ORIGINAL ARTICLE

Vasopressin gene products are colocalised with corticotrophin-releasing factor within neurosecretory vesicles in the external zone of the median eminence of the Japanese macaque monkey (*Macaca fuscata*)

Akito Otubo[1] | Natsuko Kawakami[1,2,3] | Sho Maejima[1] | Yasumasa Ueda[4,5] | John F. Morris[3] | Tatsuya Sakamoto[1] | Hirotaka Sakamoto[1,3]

[1]Ushimado Marine Institute (UMI), Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan

[2]Department of Biology, Faculty of Science, Okayama University, Okayama, Japan

[3]Department of Physiology, Anatomy & Genetics, University of Oxford, Oxford, UK

[4]Department of Physiology, Kyoto Prefectural University of Medicine, Kyoto, Japan

[5]Department of Physiology, Kansai Medical University, Osaka, Japan

Correspondence
Hirotaka Sakamoto, Ushimado Marine Institute (UMI), Graduate School of Natural Science and Technology, Okayama University, 130-17 Kashino, Ushimado, Setouchi, Okayama 701-4303, Japan.
Email: hsakamo@okayama-u.ac.jp

Abstract

Arginine vasopressin (AVP), when released into portal capillaries with corticotrophin-releasing factor (CRF) from terminals of parvocellular neurones of the hypothalamic paraventricular nucleus (PVH), facilitates the secretion of adrenocorticotrophic hormone (ACTH) in stressed rodents. The *AVP* gene encodes a propeptide precursor containing AVP, AVP-associated neurophysin II (NPII), and a glycopeptide copeptin, although it is currently unclear whether copeptin is always cleaved from the neurophysin and whether the NPII and/or copeptin have any functional role in the pituitary. Furthermore, for primates, it is unknown whether CRF, AVP, NPII and copeptin are all colocalised in neurosecretory vesicles in the terminal region of the paraventricular CRF neurone axons. Therefore, we investigated, by fluorescence and immunogold immunocytochemistry, the cellular and subcellular relationships of these peptides in the CRF- and AVP-producing cells in unstressed Japanese macaque monkeys (*Macaca fuscata*). Reverse transcription-polymerase chain reaction analysis showed the expression of both CRF and AVP mRNAs in the monkey PVH. As expected, in the magnocellular neurones of the PVH and supraoptic nucleus, essentially no CRF immunoreactivity could be detected in NPII-immunoreactive (AVP-producing) neurones. Immunofluorescence showed that, in the parvocellular part of the PVH, NPII was detectable in a subpopulation (approximately 39%) of the numerous CRF-immunoreactive neuronal perikarya, whereas, in the outer median eminence, NPII was more prominent (approximately 52%) in the CRF varicosities. Triple immunoelectron microscopy in the median eminence demonstrated the presence of both NPII and copeptin immunoreactivity in dense-cored vesicles of CRF-containing axons. The results are consistent with an idea that the AVP propeptide is processed and NPII and copeptin are colocalised in hypothalamic-pituitary CRF axons in the median

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eminence of a primate. The CRF, AVP and copeptin are all co-packaged in neurosecretory vesicles in monkeys and are thus likely to be co-released into the portal capillary blood to amplify ACTH release from the primate anterior pituitary.

1 | INTRODUCTION

The hypothalamic neuroendocrine response to stress is an important survival mechanism in vertebrates. Corticotrophin-releasing factor (CRF), a 41-amino-acid peptide, potently stimulates adrenocorticotrophic hormone (ACTH) secretion from the anterior pituitary when released into the portal capillaries of the median eminence (ME) from terminals of parvocellular neurones of the hypothalamic paraventricular nucleus (PVH).[1-3] The release of arginine vasopressin (AVP) into portal capillary plasma results in a stronger ACTH response than that obtained with CRF alone, because of the synergistic action of CRF and AVP to induce ACTH release.[4-10] A population of CRF neurones in the PVH can co-secrete AVP, and both peptides are secretagogues for ACTH in mammals, including rats[11] and humans.[12] Furthermore, the expression of the AVP gene in CRF neurones is increased under stressed conditions.[13,14] Translation of the *AVP* gene results in the formation of a propeptide which is cleaved to release the AVP hormone, but which also contains the AVP-associated neurophysin (NP) II, and a glycopeptide copeptin.[15-17] The NPII has the important function of binding/stabilising the AVP,[18] although the function of the copeptin and when and if it is proteolytically cleaved from the NPII is currently unclear.[19] In many mammals, including rats and humans, ultrastructural immunocytochemistry demonstrates that CRF and AVP are co-packaged into the same neurosecretory vesicles (NSVs) in the CRF-positive (+) and AVP+ subpopulation of fibres in the ME, indicating that they are almost certainly co-released into the portal capillary blood.[20,21] The concentration of AVP in the pituitary portal vessels increases when animals are stressed or are subjected to adrenalectomy.[22] Although, in unstressed mice, very few parvocellular neurones are doubly positive for CRF and AVP, severe stress or adrenalectomy significantly increases the ratio of double-positive cells (to half).[23] However, at least in primates, it is unknown whether CRF, AVP and the AVP-associated NPII and copeptin can all be detected in NSVs in both the cell bodies and terminal region of the CRF neurone axons. Furthermore, any clear evidence for the function of the secreted copeptin is currently lacking.[19] The present study provides the first evidence in the hypothalamus of unstressed Japanese macaque monkeys (*Macaca fuscata*) that CRF, AVP, and the AVP-associated NPII and copeptin are all co-packaged within the NSVs, and therefore that the AVP precursor protein undergoes proteolysis during the passage of NSVs from the CRH perikarya to the ME.

2 | MATERIALS AND METHODS

2.1 | Animals

Three male (2-9 years old, weight 2.4-12.6 kg) and four female (9-11 years old, weight 7.2-8.2 kg) Japanese macaque monkeys (*M. fuscata*) were used in the present study. Monkeys were maintained under a 12:12 hour light/dark cycle (lights off 8.00 PM) in a temperature-controlled (22-24°C) room. These animals were checked and shown to be free of specific pathogens. Food and water were available ad lib. All animals were kept in individual cages. The housing and experimental protocols followed the guidelines of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, and were in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Okayama University, by Kyoto Prefectural University of Medicine, and by Kansai Medical University. All efforts were made to minimise animal suffering and reduce the number of animals used in the study.

2.2 | Experimental procedures

2.2.1 | RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

One male and two female monkeys were anaesthetised with an overdose of sodium pentobarbital (50-90 mg kg^{-1} body weight), transcardially perfused with physiological saline, and killed via blood loss. The hypothalamus and cerebral cortex (temporal lobe) were quickly removed and frozen with powdered dry ice and stored at − 80°C until RNA extraction. Brains were sectioned at 100 μm thickness with a cryostat (CM3050S; Leica Microsystems, Wetzlar, Germany). Sections were then dissected at − 20°C into 2-3 mm square tissue fragments containing either the PVH or temporal cortex. Total RNA was extracted using an illustra RNAspin Mini RNA isolation kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) in accordance with the manufacturer's instructions. The concentration of total RNA was measured using a NanoDrop Lite (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was synthesised from 200 ng of total RNA with random primers using Omniscript RT kit (Qiagen, Valencia, CA, USA). The RT-PCR conditions for target genes and GAPDH as an internal control are shown in the Supporting information (Table S1). The PCR amplicons were electrophoresed on

2% agarose gels. RT-PCR studies were repeated three times using independently extracted RNA samples from different animals. Consistent results were obtained from each run.

2.2.2 | Tissue preparation for microscopic analysis

Two male and two female monkeys were anaesthetised with an overdose of sodium pentobarbital (50-90 mg kg^{-1} body weight), and transcardially perfused with physiological saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Hypothalami were immediately removed and immersed in either 4% PFA in 0.1 mol L^{-1} phosphate buffer (for immunofluorescence) or 4% PFA + 0.1% glutaraldehyde (for electron microscopic studies) for 16-24 hours at 4°C. The fixed tissue was then sectioned in the coronal plane at 30-60 µm thickness with a Linear-Slicer (PRO10; Dosaka EM, Kyoto, Japan).

2.2.3 | Immunofluorescence

The sections were rinsed with phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (PBST) three times for 5 minutes each. After blocking nonspecific binding with 1% normal goat serum and 1% bovine serum albumin (BSA) in PBST for 30 minutes at room temperature, the sections were incubated with primary rabbit antisera directed against CRF (dilution 1:20 000) or copeptin (dilution 1:10 000) and with a mouse monoclonal anti-AVP-NPII antibody (dilution 1:1000) for 4-5 days at 4°C. The rabbit polyclonal antiserum against rat/human copeptin, which was generated by us, has been characterised previously via western analysis in rats.[24] The copeptin$_{7-14}$ (ATQLDGPA) fragment, used as an antigen to produce our copeptin antiserum, is well conserved in mammals. It is identical in rats,[25] mice,[26] macaque monkeys (GenBank accession numbers XM_005568487.2 [*M. fascicularis*] and XM_011741311.2 [*M. nemestrina*]), common marmosets (GenBank accession number XM_002747402.2 [*Callithrix jacchus*]) and humans.[25] Thus, our antiserum appears to be appropriate for use in various mammalian species including primates. The mouse monoclonal NPII (PS41) antibody

has previously shown to be specific for AVP neurones in rodents.[27,28] The rabbit polyclonal antiserum against rat/human CRF (PBL rC70), which was kindly donated by Wylie Vale, has been characterised elsewhere.[29] It was directed against the rat form of CRF, which is identical to the human form, and has also been characterised in non-human primates.[30] Alexa Fluor 546-linked anti-mouse immunoglobulin G (IgG) (Molecular Probes, Carlsbad, CA, USA) and Alexa Fluor 488-linked anti-rabbit IgG (Molecular Probes) were used for detection at 1:1000 dilution. Immunostained sections were viewed by confocal laser scanning microscopy (FluoView 1000; Olympus, Tokyo, Japan). The antibodies used in the study are shown in Table 1.

The proportion of NPII-containing neurones in the CRF$^+$ neuronal population of the PVH was analysed. For each of four or five 30-µm-thick PVH sections per animal (n = 4 monkeys; two females, two males), we analysed, at 200× magnification, unit areas (630 × 630 µm^2) in the PVH containing CRF-positive neurones. In total, 87 unit areas from 17 sections were analysed. The number of the immunoreactive cells with clearly visible transected nuclei was counted.

The co-expression ratio of the immunoreactivity for CRF/NPII was also determined in sections of the ME. For each of the four to five 30-µm thick sections per animal (n = 4 monkeys; two females, two males), we analysed at 400× magnification, unit areas (50 × 50 µm^2) to determine CRF/NPII immunoreactivity. In total, 74 unit areas in the external layer and 60 unit areas in the internal layer from 17 sections were analysed. The optical density of the immunostaining of CRF (green), NPII (magenta) and the merged images (green, magenta and white) were all determined by preparing black-white images that were converted from each immunofluorescence micrograph using ImageJ, version 1.52a (National Institutes of Health, Bethesda, MD, USA). The optical density of the background labelling was estimated by comparison with similar areas of the control sections (see Supporting information, Figure S1). Each threshold optical density was determined by normalising the data to those of the control sections. The CRF/NPII-immunoreactive-fibre pixel density was semi-quantitated as the average pixel density in both the external and internal layers of the ME. The percentage of co-expression ratio was calculated in accordance with the formula: [pixel density of CRF immunoreactivity (green) + pixel density of NPII-immunoreactivity (magenta) – pixel density of the merged image (including all green,

TABLE 1 Primary antibodies used in the present study

Antigen	Description	Source, host species, cat#, or code#	Working dilution	Reference number	RRID
Copeptin	Synthetic peptide mapping at the amino acids 7-14 of human/mouse copeptin	Generated by our laboratory, rabbit polyclonal, CP8	1:10 000 (IF) 1:100 (EM)	24	AB_2722604
CRF	Human/rat CRF coupled to human α-globulins *via* bis-diazotised benzidine	Donated by Dr W. Vale, rabbit polyclonal, PBL rC70	1:20 000 (IF) 1:5000 (EM)	29,30	AB_2314234
NPII	Soluble proteins extracted from the posterior pituitary of the rat	ATCC, mouse monoclonal, PS41, CRL-1799	1:1000 (IF) 1:200 (EM)	27,28	AB_2313960

Abbreviations: ATCC, American Type Culture Collection; CRF, corticotrophin-releasing factor; EM, immunoelectron microscopy; IF, immunofluorescence; NPII, arginine vasopressin-associated neurophysin II; RRID, Research Resource Identifier.

magenta and white signals)]/pixel density of NPII immunoreactivity (magenta) × 100.

2.2.4 | Post-embedding immunoelectron microscopy

Slices of PFA + glutaraldehyde-fixed tissues containing the ME were dehydrated through increasing concentrations of methanol, flat-embedded in LR Gold resin (Electron Microscopy Sciences, Hatfield, PA, USA) and polymerised under ultraviolet lamps at − 25°C for 24 hours. Ultrathin sections (70 nm in thickness) were collected on nickel grids coated with (or without for triple labelling) a collodion film, rinsed with PBS several times, then incubated with 2% normal goat serum and 2% BSA in 50 mmol L^{-1} Tris(hydroxymethyl)-aminomethane-buffered saline (pH 8.2) for 30 minutes to block nonspecific binding. The sections were then incubated with the rabbit polyclonal antiserum against CRF (dilution 1:5000) or copeptin (dilution 1:100) and a 1:200 dilution of the PS41 mouse monoclonal antibody against NPII for 2 hours at room temperature. For double-immunoelectron microscopy, after incubation with the primary antibodies, the sections were washed with PBS, then incubated with a 1:50 dilution of a goat antibody against rabbit IgG conjugated to 10 nm gold particles (BBI Solutions, Crumlin, UK) and a goat antibody against mouse IgG conjugated to 15 nm gold particles (BBI Solutions) for 1 hour at room temperature.

Triple immunoelectron microscopy with antibodies against CRF, NPII and copeptin was performed by using the front and back of ultrathin sections mounted on nickel grids without a supporting film. First, immunocytochemistry with a pair of primary antibodies (CRF and NPII) was performed on one side of the section and detected using 15 nm (mouse) and 10 nm (rabbit) colloidal gold particles (BBI Solutions), respectively. Next, immunocytochemistry with the other primary rabbit antibody (against copeptin) was performed on the other side of the section and detected by use of 5 nm colloidal gold particles (BBI Solutions). Finally, the sections were contrasted with uranyl acetate and lead citrate and viewed using an H-7650 (Hitachi, Tokyo, Japan) or JEM-1010 (Jeol Inc., Peabody, MA, USA) electron microscope operated at 80 kV. The antibodies used in this study are shown in Table 1.

Quantification of labelled NSVs in the different varicosities was performed. At least five electron microphotographs (photographed at 5000× magnification; 3.6 × 3.6 µm²) per animal (n = 4 monkeys; two males and two females) of randomised regions in the external layer of the ME were analysed. The external layer of the ME was defined by the diameter of NSVs (approximately 100 nm) as expected for axons derived from parvocellular neurones in the PVH.[28,31] Labelled NSVs were counted manually in immunoreactive varicosities including three or more NSVs. Numbers of CRF- and NPII-specific gold particles were counted, and represented as the numbers of immunoreactive gold particles per NSV. For those varicosities in which one or more NPII-labelled NSVs could be identified (ie, excluding varicosities in which only CRF immunoreactivity could
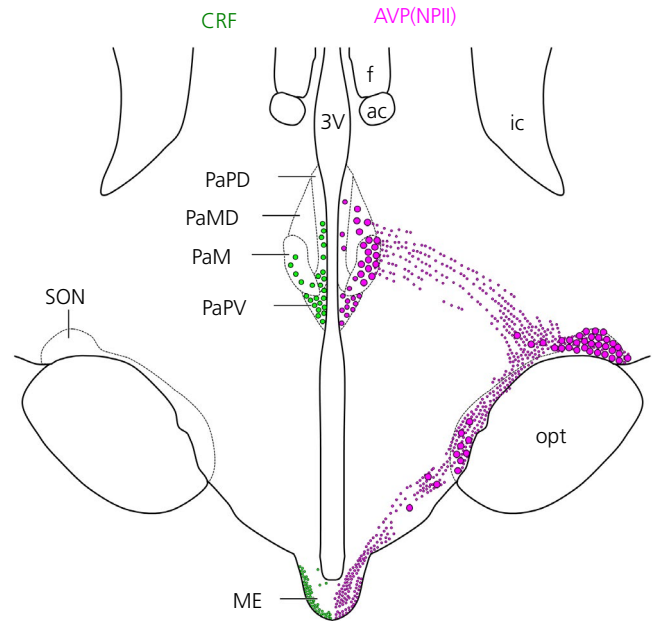

FIGURE 1 Diagram of the distribution of corticotrophin-releasing factor (CRF)- and vasopressin (AVP)-associated neurophysin (NPII)-immunoreactive neurones derived from pairs of adjacent sections immunostained using anti-CRF and anti-AVP(NPII) antibodies. CRF-expressing neurones are represented by filled (green) circles; AVP-expressing neurones are represented by open (magenta) circles. Cell bodies are represented by large circles and axonal projections are represented by small circles. 3V, third ventricle; ac, anterior commissure; f, fornix; ic, internal capsule; opt, optic tract; PaM, paraventricular hypothalamic nucleus, magnocellular part; PaMD, paraventricular hypothalamic nucleus, magnocellular part, dorsal division; PaPD, paraventricular hypothalamic nucleus, parvocellular part, dorsal division; PaPV, paraventricular hypothalamic nucleus, parvocellular part, ventral division; SON, supraoptic nucleus; ME, median eminence

be detected [CRF⁺, NPII⁻]), we calculated the proportion (%) of NSVs containing both CRF- and NPII-specific gold particles, and also the proportion (%) percentage of NSVs containing either only CRF- or only NPII-specific gold particles.

2.3 | Statistical analysis

The data are presented as the mean ± SEM and were analysed using Statcel4 (version 4, OMS publishing, Tokyo, Japan). Statistical analysis of the number of CRF- and NPII-specific gold particles was assessed via Student's *t* test.

3 | RESULTS

The expressions of *CRF*, *AVP* and *OXT* mRNAs in the PVH were examined by RT-PCR. The Supporting information (Figure S1) shows that bands were detected at the expected sizes for *CRF*, *AVP* and *OXT* genes in the PVH of both sexes. *CRF* mRNA but not *AVP* or *OXT*

mRNAs were also detected in the cerebral cortex. Almost equivalent amounts of *GAPDH* cDNA were amplified from RNA preparations among these tissues, which showed that no significant RNA degradation had occurred and a proper RT was obtained in the present study.

Figure 1 shows the topographic distribution of the neuronal components of the PVH, supraoptic nucleus, and ME derived from immunofluorescence labelling for CRF (left; green) and AVP-associated NPII (right; magenta) derived from coronal sections of the macaque hypothalamus. Neurones expressing NPII are referred to as 'AVP(NPII)' neurones.

In the PVH, a difference in size between neurones in the putative 'parvo'- and 'magnocellular' divisions of the PVH was not apparent in these primates; in both parts, the immunostained neuronal perikarya appeared to be of similar size. In the magnocellular part of the PVH (PaM), essentially no CRF immunoreactivity could be detected in the AVP(NPII)-immunoreactive neurones (Figure 2A; arrows). By contrast, in the medial parvocellular part of the PVH (PaPV), numerous neurones were CRF-immunoreactive and $39.0 \pm 0.6\%$ of the CRF$^+$ perikarya contained detectable AVP(NPII) immunoreactivity (Figure 2B; arrows). Virtually all AVP(NPII)-immunoreactive neurones in the PVH also contained copeptin immunoreactivity (Figure 3). The CRF$^+$ and AVP(NPII)$^+$ neurones were also more intermingled with magnocellular neurones in the monkey than in rodent PVH, as reported previously.[30,32] In the SON, no neurones exhibited both AVP(NPII) and CRF immunoreactivity (Figure 1). The immunofluorescence labelling for CRF and AVP(NPII) in the macaque ME shows the topographic distribution of the specific staining as reported previously (Figure 4).[33,34] The internal and external zones

were separated by a region in which the numerical density of immunoreactive processes was much lower (Figures 4 and 5A). A dense plexus of CRF$^+$ fibres was present in the external layer of the ME as reported previously,[33] and analysis of the immunoreactivity suggests that $52.3 \pm 1.5\%$ of CRF$^+$ fibres co-expressed AVP(NPII) (Figure 5A, arrowheads). By contrast, in the internal layer of the ME, AVP(NPII)$^+$ fibres were the most prominent, and only $2.8 \pm 0.3\%$ fibres contained both AVP(NPII) and CRF immunoreactivity (Figure 5A). Controls in which the primary antiserum was omitted showed no immunostaining in the ME (see Supporting information, Figure S1). Double-immunofluorescence for AVP(NPII) and the AVP-associated copeptin showed that, as expected, most AVP(NPII)$^+$ fibres in the ME also exhibited copeptin immunoreactivity in both the internal and external layers (Figure 5B). In the zone between the internal and external zones, CRF immunoreactivity was most marked (Figure 5A, merged) and, as in the PaPV, in only approximately half of the CRF-immunoreactive fibres could AVP(NPII) or copeptin immunoreactivity also be detected (Figure 5B, merged).

Electron microscopy showed that, in the external layer of the ME, the diameter of an NSV was approximately 100 nm as expected for axons derived from parvocellular neurones in the PVH (Figure 6).[28,31] Double-immunoelectron microscopy for CRF/AVP(NPII) (Figure 6) showed that, in the varicosities where both immunoreactivity was present, some individual vesicles were labelled by gold particles of both 10 nm and 15 nm, demonstrating the vesicular colocalisation of both CRF and AVP(NPII) (Figure 6A,B,B'). In some neighbouring terminals, only CRF single-positive vesicles could be detected (Figure 6A, asterisks, and C,C'). Quantification showed that the number of CRF-specific gold particles per NSV was 5.2 ± 0.5 (n = 1875 NSVs) and that
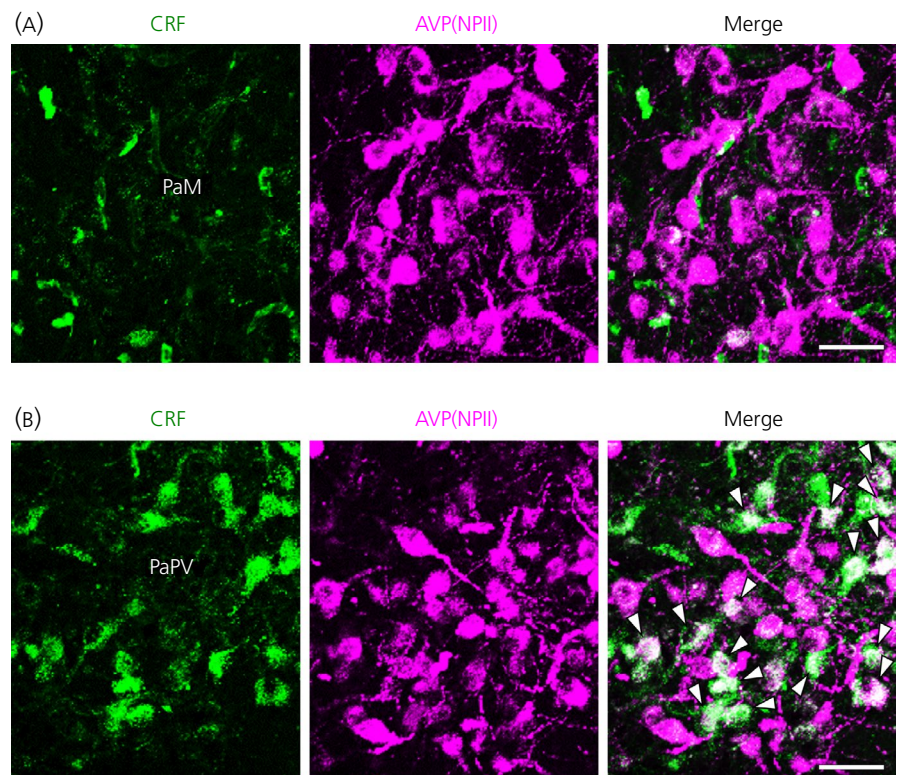

FIGURE 2 Double-label immunofluorescence for corticotrophin-releasing factor (CRF) and vasopressin (AVP)-associated neurophysin (NPII) in the paraventricular nucleus of the macaque hypothalamus (PVH). PaM, paraventricular hypothalamic nucleus, magnocellular part (A); PaPV, paraventricular hypothalamic nucleus, parvocellular part, ventral division (B). Immunoreactivity against CRF (green) and AVP(NPII) (magenta) is merged on the right (merge). Arrowheads in (B) indicate perikarya doubly immunopositive for CRF and AVP(NPII) in the PaPV. Scale bars = 50 μm
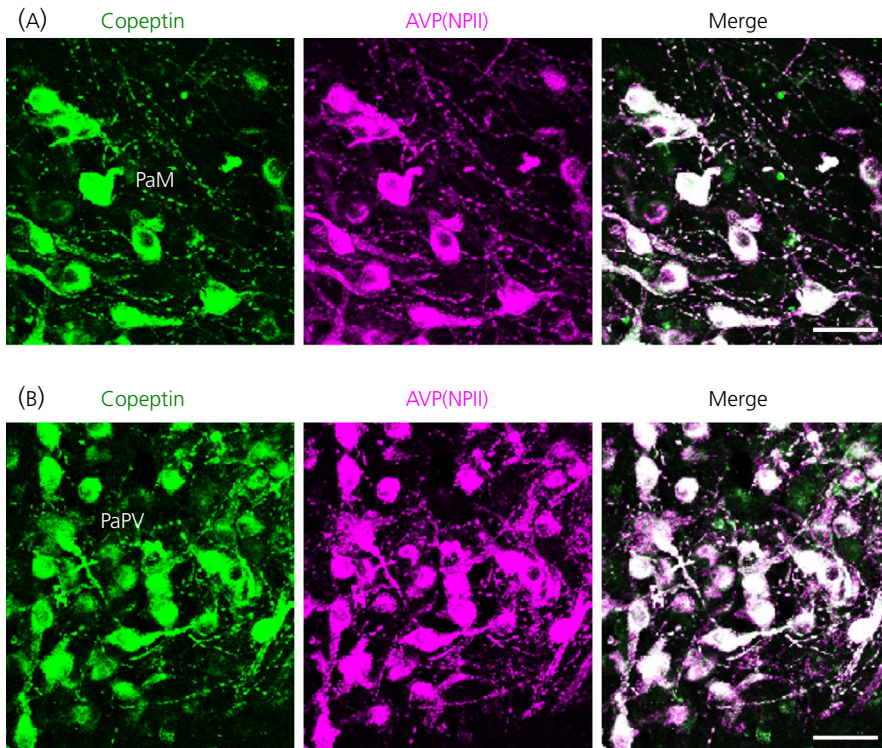
FIGURE 3 Double-label immunofluorescence for copeptin and vasopressin (AVP)-associated neurophysin (NPII) in the paraventricular nucleus of the macaque hypothalamus (PVH). PaM, paraventricular hypothalamic nucleus, magnocellular part (A); PaPV, paraventricular hypothalamic nucleus, parvocellular part, ventral division (B). Immunoreactivity against copeptin (green) and AVP(NPII) (magenta) is merged on the right (merge). The identical staining pattern between AVP(NPII) and copeptin at the cell body level is observed in the monkey PVH. Scale bars = 50 μm
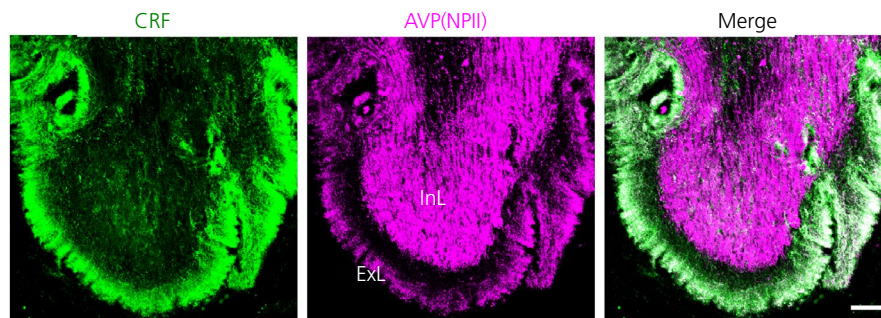
FIGURE 4 Low magnification images of macaque median eminence. Double-label immunofluorescence for corticotrophin-releasing factor (CRF) and vasopressin (AVP)-associated neurophysin (NPII) in the median eminence shows topographic distribution of the immunoidentified peptides as previously reported. Immunoreactivity against CRF (green) and AVP(NPII) (magenta) is merged on the right (merge). InL, internal layer of the median eminence; ExL, external layer of the median eminence. Scale bars = 100 μm

the number of AVP(NPII)-specific gold particles per NSV was 0.2 ± 0.0 (n = 1389 NSVs). Thus, the number of CRF-specific gold particles was significantly higher than that of AVP(NPII). In addition, the percentage of NSVs containing both CRF- and AVP(NPII)-specific gold particles located in the AVP(NPII)-immunoreactive varicosities was $12.6 \pm 1.7\%$. The percentage of NSVs containing only CRF-specific gold particles located in the AVP(NPII)-expressing varicosities was $70.4 \pm 3.1\%$, and the percentage for those containing only AVP(NPII)$^+$ was $0.6 \pm 0.4\%$. Double-labelling also showed that, in some AVP(NPII)$^+$ varicosities, NSVs were also positive for copeptin (Figure 7A,B,B'). However, no immunoreactivity for either AVP(NPII) or copeptin could be detected in many neighbouring varicosities (Figure 7A, asterisk, C,C'). Triple immunoelectron microscopy for CRF/AVP(NPII)/copeptin confirmed that the NSVs in certain neurosecretory axons contained CRF, AVP(NPII) and

copeptin immunoreactivity together (Figure 8A,B,B'), and triple-immunopositive vesicles were frequently seen. Neighbouring fibres often contained only CRF single-positive NSVs (Figure 8A, asterisk, C,C'). Very few gold particles were associated with other intra-varicosity organelles, such as the mitochondria, and background labelling over glial and endothelial elements was also extremely low. Immunoelectron microscopy studies were repeated independently at least three times using different monkeys and always produced similar results.

4 | DISCUSSION

This is the first report of the subcellular colocalisation of CRF, the AVP-associated NPII and copeptin (and therefore also AVP) in NSVs

FIGURE 5 Double-label immunofluorescence for corticotrophin-releasing factor (CRF) (A) or copeptin (B) and vasopressin (AVP)-associated neurophysin (NPII) in the macaque median eminence. Immunoreactivity against CRF (A; green) or copeptin (B; green) and AVP(NPII) (magenta) is merged in the right (overlap white). The outlined areas in merged images are enlarged. Arrowheads indicate tissue doubly immunopositive for CRF or copeptin and AVP(NPII). InL, internal layer of the median eminence; ExL, external layer of the median eminence. Scale bars = 50 μm (and 10 μm in enlarged images)
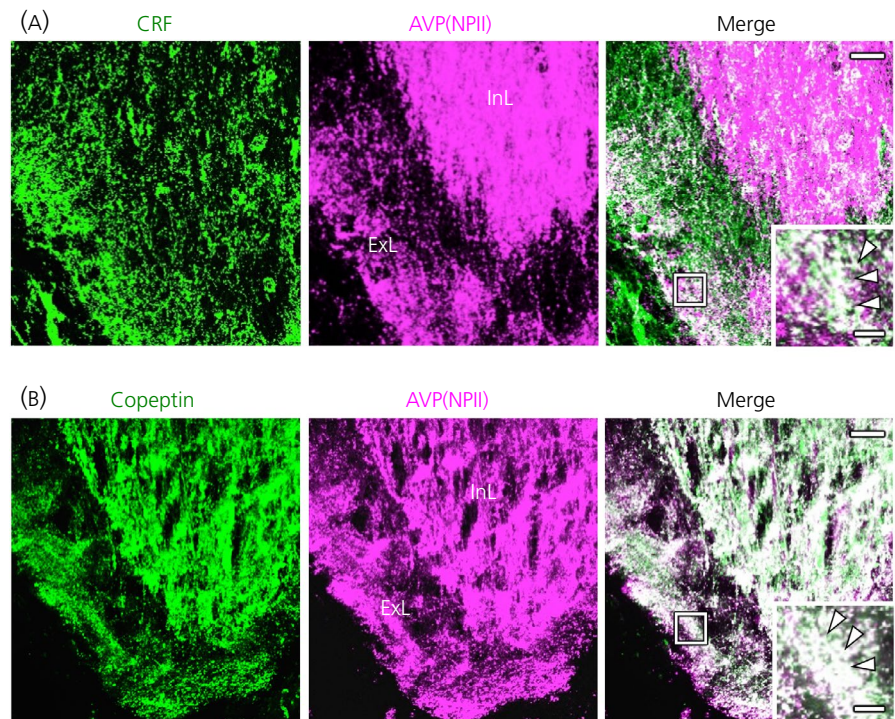
in the hypothalamic parvocellular CRF-secreting system of a primate. Our RT-PCR analysis indicates the expression of both CRF and AVP mRNAs in the monkey PVH. Earlier immunocytochemical studies have also reported that CRF and AVP can be localised to the same parvocellular neurones in the rat[20] and mouse,[23] as well as in the human PVH.[12,21,35] However, although it appears likely that CRF and the other AVP precursor components (NPII and copeptin) are co-packaged within the same NSV in the primate hypothalamus, there has thus far been no anatomical evidence.

In our unstressed monkeys, in the medial part of the PVH where the parvocellular perikarya from which axons project directly to the ME are located, approximately 61% of CRF+ perikarya did not contain immunodetectable amounts of NPII or copeptin (approximately 39% contained immunodetectable NPII). The internal zone of the ME contained only a few CRF+ fibres as expected. In the pre-terminal region between the internal and external zones of the ME, only a minority of the CRF-immunoreactive fibres contained AVP(NPII) or copeptin immunoreactivity and the overall amount of immunoreactivity was less marked, suggesting that there were fewer NSVs in the axons at this point. In the external zone of the ME close to the hypothalamic-pituitary capillaries, all three peptides (CRF, AVP and copeptin) were located in about half of the axons (approximately 52% of the CRF-immunoreactive tissue was also NPII-immunoreactive). In other adjacent axons containing CRF+ vesicles, no AVP(NPII) or copeptin could be detected. These observations are consistent with reports that, in unstressed rodents, only a minority of the CRF neurones produce significant amounts of the AVP precursor, although that either stress or adrenalectomy activates the production of AVP (and therefore the AVP precursor molecule) in the CRF neurones.[20,23] Our findings suggest that the difference in the extent to which AVP, NPII and copeptin are present in the CRF

and magnocellular systems relates primarily to the proportion of the neurones that express the AVP gene, rather than any differences in the rate of processing of the AVP precursor. In the internal zone of the ME through which the magnocellular axons pass en route to the posterior pituitary, the AVP precursor has clearly been processed sufficiently to reveal prominent AVP(NPII) and copeptin immunoreactivity in almost all of the fibres.

Both magnocellular AVP and parvocellular CRF/AVP neurones express the prohormone convertases, PC1 and PC2 in rodents.[36,37] In CRF/AVP neurones, the expression of PC1 is selectively regulated by glucocorticoids, although adrenalectomy has no effect on PC1 or PC2 levels in magnocellular neurones in rats.[37] Our finding that AVP(NPII) and copeptin immunoreactivity is somewhat more prominent in the terminal parts of the CRF-immunoreactive axons in the external ME than in their PVN perikarya could reflect the progressive proteolysis of the AVP/NPII/copeptin precursor in the CRF/AVP axons of the unstressed monkeys.

In monkeys, the axons of magnocellular AVP neurones project to the posterior pituitary via the internal zone of the ME, whereas other AVP-producing parvocellular neurones of the PVH have axons that project throughout the brain including the external zone of the ME and into the spinal cord as in other mammals.[32,38-41] In many studies, AVP has been reported to potentiate the ACTH secretory activity of CRF at the level of the ME.[7-9] The expression of AVP in the parvocellular CRF neurones is increased as a result of both adrenalectomy and stress.[42] In the unstressed rat ME, approximately 44% of the CRF-immunoreactive axons and terminals were stained for AVP. By contrast, in the adrenalectomised rat ME, almost all of the CRF-immunoreactive terminals showed strong staining for AVP, suggesting that adrenalectomy causes the transformation of a subpopulation of CRF+/AVP− axons
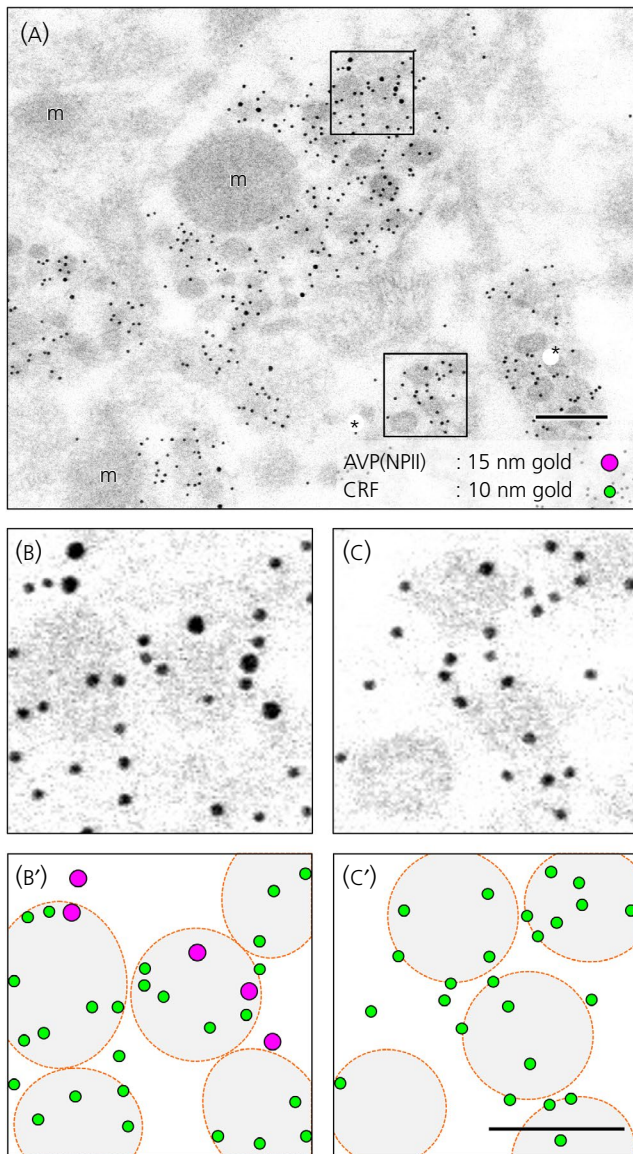
FIGURE 6 Double immunoelectron microscopy for vasopressin (AVP)-neurophysin (NPII) and corticotrophin-releasing factor (CRF) in the external zone of the macaque median eminence. In some varicosities, numerous neurosecretory vesicles were doubly immunopositive for CRF (signalled by 10-nm gold particles) and AVP(NPII) (signalled by 15-nm gold particles). The outlined area in (A) is enlarged in (B) and (C). By contrast, in other neighbouring varicosities, no AVP(NPII) immunoreactivity was detected (C). Schematic representations of the location of gold particles and neurosecretory vesicles are shown in (B') and (C'), respectively. m, mitochondrion. Scale bars = 200 nm in (A) and 100 nm in (C')
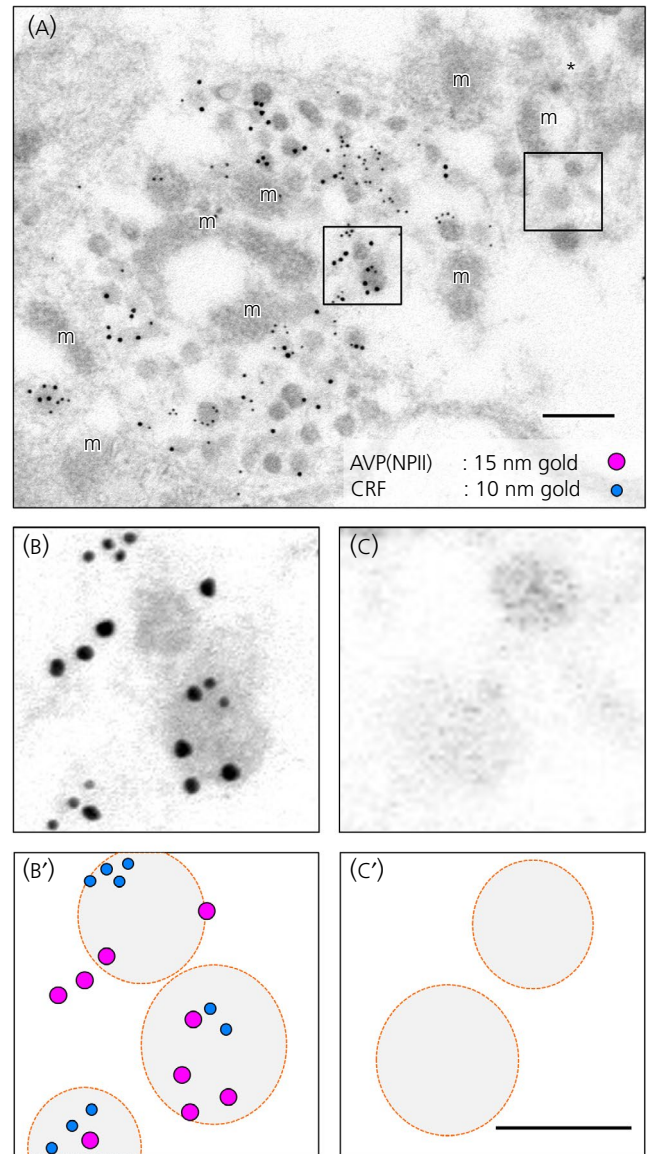
FIGURE 7 Double immunoelectron microscopy for vasopressin (AVP)-neurophysin (NPII) and copeptin in the external zone of the macaque median eminence. Numerous neurosecretory vesicles located in some of the varicosities were doubly immunopositive for copeptin (signalled by 10-nm gold particles) and AVP(NPII) (signalled by 15-nm gold particles). The outlined area in (A) is enlarged in (B) and (C). By contrast, in other neighbouring varicosities no NPII/copeptin immunoreactivity was detected (C). Schematic representations of the location of gold particles and neurosecretory vesicles are shown in (B') and (C'), respectively. m, mitochondrion. Scale bars = 200 nm in (A) and 100 nm in (C')

and terminals to ones which are CRF+/AVP+.[20] Our results indicate that, as in rodents,[20] approximately half of the CRF neurones in monkeys maintained under unstressed conditions also produce and secrete AVP, NPII and copeptin into the hypothalamic-portal capillaries where both NPII and copeptin could also influence the secretion of ACTH or other anterior pituitary hormones. The vasopressin gene in CRF neurones is switched on in stressed animals[20]; the monkeys that we studied were not stressed, and so it

is unsurprising that, in many CRF neurones, the *AVP* gene was not expressed to a detectable extent.

In the magnocellular system, any clear evidence for a function of secreted copeptin is currently lacking.[19] However, experiments seeking a possible physiological role for either NPII or copeptin have so far been confined to the systemic circulation and a potential role for either NPII or copeptin in the anterior pituitary has yet to be investigated. Our results demonstrate that CRF, AVP(NPII)
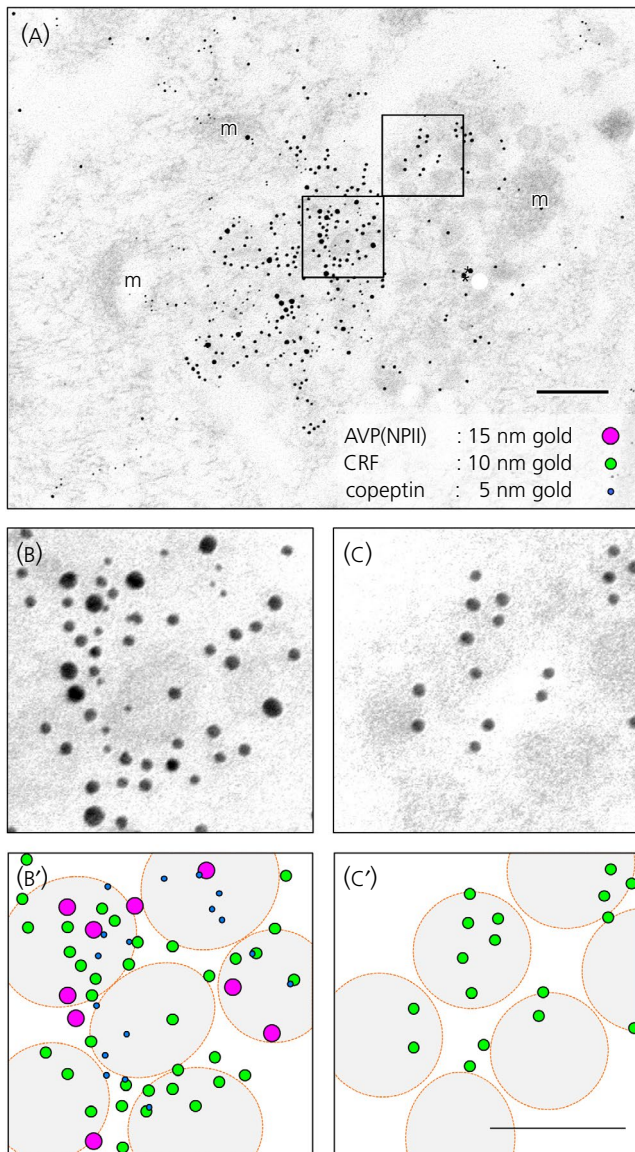
FIGURE 8 Triple immunoelectron microscopy for vasopressin (AVP)-neurophysin (NPII), copeptin, and corticotrophin-releasing factor (CRF) in the external zone of the macaque median eminence. (A) AVP(NPII) (signalled by 15-nm gold particles), CRF (signalled by 10-nm gold particles) and copeptin (signalled by 5-nm gold particles) were all detected within the varicosities. (B) Enlarged image showing that, in some varicosities, the immunoreactivity was co-associated with single neurosecretory vesicles. By contrast, in other neighbouring CRF-labelled (by 10-nm gold particles) varicosities, no AVP(NPII)/copeptin immunoreactivity was detected (C). Schematic representations of the location of gold particles and neurosecretory vesicles are shown in (B') and (C'), respectively. m, mitochondrion. Scale bars = 200 nm in (A) and 100 nm in (C')

and copeptin can all be colocalised within neurosecretory vesicles in the hypothalamic CRF-secreting system. Copeptin might therefore play a role in the hypothalamic-pituitary adrenal axis, although further studies is needed to demonstrate any such role.

In conclusion, immunofluorescence and post-embedding multiple immunoelectron microscopy revealed the subcellular colocalisation of CRF, AVP-associated NPII and copeptin in neurosecretory vesicles of the hypothalamic-pituitary CRF parvocellular perikarya and their axons in the ME of a macaque monkey. The results show that CRF, AVP and copeptin are co-packaged in NSVs in monkeys and are thus likely to be co-released into the portal capillary blood where they may synergistically modulate ACTH release in the primate anterior pituitary.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

AO and SM performed PCR experiments. AO, NK, SM, YU and HS performed immunofluorescence experiments. NK, JFM and HS performed immunoelectron microscopy analyses. YU, JFM and TS interpreted the data and provided the advice and equipment. HS wrote the paper with assistance from JFM. NK and AO contributed equally to this study. HS supervised the whole study. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

OPEN RESEARCH BADGES

This article has earned Open Data, Open Materials and Preregistered Research Design badges. Data, materials and the preregistered design and analysis plan are available at http://doi.org/10.6084/m9.figshare.12170454

DATA AVAILABILITY

The data that support the findings of this study are openly available in figshare at http://doi.org/10.6084/m9.figshare.12170454.

ORCID

Hirotaka Sakamoto https://orcid.org/0000-0002-6224-4591

REFERENCES

1. Vale W, Spiess J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science*. 1981;213:1394-1397.
2. Shibahara S, Morimoto Y, Furutani Y, et al. Isolation and sequence analysis of the human corticotropin-releasing factor precursor gene. *EMBO J*. 1983;2:775-779.
3. Rivier J, Spiess J, Vale W. Characterization of rat hypothalamic corticotropin-releasing factor. *Proc Natl Acad Sci U S A*. 1983;80:4851-4855.
4. Vale W, Vaughan J, Smith M, Yamamoto G, Rivier J, Rivier C. Effects of synthetic ovine corticotropin-releasing factor, glucocorticoids, catecholamines, neurohypophysial peptides, and other substances on cultured corticotropic cells. *Endocrinology*. 1983;113:1121-1131.
5. Rivier C, Vale W. Interaction of corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin secretion in vivo. *Endocrinology*. 1983;113:939-942.
6. Liu JH, Muse K, Contreras P, et al. Augmentation of ACTH-releasing activity of synthetic corticotropin releasing factor (CRF) by vasopressin in women. *J Clin Endocrinol Metab*. 1983;57:1087-1089.
7. Fischman AJ, Moldow RL. In vivo potentiation of corticotropin releasing factor activity by vasopressin analogues. *Life Sci*. 1984;35:1311-1319.
8. Gillies GE, Linton EA, Lowry PJ. Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. *Nature*. 1982;299:355-357.
9. Murakami K, Hashimoto K, Ota Z. Interaction of synthetic ovine corticotropin releasing factor and arginine vasopressin on in vitro ACTH release by the anterior pituitary of rats. *Neuroendocrinology*. 1984;39:49-53.
10. Hashimoto K, Murakami K, Hattori T, Ota Z. Synergistic interaction of corticotropin releasing factor and arginine vasopressin on adrenocorticotropin and cortisol secretion in *Macaca fuscata*. *Acta Med Okayama*. 1984;38:261-267.
11. Whitnall MH. Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. *Prog Neurogibol*. 1993;40:573-629.
12. Mouri T, Itoi K, Takahashi K, et al. Colocalization of corticotropin-releasing factor and vasopressin in the paraventricular nucleus of the human hypothalamus. *Neuroendocrinology*. 1993;57:34-39.
13. Bartanusz V, Jezova D, Bertini LT, Tilders FJ, Aubry JM, Kiss JZ. Stress-induced increase in vasopressin and corticotropin-releasing factor expression in hypophysiotrophic paraventricular neurons. *Endocrinology*. 1993;132:895-902.
14. Tilders FJ, Schmidt ED, de Goeij DC. Phenotypic plasticity of CRF neurons during stress. *Ann N Y Acad Sci*. 1993;697:39-52.
15. Land H, Schutz G, Schmale H, Richter D. Nucleotide sequence of cloned cDNA encoding bovine arginine vasopressin-neurophysin II precursor. *Nature*. 1982;295:299-303.
16. Davies J, Waller S, Zeng Q, Wells S, Murphy D. Further delineation of the sequences required for the expression and physiological regulation of the vasopressin gene in transgenic rat hypothalamic magnocellular neurones. *J Neuroendocrinol*. 2003;15:42-50.
17. Breslow E. Structure and folding properties of neurophysin and its peptide complexes: biological implications. *Regul Pept*. 1993;45:15-19.
18. Breslow E. Chemistry and biology of the neurophysins. *Annu Rev Biochem*. 1979;48:251-274.
19. Morgenthaler NG, Struck J, Jochberger S, Dunser MW. Copeptin: clinical use of a new biomarker. *Trends Endocrinol Metab*. 2008;19:43-49.
20. Whitnall MH, Mezey E, Gainer H. Co-localization of corticotropin-releasing factor and vasopressin in median eminence neurosecretory vesicles. *Nature*. 1985;317:248-250.

21. Bao AM, Swaab DF. Corticotropin-releasing hormone and arginine vasopressin in depression focus on the human postmortem hypothalamus. *Vitam Horm*. 2010;82:339–365.
22. Plotsky PM, Sawchenko PE. Hypophysial-portal plasma levels, median eminence content, and immunohistochemical staining of corticotropin-releasing factor, arginine vasopressin, and oxytocin after pharmacological adrenalectomy. *Endocrinology*. 1987;120:1361-1369.
23. Itoi K, Talukder AH, Fuse T, et al. Visualization of corticotropin-releasing factor neurons by fluorescent proteins in the mouse brain and characterization of labeled neurons in the paraventricular nucleus of the hypothalamus. *Endocrinology*. 2014;155:4054-4060.
24. Satoh K, Oti T, Katoh A, et al. In vivo processing and release into the circulation of GFP fusion protein in arginine vasopressin enhanced GFP transgenic rats: response to osmotic stimulation. *FEBS J*. 2015;282:2488-2499.
25. Rehbein M, Hillers M, Mohr E, et al. The neurohypophyseal hormones vasopressin and oxytocin. Precursor structure, synthesis and regulation. *Biol Chem Hoppe Seyler*. 1986;367:695-704.
26. Hara Y, Battey J, Gainer H. Structure of mouse vasopressin and oxytocin genes. *Brain Res Mol Brain Res*. 1990;8:319-324.
27. Wang H, Ward AR, Morris JF. Oestradiol acutely stimulates exocytosis of oxytocin and vasopressin from dendrites and somata of hypothalamic magnocellular neurons. *Neuroscience*. 1995;68:1179-1188.
28. Castel M, Morris JF, Whitnall MH, Sivan N. Improved visualization of the immunoreactive hypothalamo-neurohypophysial system by use of immuno-gold techniques. *Cell Tissue Res*. 1986;243:193-204.
29. Sawchenko PE, Swanson LW, Vale WW. Co-expression of corticotropin-releasing factor and vasopressin immunoreactivity in parvocellular neurosecretory neurons of the adrenalectomized rat. *Proc Natl Acad Sci USA*. 1984;81:1883-1887.
30. Foote SL, Cha CI. Distribution of corticotropin-releasing-factor-like immunoreactivity in brainstem of two monkey species (Saimiri sciureus and Macaca fascicularis): an immunohistochemical study. *J Comp Neurol*. 1988;276:239-264.
31. Shaw FD, Castel M, Morris JF. Ultrastructural characterisation of vasopressinergic terminals in the lateral septum of murine brains by use of monoclonal anti-neurophysins. *Cell Tissue Res*. 1987;249:403-410.
32. Kawata M, Sano Y. Immunohistochemical identification of the oxytocin and vasopressin neurons in the hypothalamus of the monkey (Macaca fuscata). *Anat Embryol (Berl)*. 1982;165:151-167.
33. Kawata M, Hashimoto K, Takahara J, Sano Y. Immunohistochemical demonstration of corticotropin releasing factor containing nerve fibers in the median eminence of the rat and monkey. *Histochemistry*. 1982;76:15-19.
34. Zimmerman EA, Carmel PW, Husain MK, et al. Vasopressin and neurophysin: high concentrations in monkey hypophyseal portal blood. *Science*. 1973;182:925-927.
35. Raadsheer FC, Sluiter AA, Ravid R, Tilders FJ, Swaab DF. Localization of corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus of the human hypothalamus; age-dependent colocalization with vasopressin. *Brain Res*. 1993;615:50-62.
36. Hook V, Funkelstein L, Lu D, Bark S, Wegrzyn J, Hwang SR. Proteases for processing proneuropeptides into peptide neurotransmitters and hormones. *Annu Rev Pharmacol Toxicol*. 2008;48:393-423.
37. Dong W, Seidel B, Marcinkiewicz M, Chretien M, Seidah NG, Day R. Cellular localization of the prohormone convertases in the hypothalamic paraventricular and supraoptic nuclei: selective regulation of PC1 in corticotrophin-releasing hormone parvocellular neurons mediated by glucocorticoids. *J Neurosci*. 1997;17:563-575.
38. Wagner CK, Clemens LG. Neurophysin-containing pathway from the paraventricular nucleus of the hypothalamus to a sexually dimorphic motor nucleus in lumbar spinal cord. *J Comp Neurol*. 1993;336:106-116.

39. Wagner CK, Clemens LG. Projections of the paraventricular nucleus of the hypothalamus to the sexually dimorphic lumbosacral region of the spinal cord. *Brain Res*. 1991;539:254-262.

40. Hallbeck M, Blomqvist A. Spinal cord-projecting vasopressinergic neurons in the rat paraventricular hypothalamus. *J Comp Neurol*. 1999;411:201-211.

41. Castel M, Morris JF. The neurophysin-containing innervation of the forebrain of the mouse. *Neuroscience*. 1988;24:937-966.

42. Fellmann D, Bugnon C, Bresson JL, et al. The CRF neuron: immuno-cytochemical study. *Peptides*. 1984;5(Suppl):119-133.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.