Dissertation

Translational research on neural mechanisms of male sexual function and antioxidant intervention in mammals

March, 2026

Takashi Ito

Graduate School of

Environmental, Life,

Natural Science and Technology

(Doctor's Course)

OKAYAMA UNIVERSITY

Contents

General Introduction

With the rapid ageing of the global population, extending healthy life expectancy and maintaining the quality of life (QOL) of the elderly have emerged as critical health priorities in modern medicine. Specifically in males, the age-associated decline in androgens is a primary etiological factor underlying late-onset hypogonadism (LOH) (Corona et al., 2013). LOH is a multifaceted syndrome characterized not only by diminished sexual function, including erectile dysfunction (ED) and ejaculatory disorders, but also by physical manifestations including muscle weakness and osteoporosis, as well as neuropsychiatric symptoms such as anxiety, depression, and impaired concentration. Consequently, LOH substantially compromises QOL of middle-aged and elderly men (Corona et al., 2013). Given that sexual function extends beyond mere reproductive capacity and is intricately linked to male self-esteem, psychological stability, and the maintenance of intimate relationships, its preservation and amelioration hold profound societal significance.

Currently, testosterone replacement therapy (TRT) is widely accepted as the standard therapeutic intervention for LOH (Bhasin et al., 2018). It is well documented that TRT demonstrates efficacy in improving sexual function, body composition, and psychiatric symptoms by exogenously supplementing deficient androgen levels. However, TRT is associated with several clinical challenges and limitations. First, there are potential risks of adverse effects, including erythrocytosis, exacerbation of sleep apnea syndrome, and cardiovascular complications. Second, TRT is contraindicated or requires cautious administration in patients with a history of prostate cancer or in those with benign prostatic hyperplasia (BPH) accompanied by lower urinary tract symptoms, thereby rendering a substantial proportion of patients ineligible for this treatment (Bhasin et al., 2018). Third, the administration of exogenous androgens may lead to the suppression of endogenous hormone production and the impairment of spermatogenesis (Bhasin et al., 2018). Given this context, there is an urgent need to establish novel preventive and therapeutic strategies that do not rely solely on hormone replacement, have a superior safety profile, and address the underlying pathophysiological mechanisms of the condition.

To establish novel therapeutic strategies, it is imperative to first understand the fundamental biological mechanisms underlying male sexual function. Sexual behaviour, particularly physiological events such as erection and ejaculation, is precisely orchestrated by neural circuits within the brain and spinal cord. Extensive basic research, particularly in rat models, has demonstrated that the gastrin-releasing peptide (GRP)-producing neuronal system in the lumbar spinal cord plays a pivotal role in the spinal regulation of sexual function, including erection and ejaculation (Sakamoto et al., 2008). This finding indicates that sexual function is modulated by specific neuropeptide systems at the spinal level and provides important insight into the neural mechanisms underlying sexual function. However, a major challenge in translational research lies in the species barrier between animal models and humans. It remains unclear whether the spinal neural mechanism identified in rodents is conserved and functional in primates, including humans, as its anatomical substrate has not yet been elucidated. Therefore, for successful translation to human clinical application, it is essential to verify the presence of comparable neural substrates in primates. Accordingly, in Chapter 1, this dissertation investigates the anatomical substrates underlying the neural control of male sexual function in the primate spinal cord to elucidate their evolutionary conservation across species.

Even if the universality of the neural basis is established, substantial challenges remain in developing safe therapeutic interventions to maintain or restore sexual functions. As a potential intervention strategy for LOH, this study focused on "Bee Larvae (BL)", which has been traditionally used as a natural food source for its nutritive and tonic properties. Although BL is known to be rich in nutrients, it has been reported that, unlike standard TRT, BL intake does not influence serum androgen levels (Keishi et al., 2020). This finding suggests that BL may exert ameliorative effects on sexual function and psychiatric symptoms not by directly supplementing hormones, but via an androgen-independent pathway involving mechanisms other than androgen receptor mediation. The decline in sexual function observed in LOH is postulated to stem not merely from hormonal depletion but also from the accumulation of age-related oxidative stress, which causes irreversible damage to the nervous system and vascular endothelial cells (Agarwal et al., 2006). Therefore, a therapeutic approach focused on "tissue protection and functional maintenance"—specifically, protecting the conserved neural control

mechanisms and vascular systems from oxidative stress to maximize their existing physiological capacity—may demonstrate efficacy in a hormone-independent manner. Therefore, in Chapter 2, this dissertation evaluates the effects of BL administration on LOH-associated symptoms, including sexual hypofunction and anxiety-like behaviour, using rodent models whose translational validity is supported by the findings of Chapter 1. Specifically, this chapter aims to elucidate the underlying mechanisms by examining the effects of BL administration on hormonal profiles and antioxidative neuroprotective pathways.

Building upon the foregoing background, the primary objective of this dissertation is to develop a scientifically grounded translational strategy for LOH by integrating two key perspectives: elucidation of the evolutionary conservation of the neural substrates underlying male sexual function, and evaluation of the efficacy of antioxidant-based interventions using natural products. Through this integrative framework, this study ultimately aims to establish a safe, non-hormonal therapeutic strategy for LOH that is firmly grounded in a neuroscientific rationale.

Chapter 1

The gastrin-releasing peptide system in the spinal cord controlling male sexual function and itch sensation in the Japanese macaque monkey, *Macaca fuscata*

Summary

Gastrin-releasing peptide (GRP) is widely expressed throughout the mammalian central nervous system and is implicated in various physiological processes, including feeding behavior, anxiety-like behavior, and circadian rhythms. In rodents, two distinct GRP systems have been identified in the spinal cord: one involved in itch transmission and the other in the regulation of male sexual function. However, whether these corresponding spinal GRP systems exist in primates, including humans, remains unclear. To address this gap, the present study aimed to elucidate the existence and anatomical organization of spinal GRP systems in primates, using the Japanese macaque (*Macaca fuscata*) as a model.

In this study, we first identified the ortholog gene sequences for GRP and its receptors in Japanese macaques (*Macaca fuscata*) using genomic database searches. Subsequently, bioinformatics analyses were conducted to compare the identified sequences with genomic data from a diverse range of other mammalian species. The results indicated that the Japanese macaque GRP gene sequence was consistent with its phylogenetic position. Furthermore, the analysis revealed that the GRP gene is highly conserved among primates, particularly within Old World monkeys and apes. Following the genetic identification, we performed immunohistochemical analysis for GRP using frozen sections of the Japanese macaque spinal cord. Consistent with findings in rats and mice, intense GRP immunoreactivity was commonly observed in the lumbosacral spinal cord of the macaques. Moreover, GRP-positive fibers projected to the autonomic nuclei involved in male sexual function, exhibiting a marked male-dominant sexual dimorphism, mirroring the organization seen in rodents. These findings suggest that a spinal GRP system regulating male sexual function is present in primates, analogous to the system established in rodents.

In conclusion, the present study demonstrated the existence of a spinal GRP system involved in male sexual function in the primate spinal cord. Previous studies have identified two distinct spinal GRP systems in rodents (rats and mice) and in the house musk shrew (*Suncus murinus*), a member of the order Eulipotyphla representing an early-diverging lineage of placental mammals. Collectively, the identification of the spinal GRP system in the Japanese macaque

suggests that this neural system is conserved in primates, including humans. Furthermore, these findings imply that this system represents a fundamental feature conserved across Mammalia from the perspective of molecular and functional evolution.

Introduction

Severe injuries in the lower spinal cord frequently cause sexual dysfunction in men, including erectile dysfunction (ED) and ejaculation difficulties (Brown et al., 2006; Sipski, 1998). This indicates that important neural control circuits for penile functions are located within the lower spinal cord (Breedlove, 1985; Morris et al., 2004; Matsuda et al., 2008; Breedlove & Arnold, 1983a; Breedlove & Arnold, 1983b; Sakamoto, 2012; Sakamoto, 2014). We previously demonstrated that a population of gastrin-releasing peptide (GRP) neurons in the lumbar spinal cord plays an important role in penile erection and ejaculation in rats (Sakamoto et al., 2008; Sakamoto et al., 2014). This system of GRP neurons is sexually dimorphic, being prominent in male rats but vestigial or absent in female rats (Sakamoto et al., 2008; Sakamoto et al., 2014; Sakamoto et al., 2009). Recently, we reported that this sexually dimorphic system has been identified not only in rodents but also in the Asian house musk shrew (*Suncus murinus*) (order of Eulipotyphla; formerly Insectivora) (Tamura et al., 2017). These results suggest that the sexually dimorphic spinal GRP system is general in mammals. However, little information exists on the spinal GRP system controlling male sexual function in primates, although it is clinically important to know whether the GRP system exists and has similar functions in the lower spinal cord in primates. Macaque monkeys appear to be an excellent model because they are close primate relatives to humans (Gibbs et al., 2007). Therefore, in this study, we worked to identify the sexually dimorphic spinal GRP system in primates using the Japanese macaque monkey (*Macaca fuscata*).

Materials and methods

Experimental animals

Male and female Japanese macaque monkeys, *Macaca fuscata* (*n* = 3: 3, 7, and 9-years-old males, weight 2.9–9.0 kg; *n* = 3: 8, 10, and 11-years-old females, 7.0–8.3 kg) were used in this study. Monkeys were maintained in a temperature-controlled (22–24ºC) room under a daily photoperiod of 12/12 h light/dark cycle (lights on 8:00 A.M.–8:00 P.M.). We confirmed these animals were free specific pathogens. Food and water were available *ad libitum*. All animals were kept in individual cages. The experimental protocols followed the guideline of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, and were approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Okayama University (Okayama, Japan), by Kyoto Prefectural University of Medicine (Kyoto, Japan), and by Kansai Medical University (Osaka, Japan). All efforts were made to minimize animal suffering and reduce the number of animals used in this study.

Gene database search and phylogenetic analysis

To evaluate the gene sequence of GRP precursors, the rhesus macaque monkey (*Macaca mulatta*) genomic sequence data were searched on the NCBI (www.ncbi.nlm.nih.gov) DNA database by blastn using the human nucleotide sequences of the precursors of GRP as queries. On the basis of the monkey genomic sequence data, the deduced amino acid residues were compared with other mammalian species. Alignment of the amino acid sequences of the GRP precursor protein from different species and human neuromedin B as out-group was performed using the ClustalW sequence alignment program. Phylogenetic trees were constructed using the neighbor-joining method and viewed with TreeView (Version 1.6.6). Details of the program settings are given in the legend for Figure 1.

Tissue preparation

Monkeys (males, n = 3; female, n = 3) were anesthetized with an overdose of pentobarbital sodium (90 mg/kg i.m.) and transcardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). Lumbosacral spinal cords were quickly removed and immersed in the same fixative overnight at 4ºC. After immersion in 25% sucrose in 0.1 M PB at 4ºC for cryoprotection until they sank, the preparations were quickly frozen using powdered dry ice and cut into 30 µm cross sections using a cryostat (CM3050 S, Leica, Nussloch, Germany) and stored in cryoprotectant (30% glycerol and 30% ethylene glycol in phosphate buffered saline; PBS) (pH 7.4) at –20ºC until use.

Immunocytochemistry and immunofluorescence

We performed an immunocytochemical analysis according to our established methods using cryosections (Sakamoto et al., 2008; Sakamoto et al., 2014; Tamura et al., 2017). In brief, tissue sections were first rinsed 5 times with PBS to remove the cryoprotectant. Endogenous peroxidase activity was eliminated by incubation in 1% H_2O_2 in absolute methanol for 20 min followed by three 5-min rinses with PBS. This H_2O_2 treatment process was omitted in the immunofluorescence method. After blocking nonspecific binding with 1% normal goat serum and 1% BSA in PBS containing 0.3% Triton X-100 for 30 min at room temperature, sections were incubated with primary rabbit antiserum which recognizes a 10-amino acid-peptide called neuromedin C or GRP-10 (11081-05015; AssayPro, St. Charles, MO) (1:2,000 dilution) for 3 days at 4ºC. The GRP antiserum used in this study has previously been shown to be specific for GRP in the spinal cord of rats, mice, and Asian house musk shrews (Tamura et al., 2017; Takanami et al., 2014; Satoh et al., 2015a; Satoh et al., 2015b). Immunoreactive-products were detected with a streptavidin-biotin kit (Nichirei, Tokyo, Japan), followed by diaminobenzidine (DAB) development according to our previous method (Sakamoto et al., 2008; Oti et al., 2016). GRP-immunoreactivities in the spinal cord were localized using an FSX100 microscope (Olympus, Tokyo, Japan). In addition, GRP-containing fibers were visualized with an immunofluorescence method using Alexa Fluor 488-linked anti-rabbit IgG raised in goats (1:1,000 dilution) (Molecular Probes, Eugene, OR). To determine the projection site of GRP-

containing fibers, double-immunofluorescence staining for GRP (1:1,000 dilution) and neuronal nitric oxide synthase (1:5,000 dilution) (nNOS; A-11; a mouse monoclonal antibody raised against amino acids 2–300 of nNOS of human origin, Santa Cruz Biotechnology, Santa Cruz, CA), a marker protein for neurons in the sacral autonomic nucleus (SAN), was performed as a similar way described previously in rats, mice, and Asian house musk shrews (Sakamoto et al., 2008; Sakamoto et al., 2014; Tamura et al., 2017). Alexa Fluor 546-linked anti-mouse IgG (Molecular Probes) and Alexa Fluor 488-linked anti-rabbit IgG, both raised in goats, were used at a 1:1,000 dilution for detection. Immunostained sections were imaged with a confocal laser scanning microscope (FluoView FV1000, Olympus). Immunocytochemistry studies were repeated independently at least three times using different animals and produced similar results.

Antibody specificity

The rabbit polyclonal GRP antiserum was raised against 10-amino acid GRP-10 consist of residues 20–29 of the full rat GRP. This antiserum produced identical patterns of labeling in the distribution of GRP-positive fibers in the lumbosacral spinal cord as that achieved by immunocytochemistry using the same (Takanami et al., 2014; Satoh et al., 2015a) or other antisera(Sakamoto et al., 2008; Oti et al., 2012) in rats. Control procedures for the DAB method were performed using pre-absorption of the working dilution (1:2,000) of the primary antiserum with saturating concentration of monkey $GRP_{18\text{-}27}$ (or GRP-10; corresponding to the monkey GRP deduced amino acid residues from the genomic information, see Fig 1a) antigen peptide, Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met (50 μg/mL, produced in AnaSpec; San Jose, CA) for 1 h at room temperature before use. The GRP-positive fibers were detected according to the above protocol for peroxidase immunocytochemistry.

Semi-quantitative analysis

We performed a semi-quantitative analysis of GRP expression by using the DAB-stained immunoreacted sections. To determine the density of GRP-positive fibers in the lumbosacral spinal cord (L5–S1 level), at least 5 cross sections (30-μm thick) per animal were randomly selected, and the digital images of two regions [the SAN and dorsal horn (DH)] were prepared

(magnification, ×200). The unit area (343 × 469 μm^2) was analyzed to localize the nuclei at the center of each area. The optical density of GRP staining (DAB) was determined using black-and-white images that were converted from micrographs using ImageJ software (ImageJ 1.44p; National Institutes of Health, Bethesda, MD) according to our established methods(Sakamoto et al., 2008; Sakamoto et al., 2014; Oti et al., 2016; Katayama et al., 2016). Briefly, the optical density of background labeling was estimated by comparisons with similar areas of the control sections reacted with the anti-GRP antiserum that was incubated first with an excess of peptide antigen (50 μg/mL). GRP expression was undetectable in these sections. Each threshold optical density was determined by normalizing the data to those of the preabsorbed sections. The GRP-positive-fiber pixel density was semi-quantified as the average pixel density in the SAN and DH of each animal. Micrographs were coded and evaluated without the knowledge of the experimental group designation, and the code was not broken until the analysis was complete. Data are expressed as the relative value against the mean of the DH value in males.

State of ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

Results

Sequence analysis of the monkey GRP precursor gene

To investigate the evolutionary relationships of primate GRP with other mammalian species, a phylogenetic tree based on the deduced amino acid sequence of prepro-GRP was constructed using the neighbor-joining method (Fig. 1a). According to the tree, monkey prepro-GRP at the amino acid level was grouped in the primate branch and separated from that of rodents and other mammalian species (Fig. 1a). In addition, the sequence in common marmoset, a new world monkey (*Platyrrhini*), was separated from that in old world monkeys (*Catarrhini*) and possibly proximal in the primate branch in terms of prepro-GRP (Fig. 1a). According to the deduced amino acid sequence, the macaque monkey's prepro-GRP starts with a signal peptide, followed by the bioactive $GRP_{1\text{-}27}$ (mature GRP) including the GRP-10 motif at the C-terminus of mature GRP, which is highly conserved in mammals (Fig. 1b). The mature GRP in monkeys is identical to human GRP (Fig. 1b).

GRP in the lumbosacral spinal cord of Japanese macaque monkeys

Immunocytochemical detection of GRP in the lumbosacral spinal cord (L5–S1 level) of male monkeys was performed. In the monkey lumbar and sacral cords, GRP-containing fibers were found in the dorsal gray commissure (DGC) (Fig. 2, S1 level), as in rats and mice (Sakamoto et al., 2008; Tamura et al., 2017; Oti et al., 2016). Furthermore, GRP-containing fibers appeared to project toward the SAN (*arrowheads* in Fig. 2a). We also found GRP fibers in the spinal DH of monkey lumbar and sacral cords (Fig. 2b, S1 level). Use of the pre-absorbed antibody abolished immunostaining (n = 3; 2 males and 1 female) (Fig. 2c, d). We then examined the sexually dimorphic distribution of GRP-containing fibers in the SAN of the monkey sacral cord because SAN provides autonomic preganglionic fibers to the genitalia (Morgan et al., 1981; Espinosa-Medina et al., 2016) (Fig. 3). We found male-dominant projection of GRP-positive fibers was obvious in the transition area between DGC and SAN (Fig. 3a–d). Double immunofluorescence for GRP and nNOS, a marker for autonomic preganglionic neurons (Vizzard et al., 1995), clearly showed a strong projection of GRP-positive fibers into the SAN

in males but only few fibers in females (Fig. 3e, f). Double immunofluorescence for GRP and nNOS showed that GRP-positive fibers in the SAN were more prominent in male than in female monkeys (Fig. 3g–j). Semi-quantitative analyses also supported this sex difference in the SAN but not DH (Fig. 3k, l). In addition, our GRP-immunocytochemistry did not demonstrate GRP neuronal somata in the macaque spinal cord.

Discussion

The aim of this study was to demonstrate, in a model primate, the sexually dimorphic GRP system in the spinal cord controlling penile function. To our knowledge, this is the first demonstration of this system using Japanese macaque monkeys, *Macaca fuscata*.

Phylogenetic analysis suggested that primate prepro-GRP is separated from that of other mammalian species and clustered to an independent branch as primates. Macaque (*Catarrhini*) and human prepro-GRP are very closely related, and also related to that of marmoset (*Platyrrhini*), but rather different from that of rat, and even more distinct from that of Asian house musk shrew (*Suncus murinus*) (Fig. 1b). Human GRP_{18-27} (or GRP_{20-29} in mice and rats) is a possible C-terminal fragment of mature GRP, and was identified from porcine spinal cord originally as neuromedin C (Minamino et al., 1984). A more appropriate name is perhaps GRP-10 (Takanami & Sakamoto, 2014 b). It has been reported in rodents that the second amino acid of GRP-10 is a serine(Tamura et al., 2017; Lebacq-Verheyden et al., 1988) but an asparagine exists in this location in most other mammals including humans (Spindel et al., 1984), cattle (Lemaire et al., 1989), pigs (McDonald et al., 1979), sheep (Fraser et al., 1994), dogs (Reeve et al., 1983), and guinea pigs (Shaw et al., 1987). In this study, we found that the deduced second amino acid of GRP-10 is an asparagine in macaque monkeys, and identical to those of many mammals other than rodents (Tamura et al., 2017; Lebacq-Verheyden et al., 1988). Moreover, the amino acid sequence of entire mature GRP is identical in humans and monkeys. Taken together, these results demonstrate that the GRP gene shows a high homology especially among old world monkeys and, furthermore, is well conserved among primates.

Using the Japanese macaque monkey as a non-human primate model, we demonstrated immunocytochemically that GRP-containing fibers projecting into the SAN of this primate show a remarkable male-dominant sexual dimorphism similar to that in rodents (Sakamoto et al., 2008; Sakamoto et al., 2014; Tamura et al., 2017; Oti et al., 2016). The SAN provides autonomic preganglionic fibers to the genitalia (Morgan et al., 1981; Espinosa-Medina et al., 2016). Spinal GRP-positive axons have also been shown to make direct synaptic contacts with somatic motor neurons of the spinal nucleus of bulbocavernosus (SNB) located in the sacral

spinal cord in male rats (Dobberfuhl et al., 2014; Forger & Breedlove, 1986), and GRP-sensitive receptors are expressed by rat SNB motor neurons (Sakamoto et al., 2008; Takanami & Sakamoto, 2014 b). The SNB in rats, which innervates the striated perineal muscles attached to the base of the penis (Sakamoto, 2014; Forger & Breedlove, 1986; Breedlove & Arnold, 1980; Sengelaub & Forger, 2008), is homologous with the human Onuf's nucleus, a sexually dimorphic nucleus located in the motor neuron pools of the sacral spinal cord that plays an important role in the micturition and defecatory as well as in rhythmic contractions of perineal muscles during orgasm (Onufrowicz, 1899). The number of motor neurons in Onuf's nucleus in humans is greater in men than in women (Forger & Breedlove, 1986; Onufrowicz, 1899; Nakagawa, 1980; Sato et al., 1978). Taken together with the behavioral results from our rodent studies (Sakamoto et al., 2008), our present results in a model primate strongly suggest that the sexually dimorphic spinal GRP system in primates is involved in the regulation of both autonomic and somatic penile functions. Future studies focusing on GRP receptors in the monkey spinal cord could provide new insights into the peptidergic control circuit for penile functions in primates.

Although our immunocytochemical procedure using formaldehyde-fixed tissues did not demonstrate GRP neuronal somata in the present study, when taken together with many studies in rodents (Sakamoto et al., 2008; Oti et al., 2016), it is likely that monkey GRP neurons are also located in the lumbar spinal cord. Intraspinal or intrathecal administration of colchicine could be used to facilitate the immunocytochemistry; alternatively, gene expression analyses of fresh monkey tissues could be used to localize the somata of monkey spinal GRP neurons.

GRP-containing neurons in the lumbar spinal cord highly co-express androgen receptors in rats (Sakamoto et al., 2008) and mice (Tamura et al., 2017) and the sexually dimorphic expression of GRP in the lower spinal cord is also controlled and maintained in an androgen-dependent manner in rodents (Sakamoto et al., 2014; Sakamoto et al., 2009; Oti et al., 2016). A three-year-old male monkey (possibly pre-puberty) was also used in this study. The lower optical density of GRP-positive fibers in this animal is consistent with the finding (Katayama et al., 2016) that the androgen surge during male puberty plays an important role in the development of the male-specific GRP function in the monkey spinal cord. Thus, expression of

androgen receptors would be expected to be an important characteristic of the GRP system in monkeys. Future attention should therefore be focused on the co-localization of androgen receptor/GRP in the monkey spinal cord.

Our immunocytochemical study also located GRP-immunoreactive fibers in the spinal DH of the macaque monkeys but, unlike those in the SAN they were not sexually dimorphic and, by analogy with our studies in rodents, were probably derived from dorsal root ganglion GRP neurons involved in itch (Takanami et al., 2014; Sun & Chen, 2007).

Clinical data show that ED is an increasingly common condition predicted to affect more than 300 million men worldwide by 2025 (Aytaç et al., 1999; Hehemann & Kashanian, 2016). The increasing rates may reflect the accumulating stress in society and could contribute to declining the birth rate that is becoming a serious social problem in certain societies (Hehemann & Kashanian, 2016). It has long been established that ED is a multi-factorial dysfunction, but most treatments, including sildenafil citrate, have targeted penile vasculopathy (Briganti et al., 2005). Current treatments can have unwanted side effects and, importantly, fail to address the underlying neural control problem or to protect these neural circuits from age-related degeneration. Our finding that the GRP system controlling erection and ejaculation in rodents (Sakamoto et al., 2008) is also present in primates could, in the future, provide new therapeutic approaches to sexual problems in men.

Here we demonstrate, using Japanese macaque monkeys as a model non-human primate, that the GRP system in the lower spinal cord shows a male-specific dimorphism and is likely to play an important role in penile functions in primates. The sexually dimorphic spinal GRP system controlling penile functions appeared to be general in primates, which could bridge to therapeutic treatments for sexual problems in men.

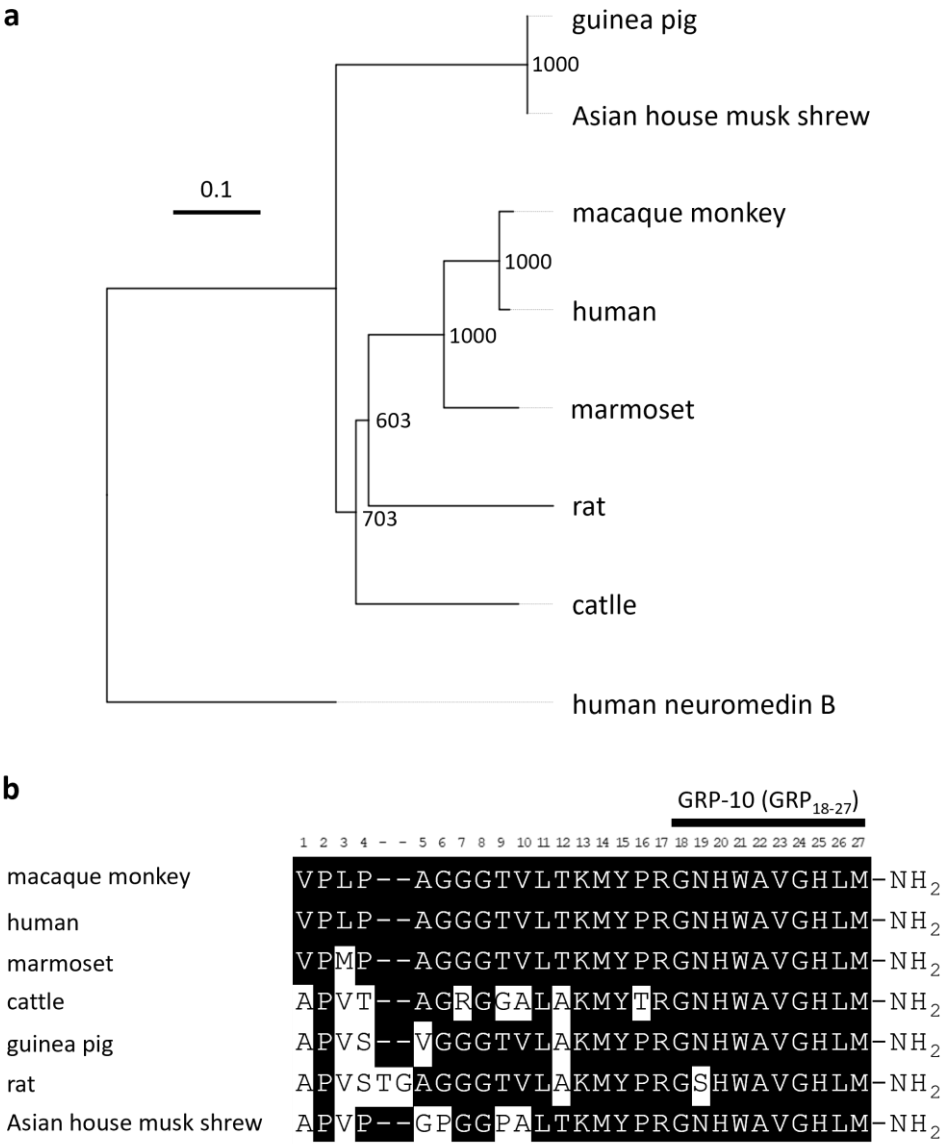

Figure 1. The evolutionary relationships of primate GRP with other mammalian species. (a) A phylogenetic tree comprising prepro-gastrin-releasing peptide (GRP) was constructed using the neighbor-joining method. Branch lengths are proportional to the number of amino acid changes on the branch. The values represent bootstrap scores for 1,000 trials, indicating the credibility of each branch. Horizontal bar indicates the genetic distance. (b) A comparison of the amino acid sequences of *Macaca* GRP with the corresponding sequences of mature GRP in mammals. Residue identity to *Macaca* GRP denoted by the highlight. Residue deletions denoted by – are inserted into some sequences to maximize structural similarity. Line-highlighted sequence indicates that the mature GRP in monkeys is identical to human GRP.

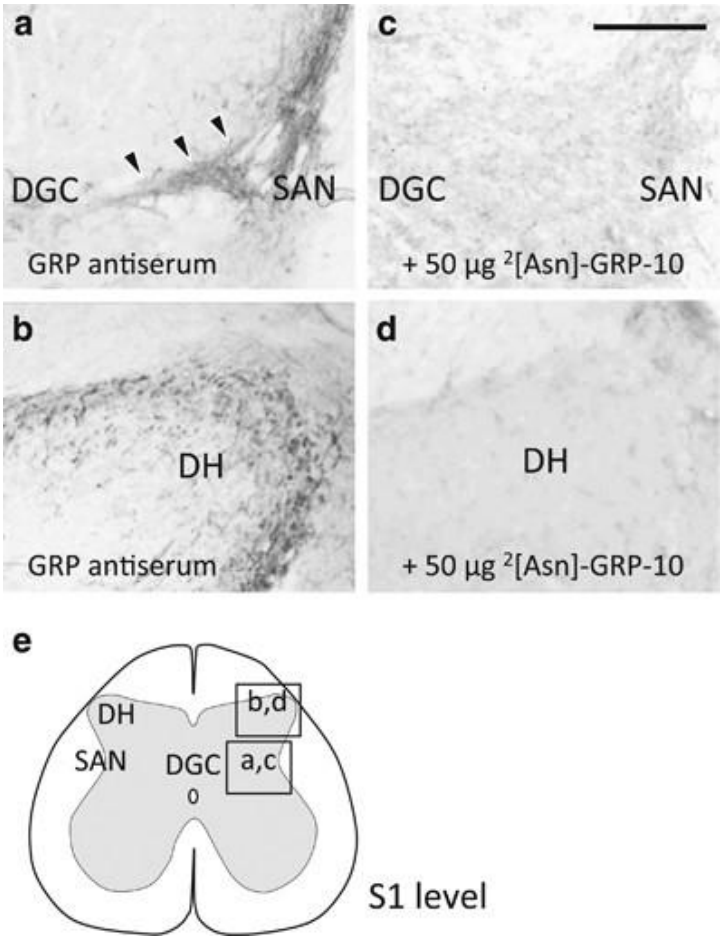

Figure 2. Immunocytochemistry analysis of gastrin-releasing peptide (GRP) in the sacral spinal cord of Japanese macaque monkeys. GRP-containing fibers in the sacral spinal cord (S1 level) were present in the dorsal gray commissure (DGC) (a), sacral autonomic nucleus (SAN) (a), and spinal dorsal horn (DH) (b) in monkeys. GRP-containing fibers projected toward the SAN is obvious (arrowheads in a). Preabsorbing the working dilution (1:2,000) of the primary GRP antiserum with a saturating concentration of GRP antigen peptide (GRP-10; 50 µg/ml) overnight at 4ºC before use eliminates the staining in the monkey spinal cord (c, d). (e) Schematic drawing of the sacral spinal cord (S1 level). Scale bar = 200 µm.

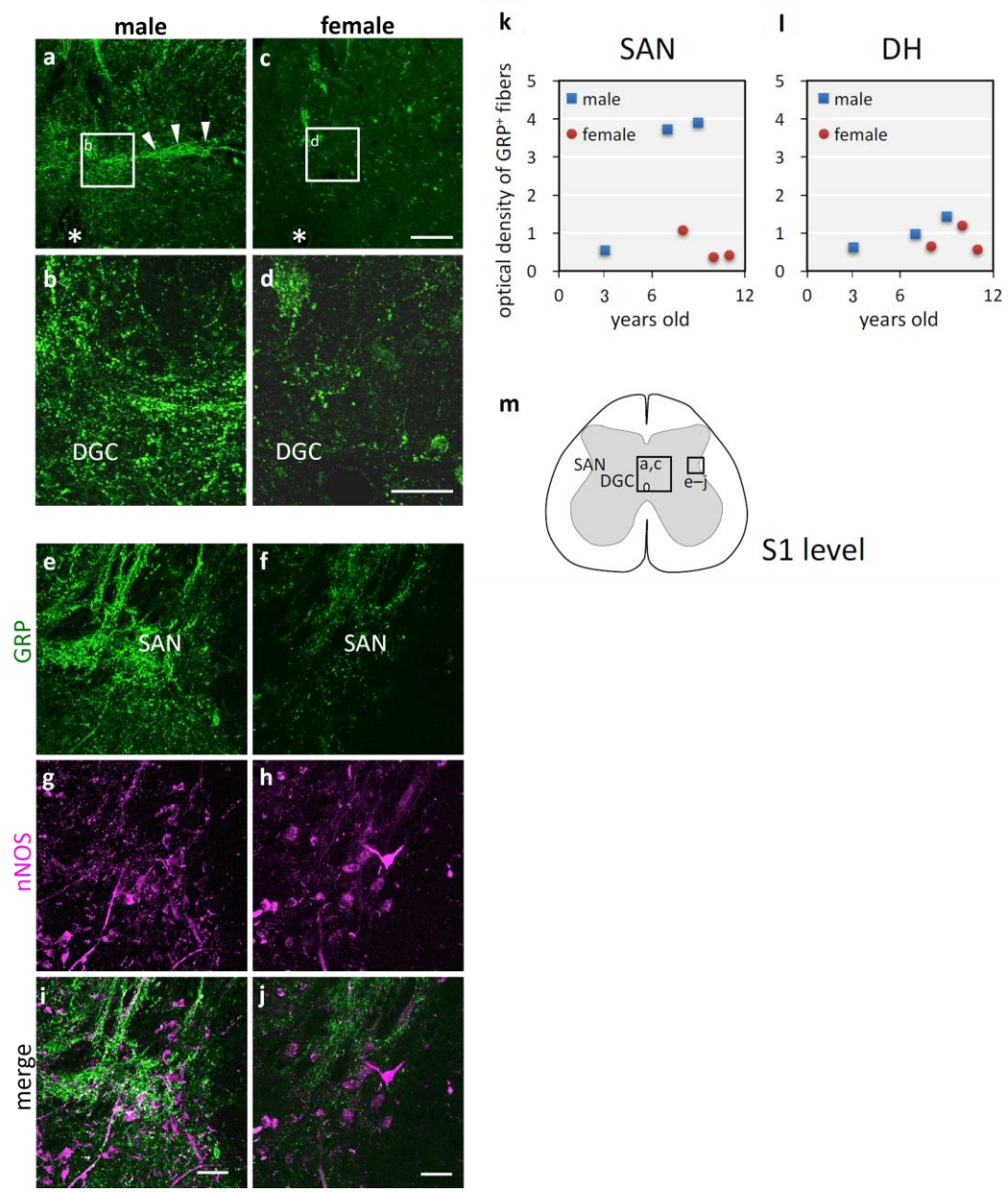

Figure 3. Immunocytochemistry analysis for gastrin-releasing peptide (GRP) in the sacral spinal cord (S1 level) of Japanese macaque monkeys. Male-specific expression for GRP is observed in the monkey sacral spinal cord (a, b). Possible GRP-containing fibers projected toward the sacral autonomic nucleus (SAN) are male-specific (a, c), and the male-dominant expression is also observed in the dorsal gray commissure (DGC) (b, d). Asterisks (*) indicate the location of the central canal in (a, c). GRP-containing fibers (green) are obviously observed in males (e) but weak in females (f). The neuronal nitric oxide synthase (nNOS) serves as a marker for SAN neurons, and the immunoreactivity for nNOS in the SAN neurons (magenta) showed no sex difference (g, h). Double immunofluorescence for GRP and nNOS revealed closely appositions of GRP-containing fibers with the cell bodies and proximal dendrites of nNOS-positive neurons in the SAN (i, j). Semi-quantitative analysis suggests that the intensity of GRP-positive fibers in the sacral spinal cord is greater in males than in females in the autonomic centers of the SAN (k) but not in the somatic sensory layers of the spinal dorsal horn (DH) (l). Schematic drawing of the sacral spinal cord (S1 level) (m). Scale bars = 200 μm in (c); 50 μm in (d, i, j).

Chapter 2

Bee Larvae Ameliorate Andropause-like Symptoms via a Hormone-Independent, Antioxidant Mechanism

Summary

Late-onset hypogonadism (LOH), also known as the male menopause, is characterized by a decline in sexual function as well as various physical and psychological symptoms, including anxiety. Although bee larvae have historically been utilized as a traditional food and medicine, their efficacy and mechanisms of action against male menopausal symptoms remain unclear. In this study, we investigated the effects of extract of bee larvae (BL) on sexual and anxiety-like behaviors using two models of the male menopause: aged rats and castrated mice.

In the aged rat model (64 weeks old), dietary BL supplementation significantly attenuated the age-associated decline in ejaculation frequency compared to controls, whereas no significant effects were observed on mount or intromission frequencies. Notably, plasma analysis after 4 weeks of treatment revealed no significant differences in testosterone or dihydrotestosterone levels between the BL and control groups. To elucidate the underlying mechanism, we evaluated sexual function using a castrated mouse model. While BL supplementation did not affect sexual behavior in intact mice, post-castration BL treatment significantly shortened intromission latency without altering mount frequency. In the elevated plus maze test, BL significantly alleviated castration-induced anxiety-like behaviors and improved exploratory activity. Furthermore, in vitro assays demonstrated that the BL extract exerts potent protective effects against oxidative stress, a pathological factor contributing to both erectile dysfunction and anxiety.

These results suggest that BL improves erectile function and anxiety via a hormone-independent mechanism, potentially by mitigating oxidative stress in vascular and neural tissues. Thus, bee larvae represent a promising functional food for ameliorating the physical and psychological symptoms of male menopause.

Introduction

In our rapidly aging society, maintaining the Quality of Life (QOL) stands as a priority in modern healthcare. Within this demographic, late-onset hypogonadism (LOH) has emerged as a critical health challenge (Corona et al., 2013). Characterized by an age-related decline in circulating androgens, LOH manifests through a broad spectrum of symptoms, ranging from diminished sexual function to physical lethargy, sleep disturbances, and psychological instability, including anxiety and depression. Notably, while sexual desire often persists in middle-aged and elderly men, the physiological decline in sexual function can severely compromise self-esteem and QOL, driving a substantial proportion of individuals to seek medical intervention (Corona et al., 2013).

Key factors contributing to the decline in male QOL include erectile dysfunction (ED) and psycho-emotional instability, particularly anxiety. Epidemiological studies indicate that the prevalence of ED increases with age, affecting approximately 40% of men in their 40s and 50% of men in their 50s (Ferrini et al., 2017). Systemic oxidative stress has been identified as a significant contributor to this age-related increase in ED (Agarwal et al., 2006). Oxidative stress plays a pivotal role in the pathogenesis of atherosclerosis, diabetes, hypertension, and hypercholesterolaemia, leading to vascular endothelial dysfunction (Agarwal et al., 2006). Notably, penile arteries, having a significantly smaller diameter than coronary or internal carotid arteries, are particularly susceptible to oxidative stress-induced endothelial damage. The resulting reduction in cavernosal blood flow is considered a primary cause of ED (Ferrini et al., 2017; Kaltsas et al., 2024). Furthermore, elevated oxidative stress is intricately linked to the pathophysiology of psychiatric symptoms, including anxiety and depression (Fedoce et al., 2018; Correia et al., 2023). Given their high oxygen consumption, cerebral neurons are highly vulnerable to oxidative stress; oxidative damage can disrupt neurotransmitter homeostasis, potentially leading to emotional dysregulation (Fedoce et al., 2018; Correia et al., 2023). Therefore, the augmentation of oxidative stress associated with ageing and androgen deficiency may serve as a common etiological factor simultaneously driving two distinct symptoms: ED and anxiety.

Bee larvae (BL), comprising both the larvae and pupae of bees, have historically been consumed worldwide as a highly nutritious food source (Guiné et al., 2022). They possess an exceptionally high nutritional value, containing a diverse array of nutrients including proteins, carbohydrates, and lipids, as well as vitamins, minerals, essential amino acids, and unsaturated fatty acids (Finke, 2005). In recent years, beyond their rich nutritional composition, studies have reported their physiological benefits, such as antioxidant and immunomodulatory effects (Moraru et al., 2024; Li et al., 2020). Furthermore, clinical studies have suggested their efficacy in improving hearing and ameliorating symptoms associated with male menopause (Aoki et al., 2012; Keishi et al., 2020). Specifically, in clinical trials targeting male menopause, supplementation with BL for 12 weeks has been observed to improve scores on the Aging Males' Symptoms (AMS) scale and the Sexual Health Inventory for Men (SHIM), while also reducing fatigue and depressive symptoms. However, the precise mechanisms underlying the functional properties of BL and their therapeutic effects on male menopausal symptoms and erectile function remain to be fully elucidated. Therefore, the present study focused on BL as a potential therapeutic agent for improving male sexual function. We aimed to verify the mechanism of action of BL, specifically focusing on the suppression of oxidative stress, using a castrated mouse model of male menopause that reproduces the decline in erectile function and anxiety-like symptoms induced by androgen deficiency.

Material and Methods

Animals and ethics

All procedures involving animals were conducted in accordance with the principles outlined in the ARRIVE guidelines. All efforts were made to minimize animal suffering and the number of animals used for the studies.

Rat experiment: The study protocol for the rat experiment was reviewed and approved by the Institutional Animal Care and Use Committee of the Animal Reproduction Institute (Approval No.: Sho-3-33) and was conducted at the same institute (Kasumigaura, Ibaraki, Japan). Wistar-Imamichi rats were procured from the Animal Reproduction Institute.

Mouse experiment: The study protocol for the mouse experiment was approved by the Animal Care and Use Committee of Okayama University (Approval No.: OKU-2023607). This experiment was conducted at Okayama University (Okayama, Japan). ICR male and female mice were purchased from Japan SLC Inc. (Shizuoka, Japan).

Preparation of bee larvae powder and diets

Enzyme-digested bee larvae (BL) powder, prepared from 19–20-day-old larvae (*Apis mellifera*) treated with protease, was supplied by Yamada Bee Company, Inc. (Okayama, Japan). The experimental diets were prepared by mixing the BL powder into a commercial basal diet, MF (Oriental Yeast Co., Ltd., Tokyo, Japan), at a concentration of 6.4% (w/w).

Rat experiment (aging model)

Experimental design and housing conditions

Wistar-Imamichi male rats (64 weeks old at the start of feeding) and female rats (9 weeks old) were procured from the Animal Reproduction Institute and used for this study. All animals were housed under controlled temperature (23 ± 3 ºC) and maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. To ensure consistent sexual performance throughout the experiment, male rats were sexually experienced before the feeding period. Healthy male rats were divided into two groups (n = 12 per group) based on equal pre-feeding copulatory

performance: the Control group (MF diet) and the BL group (BL diet). The feeding period lasted 4 weeks.

Sexual behavior test

It is well-established that sexual behavior declines with advancing age in male rats (Smith et al., 1992; Hokao et al., 1992). Sexual behavior was observed before and after the 4-week feeding period. For testing, sexually experienced male rats were paired 1:1 with sexually receptive, naïve female rats. Females were confirmed to be in behavioral estrus *via* the manual lordosis reflex on the day of testing. Observations were conducted during the dark cycle. Sexual behavior was recorded for 60 min and quantified using the following established Heimer indices: number of mounts, number of intromissions, number of ejaculations, mount latency, intromission latency, and ejaculation latency (Heimer & Larsson, 1967).

Hormone measurement

Following the final sexual behavior test, all male rats were fasted for 16 h. Blood samples were collected under isoflurane anesthesia from the caudal vena cava. Plasma was obtained and stored at −80 ºC until hormonal analysis. Plasma concentrations of testosterone (T) and dihydrotestosterone (DHT) were quantified by ASKA Pharmaceutical Co., Ltd. (Tokyo, Japan) using a highly sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS) method.

Mice experiment (castration model)

Experimental design and housing conditions

Male and female ICR mice (8–10 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were housed separately by sex (3–6 per cage) under controlled temperature (23 ± 3 ºC) and maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. To ensure consistent sexual motivation and performance, male mice were sexually pre-conditioned before the observation period; this involved providing them with at least two sexual experiences, including one ejaculation per week. This procedure was conducted to prevent the level of prior

sexual experience from confounding the experimental results. Male mice underwent castration under isoflurane anesthesia upon completion of the 12-week sexual behavior evaluation period. Following castration, the mice continued to receive their assigned BL or control diets for an additional 4 weeks (until week 16), and the elevated plus maze test was performed at the study endpoint (week 16).

Sexual behavior test

Sexual behavior tests were performed before and after castration, following the general protocol for parameter enumeration described for the *rat experiment (aging model)*. To induce sexual receptivity in stimulus females, female ICR mice were ovariectomized (OVX). Estradiol benzoate (EB, 25 μg/0.05 mL sesame oil) was subcutaneously injected 48 h prior to testing, followed by progesterone (P, 250 μg/0.05 mL sesame oil) subcutaneously injected 3–6 h before testing. Sexual behavior was recorded for 60 min. The parameters evaluated were the number of mounts, number of intromissions, mount latency, and intromission latency.

Elevated plus maze test

To evaluate anxiety-like and exploratory behaviors, the elevated plus maze (EPM) test was conducted at week 16 of the experiment. The test apparatus consisted of a plus-shaped maze elevated 75 cm above the floor, with two open arms and two closed arms (50 cm × 10 cm). The closed arms were enclosed by 40 cm high walls. Mice from each group were randomly selected and placed at the center of the maze, facing an open arm. Their behavior was recorded on video for 5 min. After each test, the apparatus was thoroughly wiped with 70% ethanol to eliminate any residual olfactory cues. The video recordings were then manually scored to quantify the following parameters: time spent in the open arms, number of entries into the open arms, and the number of head-dips from the open arms. These parameters are considered indicators of anxiety-like and exploratory behavior, respectively.

In vitro oxidative stress protection assay

Human neonatal dermal fibroblasts (NB1RGB) were obtained from RIKEN BRC through the National Bioresource Project of the MEXT, Japan. Cells were maintained in MEM α (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and used within three passages. To prepare the test sample, BL powder was suspended in water and extracted by sonication for 30 min. For the viability assay, cells were seeded into 96-well plates at a density of 1.0×10^4 cells ells per well. After 24 h of incubation, the medium was replaced with MEM α containing 0.1% FBS and the BL extract. One hour later, oxidative stress was induced by adding hydrogen peroxide (H_2O_2; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) to a final concentration of 200 μM. The cells were cultured for an additional 1 h, washed with PBS, and incubated in normal growth medium for 24 h. Cell viability was assessed using CellTiter-Glo 2.0 (Promega, Madison, WI, USA).

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism version 10.6.0 (GraphPad Software, San Diego, CA, USA), and behavioral analyses were conducted in a blinded manner where applicable. Paired t-tests were used to evaluate within-group changes (pre- vs. post-feeding for rats; pre- vs. post-castration for mice). Unpaired t-tests were used for between-group comparisons (Control vs. BL) at the study endpoints, including sexual behavior parameters (week 4 for rats; week 16 for mice), EPM indices, and hormone levels. For the in vitro assays, multiple group comparisons against the H_2O_2-alone group were performed using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. A P-value < 0.05 was considered statistically significant.

Results

Effects of BL administration on sexual behavior in aged rats

We investigated the effects of dietary BL supplementation for four weeks on sexual behavior in aged rats. Comparing sexual parameters before and after the four-week period, no significant changes were observed in the frequency or latency of mounts and intromissions in the Control group (Fig. 1). An increasing trend in ejaculation latency was observed in both groups ($P < 0.1$). Notably, while a significant decline in ejaculation frequency was observed in the Control group, no such change was detected in the BL group, indicating the preservation of this function. Furthermore, quantification of plasma androgen levels after four weeks revealed no significant differences in testosterone or dihydrotestosterone concentrations between the Control and BL groups (Fig. 2). These findings suggest that BL administration may suppress the age-associated decline in sexual behavior in rats without affecting androgen levels.

Effects of BL administration *on sexual behavior in castrated mice*

Following our findings in the aged rat model suggesting that BL may attenuate the decline in sexual behavior, we sought to verify these effects using a castrated mouse model, which reproduces a state of androgen deficiency. This model is well established for studying symptoms resembling human male menopause, as the depletion of testosterone leads to a time-dependent decline in sexual behavior. In the present study, to validate this model and assess the efficacy of BL, sexually experienced male mice were administered a BL diet for 12 weeks. Subsequently, castration was performed, and the effects were investigated for an additional 4 weeks post-surgery.

Over the 12-week pre-castration period, no significant differences were observed in body weight or food intake between the Control and BL groups (Fig. 3). Similarly, at the evaluation point 4 weeks post-castration, no significant differences were detected in these parameters. Subsequently, the effects of BL intake on sexual behavior were investigated using the frequency and latency of mounts and intromissions as key indicators. Prior to castration, no significant differences were observed between the Control and BL groups in either the frequency or latency

of mounts and intromissions. However, regarding the castration-induced decline in sexual behavior, the BL group exhibited a significant shortening of intromission latency compared to the Control group at 2 weeks post-castration (week 14) (Fig. 4). No significant differences were observed in the other parameters, specifically mount frequency, mount latency, and intromission frequency.

Effects on anxiety-like symptoms associated with male menopause

The elevated plus maze (EPM) test was conducted at 4 weeks post-castration, a time point corresponding to the established androgen depletion, to investigate the effects of BL administration on anxiety-like and exploratory behaviors (Fig. 5). Compared to the Control group, the BL group spent a significantly longer time in the open arms, indicating an alleviation of anxiety-like behaviors (Fig. 5A). In contrast, no significant difference was observed between the two groups regarding the number of entries into the open arms (Fig. 5B). Furthermore, evaluation of the frequency of head-dips, an indicator of exploratory behavior, revealed that the BL group exhibited a significantly higher frequency compared to the Control group (Fig. 5C).

Investigation of the mechanism underlying the ameliorative effects of BL on male menopausal symptoms

The results from our animal studies suggested that BL supplementation exerts ameliorative effects on erectile function and anxiety-like symptoms in male menopausal models. Consequently, we investigated the cytoprotective effects of BL against oxidative stress, a known contributing factor to the etiology of male menopausal symptoms. Exposure of NB1RGB cells to oxidative stress induced by 200 μM hydrogen peroxide H_2O_2 resulted in significant cytotoxicity; however, the addition of BL extract inhibited cell death in a dose-dependent manner (Fig. 6). Compared to the H_2O_2-treated control group, the BL extract significantly preserved cell viability at concentrations of 500 and 1000 μg/mL.

Discussion

The present study investigated the efficacy of BL on sexual function and psychological symptoms using animal models that mimic the pathology of male menopause. Our investigation using an aged rat model suggested that BL supplementation can attenuate the age-associated decline in ejaculation frequency without affecting androgen levels. Based on these findings, the primary objective of this study was to elucidate the mechanism of action of BL using a castrated mouse model, an established model of male menopause. Given that the beneficial effects of BL on sexual function were observed after a 4-week administration period in the preceding aged rat study, we selected a corresponding 4-week post-castration period as the primary evaluation window for the mouse experiments. It is well documented in rodents that testosterone levels decline rapidly following castration, leading to a time-dependent decline in sexual behavior, including decreased frequencies of mounts, intromissions, and ejaculations, as well as increased latency to intromission (Clemens et al., 1988; Smith et al., 1977: Wee et al., 1988; Hull & Dominguez, 2007). In the present study, BL administration over a 12-week period showed no effects on body weight, food intake, or sexual behavior in intact (pre-castration) mice. These results suggest that BL does not disrupt physiological homeostasis in healthy individuals but exerts its modulatory effects specifically under conditions where physiological balance is compromised, such as in male menopausal symptoms.

In the present study utilizing the castrated mouse model, a significant shortening of intromission latency, a reliable index of erectile function, was observed in the BL group compared to the Control group. This finding implies a facilitation of the initiation of erection. Conversely, no significant effects were observed on mount frequency, an indicator of sexual motivation, or on intromission frequency, which reflects the maintenance of erectile function. Collectively, these results suggest that BL does not modulate sexual desire (libido) *per se*, but rather acts specifically on the physiological onset of erectile function.

This specific effect stands in contrast to the results obtained from our preceding aged rat model. In the rat model, BL administration suppressed the age-associated decline in ejaculation frequency but did not result in significant shortening of indicators such as

intromission latency. This difference in sensitivity is likely attributable to differences in the pathological profiles of the two models; whereas the primary vulnerability in the aged rat model lies in the decline of the endurance and recovery of sexual behavior, the castrated mouse model revealed the initiation of erection as the most sensitive indicator. The effect on erectile function in the castrated mouse model suggests a temporal aspect to the efficacy of BL. Significant shortening of intromission latency was observed at 2 weeks post-castration (week 14); however, this effect was not sustained at 4 weeks post-castration (week 16), when no significant difference was observed between groups. This suggests that the action of BL may not be chronic or maintenance effect, but rather that it acts particularly against the acute physiological stress induced by androgen deficiency, potentially delaying the progression of the pathology. The erection-specific nature of the observed effects supports the hypothesis that the mechanism of action is hormone-independent. Clinical trials evaluating the impact of BL administration on sexual function have similarly reported improvements in erectile function in middle-aged and elderly men, while revealing that circulating testosterone levels remained unaffected (Keishi et al., 2020). Generally, a decline in testosterone correlates with diminished sexual motivation, leading to a decrease in mount frequency (Clemens et al., 1988). However, in the present male menopause model, no change was observed in mount frequency—an index of sexual motivation. This strongly suggests that BL does not act *via* androgen receptors but rather directly targets downstream physiological processes, particularly vascular function. It is well established that the rapid decline in androgens due to castration exerts deleterious effects on both sexual function and mental state. Specifically, androgen depletion alters dopamine metabolism in the reward system of the central nervous system (including the nucleus accumbens and ventral tegmental area), which is involved in sexual motivation and pleasure, thereby suppressing behaviors such as mounting and ejaculation (Smith et al., 1977). Concurrently, it induces a state of systemic chronic inflammation and vascular endothelial dysfunction, leading to impaired vasodilatory function of the corpus cavernosum and potentially cerebrovascular disorders (Babcock et al., 2022; Kelly & Jones, 2013). These alterations contribute to the pathogenesis of both physical (erectile) and psychological (anxiety, depression) symptoms. In our study, the BL group exhibited a suppression of the prolongation of intromission latency—an indicator of

the initiation of erectile function—while simultaneously showing a significant alleviation of castration-induced anxiety-like behaviors in the elevated plus maze test. These results suggest that BL may target a common pathology within the vascular and neural systems induced by androgen deficiency, thereby concurrently improving both erectile function and psychiatric symptoms.

Erectile dysfunction (ED) and anxiety are hallmark symptoms of male menopause, and both are intricately linked to oxidative stress (Ferrini et al., 2017; Kaltsas et al., 2024). Erectile function is critically dependent on vascular endothelial integrity, and it is well established that ageing, lifestyle-related diseases, and androgen decline exacerbate oxidative stress within vascular tissues (Ferrini et al., 2017; Agarwal et al., 2006; Kaltsas et al., 2024). Excessive oxidative stress impairs the production and bioavailability of nitric oxide (NO), a vasodilator essential for erection, thereby acting as a primary driver of ED pathophysiology. Similarly, anxiety and psychological stress are known to elevate oxidative stress levels within the brain. Cerebral neurons are particularly vulnerable to oxidative stress due to their high oxygen consumption and lipid-rich composition (Bouayed et al., 2009). Oxidative damage to neurons disrupts the homeostasis of neurotransmitter systems, such as the GABAergic and serotonergic pathways, and this is postulated to manifest as psychiatric symptoms including anxiety and depression (Bouayed et al., 2009). In models of male menopause, the coexistence of physical and psychological symptoms associated with androgen deficiency suggests a common pathological basis driven by elevated oxidative stress.

Our *in vitro* assays demonstrated that BL exerts potent protective effects against oxidative stress-induced cytotoxicity triggered by agents such as hydrogen peroxide. The potent cytoprotective activity of BL powder may be attributed to its rich nutritional profile. BL is known to contain an abundance of antioxidant components, including polyphenols and flavonoids, as well as trace elements such as zinc and selenium, which serve as essential cofactors for endogenous antioxidant enzymes (Guiné et al., 2025; Abd El-Wahed et al., 2024; Inci et al., 2023). Therefore, these constituents may have acted synergistically to enhance cellular defence mechanisms. The findings from the *in vitro* assays provide a plausible mechanistic explanation for the improvements in erectile function and anxiety-like behaviors

observed *in vivo*. Specifically, this suggests that the antioxidant properties of BL directly suppressed oxidative stress induced by androgen deficiency within both vascular tissues and the nervous system. In vascular tissues, the mitigation of oxidative stress likely restored vascular endothelial function, thereby improving blood flow to the corpus cavernosum and enhancing erectile function. In parallel, within the nervous system, the suppression of oxidative stress may have contributed to neuroprotection and the maintenance of glial (astrocyte and microglia) homeostasis. This, in turn, would restore the balance of neurotransmitters, leading to the alleviation of anxiety symptoms. Clinical research further supports the notion that antioxidant intake alleviates anxiety symptoms. A meta-analysis integrating multiple randomized controlled trials demonstrated that antioxidant supplementation significantly improves anxiety states (Wang et al., 2022); this is consistent with the results of the present study, where the antioxidant action of BL contributed to the reduction of anxiety-like behaviors. Consequently, these findings suggest that BL exerts dual beneficial effects on both physical and psychological aspects *via* antioxidant-mediated vascular and neuroprotective mechanisms.

In conclusion, the present study demonstrates that BL improves erectile function and ameliorates anxiety-like behaviors in male menopausal models via a hormone-independent mechanism. We postulate that this therapeutic efficacy is mediated by vascular and neuroprotective effects resulting from the potent antioxidant activity of BL extracts. These findings provide a scientific basis for the development of novel functional foods and therapeutic strategies targeting the comorbidities of erectile dysfunction and psychiatric symptoms associated with ageing and oxidative stress. Furthermore, BL offers a promising alternative for patients for whom testosterone replacement therapy (TRT) is contraindicated or unsuitable. Future studies are warranted to identify the specific bioactive constituents of BL and to fully elucidate their molecular mechanisms of action.

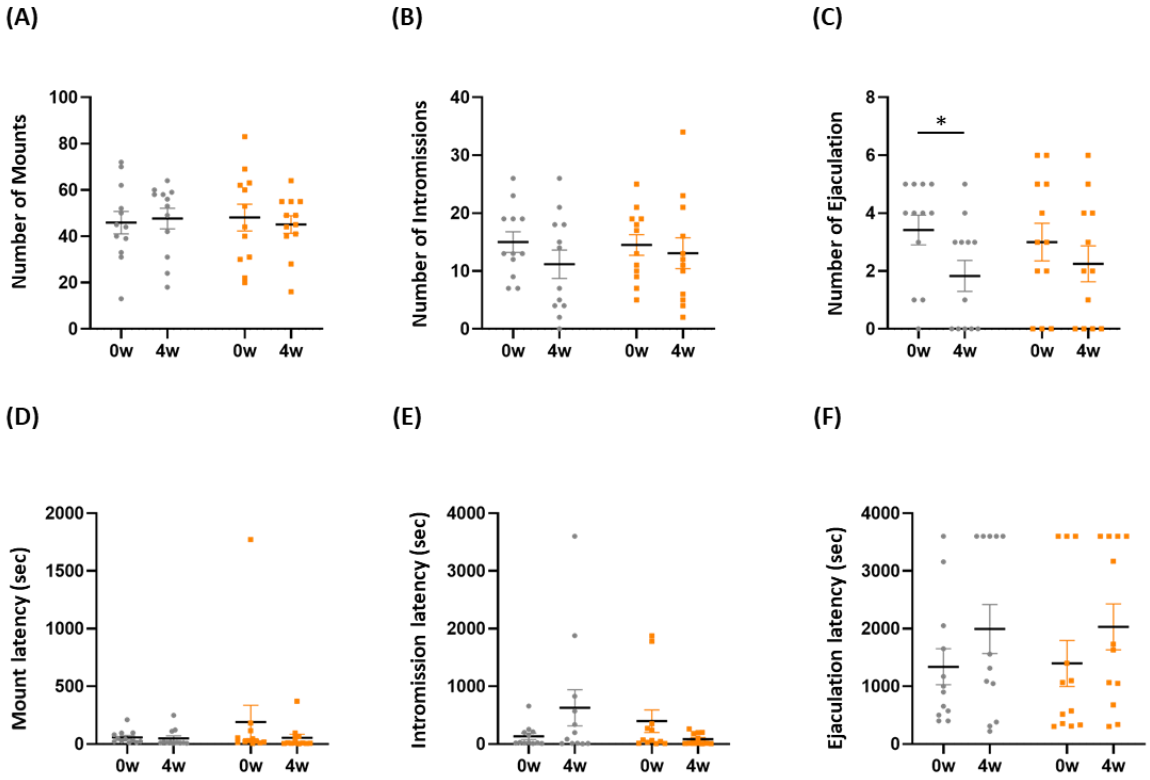

Fig. 1. Effects of administration of extracts Bee Larvae (BL) on sexual behavior parameters in aging rats.

Male Wistar-Imamichi rats (64 weeks old) were administered the Control (gray) or BL (orange) diet for 4 weeks. Sexual behavior was evaluated before and after the 4-week feeding period. (A) Mount frequency, (B) Intromission frequency, (C) Ejaculation frequency, (D) Mount latency, (E) Intromission latency, and (F) Ejaculation latency are shown. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance was determined using two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc correction. *$P < 0.05$.

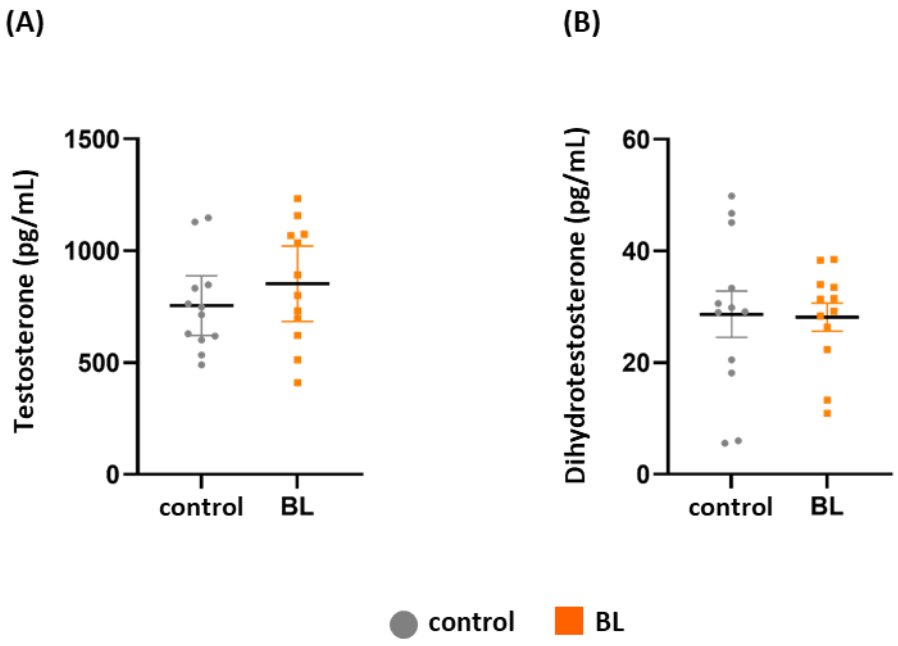

Fig. 2. Plasma androgen levels after four weeks of bee larvae (BL) extract administration in aging rats. Plasma concentrations of (A) Testosterone (T) and (B) Dihydrotestosterone (DHT) were quantified in aging male rats after 4 weeks of feeding the Control (gray) or BL (orange) diet. The hormones were quantified using the LC-MS/MS method. No significant differences were observed between the Control and BL groups for either T or DHT levels. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance was determined using Welch's t-test.

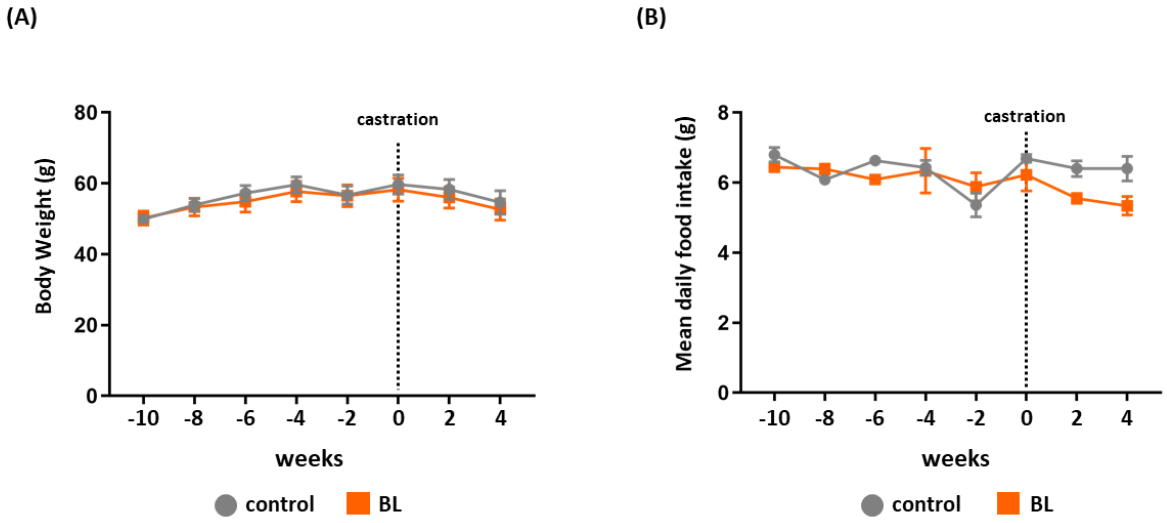

Fig. 3. Effects of bee larvae (BL) extract administration on body weight and food intake in mice.
Male mice were fed either a control diet or a BL diet for 12 weeks. The control group is represented by gray bars, while the BL-administered group is represented by orange bars. The arrow indicates the time of castration. (A) Animal body weight and (B) Food intake are shown. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance was determined using two-way analysis of variance (ANOVA).

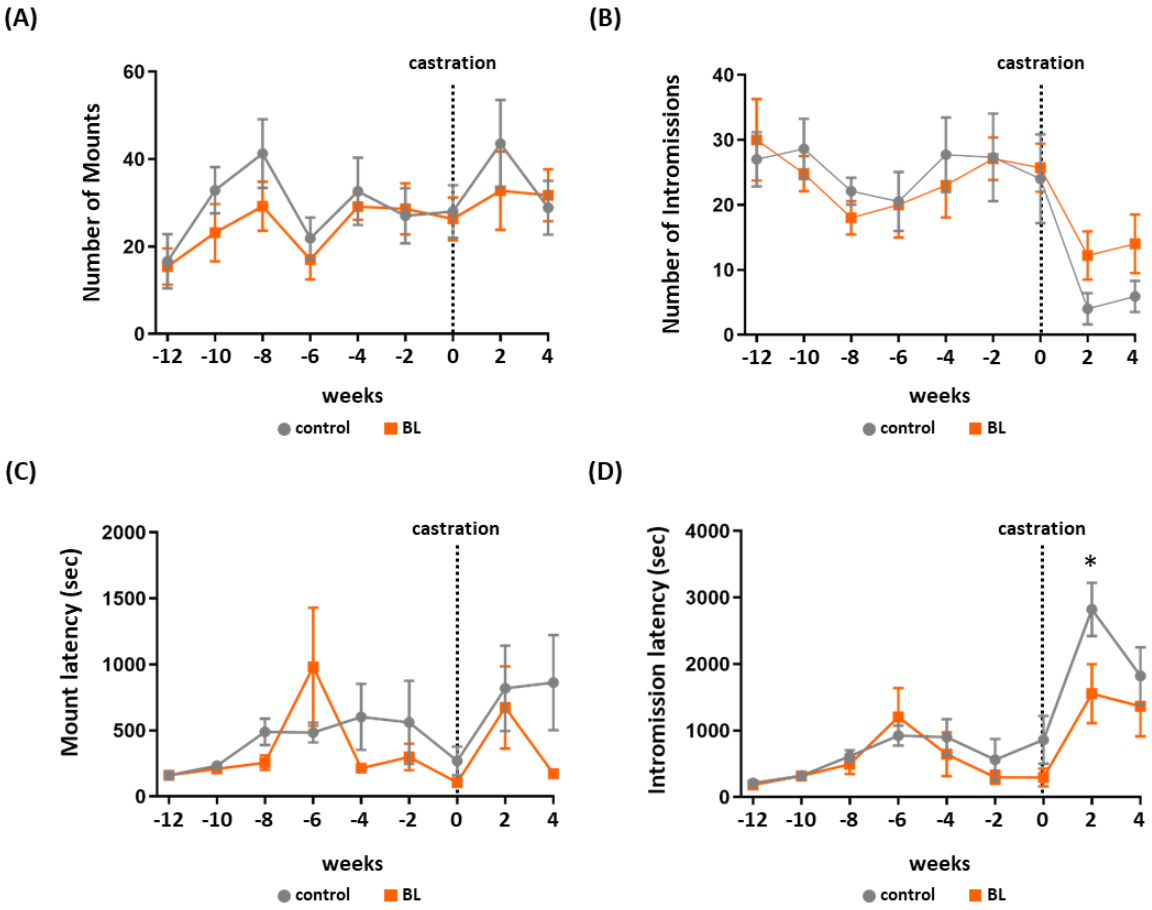

Fig. 4. Effects of bee larvae (BL) extract administration on sexual behavior in intact and castrated mice. Male mice were fed either a control diet or a BL diet for 12 weeks. Sexual behavior was evaluated before castration (weeks 0–12). Subsequently, mice were castrated, and sexual behavior was evaluated for an additional 4 weeks (weeks 12–16). The control group is represented by gray bars, while the BL-administered group is represented by orange bars. (A) Mount frequency, (B) Intromission frequency, (C) Mount latency, and (D) Intromission latency are shown. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance was determined using two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc correction. *$P<0.05$.

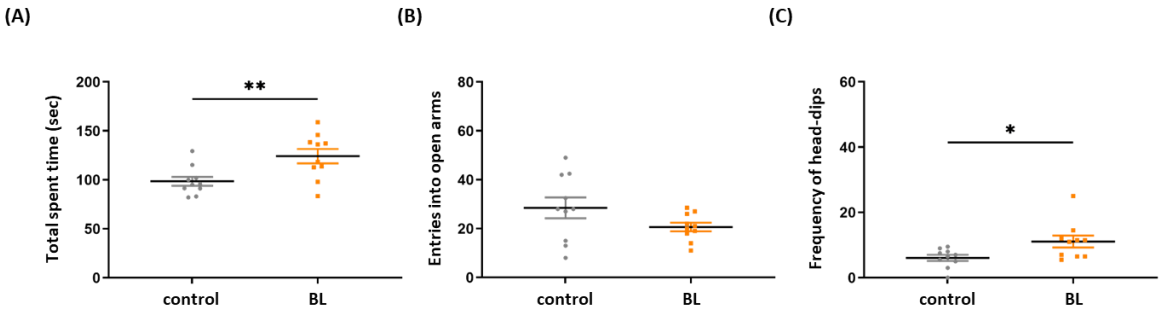

Fig. 5. Effects of bee larvae (BL) extract administration on anxiety-like and exploratory behaviors in castrated mice.

Mice were subjected to the elevated plus maze test at week 16 of the experiment. The figure shows (A) the total time spent in the open arms, (B) the number of entries into the open arms, and (C) the number of head-dips from the open arms. Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using an unpaired t-test. *$P < 0.05$, **$P < 0.01$.

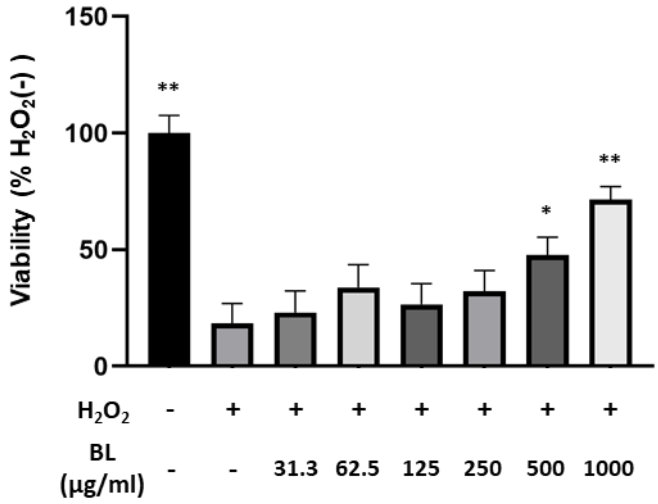

Fig. 6. Protective effect of administration of bee larvae (BL) extract against hydrogen peroxide-induced oxidative stress.

NB1RGB cells were pre-treated with BL for 24 hours. Subsequently, the cells were treated with 200 µM of hydrogen peroxide (H_2O_2) for 1 hour, washed with PBS, and cultured in fresh medium for an additional 24 hours. Cell viability was measured using the Cell Titer-Glo 2.0 assay. Data are presented as the mean ± standard error of the mean (SEM) (n = 5). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test for multiple comparisons against the H_2O_2 alone group. *$P < 0.05$, **$P<0.01$.

General discussion

The primary objective of this dissertation was to establish a scientific foundation for safe, non-hormonal therapeutic strategies targeting Late-Onset Hypogonadism (LOH) syndrome, a condition that significantly compromises the Quality of Life (QOL) of men in an increasingly super-aged society. By integrating neuroanatomical analyses in primates (Chapter 1) with pharmacological interventions using rodent models (Chapter 2), this study presents a comprehensive translational research framework that bridges the gap between basic neuroscience and clinical application. The findings from Chapter 1 demonstrated that the spinal gastrin-releasing peptide (GRP) system regulating sexual function is evolutionarily conserved in primates. This discovery provides a biological rationale affirming that functional insights derived from rodent models are not merely species-specific phenomena but are translatable and relevant to clinical contexts. Building upon this foundation, Chapter 2 demonstrated that Bee Larvae (BL) significantly improved erectile function and attenuated anxiety-like behaviours in an androgen-independent manner, effects primarily mediated through antioxidant mechanisms. Collectively, these findings suggest that protecting these evolutionarily conserved neural and vascular substrates from oxidative stress represents a viable alternative strategy to conventional testosterone replacement therapy (TRT).

Elucidating species differences in the central control mechanisms governing sexual function is imperative for the effective use of animal models. While it was well established that the gastrin-releasing peptide (GRP) system in the rodent lumbar spinal cord regulates erection and ejaculation, its existence in primates had remained unconfirmed (Sakamoto et al., 2008). In the present study, the identification of a male-dominant GRP system in the macaque spinal cord constitutes a pivotal factor bridging the gap between basic research and clinical application. The conservation of this sexually dimorphic neural control mechanism strongly suggests that the fundamental biological machinery governing male sexual function is shared across mammalian species. Therefore, this validation serves as a critical rationale supporting the hypothesis that therapeutic candidates evaluated in Chapter 2, such as BL—which demonstrated efficacy in rodent models—are likely to exert similar therapeutic effects in

humans via homologous neural circuits.

Testosterone Replacement Therapy (TRT), currently the mainstream treatment for LOH, is based on the principle of replenishing deficient hormones. Previous studies in rodents have demonstrated that GRP expression in the spinal cord is regulated in an androgen-dependent manner, and that the reduction of GRP expression levels following castration leads to a decline in male sexual function (Sakamoto et al., 2008). This suggests that the decline in sexual function in LOH pathology is underpinned by the functional downregulation of neural control mechanisms resulting from androgen deficiency. In contrast, Chapter 2 demonstrated that BL improved sexual function and anxiety-like behaviour via the regulation of oxidative stress, without restoring serum androgen levels. This functional recovery, achieved not by reversing the low-androgen state but via an androgen-independent pathway, implies a novel mechanism distinct from that of TRT. Therefore, the functional decline associated with LOH is attributable not only to the mere absence of androgen signalling but also to the cumulative damage inflicted on neural and vascular tissues by oxidative stress, which is exacerbated by androgen deficiency. Furthermore, it is plausible that BL exerts neuroprotective effects on the conserved neural substrates identified in Chapter 1. By mitigating oxidative stress, BL is postulated to maintain the integrity of the spinal GRP circuit and its downstream pathways, even under androgen-deprived conditions. This underscores the importance of an "organ-protective approach"—preserving existing neural and vascular structures—as a strategy comparable in significance to the manipulation of hormonal signalling.

Whilst the present study provides significant insights, several limitations must be acknowledged. Firstly, regarding the verification of the mechanism of action, although *in vitro* assays confirmed the potent antioxidant activity of BL, direct biochemical measurements of oxidative stress markers within *in vivo* target tissues—specifically, the spinal cord, corpus cavernosum, and specific brain regions—were not conducted. Consequently, the reduction of oxidative stress *in vivo* remains a logical inference, necessitating further empirical verification. Secondly, the specific active constituents contained within the enzyme-treated BL powder remain unidentified. The isolation and identification of the responsible bioactive compounds represent critical challenges for future quality control and potential pharmaceutical

development. Thirdly, serum testosterone levels were not directly quantified in the castrated mouse model used in this study. Although the hypothesis of hormone independence is supported by data from aged rats and clinical studies, the possibility of interactions with trace residual androgen levels cannot be entirely excluded. Finally, regarding the primate research in Chapter 1, while anatomical conservation was confirmed, functional verification—such as the pharmacological administration of GRP to macaques—was not performed due to ethical and technical constraints. Future studies addressing these gaps are essential to further enhance the translational value of this research.

In conclusion, this dissertation has elucidated that the neural basis governing male sexual function is conserved in primates and demonstrated that these functions can be maintained or improved through androgen-independent antioxidant intervention using natural products. This work represents a significant milestone in translational research, effectively linking the biological challenge of the species barrier between animal models and humans with the practical solution of "oxidative stress control." However, mechanisms warrant further investigation. Future basic research must verify, at histological and molecular levels, whether BL exerts direct antioxidant effects on the sexual function control neural network—centred on the conserved spinal GRP system—and thereby contributes to neuroprotection. Nevertheless, translating the concept of functional preservation via neuroprotection to the clinical setting presents challenges; direct histological verification of neuroprotection in humans is practically unfeasible due to its invasive nature. Consequently, future clinical trials should utilize non-invasive surrogate markers of systemic oxidative stress (such as urinary 8-OHdG) in conjunction with psychological evaluations of anxiety and sexual function. Demonstrating a correlation between the reduction of oxidative stress markers and the improvement of clinical scores would provide indirect but substantial validation for the neuroprotective mechanism proposed in this dissertation. The intervention strategy using Bee Larvae proposed herein offers a safe and sustainable approach to LOH management, particularly for patients for whom Testosterone Replacement Therapy (TRT) is contraindicated or unsuitable. By focusing on the maintenance of neural and vascular health, this study contributes to the "societal implementation" of scientifically grounded functional foods, ultimately fostering the extension

of healthy life expectancy and the enhancement of Quality of Life (QOL) for men in a super-aged society.

Acknowledgments

I would like to express my sincere gratitude to my supervisor, Prof. Hirotaka Sakamoto, for his continuous guidance and insightful discussions throughout this study. I also deeply appreciate Associate Prof. Takumi Oti for his extensive support and encouragement, which were essential to this work. My special thanks go to Prof. Tatsuya Sakamoto and Prof. Mayuko Hamada for their critical comments. I express my deepest gratitude to Dr. Nobuaki Okumura for his mentorship and continuous support since the establishment of his laboratory. Finally, I would like to thank all the members of the Laboratory of Behavioral Neuroendocrinology and the Functional Research Section of Yamada Bee Company Inc. for their cooperation.

Reference

Abd El-Wahed AA, Khalifa SAM, Aldahmash B, Zhang H, Du M, Zhao C, Tahir HE, Saeed A, Hussain H, Guo Z, El-Seedi HR (2024) Exploring the chemical constituents and nutritive potential of bee drone (Apilarnil): emphasis on antioxidant properties. Chemistry & Biodiversity 21 (2):e202400085. doi:10.1002/cbdv.202400085

Agarwal A, Nandipati KC, Sharma RK, Zippe CD, Raina R (2006) Role of oxidative stress in the pathophysiological mechanism of erectile dysfunction. Journal of Andrology 27 (3):335-347. doi:10.2164/jandrol.05136

Aoki M, Wakaoka Y, Hayashi H, Kuze B, Mizuta K, Ito Y (2012) Effect of Lyophilized Powder Made From Enzymolyzed Honeybee Larvae on Tinnitus-Related Symptoms, Hearing Levels, and Hypothalamus-Pituitary-Adrenal Axis-Related Hormones. Ear and Hearing 33 (3):430-436. doi:10.1097/AUD.0b013e318234f024

Aytaç IA, McKinlay JB, Krane RJ (1999) The likely worldwide increase in erectile dysfunction between 1995 and 2025 and some possible policy consequences. BJU International 84 (1):50-56. doi:10.1046/j.1464-410x.1999.00142.x

Babcock MC, DuBose LE, Witten TL, Stauffer BL, Hildreth KL, Schwartz RS, Kohrt WM, Moreau KL (2022) Oxidative stress and inflammation are associated with age-related endothelial dysfunction in men with low testosterone. The Journal of Clinical Endocrinology & Metabolism 107 (1):e500-e514. doi:10.1210/clinem/dgab715

Bhasin S, Brito JP, Cunningham GR, Hayes FJ, Hodis HN, Matsumoto AM, Snyder PJ, Swerdloff RS, Wu FC, Yialamas MA (2018) Testosterone Therapy in Men With Hypogonadism: An Endocrine Society Clinical Practice Guideline. The Journal of Clinical Endocrinology & Metabolism 103 (5):1715-1744. doi:10.1210/jc.2018-00229

Bouayed J, Rammal H, Soulimani R (2009) Oxidative stress and anxiety: relationship and cellular pathways. Oxidative Medicine and Cellular Longevity 2 (2):63-67. doi:10.4161/oxim.2.2.7944

Breedlove SM (1985) Hormonal control of the anatomical specificity of motoneuron-to-muscle innervation in rats. Science 227 (4692):1357-1359. doi:10.1126/science.3975621

Breedlove SM, Arnold AP (1980) Hormone accumulation in a sexually dimorphic motor nucleus of the rat spinal cord. Science 210 (4469):564-566. doi:10.1126/science.7423210

Breedlove SM, Arnold AP (1983a) Hormonal control of a developing neuromuscular system. I. Complete Demasculinization of the male rat spinal nucleus of the bulbocavernosus using the anti-androgen flutamide. The Journal of Neuroscience 3 (2):417-423. doi:10.1523/JNEUROSCI.03-02-00417.1983

Breedlove SM, Arnold AP (1983b) Hormonal control of a developing neuromuscular system. II. Sensitive periods for the androgen-induced masculinization of the rat spinal nucleus of the bulbocavernosus. The Journal of Neuroscience 3 (2):424-432. doi:10.1523/JNEUROSCI.03-02-00424.1983

Briganti A, Salonia A, Gallina A, Saccà A, Montorsi P, Rigatti P, Montorsi F (2005) Drug Insight: oral phosphodiesterase type 5 inhibitors for erectile dysfunction. Nature Clinical Practice Urology 2 (5):239-247. doi:10.1038/ncpuro0186

Brown DJ, Hill ST, Baker HW (2006) Male fertility and sexual function after spinal cord injury. Progress in Brain Research 152:427-439. doi:10.1016/S0079-6123(05)52029-6

Clemens LG, Wee BEF, Weaver DR, Roy EJ, Goldman BD, Rakerd B (1988) Retention of masculine sexual behavior following castration in male B6D2F1 mice. Physiology & Behavior 42 (1):69-76. doi:10.1016/0031-9384(88)90262-4

Corona G, Rastrelli G, Maggi M (2013) Diagnosis and treatment of late-onset hypogonadism: systematic review and meta-analysis of TRT outcomes. Best Practice & Research Clinical Endocrinology & Metabolism 27 (4):557-579. doi:10.1016/j.beem.2013.05.002

Correia AS, Cardoso A, Vale N (2023) Oxidative stress in depression: the link with the stress response, neuroinflammation, serotonin, neurogenesis and synaptic plasticity.

Antioxidants 12 (2):470. doi:10.3390/antiox12020470

Dobberfuhl AD, Oti T, Sakamoto H, Marson L (2014) Identification of CNS neurons innervating the levator ani and ventral bulbospongiosus muscles in male rats. The Journal of Sexual Medicine 11 (11):2706-2714. doi:10.1111/jsm.12418

Espinosa-Medina I, Saha O, Boismoreau F, Chettouh Z, Rossi F, Richardson WD, Brunet JF (2016) The sacral autonomic outflow is sympathetic. Science 354 (6314):893-897. doi:10.1126/science.aah5454

Fedoce AG, Ferreira F, Bota RG, Bonet-Costa V, Sun PY, Davies KJA (2018) The role of oxidative stress in anxiety disorder: cause or consequence? Free Radical Research 52 (7):737-750. doi:10.1080/10715762.2018.1475733

Ferrini MG, Gonzalez-Cadavid NF, Rajfer J (2017) Aging related erectile dysfunction—potential mechanism to halt or delay its onset. Translational Andrology and Urology 6 (1):20-27. doi:10.21037/tau.2016.11.18

Finke MD (2005) Nutrient Composition of Bee Brood and its Potential as Human Food. Ecology of Food and Nutrition 44 (4):257-270. doi:10.1080/03670240500187278

Forger NG, Breedlove SM (1986) Sexual dimorphism in human and canine spinal cord: role of early androgen. Proceedings of the National Academy of Sciences of the United States of America 83 (19):7527-7531. doi:10.1073/pnas.83.19.7527

Fraser M, McDonald TJ, Spindel ER, Fahy M, Hill D, Challis JR (1994) Gastrin-releasing peptide is produced in the pregnant ovine uterus. Endocrinology 135 (6):2440-2445. doi:10.1210/endo.135.6.7988429

Gibbs RA, Rogers J, Katze MG, Bumgarner R, Weinstock GM, Mardis ER, Remington KA, Strausberg RL, Venter JC, Wilson RK, et al. (2007) Evolutionary and biomedical insights from the rhesus macaque genome. Science 316 (5822):222-234. doi:10.1126/science.1139247

Guiné RPF, Florença SG, Barroca MJ, Costa CA (2025) Bee Brood as a Food for Human Consumption: An Integrative Review of Phytochemical and Nutritional Composition.

Insects 16 (1):796. doi:10.3390/insects16080796

Guiné RPF, Florença SG, Correia PMR, Anjos O, Coelho C, Costa CA (2022) Honey bee (Apis mellifera L.) broods: Composition, technology and gastronomic applicability. Foods 11 (18):2750. doi:10.3390/foods11182750

Hehemann MC, Kashanian JA (2016) Can lifestyle modification affect men's erectile function? Translational Andrology and Urology 5 (2):187-194. doi:10.21037/tau.2016.02.05

Heimer L, Larsson K (1967) Mating behavior of male rats after olfactory bulb lesions. Physiology & Behavior 2 (2):207-209. doi:10.1016/0031-9384(67)90035-2

Hokao R, Ito T, Noguchi J, Wakafuji Y, Agematsu Y, Saito TR, Takahashi KW, Imamichi T (1992) Effect of Age on the Copulatory Behavior of the Male Rat. Experimental Animals 41 (3):259-268. doi:10.1538/expanim1978.41.3_259

Hull EM, Dominguez JM (2007) Sexual behavior in male rodents. Hormones and Behavior 52 (1):45-55. doi:10.1016/j.yhbeh.2007.03.030

Inci H, Izol E, Yilmaz MA, Ilkaya M, Bingöl Z, Gülçin I (2023) Comprehensive phytochemical content by LC/MS/MS and anticholinergic, antiglaucoma, antiepilepsy, and antioxidant activity of apilarnil (drone larvae). Chemistry & Biodiversity 20 (7):e202300654. doi:10.1002/cbdv.202300654

Kaltsas A, Zikopoulos A, Dimitriadis F, Sheshi D, Politis M, Moustakli E, Symeonidis EN, Chrisofos M, Sofikitis N, Zachariou A (2024) Oxidative Stress and Erectile Dysfunction: Pathophysiology, Impacts, and Potential Treatments. Current Issues in Molecular Biology 46 (8):8807-8834. doi:10.3390/cimb46080521

Katayama N, Oti T, Takanami K, Sakamoto T, Sakamoto H (2016) Postnatal development of the gastrin-releasing peptide system in the lumbosacral spinal cord controlling male reproductive function in rats. Proceedings of the Japan Academy, Series B 92 (2):69-75. doi:10.2183/pjab.92.69

Keishi T, Asama T, Okumura N, Yamaki A, Miyawaki H (2020) Beneficial Effect of Protease-digested Bee Larvae on Aging Males' Symptoms, Erectile Function and Related Health

Conditions. Japanese Pharmacology and Therapeutics 48 (6):1013-1023

Kelly DM, Jones TH (2013) Testosterone: a vascular hormone in health and disease. Journal of Endocrinology 217 (3):R47-R71. doi:10.1530/JOE-12-0582

Lebacq-Verheyden AM, Krystal G, Sartor O, Way J, Battey JF (1988) The rat prepro gastrin releasing peptide gene is transcribed from two initiation sites in the brain. Molecular Endocrinology 2 (6):556-563. doi:10.1210/mend-2-6-556

Lemaire S, Trifaró JM, Chouinard L, Cécyre D, Dessureault J, Mercier P, Guevara A (1989) Structural identification, subcellular localization and secretion of bovine adrenomedullary neuromedin C [GRP-(18-27)]. Peptides 10 (2):355-360. doi:10.1016/0196-9781(89)90043-0

Li K, Sun S, Kageyama M, Xiao L, Xing G, Gao R, You F, Fu X, Zhang Z (2020) Evaluation of the immunomodulatory and anti-inflammatory activities of honey bee larva powder. Journal of Medicinal Food 23 (7):772-782. doi:10.1089/jmf.2019.4554

Matsuda K, Sakamoto H, Kawata M (2008) Androgen action in the brain and spinal cord for the regulation of male sexual behaviors. Current Opinion in Pharmacology 8 (6):747-751. doi:10.1016/j.coph.2008.08.010

McDonald TJ, Jornvall H, Nilsson G, Vagne M, Ghatei M, Bloom SR, Mutt V (1979) Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. Biochemical and Biophysical Research Communications 90 (1):227-233. doi:10.1016/0006-291X(79)91614-0

Minamino N, Kangawa K, Matsuo H (1984) Neuromedin C: a bombesin-like peptide identified in porcine spinal cord. Biochemical and Biophysical Research Communications 119 (1):14-20. doi:10.1016/0006-291x(84)91611-5

Moraru D, Alexa E, Cocan I, Obiștioiu D, Radulov I, Simiz E, Berbecea A, Grozea A, Dragomirescu M, Vintilă T, Pătruică S (2024) Chemical Characterization and Antioxidant Activity of Apilarnil, Royal Jelly, and Propolis Collected in Banat Region, Romania. Applied Sciences 14 (3):1242. doi:10.3390/app14031242

Morgan C, Nadelhaft I, de Groat WC (1981) The distribution of visceral primary afferents from the pelvic nerve to Lissauer's tract and the spinal gray matter and its relationship to the sacral parasympathetic nucleus. The Journal of Comparative Neurology 201 (3):415-440. doi:10.1002/cne.902010308

Morris JA, Jordan CL, Breedlove SM (2004) Sexual differentiation of the vertebrate nervous system. Nature Neuroscience 7 (10):1034-1039. doi:10.1038/nn1325

Nakagawa S (1980) Onuf's nucleus of the sacral cord in a South American monkey (Saimiri): its location and bilateral cortical input from area 4. Brain Research 191 (2):337-344. doi:10.1016/0006-8993(80)91285-8

Onufrowicz B (1899) Notes on the arrangement and function of the cell groups in the sacral region of the spinal cord. The Journal of Nervous and Mental Disease 26 (8):498-504.

Oti T, Satoh K, Saito K, Murata K, Kawata M, Sakamoto T, Sakamoto H (2012) Three-dimensional evaluation of the spinal local neural network revealed by the high-voltage electron microscopy: a double immunohistochemical study. Histochemistry and Cell Biology 1358 (4):693-697. doi:10.1007/s00418-012-0976-6

Oti T, Takanami K, Katayama N, Edey T, Satoh K, Sakamoto T, Sakamoto H (2016) Perinatal testosterone exposure is critical for the development of the male-specific sexually dimorphic gastrin-releasing peptide system in the lumbosacral spinal cord that mediates erection and ejaculation. Biology of Sex Differences 7:4. doi:10.1186/s13293-016-0058-x

Reeve JR Jr, Walsh JH, Chew P, Clark B, Hawke D, Shively JE (1983) Amino acid sequences of three bombesin-like peptides from canine intestine extracts. The Journal of Biological Chemistry 258 (9):5582-5588. doi:10.1016/S0021-9258(20)81930-9

Sakamoto H (2012) Brain-spinal cord neural circuits controlling male sexual function and behavior. Neuroscience Research 72 (2):103-116. doi:10.1016/j.neures.2011.11.002

Sakamoto H (2014) Sexually dimorphic nuclei in the spinal cord control male sexual functions. Frontiers in Neuroscience 8:184. doi:10.3389/fnins.2014.00184

Sakamoto H, Arii T, Kawata M (2010) High-voltage electron microscopy reveals direct synaptic inputs from a spinal gastrin-releasing peptide system to neurons of the spinal nucleus of bulbocavernosus. Endocrinology 151 (1):417-421. doi:10.1210/en.2009-0485

Sakamoto H, Matsuda K, Zuloaga DG, Hongu H, Wada E, Wada K, Kawata M (2008) Sexually dimorphic gastrin releasing peptide system in the spinal cord controls male reproductive functions. Nature Neuroscience 11 (6):634-636. doi:10.1038/nn.2126

Sakamoto H, Saito K, Marie-Luce C, Raskin K, Oti T, Satoh K, Tamura K, Sakamoto T, Mhaouty-Kodja S (2014) Androgen regulates development of the sexually dimorphic gastrin-releasing peptide neuron system in the lumbar spinal cord: Evidence from a mouse line lacking androgen receptor in the nervous system. Neuroscience Letters 558:109-114. doi:10.1016/j.neulet.2013.10.068

Sakamoto H, Takanami K, Zuloaga DG, Matsuda K, Jordan CL, Breedlove SM, Kawata M (2009) Androgen regulates the sexually dimorphic gastrin-releasing peptide system in the lumbar spinal cord that mediates male sexual function. Endocrinology 150 (8):3672-3679. doi:10.1210/en.2008-1791

Sato M, Mizuno N, Konishi A (1978) Localization of motoneurons innervating perineal muscles: a HRP study in cat. Brain Research 140 (1):149-154. doi:10.1016/0006-8993(78)90244-5

Satoh K, Takanami K, Murata K, Kawata M, Sakamoto T, Sakamoto H (2015a) Effective synaptome analysis of itch-mediating neurons in the spinal cord: A novel immunohis3tochemical methodology using high-voltage electron microscopy. Neuroscience Letters 599:86-91. doi:10.1016/j.neulet.2015.05.031

Satoh K, Takanami K, Murata K, Kawata M, Sakamoto T, Sakamoto H (2015b) Three-dimensional visualization of multiple synapses in thick sections using high-voltage electron microscopy in the rat spinal cord. Data in Brief 4:566-570. doi:10.1016/j.dib.2015.07.005

Sengelaub DR, Forger NG (2008) The spinal nucleus of the bulbocavernosus: firsts in

androgen-dependent neural sex differences. Hormones and Behavior 53 (5):596-612. doi:10.1016/j.yhbeh.2007.11.008

Shaw C, Thim L, Conlon JM (1987) Primary structure and tissue distribution of guinea pig gastrin-releasing peptide. Journal of Neurochemistry 49 (5):1348-1354. doi:10.1111/j.1471-4159.1987.tb00998.x

Sipski ML (1998) Sexual functioning in the spinal cord injured. International Journal of Impotence Research 10 (Suppl 2):S128-130.

Smith ER, Damassa DA, Davidson JM (1977) Plasma testosterone and sexual behavior following intracerebral implantation of testosterone propionate in the castrated male rat. Hormones and Behavior 8 (1):77-87. doi:10.1016/0018-506x(77)90022-8

Smith ER, Stefanick ML, Clark JT, Davidson JM (1992) Hormones and sexual behavior in relationship to aging in male rats. Hormones and Behavior 26 (1):110-135. doi:10.1016/0018-506x(92)90035-t

Spindel ER, Chin WW, Price J, Rees LH, Besser GM (1984) Cloning and characterization of cDNAs encoding human gastrin-releasing peptide. Proceedings of the National Academy of Sciences of the United States of America 81 (18):5699-5703. doi:10.1073/pnas.81.18.5699

Sun YG, Chen ZF (2007) A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. Nature 448 (7154):700-703. doi:10.1038/nature06029

Takanami K, Sakamoto H (2014) The gastrin-releasing peptide receptor (GRPR) in the spinal cord as a novel pharmacological target. Current Neuropharmacology 12 (5):434-443. doi:10.2174/1570159X12666140923201432

Takanami K, Sakamoto H, Matsuda K, Satoh K, Tanida T, Yamada S, Inoue K, Oti T, Sakamoto T, Kawata M (2014) Distribution of gastrin-releasing peptide in the rat trigeminal and spinal somatosensory systems. The Journal of Comparative Neurology 522 (8):1858-1873. doi:10.1002/cne.23506

Tamura K, Kobayashi Y, Hirooka A, Takanami K, Oti T, Jogahara T, Oda S, Sakamoto T,

Sakamoto H (2017) Identification of the sexually dimorphic gastrin-releasing peptide system in the lumbosacral spinal cord that controls male reproductive function in the mouse and Asian house musk shrew (Suncus murinus). The Journal of Comparative Neurology 525 (7):1586-1598. doi:10.1002/cne.24138

Vizzard MA, Erdman SL, de Groat WC (1995) Increased expression of neuronal nitric oxide synthase (NOS) in visceral neurons after nerve injury. The Journal of Neuroscience 15 (5):4033-4045. doi:10.1523/JNEUROSCI.15-05-04033.1995

Wang H, Jin M, Xie M, Yang Y, Xue F, Li W, Zhang M, Li Z, Li X, Jia N, Liu Y, Cui X, Hu G, Dong L, Wang G, Yu Q (2022) Protective Role of Antioxidant Supplementation for Depression and Anxiety: A Meta-analysis of Randomized Clinical Trials. Journal of Affective Disorders 319:293-300. doi:10.1016/j.jad.2022.11.072

Wee BEF, Weaver DR, Clemens LG (1988) Hormonal restoration of masculine sexual behavior in long-term castrated B6D2F1 mice. Physiology & Behavior 42 (1):77-82. doi:10.1016/0031-9384(88)90263-6