Changes of Corticotropin-Releasing Factor(CRF) and Neuropeptide Y(NPY) of Rats in Response to Footshock or Reexposure to Conditions Previously Paired with Footshock*

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족부전기충격이나 족부전기충격과 연합-학습된 조건자극에 재노출시 흰쥐뇌내 Corticotropin-Releasing Factor(CRF)와 Neuropeptide Y(NPY)의 변동에 관한 연구*

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ABSTRACT

Corticotropin-releasing factor(CRF) and neuropeptide Y(NPY) are known to play important roles in mediating stress responses and stress-related behavior. To elucidate the role of neuropeptides in response to the condition that had paired with traumatic event, we observed the changes of CRF and NPY by immunohistochemistry using a conditioned footshock paradigm. Male Sprague-Dawley rats were placed in a shuttle box and exposed to 20 pairings of a tone(< 70dB, 5sec) followed by a footshock(FS, 0.8mA, 1sec) over 60min. A second group was exposed to the tone-footshock pairings, returned to the homecage for 2days, and then reexposed to the test chamber and 20tones alone for 60min, prior to sacrifice. Control groups were : a) sacrificed without exposure to FS ; b) exposed to the tone-footshock pairings and then sacrificed two days later ; or c) exposed to the chamber and tones alone, returned to the homecage for 2days and then reexposed to the chamber and 20tones over 60min prior to sacrifice. CRF was increased in animals exposed to FS or the aversive condition(context and tone) that had paired to FS in bed nucleus of the stria terminalis (BNST) compared to the control. NPY was increased by FS in amygdala and PVN, but the condition previously associated with FS results in slight increase only in amygdala area. These results suggest that the BNST

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appears to be the mostly involved neural circuit in response to explicit cues previously paired with footshock. Moreover, this study raise the possibility that increased CRF peptide in the BNST in response to re - exposure to the aversive condition may underlie, in part, the experience of conditioned fear - related anxiety behavior.

KEY WORDS : CRF NPY · Central nucleus of amygdala · Bed nusleus of the stria termalis · Conditioned fear.

Introduction

Corticotropin-releasing factor(CRF) and neuropeptide Y(NPY) are members of neuropeptides and are known to play important roles in mediating stress responses and stress-related behavior.[1-3] For CRF, immunoreactivity is localized in extrahypothalamic structures such as the central nucleus of the amygdala(CeA), bed nucleus of the stria terminalis(BNST), locus coeruleus, and parabrachial area[4] in addition to the paraventricular nucleus of hypothalamus(PVN).[5] Anxiety or fear reactions in response to stressor are thought to be mediated by CRF in extrahypothalamic area such as BNST[6][7] and CeA,[8] whereas CRF in PVN is involved in regulation of hypothalamic-pituitary-adrenal(HPA) axis, suggesting a parallel means for mediating behavioral and endocrine responses to stressors.

On the other hand, NPY is localized in amygdala, hypothalamus, periaqueductal area, and locus coeruleus of central nervous system. Contrary to CRF, NPY is consistently reported to elicit anxiolytic-like effects, and these effects can be observed in such diverse models of anxiety as elevated plus-maze test,[3] social interaction test[9] and fear-potentiated startle.[10] Moreover, microinjection of NPY into the CeA induces anxiolytic effect with high potency, indicating the involvement of the amygdala in the mediation of the anxiolytic effect of NPY.[9][11]

Recently, it has been demonstrated that both CRF and NPY are involved in anxiety related behavior in psychological stress such as conditioned fear paradigm,[12][13] Conditioned fear is a form of Pavlovian conditioning where experimental animals are trained to associate neutral stimuli(such as tone or context) with unconditioned aversive stimuli(usually an electric footshock). Conditioned fear has been suggested as one of the animal models to elucidate the mechanism of anxiety, since classical anxiolytic drug such as benzodiazepines inhibits conditioned fear-related behavior.[14] However, it was not fully clarified whether and where both neuropeptides are changed in response to conditioned fear. To answer this question, we observed changes of CRF and NPY peptide levels in response to footshock and reexposure to conditions(tone and context) previously paired with footshock.

Methods and Material

1. Animals

Male Sprague-Dawley rats(initial weight 230 - 260 gm, Samtaco, Seoul) were received 7days before experiment and housed 4/cage under a 12hr light-dark cycle (light on at 6 : 00 A.M). Food and water were available ad libitum.

2. Apparatus

Conditioned fear were tested in a shuttle box(GEMINI avoidance system, San Diego Instruments, San Diego, USA) consisting of two enclosures(53cm(w) × 53cm (h) × 32cm(d)). The grid floor, attached to a scrambled SDI shocker(San Diego Instruments) consisted of seven parallel stainless steel rods, spaced 1.3cm apart(center to center) and measuring 4mm in diameter. The conditioning chambers were cleaned after every session with a 70% ethanol to remove olfactory cues.

3. Conditioned fear paradigm

For 3 days before experiments, rats were transported to and remained at testing room for 8h to reduce environmental novelty. In experiment, after an initial acclimatization period of 5min in a shuttle box, rats were presented with either tone(2.9kHz, 82dB) or tone-foot-

shock parings. Animals were divided into 5 groups.

1) Home cage control groups without exposure to the conditioning chamber or tone-footshock pairings(represented as control, n=4).

2) A group of rats was placed in the conditioning chamber and exposed to 20 pairings of a 5 second tone (2.9kHz, 82dB) and a 1 second of footshock(0.8mA) over 60min before sacrifice(represented as FS, n=4).

3) A group of rats exposed to the tone-footshock pairings and then sacrificed 48hr later(represented as FS-48hr, n=4).

4) A group of rats exposed to the conditioning chamber and tones alone, returned to the home cage for 48hr and then re-exposed to the chamber and 20tones over 60min before sacrifice(represented as T-T, n=4).

5) A group of rats was exposed to the tone-footshock pairings, returned to the home cage for 48 hr, and then re-exposed to the conditioning chamber and 20tones alone for 60min before sacrifice(represented as FS-T, n=4).

4. Perfusion and making a brain slice

At the end of tone or footshock exposure, rats were immediately removed and transported to an adjacent room. Rats were anesthetized with pentobarbital(100 mg/kg, i.p.), and perfused intracardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1M sodium phosphate buffer, pH7.2(PPB). Serial coronal sections (30 μm in thickness) were obtained using a freezing microtome(Sliding Microtome HM 440E, MICROM International GmbH, Walldorf, Germany), and series of every 12th section through each brain were obtained. Immunohistochemical detection of CRF or NPY was done by sequential incubations of free-floating sections in 1) CRF or NPY antiserum raised in rabbit(Peninsula, Belmont, USA, 1：5000) for 4 days at 4℃, 2) biotinylated goat anti-rabbit IgG(1：2000, Vector Laboratories, Burlingame, CA) for 90 min at room temperature(RT), and finally 3) avidin-biotin-HRP complex (1：500, Vector Elite Kit；Vector Laboratories, Burlingame, USA) for 90 min at RT. All brain slices were treated simultaneously in staining nets and dishes(Brain Research Laboratories, Newton, USA). After incubation, the sections were rinsed three times for 10min in PBS. The sections were then immersed in 0.05M Tris-HCl buffer(pH7.6) containing 0.025% 3, 3'-diaminobenzidine-4HCl(DAB；Sigma, St. Louis, USA), and 0.003% H_2O_2, for 20min at RT. The reaction was stopped by three rinses in phosphate buffered saline. Sections were washed again, mounted, and coverslipped with Permount.

5. Image capture and analysis

The staining of immunocytochemistry was quantified by densitometry with a computer-based image analysis system. The system included a CCD camera mounted on an Olympus BH2 light microscope. The camera was connected to a Macintosh computer. The images of immunostained sections were captured with the NIH-Image v1.60 software. The video signals from the camera were converted into a gray scale digital image consisting of a 640×480grid of pixels. The brightness level of each pixel ranged from 0 to 255gray levels and images were saved as TIFF files. For quantification, each area was outlined manually with the Polygon tool of the NIH-Image v1.60 software. The mean grayscale values were converted to optical density(O.D.) values with a standard O.D. calibration curve generated from eleven preset neutral density filters(Stouffer Graphics Arts Equipment, South Bend, USA) in 0.1O.D. steps from 0 to 1.0. The experimental O.D. values were within the linear range of the calibration curve.

6. Statistical analysis

For each treatment groups, the optical density measurements from both sides of each animal were analyzed, yielding 8 - 12 determinations. The results expressed as percent of control were ranked and one-way analysis of variance(ANOVA) was used to evaluate the significance among experimental groups. Post hoc testing for the ANOVAs was performed using Tukey's honestly significant difference test or Dunnett's T3, if variances were unequal among the groups, when the ANOVA indicated a significant difference in percent of control among experimental groups.

Results

Changes of CRF peptide level in BNST in response

to footshock and reexposure to conditions(tones and context) previously paired with footshock.

A one-way ANOVA revealed a statistically significant treatment effect on CRF peptide level in BNST ($F4, 39 = 7.78$, $p < 0.01$). Post hoc analysis with a Tukey's honestly significant difference revealed that CRF peptide levels of FS and FS-T were significantly increased by 64%($p < 0.01$) and 63%($p < 0.01$), respectively, in BNST compared with those of control. In addition, CRF peptide level of FS-48hr in BNST was about 41% greater than that of control($p < 0.02$). However, CRF peptide level of T-T was increased by 27% compared with that of control, although this effect was not significant(Fig. 1).

Changes of CRF peptide level in PVN in response to footshock and reexposure to conditions previously paired with footshock.

CRF peptide levels of FS and FS-T in PVN showed a tendency for a decrease, but it was not statistically significant. It is noteworthy, however, that CRF peptide level of FS-48 hr in PVN was significantly decreased compared with those of control($F4,40 = 5.11$, $p < 0.01$), FS($p < 0.01$), and FS-T($p < 0.01$)(Fig. 2).

Changes of CRF peptide level in CeA in response to footshock and reexposure to conditions previously paired with footshock CRF peptide level of FS was increased by 18% compared with control, although it was not significant. There was a smaller increase in CRF peptide levels of T-T and FS-T compared with those of control.

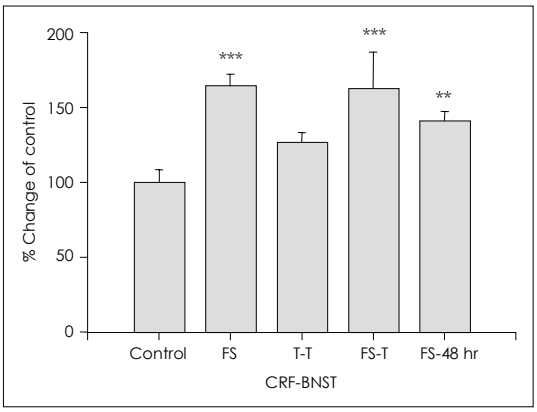

Fig. 1. Changes of CRF peptide level in BNST in response to footshock and reexposure to conditions(tones and context) previously paired with footshock (n=4 per group). ***$p < 0.01$ and **$p < 0.02$ compared to control. FS : group exposed to 20pairings of tone-footshock over 60min, T-T : group re-exposed to tones previously paired with tones and context prior to sacrifice, FS-T : group re-exposed to tones previously paired with 20tone-footshock pairings prior to sacrifice, FS-48hr : group sacrificed 48h after 20tone-footshock pairings over 60min.

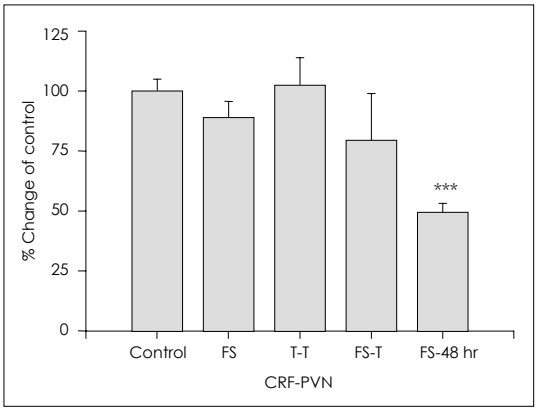

Fig. 2. Changes of CRF peptide level in PVN in response to footshock and reexposure to conditions(tones and context) previously paired with footshock (n=4 per group). ***$p < 0.01$ compared to control, FS, T-T. FS : group exposed to 20pairings of tone-footshock over 60min, T-T : group re-exposed to tones previously paired with tones and context prior to sacrifice, FS-T : group re-exposed to tones previ-ously paired with 20 tonefootshock pairings prior to sacrifice, FS-48 hr : group sacrificed 48h after 20tonefootshock pairings over 60min.

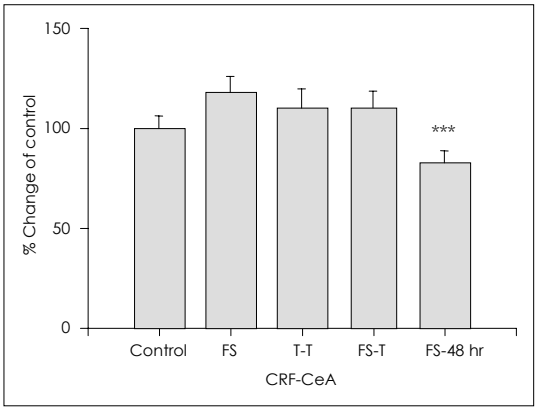

Fig. 3. Changes of CRF peptide level in CeA in response to footshock and reexposure to conditions(tones and context) previously paired with footshock (n=4 per group). ***$p < 0.01$ compared to control. FS : group exposed to 20pairings of tone-footshock over 60min, T-T : group re-exposed to tones previously paired with tones and context prior to sacrifice, FS-T : group re-exposed to tones previously paired with 20tone-footshock pairings prior to sacrifice, FS-48hr : group sacri-ficed 48h after 20tone-footshock pairings over 60min.

However, CRF peptide level of FS-48 hr in CeA was significantly decreased by 17% compared with that of control(F4,56=3.48, p<0.01)(Fig. 3).

Changes of NPY peptide level in amygdala and PVN

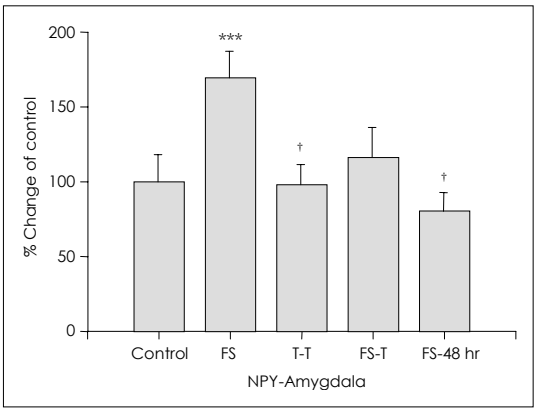

Fig. 4. Changes of NPY peptide level in the amygdala area in response to footshock and reexposure to conditions(tone and context) previously paired with footshock(n=4 per group). ***p<0.01 compared to control. †p<0.05 compared to FS. FS : group exposed to 20pairings of tone-footshock over 60min, T-T : group re-exposed to tones previously paired with tones and context prior to sacrifice, FS-T : group re-exposed to tones previously paired with 20tone-footshock pairings prior to sacrifice, FS-48hr : group sacrificed 48h after 20tone-footshock pairings over 60min.

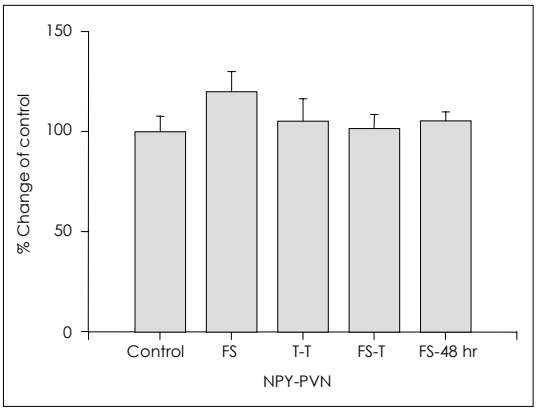

Fig. 5. Changes of NPY peptide level in PVN in response to footshock and reexposure to conditions(tones and context) previously paired with footshock (n=4per group). FS : group exposed to 20pairings of tone-footshock over 60min, T-T : group re-exposed to tones previously paired with tones and context prior to sacrifice, FS-T : group re-exposed to tones previously paired with 20tone-footshock pairings prior to sacrifice, FS-48hr : group sacrificed 48h after 20tone-footshock pairings over 60min.

in response to footshock and reexposure to conditions previously paired with footshock.

NPY peptide levels of FS in amygdala and PVN were increased significantly by 69%(F4,62=3.65, p<0.01) and 20%, respectively, compared to those of control (Figs. 4, 5). NPY peptide level of FS-48 hr in amygdala was decreased by 20% compared with that of control(Fig. 4). However, NPY peptide levels of T-T and FS-T in PVN were not different from those of control(Fig. 5).

Discussion

The results of the present study demonstrate that tone-footshock pairing alters CRF and NPY peptide level in a regionally specific manner. It increased CRF peptide level in BNST, CeA, but decreased CRF peptide level in PVN. NPY peptide level in amygdala and PVN was increased by tone-footshock pairing. Moreover, reexposure to tones and context previously paired with footshock over 60min increased CRF peptide level in BNST selectively, raising the possibility that CRF peptide in BNST plays an important role in conditioned fear-related behavior in response to conditions related with aversive exposure.

CRF is synthesized in the parvocellular paraventricular nucleus(PVN) neurons of the hypothalamus, where the highest concentrations of CRF in mammals are found. Stress is known to increase the release of CRF peptide from PVN, which, in turn, leads to activation of hypothalamic-pituitary-adrenal(HPA) axis. In the present study, exposure to tone-footshock pairings over 60min showed a tendency for a decrease in CRF peptide level of PVN, suggesting increased release of CRF into the portal hypophyseal circulation in response to tone-footshock pairings. This possibility is supported by the observation that electrical stimulation of the amygdala causes a release of CRF, resulting in a marked depletion of CRF content in median eminence.[15] It seems unlikely, however, that decreased CRF mRNA transcription account for the observed decreases in CRF peptide level of PVN in rats exposed to tone-footshock pairings. In the present study, rats were sacrificed at the end of footshock, and the duration was not suffi-

cient to induce a translation of CRF. Moreover it was reported that acute footshock either increased[16] or did not influence the level of CRF mRNA level in the PVN.[17] Moreover, reexposure alone to tones previously paired with footshock decreased CRF peptide level in PVN, which suggest that neural substrates underlying conditioned footshock may last at least for 2days. However, it is not yet clear why CRF peptide level in PVN measured at 48hr after footshock remained to be decreased compared with control and the degree of decrease was even greater than that induced by tone-footshock pairings. This suggests that the magnitude of acute footshock-induced changes in CRF peptide of PVN may be related not only to reexposure to tones previously paired with footshock but also to the elapsed time between footshock and sacrifice. Further studies will be needed to clarify the significance of this finding.

BNST is located adjacent to the septum and is known to be involved in hypothalamo-pituitary-adrenocortical (HPA) axis regulation[18][19] or in autonomic and behavioral responses of unconditioned fear or anxiety.[6][20] In addition, the BNST is strongly connected with the periaqueductal gray,[21] parabrachial area,[22] and locus coeruleus(LC),[21][23] which is thought to be involved in autonomic processes in response to stress. Given that a microinfusion of CRF into the BNST enhanced the acoustic startle reflex, while an infusion of CRF antagonist into the BNST or lesions of BNST blocked CRF-enhanced startle response, activation of the BNST by CRF would activate various brainstem target areas involved in stress and anxiety responses. Thus, BNST is presumed to be one of the main targets of elevated CSF concentration of CRF, which is frequently observed in patients with depression or PTSD.[6][7] In the present study, reexposure to tones previously paired with footshock induced the same magnitude of increase as that produced by tone-footshock pairings. Tone-footshock pairings increased the CRF peptide in the BNST, which may contribute in part to increase of anxiety. Given that Walker and Davis(1997) provided evidence suggesting that CeA preferentially mediates the expression of conditioned fear whereas the BNST is involved in the expression of unconditioned fear,[20] it is surprising that reexposure to explicit cues such as tones paired with previous footshocks increases CRF peptide level in the BNST. However, psychological stress, as well as cold stress, also increases CRF mRNA levels in the BNST,[24] suggesting that the increase of CRF peptide in the BNST may be attributable to psychological stress induced by reexposure to explicit cues paired with aversive stimuli. Interestingly, Rasmusson, et al.[25] also reported that reexposure to the conditioning chamber and tones previously paired with footshock significantly decreased BDNF mRNA of hippocampus, implying that psychological stress could decrease the expression of BDNF in hippocampus.

A number of studies support a role for the amygdala in the behavioral effects of CRF. Intracerebroventricular infusion of CRF potentiates the acoustic startle response, and lesions of CeA, but not the PVN, blocks the CRF-induced potentiation of the acoustic startle response,[6] indicating that activation of CRF receptors in the CeA and/or CRF pathways emanating from the CeA plays an important role in fear-related behavior. This may be related to the widespread projection of CRF neurons in CeA to BNST, lateral hypothalamus, midbrain central gray, raphe nuclei, parabrachial region, locus coeruleus, and the nucleus of the solitary tract.[26] In addition, microinjection into the CeA of the CRF receptor antagonist alpha-helical CRF_{9-41} reverses social stress-induced suppression of behavior in the plus-maze[27] or stress-induced freezing,[28] suggesting that CRF in the CeA has an important role in the suppression of behavior in response to stress. In the present study, exposure to tone-footshock pairings as well as either tones previously paired with tones and context or tones previously paired with footshock resulted in a small increase in CRF peptide levels of CeA. Similar to previous report in which footshock increases in CRF mRNA levels of the CeA and the BNST,[29] our results showed that footshock caused a small increase in the CRF peptide of CeA. Furthermore, rats seemed to respond to tones previously paired with footshock like a psychological stress, as indicated by increase in CRF peptide of CeA. This is supported by previous study showing that psychological components of stressor, but not physical or metabolic components of stressor, could activate the amygdaloid CRF system.[24][30] Although it

is not clear why CRF peptide levels in CeA were below control levels 48 hr after footshock, rats seemed to respond to tones previously paired with tones and context similarly to tones previously paired with footshock.

NPY is one of the most abundant peptides found in the central nervous system of mammals[31] and NPY plays an important role in the regulation of anxiety. For example, intracerebroventricular infusion of NPY[3] or microinjection of NPY into the CeA[11] causes an anxiolytic effect. From the current study, it is apparent that exposure to tone-footshock pairings increased NPY peptide levels in amygdala. Acute stress such as a single restraint is known to suppress NPY mRNA expression in amygdala,[32] providing a possible mechanism for the anxiety-promoting action of restraint stress. However, there has been no report describing effects of footshock or tone-footshock pairings on NPY peptide levels in amygdala. This increase is assumed to counteract or buffer increased CRF peptide levels in response to tone-footshock pairing,[2] since NPY has been shown to block CRF-induced anxiogenic-like behavioral effects in elevated plus maze and suppression of drinking in operant conflict test.[33] In contrast to previous report where exposure to context previously paired with footshock increased NPY peptide levels in amygdala,[13] our data showed that tones previously paired with footshock caused a small but nonsignificant increase in amygdala. This may suggest that the intensity of footshock and exposure to tones with context may influence the degree to which NPY peptide levels of amygdala increase. In the present study, footshock caused an increase of NPY peptide level in the PVN, which was consistent with previous studies demonstrating that hypothalamic NPY mRNA is increased by acute and repeated immobilization stress.[34][35] NPY in PVN is known to play a role in orexigenic action,[36][37] while the effects of intrahypothalamic NPY on emotionality have not been examined. Further studies will be needed to elucidate the specific action in emotion, besides the orexigenic action, induced by increased NPY peptide levels in the PVN of hypothalamus.

Taken together, the present study suggests that the BNST appears to be mostly involved neural circuit in response to explicit cues paired with aversive response. Moreover, this study raise the possibility that increased CRF peptide in the BNST in response to re-exposure to the conditioning chamber and tones previously paired with footshock may underlie, in part, the experience of conditioned fear-related anxiety behavior.

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