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Met-Enkephalin, Injected During the Early Phase of Stress, Attenuates Stress-Induced Increases in Noradrenaline Release in Rat Brain Regions

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TANAKA, M., Y. IDA, A. TSUDA, S. TSUJIMARU, I. SHIRAO AND M. OGUCHI. *Met-enkephalin, injected during the early phase of stress, attenuates stress-induced increases in noradrenaline release in rat brain regions.* PHARMACOL BIOCHEM BEHAV **32**(3) 791–795, 1989.—By measuring levels of 3-methoxy-4-hydroxyphenylethyleneglycol sulfate (MHPG-SO_4), the major metabolite of noradrenaline (NA), we investigated the effects of Met-enkephalin (Met-ENK) ICV injected at three different stages of stress, i.e., 0 min, 5 min, or 10 min after exposure to immobilization stress. Immobilization stress caused significant increases in MHPG-SO_4 levels in all brain regions examined, i.e., the hypothalamus, amygdala, thalamus, midbrain, hippocampus and locus coeruleus (LC), which suggests that stress increases NA release in these regions. Met-ENK at a dose of 50 μg, injected ICV immediately before stress exposure significantly attenuated stress-induced increases in MHPG-SO_4 in the amygdala, thalamus and LC, but did not have such an effect when injected either 5 min or 10 min after exposure to stress. Similarly, Met-ENK at 150 μg, injected at 0 min significantly attenuated these increases in all brain regions examined, however, it did not do so when given at 5 min or 10 min after stress initiation. The amount of defecation and the weight loss caused by stress were also significantly attenuated by Met-ENK injected but only at 0 min. These results suggest that the attenuating effect of Met-ENK on stress-induced increases in NA release is greatly affected by the time of the peptide administration and that Met-ENK might inhibit stress-induced increases in NA release in these regions by affecting the initial changes induced by stress.

Met-enkephalin Stress Noradrenaline release MHPG-SO_4 Brain regions Time of injection

BY measuring levels of noradrenaline (NA) and its major metabolite in the rat brain, 3-methoxy-4-hydroxyphenylethyleneglycol sulfate (MHPG-SO_4), we have reported that a variety of stressful stimuli cause marked increases in NA release in rat brain regions and that these increases show a variety of characteristics related to the brain regions and/or to the nature of the stressor (6–8, 11, 13–19).

Of the stressors examined to date, immobilization stress has been well-documentated to induce marked increases in NA release in extended brain regions (6, 14–16). These increases in NA release caused by immobilization stress are modified by pretreatment with drugs such as diazepam, naloxone, morphine, opioid peptides and ethanol (7, 13, 15–18).

In particular, based upon the findings that increased release of NA in brain regions such as the hypothalamus, amygdala and thalamus are enhanced by naloxone pretreatment (15,18) and attenuated by morphine (16), we have suggested that endogenous opioid peptides, released during stress, might act to attenuate stress-induced increases in NA release in these brain regions (15,16). This idea has been partly supported by the fact that Met-enkephalin (Met-ENK) injected ICV significantly attenuated stress-induced increases in NA release in these regions as well as in the midbrain, hippocampus and locus coeruleus (LC) region (17).

However, the question remains as to how Met-ENK injected exogenously, and which has a very short half-life (1, 3, 5), could attenuate these increases caused by immobilization for a comparatively long duration of 1 hour.

In order to answer this question, the present study was undertaken to investigate the effects of Met-ENK, injected at three different time-points, i.e., 0 min, 5 min or 10 min after stress exposure, on stress-induced increases in NA release in various brain regions. Moreover, the naloxone-reversibility of these actions of Met-ENK as well as the effects of this peptide on emotional responses observed during stress exposure were also investigated.

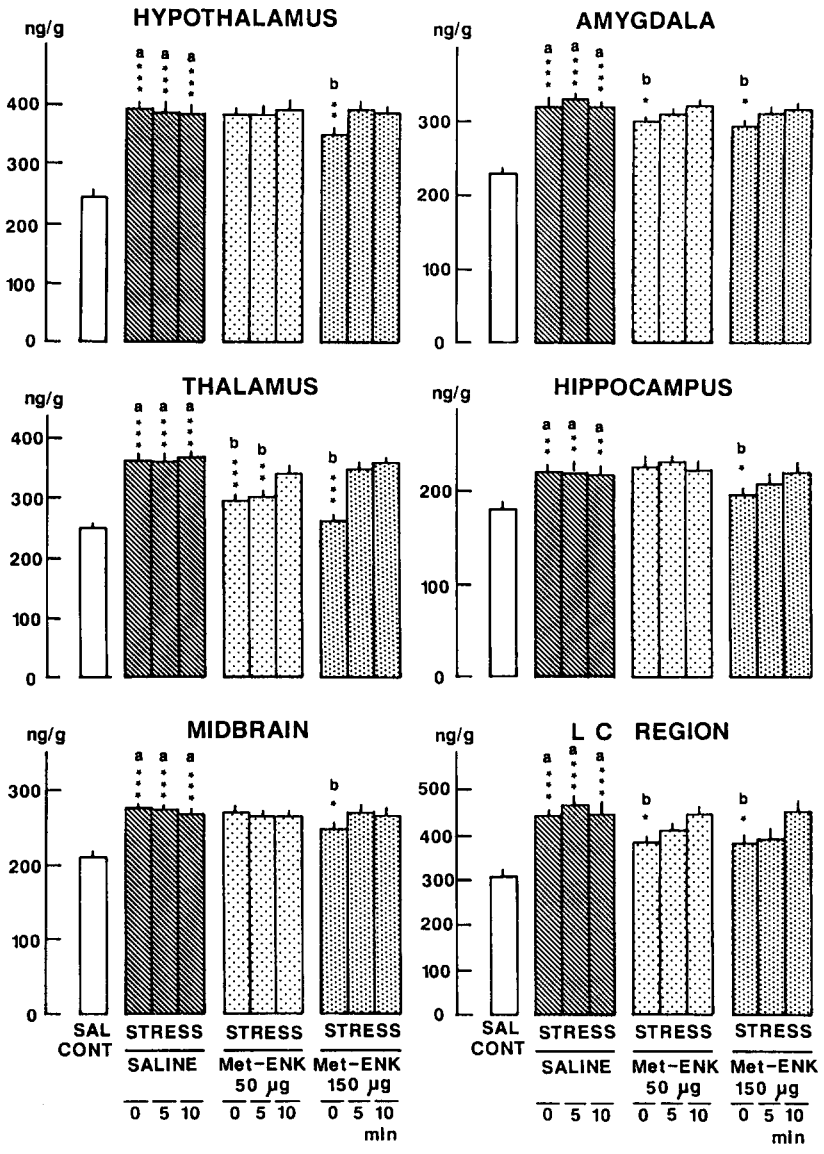

FIG. 1. Effects of Met-enkephalin, injected at three different stages, during 1-hour immobilization stress, i.e., 0 min, 5 min and 10 min after stress exposure, on changes in 3-methoxy-4-hydroxyphenylethyleneglycol sulfate (MHPG-SO_4) levels caused by stress. Each value indicates the mean ± S.E.M. of 8 rats. Abbreviations are: SAL CONT: saline control; Met-ENK: Met-enkephalin. Statistical significance; (a) vs. respective saline control group, (b) vs. respective saline stress group. $*p<0.05$, $**p<0.01$, $***p<0.001$.

METHOD

Animals

Male Wistar rats, weighing 170–190 g, were housed 4 per cage containing wood shavings at constant room temperature (24 ± 1°C) and humidity (50 ± 10%) and were allowed free access to standard chow (solid diet CE-2, Clea, Japan) and water. The animal colony was maintained on a 12-hr alternating light-dark cycle with light on at 0700 hr. All experiments were carried out between 1000 and 1400 hr, since we found no diurnal variations of either NA or MHPG-SO_4 contents during this period (10).

Stress Procedure

Immobilization stress was employed by enclosing rats in a flexible wire mesh (3 × 3 mm) initially formed into a cone which was then bent to conform to the size of the individual animals (6, 15–18).

Drugs

Met-enkephalin (Wako Pure Chemical Industries, Ltd.) and naloxone hydrochloride (a gift from Sankyo K.K.) were dissolved in physiological saline.

Experimental Procedure

The rats were anesthetized with pentobarbital and implanted with a polyethylene cannula into the right lateral ventricle 4 days

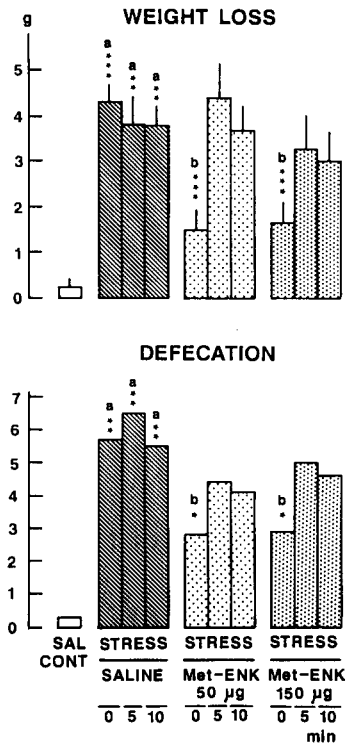

FIG. 2. Effects of Met-enkephalin, injected at three different stages during immobilization stress, i.e., 0 min, 5 min and 10 min after stress exposure, on behavioral changes caused by stress. Each value indicates the mean ± S.E.M. of 8 rats. As for defecation, the mean value is indicated. Abbreviations are; SAL CONT: saline control; Met-ENK: Met-enkephalin. Statistical significance; (a) vs. respective saline control group, (b) vs. respective saline stress group. $*p<0.05$, $**p<0.01$, $***p<0.001$.

before the experiment began according to the method of de Wied (2). We confirmed the exact position of the cannula by the fact that the cerebrospinal fluid flowed out from the cannula.

In the first study, we investigated the effects of Met-ENK injected at different time-points on changes in brain NA metabolism induced by stress. All animals, excluding the saline-injected control rats, were stressed by immobilization for 1 hour. Either Met-ENK at a dose of 50 μg or 150 μg, or physiological saline in a volume of 5.0 μl per rat, was injected ICV through the cannula within 1 min at three different time-points, i.e., 0 min, 5 min, or 10 min after stress exposure. In the 0 min group, the rats were immobilized immediately after injection, and in the 5 min and 10 min groups, the animals were first immobilized and then released from stress after 5 min or 10 min, injected ICV with saline or the respective dose of Met-ENK, and then stressed for the remaining time, i.e., 55 min and 50 min, respectively.

In the second study, in order to investigate the reversibility of the Met-ENK effects by naloxone, the rats were injected SC with either saline or naloxone at a dose of 0.5 mg/kg or 5.0 mg/kg, 5 min before ICV injection of 150 μg of Met-ENK. These animals were then stressed for 1 hour.

Behavioral Observation

We examined the number of fecal boluses deposited and body weight loss during 1 hour of immobilization stress. These measures are considered to be related to "negative emotion," i.e., "fear" of the animals exposed to stress. Control rats were derived food and water and left individually in a laboratory cage.

Tissue Preparation and Biochemical Determinations

Immediately after each treatment, the rats were sacrificed by decapitation. The brain was rapidly removed, dissected into discrete regions according to the method of Gispen *et al.* (4), excluding the locus coeruleus (LC) region, which was dissected according to the method of Reis and Ross (12), and frozen on solid CO_2. Brain regions included: hypothalamus, amygdala, thalamus, hippocampus, midbrain and LC region, since stress-induced increases in NA release were previously reported to be significantly attenuated by Met-ENK treatment in these regions (17). Brain tissues were stored at −45°C until assayed.

Only levels of MHPG-SO_4, the major metabolite of rat brain NA and indicative of brain NA release, were determined by our fluourometric method (9), since several previous reports indicate that the changes in metabolite levels are more sensitive than NA lelvels (14–16).

Statistical Analaysis

All statistical analyses for the neurochemical studies and weight loss were performed by means of the two-tailed Student's *t*-test. The Mann-Whitney U-test was used to test the significance of defecation.

RESULTS

Immobilization stress by itself, and under conditions of ICV injection with saline 0 min, 5 min or 10 min after stress exposure, significantly increased MHPG-SO_4 levels in all six brain regions examined (Fig. 1).

Met-ENK 50 μg ICV injected at 0 min but not at 5 min or 10 min after stress, significantly attenuated these increases induced by stress in the amygdala, thalamus and LC region. In the thalamus, the same dose of the peptide significantly attenuated MHPG-SO_4 increases even when injected 5 min after stress exposure.

Met-ENK at 150 μg significantly attenuated the stress-induced increases in MHPG-SO_4 levels in all brain regions examined, only when injected at 0 min (Fig. 1).

Weight loss and the number of fecal boluses deposited during 1-hour immobilization stress in the stressed rats were markedly and significantly increased as compared to those of control rats. These increases in weight loss and defecation caused by stress were significantly attenuated by both doses of Met-ENK but only when injected at 0 min (Fig. 2).

In the second study, immobilization stress again significantly increased MHPG-SO_4 levels in all brain regions examined, and these increases were also significantly attenuated by Met-ENK at 150 μg ICV injected immediately before stress exposure (Table 1). These effects of Met-ENK were antagonized by naloxone at 0.5 mg/kg or 5.0 mg/kg in all brain regions including the thalamus, wherein both doses of naloxone tended to reverse the peptide effects ($p<0.10$) (Table 1).

Met-ENK at 150 μg injected at 0 min also significantly attenuated the increases in defecation and weight loss of the stressed animals (Table 1). These effects were also antagonized by pretreatment with naloxone at both 0.5 mg/kg and 5.0 mg/kg (Table 1).

DISCUSSION

One-hour immobilization stress caused significant increases in

TABLE 1

NALOXONE REVERSIBILITY OF ATTENUATING EFFECTS OF MET-ENKEPHALIN ON STRESS-INDUCED INCREASES IN MHPG-SO_4 LEVELS (ng/g) IN THE BRAIN REGIONS AND ON NUMBER OF DEFECATION AND WEIGHT LOSS (g) DURING 1-HOUR IMMOBILIZATION STRESS

Groups	Hypothalamus	Amygdala	Thalamus	Hippocampus	Midbrain	LC Region	Weight Loss	Defecation
Saline	250.2 ± 6.63	232.6 ± 5.91	282.7 ± 9.19	209.2 ± 8.42	215.4 ± 5.77	305.2 ± 11.93	0.3 ± 0.14	0.6
Stress	431.7 ± 14.01[a]‡	335.7 ± 6.81[a]‡	373.1 ± 10.93[a]‡	301.6 ± 12.58[a]‡	281.4 ± 7.58[a]‡	434.2 ± 9.79[a]‡	5.1 ± 0.77[a]‡	4.1[a]‡
Stress Met-ENK 150 μg	379.2 ± 11.22[b]*	273.5 ± 9.31[b]‡	322.6 ± 11.90[b]†	255.1 ± 8.67[b]†	253.3 ± 8.61[b]*	386.2 ± 10.88[b]†	1.8 ± 0.60[b]†	1.8[b]*
Stress Met-ENK 150 μg NAL 0.5 mg/kg	446.5 ± 19.84[c]*	307.3 ± 10.08[c]*	347.7 ± 9.86	296.6 ± 10.82[c]†	289.8 ± 11.06[c]*	445.7 ± 15.27[c]*	2.9 ± 0.61	4.3[c]*
Stress Met-ENK 150 μg NAL 5.0 mg/kg	457.5 ± 14.95[c]‡	336.2 ± 10.54[c]‡	353.0 ± 9.96	285.5 ± 10.90[c]*	297.1 ± 11.84[c]†	499.1 ± 12.27[c]‡	3.6 ± 0.50[c]*	3.6[c]*

Each value indicates the mean ± S.E.M. of 8 rats. As for defecation, only the mean is indicated. Abbreviations are: Met-ENK: Met-enkephalin; NAL: naloxone. The rats were injected SC with saline or naloxone 5 min before ICV injection of saline or Met-enkephalin and stressed for 1 hour. Statistical significance; a: vs. saline control; b: vs. stress group; c: vs. stress + Met-ENK group. *$p<0.05$, †$p<0.01$, ‡$p<0.001$.

MHPG-SO_4 levels in all brain regions examined. This finding indicates that stress causes increases in NA release in these brain regions, as previously reported (6, 7, 14–18).

The present study again demonstrated that Met-ENK injected ICV also significantly attenuated these stress-induced increases in the metabolite levels as previously reported (17), however, the appearance of these effects was closely related to the time when the peptide was injected. Met-ENK injected ICV prior to and within 5 min of stress exposure, mostly effectively attenuate these stress-induced increases. However, if the peptide was injected 10 min after the initiation of stress, the attenuating effect of Met-ENK disappeared. The differences in the effects between 50 and 150 μg of the peptide seems to reflect dose-dependency rather than a simple diffusion problem. The present result indicates that Met-ENK, injected only during the early phase of stress, attenuates stress-induced increases in NA release in various brain regions.

Interestingly, these neurochemical findings of Met-ENK parallel the findings concerning the behavioral effects of this peptide that Met-ENK, injected at 0 min but not at 5 min or 10 min after exposure to stress, significantly attenuated both the increased defecation and weight loss caused by immobilization stress.

It is further suggested that there exists a critical period for injection of this peptide during stress exposure outside which stress-attenuating effects are not observed. The possibility exists that Met-ENK attenuates stress-induced increases in NA release in several brain regions by interfering with the initial influence of stress, which might then result in attenuation of subsequent changes caused by stress. This idea may explain why the peptide, which has a short half-life, attenuates changes caused by stress over a 1-hour period. This hypothesis is supported by a previous report that naloxone, given only during the early stage of stress, enhanced stress-induced increases in NA release in several brain regions (18). Together with these findings, it is further suggested that endogenous opioid peptides, released primarily during the initial phases of stress, have a critical role in attenuating subsequent stress-induced physiological (pathological) changes including the neurochemical and behavioral changes.

The attenuating effects of Met-ENK on both neurochemical and behavioral changes caused by stress were antagonized by pretreatment with naloxone, which indicates that these effects are mediated via opioid receptors.

Taken together, our neurochemical and behavioral findings suggest that attenuation of stress-induced increases in NA release in brain regions such as the hypothalamus, amygdala and LC region might be closely related to the relief of "negative emotions" such as fear and/or anxiety of animals exposed to stress.

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REFERENCES

1. Craves, F. B.; Law, P. Y.; Hunt, C. A.; Loh, H. H. The metabolic disposition of radiolabelled enkephalins in vitro and in situ. J. Pharmacol. Exp. Ther. 206:492–506; 1978.
2. De Wied, D. Behavioral effects of intraventricularly administered vasopressin and vasopressin fragments. Life Sci. 19:685–690; 1976.
3. Dupont, A.; Cusan, L.; Garon, M.; Alvarado-Urbina, G.; Labrie, F. Extremely rapid degradation of [^{3}H]methionine-enkephalin by various rat tissues *in vivo* and *in vitro*. Life Sci. 21:907–914; 1977.
4. Gispen, W. H.; Schotman, P.; de Kloet, E. R. Brain RNA and hypophysectomy. Neuroendocrinology 9:285–296; 1972.
5. Hartvig, P.; Nagren, K.; Lundberg, P. O.; Muhr, C.; Terenius, L.; Lundqvist, H.; Larkfors, L.; Langstrom, B. Kinetics of four ^{11}C-labelled enkephalin peptides in the brain, pituitary and plasma of Rhesus monkeys. Regul. Pept. 16:1–13; 1986.
6. Ida, Y.; Tanaka, M.; Tsuda, A.; Kohno, Y.; Hoaki, Y.; Nakagawa, R.; Iimori, K.; Nagasaki, N. Recovery of stress-induced increases in

noradrenaline turnover is delayed in specific brain regions of old rats. Life Sci. 34:2357–2363; 1984.
7. Ida, Y.; Tanaka, M.; Tsuda, A.; Tsujimaru, S.; Nagasaki, N. Attenuating effect of diazepam on stress-induced increases in noradrenaline turnover in specific brain regions of rats: Antagonism by Ro 15-1788. Life Sci. 37:2491–2498; 1985.
8. Iimori, K.; Tanaka, M.; Kohno, Y.; Ida, Y.; Nakagawa, R.; Hoaki, Y., Tsuda, A.; Nagasaki, N. Psychological stress enhances noradrenaline turnover in specific brain regions in rats. Pharmacol. Biochem. Behav. 16:637–640; 1982.
9. Kohno, Y.; Matsuo, K.; Tanaka, M.; Furukawa, T.; Nagasaki, N. Simultaneous determination of noradrenaline and 3-methoxy-4-hydroxyphenylethyleneglycol sulfate in discrete brain regions of the rat. Anal. Biochem. 97:352–358; 1979.
10. Kohno, Y.; Tanaka, M.; Nakagawa, R.; Toshima, N.; Takeda, S.; Nagasaki, N. Study on diurnal variation of noradrenaline release in three brain regions of rats. Kurume Med. J. 27:227–232; 1980.
11. Nakagawa, R.; Tanaka, M.; Kohno, Y.; Noda, Y.; Nagasaki, N. Regional responses of rat brain noradrenergic neurones to acute intense stress. Pharmacol. Biochem. Behav. 14:729–732; 1981.
12. Reis, D. J.; Ross, R. A. Dynamic changes in brain dopamine-β-hydroxylase activity during anterograde and retrograde reactions to injury of central noradrenergic axons. Brain Res. 57:307–326; 1973.
13. Shirao, I.; Tsuda, A.; Ida, Y.; Tsujimaru, S.; Satoh, H.; Oguchi, M.; Tanaka, M.; Inanaga, K. Effect of acute ethanol administration on noradrenaline metabolism in brain regions of stressed and nonstressed rats. Pharmacol. Biochem. Behav. 30:769–773; 1988.
14. Tanaka, M.; Kohno, Y.; Nakagawa, R.; Ida, Y.; Iimori, K.; Hoaki, Y.; Tsuda, A.; Nagasaki, N. Time-related differences in noradrenaline turnover in rat brain regions by stress. Pharmacol. Biochem. Behav. 16:315–319; 1982.
15. Tanaka, M.; Kohno, Y.; Nakagawa, R.; Ida, Y.; Iimori, K.; Hoaki, Y.; Tsuda, A.; Nagasaki, N. Naloxone enhances stress-induced increases in noradrenaline turnover in specific brain regions in rats. Life Sci. 30:1663–1669; 1982.
16. Tanaka, M.; Kohno, Y.; Tsuda, A.; Ida, Y.; Iimori, K.; Hoaki, Y.; Nagasaki, N. Differential effects of morphine on noradrenaline release in brain regions of stressed and non-stressed rats. Brain Res. 275: 105–115; 1983.
17. Tanaka, M.; Tsuda, A.; Ida, Y.; Ushijima, I.; Tsujimaru, S.; Nagasaki, N. Methionine-enkephalin inhibits stress-induced increases in noradrenaline turnover in brain regions of rats. Jpn. J. Pharmacol. 37:117–119; 1985.
18. Tanaka, M.; Ida, Y.; Tsuda, A. Naloxone, given before but not after stress exposure, enhances stress-induced increases in noradrenaline release in rat brain regions. Pharmacol. Biochem. Behav. 29:613–616; 1988.
19. Tsuda, A.; Tanaka, M.; Kohno, Y.; Ida, Y.; Hoaki, Y.; Iimori, K.; Nakagawa, R.; Nishikawa, T.; Nagasaki, N. Daily increases in noradrenaline turnover in brain regions of activity-stressed rats. Pharmacol. Biochem. Behav. 19:393–396; 1983.