Specific expression of an oxytocin-enhanced cyan fluorescent protein fusion transgene in the rat hypothalamus and posterior pituitary

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Abstract

We have generated rats bearing an oxytocin (OXT)-enhanced cyan fluorescent protein (eCFP) fusion transgene designed from a murine construct previously shown to be faithfully expressed in transgenic mice. *In situ* hybridisation histochemistry revealed that the *Oxt-eCfp* fusion gene was expressed in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) in these rats. The fluorescence emanating from eCFP was observed only in the SON, the PVN, the internal layer of the median eminence and the posterior pituitary (PP). In *in vitro* preparations, freshly dissociated cells from the SON and axon terminals showed clear eCFP fluorescence. Immunohistochemistry for OXT and arginine vasopressin (AVP) revealed that the eCFP fluorescence co-localises with OXT immunofluorescence, but not with AVP immunofluorescence in the SON and the PVN. Although the expression levels of

the *Oxt-eCfp* fusion gene in the SON and the PVN showed a wide range of variations in transgenic rats, eCFP fluorescence was markedly increased in the SON and the PVN, but decreased in the PP after chronic salt loading. The expression of the *Oxt* gene was significantly increased in the SON and the PVN after chronic salt loading in both non-transgenic and transgenic rats. Compared with wild-type animals, euhydrated and salt-loaded male and female transgenic rats showed no significant differences in plasma osmolality, sodium concentration and OXT and AVP levels, suggesting that the fusion gene expression did not disturb any physiological processes. These results suggest that our new transgenic rats are a valuable new tool to identify OXT-producing neurones and their terminals.

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Introduction

The neurohypophyseal hormones arginine vasopressin (AVP) and oxytocin (OXT) are mainly synthesised in discrete groups of magnocellular neurosecretory cells (MNCs) that are located in the hypothalamus. The gene expression, synthesis and secretion of OXT and AVP in the hypothalamo-neurohypophyseal system (HNS) have been well studied, and this system is an excellent model to investigate the fundamental regulation and functions of the neuroendocrine systems (Burbach *et al.* 2001).

Recently, transgenic animals have been widely used in the field of neuroendocrinology to understand both the

physiological roles and the regulation of the neurohypophyseal hormones (Murphy & Wells 2003, Young & Gainer 2003). For example, we have described the generation and characterisation of rats that faithfully express an AVP-enhanced green fluorescent protein (eGFP) fusion transgene (*Avp-eGfp*; Ueta *et al.* 2005). In these rats, eGFP fluorescence was observed in the supraoptic nucleus (SON), the suprachiasmatic nucleus (SCN), the paraventricular nucleus (PVN), the median eminence (ME) and in their axon terminals in the posterior pituitary (PP) under euhydrated conditions (Ueta *et al.* 2005). The level of *Avp-eGfp* fusion gene expression was markedly increased after dehydration and chronic salt loading in the SON and the PVN, but not in the SCN (Ueta *et al.* 2005, Fujio *et al.* 2006).

It is very interesting to note that compared with the endogenous *AVP* gene, the response of the fusion gene to physiological stimuli such as osmotic challenge (Ueta *et al.* 2005, Fujio *et al.* 2006), stressful (Shibata *et al.* 2007) and other conditions (Ueta *et al.* 2008, Suzuki *et al.* 2009) was exaggerated.

Another important HNS hormone is OXT, which is mainly expressed in a different set of MNCs from AVP (Burbach *et al.* 2001). The characteristics of OXT-synthesising and -secreting cells and the release of OXT under different physiological conditions have been studied widely (Cazalis *et al.* 1985, Dayanithi *et al.* 1986, 2000, 2008, Ludwig *et al.* 2002). Young *et al.* (1999) have previously described OXT gene regulatory sequences capable of directing the expression of GFP to mouse OXT neurones and terminals (Young *et al.* 1999, Zhang *et al.* 2002). Based on these data, we have now generated transgenic rats to visualise OXT-producing MNCs and terminals using a fusion gene consisting of OXT regulatory and coding sequences in frame with an enhanced eCFP, which is one of the various GFP spectral variants (Hadjantonakis & Nagy 2001). In these rats, we have studied physiological responses to osmotic stimulation.

Materials and Methods

Animals

Non-transgenic and heterozygous transgenic Wistar rats were bred and housed under normal laboratory conditions (12 h light:12 h darkness, and 0700–1900 h lights on) with free access to food and drinking water. All experimental procedures in this study were performed 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. For fluorescent microscopic observation for eCFP fluorescence, *in situ* hybridisation histochemistry for *eCfp*, *Oxt* and *Avp* mRNAs, and analysis in plasma under normal conditions and after chronic salt loading, 1–3-month-old OXT-eCFP transgenic and control non-transgenic age-matched male and female rats were used.

Constructs for microinjection

In the *Oxt-eCfp* transgene, the eCFP coding region is in frame in the middle of exon III, after the OXT and bulk of the neurophysin coding regions (Fig. 1A; Young *et al.* 1999, Zhang *et al.* 2002). A *HincII*–*SphI* fragment containing the nucleotide region –471 to 1640 of the mouse *Oxt* gene from plasmid VPOT8.3p2 was ligated into a modified multiple cloning region of pSP72 (Promega) at the *HincII*–*SphI* sites. A second polylinker was placed into exon III of the *Oxt* gene at nucleotide positions 756–766 using *Eco47III* and *PfMI*. The *SmaI*–*NotI* fragment of *eCfp* was inserted into the second multiple cloning region using

Eco47III and *NotI*, placing *eCfp* at the end of the coding region (Young *et al.* 1999).

The *Oxt-eCfp* fusion gene was validated by sequencing. The transgene fragment was microinjected into the pronuclei of fertilised oocytes obtained from the Wistar rats. The transgenic founders were identified by Southern blot analysis using genomic tail DNA with a ³²P-labelled eCFP probe. The founders were bred and F1 rats were screened by PCR analysis of genomic DNA extracted from the rats' tails. The PCR was performed using the oligonucleotide primers (sense sequence: 5'-CAC CAT CTT CTT CAA GGA CGA C-3' and antisense sequence: 5'-ATG ATA TAG ACG TTG TGG CTG TTG T-3').

Frozen and/or 4% paraformaldehyde-fixed brain sections obtained from eCFP-positive rats (F1) were observed under the fluorescence microscope (Eclipse E600, Nikon, Tokyo, Japan) with a CFP filter (Nikon) for screening whether eCFP was visually expressed in the hypothalamus and pituitary gland. Fluorescent microphotographs were taken with a digital camera (COOLPIX 990, Nikon).

Immunohistochemistry for OXT and AVP

The animals were deeply anaesthetised with sodium pentobarbital (50 mg/kg) and were perfused transcardially with 0.1 M phosphate buffer (PB; pH 7.4) containing heparin (1000 U/l) followed by 4% (wt/vol) paraformaldehyde. The brains were then removed and cut into three blocks. The blocks which included the hypothalamus were postfixed with the same fixative for 48 h at 4 °C. The tissues were then immersed in 20% (wt/vol) sucrose in 0.1 M PB for 48 h at 4 °C for cryoprotection, quickly frozen using powdered dry ice and sectioned at 30 µm on a cryostat (Leica CM3050, Nussloch, Germany). The sections were rinsed twice with 0.1 M PBS containing 0.3% (vol/vol) Triton X-100. The floating sections were incubated with a primary OXT antibody (Chemicon International, Inc., Temecula, CA, USA; diluted 1:8000) or AVP antibody (Chemicon International, Inc.; diluted 1:8000) for 3 days at 4 °C. After washing for 20 min in 0.1 M PBS solution containing 0.3% (vol/vol) Triton X-100, the sections were incubated for 2 h with Alexa Fluor 546-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; diluted 1:1000). After a final washing with 0.1 M PBS, sections were mounted onto the cover glasses and coverslipped with hydrophilic mounting media (Tris-HCl containing polyvinyl alcohol and glycerol). The sections were observed under a confocal laser scanning microscope (LSM 510, Carl Zeiss Co. Ltd, Jena, Germany).

Experimental procedures

In the first experiment, we examined the effects of chronic salt loading on eCFP fluorescence in the PVN, the SON, the ME and the PP in OXT-eCFP transgenic rats. The control group consisted of six OXT-eCFP animals. Unless otherwise

Figure 1 (A–C) Structure of the oxytocin (OXT)-enhanced cyan fluorescent protein (eCFP) transgene (A) and representative autoradiographs of brain sections hybridised to a ^{35}S -labelled oligodeoxynucleotide probe for *eCfp* mRNA in a transgenic rat (B and C). (D) Enlargement of the boxed area in B. (E) Enlargement of the boxed area in C. White is the most intense signal, and black is the least intense signal. (F–K) Endogenous fluorescence of eCFP in the paraventricular nucleus (PVN; F and I). (G) OXT antibodies were visualised as red fluorescence, using an Alexa 546-conjugated secondary antibody. (J) Arginine vasopressin (AVP) antibodies were visualised as red fluorescence, using an Alexa 546-conjugated secondary antibody. (H) Merged view of fluorescence of eCFP, and specific OXT is seen as white. (K) Merged view of fluorescence of eCFP, and specific AVP is seen as white. Scale bars, 1 mm (B and C), 0.5 mm (D and E) and 50 μm (F–K). O, oxytocin; V, vasopressin; OT, optic tract.

stated, all animals used in this study were allowed free access to tap water and dry food for 10 days. The salt-loaded OXT-eCFP group ($n=6$) was allowed free access to tap water and dry food for the first 5 days, and then the rats were given 2% (wt/vol) salt solution instead of tap water for the next 5 days. Next, the rats were deeply anaesthetised by

i.p. administration of sodium pentobarbital (50 mg/kg). After perfusion with 4% (wt/vol) paraformaldehyde, the brains and pituitaries were removed carefully.

In another set of experiments in which the effects of chronic salt loading on the expressions of the *eCfp*, *Oxt* and *Avp* genes in the SON and the PVN in OXT-eCFP transgenic rats

were examined, 12 non-transgenic male and 12 female rats and 12 transgenic male and 12 female rats were used. Control groups of six non-transgenic male and six female rats were maintained as described earlier. The salt-loaded groups of six non-transgenic male and six female rats were allowed free access to tap water and dry food for the first 5 days, and then for the last 5 days, 2% (wt/vol) salt solution was substituted with drinking water. Control groups of six OXT-eCFP transgenic male and six female rats were allowed free access to tap water and dry food for 10 days. The salt-loaded groups of six OXT-eCFP transgenic male and six female rats were treated similar to the salt-loaded groups of non-transgenic rats. At the end of the experiment, rats were decapitated, and the trunk blood was collected. The brains were removed carefully and frozen immediately and were stored at -80°C until *in situ* hybridisation analysis. Plasma OXT and AVP in non-transgenic and transgenic rats under normal conditions and after chronic salt loading were measured by RIA (see below). Plasma osmolality and plasma concentrations of sodium were measured by conventional methods (see below).

Finally, to examine the effects of chronic salt loading on body fluid homeostasis in OXT-eCFP transgenic rats, groups of six non-transgenic male and six female rats and groups of six OXT-eCFP transgenic male and six female rats were allowed free access to tap water and dry food for the first 5 days. For the last 5 days, 2% (wt/vol) salt solution was substituted with drinking water in each group. From the beginning of the observation, body weight was measured at 1800 h (every 24-h period) for each rat housed in a standard metabolic cage.

Slice preparation and dissociation of OXT-eCFP transgenic rat SON neurones

The protocol for slice preparation and the dissociation of transgenic rat SON neurones has been described previously in detail (Ohbuchi *et al.* 2009). Briefly, young adult male OXT-eCFP transgenic rats (80–150 g) were killed by decapitation, and the brains were quickly and carefully removed and placed in ice-cold artificial cerebrospinal fluid containing (in mM) 124 NaCl, 5 KCl, 1.3 MgSO_4 , 1.24 KH_2PO_4 , 2 CaCl_2 , 25.9 NaHCO_3 and 10 glucose (297–303 mOsm/l; pH 7.4) bubbled with 95% (vol/vol) O_2 :5% (vol/vol) CO_2 . After a block containing the hypothalamus was cut from the brain, coronal slices (150 μm) containing SON were cut from the block by a vibratome-type slicer (Linear slicer Pro 7, DSK, Kyoto, Japan). The slices were trimmed carefully with a circular punch (inner diameter 1.8 mm) and stored in bubbled artificial cerebrospinal fluid at room temperature ($22\text{--}24^{\circ}\text{C}$) for 1 h until they were used for enzyme digestion. Enzymatic dissociation was carried out by incubating the tissue pieces in oxygenated HEPES-buffered solution (HBS) containing (in mM) 140 NaCl, 5 KCl, 1.2 KH_2PO_4 , 2 CaCl_2 , 1.2 MgCl_2 , 10 glucose and 10 HEPES (pH 7.4) supplemented with 0.5 mg/ml of deoxyribonuclease I (Sigma) and 20 units/ml papain for 50 min at 30°C . The tissue pieces were then rinsed

with HBS and dissociated mechanically with fire-polished glass pipettes. The cell suspension was plated onto cover glasses. The cover glasses containing the plated neurones were placed in a glass-bottomed chamber and were continuously perfused with HBS at a rate of 1.5 ml/min. The cells were observed under a fluorescence microscope (Eclipse TE2000-U, Nikon) with a CFP filter (Nikon).

Isolation of nerve terminals from the neurohypophyses

Two transgenic or non-transgenic rats were used for each preparation. After decapitation with a guillotine, the neurohypophyses devoid of the pars intermedia were taken for terminal preparation as described previously (Cazalis *et al.* 1987, Ueta *et al.* 2005). Briefly, after removal of the anterior and the intermediate lobe of the pituitary, the neurohypophyses were dissociated in Locke's buffer that contained (in mM) 140 NaCl, 5 KCl, 1.2 MgCl_2 , 1.8 CaCl_2 , 10 glucose and 10 HEPES-Tris (pH 7.25); the osmolality was between 298 and 302 mOsm/l. The homogenised neurohypophyses were spun at 100 g for 1 min with the supernatant and were then spun at 2400 g for 4 min. The resulting pellet was then resuspended in normal Locke's buffer at 37°C . This preparation contained pure terminals with different diameters from 2 to 10 μm (Nordmann & Dayanithi 1988). One hour later, the terminals were observed under a fluorescence microscope (Eclipse TE2000-U, Nikon) with a CFP filter (Nikon).

In situ hybridisation histochemistry for eCfp, Oxt and Avp mRNAs

Cryostat sections were cut into 12- μm slices and thaw-mounted on gelatin/chrome alum-coated slides. The locations of the hypothalamic areas including the SON and the PVN were determined according to coordinates given by the rat brain atlas (Paxinos & Watson 1986). The pituitaries were also cut into 12- μm slices and thaw-mounted on gelatin/chrome alum-coated slides. ^{35}S 3'-end-labelled deoxyoligonucleotides complementary to transcripts encoding *eCfp*, *Oxt* and *Avp* were used (*eCfp* probe sequence: 5'-GTG CGC TCT TGG ACA TAG CCT TCG GGC ATG GCG GAC TTA A-3'; *Oxt* probe sequence: 5'-CTC GGA GAA GGC AGA CTC AGG GTC GCA GGC-3'; and *Avp* probe sequence: 5'-CAG CTC CCG GGC TGG CCC GTC CAG CT-3'). The probe was 3'-end-labelled using terminal deoxynucleotidyl transferase and [^{35}S] dATP. Each labelled probe was counted. Probe for the *eCfp* transcripts was labelled as $4.6 \pm 0.6 \times 10^5$ c.p.m./ μl ($n=5$). Probe for the *Oxt* transcripts was labelled as $3.9 \pm 0.7 \times 10^5$ c.p.m./ μl ($n=3$). Probe for the *Avp* transcripts was labelled as $3.8 \pm 0.5 \times 10^5$ c.p.m./ μl ($n=3$). The *in situ* hybridisation protocol has been described in detail previously (Ueta *et al.* 1995). Briefly, sections were fixed in 4% (wt/vol) formaldehyde for 5 min and incubated in saline containing 0.25% (vol/vol) acetic anhydride and 0.1 M triethanolamine for 10 min and were then dehydrated, delipidated in chloroform and

partially rehydrated. Hybridisation was carried out overnight at 37 °C in 45 µl of hybridisation buffer under a Nescofilm (Bando Kagaku, Osaka, Japan) coverslip. A total count of 1×10^6 c.p.m. for *eCfp* transcripts and 5×10^5 c.p.m. for *Oxt* and *Avp* transcripts per slide was used. After hybridisation, sections were washed for 1 h in four changes of $1 \times \text{SSC}$ (150 mM NaCl and 15 mM sodium citrate) at 55 °C and for an additional 1 h in two changes of $1 \times \text{SSC}$ at room temperature. Hybridised sections including the hypothalamus were examined by autoradiography (Hyperfilm, Amersham) for 2 weeks for the eCFP probe, 12 h for the OXT probe and 8 h for the AVP probe. The resulting images were analysed by computerised densitometry using a MCID imaging analyser (Imaging Research Inc., Cambridge, UK). The mean optical densities (ODs) of autoradiographs were measured by comparing them with simultaneously exposed ^{14}C -labelled microscale samples (Amersham), and they are presented in arbitrary units compared to the mean OD obtained from control rats.

RIA for OXT and AVP in plasma

Plasma concentrations of OXT and AVP were determined by RIA with specific anti-OXT and anti-AVP antisera as described previously (Onaka & Yagi 1990). Coefficients of inter- and intra-assay variations were 10 and 5% for OXT and 14 and 6% for AVP respectively. The minimum detection limit was 2 pg/ml for OXT and 0.5 pg/ml for AVP. Cross-reactivity of the anti-AVP antibody for OXT and that of the anti-OXT antibody for AVP were $<0.01\%$ (Higuchi *et al.* 1985).

Measurements of osmolality and sodium concentrations in plasma

Plasma concentrations of Na^+ were measured using conventional methods. The osmolalities of plasma were measured in 15-µl samples using a microsample osmometer (Model 110, Fiske Associates, Norwood, MA, USA).

Statistical analysis

The mean $\pm$ S.E.M. deviation from control (%) was calculated from the results of the *in situ* hybridisation histochemistry study. Each group within an experiment was compared with the control group. The results of RIA, the measurements of osmolality and sodium in plasma and body weight were also expressed as the mean $\pm$ S.E.M. The data were analysed using a one-way fractional ANOVA followed by Fisher's PLSD test for multiple comparisons. The statistical significance was set at $P < 0.05$.

Results

Production of transgenic rats

The purified *Oxt-eCfp* transgene was injected into the pronuclei of 665 fertilised oocytes obtained from the Wistar rat donors. A total of 45 pups were obtained. Eight transgenic founders (six males and two females) were identified by Southern blot analysis using genomic tail DNA with a ^{32}P -labelled eCFP probe. The copy numbers of the *eCfp* gene were between 3 and 300. Eight founders were bred and

Figure 2 Endogenous fluorescence of enhanced cyan fluorescent protein (eCFP) in the SON (A), the PVN (B), the ME (C) and the PP (D). Effects of salt loading for 5 days on the eCFP fluorescence of the SON (E), the PVN (F), the ME (G) and the PP (H). Visually identified neurones with high-intensity endogenous fluorescence of eCFP in a freshly isolated supraoptic nucleus neurone (I and J) and axon terminals (K and L). Under light (I and K) and fluorescent microscopy (J and L). Scale bars, 50 µm. OT, optic tract; 3V, third ventricle; AP, anterior pituitary.

F1 rats were screened by PCR analysis of genomic DNA extracted from the rats' tails. The PCR product was identified as the band of 1.6 kbp in agarose gels.

Frozen brain sections that were obtained from eCFP-positive rats (F1) were examined with a fluorescent microscope. Two lines (#88-1 and #98-3) demonstrating robust expression of eCFP in the SON and the PVN were selected for physiological experiments and breeding. The copy numbers of the *eCfp* gene in #88-1 and #98-3 were 3 and 300 respectively. In the present study, one line (#98-3) was used.

Distribution of *eCfp* mRNA in the central nervous system

In situ hybridisation histochemistry revealed that *eCfp* mRNA was localised in the SON (Fig. 1B and D) and the magnocellular and parvocellular divisions of the PVN

(Fig. 1C and E). There was no ectopic expression of the *eCfp* gene elsewhere in the central nervous system (CNS; Fig. 1B–E).

Immunostaining for OXT and AVP

Most eCFP-expressing MNCs also exhibited OXT-immunoreactivity (OXT-ir) in the SON and the PVN (more than 90%; Fig. 1F–H). No eCFP-expressing MNCs exhibited AVP-ir in the SON and the PVN (Fig. 1I–K).

Effects of chronic salt loading on eCFP fluorescence in the hypothalamus of OXT-eCFP transgenic rats

eCFP fluorescence was observed throughout the SON (Fig. 2A). There were scattered eCFP-expressing neurones in the parvocellular division of the PVN and abundant

Figure 3 (A–H) Representative autoradiographs of brain sections hybridised to ^{35}S -labelled oligodeoxynucleotide probe for enhanced cyan fluorescent protein (*eCfp*) mRNA in transgenic rats. The expression of the *eCfp* gene was detected in the SON (A–D) and the PVN (E–H). Sections A and E were obtained from non-salt-loaded male rats. Sections B and F were obtained from salt-loaded male rats. Sections C and G were obtained from non-salt-loaded female rats. Sections D and H were obtained salt-loaded female rats. The changes in *eCfp* mRNA levels in the SON (I) and PVN (J) after salt loading for 5 days in OXT-eCFP transgenic rats. The number of the non-salt-loaded (Control) or salt-loaded male and non-salt-loaded (Control) or salt-loaded female rats each was 6 respectively. White is the most intense signal, and black is the least intense signal. Scale bars, 1 mm (A–D) and 0.5 mm (E–H).

fluorescence of eCFP in the magnocellular division of the PVN (Fig. 2B). In the internal layer of the ME, intense fluorescence of eCFP was also observed (Fig. 2C). In the PP, the fluorescence of eCFP was clearly observed in the axon terminals (Fig. 2D). After salt loading for 5 days, the fluorescence of eCFP was remarkably increased throughout the SON in OXT-eCFP transgenic rats (Fig. 2E), and in the majority of the magnocellular neurones and several parvocellular neurones in the PVN in OXT-eCFP transgenic rats (Fig. 2F). However, the fluorescence of eCFP was decreased in the ME and in the PP (Fig. 2G and H).

Visualisation of eCFP expression in dissociated SON neurones and nerve terminals from the neurohypophyses

Dissociated SON neurones and axon terminals are widely used for many physiological studies using electrophysiological, fluorescence photometry and peptide release techniques, but usually the cells are identified only after the recordings are made (Dayanithi *et al.* 1996, Wang *et al.* 1999, Widmer *et al.* 2003). Here, we have succeeded in observing eCFP expression in half of the population of freshly dissociated SON neurones (Fig. 2I and J) and axon terminals (Fig. 2K and L).

Effects of chronic salt loading on the expression of the eCfp gene

There were no differences in the expression of the *eCfp* gene in the SON and the PVN between male and female transgenic rats under normal conditions (Fig. 3A, C, E, G, I and J). After salt loading for 5 days, in some male transgenic rats, the expression of the *eCfp* gene in the SON and the PVN was significantly increased (Fig. 3B, F, I and J). Under similar salt-loaded conditions, female transgenic rats did not show an increase in the expression of the *eCfp* gene in the SON and the PVN (Fig. 3D and H–J). It should be noted that the expression of the *eCfp* transgene in the SON and the PVN varies widely as determined by *in situ* hybridisation (Fig. 3A–J).

Effects of chronic salt loading on the expressions of the *Oxt* and *Avp* genes in the hypothalamus of non-transgenic and transgenic rats

There were no differences in the expression of the *Oxt* gene in the SON and the PVN between non-transgenic and transgenic rats under normal conditions (Fig. 4A, C, E, G and I–L). After salt loading for 5 days, the expression of the *Oxt* gene in the SON and the PVN was significantly increased in

Figure 4 (A–H) Representative autoradiographs of brain sections hybridised to ³⁵S-labelled oligodeoxynucleotide probe for oxytocin (*Oxt*) mRNA in OXT-enhanced cyan fluorescent protein (eCFP) transgenic rats. The expression of the *Oxt* gene was detected in the SON (A–D) and the PVN (E–H). Sections A and E were obtained from non-salt-loaded male transgenic rats. Sections B and F were obtained from salt-loaded male transgenic rats. Sections C and G were obtained from non-salt-loaded female transgenic rats. Sections D and H were obtained from salt-loaded female transgenic rats. Scale bars, 1 mm (A–D) and 0.5 mm (E–H). Changes in *Oxt* mRNA levels in the SON (I and J) and the PVN (K and L) after salt loading for 5 days in non-transgenic rats and OXT-eCFP transgenic rats. The number of non-transgenic (eCFP(–)) non-salt- or salt-loaded male, non-transgenic (eCFP(–)) non-salt- or salt-loaded female, (eCFP(+)) non-salt- or salt-loaded male, and (eCFP(+)) non-salt- or salt-loaded female rats each was 6 respectively. **P* < 0.05 and ***P* < 0.01 versus control of each group (non-transgenic and transgenic).

both non-transgenic and transgenic rats (Fig. 4B, D, F and H–L). There were no differences in the expression of the *Oxt* gene in the SON and the PVN between non-transgenic and transgenic rats after salt loading for 5 days (Fig. 4A–L). No differences were observed between male and female animals studied in these conditions (Fig. 4A–L).

There were no differences in the expression of the *Avp* gene in the SON and the PVN between non-transgenic and transgenic rats under normal conditions (Fig. 5A, C, E, G and I–L). After salt loading for 5 days, the expression of the *Avp* gene in the SON and the PVN was significantly increased in both non-transgenic and transgenic rats (Fig. 5B, D, F and H–L). There were no differences in the expression of the *Avp* gene in the SON and the PVN between non-transgenic and transgenic rats after salt loading (Fig. 5A–L). No differences were observed between male and female animals studied in these conditions (Fig. 5A–L).

Effects of chronic salt loading on plasma OXT, AVP, osmolality and Na⁺ in non-transgenic and transgenic rats

There were no differences in plasma osmolality between non-transgenic and transgenic rats under normal conditions

(Fig. 6A and B). After salt loading for 5 days, the plasma osmolality was significantly increased in both non-transgenic and transgenic rats (Fig. 6A and B). No difference was observed between male and female animals studied under these parameters. Plasma sodium concentration was not altered in either non-transgenic or transgenic rats under normal conditions (Fig. 6C and D). However, after salt loading for 5 days, plasma sodium concentrations were significantly increased in both non-transgenic and transgenic rats (Fig. 6C and D).

There were no differences in plasma OXT levels between non-transgenic and transgenic rats under normal conditions (Fig. 6E and F). After salt loading for 5 days, plasma concentrations of OXT did not change in either non-transgenic or transgenic rats (Fig. 6E and F). There were no differences in plasma AVP levels between non-transgenic and transgenic rats under normal conditions (Fig. 6G and H). After salt loading for 5 days, the plasma AVP concentrations were significantly increased in both non-transgenic and transgenic rats (Fig. 6G and H). There were no differences in plasma AVP concentrations between non-transgenic and transgenic rats after salt loading for 5 days (Fig. 6G and H). No differences were observed between males and females studied in these conditions/parameters.

Figure 5 (A–H) Representative autoradiographs of brain sections hybridized to ³⁵S-labelled oligodeoxynucleotide probe for arginine vasopressin (*Avp*) mRNA in oxytocin (OXT)-enhanced cyan fluorescent protein (eCFP) transgenic rats. The expression of the *Avp* gene was detected in the SON (A–D) and the PVN (E–H). Sections A and E were obtained from non-salt-loaded male transgenic rats. Sections B and F were obtained from salt-loaded male transgenic rats. Sections C and G were obtained from non-salt-loaded female transgenic rats. Sections D and H were obtained from salt-loaded female transgenic rats. White is the most intense signal, and black is the least intense signal. Scale bars, 1 mm (A–D) and 0.5 mm (E–H). Changes in *Avp* mRNA levels in the SON (I and J) and the PVN (K and L) after salt loading for 5 days in non-transgenic rats and OXT-eCFP transgenic rats. For each experimental condition, six rats were used. **P*<0.05 and ***P*<0.01 versus control of each group (non-transgenic and transgenic).

Figure 6 Plasma osmolality (A and B), Na⁺ (C and D), oxytocin (OXT; E and F) and arginine vasopressin (AVP; G and H) in 5-day salt-loaded non-transgenic and OXT-enhanced cyan fluorescent protein (eCFP) transgenic rats. The number of eCFP(-), eCFP(+) non-salt- or salt-loaded male or female rats each was 6. * $P < 0.05$ and ** $P < 0.01$.

Discussion

Transgenic animals are widely used to understand the physiological role and regulation of neuroendocrine genes (Murphy & Wells 2003, Young & Gainer 2003). Most transgenic studies have been performed on mice, but it is the rat model that has been used more widely in physiological studies, particularly in the fields of endocrinology and neuroendocrinology. The anatomy of the rat brain is well mapped, and the structure, function and regulatory mechanisms of the rat CNS have been defined clearly (Young *et al.* 1999, Young & Gainer 2003). With the elucidation of the sequence of the rat genome (Rat Genome Sequencing Project Consortium 2004), the utility of the rat as an experimental model is further enhanced (Abbott 2004, Lindblad-Toh 2004, Cozzi *et al.* 2008, Mashimo & Serikawa 2009).

Standard methods to identify neurones expressing particular genes are cumbersome, and they are usually carried out retrospectively on dead tissue. In order to improve visualisation and to offer the prospect of identifying specific cell type in live tissue, a fluorescent marker, eGFP, can be expressed transgenetically. For example, eGFP has been expressed in GnRH neurones in transgenic mice (Kato *et al.* 2003) and rats (Spergel *et al.* 1999). In mice, Young *et al.* (1999) succeeded in expressing the *eGfp* gene in OXT-secreting neurones; eGFP fused to the C-terminus of the OXT preprohormone was expressed selectively in OXT MNCs under transgenic *Oxt* gene control (Young *et al.* 1999, Zhang *et al.* 2002).

We have already described the generation of novel transgenic rats expressing an *Avp-eGfp* fusion gene

(Ueta *et al.* 2005). These rats have enabled the direct identification of living AVP neurones and have been used to study various physiological aspects (Ueta *et al.* 2005, 2008, Fujio *et al.* 2006, Shibata *et al.* 2007, Suzuki *et al.* 2009). Likewise, we have now, and for the first time, succeeded in generating transgenic rats that express an *Oxt-eCfp* fusion gene in the SON and the PVN. The eCFP fluorescence was observed in the SON, PVN, internal layer of the ME and PP. *In situ* hybridisation histochemistry revealed that there was no expression of the *eCfp* gene elsewhere in the brain at the level of the SON and the PVN (Fig. 1B–E).

We then sought to identify the specific neurochemical phenotype of the cells expressing eCFP in the hypothalamus. Using a specific antibody for OXT, we have shown that almost all OXT-containing neurones express eCFP. Conversely, in the PVN, no eCFP-expressing neurones exhibited AVP-ir (Fig. 1F–K). In the SON, comparable results were obtained. These results suggest that almost all eCFP-expressing neurones in the SON and the PVN express OXT, and they are consistent with previous results showing generally mutually exclusive expression of AVP and OXT in different magnocellular neurones (Mohr *et al.* 1988, Kiyama & Emson 1990), with little eCFP expression in AVP cells.

The fluorescence of eCFP in the SON and the PVN was clearly observed during fluorescent microscopy. The intensity of the eCFP fluorescence in the SON and the PVN was dramatically increased in the SON and the PVN after salt loading, suggesting the activation of biosynthesis in parallel with the endogenous *Oxt* gene (Yue *et al.* 2008). On the other hand, the intensity of the internal layer of the ME and the PP was markedly decreased after salt loading (Fig. 2A–H).

This likely indicates that like endogenous OXT, eCFP is released into the systemic circulation. In addition, it was easy to identify single OXT-eCFP-expressing cells dissociated from the SON by fluorescence.

Our previous studies have demonstrated that in the SON and the PVN, the response of the *Avp-eCfp* fusion gene to physiological stimuli was exaggerated (Fujio *et al.* 2006, Shibata *et al.* 2007). In the present study, we also examined the effects of salt loading on the expression of the *Oxt-eCfp* fusion gene in the SON and the PVN. Unexpectedly, the expression level of the *Oxt-eCfp* gene showed a relatively wide range both under euhydrated conditions and following salt loading (Fig. 3). Thus, it was difficult to evaluate the response of the *Oxt-eCfp* fusion gene in the SON and the PVN to salt loading. The reason for this remains unclear, but it may be related to the use of a murine *Oxt* transgene (Young *et al.* 1999, Zhang *et al.* 2002) in a rat host. It is also possible that the transgene is susceptible to varying amounts of epigenetic regulation (e.g. methylation). The variability of transgene expression will limit the physiological studies, such as the effect of osmotic challenge on the expression of the transgene.

In the present study, we examined whether fusion gene expression disturbed endogenous *Oxt* gene expression, and physiological response of the transgenic rats to salt loading. The results showed that transgene had no effect on host mRNA synthesis, and that expression of the *eCfp* gene had no effect on the regulation of the endogenous OXT system in the transgenic rats under normal and salt-loaded conditions. This means that the transgenic rats are suitable substrates for physiological studies such as identification of OXT cells isolated from the hypothalamus.

Finally, we showed that single cells isolated from the SON have sufficient eCFP fluorescence intensity to enable their identification in an *in vitro* preparation. In our preparations, similar eCFP fluorescence could be observed in the isolated terminals of the neurohypophysis (Fig. 2K and L). These dispersed neurone and terminal preparations have been widely used for the studies in cell-specific physiology (Lambert *et al.* 1994, Dayanithi *et al.* 2000, Widmer *et al.* 2003). The ability to directly identify OXT neurones in these preparations will be invaluable in electrophysiological and calcium imaging studies in living cells.

In conclusion, we have generated new transgenic rats that express an *Oxt-eCfp* fusion gene in the HNS. These rats demonstrate robust fluorescence, specifically in OXT-secreting neurones of the SON, the PVN and the PP. For the first time, living oxytocinergic neurones can be directly identified by virtue of their inherent fluorescence.

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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