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.

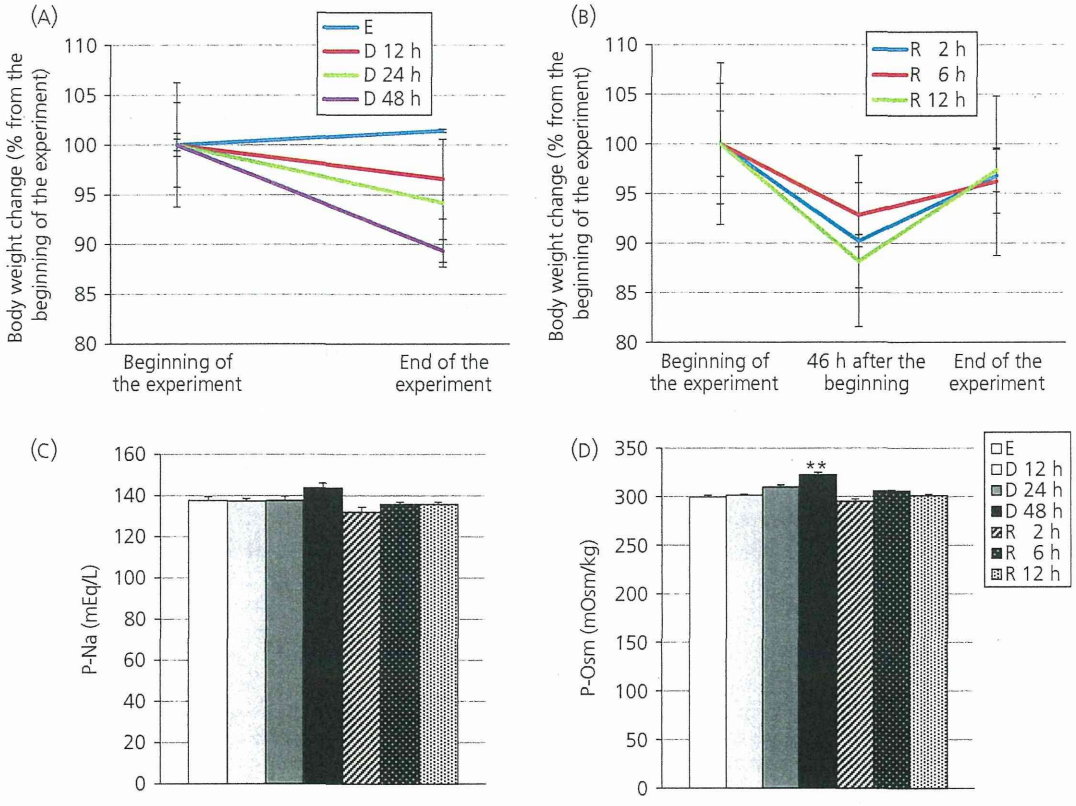

Fig. 1. Body weight changes and plasma measurements in male rats. (A) Body weight changes on euhydration and 12, 24 and 48 h of dehydration. (B) Body weight changes on 2, 6 and 12 h of rehydration. All data are presented as a percentage of the weights at the beginning of the experiment. (C) Plasma sodium 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).

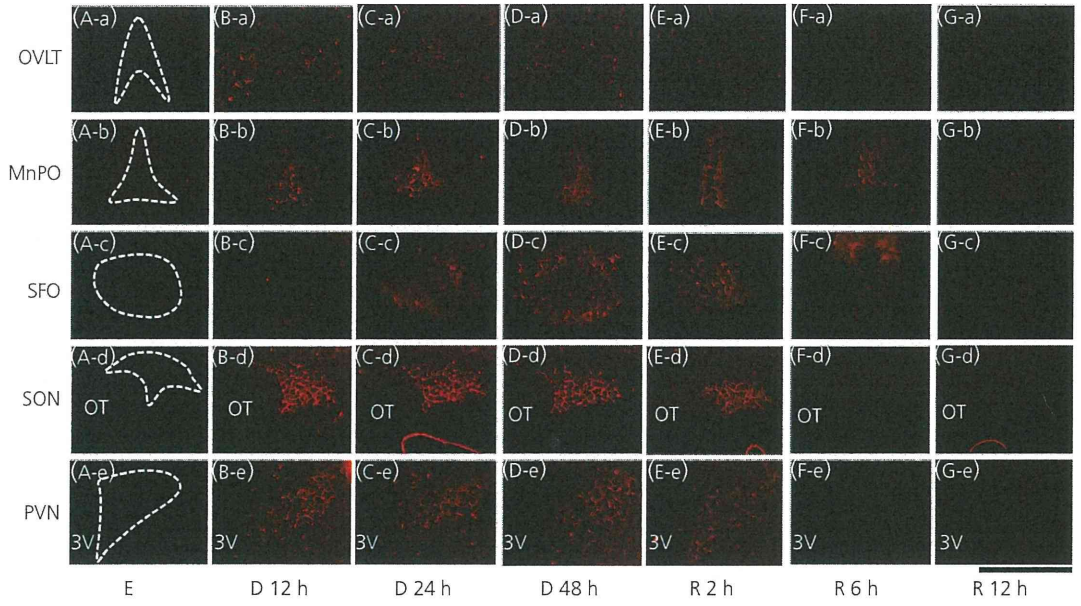

Fig. 2. Digital images of representative examples of monomeric red fluorescent protein 1 (mRFP1) expression patterns in the forebrain in male rats. The white dotted lines show the location of analysis (A-a–A-e). The conditions used in these studies are shown at the bottom of each vertical column, and 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

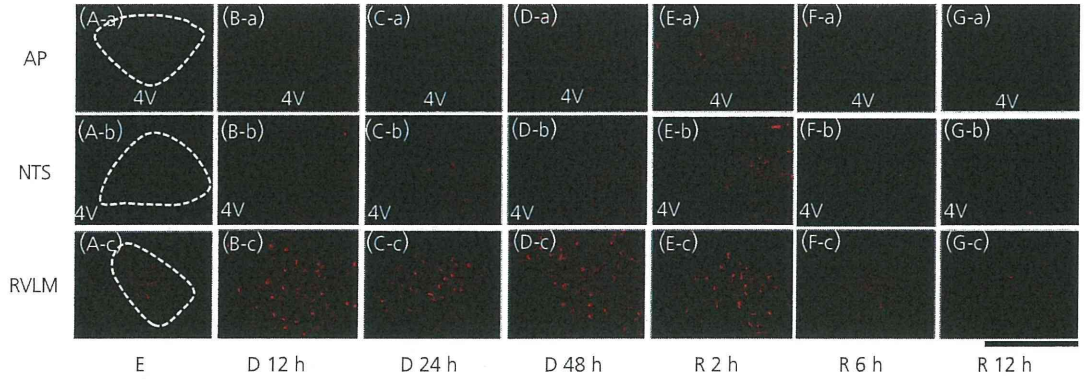

Fig. 3. Digital images of representative examples of monomeric red fluorescent protein 1 (mRFP1) expression patterns in the brainstem in male rats. The white dotted lines show the location of analysis (A-a–A-c). The conditions used in these studies are shown at the bottom of each vertical column, and 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.

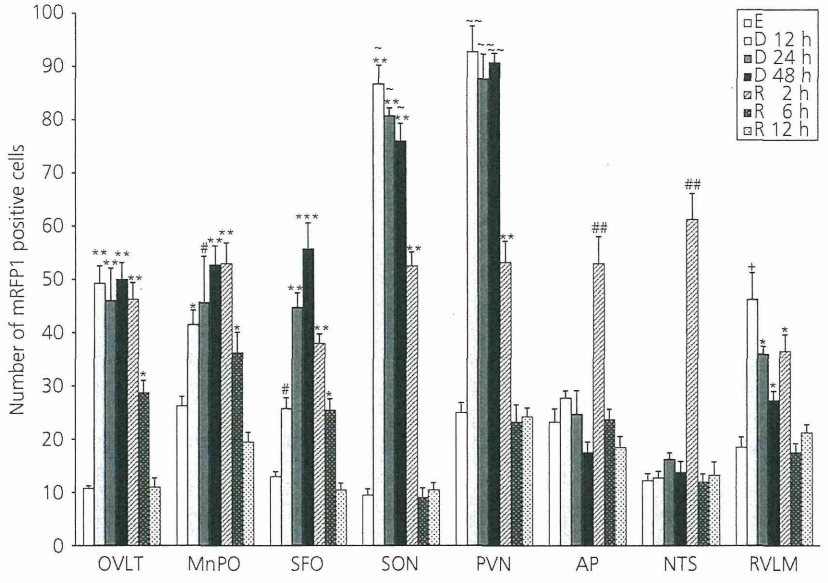

Fig. 4. Changes in number of monomeric red fluorescent protein 1 (mRFP1) positive cells in the forebrain and brainstem in male rats. E, *ad lib.* to water; D 12 h, water deprivation for 12 h; D 24 h, water deprivation for 24 h; D 48 h, water deprivation for 48 h; R 2 h, water deprivation for 46 h + *ad 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. ~P < 0.05 versus R 2 h. ~~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. 5D) 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

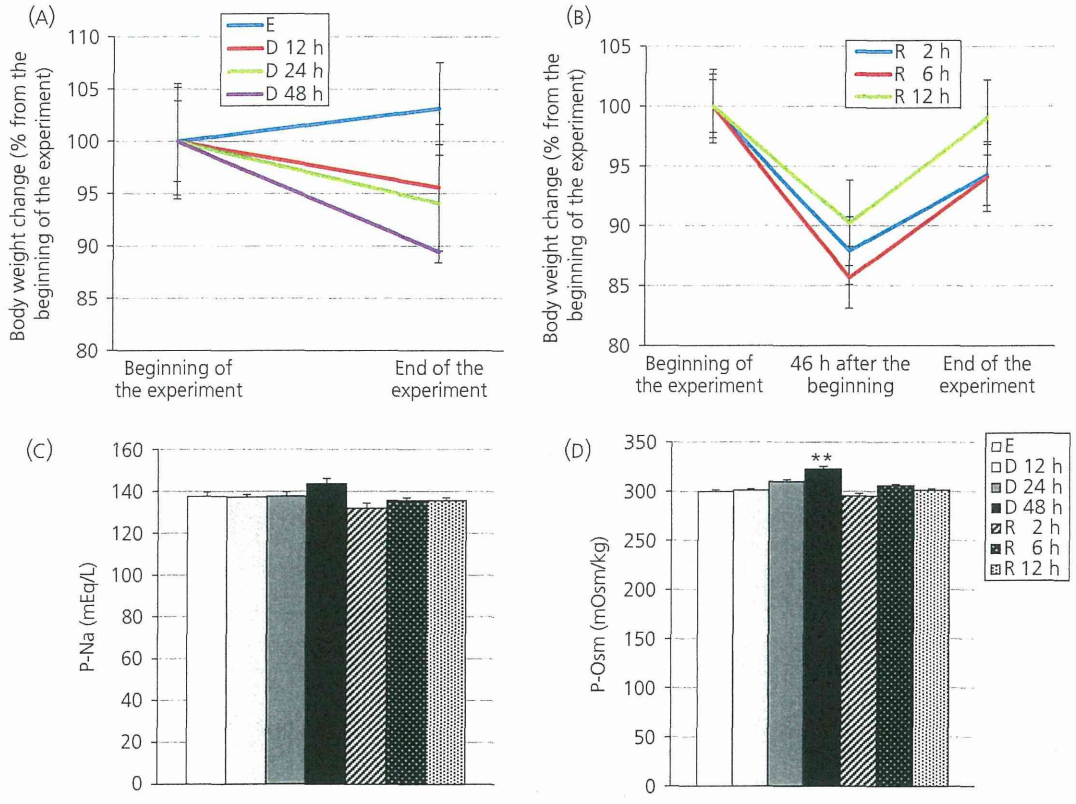

Fig. 5. The body weight change and plasma measurements in female rats. (A) Body weight changes on euhydration and 12, 24 and 48 h of dehydration. (B) Body weight changes on 2, 6 and 12 h of rehydration. All data are presented as percentages from the beginning of the experiments. (C) Plasma sodium concentration (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.

cells increase in the AP (Figs 7E-a and 8) and the NTS (Figs 7E-b and 8).

In the RVLM, the number of mRFP1 positive cells following 2 h of rehydration (Fig. 7E-c) were not statistically changed compared to dehydration (Fig. 8). After 6 h of rehydration, basal, euhydrated levels of mRFP1 expression were restored in all brainstem regions (Figs 7F-a–F-c and 8) and this low level of expression was maintained to the 12-h rehydration time point (Figs 7G-a–G-c and 8). Similar results were seen in female rats, with no significant differences identified between the sexes.

Expression patterns of c-Fos-LI positive cells in the brain and brainstem

Expression of c-Fos-LI cells in male and female rats was observed in the OVLT, MnPO, SFO, SON, PVN, AP, NTS and RVLM in euhydrated rats, 48 h dehydrated rats, and in rats dehydrated for 46 h then rehydrated for 2, 6 or 12 h (Fig. 9). Quantification of transgene expression involved counting the number of c-Fos-LI positive cells. In all the regions examined, very few c-Fos-LI positive cells were seen in the euhydrated state (Fig. 9A-a–A-h, D-a–D-h). Dehydration resulted in a significant increase in the number of c-Fos-LI positive cells in all the observed regions (Fig. 9B-a–B-h, E-a–E-h). Then the number of the cells returned to the euhydrated level after 12 h of rehydration (Fig. 9C-a–C-h, F-a–F-h). There were no significant differences identified between the sexes.

Discussion

The present study has revealed the effects of chronic osmotic stimulation and subsequent water rehydration on mRFP1 fluorescence in specific forebrain and the brainstem regions of male and female c-*fos*-mRFP1 transgenic rats. We observed strong mRFP1 signals after dehydration in all the osmosensitive areas, as previously reported in Fos immunostaining studies (8–10). This may suggest that the mRFP1 signals have at least equal sensitivity or more to immunostaining for Fos protein. However, the assessment of mRFP1 expression is relatively straightforward, and acts as a facile and sensitive proxy by which to quantify neuronal activity in osmosensitive neurones.

In the male and female forebrain SON and PVN, the hindbrain RVLM, and in the OVLT (a forebrain circumventricular organ, CVO), 12 h of dehydration resulted in a massive and significant increase in the number of mRFP1 positive cells, and this did not change at

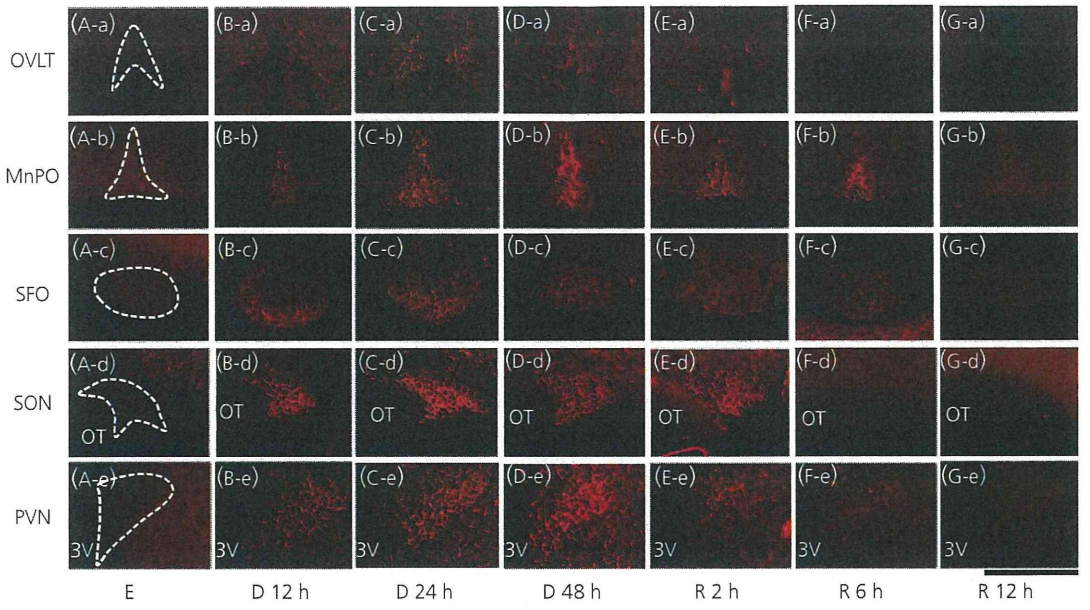

Fig. 6. Digital images of representative examples of monomeric red fluorescent protein 1 (mRFP1) expression patterns in the forebrain in female rats. The white dotted lines show the location of analysis (A-a–A-e). The conditions used in these studies are shown at the bottom of each vertical column, and 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.

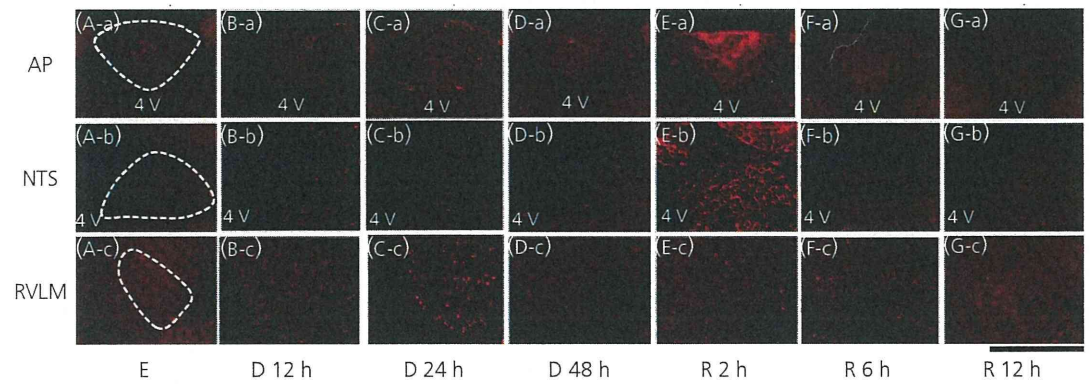

Fig. 7. Digital images of representative examples of monomeric red fluorescent protein 1 (mRFP1) expression patterns in the brainstem in female rats. The white dotted lines show the location of analysis (A-a–A-c). The conditions used in these studies are shown at the bottom of each vertical column, and 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; AP, area postrema; NTS, nucleus of the solitary tract; RVLM, rostral ventrolateral medulla. Scale bar = 500 μm.

24 or 48 h of dehydration. Thus, regardless of the degree of progressive bodily water depletion, there is no change in the number of mRFP1 positive cells beyond the initial induction over the first 12 h of the stimulus. This is also the case for the female forebrain OVLT CVO. However, in contrast, progressive dehydration induced a gradual increase in mRFP1 positive cells in the male MnPO and SFO forebrain CVOs. The physiological significance of this sexual dimorphism remains to be determined. Note that we studied randomly cycling female transgenic rats, and we concede that oestrous cycle differences may skew our results to an unknown extent. Several studies report that there are oestrogen receptors in the MnPO and SFO (11,12). Oestrogen may attenuate Fos induction in the MnPO and SFO after physiological stimuli such as dehydration.

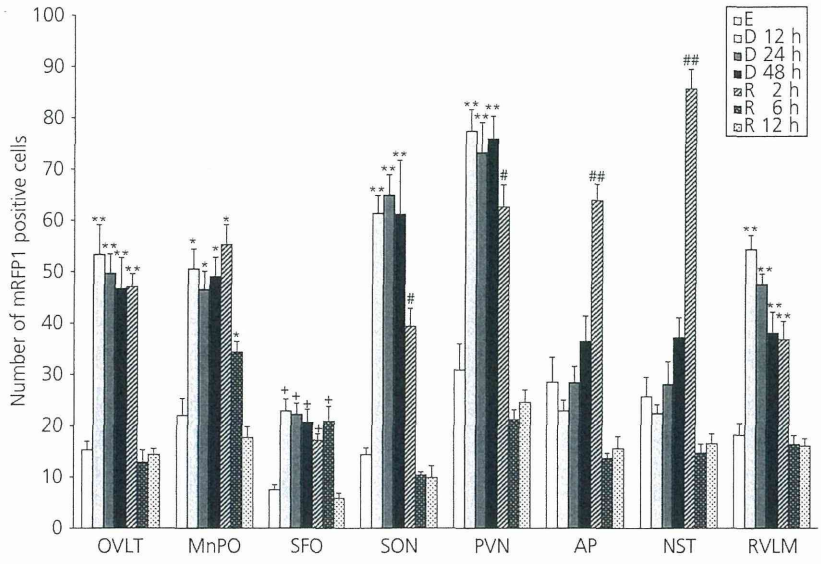

Fig. 8. Changes in number of monomeric red fluorescent protein 1 (mRFP1) positive cells in the forebrain and brainstem in female rats. E, *ad lib.* to water (n = 6); D 12 h, water deprivation for 12 h (n = 6); D 24 h, water deprivation for 24 h (n = 6); D 48 h, water deprivation for 48 h (n = 7); R 2 h, water deprivation for 46 h + *ad lib.* to water for 2 h (n = 12); R 6 h, water deprivation for 46 h + *ad lib.* to water for 6 h (n = 6); R 12 h, water deprivation for 46 h + *ad lib.* to water for 12 h (n = 6). $^{*}P < 0.05$ versus E and R 12 h. $^{**}P < 0.01$ versus E, R 6 h and R 12 h. $^{+}P < 0.05$ versus E and R 12 h. $^{\#}P < 0.05$ versus E, R 6 h and R 12 h. $^{\#\#}P < 0.01$ versus all other groups. 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. Data are presented as the mean ± SEM.

Strikingly, dehydration had no inductive effect on mRFP1 expression in the NTS. Gottlieb *et al.*(8) have reported an increase in *c-fos* in the NTS and AP after 48 h dehydration, which is consistent with our c-Fos-LI study (Fig. 9). The rats that they used were Sprague-Dawley rats (250–350 g body weight). It is likely that strain and weight differences contribute to the differences between their experiments and ours. For example, water deprivation for 48 h may develop different degrees of hypovolemia, which resulted in differential mRFP1 expression in the NTS and AP (14).

Similarly, and in contrast to the forebrain CVOs, including the osmosensitive SFO, the AP, a hindbrain CVO, does not up-regulate mRFP1 as a consequence of chronic dehydration. We have previously used Affymetrix microarray expression profiling (Affymetrix, Santa Clara, CA, USA) to compare those genes that are significantly regulated by chronic (72 h) dehydration in the SFO (15) and AP (16). Interesting, many more genes (305) were regulated in the SFO compared to the AP (53), suggesting that the SFO is much more sensitive to chronic water deprivation that the AP. Furthermore, the genes regulated by chronic dehydration in the SFO may be transcriptional targets of *c-fos*.

As well as documenting the effects of chronic dehydration on mRFP1 expression in the brains of our transgenic rats, we also investigated the consequences of 2, 6 and 12 h of rehydration. Strikingly, and in marked contrast to the lack of responses to chronic dehydration, 2 h of rehydration induced a massive but transient increase in mRFP1 expression in the AP and NTS; by 6 h, expression was back to basal levels. The NTS is involved in gustatory processing (17) and we speculate that the NTS shows increases in mRFP1 positive cells associated with water intake. The NTS receives afferent projections from other brain regions and a variety of organ systems (6,18), which may also play a role in the expression of mRFP1 after rehydration. The privileged location of the AP outside of the blood–brain barrier makes this sensory circumventricular organ a vital player in the control of autonomic functions by the central nervous system (19). It has been described that the AP is involved in nausea and vomiting (20). Although details of the mechanism are unknown, it may be presumed that the AP responds by nausea that occurs after rehydration.

After 2 h of rehydration, the number of mRFP1 positive cells in the OVLT, MnPO and SFO was as same as in the dehydrated state. These three forebrain CVOs have been described to contain osmosensitive neurones that project to the SON and PVN, and they play an important role in cardiovascular regulation and body fluid homeostasis (5,21,22). It has been suggested that Fos immunostaining in the OVLT and MnPO after rehydration is a result of sustained activation of the renin–angiotensin system (23).

The number of mRFP1 positive cells returned to the euhydrated state in the SON and PVN after 6 h of rehydration, and were attenuated in the OVLT and MnPO, then completely returned to the basal euhydrated levels after 12 h of rehydration in all the areas we observed. Previous studies suggested that the number of cells that were immunostained for Fos in the SON returned to the euhydrated levels after only 2 h of water intake (8). It is thus possible that our mRFP1 Fos-surrogate has a different half-life to the native Fos protein, or that our transgene is subject to marginally different regulatory control.

Previous studies have revealed that the MnPO is involved noradrenergic control of body fluid volume, and it has been suggested

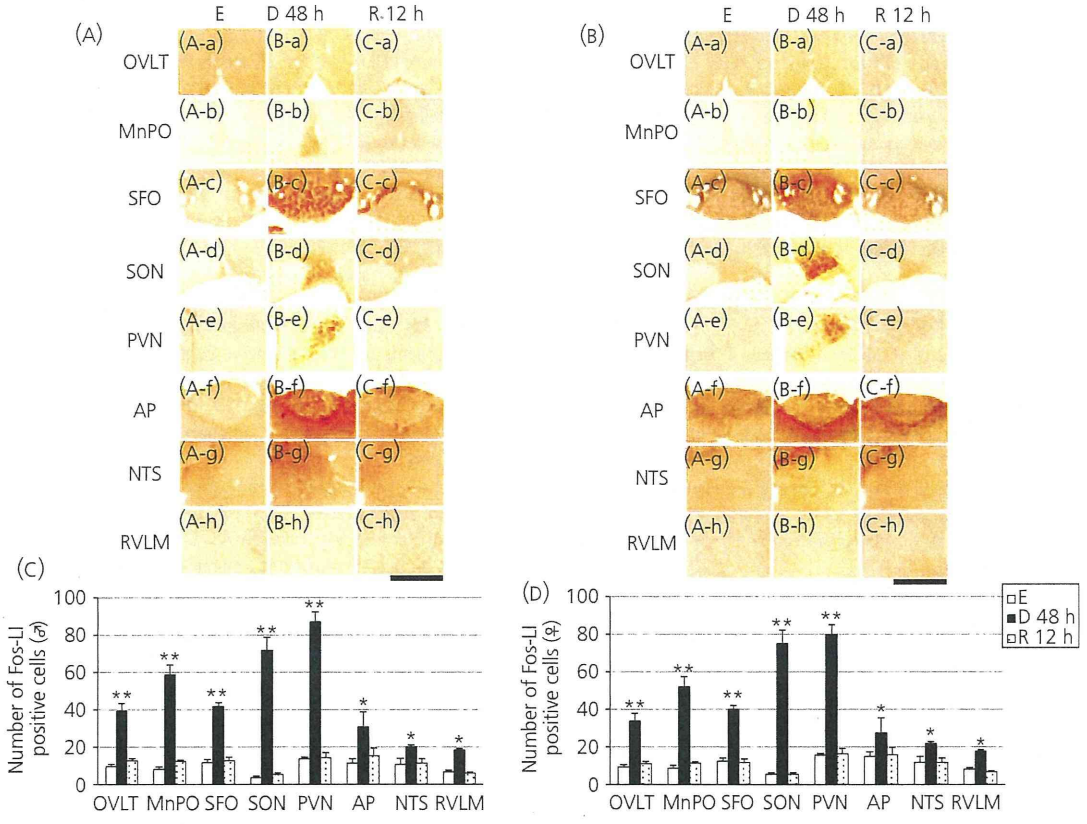

Fig. 9. Immunostaining for Fos protein in the brain and brainstem. (A) Digital images representing c-Fos immunostaining in the brain and brainstem in male Wistar rats. (B) Digital images representing c-Fos immunostaining in the brain and brainstem in female Wistar rats. (C) Quantification of number of c-Fos-LI cells in male Wistar rats. (D) Quantification of number of c-Fos-LI cells in female Wistar rats. E, *ad lib.* to water (male; n = 3, female; n = 3); D 48 h, water deprivation for 48 h (male; n = 3, female; n = 3); R 12 h, water deprivation for 46 h + *ad lib.* to water for 12 h (male; n = 3, female; n = 3). *$P < 0.05$ versus E and R 12 h. **$P < 0.01$ versus E and R 12 h. Scale bar = 500 μm. 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. Data are presented as the mean ± SEM.

that the system may play an important role in the elicitation of hypovolaemia-induced dipsogenic response (24). The SFO has been described as an integral player in fluid homeostasis, and has been implicated in the *de novo* synthesis of angiotensin II (25). The increases of mRFP1 positive cells in the RVLM may indicate the activation of baroreceptor reflex. It is expected that the blood pressure changed by water deprivation and rehydration, and these may the cause of increases of the mRFP1 positive cells in the RVLM.

In conclusion, we determined the expression patterns of mRFP1 in the transgenic central nervous system after osmotic stimuli. Both acute and chronic osmotic stimulation caused the induction of mRFP1 fluorescence in osmosensitive areas in c-*fos*-mRFP1 transgenic rats. Our results are similar to previous studies of Fos immunostaining (8–10) and it was demonstrated to be of at least equivalent sensitivity compared to native Fos protein detection. Thus, the c-*fos*-mRFP transgenic rats are useful animal model for various physiological studies including the central responses to acute and chronic osmotic challenges. Coupled with deep-brain 'optrode' detection of mRFP1 (26), it may be possible to detect neuronal activation in living, conscious animals.

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ORIGINAL PAPER

Effects of food deprivation on the hypothalamic feeding-regulating peptides gene expressions in serotonin depleted rats

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Abstract We examined the effects of serotonin (5-HT) depletion induced by peripheral injection of 5-HT synthesis inhibitor *p*-chlorophenylalanine (PCPA) on the expression of feeding-regulating peptides expressions by using in situ hybridization histochemistry in adult male Wistar rats. PCPA pretreatment had no significant effect on basal levels of *oxytocin*, *corticotropin-releasing hormone* (*CRH*), *thyrotropin-releasing hormone* (*TRH*), *pro-opiomelanocortin* (*POMC*), *cocaine and amphetamine-regulated transcript* (*CART*), *neuropeptide-Y* (*NPY*), *agouti-related protein* (*AgRP*), *melanin-concentrating hormone* (*MCH*) or *orexin* in the hypothalamus. Food deprivation for 48 h caused a significant decrease in *CRH*, *TRH*, *POMC*, and *CART*, and a significant increase in *NPY*, *AgRP* and *MCH*. After PCPA treatment, *POMC* and *CART* did not decrease despite food deprivation. *NPY* was significantly increased by food deprivation with PCPA, but was attenuated compared to food deprivation without PCPA. These results suggest that the serotonergic system in the hypothalamus may be involved in the gene expression of *POMC*, *CART*, and *NPY* related to feeding behavior.

Keywords Serotonin · Feeding · Neuropeptides · Hypothalamus · *p*-chlorophenylalanine

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Introduction

Many physiological behaviors, including emotion, memory, learning, awakening, attention, thermoregulation, and feeding, are manipulated by the serotonergic system [1–4]. With regard to feeding behavior, although its regulation by serotonin (5-HT) is broadly well described, the mechanisms or central pathways that mediate this behavior are still poorly understood. While current approaches to identifying the mechanisms of feeding regulation have focused on feeding-regulating neuropeptides [5], possible interactions between monoamine and neuropeptides in appetite regulation have been clarified [6, 7].

p-chlorophenylalanine (PCPA) depletes brain serotonin (5-HT) by synthetic inhibition [8, 9], of which systemic pretreatment for 2 days resulted in 95 % depletion in hypothalamic serotonin [10]. This pharmaceutical enables us to explicate the interactions between 5-HT and feeding-regulating neuropeptides. Generally, anorexigenic peptides, such as corticotropin-releasing hormone (CRH) [11] and thyrotropin-releasing hormone (TRH) [12, 13], are down-regulated by food deprivation; conversely, orexigenic peptides, such as neuropeptide-Y (NPY) [14] and melanin-concentrating hormone (MCH) [15], are up-regulated. If the feeding-regulating neuropeptides do not