Proteomic search and functional analysis of sexually dimorphic proteins in the anteroventral periventricular nucleus

前腹側脳室周囲核における性差発現タンパク質の探索と機能解析

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CONTENTS

ABSTRACT

Sexual dimorphism in the hypothalamus is caused by testicular androgens and occurs from the late embryonic stage to the first postnatal week. In the anteroventral periventricular nucleus (AVPV), one of the sexual dimorphic nuclei, females have more total dopaminergic, and kisspeptin-immunoreactive (kisspeptin-ir) neurons than do males, whereas the sexually dimorphic nucleus of the preoptic area (SDN-POA) has a larger volume in males than in females. However, the molecular mechanisms underlying sexual differentiation are not fully understood. The aims of the present study were to identify proteins involved in the sexual differentiation of the AVPV in rats using proteomics analysis, and to reveal the function of these proteins using gene-deficient mice. I compared the expression of proteins in the AVPV in males and females on postnatal day 1 (PD1) using two-dimensional fluorescence difference gel electrophoresis followed by MALDI-TOF-MS. I identified a number of protein spots with sexually dimorphic expression, one of which was identified as collapsin response mediator protein-4 (CRMP4). Real-time RT-PCR analysis showed that the expression level of CRMP4 mRNA in the AVPV was higher in males than in females on PD1. However, no difference in CRMP4 expression was detected on PD6. Prenatal androgen treatment increased CRMP4 mRNA expression in the female AVPV on PD1 to the same level as in males. Next, I used CRMP4-knockout (CRMP4-KO) mice to determine the *in vivo* function of CRMP4 in the AVPV. CRMP4-KO did not change the number of kisspeptin-ir neurons in the adult AVPV in either sex. However, the number of tyrosine hydroxylase-immunoreactive (TH-ir) neurons was larger in the AVPV of adult female CRMP4-KO, compared to adult female CRMP4-wild type (CRMP4-WT) mice. No

significant difference in the number of TH-ir neurons was detected in both sexes and genotypes on embryonic day 15 (E15). However, a female-specific increase of TH-ir neurons was observed in CRMP4-KO mice on PD1, a time when sex differences found in adult wild type mice are not yet apparent. These results suggest that CRMP4 regulates the number of TH-ir neurons in the female AVPV.

ABBREVIATION

2D: two-dimensional

2D-DIGE: two-dimensional difference gel electrophoresis

3V: third ventricle

AH: anterior hypothalamus

AP: alkaline phosphatase

ARC: arcuate nucleus

AVPV: anteroventral periventricular nucleus

BrdU: bromodeoxyuridine

CHAPS: 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonate

CRMP: collapsin response mediator protein

DIG: digoxigenin

E: embryonic day

E_2: estradiol

ER: endoplasmic reticulum

Gn: gonadotropin

GnRH: gonadotropin-releasing hormone

Grp75: 75kDa glucose-regulated protein

IPG: immobilized pH gradient

ir: immunoreactive

KO: knockout

LH: luteinizing hormone

MPNc: medial preoptic nucleus

OVLT: organum vasculosum of the lamina terminalis

PBS: phosphate buffered saline

PD: postnatal day

PeN: periventricular nucleus

PNP: purine nucleoside phosphorylase

POA: preoptic area

SDN-POA: sexually dimorphic nucleus of the preoptic area

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SO: stratum oriens

SSC: saline-sodium citrate

TP: testosterone propionate

TRIP: TNF receptor-associated factor 2-inhibiting protein

TH: tyrosine hydroxylase

WT: wild type

ZVAD: N-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone

INTRODUCTION

Structural and functional sex differences have been reported in several brain regions. Among these regions, the hypothalamus exhibits sex differences throughout vertebrate species, including mammals. Although many studies have reported sex differences in the hypothalamus, there are a limited number of studies that have examined the molecular and cellular mechanisms underlying these differences. In this thesis, I examined proteins which are thought to guide hypothalamic sex differentiation, focusing on the rodent anteroventral periventricular nucleus (AVPV) due to its important role in regulating the pulsatile secretion of gonadotropin (Gn).

Functions of the hypothalamus

The hypothalamus exhibits sex differences in functions related to reproductive behavior and Gn secretion. It is well accepted that sex differences in the neuroendocrine system and the sexual behavior are induced by androgens during the perinatal period. For example, despite the male genotype, orchidectomy during the perinatal periods alters the male-specific Gn secretion pattern and produces phenotypically female sexual behavior in adult rats (Pfeiffer, 1936). In contrast, testosterone injection in female rats during the perinatal period extinguishes the estrous cycle and induces male sexual behavior (Takasugi, 1952). These findings suggest that sexual differentiation of the neuroendocrine system regulating Gn secretion and the regulation of sexual behavior are determined by perinatal exposure, or lack thereof, to androgens.

The role of the AVPV in sex differentiation

The AVPV is a small nucleus along the third ventricle in the hypothalamus and is

located immediately caudal to the organum vasculosum of the lamina terminalis (OVLT, Fig. 1). The AVPV plays critical roles in regulating the pulsatile secretion of Gn in females (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). In adults, the AVPV has a larger nuclear volume and higher neuronal packing density in females than in males (Fig. 2; Bleier et al., 1982; Sumida et al., 1993). In addition, in comparison to males, females have 3-4 times more dopaminergic neurons (Simerly et al., 1985a), 10-20 times more kisspeptin-immunoreactive (kisspeptin-ir) neurons (Clarkson and Herbison, 2006; Kauffman et al., 2007), and nearly twice as many GABAergic neurons (Ottem et al., 2004) in the AVPV.

Dopaminergic neurons in rat AVPV were the first to be identified as showing sexual dimorphism (Simerly et al., 1985a; Semaan et al., 2010). Dopaminergic neurons are more abundant in females than in males, and a similar sex difference has been shown in the mouse AVPV (Simerly et al., 1985a, b; Simerly et al., 1997; Forger et al., 2004). In female rats, approximately 80% of the kisspeptin neurons in the AVPV and preoptic periventricular nucleus (PeN) co-express mRNA of tyrosine hydroxylase (TH), which is an enzyme involved in catecholamine synthesis (Semaan et al., 2010). In contrast, only approximately 30% of kisspeptin neurons in the AVPV co-express TH mRNA in female mice (Kauffman et al., 2007). Therefore, there is a species difference in the co-expression rate of TH mRNA and kisspeptin in the female AVPV between rats and mice.

Kisspeptin stimulates the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus and plays a critical role in regulating reproductive function (Fig. 3; Simerly, 1998, Simerly, 2002; Gottsch et al., 2004; Irwig et al., 2004; Matsui et al., 2004; Kinoshita et al., 2005; Navarro et al., 2005). A number of studies have shown that

there are many kisspeptin neurons in the AVPV and the arcuate nucleus (ARC) in female rats and mice (Smith et al., 2005; Clarkson and Herbison, 2006; Adachi et al., 2007; Kauffman et al., 2007). However, in males, while kisspeptin neurons are located in the ARC, few are found in the AVPV. A small periventricular column located immediately caudal to the OVLT corresponds to the AVPV. Electrolytic lesions applied to this structure or to the medial preoptic nucleus lead to the loss of estrus cycle (persistent estrus) and failure of the steroid-induced luteinizing hormone (LH) surge (Wiegand et al., 1980). Kauffman et al. (2007) showed that testosterone propionate (TP)-treated neonatal female rats display a male-like pattern of reduced expression of *Kiss1*, which encodes kisspeptin in the AVPV. In addition, loss of kisspeptin receptor function in humans and rodents causes hypogonadism (de Roux et al., 2003, Seminara et al., 2003). Taken together, these findings suggest that estrogen converted from fetal or neonatal testicular androgen causes loss of kisspeptin expression in the AVPV and GnRH surge in male rats (Kauffman et al., 2007).

Neurogenesis and sexual differentiation of the AVPV

The mechanism regulating sexual differentiation in the AVPV has been investigated with special reference to neurogenesis, neuronal death, and neuronal migration. In an experiment using bromodeoxyuridine (BrdU), it was found that neurogenesis of the rat AVPV occurred during a limited period, from embryonic day 13 (E13) to E18, and was not affected by the treatment with TP (Nishizuka, 1993). In contrast, there is no report about neurogenesis in the mouse AVPV.

Apoptosis and sexual differentiation of the AVPV

Neuronal death is classified into two major types - necrosis and apoptosis. Necrosis is

caused by extracellular factors, such as the deficiency of nutrition and external injury. In contrast, apoptosis is regulated by intracellular factors. Apoptosis is mainly regulated by two different processes, the mitochondrial pathway and the endoplasmic reticulum (ER) pathway. The mitochondrial pathway is well characterized and regulates apoptosis by the Bcl-2 family. Among the Bcl-2 family, Bcl-2 suppresses apoptosis through the inhibition of cytochrome C release from mitochondria, while Bax promotes apoptosis by inducing cytochrome C release. The released cytochrome C then activates caspase 9 and caspase 3 (Kluck et al., 1997; Li et al., 1997; Eskes et al., 1998; Hu et al., 1999). Initiation of the ER pathway is caused by ER stress, i.e., from the accumulation of misfolded proteins (Martinez et al., 2010; Tabas and Ron, 2011; Jing et al., 2012). The crosstalk between the mitochondrial pathway and the ER pathway plays an important role in ER stress-mediated apoptosis. The cytochrome C-dependent mitochondrial pathway is activated by ER stress. In addition, the ER pathway activates caspase 12 without cytochrome C (Martinez et al., 2010). Besides the mitochondrial and ER pathways, the death ligand pathway is known as an extrinsic pathway. The death ligand pathway causes apoptosis when extracellular death ligands, such as Fas ligand, bind cell membrane receptors. After the receptor binding, caspase 8 and subsequently caspase 3 are activated, which induces apoptosis (Kreuz et al., 2004; Pellegrini et al., 2005).

Several studies have examined apoptosis in the AVPV of rats and mice. The number of apoptotic cells in the AVPV is higher in male rats than in female rats on postnatal day 2 (PD2). By PD5, however, sex differences in apoptosis are no longer detected (Yoshida et al., 2000). These results suggest that apoptosis during the early neonatal period is involved in sexual differentiation of the rat and mouse AVPVs. The Bcl-2 family in the mitochondrial pathway is involved in the sexual differentiation of the

AVPV. In the rat AVPV on PD1, the expression level of Bax is higher in males than females, whereas that of Bcl-2 is higher in females than in males (Tsukahara et al., 2006). In mice, the null mutation of *Bax* gene completely eliminates the sex difference in the total neuron number of the AVPV (Forger et al., 2004), whereas Bcl-2 overexpression significantly increases the overall neuron density of the male AVPV (Zup et al., 2003). These results suggest that the total number of neurons and the neuron density in the AVPV are negatively regulated by Bax and positively by Bcl-2. Krishnan et al. (2009) examined apoptosis-related genes in the rat AVPV by DNA microarray and identified 23 genes, including *TNF receptor-associated factor 2-inhibiting protein* (*TRIP*). TRIP inhibits *bcl-2* gene expression and is higher in the male than in the female AVPV on PD2. In the rat AVPV, regardless of sex, 70% of all AVPV GABAergic neurons contained TRIP mRNA on PD2, but the mean cellular levels of TRIP mRNA were significantly higher in GABAergic neurons of males than females. Therefore, sex differences of GABAergic neuron number in the rat AVPV may be regulated by TRIP through Bcl-2 in the mitochondrial pathway.

Recently, it has been suggested that sex differences in the numbers of TH-ir neurons and kisspeptin-ir neurons in mouse AVPV are regulated by a pathway independent of the Bcl-2 family (Zup et al., 2003; Forger et al., 2004; Semaan et al., 2010). It was reported that sex differences of TH-ir neuron number in mouse AVPV are not affected by *Bax* gene deletion (Forger et al., 2004) and *Bcl-2* overexpression (Zup et al., 2003).

Sex steroid hormones may regulate apoptosis in the AVPV. In the rat AVPV, the percentage of degenerating cells in TP-treated females was significantly higher than in normal females (Murakami and Arai, 1989). In the female mouse AVPV, estradiol (E_2) treatment on PD2 increased the number of apoptotic cells on PD3. However, the

number of apoptotic cells in E_2-treated female decreased to that of oil-treated control by PD6 (Waters and Simerly, 2009). Moreover, in rats, E_2 treatment reduced the number of TH-ir neurons *in vivo* (Simerly and Swanson, 1987; Simerly, 1989; Waters and Simerly, 2009), and simultaneous application of E_2 and estrogen receptor antagonist, ICI 182,780, blocked alterations in TH-ir neuron number in AVPV explants *in vitro* (Waters and Simerly, 2009). These results suggest that TH-ir neuron number is regulated by E_2 through estrogen receptors. To investigate caspase-dependent TH-ir neuron loss, the pancaspase inhibitor ZVAD (N-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone) was used to rescue TH-ir neurons from E_2-mediated reduction in number (Waters and Simerly, 2009). These results suggest that E_2 regulates TH-ir neuron numbers by apoptosis of the pathway involving caspase in the AVPV.

Neuronal migration and sexual differentiation of the AVPV

The neuronal migration of the preoptic area/anterior hypothalamus (POA/AH), including the AVPV and sexually dimorphic nucleus (SDN)-POA, has been investigated (Henderson et al., 1999; Knoll et al., 2007). Knoll et al. (2007) examined sex differences in neuronal movement using live-cell fluorescence video microscopy in organotypic brain slices. Neurons from E14 mice displayed significant sex differences in their basal neuronal movement characteristics in the POA/AH. Cells in female slices migrated nearly three times as fast as those in males. In addition, exposure to E_2 decreased the rate of motion of cells located in the dorsal POA/AH but increased the frequency of movement in cells located more ventrally (Knoll et al., 2007). This study demonstrates that the effect of E_2 is specific to the POA region. Although two mechanisms of sexual differentiation, apoptosis and migration, have been suggested

from these studies, the molecular regulation of sexual differentiation in the AVPV remains to be elucidated.

Sexual differentiation of the SDN-POA

The SDN-POA was discovered in the rat medial POA by Gosrki et al. (Gorski et al., 1978). The SDN-POA lies caudal to the AVPV (Fig. 1) and exhibits sex differences. In contrast to the AVPV, the SDN-POA is 5-7 times larger in volume and has more neurons in male rats as compared with females (Gorski et al., 1978, 1980). Additionally, the volume of the calbindin-D28K-ir region in the SDN-POA is 2-4 times larger in males than in females (Simerly et al., 1990, Sickel and McCarthy, 2000, Orikasa et al., 2007). Although the morphological sex differences are well established, the physiological role of SDN-POA in males and females has been only partly determined. In sexually experienced adult male rats, male sexual behaviors are lost after extensive lesions to the POA, but not after small lesions limited to the SDN-POA.

Sex differences of the neuronal number in SDN-POA may result from apoptosis in the early postnatal period. In the central division of the medial preoptic nucleus (MPNc), a major component of the SDN-POA, the number of apoptotic cells is greater in female than in male rats between PD7 and PD10 (Davis et al., 1996), and the number of apoptotic cells reaches peak on PD8 in the female MPNc (Chung et al., 2000). Thus, sex differences in the number of apoptotic cells are negatively correlated with those in the number of neurons in adulthood. In contrast to the AVPV, apoptosis is suppressed by E_2 in SDN-POA (Arai et al., 1996; Davis et al., 1996).

Collapsin response mediator protein

In the present proteomics analysis, I identified some proteins, including CRMP4,

which showed sex differences in amount of expression in the AVPV. CRMP was originally identified as a signaling molecule of semaphorin3A (Goshima et al., 1995). The CRMP family consists of five cytosolic proteins, CRMP1-5, all of which are highly expressed in the developing and adult nervous system (Minturn et al., 1995; Byk et al., 1996; Hamajima et al., 1996; Wang and Strittmatter, 1996; Fukada et al., 2000; Inatome et al., 2000; Yuasa-Kawada et al., 2003; Tsutiya and Ohtani-Kaneko, 2012). Unphosphorylated CRMP2 binds to tubulin heterodimers and promotes microtubule assembly, whereas phosphorylation of CRMP2 by Rho/ROCK kinase, cyclin-dependent kinase-5, and glycogen synthase kinase-3® suppresses the binding affinity of CRMP2 to tubulin (Fukata et al., 2002; Uchida et al., 2005). Recently, several studies using gene-KO mice demonstrated that the loss of CRMP1, 3, or 5 causes impairment of cell migration, dendritic patterning, and dendritic spine formation (Charrier et al., 2006; Yamashita et al., 2006, 2007, 2011; Su et al., 2007; Quach et al., 2008). In the cerebellar explants of CRMP1-KO mice, radial migration was retarded (Yamashita et al., 2006). CRMP1-KO mice also showed a decrease in the number of granule cells migrating out of explants of developing cerebellum (Charrier et al., 2006; Yamashita et al., 2006). In CRMP3-KO mice, apical dendrites of hippocampal CA1 neurons displayed a reduction in length and branching points (Quach et al., 2008). Their basal dendrites also exhibited a reduction in length with alteration in soma stem distribution and increased number of thick dendrites localized in stratum oriens (SO). Furthermore, mushroom and finger spine lengths were shorter in CRMP3-KO than WT mice in SO and stratum radiatum (Quach et al., 2008). The CRMP5-KO mice revealed aberrant dendrite morphology in cerebellar Purkinje cells (Yamashita et al., 2011). In these mice, soma size and primary dendrite diameter of the Purkinje cells were decreased and the induction of long-term

depression of excitatory synaptic transmission between parallel fibers and Purkinje cells was lost (Yamashita et al., 2011). These results suggest that CRMPs play important roles in cell migration, dendritic patterning, and dendritic spine formation during brain development. The present study highlighted CRMP4 as one of the sexually dimorphic proteins in the AVPV; however, the role of CRMP4 is less well understood.

Purpose of the present study

The AVPV is a hypothalamic nucleus that shows sexual dimorphism in rodents. The numbers of total neurons, dopaminergic neurons, and kisspeptin-ir neurons are higher in females than in males. The purpose of the present study was to identify proteins involved in the sexual differentiation of the AVPV in rats using proteomics analysis, and to reveal the functions of these proteins using gene-deficient mice. To this end, I first searched for proteins exhibiting sexual differences in expression in the rat AVPV using proteomics analysis, and found that CRMP4 is a sexually dimorphic protein in the AVPV on PD1. Next, the effect of androgen treatment on the expression of CRMP4 mRNA was examined. Finally, I analyzed the role of CRMP4 in the generation of sex differences in the AVPV by using gene-deficient mice.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were used for the proteomics analysis and in subsequent experiments including prenatal hormone treatment. Pregnant rats purchased from Japan SLC (Hamamatsu, Japan) were maintained at the University of Tsukuba animal care facility under a 12-hour light/12-hour dark cycle at 23±1°C, with free access to water and food. Female and male newborns were used on PD1 (day of birth) or PD6. All animal experiments were approved and conducted according to the Guidelines of University of Tsukuba for the Care and Use of Experimental Animals.

Crmp4 gene-deficient mice were generated as previously described (Niisato et al., 2012). Age-matched wild type littermates were used as controls. The mice were housed in a standard mouse facility and allowed access to autoclaved diet and water under a 12-/12-hour light/dark cycle at 24±1°C. All procedures were performed according to the guidelines outlined in the Institutional Animal Care and Use Committee of Yokohama City University School of Medicine.

Throughout all experiments, efforts were made to minimize the number of animals used and their suffering.

Tissue samples for proteomics analysis and real-time PCR

In order to identify proteins showing sex differences in expression during AVPV development, a protein sample extracted from the AVPV was subjected to proteomic analysis. In this study, I searched for proteins exhibiting sex differences on PD1 (early neonatal stage), focusing on the proteins whose sex differences in neuronal cell death

have been reported. The sex differences in neuronal cell death were no longer detected on PD6. Thus, I focused on proteins whose expression levels were no longer sexually dimorphic at PD6. First, on PD1, newborns (15 males and 18 females) were anesthetized by subcutaneous injection of sodium pentobarbital and their brains were removed. The AVPV was identified according to previously described procedures (Tsukahara et al., 2008). Frontal slices (300 μm thick) of the brain were cut at the level of the optic chiasm using a microslicer (Dosaka EM, Kyoto, Japan), and tissue fragments of the AVPV were immediately isolated from the slices under a stereo-microscope using a stainless steel tube (inner diameter, 0.65 mm). The tissue fragments were then homogenized in 50 μl of lysis buffer (30 mM Tris buffer (pH 8.5) containing 2 M thiourea, 7 M urea, and 4% CHAPS). For mRNA extraction, AVPV tissue fragments were sampled from newborns (6 males and 6 females) and homogenized in 50 μl of RNAlater (Ambion, Austin, TX). These samples were immediately frozen and kept at -80°C until use. To confirm the location of the isolated tissue, the remaining brain slices were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), stained with Hoechst 33258 (Molecular Probes, Inc., Eugene, OR) and subjected to fluorescence microscopy to obtain images.

Protein and mRNA samples were prepared from the AVPV tissue fragments on PD6 (9 females and 9 males for the preparation of protein samples; 6 males and 6 females for the preparation of mRNA samples). In addition, proteins and mRNAs were also extracted from SDN-POA fragments on PD1 (15 males and 18 females for protein extraction; 4 males and 4 females for mRNA extraction) and PD6 (9 males and 9 females for protein; 6 males and 6 females for mRNA extraction) to compare mRNA expression levels of the identified proteins between the AVPV and the SDN-POA, as

well as between PD1 and PD6.

Two-dimensional difference gel electrophoresis for proteomics analysis

For two-dimensional (2D)-DIGE analysis, protein extracts (2.5 μg) were incubated with 1 nM Tris (2-carboxyethyl) phosphine hydrochloride (Sigma, St Louis, MO) at 37°C in the dark for 1 hour. Then, the reduced samples were labeled with 2 mM of Cyanine dye 5 (Cy5, Cy Dye DIGE Fluor Labelling kit for Scarce Sample, GE Healthcare) according to the manufacturer's instructions. For the in-gel standards, equal aliquots (2.5 μg) of each sample (AVPV on PD1 and PD6; SDN-POA on PD1 and PD6) were pooled and labeled with Cy3. The reaction mixture was incubated at 37°C for 30 minute. The labeling reaction was terminated by the addition of lysis buffer (30 mM Tris-HCl, 2 M thiourea, 7 M urea, 4% CHAPS, 130 mM dithiothreitol, 2% immobilized pH gradient (IPG) buffer pH 4-7 (GE Healthcare), pH 8.5). All the labeling procedures were carried out in the dark. Next, the Cy3-labeled internal standard sample and each of the individual Cy5-labeled protein samples were mixed and loaded onto a 24-cm Immobiline Dry-strip covering the range of pH 4 to pH 7 (GE Healthcare) for isoelectric focusing using the IPGphor Isoelectric Focusing System (GE Healthcare). Separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then performed with the Ettan DALTsix Electrophoresis System (GE Healthcare) according to a previous report (Fujisawa et al., 2008). For mass spectrometry analysis, the protein samples (50 μg) were separated by 2D gel electrophoresis.

Proteomic image analysis

The separated labeled proteins on the 2D gels were scanned at 100-μm resolution using an image analyzer (Typhoon 9400 Imager, GE Healthcare). Matching between

gels was done by means of landmarking spots in the internal standard images for each gel. To compare spot intensities between the sexes, the Cy5-fluorescent intensities of the protein spots were normalized with the Cy3-fluorescent intensities of identical spots using Progenesis PG240 software (Nonlinear Dynamics, Newcastle, UK) program, and normalized Cy5-intensities were used in statistical analyses.

In-gel digestion, mass determination, and protein identification

2D gel fragments (approx. 1 mm in diameter) corresponding to the protein spots of interest were picked up with Xcise (Proteome Systems Ltd. and Shimadzu-Biotech, Kyoto, Japan), and peptide samples were prepared according to a previously described procedure (Fujisawa et al., 2008). In brief, the gel fragment was cut into small pieces, decolored, and incubated in a trypsin solution (0.02 μg/μl trypsin (Promega, Madison, WI)) at 37°C for 16 hours for digestion of the contained proteins. The digested peptides were extracted from the gel pieces with trifluoroacetic acid (Sigma) and acetonitrile. After four extraction cycles, the supernatant was filtered and concentrated in an evaporator. The peptide sample solution was stored at -20°C until the mass spectrometry analysis. The digested peptides in the samples were analyzed using a MALDI-TOF/TOF mass spectrometer (Ultraflex, Bruker Daltonics, Germany) or HCTultra ETD II system (Bruker Daltonics). The identification of peptides (proteins) was carried out by a search against the NCBI database with Mascot software (Matrix Science Ltd., London, U.K.).

Quantitative real-time RT-PCR

Total RNA was extracted from tissue samples immersed in RNAlater (Ambion) using an RNeasy kit (QIAGEN, Valencia, CA), and then converted to cDNA using a reverse transcription kit (QIAGEN). The synthesized cDNA was used as a template in

the following PCR. Real-time RT-PCR analysis was performed using a Thermal Cycler Dice Real Time System TP800 (Takara Bio, Ootsu, Japan) according to the manufacturer's protocol. Gene expression levels were normalized with those of 18S rRNA measured simultaneously. Primers used for gene amplification are listed in Table 1. The amplification was carried out in a 20-µl volume containing 1 µl of cDNA, 200 nM of each primer pair and SYBR Premix Ex Taq (Takara Bio). The reaction mixture was subjected to 40 cycles of amplification, followed by post-PCR fluorescence melting curve analysis.

Treatment with TP

Pregnant rats were subcutaneously injected with 0.2 ml sesame oil vehicle containing TP (2 mg/day, 2 females) or 0.2 ml vehicle alone (2 females) daily from gestation days 14 to 18. On PD1, newborns (male oil: n=4, female oil: n=9; male TP: n=5, female TP: n=10) were decapitated, and the brains were quickly removed. The AVPV was isolated from the brain, and the amount of CRMP4 mRNA was analyzed by real-time RT-PCR according to the method described above.

***In situ* hybridization**

In situ hybridization was carried out according to a previous report (Kanda et al., 2010), with a slight modification. In brief, cDNA containing 1792 bp of *Rattus norvegicus* CRMP4 (GenBank: AF389425.1) was incubated overnight with Xba I (Roche Diagnostics, Tokyo, Japan) or Hind III (Roche Diagnostics) in 1× SuRE/Cut Buffer H (Roche Diagnostics) at 37°C to prepare template DNA for the generation of an antisense or sense probe. To generate the digoxigenin (DIG)-labeled antisense or sense probe, the template DNA was incubated with T7 (for anti-sense probe) or T3 (for sense

probe) RNA polymerase (Roche Diagnostics).

Newborn Sprague-Dawley rats (3 males and 3 females) were anesthetized on PD1 and transcardially perfused with 4% paraformaldehyde in 0.1 M PBS, and the brains were removed. Serial frontal cryosections (16-μm thick) of the brain were cut with a cryostat (CM-3050-S; Leica Microsystems, Tokyo, Japan) and thaw-mounted on MAS-coated glass slides (Matsunami, Osaka, Japan). Brains of male and female WT mice on PD1 were removed after anesthesia and fixed with 4% paraformaldehyde. Under pentobarbital anesthesia, the brains of female and male WT mice on PD8 were removed following intracardiac perfusion with saline followed by 4% paraformaldehyde. The brains were then sectioned with the cryostat. After alkaline phosphatase (AP) activity was quenched with 0.2 N HCl, sections were postfixed with 4% paraformaldehyde, treated with proteinase K (Promega, Tokyo, Japan), and acetylated with 0.25% (v/v) acetic anhydride (Wako, Osaka, Japan) in 0.1 M triethanolamine (Sigma Aldrich, Tokyo, Japan). The sections were then incubated with prehybridization solution, and hybridized with 1 μg/mL DIG-labeled antisense cRNA probe. A sense probe was used as a negative control. After hybridization, the sections were washed twice with 2× saline-sodium citrate (SSC; Sigma) and treated with 20 μg/mL RNaseA (Sigma) in TNE (10 mM Tris-HCl, 0.5 M NaCl, and 1 mM EDTA, pH7.4; Sigma). After soaking in TNE buffer and washing with 2× SSC and then 0.5× SSC, the sections were incubated with AP-conjugated anti-DIG antibody (1:1000; Roche Diagnostics) and treated with a chromogen solution until a signal became visible. Sections were dehydrated, embedded with Entellan, and observed microscopically (AXIO Imager A1, Zeiss, Jena, Germany).

Western blot analysis

In order to confirm the deficiency of CRMP4 protein, western blot analysis was carried out using CRMP4-KO mice. Whole brains (WT male, n=4; WT female, n=4; CRMP4-KO male, n=4; CRMP4-KO female, n=4) were homogenized and separated by electrophoresis on a 12% SDS-PAGE. Proteins were electrophoretically transferred from the gel to a polyvinylidene difluoride membrane (Immobilon-P transfer membranes, Merk Millipore, Darmstadt, Germany). Membranes were incubated with the polyclonal CRMP4 antibody (1:2500; Chemicon, Temecula, U.S.A.) and then the AP-conjugated secondary antibody (1:1000; Abcam, Cambridge, U.K.). CRMP4 was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP). Image analysis was performed using the ImageJ 1.41 (National Institutes of Health, U.S.A.).

Morphological comparison of the AVPV among CRMP4-KO and WT mice

Male and female CRMP4-KO and WT littermates on E15 (WT male, n=4; WT female, n=6; CRMP4-KO male, n=6; CRMP4-KO female, n=6) were isolated from the anesthetized pregnant mice. Brains of PD1 mice (WT male, n=3; WT female, n=3; KO male, n=3; KO female, n=3) were isolated after anesthesia. Eight-week-old (8w, adulthood) mice (WT male, n=4; WT female, n=6; KO male, n=6; KO female, n=6) were anesthetized and subjected to perfusion fixation with saline, followed by 4% paraformaldehyde. Brains were removed, postfixed overnight, and cryoprotected in 20 % sucrose in 0.1 M PBS at 4°C overnight before sectioning on a cryostat. Serial frontal frozen sections of the brains (PD1 and adult) were cut at a thickness of 16 μm and mounted on MAS-coated glass slides. Serial frontal frozen sections (12 μm thickness) of brains from E15 fetuses were obtained. To determine the sex of samples,

cDNA from each sample was analyzed for *Zfy* expression.

For the purpose of cytoarchitecture determination, a series of every third section from each brain was stained with cresyl violet (Muto Pure Chemicals, Tokyo, Japan). This was used to measure AVPV size and cell numbers. Other sections were immunohistochemically stained with rabbit polyclonal antibody against TH (ab621, 1:2000 dilution; Abcam) or rabbit polyclonal antibody against kisspeptin 10 (kp10, 1:1000 dilution, AB9754; Chemicon), in combination with Hoechst 33258, or used for double immunostaining with a chicken polyclonal antibody against TH (1: 2000 dilution, NBP2-10493; Novus Biologicals, Littleton, CO) and a polyclonal rabbit antibody against CRMP4 (1:5000 dilution, AB5454; Millipore, Billerica, MA). After incubation with the primary antibodies, the sections were reacted with anti-rabbit IgG conjugated with Alexa Fluor 488 or Alexa Fluor 555 (1:500; Invitrogen, Carlsbad, U.S.A.). For double immunostaining, anti-chicken IgG conjugated with Alexa Fluor 555 and anti-rabbit IgG conjugated with Alexa Fluor 488 were used as secondary antibodies. AVPVs were identified according to the standard mouse brain atlas (Allen Reference Atlas and Atlas of the Developing Mouse Brain). The shape of the third ventricle (3V), anterior commissure, and optic chiasm were used as landmarks to determine the AVPV. For enumeration of total neurons, TH-ir, and kisspeptin-ir neurons, cells with a clearly visible nucleus were enumerated in the AVPV. For adult and PD1 AVPVs, measurements were performed in 6 or 7 sections from a series of every third section (16-μm thickness) from each brain. For AVPVs on E15, measurements were performed in 5 or 6 sections from a series of every third section (12-μm thickness) from each brain.

Statistical analysis

Density of protein spots and expression levels of mRNA were compared between sexes using Student's *t*-test (paired, $P<0.05$). TP treatment experiments and experiments of CRMP4-KO mice were analyzed using a two-way ANOVA. In all analyses, ANOVA was followed by Scheffe's *post hoc* test for multiple comparisons ($P<0.05$).

RESULTS

Analysis of sexually dimorphic proteins in the AVPV on PD1 with 2D-DIGE

In order to characterize the proteins related to AVPV sex differentiation, I compared the expression of each protein spot in Cy5-labeled male or female AVPV samples with that in Cy3-labeled in-gel standards on 2D-DIGE gels (Fig. 4A). The samples used in this experiment were extracted on PD1, the early phase of the sex differentiation. Electrophoresis was performed on each male and female sample (from 15 and 18 samples, respectively), which provided 6 gel images in total. Approximately 1,000 protein spots were matched among the 6 gel images. From matched spots, I selected 9 protein spots (Fig. 4B) whose intensity exhibited a sex-difference of greater than 1.3-fold and was statistically significant ($P<0.05$, Student's *t*-test). Among the 9 spots, the intensity of 6 spots was greater in females than in males and the intensity of the remaining 3 was greater in males than in females (Table 2). I subsequently attempted to identify the proteins of the 9 spots using mass spectrometric analysis, and successfully identified the proteins in 6 spots (Table 2). Among these spots, two cytoskeletal proteins, γ-actin and α-internexin were identified. The other four spots were identified as stress-70 protein mitochondrial precursor (75 kDa glucose-regulated protein; GRP75), purine nucleoside phosphorylase, heterogeneous nuclear ribonucleoprotein K and CRMP4. The proteins in three remaining spots could not be identified.

Comparison of mRNA expression of identified proteins in female and male AVPVs on PD1

Relative gene expression levels of the identified 6 proteins were compared between the sexes in the AVPV on PD1 using real-time RT-PCR. The mRNA levels of

α-internexin and CRMP4 were significantly higher in males than in females, whereas those of the other 4 proteins did not show significant sex differences (Table 2, mRNA). I, thus, identified α-internexin and CRMP4 as proteins with sexually dimorphic expression at the level of protein and mRNA. In the present study, I focused on CRMP4, due to the important roles of the CRMP family in neural development (Nishimura et al., 2003; Yamashita et al., 2006, 2007, 2011; Yamashita and Goshima, 2012).

Absence of sex differences in CRMP4 expression in the AVPV on PD6

It has been known that the sexual differentiation occurs in the AVPV from the late fetal stage to the first postnatal week (MacLusky and Naftolin, 1981). Thus, I examined CRMP4 expression levels in the AVPV on PD6, near the end of sexual differentiation. Pooled protein samples extracted from the AVPV on PD6 (each from 3 males or 3 females) were quantitatively compared using 2D-DIGE (Fig. 5A). As described above, the intensity of the CRMP4 spot on PD1 (spot No. 787 in Fig. 5A and in Table 2) was statistically higher in males than in females (Fig. 5B, left). In addition, the other spots that were also identified as CRMP4 tended to have higher expression in males on PD1 (Fig. 6). However, the intensity of the CRMP4 spot on PD6 (spot No. 2327 in Fig. 5A right) showed no apparent sex difference (Fig. 5B, right).

Next, I measured the expression levels of the CRMP4 mRNA in the PD6 AVPV using real-time RT-PCR (Fig. 5C). The sex difference in the CRMP4 mRNA expression levels observed on PD1 (Fig. 5C, left) was no longer detected on PD6 (Fig. 5C, right). Therefore, the relative up-regulation of CRMP4 in males occurred specifically on PD1 and not on PD6.

Expression of CRMP4 protein and mRNA in the SDN-POA on PD1 and PD6

I examined protein and mRNA expression of CRMP4 in the SDN-POA of both sexes on PD1 and PD6. 2D-DIGE analysis showed that no differences were observed between males and females in CRMP4 spot intensity on PD1 and PD6 (Fig. 7A). Moreover, real-time RT-PCR analysis revealed that CRMP4 mRNA levels were not significantly different between the sexes in the SDN-POA on PD1 and PD6 (Fig. 7B).

Prenatal TP-treatment suppresses sex differences in CRMP4 mRNA expression on PD1

To investigate hormonal effects on the expression of CRMP4 mRNA in the AVPV, TP-containing sesame oil, or sesame oil alone (as a control), was injected into pregnant rats and RNA was extracted from the AVPV on PD1. CRMP4 mRNA expression level in the control males was significantly higher than that of control females (Fig. 8). Prenatal TP-treatment had no effect on the expression of CRMP4 mRNA in males. In contrast, TP-treatment significantly increased the expression of CRMP4 mRNA in females, and eliminated the sex difference in the expression of CRMP4 mRNA on PD1.

Deletion of *crmp4* gene affects the number of TH-ir neurons in adult female AVPV

CRMP4-KO mice were previously established (Niisato et al., 2012). I compared sexually dimorphic morphological features of the AVPV between WT and CRMP4-KO mice at 8 weeks of age. As shown in Table 3, there was a tendency that the size of the AVPV was larger in WT females than in WT males, although the difference was not significant ($P = 0.073$, WT male, n=4; WT female, n=6, Scheffe's *post hoc* test). Deletion of *crmp4* gene did not induce significant changes in the size of the AVPV. Additionally, sex differences in the number of AVPV neurons identified by Nissl

staining were detected in WT mice, as well as CRMP4-KO mice (Table 3). In CRMP4-KO mice, the number of AVPV neurons was not significantly different as compared with that in WT mice.

Deletion of *crmp4* gene alters the number of TH-ir, but not kisspeptin-ir neurons in the adult female AVPV

I performed western blot analysis using an anti-CRMP4 antibody of CRMP4-WT and CRMP4-KO mouse brains, and confirmed that CRMP4 protein was absent in the CRMP4-KO brain (Fig. 9). I then determined the numbers of kisspeptin-ir and TH-ir neurons in the adult AVPV of WT and CRMP4-KO mice. As previously reported (Clarkson and Herbison, 2006; Kauffman et al., 2007), the number of kisspeptin-ir neurons in the AVPV was much higher in WT females than in WT males (Fig. 10A left, 10B). Deletion of the *crmp4* gene did not affect the number of kisspeptin-ir neurons in either sex (Fig. 10A, 10B).

In contrast, *crmp4* gene deficiency altered the number of TH-ir neurons in the female AVPV. Consistent with the previous reports (Simerly et al., 1985a, b), the number of TH-ir neurons in the AVPV of adult WT mice was significantly greater in females than in males (Fig. 11A left, 11B). Deletion of the *crmp4* gene did not affect the number of TH-ir neurons in males (Fig. 11A right, 11B). However, the number of TH-ir neurons was significantly larger in CRMP4-KO females, as compared with WT females (Fig. 11A right, 11B).

Deletion of *crmp4* gene specifically affects the development of TH-ir neurons in the female AVPV

In order to examine the developmental effects of CRMP4-KO on TH-ir neurons, I

determined the number of TH-ir neurons in the AVPV of both sexes in WT and CRMP4-KO mice at E15 and PD1 (Fig. 12). On E15, no significant difference in the number of TH-ir neurons was detected between WT and CRMP4-KO mice or between females and males (Figs. 12A, 13). The numbers of TH-ir neurons in WT mice of both sexes and CRMP4-KO males (Fig. 12B) remained unchanged from E15 to PD1 (Figs. 12, 13). However, a dramatic increase in the number of TH-ir neurons was detected in female CRMP4-KO mice on PD1, producing a statistically significant sex difference in CRMP4-KO mice on PD1 (Figs. 12, 13).

After PD1, the number of TH-ir neurons was decreased in male WT mice. In contrast, the number of TH-ir neurons was similar in female WT mice at PD1 and adulthood (Figs. 11, 13). Thus, a sex difference in the number of TH-ir neurons was observed in WT mice (Figs. 11, 13). Although the number of TH-ir neurons in both sexes of CRMP4-KO mice was smaller at adulthood than on PD1 (Figs. 11, 13), the number of TH-ir neurons was higher in adult female CRMP4-KO mice than in adult male CRMP4-KO mice (Figs. 11, 13). Lastly, female CRMP4-KO mice had significantly more TH-ir neurons than did female WT mice (Figs. 11, 13).

Expression of CRMP4 mRNA and colocalization of CRMP4 and TH in the PD1 mouse AVPV

As shown in Fig. 14, CRMP4 mRNA was detected in the mouse AVPV by *in situ* hybridization on PD1 (Fig. 14A). Hybridization signals were detected as black dots in the cytoplasm of many cells within the AVPV on PD1 (insert in Fig. 14A). Sex differences in the expression of CRMP4 mRNA were observed on PD1 (Fig. 14A) but not on PD8. Hybridization signals were very weak on PD8, comparable to the other areas of the mouse preoptic region, as reported previously (Tsutiya and Ohtani-Kaneko,

2012). I assessed colocalization of CRMP4 and TH in the male AVPV (Fig. 14C-F) using double immunostaining with the CRMP4 and the TH antibodies, and found that TH-ir cells were immunoreactive for CRMP4 (arrows in Fig. 14F), though many TH-negative cells were also CRMP4 immunoreactive.

DISCUSSION

A proteomics approach (2D-DIGE followed by MALDI-TOF-MS) was used to identify sexually dimorphic expression of CRMP4 in the AVPV. The sexually dimorphic expression was identified on PD1, during the development of sex differences in the AVPV, but not on PD6 when AVPV sexual differentiation is mostly completed. Consistent with the changes in CRMP4 protein levels, the expression of CRMP4 mRNA was also up-regulated in the male AVPV on PD1, but not on PD6. Furthermore, sexually dimorphic expression was not found in CRMP4 protein and mRNA in the SDN-POA at both PD1 and PD6. This is the first study that demonstrates sexual dimorphic expression of CRMP4 in the AVPV. In the present study, CRMP4-KO mice with depletion of the *crmp4* gene did not affect the number of kisspeptin-ir neurons, which is known to be sexually dimorphic in the AVPV. However, loss of the *crmp4* gene caused a female-specific increase in the number of TH-ir neurons in neonatal and adult mice. These results suggest a new role for CRMP4 in sex-dependent regulation of TH-ir neurons in the AVPV.

Different mechanisms involved in determining the neuronal subpopulations in the AVPV

Both the volume and number of neurons in the female AVPV are more than twice as those of the male AVPV in rodents (Bleier et al., 1982; Ito et al., 1986; Sumida et al., 1993). The number of neurons in brain nuclei is determined by a variety of factors that regulate neurogenesis, neuronal migration, and apoptosis during development. An examination of Bax-KO mice and transgenic mice with Bcl-2-overexpression showed that the sexual dimorphism in the total neuron number in the AVPV was absent in these

mice (Zup et al., 2003; Forger et al., 2004). These studies suggest that apoptosis through the Bcl-2 family is involved in the regulation of the total number of neurons in the AVPV.

Sexual dimorphism in the AVPV has also been found in neuronal subpopulations; a female-dominant sex difference was previously showed in the number of kisspeptin-ir neurons (10-20 fold difference) in the AVPV and the PeN (Clarkson and Herbison, 2006), as well as TH-ir neurons (3-4 fold difference) in the AVPV (Simerly, 1985a). In Bax-KO mice, the sexual dimorphism in the number of kisspeptin-ir neurons was lost in the AVPV, but that of TH-ir neurons was maintained (Forger et al., 2004; Semaan et al., 2010). These results suggest that apoptosis through Bax regulates the number of kisspeptin-ir neurons but not TH-ir neurons. Additionally, overexpression of Bcl-2 does not affect the number of TH-ir neurons (Zup et al., 2003). This result demonstrates the heterogeneity of the mechanisms involved in regulating cell numbers in the AVPV. Bcl-2 and Bax may regulate sexual dimorphism of the neuronal density and the number of kisspeptin-ir neurons in the AVPV, whereas the Bcl-2 family does not regulate sex differences in TH-ir neuronal numbers. The present results show differences in effects of *crmp4* gene deficiency between kisspeptin-ir neurons and TH-ir neurons and the heterogeneity of the mechanisms determining the cell numbers of neuronal subpopulations in the AVPV.

Role of CRMP4 in the development of the AVPV

As described in the introduction, several studies have demonstrated that the loss of CRMP1, 3 and 5 causes impaired cell migration, dendritic patterning and dendritic spine formation (Charrier et al., 2006; Yamashita et al., 2006, 2007, 2011; Su et al.,

2007; Quach et al., 2008). Based on these studies describing the roles of the CRMP family in neuronal development, I focused on CRMP4 among the sexually dimorphic proteins identified in the present proteomics analysis.

The CRMP4 mRNA is initially detected in the rat brain on E13, increases transiently until PD1, and then decreases rapidly to the adult level (Wang and Strittmatter, 1996). However, certain brain regions where neurogenesis is maintained throughout adulthood, including the dentate gyrus (Bayer et al. 1982; Bayer, 1982), retain CRMP4 expression until adulthood (Nacher et al., 2000; Tsutiya and Ohtani-Kaneko, 2012). Therefore, it is suggested that CRMP4 may have roles in neuronal development in many brain regions during embryonic and early postnatal stages, as well as in adult neurogenic regions.

CRMP4 appears in growing axons of dorsal root ganglion neurons (Minturn et al., 1995), and has a regulatory role in actin cytoskeleton dynamics, which may be related to growth cone collapse (Rosslenbroich, 2005). Alabed et al. (2007) found that short interfering RNA-mediated knockdown of CRMP4 promotes prominent process outgrowth of HEK293T cells. Niisato et al. (2012) recently generated CRMP4-KO mice in order to study the *in vivo* roles of CRMP4 and identified an increased proximal bifurcation of apical dendrites in CA1 pyramidal neurons of these mice. They also found increased dendritic branching in cultured hippocampal neurons of CRMP4-KO mice, as well as in cultured cortical neurons treated with CRMP4 short hairpin RNA (shRNA). In addition, other research groups have shown that CRMP4 is involved in the developmental process as well as in degenerative or regenerative processes of neurites in adulthood (Nacher et al., 2000; Franken et al. 2003; Fujisawa et al., 2008; Liu et al., 2009; Duplan et al., 2010; Jang et al., 2010). Furthermore, Franken et al. (2003) showed the involvement of CRMP4 in naturally occurring cell death during cortical

development.

The present study demonstrated increased TH-ir neurons in the AVPV of female CRMP4-KO mice. CRMP4 may regulate the number of TH-ir neurons through several processes: neurogenesis, neuronal migration, and apoptosis. In addition, the possibility cannot be excluded that the increase of TH-ir neuronal numbers is due to the increased expression of TH. If this holds true, CRMP4 may regulate the expression of TH.

An examination of neurogenesis showed cell labeling in the adult AVPV when BrdU was injected into pregnant rats during E13-E18 but not during E10-E12, E19-E20, or on PD1 (Nishizuka et al., 1993). These results suggest that neurogenesis in the rat AVPV occurs during a limited period between E13 and E18. The exact day for the neurogenesis in the mouse AVPV is unknown. Considering the rat data described above (Nishizuka et al., 1993), neurogenesis in the mouse AVPV may occur during the embryonic stages. I found that the numbers of TH-ir neurons on E15 were the same in both sexes of WT or CRMP-KO mice, and their sexual differentiation in numbers of TH-ir neurons emerged in CRMP4-KO mice on PD1. Based on these findings, CRMP4 may regulate the proliferation of TH-ir neurons after E15. Therefore, a close examination using BrdU-administered CRMP4-KO mice is needed to address the question whether CRMP4 controls the number of TH-ir neurons via a neurogenic pathway.

Some studies have also demonstrated that CRMP4 is involved in apoptosis. Franken et al. (2003) showed that CRMP4 is involved in naturally occurring cell death during cerebral cortex development. Recent studies show that CRMP4 acts as a pro-apoptotic factor in apoptotic neurons, i.e., motoneurons of mutant superoxide dismutase 1 mice (Duplan et al., 2010). Moreover, calpain truncated CRMP4 induces apoptosis in

cerebellar granule neurons (Liu et al., 2009). It is also known that calpain activates caspase 12 and induces apoptosis through the ER pathway (Martinez et al., 2010). Taken together, these findings suggest that CRMP4 is involved in the regulation of TH-ir neuron numbers via an apoptotic pathway.

In addition to proliferation and apoptosis, neuronal migration might play a role in CRMP4-mediated regulation of TH-ir neuron number in the AVPV. It has been demonstrated that CRMP1 and CRMP2 are involved in the signal transduction pathway that mediates neuronal migration in the cerebral cortex (Yamashita et al., 2006; Ip et al., 2011). These studies suggest that CRMP4 could regulate TH-ir neuron number by controlling their migration.

Because the mechanisms underlying the regulation of the number of TH-ir neurons in male and female mice by CRMP4 remain undetermined, the role of CRMP4 should be examined from various perspectives, including proliferation, apoptosis, and migration. Finally, most of AVPV cells including TH-ir neurons have CRMP4 immunoreactivity, suggesting that CRMP4 could affect the characteristics of diverse range of cells in the AVPV.

Speculations as to why TH-ir neurons increased only in CRMP4-KO female mice

I identified increased expression of CRMP4 mRNA in the AVPVs of control male rats and TP-treated female rats. Inexplicably, the deletion of *crmp4* gene increased the number of TH-ir neurons only in female mice, suggesting that CRMP4 is involved in limiting TH-ir neuronal density in the female mouse AVPV. One possible explanation for this discrepancy is that the CRMP4-dependent pathway regulating the number of TH-ir neurons may be masked in males by a more potent cell death pathway induced by

testosterone during the perinatal period. This potent cell death pathway might lead to decreased male TH-ir neurons irrespective of CRMP4. On the other hand, in females, the CRMP4-dependent regulatory pathway might be able to function because of the absence of testosterone.

Other sexually dimorphic proteins identified by proteomics analysis

The present proteomics study identified other sexually dimorphic proteins, including the cytoskeletal protein γ-actin, GRP75, and purine nucleoside phosphorylase (PNP). Mutations in the *γ-actin* gene are associated with hearing loss (Zhu et al 2003). It is possible that γ-actin plays an important role in development. It is known that actin has at least 17 kinds of post-translational modification. Arginylation promotes the polymerization of actin and glutathionylation decreases the polymerization. Phosphorylation promotes the polymerization of actin depending on the residue, or decreases the polymerization (Terman and Kashina, 2013). The deficit of arginylation of β-actin which is the same cytoskeleton as γ-actin, decreases cell movement (Karakozova et al., 2006; Kurosaka et al., 2010). Glutathionylation of actin protects neurons from oxidant stress, and is participating in neuronal survival (Sparaco et al., 2006). Thus, the post-translational modification of actin influences neuronal migration and neuronal survival. In the present study, the density of protein spot of γ-actin was increased in the female without changes in the expression of the mRNA. It is possible that the isoelectric point was changed by these post-translational modifications. In this study, the expression of PNP was higher in the female AVPV than in males. It has been reported that PNP suppresses apoptosis in chronic lymphocytic leukemia cells (Balakrishnan et al., 2006). Therefore, PNP could suppress apoptosis in the female

AVPV. GRP75 is an important molecular chaperone that belongs to the heat shock protein 70 family and resides predominantly in mitochondria. GRP75 has been suggested to suppress apoptosis through inhibition of Bax in PC12 cells (Yang et al., 2008, 2011). In the present study, the expression of GRP75 was also higher in the females AVPV than in males. Thus, GRP75 may increase the number of cells in female AVPV through inhibition of Bax. Further study is needed to investigate the precise roles of these proteins in the sexual differentiation of the AVPV.

Technical considerations for 2D-DIGE

Some remarks should be added on various technical considerations concerning the 2D-DIGE method. Several methods have been used to identify molecules underlying the sexual differentiation of the hypothalamus (Yonehara et al., 2003; Krishnan et al., 2009). DNA microarray was used to compare male and female gene expression patterns in the hypothalamus on PD5 (Yonehara et al., 2003). It was shown that the mRNA expression of GAD65 and Coro1b was higher in males than in females, whereas that of TrkR2 and COL3A1 was higher in females. Krishnan et al. (2009) also used DNA microarray to examine apoptosis-related genes and identified 23 genes, including *TRIP*, which showed sex differences in the AVPV on PD2. Interestingly, none of these gene products were identified in the present study. There may be several reasons for the differences between the above genes identified with microarray and proteins identified with proteomics here. One of the reasons is that different hypothalamic nuclei were examined. Yonehara et al. (2003) examined the entire hypothalamic nuclei, while others (Krishnan et al., 2009; present study) focused on the AVPV. It was reported that different mechanisms are involved in mediating sexual differentiation among the

hypothalamic nuclei (Schwarz and McCarthy, 2008a, b). The second reason is that different developmental stages were examined. The present study focused on PD1 for two reasons. First, previous studies have shown that there are more apoptotic cells in the AVPV of male rats than female rats on PD2. Second, there are significant sex differences in the levels of Bcl-2 (female > male) and Bax (female < male) proteins in the AVPV of PD1 rats (Yoshida et al., 2000; Tsukahara et al., 2006). Finally, the present 2D-DIGE analysis examined the expression of proteins in the pH range of 4-7. As a result, proteins with the isoelectric points outside this range were not characterized. For example, the theoretical isoelectric point of TRIP is 8.93; therefore, TRIP could not be identified in the present analysis.

It is known that CRMP4 and heterogeneous nuclear ribonucleoprotein K are phosphorylated (Rembutsu et al., 2008; Kimura et al., 2010). Post-translational modification is a very important process for controlling the function of these proteins. Therefore, it is necessary to carry out further analyses to understand the function of post-translational modifications of these proteins

.

Conclusions

In the present study, I aimed to elucidate the mechanisms involved in mediating sexual dimorphism in the AVPV. First, I used proteomic analysis to show that CRMP4 is a sexually dimorphic protein in the AVPV during the critical developmental period in a stage- and region-specific manner. Next, I demonstrated that the sex differences in CRMP4 expression are dependent on androgens during the critical period. Finally, I

used CRMP4-KO mice to show that *crmp4* gene-deficiency increased the number of TH-ir neurons in neonatal and adult female animals. The findings in the present study show a new role for CRMP4 in sex-dependent regulation of TH-ir neurons in the AVPV. Although I could not find molecules that are responsible for AVPV sexual dimorphism, the present study is the first to show the involvement of CRMP4 in controlling the number of TH-ir neurons as well as the androgen dependency of its control. This study provides important information regarding the mechanisms underlying the formation of this sexually dimorphic nucleus as well as the molecular mechanisms mediating hormonal regulation of brain development.

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Table 1

Primers used in this study

Gene	Primer sequence (5'-3')	Product size (bp)	Reference
α-internexin	Forward: TTCGGGAATACCAGGACTTG Reverse: AATCGTGTCTCTTCGCCTTC	94	NM_019128.4
collapsin response mediator protein-4	Forward: TGACCGAGGCCTATGAAAAG Reverse: GGTGATGTCCACATGCAAAG	80	NM_012934.1
heterogeneous nuclear ribonucleoprotein K	Forward: CCCTAACACCGAAACCAATG Reverse: CAGCATTCTTGCTCTGAAGC	134	NM_057141.1
purine nucleoside phosphorylase	Forward: TGATCCGTGACCACATCAAC Reverse: ATCCCGGTCATAAGCATCAG	116	NM_001106031.1
γ -actin	Forward: CTATTGAGCACGGCATTGTC Reverse: TGTTAGCTTTGGGGTTCAGG	138	XM_001081785.1
stress-70 protein, mitochondrial precursor (75 kDa glucose-regulated protein)	Forward: GTTATGGAGGGCAAACAAGC Reverse: GACAGCTTGTCGTTTTGCTG	126	NM_001100658.1
18s rRNA	Forward: AAGTTTCAGCACATCCTGCGAGTA Reverse: TTGGTGAGGTCAATGTCTGCTTTC	140	NM_213557.1

Table 2

Identified proteins with sex differences in the AVPV on PD1

Spot No.	Accession No.	Protein names	Molecular Weight (kD)	pI (pH)	Protein spots		mRNA	
					Difference (♀/♂)	P-value (t-test)	Difference (♀/♂)	P-value (t-test)
461	gi|109492380	γ -actin	82.614	5.6	1.35	0.008	0.81	0.131
593	gi|116242506	stress-70 protein, mitochondrial precursor (75 kDa glucose-regulated protein)	74.852	5.4	1.36	0.001	0.93	0.453
922		Unidentified	59.68	6.1	1.67	0.01	-	-
1437	gi|157822819	purine nucleoside phosphorylase	38.255	6.5	1.59	0.004	0.94	0.735
1784		Unidentified	25.853	5.6	4.63	0.01	-	-
1816		Unidentified	24.796	6.5	3.28	0.002	-	-
773	gi|149039794	heterogeneous nuclear ribonucleoprotein K	66.790	5.2	0.69	0.04	0.93	0.388
774	gi|55621	α-internexin	66.420	5.3	0.74	0.028	0.75	0.049
787	gi|25742568	collapsin response mediator protein-4 (CRMP4)	65.777	6.1	0.60	0.038	0.75	0.015

Table 3

Nuclear size and cell number in the AVPV of wild type (WT) and CRMP4-knockout (CRMP4-KO) mice

Cell type	Size (μm^2)	Cell number
WT male (n=4)	535240 ± 57726	1518 ± 78
WT female (n=6)	801460 ± 84252 n.s.	2784 ± 324*
CRMP4 KO male (n=6)	650560 ± 71414	1922 ± 236
CRMP4 KO female (n=6)	840173 ± 70744 n.s.	3154 ± 380*

n.s.; Not different from males of the same genotype.

*Significantly different from males of the same genotype.

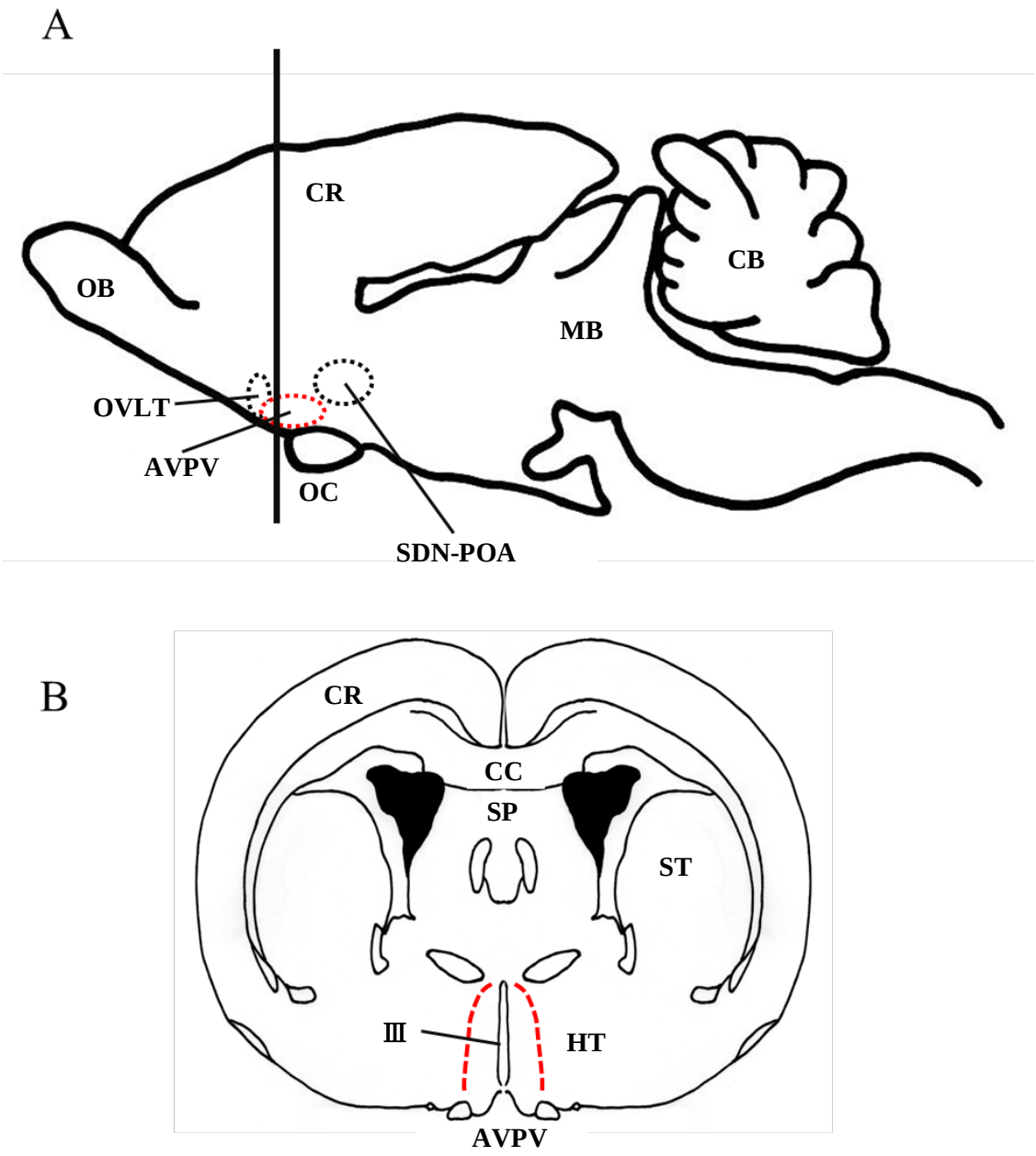

Figure 1. Diagrams showing the location of AVPV. A: Sagittal section of the rat brain showing the location of AVPV (red dotted lines) and SDN-POA (dotted lines). B: Frontal section at the plane shown by the line in A. III, third ventricle; CB, cerebellum; CC, corpus callosum; CR, cerebral cortex; HT, hypothalamus; MB, midbrain; OB, olfactory bulb; OC, optic chiasm; OVLT, organum vasculosum of the lamina terminalis; SP, septum; ST, striatum.

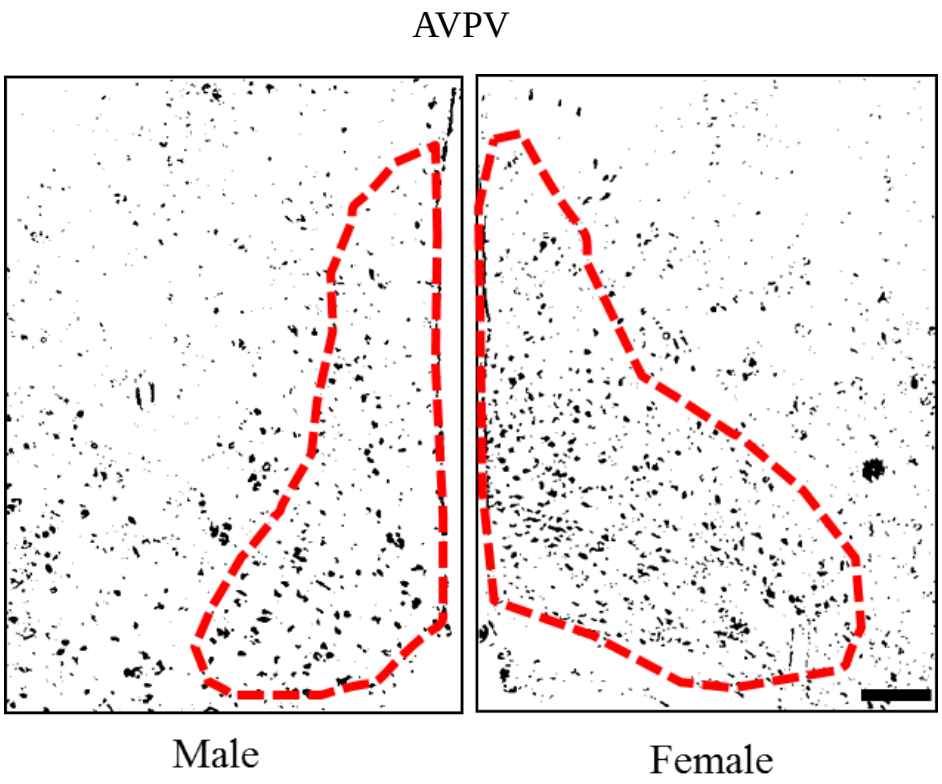

Figure 2. Photomicrographs showing the frontal section of the mouse AVPV stained with cresyl violet. Red dotted lines show the AVPV outline. Scale bar: 100 μm.

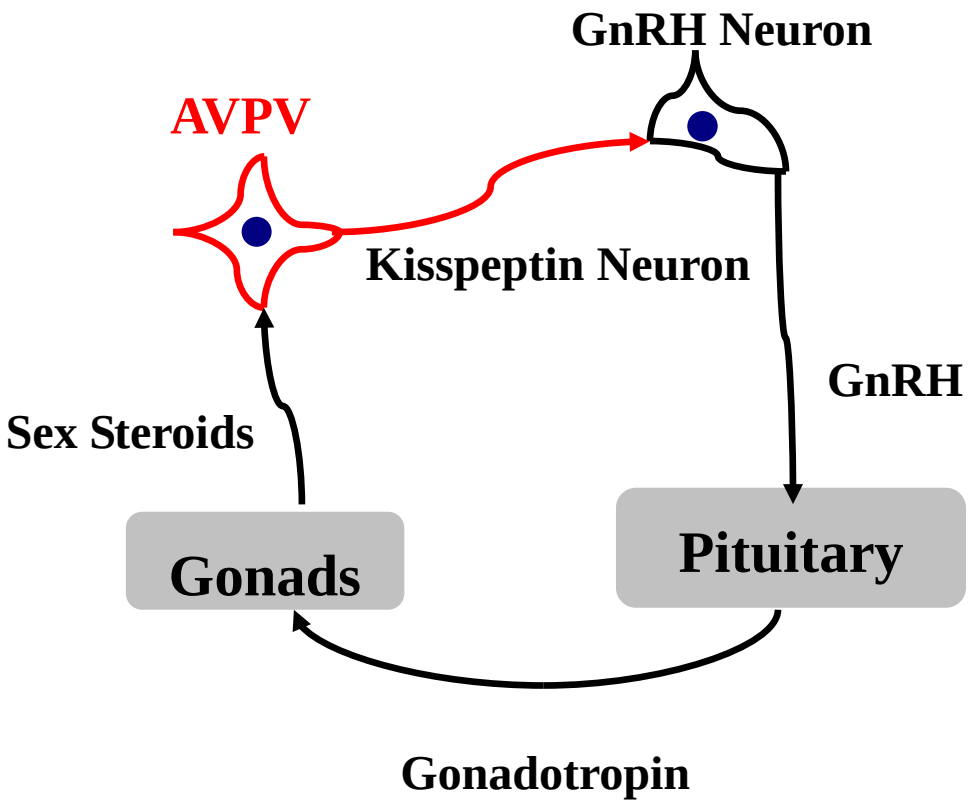

Figure 3. Schematic diagram showing positive feedback of GnRH through the kisspeptin neuron in the AVPV.

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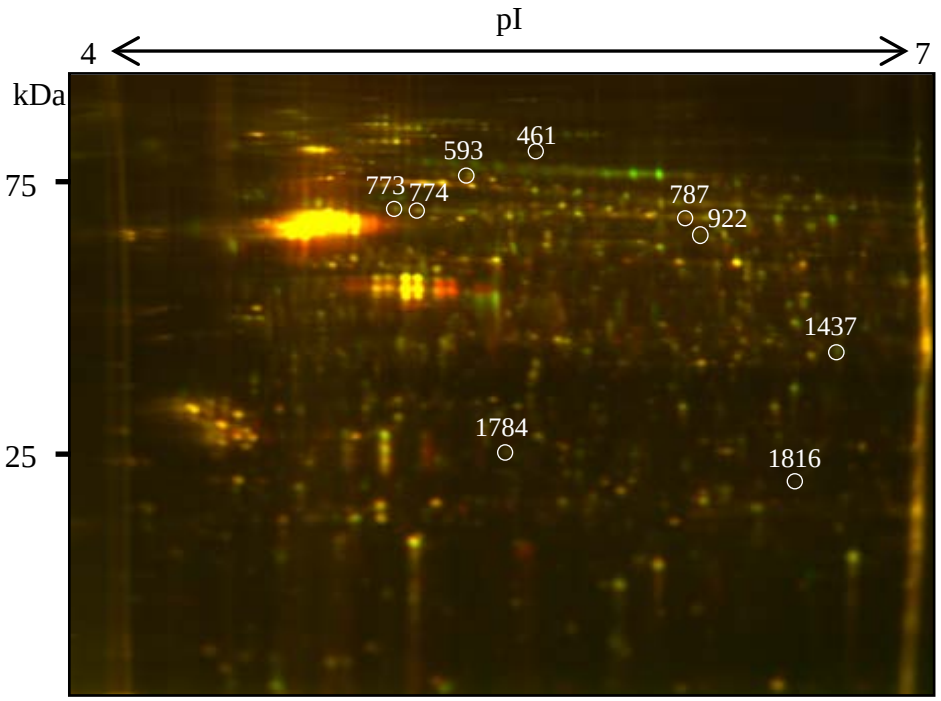
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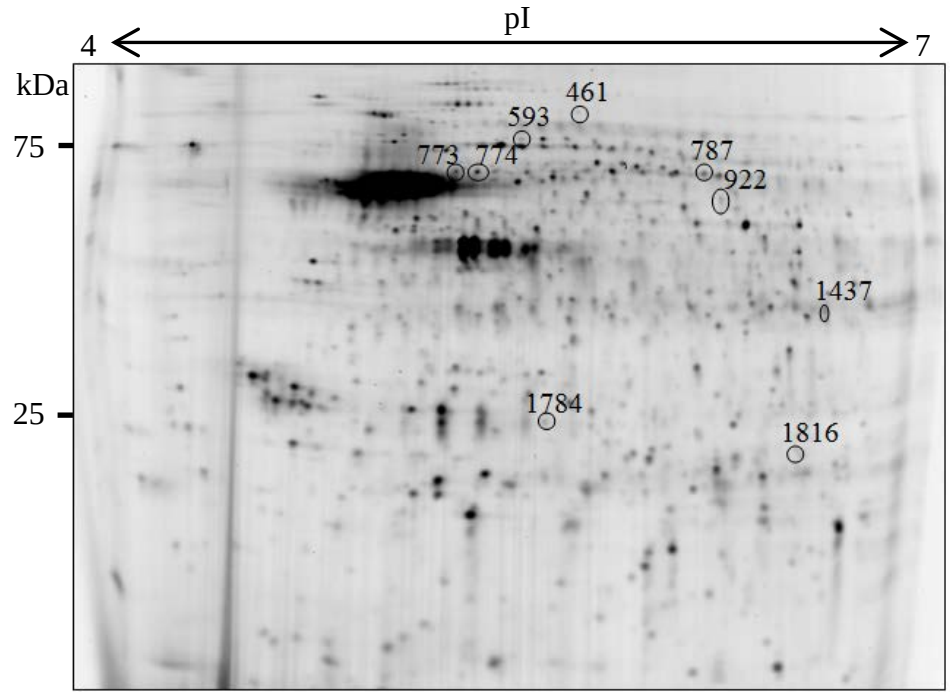
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Figure 4. (A) A representative two-dimensional difference gel electrophoresis (2D-DIGE) image. Proteins extracted from the female or male rat anteroventral periventricular nucleus (AVPV) were labeled with Cy5, and equal amounts of protein were labeled with Cy3; these Cy3-labeled proteins were used as an in-gel standard to quantify differences between female and male samples. These 2 differentially labeled samples were separated by isoelectric focusing and subsequent 12.5% SDS-PAGE. Protein spots exhibiting differential intensities compared to those of the in-gel standard were visualized as red (higher intensity) or green (lower intensity), depending on the degree of change. Protein spots that showed little difference appear yellow in color. (B) Detection of sexually dimorphic proteins in the AVPV by 2D-DIGE. Circled and numbered protein spots indicate sexually dimorphic proteins that were processed for further identification by MALDI-TOF-MS.

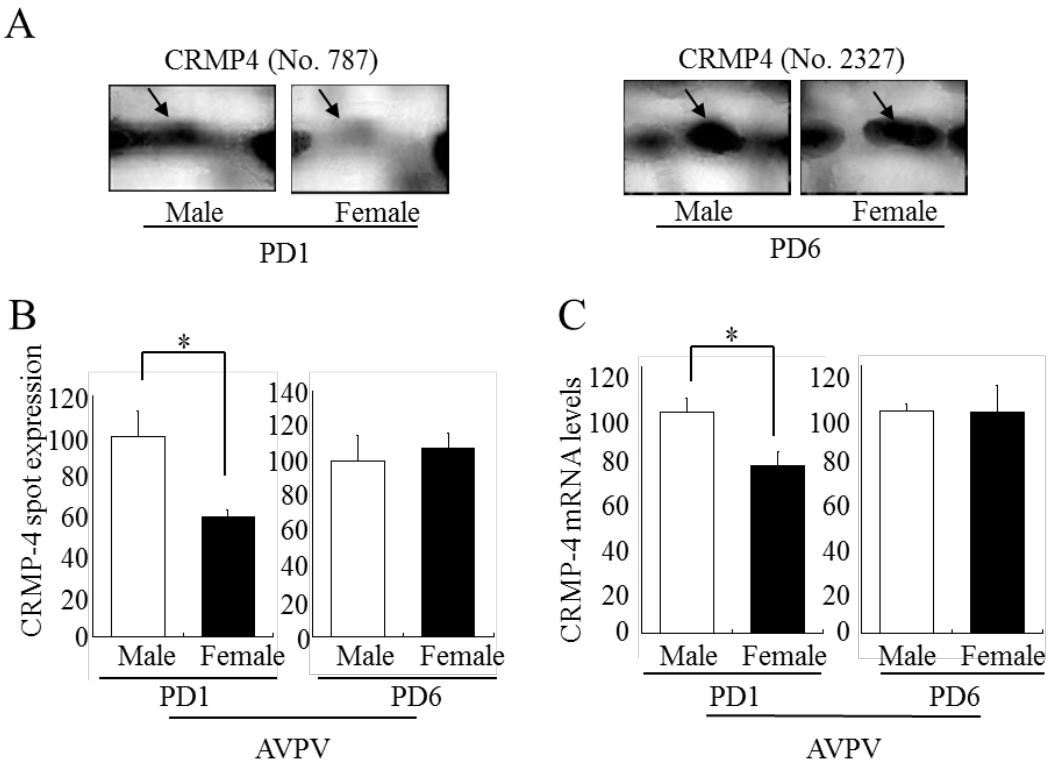

Figure 5. (A) Paired images of 2D-DIGE spots identified as CRMP4 in male and female rats on PD1 (left) and PD6 (right). (B, C) Relative expression of CRMP4 protein (B) and CRMP4 mRNA (C) in the male and female AVPV on PD1 (left) and PD6 (right). The relative levels of CRMP4 protein and mRNA in females were calculated by comparison with CRMP4 expression in males. Values (100%) are expressed as means ± SEMs. Asterisks indicate significant differences between sexes (Student's *t*-test, $P<0.05$).

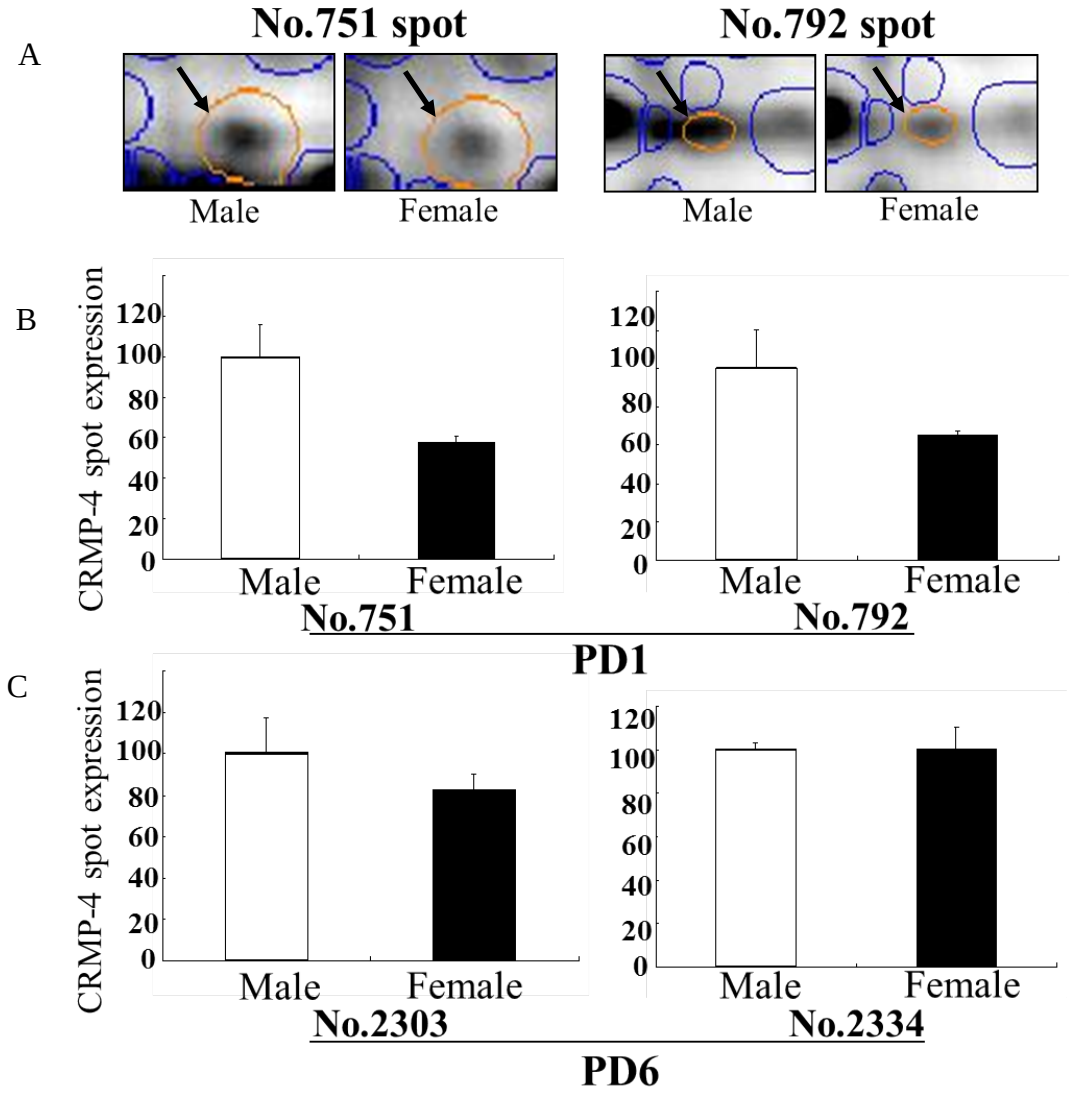

Figure 6. (A) Paired images of 2D-DIGE spots identified as CRMP4 in male and female rats on PD1 (B) and PD6 (C). The relative levels of CRMP4 protein in female AVPV were calculated by comparison with CRMP4 expression in males. Values (100%) are expressed as means ± SEMs.

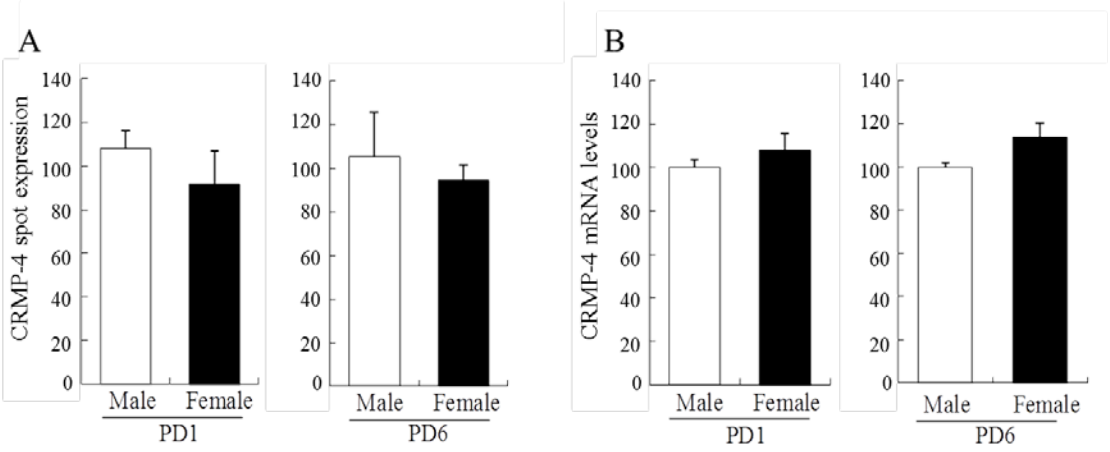

Figure 7. Expression of CRMP4 protein and mRNA in the SDN-POA of male and female rats on PD1 and PD6. Relative expression levels of the CRMP4 spot (A) and CRMP4 mRNA (B) in the SDN-POA of male and female rats on PD1 and PD6. CRMP4 protein and mRNA expressions in the SDN-POA on PD1 and PD6 showed no sex difference. Values are indicated as means ± SEMs.

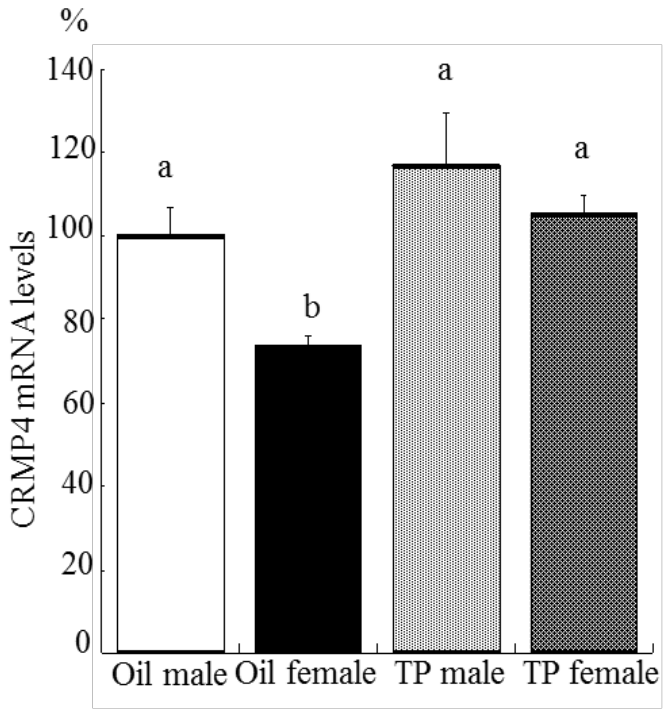

Figure 8. Effect of testosterone propionate (TP) on CRMP4 mRNA expression. Pregnant rats were injected daily with TP or sesame oil (Oil) from day 14 to 18 of gestation, and the relative expression of CRMP4 mRNA in the AVPV was determined on PD1 by real-time RT-PCR. Expression levels of CRMP4 mRNA were calculated by comparing to the levels of control males (Oil male). TP treatment significantly increased CRMP4 mRNA in females. Values are indicated as means ± SEMs. Different letters (a, b) indicate a significant difference by the Scheffe's *post hoc* test ($P < 0.05$).

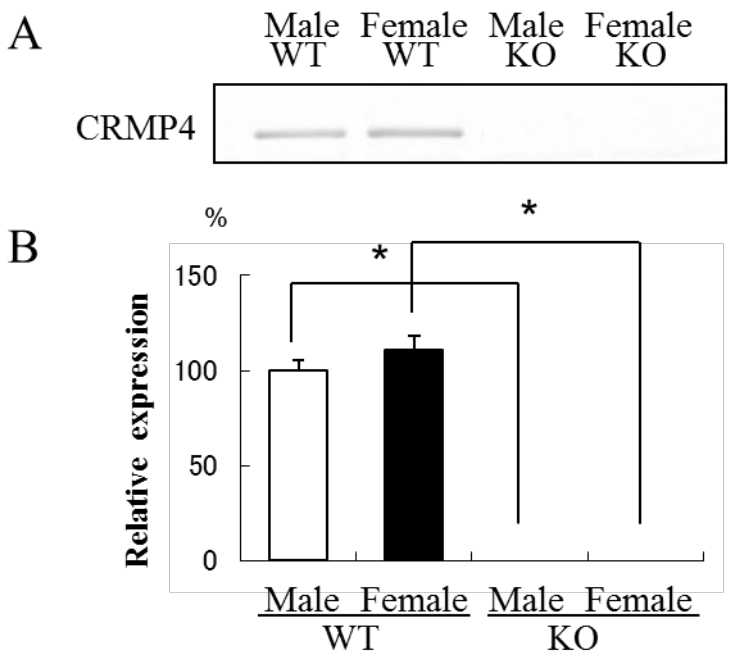

Figure 9. CRMP4 protein expression in whole brains of adult CRMP4 WT and KO mice. (A) Western blot analysis of male and female WT and KO mice. (B) The expression of CRMP4 was virtually absent in male and female KO mice. Values are indicated as means ± SEMs. Asterisks indicate significant differences between sexes (Student's *t*-test, $P<0.05$).

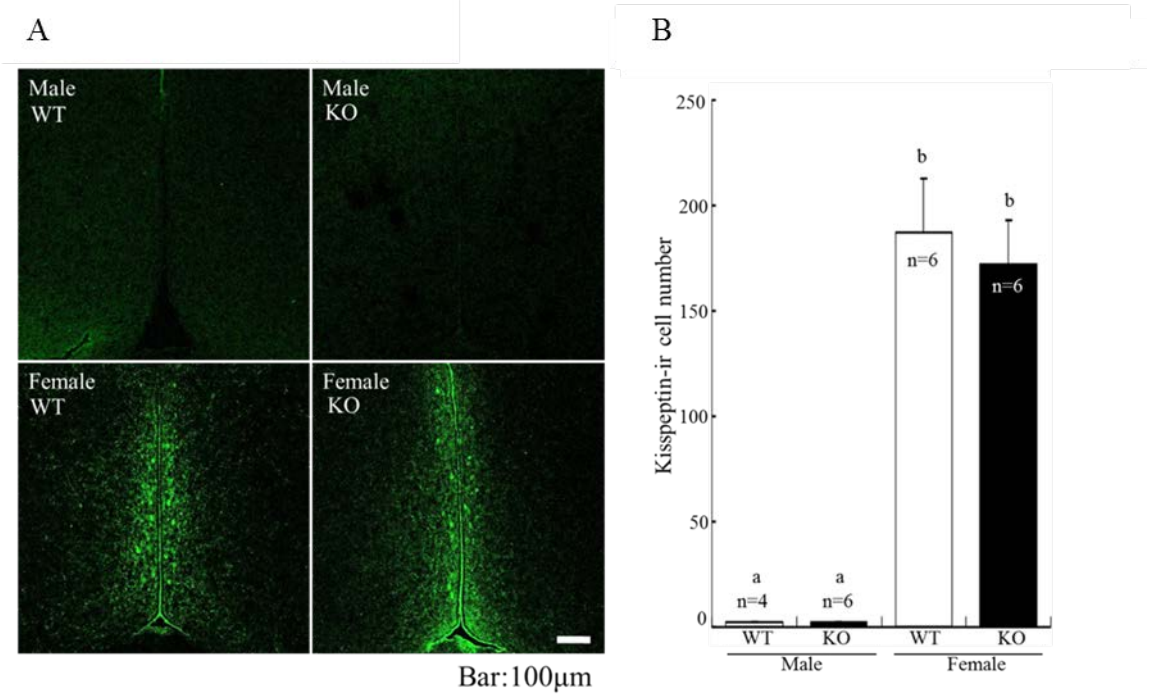

Figure 10. Effect of CRMP4-KO on the number of kisspeptin-ir neurons in the AVPV of adult mice. (A) Representative micrographs showing kisspeptin-ir neurons in male and female AVPVs of WT and CRMP4-KO mice. Scale bar: 100 μm. (B) The number of kisspeptin-ir neurons was greater in WT and KO female mice than in WT and KO male mice. CRMP4-KO did not affect the number of kisspeptin-ir neurons in either sex. Values are expressed as means ± SEMs. Different letters (a, b) indicate significant differences (two-way ANOVA with sex and genotype as the independent factors, followed by Scheffe's *post hoc* test for multiple comparisons, $P < 0.05$).

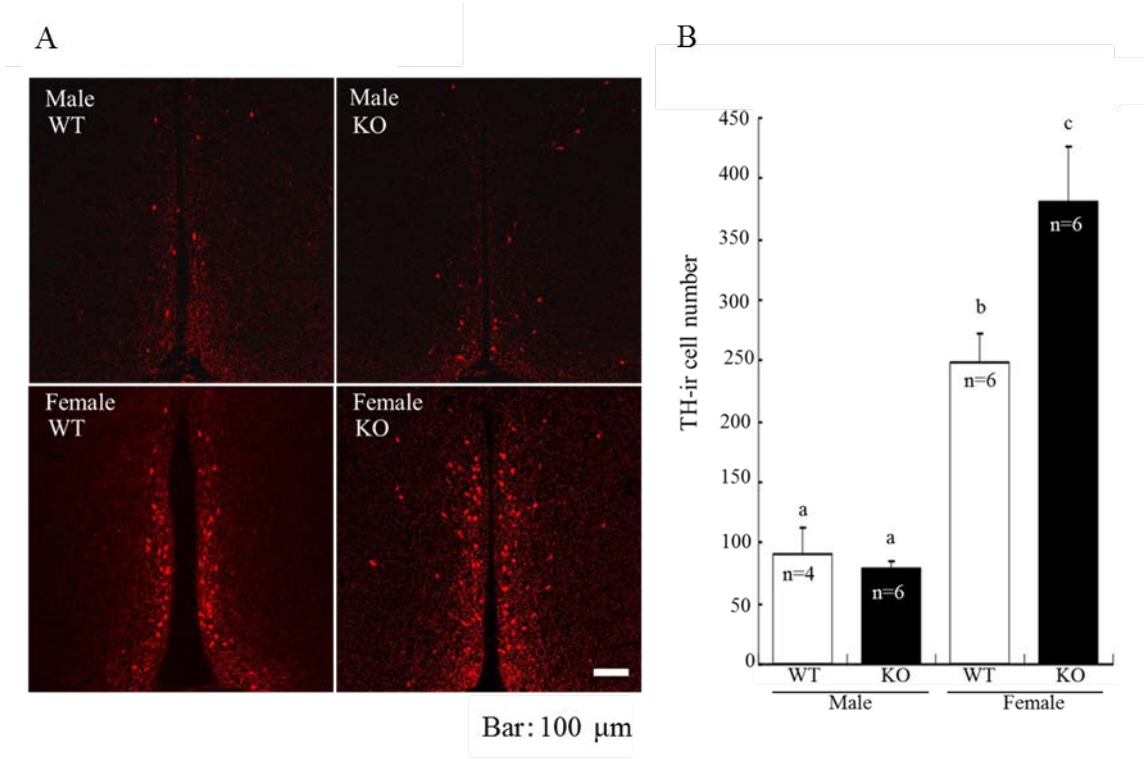

Figure 11. Effect of CRMP4 KO on the number of TH-ir neurons in the AVPV of adult mice. (A) Representative micrographs showing TH-ir neurons in male and female AVPVs of WT and CRMP4-KO mice. Scale bar: 100 μm. (B) The number of TH-ir neurons was greater in WT and KO female mice than in WT and KO male mice. CRMP4-KO increased the number of TH-ir neurons in females (right) but not in males (left). Values are expressed as means ± SEMs. Different letters (a, b, c) indicate significant differences (two-way ANOVA with sex and genotype as the independent factors, followed by Scheffe's *post hoc* test for multiple comparisons, $P< 0.05$).

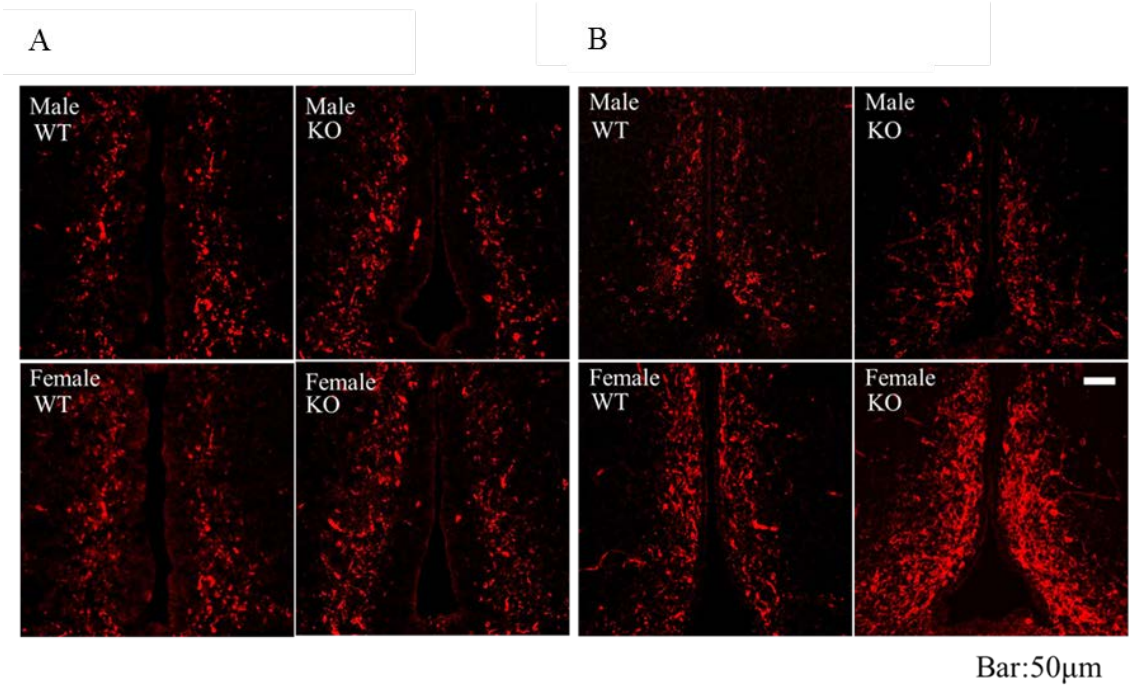

Figure 12. Effect of CRMP4 KO on the number of TH-ir cells in the developing AVPVs. Micrographs showing TH-ir neruons in the male and female AVPVs of WT and KO mice on E15 (A) and PD1 (B). Scale bar: 50 μm.

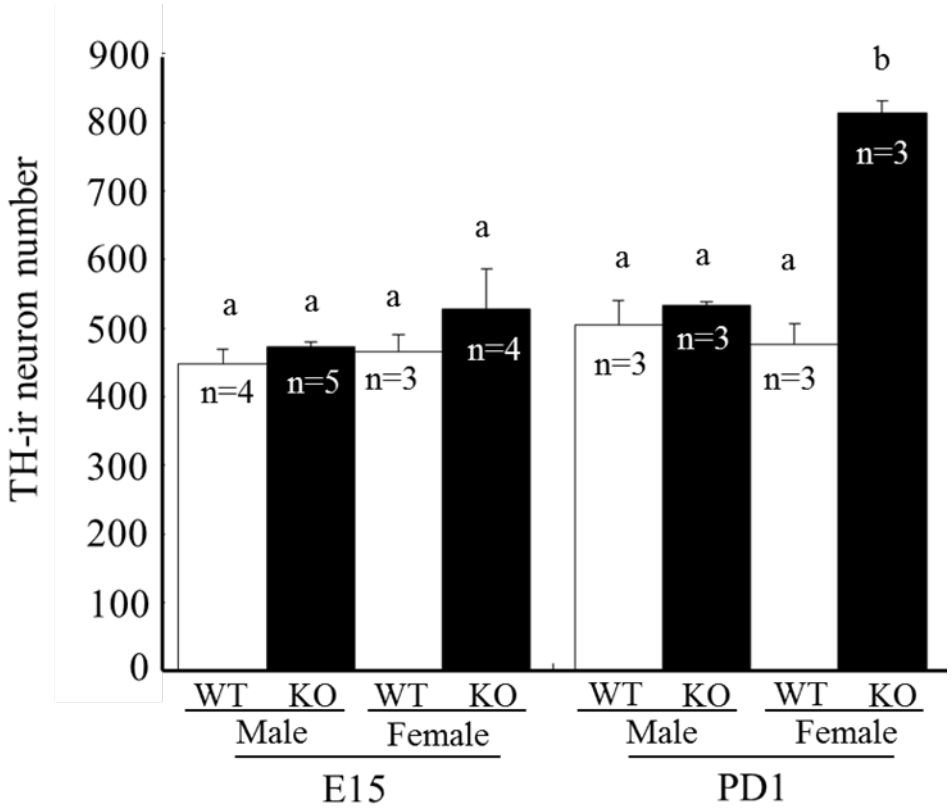

Figure 13. Changes in the number of TH-ir neurons in the AVPVs of WT and KO mice during development. TH-ir neurons in female and male AVPVs of WT and KO mice were enumerated on E15 and PD1. No significant differences were found in the number of TH-ir neurons in male versus female mice or in WT versus CRMP4-KO mice on E15. A female-specific increase in the number of TH-ir neurons was found in CRMP4-KO mice on PD1, when the sex difference had yet to be observed in WT mice. Values are expressed as means ± SEMs. Different letters (a, b) indicate significant differences (two-way ANOVA with sex and genotype as the independent factors, followed by Scheffe's *post hoc* test for multiple comparisons, $P < 0.05$).

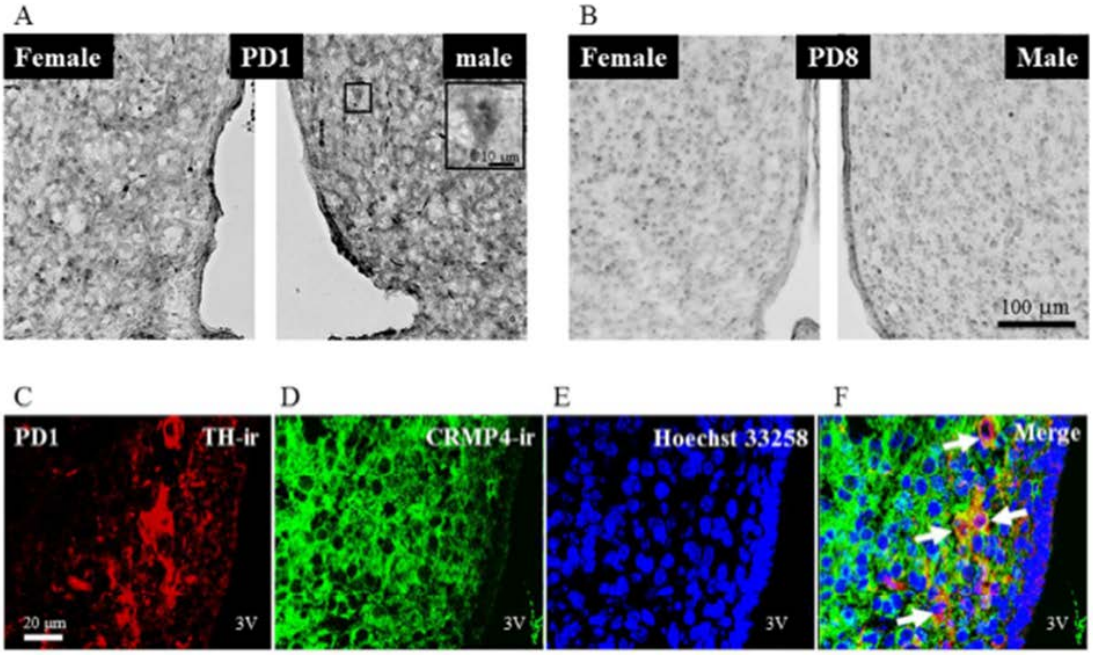

Figure 14. The expression of CRMP4 mRNA in female and male WT mouse AVPVs, and colocalizaton of TH-immunoreactivity with CRMP4. Representative micrographs of *in situ* hybridization showing the expression of CRMP4 mRNA in the AVPVs of male and female mouse brains on PD1 (A) and PD8 (B). Scale bar: 100 μm. (C-F) Double immunostaining for TH and CRMP4. Scale bar: 20 μm. 3V: third ventricle. (C) TH-immunoreactive (TH-ir, red). (D) CRMP4-ir (green). (E) Hoechst 33342 (blue). (F) Merged image.

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