FULL PAPER *Theriogenology*

Unpredictable Chronic Stress-Induced Reproductive Suppression Associated with the Decrease of Kisspeptin Immunoreactivity in Male Mice

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ABSTRACT. Environmental stress affects various parts of mammals typically through the circulation of stress hormones. It has been identified as one of the possible reasons for male reproductive difficulties, but the complex mechanisms responsible for stress-induced reproductive suppression are poorly understood. Here, we examined the relationship between chronic environmental stress and hypothalamic kisspeptin, a recently discovered upstream regulator of the reproductive endocrine feedback system. We studied male mice under an unpredictable chronic stress procedure to replicate the situation of animals under chronic stress. Histological and immunohistochemical analyses were performed focusing on kisspeptin neurons in the arcuate hypothalamic nucleus (ARC) and DNA fragmented cells in seminiferous tubules. Although the ARC was not morphologically altered in either the stressed or non-stressed group, granular kisspeptin immunoreactivities decreased slightly in the stress group. In the testes of the stress group, several signs of testicular degeneration were observed, including increased numbers of ssDNA-positive cells per seminiferous tubule, thinning, vacuoled seminiferous epithelia and multinucleated giant cells. The decreases in kisspeptin in the stress group might be due to other hypothalamic peptides, such as corticotropin-releasing hormone and leptin, whose receptors are known to coexpress in the ARC. In addition, environmental stress directly and indirectly affects testicular function through stress hormones and gonadotropins. In summary, our findings enhance the understanding of stress-induced reproductive suppression possibly mediated by kisspeptin in the ARC.

KEY WORDS: environmental stress, kisspeptin, male infertility, reproductive function, testis

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Since the beginning of life on earth, living things have been surrounded by various types of stress from their environment — physical, chemical, biological, psychological and social stress — and they have survived by adapting to the stress in order to maintain homeostasis. After Hans Selye defined stress as the "non-specific response of the body to any demand for change" in the 1920s, the effects of stress were examined from several different angles. It is now well known environmental stress has various adverse effects on mammals including on the immune system [16], the autonomic nervous system [32] and the endocrine system [13], mainly by activating the hypothalamic-pituitary-adrenal (HPA) axis and the subsequent release of several stress hormones.

Reproductive function is a potential target of environmental stress through the reproductive endocrine feedback system, which is called the hypothalamic-pituitary-gonadal (HPG) axis [34, 44]. In fact, hypothalamic gonadotropin-releasing hormone (GnRH) provokes the pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which stimulate Leydig cells and Sertoli cells in males and granulosa cells and follicles in females to release sex steroids and the gonadal hormone inhibin. The stress hormones cause multiple suppressions of the HPG axis. For example, glucocorticoids from the adrenal cortex and corticotropin-releasing hormone (CRH) from the hypothalamus inhibit GnRH, LH and FSH secretion [35, 47].

The feedback system of the HPG axis is not fully understood, because there are no sex steroid receptors on GnRH neurons. Kisspeptin, a novel neuropeptide encoded by the *Kiss1* gene, acts as an upstream regulator of the HPG axis and was identified as an endogenous ligand of G-protein-coupled receptor 54 (GPR54/Kiss1r) in 2001 [19, 31]. In studies of *Kiss1*- or *Kiss1r*- deficient mice [20, 37] and patients with idiopathic hypogonadotropic hypogonadism who have a mutation of *KISS1R* [8], kisspeptin proved to be critical to the onset of puberty [14] and to have a potent stimulatory effect of GnRH. Kisspeptin neurons, which express sex steroid receptors, are localized mainly in the two hypothalamic regions. They project fibers to the medial preoptic area (mPOA), which contains most of the GnRH neurons.

First, kisspeptin neurons in the anteroventral periventricular

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nucleus (AVPV), which is known as the sexually dimorphic region, regulate the positive feedback of sex steroids and are rarely observed in males, unlike females. Second, kisspeptin neurons in the arcuate hypothalamic nucleus (ARC) mediate the negative feedback in both males and females. Upstream signalings of the HPG axis such as that provided by kisspeptin are currently attracting much attention in the broad biological area.

Morgan and Tromborg [27] pointed out that zoo animals are subjected to various stressors and that many zoo animals are experiencing reproductive difficulties. In particular, the levels of fecal glucocorticoids and estrogen in captive cheetahs are significantly higher than those of cheetahs in the wild [42]. Seminiferous degenerations frequently occur in captive gorillas [9]. Similarly, the infertility rates in humans have also gradually increased, caused in part by drastic decreases in sperm quality [3, 15]. Environmental stress is one of the possible reasons for these infertility problems [38], but the mechanisms underlying environmental stress-associated reproductive failure are still unclear.

The purpose of the present study was to investigate the central and peripheral reproductive effects of environmental stress in mature male mice. We applied an unpredictable chronic stress procedure to adult male mice, which are often used in the assessment of antidepressant medications [25]. We performed histological and immunohistochemical analyses focusing on the expression of hypothalamic kisspeptin to understand the comprehensive effect of environmental stress.

MATERIALS AND METHODS

Experimental animals: Eight-week-old male C57BL/6NCrSlc mice were purchased from Japan SLC (Hamamatsu, Japan). We divided the group of mice into control and stress groups (n=6 mice each) by weight, with no significant weight difference between the groups. Each group was maintained in a 40.5 × 20.5 × 18.5 cm cage under controlled conditions of temperature (22 ± 2°C) and humidity (50 ± 10%) on a 12-hr light/dark cycle. All mice had *ad libitum* access to food (Labo MR Stock: Nihon Nosan Co., Yokohama, Japan) and filtered water. This study was approved by the Institutional Animal Care and Use Committee (Permission number: 24-10-03) and carried out according to the Kobe University Animal Experimental Regulation.

Stress procedure: All mice were allowed to acclimate to their home cages for 1 week prior to the initiation of experiments. The stress-group mice were subjected to different stressors as described by Molina *et al*. [26] and Murua *et al*. [28] with some modifications. The following six stressors were used: 5 min forced swimming in water at room temperature (RT), 24 hr food and water deprivation, continuous overnight illumination, 30 min horizontal cage shaking (80 rpm), 24 hr switching of cagemates (being housed with another mouse) and 24 hr wet bedding. To maximize the unpredictability of this paradigm, the stress group mice were randomly subjected to one stressor per day at varying times for 4 weeks. The control-group mice were kept in the same environment and experimental period without being subjected to any of the stressors. All mice were weighed once a week throughout the 4 weeks experimental period.

Tissue preparation: On the day following the last stress exposure, all mice were deeply anesthetized with diethyl ether and transcardially perfused with 0.9% normal saline, followed by perfusion with ice-cold 4% paraformaldehyde in phosphate buffer. The brains and testes were excised, weighed, postfixed with the same fixative overnight at 4°C, dehydrated through a graded series of ethanol followed by xylene and embedded in paraffin. Serial sections were then cut (testes, 4 μm thick; brains, 10 μm thick), and each tissue section was mounted on a slide glass (Platinum; Matsunami Glass, Kishiwada, Japan) for the following steps.

Histological and immunohistochemical analyses: After deparaffinization and hydration, for a general histological analysis, testis sections were stained with hematoxylin and eosin (HE; Merck KGaA, Darmstadt, Germany) following the manufacturer's instructions, and brain sections were stained with 0.1% cresyl fast violet solution, which visualizes Nissl's substance.

To detect kisspeptin immunoreactivity in the hypothalamus and the localization of DNA fragmented cells in the testis, we performed the following immunohistochemistry protocol. The brain sections were incubated in 25 μg/m*l* Proteinase K (Takara Bio, Otsu, Japan) at 37°C for 10 min for antigen retrieval. The sections were immersed in absolute methanol and 0.5% H_2O_2 for 30 min, respectively, at RT to quench the endogenous peroxidase activity. The sections were incubated with Blocking One Histo (Nacalai Tesque, Kyoto, Japan) for 1 hr at RT for protein blocking and then incubated with the rabbit polyclonal AC#566 kisspeptin antibody [10] (a generous gift from Dr. A. Caraty, INRA, France) diluted 1:16,000 and the rabbit polyclonal antibody against single-stranded DNA (ssDNA: 18731; IBL Co., Fujioka, Japan) diluted 1:1,000 in phosphate buffered saline (PBS) for 18 hr at 4°C.

After being washed with PBS, the sections were reacted with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer in tris (hydroxymethyl) aminomethane-HCl buffer (EnVision+; Dako, Glostrup, Denmark) for 1 hr at RT. Immunoreactivity was then detected by incubation with 3,3′-diaminobenzidine solution (EnVision+ kit/HRP[DAB], Dako). The sections were then rinsed with distilled water and counterstained lightly with hematoxylin solution for 1 min. Next, the sections were placed in a graded series of ethanol, dehydrated with absolute ethanol, cleared by xylene and coverslipped with Eukitt (O. Kindler GmbH, Freiburg, Germany).

Immunohistoplanimetry: To analyze the frequency of DNA fragmented cells in the testes, we randomly selected three sections (200 seminiferous tubules) from all mice and counted the number of DNA fragmented cells located in the seminiferous tubules. The numbers per seminiferous tubule of each group were then calculated.

Statistical analysis: Statistical analyses were performed with Excel Statistics 2012 (SSRI version 1.00, Tokyo, Japan).

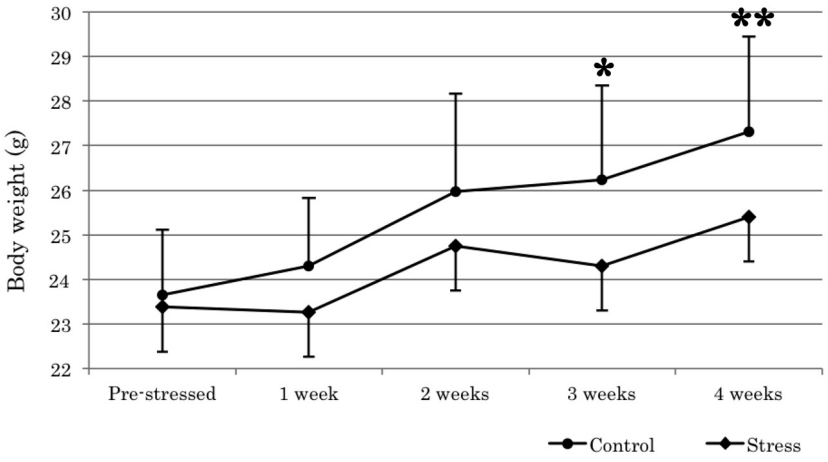

Fig. 1. Unpredictable chronic stress suppressed the body weight gain of the mice. All mice gained weight gradually throughout the 4 weeks experimental period, but there were significant differences between the body weights of the control and stress groups at 3 weeks and 4 weeks. Values are presented as the mean ± SD (n=6 mice each). *P<0.05; **P<0.01.

The numbers of ssDNA-positive cells and the brain and testis weights were analyzed using the independent two-tailed *t*-test and the Mann-Whitney U-test. The results were considered significant when the *P*-value was less than 0.05.

RESULTS

Body and organ weights: The weekly changes of the body weights of the mice are shown in Fig. 1. The body weights of the stress group at 3 weeks (24.30 ± 1.33 g) and 4 weeks (25.41 ± 0.81 g) were significantly lower than those of the control group (3 weeks, 26.24 ± 2.11 g; P<0.05; 4 weeks, 27.32 ± 2.15 g; P<0.01). The brain and testis weights at 4 weeks are shown in Fig. 2. Although the testis weights of the stress group decreased significantly compared to those of the control group (Fig. 2A, control: 100 ± 4 mg; stress: 92 ± 11 mg; P<0.01), no significant between-group difference was observed in the brain weight (Fig. 2B, control: 462 ± 18 mg; stress: 452 ± 22 mg).

Histological and immunohistochemical findings of testis and brain: In the control group, the testes showed normal cell arrangement and spermatogenesis in the seminiferous tubules (Fig. 3A). In the stress group, the testes showed thin and vacuoled germ cell layers and enlarged tubular lumen due to the decrease in the number of germ cells, and multinucleated giant cells caused by the degeneration of germ cells were observed (Fig. 3B). There was no histological difference between the Leydig cells in the testicular interstitial tissues of the stress group and those of the control group. The localization of DNA fragmented cells was visualized (Fig. 3C and 3D). Only a few ssDNA-positive spermatogonia were detected in the control group testes, but some aggregations of ssDNA-positive spermatogonia and spermatocytes surrounding Sertoli cells were observed in the stress group testes. The numbers of ssDNA-positive spermatogonia and spermatocytes per seminiferous tubule of the stress group increased significantly relative to those of the control group (Fig. 3E, control: 0.302 ± 0.127; stress: 0.554 ± 0.447; P<0.05).

In the cresyl fast violet-stained sections, the ARCs were composed of round or ovoid cells with short dendrites. There was no marked morphological difference in the ARC between the control and stress groups (Fig. 4A and 4B). The kisspeptin immunoreactivity was granularly detected in axons and dendrites over the entire area of the ARC in both groups (Fig. 4C–4F). The numbers of kisspeptin neurons were uncountable because of the unclear boundary of the individual neurons, but we observed a decreasing trend of granular kisspeptin immunoreactivity in the ARC of the stress group.

DISCUSSION

We examined the hypothalamic and testicular effects of chronic environmental stress on male reproductive function in mice. Stimulatory effects of kisspeptin on gonadotropins and GnRH were well confirmed in studies using the administration of the synthetic peptide and its antagonist to rodents [6, 11, 21]. However, there are few reports regarding whether kisspeptin is involved in stress-induced reproductive suppression. Most kisspeptin neurons in the ARC colocalize with corticotropin-releasing hormone receptor 1 (CRH-R1) [41], suggesting that the stress hormone could disturb the functioning of the kisspeptin neurons directly. Three different stress paradigms known to inhibit LH pulsed secretion also suppressed both *Kiss1* and *Kiss1r* mRNA expression [17], whereas alterations of kisspeptin at the peptide level have not been previously reported, to the best of our knowledge.

In the present study, kisspeptin in the ARC was immunohistochemically visualized as diffuse granule-like particles. Neuropeptides, like kisspeptin, are synthesized as precursors in the endoplasmic reticulum in the neuron cell

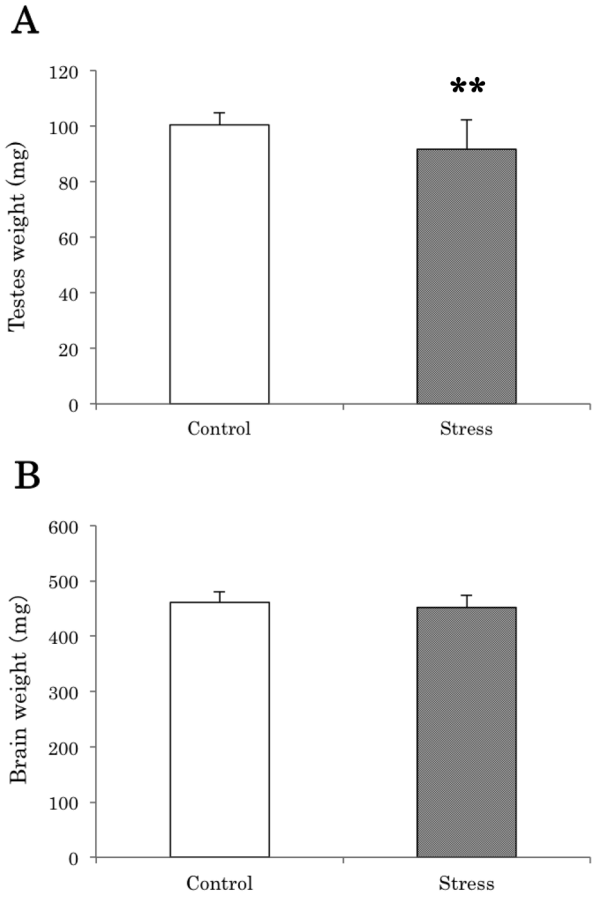

Fig. 2. Effect of unpredictable chronic stress on absolute weights of brain and testes at 4 weeks. (A) The average testis weight of the stress group was significantly lower than that of the control group. (B) There was no significant difference between brain weights of both groups. Values are presented as the mean ± SD (n=6 mice each). ***P*<0.01.

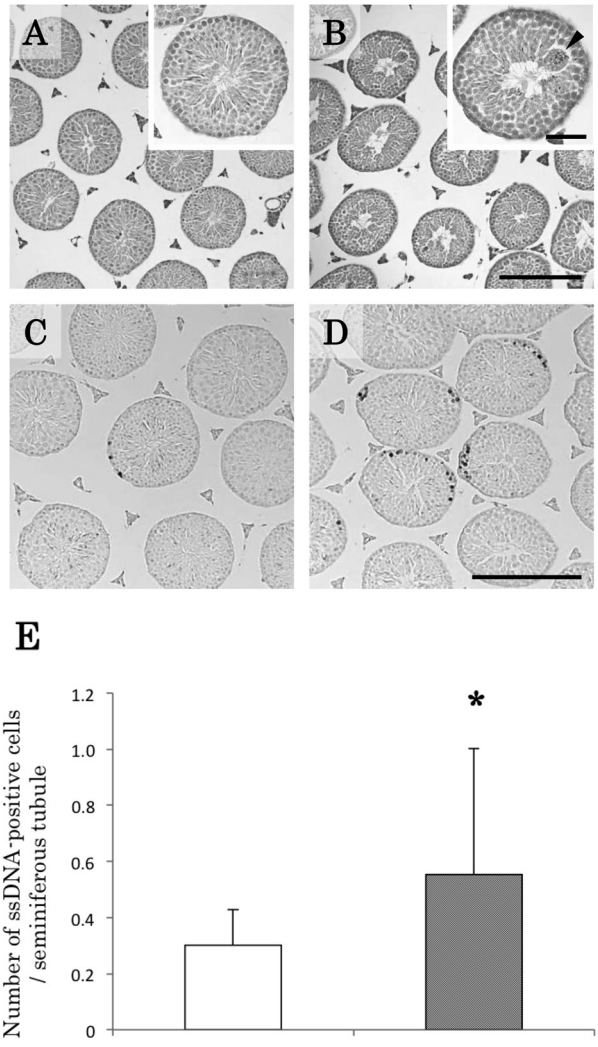

Fig. 3. Representative histology and immunohistochemistry of the testis in the control and stress group mice. (A) In the control group, normal cell arrangements were present in the seminiferous tubules. (B) In the stress group, multinucleated giant cells (arrowhead) and vacuolization were occasionally found in the thinned seminiferous epithelia. Sperm were seen in the lumen of seminiferous tubules in both groups. HE staining. (C) Immunoreactivity for single-stranded DNA was rarely detected in the nuclei of the spermatogonia of the control group testes. (D) Aggregations of ssDNA-positive spermatogonia and spermatocytes were observed in the stress group testes. Bar=200 μm (inset: 50 μm). (E) The number of ssDNA-positive cells significantly increased in the stress group compared to that of the control group. Values are presented as the mean ± SD (n=6 mice each). **P*<0.05.

body. They are modified to active forms and transported to the axon terminal as larger vesicles than those of neurotransmitters. Therefore, the granular immunoreactivity of kisspeptin detected in the ARC in the present study indicates stained precursors and transport vesicles. Similar staining was observed in the previous study using the same antibody [4]. The decreased kisspeptin immunoreactivity observed in the present stress-group mice is in agreement with previous research [17]. Although the kisspeptin expression in the mPOA (the distributional area of the axon terminals) remains to be investigated, decreasing the number of positive granules of kisspeptin in the ARC would result in the inhibition of kisspeptin secretion.

Other neuropeptides may synergistically affect kisspeptin expression and stress-induced reproductive suppression. For example, gonadotropin-inhibitory hormone (GnIH), which has an effect that is the opposite of that of kisspeptin, also mediates the stress condition to the HPG axis. Acute and chronic immobilization stress increase the expression of *GnIH* mRNA in the dorsomedial hypothalamic nucleus, in which the glucocorticoid receptor is localized [18], implying that there may be a number of stress input systems to the HPG axis.

In addition, nutritional states are known to affect reproduction. Feeding-related peptides in the hypothalamus,

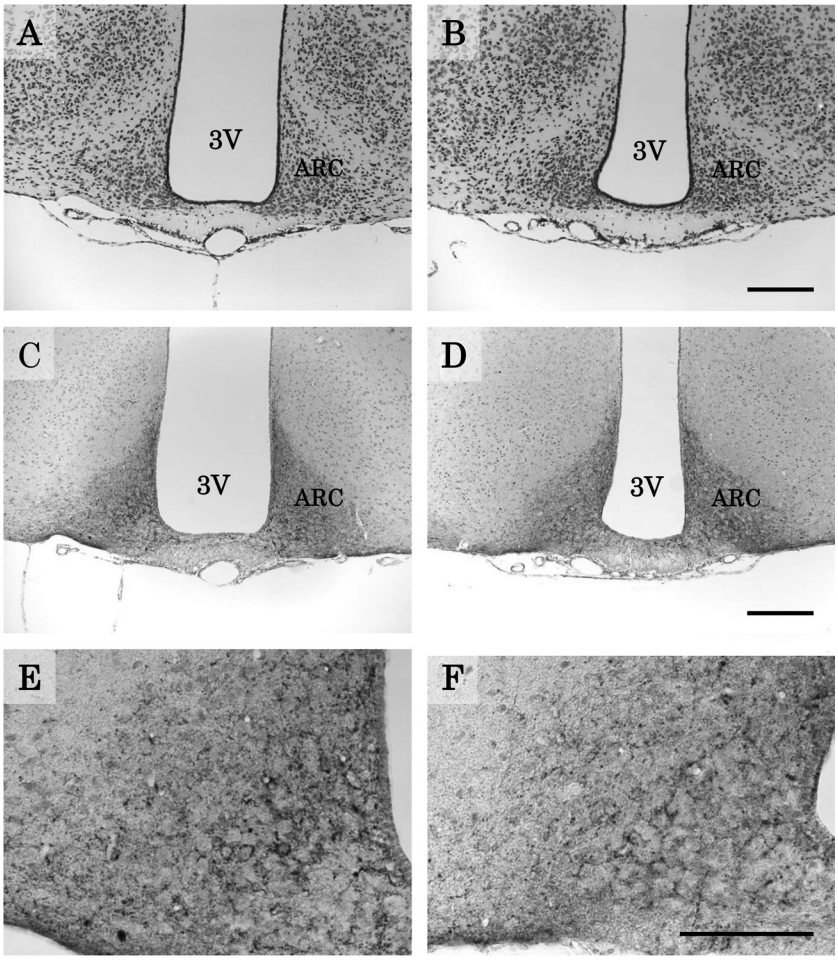

Fig. 4. Representative histology and immunohistochemistry of the brain in the control and stress-group mice. Coronal photomicrographs show no morphological differences between the ARC of the control (A) and stress (B) groups (ARC: arcuate hypothalamic nucleus. 3V: third ventricle. Bar=200 μm). Kisspeptin neurons in the ARC were similarly detected in the control (C) and stress (D) groups. At high magnification: the granular kisspeptin immunoreactivity decreased slightly in the brain sections from the stress group (F) compared to that of the control group (E). Bar=100 μm.

such as leptin, Neuropeptide Y, α-melanocyte-stimulating hormone and insulin-like growth factor 1, regulate *Kiss1* mRNA expression [29]. In particular, *Kiss1* mRNA decreases without a sufficient leptin concentration. Leptin from adipose cells can be expected to be a direct mediator of nutritional effects on kisspeptin neurons, because approx. 40% of the kisspeptin neurons in the ARC have leptin receptors [39]. It cannot be denied that the stress-induced suppression of body weight gain in the present study's stress group may be attributable to poor nutrition, causing kisspeptin to decrease through leptin signaling.

In the testes of the mice, we found that the number of DNA fragmented cells per seminiferous tubule was significantly higher in the stress group, corresponding to earlier research concerning stress-induced testicular germ cell apoptosis [48]. It is well known that spermatogenesis depends mainly on FSH signaling in Sertoli cells [36]. On the other hand, testicular steroidogenesis is regulated by LH signaling, which activates steroidogenic acute regulatory (StAR) protein and steroidogenic enzymes, such as cytochrome P450 side-chain cleavage enzyme (P450scc) [40]. The protein and mRNA levels of StAR and P450scc in Leydig cells *in vitro* dose-dependently decreased by corticosterone or diethylstilbestrol, indicating that they are early targets of environmental factors in the testes [23, 33, 45].

Previous works using rodents under low LH and FSH conditions created by estradiol and diethylstilbestrol administration [2, 46] suggested that the thinning seminiferous epithelia observed in both those studies and the present study may be due to a deficiency of both hormones. In addition, *in vitro* studies showed that spermatogonia in their seventh generation bound to be primary spermatocytes

would die by apoptosis without a sufficient FSH/prolactin ratio and meiosis initiation factors from Sertoli cells [1, 49]. Although testicular apoptosis is known to occur rarely under the physiological condition in mature males, the aggregated distribution of ssDNA-positive spermatogonia around certain Sertoli cells in the present study's stress-group mice supports this theory.

The multinucleated giant cells observed in our stress-group mice are similar to those seen in the seminiferous tubules of gorillas kept in zoos [9]. Chronic underexercise and nutritional and psychological stress are thought to be causes of testicular damages in gorillas [30]. Although the relationship between male infertility in humans and environmental stress is not yet understood, these critical conditions in zoo animals may reflect those in humans. We hope that further efforts to promote environmental enrichment for zoo animals will be made.

Regarding testicular oxidative stress, an imbalance between the generation of reactive oxygen species (ROS) and scavenging antioxidative enzymes should also be noted. Redundant ROS mainly attack sperm DNA and the cell membrane, and thus, testicular oxidative stress is associated with male infertility from the clinical perspective [43]. The origins of oxidative stress range widely and are not fully identified, but recent studies demonstrated that corticosterone administration and restraint stress induced oxidative stress in chicken and rat [22, 50].

In general, stress is thought to suppress food intake and body weight. The weekly differences in body weight between the present control and stress groups gradually widened throughout the experiment and became significant at 3 to 4 weeks. The metabolic suppression is a common symptom resulting from an unpredictable chronic stress procedure [5, 24]. Such procedures are also known to increase the plasma corticosterone level [5, 7] and decrease sexual behaviors [12]. Therefore, the mice in the present stress group could be an animal model of reproductive suppression by environmental stress. The present findings are worthwhile, because detailed research about the reproductive effects of this unpredictable chronic stress procedure has not been conducted.

Taken together, the findings in the present experiment demonstrated that even a mild degree of environmental stress can affect male reproduction. To our knowledge, this study provides the first evidence of an association between stress-induced reproductive suppression and reduced kisspeptin immunoreactivity at the peptide level. The testicular degeneration with an increased number of DNA fragmented cells occurred through these possible mechanisms. These effects of environmental stress might be crucial at the juvenile, fetal and organogenesis stages with their greater susceptibility. Further studies are needed to clarify the multilevel reproductive suppression caused by environmental stress.

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REFERENCES

1. Abé, S. 2004. Hormonal control of meiosis initiation in the testis from Japanese newt, *Cynops pyrrhogaster*. *Zoolog. Sci.* **21**: 691–704. [Medline] [CrossRef]
2. Blanco-Rodríguez, J. and Martínez-García, C. 1997. Apoptosis pattern elicited by oestradiol treatment of the seminiferous epithelium of the adult rat. *J. Reprod. Fertil.* **110**: 61–70. [Medline] [CrossRef]
3. Carlsen, E., Giwercman, A., Keiding, N. and Skakkebaek, N. E. 1992. Evidence for decreasing quality of semen during past 50 years. *BMJ* **305**: 609–613. [Medline] [CrossRef]
4. Clarkson, J., d'Anglemont de Tassigny, X., Colledge, W. H., Caraty, A. and Herbison, A. E. 2009. Distribution of kisspeptin neurones in the adult female mouse brain. *J. Neuroendocrinol.* **21**: 673–682. [Medline] [CrossRef]
5. Cox, B. M., Alsawah, F., McNeill, P. C., Galloway, M. P. and Perrine, S. A. 2011. Neurochemical, hormonal, and behavioral effects of chronic unpredictable stress in the rat. *Behav. Brain Res.* **220**: 106–111. [Medline] [CrossRef]
6. d'Anglemont de Tassigny, X., Fagg, L. A., Carlton, M. B. and Colledge, W. H. 2008. Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. *Endocrinology* **149**: 3926–3932. [Medline] [CrossRef]
7. Detanico, B. C., Piato, A. L., Freitas, J. J., Lhullier, F. L., Hidalgo, M. P., Caumo, W. and Elisabetsky, E. 2009. Antidepressant-like effects of melatonin in the mouse chronic mild stress model. *Eur. J. Pharmacol.* **607**: 121–125. [Medline] [CrossRef]
8. de Roux, N., Genin, E., Carel, J. C., Matsuda, F., Chaussain, J. L. and Milgrom, E. 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc. Natl. Acad. Sci. U.S.A.* **100**: 10972–10976. [Medline] [CrossRef]
9. Enomoto, T., Matsubayashi, K., Nakano, M., Fujii-Hanamoto, H. and Kusunoki, H. 2004. Testicular histological examination of spermatogenetic activity in captive gorillas (Gorilla gorilla). *Am. J. Primatol.* **63**: 183–199. [Medline] [CrossRef]
10. Franceschini, I., Lomet, D., Cateau, M., Delsol, G., Tillet, Y. and Caraty, A. 2006. Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. *Neurosci. Lett.* **401**: 225–230. [Medline] [CrossRef]
11. Gottsch, M. L., Cunningham, M. J., Smith, J. T., Popa, S. M., Acohido, B. V., Crowley, W. F., Seminara, S., Clifton, D. K. and Steiner, R. A. 2004. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology* **145**: 4073–4077. [Medline] [CrossRef]
12. Grønli, J., Murison, R., Fiske, E., Bjorvatn, B., Sørensen, E., Portas, C. M. and Ursin, R. 2005. Effects of chronic mild stress on sexual behavior, locomotor activity and consumption of sucrose and saccharine solutions. *Physiol. Behav.* **84**: 571–577. [Medline] [CrossRef]
13. Habib, K. E., Gold, P. W. and Chrousos, G. P. 2001. Neuroendocrinology of stress. *Endocrinol. Metab. Clin. North Am.* **30**: 695–728. [Medline] [CrossRef]
14. Han, S. K., Gottsch, M. L., Lee, K. J., Popa, S. M., Smith, J. T., Jakawich, S. K., Clifton, D. K., Steiner, R. A. and Herbison, A. E. 2005. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset

of puberty. *J. Neurosci.* **25**: 11349–11356. [Medline] [CrossRef]
15. Joffe, M. 2010. What has happened to human fertility? *Hum. Reprod.* **25**: 295–307. [Medline] [CrossRef]
16. Khansari, D. N., Murgo, A. J. and Faith, R. E. 1990. Effects of stress on the immune system. *Immunol. Today* **11**: 170–175. [Medline] [CrossRef]
17. Kinsey-Jones, J. S., Li, X. F., Knox, A. M., Wilkinson, E. S., Zhu, X. L., Chaudhary, A. A., Milligan, S. R., Lightman, S. L. and O'Byrne, K. T. 2009. Down-regulation of hypothalamic kisspeptin and its receptor, Kiss1r, mRNA expression is associated with stress-induced suppression of luteinising hormone secretion in the female rat. *J. Neuroendocrinol.* **21**: 20–29. [Medline] [CrossRef]
18. Kirby, E. D., Geraghty, A. C., Ubuka, T., Bentley, G. E. and Kaufer, D. 2009. Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats. *Proc. Natl. Acad. Sci. U.S.A.* **106**: 11324–11329. [Medline] [CrossRef]
19. Kotani, M., Detheux, M., Vandenbogaerde, A., Communi, D., Vanderwinden, J. M., Le Poul, E., Brézillon, S., Tyldesley, R., Suarez-Huerta, N., Vandeput, F., Blanpain, C., Schiffmann, S. N., Vassart, G. and Parmentier, M. 2001. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J. Biol. Chem.* **276**: 34631–34636. [Medline] [CrossRef]
20. Lapatto, R., Pallais, J. C., Zhang, D., Chan, Y. M., Mahan, A., Cerrato, F., Le, W. W., Hoffman, G. E. and Seminara, S. B. 2007. Kiss1−/− mice exhibit more variable hypogonadism than Gpr54−/− mice. *Endocrinology* **148**: 4927–4936. [Medline] [CrossRef]
21. Li, X. F., Kinsey-Jones, J. S., Cheng, Y., Knox, A. M., Lin, Y., Petrou, N. A., Roseweir, A., Lightman, S. L., Milligan, S. R., Millar, R. P. and O'Byrne, K. T. 2009. Kisspeptin signaling in the hypothalamic arcuate nucleus regulates GnRH pulse generator frequency in the rat. *PLoS ONE* **4**: e8334. [Medline] [CrossRef]
22. Lin, H., Decuypere, E. and Buyse, J. 2004. Oxidative stress induced by corticosterone administration in broiler chickens (Gallus gallus domesticus) 1. Chronic exposure. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **139**: 737–744. [Medline] [CrossRef]
23. Maeda, N., Okumura, K., Tanaka, E., Suzuki, T., Miyasho, T., Haeno, S., Ueda, H., Hoshi, N. and Yokota, H. 2013. Downregulation of cytochrome P450scc as an initial adverse effect of adult exposure to diethylstilbestrol on testicular steroidogenesis. *Environ. Toxicol.* **20**: 10.1002/tox.21875. [Medline]
24. Matthews, K., Forbes, N. and Reid, I. C. 1995. Sucrose consumption as an hedonic measure following chronic unpredictable mild stress. *Physiol. Behav.* **57**: 241–248. [Medline] [CrossRef]
25. Mineur, Y. S., Belzung, C. and Crusio, W. E. 2006. Effects of unpredictable chronic mild stress on anxiety and depression-like behavior in mice. *Behav. Brain Res.* **175**: 43–50. [Medline] [CrossRef]
26. Molina, V. A., Volosin, M., Cancela, L., Keller, E., Murúa, V. S. and Basso, A. M. 1990. Effect of chronic variable stress on monoamine receptors: influence of imipramine administration. *Pharmacol. Biochem. Behav.* **35**: 335–340. [Medline] [CrossRef]
27. Morgan, K. N. and Tromborg, C. T. 2007. Sources of stress in captivity. *Appl. Anim. Behav. Sci.* **102**: 262–302. [CrossRef]
28. Murua, V. S., Gomez, R. A., Andrea, M. E. and Molina, V. A. 1991. Shuttle-box deficits induced by chronic variable stress: reversal by imipramine administration. *Pharmacol. Biochem. Behav.* **38**: 125–130. [Medline] [CrossRef]
29. Navarro, V. M. and Tena-Sempere, M. 2012. Neuroendocrine control by kisspeptins: role in metabolic regulation of fertility. *Nat. Rev. Endocrinol.* **8**: 40–53. [Medline] [CrossRef]
30. Ochiai-Ohira, T., Kurashima, O., Akami, R., Hasegawa, T., Hirai, M., Matsuzawa, T. and Yoshikawa, Y. 2006. Past and present of great apes in Japan. *Primate Rep.* **22**: 123–136. [CrossRef]
31. Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., Terao, Y., Kumano, S., Takatsu, Y., Masuda, Y., Ishibashi, Y., Watanabe, T., Asada, M., Yamada, T., Suenaga, M., Kitada, C., Usuki, S., Kurokawa, T., Onda, H., Nishimura, O. and Fujino, M. 2001. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* **411**: 613–617. [Medline] [CrossRef]
32. Pike, J. L., Smith, T. L., Hauger, R. L., Nicassio, P. M., Patterson, T. L., McClintick, J., Costlow, C. and Irwin, M. R. 1997. Chronic life stress alters sympathetic, neuroendocrine, and immune responsivity to an acute psychological stressor in humans. *Psychosom. Med.* **59**: 447–457. [Medline] [CrossRef]
33. Rengarajan, S. and Balasubramanian, K. 2008. Corticosterone induces steroidogenic lesion in cultured adult rat Leydig cells by reducing the expression of star protein and steroidogenic enzymes. *J. Cell. Biochem.* **103**: 1472–1487. [Medline] [CrossRef]
34. Rivier, C. and Rivest, S. 1991. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. *Biol. Reprod.* **45**: 523–532. [Medline] [CrossRef]
35. Rivier, C., Rivier, J. and Vale, W. 1986. Stress-induced inhibition of reproductive functions: role of endogenous corticotropin-releasing factor. *Science* **231**: 607–609. [Medline] [CrossRef]
36. Sairam, M. R. and Krishnamurthy, H. 2001. The role of follicle-stimulating hormone in spermatogenesis: lessons from knockout animal models. *Arch. Med. Res.* **32**: 601–608. [Medline] [CrossRef]
37. Seminara, S. B., Messager, S., Chatzidaki, E. E., Thresher, R. R., Acierno, J. S. Jr., Shagoury, J. K., Bo-Abbas, Y., Kuohung, W., Schwinof, K. M., Hendrick, A. G., Zahn, D., Dixon, J., Kaiser, U. B., Slaugenhaupt, S. A., Gusella, J. F., O'Rahilly, S., Carlton, M. B., Crowley, W. F. Jr., Aparicio, S. A. and Colledge, W. H. 2003. The GPR54 gene as a regulator of puberty. *N. Engl. J. Med.* **349**: 1614–1627. [Medline] [CrossRef]
38. Sheiner, E. K., Sheiner, E., Carel, R., Potashnik, G. and Shoham-Vardi, I. 2002. Potential association between male infertility and occupational psychological stress. *J. Occup. Environ. Med.* **44**: 1093–1099. [Medline] [CrossRef]
39. Smith, J. T., Acohido, B. V., Clifton, D. K. and Steiner, R. A. 2006. KiSS-1 neurons are direct targets for leptin in the ob/ob mouse. *J. Neuroendocrinol.* **18**: 298–303. [Medline] [CrossRef]
40. Stocco, D. M. 2001. StAR protein and the regulation of steroid hormone biosynthesis. *Annu. Rev. Physiol.* **63**: 193–213. [Medline] [CrossRef]
41. Takumi, K., Iijima, N., Higo, S. and Ozawa, H. 2012. Immunohistochemical analysis of the colocalization of corticotropin-releasing hormone receptor and glucocorticoid receptor in kisspeptin neurons in the hypothalamus of female rats. *Neurosci. Lett.* **531**: 40–45. [Medline] [CrossRef]
42. Terio, K. A., Marker, L. and Munson, L. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (Acinonyx jubatus) based on adrenal morphology and function. *J. Wildl. Dis.* **40**: 259–266. [Medline] [CrossRef]
43. Tremellen, K. 2008. Oxidative stress and male infertility — a clinical perspective. *Hum. Reprod. Update* **14**: 243–258. [Medline] [CrossRef]

44. Tsigos, C. and Chrousos, G. P. 2002. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *J. Psychosom. Res*. **53**: 865–871. [Medline] [CrossRef]
45. Warita, K., Mitsuhashi, T., Sugawara, T., Tabuchi, Y., Tanida, T., Wang, Z. Y., Matsumoto, Y., Yokoyama, T., Kitagawa, H., Miki, T., Takeuchi, Y. and Hoshi, N. 2010. Direct effects of diethylstilbestrol on the gene expression of the cholesterol side-chain cleavage enzyme (P450scc) in testicular Leydig cells. *Life Sci.* **87**: 281–285. [Medline] [CrossRef]
46. Warita, K., Okamoto, K., Mutoh, K., Hasegawa, Y., Yue, Z. P., Yokoyama, T., Matsumoto, Y., Miki, T., Takeuchi, Y., Kitagawa, H., Sugawara, T. and Hoshi, N. 2008. Activin A and equine chorionic gonadotropin recover reproductive dysfunction induced by neonatal exposure to an estrogenic endocrine disruptor in adult male mice. *Biol. Reprod.* **78**: 59–67. [Medline] [CrossRef]
47. Williams, C. L., Nishihara, M., Thalabard, J. C., Grosser, P. M., Hotchkiss, J. and Knobil, E. 1990. Corticotropin-releasing factor and gonadotropin-releasing hormone pulse generator activity in the rhesus monkey. Electrophysiological studies. *Neuroendocrinology* **52**: 133–137. [Medline] [CrossRef]
48. Yazawa, H., Sasagawa, I., Ishigooka, M. and Nakada, T. 1999. Effect of immobilization stress on testicular germ cell apoptosis in rats. *Hum. Reprod.* **14**: 1806–1810. [Medline] [CrossRef]
49. Yazawa, T., Yamamoto, T., Jin, Y. and Abé, S. 2002. Follicle-stimulating hormone is indispensable for the last spermatogonial mitosis preceding meiosis initiation in newts (Cynops pyrrhogaster). *Biol. Reprod.* **66**: 14–20. [Medline] [CrossRef]
50. Zafir, A. and Banu, N. 2009. Modulation of *in vivo* oxidative status by exogenous corticosterone and restraint stress in rats. *Stress* **12**: 167–177. [Medline] [CrossRef]