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Effect of Autoanalgesia on CNS Enkephalin Receptors

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DEVRIES, G. H., W. T. CHANCE, W. R. PAYNE AND J. A. ROSECRANS. *Effect of autoanalgesia on CNS enkephalin receptors*. PHARMAC. BIOCHEM. BEHAV. 11(6) 741–744, 1979.—Fear conditioning to foot shock (15 sec/day, 12 days) elicited autoanalgesia in 10 male (Sprague Dawley) rats, while 17 non-shock control rats exhibited no analgesia as measured by the tail-flick assay. The binding of ^{3}H-leu-enkephalin to synaptosomal prepartions isolated from fear conditioned (experimental) and control animals was analyzed. At leu-enkephalin concentrations of 10^{-9} M or less, both synaptosomal preparations demonstrated high affinity binding with dissociation constants on the order of 10^{-10}. Binding of leu-enkephalin could not be displaced by a hundred-fold excess of naloxone at leu-enkephalin concentrations less than 10^{-9} M. However, the ability of naloxone to compete with leu-enkephalin for binding sites progressively increased at concentrations greater than 10^{-9} M leu-enkephalin. At these ligand concentrations, the competition of naloxone for leu-enkephalin binding sites was more dramatic in the control than in the experimental animals. These data support the existence of two classes of receptors for leu-enkephalin, one of which is not blocked by opiate antagonists. Furthermore, changes in binding capacity associated with autoanalgesia produced by conditioned fear are consistent with the hypothesis of endogenous release of opiate-like peptides in response to stress.

Conditioned fear Autoanalgesia Leu-enkephalin Naloxone and opiate receptors

ANTINOCICEPTION, as assessed by the tail-flick procedure, can be reliably elicited by acute stress [1] or classically-conditioned fear [2]. Changes in opiate [3,4] and opioid [5] binding to brain homogenate have been reported to parallel behaviorally-induced antinociception (autoanalgesia). The role of the opiate receptor in mediating this analgesic effect may be questioned however since opiate antagonists are relatively ineffective in blocking this behavioral response [1,6]. In addition, *in vitro* assessment of biologically significant opioid binding is particularly difficult, due to the nonavailability of specific opiate antagonists or nonactive stereoisomers. In the present communication we provide evidence that ^{3}H leu-enkephalin binds to a class of high affinity receptors in synaptosomal preparations. While naloxone has no effect on this binding it can compete for the binding of leu-enkephalin to synaptosomal receptors of lower affinity. Classical conditioning of fear induces analgesia and concomitant changes in the binding capacity and affinity of the synaptosomal receptors for leu-enkephalin. These binding changes may be responsible for autoanalgesia observed within the fear conditioning paradigm.

METHOD

As previously described [2, 3, 5], autoanalgesia was elicited by shocking (15 sec/day; 0.8 mA) 10 adult, male, Sprague-Dawley rats 10 sec after the determination of their tail-flick latencies. Thus, fear was being conditioned to the environmental stimuli associated with the procedure of assessing antinociception. Seventeen control rats were handled in a similar manner, but were never shocked. During these tests, the tail-flick apparatus was adjusted to elicit response latencies of 3 to 4 sec in control subjects and a 9 sec cut-off response criterion was maintained. In order to assess acquisition of autoanalgesia, antinociception was assessed (prior to the 15 sec shock) on each of 12 consecutive days under blind conditions. On the day following the last shock, antinociception was again assessed and each rat was immediately sacrificed by decapitation. The brains were rapidly removed, frozen in liquid nitrogen, and stored at −80°C until the synaptosomal fractions were isolated as previously described [5].

The binding of leu-Enk was studied at a concentration range from 10^{-10} M to 10^{-7}M. All binding in this study is expressed as fmoles leu-Enk bound per the amount of synaptosomal fraction derived from one mg of brain wet weight. Binding assays were conducted as previously described [5] using leu-Enk obtained from New England Nuclear, Inc. (specific activity=21.0 ci/mmole). The polypropylene centrifuge tubes (16×98 mm) containing the labeled ligand and synaptosomal membrane were gently agitated in an ice bath during the 60 min binding assay to keep the membranes in suspension. After the binding was complete, 10 ml of 0.05 M Tris buffer pH 7.3 was added, followed by centrifugation at 37,000 xg for 15 min. The synaptosomal pellet was washed once with 15 ml of the same buffer, centrifuged as before, and the synaptosomal pellet was solubilized by heating at 90° in 1 ml of 3% sodium dodecyl

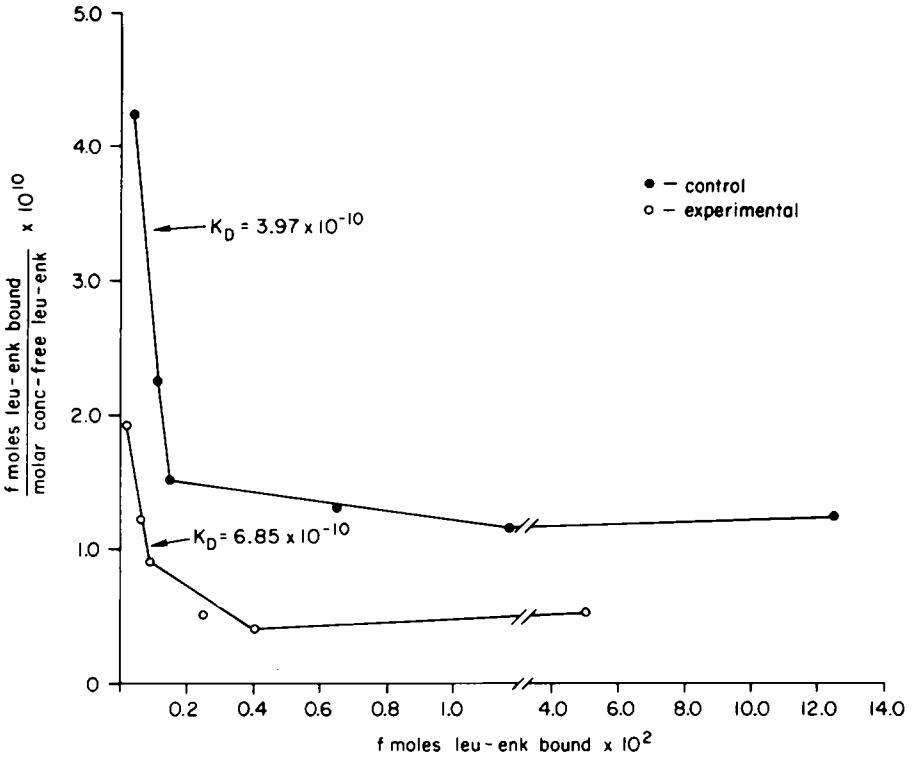

FIG. 1. Scatchard plot analysis of binding of ^{3}H-leu-enkephalin (leu-Enk) to rat CNS synaptosomal fractions prepared from control and experimental (fear-conditioned) animals. The data represents the average of nine control animals and six experimental animals. Binding is expressed as femtomoles/total synaptosomal fraction derived from 1 mg wet weight of brian.

sulfate, 10 mM Na_2CO_3. The radioactivity determined by liquid scintillation counting using 10 ml of Hydromix (Yorktown) and an Intertechnique Model 4100 Scintillation Counter. In order to evaluate the competition of naloxone for enkephalin binding sites, samples were preincubated for 15 min with a 100-fold excess of naloxone (relative to leu-Enk) followed by addition of the radioactive ligand.

RESULTS AND DISCUSSION

Figure 1 shows a Scatchard plot analysis of the binding of leu-Enk to the pooled synaptosomal fractions. It is evident from the break in the curve that at least two classes of sites are binding leu-enkephalin: a group of high affinity receptors (HAR) in the concentration range 10^{-10} M to 10^{-9} M ligand and a group of receptors with a lower affinity which are evident at ligand concentrations greater than 10^{-9} M. Note that the break in the Scatchard plot for control animals is more abrupt and has a lesser slope than the Scatchard plot for the binding of leu-Enk in the experimental animals which is more curvilinear. This indicates that the receptors which are observed at higher ligand concentrations have a lower affinity and greater capacity in the control animals relative to the experimental animals. However, the affinity of the HAR is similar in both the experimental and control groups of animals. When the leu-enkephalin binding to synaptosomal preparations from the control animals is carried out in the presence of a 100 fold excess of naloxone, the dissociation constant for the binding of leu-enkephalin to HAR is decreased from 3.97×10^{-10} to 2.86×10^{-10}. Under these conditions the maximal binding capacity of the HAR (as determined from the Scatchard plot) was also decreased from 14.31 f moles per mg synaptosomal protein to 9.31 f moles per mg synaptosomal protein (determined from Scatchard plot; data not shown). However, in the presence of naloxone and with ligand concentrations from 1×10^{-9} M to 1×10^{-8} M there was decrease in the dissociation constant (from 3.10×10^{-8} to 4.22×10^{-9}) and a striking decrease in binding capacity (from 334.03 f moles per mg synaptosomal protein to 38.68 f moles per mg synaptosomal protein). In contrast, the experimental synaptosomal preparations showed no significant change in either binding strength or binding capacity in the presence of a 100 fold excess of naloxone at leu-Enk concentrations from 1×10^{-9} M to 1×10^{-8} M. Other experiments were carried out with synaptosomal preparations isolated from fear conditioned animals using leu-Enk in concentrations from 1×10^{-10} M to 1×10^{-8} M in the presence of a one hundred fold excess of naloxone (data not shown). The resulting Scatchard plot indicated a single class of binding sites were interacting with the leu-Enk under these conditions. The binding strength and binding capacity of these receptors over the entire concentration range were identical to those of the receptors observed in the experimental animals in the absence of naloxone at leu-Enk concentrations of 1×10^{-9} M or less. These results are consistent with the view that leu-enkephalin binds to at least two kinds of receptors. The low capacity high affinity receptor observed at lower ligand concentrations may repre-

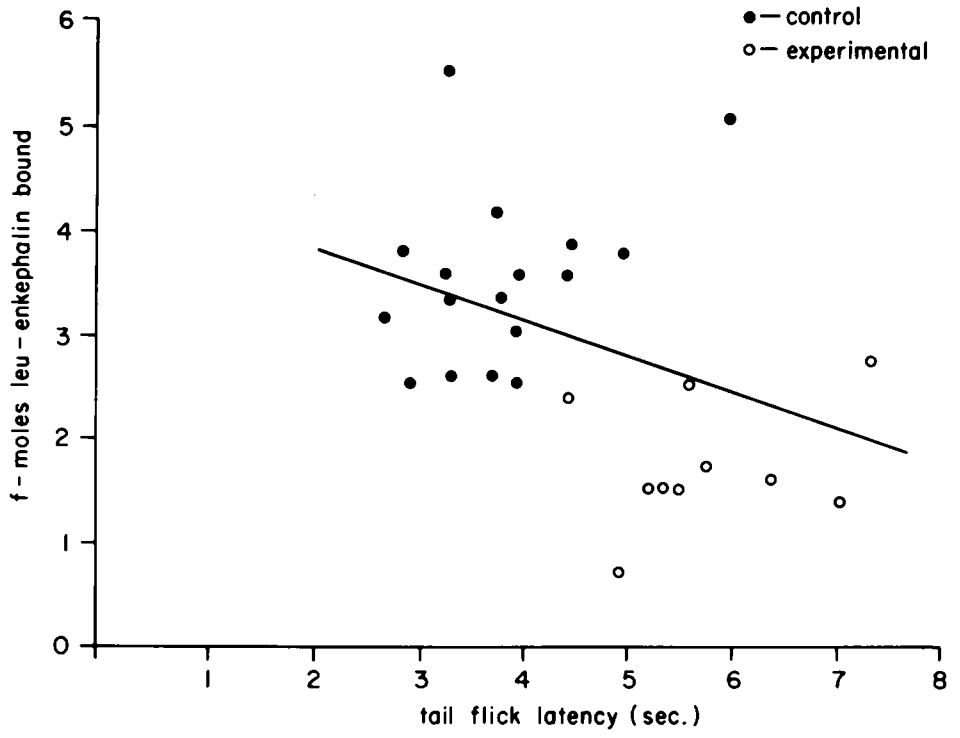

FIG. 2. Correlation of binding of ^{3}H-leu-enkephalin (1×10^{-10} M) expressed as femtomoles/total synaptosomal fraction derived from 1 mg wet weight of brain with tail-flick latency (sec). Each experimental subject (filled circles) had undergone 12 prior tests of antinociception, each followed 10 sec later by 15 sec of footshock (0.9 mA). The control rats (open circles) were handled similarly, but were never shocked. On the 13th day each of the presented tail-flick latencies were determined and each rat was immediately sacrificed for determination of individual binding. The significant decrease in binding ($p<0.01$) in the experimental group paralleled their increase in tail-flick response latency ($p<0.01$). The significant negative correlation ($r=0.04$; $p<0.05$ between binding and tail-flick latency for both groups suggests that the greater the amount of endogenous ligand bound (as indicated by less binding of leu-Enk) the greater the analgesic score (as indicated by increased tail-flick latencies) would be.

sent the receptor which specifically interacts with leu-Enk. Naloxone cannot effectively compete with leu-enkephalin for this receptor. Leu-Enk also interacts with a opiate type of receptor with a lower binding affinity and associated higher capacity for leu-Enk binding. Naloxone can compete with leu-Enk for binding at this receptor. It is possible that the marked changes observed in the Scatchard plot for leu-Enk binding to synaptosomal preparations of fear conditioned animals (shown in Fig. 1) are related to the endogenous release of leu-enkephalin and other opiate like substances by the fear-conditional animal. The decreased binding capacity for leu-Enk observed with the experimental animals may be related to prior occupancy by endogenously released leu-Enk. A hundred-fold excess of naloxone does not change the characteristics of the Scatchard plot for the binding of leu-enkephalin to the synaptosomal preparations isolated from fear-conditioned animals while these conditions cause the Scatchard plot for control synaptosomal preparations to resemble the experimental Scatchard plot. These observations are consistent with the view that naloxone sensitive sites in the synaptosomal preparations isolated from the fear conditioned animals are blocked in some way. This same effect can be mimiced by treating control synaptosomal preparations with excess naloxone. Obviously, the release of leu-Enk alone cannot be the sole basis for the observed effect since it would primarily alter the binding capacity and binding affinity observed at the lowest ligand concentrations. In fact, the most dramatic changes induced by fear-conditioning are evident in the high capacity low affinity part of the binding curve.

The relationship of individual binding of leu-Enk to each animal's tail-flick latency is illustrated in Fig. 2. These values for individual binding were obtained at a concentration of leu-Enk (1×10^{-10} M) which is not antagonized by naloxone, suggesting that, at this concentration, these receptors are specific for leu-Enk. At 1×10^{-10} M there was a significant difference in binding capacity between control ($3.75 \pm 0.60\times10^{-2}$ fmoles leu-Enk/mg synaptosome and experimental ($1.50 \pm 0.33\times10^{-2}$ fmoles leu-Enk/mg synaptosome) groups, $t(25)=6.06$; $p<0.01$. Although there was no difference in tail-flick latencies on Day 1, the latencies of the fear-conditioned subjects gradually increased each day to a mean value of 6.73 sec, controls - 3.75 sec; $t(25)=5.51$; $p<0.01$, on the day of sacrifice. There was also a significant negative correlation, $r=-0.40$; $p<0.05$, between each animal's tail-flick latency and binding score. This negative

relationship suggests a lower availability of binding sites in animals with longer latencies, possible due to the prior release of an endogenous ligand. These data are similar to our previous results [5] using leu-Enk at 2×10^{-8} M.

We believe that these data support the existence of at least two classes of receptors for leu-Enk in the CNS. The binding of leu-enkephalin to the first class of receptors can be displaced by naloxone while the other class of receptors are not blocked by naloxone and appear to have a much lower affinity for enkephalin. These results are similar to recent reports [7,8] suggesting multiple receptor sites for leu-Enk, some of which were not affected by narcotic antagonists. Conditioned fear induced dramatic changes in the binding affinity and capacity of both classes of receptors probably as a consequence of the release of an endogenous opiate-like peptide. The neuroanatomical localization of each class of receptor has not been established in the CNS. Therefore, even though there is a striking decrease in binding capacity observed for the low affinity binding of leu-Enk in the fear conditioned animals, the 50% decrease in HAR binding capacity under these conditions could be of greater importance if the localization of the HAR is restricted to pathways which are of functional significance for autoanalgesia.

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