Cellular Distribution, Regulated Expression, and Functional Role of the Anorexigenic Peptide, NUCB2/Nesfatin-1, in the Testis

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Nesfatin-1, product of the precursor NEFA/nucleobindin2 (NUCB2), was initially identified as anorectic hypothalamic neuropeptide, acting in a leptin-independent manner. In addition to its central role in the control of energy homeostasis, evidence has mounted recently that nesfatin-1 is also produced in peripheral metabolic tissues, such as pancreas, adipose, and gut. Moreover, nesfatin-1 has been shown to participate in the control of body functions gated by whole-body energy homeostasis, including puberty onset. Yet, whether, as is the case for other metabolic neuropeptides, NUCB2/nesfatin-1 participates in the direct control of gonadal function remains unexplored. We document here for the first time the expression of NUCB2 mRNA in rat, mouse, and human testes, where NUCB2/nesfatin-1 protein was identified in interstitial mature Leydig cells. Yet in rats, NUCB2/nesfatin-1 became expressed in Sertoli cells upon Leydig cell elimination and was also detected in Leydig cell progenitors. Although NUCB2 mRNA levels did not overtly change in rat testis during pubertal maturation and after short-term fasting, NUCB2/nesfatin-1 content significantly increased along the puberty-to-adult transition and was markedly suppressed after fasting. In addition, testicular NUCB2/nesfatin-1 expression was up-regulated by pituitary LH, because hypophysectomy decreased, whereas human choriogonadotropin (super-agonist of LH receptors) replacement enhanced, NUCB2/nesfatin-1 mRNA and peptide levels. Finally, nesfatin-1 increased human choriogonadotropin-stimulated testosterone secretion by rat testicular

explants ex vivo. Our data are the first to disclose the presence and functional role of NUCB2/nesfatin-1 in the testis, where its expression is regulated by developmental, metabolic, and hormonal cues as well as by Leydig cell-derived factors. Our observations expand the reproductive dimension of nesfatin-1, which may operate directly at the testicular level to link energy homeostasis, puberty onset, and gonadal function.

Abbreviations: EDS, Ethylene dimethane sulfonate; hCG, human choriogonadotropin; HPG, hypothalamic-pituitary-gonadal; HPX, hypophysectomized; IHC, immunohistochemical; IR, immunoreactivity; NUCB2, NEFA/nucleobindin2; RP, ribosomal protein.

Reproduction is under the control of a wide array of regulatory signals that integrate at the so-called hypothalamic-pituitary-gonadal (HPG) axis. Within this axis, gonadal function is driven by the dynamic interplay of numerous endocrine factors, including prominently pituitary gonadotropins, and locally born signals (1,2). Among its major external regulators, the function of the HPG axis is metabolically gated, and conditions of metabolic stress and disorders of body energy homeostasis, ranging from anorexia to obesity, are known to impact, directly and/or indirectly, upon gonadal function (3–5). The molecular and neuroendocrine basis for such a close association between energy balance and fertility have begun to be deciphered only during the last two decades, when the reproductive actions of various key players in energy and metabolic homeostasis have been demonstrated in various species, including rodents and primates (4). Of note, such analyses have revealed the considerable complexity of the metabolic-reproductive interactions. As a paradigmatic example, leptin, an adipose signal of energy abundance, has been shown to play a major permissive role in puberty onset and the gating of reproductive function, acting mainly at central hypothalamic levels (6). Yet detailed analyses of the reproductive effects and sites of action of leptin revealed direct actions of leptin on the gonads that include inhibition of steroidogenesis at high concentrations (6–

8), a phenomenon that may contribute to the state of hypogonadism frequently linked to morbid obesity.

The testis is a complex endocrine organ where different cell types, including interstitial Leydig cells as well as Sertoli and germ cells in the seminiferous tubules, interact to ensure proper hormonal secretion (mainly androgens) and sperm production, under the control of numerous endocrine, paracrine, and autocrine regulators (9–11). In recent years, it has been conclusively demonstrated that different factors with key roles in body energy homeostasis are involved in the direct regulation of testicular function (6,7,12). These include not only leptin but also other adipokines, such as resistin and adiponectin (13,14), gut hormones such as ghrelin (15), and neuropeptides such as orexin-A (16,17). Such factors have been demonstrated to have different regulatory actions (*e.g.* inhibition or stimulation of testosterone secretion), patterns of expression (*e.g.* in Leydig cells and/or the seminiferous epithelium) and cellular targets. Complete elucidation of the metabolic factors expressed and/or acting in the testis would help to enlarge our understanding of the mechanisms for the integral control of energy homeostasis and reproductive function and their potential pathophysiological alterations.

In 2006, nesfatin-1 was identified in the rat as a secreted hypothalamic neuropeptide with the capacity to suppress food intake, putatively involved in the physiological control of body weight (18). Nesfatin-1 is an 82-amino-acid fragment cleaved from a common 398-amino-acid precursor that gives rise also to nesfatin-2 and nesfatin-3 (19,20). However, pharmacological analyses revealed that only nesfatin-1 is endowed with significant anorectic activity after central or systemic injection in rodents (18,21). The precursor of nesfatin-1 is encoded by the nucleobindin 2 gene (*NUCB2*), termed also *NEFA* (for DNA-binding/EF-hand/acidic protein); nesfatin standing for *NEFA*/*NUCB2*-encoded satiety- and fat-influencing protein (18,20). In keeping with its proposed role in body weight

homeostasis, expression of NUCB2/nesfatin-1 has been documented in hypothalamic areas with essential roles in food intake control (18,22–25), and its levels are negatively regulated by fasting (26). Interestingly, the anorexigenic actions of nesfatin-1 are independent of leptin signaling but likely involve the interplay with other central transmitters, such as oxytocin, cholecystokinin, neuropeptide Y, and melanocortins, as evidenced by a wealth of expression and functional analyses in rodents (21–23,25,27–29). Besides its major hypothalamic actions, NUBC2/nesfatin-1 is expressed also in several peripheral tissues with key roles in metabolism and energy balance, such as the adipose, the gut, and the endocrine pancreas (19,30–33), and its circulating levels are altered in metabolic perturbations, such as obesity and type 2 diabetes (33). In fact, recent rodent data suggest that nesfatin-1 participates in the control of stimulated insulin secretion and whole-body energy homeostasis (34).

In line with its role as metabolic integrator, recent experimental evidence has suggested that NUCB2/nesfatin-1 may also participate in the control of key facets of reproduction, such as puberty onset and gonadotropin secretion (26), therefore providing complementary mechanisms for the metabolic gating of fertility. To our knowledge, however, the possibility of the direct expression and/or actions of NUCB2/nesfatin-1 in the gonads remains virtually unexplored. To our knowledge, our study is the first to document the pattern of cellular distribution, regulated expression, and putative functional role of nesfatin-1 in the testis, with significant commonalities (in terms of expression) between human, rat, and mouse testes, which strongly suggest the conserved role of this newly discovered molecule in the metabolic control of gonadal function in mammals.

Materials and Methods

Experimental procedures were conducted in Wistar male rats, bred in the vivarium of the University of Córdoba. In addition, testicular tissue samples from C57BL6 mice and archival human testicular sections obtained from the Department of Pathology of the University of Córdoba were used. Experimental procedures were approved by the Córdoba University Ethical Committee, and those involving animal manipulation were conducted in accordance with the European Union normative for use of experimental animals. Rat nesfatin-1 (1–82) (molecular weight 9582.80, verified by mass spectrometry; ≥95% purity) was purchased from Phoenix Pharmaceuticals Ltd. (Belmont, CA; Ref. no. 003-22B). Hypophysectomized (HPX) rats were purchased from Charles River (Barcelona, Spain). FSH (Gonal-f) and human choriogonadotropin (hCG; Profasi) were obtained from Serono (Madrid, Spain). For *in vivo* experiments, FSH and hCG were dissolved in saline immediately before use. For *in vitro* experiments, nesfatin-1 and hCG were dissolved in DMEM with 4.5 g/liter glucose and without L-glutamine and phenol red (DMEM) (BioWhittaker, Verviers, Belgium) immediately before use. Ethylene dimethane sulfonate (EDS) was synthesized in our laboratory as described in detail elsewhere and dissolved in dimethylsulfoxide/water (1:3 vol/vol).

For rat/mouse expression analyses involving RT-PCR or Western blot, testes were dissected out from the epididymis and surrounding fat immediately upon decapitation of the animals, frozen in liquid nitrogen, and stored at −80 C until used for RNA or protein isolation. Tissue collection and processing for immunohistochemical analyses was conducted as described in the following sections.

Experimental designs

To document the expression of NUCB2/nesfatin-1 in the mammalian testes, in experiment 1, a combination of RT-PCR, Western blot, and immunohistochemical (IHC) analyses were implemented in freshly isolated or archival testicular specimens from adult rats, mice, and humans. Groups (n = 4) of adult male rats and mice were killed by decapitation, and the testes were immediately excised for storage and subsequent processing for RNA (rat and mouse) or protein (rat) isolation, as described in the previous section. In addition, a subset of samples was used for IHC analyses of NUCB2/nesfatin-1 staining, as described in the section *Testicular IHC*. Commercial human testicular RNA (purchased from Ambion Inc., Woodward, TX) and archival human testicular sections were also included in the analysis for comparative purposes. On the latter, only sections from healthy donors, used in a previous immunohistochemical study from our group assessing the expression patterns of ghrelin receptor in human testicular tissue, were used for detailed description of samples, see Ref. 35).

Given the prominent location of NUCB2/nesfatin-1 in mature Leydig cells within adult testes from the various species under analysis, in experiment 2, specific expression analyses were conducted in a model of selective ablation of mature Leydig cells *in vivo*, by means of systemic administration of the cytotoxic compound, EDS, to adult male rats. Groups of male rats (n = 5) were ip injected with a single bolus of EDS (75 mg/kg), following previously published protocols (15,36). The animals were killed for testicular sampling on d 3 and 5 after administration of the toxicant, a time window when mature Leydig cells have been reported to be completely eliminated from the testicular interstitium (36). This contention was confirmed by histological analysis of testicular sections from EDS-treated rats, 3 d after administration of the cytotoxic compound. Upon decapitation of the animals, the testes were excised for storage and subsequent processing

for RNA isolation (at 3 and 5 d after EDS) or IHC (at d 3 after EDS), as described above. Male rats injected with vehicle were sampled in parallel (on d 3) for comparative, control purposes.

Initial evidence for the conserved expression of NUCB2/nesfatin-1 in rat, mouse, and human testes prompted further analyses on potential developmental changes that were conducted mainly in the rat. To this end, in experiment 3, groups (n = 6–8) of pubertal (39 d old) and adult (>75 d old) male rats were killed by decapitation, and the testes were immediately excised for storage and subsequent processing for RNA or protein isolation, as described above. In addition, IHC analyses for NUCB2/nesfatin-1 were conducted in testicular sections from immature (20 d old) rats as well as in archival samples of fetal human testes obtained from the Pathology Department of the University of Córdoba.

In addition, regulation of testicular expression of NUCB2/nesfatin-1 by metabolic cues was explored in experiment 4. Groups (n = 6–8) of pubertal (39-d-old) and adult (>75-d-old) male rats were subjected to food deprivation for 36 h (pubertal) or 48 h (adult), as standard protocols of metabolic distress, in keeping with previous references. The animals were killed by decapitation at the end of the fasting periods, and testes were processed for RNA and protein analyses, as described in previous experiments. Groups of animals fed *ad libitum* served as controls.

Hormonal regulation of testicular NUCB2/nesfatin-1 expression was explored in experiment 5. To this end, groups of long-term HPX male rats (n = 5; 4 wk after HPX) were daily injected for 1 wk with vehicle or subjected to standard protocols of hCG (50 IU/24 h) or FSH (12.5 IU/24 h) supplementation, or a combination of both, following previously published protocols (14,17). At the end of the treatments, the animals were killed by decapitation and testes were processed as described in previous experiments. Groups of intact animals served as controls.

Finally, in experiment 6, the ability of nesfatin-1 to directly modulate testicular testosterone secretion was assessed using static incubations of fragments of testis tissue obtained from adult (>75 d old) male rats. The effects of nesfatin-1 were tested both in basal conditions and after costimulation with an effective dose of hCG. General procedures for tissue collection and incubation are described below.

Static incubation of testicular tissue

For the analysis of the direct effects of nesfatin-1 on testosterone secretion (experiment 6), incubation of testicular tissue was carried out as described previously, with minor modifications (13,15,17). Groups of adult male rats (n = 6 per group) were killed by decapitation, and the testes were immediately removed, decapsulated, and cut into two halves of approximately equal size (mean 775.9 ± 14.7 mg/piece; no significant difference between groups). Hemi-testes were incubated in 2 ml DMEM in a Dubnoff shaker (60 cycles/min) at 32 C under an atmosphere of 5% CO_2/95% O_2. After preincubation for 1 h, the media were replaced with either fresh medium or medium containing synthetic peptide nesfatin-1 (10^{-7}M), each group being composed of 12 independent hemi-testicular samples. In addition, to test the ability of nesfatin to modulate stimulated testosterone secretion, hemi-testes were stimulated with nesfatin-1 (10^{-7}M) plus hCG (10 IU/ml) or with hCG alone. After 90 and 180 min, aliquots were taken from the incubation media for testosterone measurement, as described below. The levels of testosterone in the media are expressed as normalized values per gram incubated tissue.

RNA analysis by semiquantitative RT-PCR

Total RNA was isolated from rat and mouse testicular samples using the TRIsure reagent (Bioline, Taunton, MA), following the instructions of the manufacturer. Commercial human testicular RNA samples were also subjected to analysis. *NUCB2* mRNA levels

were assayed by RT-PCR, according to previous references (26). For analysis of rat and mouse samples, the following primer pair was used: *rNef* forward (5′-GAG GAG ATA AGG AGC GGG AGG C-3′) and *rNef* reverse (5′-ATG TGT CAG GAT TCT GGT GGT TCA-3′), with a predicted amplicon size of 208 bp. RT-PCR analyses in human samples were conducted using the following primer pair: *hNef* forward (5′-ATT CAC CCT GTG GAA AGT GCG A-3′) and *hNef* reverse (5′-TTG TTG CCG CTT TGA TTA GCA T-3′), with a predicted amplicon size of 382 bp. General procedures for RT-PCR were conducted as described previously (26). Cycling PCR conditions consisted in a first denaturing cycle at 97 C for 5 min, followed by 27 cycles of amplification, defined by denaturation at 96 C for 30 sec, annealing at 63 C for 30 sec, and extension at 72 C for 1 min. A final extension cycle of 72 C for 10 min was included. The number of PCR cycles was defined at optimization assays, as to allow amplification within the exponential phase (data not shown). As internal control, amplification of a 240-bp fragment of ribosomal protein (RP) S11 mRNA or a 290-bp fragment of RP-L19 mRNA was carried out in parallel in each sample, as described elsewhere (37,38). Specificity of PCR products was confirmed by direct sequencing. Quantification of intensity of RT-PCR signals was carried out by densitometry scanning using an image analysis system (1-D Manager; TDI Ltd., Madrid, Spain), and values of the specific target were normalized to those of internal controls to express arbitrary units of relative expression. In all assays, liquid controls and reactions without reverse transcription resulted in negative amplification.

Testicular IHC

Testicular specimens were excised from prepubertal (20 d old) and adult rats as well as adult mice immediately upon decapitation of the animals (n = 5–6). The tissues were fixed in Bouin's solution for 24 h, and slabs containing testes were processed for paraffin embedding. In addition, archival human testicular sections fixed in Bouin's and embedded

in paraffin were also used. Six-micrometer-thick sections were cut and processed for immunohistochemical detection of NUCB2/prepronesfatin, using a specific polyclonal antibody obtained from Phoenix (Ref. no. H-003-22), in keeping with our previous references (19,26). After dewaxing and rehydration in graded ethanol series, sections were incubated in 2% hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase. After being washed in distilled water, sections were immersed in 10 mM citrate buffer and autoclaved at 1.5 atmospheres for 3 min. Thereafter, sections were allowed to cool at room temperature, washed in PBS, blocked with normal rabbit serum, and incubated overnight with the primary antibody (diluted 1:300). The sections were then processed according to the avidin-biotin-peroxidase complex (ABC) method, following the instructions of the manufacturer. Finally, sections were counterstained with hematoxylin. As control for specificity of detection, IHC reactions were carried out after preabsorption of the primary antiserum overnight at 4 C with nesfatin-1 peptide (Phoenix). In addition, negative controls were run routinely in parallel by replacing the primary antibody by preimmune serum.

Protein analysis by Western blot

Total protein lysates (30 μg) obtained from different testicular specimens were subjected to SDS-PAGE on 10% polyacrylamide gels, and electrotransferred on polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). Membranes were probed for 16 h at 4 C in the presence of a 1:1000 dilution of the primary antibody against rat NUCB2/nesfatin-1, in keeping with our previous studies (26). Protein detection was performed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Inmobilion, Millipore). At least four testicular specimens were included per group. The antiserum against rat NUCB2/prepronesfatin H-003-22 (Phoenix) was used. As internal control, tubulin protein content per lane was assayed

using a similar protocol and primary rabbit antiserum (dilution 1:10,000) against rat tubulin from AbCam Inc. (Cambridge, UK).

Hormone measurements

Testosterone levels in static incubation media were measured using a commercial kit from MP Biomedicals (Costa Mesa, CA). All medium samples were measured in the same assay. The sensitivity of the assay was 1 ng/ml, and the intraassay coefficient of variation was 4.5%. Serum LH and FSH levels were determined in a volume of 25–50 μl using a double-antibody method and RIA kits supplied by the National Institutes of Health (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 and FSH-I-9 were labeled with ^{125}I using Iodogen tubes, following the instructions of the manufacturer (Pierce, Rockford, IL). Hormone concentrations were expressed using reference preparations LH-RP-3 and FSH-RP-2 as standards. Intra- and interassay coefficients of variation were, respectively, less than 8 and 10% for LH and less than 6 and 9% for FSH. The sensitivity of the assay was 5 pg/tube for LH and 20 pg/tube for FSH. All samples were measured in the same assay; accuracy of hormone determinations was confirmed by assessment of rat serum samples of known hormone concentrations.

Presentation of data and statistics

Hormone determinations were conducted in duplicate; $n \geq 6$ samples per group for serum determinations, $n = 12$ samples per group for medium measurements. RT-PCR analyses were carried out in duplicate from at least four to five RNA samples per group. Western blots were carried out in duplicate; $n \geq 4$ protein samples per group. Hormonal and semiquantitative RNA data are presented as mean ± SEM. Results were analyzed using Student's *t* test or ANOVA followed by Student-Newman-Keuls multiple-range test

(SigmaStat version 2.0; Jandel Corp., San Rafael, CA). $P \leq 0.05$ was considered significant.

Results

Expression and cellular distribution of NUCB2/nesfatin-1 in the testis

The presence and cellular distribution of NUCB2/nesfatin-1 was explored at the mRNA and protein levels in the testes from two representative rodent species, namely rat and mouse, as well as in human testicular archival samples. RT-PCR analyses using specific primer pairs and optimized conditions for semiquantitative amplification revealed that NUCB2 mRNA is expressed in adult testes from rats, mice, and humans; amplicons of expected size were obtained by PCR assays, whose identity was confirmed by direct sequencing (Fig. 1, *left panel*). In good agreement, initial Western blot analyses demonstrated the presence of a 42-kDa protein, corresponding in size to that predicted for the mature nesfatin precursor, in two independent testicular specimens from adult rats; similar protein products were detected in samples from rat gastric mucosa and hypothalamus, taken as positive controls (Fig. 1, *left panel*). Of note, an additional protein band of approximately 50 kDa was detected in testicular and gastric samples, whose identity is yet to be defined. Further semiquantitative analyses on NUCB2/nesfatin-1 protein levels focused on the 42-kDa species.

Complementary IHC analyses for detection of NUCB2/nesfatin-1 immunoreactivity (IR) were carried out in testicular sections from adult rats, mice, and humans, with strong IR being detected in the three species. In rat testicular sections (Fig. 1, B–D), strong NUCB2/nesfatin-1 IR was observed in the testicular interstitium of adult rats, where specific staining was clearly visible in the cytoplasm of mature Leydig cells. In contrast, blood vessels and other interstitial cell types such as testicular macrophages were

negative. In addition, NUCB2/nesfatin-1 signal was negligible (and undistinguishable from the background) in the seminiferous tubules. Similar patterns of cellular distribution of NUCB2/nesfatin-1 IR were detected in mouse and human testes (Fig. 1, E and F, respectively), with strong signal being detected in mature Leydig cells, but not in the tubular compartment or any other interstitial cell type, of adult specimens. No immunostaining for NUCB2/nesfatin-1 was detected in negative control sections from rat testis (Fig. 1A).

Given the prominent expression of NUCB2/nesfatin-1 in mature Leydig cells, further analyses were conducted in a rat model of selective elimination of this cell population *in vivo*, by means of administration of the cytotoxic drug EDS. In keeping with previous literature, systemic administration of a single bolus of EDS caused the rapid and specific ablation of mature Leydig cells from the testicular interstitium of adult male rat, as documented by histological assays of testicular sections from rats 3 d after injection of the toxicant. Despite its predominant expression in adult-type Leydig cells, and the effectiveness of the treatment in terms of cell ablation as confirmed by histological examination, EDS administration failed to overtly decrease NUCB2 mRNA levels in testicular samples on d 3 and 5 after EDS injection (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). IHC analyses revealed that elimination of mature Leydig cells (and hence of the associated IR) did induce a shift in the pattern of cellular distribution of NUCB2/nesfatin-1 immunostaining, so that prominent IR signals became clearly detectable in the seminiferous epithelium in the cytoplasm of presumable Sertoli cells, which were negative in control conditions (Fig. 2, A and B). The most intense immunostaining was present in stages XII-II; of note, although the stages of the seminiferous epithelium are disrupted after selective depletion of mature Leydig cells, most stages are still

recognizable on d 3 after EDS treatment. In addition, and in keeping with previous observations, histological analyses confirmed that elimination of mature Leydig cells induced the appearance of fibroblast-like cells in the testicular interstitium, which were found in perivascular or peritubular locations and displayed irregular nuclei, elongated cytoplasm, and frequent mitotic figures. These morphological features are suggestive of presumptive Leydig cell precursors that have been reported to proliferate on d 3 after EDS treatment. These progenitors remain largely undifferentiated at this early maturational stage, yet they consistently showed strong NUCB2/nesfatin-1 IR (Fig. 2, C–E).

Developmental and metabolic regulation of NUCB2/nesfatin-1 expression in the testis

Based on the compelling evidence for the expression of NUCB2/nesfatin-1 in the testis from various mammalian species, analyses were implemented to evaluate potential developmental changes in its mRNA/protein levels. RT-PCR analyses in the rat, comparing pubertal and adult testes, failed to demonstrate overt changes in the relative NUCB2 mRNA levels between these two stages. In clear contrast, although Western blot analyses documented the presence of NUCB2/nesfatin-1 protein in both pubertal and adult testes, they also evidenced a substantial enhancement (~10-fold increase) of the protein levels along the pubertal-to-adulthood transition (Fig. 3, *upper panel*).

In addition, IHC analyses confirmed the presence of NUCB2/nesfatin-1 in immature testis, from prepubertal (20 d old) rats as well as in human fetal testis, where in keeping with adult data, NUCB2/nesfatin-1 IR was prominently located in Leydig cells at different stages of differentiation. In detail, in prepubertal rat testis, strong immunostaining was present in both mature fetal-type Leydig cells, which appeared as clumps of vacuolated cells, and immature adult-type Leydig cells, which displayed scarce

cytoplasm and frequent mitotic figures (Fig. 3, A–C). Similarly, NUCB2/nesfatin-1 IR was present in both fetal and immature adult-type Leydig cells (Figure 3, D and E).

In addition to its developmental regulation, the potential modulation of NUCB2/nesfatin-1 expression by energy status and fuel reserves was evaluated in a model of acute metabolic stress by short-term fasting. Both pubertal and adult male rats were subjected to standard protocols of food deprivation (36 h in pubertal, 48 h in adult animals), which resulted in a significant decrease in body weight at both ages: 93.2 ± 2.35 *vs.* 115.15 ± 1.57 g body weight in pubertal males fed *ad libitum* and 352.3 ± 7.67 *vs.* 384.3 ± 12.51 g body weight in adult males fed *ad libitum* ($P < 0.05$). Similarly, these fasting protocols induced a significant reduction of serum LH and/or FSH levels: LH in pubertal rats was 0.32 ± 0.12 *vs.* 0.41 ± 0.06 ng/ml in control animals fed *ad libitum*; LH in adult rats was 0.48 ± 0.16 *vs.* 1.18 ± 0.22 ng/ml in control animals fed *ad libitum* ($P < 0.05$); FSH in pubertal rats was 2.09 ± 0.58 *vs.* 4.46 ± 0.86 ng/ml ($P < 0.05$); and FSH in adult rats was 5.47 ± 0.62 *vs.* 6.1 ± 0.59 ng/ml in control animals fed *ad libitum*. In these settings, RT-PCR analyses demonstrated that relative levels of NUCB2 mRNA in rat testis do not apparently change during this fasting period. However, NUCB2/nesfatin-1 protein levels, as assayed by Western blot, significantly dropped in fasted animals (~4-fold decrease), at both ages tested (Fig. 4, A and B).

Gonadotropic regulation of NUCB2/nesfatin-1 expression in the rat testis

Given the major roles of pituitary gonadotropins in driving testicular trophic maturation and function, gonadotropic regulation of NUCB2/nesfatin-1 expression was explored in a rat model of HPX, with or without gonadotropin replacement. Efficiency of the HPX and/or hormonal replacement procedures was documented by corresponding changes in testicular and sex accessory organ weights in the different groups (see Supplemental Table 1). In HPX rats, the marked drop of testicular mass and function was associated

with a significant reduction in NUCB2 mRNA levels, which fell to nearly half of those of intact control males. Hormonal replacement with effective doses of hCG for 1 wk (see Supplemental Table 1) rescued the relative levels of NUCB2 mRNA in the testis. In contrast, FSH administration to HPX rats, despite inducing some recovery of testicular weight, was unable to significantly enhance NUCB2 mRNA levels. Finally, combined replacement with hCG and FSH was as effective as hCG alone in normalizing testicular expression of NUCB2 mRNA (Fig. 5A). Parallel changes were detected for testicular NUCB2/nesfatin-1 peptide content in testicular samples from the above experimental groups. Thus, HPX resulted in a dramatic drop in prepronesfatin protein levels that were partially restored by hCG replacement. In contrast, FSH administration to HPX male rats resulted only in marginal, nonsignificant stimulatory responses, whereas the combined administration of hCG and FSH, despite promoting complete normalization of testicular weight, did not increase relative NUCB2/nesfatin-1 content over the values induced by hCG alone (Fig. 5B).

Regulation of testicular testosterone secretion by nesfatin-1

Finally, the potential role of nesfatin-1 in the control of testicular function was addressed using a static incubation system. Considering the previous evidence on the ability of various metabolic hormones and neuropeptides to directly modulate basal and/or hCG-stimulated testosterone secretion, and given the proven expression of NUCB2/nesfatin-1 in Leydig cells, changes in testosterone secretion after challenge *ex vivo* with an effective dose of nesfatin-1, either alone or in the presence of a stimulatory dose of hCG, were monitored as readout, with testosterone measurement being conducted at 90 and 180 min after the initiation of hormonal challenges. Basal testosterone secretion remained unaffected after stimulation with 10^{-7}M nesfatin-1 at both time points, whereas hCG stimulation resulted in an approximately 10-fold increase over corresponding basal levels.

Of note, however, costimulation with nesfatin-1 plus hCG resulted in further augmentation of testosterone concentrations in the incubation media, which were approximately 60% higher than in media from testes incubated with hCG alone (Fig. 6).

Discussion

In the last few years, NUCB2/nesfatin-1 has been recognized as hypothalamic satiety factor that, acting in a leptin-independent manner, participates in the central control of appetite (18,21). In addition, very recent experimental evidence has demonstrated that NUCB2/nesfatin-1 is expressed in a number of key metabolic tissues and that nesfatin-1 operates as putative regulator of gut motility, insulin secretion, glucose levels, and whole-body energy homeostasis (19,20,34). Our present data expand our knowledge on the sites of expression and putative functional roles of NUCB2/nesfatin-1, with the demonstration of its regulated expression and action in the testis. Such a reproductive dimension resounds with recent findings on the role of central nesfatin-1 signaling in the central control of puberty and, eventually, the gonadotropic axis (19,26) and adds further strength to the proposal that NUCB2/nesfatin-1 may contribute to the integral coupling of body energy homeostasis and key aspects of reproduction, such as puberty onset and testicular function.

Testicular expression of NUCB2/nesfatin-1 was assessed by a combination of analytical approaches, which allowed us to provide a comprehensive account of its distribution and quantitative features. Testicular expression of NUCB2 gene was confirmed by RT-PCR and direct sequencing, whereas the presence of 42-kDa prepronesfatin in the testis of various mammalian species was documented by Western blot. Of note, our analyses evidenced the expression of an additional immunoreactive protein product of approximately 50 kDa, detected also in the gastric mucosa, used as positive control, in

keeping with previous reports (32,39). Admittedly, the nature of such a variant is yet to be defined, and whether it arises from alternative splicing NUCB2 or posttranslational processing of prepronesfatin warrants further investigation. Of note, however, its levels did not overtly fluctuate in the different experimental settings (data not shown); for this reason, it was not considered for semiquantitative analysis. In addition, it is worth mentioning that our protein analyses did not detect mature nesfatin-1 peptide in whole testicular fragments, in line with previous observations in rat brain and peripheral tissues (26,32,39), a finding that might suggest a postsecretory cleavage/regulation of NUCB2 (32), which is yet to be confirmed.

Our IHC studies demonstrated that NUCB2/nesfatin-1 peptide is confined to testosterone-producing Leydig cells within the adult testes, with roughly similar patterns in rats, mice, and humans. However, its presence was also observed in immature and fetal-type Leydig cells at earlier developmental stages and, curiously, become detectable in Sertoli cells within the seminiferous tubules in experimental (rat) models of selective elimination of mature Leydig cells. The former would suggest that acquisition of NUCB2/nesfatin-1 expression is a sign, and hence a robust marker, of early differentiation into the Leydig cell lineage, which is in clear contrast with the profile of other Leydig cell products with putative metabolic functions, such as ghrelin, which is predominantly expressed in fully differentiated stages (40). The latter observation implies that Leydig cell-secreted factors might be responsible for the tonic repression of NUCB2/nesfatin-1 expression in Sertoli cells in normal conditions, a finding of as yet unknown physiological relevance. In this context, the role, if any, of testosterone (as a major Leydig cell product) in the control of NUCB2/nesfatin-1 expression in Sertoli cells warrants specific investigation. Alternatively, elevated gonadotropin levels after selective elimination of Leydig cells might theoretically contribute to this phenomenon (36). However, this possibility seems

unlikely because FSH, as a major gonadotropic regulator of Sertoli cell function (11), failed to induce significant changes in terms of testicular NUCB2/nesfatin-1 mRNA or peptide levels in HPX rats. Complementary *in vitro* studies, using primary cultures or cell lines, may help to further characterize the expression patterns and eventual regulatory signals of NUCB2/nesfatin-1 in Sertoli cells.

Our semiquantitative analyses revealed that testicular expression of NUCB2/nesfatin-1 is robustly regulated by developmental and nutritional cues. Thus, a marked 10-fold increase in prepronesfatin levels took place during the transition between the pubertal and adult stages of testis maturation. Conversely, testicular NUCB2/nesfatin-1 peptide content was severely decreased after short-term fasting in both pubertal and adult rats. Such profiles are strikingly similar to those detected recently by our group at the rat hypothalamus, where prepronesfatin levels were significantly elevated during the female pubertal transition and were markedly suppressed by acute fasting (26). Of note, the current changes in testicular expression were observed only at the protein level, with substantial modifications in the abundance of the 42-kDa NUCB2/nesfatin-1 product being detected in Western blot analyses. In contrast, relative NUCB2 mRNA levels failed to change under the same experimental conditions. These observations demonstrate differential regulatory mechanisms for NUCB2/nesfatin-1 mRNA and peptide expression, with more constitutive mRNA levels and more readily modulated prepronesfatin content. The underlying molecular mechanism for such a preferential regulation at the translational level remains to be elucidated.

Both developmental and metabolic-induced changes in NUCB2/nesfatin-1 peptide concentrations in the testis may be caused by changes in circulating LH levels during pubertal maturation and fasting, as illustrated by our results from HPX experiments, where hCG, as a super-agonist of LH receptors, was able to consistently stimulate the

testicular levels of the peptide. This action is in good agreement with the selective expression of LH receptors in Leydig cells within the testis (9). However, the contribution of other regulatory signals cannot be ruled out. For instance, a marked suppression of prepronesfatin content was detected in the testes of pubertal males after short-term fasting even before an overt decrease in LH concentrations could be detected, therefore suggesting the potential involvement of other hormonal or nutritional factors. Notwithstanding this, it is also worth noting that in HPX animals, a concomitant reduction of both mRNA and peptide levels of NUCB2/ nesfatin-1 was observed, which is in contrast with the peptide-only changes detected in the developmental and fasting studies mentioned above. It is reasonable to speculate that although variations within a more physiological range may have an impact only on the posttranscriptional regulation of the peptide content, the extreme hormonal challenge resulting from HPX may perturb both transcriptional and posttranscriptional regulatory mechanisms. In addition, the potential contribution of changes in pituitary hormones other than gonadotropins after HPX cannot be ruled out either.

Our functional analyses revealed not only that the testicular expression of NUCB2/nesfatin-1 is finely regulated but also that nesfatin-1 is provided with discernible functions within the male gonad, because it enhanced hCG-stimulated testosterone secretion by rat testicular explants. Admittedly, exploration of additional functional roles of nesfatin-1 in the testis was not conducted here, due in part to the fact that the cognate nesfatin receptor, and hence its potential distribution within the gonads, remains unknown. Nonetheless, comparison of present data with our previous findings on the direct actions of several metabolic signals in the testis allows us to delineate an intricate network of regulatory signals of both a stimulatory and inhibitory nature. Thus, similar to nesfatin-1, resistin and orexin-A have been shown to operate as putative stimulators of

testicular testosterone secretion (14,17), whereas leptin, ghrelin, and adiponectin conduct predominant inhibitory actions (13,15,41). The physiological roles, however, of some of the above signals with similar net effects in terms of testosterone secretion are likely to be different. For instance, although nesfatin-1 increased hCG-stimulated, but not basal, testosterone secretion (see Fig. 6), orexin-A was able to enhance only basal testosterone release *in vitro* (17). In any event, the specific features of NUCB2/nesfatin-1, as a factor selectively expressed by Leydig cells under the stimulatory influence of LH and with positive effects on testosterone secretion, make it tempting to propose an autoregulatory loop whereby Leydig cell-derived nesfatin-1 may contribute to the dynamic regulation of testicular testosterone production during development and under metabolic stress. In addition, our present findings open up the possibility that circulating nesfatin-1, whose levels fluctuate according to whole-energy status, may have a direct impact on testicular steroidogenic function, a possibility that needs to be experimentally confirmed.

Recent data have demonstrated that, in addition to specific neuronal populations, the endocrine X/A cells of the gastric mucosa, as a major source of circulating ghrelin, coexpress also NUCB2/nesfatin-1 (32,42). Our present findings, in conjunction with previous observations from our group on the specific expression of ghrelin in Leydig cells in rodent and primate testis (15,35), allow us to draw a striking parallelism between these two endocrine cell types. Yet our data do not provide direct evidence for the potential contribution of the testis to the pool of circulating nesfatin-1, although our preliminary evidence indicates that its serum levels in adult male rats are higher than in pair-aged females (our unpublished data). Of note, as is the case also in the stomach, the expression profiles of ghrelin and NUCB2/nesfatin-1 in Leydig cells appear to be clearly different; *e.g.* whereas prepronesfatin immunoreactivity becomes detectable at early stages of Leydig cell differentiation (present data), acquisition of ghrelin expression takes

place in this cell population at the final maturational stages (40). Similarly, although ghrelin and nesfatin-1 from gut origin seem to reciprocally operate upon the centers controlling appetite (19), the locally produced peptides may contribute to the autoregulation of Leydig cell secretion of testosterone in a similar yin-yang manner. Admittedly, however, testicular expression of both factors is under the positive regulation of LH (Fig. 5) (15), therefore suggesting a complex regulatory dynamics of these molecules within the same cell type. Of additional note, the joint expression of ghrelin and nesfatin-1 has been also suggested in the pancreas and the adipose tissue. To our knowledge, however, colocalization of both factors within the same cell type in those organs has not been reported to date.

In conclusion, we provide here the first thorough characterization of the patterns of cellular distribution and regulation of NUCB2/nesfatin-1 in the mammalian testis. Our data disclose the specific expression of NUCB2/nesfatin-1 in Leydig cells, which is under the control of developmental, nutritional, and gonadotropic cues. In addition, our studies unveil the potential role intratesticular signals, of as yet unknown nature, in the local control of NUCB2/nesfatin-1 expression and strongly suggest that nesfatin-1 may operate as a local regulator of essential testicular functions, such as testosterone secretion. Our present findings refine our knowledge on the putative reproductive roles of nesfatin-1, as a modulator of different elements of the HPG axis, from the central pathways involved in (female) puberty onset to key testicular cell populations. These observations are potentially relevant for a better understanding of the complex neuroendocrine mechanisms whereby body energy status and fertility are functionally coupled and expand the characterization of the repertoire of biological actions of nesfatin-1, as a potential therapeutic target in the management of body weight disorders.

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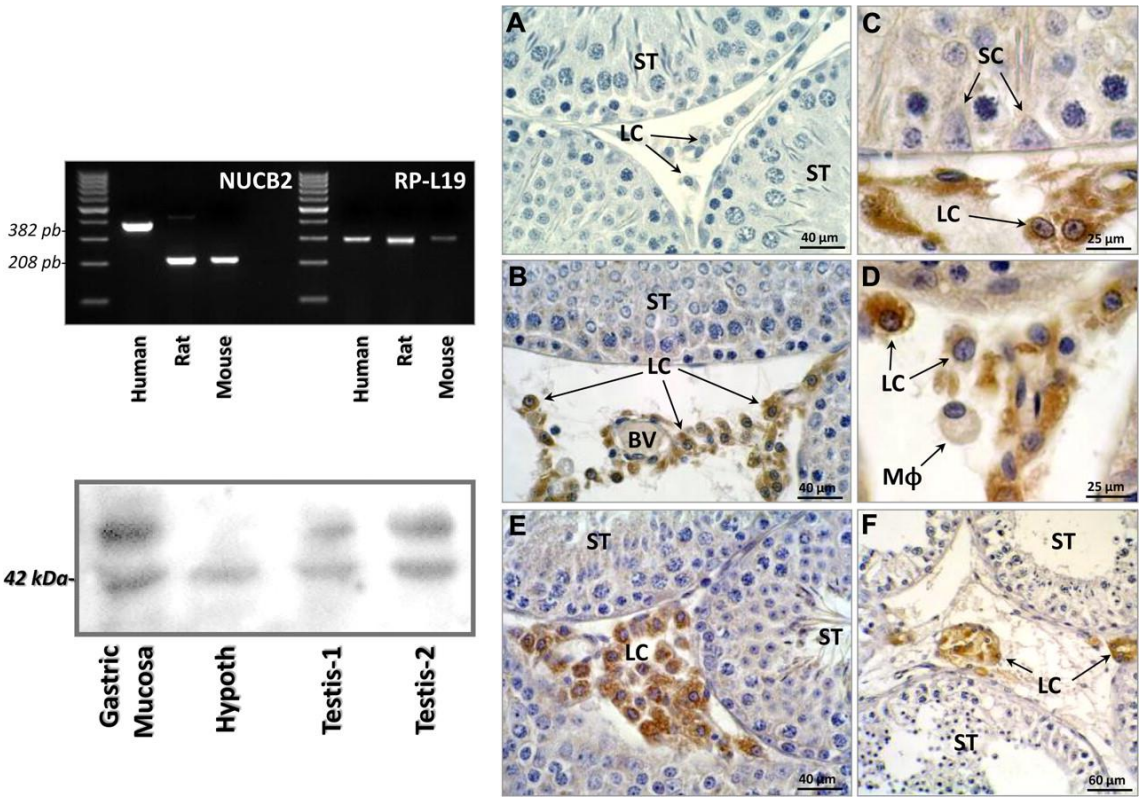

Fig. 1. Expression of NUCB2/nesfatin-1 in the mammalian testis. Shown in the left panels are representative RT-PCR (top) and Western blot (bottom) of NUCB2/nesfatin-1 expression in the testis. RT-PCR assays confirmed expression of NUCB2 mRNA in testis from rat, mouse, and human species. Protein analyses in rat tissues demonstrated the presence of prepronesfatin (predicted size, 42 kDa) in two independent testicular specimens from adult animals as well as in the hypothalamus and gastric mucosa, taken as positive controls. An additional protein product of approximately 50 kDa was detected in Western blots in gastric and testicular samples, whose nature is yet to be defined. In the right panels, representative IHC analyses are presented of nesfatin IR in adult rat (B–D), mouse (E), and human (F) testes. A representative negative control section from rat testis is shown in A. In immunostained sections (B), strong nesfatin IR was present in the cytoplasm of Leydig cells (LC) but was absent and/or negligible in blood vessels (BV), Sertoli cells in the seminiferous tubules (SC in panel C) as well as in interstitial macrophages (Mϕ in panel D). Nesfatin IR was also clearly detected in mouse (E) and human (F) Leydig cells, with roughly similar distribution patterns. Hematoxylin was used for counterstaining. ST, Seminiferous tubules.

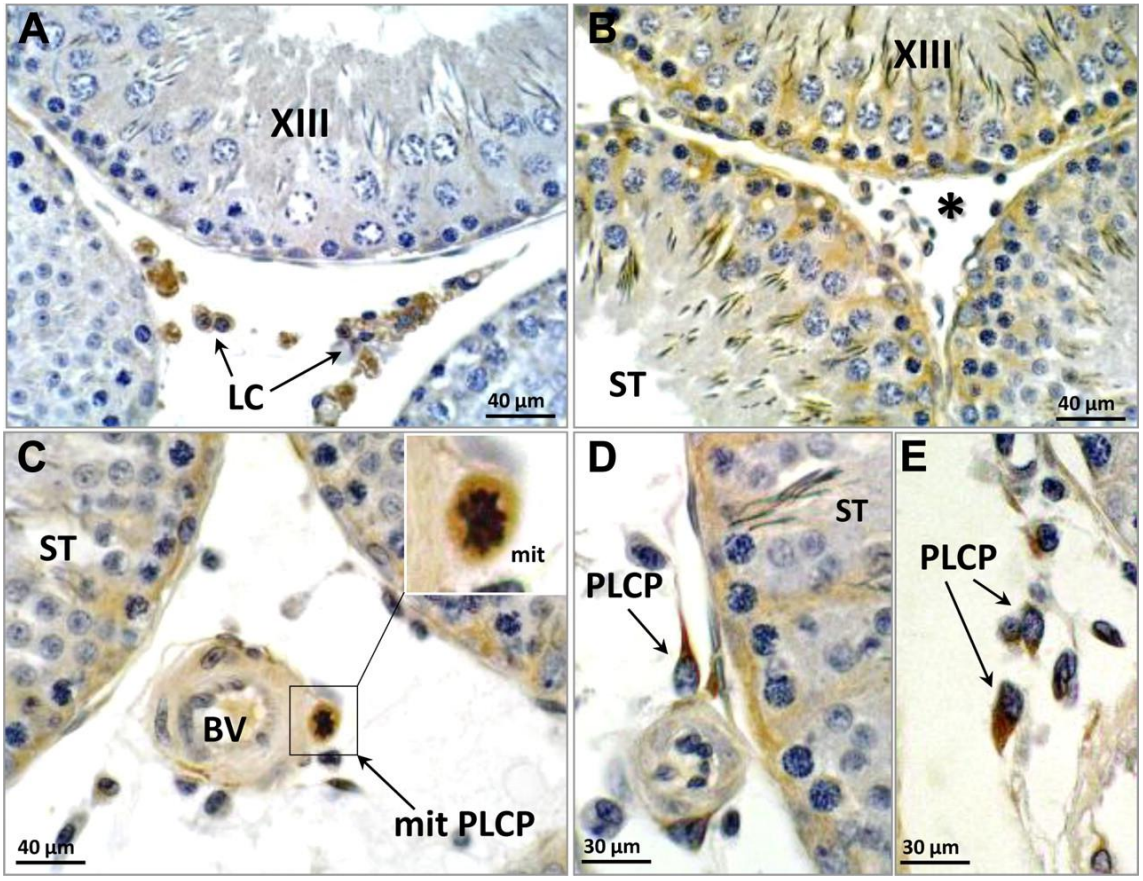

Fig. 2. Testicular expression of NUCB2/nesfatin-1 after elimination of mature Leydig cells. Nesfatin IR, as studied by IHC analyses, in the testes from adult rats, on d 3 after systemic administration of the Leydig cell cytotoxicant EDS. In vehicle-injected rats (A), nesfatin IR was present in interstitial Leydig cells (LC) and barely detectable in the seminiferous tubules (ST, stage XIII). After EDS treatment (B), Leydig cells were absent in the interstitium (asterisk), in keeping with the reported effects of the toxicant, but nesfatin IR became clearly evident in Sertoli cells, particularly in still recognizable stages XII-II. In addition, nesfatin IR was present in the cytoplasm of undifferentiated, fibroblast-like presumptive Leydig cell precursors (PLCP in C–E), some of which showed mitotic features (mit-PLCP in panel C). BV, Blood vessels.

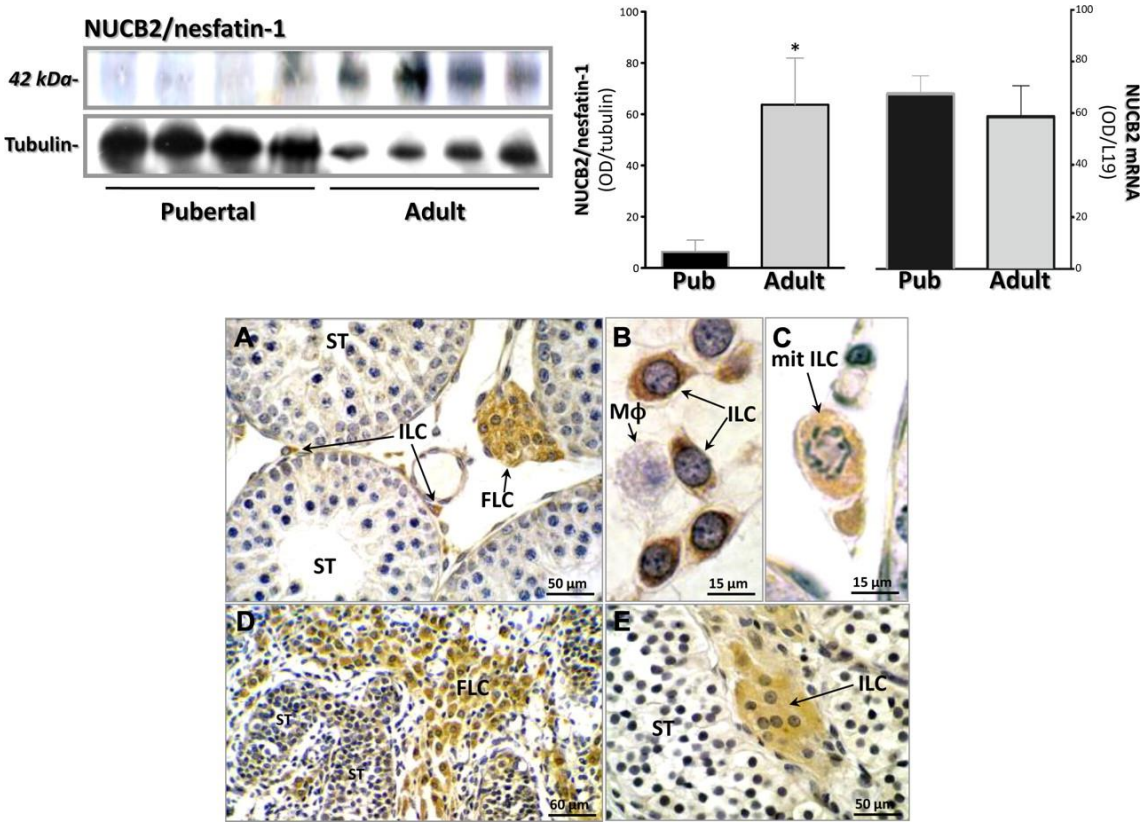

Fig. 3. Developmental regulation of NUCB2/nesfatin-1 in the testis. In the upper panel, testicular expression levels of NUCB2/nesfatin-1 in pubertal and adult males are shown. Representative Western blots of four independent specimens for each age point are shown. In addition, semiquantitative data for protein and mRNA levels, as estimated by Western blot and RT-PCR assays, are also presented (*, $P < 0.05$ vs. pubertal group; Student's t test). In the lower panel, IHC analyses are shown of nesfatin IR in Leydig cell types at early (prepubertal) differentiation stages. In prepubertal rats (A–C), nesfatin IR was present in fetal (FLC) and immature adult-type (ILC) Leydig cells. Immunostaining was strong in immature Leydig cells (ILC in panel B) and absent in interstitial macrophages (Mϕ). Mitotic immature Leydig cells (mit-ILC in panel C) were frequently observed. Nesfatin IR was also present in human fetal (FLC in panel D) and immature (ILC in panel E) Leydig cells. ST, Seminiferous tubules.

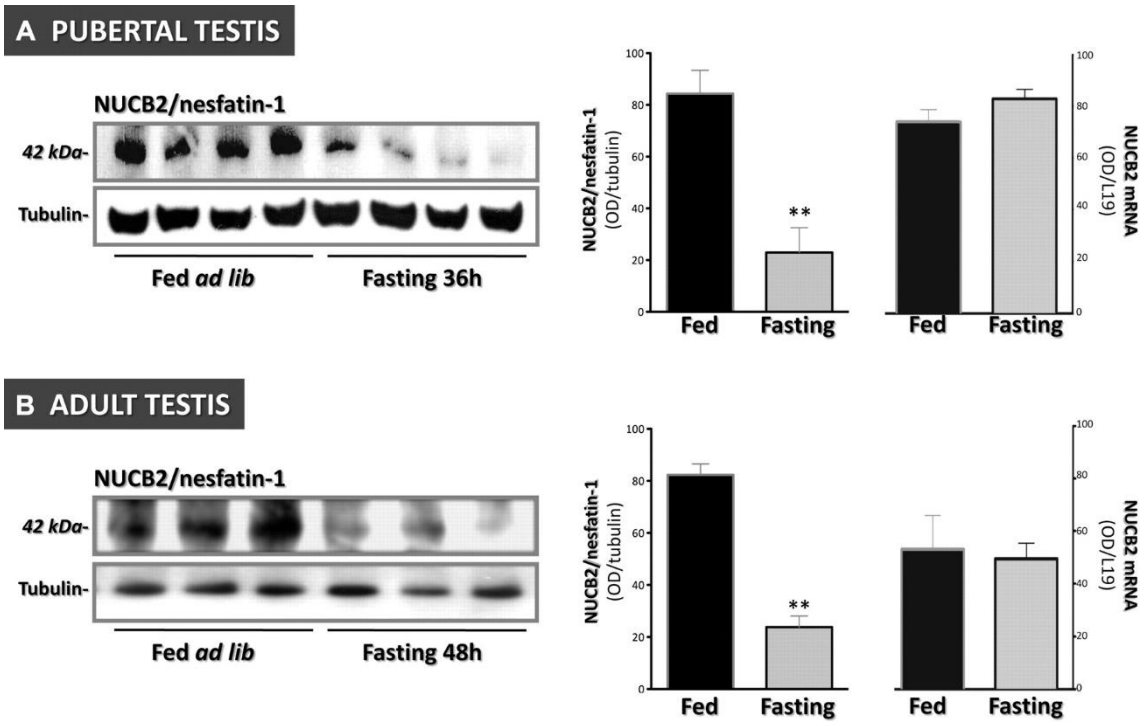

Fig. 4. Metabolic regulation of testicular expression of NUCB2/nesfatin-1: effects of acute fasting. Analyses are shown of testicular expression levels of NUCB2/nesfatin-1 in pubertal (upper panel) and adult (lower panel) males after metabolic challenge by short-term fasting. Representative Western blots of three to four independent specimens for each age point (pubertal vs. adult) and experimental condition (fed ad libitum vs. fasting) are shown. In addition, semiquantitative data for protein and mRNA levels, as estimated by Western blot and RT-PCR assays, are also presented. **, $P < 0.01$ vs. corresponding control group fed ad libitum (Student's t test).

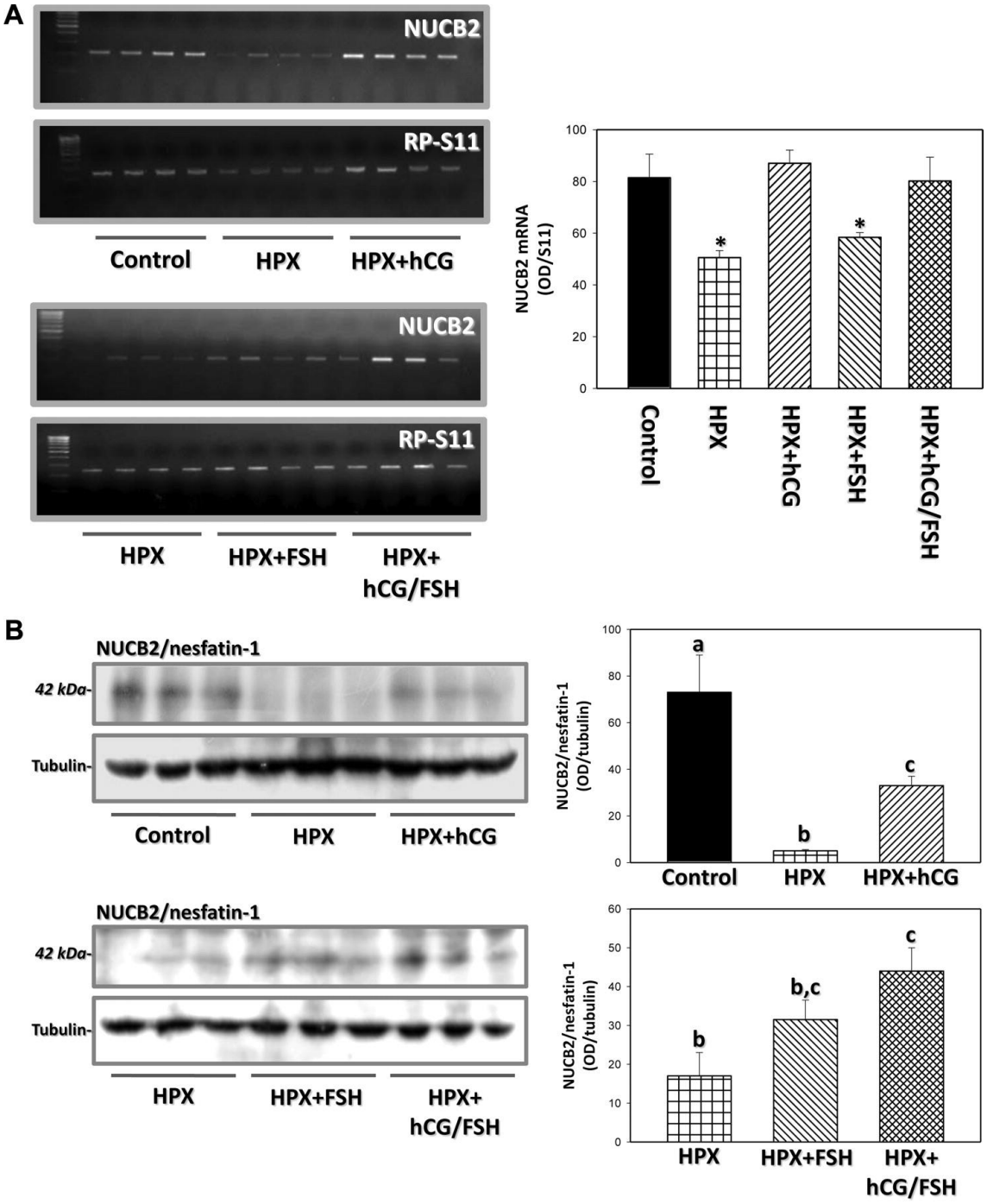

Fig. 5. Gonadotropic regulation of testicular expression of NUCB2/nesfatin-1. Analyses are shown of testicular expression levels of NUCB2 mRNA (A) and prepronesfatin protein (B) in control and HPX male rats; the latter includes rats without hormonal treatment (HPX) or submitted to replacement for 1 wk with hCG (HPX+hCG), FSH (HPX+FSH), or a combination of both (HPX+hCG/FSH). Representative RT-PCR assays and Western blots of three to four independent specimens for each experimental condition are shown. In addition, semiquantitative data for mRNA and protein levels are also presented. For semiquantitative RNA data: *, $P < 0.05$ vs. corresponding control group (ANOVA followed by Student-Newman-Keuls multiple-

range test). For semiquantitative protein data, bars with different letters are statistically different (ANOVA followed by Student-Newman-Keuls multiple-range test).

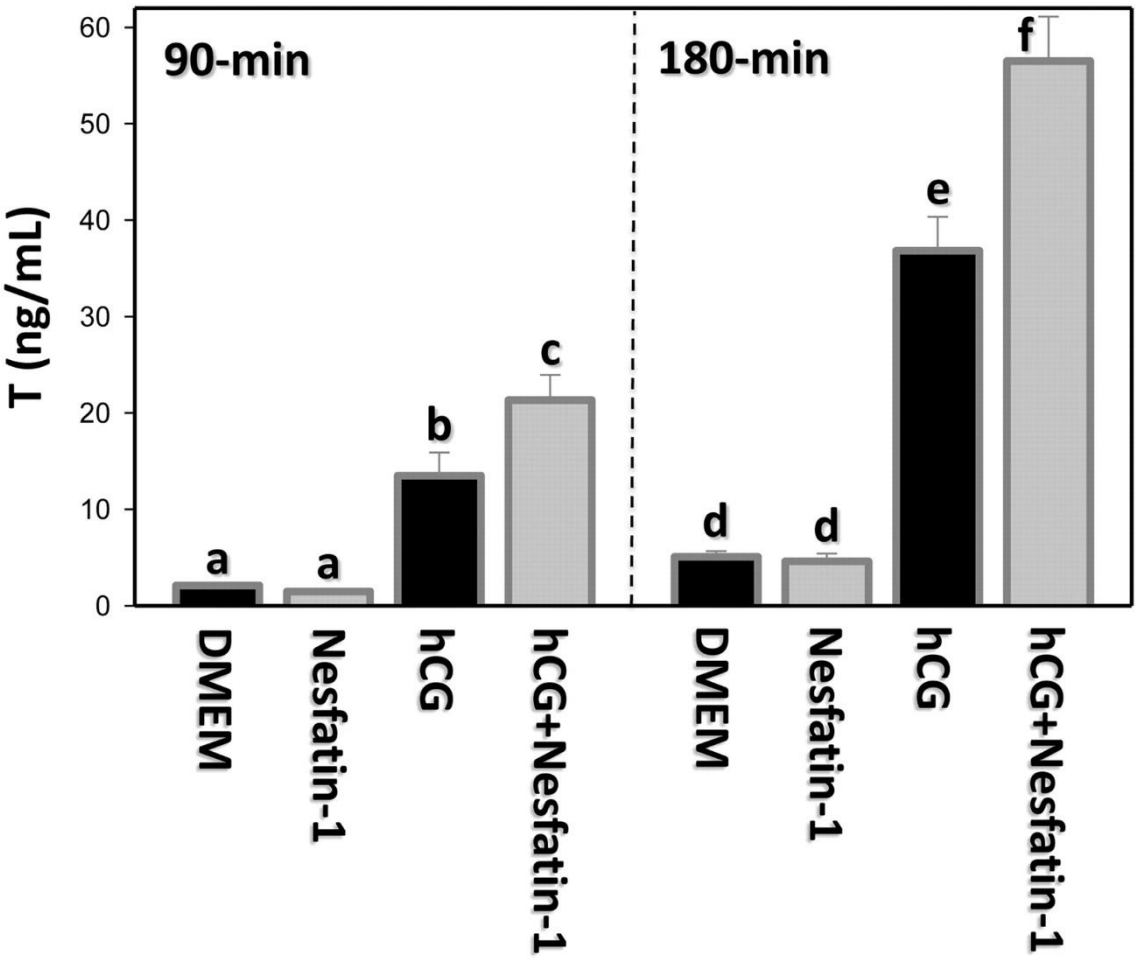

Fig. 6. Modulation of testosterone secretion by nesfatin-1: effects in basal and hCG-stimulated conditions. Slices of testicular tissue were incubated with an effective dose of 10^{-7}M nesfatin-1, either in basal conditions or after the combined challenge with hCG (10 IU/ml). Groups incubated with medium (DMEM) or hCG alone served as corresponding controls. The concentration of testosterone (T) in the media was monitored after 90 and 180 min incubation. Data are expressed as mean ± SEM (n = 10–12 samples per group). Bars with different letters are statistically different (ANOVA followed by Student-Newman-Keuls multiple-range test).