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Muscarinic M₂ receptor promotes vasopressin synthesis in mice supraoptic nuclei

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Abstract

Muscarinic acetylcholine receptors have been suggested to be implicated in arginine–vasopressin secretion because intracerebroventricular muscarinic agonist administration induces arginine–vasopressin release into the circulation. Although which subtype is involved in the regulation of arginine–vasopressin secretion is unclear, M₂ receptors have been reported to be highly expressed in the hypothalamus. In the present study, M₂ receptor-knockout mice were used to elucidate whether M₂ receptor regulates arginine–vasopressin synthesis in the paraventricular nuclei and supraoptic nuclei of the hypothalamus. The number of arginine–vasopressin-immunoreactive neurons in M₂ receptor-knockout mice was significantly decreased in the supraoptic nuclei, but not in the paraventricular nuclei compared with wild-type mice. Plasma arginine–vasopressin level in M₂ receptor-knockout mice was also significantly lower than in the wild-type mice. Urinary volume and frequency as well as water intake in M₂ receptor-knockout mice were significantly higher than those in wild-type mice. The V₂ vasopressin receptor expression in kidneys of M₂ receptor-knockout mice was comparable with that of wild-type mice, and increased urination in M₂ receptor-knockout mice was significantly decreased by administration of desmopressin, a specific V₂ receptor agonist, suggesting that V₂ receptors in the kidneys of M₂ receptor-knockout mice are intact. These results suggest that M₂ receptors promote arginine–vasopressin synthesis in the supraoptic nuclei and play a role in the regulation and maintenance of body fluid.

Key Words

- ▶ cholinergic receptor
- ▶ muscarinic receptor
- ▶ arginine–vasopressin
- ▶ magnocellular neuron
- ▶ supraoptic nucleus

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Introduction

Arginine–vasopressin (AVP), an antidiuretic hormone, is synthesized by magnocellular neurosecretory cells (MNCs) in the paraventricular nuclei (PVN) and supraoptic nuclei (SON) of the hypothalamus and subsequently released from nerve terminals in the posterior pituitary into the blood circulation. AVP maintains body fluid homeostasis

by stimulating water reabsorption via vasopressin V₂ receptors in the renal collecting tubule. Synthesis and secretion of AVP are regulated by complex mechanisms that are mediated by various neurotransmitters. Involvement of cholinergic neurons in AVP production is well documented. Some pharmacological studies have

shown that nicotinic receptors induce MNC excitation and promote AVP release (Armstrong 1982, Hatton *et al.* 1983, Zaninetti *et al.* 2002), whereas other studies have revealed the participation of muscarinic receptors in AVP release (Iitake *et al.* 1986, Michels *et al.* 1991, Shen & Sun 1995). Particularly in the SON, cholinergic neurons project to MNCs (Rao *et al.* 1987, Wang *et al.* 2015), and muscarinic receptors are expressed in this region (Rotter *et al.* 1979, Meeker *et al.* 1988). Moreover, microinfusion of muscarinic agonist into the SON increases c-fos expression in MNCs (Shen & Sun 1995) and decreases urinary volume (Mori *et al.* 1994), suggesting muscarinic regulation of AVP release in the SON region.

Plasma hyperosmolality is known as a physiological stimulation that promotes AVP secretion. The subfornical organ (SFO) and median preoptic nucleus of the hypothalamus are regions controlling plasma osmolality and drinking behavior (Leng *et al.* 1989, Fitzsimons 1998). Anatomical evidence showed that these regions project cholinergic nerve terminals to the PVN and SON (Oldfield *et al.* 1991, Xu *et al.* 2003), and it was indicated that they activate AVP neurons via muscarinic receptors, but not nicotinic receptors (Xu *et al.* 2001a). In addition, AVP secretion is also related to blood pressure, which is regulated by the cardiovascular system. Previous studies have demonstrated that muscarinic receptor activation in the bed nucleus of the stria terminalis (BNST) and the posterior hypothalamic nuclei caused a pressor response (Xiao & Brezenoff 1988, Martin 1996, Alves *et al.* 2007, 2011).

Muscarinic receptors are classified into five subtypes, M₁ to M₅, and colocalized in various organs. However, which subtype is implicated in AVP production remains to be identified because no reagent has a specific affinity for each subtype. Although the M₂ receptor is known to be abundantly expressed in the hypothalamus (Vilaró *et al.* 1993, Wei *et al.* 2002, Oki *et al.* 2005), and previous reports have shown that the M₂ receptor in the brain induced drinking behavior (Hagan *et al.* 1987) and a pressor response resulted from increase in circulating AVP (Brezenoff *et al.* 1988, Xiao & Brezenoff 1988, Alves *et al.* 2007), its detailed function is unclear.

In the present study, transgenic mice lacking the M₂ subtype of muscarinic receptor were used to elucidate if the M₂ receptor regulates AVP production. We assessed the capability of AVP synthesis and secretion by counting the number of AVP-immunoreactive (AVP-IR) neurons in the PVN and SON and measuring plasma AVP levels. Furthermore, AVP-mediated antidiuresis was estimated from parameters of drinking and urination. The results suggest that M₂ receptors contribute to AVP synthesis in the SON.

Materials and methods

Animals

Female M₂ receptor-knockout (M₂KO) mice (3–4 months old), weighing 25.1±1.0 g (mean±S.E.M.), and their corresponding wild-type (WT) mice, weighing 27.9±1.0 g, were used in all experiments. The generation of M₂KO mice has been described previously (Gomez *et al.* 1999, Struckmann 2003). The genetic background of mice used in the present study was 129J1 (50%)×CF1 (50%) for the M₂KO and WT mice. The animals were housed in a temperature-controlled room at 22–25°C under a 12-h light/darkness cycle (07:00–19:00h). The animals were allowed to access the chow and distilled water *ad libitum*. All experimental procedures were performed based on the guidelines approved by the local animal ethics committee of the Faculty of Applied Biological Science, Gifu University.

AVP immunohistochemistry

The experiment was performed between 09:00 and 12:00h to avoid the diurnal variations in AVP synthesis. The animals were anesthetized with ketamine (80mg/kg, i.p.) and xylazine (10mg/kg, i.p.) and immediately perfused intracardially with heparinized saline, followed by 4w/v% paraformaldehyde in 0.1-M phosphate buffer (Wako Pure Chemical Industries). The brain was removed and postfixed in the same fixative overnight at 4°C. The tissues were dehydrated in a graded series of ethanol, cleared in xylene and paraffin embedded. Coronal sections of 5-μm thickness containing the PVN and/or SON were cut between the optic chiasm and median eminence and collected in five alternate slices. They were deparaffinized in xylene and rehydrated in a graded series of ethanol. The sections were rinsed thrice for 5 min in PBS with 0.1 v/v% Triton X-100 (PBST) and were incubated for 30 min at room temperature with blocking buffer, including 0.1 v/v% normal goat serum, 1 v/v% bovine serum albumin and 0.1 w/v% sodium azide. After being rinsed in PBST, they were incubated overnight at 4°C with rabbit anti-AVP polyclonal antibody (1:2500, AB1565, Chemicon International). They were rinsed in PBST and incubated for 30 min at room temperature with biotinylated anti-rabbit IgG antibody (1:1000, Sigma-Aldrich), and then for 30 min at room temperature with peroxidase-labeled avidin–biotin complex (1:400, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA). AVP immunoreactivity was visualized in

Tris-HCl containing 0.02 w/v% diaminobenzidine and 0.003 w/v% H₂O₂ for 10 min, and the sections were subsequently stained with hematoxylin as counterstain. After being dehydrated in a graded series of ethanol and cleared in xylene, they were mounted with coverslips.

To count the number of AVP-IR neurons, digitalized images, including the PVN and/or SON area were obtained by using a digital camera (Pro 600ES, Pixera Corporation, Santa Clara, CA, USA) installed on a microscope. To discriminate between the AVP-positive neurons and other neurons, a threshold value for the intention of immunoreactivity was set by using the image software ImageJ (National Institutes of Health, Bethesda, MD, USA). The numbers of AVP-IR neurons were counted from an observer's eyes. The magnocellular region of the PVN was divided based on the mouse brain in stereotaxic coordinates by Paxinos & Franklin (2004). The differences between the right and left regions of each nucleus were not distinguished.

Plasma AVP level

Mice were anesthetized with diethyl ether. Blood samples were collected via the heart in chilled tubes containing ethylene diamine tetraacetic acid and were then centrifuged (2000g, 10 min). Supernatants were left at -20°C and used to quantify plasma AVP levels by radioimmunoassay consigned to Monolis Co., Ltd. (Tokyo, Japan).

Drinking and urination test

Mice were acclimatized in metabolic cages for a day before an experimental day. Measurements of water intake, urinary volume and urinary frequency were obtained by putting the upper chambers of the metabolic cages (3600M021, Tecniplast, VA, Italy) on a windbreaking holder covering an electronic balance. A metal mesh placed at the bottom of the upper chamber was subjected to water-repellent treatment. Urinary parameters were recorded at every 1 s by using a measurement software (Salto In TIMER, Sartorius Mechatronics, Göttingen, Germany), which was installed in a computer connected to an electronic balance. Data were determined as urination when the recorded value was higher than 0.100 g, following the report by Yamamoto *et al.* (2009). If plurality of data recorded as urination was obtained within 10 s, they were decided as a single urination. Water intake was estimated by comparing a weight of the water bottle before and after measurements. These experiments

were conducted from 19:00 to 13:00 h the following day. The animals were not allowed to take chow during the experiments.

Blood chemistry

Blood samples were collected via a caudal vein in chilled tubes containing heparin, which were then centrifuged (1500g, 5 min). Serum was analyzed for biochemistry parameters by the biochemical analyzer (FUJI DRI-CHEM 7000V, FUJIFILM, Tokyo, Japan): blood urea nitrogen (BUN), glucose (GLU), sodium (Na), potassium (K), calcium (Ca). The approximate values of plasma osmolality were calculated by substituting the values of Na, K, GLU and BUN for the following formula (Dorwart & Chalmers 1975):

$$\text{Calculated osmolality} = 2 [\text{Na}] + 2 [\text{K}] + [\text{GLU}] / 18 \\ + [\text{BUN}] / 2.8 \text{ (mosmol / kg)}$$

Urine analysis

Urine collected by manual compression of the urinary bladder was centrifuged (1500g, 5 min), and the supernatant was then analyzed for urine-specific gravity by a refractometer (UR-JE, ATAGO, Tokyo, Japan).

Testing for the presence of hemoglobin, bilirubin, urobilinogen, ketones, proteins, glucose and nitrite was performed using urine test strips (Uropaper III, Eiken Chemical, Tokyo, Japan).

Histopathological analysis

The animals were killed by cervical dislocation, and the following organs were removed: kidney, urinary bladder, thyroid glands, adrenal glands, liver and heart. These organs were trimmed and fixed in 4 w/v% paraformaldehyde in 0.1-M phosphate buffer overnight at 4°C. The tissues were processed for paraffin sectioning as mentioned above and cut into 4-μm sections. The sections were stained with hematoxylin and eosin and then mounted with coverslips. They were used to detect pathological lesions by using a light microscope.

V₂ immunohistochemistry

Paraffin sections of the kidney were made as mentioned earlier. Immunohistochemistry of vasopressin V₂ receptors (V₂R) was performed to confirm the presence

of V₂R similar to that of AVP. The rabbit anti-V₂ receptor polyclonal antibody (1:100, Bioss, Woburn, MA, USA) was used as a primary antibody. The second antibody was the HRP-labeled anti-rabbit antibody (1:1, En Vision System HRP, Dako).

Enzyme-linked immunosorbent assay

ELISA was conducted to evaluate the V₂R expression in the kidney by using anti-V₂R antibody. The kidneys were homogenized with 1-mL CellLytic MT (Sigma-Aldrich) per 50-mg tissue. After centrifugation (15,000g, 10 min), the supernatants were collected as sample solutions.

For calibration curve construction, the BSA solution was diluted with distilled water to appropriate concentrations, and the absorbance was measured at 595 nm by using a spectrophotometer (U-1100, HITACHI). Absorbance was measured by using the protein assay CBB solution (Nakalai-tesque, Kyoto, Japan) and by following the manual. The sample absorptions were measured similarly, and the protein concentrations were calculated from the calibration curve.

The sample solutions diluted (10 µg/mL) with 0.05-M carbonate–bicarbonate buffer (pH 9.6) were dispensed (50 µL/well) into microtiter plates with 96 wells and incubated overnight at 4°C. After being washed with PBST, they were blocked with PBS containing 3 w/v% skim milk for 1 h at 37°C. After washing, they were incubated with rabbit anti-V₂ receptor polyclonal antibody (1:30; Santa Cruz Biotechnology) for 1 h at 37°C. The second antibody used in the immunohistochemistry of V₂R (1:1) was added (50 µL/well) and incubated for 1 h at 37°C after washing. After being washed six times, the substrate solution was added (100 µL/well) to the wells, and the plates were incubated for 1 h at room temperature in the dark. After the substrate reaction was stopped, the absorbance was read at 415 nm with a microplate reader (model 680, Bio-Rad Laboratories).

Desmopressin test

Desmopressin, a synthetic AVP analog with a high affinity for V₂R, was used to evaluate V₂R reactivity in the kidneys. The animals were loaded with distilled water (15 mL/kg, p.o.) and administered with desmopressin acetate hydrate (1 µg/kg, i.p., Kyowa Hakko Kirin, Tokyo, Japan). Thereafter, urinary volumes were recorded for 5 h while avoiding of food and water intake. Control groups were treated with saline (5 mL/kg, i.p.) instead of desmopressin.

Statistical analysis

Data were presented as the mean ± S.E.M. Student *t*-test was used to determine significance. Values were considered significant if *P* < 0.05.

Results

AVP-IR neurons in the PVN and SON

AVP-IR somata and fibers were observed in the PVN and SON of the hypothalamus in both types of mice (Fig. 1). The number of AVP-IR neurons in the PVN of M₂KO mice was similar to that of WT mice (Fig. 1A and B). However, in the SON of M₂KO mice, the number of AVP-IR neurons seemed to be decreased, and their individual size appeared smaller than in WT mice (Fig. 1C and D). A few small perikarya showing AVP immunoreactivity were observed in the parvocellular region of the PVN in both mice groups (not shown).

The number of AVP-IR perikarya was not different between the PVN of both strains (343.2 ± 45.2, *n* = 5, vs 313.8 ± 37.5, *n* = 5; *P* > 0.05; Fig. 2A). In contrast, AVP-immunostained perikarya were significantly decreased in the SON of M₂KO mice compared with that in WT mice (2353.8 ± 245.4, *n* = 5, vs 898.2 ± 142.4, *n* = 5; *P* < 0.01; Fig. 2B).

Plasma AVP level and antidiuresis

As shown in the Fig. 3, plasma AVP concentration obtained from M₂KO was only approximately 30% of that from WT (1029.9 ± 233.8 pg/mL, *n* = 10, vs 341.8 ± 105.7 pg/mL, *n* = 8; *P* < 0.05).

The absence of M₂ receptors induced remarkable increases in drinking and urination. Water intake in the M₂KO group was 9.6-fold higher than in the WT group (0.69 ± 0.12 g, *n* = 27, vs 10.0 ± 0.80 g, *n* = 8; *P* < 0.01; Fig. 4A). Urinary volume in M₂KO mice was 14.5-fold higher than in WT mice (5.6 ± 0.33 g, *n* = 27, vs 43.5 ± 1.8 g, *n* = 8; *P* < 0.01; Fig. 4B), and the frequency was also 7.8-fold higher than in WT mice (1.2 ± 0.13, *n* = 27, vs 11.5 ± 0.80, *n* = 8; *P* < 0.01; Fig. 4C).

Plasma biochemistry and osmolality

From the results of serum chemistry analysis, no significant differences in all parameters were found between the M₂KO and WT groups (Table 1). Plasma osmolality calculated from the values of Na, K, GLU and BUN showed plasma hyperosmolality in M₂KO mice (325.6 ± 1.4, *n* = 13, vs 331.3 ± 2.3 mosmol/kg, *n* = 8; *P* < 0.05; Fig. 5).

Figure 1

Localization of AVP-IR cells in the PVN (A and B) and SON (C and D) of WT (A and C) and M₂KO mice (B and D). AVP immunoreactivity was detected in perikarya and fibers. 3V, third ventricle; OC, optic chiasm. Scale bar indicates 30 μm. A full color version of this figure is available at <https://doi.org/10.1530/JOE-17-0630>.

In the M₂KO group, urine-specific gravity was significantly decreased compared with the WT group (1.07 ± 0.03 , $n=27$, vs 1.04 ± 0.05 , $n=8$; $P<0.05$). None of the pathological components comprising hemoglobin, bilirubin, urobilinogen, ketones, proteins, glucose and nitrite were detected in the urine of both mice groups.

Morphologic pathology

Microscopic examination of the kidney, urinary bladder, thyroid glands, adrenal glands, liver and heart revealed no pathological changes in all organs of both strains (data not shown). No macroscopic lesions were found in both strains (data not shown).

Expression and reactivity of V₂R

The V₂R immunoreactivity in the kidney of M₂KO mice was found in the tubular epithelial cells (Fig. 6A(b)). This finding was similar to that of WT mice (Fig. 6A(a)). The expression levels of V₂R in the kidney were shown as the absorbance obtained by ELISA. They were not significantly different between both strains (0.756 ± 0.058 , $n=6$, vs 0.923 ± 0.067 , $n=6$; $P>0.05$; Fig. 6B).

The urinary volume of the WT group was decreased by desmopressin administration. Although the urinary volume of the control group in the M₂KO group was twice as much as that in WT, desmopressin administration to the M₂KO group decreased the value to almost the same level with WT group treated with desmopressin

(WT: 0.63 ± 0.17 g, $n=5$ vs 0.06 ± 0.06 g, $n=6$; $P<0.05$; M₂KO: 1.19 ± 0.10 g, $n=5$ vs 0.08 ± 0.09 g, $n=6$; $P<0.05$; Fig. 7).

Discussion

The present study suggests that the lack of M₂ receptors is involved in the decrease of AVP synthesis and secretion into the systemic circulation. These results supported the previous reports indicating that muscarinic receptor activation in the brain promoted AVP secretion (Iitake *et al.* 1986, Gribkoff *et al.* 1988, Michels *et al.* 1991, Ghamari-Langroudi & Bourque 2004). The AVP-IR neurons in the SON of M₂KO mice were significantly

Figure 2

Number of AVP-IR cells in the PVN (A) and SON (B) of WT ($n=5$) and M₂KO mice ($n=5$). A significant difference in the SON is found between WT and M₂KO groups, but not in the PVN. * $P<0.05$ vs WT group.

Figure 3

Plasma AVP level in WT and M₂KO mice. The value in M₂KO mice (*n*=8) is significantly lower than that in WT mice (*n*=10). **P*<0.05 vs WT group.

less than that of WT mice, although the decreased AVP-IR neurons' number does not always mean that AVP synthesis is decreased in the SON. However, the plasma AVP level in M₂KO mice was significantly lower than that in the WT mice. These findings suggested that M₂ receptors can promote AVP synthesis in the SON. On the other hand, the number of AVP neurons in the PVN of the M₂KO group was comparable to that of the WT group. There are several reports suggesting functional differences between the PVN and SON (Shen *et al.* 1992, Takahashi *et al.* 2001). Alves *et al.* have demonstrated that an increase of AVP secretion in response to microinjection of carbachol, a muscarinic agonist, into the BNST was blocked by prior injection of a neurotransmission blocker cobalt chloride into the SON but not into the PVN (Alves *et al.* 2011). They have also reported that a cardiovascular effect induced by carbachol into the BNST was blocked by

Figure 4

Effect of M₂ deficiency on urinary volume (A), urinary frequency (B) and water intake (C). Recordings are performed for 18h while avoiding food and drinking. In all parameters, M₂KO mice (*n*=8) showed significantly higher values than WT mice (*n*=27). **P*<0.05 vs WT group.

pretreatment with M₂ receptor antagonist into the BNST or i.v. pretreatment with the AVP receptor antagonist (Alves *et al.* 2007). These findings imply that M₂ receptors in the BNST might involve neuronal pathway to the SON to mediate AVP secretion into the blood. Similar to these studies, the present results suggest that M₂ receptors are involved in AVP synthesis regulation in the SON.

Some previous reports suggested that water drinking was regulated by muscarinic receptors (Mangiapane & Simpson 1979, Hagan *et al.* 1987, Takahashi *et al.* 2001). If a deficiency of the M₂ receptor facilitates drinking followed by polyuria, and excessive increase of body fluid consequently inhibits AVP secretion, plasma osmolality in M₂KO mice should be decreased. In contrast, if a decrease of AVP synthesis induced by the loss of M₂ receptors attenuates antidiuretic effects, which result in polyuria–polydipsia, plasma osmolality in M₂KO mice should be increased. The significant increase in plasma osmolality in the M₂KO group supports the latter hypothesis.

A plasma osmolality is strictly controlled in a narrow range; hence, increased osmolality in plasma potently stimulates the promotion of AVP synthesis and secretion (Martin 1996). Nevertheless, the number of AVP-positive neurons in the PVN of M₂KO mice was not significantly different from that of WT mice. This suggests that the SON may play a predominant role in AVP secretion into the blood circulation. Magnocellular AVP neurons secrete AVP not only from axon terminal into the blood stream but also from somata and dendrites into the extracellular fluid of the SON and PVN (Pow & Morris 1989, Landgraf & Ludwig 1991, Ludwig *et al.* 1994). It has been observed that certain stressors such as forced swimming and social defeat induced increase of mRNA level and intranuclear AVP secretion in only the PVN but not the SON (Wotjak *et al.* 1996, 2001). Nonetheless, such stressors did not increase plasma AVP level. Thus, intranuclear secretion of AVP in the PVN seems to be involved in stress responses. In addition, some reports have indicated that the SON may take more responsibility for responses to stimuli such as hyperosmolality (Sherman *et al.* 1986, da Silveira *et al.* 2007) and hypovolemia (Shen *et al.* 1992). Therefore, magnocellular AVP neurons of the PVN are more likely to participate in stress responses than those of the SON. Conversely, AVP synthesized in the SON seems to be closely related to the homeostasis of body fluids rather than stress responses. However, there might be possibilities that we were not able to distinguish magnocellular neurons from parvocellular neurons precisely and that the difference, which should have been detected in the PVN between WT and M₂KO

Table 1 Blood serum chemistry of WT (*n*=12–22) and M₂KO (*n*=7–9) mice.

	BUN (mg/dL)	GLU (mg/dL)	Na (mEq/L)	K (mEq/L)	Ca (mEq/L)
WT	20.8±0.4	141±1.4	152±0.3	5.2±0.3	9.0±0.3
M ₂ KO	23.0±0.5	148±2.4	153±0.4	6.2±0.5	8.8±0.5

There was no significant difference between these mice strains.

groups was masked. Indeed, accurate discrimination of immunopositive MNCs from the other cells was impossible due to gathering densely even though we referred to a mice brain atlas. Further consideration is needed to conclude if the M₂ receptor affects AVP synthesis and secretion in the PVN. Additionally, AVP synthesis inhibition by a lack of M₂ receptors might antagonize AVP synthesis driven by increase of plasma osmolality. For elucidating this hypothesis, to assess the reactivity to a physiological stimulation, such as hypertonic saline administration, is necessary.

M₂KO mice display some phenotypes in their physiological characteristics due to lacking M₂ receptors from birth (Gomez *et al.* 1999, Bymaster *et al.* 2003, Zhang 2006). Therefore, we assessed if some specific organs have specific lesions. The urinary bladder and heart are known as peripheral organs that highly express M₂ receptors. However, they had no pathological finding. In fact, previous studies using M₂KO mice have reported that a loss of M₂ receptors did not influence of bladder contraction potently (Igawa *et al.* 2004, Uchiyama & Chess-Williams 2004, Ehlert *et al.* 2005), and basal heart rate and blood pressure in M₂KO mice were also not significantly different from that of WT (LaCroix *et al.* 2008). Moreover, the thyroid and adrenal glands also had no specific lesion. In addition, the liver, where AVP is metabolized, seemed not to be different between the WT and M₂KO groups. We concluded that these organs were likely not to have a crucial effect on decrease of AVP synthesis observed in M₂KO mice although the other inspections, such as endocrinologic or physiologic tests, were not conducted in the present study. However, we could not contradict a possibility that the number of MNC was diminished in M₂KO mice or that the intensity of immunostaining in perikarya was decreased by accelerated axonal transportation of AVP peptides from somata to terminals. Further experiments are needed to inspect these possibilities.

In M₂KO mice, water intake, urinary volume and urinary frequency were significantly increased, and urine-specific gravity was decreased compared with those of WT mice. Thus, M₂KO mice had symptoms, such as diabetes insipidus. Although some other diseases, such as diabetes, psychogenic polydipsia, hypercalcemia and hypokalemia

are also known to represent a polyuria–polydipsia (Alves *et al.* 2007), they were denied by considering that the values of glucose, calcium and potassium in M₂KO mice were not significantly different from that of WT mice besides higher plasma osmolality and lower urine-specific gravity.

Pathohistologic findings were not detected in the kidney and other organs, and no pathological contents were detected in the urine in both mice groups. For a possibility of functional change of V₂R as nephrogenic diabetes insipidus, V₂R was sufficiently expressed in the kidney of both mice types and decreasing urinary volume after DDAVP treatment confirmed that V₂R in M₂KO mice had substantial reactivity to induce an antidiuretic effect. These findings suggest that AVP syntheses/secretion mediated by M₂ receptors has a crucial role in maintaining body fluid homeostasis.

The M₂ receptor is known as a Gi/o type of G protein-coupled receptor, which commonly yields an inhibitory effect on neurotransmitter release. In the auditory cortex and the hippocampus in the brain, it has been reported that the M₂ receptor inhibited γ -amino butyric acid (GABA) release from presynaptic endings of GABAergic neurons and then indirectly caused excitation in postsynaptic neurons (Fukudome *et al.* 2004, Salgado *et al.* 2007). Indeed, GABAergic regulation in the SON (Decavel & Van Den Pol 1990, Pirker *et al.* 2000) and existence of

Figure 5

Plasma osmolality in WT and M₂KO mice. The values are calculated by using the value of sodium, potassium, glucose and blood urea nitrogen obtained from blood tests: Calculated osmolality = 2 [Na] + 2 [K] + [GLU]/18 + [BUN]/2.8 (mosmol/kg). The value in M₂KO mice is significantly higher than that in WT mice (325.6 ± 1.4, *n* = 13; vs 331.3 ± 2.3, *n* = 8). **P* < 0.05 vs WT group.

Figure 6

Expression of V_2 receptor in kidney. Immunohistochemistry for V_2 receptor (A). V_2 R immunoreactivity is detected in the tubular epithelial cells of WT (a) and M₂KO mice (b). No signal is detected without the primary antibody in WT mice (c). Scale bar indicates 50 μm. Arrows show immunoreactive cells to anti- V_2 R antibody. Expression level of V_2 R obtained from ELISA (B). No significant difference is found between both mice groups (WT, $n=6$; KO, $n=6$). A full color version of this figure is available at <https://doi.org/10.1530/JOE-17-0630>.

cholinergic nerves and muscarinic receptors around the SON (Meeker *et al.* 1988) have also been well documented. Moreover, Wang *et al.* found that two types of choline acetyltransferase (ChAT)-containing neurons, which were large, brightly fluorescent and small, dimly fluorescent, in the hypothalamus of transgenic rats of which ChAT was tagged with a fluorescent protein (Wang *et al.* 2015). These large ChAT neurons were observed only near the SON but not the PVN, implying existence of predominant cholinergic regulation in the SON than in the PVN. M_2 receptor is likely the most common subtype among muscarinic receptors in the hypothalamus (Vilaró *et al.* 1993, Wei *et al.* 2002, Oki *et al.* 2005) and the SON is the hypothalamic nuclei, which highly expresses muscarinic

Figure 7

Effect of desmopressin on urinary volume in WT and M₂KO mice. Animals are loaded with distilled water (15 mL/kg, p.o.) and administered with saline (5 mL/kg, i.p.) or desmopressin acetate hydrate (1 μg/kg, i.p.) before recording. Urinary volumes are recorded for 5 h while avoiding of food and drink. Control, saline-injected group; DP, desmopressin-injected group. Urinary volume of DP group is significantly decreased compared with the control group in both strains (WT, control, $n=5$; DP, $n=6$; KO, control, $n=5$; DP, $n=6$). * $P<0.05$ vs control of the corresponding mice group, # $P<0.05$ vs control of the WT mice group.

receptors (Rotter *et al.* 1979). However, we were not able to find any reports referring to the difference in expression of the M_2 receptor between the PVN and SON. In our preliminary experiment with WT mice (Nagano H, Matsuyama H, Iino S, Hashimoto T & Unno T, unpublished observations), immunoreactivity for the M_2 receptor were observed throughout the hypothalamus as reported previously (Vilaró *et al.* 1993), and it did not appear that there is notable difference between them. It might also be considered that M_2 receptors expressed in other regions have an important role. For example, SFO, which transfers signal of hyperosmolality to the PVN and SON, has also been well documented to be activated via muscarinic receptors (Mangiapane & Simpson 1979, 1983, Xu *et al.* 2001a, Honda *et al.* 2003). Particularly, the M_2 receptor around the perifornical area is suggested to promote drinking (Hagan *et al.* 1987). Based on other studies, muscarinic stimulation activates SFO neurons by decreasing GABA release, although the subtype has not been defined (Xu *et al.* 2001b). In sum, the M_2 receptor may be expressed on GABAergic neurons rather than the membrane of MNCs and related to some signal transduction to promote AVP synthesis by decreasing GABA release from presynaptic endings. Future study should focus on which part of those regions is essential for regulation of AVP synthesis in the SON.

In summary, the present study suggested that the M_2 receptor promotes AVP synthesis by MNCs in the SON region, and subsequently, plays a crucial role in

maintaining body fluid homeostasis by controlling plasma AVP concentration. This conclusion is likely to contribute to developing new therapeutic strategies against central diabetes insipidus.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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