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

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 (litake 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 terminals (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 (Gomeza et al. 1999, Struckmann 2003). The genetic background of mice used in the present study was 129J1 (50%) × C57Bl/6J (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 (07:00–19:00 h). 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 12:00 h to avoid the diurnal variations in AVP synthesis. The animals were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and immediately perfused intracardially with heparinized saline, followed by 4 w/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% dianminobenzidine 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 (2000 g, 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 (1500 g, 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):

\[
\text{Calculated osmolality} = 2 \left[ \frac{\text{Na} + 2 \times \text{K}}{18} + \frac{\text{BUN}}{2.8} \right] (\text{mosmol} / \text{kg})
\]

**Urine analysis**

Urine collected by manual compression of the urinary bladder was centrifuged (1500 g, 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 V$_3$R similar to that of AVP. The rabbit anti-V$_3$ 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 V$_3$R expression in the kidney by using anti-V$_3$R antibody. The kidneys were homogenized with 1-mL CellLytic MT (Sigma-Aldrich) per 50-mg tissue. After centrifugation (15,000 g, 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 for 1 h at 37°C after washing. After being washed six times, the substrate solution was used in the immunohistochemistry of V$_3$R antibody. The kidneys were blocked with PBS containing 3 w/v% skim milk (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 V$_3$R, was used to evaluate V$_3$R 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 M$_2$KO mice was similar to that of WT mice (Fig. 1A and B). However, in the SON of M$_2$KO 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 M$_2$KO mice compared with that in WT mice ($235.3±245.4$, $n=5$, 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 M$_2$KO was only approximately 30% of that from WT (1029.9 ± 233.8 pg/mL, $n=10$, vs 3418.7 ± 105.7 pg/mL, $n=8$: $P<0.05$).

The absence of M$_2$ receptors induced remarkable increases in drinking and urination. Water intake in the M$_2$KO group was 9.6-fold higher than in the WT group ($69±0.12g$, $n=27$, vs $10.0±0.80g$, $n=8$: $P<0.01$; Fig. 4A). Urinary volume in M$_2$KO mice was 14.5-fold higher than in WT mice ($5.6±0.33g$, $n=27$, vs $43.5±1.8g$, $n=8$: $P<0.01$; 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 M$_2$KO and WT groups (Table 1). Plasma osmolality calculated from the values of Na, K, GLU and BUN showed plasma hyperosmolality in M$_2$KO mice ($325.6±1.4$, $n=13$, vs $331.3±2.3$ mosmol/kg, $n=8$: $P<0.05$; Fig. 5).
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
Effect of M₂ deficiency on urinary volume (A), urinary frequency (B) and water intake (C). Recordings are performed for 18 h 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.
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 (Gomeza 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 synthesis/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

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

<table>
<thead>
<tr>
<th></th>
<th>BUN (mg/dL)</th>
<th>GLU (mg/dL)</th>
<th>Na (mEq/L)</th>
<th>K (mEq/L)</th>
<th>Ca (mEq/L)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>20.8 ± 0.4</td>
<td>141 ± 1.4</td>
<td>152 ± 0.3</td>
<td>5.2 ± 0.3</td>
<td>9.0 ± 0.3</td>
</tr>
<tr>
<td>M₂KO</td>
<td>23.0 ± 0.5</td>
<td>148 ± 2.4</td>
<td>153 ± 0.4</td>
<td>6.2 ± 0.5</td>
<td>8.8 ± 0.5</td>
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There was no significant difference between these mice strains.

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.
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. M₂ 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 receptors (Rotter et al. 1979). However, we were not able to find any reports referring to the difference in expression of the M₂ 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 M₂ 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 M₂ 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 M₂ 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 M₂ 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 M₂ receptor promotes AVP synthesis by MNCs in the SON region, and subsequently, plays a crucial role in

Figure 6
Expression of V₂ receptor in kidney. Immunohistochemistry for V₂ receptor (A). V₂R 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-V₂R antibody. Expression level of V₂R 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.

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.
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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