



Molecular morphological study of the combined effects of environmental factors on the reproductive function and stress reactivity

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Doctoral Dissertation

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Department of Animal Science,
Graduate School of Agricultural Science, Kobe University

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博士論文

Molecular morphological study of
the combined effects of environmental factors
on the reproductive function and stress reactivity
環境要因が生殖機能およびストレス反応系に与える
複合影響に関する分子形態学的研究

January, 2017

Department of Animal Science,
Graduate School of Agricultural Science, Kobe University

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PREFACE

One urgent and largely unresolved issue in modern society is the increase in reproductive, metabolic, immune and psychiatric disorders due to changes in a wide range of environmental factors. In general, threats to human health are attributable to interactions between genetic and environmental factors that lead to variations in phenotypes. Although the myriad environmental factors can be divided into two categories—non-biological factors such as exposure to chemicals, radiation and climate and biological factors such as conditions of stress, nutrition and infection—the mechanisms by which these factors interact with our genes to give rise to disorders are complex and vary widely.

For example, while environmental stressors are often invisible, they can clearly damage the brain and whole body through activation of the hypothalamic-pituitary-adrenal (HPA) axis and subsequent release of several stress hormones. The increased psychological and social stressors in modern life are now well known as major risk factors for infertility and mental illness. However, the mechanisms by which stressors make us vulnerable to other environmental factors are not fully understood.

At the same time, the enormous quantities of novel chemicals we have been creating to improve our life are having unpredictable effects on our health. As Rachel Carson first discussed in the *Silent Spring* in 1962, industrial and agricultural chemicals that included dichloro-diphenyl-trichloroethane as an ingredient were not always useful and safe. As Theo Colborn subsequently pointed out in *Our Stolen Future* in 1996, chemicals such as polychlorinated biphenyls and dioxins mimic sex steroids *in vivo* and thus are classified as

endocrine-disrupting chemicals (EDCs); these agents cause morphological abnormalities of the reproductive organs of wild animals and have also become a public health issue.

In the case of newer chemicals, however, the risk assessments are not always keeping pace with the research and development. In fact, identifying risky chemicals has become a kind of cat-and-mouse game, since the onset of toxicity is not always dose-dependent, and is often affected by specific or individual chemical sensitivities and the point of exposure. Moreover, the combined effects of these chemicals are often unknown. Thus while three typical EDCs (bisphenol A, di-2-ethylhexyl phthalate, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) have countervailing effects when used in combination [Tanida *et al.*, 2009], the effect of exposure to four antiandrogens was shown to be much greater than the sum of the individual effects of each drug [Christiansen *et al.*, 2009]. The conventional ways of safety evaluation only for single compound were not able to reflect the actual combined exposure condition. As the World Health Organization described when introducing their new framework for cumulative health risk assessment [Meek *et al.*, 2011], methods for evaluating the interactive effects of multiple chemicals and other environmental factors will be urgently needed in the future.

Although it is impossible to assess the effects of all suspected environmental factors, the sets of processes of various influences by environmental factors through molecular, cellular, tissue and individual changes are partly common among them. Therefore, our primary tasks are (i) revealing the individual mechanisms and pathways, and (ii) developing common toxicological endpoints and predictive biomarkers for the adverse effects of environmental factors. In this

study, I examined the reproductive and behavioral effects of environmental stress, environmental chemicals, or both to understand the universal mechanisms of the harmful effects of environmental factors.

Chapter I

Unpredictable chronic stress-induced reproductive suppression associated with the decrease of kisspeptin immunoreactivity in male mice

Introduction

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 [Khansari *et al.*, 1990], the autonomic nervous system [Pike *et al.*, 1997] and the endocrine system [Habib *et al.*, 2001], 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 [Rivier and Rivest, 1990; Tsigos and Chrousos, 2002]. 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 [Rivier *et al.*, 1986; Williams *et al.*, 1990].

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 [Ohtaki *et al.*, 2001; Kotani *et al.*, 2001]. In studies of *Kiss1*- or *Kiss1r*- deficient mice [Seminara *et al.*, 2003; Lapatto *et al.*, 2007] and patients with idiopathic hypogonadotropic hypogonadism who have a mutation of *KISS1R* [de Roux *et al.*, 2003], kisspeptin proved to be critical to the onset of puberty [Han *et al.*, 2005] 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 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 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 [Terio *et al.*, 2004]. Seminiferous degenerations frequently occur in captive gorillas [Enomoto *et al.*, 2004]. Similarly, the infertility rates in humans have also gradually increased,

caused in part by drastic decreases in sperm quality [Carlsen et al., 1992; Joffe, 2010]. Environmental stress is one of the possible reasons for these infertility problems [Sheiner *et al.*, 2003], 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 [Mineur *et al.*, 2006]. 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 ($22 \pm 2^{\circ}\text{C}$) and humidity ($50 \pm 10\%$) 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: 24-15) 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.* and Murua *et al.* 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/ml 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% H₂O₂ 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 [Franceschini *et al.*, 2006] (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). 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. I-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. I-2. Although the testis weights of the stress group decreased significantly compared to those of the control group (Fig. I-2A, control: 100 ± 4 mg; stress: 92 ± 11 mg; $p < 0.01$), no significant between-group difference was observed in the brain weight (Fig. I-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. I-3A). In the stress group, the testes showed thin and vacuolated 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. I-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. I-3C and D). 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. I-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. I-4A and B). The kisspeptin immunoreactivity was granularly detected in axons and dendrites over the entire area of the ARC in both groups (Fig. I-4C–F). 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 [Gottsch *et al.*, 2004; d'Anglemont de Tassigny *et al.*, 2008; Li *et al.*, 2009]. 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) [Takumi *et al.*, 2012], 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 [Kinsey-Jones *et al.*, 2009], 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 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 [Clarkson *et al.*, 2009]. The decreased kisspeptin immunoreactivity observed in the present stress-group mice is in agreement with previous research [Kinsey-Jones *et al.*, 2009]. 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 [Kirby *et al.*, 2009], 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, such as leptin, Neuropeptide Y, α -melanocyte-stimulating hormone, and insulin-like growth factor 1, regulate *Kiss1* mRNA expression [Navarro and Tena-Sempere, 2012]. 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 [Smith *et al.*, 2006]. 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 [Yazawa *et al.*, 1999]. It is well known that spermatogenesis depends mainly on FSH signaling in Sertoli cells [Sairam and Krishnamurthy, 2001]. 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) [Stocco, 2001]. 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 [Rengarajan and Balasubramanian, 2008; Warita *et al.*, 2010; Maeda *et al.*, 2013].

Previous works using rodents under low LH and FSH conditions created by estradiol and diethylstilbestrol administration [Blanco-Rodríguez and Martínez-García, 1997; Warita *et al.*, 2008] 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 [Yazawa *et al.*, 2002; Abé, 2004]. 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 [Enomoto *et al.*,

2004]. Chronic underexercise and nutritional and psychological stress are thought to be causes of testicular damages in gorillas [Ochiai-ohira *et al.*, 2006]. 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 [Tremellen, 2008]. 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 [Lin *et al.*, 2004; Zafir and Banu, 2009].

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 [Matthews *et al.*, 1995; Cox *et al.*, 2011]. Such procedures are also known to increase the plasma corticosterone level [Detanico *et al.*, 2009; Cox *et al.*, 2011] and decrease sexual behaviors [Grønli *et al.*, 2005]. 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.

Summary

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, vacuolated 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.

Figures and Figure legends

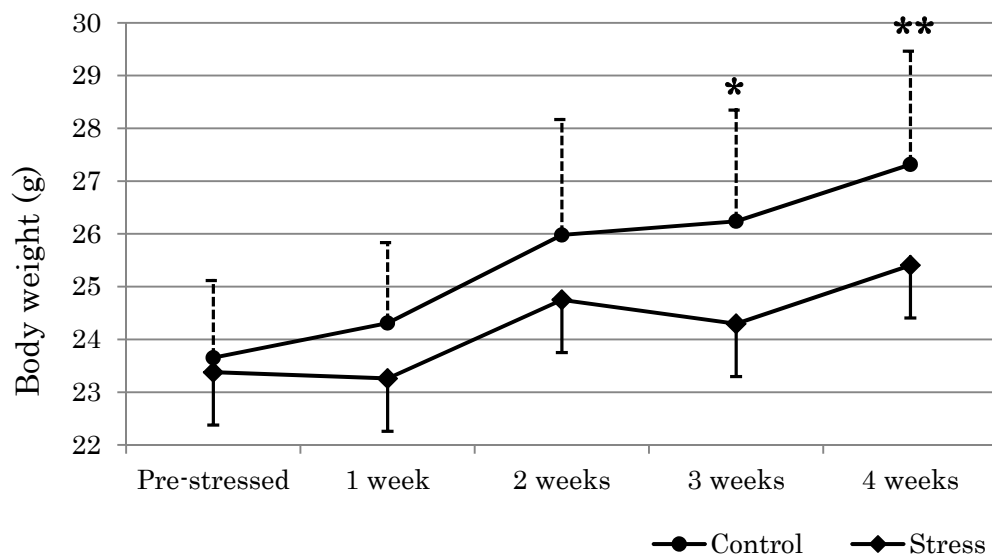


Fig. I-1. Unpredictable chronic stress suppressed the body weight gain of the mice. All mice gained weight gradually throughout the 4 weeks experimental period, but there were significant differences between the body weights of the control and stress groups at 3 weeks and 4 weeks. Values are presented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

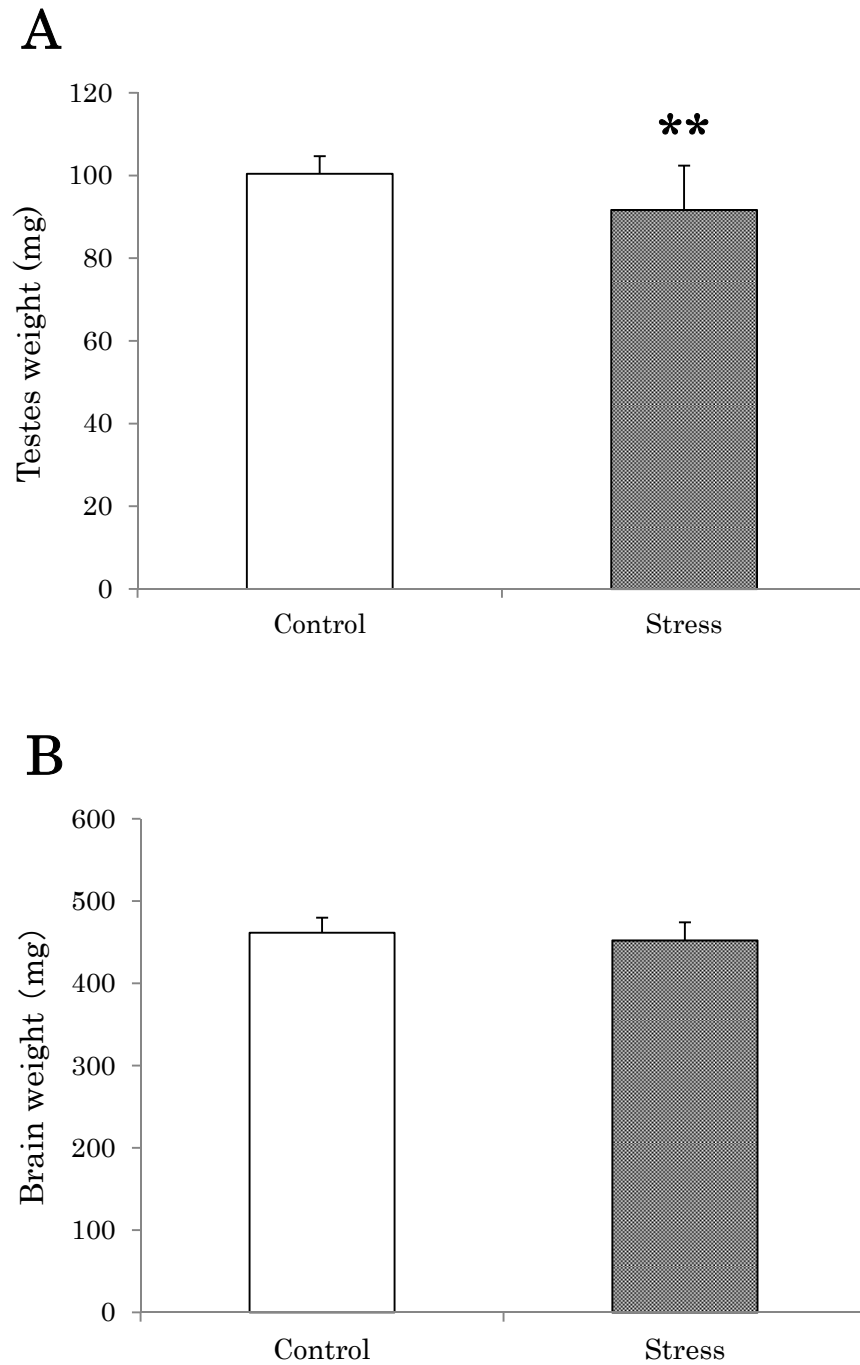


Fig. I-2. Effect of unpredictable chronic stress on absolute weights of brain and testes at 4 weeks. (A) The average testis weight of the stress group was significantly lower than that of the control group. (B) There was no significant difference between brain weights of both groups. Values are presented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

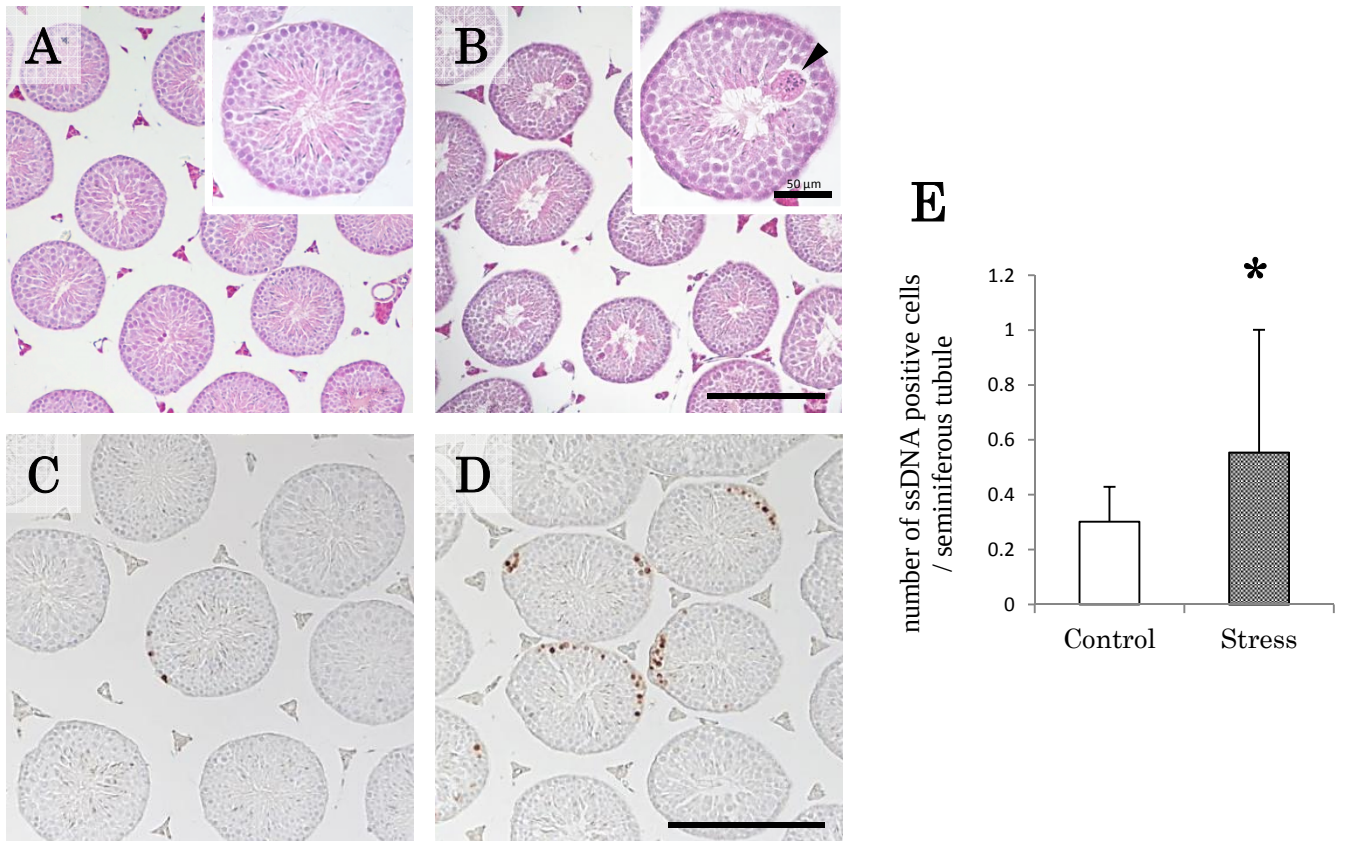


Fig. I-3. Representative histology and immunohistochemistry of the testis in the control and stress group mice. (A) In the control group, normal cell arrangements were present in the seminiferous tubules. (B) In the stress group, multinucleated giant cells (arrowhead) and vacuolization were occasionally 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 μ m (inset: 50 μ m). (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 $\pm$ SD (n=6 mice each). * p <0.05.

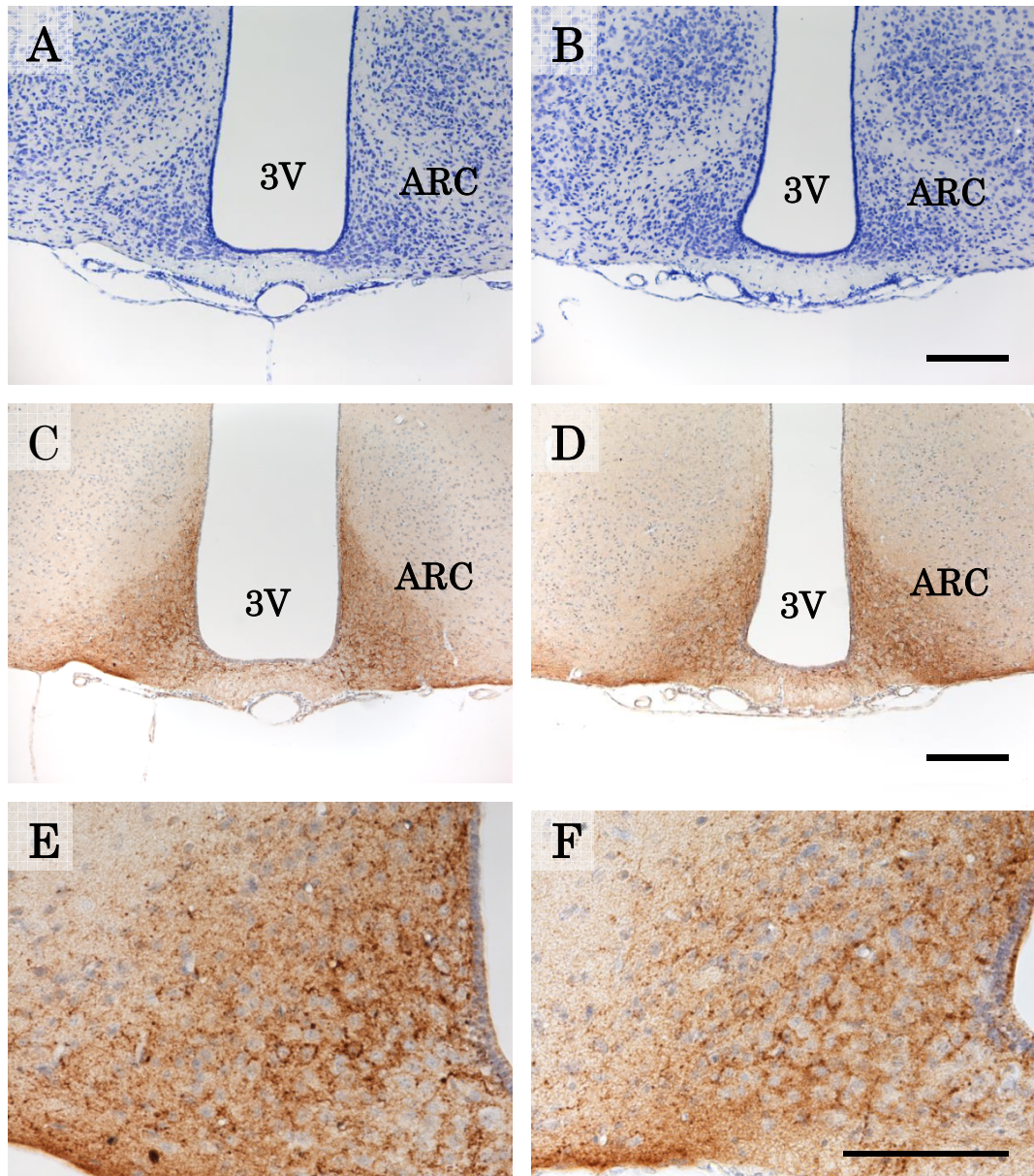


Fig. I-4. Representative histology and immunohistochemistry of the brain in the control and stress-group mice. Coronal photomicrographs show no morphological differences between the ARC of the control (A) and stress (B) groups (ARC: arcuate hypothalamic nucleus. 3V: third ventricle. Bar = 200 μ m). Kisspeptin 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 μ m.

Chapter II

The combined effect of clothianidin and environmental stress on the behavioral and reproductive function in male mice

Introduction

Neonicotinoids, which were developed in the 1990s, have been some of the most widely used pesticides, accounting for over 25% of the world market share with rapidly increasing sales. They act as agonists to the nicotinic acetylcholine receptors (nAChRs) of insects with much higher affinity than to those of mammals, resulting in death of insects from abnormal excitability in their cholinergic nervous system. However, with the recent expanding use of neonicotinoids in farming, forestry and the building industry, an unanticipated phenomenon called honeybee colony collapse disorder (CCD) has inflicted great damage on crop production in many parts of the world [Potts *et al.*, 2010]. Some neonicotinoids were proved to disturb the foraging and homing behaviors of honeybees, weakening their colonies [Henry *et al.*, 2012; Whitehorn *et al.*, 2012; Gill *et al.*, 2012]. Neonicotinoids have thus attracted much attention as a strongly suspected agent of CCD.

In experimental animals, neonicotinoids were immediately absorbed and largely excreted into the urine with 2–6 hr half-life *in vivo* [Ford and Casida, 2006a]. An *in vitro* study showed that mammalian nAChRs also have some affinity for neonicotinoids, although at much lower levels compared to insect nAChRs [Tomizawa and Casida, 2005]. In neonatal rats, several neonicotinoids cause excitation of cerebellar neurons through their nAChRs [Kimura-Kuroda *et al.*, 2012], suggesting further unpredictable adverse effects of neonicotinoids on vertebrates. According to those reports, the European Union (EU) issued a decision in 2013 to ban the use of three neonicotinoids for a fixed period of two years as a precautionary principle. Contrary to this, in Japan, the residue standards

for neonicotinoids, which are several or hundreds of times higher than the corresponding standards in Europe and the United States, are being relaxed by the Ministry of Health, Labour and Welfare. An increasing number of research studies demonstrate that neonicotinoids are definitely harmful to mammals, birds, fishes and insects, and there is an urgent need for decisive information to judge the risk of neonicotinoids.

In particular, clothianidin (CTD), the sixth-developed neonicotinoid, is a white crystalline compound with the molecular weight of 249.68 and the lowest water solubility (0.327 g/l) among the neonicotinoids. A research about metabolic fate of neonicotinoids revealed that CTD was comparatively persistent in mammal brain [Ford and Casida, 2006b]. CTD also accelerates the responses to acetylcholine in HEK cells expressing human nAChRs $\alpha 4\beta 2$ [Li *et al.*, 2011], implying an excitatory effect of CTD on mammalian brains. The reproductive function also seems to be a target of CTD; this neonicotinoid reduced the weights of reproductive organs and semen quality parameters of immature and mature male rats [Bal *et al.*, 2012, 2013]. We recently reported that low-dose CTD affects avian reproductive functions through oxidative stress, and this effect could be more severe in birds that have higher sensitivities [Tokumoto *et al.*, 2013; Hoshi *et al.*, 2014].

As described above, the typical targets of neonicotinoids are nAChRs, which are expressed throughout the body, especially in postsynaptic neuromuscular junctions, autonomic ganglions and the central nervous system for cholinergic neurotransmission by acetylcholine. These ion channel-linked receptors, are a circular homo- or hetero-pentamer composed of dozens of subunits determining

their physiological properties. In mammal brain, $\alpha 7$ homomer and $\alpha 4\beta 2$ heteromer play important roles as regulators of intraneuronal signaling and neurotransmitter releases. Phenotypes of knockout models indicated that nAChRs are involved in emotional and anxiety-like behaviors, along with some psychiatric disorders [Picciotto *et al.*, 2001; Drago *et al.*, 2003].

It recently became apparent that the expressions of nAChRs are influenced by environmental stress. For example, immobilize stress decreases the expression of nAChRs $\alpha 7$ in the hippocampus of mature rats [Hunter *et al.*, 2010], whereas, prenatal stress in rats increases the expression of nAChRs in the hippocampus of their adult offspring [Schulz *et al.*, 2013]. We thus hypothesized that environmental stress altered the sensitivity to neonicotinoids by modifying the cholinergic system.

The purpose of the present study was to investigate the effect of chronic combined exposure to CTD and environmental stress. The oral administration method is known to add extraordinary stress to experimental animals with a transient secretion of glucocorticoids [Balcombe *et al.*, 2004]. To assess the effects of CTD and environmental stress separately, we chose a non-invasive CTD administration method using commercial oral rehydration gels. We conducted an open field test to detect behavioral changes following this combined exposure, and histological and immunohistochemical analyses were performed focusing on the expression of the antioxidant enzymes in testes.

Materials and methods

Experimental animals

Male C57BL/6NCrSlc mice (8 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). All mice were maintained in 40.5 × 20.5 × 18.5 cm individual ventilated cages (Sealsafe Plus Mouse; Tecniplast, Buguggiate, Italy) under controlled temperature (23 ± 2°C) and humidity (50 ± 10%) on a 12-hr light/dark cycle at the Kobe University Life-Science Laboratory with *ad libitum* access to a pellet diet (DC-8; Clea Japan, Tokyo, Japan) and filtered water. This study was approved by the Institutional Animal Care and Use Committee (Permission #24-10-03) and carried out according to the Kobe University Animal Experimental Regulation.

CTD purification and HPLC analysis

Water-soluble Dantotsu[®] (involving 16% of CTD; Sumitomo Chemical Co., Tokyo, Japan), donated by Sado City (Niigata, Japan), was washed with 10 times the amount of distilled water to remove the surfactant activating and granulating agents. After being left to stand for at least 48 hr, the supernatant was removed. This step was repeated five times, and then, the white precipitate was collected and air-dried naturally for a week. The content rate of CTD in the white precipitate was measured by a LaChrom high-performance liquid chromatography (HPLC) system (interface L-7000, pump L-7100, auto sampler L-7200, column oven L-7350 and UV-VIS detector L-7420; Hitachi, Tokyo, Japan) using a Capcell Pak C18 UG120 column (5 µm particles, 4.6 × 250 mm; Shiseido, Tokyo, Japan).

CTD standard (>99.8%; Wako Chemical, Osaka, Japan) and the obtained white

powder were completely dissolved in dimethyl sulfoxide (DMSO) and then serial-diluted with a mobile phase consisting of 55% acetonitrile in 50 mM potassium phosphate buffer (pH 3.0), followed by filtration with a 0.20 µm syringe driven filter unit (Millex-LG; Millipore, Billerica, MA, U.S.A.). The column maintained at 40°C was eluted with the mobile phase at a flow rate of 1.0 ml/min. After the column was equilibrated, 10 µl of the samples was injected into the HPLC system. We monitored the resultant chromatograph at the wavelength of 260 nm and then calculated the CTD content of the obtained white powder from the calibration curve created by the peak areas and heights of the CTD standard. A single peak was observed in the samples from the white precipitate with the same retention time as that of the CTD standard. A linear calibration curve ($R^2 = 0.999$) created from samples serial-diluted with the CTD standard showed that our purification makes the content rate of CTD increased from 14–16% to 93–97% by weight.

CTD administration and stress exposure

All mice were allowed to acclimate to their home cages for a week prior to the initiation of experiments. We divided the mice into eight groups (n=5 mice in each cage): CTD-0 (Control), CTD-10 (10 mg/kg/day), CTD-50 (50 mg/kg/day), and CTD-250 (250 mg/kg/day) with the presence or absence of stress exposure. As a substitute for filtered water for the mice, we used the MediGel[®] Sucrarose 2 oz cup (ClearH2O, Portland, ME, U.S.A.), which is a flavored thermoreversible hydration gel matrix. The amounts of the purified CTD for the respective administration groups were calculated from the CTD purity (95%), daily gel

intake (5 g/day/mouse), total gel weight (60 g; excluding the package weight) and average mouse weight (24 g; weighed at initiation of experiments). These amounts of CTD were completely dissolved in 600 μ l DMSO (1% volume of a gel) and injected into the gels and then double-boiled at 60°C followed by shaking to ensure that the CTD was diffused well. For the CTD-0 group, the same volume of DMSO without the purified CTD was injected into the gels. All gels were strapped with cable ties under the grate of the cage lid to prevent contamination with the beddings and the excretions. All gels were weighed daily to estimate the amounts of the putative CTD exposure. In the four stress groups, the mice were subjected to an unpredictable chronic stress procedure as described in our earlier report [Hirano *et al.*, 2014] with some modifications. Briefly, 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 mice were randomly exposed to two mild stressors per day at varying times for 4 weeks.

Behavioral analysis

On the last day of the 4 week experimental period, an open field test was conducted during the light phase to evaluate the locomotor activity and the anxiety-like behavior of the mice. Briefly, the mouse was placed on the corner of an open field (60 × 60 × 30 cm) with LED illumination. All of the mouse's activities were recorded by a video camera for the subsequent 10 min, and we then

analyzed the total distance traveled and the time spent in the center zone (30 × 30 cm) using SMART video tracking software V3.0 (San Diego Instruments, San Diego, CA, U.S.A.).

Tissue preparation

On the day after the completion of the 4 weeks of combined exposure to CTD and stress, 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 testes were excised, weighed and postfixed with the same fixative overnight at 4°C. The testes were dehydrated through a graded series of ethanol followed by xylene and embedded in paraffin. Serial sections of testes were then cut at 4 µm thickness on a sliding microtome (SM2000R; Leica Microsystems, Wetzlar, Germany) and mounted on slide glasses (Platinum Pro; Matsunami Glass Ind., Kishiwada, Japan). All sections were stored at -30°C until use for the following steps.

Histological and immunohistochemical analyses

For the general histological analysis, testis sections were stained with hematoxylin and eosin (HE; Merck KGaA, Darmstadt, Germany) after their deparaffinization and hydration, following the manufacturer's instructions. To detect antioxidant enzymes in the testes, we performed the following immunohistochemistry protocol. The sections were immersed in absolute methanol and 0.5% H₂O₂ for 30 min, respectively, at RT to quench the endogenous peroxidase activity. They were then incubated with Blocking

OneHisto (Nacalai Tesque, Inc., Kyoto, Japan) for 1 hr at RT for protein blocking and then incubated with the rabbit polyclonal anti-GPx4 antibody (Item No. 10005258; Cayman Chemicals, Ann Arbor, MI, U.S.A.) diluted 1:8,000 in phosphate buffered saline with 0.05% Tween-20 (PBST; pH 7.4) for 18 hr at 4°C.

After being washed with PBST, 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).

Statistical analysis

Statistical analyses were performed with Excel Statistics 2012 (SSRI version 1.00, Tokyo, Japan). In the behavioral analyses, outliers more distant than 1.5 interquartile ranges from the upper or lower quartile were omitted. All data were analyzed by two-way ANOVA (CTD × stress) followed by the Tukey-Kramer's post hoc test. The results were considered significant when the *p*-value was less than 0.05.

Results

Gel intake, body and testis weights

The daily gel consumption, body weights and testes weights are shown in Fig. II-1, and all measured values are summarized in the Table. CTD significantly suppressed the daily gel intake in all CTD administration groups compared to the non-CTD administration groups (Fig. II-1A). Although the daily intake was significantly inhibited in the stress groups, there was little difference if the values in the days of food deprivation in our stress protocol were omitted (Table). As a result, the body weights of mice reflected the same tendency of the gel intake. The body weights were suppressed in all stress groups relative to the non-stressed groups at the same dose of CTD (Fig. II-1B). The body weights of the CTD-250 groups were significantly lower than those of the other three dose groups. On the other hand, the absolute weights of the testes were decreased by the stress procedure in the CTD-0, CTD-50 and CTD-250 groups (Fig. II-1C). No interaction effects were detected in any of the measured values.

Open field test

The representative trajectory maps (Fig. II-2A) revealed that the mice with the combined CTD exposure and stress procedure tended to walk alongside the walls. CTD and the stress procedure had insignificant impacts on the locomotor activities measured by the total distances traveled (Fig. II-2B). By contrast, the anxiety-like behaviors that avoiding being in the center zone were elevated by the stress procedure and were significantly different between the CTD-0 groups (Fig. II-2C). CTD also enhanced the anxiety-like behaviors in a dose-dependent manner,

and in the non-stressed condition, the difference was significant in the CTD-10 and CTD-250 groups compared to the CTD-0 group.

Histological and immunohistochemical findings

The general histological analyses of the testes by HE staining are shown in Fig. II-3. Although there were seminiferous tubules densely arranged with germ cells and sperms in the testes of the control group, several testicular signs of toxicity were observed in combined exposure groups. Multinucleated giant cells were occasionally observed in the seminiferous tubules of the mice in the stress groups. In addition, variably sized vacuolization were scattered at the bottom of seminiferous tubules of the CTD-administered groups. In particular, seriously degenerated seminiferous tubules, from which almost all germ cells had dropped off, were found in the CTD-250 groups. There was no marked histological difference between the Leydig cells in the testicular interstitial tissues of any of the experimental groups.

The results of the immunohistochemical analyses of the testes visualizing GPx4 are shown in Fig. II-4. In the control group, strong GPx4 immunoreactivity was detected in the sperm, and diffuse GPx4 immunoreactivity was seen in the cytoplasm of spermatids. The intensity of GPx4 immunoreactivity in the sperm was decreased by stress, and the cytoplasmic immunoreactivity in the spermatids was dose-dependently decreased by CTD. In addition, abnormal immunoreactivity of GPx4 in Sertoli cells was detected in the combined exposure groups, and it became strong in degenerated seminiferous tubules in the CTD-50+stress group and the CTD-250 groups.

		Groups							
		CTD-0	CTD-0 +Stress	CTD-10	CTD-10 +Stress	CTD-50	CTD-50 +Stress	CTD-250	CTD-250 +Stress
Body weight (g)		28.62 ± 1.66	26.48 ± 0.72	26.98 ± 0.99	25.36 ± 1.22	27.50 ± 0.82	25.24 ± 0.78	25.82 ± 0.99	23.76 ± 0.40
Gel intake / day (g)		6.13 ± 1.37	5.64 ± 2.80 [6.66 ± 2.56]	4.41 ± 0.84	2.85 ± 1.77 [3.73 ± 1.59]	4.60 ± 1.59	3.55 ± 2.09 [4.65 ± 1.79]	3.64 ± 0.64	2.38 ± 1.42 [3.16 ± 1.09]
Putative exposure (mg / kg / day)		0	0	8.82 ± 1.68	5.70 ± 3.54 [7.46 ± 3.18]	46.0 ± 15.9	35.5 ± 20.9 [46.5 ± 17.9]	182 ± 32	119 ± 71 [158 ± 54.5]
Testis weight	(mg) (%)	99.6 ± 5.5 0.349 ± 0.027	88.2 ± 11.8 0.311 ± 0.048	95.7 ± 9.6 0.355 ± 0.004	90.5 ± 9.7 0.356 ± 0.028	100.3 ± 9.8 0.364 ± 0.030	81.1 ± 8.2 0.321 ± 0.030	97.5 ± 7.8 0.377 ± 0.021	87.5 ± 9.7 0.368 ± 0.038

Table. Summarized table of all measured values in this experiment. The values in square brackets were presented the averages with removal of values in the deprivation days from the calculations. Values are presented as the mean ± SD (n=5 mice each).

Discussion

We verified the combined effects of CTD and stress on the reproductive and behavioral function in mature male mice by a non-invasive administration method. There are several advantages of the gels we used; compared to baking pellets, the gels can uniformly deliver the test articles to animals without exposing them to high temperature. The gels could not completely cloak the bad palatability of CTD as reported earlier using similar gels mixed with an aromatase inhibitor, anastrozole [Overk *et al.*, 2012]; however, the gel consumption observed in combined exposure groups was enough for maintaining the health of the mice. In our preliminary experiments, we initially suspected that the CTD avoidance effect was attributable to any accessory constituents of the commercial pesticide containing CTD, but CTD itself appeared to have some repellent effects on mice. In agricultural fields, CTD-containing pesticides are used at dilutions from 1:20 to 1:4,000 for treating seeds and plants. Our purification protocol and the water solubility of CTD imply that the white precipitate resulting from the recommended concentration of CTD products is particularly dangerous for pesticide users.

In the present study, both CTD and stress concurrently affected the behavioral functioning of mature male mice. The unpredictable stress model, which is often used in the assessment of antidepressant medications, evidently raised the anxiety level in the mice. CTD dose-dependently induced anxiety alone, but there was little difference in this measure among the combined exposure groups. To the best of our knowledge, the behavioral effects of CTD in mature mice have not been previously reported.

There are only a few reports about the behavioral effects of neonicotinoids on mammals. Several parameters of behavioral developmental tests that evaluated the sense of equilibrium, coordinated movement and muscular power of offspring were changed by maternal exposure to low-dose CTD, with a sex difference [Tanaka, 2012a, b]. Thiamethoxam (TMX, 100 mg/kg/day) significantly reduced the locomotor activity of rats in an open field test and induced anxiety in an elevated plus-maze test while suppressing acetylcholinesterase (AChE) activity in three brain regions [Rodrigues *et al.*, 2010]. Considering that TMX is metabolized to CTD in mammals, our findings regarding the anxiogenic effects of CTD sufficiently agree with the findings of these prior studies.

Although the mechanisms of the behavioral effects of neonicotinoids remain unclear, cholinergic systems and downstream neurotransmitters are undoubtedly involved. An *in vivo* study revealed that CTD directly injected into the rat brain evoked a striatal dopamine surge through nAChRs [Faro *et al.*, 2012]. Early types of neonicotinoids caused a subsequent desensitization of nAChRs as well as immediate neuronal excitation [Kimura-Kuroda *et al.*, 2012]. The behavioral consequences of chronic nicotine intake are also attributed to both the upregulation and desensitization of nAChRs [Picciotto *et al.*, 2008]. The anxiety-like behaviors in several behavioral tests are part of the phenotype of nAChRs subunit $\alpha 4$ knockout mice [Ross *et al.*, 2000], implying that the anxiogenic effect of CTD observed in the present study was presumably triggered by the chronic desensitization of nAChRs.

The *CHRNA7* coding $\alpha 7$ subunit of nAChRs contains a glucocorticoid response element [Leonard *et al.*, 2002], which may explain the decreasing nAChRs $\alpha 7$

expression by chronic immobilization stress [Hunter *et al.*, 2010]. These observations may support the hypothesis that the behavioral effects of CTD and stress partly share a common mechanism mediating weakened cholinergic activities, such as the desensitization of nAChRs, the suppressed level of AChE and the disturbance of the release of downstream neurotransmitters. In the CTD-250+stress groups, the stress procedure and the CTD-induced feeding suppression presumably fully suppressed the cholinergic activities, which made the anxiogenic effect of CTD invisible.

On the other hand, we found that the chronic exposure to CTD and the stress influenced the testes in different ways. The stress procedure significantly decreased the testicular weight in addition to causing the emergence of the multinucleated giant cells, and both of these are common toxicant observations in testes produced by an abnormal meiotic division of germ cells. CTD did not show an impact on the testicular weight, whereas it dose-dependently degenerated the seminiferous epithelia. In particular, the Sertoli cell-only seminiferous tubules found in the CTD-250 groups were unexplainable by CTD-induced chronic nutritional deprivation. In the clinical field, Sertoli cell-only syndrome is known to result from partial deletions of the azoospermia factor region on the Y-chromosome, which is one of the markers of male infertility. From the toxicological perspective, germ cells are vulnerable to damage from exogenous chemicals, and they are easily phagocytized by Sertoli cells. Several endocrine-disrupting compounds (EDCs) including diethylstilbestrol and flutamide induce such a destruction of seminiferous tubules [Atanassova *et al.*, 1999; Foster and Harris, 2005; Warita *et al.*, 2008]. An increasing number of

vacuolizations in seminiferous epithelia are also caused by the absence of germ cells, suggesting that they are the first target of CTD in the testes through the blood-testis barrier. This corresponds to the CTD dose-dependent increase of single-stranded DNA immunopositive germ cells observed in the testes of quails [Tokumoto *et al.*, 2013; Hoshi *et al.*, 2014].

GPx4 (also called phospholipid-hydroperoxide glutathione peroxidase as a member of the glutathione peroxidase family) is an antioxidant enzyme expressed in every mammalian organ. There are three types of transcripts from the same *GPx4* gene: mitochondrial, non-mitochondrial and nuclear types were identified. The mitochondrial and non-mitochondrial types in particular are expressed in spermatocytes, and GPx4 is the principal antioxidant enzymes in the testes with 30 times greater expression in the testes compared to other organs. Notably, in sperm, GPx4 is responsible for the abilities to scavenge approx. one-half of the hydrogen peroxide and 90% of the phospholipid hydroperoxides [Imai *et al.*, 2001].

Testis-specific GPx4 knockout mice showed a phenotype of infertility with degenerated testicular tissue and morphological aberration of sperm [Imai *et al.*, 2009]. The antigen of the polyclonal antibody used in this study is a common sequence among the three types of mouse GPx4, and thus, we could not classify the types of GPx4 immunoreactivity. The weakened GPx4 immunoreactivity in the lumen of seminiferous tubules presumably resulted from the decreases in the number and quality of the sperm.

Neonicotinoids are known to have strong oxidizing properties that affect the liver and the reproductive and central nervous systems [Kapoor *et al.*, 2011;

Duzguner and Erdogan, 2012]. The present report is the first to describe the localized decrease of antioxidant enzymes by CTD in the testes of a mammal. Similarly, oxidative effects of psychological stress via stress hormones have been reported [Lin *et al.*, 2004; Zafir and Banu, 2009]. Although a constant level of reactive oxygen species is necessary for the proliferation of spermatogonial stem cells [Morimoto *et al.*, 2013], the present results also showed that oxidative mechanisms partly explain how stress impacts the reproductive function cooperatively with CTD.

Although there are no reports of plentiful expressions of GPx4 in Sertoli cells, strong ectopic expression of antioxidant enzymes was observed in Sertoli cells in degenerated seminiferous tubules in the CTD-250 groups. Because GPx4 mRNA was not expressed in Sertoli cell in rat [Mori *et al.*, 2001], this unexpected finding may have originated from phagocytosed germ cells damaged by CTD. Baek *et al.* investigated whether GPx4 mRNA in the testes was altered by several EDCs, implying the measurements of oxidative conditions provide an indication of the testicular toxicity by environmental factors.

In the present study, although no remarkable interaction effect was detected, we investigated whether CTD and stress affected the behavioral and reproductive functions additively rather than synergistically in adult mice. The endogenous nAChRs agonist Lynx1 was recently reported to serve as a molecular break system for brain plasticity [Morishita *et al.*, 2010]. The developmental effects of neonicotinoids in juvenile and fetal periods remain to be determined. Neonicotinoids and their metabolites are detected at high frequency in urine samples from Japanese people [Ueyama *et al.*, 2014]. Human exposure to CTD at

a high concentration as high as that of the present CTD-250 groups does not occur naturally, but the precipitates that result from the low water solubility of CTD are very dangerous. In addition, a low concentration of CTD could become harmful under a stressed condition, such as fasting.

The finding that one of the metabolites of imidacloprid (IMI) seriously disturbs MAPK/ERK signaling compared to IMI itself [Tomizawa and Casida, 2002] suggests that the safety coefficient ($\times 100$) for calculating the acceptable daily intake is not satisfactory. Taking the results of the present study as an example, it is clear that further research is needed to identify the toxicant mechanisms of environmental chemicals in order to protect humans and animals with high sensitivities under stressed conditions.

Summary

Neonicotinoids, some of the most widely used pesticides in the world, act as agonists to the nicotinic acetylcholine receptors (nAChRs) of insects, resulting in death from abnormal excitability. Neonicotinoids unexpectedly became a major topic as a compelling cause of honeybee colony collapse disorder, which is damaging crop production that requires pollination worldwide. Mammal nAChRs appear to have a certain affinity for neonicotinoids with lower levels than those of insects; there is thus rising concern about unpredictable adverse effects of neonicotinoids on vertebrates. We hypothesized that the effects of neonicotinoids would be enhanced under a chronic stressed condition, which is known to alter the expression of targets of neonicotinoids, *i.e.*, neuronal nAChRs. We performed immunohistochemical and behavioral analyses in male mice actively administered a neonicotinoid, clothianidin (CTD; 0, 10, 50 and 250 mg/kg/day) for 4 weeks under an unpredictable chronic stress procedure. Vacuolated seminiferous epithelia and a decrease in the immunoreactivity of the antioxidant enzyme glutathione peroxidase 4 were observed in the testes of the CTD+stress mice. In an open field test, although the locomotor activities were not affected, the anxiety-like behaviors of the mice were elevated by both CTD and stress. The present study demonstrates that the behavioral and reproductive effects of CTD become more serious in combination with environmental stress, which may reflect our actual situation of multiple exposure.

Figures and Figure legends

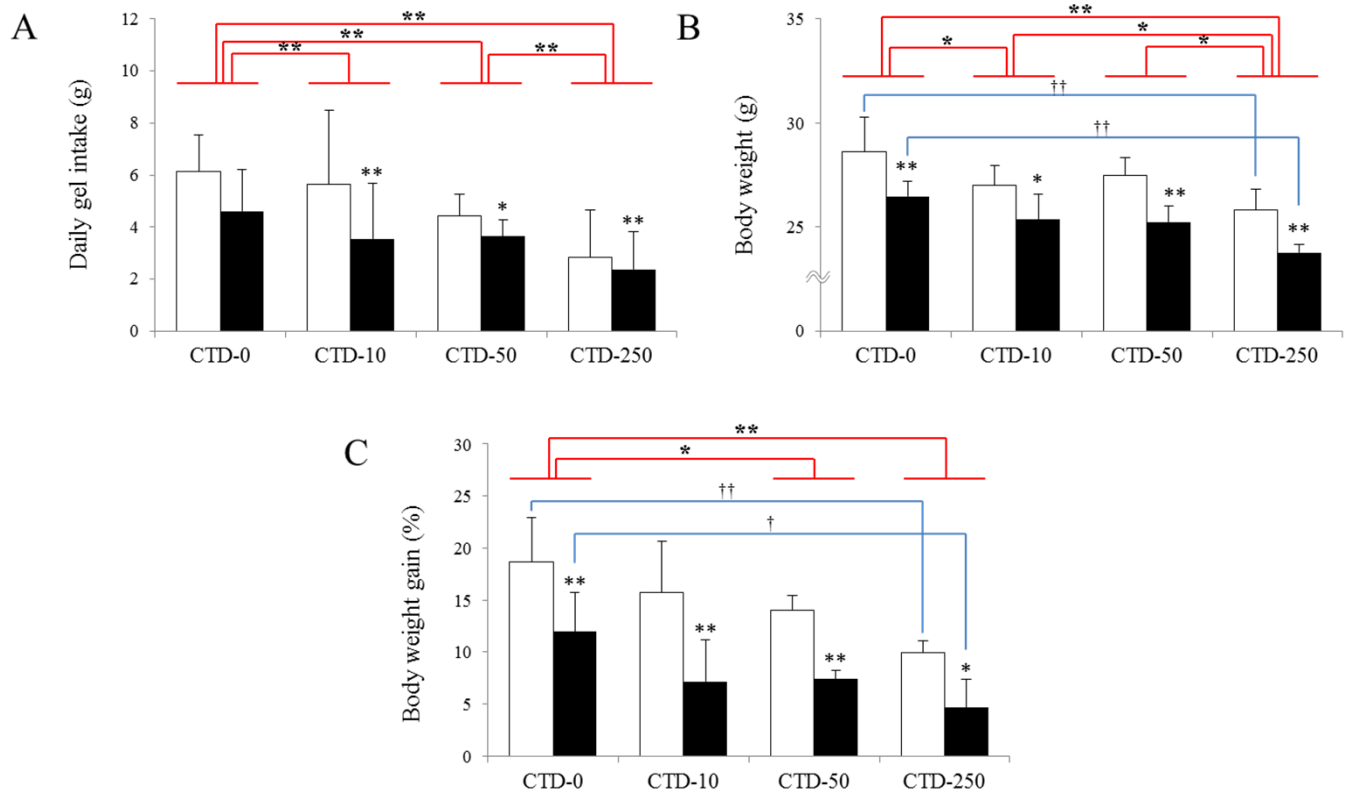


Fig. II-1. Effects of combined exposure on the daily gel intake (A), body weights (B) and testis weights (C) in the non-stressed (open column) and stress groups (closed column). The two-way ANOVA showed significant main effects for the CTD and stress ($p<0.01$), but the interaction effects were not significant in gel intake and body weights. (A) CTD dose-dependently inhibited the gel intake, and the inhibition was significant in all CTD-administration groups compared to the CTD-0 groups. The stress procedure significantly inhibited the gel intake in each CTD-administration group. (B) The body weights of the CTD-250 stress and non-stressed groups were significantly lower than those of the other dose groups, in addition to the significant difference between the CTD-0 and CTD-10 groups. The post hoc analyses also revealed that there were significant differences between the CTD-0 and CTD-250 stress and non-stressed groups. (C) The two-way ANOVA showed a significant main effect for stress ($p<0.01$), but the interaction effect was not significant in the absolute weights of testes. The stress procedure significantly suppressed the absolute testes weights in all stress groups, except the CTD-10 group. Values are mean $\pm$ SD ($n=5$ mice each). * $p<0.05$, ** $p<0.01$.

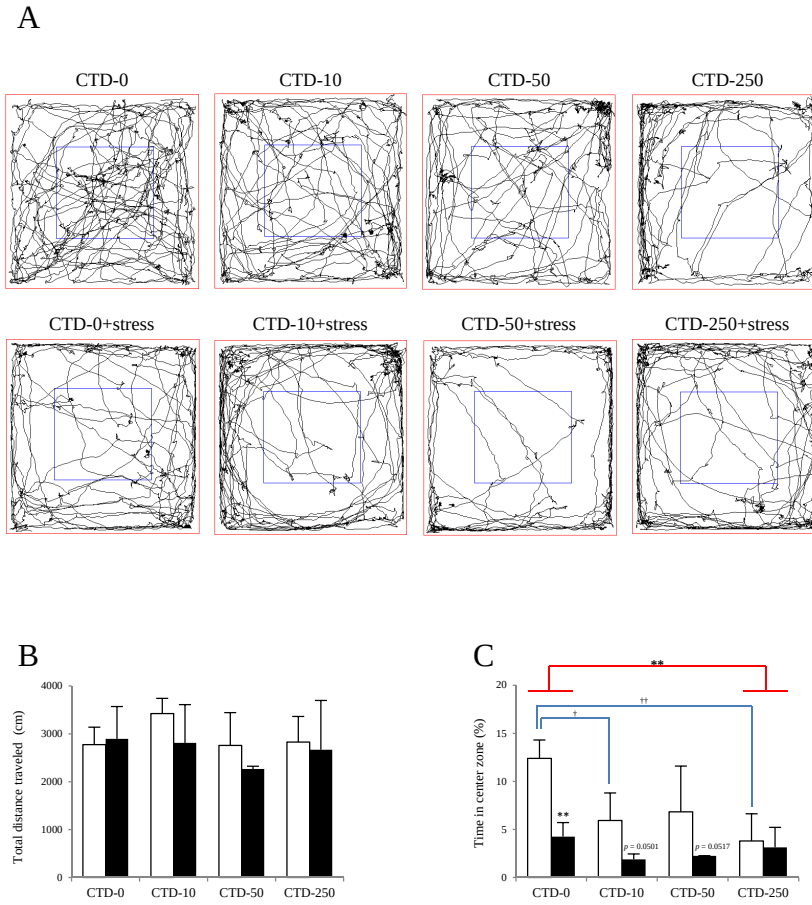


Fig. II-2. Behavioral effect of combined exposure of CTD and stress in the open field activity in the non-stressed and stressed groups. (A) A representative trajectory map of the mice as illustrated by the video tracking software. The exploratory behaviors in the center zone (30 × 30 cm) of the open field (60 × 60 cm) were dose-dependently suppressed by CTD compared to the non-administration groups. (B) Total distances traveled in the open field of the non-stressed (open columns) and stressed groups (closed columns). No marked difference was detected by two-way ANOVA in the total distances traveled. (C) Time spent in the center zone in the open field of the non-stressed (open columns) and stressed groups (closed columns). Two-way ANOVA showed significant main effects for CTD ($p<0.05$) and stress ($p<0.01$), but the interaction effect was not significant. CTD significantly inhibited the times spent in the center zone in the CTD-250 groups compared to the non-CTD administration groups. A significant anxiogenic effect of the stress procedure was observed between the CTD-0 groups. In the non-stressed condition, there were significant differences in the CTD-10 and CTD-50 groups compared to the CTD-0 group. Values are mean $\pm$ SD ($n=5$ mice each). * $p<0.05$, ** $p<0.01$.

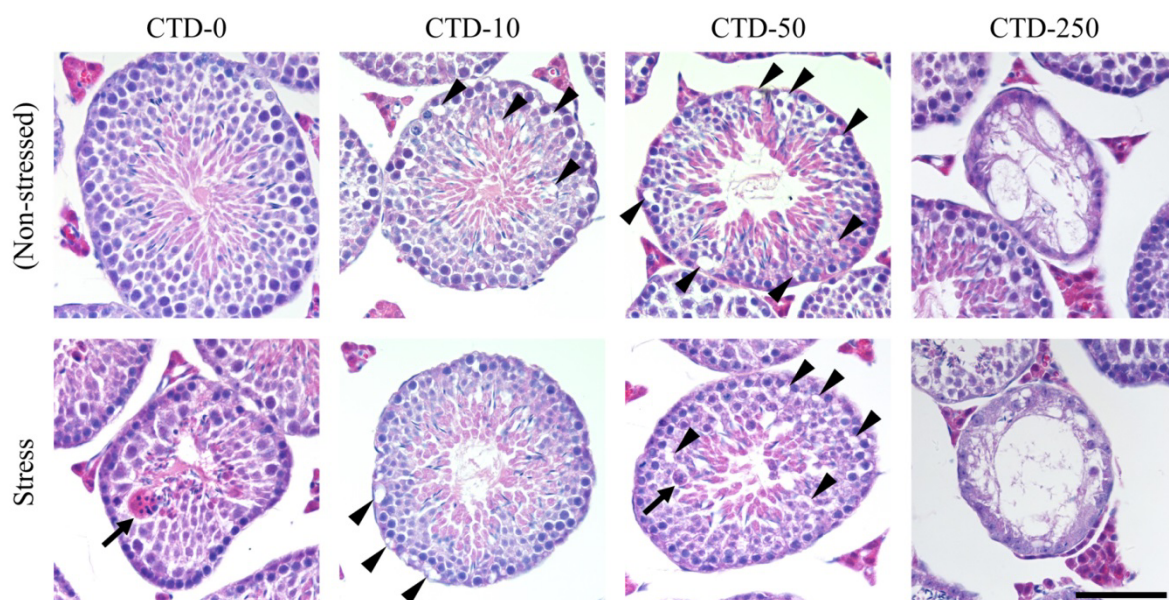


Fig. II-3. Representative histology of the testis in the non-stressed and stressed groups. The seminiferous tubules of the control group showed robust spermatogenesis with densely stacked germ cells. Multinucleated giant cells (arrow) were occasionally observed in the testes of the non-CTD stressed groups. In the CTD groups, dose-dependent vacuolated degeneration (arrowhead) of seminiferous tubules was observed. Degenerated seminiferous tubules only composed of Sertoli cells were present in the CTD-250 groups. Bar = 100 μ m.

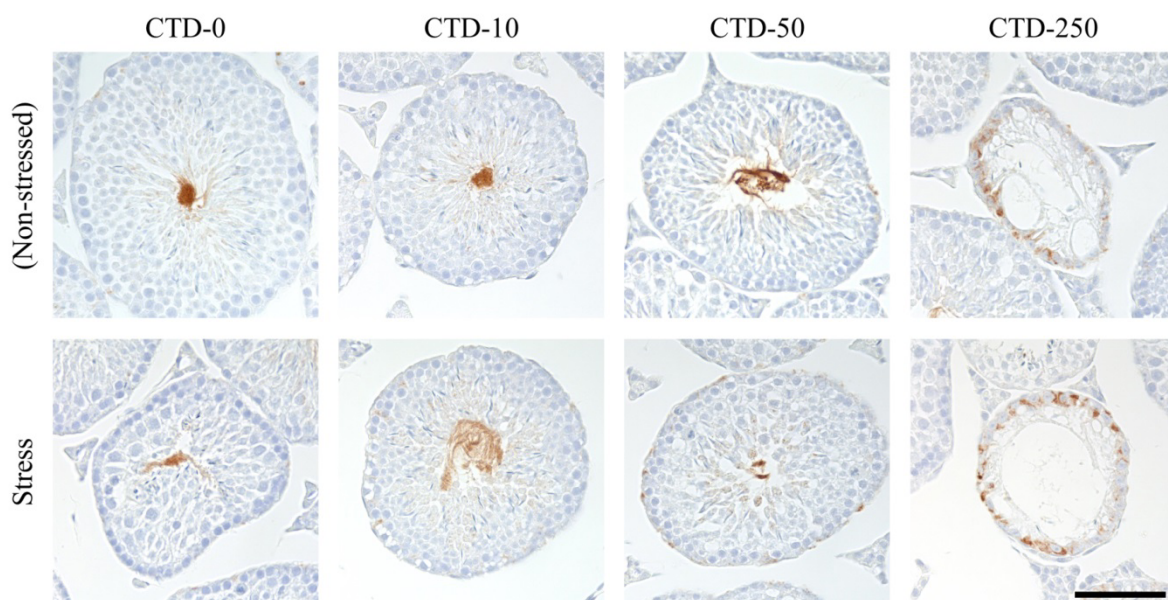


Fig. II-4. Representative immunohistochemistry for GPx4 of the testis in the non-stressed and stressed groups. GPx4 immunoreactivities were detected in sperms and spermatids in the testes of the control group. In the testes of the stressed groups, reduced immunoreactivity of GPx4 in sperm was observed. In the CTD groups, spermatids showed weakened immunoreactivity of GPx4. Sertoli cells ectopically expressed GPx4 in degenerated seminiferous tubules of the CTD-50+stress group and the CTD-250 groups. Bar = 100 μ m.

Chapter III

**Low doses of a neonicotinoid pesticide, clothianidin, acutely
induce anxiety-related behavior with human-audible
vocalizations in male mice in a novel environment**

Introduction

Neonicotinoids are widely used systemic pesticides characterized by high water solubility, residual efficacy and selective toxicity for insects. They have agonistic effects on—and high affinity for—the nicotinic acetylcholine receptors (nAChRs) of insects [Tomizawa and Casida, 2005], but thought to be safe for humans and other vertebrates. Nevertheless, a pioneering study demonstrated that acetamiprid (ACE) and imidacloprid (IMI), earlier types of neonicotinoids, induced an excitatory calcium influx mediated by mammal nAChRs into cerebellar neurons from neonatal rats [Kimura-Kuroda *et al.*, 2012], raising the question of whether neonicotinoids would affect the nervous systems of vertebrates. At the same time, it has been reported that neonicotinoids have adverse effects on the reproductive, immune and nerve functions of non-target species such as fish, amphibians, reptiles, birds and mammals [Tokumoto *et al.*, 2013; Hoshi *et al.*, 2014; Gibbons *et al.*, 2015].

Clothianidin (CTD) was developed in 2001 and is thus one of the latest neonicotinoids; it is used for pest control and seed treatment around the world. Unlike IMI, which suppresses the responses to acetylcholine (ACh) in HEK cells expressing human $\alpha 4\beta 2$ nAChRs, CTD augments and sustains the responses to ACh [Li *et al.*, 2011]. In addition, *in vivo* studies showed that CTD triggered transient dopamine releases through extracellular calcium influx in the rat striatum [Falo *et al.*, 2012], and changed several developmental behaviors of the offspring following maternal exposure [Tanaka, 2012]. We recently reported that subchronic exposure of CTD resulted in anxiety-like behavior in mature male mice in an open field test, which became more serious under mildly stressed conditions [Hirano *et*

al., 2015]. Taken together, these facts indicate that CTD exerts excitatory effects on the neurobehavioral function of mammals at both the cellular and individual level.

The endogenous target of neonicotinoids, nAChRs are ion channel-linked receptors that are partially conserved during the evolution from invertebrates to vertebrates [Bossy *et al.*, 1988]. Their properties vary according to their subunit compositions, and they play diverse roles such as controlling the inflammation and immune responses in the peripheral tissues, in addition to their primary role of transmitting nerve impulses by acetylcholine [Wang *et al.*, 2003]. There are two major types of neural nAChRs in the mammalian brain, the $\alpha 7$ homopentamers and $\alpha 4\beta 2$ heteropentamers, and both types are expressed in the olfactory bulbs, cortex, striatum, hippocampus, thalamus, hypothalamus, amygdala, midbrain, cerebellum and pons on the central nervous system [Gotti *et al.*, 2006]. They function not only in cholinergic neurotransmission at the postsynaptic membranes, but also modulate the release of other neurotransmitters at the presynaptic terminals [Marchi and Grilli, 2010]. Disturbances of the signals mediated by nAChRs have been associated with psychiatric disorders including Alzheimer's disease, schizophrenia, depression, attention deficit hyperactivity disorder and nicotine addiction [Taly *et al.*, 2009], and thus agents that disturb these signals could pose a significant risk to public health.

As previously mentioned, there are different types of nAChRs broadly expressed in various brain regions, and it remains unclear which brain regions neonicotinoids may affect. Therefore, the purposes of this study were (i) to elucidate whether acute CTD exposure has anxiogenic effects in a novel

environment in mammals, and (ii) to reveal the possible target regions of the mammalian brains related to the neurobehavioral effects of neonicotinoids. To this end, we conducted behavioral and neuroactivity analyses focusing on anxiety-related phenotypes such as the abnormal vocalizations observed in mice administered CTD mice during the elevated plus-maze test.

Materials and methods

Experimental animals and procedure

Male C57BL/6N mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were group-housed in 40.5 × 20.5 × 18.5 cm individually ventilated cages (Sealsafe Plus Mouse; Tecniplast, Buguggiate, Italy) under controlled temperature (23 ± 2°C) and humidity (50 ± 10%) on a 12-h light/dark cycle at the Kobe University Life-Science Laboratory with *ad libitum* access to a pellet diet (DC-8; Clea Japan, Tokyo, Japan) and filtered water. This study was approved by the Institutional Animal Care and Use Committee (Permission #26-05-07) and carried out according to the Regulation on Animal Experimentation at Kobe University. At 9–10 weeks of age, mice were orally administered CTD at a dose of 0, 5 or 50 mg/kg body weight suspended in 0.5% carboxymethylcellulose (10 mL/kg) as vehicle with reference to the no-observed-adverse-effect level (NOAEL) of 47.2 mg/kg from general toxicity studies in mice (Fig. 1). These groups were defined as the control, CTD-5 and CTD-50 groups, respectively. All chemicals were administered to mice under isoflurane anesthesia to minimize handling stress.

Elevated plus maze test

After the CTD administration, all mice in their home cages were allowed to acclimate to the conditions in a dimly-illuminated experimental room (10–20 lux, 23 ± 2°C) for 1 h. The elevated plus-maze test was then conducted as described previously [Walf and Frye, 2007] with some modifications. Briefly, the apparatus was constructed of white acrylic plates and consisted of two opposite open arms

(30 × 5 cm) and two opposite enclosed arms (the same size with walls 15 cm high) extending from a common central platform (5 × 5 cm) (Tom Product, Tokyo, Japan). Mice were individually placed on the central platform facing an open arm, and all their activities were recorded by a video camera for the subsequent 10 min. The total distance traveled, the total number of arm entries, the percentage of open arm entries, and the percentage of time spent in open arms were scored by using Image EP software [Komada *et al.*, 2008].

Vocalization analysis

All sounds during each elevated plus-maze test were recorded by a PCM recorder (96 kHz sampling rate, 24 bit depth) to visualize the human-audible abnormal vocalizations of mice. Frequency spectrograms (hamming window, FFT length = 256, frame size = 100%, overlap = 75%, cut-off frequency <3 kHz) were made by using SAS Lab software (Avisoft Bioacoustics, Berlin, Germany). Human-audible vocalizations were manually checked and counted for each minute of the behavioral tests.

Neuroactivity analysis

Two hours after the behavioral test, all mice were deeply anesthetized with isoflurane and transcardially perfused with 0.9% normal saline, then with ice-cold 4% paraformaldehyde in phosphate buffer. The brains were cryoprotected in ascending solutions of sucrose (10%, 20%, 30%) in 0.1 M phosphate buffer (PB) overnight and then frozen in liquid nitrogen in an embedding solution consisting of Tissue-Tek® O.C.T. compound (Sakura Finetek, Tokyo, Japan): 30% sucrose in

0.1 M PB = 1: 2. Coronal sections (−1.00 mm to −1.70 mm from the bregma) cut at 10 µm thickness with 120 µm intervals on a cryostat (CM1950; Microsystems, Wetzlar, Germany) were mounted on slide glasses precoated with 2% 3-aminopropyltriethoxysilane (Shin-Etsu Chemical Co., Tokyo, Japan).

The sections were immersed in absolute methanol and 0.5% H₂O₂ for 30 min, respectively, at room temperature (RT) to quench the endogenous peroxidase activity. They were then incubated with Blocking OneHisto (Nacalai Tesque Inc., Kyoto, Japan) for 1 h at RT for protein blocking, followed by incubation with rabbit polyclonal anti-c-fos antibody (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted 1:2,000 in phosphate buffered saline with 0.05% Tween-20 (PBST; pH 7.4) for 18 h at 4°C. After being washed with PBST, 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 h at RT. Immunoreactivity was then detected by incubation with 3,3'-diaminobenzidine solution (EnVision+ kit/HRP[DAB]; Dako). Next, the sections were placed in a graded series of ethanol, dehydrated with absolute ethanol, cleared in xylene and coverslipped with Eukitt mounting medium (O. Kindler GmbH, Freiburg, Germany). We determined the brain regions anatomically according to a brain atlas and calculated the number of c-fos immunopositive nuclei per area by using ImageJ software. At least 3 sections per mouse were analyzed in this way.

Statistical analysis

Statistical analyses were performed with Excel Statistics 2012 (SSRI version

1.00; SSRI, Tokyo, Japan). In the behavioral analyses, outliers more distant than 1.5 interquartile ranges from the upper or lower quartile were omitted. All data were analyzed by one-way ANOVA followed by the Dunnett's post hoc test. The results were considered significant when the p -value was less than 0.05.

Results

Behavioral effects of acute CTD in the elevated plus maze test

To measure the anxiogenic effect of CTD in a novel and stressful environment, all mice were subjected to the elevated plus-maze test. There were no marked physical signs of acute toxicity in the CTD-administered mice during the 1-h period spent in their home cages before the test. The representative trajectory maps showed that the CTD-administered mice seldom walked in the open arms (Fig. 2A). The locomotor activities measured by the total distance traveled and the total number of arm entries were not changed in the CTD-5 groups, but were significantly lower in the CTD-50 group than the control group (Fig. 2B, C). At the same time, the open arm activities (the percentage of time spent in the open arms and the percentage of open arm entries) were significantly decreased in both the CTD-5 and CTD-50 groups, indicating an increased level of anxiety (Fig. 2D, E). In addition, mice in the CTD-50 group showed several signs of excessive anxiety, such as teeth chattering, freezing and abnormal vocalization during the behavioral test.

Vocalization analysis

All mice in the CTD-50 group spontaneously emitted chirp-like vocalizations on the elevated plus-maze when they were selecting arms on the center platform or looking down from open arms (Supplementary movie). The spectrogram revealed that the human-audible vocalizations produced by mice consisted of harmonics at a constant frequency between 4 and 16 kHz with durations between 75 and 120 msec (Fig. 3A). There were 70.6 ± 19.5 times of vocalizations in the

elevated plus-maze test in the CTD-50 group, but no vocalizations in the control and CTD-5 groups (Fig. 3B). It was also noted that most of the vocalizations were observed immediately after the elevated plus-maze test started and decreased drastically within 1 min (Fig. 3C).

The effects of CTD on c-fos expression induced by the elevated plus maze test

To identify the brain regions involved in excessive behavioral responses to the elevated plus-maze test in the CTD-50 group, neuroactivity analyses visualizing c-fos immunoreactivity were performed (Fig. 4). There were c-fos immunoreactive cells detected in parts of the cortex, hippocampus, thalamus, amygdala and hypothalamus—namely, the piriform cortex, hippocampal CA1 and CA3 regions, dentate gyrus of the hippocampus (DG), paraventricular thalamic nucleus (PVT), habenular nucleus, laterodorsal thalamic nucleus, medial nucleus of amygdala, paraventricular hypothalamic nucleus, dorsomedial hypothalamic nucleus, and ventromedial hypothalamic nucleus—in both the control and CTD-50 groups, which was in agreement with the previous report [Silveira *et al.*, 1993]. Notably, in PVT (Fig. 4A–C) and the medial blade of DG (Fig. 4D–F), there were significant increases in the c-fos immunoreactive nucleus per area in the CTD-50 group compared to the control group.

Discussion

In this study, we demonstrated the acute effect of CTD at the NOAEL dose on the behavioral responses to a novel environment and explored the brain regions related to the anxiety-like behaviors in the elevated plus-maze test. Intraperitoneally-administered CTD (20 mg/kg) was immediately absorbed and was detected in the mouse brain at a level on the order of ppm for at least 4 h [Ford and Casida, 2006]; therefore, CTD was considered to be present in the brains of CTD-administered mice at a concentration that was previously shown to be sufficient to cause neuronal excitation *in vitro* [Kimura-Kuroda *et al.*, 2012]. The behavioral effects observed in this study strongly suggest that low-dose CTD has anxiogenic effects in mammals. These findings agree closely with a previous study on the behavioral effects of thiamethoxam (TMX, 100 mg/kg/day), a metabolic precursor of CTD, which was found to decrease locomotor activity in an open field test and open arm activity in an elevated plus-maze test [Rodrigues *et al.*, 2010].

As mentioned above, neural nAChRs are involved with emotional reactions such as mood and anxiety [Picciotto *et al.*, 2015]. Nicotine, a typical agonist of nAChRs, exerts both anxiolytic and anxiogenic effects depending on the dose of exposure, which are known to be largely mediated through the $\alpha 4\beta 2$ type of nAChRs [Anderson and Brunzell, 2015]. Both the $\alpha 4$ subunit knock-out mice [Ross *et al.*, 2000] and hypersensitive $\alpha 4$ receptor mice [Labarca *et al.*, 2001] have been reported to show strong anxiety in the elevated plus-maze test. Importantly, continuous exposure to a nicotinic agonist at a nanomolar concentration strongly desensitizes nAChRs following transient activation

[Giniatullin *et al.*, 2005], implying that the anxiogenic effects of CTD would be attributed to a decrease in the sensitivity of nAChRs to agonists, as with signal blockades by antagonists, rather than to the instantaneous excitation of neurons.

One of the most distinctive findings in this study was the observation of human-audible vocalization behaviors in the CTD-50-group mice during the elevated plus-maze test. To the best of our knowledge, there have been no reports of mice expressing such a pronounced phenotype on this behavioral test. It is well known that adult experimental mice rarely make chirping sounds in the human-audible range except under stressful and crisis situations [Sánchez, 2003]. According to the latest reports, the vocalizations of mice are indicators of emotional states and can be classified by frequency as follows: (i) ultrasonic vocalizations (>20 kHz) are used to communicate with others such as in mother-pup and male-female interactions; (ii) mid-frequency vocalizations (12 kHz) are observed under restraint-stress conditions; and (iii) low-frequency harmonics (5–40 kHz, broad harmonic structure) are emitted during strong pain or aggressive encounters [Scattoni *et al.*, 2009; Grimsley *et al.*, 2016]. The latter two types of vocalizations are also described as “distress calls” of mice experiencing negative emotions and are similar to the 22 kHz ultrasonic vocalizations of rats in stressful situations. Although the human-audible chirps made by CTD-administered mice in this study were characterized by a low and harmonic frequency (4–16 kHz) that was similar to that of mice making distress calls, they might be categorized as another type of mouse vocalization in an aversive behavioral context. Taken together, these findings indicate that CTD rendered mice irritable in the presence of what would otherwise have been a tolerable level

of stress, leading them to emit novel human-audible calls as signs of excessive stress or anxiety early in the course of the elevated plus-maze test.

It remains a critical question which neural circuits were involved in the unique behavioral changes induced by CTD. Cholinergic neurons in the mammalian brain are divided into two major populations: (i) neurons of the basal forebrain region, which mainly project to the olfactory bulbs, frontal cortex, amygdala, and hippocampus, and (ii) those of the pontomesencephalic tegmental region, which project to the thalamus, hypothalamus, cerebellum, midbrain and pons [Woolf, 1991]. In these possible target regions receiving cholinergic innervations and expressing nAChRs, we demonstrated that the level of excitability of parts of the thalamus and hippocampal areas was elevated in response to the novel environment after CTD administration, suggesting that the cholinergic signals from both the major populations could be disrupted by CTD.

PVT, a small dorsal nucleus of thalamus, has recently been shown to modulate negative emotional behaviors such as fear, anxiety and stress response [Hsu *et al.*, 2014]. Consistent with our results, rodent studies showed that various stressors and nicotine both activated the PVT [Bubser and Deutch, 1999], and the c-fos expressions induced by restraint stress were much greater under nicotine treatment [Pagliusi *et al.*, 1996]. The PTV anatomically receives the afferent fibers from the brainstem, diencephalon and telencephalon, and project to the forebrain regions [Kirouac, 2015]. The main role of the PVT could be described as “coordinating” the emotional responses that integrate information from neurotransmitters and neuropeptides including serotonin, dopamine, norepinephrine, corticotropin releasing hormone and orexin from the caudal brain regions. In addition,

according to lesion studies, the PVT is required for down-regulating the stress response [Bhatnagar *et al.*, 2002; Spencer *et al.*, 2004]. Moreover, a study in *Nature* described that the PVT-amygdala pathway controls the fear circuit [Penzo *et al.*, 2015]. Although there were no changes of the nervous activities in amygdaloidal regions by CTD, the activation of PVT and subsequent enhancement of the input to PVT-innervating regions such as the central nucleus of the amygdala, bed nucleus of the stria terminalis and nucleus accumbens may be among the factors involved in the emotional changes observed in CTD-administered mice.

The hippocampus is important not only for spatial and episodic memory, but also stress responses. Cholinergic blockade in the hippocampus activates the physical-stress response of the hypothalamic-pituitary-adrenal axis [Bhatnagar *et al.*, 1997]. Either region-specific administration of nicotine or knockout of acetylcholinesterase in the hippocampus results in an elevated level of anxiety in rodents [Ouagazzal *et al.*, 1999; Mineur *et al.*, 2013], suggesting that abnormal states of cholinergic signaling in the hippocampus result in aversive behaviors similar to those observed in the present study. In particular, the DG is described as an input region of hippocampal formation [Jonas and Lisman, 2014] and is involved in an event called adult neurogenesis in the granular cell layer, which is vulnerable to environmental chemicals and stressors [Heine *et al.*, 2004; Kobayashi *et al.*, 2015]. Anatomical studies have shown that the DG is innervated by multiple neurotransmitters that project to the hippocampal CA3 regions. The cholinergic afferents from the medial septal nucleus and nucleus of the diagonal band of Broca are important as excitatory inputs to the DG [Amaral *et al.*, 2007].

In this study, mice in the CTD-50 group showed an increase in the number of c-fos-positive nuclei in granule cells in the DG, which has been shown to express the functional $\alpha 7$ type of nAChRs from an immature stage [John *et al.*, 2015], and this change may have had an impact on the entire hippocampus and contributed to the behavioral effects of neonicotinoids.

According to the latest studies, other brain regions may also be targeted by neonicotinoids. Kimura-Kuroda *et al.* (2016) recently reported an alteration in the transcriptome of cerebellar cultures exposed to ACE and IMI, including changes in genes essential for brain development. Terayama *et al.* (2016) revealed that ACE decreased the $\beta 2$ type of nAChRs in many brain regions and tended to be residual in the midbrain. In other studies, stress-induced 22 kHz vocalizations of rats were attributed to the activation of cholinergic neurons in the midbrain and pons [Kroes *et al.*, 2007; Borges *et al.*, 2010]. Considering all of these evidences, it cannot be denied that the cerebellum and midbrain innervated by cholinergic projection could also be upstream targets of neonicotinoids.

As noted before, the cholinergic signals in the brain are important for stress responses [Picciotto *et al.*, 2012]. For instance, nicotine is known to enhance the responses to various stressors [Yu and Sharp, 2012]. However, in the risk assessment for new chemical substances, there have been no studies measuring their disruption of the stress reactivity in mammals. This is crucial information, since excessive stress responses are known to suppress the physical condition of mammals, including impairments in reproductive, immune and metabolic function [Chrousos and Gold, 1992; Hirano *et al.*, 2014], and thus an increase in the threshold for novel environments and the activation of stress reactivity may be

helpful endpoints when evaluating the systemic effects of environmental factors.

In the current study, we present conclusive evidence that CTD at a NOAEL dose activates neural circuits controlling the stress reactivity, including the PVT and DG, resulting in excessive anxiety-like behavior in the elevated plus-maze test. Epidemiological studies revealed that the detection rates and amounts of neonicotinoids in urine in Japanese adults became increased in proportion to the domestic usage [Ueyama *et al.*, 2015], suggesting that further information is needed for the proper global risk assessment of neonicotinoids. These results should make several contributions toward evaluating the neurobehavioral effects of neonicotinoids at the actual dose level of exposure.

Summary

Neonicotinoids are novel systemic pesticides acting as agonists on the nicotinic acetylcholine receptors (nAChRs) of insects. Experimental studies have revealed that neonicotinoids pose potential risks for the nervous systems of non-target species, but the brain regions responsible for their behavioral effects remain incompletely understood. This study aimed to assess the neurobehavioral effects of clothianidin (CTD), a later neonicotinoid developed in 2001 and widely used worldwide, and to explore the target regions of neonicotinoids in the mammalian brain. A single-administration of CTD to male C57BL/6N mice at or below the no-observed-adverse-effect level (NOAEL) induced an acute increase in anxiety during the elevated plus-maze test. In addition, mice in the CTD-administered group spontaneously emitted human-audible vocalizations (4–16 kHz), which are behavioral signs of aversive emotions, and showed increased numbers of c-fos immunoreactive cells in the paraventricular thalamic nucleus and dentate gyrus of the hippocampus. In conclusion, mice exposed to low-dose CTD were rendered vulnerable to a novel environment via the activation of thalamic and hippocampal regions related to stress responses. These findings should provide critical insight into the central effects of neonicotinoids on vertebrates.

Figures and Figure legends

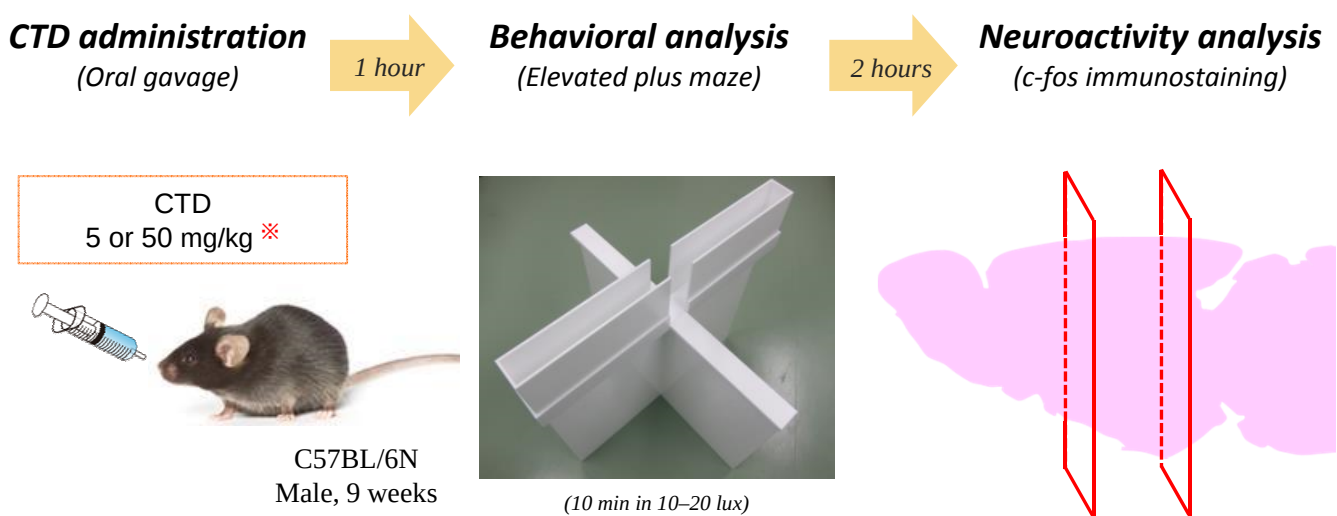


Fig. III-1. Scheme showing the overall experimental design. One hour after oral administration of CTD (0, 5, 50 mg/kg) to mice, behavioral effects were assessed by an elevated plus-maze test for 10 min. After 2 h of the behavioral test, mice were sacrificed for the neuroactivity analysis.

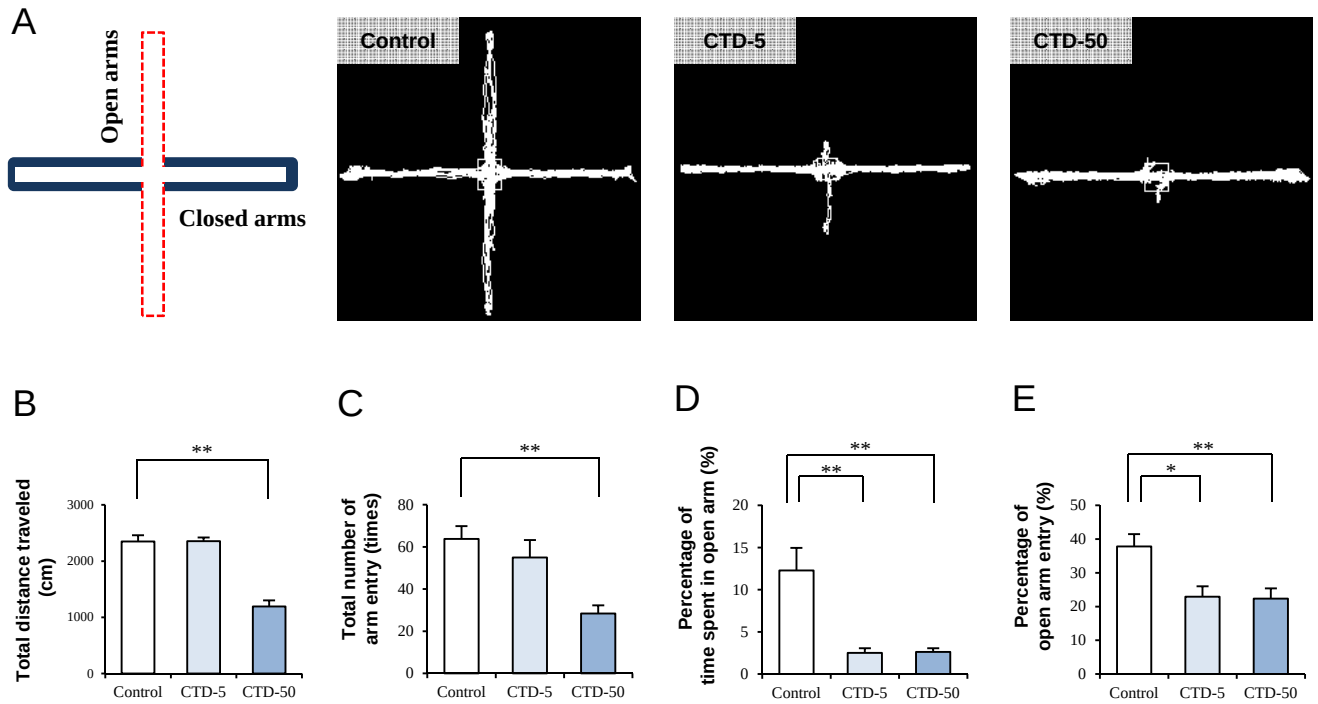


Fig. III-2. Behavioral effect of acute CTD observed in the elevated plus-maze test. (A) representative trajectory maps showed that the exploratory behaviors in the open arms were suppressed in the CTD-administered groups compared to the control group. (B) The total distance traveled and (C) the total number of arm entries were not changed in the CTD-5 groups, but were significantly lower in the CTD-50 group relative to those of the control group. (D) The percentage of time spent in the open arms and (E) the percentage of open arm entries were significantly lower in both the CTD-5 and CTD-50 groups than the control group. Values are presented as the mean $\pm$ SE (n =10–14 mice per group). * p <0.05, ** p <0.01.

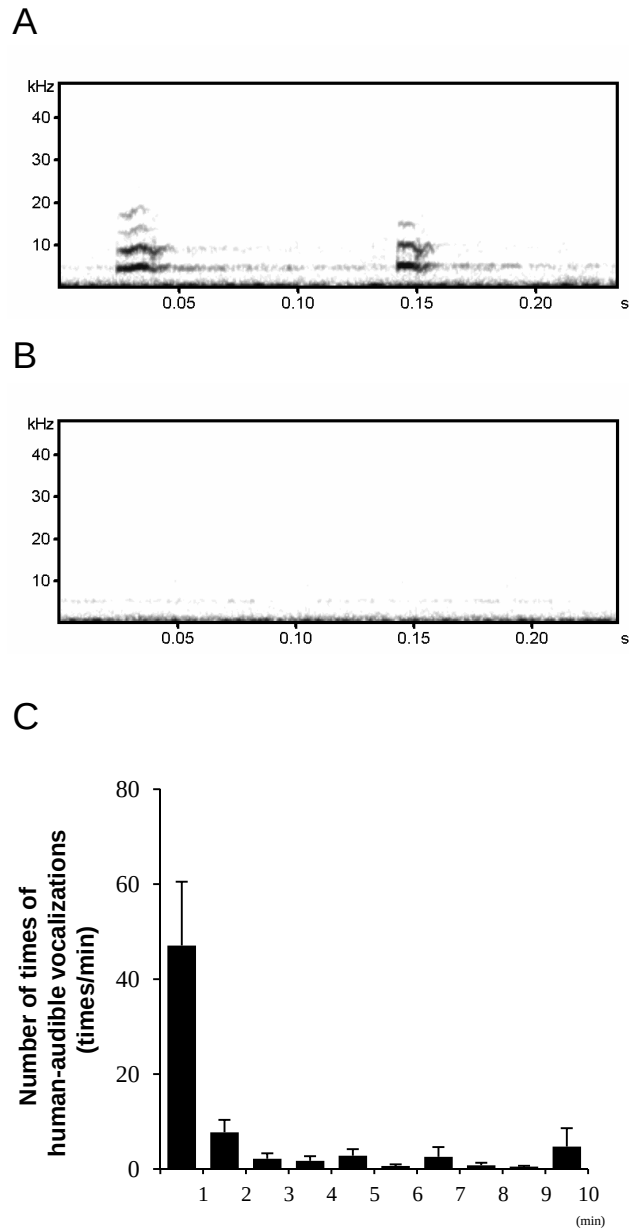


Fig. III-3. Representative spectrograms of human-audible vocalizations emitted by mice in the CTD-50 group during the elevated plus-maze (A); no vocalizations were emitted in the control group (B). The frequency distribution of the vocalizations was in the human-audible frequency range (<20 kHz). (C) Histogram showing the number of the vocalizations observed during the elevated plus-maze test. Values are presented as the mean $\pm$ SE (n =10 mice in the CTD-50 group).

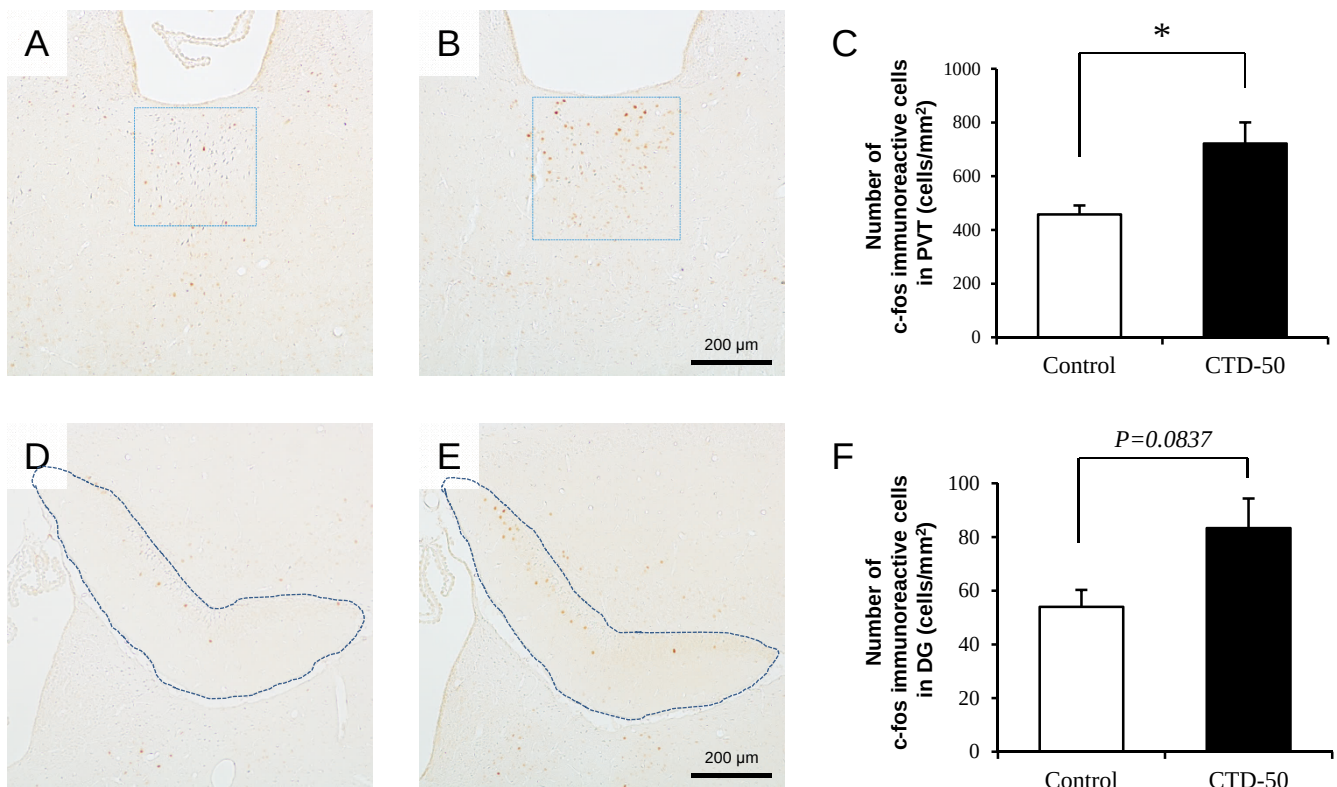


Fig. III-4. Representative histology of the c-fos immunoreactivities in the paraventricular thalamic nucleus (PVT, surrounded by a dotted square) (A, B) and hippocampal dentate gyrus (DG, surrounded by a dotted line) (D, E) of the control (A, D) and CTD-50 (B, E) groups. The number of c-fos-immunoreactive cells in PVT (C) and DG (F) were significantly increased in the CTD-50 group compared to the control group. Values are presented as the mean $\pm$ SE (n =6 mice per group). * $p<0.05$. Bar = 100 μ m.

CONCLUSION

There will continue to be produced enormous novel chemicals in the future; therefore, developing new endpoints and methods of risk assessment for adverse effects of environmental factors were needed under the present circumstances. In this study, I have assessed the broad effects of environmental factors, *i.e.*, environmental stressors and chemicals, and revealed novel targets of them.

In the Chapter I, I have assessed the systemic effect of the chronic mild stress to understand the mechanism of reproductive suppression under stressed conditions. Importantly, there are several signs of testicular degeneration, including increased numbers of ssDNA-positive cells per seminiferous tubule, thinning, vacuolated seminiferous epithelia and multinucleated giant cells were observed in the stressed group with the decreasing of immunoreactivities of kisspeptin in hypothalamus. These results suggest that environmental stress input to the reproductive axis by an upstream neuropeptide, kisspeptin as a target [Hirano *et al.*, *J. Vet. Med. Sci.*, 76: 1201–1208. 2014].

Chapter II demonstrates that both environmental chemical and stress could additively affect both the reproductive and behavioral function of male mice. I have assessed the effects of a new environmental chemical, one of neonicotinoid pesticides, clothianidin (CTD), under the stressed condition. We observed vacuolated seminiferous epithelia and decreased antioxidant enzymes, glutathione peroxidase 4, in the testes of the CTD-treated mice. In an open field test, anxiety-like behavior appeared to increase most in the CTD + stress mice. These results suggest that broad effects of environmental chemicals could be more serious under environmental stress [Hirano *et al.*, *J. Vet. Med. Sci.*, 2015, 77:

1207–1215, 2015].

In the Chapter III, I have analyzed the anxiety-like behavior induced by CTD in novel environment, and explored the target brain region associated with neurobehavioral effects of neonicotinoid pesticides. A single administration of CTD led to decreases in the total distance traveled and the percentage of open arm entry in addition to the occurrence of abnormal vocalization (4–16 kHz) during the elevated plus maze test. The numbers of c-fos immunoreactive cells were increased in several brain regions receiving neural input regarding aversive emotion including the paraventricular thalamic nucleus and the dentate gyrus of the hippocampus, suggesting an exaggerated response to stress from a novel environment. These results suggest that a low dose of neonicotinoids induced various neurobehavioral changes, possibly mediated by activating broad neural circuits including the thalamus and hippocampus.

In conclusion, broad effects of environmental factors are commonly mediated by stress response in the mammal brain. I pointed out the mechanism of combined effect, which environmental chemicals disrupt the stress reactivity, suggesting that the “stress reactivity” should be a novel endpoint to detect the effects of environment chemicals.

SUMMARY IN JAPANESE

種々の環境要因は生体に多大な影響を与え、その表現型をも変化させる。とくに現代においては、次々と生み出される無数の化学物質は生体に予期せぬ影響を与え続け、その評価並びにそれに基づく適切な対処は「持続可能な発展」を目指す人類にとって今後も直面し続ける意義深いテーマである。また、環境化学物質に対する既存の影響評価は物質単独の作用を解析するものが主であったが、2009年にWHOが複数化学物質への複合曝露に対する評価枠組み(Risk Assessment of Combined Exposures to Multiple Chemicals: A WHO/IPCS Framework)を公表する等、他の化学物質や環境要因との複合影響の存在が近年大きな注目を集めている。

一方、近年社会問題となっている精神疾患や不妊増加の原因としては、生来の遺伝的要因に加えて、環境化学物質や心理的ストレス曝露による環境要因が挙げられる。しかしながら、一卵性双生児が同じ疾患を発症しない例や、特定の高感受性集団においてのみ影響が顕在化する化学物質過敏症等の例が示すように、発症メカニズムとして、個々のもつ素因に様々なストレスが複合し、閾値を越えた際に症状が顕在化する「ストレス脆弱性仮説」が支持されている。このような状況の中、毒性学分野においては、環境要因による有害影響のメカニズムやパスウェイを分子、細胞、組織、個体レベルで解明することで上流イベントにおける共通性を見出し、エンドポイントやバイオマーカーを新規開発することでより予測的な影響評価を行う必要性が指摘されている。そこで本研究では、環境ストレス、並びに新規環境化学物質であるネオニコチノイドの単独、あるいは複合影響評価を行い、影響メカニズムの解明と新規エンドポイントの探索を試みた。

第I章では、さまざまな環境ストレスに慢性的に曝露されている生物の代表として、うつ病のモデル動物として薬理試験等に用いられる慢性予測不能ストレス

(Unpredictable Chronic Stress)に曝露した 8 週齢のマウスを用い、特に性成熟以降の雄マウスにおいて、慢性予測不能ストレスの曝露が如何にして視床下部－下垂体－性腺(HPG)系のフィードバック機構を破綻させ生殖機能を低下させるのかを解明すべく、その影響を組織学的、内分泌学的に検討した。

ストレス群において、精巣重量は対照群と比較して有意に減少した。ストレス群の脳では、視床下部弓状核における神経細胞体の大きさ、形状、細胞密度、配列に差はみられなかったが、キスペプチン陽性反応はわずかに減弱した。ストレス群の精巣では、精上皮の菲薄化、多核巨細胞の出現、ssDNA 陽性細胞の増加等の精巣毒性所見が観察された。キスペプチンの減弱所見は環境ストレスによる慢性的なストレスホルモンの分泌増加によるものと推察され、これにより FSH, LH の分泌低下を引き起こし、その結果、精巣における生殖細胞が減少したと考えられた。以上より、環境ストレスは成熟雄マウスの生殖機能に影響を及ぼすことが明らかになった。

1990 年代にニコチンの構造を基に開発されたネオニコチノイドは、昆虫のニコチン性アセチルコリン受容体(nAChRs)を標的とし、持続的神経興奮を惹起させる選択的アゴニスト作用を示すため、農薬及び家庭用殺虫剤等の成分として使用量が急激に増加している。ところが近年、ネオニコチノイドは僅かながらも哺乳類型 nAChRs に対する結合能を持ち、ラット新生子小脳顆粒細胞に対してもニコチン曝露時と類似した異常性興奮反応を引き起こすことが示され、ADHD、うつ、学習障害との関与並びにさらなる不測の影響を昆虫以外へ与える可能性が懸念されている。これまでに当研究室では、新潟県佐渡市におけるトキの繁殖障害問題を受け、ニホンウズラを用いてネオニコチノイド系農薬の一種クロチアニジン(CTD)が酸化ストレスを介して鳥類の生殖機能を障害することを報告した。しかし、哺乳類に対する影響を調べた報告は非常に少なく、依然として安全性

は十分に証明されていない。2013 年 12 月, EU は予防原則に基づきネオニコチノイド系農薬 3 種を暫定使用禁止としたが, 我が国においては農作物中の残留基準値を現状よりも緩和しようとする相反した対応がみられ, その影響評価は国際レベルで急務であると考えられる。ネオニコチノイド系農薬の標的である nAChRs は神経筋接合部並びに生殖中枢である視床下部を含む脳全域に分布する。また, 海馬等の脳各部において nAChRs を構成するサブユニットの発現はストレス曝露により変化し, その影響は世代を超えて受け継がれる。そこで第 II 章では, ストレスがネオニコチノイドの標的である nAChRs 発現を変化させ, 生殖機能及び中枢神経系に対して相乗的に作用するとの仮説を立て, その影響を検証した。

市販のダントツ水溶剤(住友化学)を蒸留水で繰り返し洗浄することで, 製品中に含まれる CTD を高純度(93~97%)に精製することに成功した。上記の CTD を一定量配合(0, 10, 50, 250 mg/kg/day, 以下各 CTD-0, 10, 50, 250 群とする)した経口補水ゲルを 8 週齢雄 C57BL/6 マウスに与え, 6 種の軽度ストレスより 2 つを毎日順序無作為に与える慢性予測不能ストレス(Unpredictable Chronic Stress)に 4 週間複合曝露した。曝露期間中のゲル摂取量を 1 日毎に測定し, 曝露最終日に 10 分間の Open Field 試験における総移動距離及び中心区画滞在時間を計測した。その後, それぞれのマウスを 4%PFA により灌流固定し, 摘出した精巢を重量測定及び組織学的解析に供した。

その結果, CTD 及びストレスの複合曝露によりマウスのゲル摂取量は有意に減少し, それに伴って採材時体重の有意な減少がみられた。Open field 試験における自発運動量の指標となる総移動距離は, CTD 及びストレスの曝露により変化しなかった。一方で, 不安様行動を示す個体では値が減少する中央区画滞在時間は, CTD 及びストレスの単独及び複合曝露によって減少した。

精巣重量はストレス曝露により有意に減少したが CTD 曝露による影響はみられなかった。しかしながら、HE 染色による一般組織化学的解析の結果、ストレス曝露群においては多核巨細胞の出現、CTD 曝露群の精巣においては生殖細胞脱落による精上皮空胞変性等の精巣毒性所見が観察された。さらに、CTD-250 群においては精細管内腔の生殖細胞のほとんどが脱落したセルトリ細胞のみで構成される精細管が散見された。精巣における主要な抗酸化酵素である glutathione peroxidase 4 (GPx4) 及び manganese superoxide dismutase (Mn-SOD) の局在を免疫組織化学的に検出したところ、GPx4 は精子及び精子細胞の細胞質に強い陽性反応がみられ、Mn-SOD は精子、精子細胞、精母細胞及びライディッヒ細胞の細胞質に陽性反応がみられた。CTD 及びストレス曝露群の精巣においては、これらの抗酸化酵素の陽性反応減弱が認められ、とくに CTD-250 群のセルトリ細胞においては異所性陽性反応がみられた。

以上の結果から、CTD とストレスは行動及び生殖機能に対し相加的に影響を与えることが示された。脳内においてニコチン等の nAChRs リガンドは、その脱感作及び活性変化を引き起こすことで様々な行動影響を及ぼすことが知られている。本研究においても、慢性的な CTD 及びストレス複合曝露は、nAChRs の脱感作及び nAChRs 発現減少を介したコリン作動性伝達減弱を引き起こし、不安様行動を惹起する可能性が考えられた。一方精巣においては、生殖細胞が CTD やストレスによる酸化ストレスの第一標的となり、内腔へ脱落、もしくはセルトリ細胞に貪食されることで精巣組織構造に異常を引き起こすことが示唆された。

さらに第Ⅲ章では、CTD の急性投与により誘発される不安に関する詳細な行動解析を行い、行動影響発現に関与する脳領域を探索することを目的とした実験を行った。成獣雄マウスに 5 もしくは 50 mg/kg の CTD を単回経口投与し、投与 1 時間後に高架式十字迷路試験による急性行動影響解析を行った。CTD

5 mg/kg 投与群においては、値の減少が不安様行動の指標となる Open arm 滞在時間および侵入回数の減少がみられた。加えて、CTD 50 mg/kg 投与群においては総移動距離の減少ならびに「負」の情動応答であるとされる異常啼鳴(4-16 kHz)が観察された。c-fos 発現を指標とした神経活動性解析の結果、CTD 投与群においては「負」の情動入力を受ける視床室傍核や海馬歯状回の過剰な活性化がみられ、新規環境に対するストレス応答性が向上している可能性が示唆された。

以上の結果から、ネオニコチノイドは NOAEL 以下の濃度であっても曝露時期や期間によりさまざまな行動学的影響(不安様行動の亢進、異常啼鳴、自発運動量の増加等)を及ぼすことが示された。ネオニコチノイドの標的となる nAChRs は脳全域に分布するが、本研究からも海馬、視床、中脳等の広範な脳領域が標的となっている可能性が明らかとなった。

本研究は環境要因による複合影響の一例として、ネオニコチノイド系農薬及びストレスが相加的に影響することを検証し、環境化学物質及びストレス感受性における個体差を説明する糸口を与える初めての成果である。さらに、従来認識されていなかった複数の環境要因間に存在する複合影響を初めて提起し、環境化学物質により生体のストレス反応性が上昇するという観点から複合影響メカニズムの一端を解明した。さらに将来的には「ストレス応答系の攪乱」は重要な新規エンドポイントとなる可能性を示し、今後も生み出され続ける新規環境化学物質に対する新規リスク評価システムおよびバイオマーカーの開発も期待でき、環境医科学、薬学および獣医学を通じてヒトを含む哺乳動物の保全に大きく貢献できると思われる。

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