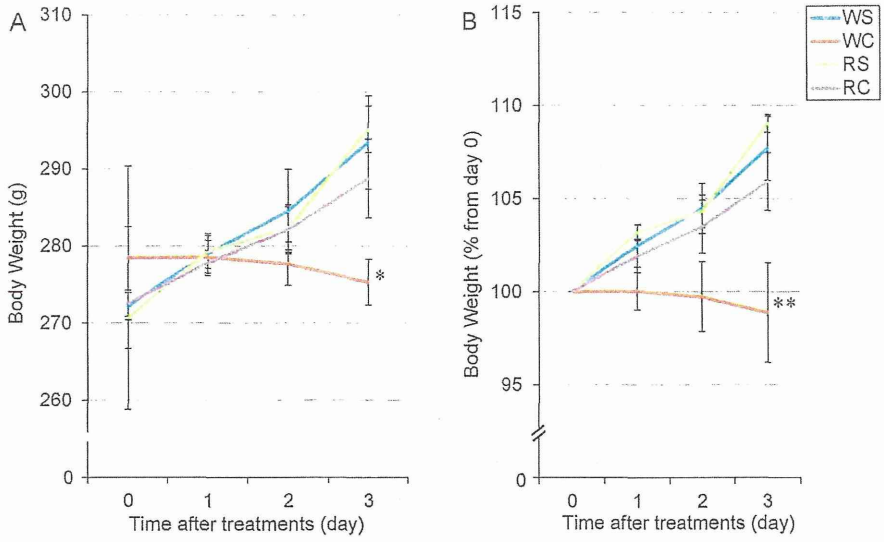

Fig. 1. Changes in body weight. (A) Changes in body weight from day −1 to day 3. (B) Changes in body weight from day 0 (percentage comparison from day 0). Body weight of WS, RS and RC gradually increased during the experiment, whereas that of WC (red line) gradually decreased. Data are presented as mean ± SEM. $^{*}P < 0.05$ *vs.* all other groups. $^{**}P < 0.01$ *vs.* all other groups.

Cumulative food intake was measured from day −1 to day 3 (Fig. 2C). At day 1, food intake in RC (purple line) and WC (red line) significantly decreased compared to the other two groups (Fig. 2C). After day 2, food intake in WC significantly decreased compared to all the other groups (Fig. 2C). Food intake per day from day 0 to day 3 is also shown in the graph (Fig. 2D). Food intake per day in WC significantly decreased compared to WS. There were no statistical differences among WS, RS and RC.

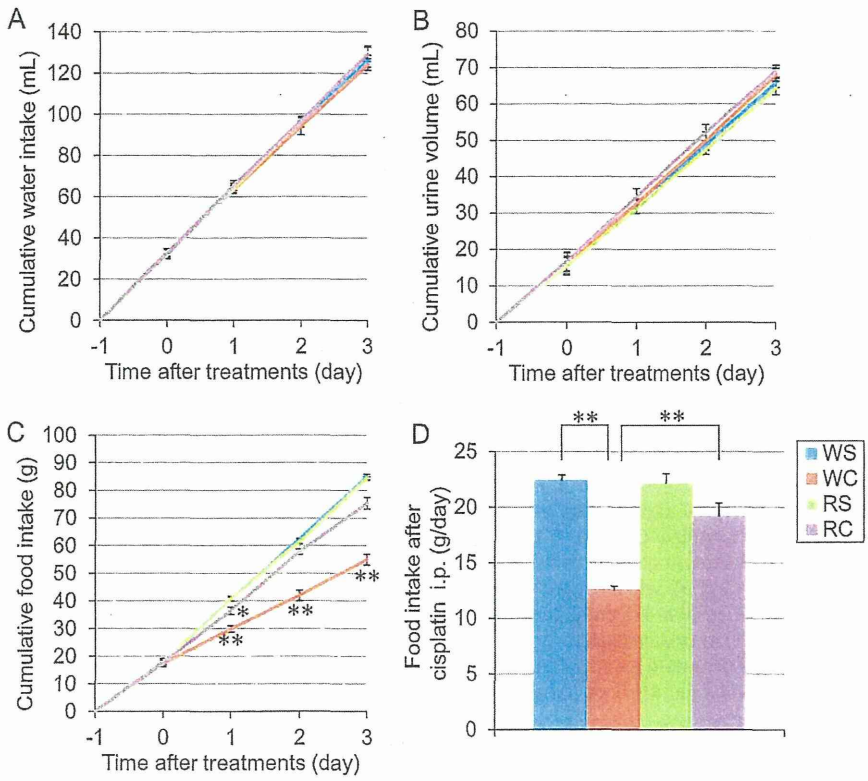

Fig. 2. Cumulative water intake, urine volume and food intake. (A) Cumulative water intake from day −1 to day 3. (B) Cumulative urine volume from day −1 to day 3. No statistical differences were seen in cumulative water intake and cumulative urine volume among all experimental groups. (C) Cumulative food intake from day −1 to day 3. (D) Food intake per day after administering cisplatin (day 0). Data are presented as mean ± SEM. $^{*}P < 0.05$ *vs.* all other groups. $^{**}P < 0.01$ *vs.* all other groups.

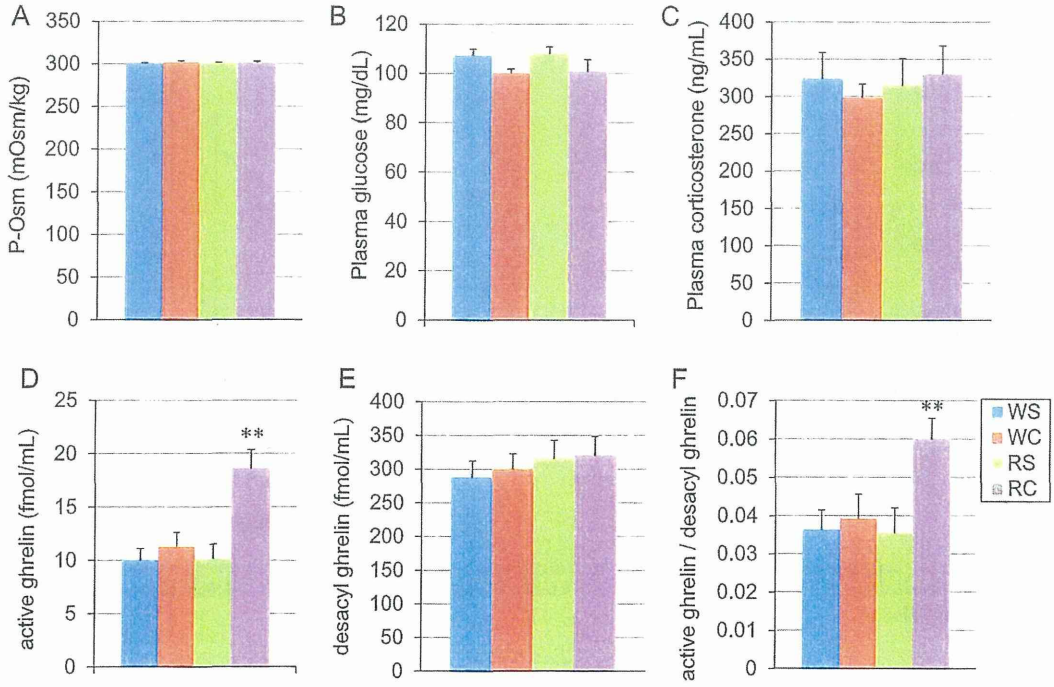

Fig. 3. Plasma measurements. (A) Plasma osmolality, (B) plasma glucose, (C) plasma corticosterone, (D) plasma active ghrelin, (E) plasma desacyl ghrelin, and (F) the ratio of plasma active and desacyl ghrelin. $^{**}P<0.01$ *vs.* all other groups. Data are presented as mean ± SEM.

3.3. Plasma measurement

We did plasma measurements of P-Osm (Fig. 3A), plasma glucose (Fig. 3B), plasma corticotrophin (Fig. 3C), plasma active ghrelin (Fig. 3D), and plasma desasyl ghrelin (Fig. 3E). The ratio of active and desasyl ghrelin is demonstrated in Fig. 3F. There were no significant differences in P-Osm, plasma glucose and plasma corticotrophin among all the experimental groups. Plasma active ghrelin dramatically increased only in RC (Fig. 3D). There were no statistical differences in desacyl ghrelin in all among the groups (Fig. 3E). Understandably, the ratio of active and desasyl ghrelin dramatically increased only in WC (Fig. 3F).

3.4. Feeding-regulating peptides in the hypothalamus

Feeding-regulating peptides in the hypothalamus were measured by *in situ* hybridization histochemistry followed by quantification using MCID. The expression of *oxytocin* mRNA signals in the PVN was comparable among all the experimental groups (Figs. 4A-a–A-d and 5). *CRH* mRNA in the PVN in WC significantly decreased compared to all the other groups (Figs. 4B-a–B-d and 5). *POMC* and *CART* mRNA in the ARC in WC significantly increased compared to all the other groups (Figs. 4A, C-a–C-d, D-a–D-d and 5). *NPY* mRNA in the ARC in WC significantly increased compared to all the other groups (Figs. 4E-a–E-d and 5), whereas that of *AgRP* in the ARC in WC was comparable (Figs. 4F-a–F-d and 5). *MCH* and *orexin* mRNA in the LHA in WC significantly increased compared to all the other groups (Figs. 4G-a–G-d, H-a–H-d and 5). There were no statistical differences among WS, RS and RC in all the peptides that were examined in this study.

4. Discussion

Traditional Japanese herbal medicines (kampo) are widely prescribed, and RKT is one of the most popularly prescribed kampo in Japan. However, although most kampo are used empirically, their mechanism is unclear. Although there has been no exception regarding RKT, its mechanism has been gradually proposed in recent years. In this study, we confirmed the efficacy of RKT on cisplatin-induced anorexia, and examined the feeding-regulating peptides in the hypothalamus in cisplatin-treated and cisplatin-RKT-treated rats.

As shown in Figs. 1 and 2C, the results of body weight and cumulative food intake showed a gradual increase in WS, RS and RC, whereas cumulative food intake in WC was less than those three groups and body weight decreased. These were observed at least three days after administering cisplatin or saline. Ghrelin is produced in the stomach and localized to neurons in the hypothalamic ARC [8,11]. It has been revealed that RKT is a ghrelin enhancer [3,19,26], thus indicating increased food intake and body weight. Cisplatin-induced anorexia may also be associated with reduced hypothalamic ghrelin secretion [26]. Taken together with our results, this is true under cisplatin-administered conditions but not under saline-treated conditions. In other words, administration of RKT alone does not increase body weight and food intake. It is indicated that even though ghrelin secretion is increased by RKT, food intake does not increase unless under pathological conditions.

Cumulative water intake and cumulative urine volume were the same extent in all the groups in our study (Fig. 2A and B). Although there have been reported cases of the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) induced by cisplatin [1,5,21], it is suggested that cisplatin and/or RKT have little effect on body fluid regulation within a few days after administration. The same level of P-Osm (Fig. 3A) among the groups also supports this hypothesis.

Cumulative food intake in WS and RS did not differ at all (Fig. 2C). However, cumulative food intake in WC at day 1, day 2 and day 3 significantly decreased in comparison with all the other groups

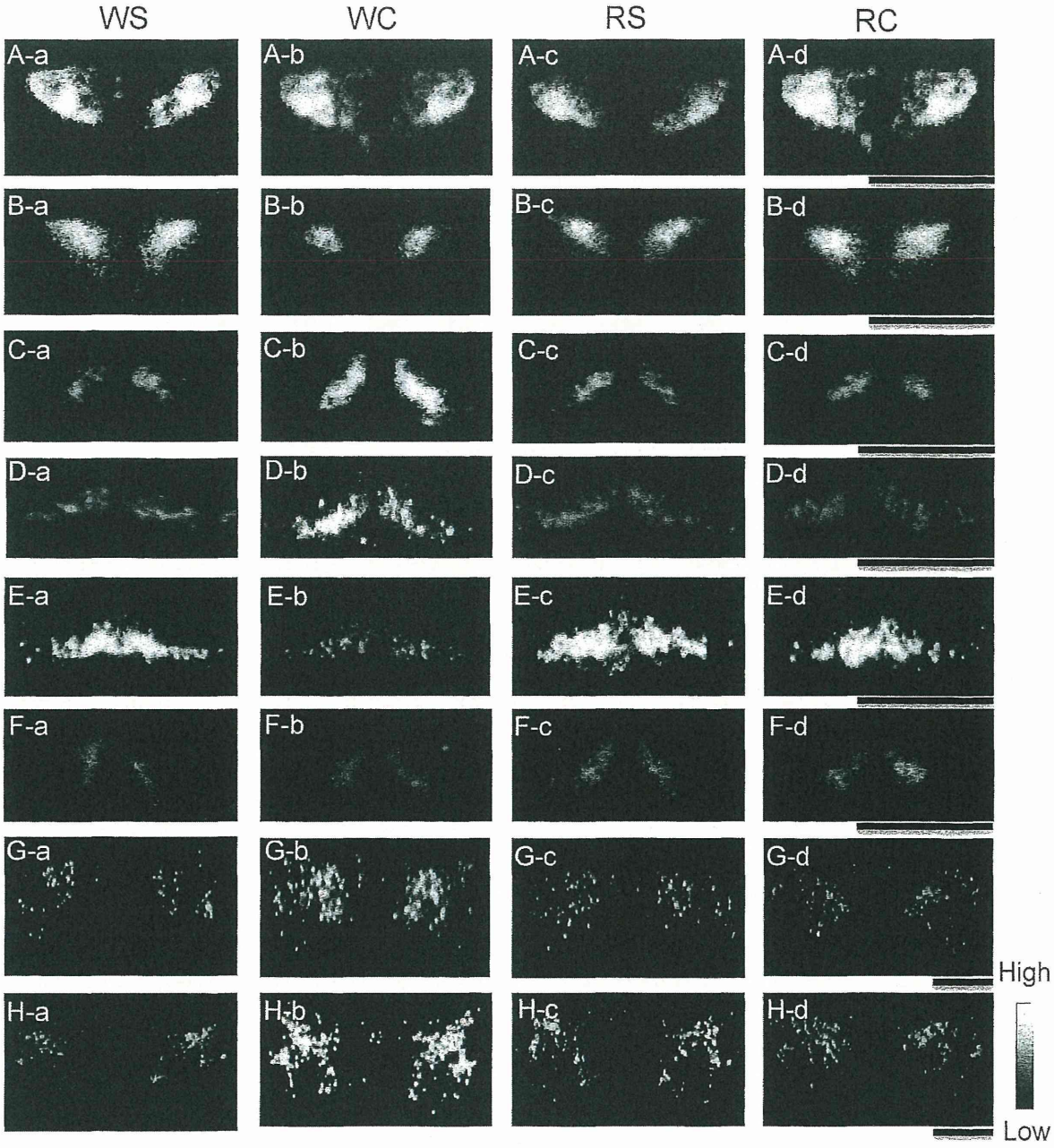

Fig. 4. Digital images of ISH of feeding-regulating peptides. (A) Digital images of *in situ* hybridization histochemistry of *oxytocin* in the PVN (A-a–A-d), *CRH* in the PVN (B-a–B-d), *POMC* in the ARC (C-a–C-d), *CART* in the ARC (D-a–D-d), *NPY* in the ARC (E-a–E-d), *AgRP* in the ARC (F-a–F-d), *MCH* in the LHA (G-a–G-d), and *orexin* in the LHA (H-a–H-d).

(Fig. 2C). The administration of RKT with cisplatin canceled the decreased food intake induced by cisplatin. This may indicate that RKT does not affect food intake in a non-pathological state.

In plasma measurements, P-Osm did not differ among the groups, as mentioned above. It is expected that plasma glucose levels were decreased as a result of reduced food intake in WC (Fig. 2D). There may be a possibility that other factors are involved in glucose metabolism, or that, as a result of stress response by administration of cisplatin, plasma glucose levels were comparable to other groups despite decreased food intake in WC. Needless to say, Li et al. isolated adrenocorticotrophic hormone from sheep pituitary [9], and it is widely recognized as a hormone which is secreted by various kinds of stress. Although it is expected that plasma corticotrophin levels might have increased in WC because of the stress which was induced by cisplatin administration, they did not differ among all the groups, whereas *in situ* hybridization of *CRH* in the PVN decreased in WC (Figs. 4B-b and 5). Perhaps there may be a very little change that cannot be captured in peripheral plasma even if there is a significant change in central nervous system. Interestingly, plasma active ghrelin neither decreased by cisplatin treatment nor by RKT treatment, however, it dramatically increased by RKT with cisplatin treatment (Fig. 3D-3F), which indicating that RKT acts only in pathological state but not in physiological condition. Although several studies have demonstrated that

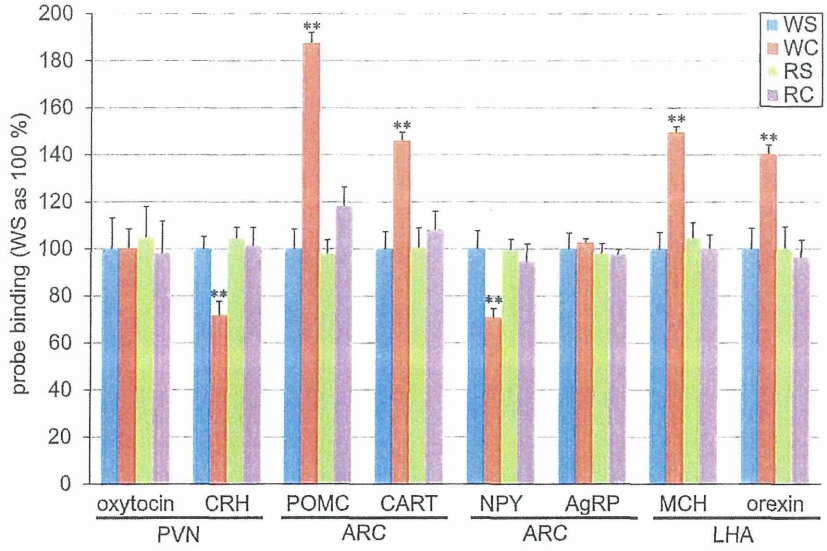

Fig. 5. Quantification of ISH of feeding-regulating peptides. Quantification of mRNA signals using MCID. WS data are presented as 100%. Signals of *oxytocin* in the PVN and *AgRP* in the ARC did not differ among all experimental groups. Signals of *POMC* in the ARC, *CART* in the ARC, *MCH* in the LHA and *orexin* in LHA in WC increased significantly compared to all other groups. On the other hand, signals of *CRH* in the PVN and *NPY* in the ARC significantly decreased in WC compared to all other groups. There were no statistical differences among WS, RS and RC in all examined peptides. Scale bar; 1 mm. Data are presented as mean ± SEM. $^{*}P < 0.05$ *vs.* all other groups. $^{**}P < 0.01$ *vs.* all other groups.

cisplatin provoke anorexia *via* reduced hypothalamic ghrelin secretion [25,26], cisplatin alone did not reduce plasma active ghrelin according to our study. Because our result is based on whole body plasma measurements, cisplatin may reduce the hypothalamic ghrelin secretion which cannot be captured by plasma measurements.

With respect to *in situ* hybridization histochemistry of feeding-regulating peptides in the hypothalamus, as shown in Figs. 4 and 5, the *oxytocin* probe binding in the PVN were comparable among all the experimental groups. It seems likely that cisplatin and RKT have little involvement with oxytocin transcription, or that a significant change cannot be detected as a state of mRNA. Further study, such as comparison with heteronuclear RNA expression, is needed.

CRH probe binding in the PVN significantly decreased in WC, and *MCH* and *orexin* probe binding in the LHA significantly increased in WC compared to all the other groups. CRH is recognized as an anorexigenic peptide, and MCH and orexin are recognized as orexigenic peptides [12]. We believe that these changes were a result of decreased appetite induced by cisplatin.

POMC and *CART* probe binding in the ARC were significantly increased, and *NPY* probe binding in the ARC significantly decreased. POMC and CART are recognized as anorexigenic peptides, and NPY is an orexigenic peptide [12]. These results indicate that cisplatin induces anorexia by increasing *POMC* and *CART* and decreasing *NPY* in the ARC. It seems likely that RKT affects the decrease of *POMC* and *CART* and the increase of *NPY* in the ARC under cisplatin-administered conditions, however there were no differences between WS and RS in our study, which suggests that RKT does not directly affect *POMC*, *CART* and *NPY* in the ARC without cisplatin. NPY, which is located downstream of the control of a number of feeding-regulating peptides, plays an important role in controlling feeding action [14,18]. It has been reported that centrally administered ghrelin stimulates food intake and weight gain by inducing NPY production in the ARC [13]. Furthermore, cisplatin-induced anorexia is involved in reduced ghrelin secretion [26], and the *CART* in the ARC has been inhibited by ghrelin [2]. Taken together with our results, cisplatin induces anorexia not only by reducing *NPY* in the ARC by reduced ghrelin secretion but also by increasing *POMC* and *CART* transcripts in the ARC. RKT may increase ghrelin secretion in the hypothalamus [26], thus canceling these reactions. It is not clear whether RKT affects the increase of *POMC* and *CART* directly or not. Ghrelin may have a potential role in regulating these peptides which were examined in this study.

AgRP probe binding in the ARC was comparable in all the experimental groups. It has been reported that ghrelin regulates not only *NPY* but also *AgRP* in the ARC [10]. We expected that *NPY* and *AgRP* would change similarly; however, they did not change in the same way. There may be other factors regulating AgRP except ghrelin when administering cisplatin.

The reason why the feeding-regulating peptides in RS were comparable to those in WS remains unclear. These reactions were not observed by administering RKT alone. There may have been involvement of other feeding-regulating peptides which were not investigated in this study when administering RKT alone. Further study is needed to elucidate the anti-anorexigenic mechanism of RKT.

In conclusion, we confirmed that cisplatin-induced anorexia is attenuated by RKT in rats. We revealed that cisplatin increases *POMC* and *CART* and decreases *NPY* in the ARC, which may also be one of the causes of anorexia, whereas intragastric RKT administration with cisplatin i.p. attenuates these reactions. Our study suggests that RKT may have therapeutic potential for cisplatin-induced anorexia.

Acknowledgements

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ORIGINAL ARTICLE

A c-fos-Monomeric Red Fluorescent Protein 1 Fusion Transgene is Differentially Expressed in Rat Forebrain and Brainstem after Chronic Dehydration and Rehydration

M. Yoshimura*, J. Ohkubo*, A. Katoh*, M. Ohno*, T. Ishikura*, T. Kakuma†, H. Yoshimatsu†, D. Murphy‡ and Y. Ueta*

*Department of Physiology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan.

†Department of Internal Medicine 1, School of Medicine, Oita University, Yufu, Japan.

‡Molecular Neuroendocrinology Research Group, The Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol, Bristol, UK.

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We have previously shown that an acute osmotic stimulation induces the expression of a c-*fos* and monomeric red fluorescent protein 1 (mRFP1) fusion transgene in osmosensitive rat brain areas, including the supraoptic (SON) and paraventricular nuclei (PVN). However, the effects of chronic stimuli, such as dehydration, have not been investigated. In the present study, the expression patterns of the c-*fos*-mRFP1 fusion gene in the forebrain and the brainstem of male and female transgenic rats were studied in seven experimental groups: *ad lib.* water (euhydration), water deprivation for 12, 24 or 48 h (dehydration) and water deprivation for 46 h + *ad lib.* water for 2, 6 or 12 h (rehydration). The number of cells that express nuclear mRFP1 fluorescence was quantified in the hypothalamus, the circumventricular organs and the brainstem. Compared to the euhydrated state, the number of transgene expressing cells significantly increased in all forebrain areas and in the rostral ventrolateral medulla after dehydration and 2 h of rehydration. In the nucleus of the solitary tract and area postrema, the number of mRFP1 fluorescent cells was markedly increased after 2 h of rehydration. Although the number of mRFP1 fluorescent cells in the organum vasculosum laminae terminalis, median preoptic nucleus and subfornical organ remained significantly increased after 6 h of rehydration, reaching control levels after 12 h of rehydration, the number of mRFP1 fluorescent cells in the SON and the PVN reached control levels after 6 h of rehydration. There were no significant differences between male and female rats. These results show that the expression of the c-*fos*-mRFP1 fusion gene changes in the forebrain and the brainstem not only after acute osmotic stimulation, but also after chronic osmotic stimulation. Interestingly, these studies reveal the differential activation of different neuronal groups over the time course of dehydration and rehydration.

Key words: c-*fos*, red fluorescent protein, osmotic stimulation, brain, transgenic rat

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Correspondence to:
Yoichi Ueta, Department of Physiology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807-8555, Japan
(e-mail: yoichi@med.uoeh-u.ac.jp).

The expression of the c-*fos* gene, assayed at the RNA or the protein level, has been widely used as a marker of the neuronal activity elicited in the central nervous system by various physiological stimuli (1–6). Previous studies have used electrophysiological methods (7) and *in situ* hybridisation histochemistry for c-*fos* mRNA (8), or immunocytochemistry for Fos and Fos-related proteins (9–13), to reveal a network of osmosensitive areas in the brain involved in body fluid regulation. Recently, we have described the generation of a transgenic rat line that expresses a c-*fos* and monomeric red fluorescent protein 1 (mRFP1) fusion gene (7). Acute osmotic stimulation of these transgenic rats induced the expression of the c-*fos*-mRFP1 fusion gene in the osmosensitive areas, including the supraoptic (SON) and paraventricular nuclei (PVN), the organum vasculosum of the lamina terminalis (OVLT), the median preoptic nucleus (MnPO) and the subfornical organ (SFO) (7). In the brainstem, acute osmotic stimulation also induced c-*fos*-mRFP1 fusion gene expression in the nucleus of the solitary tract (NTS) and rostral ventrolateral medulla (RVLM) of the brainstem but not in the area postrema (AP) (M. Y. and Y. U.'s unpublished data). Thus, in the present study, we examined the effects of the chronic osmotic

stimulus of dehydration on mRFP1 signals in hypothalamic and brainstem regions. We also investigated the effect of subsequent rehydration, and compared male and female subjects.

Materials and methods

Animals

Adult male c-*fos*-monomeric RFP1 transgenic rats (320–550 g body weight) and randomly cycling adult female c-*fos*-monomeric RFP1 transgenic rats (290–370 g body weight) were individually housed and maintained under a 12 : 12 h light/dark cycle (lights on 07.00 h) at 23–25 °C. All experiments were performed in strict accordance with guidelines on the use and care of laboratory animals issued by the Physiological Society of Japan, and were approved by the Ethics Committee of Animal Care and Experimentation of University of Occupational and Environmental Health.

Experimental procedures

All transgenic rats had access to *ad lib.* food throughout the experiments. Rats were divided into seven groups: euhydration (*ad lib.* to water, male: n = 4, female: n = 6), 12 h of dehydration (water deprivation for 12 h, male: n = 4, female: n = 6), 24 h of dehydration (water deprivation for 24 h, male: n = 4, female: n = 6), 48 h of dehydration (water deprivation for 48 h, male: n = 4, female: n = 7), 2 h of rehydration (water deprivation for 46 h + *ad lib.* to water for 2 h, male: n = 4, female: n = 12), 6 h of rehydration (water deprivation for 46 h + *ad lib.* to water for 6 h, male: n = 4, female: n = 6) and 12 h of rehydration (water deprivation for 46 h + *ad lib.* to water for 12 h, male: n = 4, female: n = 6). Body weights were measured at the beginning of the experiments, 46 h after the onset of dehydration, and at the end of the experiments after different lengths of dehydration and rehydration. After treatments, rats were deeply anaesthetised with pentobarbital sodium (pentobarbital; 50 mg/kg i.p), followed by perfusion for tissue fixation. Blood samples were taken transcardinally during perfusion, and were collected into chilled reaction tubes (Greiner Bio-One, Kremsmuenster, Austria) containing heparin. Blood samples were centrifuged at 1000 ***g*** for 10 min at 4 °C. After the blood was centrifuged, a sample of plasma (150 μm) was taken for measuring plasma sodium concentration (P-Na) (SRL, Tokyo, Japan) and 15 μm was used for measuring plasma osmolarity (P-Osm) using a ONE-TEN osmometer (FISKE, Norwood, MA, USA). The rats were perfused transcardially with 0.1 M phosphate-buffered saline (PBS) (pH 7.4) containing heparin (1000 U/l) followed by 4% (wt/v) paraformaldehyde. The brain and brainstem were then removed and divided into three blocks. The blocks were postfixed with the same fixative for 48 h at 4 °C. The tissues were then immersed in 20% (wt/v) sucrose in 0.1 M PBS for 48 h at 4 °C for cryoprotection. Then, the tissues were cut into 40 μm sections using a microtome (Komatsu Electronics, Hiratsuka, Japan). After being cut, they were stored in 0.1 M PBS at 4 °C.

Fluorescent microscopic observation

The sections were mounted onto the glass slides and directly observed by fluorescence microscopy (Eclipse E 600; Nikon, Tokyo, Japan) with a RFP filter (Nikon) to investigate c-*fos*-mRFP1 expression. We counted the cells that express nuclear mRFP1 fluorescence in each area of each divided group. Digital images of each section were captured. Printed images were blinded and RFP positive cells were counted by two people to avoid bias. Double-counting was carefully avoided by marking the cell. The observed areas were the OVLT, MnPO, SFO, SON, PVN, AP, NTS and RVLM.

Statistical analysis

All data were analysed by one-way ANOVA with the Student–Newman–Keul's t-test (ORIGIN PRO, version 8.5J; Lightstone, Tokyo, Japan). $P < 0.05$ was considered statistically significant. All data are presented as the mean ± SEM.

Immunohistochemistry for c-Fos

We examined c-Fos like immunoreactivity (LI). Adult male (350–400 g body weight) and female (250–300 g body weight) Wistar rats were divided into three groups: water *ad lib.* (E, male: n = 3, female: n = 3), water deprived for 48 h (48 h WD, male: n = 3, female: n = 3) and rehydration for 12 h after 46 h of water deprivation (R, male: n = 3, female: n = 3). Deeply anaesthetised animals were perfused transcardially with 0.1 M phosphate buffer (PB) (pH 7.4) containing heparin (1000 U/l saline) followed by 4% (w/v) paraformaldehyde and 0.2% (w/v) picric acid in 0.1 M PB. The brains were then removed and divided into three blocks that included the forebrain, hypothalamus and brain stem. The blocks were postfixed with 4% (w/v) paraformaldehyde and 0.2% (w/v) picric acid in 0.1 M PB for 48 h at °C. The tissues were then cryoprotected in 20% (w/v) sucrose in 0.1 M PB for 48 h at °C. Serial sections of 40 μm were cut using a microtome. The sections were rinsed twice with 0.1 M PBS containing 0.3% Triton X-100 and incubated in 0.1 M PBS containing 0.3% (v/v) Triton X-100 with 1% (v/v) hydrogen peroxidase for 60 min. They were then rinsed twice with 0.1 M PBS containing 0.3% (v/v) Triton X-100. The floating sections were incubated with a primary anti-c-Fos antibody (Sigma-Aldrich Japan Co. LLC., Tokyo, Japan) at a dilution of 1 : 500 in 0.1 M PBS containing 0.3% (v/v) Triton X-100 at 4 °C for 4 days. After washing for 20 min in 0.1 M PBS containing 0.3% (v/v) Triton X-100, the sections were incubated for 120 min with a biotinylated secondary antibody solution (dilution 1 : 250) and finally with an avidin-biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA) for 120 min. The peroxidase in the sections was visualised with 0.02% (w/v) diaminobenzidine in a Tris buffer containing 0.05% (v/v) hydrogen peroxide for 1.5 min. The sections were mounted onto gelatin-coated slides, air dried, dehydrated in 100% (v/v) ethanol, cleared using xylene, and then finally coverslipped and examined under a light microscope. The presence of a dark brown labelled cell was judged to be indicative of c-Fos-LI-positive cells. We took digital images of each section per area and printed out to paper in expansion size. Then, the printed papers were blinded and c-Fos-LI positive cells were counted by two people to avoid bias. To prevent double-counting, we checked the cross mark in the printed paper every time we count the c-Fos-LI positive cell.

Results

Body weight changes of the male c-*fos*-mRFP1 transgenic rats

The body weight of male euhydrated rats increased during the experimental period (Fig. 1A). Body weight decreased during dehydration (Fig. 1A) but increased again with rehydration (Fig. 1B). All data are presented as a percentage of the weight at the beginning of the experiments.

Plasma measurements of the male c-*fos*-mRFP1 transgenic rats

Water deprivation for 48 h significantly increased P-Osm ($P < 0.01$ versus all other groups; Fig. 1C) but did not increase plasma sodium concentration (Fig. 1D) compared to the euhydrated state.

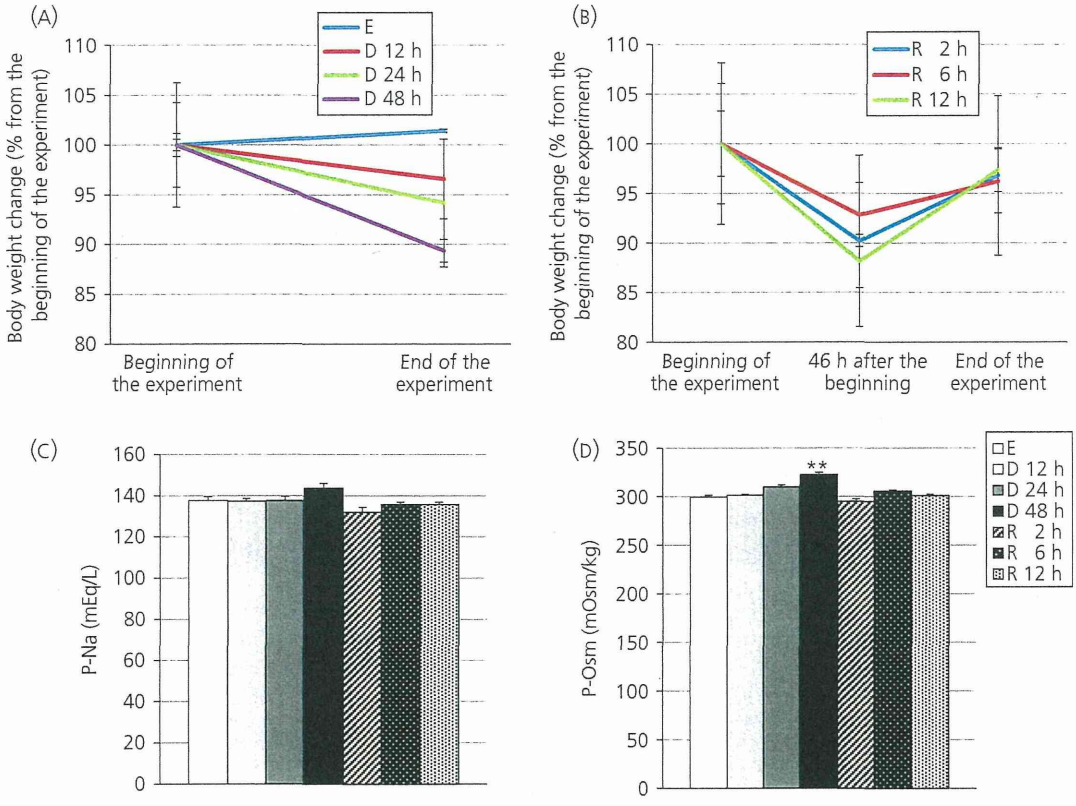

Fig. 1. Body weight changes and plasma measurements in male rats. (A) Body weight changes on euhydration and 12, 24 and 48 h of dehydration. (B) Body weight changes on 2, 6 and 12 h of rehydration. All data are presented as a percentage of the weights at the beginning of the experiment. (C) Plasma sodium concentration (P-Na) in male rats, (D) Plasma osmolality (P-Osm) in male rats. E, *ad lib.* to water; D 12 h, water deprivation for 12 h; D 24 h, water deprivation for 24 h; D 48 h, water deprivation for 48 h; R 2 h, water deprivation for 46 h + *ad lib.* to water for 2 h; R 6 h, water deprivation for 46 h + *ad lib.* to water for 6 h; R 12 h, water deprivation for 46 h + *ad lib.* to water for 12 h. **$P < 0.01$ versus all other groups. All data are presented as the mean ± SEM.

Expression patterns of mRFP1 in the male forebrain

Expression of mRFP1 in male rats was observed in the OVLT, MnPO, SFO, SON and PVN of euhydrated, 12-, 24- or 48-h dehydrated rats, and in rats dehydrated for 46 h then rehydrated for 2, 6 or 12 h (Fig. 2). Quantification of transgene expression (Fig. 4) involved counting the number of mRFP1 positive cells contained within the respective nuclei delineated by the dotted white line (Fig. 2A-a–A-e).

In all forebrain regions examined, very few mRFP1 positive cells were seen in the euhydrated state (Figs 2A-a–A-e and 4). However, dehydration resulted in a significant increase in the number of mRFP1 positive cells (Figs 2B-a–B-e, C-a–C-e, D-a–D-e and 4). In particular, in the SON and the PVN, mRFP1 positive cells were observed throughout the whole SON and the magnocellular divisions of the PVN (Fig. 2B-e, C-e, D-e).

At the 2-h rehydration time point, although transgene expression is falling in the SFO, SON and PVN (Figs 2E-c–E-e and 4), the number of mRFP1 positive neurones in the OVLT and MnPO remain at dehydrated levels (Figs 2E-a, E-b and 4). In the SON and PVN, mRFP1 expression reached euhydrated levels by 6 h of rehydration (Figs 2F-d, F-e and 4), whereas the number of mPFP1 positive cells in the OVLT, MnPO and SFO remained significantly elevated (Figs 2F-a–F-c and 4). Following 12 h of rehydration, basal, euhydrated levels of mRFP1 expression were restored in all forebrain regions (Figs 2G-a–G-e and 4). However, the time course of this decline was not uniform.

Expression patterns of mRFP1 in the male brainstem

Expression of mRFP1 in male rats was observed in AP, NTS and RVLM of euhydrated, 12-, 24- or 48-h dehydrated rats, and in rats dehydrated for 46 h then rehydrated for 2, 6 or 12 h (Fig. 3). Quantification of transgene expression involved counting the number of mRFP1 positive cells (Fig. 4) contained within respective nuclei delineated by the dotted white line (Fig. 3A-a–A-c). In all brainstem regions examined, very few mRFP1 positive cells were seen in the euhydrated state (Figs 3A-a–A-c and 4). Dehydration resulted in a significant increase in the number of mRFP1 positive cells in the RVLM (Figs 3B-c, C-c, D-c and 4) but not in the AP (Figs 3B-a, C-a, D-a and 4) nor the NTS (Figs 3B-b, C-b, D-b and 4). Only after 2 h of rehydration did the number of mRFP1 positive cells increase in the AP (Figs 3E-a and 4) and the NTS (Figs 3E-b and 4).

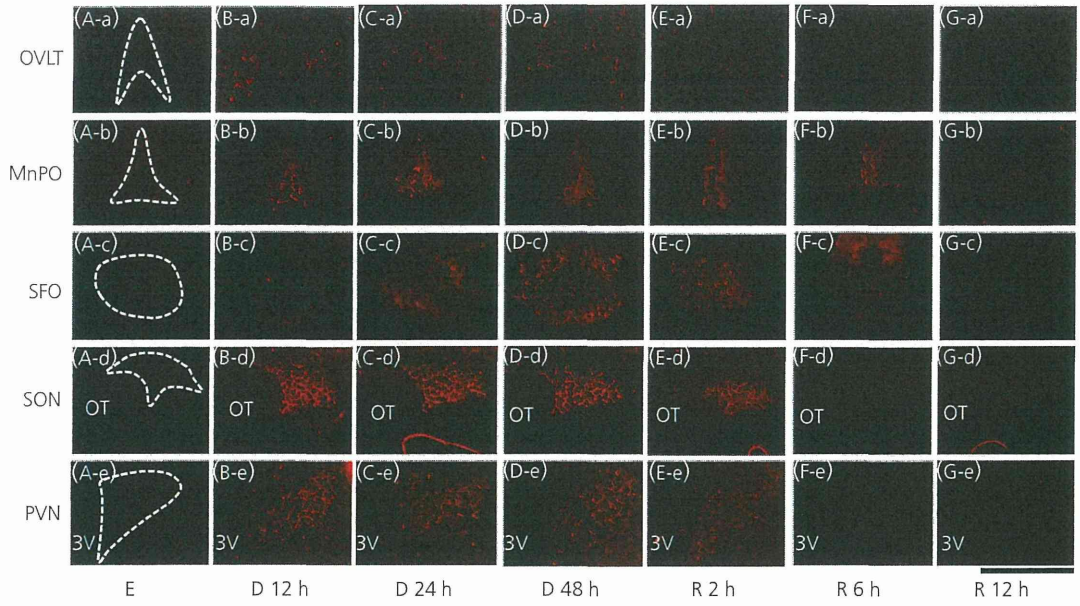

Fig. 2. Digital images of representative examples of monomeric red fluorescent protein 1 (mRFP1) expression patterns in the forebrain in male rats. The white dotted lines show the location of analysis (A-a–A-e). The conditions used in these studies are shown at the bottom of each vertical column, and the brain areas being assayed are shown on the left. E, *ad lib.* to water; D 12 h, water deprivation for 12 h; D 24 h, water deprivation for 24 h; D 48 h, water deprivation for 48 h; R 2 h, water deprivation for 46 h + *ad lib.* to water for 2 h; R 6 h, water deprivation for 46 h + *ad lib.* to water for 6 h; R 12 h, water deprivation for 46 h + *ad lib.* to water for 12 h; OT, optic tract; 3V, third ventricular; OVLT, organum vasculosum of the lamina terminalis; MnPO, median preoptic nucleus; SFO, subfornical organ; SON, supraoptic nucleus; PVN, paraventricular nucleus. Scale bar = 500 μm.

In the RVLM, the number of mRFP1 positive cells following 2 h of rehydration (Fig. 3E-c) was not statistically changed compared to dehydration (Fig. 4). After 6 h of rehydration, basal, euhydrated levels of mRFP1 expression were restored in all brainstem regions (Figs 3F-a–F-c and 4) and this low level of expression was maintained to the 12-h rehydration time point (Figs 3G-a–G-c and 4).

Body weight changes of the female *c-fos*-mRFP1 transgenic rats

The body weight of male euhydrated rats increased during the experimental period (Fig. 5A). Body weight decreased during dehydration (Fig. 5A) but increased again with rehydration (Fig. 5B). All

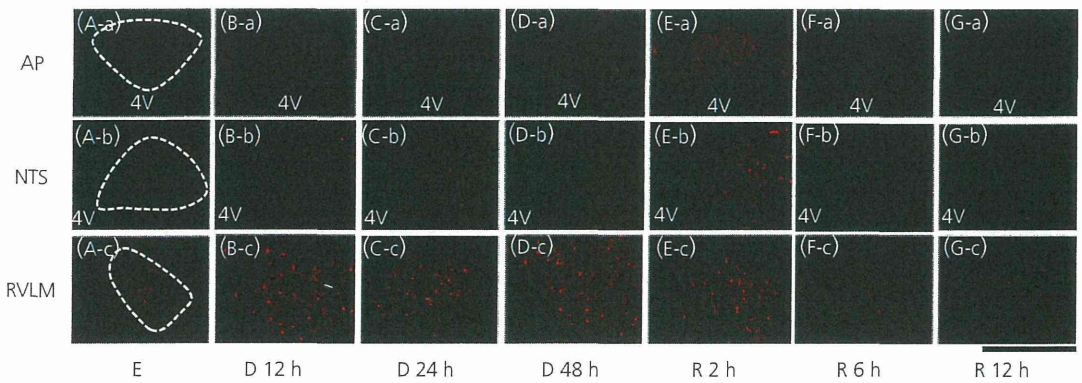

Fig. 3. Digital images of representative examples of monomeric red fluorescent protein 1 (mRFP1) expression patterns in the brainstem in male rats. The white dotted lines show the location of analysis (A-a–A-c). The conditions used in these studies are shown at the bottom of each vertical column, and the brain areas being assayed are shown on the left. E, *ad lib.* to water; D 12 h, water deprivation for 12 h; D 24 h, water deprivation for 24 h; D 48 h, water deprivation for 48 h; R 2 h, water deprivation for 46 h + *ad lib.* to water for 2 h; R 6 h, water deprivation for 46 h + *ad lib.* to water for 6 h; R 12 h, water deprivation for 46 h + *ad lib.* to water for 12 h; 4V, fourth ventricular. Scale bar = 500 μm.

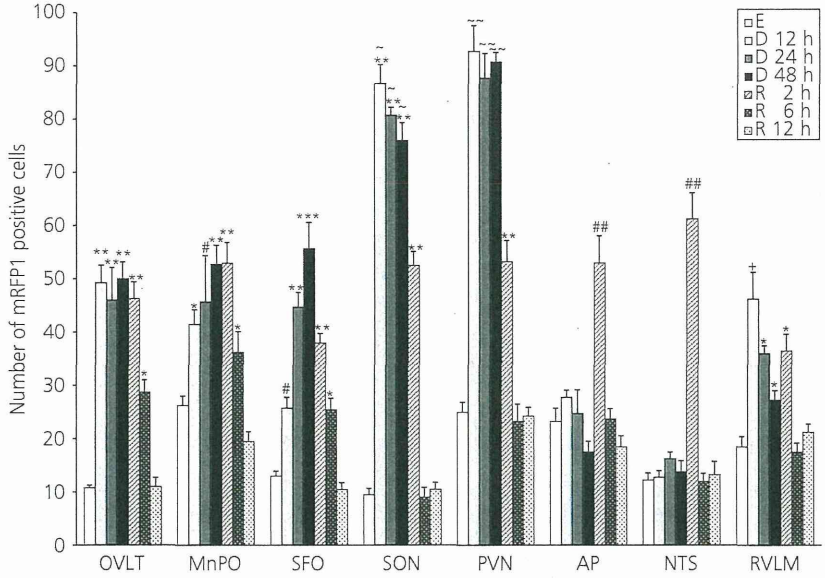

Fig. 4. Changes in number of monomeric red fluorescent protein 1 (mRFP1) positive cells in the forebrain and brainstem in male rats. E, *ad lib.* to water; D 12 h, water deprivation for 12 h; D 24 h, water deprivation for 24 h; D 48 h, water deprivation for 48 h; R 2 h, water deprivation for 46 h + *ad lib.* to water for 2 h; R 6 h, water deprivation for 46 h + *ad lib.* to water for 6 h; R 12 h, water deprivation for 46 h + *ad lib.* to water for 12 h. Each group, n = 4. *P < 0.05 versus E and R 12 h. **P < 0.01 versus E, R 6 h and R 12 h. ***P < 0.01 versus E, D 12 h, R 6 h and R 12 h. $^{+}$P < 0.05 versus E, R 6 h and R 12 h. OVLT, organum vasculosum of the lamina terminalis; MnPO, median preoptic nucleus; SFO, subfornical organ; SON, supraoptic nucleus; PVN, paraventricular nucleus; AP, area postrema; NTS, nucleus of the solitary tract; RVLM, rostral ventrolateral medulla. $^{\#}$P < 0.05 versus R 12 h. $^{\#\#}$P < 0.01 versus all other groups. $^{\sim}$P < 0.05 versus R 2 h. $^{\sim\sim}$P < 0.01 versus E, R 2 h, R 6 h and R 12 h. Data are presented as the mean ± SEM.

data are presented as a percentage of the weight at the beginning of the experiments.

Plasma measurements of the female *c-fos*-mRFP1 transgenic rats

Water deprivation for 48 h significantly increased P-Osm (P < 0.01 versus all other groups; Fig. 5c) but did not increase plasma sodium concentration (Fig. 5b) compared to the euhydrated state. There were no other statistically differences between male or female groups.

Expression patterns of mRFP1 in the female forebrain

Expression of mRFP1 in female rats was observed in OVLT, MnPO, SFO, SON and PVN of euhydrated, 12-, 24- or 48-h dehydrated rats, and in rats dehydrated for 46 h then rehydrated for 2, 6 or 12 h (Fig. 6). Quantification of transgene expression (Fig. 8) involved counting the number of mRFP1 positive cells contained within respective nuclei delineated by the dotted white line (Fig. 6A-a–A-e).

In all forebrain regions examined, very few mRFP1 positive cells were seen in the euhydrated state (Figs 6A-a–A-e and 4). However, dehydration resulted in a significant increase in the number of mRFP1 positive cells (Figs 6B-a–B-e, C-a–C-e, D-a–D-e and 8). In particular, in the SON and the PVN, mRFP1 positive cells were observed throughout the whole SON and the magnocellular divisions of the PVN (Fig. 6B-e, C-e, D-e).

At the 2-h rehydration time point, although transgene expression is falling in the SFO, SON and PVN (Figs 6E-C–E-e and 8), the number of mRFP1 positive neurones in the OVLT and MnPO remain at dehydrated levels (Figs 6E-a, E-b and 8). In the SON and PVN, mRFP1 expression reached euhydrated levels by 6 h of rehydration (Figs 6F-d, F-e and 8), whereas the number of mPFP1 positive cells in the OVLT, MnPO and SFO remained significantly elevated (Figs 6F-a–F-c and 8). After 12 h of rehydration, basal, euhydrated levels of mRFP1 expression were restored in all forebrain regions (Figs 6G-a–G-e and 8). However, the time course of this decline was not uniform. There were significant differences in expression only in the MnPO and SFO between males and females.

Expression patterns of mRFP1 in the brainstem

Expression of mRFP1 in female rats was observed in AP, NTS and RVLM of euhydrated, 12-, 24- or 48-h dehydrated rats, and in rats dehydrated for 46 h then rehydrated for 2, 6 or 12 h (Fig. 7). Quantification of transgene expression (Fig. 8) involved counting the number of mRFP1 positive cells contained within respective nuclei delineated by the dotted white line (Fig. 7A-a–A-c). In all brainstem regions examined, very few mRFP1 positive cells were seen in the euhydrated state (Figs 7A-a–A-c and 8). Dehydration resulted in a significant increase in the number of mRFP1 positive cells in the RVLM (Figs 7B-c, C-c, D-c and 8) but not in the AP (Figs 7B-a, C-a, D-a and 8), nor the NTS (Fig. 7 -b, C-b, D-b and 8). Only after 2 h of rehydration did the number of mRFP1 positive