Centrally administered adrenomedullin 5 activates oxytocin-secreting neurons in the hypothalamus and elevates plasma oxytocin level in rats

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Abstract

We examined the effects of intracerebroventricular (i.c.v.) administration of adrenomedullin 5 (AM5) in the brain of conscious rats. We used porcine AM5 in the present study because rat AM5 has not been detected. We observed Fos-like immunoreactivity (LI) in the hypothalamus and brainstem of conscious rats after i.c.v. administration of AM5 (2 nmol/rat). Fos-LI, measured at 90 min post-AM5 injection, was observed in various brain areas, including the supraoptic (SON) and the paraventricular nuclei (PVN). Dual immunostaining for Fos/oxytocin (OXT) and Fos/arginine vasopressin (AVP) revealed that OXT-LI neurones predominantly colocalised Fos-LI compared with AVP-LI neurones in the SON and the PVN. Plasma OXT levels were significantly increased 5 min after i.c.v. administration of AM5 (1 nmol/rat) compared with vehicle and remained elevated in samples taken at 10, 15 and 30 min without changes in plasma AVP levels at any time. In situ hybridisation histochemistry showed that i.c.v. administration of AM5 (0.2, 1 and 2 nmol/rat) caused a marked induction of the expression of the c-fos gene in the SON and the PVN. This induction was significantly but not completely reduced by pretreatment with both the calcitonin gene-related peptide (CGRP) antagonist CGRP-(8-37) (3 nmol/rat) and the AM receptor antagonist AM-(22-52) (27 nmol/rat). Although porcine AM5 has not been detected yet in the brain, these results suggest that centrally administered porcine AM5 may activate OXT-secreting neurosecretory cells in the hypothalamus partly through AM/CGRP receptors and elicit secretion of OXT into the systemic circulation in conscious rats.
Adrenomedullin (AM), which was discovered from the human pheochromocytoma, is a multi-functional peptide that belongs to the calcitonin gene-related peptide (CGRP) family that is composed of CGRP, AM and amylin (López & Martinez 2001, Brain & Grant 2004, Muff et al. 2004). In teleost fish, AM peptides were identified as five AMs (AM1-5), and they form an independent subfamily (Ogoshi et al. 2003). Takei et al. (2004a) searched the orthologs of the AMs in the genome and established sequence tag (EST) databases and identified AM2 and AM5 genes in mammals (Takei et al. 2004a, Ogoshi et al. 2006). AM2 is identical to intermedin, which was discovered by Roh et al. (2004). AM, AM2 and AM5 in mammals are respectively identical to AM1, AM2 and AM5 in teleost fish.

AM and AM2 are found in various peripheral organs and the central nervous system (CNS) (Takahashi et al. 1997, Cameron et al. 1998, Roh et al. 2004, Takei et al. 2004a, Taylor et al. 2005, Takahashi et al. 2006). They have been well known as multi-functional peptides, for example, in the cardiovascular system, neuroendocrine system and ingestive behaviours. Intracerebroventricular (i.c.v.) administration of AM caused hypertension and tachycardia, whereas peripheral administration of AM resulted in vasodilatation and hypotension (Kitamura et al. 1993, Takahashi et al. 1994, Saita et al. 1998). I.c.v. administration of AM caused behavioral responses in rats such as inhibition of water consumption (Murphy et al. 1995), appetite for salt (Samson et al. 1997) and feeding (Taylor et al. 1996). I.c.v. administration of AM also results in neuroendocrine responses such as the inhibition of osmotic and hypovolemic stimuli-induced secretion of arginine vasopressin (AVP) (Yokoi et al. 1996). Central
administration of AM activated oxytocin (OXT)-secreting neurones in the supraoptic (SON) and the paraventricular nuclei (PVN) (Serino et al. 1999, Ueta et al. 2000), and markedly increased plasma OXT level in rats (Serino et al. 1999). AM2 also has effects similar to those of AM on the cardiovascular system, neuroendocrine system and ingestive behaviours (Lin et al. 2005, Hashimoto et al. 2005, Taylor et al. 2005, Taylor et al. 2006).

The CGRP family peptides exhibit biological effects principally through the complex of the calcitonin receptor (CTR) or calcitonin receptor-like receptor (CLR) associated with one of the three receptors activity-modifying proteins (RAMPs); CLR-RAMP1 is a receptor for CGRP, CLR-RAMP2/3 for AM, CLR-RAMP3 for AM2, and CTR-RAMPs for amylin (Brain & Grant 2004; Conner et al. 2004). They activate a cAMP-dependent pathway via those receptor complexes to mediate biological actions. AM2 has lower affinity than AM to the CLR-RAMP2 complex, whereas the central action of AM2 was more potent than AM in conscious rats (Hashimoto et al. 2007). Further, the central AM2 effect was not blocked completely by both AM-(22-52) and CGRP-(8-37), antagonists for the CGRP family receptors (Hashimoto et al. 2007). Therefore, it is possible that an additional receptor specific for AM2 may exist in the rat.

Since AM and AM2 have many effects on the CNS in mammals, AM5, which is a newly discovered 50 amino-acid peptide identical to fish AM5, may also have similar actions on the CNS through the CLR/CTR-RAMPs complexes. However, it remains unknown whether the AM5/its receptor genes are expressed as functional proteins in mammals.

In the present study, we examined the effects of central administration of AM5 on the CNS in conscious rats, using immunohistochemistry for Fos, in situ hybridisation
histochemistry (ISH) for $c$-fos mRNA and radioimmunoassay (RIA) for plasma OXT and AVP. Furthermore, we investigated whether those actions of centrally administered AM5 are mediated by the CGRP family receptor complexes.

Material and methods

Animals

Adult male Wistar rats weighing 200-250 g were housed individually in plastic cages in an air-conditioned room (24±1°C) under a 12:12-h light (0700-1900)-dark (1900-0700) cycle. Food and water were available ad libitum. All procedures in the present study were done in accordance with the guidelines on the use and care of laboratory animals as set out by the Physiological Society of Japan and under the control of the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan.

Surgical procedures

The Animals to be i.c.v. administered AM5 or 0.9% saline (vehicle) were implanted with stainless steel cannula aimed at the lateral ventricle. For implantation surgery, the animals were anaesthetised with pentobarbital sodium (50 mg/kg body wt i.p. injection) and then placed in a stereotaxic frame. A stainless steel guide cannula (550 µm outer diameter, 10 mm length) was implanted stereotaxically at the following coordinates: 0.8 mm posterior to the bregma, 1.4 mm lateral to midline, and 2.0 mm below the surface of the left cortex, such that the tip of the cannula was 1.0 mm above the left cerebral
ventricle (Paxinos & Watson, 1986). Two stainless steel anchoring screws were fixed to
the skull, and the cannula was secured in place by acrylic dental cement. The animals
were then returned to their cages and allowed to recover for at least 7 days. They were
then handled every day and housed in cages before the start of the experiments.

Central administration of AM5 and vehicle

For i.c.v. administration of AM5 or vehicle, a stainless steel injector (300 µm outer
diameter) was introduced through the cannula at a depth of 1.0 mm beyond the end of
the guide. The total volume of injected solution of AM5 and vehicle into the lateral
ventricle was 5 µl. Porcine AM5 was kindly provided by Peptide Institute (Minoh,
Japan). Rat AM, human CGRP-(8-37), and human AM-(22-52) were all purchased from
the Peptide Institute. The AM5, AM, CGRP-(8-37), and AM-(22-52) were dissolved in a
pyrogen-free sterile 0.9% saline solution (Otsuka Pharmaceutical Co., Ltd, Tokyo,
Japan).

Experimental procedures

Studies of the colocalisation of c-fos activity with OXT or AVP were performed on
animals that were infused with AM5 (2 nmol/rat) or vehicle (n = 3 or 5 in each group).
After i.c.v. administration of the solution (90 min), the animals were anaesthetised
deeply (pentobarbital sodium, 75 mg/kg body w.t. i.p.), perfused with fixative, and
processed for immunohistochemistry of Fos, OXT, and AVP (see below).

Animals used for ISH for c-fos mRNA were decapitated 5, 15, 30, 60 or 180 min after
i.c.v. administration of AM5 (1 nmol/rat) or vehicle (n = 7-8 in each group) in conscious
rats for time-course studies, or at 30 min after i.c.v. administration of AM5 (0.2, 1 and 2
nmol/rat) or vehicle (n = 7-8 in each group) in conscious rats for dose-response studies. The brains were rapidly removed and placed on powdered dry ice for ISH for c-fos mRNA (see below).

For studies of the circulating levels of OXT and AVP, animals were decapitated 5, 15, 30, 60 or 180 min after i.c.v. administration of AM5 (1 nmol/rat) or vehicle (n = 7-8 in each group) in conscious rats for time-course studies, and 30 min after i.c.v. administration of AM5 (0.2, 1 and 2 nmol/rat) or vehicle (n = 7-8 in each group) in conscious rats for dose-response studies. Trunk blood was collected for measuring plasma concentrations of OXT and AVP, using RIA.

For studies of CGRP or AM receptor antagonist action, animals received i.c.v. administration of 5 µl 0.9% saline (vehicle), CGRP-(8-37) (3 nmol/rat) in 5 µl vehicle, or AM-(22-52) (27 nmol/rat) in 5µl vehicle (n = 7-8 in each group). 10 min after infusion of the antagonist or vehicle, rats were given a second i.c.v. injection containing 5 µl 0.9% saline (vehicle) or AM5 (1 nmol/rat) in 5µl vehicle and decapitated 30 min after the second infusion. The brains were removed and placed on powdered dry ice for ISH for c-fos mRNA.

Colocalisation of Fos-like immunoreactivity (LI) with OXT-LI or AVP-LI
The deeply anaesthetised animals were perfused transcardially with a 0.1 M phosphate buffer (PB, pH 7.4) containing heparin (1,000 U/l saline) followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB. Then the brains were removed, coronally cut, and divided into three blocks (forebrain, hypothalamus, and brainstem). The blocks were postfixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB for 48 h at 4°C and then cryoprotected in 20% sucrose in 0.1 M PB for another 48 h at
4°C. Serial sections of either 40 µm for immunostaining for Fos or 30 µm for staining for Fos and OXT/AVP were cut using a microtome. The sections were rinsed twice with a 0.1 M phosphate buffered saline (PBS) containing 0.3% Triton X-100, incubated in 0.1 M PBS containing 0.3% Triton X-100 with 1% hydrogen peroxidase for 60 min, and then rinsed twice with 0.1M PBS containing 0.3% Triton X-100. Floating sections were incubated with a primary Fos antibody (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:5000 in 0.1 M PBS containing 0.3% Triton X-100 at 4°C for 4 days. After being washed for 20 min in 0.1 M PBS containing 0.3% Triton X-100, the sections were incubated for 120 min with a biotinylated secondary antibody solution (1:250), and finally with an avidin-biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 120 min. The peroxidase in the sections was visualised with 0.02% diaminobenzidine in a Tris buffer containing 0.05% hydrogen peroxidase for 3 min. In the dual staining for OXT or AVP, the sections were subsequently incubated in OXT antibody (Chemicon International, Temecula, CA; diluted 1:5,000) or AVP antibody (Incstar, Stillwater, MN; diluted 1:10,000) for 3 days at 4°C. The avidin-biotin peroxidase complex was made visible by using nickel sulfate. Sections were mounted on gelatin-coated slides, air-dried, dehydrated in 100% ethanol, cleared with xylene, and then finally covered with a coverslip. The presence of a dark brown label that appeared in the round structures was judged as indicative of Fos-LI-positive nuclei (Ueta et al. 1995), and that of a violet label that appeared in the spindle-shaped structures was judged as indicative of OXT- or AVP-LI. Details of the immunohistochemistry have been published elsewhere (Ison et al. 1993, Onaka et al. 1995). To count the double-labelled cells, three serial sections that included the SON and the PVN per animal were chosen and counted under a light microscope by two
independent investigators.

**ISH for c-fos mRNA**

ISH was performed on frozen 12-µm-thick coronal brain sections cut on a cryostat at -20°C, thawed, and mounted on gelatin/chrome alum-coated slides. The brain tissue was stored at -80°C before cutting. The locations of the SON, the PVN and the nucleus tractus solitarius (NTS) were determined according to coordinates given by the atlas of Paxinos & Watson (1986). The sections including the SON were chosen from plate 17 and the sections including the PVN were chosen from plate 18 in the atlas. Ten sets of two sections containing the SON and the PVN were used from each rat to measure the density of autoradiography. The slides were warmed to room temperature, allowed to dry for 10 min, and then fixed in 4% formaldehyde in PBS for 5 min. They were then washed two times in PBS and incubated in 0.9% NaCl containing 0.25% acetic anhydride (vol/vol) and 0.1 M triethanolamine at room temperature for 10 min. The sections were dehydrated using a series of 70% (1 min), 80% (1 min), 95% (2 min), and 100% (1 min) ethanol solutions consecutively and delipidated in 100% chloroform for 5 min. The slides were then partially rehydrated first in 100% (1 min) and then 95% (1 min) ethanol and allowed to air-dry briefly.

Hybridisation was performed at 37°C overnight in a 45-µl buffer solution consisting of 50% formamide and 4×saline sodium citrate (SSC; 1×SSC = 150 mM NaCl and 15 mM sodium citrate), which contained 500 µg/ml sheared salmon sperm DNA (Sigma, St. Louis, MO), 250 µg/ml baker’s yeast total RNA (Roche Molecular Biochemicals, Mannheim, Germany), 1×Denhardt’s solution, and 10% dextran sulfate (500,000 molecular weight; Sigma). The hybridisation was performed under a Nescofilm (Bando...
Chemical IMD, Osaka, Japan) coverslip. A $^{35}$S-3'-end-labelled deoxyoligonucleotide that was complementary to transcripts coding for c-fos (bases 138-185 of rat c-fos nucleotides) was used. The specificity of the probe has been described previously (Harbuz et al. 1993). A total of $1\times10^6$ cpmp/slide for c-fos anti-sense transcripts was used. After hybridisation, the sections were washed for 1 h in four separate 1×SSC rinses at 55°C and for another hour in two changes of 1×SSC at room temperature. All independent experimental sections were treated simultaneously to minimise the variable effects of hybridisation and wash stringency. Hybridised sections containing the SON and the PVN were apposed to autoradiography film (Hyperfilm; Amersham, Buckinghamshire, UK) for 2-3 weeks for c-fos transcripts. The autoradiographic images were quantified using a MCID imaging analyzer (Imaging Research, St. Catherines, Ontario, Canada). The images were captured by a chargecoupled device camera (DAGE-MTI, Michigan City, IN, USA) with ×40 magnification. The mean optical density (OD) of the autoradiographs was measured by comparing them with simultaneously exposed $^{14}$C microscale samples (Amersham). $^{14}$C was used as the standard for quantification of the OD of the autoradiographs for ISH. The standard curve was fitted by the OD of the $^{14}$C microscale on the same film. Slides hybridised with the c-fos probe were dipped in a nuclear emulsion (K-5; Ilford, Cheshire, UK) and exposed for a further 6 weeks.

**RIA for OXT and AVP**

The plasma concentrations of OXT and AVP were determined by RIA with specific anti-OXT and anti-AVP antisera, as described previously (Higuchi et al. 1985, Hyodo et al. 2004). The coefficients of inter- and intraassay variations were 9.7 and 4.7% for
OXT and 7.3 and 3.2% for AVP, respectively. The minimum detection limit was 0.76 fmol/ml for OXT and 0.68 fmol/ml for AVP.

Statistical analysis

A mean deviation from control (percentage) ± S.E.M. was calculated from data obtained from the measurement of the plasma OXT and AVP levels, immunohistochemistry for Fos, and in situ hybridisation for c-fos mRNA. Each group within an experiment was compared with the control group. The data were analyzed using a one-way fractional ANOVA followed by a Bonferroni correction for multiple comparisons. The statistical significance was set at $P < 0.05$.

Results

Functional mapping by Fos expression

Many intense Fos-LI were found in various regions of the brain 90 min after i.c.v. administration of AM5 (2 nmol/rat) (Fig. 1, right side of each brain section). On the other hand, only a small number of Fos-LI was observed in the CNS after i.c.v. administration of vehicle (Fig. 1, left side of each brain section). In the forebrain, many Fos-LI were localised to the piriform cortex and the central amygdaloid nucleus (CeA) (Fig. 2, H). In the hypothalamus, many Fos-LI were observed in the medial preoptic nucleus, the SON (Fig. 2, B), the PVN (Fig. 2, D), the hypothalamic arcuate nucleus (Arc) (Fig. 2, F), the periventricular regions of the third ventricle, and the ventromedial hypothalamic nucleus (VMH). In the midbrain and the brainstem, there were many
Fos-LI in the locus coeruleus (LC) (Fig. 2, J), the area postrema (AP) and the nucleus of the nucleus tractus solitaries (NTS) (Fig. 2, L). On the other hand, only a few Fos-LI were observed in the corresponding areas in the controls injected i.c.v. with a vehicle (Fig. 2, A, C, E, G, I and K).

Colocalisation of Fos-LI and OXT-LI or AVP-LI in the SON and the PVN

In the SON, OXT-LI cells predominated in the nuclear Fos-LI region compared with AVP-LI cells (Fig. 3, A-D and 4). Fos-LI cells in the magnocellular parts of the PVN after i.c.v. administration of AM5 (2 nmol/rat) colocalised to OXT-LI rather than AVP-LI cells (Fig. 3, E-H and 5). The numerous Fos-LI in the parvocellular parts of the PVN were reactive for neither AVP nor OXT (Fig. 3, E-H). Taking the total OXT-LI-positive cells counted in each nucleus as 100%, the percentage of Fos-LI-positive cells (n = 5) was 82.0 ± 3.7% in the SON and 87.3 ± 9.3% in the PVN after i.c.v. administration of AM5 (2 nmol/rat). Similarly taking the total AVP-LI-positive cells counted in each nucleus as 100%, the percentage of Fos-LI-positive cells (n = 5) was 6.1 ± 0.8% in the SON and 12.2 ± 3.2% in the PVN after i.c.v. administration of AM5 (2 nmol/rat) (data not shown).

Effects of i.c.v. administration of AM5 on c-fos gene induction in the SON and the PVN

ISH revealed that c-fos gene expression in the SON and the PVN was significantly increased in a dose-related manner 30 min after i.c.v. administration of AM5 (0.2, 1 and 2 nmol/rat; Fig. 6, A and B). The i.c.v. administration of AM5 (1 nmol/rat) caused a significant increase in the expression of the c-fos gene from 5 to 60 min in the SON and the PVN (Fig. 6, C and D). The i.c.v. administration of AM5 induced the expression of
the c-fos gene to its greatest degree at 30 min in the SON and the PVN (Fig. 6, C and D). The expression of the c-fos gene in the SON and the PVN after i.c.v. administration of the vehicle did not change significantly (Fig. 6, A-D).

Effects of i.c.v. administration of AM5 on plasma concentrations of OXT and AVP

The plasma concentrations of OXT and AVP measured 30 min after i.c.v. administration of AM5 (0.2, 1 and 2 nmol/rat) or vehicle showed large, significant increases in plasma OXT levels at all AM5 doses (Fig. 7, A) but no significant changes in plasma AVP levels (Fig. 7, B). The concentrations of plasma OXT and AVP were measured 5, 15, 30, 60 and 180 min after i.c.v. administration of AM5 (1 nmol/rat) or vehicle. The concentrations of plasma OXT were profoundly increased 5, 15 and 30 min after i.c.v. administration of AM5 (1 nmol/rat) compared with vehicle (Fig. 7, C). The concentrations of plasma AVP were not increased at any of the sample times after i.c.v. administration of AM5 (1 nmol/rat) compared with vehicle (Fig. 7, D).

Effects of i.c.v. pretreatment with CGRP and AM receptor antagonists on AM5-induced c-fos gene expression in the SON and the PVN

ISH revealed that the c-fos gene expression in the SON and the PVN was significantly increased after i.c.v. administration of either AM5 (1 nmol/rat) or AM (1 nmol/rat) with pretreatment of vehicle (Fig. 8, A, B, D and I). AM-(22-52) (27 nmol/rat; AM receptor antagonist) or CGRP-(8-37) (3 nmol/rat; CGRP receptor antagonist) attenuated the effect of i.c.v. administration of either AM5 or AM (Fig. 8, A, B, E, F, J and K). With a pretreatment of a combination of the antagonists, the c-fos gene expression in the SON and the PVN was completely suppressed after i.c.v. administration of AM (Fig. 8, A and
B). By contrast, even when the combination of antagonists was applied, i.c.v. administration of AM5 was still able to induce a significant increase in the c-fos gene expression in the SON and the PVN (Fig. 8, A, B, G and L).

Discussion

AM5, which is a 50 amino-acid peptide, is a newly identified member of the CGRP family, and its effects in the CNS remain unknown. In the present study, we provide the first evidence showing that centrally administered AM5 activates various regions in the rat brain. Especially, we showed, by using dual immunostaining for Fos/OXT, ISH and RIA, that centrally administered AM5 markedly activates OXT-secreting neurones in the SON and the PVN and induces increases in the concentration of plasma OXT as shown previously with AM and AM2 in conscious rats (Serino et al. 1999, Hashimoto et al. 2005). Furthermore, we demonstrated that the effects were significantly reduced by pretreatment with AM-(22-52) and/or CGRP-(8-37). These results suggest that centrally administered AM5 elicits its action partly through the CGRP and/or AM receptors in conscious rats.

The present study indicates a possibility that AM5 elicits its effects as a neurotransmitter or neuromodulator in the rat neuroendocrine system, particularly in OXT-secreting neurones of the hypothalamus. Although we began this study in anticipation of detection of AM5 in the rat brain because the other members of AM family, AM and AM2, exist in the rat brain, there is no evidence the AM5 exists in the CNS to date. In pigs, the AM5 gene was expressed abundantly in the spleen and thymus,
slightly in the adrenal and pituitary, and undetectable in the brain (Takei et al. 2008). However, it remains a possibility that AM5 may exist in some restricted regions of the pig brain because they examined only a small part of the pig brain, including the ventral hypothalamic area (including the SON, organum vasculosum laminae terminalis and median eminence) (Takei et al. 2008). Further studies such as ISH and RT-PCR using different parts of the brain may reveal the existence of AM5 in the pig brain. As an alternative route of central action, peripheral AM5 may act on the CNS via the circumventricular organs, which lack a blood brain barrier, to effect CNS functions.

In the present study, porcine AM5 was administered in the rat brain. AM5 sequences are highly conserved within some mammalian species. Takei et al. (2008) described that they identified AM5 sequences in the genome database of ungulates (artiodactyls: pig, ox, and sheep; and perissodactyls: horse), carnivores (dog and cat), and primates (prosimians: Tupaia belangeri, and simians: Macaca mulatta), but they could not identify such sequences in the genome database of rodents (mice and rats). They also described that an AM5-like sequence exists in the human and chimpanzee, but the sequence changes in the middle of the mature peptide because of the deletion of two nucleotides. In addition, AM5-like sequence is detected in the EST database of guinea pigs which belong to rodents (Wong, M. K. S. and Takei, Y, unpublished data). Thus, we cannot exclude the possibility that the AM5 gene still exists in a considerably altered form in the rat genome, or the gene has lost its function recently but the AM5 receptor is still functioning in the rat.

In this study, we showed that centrally administered porcine AM5 activates various regions in the rat brain and increases the concentration of plasma OXT in conscious rats. It can be considered that AM5 elicited its effects through AM and/or AM2 receptors.
AM5, as well as AM and AM2, has a characteristic structure which includes an intramolecular ring structure of six amino acid residues flanked by a single disulfide bond and amidated carboxyl ends (Takei et al. 2004b, Ogoshi et al. 2006). In the present study, the effects of centrally administered AM5 on plasma OXT levels were similar to the effects of AM and AM2 (Serino et al. 1999, Hashimoto et al. 2005). Centrally administered AM5 activated almost the same brain regions that were activated by AM and AM2 in conscious rats (Ueta et al. 2001, Hashimoto et al. 2005). The pretreatment with the AM receptor antagonist, AM-(22-52) and CGRP-(8-37), reduced the effects of AM5. These findings suggest that AM5 exerted its effects at least partly through the AM and AM2 receptors. It was shown that AM5 has a low affinity to the complex of CLR associated with RAMP2, and both CLR and RAMP2 mRNA are detected in the hypothalamus in rats (Oliver et al. 2001, Stachniak & Krukoff 2003). It is possible that AM5 elicits its effects through the CLR and RAMP2 complex.

On the other hand, our results raised the possibility of the presence of AM5-specific receptor. As shown in this study, centrally administered AM5 caused quick increases in plasma OXT concentration with maximum at 5 min, while AM and AM2 caused the maximal increases after 10 and 30 min respectively (Serino et al. 1999, Hashimoto et al. 2005). Similar differences in the time course were reported with changes of arterial pressure and heart rate after i.c.v. administration of AM and AM5 in urethane-anaesthetised rats (Takei et al. 2008). These differences indicate that AM5 may elicit its actions through a receptor different from the AM and AM2 receptor. The presence of AM5 receptor distinct from the AM receptor has also been suggested in the eel where cardiovascular (Nobata et al. 2008) and osmoregulatory (Ogoshi et al. 2008) actions of AM5 is much more potent than AM(1), although affinity of AM5 to the
CLR-RAMP complexes are apparently lower than that of AM(1) (Nag et al. 2005). If such AM5-specific receptor also exists in mammals, AM5 may have acted on it in the rat brain in the current study as it may still be retained in the rat.

The previous studies revealed that various stressors, for example intravenous injection of lipopolysaccharide, restrain stress, 24 hours dehydration, reduce expression of preproadrenomedullin (ppAM) gene which encodes AM in rat brain (Shan et al. 2001). A decrease in blood pressure also reduces ppAM mRNA expression in the hypothalamus (Stachniak et al. 2003). Furthermore Chen et al. reported that the concentration of AM in the rat cerebrospinal fluid was increased after salt loading and systemic administration of lipopolysaccharide (2004). It was also reported that focal stroke upregulates the AM mRNA expression in rat brain (Feuerstein et al. 1997). These results suggest that inflammation, focal brain ischemia and change of blood pressure and fluid electrolyte regulate AM expression in the CNS.

Verbalis et al. (1995) demonstrated that i.c.v. administration of OXT inhibits food intake in fasted rats and NaCl intake in hypovolemic rats. Both AM and AM2 may be considered to have effects on the central regulation of salt appetite, feeding, and water balance (Taylor et al. 1996, Samson et al. 1997, Taylor et al. 2005). In the present study, centrally administered AM5 activated the various brain regions such as the SON, the PVN, the Arc and the VMH that are related to feeding behaviour and water balance. Thus, it is possible that AM5 also has effects on the central regulation of salt appetite, feeding, and water balance, as did AM and AM2. Fos-LI cells were also seen in the LC, the CeA, the AP and the NTS. It is known that there is a connection between the LC and the CeA for the transmission of sympathetic signals (Petrov et al. 1996), and AM5-activated neurones in the LC and the CeA may be involved in the pathway of
sympathetic signals. It is also known that the AP and the NTS are involved in the regulation of cardiovascular system (Bishop et al. 1993, Lawrence et al. 1996). Allen and Ferguson (1996) showed in an in vitro study that AM excites AP neurones directly and modulates the activity of the NTS neurones indirectly. Cui et al. (2008) reported that microinjection of AM2 into the NTS elevates arterial pressure and heart rate in urethane-anaesthetised rats. Further, Takei et al. (2008) reported that AM5 injected into the cerebral ventricle dose-dependently increased arterial pressure and heart rate in urethane-anaesthetised rats. These results suggest that centrally administered AM5 is involved in the cardiovascular system in rats.

In conclusion, the present study showed that centrally administered AM5 induced the expression of the c-fos gene in the SON and the PVN, and this induction was significantly reduced by pretreatment with both the CGRP and AM receptor antagonists. Therefore, we presume that central AM5 activates OXT-secreting neurones in the SON and the PVN partly through the CGRP and/or AM receptor. Further study is required to explore the possibility that unknown specific receptors for AM5 may exist in the CNS.

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Figure Legends

Fig. 1
Distribution of Fos-LI in the central nervous system 90 min after i.c.v. administration of adrenomedullin 5 (AM5) (2 nmol/rat; red dots on right side) or vehicle (black dots on left side) in a conscious rat. Drawings (A-L) represent coronal brain section rostral to caudal through the forebrain and brainstem. AP, area postrema; Arc, arcuate hypothalamic nucleus; CeA, central amygdaloid nucleus; DR, dorsal raphe nucleus; LC, locus coeruleus; LV, lateral ventricle; MD, mediodorsal thalamic nucleus; ME, median eminence; Mve, medial vestibular nucleus; opt, optic tract; ox, optic chiasma; Pir, piriform cortex; POA, preoptic area; PV, paraventricular thalamic nucleus; PVN, paraventricular nuclei; SON, supraoptic nuclei; SuM, supramammillary nucleus; VMH, ventromedial hypothalamic nucleus. Bar indicates 300 µm.

Fig. 2
Photomicrographs showing changes in Fos-like immunoreactivity (LI) in the SON (A and B), the PVN (C and D), the Arc (E and F), the CeA (G and H), the LC (I and J), and the AP and NTS (K and L). Bars indicate 100 µm.

Fig. 3
Coexistence of Fos-LI and oxytocin (OXT; A & E)-/arginine vasopressin (AVP; C & G)-LI in the SON and the PVN of rats 90 min after i.c.v. administration of AM5 (2 nmol/rat). B and D, Enlargements from the boxed areas in panels A and C. F and H, Enlargements from the boxed areas in panels E and G. Black arrowheads indicate
coexistence of nuclear Fos-LI and OXT- or AVP-LI. White arrowheads indicate OXT- or AVP-LI without Fos-LI.

Fig. 4
Topographical mapping of Fos-LI and OXT-LI or AVP-LI in the SON 90 min after i.c.v. administration of vehicle (A and C) or AM5 (2 nmol/rat: B and D). A and B: coexistence of Fos-LI and OXT-LI (▲). C and D: coexistence of Fos-LI and AVP-LI (■). Dots, Fos-LI-positive cells; △, OXT-LI-positive cells; □, AVP-LI-positive cells. In each panel, two 30-µm-thick coronal sections from the SON were selected. Bar indicates 50 µm.

Fig. 5
Topographical mapping of Fos-LI and OXT-LI or AVP-LI in the PVN 90 min after i.c.v. administration of vehicle (A and C) or AM5 (2 nmol/rat: B and D). A and B: coexistence of Fos-LI and OXT-LI (▲). C and D: coexistence of Fos-LI and AVP-LI (■). Dots, Fos-LI-positive cells; △, OXT-LI-positive cells; □, AVP-LI-positive cells. In each panel, two 30-µm-thick coronal sections from the PVN were selected. Bar indicates 50 µm.

Fig. 6
Effects of i.c.v. administration of vehicle or AM5 (0.2, 1, and 2 nmol/rat) on c-fos transcript prevalence in the SON (A and C) and the PVN (B and D). A and B: dose-response effects of i.c.v. administration of vehicle or AM5 (0.2, 1, and 2 nmol/rat) on c-fos transcript prevalence. C and D: time-course effects of i.c.v.
administration of vehicle or AM5 (1 nmol/rat) on *c-fos* transcript prevalence. Values 
represent the mean ± S.E.M. (*n* = 7-8). **P < 0.01, compared with vehicle-administered 
rats.

**Fig. 7**

Effects of i.c.v. administration of AM5 (0.2, 1, and 2 nmol/rat) or vehicle on plasma 
concentrations of OXT (A and C) and AVP (B and D) in conscious rats. A and B: all 
rats were decapitated 30 min after i.c.v. administration of the AM5 (0.2, 1, and 2 
nmol/rat) or vehicle. C and D: rats were decapitated 5, 15, 30, 60 and 180 min after 
i.c.v. administration of the AM5 (1 nmol/rat) or vehicle. Data for plasma 
concentrations of OXT and AVP are expressed as means ± S.E.M. (*n* = 7-8 rats). 
*P < 0.05 and **P < 0.01, compared with vehicle-administered rats.

**Fig. 8**

Effects of i.c.v. administration of vehicle, AM (1 nmol/rat) or AM5 (1 nmol/rat) on 
c-*fos* transcript prevalence in the SON (A and C-G) and the PVN (B and H-L) after 
pretreatment with calcitonin gene-related peptide (CGRP) antagonist [CGRP-(8-37), 
3 nmol/rat] and/or AM antagonist [AM-(22-52), 27 nmol/rat]. Representative images 
of emulsion-dipped sections hybridised to a $^{35}$S-labelled oligodeoxynucleotide probe 
for *c-fos* mRNA in the SON and the PVN 30 min after i.c.v. administration of vehicle 
(C and H) or AM5 (1 nmol/rat) (D-G and I-L). Panel C, D, H and I are sections of 
pretreatment with vehicle, E and J are sections of pretreatment with AM-(22-52) (27 
nmol/rat), F and K are sections of pretreatment with CGRP-(8-37) (3 nmol/rat), G 
and L are sections of pretreatment with AM-(22-52) (27 nmol/rat) and CGRP-(8-37)
(3 nmol/rat). Values represent means ± S.E.M. (n=7-8). $P<0.01$, compared with vehicle-administered rats (**) and compared with saline + AM5-administered rats (††). Bars indicate 100 µm.
Fig. 3

A

OX

B

C

D

OX

E

3V

F


G

3V

H
Fig. 4

A Vehicle

B AM5 (2nmol)

- Fos
- OXT
- Fos/OXT
Fig. 5 (continued)

C  Vehicle  D  AM5 (2nmol)

- Fos
- AVP
- Fos/AVP

3V  3V  3V  3V
Fig. 6

A

Time after i.c.v. injection (min)

B

C

D

Time after i.c.v. injection (min)
**Fig. 7**

(A) Plasma OXT level (fmol/ml) vs Time after i.c.v. injection (min)

(B) Plasma AVP level (fmol/ml) vs Time after i.c.v. injection (min)

(C) Plasma OXT level (fmol/ml) vs Time after i.c.v. injection (min)

(D) Plasma AVP level (fmol/ml) vs Time after i.c.v. injection (min)

- **vehicle**
- **AM5 0.2 nmol**
- **AM5 1 nmol**
- **AM5 2 nmol**

**Note:** The graphs show the concentration changes over time for OXT and AVP levels in response to different doses of AM5.
Fig. 8

A

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<th>AM22-52</th>
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B

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</tr>
</tbody>
</table>

SON

vehicle + vehicle

vehicle + AM5

AM22-52 + AM5

CGRP8-37 + AM5

AM22-52 + CGRP8-37 + AM5

PVN

vehicle + vehicle

vehicle + AM5

AM22-52 + AM5

CGRP8-37 + AM5

AM22-52 + CGRP8-37 + AM5

Bar scale

Dex/vehicle

Dex/AM5

Dex/AM22-52

Dex/AM22-52 + CGRP8-37

Dex/AM22-52 + AM5

Dex/CGRP8-37 + AM5