in TE Buffer (50 mMTris Base, 1 mM EDTA, 0.5% Tritón X-100, pH 8.0) at room temperature for 10 min. The slides were then blocked with 1% Bovine Serum Albumin (BSA) in PBS at room temperature for 30 min. Then, they were incubated at 37°C for 1h with polyclonal antibodies against β-NGF (dilution 1:1500, sc-548) or TrKA (dilution 1:100, sc-118) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with specific antibodies, sections were incubated for 20 min with a 1:200 dilution of the biotinylated anti-rabbit IgG antibody (B8895, Sigma). Following three washes with 0.02% Tween in PBS, slides were incubated with a 1:500 dilution of ExtraAvidin-Alkaline Phosphatase Conjugates (Sigma) for 30 min. and then incubated with SigmaFast substrate (BCIP/NBT, B5655, Sigma) until color development. Sections were counterstained

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with Nuclear Fast Red (N3020, Sigma), dehydrated and mounted. The controls sections were processed by replacing primary antibody with blocking buffer.

Slides were observed using a Leica DM 500 light microscope and images were captured with a Leica ICC50 HD camera using LASZ Leica Inc. Software. Staining and image acquisition were performed in parallel for the entire set. Identical image acquisition settings and exposure times were applied.

4.2 Experiment 2. Presence of β-NGF and TrKA in sperm cells

4.2.a Animals

Five llama males between 6 and 10 years old from the Institute of Research and Technology for Animal Reproduction of the Faculty of Veterinary Sciences at the University of Buenos Aires (Buenos Aires, Argentina) localized at 34° 36′ latitude and 58° 26′ longitude on the sea level were used for this experiment. Animals were kept on natural pasture (supplemented with bales of alfalfa), and water was provided *ad libitum*. Semen was collected during the spring of 2017. Epididymal sperm were obtained from 3 male llamas between 3 and 4 years old from a local abattoir (Bella Vista, Tucumán, Argentina).

4.2.b Semen collection

Semen collections were carried out using electroejaculation (EE) under general anesthesia with 0.2 mg/kg of xylazine IV (Xilazina®, Vetec, Argentina) and 1.5 mg/kg of ketamine IV (Ketamina®, ClínicaEquina, PRO-SER, Argentina), according to the technique described by Director et al. (2007). As EE requires general anesthesia,

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collection was performed every 15 days on individual randomly selected males. All procedures (protocol 2014/16) were approved by the Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences at the University of Buenos Aires.

4.2.c Sperm isolation from semen samples

Semen samples (n = 7) were diluted with saline solution (SS) and centrifuged at 800g for 10 min at room temperature to remove the seminal plasma. This procedure was repeated three times.

Sperm smears were prepared on a positively-charged slide, and air-dried slides were then fixed with Carnoy's solution (methanol/acetic acid 3:1) for 2 h at room temperature.

To induce the AR, an aliquot of washed sperm from each ejaculated was incubated with TALP–BSA (6 mg/ml) for 3 h at 38°C with 5% CO_2 and 100% humidity and was then incubated under the same conditions for 1 h using 5 μM calcium ionophore (A23187) according to Carretero et al. (2015). Finally, sperm were centrifuged (800g for 8 min) and washed twice with SS and then suspended in this medium. Sperm smears were fixed on positively-charged slides as previously described. Some of them were used to control AR by the FITC-PNA/PI (Fluorescein isothiocyanate - Arachishypogaea agglutinin/ Propidium iodide) staining technique (Carretero et al., 2015).Therefore, two sperm populations (acrosome and non-acrosome reacted) were used from semen samples for immunofluorescences studies.

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4.2.d Epididymal sperm

Epididymis (n = 3) obtained post-mortem were excised to obtain sperm cells, which were suspended in 5 ml of SS and centrifuged at 1,000g for 10 min at room temperature. The supernatant was removed, and the sperm were suspended in 200 μl of SS. The smears were performed as described above.

4.2.e Immunofluorescence

Slides containing the epididymal, AR and non-AR sperm were washed with PBS and treated with Proteinase K solution (10 μg/μl inTE Buffer, pH 8.0) for antigen retrieval. Followed by incubation in Buffer TE at room temperature for 10 min, the slides were treated with 0.3% Triton X-100 (Sigma) for 5 min at room temperature. They were then blocked with BSA (1%) in PBS at room temperature for 30 min and incubated with the primary antibody solutions (anti- β-NGF or anti-TrKA, as appropriate) at 37°C for 1 h. Following three washes with 0.02% Tween in PBS, slides were incubated for 20 min with a 1:200 dilution of the biotinylated anti-rabbit IgG antibody. Slides were exposed to streptavidin conjugated with fluorescein isothiocyanate (FITC, 1:2000; Sigma) for 30 min at room temperature in a dark chamber, and then washed with 0.02% Tween in PBS. For negative controls, slides were incubated with BSA diluted with PBS instead of primary antibody. Photomicrographs were taken using a confocal microscope (Olympus FV300).

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4.3. Experiment 3. Detection of β-NGF in sperm-adsorbed proteins

4.3.a Animals

Fertile male llamas (*Lama glama*) between 3 and 8 years old were used in this experiment (n = 9). The animals were kept at the experimental farm of Instituto Nacional de Tecnología Agropecuaria (INTA) in Abra Pampa (Jujuy, Argentina), located in the high Andean Plateau of northwest Argentina at 3484 m above sea level (22° 49′S latitude and 65° 47′W longitude).

4.3.b Semen collection

Semen was obtained by using a modified bovine artificial vagina (20 cm in length and filled with water at 39°C) after 5 days of sexual abstinence, according to Giuliano et al. (2008). Semen was collected using a long plastic sleeve sealed at one end, and inserted within the latex inner lining of the artificial vagina. A teaser female was used for the collections. Duration of each collection was 20–25 min.

4.3.c Sperm-adsorbed protein extraction

Sperm-adsorbed proteins were obtained as previously described by Zampini, Sequeira, Argañaraz, & Apichela (2017). Briefly, pools of three semen samples were diluted five-fold and washed three times with HBSS (25 mM Hepes, 130 mM NaCl, 5 mM KCl, 0.36 mM NaH_2PO_4, 0.49 mM $MgCl_2$, and 2.4 mM $CaCl_2$; pH 7.4, 290 mOsm/kg) to remove seminal plasma. Washed sperm were re-suspended in HBSS with 1X protease inhibitor cocktail (Sigma). An equal volume of 1M KCl in HBSS was added, and sperm proteins were extracted by gentle mixing on a rotary shaker for 1 h at 4°C.

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Spermatozoa were then removed by centrifugation at 6,000g for 10 min, and discarded. Extracts were clarified by filtration (0.2-μm cellulose acetate). A series of centrifugations in 3kDa cellulose filters (Amicon, Lexington, MA) were performed at 9,000g for 5 min at 4°C, re-diluting in HBSS every centrifugation to reduce the salts and to concentrate the sample. Protein content was determined using a Micro BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) with a bovine serum albumin standard. Aliquots of clarified seminal plasma were stored at -70°C until needed.

4.3.d Polyacrylamide gel electrophoresis

SAP samples were separated by denaturing polyacrylamide gel electrophoresis, according to Gevaert &Vandekerckhove (2000). Briefly, 30 μg of total protein was diluted (v/v) with sample buffer (0.1M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 1% β–Mercaptoethanol, 30% glycerol, and 0.05% bromophenol blue), and loaded onto a 15% polyacrylamide resolving gel with a 4% stacker. Molecular masses were determined by running protein markers (Page Ruler Unstained Broad Range Protein Ladder, Thermo Fisher Scientific, Rockford, IL) covering the range of 5–250 kDa. Gels were run in a PROTEAN II xi Cell (Bio-Rad, Hercules, CA) at 150 V for 1.5 h at room temperature. The separated proteins were stained with colloidal Coomassie Blue G-250 (Sigma) (Neuhoff et al., 1990). Coomassie Blue Gel images were obtained using a Pentax Optio M 90 camera (Pentax, Milan, Italy). GelAnalyzer, version 2010a, was used to determine the molecular weight of the detected bands on the digitized images.

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4.3.e Western blot

Sixteen μg of SAP, obtained as indicated before, was subjected to 15% SDS-PAGE along with 100 ng of recombinant human β-NGF (R&D Systems, Minnesota, USA). Then, proteins were transferred onto PVDF membranes (Immobilon-P, Merck KGaA, Darmstadt, Alemania). Semidry electroblotting was carried out on a Trans-Blot SD semi-dry transfer cell (Bio-Rad; Richmond, CA) and run at 20 V for 30 min. The membranes were blocked in 5% BSA in HBS-T (130 mM NaCl, 5 mM KCl, 1.36 mM Na_2HPO_4, 2.4 mM$CaCl_2$, 25 mM HEPES, 0.49 mM$MgCl_2$, 0.1% Tween 20) for 30 min at 37°C and then overnight at 4°C. Afterwards membranes were incubated with polyclonal antibody against β-NGF (dilution 1:2000, sc-548) (Santa Cruz Biotechnology) for 2 h at 37ºC, followed by incubation with the secondary antibody for1 h at 37°C (1:1000) (biotinylated anti-rabbit IgG antibody, B8895, Sigma). Finally, membranes were incubated for 30 min with alkaline phosphatase-linked ExtrAvidin (1:5,000, Sigma) and protein bands were developed using the SigmaFast substrate (BCIP/NBT, Sigma). Membrane digital images were obtained with an Olympus C5060 Wide Zoom color digital camera (Olympus). Molecular weight marker proteins (Page Ruler Unstained Broad Range Protein Ladder, Thermo Fisher Scientific, Rockford, IL) were run on a separate lane to determine the molecular weights of the immunostained bands. The primary antibody omitted from the staining reaction was used as the control.

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4.3.f Mass spectrometry

Bands immunostained for β-NGF were excised from a colloidal Coomassie Blue stained polyacrylamide gel for characterization using matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS), performed on an Ultraflex II TOF/TOF (Bruker Daltonics, Bremen, Germany) mass spectrometer at the CEQUIBIEM mass spectrometry facility (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina). Proteins were identified by peptide-mass fingerprinting with MASCOT v. 2.2.03. Fragmentation was carried out with the most intense peaks (MS/MS). When possible, MS and MS/MS data were combined for one or more peptide searches. De novo sequencing was inferred from BLAST results when peak fragmentation was allowed. The percentage of protein coverage was determined for each band using the MASCOT search.

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FIGURES

Figure 1. Semi-quantitative analysis of (a) *TrKA* and (b) *β-NGF* transcription in testis (T), prostate (P), bulbourethral gland (BG) and epididymis (head [HE], body [EB], and tail [ET]). Relative mRNA expression, normalized to *ACTB* mRNA levels, is shown (mean ± standard error; n = 3). Significant differences are indicated with different letters ($p < .05$).

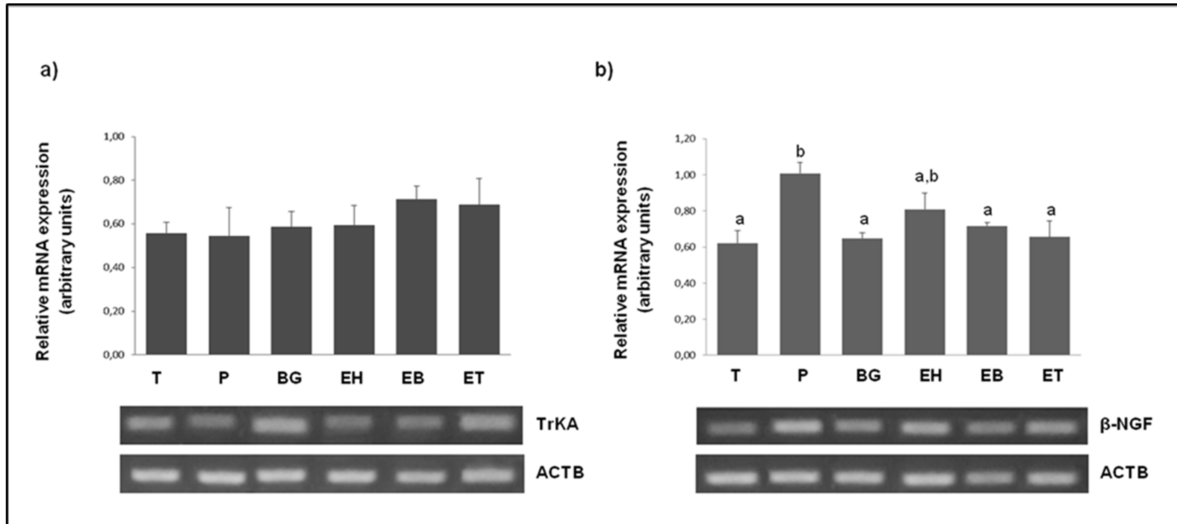

Figure 2. Localization of β-NGF in llama male reproductive organs by immunohistochemistry. No mark was observed in testis, epididymis (head, body, and tail) and bulbourethral gland (a-e). Strong immunoreaction was observed in the cytoplasm of the prostate gland cells (f). Scale bar, 50 μm.

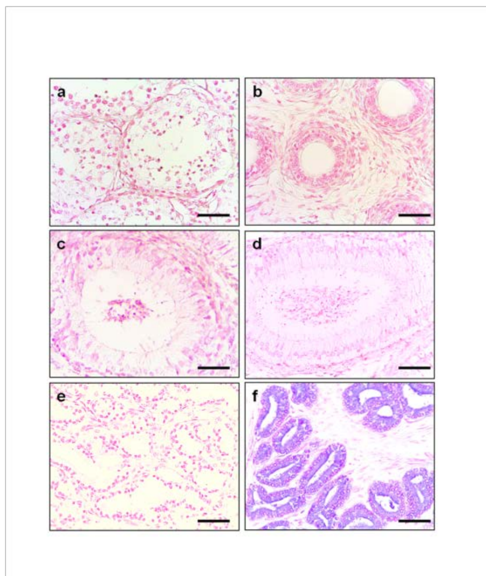

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Figure 3. Immunohistochemical reactivity for TrKA in llama male reproductive organs. Positive signals are localized in testis (a), epididymis head (b), body (c) and tail (d); bulbourethral gland (e) and prostate (f). Scale bar, 50 µm.

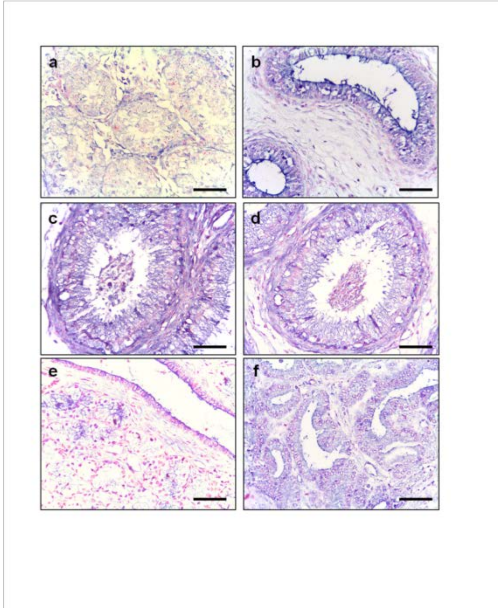

Figure 4. Immunolocalization of TrKA (a-c) and β-NGF (d-f) in llama sperm by confocal microscopy. TrkA was localized in the middle piece of (a) ejaculated non-acrosome reacted (AR), (b) AR induced and (c) epididymal sperm. β-NGF was localized in middle piece of (d) non-AR and (e)AR ejaculated sperm. No signal was detected in (f) epididymal sperm. Negative controls consisted of sperm incubated with BSA (g-i). Insets correspond to bright field microscopy. Scale bar, 10 µm.

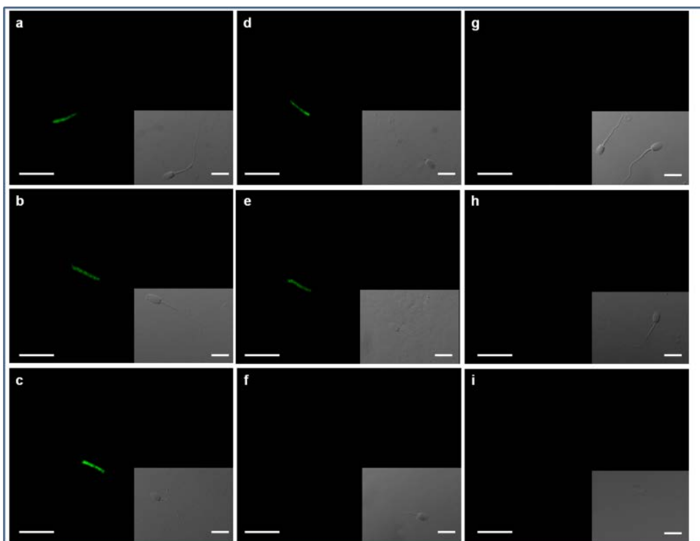

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Figure 5. (a) Western blotting of β-NGF protein in sperm-adsorbed protein (SAP). As positive control, 100 ng of recombinant human β-NGF (RH- β-NGF) was run. (b) For negative control primary antibody was replaced by BSA in HBS-T.

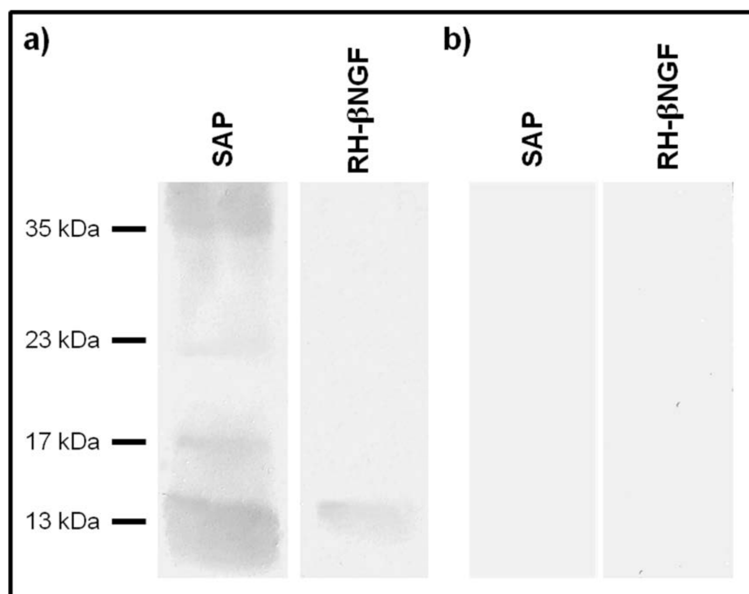

Table 1. Identity of SAP bands.

Identity	Accession Nº	Species	EPMW kDa	TPMW kDa	Coverage (%)	MP	S/SL	E
Beta-nerve growth factor precursor	EPY74508.1	*Camelus ferus*	39	27	54	18	107/75	3.6e-05
Beta-nerve growth factor precursor	EPY74508.1	*Camelus ferus*	23	27	56	20	100/75	0.00019
Beta-nerve growth factor precursor	EPY74508.1	*Camelus ferus*	13	27	51	16	116/75	4.6e-06

EPMW: experimental protein molecular weight, **TPMW:** theoretical protein molecular weight, **MP:** matched peptides, **S/SL:** score/significance level, **E:** expectation value.

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Table 2. Specific primers used in RT-PCR.

Primer name	Sequences 5´-3´	Product Length (bp)	GenBank accession number
NGF forward	TGCTGGGAGAGGTGAACATT	147	XM_006213697.2
TrKA forward	GCTTCATCTTCACCGAGTTCCT	114	XM_015248158.1
ACTB forward	GCGGGACCACCATGTACC	183	XM_006210388.1

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