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Research Article

The IL-27 component EBI-3 and its receptor subunit IL-27Rα are essential for the cytoprotective action of humanin on male germ cells†

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Abstract

Humanin (HN) is a mitochondrial-derived peptide that protects many cells/tissues from damage. We previously demonstrated that HN reduces stress-induced male germ cell apoptosis in rodents. HN action in neuronal cells is mediated through its binding to a trimeric cell membrane receptor composed of glycoprotein 130 (gp130), IL-27 receptor subunit (IL-27R, also known as WSX-1/TCCR), and ciliary neurotrophic factor receptor subunit (CNTFR). The mechanisms of HN action in testis remain unclear. We demonstrated in ex-vivo seminiferous tubules culture that HN prevented heat-induced germ cell apoptosis was blocked by specific anti-IL-27R, anti-gp130, and anti-EBI-3, but not by anti-CNTFR antibodies significantly. The cytoprotective action of HN was studied by using groups of *il-27r*$^{-/-}$ or *ebi-3*$^{-/-}$ mice administered the following treatment: (1) vehicle; (2) a single intraperitoneal (IP) injection of HN peptide; (3) testicular hyperthermia; and (4) testicular hyperthermia plus HN. We demonstrated that HN inhibited heat-induced germ cell apoptosis in wildtype but not in *il-27r*$^{-/-}$ or *ebi-3*$^{-/-}$ mice. HN restored heat-suppressed STAT3 phosphorylation in wildtype but not *il-27r*$^{-/-}$ or *ebi-3*$^{-/-}$ mice. Dot blot analyses showed the direct interaction of HN with IL-27R or EBI-3 peptide. Immunofluorescence staining showed the co-localization of IL-27R with HN and gp130 in Leydig cells and germ cells. We conclude that the anti-apoptotic effects of HN in mouse testes are mediated through interaction with EBI-3, IL-27R, and activation of gp130, whereas the role of CNTFR needs further studies. This suggests a multicomponent tissue-specific receptor for HN in the testis and links HN action with the IL-12/IL-27 family of cytokines.

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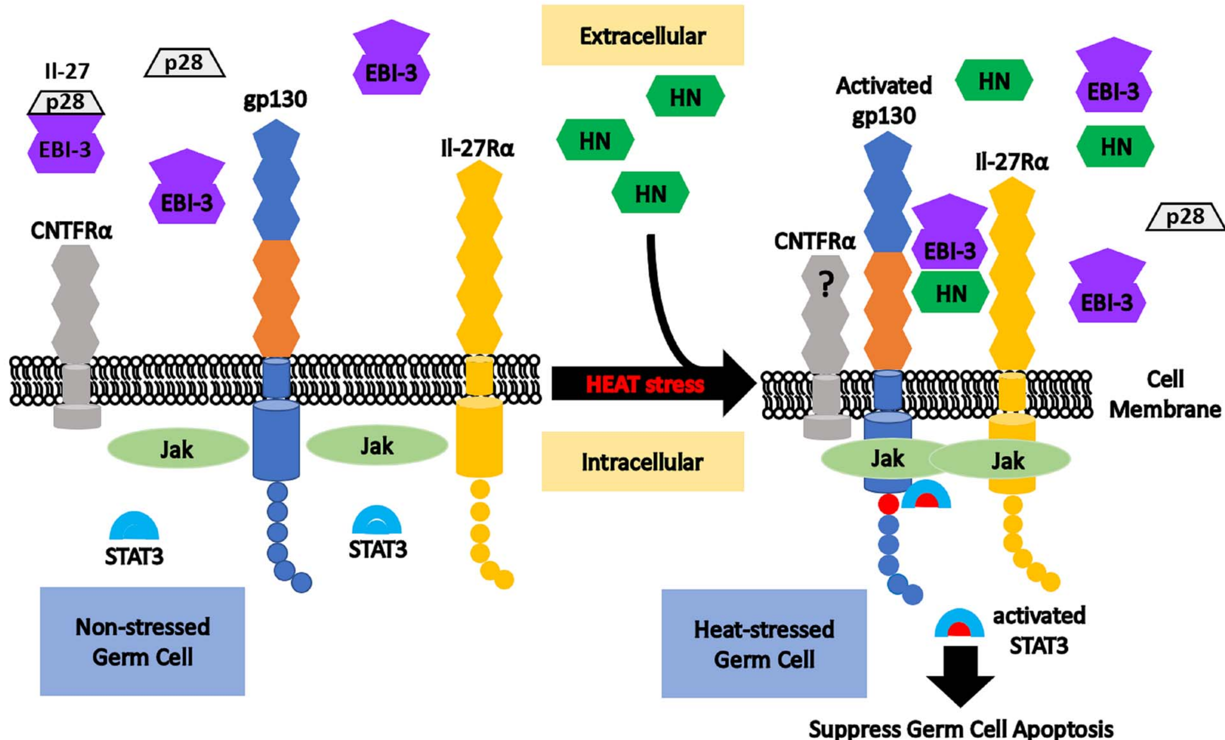
Summary sentence

The cytoprotective effects of HN in mouse testes are mediated via binding with EBI-3, IL-27R, and activation of gp130. This suggests there is a multicomponent tissue-specific HN receptor in the testis and links HN with the IL-12/IL-27 family of cytokines.

Graphical Abstract



Model of humanin (HN) interaction with EBI-3, IL-27Rα, and gp130 in male germ cells at baseline and after heat induced stress. Under nonstressed condition (left part), free EBI-3 may bind with p28 to form cytokine IL-27, while IL-27Rα and gp130 may combine with each other to form the IL-27 receptor. CNTFRα may bind gp130 and IL-27Rα to form the HN membrane receptor in neuronal cells. After heat stress in the testis, exogenous HN binds with EBI-3 and interacts with gp130 plus IL-27Rα to activate the downstream Jak/STAT3 pathway. Activated STAT3 suppresses heat-induced male germ cell apoptosis. In our study, the role of CNTFRα cannot be concluded definitely. Our data suggest a different multicomponent and tissue-specific receptor for HN in the testis and linking HN action with the IL-12/IL-27 family of cytokines.

Key words: humanin, spermatogenesis, apoptosis, EBI-3, IL-27Rα, STAT3, humanin-receptor, signal transduction, mouse.

Introduction

Germ cell apoptosis occurs spontaneously during spermatogenesis or can be induced in mice [1–7], rats [8–14], monkeys [15, 16], and men [17–19] by a variety of apoptotic stimuli including testicular hyperthermia, insulin-like growth factor binding protein 3 (IGFBP-3), gonadotropin-releasing hormone antagonist (GnRH-A) and testosterone (T) (suppressing both endogenous gonadotropins and subsequently intratesticular T) [20, 21], or chemotherapies [22–25]. HN is an anti-apoptotic, putatively mitochondrial-derived peptide that protects cells from stress/injury in neuronal tissue [26–34], heart and vasculature [35–38], blood-derived cells [39], pancreas [40], and the testis [22, 24, 25, 41–43]. We have previously demonstrated that HN partially prevents GnRH-A or chemotherapy induced male germ cell apoptosis in rodents [22, 24, 25, 41–43].

A previously proposed mechanism of HN action on prevention of neuronal cell death is through binding to a tripartite neuro-cytokine-related membrane receptor composed of glycoprotein 130 (gp130), IL-27 receptor subunit α (IL-27Rα, also known as WSX-1 or T cell

cytokine receptor, TCCR), and ciliary neurotrophic factor receptor subunit α (CNTFRα). This trimeric receptor activates the STAT3 signaling pathway [31, 44–46]. IL-27Rα is found in immune cells, CNTFRα in neuronal cells, whereas gp130 is the essential subunit for multiple cytokine receptors belonging to the IL-6/IL-12 family of which IL-27 is a member [47]. It remains unknown whether the three components IL-27Rα/gp130/CNTFRα of HN receptor are required for the cytoprotective action of HN in other tissues including male germ cells, or whether HN interacts with other interleukin receptor subunits to mediate its effects. IL-27Rα and gp130 are both subunits of IL-27 receptor.

The cytokine IL-27 is a heterodimeric cytokine consisting of two independent subunits: p28 (also known as IL-30) and Epstein–Barr virus-induced gene 3 (EBI-3). EIB-3 predominantly binds to p28 but also binds to P35 subunit of IL-12, and p28 signals independently of EBI3 [48]. IL-27 is structurally related to IL-6 or IL-12 cytokine families [48–57]. Activation of the IL-27 receptor complex consisting of IL-27Rα subunit and gp130 [48–50] stimulates the downstream signaling pathway involving the transcription factors termed signal transducer and the activator of transcription-1 (STAT1) and STAT3 [50, 51, 54, 55]. IL-27 may show diverse immune regulatory activities under different conditions [55] and has been demonstrated to have dual roles in both the induction and inhibition of inflammation [56, 57] as well as in cancer biology [48–50, 52].

The present studies utilized experimental models previously established in our laboratory where HN prevented transient hyperthermia-induced apoptosis of germ cells in mouse ST cultures (*ex vivo*) [22] and in mice (*in vivo*). Our goal was to examine whether HN action on the male germ cells required: (1) binding to its proposed IL-6/IL-12-like trimeric receptor; and (2) interaction with IL-27 component EBI-3. Our data suggest that (1) the cytoprotective effects of HN on male germ cells are mediated via membrane receptor subunits gp130 and IL-27Rα (and activating STAT3 phosphorylation), while the role of CNTFRα is not clear; and (2) the interaction of HN with EBI-3 is necessary for the protective effect of HN against heat-induced male germ cell apoptosis. We conclude that the physical interaction of HN with EBI-3 and binding to membrane receptor subunits gp130 and IL-27Rα plays a predominant role for the cytoprotective action of HN against heat-induced male germ cell apoptosis. This is the first report that the IL-27 component, EBI-3, is involved in HN's action which further suggests connections between HN and cytokine network.

Material and methods

Ethics statement

Animal handling and experimentation were in accordance with the recommendation of American Veterinary Medical Association and were approved by the animal care and use review committee at the Lundquist Institute at the Harbor-University of California, Los Angeles Medical Center.

Animal experimental protocol

Adult (12 week old) male wild-type mice (C57BL6/J) from Jackson Lab (Bar Harbor, Maine) were used for *ex vivo* seminiferous tubule culture study. Adult male *il-27α* knockout mice (gift from Dr Chris Saris, Amgen, Thousand Oaks, CA) and age-matched wild-type (*C57BL6/N*) mice from Jackson Lab were used for studying the role of IL-27Rα in HN's cytoprotective effects on germ cells. Adult male *ebi-3* knockout and age-matched wild-type (*C57BL6/NJ*) mice from Jackson Lab were used for studying whether EBI-3 potentiates HN's cytoprotective actions on male germ cells. All mice were housed in a standard animal facility under controlled temperature (22 °C) and photoperiod of 12 h of light and 12 h of darkness with free access to food and water.

To investigate the functional roles of HN membrane receptor subunits IL-27Rα, GP-130, and CNTFRα in the HN's cytoprotective action on male germ cells, we performed experiments using IL-27Rα, GP-130, and CNTFRα blocking antibodies in an *ex vivo* ST culture system. Ten to twelve ST segments from one wild-type mouse were immersed into the culture medium in triplicates for each experiment and exposed to heat stress at 43 °C for 15 min in the presence and absence of HN and different blocking antibodies to the components of the putative HN receptor. Each experiment was repeated 6–8 times with the same age mice. The role of IL-27 component EBI-3 in the HN's cytoprotective activity was studied using the same ST *ex vivo* culture system.

For the *in vivo* studies of the contribution of IL-27Rα or EBI-3 to HN action, age-matched adult male *il-27rα* (12–28 weeks old) or *ebi-3* (12 weeks old) knockout or the corresponding wild-type mice were divided into four groups ($n = 4$ or 5/group per experiment) and received one of the following treatments: (1) vehicle (control); (2) a single IP injection of HN peptide at a dose of 40-mg/kg body weight (BW) (HN); (3) testicular hyperthermia (43 °C for 15 min; Heat); and (4) testicular hyperthermia plus HN IP injection (Heat + HN). All animals were sacrificed 6 h after treatment.

Tissue preparation

Both control and experimental animals were injected with heparin (130 IU/100 g BW, IP) 15 min before a lethal injection of sodium pentobarbital (200-mg/kg BW, IP) to facilitate testicular perfusion using a whole-body perfusion technique [13]. After perfusion with saline, one testis was removed and weighed. Portions of testicular parenchyma were snap frozen in liquid nitrogen and stored at −80 °C for western blotting. The contralateral testis was fixed by vascular perfusion with Bouin's solution, collected, weighed, and processed for routine paraffin embedding for either *in situ* detection of apoptosis or co-immunofluorescence staining.

ST culture and flow cytometry assessment

Mice were injected with a lethal dose of sodium pentobarbital (200-mg/kg BW, IP) and the testes were harvested. Testicular tissues were microdissected in Petri dishes containing tissue culture medium (Nutrient Mixture Ham's F10; Invitrogen, Paisley, UK), supplemented with 0.1% of human serum albumin (Sigma-Aldrich Co LLC, St. Louis, MO) and 10-mcg/ml gentamicin (Invitrogen, Carlsbad, CA) [14]. Light segments of ST (∼2 mm in length, containing early and late Stages XI–IV of the seminiferous epithelium, susceptible to heat stress) were isolated under the microscope and transferred to 6-well culture plates containing 2 ml/well serum-free culture medium. ST (10–12 segments per well, in triplicates) were heated at 43 °C for 15 min to induce germ cell apoptosis. Heat-treated groups were incubated with (1) HN (10 mcg/ml); (2) scrambled peptide (SP) (10 mcg/ml); (3) HN (10 mcg/ml) + anti-gp130 neutralization antibody (1.0 mcg/ml, Cat#AF468, R&D systems, Minneapolis, MN); (4) HN (10 mcg/ml) + anti-IL-27Rα antibody (1.0 mcg/ml, gift from Dr Masaaki Matsuoka, Tokyo, Japan); or (5) HN (10 mcg/ml) + anti-CNTFRα neutralization antibody (1.0 mcg/ml, Human Cat#AF-303-NA, or Rat Cat#AF-559-NA R&D

systems, Minneapolis, MN); and (6) HN (10 mcg/ml) + anti-EBI-3 antibody (1.0 mcg/ml, Cat#124694, Abcam plc, Cambridge, MA). (7) HN (10 mcg/ml) + goat IgG (1.0 mcg/ml, Cat#AB-108-C, R&D systems, Minneapolis, MN) or HN (10 mcg/ml) + rabbit IgG (1.0 mcg/ml, Cat#AB-105-C, R&D systems, Minneapolis, MN) were used as negative control for the neutralization antibodies described above. The plates were then incubated at 34 °C for 16 h with 5% CO_2.

After 16-h incubation, segments of ST from all the groups were collected from each well, digested into single cell suspension, and analyzed by flow-cytometry to detect germ cell apoptosis. In brief, seminiferous tubule fragments were digested by 0.25% collagenase, filtered by the cell strainer (BD Falcon REF352340, BD Biosciences, San Jose, CA), and collected with a new set of tubes. After centrifuge and washing twice with PBS for 10 min, cells were resuspended into 100 mcL of binding buffer and stained with 5-mcL Annexin V conjugated APC (BD Biosciences, San Jose, CA) and 5 mcL of 7-AAD (BD Biosciences, San Jose, CA) in the dark for 15 min on ice. Tubes with nonstained cells or stained with APC only or 7-AAD only were used as background controls; 400-mcL binding buffer was added to each tube and then subjected to flow cytometry analysis (BD FACSCalibur Flow Cytometer, BD Biosciences, San Jose, CA).

Western blotting analysis

Western blotting was performed as described previously [58, 59]. In brief, proteins were denatured and separated by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system (Invitrogen, Carlsbad, CA). After transferring, the Immuno-blot PVDF Membrane (Bio-Rad, Hercules, CA) was blocked for 1 h and then probed using anti-STAT3 (Cat#9139) or anti-pSer727 STAT3 (Cat# 9134, Cell signaling Technology, Inc., Beverly, MA) overnight at 4 °C with constant shaking. After washing, membrane was then incubated with an anti-mouse (for STAT3 antibody, Cat#sc-2069, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-rabbit (Cat#NA934V, for all other antibodies, Amersham Biosciences, Piscataway, NJ) IgG-HRP secondary antibody. All antibodies were diluted in blocking buffer. For immunodetection, the membrane was incubated with enhanced chemiluminescence solutions per the manufacturer's specifications (Amersham Biosciences, Piscataway, NJ) and exposed to Hyperfilm ECL (Denville Scientific Inc., Metuchen, NJ).

Assessment of apoptosis

In situ detection of cells with DNA strand breaks was performed in Bouin's-fixed, paraffin-embedded testicular sections by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) technique [60, 61] using an ApopTag-peroxidase kit (Chemicon International, Inc., Temecula, CA). Enumeration of the nonapoptotic Sertoli cell nuclei with distinct nucleoli and apoptotic germ cells was quantified at stages I–IV (early stages) and stages XI–XII (late stages) of the seminiferous epithelial cycle using an Olympus BH-2 microscope (New Hyde Park, NY). Stages were identified according to the criteria proposed by Russell et al. [62] for paraffin sections. The rate of germ cell apoptosis (apoptotic index) was expressed as the number of apoptotic germ cells per Sertoli cells [60].

Dot blotting assessment

Dot blotting was used to determine *in vitro* direct HN interaction with the IL-27 receptor subunit IL-27Rα; IL-27 component EBI-3, p28, and IL-12 family components p35 and p40 [63]. Peptides of interest and respective controls were spotted onto nitrocellulose (NC) membrane (Pierce, Rockford, IL) at 1 mcL per spot and then allowed to dry. In IL-27Rα experiment, the peptides spotted on membrane included: HN peptide (1, 2, and 5 nmol, GeneMed Synthesis Inc., San Antonio, TX), SP (1, 2, and 5 nmol, a nonbinding partner of HN used as negative control; GeneMed Synthesis Inc., San Antonio, TX), BAX (mouse full long sequence used as positive control, 1, 2, and 5 mcg, ProSpec-Tany TechnoGene Ltd. Rehovot, Israel), IL-27Rα (1, 2, and 5 mcg, with Human IgG Fc tail, Sigma-Aldrich, MO), and Human IgG Fc (1, 2, and 5 mcg, negative control for IL-27Rα peptide tail, Sigma-Aldrich, MO). In the EBI-3, p28, p35, and p40 experiment, the peptides spotted on membrane included: HN peptide (1, 2, and 5 nmol), SP (1, 2, and 5 nmol, negative control; GeneMed Synthesis Inc., San Antonio, TX), BAX (0.1, 1, and 5 mcg, positive control), EBI-3 (with Human IgG Fc tail), p28, p35, and p40 (0.1, 1, and 5 mcg, all purchased from Sigma-Aldrich, MO), and Human IgG Fc (0.1, 1, and 5 mcg, negative control for EBI-3 peptide tail, Sigma-Aldrich, MO). After blocking the nonspecific sites by 0.2% I-block (Applied Biosystems, Foster City, CA) for 3 h at room temperature, the NC membranes were incubated with test peptides (HN peptide, 5 mcg/ml) overnight at 4 °C with gentle rocking. After washing in 0.3% Tween 20 in Tris-buffered saline, the NC membranes were incubated with the primary antihuman HN antibody (Cat#H2414, Sigma-Aldrich Co LLC, St. Louis, MO) overnight 4 °C with gentle rocking. Following incubation with secondary antirabbit antibody (Cat#NA934V, Amersham Biosciences, Piscataway, NJ), the membranes were washed, incubated with enhanced chemiluminescence solutions per the manufacturer's specifications (Amersham Biosciences, Piscataway, NJ), and exposed to Hyperfilm ECL (Denville Scientific Inc., Metuchen, NJ).

Immunofluorescence analyses

Co-localization of IL-27Rα with humanin or gp130 in the testis was detected by confocal microscopy using double immunostaining as previously described [59]. In brief, after deparaffinization and rehydration, testicular sections were treated with blocking serum at room temperature. After washing the slides, sections were incubated with the affinity purified antihumanin antibody (1:100, gift from Dr Pinchas Cohen) or anti-gp130 antibody (Cat#sc-656, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h followed by goat antirabbit Alexa 633-labeled secondary antibody (Cat#A-21070, Invitrogen, Carlsbad, CA) for 30 min. The sections were then incubated with a goat polyclonal anti-IL-27Rα antibody (Cat#sc-47065, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight followed by donkey antigoat FITC secondary antibody (Cat#sc-2024, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min. For negative controls, sections were treated only with secondary antibody, and no signals were detected (Figure 6, bottom panel). *il-27rα* knockout mouse testis slide was used as negative control for determining IL-27Rα antibody specificity and no staining signals were detected (data not shown). Con-focal imaging was performed using a Leica TCS-SP-MP con-focal microscope equipped with a 488-nm argon laser for the excitation of green fluorophores (FITC) and a 633-nm helium-neon laser for the excitation of red fluorophores (Alex 633).

Statistical analysis

Statistical analyses were performed using the SigmaStat 12.0 Program (Systat Software Inc., San Jose, CA). The Student–Newman–Keuls test after one-way repeated measures ANOVA was used to test

for statistical significance. Differences were considered significant if $P < 0.05$.

Results

IL-27Rα and gp130 neutralizing antibodies blocked HN's effect on heat-induced germ cell apoptosis

Heat-induced male germ cell apoptosis in *ex vivo* seminiferous tubule culture was significantly decreased despite variation from different experiments by co-incubation with 10-mcg/ml HN peptide but not by SP (Figure 1A–D). Anti-gp130 (Figure 1A) antibody significantly blocked the protective effect of HN peptide on apoptosis ($P < 0.05$), whereas goat IgG had no effect on germ cell apoptosis. Anti-IL-27Rα (Figure 1B) antibody significantly blocked the protective effect of HN peptide on germ cell apoptosis ($P < 0.05$). In contrast, anti-CNTFRα neutralizing antibodies (either rat or human specific) similar to Immunoglobulin G did not significantly decrease the cytoprotective effect of HN on germ cells (Figure 1C and D). The specificity of both anti-CNTFRα receptor antibodies was demonstrated as both antibodies neutralized the effect of CNTF peptide in neuroendocrine beta cells (NIT-1 cell line) and ST (see Supplementary Figure S1A and B).

EBI-3 antibody blocked HN effect on heat-induced germ cell apoptosis

Heat-induced male germ cell apoptosis in *ex vivo* seminiferous tubule cultures was significantly decreased by co-incubation with 10-mcg/ml HN peptide but not by SP (Figure 1E). Anti-EBI-3 (Figure 1E) antibody significantly blocked the protective effect of HN peptide on apoptosis ($P < 0.05$), whereas rabbit IgG had no effect on germ cell apoptosis.

HN prevents heat-induced male germ cell apoptosis in wild-type mice but not in il-27rα or ebi-3 knockout animals

In *il-27rα* knockout versus wild-type mice experiment, testicular hyperthermia increased germ cell apoptosis primarily at early and late stages of seminiferous epithelial cycles in *wt* mice (Figure 2A, left panel: 0.79 ± 0.10, TUNEL positive germ cell/Sertoli cell; $P < 0.01$ compared with *wt* control group, 0.17 ± 0.03). HN partially suppressed heat-induced germ cell apoptosis (Figure 2A, left panel: 0.38 ± 0.05, $P < 0.01$ compared with heat-treated group). In *il-27rα* knockout mice, heat significantly induced germ cell apoptosis (Figure 2A, right panel: 0.77 ± 0.10; $P < 0.01$ compared with knockout non-heated group, 0.09 ± 0.02) mainly at early and late stages. HN was not effective in preventing heat-induced apoptosis (Figure 2A, right panel: 0.56 ± 0.06, $P > 0.05$ compared with heat treatment group).

In *ebi-3* knockout versus wild-type mice, testicular hyperthermia increased germ cell apoptosis primarily at early and late stages of seminiferous epithelial cycles in *wt* mice (Figure 2B, left panel: 0.34 ± 0.03 TUNEL positive germ cell/Sertoli cell; $P < 0.01$ compared with *wt* control group, 0.04 ± 0.01). Heat-induced germ cell apoptosis was partially inhibited by synthetic HN administration in *wt* mice (Figure 2B, left panel: 0.15 ± 0.02, $P < 0.01$ compared with heat-treated group). In *ebi-3* knockout mice, heat significantly induced germ cell apoptosis (Figure 2B, right panel: 0.30 ± 0.03; $P < 0.01$ compared with knockout nonheated group, 0.07 ± 0.01) mainly at early and late stages; while HN did not prevent heat-induced apoptosis (Figure 2B, right panel: 0.30 ± 0.04, $P > 0.05$ compared with heat treatment group).

HN treatment restored heat-suppressed STAT3 phosphorylation

STAT3 has 2 phosphorylation sites—Ser727 and Tyr705. Phosphorylation of these 2 sites leads to the activation of STAT3. Immunoblot analyses on testis homogenates showed that STAT3 expression did not significantly change with HN treatment under basal conditions in both *wt* and *il-27rα* knockout mice (Figure 3A and B). Heat treatment suppressed Ser727-phosphorylated STAT3 in testes in both the *wt* and *il-27rα* knockout mice ($P < 0.05$, Figure 3A and B). HN treatment restored the expression of Ser727-phosphorylated STAT3 that was suppressed by heat treatment ($P < 0.05$) in the testes of *wt* mice (Figure 3A) but not in *il-27rα* knockout animals after heat exposure (Figure 3B).

Immuno-blot analyses on testis homogenates also confirmed the similar changes of STAT3 phosphorylation in *wt* and *ebi-3* knockout mice ($P < 0.05$, Figure 4A and B). STAT3 expression had no significant change with HN treatment under basal conditions in both *wt* and *ebi-3* knockout mice (Figure 4A and B). Heat treatment suppressed Ser727-phosphorylated STAT3 in testes in both the *wt* and *ebi-3* knockout mice ($P < 0.05$, Figure 4A and B). HN treatment restored the expression of Ser727-phosphorylated STAT3 that was suppressed by heat treatment ($P < 0.05$) in the testes of *wt* mice (Figure 4A) but not in *ebi-3* knockout animals after heat exposure (Figure 4B).

Direct interaction between HN and IL-27Rα

To determine if HN interacts directly with IL-27Rα *in vitro*, we performed dot blot experiments where increasing concentrations of HN, SP, BAX, IL-27Rα, and human IgG Fc fragment were spotted on a membrane and then incubated sequentially with HN, rabbit anti-HN antibody, antirabbit secondary antibody, and ECL plus reporting reagent. Figure 5A demonstrates that HN interacted with BAX (positive control) and IL-27Rα but not with SP and human IgG Fc (negative controls).

Direct interaction between HN and EBI-3 but not IL-27 or IL-12 cytokine components

To examine whether HN interacts directly with EBI-3 *in vitro*, we performed dot blot experiments where increasing concentrations of HN, SP, BAX, EBI-3 (IL-27 component), p28 (IL-27 component), p35, p40 (other components of the IL-12 cytokine family), and human IgG Fc fragment were spotted on a membrane and then processed. Figure 5B demonstrates that HN interacted with BAX (positive control) and EBI-3 but not with p28, p35, p40, SP, and human IgG Fc (negative controls).

Co-localization of HN with IL-27Rα and IL-27Rα with gp130 in testis

Testicular sections from all treatment groups were immunostained for HN and IL-27Rα. As shown in Figure 6 upper panel, after heat exposure, IL-27Rα and HN co-localized both in germ cells and Leydig cells (see insets). IL-27Rα also co-localized with gp130 in Leydig cells and germ cells (Figure 6, middle panel, see insets). There was no staining when primary antibodies to HN and IL-27Rα were not used (Figure 6, bottom panel). We could not perform HN, IL-27Rα, and gp130 co-staining with EIB-3 because of the limited

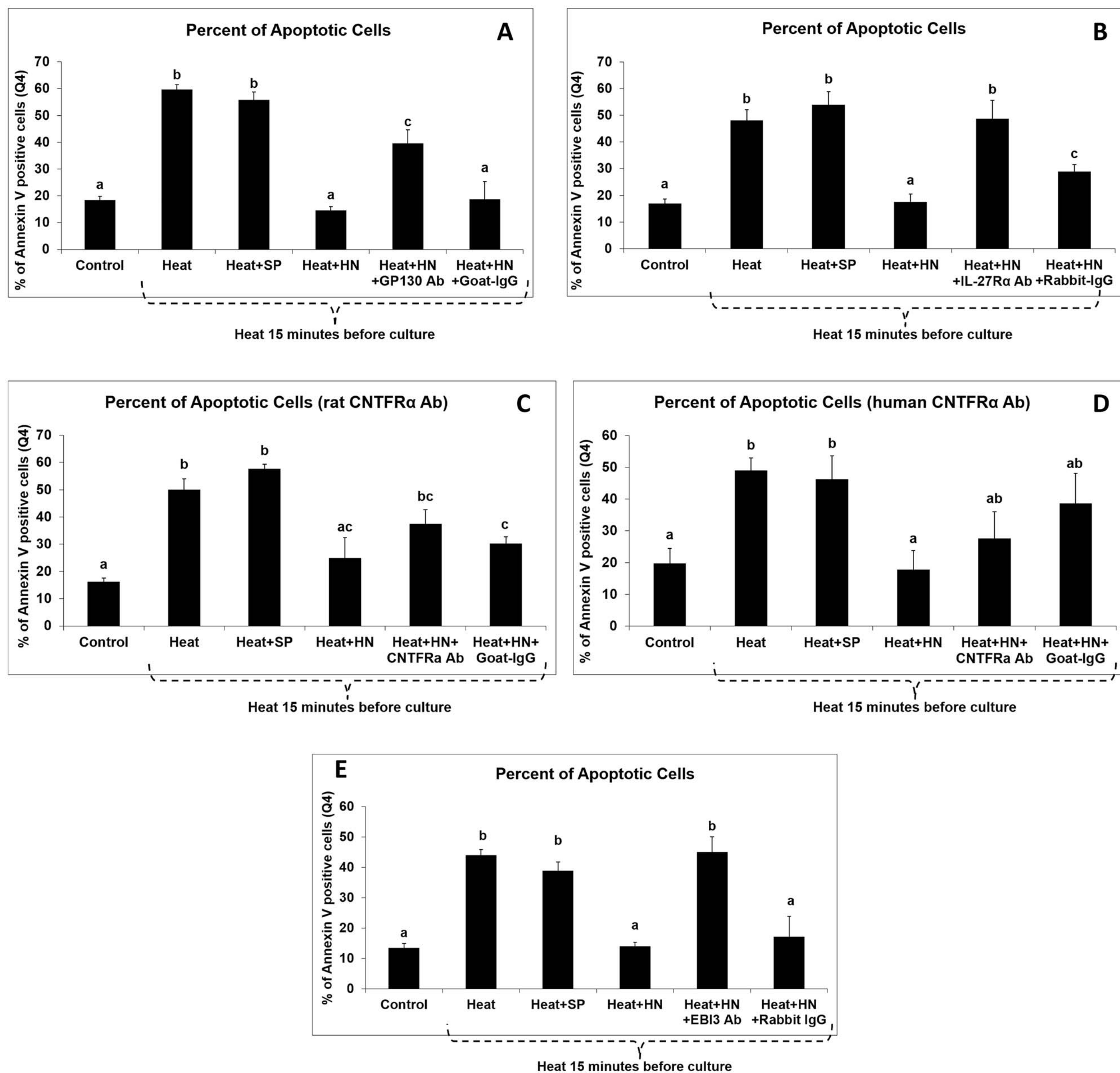


Figure 1. Effect of anti-gp130, anti-IL-27Rα, anti-CNTFRα, and anti-EBI-3 antibodies on the cytoprotective effect of HN against heat-induced germ cell apoptosis. Mouse ST were treated with vehicle (Control), heat (Heat), heat plus SP (Heat + SP), heat plus HN peptide (Heat + HN), heat plus HN peptide plus anti-gp130 (Heat + HN + gp130) (A), or heat plus HN peptide plus anti-IL-27Rα (Heat + HN + IL-27Rα) (B), or heat plus HN peptide plus anti-CNTFRα antibodies (Heat + HN + CNTFRa) (C and D), or heat plus HN peptide plus anti-EBI-3 (Heat + HN + EBI-3) (E) as described in experimental procedures. Apoptotic cell numbers were determined by flow-cytometry with double staining with 7-AAD and Annexin V conjugated APC. Compared with negative control, anti-gp130 (A), anti-IL-27Rα (B), anti-EBI-3 (E), but not anti-CNTFRα (C and D) antibodies, blocked the protective effect of HN on heat-induced germ cell apoptosis. Heat + HN peptide + normal IgG (Heat + HN + IgG) was used as negative control. Values are means ± SEM. Means with unlike superscripts are significantly (P < 0.05) different.

availability of secondary antibodies selection from different species to avoid cross reactivity with endogenous mouse immunoglobulin G (IgG).

Discussion

HN is an endogenous peptide that protects against Alzheimer Disease (AD)-related and non-AD-related neuronal cell death [26–34]. A putative trimeric receptor was demonstrated in the neuronal cell membranes, which mediates the neuroprotective activity of HN. This trimeric receptor belongs to the IL-6 neuro-cytokine receptor family (with three subunits: CNTFRα, IL-27Rα, and gp130) and mediates its action via the STAT3 phosphorylation [44–46, 64]. Glycoprotein 130 (gp130) is a common transmembrane subunit of many cytokine receptors belonging to the IL-6/IL-12 receptor family of which IL-27 is a member. IL-27 has two components—p28 and EBI-3. The IL-27Rα/gp130 heterodimeric receptor com-

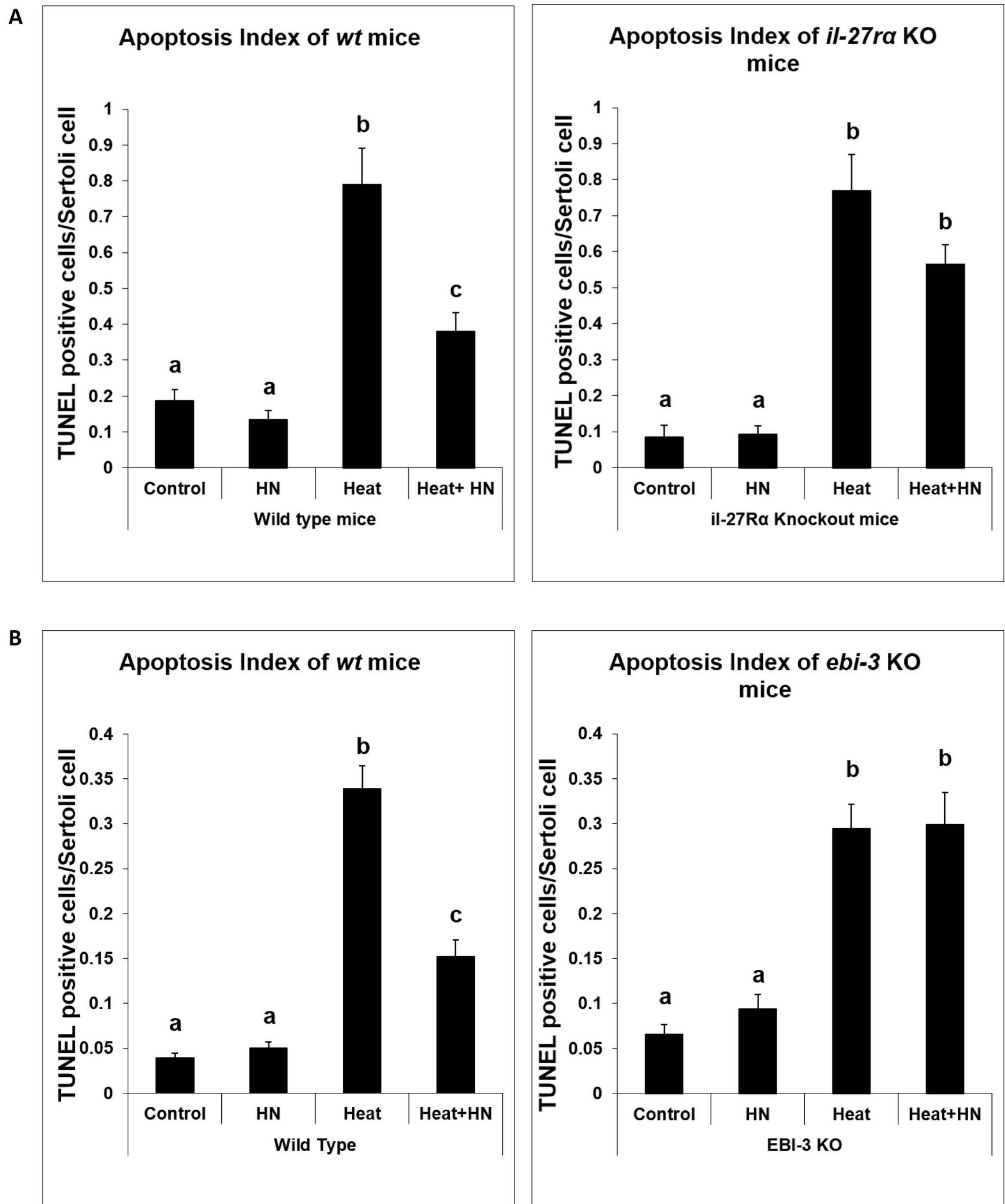


Figure 2. Effect of HN on heat-induced male germ cell apoptosis in wild-type and *il-27rα* or *ebi-3* knockout mice. Both wild-type and *il-27rα* or *ebi-3* knockout mice were treated with vehicle (Control), Humanin peptide (HN), heat (Heat), and heat plus HN peptide (Heat + HN) as described in experimental procedures. Male germ cell apoptosis was detected by TUNEL staining and expressed as the number of TUNEL positive germ cells per Sertoli cell. HN prevented heat-induced male germ cell apoptosis in wild-type mice (A and B, left panel, $P < 0.05$), whereas in *il-27rα* knockout animals, HN did not significantly prevent heat-induced male germ cell apoptosis (A, right panel, $P > 0.05$). In *ebi-3* knockout animal, HN also did not prevent heat-induced male germ cell apoptosis (B, right panel, $P > 0.05$).

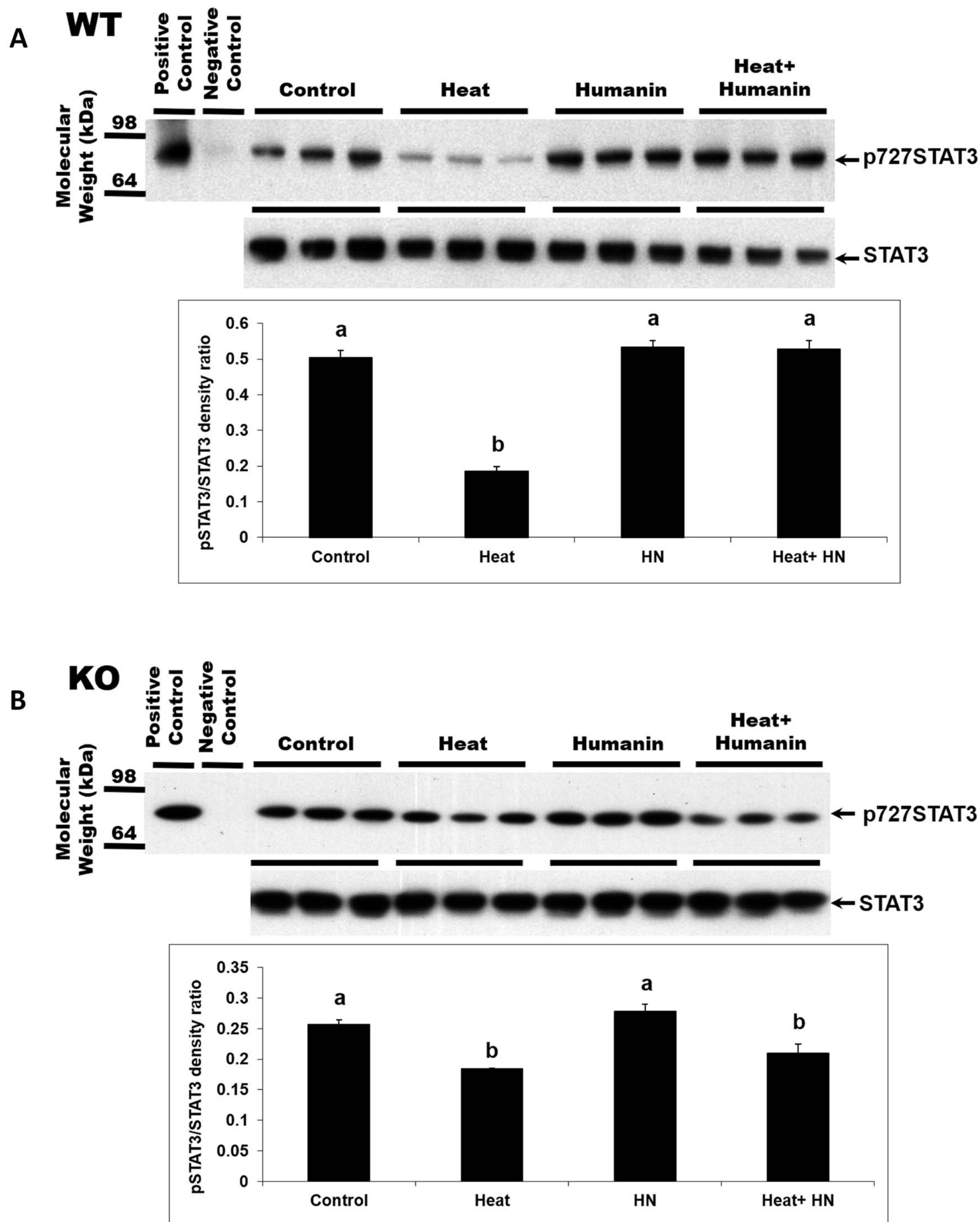


Figure 3. Effect of HN on the heat-suppressed STAT3 phosphorylation in wild-type and *il-27rα* knockout mice. STAT3 phosphorylation of total testicular lysates was detected by western blot. Mice were treated with vehicle (Control), HN peptide (HN), heat (Heat), and heat plus HN peptide (Heat + HN) as described in experimental procedures. (A) In wild-type mice, phosphorylated STAT3 (p727) decreased after heat treatment ($P < 0.05$). HN combined with heat treatment restored STAT3 phosphorylation. (B) In *il-27rα* knockout mice, phosphorylated STAT3 decreased after heat treatment. HN combined with heat treatment did not restore STAT3 phosphorylation to the control or HN treated levels. Values are means ± SEM. Means with unlike superscripts are significantly ($P < 0.05$) different.

plex plays a critical role in cytokine IL-27 signal transduction through STAT1 and 3 mainly [65–68]. CNTF is another component of IL-6/IL-12 heterodimeric cytokine family that activates a receptor comprising of a glycosylphosphatidylinositol-anchored nonsignaling subunit, CNTFRα, and two signaling transmembrane chains—leukemia inhibitory factor receptor b (LIFRb) and gp130 [69–73]. Binding of IL-6/IL-12 family cytokines to their receptors triggers intracellular signal cascades including Janus kinase (JAK)/STAT signaling pathways [71, 74, 75]. HN-induced anti-apoptotic effects in AD *in vitro* and *in vivo* depend on the activation

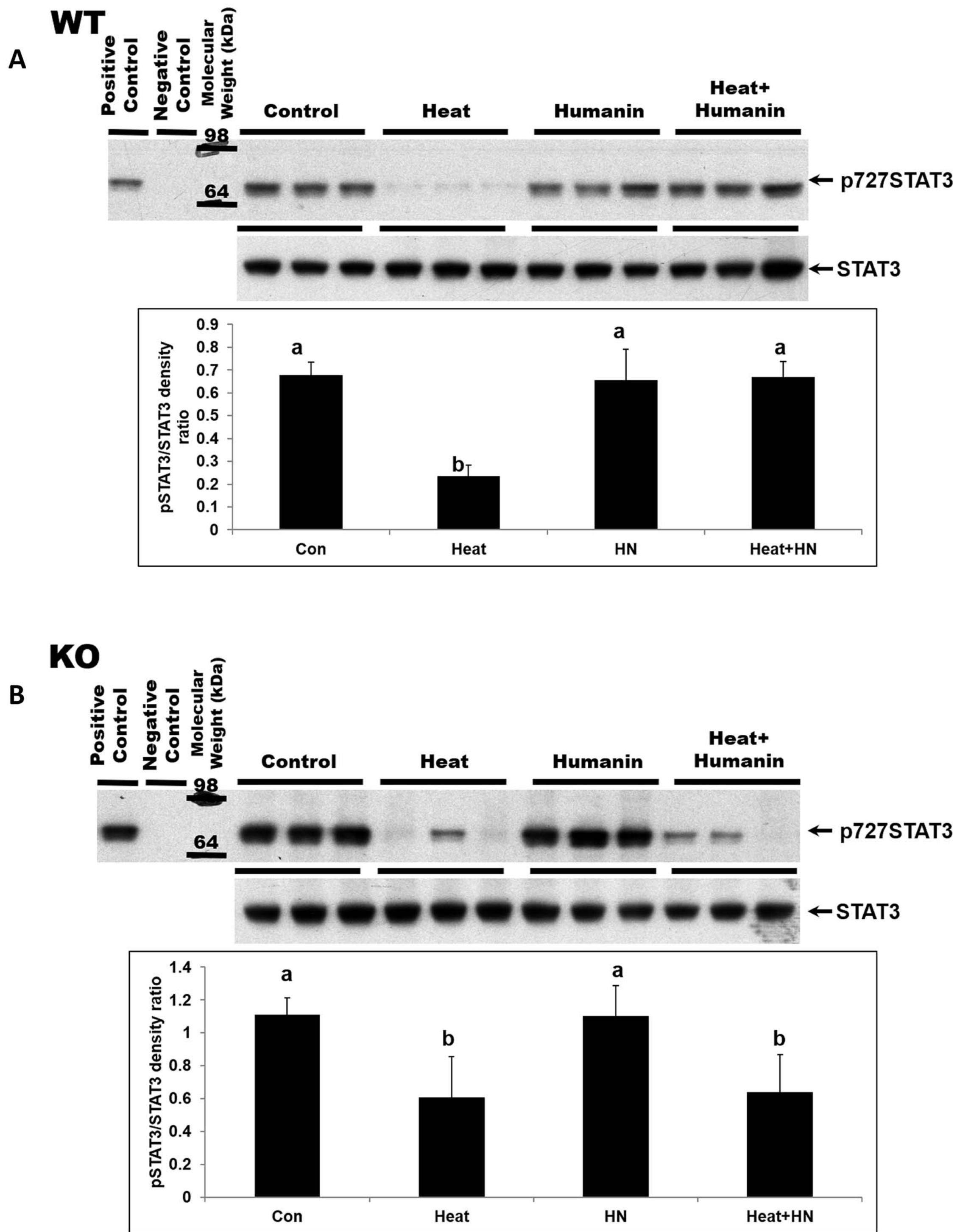


Figure 4. Effect of HN on the heat-suppressed STAT3 phosphorylation (p727) in wild-type and *ebi-3* knockout mice. STAT3 phosphorylation of total testicular lysates was detected by western blot. Mice were treated with vehicle (Control), HN peptide (HN), heat (Heat), and heat plus HN peptide (Heat + HN) as described in experimental procedures. (A) In wild-type mice, phosphorylated STAT3 decreased after heat treatment ($P < 0.05$). HN combined with heat treatment restored STAT3 phosphorylation. (B) In *ebi-3* knockout mice, phosphorylated STAT3 decreased after heat treatment. HN combined with heat treatment did not restore STAT3 phosphorylation to the control or HN treated levels. Values are means ± SEM. Means with unlike superscripts are significantly ($P < 0.05$) different.

of STAT3 [27, 31, 44–46]. Binding of HN to the proposed trimeric receptor has only been shown in neuronal cells. As IL-27Rα and gp130 are both IL-27 receptor subunits, we studied the role of the IL-12 heterodimeric cytokine components EBI-3, p28, p35, and p40 in the cytoprotective action of HN in the testis. HN has also been shown to have other mechanisms of action. Exogenous HN

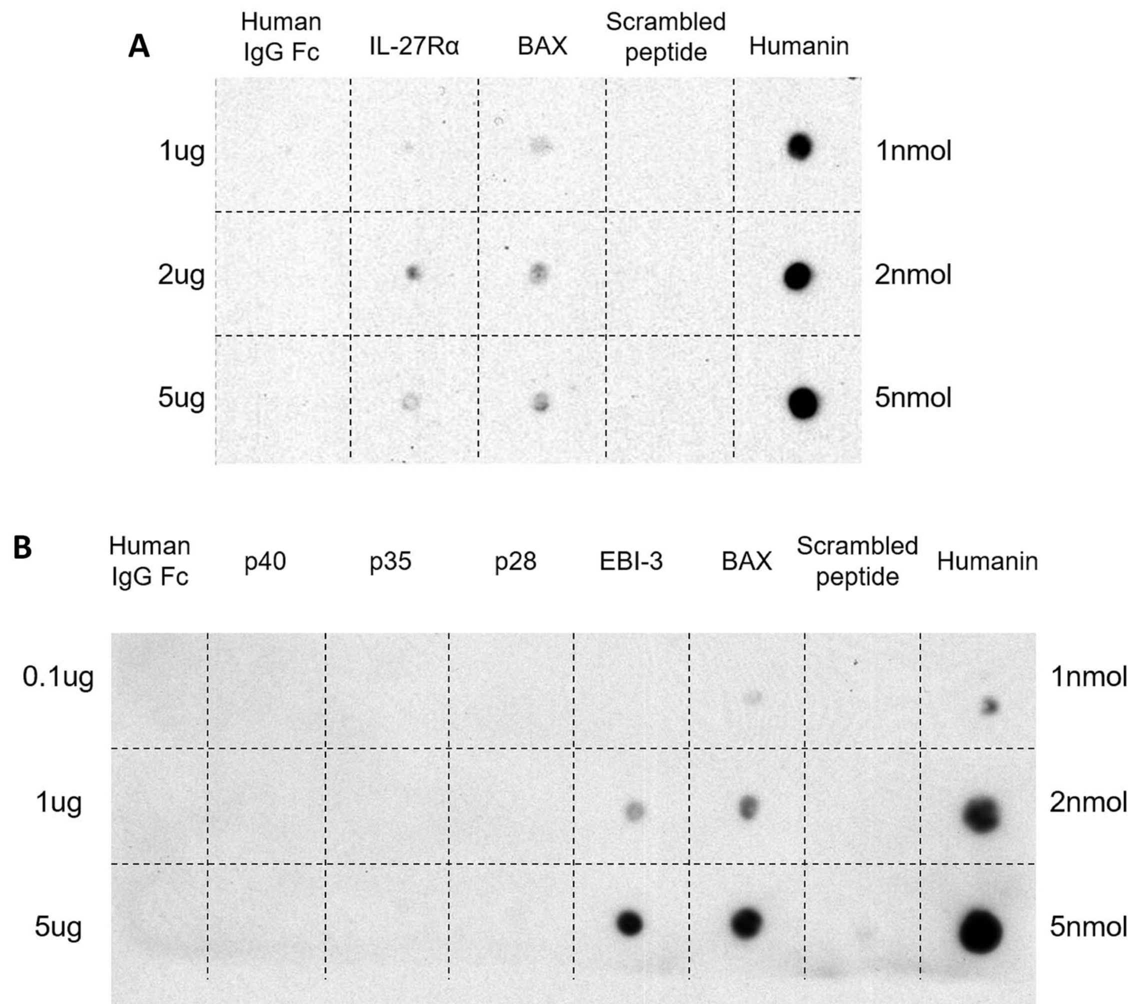


Figure 5. HN and IL-27Rα or EBI-3 interaction detected by dot blot. (A) The peptides used for dot blots included: humanin (HN 1, 2, and 5 nmol), SP (1, 2, and 5 nmol, peptide with the same amino acid as HN but scrambled was used as negative control), BAX (1, 2, and 5 mcg, positive control), IL-27Rα (1, 2, and 5 mcg), and human IgG Fc (1, 2, and 5 mcg, negative control for IL-27Rα). Dot blots confirmed the interaction between HN and IL-27Rα. (B) The peptides used for dot blots included: humanin (HN 1, 2, and 5 nmol), SP (1, 2, and 5 nmol, negative control), BAX (0.1, 1, and 5 mcg, positive control), EBI-3 (0.1, 1, and 5 mcg), p28 (0.1, 1, and 5 mcg), p35 (0.1, 1, and 5 mcg), p40 (0.1, 1, and 5 mcg), and human IgG Fc (0.1, 1, and 5 mcg, negative control for EBI-3). Dot blots confirmed the specific interaction between HN and EBI-3.

can be taken up by cells in culture and localizes in cytoplasmic compartments and mitochondria [76]. HN acts intracellularly by binding to BAX preventing its translocation to the mitochondria to initiate apoptosis [77]. HN also binds intracellularly to IGFBP-3 which may prevent IGFBP-3 action [78]. Additionally, HN has been shown to bind the formyl peptide receptor-like receptor [79].

In this study, we found that HN prevented heat-induced male germ cell apoptosis both *ex vivo* and *in vivo*. HN restored heat-suppressed STAT3 phosphorylation in the testis. This is consistent with our previous finding that HN prevented GnRH-A-induced apoptosis by reversing the GnRH-A-suppressed STAT3 phosphorylation [41]. Our prior data support that the anti-apoptotic effect of HN in male germ cell acts via STAT3 phosphorylation suggesting that HN may interact with the cell membrane cytokine receptor complex CNTFRα/WSX1/gp130 to activate the downstream STAT3 pathway [30, 44–46].

Using *ex vivo* mouse seminiferous tubule culture system, in this study, we showed that neutralizing antibodies against gp130 and IL-27Rα prevented the cytoprotective action of HN in reducing heat-induced male germ cell apoptosis. We used the *il-27rα* knockout mice to further validate the role of IL-27Rα in the cytoprotective effect of HN in heat-induced male germ cell apoptosis. HN prevented heat-induced male germ cell apoptosis via restoring STAT3 phosphorylation in wild-type mouse. In *il-27rα* knockout animals, HN was able to neither protect male germ cells from heat-induced apoptosis nor restore STAT3 phosphorylation. We further confirmed our finding by showing the direct interaction of HN and IL-27Rα in dot blot assay. Immunofluorescent co-staining studies support that HN and IL-27Rα co-localized in germ cells and Leydig cells after heat treatment. We also found that IL-27Rα and gp130 co-localized in germ cells and Leydig cells. However, two neutralizing antibodies against CTNFRα did not significantly change the cytoprotective action of HN against heat-induced germ cell death. We did not further investigate the role of CTNFRα as null mutant mice are not viable [80] and we did not generate a testis-targeted null mutant or a knock-down animal to extend the studies. From our observation,

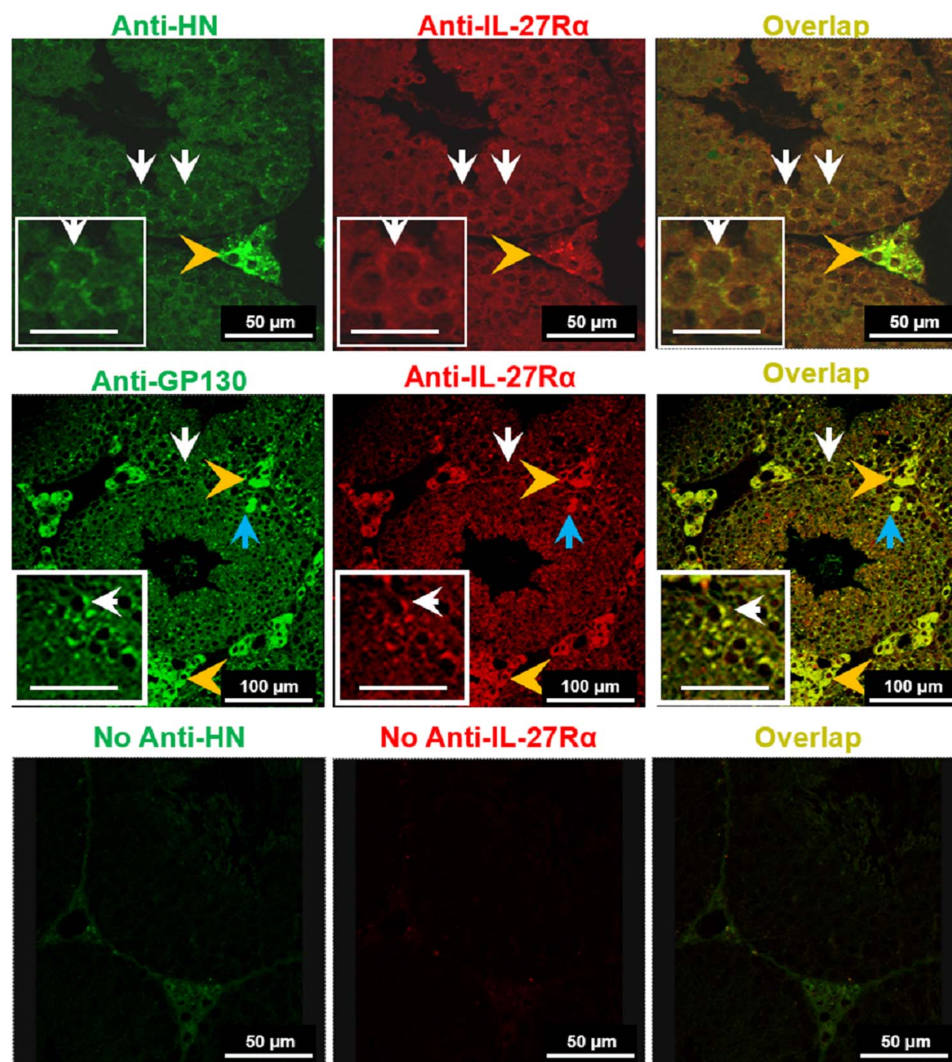


Figure 6. Co-localization of HN and IL-27Rα or IL-27Rα with gp130 in testes. Con-focal images of mouse testis from heat plus HN-treated mice exhibited HN immunoreactivity (green), IL-27Rα (red), and co-localization of HN and IL-27Rα (yellow) in both Leydig cells (Upper panel, Scale bar, 50 μm) and germ cells (Upper panel insets, Scale bar, 25 μm). Middle panel showed con-focal images of testis from heat plus HN-treated mouse exhibited gp130 (green), IL-27Rα (red), and co-localization of gp130 and IL-27Rα (yellow) in both Leydig cells (Middle panel, Scale bar, 100 μm) and germ cells (Middle panel inserts, Scale bar, 50 μm). [Gold arrowheads point to Leydig cells; white arrowheads germ cells; and light blue arrowheads apoptotic germ cells.] Bottom panel showed negative controls with no anti-HN antibody (secondary antibody label green), no anti-IL-27Rα antibody (secondary antibody label red), and co-localization showing no florescent signal in germ cells and weak background signal in Leydig cells (Bottom panel, Scale bar, 50 μm).

HN predominantly interacts with gp130 and IL-27Rα and activates STAT3 to exert its anti-apoptotic action on male germ cells under stress.

In addition to requiring the IL-27 heterodimeric cytokine receptor subunits gp130 and IL-27Rα, our results suggested that IL-27 cytokine component EBI-3 may play an important role in the action of HN. We demonstrated that EBI-3 blocking antibody suppressed the cytoprotective action of HN on male gem cells in *ex vivo* mouse seminiferous tubule cultures. Our subsequent experiment with *ebi-3* knockout mice provided further evidence that without EBI-3 expression, the cytoprotective effect of HN in heat-induced male germ cell apoptosis was compromised. In *ebi-3* knockout animals, HN was unable to protect heat-induced male germ cell apoptosis and restore STAT3 phosphorylation. The direct and specific interaction of HN and EBI-3 was confirmed in dot blot assay, whereas p28, p35, and p40, the other components of IL-12 cytokine family, had no interaction with HN.

Our results in the present study support that EBI-3 is an interacting partner facilitating HN interaction with gp130/IL-27Rα receptor and activating the downstream STAT3 pathway to mediate its cytoprotective action in male germ cells. EBI-3 is one of the component of IL-27, the other one is p28. Membrane bound IL-27Rα and gp130 form the IL-27 receptor [49]. The interaction between EBI-3 and gp130/IL-27Rα had been shown to be essential for IL-27 and its receptor mediated signaling pathway in immune cells [48, 56, 81]. It is known that EBI-3 functions as a soluble cytokine-receptor-like molecule and together with p28 (a cytokine like peptide) forms the heterodimeric cytokine IL-27 that modulates both T and B cells through heterodimer receptor consisting of two subunits, gp130 and IL-27Rα [56, 82]. IL-27 binding with the

gp130/IL-27Rα complex activates the JAK/STAT signaling pathway, which mainly involves STAT1 and STAT3 phosphorylation [50]. IL-27R has immune-enhancing activities such as mediating Th1 polarization [65, 83], inducing IFN-γ production [84], promoting IL-10 production [85], and increasing cytotoxic T lymphocyte generation and proliferation [86, 87]. IL-27 also has anti-inflammatory and immune-regulatory functions including inhibition of Th2, innate lymphoid cell-2 (ILC2), and Th17 responses [88, 89]; induction of type-1 regulatory (Tr1) T cells and the upregulation of PD-L1 [90, 91]; and expression of IL-10 [92–94]. Importantly, HN functionally related molecules, EBI-3, IL-27Rα, and gp130, are key members of IL-27 and IL-27R complex and may also be involved in cytokines IL-6/IL-12 function. Thus, our results suggest that HN may be a cytokine involved in immune regulation in response to stresses.

The mechanism of HN action may include the binding to cell surface receptors activating intracellular signaling pathways and/or directly be taken up into cytoplasmic compartment activating the mitochondria-mediated anti-apoptotic cascades [38]. Our present data showed that EBI-3 (an IL-27 component) and IL-27Rα and gp130 (both IL-27 receptor subunits) are components of HN-receptor complex in male germ cells that mediates the cytoprotective effect of HN, although the role of CNTFRα cannot be completely excluded. Because gp130 is the common subunit in IL-6 receptor family for signal transduction [47], the membrane receptor subunit IL-27Rα and IL-27 component EBI-3 may play more specific roles in HN protective effects in mouse testis. While our data are compelling, we cannot exclude that other subunit(s) may be involved in HN receptor assembling. The difference in HN receptor subunits between neuronal and male germ cell suggests that HN may have tissue-specific receptor subunits in different organs. Taking into consideration our published data and work from other group demonstrating that HN binds IGFBP-3 [78] and BAX [77, 95, 96], we conclude that the mechanism of anti-apoptotic effect of HN on male germ cells is mediated through both membrane receptor and mitochondria-associated pathways. We speculate that tissue-specific HN membrane receptors may be a target of drug development where agonists may protect male germ cells from stress-induced death (e.g. testicular hyperthermia, hormonal deprivation, and chemotherapeutic agents), and antagonists may accelerate germ cell death for male contraception development.

In summary, we have demonstrated that the cytoprotective action of HN on heat-induced male germ cell apoptosis is mediated by interactions with the IL-27 component EBI-3, and predominantly with a receptor complex composed of the membrane bound subunits IL-27Rα and gp130 and subsequent activation of STAT3 signaling pathways. HN, the mitochondrial-derived small anti-apoptotic peptide, is important in the regulation of germ cell homeostasis, balancing germ cell proliferation against germ cell death which may be important in testicular stress and male fertility. Our data also suggest that HN may be a cytokine interacting with the IL-6/IL-12 family proteins to exert different actions in different tissues.

Supplementary material

Supplementary material is available at *BIOLRE* online.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

YJ, RSS, and CW designed research; YJ, YL, PS, and JD-J performed experiment and analyzed data; YJ, YL, RSS, PC, and CW wrote the paper. All authors approved the final version of the manuscript.

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