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The Influence of Conditioned Fear-Induced Stress on the Opioid Systems in the Rat

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PRZEWŁOCKA, B., A. SUMOVÁ AND W. LASOŃ. *The influence of conditioned fear-induced stress on the opioid systems in the rat.* PHARMACOL BIOCHEM BEHAV **37**(4) 661–666, 1990.—In this study the rats were repeatedly placed in a conditioning box, and 30 min later were subjected to a mild foot-shock. Anticipation of painful stimuli resulted in development of antinociception before a painful stimulus was applied. This conditioned fear-induced antinociception was antagonized by naloxone (1 mg/kg IP), as well as by ipsapirone (10 mg/kg IP), as measured by a tail-flick test. Stressed rats were hypersensitive to the analgesic action of morphine (1 mg/kg SC), but not to the specific κ agonist U69,593 (0.1 mg/kg SC). In order to determine the involvement of the proopiomelanocortin and prodynorphin systems in stress we measured levels of their representative peptides β-endorphin and α-neoendorphin using selective RIAs. Biochemical data showed that conditioned stress evoked a marked decrease in the β-endorphin level in the hypothalamus and both lobes of the pituitary, together with a three-fold increase in the peptide level in the plasma. In contrast, the level of α-neoendorphin in the hypothalamus, pituitary and spinal cord remained unchanged. Only in the plasma a decrease in that peptide content was found. Furthermore, in vitro studies showed that the spontaneous and K^+- stimulated release of β-endorphin from the hypothalamus of rats which had been exposed to a conditioned stimulus was enhanced, whereas the release of α-neoendorphin from that tissue was attenuated. These results suggest a major role of the proopiomelanocortin system and, to the lesser extent, of the prodynorphin one in the mechanism of a conditioned fear-induced stress.

Stress α-Neoendorphin β-Endorphin Analgesia Tail-flick

THE role of endogenous opioid systems in stress, in particular in the stress-induced analgesia, has been frequently investigated (1, 12, 19). It has been reported that analgesia may be evoked in animals not only by a direct painful stimulus but also by exposure to the environment in which they previously experienced pain. Such a situation, known as conditioned fear-induced stress or "anticipation stress" (2,9), seemed to us a particularly valuable model for studying involvement of endogenous opioid peptides in stress phenomena for the following reasons. Firstly, the stress-induced behavioral and biochemical changes found in this model are not directly influenced by physical painful stimuli, such as, e.g., foot-shock. Secondly, the foot-shock used for conditioning purposes is mild and short-lasting, which is an important ethical aspect of chronic stress studies. Other stress models are known to produce mixed opioid/nonopioid responses (1, 11, 22). However, there are few data available concerning mechanisms of the conditioned stress-induced analgesia. Biochemical studies showed that in this stress model the binding of [^{3}H]Leu-enkephalin as well as of [^{3}H]naloxone was decreased in membrane fractions obtained from defined brain regions of stressed animals (2,21), which might indicate involvement of an opioid mechanism. No data have been available concerning the effect of this stress model on endogenous opioid peptide systems. Therefore, we studied involvement of opioid mechanisms in the development of stress-induced analgesia, and examined changes in the level and release of β-endorphin (β-END) and α-neoendorphin (α-NEO), peptides representing two endogenous opioid peptide systems: the proopiomelanocortin and the prodynorphin ones.

METHOD

Experimental Procedure

Male Wistar rats, weighing 200–220 g, were housed in groups of 8 to a cage under a constant light-dark cycle (light on between 08.00 and 20.00 h), with free access to food and water. They were adapted to handling before the experiment. The experiments were made between 9 a.m. and 2 p.m.

Conditioned fear-induced stress was achieved by pairing a distinctive environment (foot-shock cage) with foot-shock. The rats were transferred from the home cage to a foot-shock cage (dimen-

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sions: 40 × 20 × 40) with an electrical grid as the floor, in which they were left to rest for 30 min. After that time a foot-shock (1 mA, 60 s) was delivered. Control rats were also put into the foot-shock cage for 30 min, but no foot-shock was applied. That procedure was repeated for 8 days. On the 9th day (foot-shock was not applied) the rats were used for estimation of the effect of drugs on the fear-induced antinociception, or were sacrificed for a biochemical study at 15 min after placing them in the foot-shock cage or 24 h later.

Analgesia Measurements

To estimate the development of antinociception, every day the animals were placed in the foot-shock cage, were then taken out from it after a 15-min anticipation period. Afterwards, after the tail-flick test, they were put back into the cage and a foot-shock was delivered. On the 9th day the effect of drugs on the tail-flick latency was measured at the same time. Only for naloxone the tail-flick test was repeated three times: at 5, 15 and 30 min after putting the rats into the foot-shock cage. The drugs were administered at the following doses and times before putting the rats into the foot-shock cage: ipsapirone (10 mg/kg IP, 15 min), morphine (1 mg/kg SC, 30 min), U69,593 (0.1 mg/kg SC, 15 min), naloxone (1 mg/kg IP, 10 min).

The tail-flick test was applied with an analgesia meter apparatus (IITC, Inc., USA). Each animal was gently restricted by hand, and radiant heat was directed to the animal's tail, approx. 2.5–3 cm from its tip. The cut-off time was 8 s. The measurements were taken 3 times at 15-second intervals and the mean was used for calculations. The intensity of the light stimulus was adjusted to produce an average baseline of 1.5–2 s.

Release of Opioid Peptides From Hypothalamic Slices

For the release study a basket made from cut up plastic tubes with nylon mesh serving as a bottom was used. After decapitation the hypothalamus of rats was rapidly dissected. Slices (500 μm) were obtained using a McIlwain tissue chopper, and were then transferred to baskets and then placed in a beaker containing an oxygenated Krebs-bicarbonate solution, modified to contain the following: 127 mM NaCl, 3.85 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM KH_2PO_4, 1.18 mM $MgSO_4$, 20 mM $NaHCO_3$, 11 mM D-glucose, BSA (1 mg/ml), bacitracin at 30 μg/ml. After a 30-min washout, each basket was transferred for 10 min to 6 polyethylene tubes containing 2 ml of oxygenated Krebs solution. The third and the sixth tubes contained additionally 24 and 57 mM K^+, respectively. RIAs of the released α-neoendorphin and β-endorphin were performed directly in the Krebs solution in the presence of poly-l-lysine and PMSF (20 μM and 1 mM, respectively).

RIA Assay Conditions

After decapitation, the animal brain, pituitary and spinal cord were quickly removed. The hypothalamus and the anterior and neurointermediate lobes of the pituitary were dissected; the spinal cord was divided into 3 parts corresponding to cervical, thoracic and lumbar sections. The trunk blood was collected in ice-cold tubes containing 15 mg of EDTA, and was immediately centrifuged for 15 min at 10,000 × g at 4°C.

Tissue parts were weighed and then incubated in 0.1 N HCl (5 times the volume of the tissue) for 10 min at 95°C. After homogenization, the homogenates were centrifuged at 10,000 × g for 15 min, and the supernatants were adjusted to pH 7.5. After further centrifugation aliquots of the supernatants were appropriately diluted and assayed for ir-α-NEO and ir-β-END according to the previously described procedures (6,13).

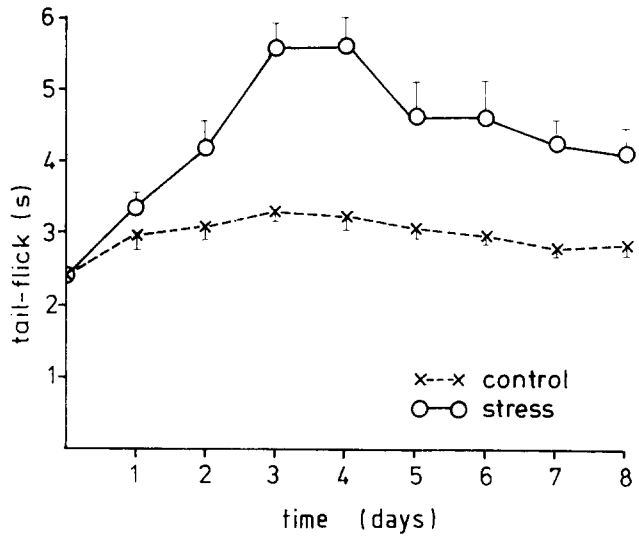

FIG. 1. The influence of everyday conditioned stress on antinociception measured by the tail-flick test 15 min after placing the rat into the foot-shock cage. Each point represents the mean ± SEM for groups of 8 rats.

Estimation of the levels of ir-α-NEO and ir-β-END in the trunk blood was carried out using RIA after ethanol extraction (6).

Antisera

All the antisera used in this study were obtained from Prof. A. Herz, Martinsried, FRG.

The antiserum directed against the synthetic α-NEO recognized α- and β-neoendorphin with high avidity (10% cross-reactivity), but did not cross-react with dynorphin A, Met- or Leu-enkephalin or β-endorphin (less than 0.01%) (13).

The antiserum directed against the human β-END recognized the rat β-END, but did not cross-react with Leu-enkephalin, dynorphin or α-neoendorphin (6).

Statistics

The results were statistically assessed by a two-way analysis of variance (ANOVA), and the intergroup differences were analysed by Duncan's test.

Drugs

Drugs used were ipsapirone (Troponwerke), morphine (Kutno), naloxone (Sigma), and U69,593 (Upjohn Company).

RESULTS

Development of the Stress-Induced Analgesia

Figure 1 shows the time-course of the stress-induced analgesia. On the first day no changes in tail-flick latencies were observed. A marked analgesia was observed in rats subjected to stress on the 3rd day; it persisted till the end of the experiment. During a 30-min period of waiting for the foot-shock, the strongest analgesia was found after 15 min (Fig. 2). The analgesia was blocked by naloxone (1 mg/kg IP) injected 10 min before the rats were exposed to stress (Fig. 2).

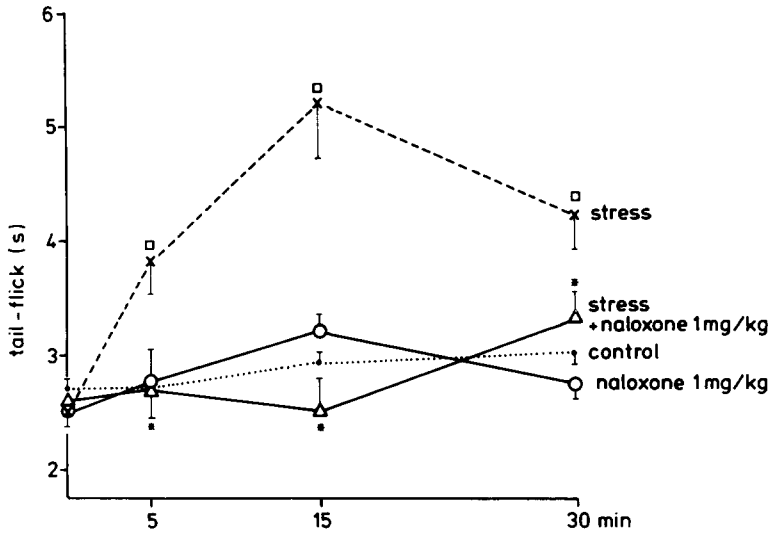

FIG. 2. The effect of naloxone on conditioned stress-induced antinociception measured by the tail-flick test 5, 15 and 30 min after placing the rat into the foot-shock cage on the 9th day of the experiment. The values are the mean ± SEM for groups of 6 rats. *$p<0.05$ vs. stress, #$p<0.05$ vs. control, Duncan's test.

The Effect of Morphine, U69,593 and Ipsapirone on the Stress-Induced Analgesia

In nonstressed rats the used substances did not significantly influence the tail-flick latency. Animals subjected to a conditioned stress showed a significantly enhanced analgesic response to morphine, but not to the specific κ agonist U69,593. On the other hand, the analgesia was blocked by the $5HT_{1A}$ agonist ipsapirone (Fig. 3).

The Effect of Conditioned Stress on In Vitro Release of ir-α-NEO and ir-β-END From the Hypothalamus

In the control group both 24 and 57 mM K^+ increased ca. 8 times the ir-α-NEO release, as compared with the basal value. The conditioned stress had no effect on the basal ir-α-NEO release, but significantly decreased the K^+-stimulated release of the peptide; 24 h after the stress the stimulated release was significantly higher, but did not reach control values. In contrast to the control group, during the stress and 24 hours later a significant difference was observed in the ir-α-NEO release stimulated by 24 and 57 mM K^+, respectively (Fig. 4).

In the control group both 24 and 57 mM K^+ increased ca. 1.5 times the ir-β-END release, as compared with the basal value. The basal release of the peptide increased during conditioned stress, and was back to control values 24 hours later. During the stress the stimulated release of ir-β-END was significantly elevated only when a 57 mM K^+ pulse was applied. No changes in the basal release and decrease (significant for 24 mM K^+) in the stimulated release were found 24 h after the stress (Fig. 4).

The Effect of Conditioned Stress on the Levels of ir-α-NEO and ir-β-END in the Hypothalamus and Pituitary

No significant changes were detected in the content of ir-α-NEO in the hypothalamus and both lobes of the pituitary in rats which had been exposed to conditioned stress, as compared with the control. However, 24 h later a significant decrease in the ir-α-NEO level in the hypothalamus and neurointermediate lobe of the pituitary was observed (Fig. 5).

The levels of ir-β-END in the hypothalamus and the anterior as well as the neurointermediate lobe of the pituitary were significantly decreased in rats subjected to stress. At 24 h after the stress those levels were back to control values (Fig. 5).

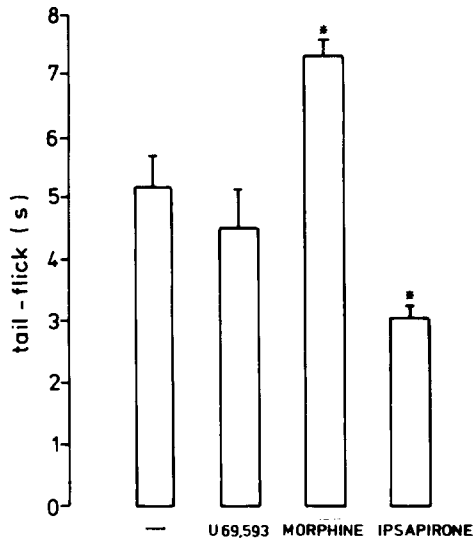

FIG. 3. The effect of U69,593 (0.1 mg/kg, SC), morphine (1 mg/kg, SC) or ipsapirone (10 mg/kg, IP) on conditioned stress-induced antinociception measured by the tail-flick test 15 min after placing the rat into the foot-shock cage on the 9th day of the experiment. The values are the mean ± SEM for groups of 6 rats. *$p<0.05$ vs. stress alone, Duncan's test.

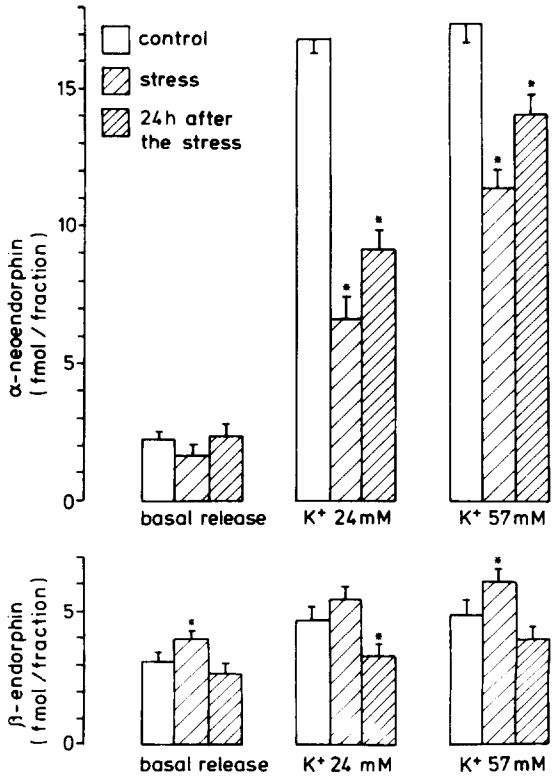

FIG. 4. The effect of conditioned stress on ir-α-NEO and ir-β-END basal and K^+-stimulated release from the hypothalamic slices of rats killed 15 min after placing the rat into the foot-shock cage on the 9th day of the experiment or 24 hours later. The values are the mean ± SEM for 6–11 measurements. *$p<0.05$ vs. respective control, Duncan's test.

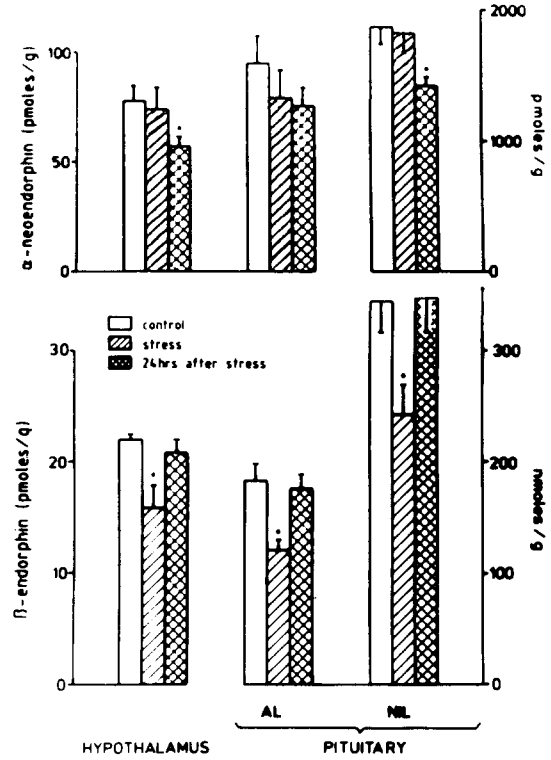

FIG. 5. The effect of conditioned stress on ir-α-NEO and ir-β-END levels in the hypothalamus, anterior (AL) and neurointermediate (NIL) lobes of the pituitary of rats killed 15 min after placing the rat into the foot-shock cage on the 9th day of the experiment or 24 h later. The values are the mean ± SEM for groups of 8 rats. *$p<0.05$ vs. respective control, Duncan's test.

The Effect of Conditioned Stress on the Level of ir-α-NEO in the Spinal Cord

Table 1 shows the ir-α-NEO level in various sections of the spinal cord. No significant changes were found in that tissue after the stress, as compared with the control group.

The Effect of Conditioned Stress on the Levels of ir-β-END or ir-α-NEO in the Plasma

The stress evoked a marked increase in the ir-β-END level. At 24 h after the stress the level of the peptide decreased, but was still higher than in the control group. In contrast, that kind of stress evoked a significant decrease in the ir-α-NEO plasma level, which was back to the control value 24 h after the stress (Table 2).

DISCUSSION

The present study indicates that endogenous opioid systems contribute to the conditioned stress-induced analgesia. The analgesia observed in this model was reversed by naloxone, this finding being in agreement with the previous report (9). The analgesia was also antagonized by the partial $5HT_{1A}$ agonist ipsapirone. The role of the particular 5-HT receptor subtypes and their interaction with opioids is still unclear. The efficacy of ipsapirone in blocking the conditioned fear-induced antinociception may result from anxiolytic properties of the drug. Secondly, the opioid mechanism may also be involved, as $5\text{-}HT_{1A}$ agonists have recently been reported to act as functional antagonists of the opiate-induced analgesia (3,15). Furthermore, our current results indicate that rats subjected to that stress showed supersensitivity to the analgesic action of morphine. These results are in line with the observation (7,8) that rats subjected to inescapable stress become hypersensitive to the analgesic effect of morphine. Moreover, the enhanced antinociceptive effect of morphine may result from an additive analgesic effect of this substance and the endogenous opioids released during stress. In fact, we observed an enhanced release of ir-β-END from the hypothalamus of rats which had been exposed to conditioned stress, which suggests a higher activity of proopiomelanocortin-containing neurons. Indeed, the marked decrease in the ir-β-END level in structures of the hypothalamo-pituitary axis, accompanied with an increase in the peptide level

TABLE 1

THE LEVEL OF ir-α-NEO IN DIFFERENT PARTS OF THE SPINAL CORD OF RATS KILLED 15 MIN AFTER PLACING THEM IN A FOOT-SHOCK CAGE ON THE 9th DAY OF THE EXPERIMENT

	Cervical	Thoracic	Lumbar
Control	16.0 ± 0.6	16.0 ± 1.8	15.3 ± 1.0
Conditioned Stress	17.5 ± 1.2	13.8 ± 0.9	16.3 ± 2.0

The values are the mean ± SEM for groups of 8 rats each, and are expressed as pmol/g of wet tissue.

TABLE 2

THE LEVEL OF ir-α-NEO AND ir-β-END IN THE PLASMA OF RATS KILLED 15 MIN AFTER PLACING THEM IN A FOOT-SHOCK CAGE ON THE 9th DAY OF THE EXPERIMENT OR 24 H LATER

	Control	Stress	24 h After the Stress
ir-α-NEO	67.1 ± 7.2	46.4 ± 5.5*	68.9 ± 7.8
ir-β-END	28.6 ± 7.6	69.3 ± 6.1*	36.8 ± 8.4

The values are the mean ± SEM for groups of 8 rats each.
*$p<0.05$ vs. control, Duncan's test.

in the plasma during stress, speaks further for activation of the proopiomelanocortin system. The coexistence of β-END in the anterior pituitary corticotrophs and its cosecretion with ACTH in response to various stressful stimuli (5) are the clues that suggest a role of the pituitary β-END in the stress-induced analgesia. Although the role of the proopiomelanocortin system in the stress-induced analgesia is still a matter of controversy (12,16), attenuation of this type of analgesia after hypophysectomy or lesion of the nucleus arcuatus (10,14) favours such an interpretation. Furthermore, β-END shows high affinity towards both μ and δ receptors which are reported to be critically involved in the conditioned stress-induced antinociception (4). Thus, activation of this opioid system may be responsible for the analgesic effect of the conditioned stress observed in our study. The occupation of μ and δ receptors by the endogenous ligand during repeated stress might explain the decrease in [^{3}H]naloxone and [^{3}H]Leu-enkephalin binding found in the brain membranes of stressed animals.

Interestingly, in contrast to pronounced changes in the proopiomelanocortin system, conditioned stress did not influence the tissue level of ir-α-NEO, a peptide which might be regarded as a marker of the prodynorphin neuron activity. The lack of changes in the hypothalamus and pituitary ir-α-NEO levels, together with a fall in this peptide plasma level, suggests that the prodynorphin system activity remains unchanged, or is even inhibited during conditioned stress. The latter suggestion is supported by attenuation of the stimulated release of ir-α-NEO from the hypothalamic slices of stressed rats. It is noteworthy that even 24 h after the stress the release of ir-α-NEO was still attenuated and accompanied with a decrease in the peptide level in the hypothalamus and neurointermediate lobe of the pituitary. These facts suggest that conditioned stress may lead to a delayed inhibition of the prodynorphin system activity. The fact that the specific κ agonist U69,593 does not influence the conditioned stress-induced antinociception in our and other authors' studies (4) supports the assumption that prodynorphin-derived peptides which are considered to be endogenous ligands for κ receptors play no important role in the stress-induced antinociception.

It has been well documented that after an intensive foot-shock and chronic pain dynamic changes occur in the spinal cord content of prodynorphin peptides (17, 18, 20). In this study, conditioned stress did not evoke any significant changes in the spinal ir-α-NEO level. This discrepancy might result from a restricted use of painful stimuli in conditioned stress, in contrast to the above models in which a prolonged painful stimulation was used. Although we applied the foot-shock repeatedly for the conditioning of rats, the electric stimulus was short-lasting and of low intensity, and did not evoke itself any changes in the spinal ir-α-NEO level.

In conclusion, our data suggest involvement of the opioid peptide system(s) in the mechanism of the analgesic effect of a conditioned fear-induced stress. Furthermore, the proopiomelanocortin system activation evidenced in this study may play some role in other behavioral and physiological changes known to be associated with the conditioned fear-induced stress, such as freezing or cardiopulmonary changes. In contrast to β-END, the release and levels of α-NEO seem to be decreased during stress, which suggests a reverse regulation of the prodynorphin and proopiomelanocortin systems.

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