Prolactin-Releasing Peptide as a Novel Stress Mediator in the Central Nervous System*

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ABSTRACT

A1/A2 noradrenergic neurons in the medulla oblongata are well known to mediate stress signals in the central nervous system. Stress activates A1/A2 noradrenergic neurons, and then noradrenaline (NA) stimulates ACTH secretion through hypothalamic CRH. On the other hand, PRL-releasing peptide (PrRP) was recently isolated and was found to be produced by some A1/A2 neurons and the dorsomedial hypothalamic nucleus. We previously demonstrated that PrRP neurons make synapse-like contact with hypothalamic CRH neurons. In fact, we demonstrated that the central administration of PrRP stimulates CRH-mediated ACTH secretion. Furthermore, it has been reported that PrRP neurons in A1/A2 cell groups are colocalized with tyrosine hydroxylase (TH), which is known as the marker enzyme of catecholaminergic neurons. These data strongly suggest that PrRP is related to stress-responsive signal transduction, and PrRP and NA cooperatively modulate the hypothalamo-pituitary-adrenal axis. We therefore examined the effect of water immersion-restraint stress on c-Fos protein accumulation in PrRP- and TH-immunoreactive neurons. The synergistic effects of PrRP and NA on plasma ACTH elevation were also examined. The results clearly showed that c-Fos protein accumulation dramatically increased in the nuclei of A1/A2 and dorsomedial hypothalamic nucleus PrRP neurons. In addition, it was revealed that c-Fos protein was specifically expressed in the PrRP/TH double positive cells in the A1/A2 cell groups. We also demonstrated that the central administration of PrRP and NA in combination at subactive (noneffective) doses clearly induced plasma ACTH elevation. Here we report that PrRP is a novel and important mediator of the hypothalamo-pituitary-adrenal axis for the stress response. (Endocrinology 142: 2032–2038, 2001)

PRL-RELEASING PEPTIDE (PrRP) was recently isolated as a ligand of an orphan seven-transmembrane domain receptor (hGR3) (1). PrRP is known to stimulate PRL release both in vitro (1) and in vivo (2, 3). It has been demonstrated that PrRP-producing cells exist in the dorsomedial hypothalamic nucleus (DM) and in the A1 region of the ventrolateral reticular formation and the A2 region of the nucleus of the solitary tract in medulla oblongata (4–11). These PrRP-producing neurons extend their axons to magnocellular and paraventricular neurosecretory cells in the paraventricular hypothalamic nucleus (PVH) and then make synapse-like contact with these cell bodies (4, 12), in which PrRP receptors are known to exist (10). These morphological data strongly suggest that PrRP plays an important biological role in the neuroendocrine system. In fact, we previously found that intracerebroventricular (icv) administration of PrRP significantly increases plasma oxytocin and vasopressin secretion from magnocellular neurosecretory cells in the PVH (13). In addition, we recently found that central administration of PrRP stimulates ACTH and β-endorphin secretion via CRH from the paraventricular neurosecretory cells in the PVH (12). On the other hand, noradrenergic neurons that project to the PVH are located in A1/A2 cell groups in the medulla oblongata, and a minor portion are found in the locus coeruleus (A6 cell group). These noradrenergic neurons are well known to mediate stress signals in the central nervous system (CNS) (14–19). In fact, lesions of catecholaminergic cell groups in the brainstem or their ascending fibers block or reduce stress-induced changes in the hypothalamo-hypophyseal system (15, 20, 21). In addition, it is commonly accepted that stress activates A1/A2 noradrenergic neurons (18), and noradrenaline (NA) stimulates ACTH secretion through a hypothalamic CRH (19). Interestingly, it has been reported that PrRP neurons in A1/A2 cell groups are colocalized with NA in A1/A2 cell groups (9–11). These data indicate that PrRP mediates stress signals in the CNS as well as NA. The colocalization of PrRP and NA in the same neurons also suggests their synergistic effects. Therefore, we examined the effect of stress on immediate response gene (c-Fos) accumulation in A1/A2 PrRP neurons. The synergistic effects of PrRP and NA on CRH-mediated ACTH secretion were also analyzed in this study. We report here that stress is a potent activator of PrRP neurons, and that PrRP is an important stress mediator in the CNS.

Materials and Methods

Animals

Adult Wistar male rats were housed in group cages illuminated from 0800–2000 h (12-h cycle). Room temperature varied from 21–24 C. Tap water and laboratory chow were available ad libitum. All procedures

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were performed in accordance with institutional guidelines for animal
care at Saitama University and Takeda Chemical Industries Co., Ltd.

**Immunocytochemistry**

Proteins were localized as previously described (4, 12, 13). Briefly, the
animals were deeply anesthetized and fixed with 5% acrolein in 0.07 m
phosphate buffer (pH 7.4). Frozen sections (40 μm) were prepared from
the brains. A mouse monoclonal antibody (P2L-1T) and rabbit poly-
clonal anti-bovine PrRP (no. 8, provided by Takeda Chemical Industries
Co., Ltd.) were used as primary antibodies for PrRP. Mouse monoclonal
antitryptase hydroxylase clone TH2 (Sigma, St. Louis, MO) and rabbit polyclonal anti-c-Fos (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)
were used for the detection of tryptase hydroxylase (TH) and c-Fos,
respectively. For fluorescence immunocytochemistry for TH and PrRP
(shown in Fig. 1), PrRP (labeled with no. 8) and TH were visualized,
respectively, with Alexa488-conjugated goat antimouse IgG (Molecular
Probes, Inc., Eugene, OR) in red and Alexa594-conjugated goat antirab-
bit IgG (Molecular Probes, Inc.) in green. For the double staining of PrRP
and c-Fos (shown in Fig. 2), PrRP (labeled with P2L-1T) and c-Fos were
visualized, respectively, with diaminobenzidine (DAB) in brown and
cobalt-DAB in black after labeling with peroxidase by the avidin-biotin-peroxidase complex method. For the triple staining of PrRP, TH, and
c-Fos (shown in Fig. 4), PrRP (labeled with no. 8) and TH were visu-
alized, respectively, with Alexa488-conjugated goat antimouse IgG (Mo-
lecular Probes, Inc.) in red and Alexa594-conjugated goat antirabbit IgG
(Molecular Probes, Inc.) in green, and c-Fos was labeled with peroxidase
by the avidin-biotin-peroxidase complex method and then stained with
DAB in brown, which changed to blue under a confocal laser
microscope.

For morphometry, a complete series of frontal sections (40 μm each)
from the caudal end of the area postrema to 1.4 mm posteriorly were
analyzed. All neurons in the lateral half of each nuclear area were
counted. To avoid double counting, only neurons with a complete nu-
cleus were counted.

**Water immersion-restraint stress**

Water immersion-restraint stress was performed as previously de-
scribed (22). Rats were immobilized in stainless restrainers (Natsume,
Tokyo, Japan) with the lower half of their bodies immersed in water.

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![Fig. 1. Photomicrographs showing the double fluorescence immunostaining to locate PrRP and TH in the A1/A2 cell groups. PrRP and TH are stained red and green, respectively, and the double positive neurons are shown in yellow. A–C, Photomicrographs of the A1 region. D–F, Photomicrographs of the A2 region. A and D, Photomicrographs showing only PrRP staining. B and E, Photomicrographs showing only TH staining. C and F, Photomicrographs showing both PrRP and TH staining. G and H, High magnification photomicrographs of C and F, respectively. All PrRP-ir neurons were colocalized with TH in the A1 (A–C and G) and A2 (D–F and H) cell groups. However, TH-ir neurons did not always coexist with PrRP in the A2 cell groups (D–F and H). Arrows indicate the PrRP-negative/TH-positive neurons. Scale bars, 100 μm (A–F) and 50 μm (G and H).](https://academic.oup.com/endo/article-abstract/142/5/2032/2989363)
(25 ± 1 C). They were decapitated 2 or 6 h after the onset of immobilization. Intact (nonstressed) rats were used as a control group.

**Synthetic peptide**

Rat PrRP31 was synthesized using a combination of recombinant DNA technology and a cysteine-specific cyanlation reaction (23).

**Intracerebroventricular administration, blood sampling, and measurement**

For icv administration of PrRP, male Wistar rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and then fixed on a stereotaxic apparatus (Narishige, Tokyo, Japan) with the incisor bars adjusted to 3.3 mm below the interaural line. A stainless steel guide cannula (id, 0.4 mm; od, 0.5 mm; AG-8, Eicom, Kyoto, Japan) was inserted into the right lateral ventricle. The stereotaxic coordinates, set according to the atlas of Paxinos and Watson (24), were: anterior-posterior, 7.7 mm above the interaural line; lateral, 1.8 mm from the midline; and height, 6.8 mm above the interaural line. The cannula was fixed to the skull with acrylic dental cement and screws. The cannula-implanted rats were housed as described above for at least 7 days after the operation. PrRP31, NA, or PBS containing 0.5% BSA was injected into the right lateral ventricle. Blood samples were collected via the venous catheter in ice-cooled tubes containing 0.01 M EDTA, 300 kallikrein inhibitor units/ml aprotinin, and 2.5 × 10^4 M o-phenanthroline at 15 min pre- and post-icv injection. Plasma was separated from blood and stored at −80 C until the measurement of ACTH. The plasma ACTH concentration was measured with a RIA kit for ACTH (Mitsubishi Yuka Co., Tokyo, Japan). All plasma ACTH measurements were performed between 0900 and 1200 h to avoid the influence of the circadian rhythm. The entire procedure has been described previously (12, 13).

**Statistical analysis**

The data were analyzed by one-way ANOVA with repeated measurements, and differences between treatment groups were evaluated using Dunnett’s multiple test. The statistical significance level was set at P < 0.05.

**Results**

**Double immunocytochemistry with PrRP and TH**

To determine the relationship between PrRP and A1/A2 NA cell groups, we examined the localization of immunoreactive PrRP and TH in A1/A2 cell groups by fluorescence immunocytochemistry. As previously reported (9–11), the
PrRP-immunoreactive (ir) neurons were colocalized with TH-ir neurons in the A1/A2 cell groups (Fig. 1). In these areas PrRP-ir neurons were always coexpressed with TH-ir neurons (data not shown). Inversely, most TH-ir neurons in A1 cell groups were also positive for PrRP (Fig. 1, A–C and G), but TH-ir neurons in A2 cell groups were not always positive for PrRP (Fig. 1, D–F and H). The results of morphometry are shown in Table 1. In nonstressed rats, the percentages of PrRP-ir cells among the total number of TH-ir cells were 98.4 ± 2.8% and 81.7 ± 3.2% in the A1 and A2 regions, respectively. These values did not significantly change after exposure to water immersion-restraint stress for 2 h in A1/A2 cell groups (Table 1).

Effect of water immersion-restraint stress on the c-Fos expression in PrRP neurons

The immediate response gene c-Fos was used as a marker of neural activation in this study. Rats exposed to nonstress and water immersion-restraint stress for 2 and 6 h were analyzed. Double immunocytochemistry for PrRP and c-Fos clearly showed that water immersion-restraint stress dramatically increased the number of c-Fos-positive nuclei in A1/A2 and DM PrRP-ir neurons (Fig. 2). Morphometry showed that the percentage of c-Fos expression in the PrRP-ir cells of A1/A2 cell groups was significantly increased by water immersion-restraint stress for 2 h compared with that in nonstressed rats, i.e. 8.1-fold (P < 0.01) in A1 and 3.3-fold (P < 0.01) in A2 neurons (Fig. 3). After 6-h stress exposure, it became 8.3-fold (P < 0.01) in A1 and 3.5-fold (P < 0.01) in A2 neurons. However, the c-Fos activation in DM was weaker than that in A1/A2 cell groups, i.e. 1.7-fold (P < 0.01) at 2 h and 1.6-fold (P < 0.01) at 6 h.

Triple immunocytochemistry for PrRP, TH, and c-Fos in A1/A2 cell groups after exposure to water immersion-restraint stress

Triple immunocytochemistry for PrRP, TH, and c-Fos in A1/A2 cell groups clearly showed that most PrRP/TH double positive neurons also become positive for c-Fos under water immersion-restraint stress compared with those in nonstressed rats (Fig. 4, A and B). However, most PrRP-negative/TH-positive neurons in A2 cell groups were not reactive to c-Fos (Fig. 4, C–F), i.e. PrRP-containing NA neurons responded specifically to water immersion-restraint stress in the A1/A2 cell groups. The results of morphometric analysis are shown in Table 2. Morphometry showed that the percentage of c-Fos expression in PrRP/TH-double positive neurons was 17.8 ± 5.0% in nonstressed rats in the A2 region, and that of PrRP-negative/TH-positive neurons was 0.5 ± 0.8%. In the A1 region, the percentage of c-Fos expression in PrRP/TH double positive neurons was 10.8 ± 13.1% in nonstressed rats. On the other hand, the percentage of c-Fos expression PrRP/TH double positive neurons was 90.5 ± 6.2% after exposure to water immersion-restraint stress in the A2 region, whereas that of PrRP-negative/TH-positive neurons was 2.2 ± 2.2%. In the A1 region, the percentage of c-Fos expression in PrRP/TH double positive neurons was 87.9 ± 6.3% after exposure to water immersion-restraint stress. The value for PrRP-negative/TH-positive neurons in the A1 region was not determined because there are almost no PrRP-negative/TH-positive neurons in the A1 region.

Effects of icv administration of PrRP and NA on the plasma ACTH level

The colocalization of NA and PrRP in the A1/A2 cell groups suggests the synergistic actions of these factors on the hypothalamo-pituitary-adrenal axis. We therefore performed coadministration (icv) of subjective (noneffective) doses of PrRP (1 nmol) and NA (0.1 nmol) to examine their synergistic effects on the plasma ACTH level. Blood was collected 15 min pre- and post-icv injection, and the blood collected 15 min before icv injection was used for measurement of basal ACTH levels in individual rats. PrRP (1 nmol) and NA (0.1 nmol) had no effect compared with basal plasma ACTH levels. In contrast, coadministration of PrRP (1 nmol) and NA (0.1 nmol) significantly increased (3.2-fold; P < 0.01) the plasma ACTH level compared with basal plasma ACTH levels (Fig. 5). These data suggest that PrRP and NA act synergistically to induce CRH-mediated plasma ACTH elevation.

Discussion

It is generally accepted that A1/A2 NA neurons extend their axons to the PVH and play an important role as stress mediators. These NA stimulate ACTH secretion through hypothalamic CRH (14–21); A1 NA neurons particularly promote vasopressin and oxytocin secretion (25–27). On the other hand, it was recently revealed that PrRP-producing cells exist in the DM in the hypothalamus and the A1/A2 region of the medulla oblongata (4). The axons of PrRP neurons project to the PVH (4), and central administration of PrRP clearly stimulates oxytocin and vasopressin secretion (13) and CRH-mediated ACTH secretion (12). In addition, it has been reported that A1/A2 PrRP neurons as well as the DM are retrogradely labeled after tracer injections in the PVH (11), which indicates that PrRP neurons in A1/A2 regions and also the DM are in direct contact with neurons in the PVH. These locations and the functional coincidence between NA and PrRP neurons strongly suggest that PrRP as well as NA may be related to stress responses in the CNS.

In this study we confirmed previous reports (9–11) showing that PrRP and TH are colocalized in A1/A2 cell groups (Fig. 1). Morphometry showed that PrRP-ir neurons were colocalized, with 81.7 ± 3.2% of the TH-ir neurons in the A2 region and 98.4 ± 2.8% of those in the A1 region (Table 1). Morales et al. reported that some TH-ir neurons in the A1/A2

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**TABLE 1.** Percentages of PrRP-ir cells among the total numbers of TH-ir cells

| Region | Nonstress | Stress, 2 h |
|--------|-----------|------------|
| A1     | 98.4 ± 2.8| 81.7 ± 3.2 |
| A2     | 98.1 ± 1.7| 76.2 ± 4.9 |

In the A2 region, TH-ir cells were divided into two subpopulations, PrRP/TH-double positive neurons and PrRP-negative/TH-positive neurons. The percentage of PrRP-ir neurons among the total number of TH-ir cells is not significantly different between nonstressed rats and rats exposed to water immersion-restraint stress for 2 h. Values are the mean ± SEM (n = 3).
regions also contain PrRP messenger RNA, i.e. 36.6 ± 13.7% of the TH-ir neurons in the A2 region and 35.2 ± 9.4% of those in the A1 cell groups are positive for PrRP messenger RNA (11). Their results were different from our data; however, this may be due to the different detection methods. At least both sets of data show that NA neurons in the A2 cell group are divisible into two subpopulations, i.e. PrRP-positive and -negative neurons. To determine whether these PrRP neurons respond to stress, we examined c-Fos expression in PrRP neurons under stress. Our results clearly indicated that water immersion-restraint stress activates A1/A2 PrRP-ir neurons. In addition, triple immunostaining for PrRP, TH, and c-Fos in the A1/A2 cell groups showed that PrRP/TH double positive and PrRP-negative/TH-positive neurons are distinct in the response to water immersion-restraint stress. Morphometry after triple immunostaining for PrRP, TH, and c-Fos also indicated that the percentage of c-Fos expression in PrRP/TH double positive neurons was 90.5 ± 6.2% after
plasma ACTH elevation. Values are the mean ± SEM (n = 6). *P < 0.01 vs. nonstress (A1, PrRP+/TH−). b P < 0.01 vs. nonstress (A2, PrRP+/TH−). c P < 0.01 vs. stress, 2 h (A2, PrRP+/TH−).

In the A1 and A2 regions, c-Fos protein expression was significantly increased in PrRP+/TH− neurons after exposure to water immersion-restraint stress. In the A2 region, PrRP+/TH− neurons specifically expressed c-Fos protein compared with PrRP−/TH− neurons after exposure to water immersion-restraint stress for 2 h. The value for PrRP+/TH− neurons in the A1 region was not determined because there were almost no PrRP+/TH− neurons in the A1 region. Values are the mean ± SEM (n = 3). NE, Not examined.

On the other hand, we previously demonstrated that PrRP neurons in the brainstem are related to suppression of food intake during an acute stress response. However, further study is needed to clarify the functional difference may exist between A1 and A2 PrRP neurons predominantly responded to water immersion-restraint stress.

As a result, we demonstrated that the central administration of PrRP (1 nmol) and NA (0.1 nmol) in combination at subactive doses clearly induced plasma ACTH elevation. This clearly showed that PrRP and NA cooperatively stimulate the hypothalamo-pituitary-adrenal axis.

Some functions of PrRP in the CNS (12, 13, 29–31) have been reported; however, the biological significance of A1/A2 PrRP neurons has not been discussed. In this study we first found that PrRP neurons in the brainstem are related to stress. We also showed that PrRP synergistically acts with NA to induce ACTH secretion. Our data showed that a subpopulation of A1/A2 NA cells expressing PrRP plays a more integral and specific role in stress responses in the CNS compared with neighboring non-PrRP-containing NA neurons. This novel stress-related signal pathway is schematically illustrated in Fig. 6. We believe that our findings suggest the biological significance of PrRP in the CNS. This may be supported by the previous report that icv administration of PrRP increases blood pressure (31) and plasma oxytocin and vasopressin levels (13), which are known to be common stress responses (32, 33). PrRP maybe regulate these stress-relating phenomena as a stress mediator.

The functional differences between PrRP-producing nuclei are not been understood at present. However, it is also known that A1 NA neurons mainly project to the magnocellular division of the PVH and regulate vasopressin and oxytocin neurons, whereas A2 NA neurons mainly project to the parvocellular division of the PVH and regulate CRH neurons (25–27). This indicates the possibility that a functional difference may exist between A1 and A2 PrRP neurons as well as NA. In addition, the c-Fos expression study showed that not only A1/A2 cell groups but also the PrRP-positive cell group in the DM respond to stress stimuli. To explain this phenomenon it is noteworthy that the DM is known to be related to food intake (34), and that PrRP suppresses food intake (30). This suggests that the DM may be related to suppression of food intake during an acute stress response. However, further study is needed to clarify the details of PrRP related-stress signal transduction.

**TABLE 2.** Percentages of c-Fos protein expression in PrRP/TH-double positive (PrRP+/TH+) and PrRP-negative/TH-positive (PrRP−/TH+) neurons

|       | A1 | A2 |
|-------|----|----|
|       | PrRP+/TH+ | PrRP+/TH− | PrRP−/TH+ | PrRP−/TH− |
| Nonstress | 10.8 ± 13.1 | NE | 17.8 ± 5.0 | 0.5 ± 0.8 |
| Stress, 2 h | 87.9 ± 6.3a | NE | 90.5 ± 6.2b | 2.2 ± 2.2c |

Fig. 5. Effect of coadministration of PrRP and NA into the right lateral ventricle on ACTH release in conscious male rats. The plasma ACTH levels, pretreatment (pre) and 15 min after icv injection (15 min) were compared. The icv administration of 0.5% BSA in PBS (control), 1 nmol PrRP, or 0.1 nmol NA had no effect on ACTH elevation. However, coadministration of 1 nmol PrRP and 0.1 nmol NA induced a large increase in the plasma ACTH level, which suggested that PrRP and NA acted synergistically to induce CRH-mediated plasma ACTH elevation. Values are the mean ± SEM (n = 4). *P < 0.01 vs. pre.

Fig. 6. Schematic representation showing the stress-related function of PrRP. PrRP coexists with medullary A1/A2 NA neurons, and these cells are peculiarly activated by stress. PrRP and NA are discharged from the neural terminals of the PVH and act synergistically to induce CRH-mediated ACTH elevation. ME, Median eminence.

hypothalamic CRH. As a result, we demonstrated that the central administration of PrRP (1 nmol) and NA (0.1 nmol) in combination at subactive doses clearly induced plasma ACTH elevation. This clearly showed that PrRP and NA cooperatively stimulate the hypothalamo-pituitary-adrenal axis.
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