



Research paper

Nasal delivery of nerve growth factor rescue hypogonadism by up-regulating GnRH and testosterone in aging male mice



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ABSTRACT

Background: Nerve growth factor (NGF) plays essential roles in regulating the development and maintenance of central sympathetic and sensory neurons. However, the effects of NGF on hypogonadism remain unexplored.

Methods: To assess the effects of NGF on hypogonadism, we established a convenient and noninvasive way to deliver NGF to the hypothalamus by spraying liposome-encapsulated NGF into the nasal cavity. The ten-month-old aging male senescence accelerate mouse P8 (SAMP8) mice with age-related hypogonadotropic hypogonadism were used to study the role of NGF in hypogonadism. The age-matched accelerated senescence-resistant mouse R1 (SAMR1) served as a control. The ten-month-old SAMP8 mice were treated with NGF twice per week for 12 weeks. Sexual hormones, sexual behaviors, and fertility were analyzed after NGF treatment. And the mechanisms of NGF in sex hormones sexual function were also studied.

Findings: NGF could enhance the sexual function, improve the quality of the sperm, and restore the fertility of aging male SAMP8 mice with age-related hypogonadism by activating gonadotropin-releasing hormone (GnRH) neuron and regulating secretion of GnRH. And NGF regulated the GnRH release through the PKC/p-ERK1/2/p-CREB signal pathway.

Interpretation: These results suggest that NGF treatment could alleviate various age-related hypogonadism symptoms in male SAMP8 and may be usefulness for age-related hypogonadotropic hypogonadism and its related subfertility.

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1. Introduction

Adequate testosterone is essential for spontaneous erections and sexual desire, sperm differentiation and maturation, and maintenance of reproductive function [1]. However, studies have documented significant decreases in the circulating level of testosterone with advancing age [2, 3]. These decreases may lead to typical age-associated symptoms, including sexual dysfunction, low libido, and the decline in sperm quality, muscle mass, and strength [4, 5]. In clinical settings,

testosterone therapy is used to increase the androgen levels with exogenous testosterone. It can alleviate the detrimental effects of insufficient androgen levels in aging men [6, 7]. However, the most relevant issue with exogenous testosterone is infertility due to suppression of spermatogenesis. This also causes testicular atrophy, which is a major concern for many men. It has become essential to find an alternative therapy for testosterone supplementation to meet the needs of fertility.

The biosynthesis of testosterone is regulated by the HPG axis. Hypothalamic GnRH is the master regulator of the HPG axis. GnRH produced by the hypothalamus stimulates the pituitary gland to synthesize and release luteinizing hormone (LH) and follicle stimulating hormone (FSH), which are required for the testis to produce both testosterone and mature sperm [8]. The integrity of HPG axis is absolutely essential to development, maturation, and maintenance of reproductive systems

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Research in context

Evidence before this study

Nerve growth factor (NGF) the classical neurotrophic factor, regulates the development and maintenance of sympathetic and sensory peripheral neurons and central cholinergic neurons. We have demonstrated that NGF could attenuate the disease progression of senescence accelerate mouse P8 (SAMP8), an age-associated Alzheimer's disease (AD) animal model, by reducing the level of amyloid- β peptide (A β) generation in the brains. Surprisingly, we noticed that NGF treatment dramatically increased serum testosterone concentrations. This finding raises the possibility that NGF treatment may become a potential therapy for age-related hypogonadism.

Added value of this study

Age-related hypogonadotropic hypogonadism is associated with a decline in androgen levels and fertility. Here, we reported an alternative therapy of NGF nasal delivery, which could ameliorate the reproductive function of SAMP8 mice with age-related hypogonadotropic hypogonadism and robustly enhance sexual function, improve the quality of sperm, and restore fertility by stimulating the activity of the hypothalamic GnRH neurons via PKC/p-ERK1/2/p-CREB signaling pathway.

Implications of all the available evidence

Our data suggest that NGF treatment could alleviate various age-related hypogonadism symptoms in male SAMP8 and may be usefulness for age-related hypogonadotropic hypogonadism and its related subfertility.

and maintenance of sexual functions [9]. Alterations of GnRH secretion are proved to be associated with male hypogonadism and infertility [10, 11].

The central role played by GnRH in male reproductive dysfunctions has been supported by the observation that infertility associated with hypogonadotropic hypogonadism can be mitigated by treatment with pulsatile GnRH [12–14]. It has been reported that the amino acid neurotransmitters gamma-aminobutyric acid and glutamate acid [15], anti-Müllerian hormone [16], insulin-like growth factor-I [17, 18], basic fibroblast growth factor [19, 20], transforming growth factor [21], leptin [22], and kisspeptin [23, 24] are capable of affecting GnRH neural functions, and helping to maintain the reproductive dynamic balance. In this way, increasing pulsatile GnRH synthesis and release may be an ideal therapy for the age-related decline in testosterone levels with physiological patterns.

NGF, the well-characterized neurotrophic factor in the neurotrophic protein family, has very important roles in the survival, growth, and maintenance of various neurons in the mammalian nervous system [25, 26]. In our previous studies, we have confirmed that NGF could attenuate the disease progression of SAMP8, an age-associated Alzheimer's disease (AD) animal model, by reducing the level of amyloid- β peptide (A β) generation in the brains [27]. However, the most notable finding of our studies was that NGF treatment not only improved the brain function, but also increased LH and testosterone concentrations to a considerable extent compared to the control counterpart strain, SAMR1 (one strain of accelerated senescence-resistance mouse) at the same age. We suggest that a deficiency in NGF signaling in the brain may play a role in the age-related decline in testosterone and that NGF supplementation may be an approach to

promote testosterone production and restore the fertility of aging male mice by increasing the pulsatile synthesis and release of GnRH.

Here we report that NGF can robustly enhance sexual function, improve sperm quality and restore the fertility of aging male mice by stimulating the activity of hypothalamic GnRH neurons via the TrkA signaling pathway.

2. Materials & methods

2.1. Animals

Adult female BALB/c mice (10 weeks) were purchased from the Experimental Animal Center of Guangdong Province. Aging male SAMP8 and SAMR1 mice (8 months) were obtained from the Third Affiliated Hospital of Tianjin University of Traditional Chinese Medicine. All of the animals were acclimatized at least 7 days under a 12-h light/dark cycle with ad libitum access to food and water in a controlled temperature ($24 \pm 2^\circ\text{C}$) with relative humidity (50%–60%) before the experiments. The SAMP8 and SAMR1 mice were age-matched, housed under phytoestrogen-free conditions, and except where otherwise stated, singly caged during experimentation to prevent them from fighting. All experiments were conducted according to the National Institute of Health guidelines on the care and use of animals and approved by the Institutional Animal Care and Use Committee of Jinan University.

2.2. Reagents

Recombinant mouse nerve growths factor (NGF) (R&D Systems, Abingdon, UK) was dissolved in 0.1 M PBS to create an experimental dosage solution for use in drug administration. Unloaded liposomes were provided by the Biopharmaceutical Research and Development Center of Jinan University (Guangzhou, China). GnRH antagonist cetrorelix acetate (Sigma, St. Louis, MO, US) was dissolved in methanol (Sigma) and K252a (Sigma) was dissolved in DMSO (Sigma). All of the corresponding solvents were used as the control.

2.3. Lip-NGF of nasal administration

NGF-encapsulated with liposomes were performed before nasal administration. In brief, liposomes were produced based on the method [28, 29]. Unloaded liposomes and NGF prepared in PBS were mixed for 30 min in a mass ratio of 2:1 at the temperature of 25–30 °C. In the text, in vivo NGF with intranasal administration refer to the mixture of liposomes and NGF unless otherwise noted. According to a procedure modified from the method of Capsoni [30, 31], the mixture or the solvent was delivered intranasally to mice in a volume of 18 μl with 6 rounds of dropping in alternate nostrils at 2-min intervals.

2.4. Intracerebroventricular (i.c.v.) injections

For one single i.c.v. injection into the third ventricle (bregma, -1.5 mm lateral, -1.8 mm posterior, -2.5 mm deep), mice were placed under anesthesia with 1% pentobarbital sodium. NGF or K252a was dissolved at an experimental concentration in a final volume of 3 μl per mouse and flowed at a rate of 1 $\mu\text{l}/\text{min}$ using a stereotaxic apparatus (RWD Life Science, China). The microsyringe was kept in place for 5 min and then extracted at a rate of 1 mm/min.

2.5. Hormone concentrations of serum

Blood samples were collected via retinal venous plexus and centrifuged at 8000 rpm for 10 min. Serum was collected and stored at -80°C . The concentrations of testosterone in serum and intratesticular testosterone were assayed by ^{125}I -testosterone Coat-A-Count RIA kits (Beijing North Institute of Biological Technology, China). Serum LH and FSH levels were measured using a sensitive sandwich ELISA kit

(Shanghai Institute of Biotech, China) or competitive ELISA kit (CUSABIO, Wuhan, China), respectively, in accordance with the manufacturer's instructions.

2.6. Sexual behavior test

The sexual experience of SAMR1 and SAMP8 male mice was performed with the method at 19:00–22:00 [32]. 2-month-old virgin BALB/c female mice were pretreated with a subcutaneous (s.c.) injection of estradiol benzoate (50 µg) 72 h before testing, followed by s.c. injection of progesterone (500 µg) 4 h before testing to induce regular sexual receptivity. To stabilize their sexual behavior, every male was preplaced in a plexiglas cage (20 cm × 20 cm × 20 cm) for 5 min to acclimatize. Then, a male and a stimulus female were placed into the test cage at random for 20 min, and the sexual interaction between the male and sexually receptive female was recorded by infrared camera. The male reproductive behaviors scored were anogenital sniffing of the female, mounting, intromission and ejaculation. The measures scored were latency of mounting (duration of anogenital sniffing before first mounting), times of mounting in 20 min, latency of ejaculation (calculated by subtracting latency of mounting from duration of anogenital sniffing before first ejaculating), and times of ejaculation in 20 min. If a subject did not perform a behavior during the hour-long test, a maximum latency value of 1200 s was assigned for that behavior.

2.7. Mating with female test

After sexual behavior, the ability of SAMR1 and SAMP8 to cause confirmed pregnancies were determined by matching with adult estrus BALB/c females for 10 d, and then recording the number of breeding females at 14 d to determine whether the male was fertile.

2.8. Immunofluorescence of tissue

Treated mice were killed by cervical dislocation after blood collection, perfused with 0.9% saline flush, and fixed with 4% paraformaldehyde for 48 h. Tissue was placed in O.C.T. media after a sequential sucrose gradient. Contiguous coronal sections taken across the hypothalamus were processed for double-immunofluorescence using a rabbit polyclonal antibody for GnRH and goat polyclonal antibody for TrkA monoclonal antibody, anti-mouse, or anti-rabbit. IgG-HRP secondary antibody, respectively. The immunofluorescence of NGF receptors in cells was measured with primary antibodies of TrkA and 75 kDa pan neurotrophin receptor (p75). Sections of the testis were analyzed with the primary antibodies of SYCP3. Details of the primary antibody were listed in Table S21. Specific fluorescence was visualized by a confocal microscopy (Carl Zeiss LSM-700, Germany).

2.9. Epididymal sperm collection and processing

Sperm masses were obtained from the epididymis cauda using a previously described protocol [33]. Briefly, one epididymis cauda was removed and placed gently at the bottom of a 1.5-ml microcentrifuge tube containing 1 ml Ham's F10 (Life Technologies, Carlsbad, CA, US), then cut off fully and incubated for about 20 min at 35 °C for sperm moving to the medium. Sperm suspension was then loaded in a sperm counting chamber with CASAS (Malang ML-608JZII, China). Images of 40–50 areas of the chamber (about 1000 sperms) were recorded randomly to analyze the number, motility, and viability of sperm in each suspension.

2.10. NGF conjugation with dye Alexa Fluor® 647 and imaging of brain

NGF was labeled with Alexa Fluor® 647 following the specification of a Microscale Protein Labeling Kit (Life Technologies). In brief, we mixed 100 µl NGF (1 µg/µl) with 1 M sodium bicarbonate solution and

added 6.53 µl of Alexa Fluor 647 (7.94 nmol/µl) based on the instructions. We mixed these components thoroughly, then incubated the reaction mixture for 15 min at room temperature. Purified dye-labeled protein was obtained in a collection tube. We pipetted no >50 µl of the conjugate reaction mixture and centrifuged it at 16,000 ×g for 1 min. Then, the conjugates were analyzed spectrophotometrically with a NanoDrop ND-1000 spectrophotometer. The conjugate samples were read at 280 and 650 nm to calculate the concentrations of the protein and degree of labeling. Finally, we intranasally administered the labeled NGF (200 µg/kg) in adult mice. After 1 h circulation, the intact brain tissues were collected and embedded in OCT-embedding medium frozen at –80 °C. Contiguous coronal frozen sectioning across the hypothalamus was made, and imaging was performed with a Leica laser scanning confocal microscope (LSCM: Leica TCS SP8, Germany).

2.11. Cell culture and treatments

Immortalized hypothalamic neurons (GT1-7 cells) were provided gratis by the Shanghai Clinical Center for Endocrine and Metabolic Diseases, Shanghai Jiaotong University School of Medicine (Shanghai, China). GT1-7 cells were cultured in DMEM (Life Technologies) with 4.5 g/l glucose, L-glutamine, and sodium pyruvate supplemented with 10% FBS (Life Technologies) in 5% CO₂/95% O₂ at 37 °C. Cells were seeded into 6-well plates (1 × 10⁶ [6]), allowed to adhere 24 h to crosslinking 80–90%, and media was replaced with serum free media of Opti-MEM (Life Technologies) to deprivation 2 h before NGF treatment. Then, cells were treated with different concentrations of NGF (10, 50, and 100 ng/ml), and conditioned media and cells were obtained to detect the change of GnRH secretion and expression of signal pathway separately. To further examine whether the activation of these signaling molecules is indeed involved in the NGF-induced GnRH secretion, the activation of these molecules was blocked by specific inhibitors. The PKC inhibitor Go6983 (Sigma) and p-ERK1/2 inhibitor PD98059 (Sigma) dissolved in DMSO were used to block PKCα. These compounds were further diluted in DMEM as needed before a final dilution of 1:1000 in media (0.1% DMSO final). In this experiment, cells were treated with NGF (100 ng/ml) and simultaneously coupled with an inhibitor, Go6983 (10 µM) or PD98059 (50 µM), for 2 h or with the vehicle alone. Conditioned medium was collected to measure the GnRH concentration. After 4 h of treatment, cells were collected to analyze the expression of p-ERK1/2 after treatment for 2 h.

2.12. Static culture secretion experiments of GnRH

For the secretion study, GT1-7 cells were seeded into 6-well plates and allowed to adhere overnight. Media was changed to Opti-MEM. The cells were allowed to sit for 2 h and then treated with either the vehicle or (10, 50, or 100 ng/ml) NGF. The supernatant was then transferred to a clean 4-ml round-bottom microcentrifuge tube and frozen at –80 °C. Samples were lyophilized using a speedvac, and dried samples were resuspended with 0.1 M PBS (pH 7.4) by vortexing and pipetting on a rocking shaker at 4 °C overnight. The GnRH content could be assayed using an enzyme-linked immunosorbent assay (ELISA, CUSABIO) according to the manufacturer's instructions.

2.13. RNA isolation and real-time PCR analysis

For gene expression analyses, mRNA was obtained from the hypothalamus tissue of adult mice and GT1-7 cells using RNeasy Plus Mini Kits (Qiagen, Chatsworth, CA, US). We used 1000 ng total mRNA as the template for cDNA synthesis primed with random hexamers (Bio-Rad, Richmond, CA, US). Then, the reaction mixture was diluted at 1:2, and 2 µl of the diluted template was used per 20 µl of the real-time quantitative PCR assay using the Bio-Rad SsoAdvanced™ Universal SYBR (Bio-Rad 172–5274, Mississauga, ON, Canada). The Bio-Rad CFX Connect Real-Time system and the Bio-Rad CFX Manager Software

(version 2.0) were used to collect the PCR data. Results are presented as linearized values normalized against β -actin. The primers (Table S2) were synthesized by the Beijing Genomic Institute (BGI).

2.14. Western blot analysis

All of the protein samples from the cells and testes were normalized for protein concentration and placed on a 10% SDS-PAGE gel. For immunoblotting analysis, proteins in the SDS gels were transferred to a 0.22- μ M polyvinylidene difluoride (PVDF) membrane by an electroblot apparatus. Membranes were incubated with primary antibodies (Table S1) at 4 °C overnight. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, the membranes were detected with Immobilon™ Western chemiluminescent HRP substrate (Millipore Corporation, Bedford, MA, US). Protein expression was normalized to GAPDH.

2.15. Luciferase assays

For transient transfections, approximately 24 h before transfection, GT1-7 cells were seeded into 24-well plates at a density of 150,000 cells per well. Cells were transfected with plasmid DNA using Attractene Transfection Reagent (Promega, WI, US) according to the manufacturer's protocol with a 3:1 ratio of Fugene to DNA. Approximately 24 h after transfection of pGL-basic-*Gnrh1* and pcDNA3.1-*Creb1* or pcDNA3.1, GT1-7 cells were switched to Opti-MEM containing NGF (100 ng/ml) for 12 h, or at the same time coupled with p-CREB inhibitor 666–15 (50 μ M, R&D system) for 12 h. Luciferase values were measured with a Dual-Luciferase® Reporter Assay System (Promega) and measured with 1420 Multilabel counter (PerkinElmer, USA), internally normalized using a pRL-tk reporter for transfection efficiency. Values shown are the change of luciferase values relative to the control (only transfection with pGL-basic-*Gnrh1* and pcDNA3.1).

2.16. Statistical analysis

All of the data were analyzed using Prism 5 (GraphPad Software) and they are expressed as mean \pm SEM. Data were compared by a two-tailed unpaired Student's *t*-test or one-way analysis of variance for multiple comparisons. Differences were considered significant at $P < .05$ in all of the cases. The number of biologically independent experiments and the ages and sexes of the animals are indicated in the preamble.

3. Results

3.1. NGF promoted sexual hormone secretion and enhanced sexual motivation and performance of aging male SAMP8 mice

Male aging is associated with hypogonadism, which is characterized by low serum testosterone and three sexual symptoms (erectile dysfunction, loss of libido, and reduced frequency morning erections) [34–36]. In our study, we found that 10-month-old SAMP8 mice began to display significantly lower levels of both the circulating LH and testosterone than the age-matched controls, SAMR1 mice (Fig. 1b–c). We also analyzed the behavioral changes in sexual motivation and performance that are known to be directly related to lower serum testosterone concentration. As a result, we observed that 10-month-old aging male SAMP8 mice had weakened sexual motivation and performance with significantly reduced mounting times, ejaculation times, and pregnancy rate; and greatly increased latency period post ejaculation compared with the SAMR1 controls (Fig. 1d–g). These ten-month-old aging male SAMP8 mice also showed a significant decline in *Gnrh1* and *Ngf* mRNA in the hypothalamus (Fig. 1h–i). These results indicated that 10-month-old aging male SAMP8 mice had low GnRH, LH, testosterone levels and reduced sexual behavior similar to the syndrome of hypogonadotropic hypogonadism. Therefore, ten-

month-old aging male SAMP8 mice were used to study the role of NGF in hypogonadism.

We first assessed the changes in male sexual hormone and behaviors after the intranasal administration of NGF. Administration of NGF (12.5, 25, 50, or 100 μ g/kg) twice per week for 12 weeks in SAMP8 mice (ten months) resulted in a significant increase in serum LH, FSH, serum testosterone and the intratesticular testosterone concentrations (Fig. 2a–d). Accompanied by the elevating in gonadal hormones, the sexual behaviors, including ejaculation latency, mounting times, and ejaculation times were all restored to the levels of the age-matched control (SAMR1) animal (Fig. 2e–g). These results indicated that intranasal administration of NGF could normalize the sex hormones and sexual behaviors.

3.2. NGF rescued aging male SAMP8 fertility and restored impaired spermatogenesis

In addition to sex behaviors, aging is also associated with the decline in sperm quality and fertility. Mating of SAMP8 and SAMR1 mice with estrous female mice confirmed that SAMP8 mice with hypogonadotropic hypogonadism had significantly lower fertility than age-matched SAMR1 control (Fig. 1g). But the administration of NGF (25 μ g/kg and above) for 12 weeks produced a significant increase in pregnancy rates and the weight of seminal vesicle over corresponding levels for the age-matched SAMR1 controls (Fig. 3a–b). The restoration of sperm quality and number was also confirmed by functional assays of the cauda epididymal sperms, and by morphological analysis of epididymal sections. The seminal vesicle weight, sperm vitality, sperm survival rate of the cauda epididymal (Fig. 3c–d) and the number of sperms in the epididymis (Fig. 3e) significantly increased in SAMP8 mice treated with NGF. Testicular atrophy and reduced germ cell numbers of SAMP8 mice with hypogonadotropic hypogonadism were largely rescued by NGF (Fig. 4a). Detailed analysis of the markers of undifferentiated spermatogonia (*GFR α 1* and *Stra8*) and differentiating spermatogonia (*SYCP3*) suggested that NGF might specifically stimulate the differentiation of spermatogonia cells (Fig. 4b–f).

Overall, these results suggested that NGF intranasal administration was a highly effective method that could not only restore serum sex hormones but also rescue fertility and alleviate impaired spermatogenesis in male SAMP8 mice with hypogonadotropic hypogonadism. However, the underlying mechanism by which nasally delivered NGF can increase levels of sexual hormone in male mice still needs to be investigated further.

3.3. NGF stimulated LH secretion by acting on the GnRH neuron of the hypothalamus of SAMP8 mice with hypogonadotropic hypogonadism

It has been reported that NGF could transport to the cholinergic basal forebrain following nasal administration [37]. In our study, to examine whether NGF could be delivered into hypothalamus via the intranasal administration, NGF labeled by a red tracing dye Alexa Fluor® 647. Then the SAMP8 mice treated with the labeled NGF via the intranasal administration. We found that NGF could distribute in several brain regions, including the cortex (Fig. 5b–c) and hypothalamus where GnRH neurons located (Fig. 5d–e). The result demonstrated that NGF could be delivered into the brain by intranasal administration.

To rule out possibility that NGF regulated LH and testosterone secretion with systemic blood circulation, we treated SAMP8 mice with a single use of NGF via peripheral intravenous injection (i.v.) and central intracerebroventricular injection (i.c.v.). The results indicated that i.c.v. injection of NGF elevated the levels of serum LH, FSH and testosterone significantly (Fig. 6a–c), but the i.v. injection did not trigger any changes in the HPG axis even at the doses of NGF as high as 300 μ g/kg (Fig. 6d–e). These results indicated that exogenously NGF regulation of LH and testosterone secretion on the hypothalamus or pituitary. To identify which one is the target of NGF, we administered a GnRH antagonist (cetorelix

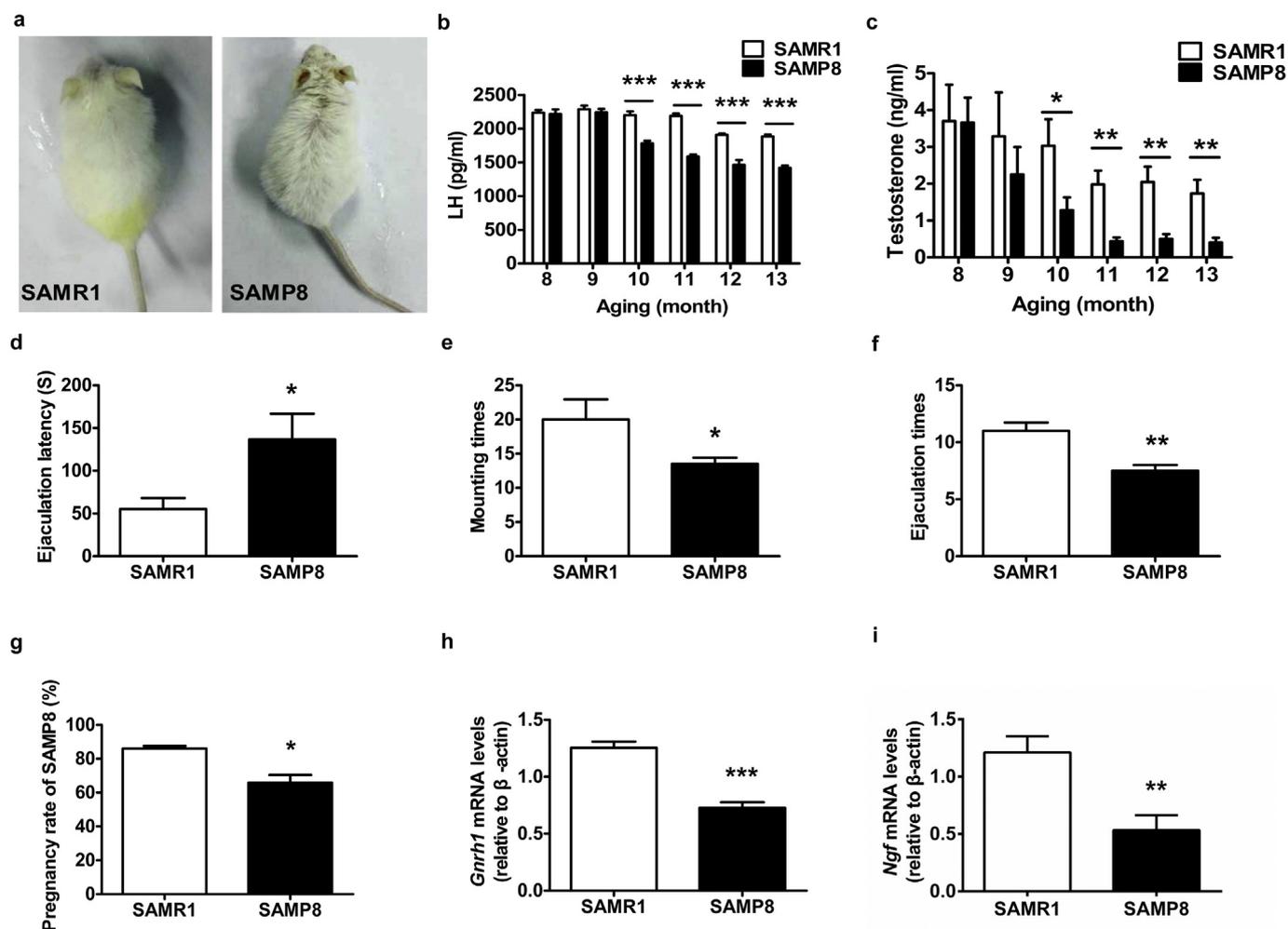


Fig. 1. LH and testosterone deficiency cause loss of sexual preference and fertility impairment in aging male SAMP8 mice (10 months). (a) Appearance of male SAMR1 and SAMP8 mice at 10 months of age. (b) Serum LH level of male SAMR1 and SAMP8 mice at different ages ($n = 8$). (c) Serum testosterone levels of male SAMR1 and SAMP8 mice at different ages ($n = 8$). (d–f) Measures of sexual behavior of SAMR1 and SAMP8 mice at 10 months of age in 20-min mating tests with sexually receptive females ($n = 8$). (d) Ejaculation latency, and (e) mounting times, and (f) ejaculation times. (g) The pregnancy rate of SAMR1 and SAMP8 mice at 10 months were determined by matching with estrus BALB/c females for 10 d ($n = 8$). (h, i) Changes in *Gnrh1* and *Ngf* mRNA in male SAMR1 and SAMP8 mice at 10 months of age ($n = 6$). Values are here expressed as means \pm SEM. The values were analyzed using unpaired Student's *t*-test. *** $P < .001$, ** $P < .01$, * $P < .05$ relative to SAMR1 mice.

acetate; 0.5 mg/kg) intraperitoneal injection 30 min before NGF i.c.v. administration. The data suggested that the effects of NGF stimulating LH and testosterone secretion were completely blocked by cetrorelix treatment (Fig. 7a–b). This result verified the target activated by NGF was hypothalamus not pituitary in the HPG axis.

To verify whether NGF could act on GnRH neurons in the hypothalamus, preoptic area (POA) and medial basal hypothalamus (MBH) explants of hypothalamus were separated from ten-month old aging male SAMP8 mice. The explants were treated with NGF for 6 h before the using of enzyme-linked immunosorbent assay (ELISA) to measure the amount of GnRH release. The result showed that NGF significantly increased GnRH release when compared with vehicle-treated explants (Fig. 7c–d). Additionally, we analyzed the expression of *Gnrh1* in the hypothalamus. The result showed that the expression of *Gnrh1* was upregulated significantly at the time of 6 h after NGF administration, which returned to baseline by 12 h (Fig. 7e). These data suggested that NGF promoted LH and testosterone secretion by activating GnRH neurons and increasing GnRH release.

As we know, NGF mediates its biological activity through the TrkA or p75 receptors signal. To determine which receptor is target of NGF, double-immunofluorescence experiments were performed on the hypothalamic section. We found GnRH (Green) and TrkA (Red) to be co-expressed in the hypothalamic GnRH neurons (Fig. 7h–k), but we did

not detect any co-expression of GnRH and p75 receptor (Fig. 7l–o). We then determined whether TrkA blockage could affect GnRH neuronal activation by the administration of TrkA inhibitor (K252a) before NGF administration. As expected, the results showed the effect of NGF on LH and testosterone secretions could be neutralized by K252a (Fig. 7f–g), indicating that the effects of NGF on HPG axis were through acting on its classic Trk receptor signaling.

3.4. Effects of NGF on GnRH involved the PKC/p-ERK1/2/p-CREB signaling pathway

To identify the signaling pathway by which NGF regulates GnRH secretion, we utilized the GT1-7 cell, an adult GnRH neuronal cell line that expresses the NGF receptor (TrkA) and is capable of producing GnRH (Fig. S1). NGF, at 50 ng/ml, rapidly upregulated *Gnrh1* mRNA expression by 30 min after treatment (Fig. 8a), which resulted in a significant increase in GnRH release by 4 h (Fig. 8b). These findings supported the observations that NGF increased GnRH secretion in vivo by acting on GnRH neurons via activating TrkA signaling.

We then determined whether PKC and MAPK pathways were involved in the induction of GnRH production by NGF [38]. With NGF treatments, the expression of PKC, p-PKC, p-ERK1/2, and p-CREB Ser-133 markedly increased by 2 h (Fig. 8c, Fig. S2a–d). And the expression

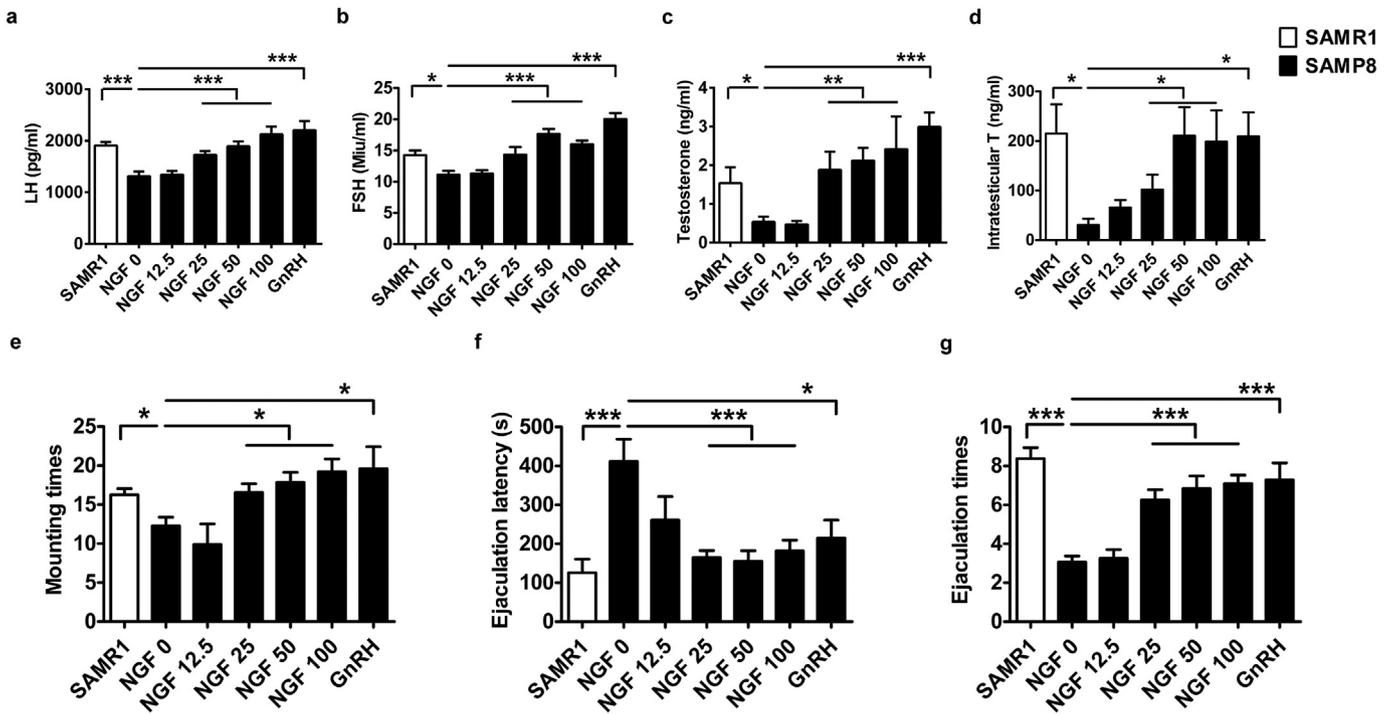


Fig. 2. Intranasal administration of NGF promoted sexual hormone secretion, enhanced sexual motivation and performance of aging male SAMP8 mice (10 months). (a, b) Serum gonadotropin levels of male SAMR1 and SAMP8 mice after NGF or GnRH treatment for 12 weeks ($n = 8$). (c) Serum testosterone levels of male SAMR1 and SAMP8 mice after NGF or GnRH treatment for 12 weeks ($n = 8$). (d) Intratesticular testosterone levels of male SAMR1 (10 months) and SAMP8 (10 months) mice after NGF or GnRH treatment for 12 weeks ($n = 8$). (e–g) Measures of sexual behavior of SAMR1 and SAMP8 (10 months) mice in 20-min long mating tests with sexually receptive female after NGF or GnRH treatment for 12 weeks ($n = 8$). (e) Mounting times, (f) mean ejaculation latency, and (g) ejaculation times. Values are expressed as means \pm SEM. The data were analyzed using one-way ANOVA analysis and the sexual behavior and mating tests were replicated at three times. *** $P < .001$, ** $P < .01$, * $P < .05$ compared to the SAMP8 mice.

PKC isoforms, such as conventional/classical PKCs (α , β and γ), novel PKCs (PKC δ) were also analyzed. Results showed NGF upregulated the expression of PKC α , and PKC β and had no influence in PKC γ and PKC δ expression (Fig. S2e–i). To further determine whether these signaling molecules were indeed involved in the NGF-induced GnRH secretion, we blocked their activation using specific inhibitors. Blocking PKC

with the PKC inhibitor Go6983 significantly attenuated GnRH secretion (Fig. 8d) and downregulation of PKC, p-ERK1/2, and p-CREB expression (Fig. S2j–k). Blocking ERK1/2 phosphorylation with an ERK inhibitor (PD98059) also inhibited NGF-induced GnRH release (Fig. 8e). Therefore, we concluded that NGF binding TrkA regulated the secretion of GnRH involving PKC/p-ERK1/2/p-CREB signaling pathway.

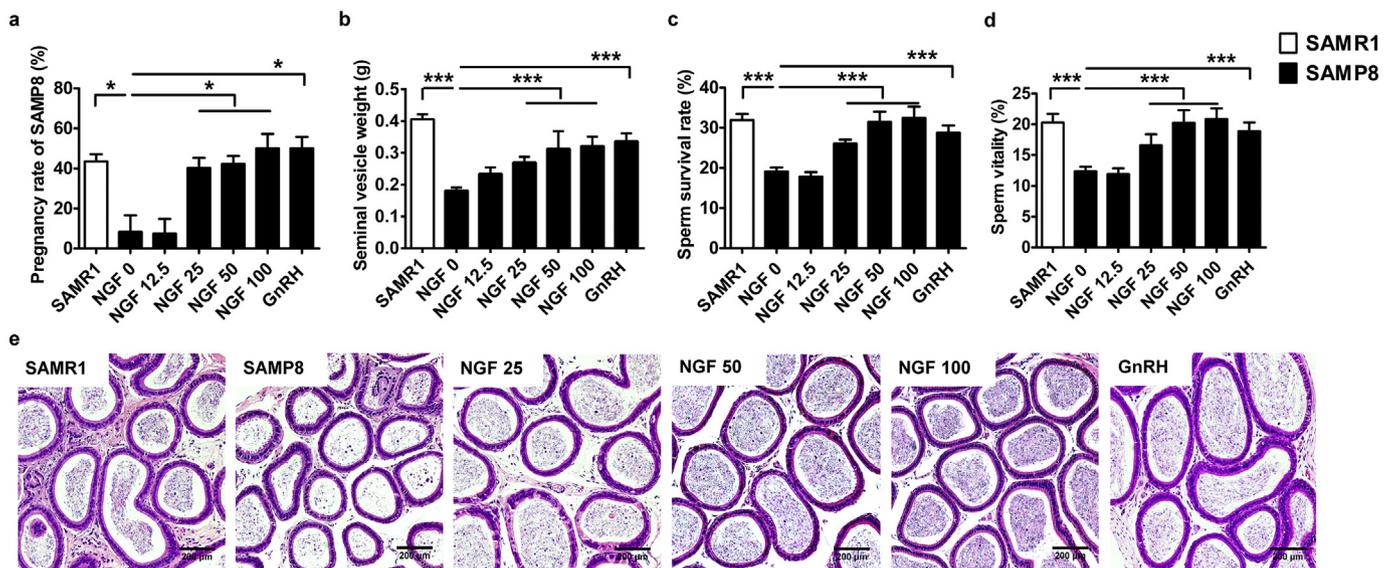


Fig. 3. Intranasal administration of NGF rescues fertility in aging SAMP8 mice (10 months). (a) Pregnancy rate of SAMR1 (10 months) and SAMP8 (10 months) mice after NGF therapy or GnRH treatment for 12 weeks ($n = 8$). (b) The seminal vesicle weight of SAMR1 (10 months) and SAMP8 (10 months) mice after treatment ($n = 8$). (c, d) Survival and vitality of sperms in the cauda epididymidis in treated mice ($n = 8$). The data were analyzed for more sperm counts over 1000 with CASAS. (e) Representative electron micrographs of sperms in epididymis of SAMR1 (10 months) and SAMP8 (10 months) mice after administration ($n = 8$). Values are expressed here as means \pm SEM. The data were analyzed using one-way ANOVA analysis. *** $P < .001$, ** $P < .01$, * $P < .05$ compared to the SAMP8 mice.

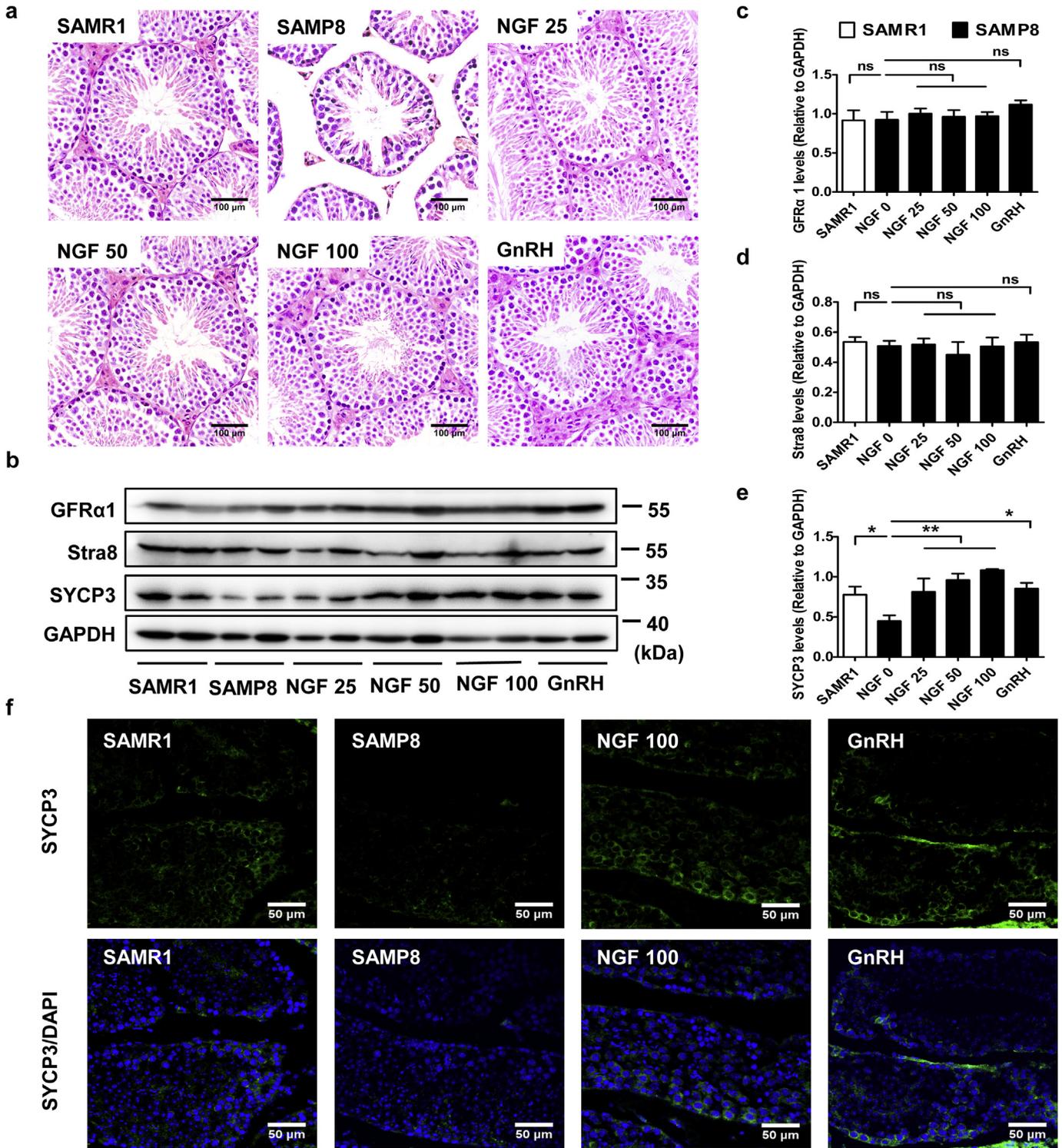


Fig. 4. Intranasal administration of NGF promoted the differentiation of spermatogonia in aging SAMP8 mice (10 months). (a) Representative histopathology of testes in SAMP8 mice after NGF therapy or GnRH treatment for 12 weeks. (b) Representative photographs of GFRα1, Stra8 and SYCP3 expression in the testis after administration (n = 8). (c-e) Grayscale value of Western blots of GFRα1, Stra8 and SYCP3. (f) The meiotic spermatocytes in testis were assessed by immunofluorescence staining with anti-SYCP3 (green) in the treated mice. Nucleus were dyed with DAPI staining (blue), scale bar = 50 μm. Values (c-e) are expressed as means ± SEM. The Western blots were performed in triplicate. The data were analyzed using one-way ANOVA analysis. ***P < .001, **P < .01, *P < .05 and compared to the values for SAMP8 mice.

Unlike PKC and ERK1/2, CREB is a stimulus-induced transcription activator, which could act on transcription directly. We found there were putative CREB binding sites within the *Gnrh1* promoter region by the Genomatix database. We examined the direct effect of *Creb1* on *Gnrh1* promoter activity by transfecting GT1-7 cells with a -2 Kb GnRH promoter-driven luciferase reporter (pGL-basic-*Gnrh1*) plasmid.

Treatment with NGF or transfection with *Creb1* significantly increased the activity of the *Gnrh1* promoter. Treating the cells with *Creb1* inhibitor 666-15 could attenuate the effect of NGF. These data indicated CREB played an important role in NGF-inducing *Gnrh1* gene transcription (Fig. 8f-g).

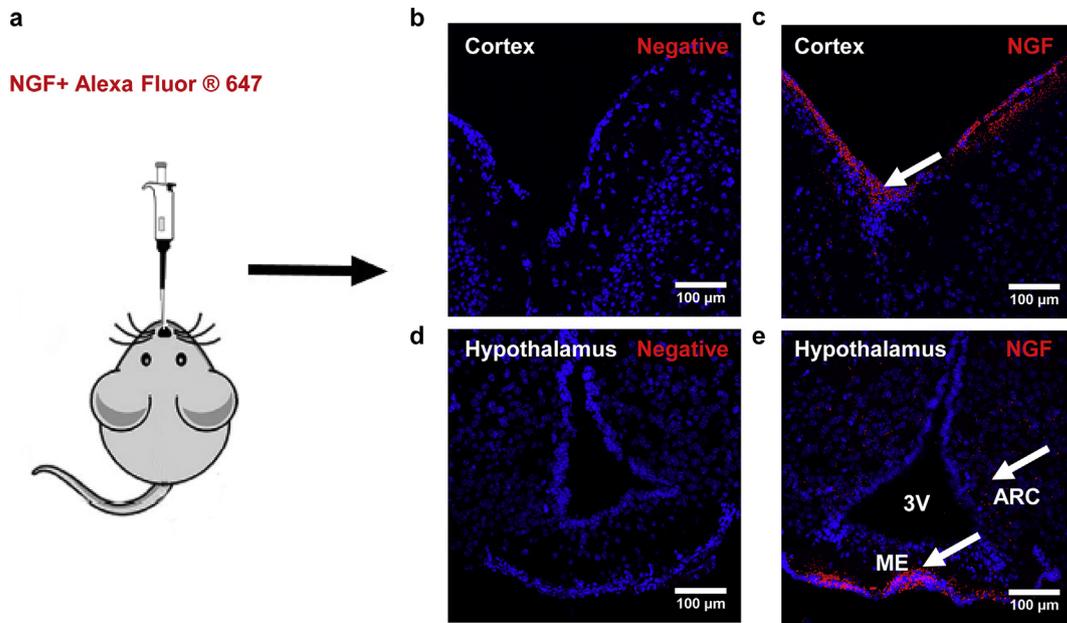


Fig. 5. Distribution of NGF after a single intranasal treatment in SAMP8 mice (10 months). (a) Schematic representation of NGF combined with Alexa Fluor® 647 (Red) nasal administration. Distribution of NGF combined with Alexa Fluor® 647 (Red) 1 h after a single intranasal treatment in cortex (b, c) and hypothalamus (d, e). ARC, arcuate nucleus. ME, hypothalamic median eminence; 3 V, third ventricle. The negative refer to the group given 0 $\mu\text{g}/\text{kg}$ NGF.

4. Discussion

Currently, testosterone therapy is one choice of hypogonadism treatment with recommendations and guidelines set by the American Association of Clinical Endocrinologists and the Endocrine Society. Due to the feedback inhibitory mechanism of HPG axis, exogenous testosterone therapy may inhibit intratesticular testosterone production and

further repress spermatogenesis. In this way, GnRH pulsatile release therapy may be safer and more effective than testosterone therapy. Here, we report an alternative approach to NGF intranasal administration, which could promote testosterone production and restore the fertility of aging male mice by activating the GnRH neuron and increasing the GnRH release.

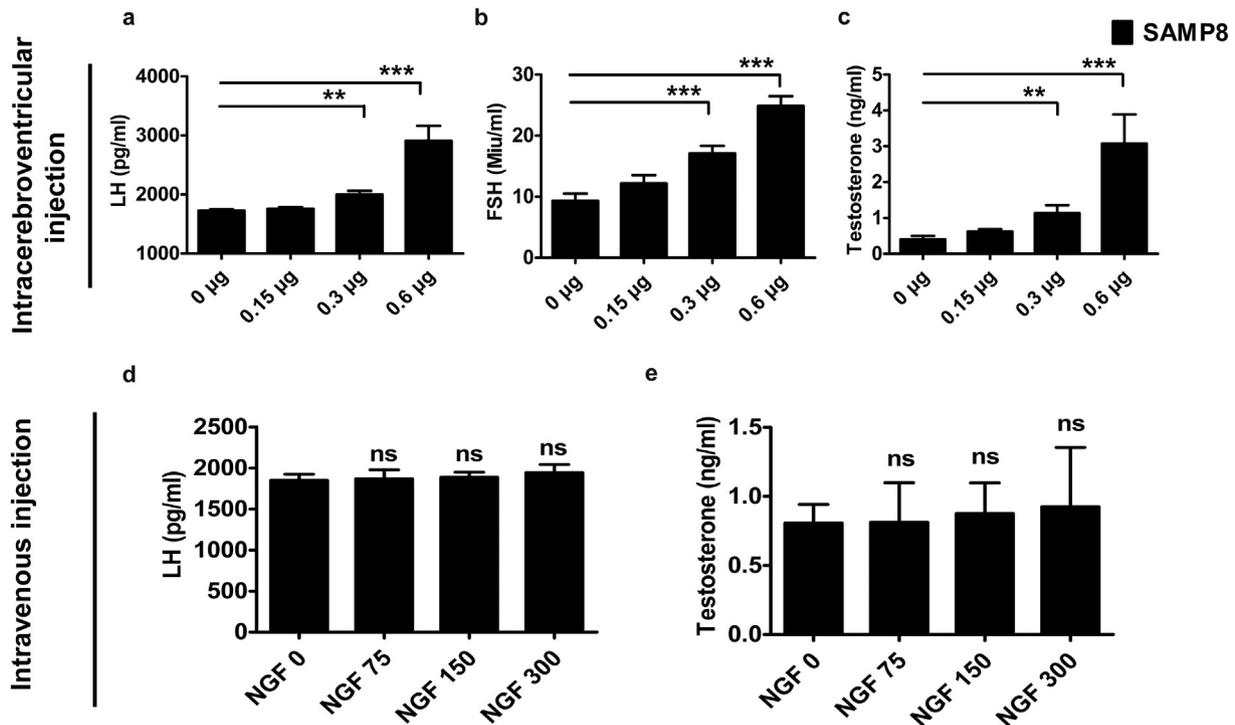


Fig. 6. Different effects of a single i.c.v. and i.v. injection of NGF on serum sex hormone in SAMP8 mice (10 months). (a, b, c) The sera were collected and serum LH, FSH and testosterone were measured 24 h after a single i.c.v. injection with increasing dose of NGF or its vehicle (0 μg) (n = 8). (d, e) The serum LH and testosterone levels were measured at 24 h after a single i.v. injection of NGF (n = 8). Values are expressed as means \pm SEM. The data were analyzed using one-way ANOVA analysis. *** $P < .001$, ** $P < .01$, * $P < .05$ compared to the vehicle (0 μg) and performed in triplicate.

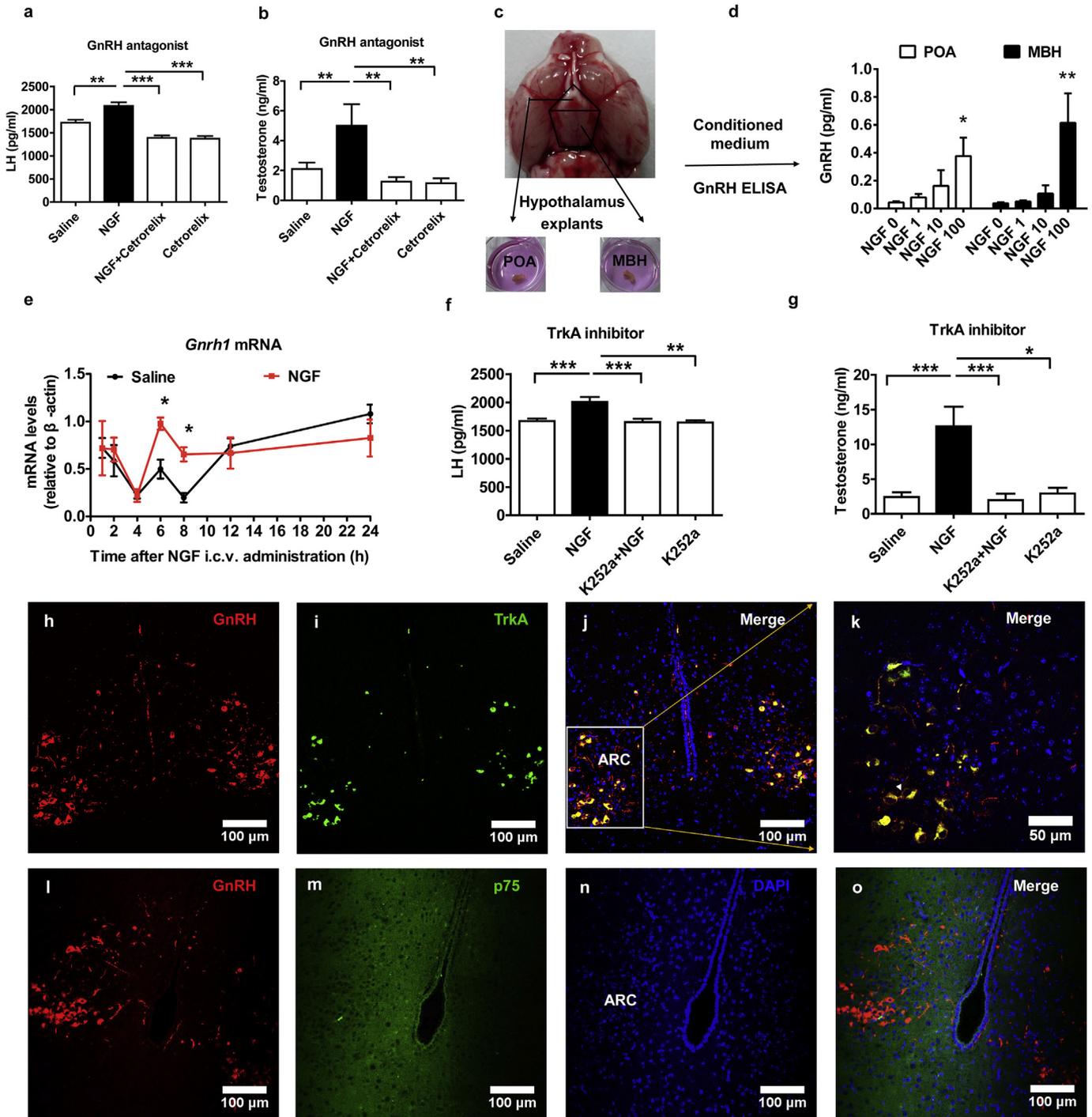


Fig. 7. NGF acts on the GnRH neuron of the hypothalamus in SAMP8 mice (10 months). (a, b) Effects of NGF (100 µg/kg) or solvent, coupled with pretreatment with a GnRH antagonist cetrorelix, or alone (500 µg/kg, delivered, s.c, n = 8) on serum LH and testosterone concentration at 24 h after NGF injection. (c) Schematics illustrating POA and MBH dissection of explant preparation. POA, preoptic area. MBH, medial basal hypothalamus. (d) Quantification of GnRH secretion from POA and MBH explants in hypothalamus stimulated with NGF (1, 10, or 100 ng/ml) (n = 6). (e) The changes in *Gnrh1* mRNA expression of hypothalamus after NGF treatment (100 µg/kg) or solvent, coupled with pretreatment with a TrkA inhibitor K252a or alone (100 µg/kg, delivered, i.c.v., n = 8) on serum LH and testosterone concentration at 24 h after NGF injection. (f, g) The effect of NGF treatment (100 µg/kg) or solvent, coupled with pretreatment with a TrkA inhibitor K252a or alone (100 µg/kg, delivered, i.c.v., n = 8) on serum LH and testosterone concentration at 24 h after NGF injection. (h-o) Confocal representative photomicrographs showing co-localization of GnRH (Red) and TrkA (Green) (h-k) or p75 (Green) (l-o) in coronal hypothalamic sections in SAMP8 mice. Values are expressed as means ± SEM. Three independent replicates were performed. The data were analyzed using one-way ANOVA analysis. ****P* < .001, ***P* < .01, **P* < .05.

NGF, the classical neurotrophic factor, regulates the development and maintenance of sympathetic and sensory peripheral neurons and central cholinergic neurons. It has been shown to play important roles in neuronal development, plasticity, and therapies for neurodegenerative diseases, such as AD [39, 40]. There is accumulating evidences that NGF may not only affect the nervous system but also regulate the development of cells in the cardiovascular, immune, and endocrine

systems. However, the relationship between NGF and GnRH regulation has not been studied before.

In our previous studies of an AD animal model (SAMP8 mice), we found that NGF treatment moderately improved brain functions by reducing the generation of Aβ [27]. We also noticed that NGF treatment dramatically increased serum testosterone concentrations. This finding raises the possibility that NGF treatment may become a

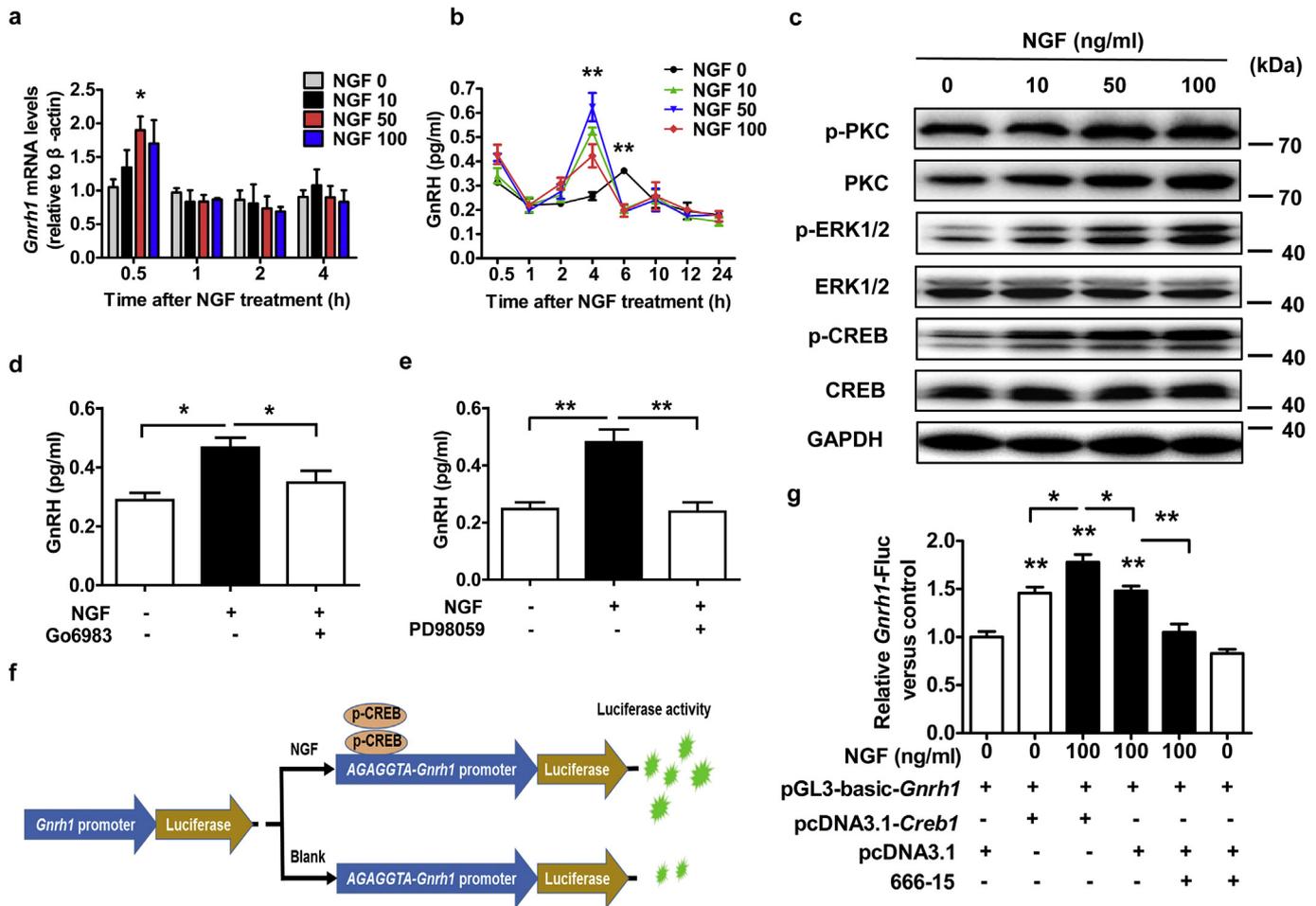


Fig. 8. NGF increased the transcription and secretion of GnRH by the PKC and MAPK signaling pathway in GT1-7 cells. (a) Time-course effects of NGF on *Gnrh1* mRNA expression of GT1-7 cells after NGF treatment. (b) Time-course effects of NGF on GnRH production in conditioned media of GT1-7 cells with ELISA after NGF treatment. (c) The changes in the expression of p-PKC/PKC/p-ERK1/2/p-CREB signal pathway after NGF treatment for 2 h. (d, e) Effects of NGF or coupled with PKC inhibitor Go6983 (10 μ M) or p-ERK inhibitor PD98059 (50 μ M) on GnRH production in GT1-7 cells. Conditioned media were collected to measure the GnRH concentration with ELISA after NGF treatment for 4 h. (f) Schematic diagram showing NGF-induced expression of *Gnrh1* via the upregulation of p-CREB to binding of AGAGGTA into the promoter of *Gnrh1*. (g) Effect of NGF or CREB on GnRH promoter activity. Luciferase values were measured after GT1-7 cells were transiently transfected with pGL3-basic-*Gnrh1* and pcDNA3.1-*Creb1* or pcDNA3.1 for 24 h and treated with NGF, or coupled with the pretreatment of p-CREB inhibitor 666-15 (50 μ M) for 12 h. Here values are expressed as means \pm SEM. The data were analyzed using one-way ANOVA analysis. *** $P < .001$, ** $P < .01$, * $P < .05$ compared to the control (vehicle), for $n = 3$ independent experiments.

potential therapy for hypogonadism. However, there is no standard animal model for age-related hypogonadism which is characterized by the low sexual hormone, sexual behavior, and fertility. Therefore low sexual hormone, sexual behavior, and fertility were used as model-selection criteria. We found that ten-month-old aging male SAMP8 mice displayed age-associated features of accelerated aging, including loss of activity, lack of hair and glossiness, which consisted with the previous research [41]. But above all, these mice had low *Gnrh1* mRNA, LH, testosterone levels, reduced sexual behavior and fertility similar to the syndrome of age-related hypogonadotropic hypogonadism, which is consistent with a previous report that testosterone decreases markedly in male SAMP8 mice between 4 and 12 months of age [42]. So ten-month-old aging male SAMP8 mice were used as an animal model for studying age-related hypogonadotropic hypogonadism. SAMR1 served as a control in that it was a related mouse strain that had normal aging process and did not show accelerated aging, but had a background similar to that of the SAMP8 strain.

SAM mouse has been developed from the AKR/J strain. Litters with severe exhaustion were selected as the progenitors of the senescence-prone series (P series). Litters in which the aging process was normal were selected as progenitors of the senescence-resistant series (R series). SAMP8 exhibits characteristic disorders such as learning and

memory deficits in old age. SAMR1 is normal aging with non-thymic lymphoma and non-histiocytic sarcoma. Recently, SAMP8 have also been widely used in age-related cardiac dysfunction and immune dysregulation [43, 44]. Moreover, it has been reported female SAMP8 mice are a model of midlife human female reproductive aging [45]. Nevertheless, SAMP8 is a particular mouse strain. In our study, normal inbred aging mice (Kunming mice, 16 months) were also have reduced testosterone associated with aging (Fig. S3a) and used to assess the role of NGF in hypogonadism. The data showed that NGF could also increase the serum testosterone and improve the sperm quality significantly (Fig. S3b–d). Therefore, NGF could be one potential treatment for age-associated declines in testosterone. But the normal aging process would result in significant individual differences and the recurrence may be low. Therefore, normal inbred aging mice (Kunming mice, 16 months) were not used as a model in this study. Besides, SAMR1 mice (ten months) and three-month-old Kunming mice (young) were also used to study the effect of NGF on hypogonadism. The effects are similar to SAMP8. Serum testosterone level significantly increased 12 h after the administration of NGF, but the testosterone returned to baseline by 24 h in SAMR1 mice and the young Kunming mice (Fig. S3e–f). The reason may be that testosterone value within feedback effective range, and testosterone level could return to the intact range. Therefore, we believe NGF is useful for hypogonadism. But

more research is needed to understand the effect of NGF on hypogonadism by using more animal models.

Subsequently, we study the mechanisms of NGF in sex hormones sexual function. Results suggested NGF enhanced sexual function, improved the quality of the sperm, and restored the fertility of aging male mice by activating GnRH neurons and regulating the secretion of GnRH. Spermatogenesis and sexual function are under the control of pituitary hormones FSH and LH, which are directly regulated by hypothalamic GnRH. This makes GnRH become a key point for HPG axis regulation. In this study, we focused on the mechanisms by which NGF regulates the release of GnRH and testosterone synthesis. First, in order to rule out the possibility that NGF acted on the Leydig cells, which produce most of the body's testosterone (>95%) and regulate testosterone secretion, we treated mice with NGF via i.v. and i.c.v. injection separately. The results showed that there was significant increase in serum testosterone, LH, and FSH associated with i.v.c. administration, but i.v. injection produced no difference. These results indicated that exogenous NGF administration could regulate testosterone secretion not by directly affecting the Leydig cells in the testis but rather by activating the hypothalamus. Second, using GnRH antagonist (cetrorelix acetate) blocking experiments, we proved again that the effects of NGF stimulating LH and testosterone secretion were mediated by GnRH neuronal activity of hypothalamus, and were not caused by the direct regulation of pituitary gland. Specifically, we found hypothalamic GnRH neurons to have high expression of the TrkA receptor, and a low expression of the p75 receptor *in vivo* and *in vitro*. The effect of NGF on LH and testosterone secretions could be neutralized by TrkA inhibitor indicating that the effects of NGF on HPG axis were through acting on Trk receptor signaling.

NGF/TrkA signal transduction pathways in survival and differentiation of neurons involved in Ras-MAPK, PI3K-Akt, and PLC γ -PKC pathways [46]. PKC second messengers and MAPK pathway have been implicated in the regulation of GnRH expression and secretion in GT1-7 cells [47, 48]. In our study, we demonstrated NGF binding TrkA regulated the secretion of GnRH involving PKC/p-ERK1/2/p-CREB signaling pathway. Then the effects of NGF on promoting CREB phosphorylation can lead to its binding to the *Gnrh1* promoter and enhance the transcription of the *Gnrh1*. However, we do not rule out the possibility that NGF influences GnRH secretion indirectly with paracrine pathway because of the complex networks between neurons of the hypothalamus. The precise regulatory mechanisms of NGF in age-related hypogonadotropic hypogonadism deserve further investigation.

Based on our results, it seemed that NGF intranasal administration could become an alternative therapy for hypogonadism and replace exogenous testosterone treatment. The largest obstacle is how to deliver NGF to the hypothalamus through the blood-brain barrier. In our previous study, we explored a very simple, safe, and convenient way to deliver pharmaceutical proteins, such as acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) to the brain, which is to spray the protein into the nasal cavity [49–51]. This intranasal administration plus the liposome encapsulation of protein greatly increased the stimulating effect of that agent on gonadotropin levels (Fig. S4). By using this noninvasive method, we evaluated the therapeutic function of NGF in restoring the age-related loss of reproductive functions in SAMP8 mice. We confirmed that the ten-month-old SAMP8 mice had lower levels of testosterone, sexual behavior, and pregnancy rate than age-matched control SAMR1 mice. Intranasal administration of NGF for 12 weeks systemically improved all of the aspects of reproductive functions, including increased serum LH, FSH, and testosterone concentrations, facilitated sperm production, and improved reproductive behaviors and the ability to impregnate the female partners.

Metabolic dysfunction, cognitive impairment, and testosterone deficiency are common hallmarks of aging in male animals [52–54]. Through our studies, we also found NGF treatment to have more advantages than testosterone treatment, especially in aging mice. Our results showed that NGF treatment could not only increase serum testosterone

and rescue reproductive function, but also decrease the level of serum triglycerides (Fig. S5a), and improve movement behavior (Fig. S5b–e).

In conclusion, we have reported a novel role of NGF in regulating GnRH release and testosterone synthesis by activating the TrkA signaling, which can robustly enhance the sexual function, improve the quality of the sperm and restored the fertility of aging male mice. NGF intranasal administration will be potentially developed to an alternative therapy for hypogonadotropic hypogonadism and its related subfertility.

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Conflicts of interest

There is no conflict of interest for all authors who share data and materials in this article.

Author contributions

Y.Y. and H.Y.D. designed research; L.J., Z.T.T. and Y.Y. performed research; L.Q.L., Y.D., X.Q. and S.Z.J. contributed new reagents or analytic tools; L. J. and Y.Y. analyzed data and wrote the paper; C.H.L., S.Z.J., G.R.S. and X.W. revised the manuscript; H.Y.D. supervised the experiments, revised and approved the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.08.021>.

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