Changes in ^{3}H-leucine Enkephalin Binding in Spinal Cord of Frog After Dorsal Rhizotomy, Cordotomy, Transcutaneous Stimulation and Temperature Variations: Correlation with Nociceptive Sensitivity

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Abstract. In the frog spinal cord about 50 % of the ^{3}H-leucine enkephalin (^{3}H-LE) binding sites (b.s.) were blocked by an endogenous ligand. Three days after deafferentation and cordotomy the number of free b.s. increased by 44 and 56 %, respectively. In spinal frogs the threshold of the flexor reflex responses evoked by nociceptive stimuli decreased. More than 7 days after deafferentation and cordotomy the number of both total and free ^{3}H-LE b.s. decreased, while the threshold of the flexor reflex responses returned to that before spinalization. Transcutaneous electrical stimulation (TES) of the hind limbs (30 Hz, 5 minutes) in frogs spinalized 3 hours earlier increased ^{3}H-LE binding at low intensities of stimulation (0.2 mA) and decreased the threshold of the flexor reflex responses. TES at higher intensities (1.0 mA) decreased ^{3}H-LE binding and increased the threshold. Three days after spinalization TES even at low intensity diminished 3H-LE binding and raised flexor reflex threshold. A decrease in the number of free ^{3}H-LE b.s. was found when the frog body temperature was elevated (from 15 to 24 °C) or lowered (from 15 to 1 °C) for 14 days and was accompanied by an increase in flexor reflex threshold. The data suggest the existence of an endogenous opioidergic system in the frog spinal cord which has a high degree of tonic activity.

Key words: Enkephalin binding — Frog spinal cord — Deafferentation — Cordotomy — Electrical stimulation

Introduction

In the past decade it has been accepted that endogenous opiate-like peptides have a modulatory role in the neural processing of pain and that various opiate-like peptides are released in increased amounts following painful stimulation and other stressful situations (for review see Kosterlitz and McKnight 1981; Yaksh 1981; Bonica et al. 1983). The density of opioid receptors and their endogenous ligands

in the spinal cord has been demonstrated to be high in the region where the primary afferents terminate, i.e. in the upper laminae (I—III) in the dorsal horn of mammals (Kuhar et al. 1973; Elde et al. 1976; LaMotte et al. 1976; Atweh and Kuhar 1977; Hökfelt et al. 1977; Simantov et al. 1977; Hunt et al. 1980; Glazer and Basbaum 1981) as well as in the upper dorsal horn of lower vertebrates including the frog (Hájek and Syková 1981; Lorez and Kemali 1981; Naik et al. 1981).

Chance et al. (1978) showed that decreased binding by the rat brain homogenate of exogenously added radiolabelled enkephalin accompanied conditioned fear-induced antinociception. They reported that differences in receptor occupation by an endogenous ligand were responsible for the difference in binding of labelled ligand since the observed difference in binding was abolished by preincubation of the crude membrane fraction at 37 °C. During preincubation the endogenous ligands are degraded by specific peptidases (Creese et al. 1975). The number of free binding sites therefore probably reflects the rapid changes in spontaneous release of the endogenous ligand, whereas the total number of sites disclosed by preincubation may reflect the turnover of the receptor protein. The ratio between the number of free receptors and receptors blocked by the endogenous ligand might represent "basal" or "resting" opioidergic tone.

In this study changes in the activity of the opioidergic system in frog spinal cord were investigated. We measured the specific ^{3}H-leucine enkephalin (^{3}H-LE) binding in crude membrane fractions of frog spinal cord and changes after a variety of experimental manipulations, i.e. after spinal cord transection, dorsal rhizotomy, transcutaneous electrical stimulation, and changes in body temperature. We used the *in vitro* receptor binding technique, which involves incubation of half membrane fraction at 0 °C as an indirect method for evaluating the *in vivo* functional state of the opiate receptor population — presumably occupation of receptors by an endogenous ligand (Chance et al. 1978; Cesselin et al. 1980; Christie et al. 1981). The observed values were compared with ^{3}H-LE binding following preincubation of the second half of the membrane fraction at 20 °C in order to allow liberation and/or degradation of the bound endogenous opiate-like ligand (Chance et al. 1978) and thus to obtain data about the total binding capacity of the membrane fraction. The concomitant changes in threshold for flexor reflex responses evoked by nociceptive stimulation were studied and used to indicate altered pain sensitivity.

Materials and Methods

Preparation. Experiments were performed on more than 200 frogs *(Rana temporaria)* during autumn, winter and spring. Dorsal laminectomy was performed in frogs anaesthetized with ether. For spinalization two spinal segments were exposed and the spinal cord was cut with fine scissors at the level of the dorsal root III entry. Bleeding was stopped with gelfoam and the skin sutured. For dorsal root

section the lumbar spinal cord was exposed and dorsal roots VII, VIII, IX and X were cut bilaterally under the microscope. The cord was covered with gelfoam and washed with Ringer solution, the back muscles were replaced and the skin sutured.

After an appropriate interval, the frogs were anaesthetized with ether and the spinal cord below the transection or the deafferented lumbar segment were removed in about one minute and kept in ice-cold Tris-HCl buffer for binding studies. The same sections of the spinal cord removed from intact frogs, which were kept at a similar temperature (13—16 °C) and conditions to the operated animals, served as controls. To study the effect of a range of temperatures on enkephalin binding the animals were kept for 14 days at constant temperatures (15 °C, 7 °C, 1 °C) or at room temperature (24 °C). Care was taken to ensure that all animals were exposed to similar lighting schedules.

Assesment of changes in flexor reflex threshold. To determine changes in the animal's sensitivity to nociceptive stimuli we tested the threshold for the flexor reflex evoked by immersion of the hind limb paws in hot water. The paws were immersed until withdrawn in a series of beakers with water temperature increasing by 0.5—1.0 °C. The hind limb was then immediately washed in water of the same temperature as that at which the frogs were kept for at least 14 days before the experiment. The immersion periods were limited to 15 s to prevent irreversible skin damage. The threshold for the flexor reflex was defined as the lowest temperature which repeatedly produced a flexor response, usually accompanied by vocalization. To minimize accomodation the frogs were exposed to different temperatures in random order.

Transcutaneous electrical stimulation (TES). The skin was electrically stimulated at a frequency of 30 Hz and intensity 0.1—1.0 mA (duration of pulses 1 ms) via bipolar silver electrodes lightly pressed onto the skin surface of the thigh (interelectrode distance 5—10 mm). The actual intensity of stimulation was calculated from the voltage drop across a resistance of 100 Ω measured on an oscilloscope. Intact frogs which were kept unrestrained tolerated TES up to an intensity of 0.8 mA, and usually no escape reaction was observed, only light muscle contractions. At higher intensities the animals tried to escape. Evoked potentials were recorded from dorsal root VIII or IX during TES. With a TES intensity of 0.2—0.8 mA only low threshold, large myelinated fibres with conduction velocity about 20 m . s^{-1} were excited. At higher stimulation intensities also fibres with slow conduction velocity about 9 m . s^{-1}, which are probably the myelinated pain afferents (Aδ), were excited.

Subcellular fractionation. In order to eliminate interference by endogenous ligand(s) present in the tissue which would appear in the rough homogenate and compete with ^{3}H-LE for binding, a crude membrane fraction was used for binding studies. This approach (Simantov et al. 1978) is generally preferred to the rough homogenates which were originally used (Lord et al. 1977). Isolated spinal cords were homogenized in a glass-teflon homogenizer in about 20—50 volumes of Tris-HCl buffer (50 mmol . l^{-1}, 0 °C, pH 7.4). A crude membrane fraction was then prepared from a $1000 \times g$ supernatant (0 °C, 5 minutes) by centrifugation at $20{,}000 \times g$ (0 °C for 15 minutes). The resulting sediment was resuspended in the original volume of Tris-HCl buffer.

^{3}H-leucine enkephalin binding. Fifty μl aliquots of the suspension were incubated for 2 hours in triplicate in ice-cold Tris-HCl buffer (50 mmol . l^{-1}, pH 7.4 — Simantov et al. 1978) containing labelled ligand (^{3}H-Tyr-leucine enkephalin, specific activity 38 Ci . mmol . l^{-1}). The total volume of incubation medium was 0.5 ml. The concentration of ^{3}H-LE ranged from 0.5 to 200 nmol . l^{-1} and binding was expressed using either the semilogarithmic plot (Klotz 1982) or the Scatchard plot. Binding curves were fitted to experimental data using analysis system "Statistics" on a Hewlett-Packard 9862A Calculator Plotter. Nonspecific binding was estimated by adding unlabelled LE in a concentration 2 μmol . l^{-1}. Specific binding was then determined as the difference between radioactivity in the absence and in the

presence of unlabelled ligand and expressed either in terms of mol . g^{-1} protein or as a percentage of the corresponding controls. Nonspecific binding represented 20—40% of the total binding and increased linearly with the ^{3}H-LE concentration over the whole concentration range studied. Membrane fractions after labelling were isolated by rapid filtration through Whatman CF/C glass microfibre filters and washed with 3 aliquots (5 ml each) of ice-cold Tris buffer. Filters were transferred into scintillation vials and counted in Bray scintillation fluid with an efficiency of 27—30%. The enkephalin binding sites labelled during incubation of membrane fraction in ice-cold buffer were designated as free enkephalin binding sites.

The number of free enkephalin binding sites was compared with the total number of enkephalin binding sites which was determined after the preincubation of membrane fraction in Tris-HCl buffer (pH 7.4) at 20 °C for 30 minutes. This procedure presumably led to the liberation and/or degradation of previously bound endogenous opiate-like ligand to enkephalin sites (Chance et al. 1978). After another centrifugation, the preincubated membrane fraction was again resuspended in Tris-HCl buffer (50 mmol . l^{-1}) containing labelled ligand, and incubated for 2 hours in cold. Subtraction of free from total binding sites indicated the amount of receptor sites blocked by endogenous ligand. Protein determinations were carried out on each tissue sample by the Lowry micromethod (Lowry et al. 1951). Student's t-test was used to calculate the statistical differences between control and experimental samples.

Chemicals. Labelled leucine enkephalin (^{3}H-LE) was obtained from the Isotope Laboratory, Biological Centre of Hungarian Academy of Sciences, Szeged, Hungary. Leucine and methionine enkephalin were from Sigma Chemical Company (U.S.A.), morphine hydrochloride from Spofa (Czechoslovakia), bacitracin from Serva (F.R.G.). Naloxone was a gift by Endo Laboratories (U.S.A.). All other chemicals used were of analytical grade.

Results

^{3}H-LE binding sites in membrane fractions of the spinal cord.

^{3}H-LE was found to bind specifically to crude membrane fractions of the frog spinal cord. This specific ^{3}H-LE binding increased after preincubation of membrane fractions at 20 °C (Fig. 1 and Fig. 2) which suggests that some of the binding sites previously occupied by the enkephalin-like endogenous ligand might be disclosed by this procedure (Chance et al. 1978). The number of available binding sites increased gradually during 5 to 20 minutes of preincubation, while longer preincubation did not farther increased ^{3}H-LE binding. The specific binding of ^{3}H-LE in non-preincubated membrane fraction (^{3}H-LE binding to free sites) as well as after preincubation at 20 °C for 30 minutes ("total" ^{3}H-LE binding) was studied in the concentration range 0.5—200 nmol . l^{-1} and the data were plotted on a semilogarithmic binding curve. The double-sigmoid shape of both curves, which represent either "total" ^{3}H-LE binding or ^{3}H-LE binding to free binding sites, suggests the presence of two types of ^{3}H-LE binding sites in frog spinal cord (Figs. 1, 2 and 4). Similar conclusions could be drawn from a Scatchard plot (not shown), the curvilinear manifestation of which indicated the presence of more than one, probably two, binding sites (Titeler 1981). The B_{max} (maximum binding

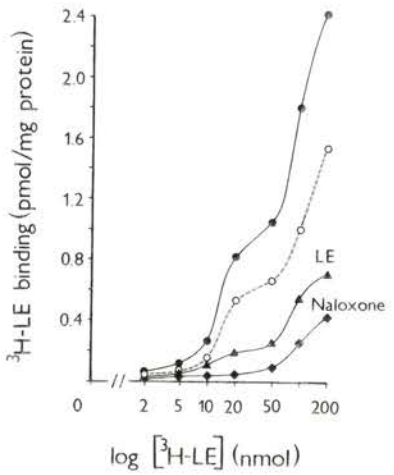

Fig. 1. Binding isotherms of specific ^{3}H-LE binding to frog spinal cord membrane fractions at ligand concentrations ranging from 2 to 200 nmol . l^{-1}. Open circles — ^{3}H-LE binding to non-preincubated membranes, full circles — ^{3}H-LE binding after preincubation at 20 °C. Full triangles — the effect of leucine-enkephalin, LE, (10 μmol . l^{-1}) and full squares — the effect of naloxone (1 μmol . l^{-1}) when added to the medium during preincubation. The values shown are from a single representative experiment performed on membrane fractions from 5 pooled spinal cords. Each point was assayed in triplicate and results are mean values.

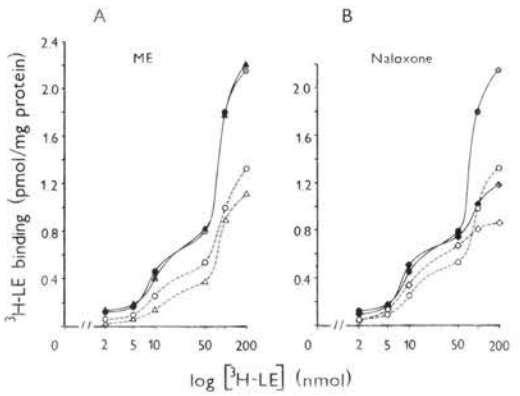

Fig. 2. Binding isotherms of specific ^{3}H-LE binding to frog spinal cord membrane fractions at ligand concentrations ranging from 2 to 200 nmol . l^{-1}. Membrane fractions were prepared from isolated spinal cords they had been superfused for 30 minutes at 20 °C either with oxygenated Ringer solution (circles) or with Ringer solution containing methionine enkephalin (ME, 10 μmol . l^{-1} — triangles), or naloxone (1 μmol . l^{-1} — squares). Membrane fractions were prepared from 4 cords in each sample. Open symbols — ^{3}H-LE binding in non-preincubated membrane fractions, full symbols — ^{3}H-LE binding after preincubation of membranes at 20 °C.

capacity) and K_d (affinity constant) were not calculated since we did not succeed in obtaining complete saturation of the second binding site (see Discussion). It is evident that the first ^{3}H-LE binding site had a higher affinity and lower total binding capacity than the second ^{3}H-LE binding site. It is however apparent that the second binding site was characterized by a higher binding capacity but lower affinity for the ^{3}H-LE.

After preincubation of membrane fractions at 20 °C, the total binding increased over the whole concentration range, but no apparent changes in the affinity of ^{3}H-LE binding sites were observed (see Fig. 1). After preincubation of membrane fraction at 20 °C and recentrifugation, membrane sediment and supernatant were obtained. The supernatant decreased ^{3}H-LE binding in non-preincubated as well in preincubated membrane fractions, when added together with ^{3}H-LE in the amount which was previously removed from the respective fraction (Table 1). In addition when the membrane fractions were preincubated in the presence of peptidase inhibitor bacitracin the typical increase in binding capacity was attenuated or did not occur at all, i.e. ^{3}H-LE binding remained equal to that found in non-preincubated fractions. At concentrations of 0.5, 1.0 and 2.0 nmol . l^{-1} bacitracin inhibited the increase in ^{3}H-LE binding by 21.9 ± 2.1, 28.9 ± 1.9 and 100%, respectively (mean ± S.E., $n = 4$, ^{3}H-LE concentration 5 nmol . l^{-1}). The results were essentially the same for ^{3}H-LE concentration of 50 nmol . l^{-1}.

Localization of ^{3}H-LE binding sites in the spinal cord.

To determine the approximate localization of opioid binding sites the ^{3}H-LE binding was tested in membrane fractions from dorsal and ventral halves of the spinal cord ($n = 9$). Using labelled ligand at concentrations of 5 or 20 nmol . l^{-1} it was found that most of the radioactive ligand was bound to the non-preincubated fraction from the dorsal half of the spinal cord (70.9 ±2.7% and 69.7 ±1.5%, respectively), however, a considerable proportion of the ^{3}H-LE binding was also found in the ventral halves. When measured after preincubation of the membrane fractions at 20 °C, quantitatively similar results were obtained.

Changes in ^{3}H-LE binding site occupancy.

The changes in ^{3}H-LE binding sites occupancy were studied in experiments where unlabelled leucine enkephalin (LE, 10 μmol . l^{-1}) or naloxone (1 μmol . l^{-1}) were added to membrane fractions during preincubation at 20 °C. Fig. 1 shows a typical result of such an experiment ($n = 4$). Whereas the preincubation of membrane fraction in Tris buffer increases (the number of binding sites available for ^{3}H-LE, the addition of either LE or naloxone decreases the number of available binding

Table 1. Inhibition of ^{3}H-LE binding by addition of supernatant obtained from spinal cords by preincubation of membrane fractions at 20 °C.

	^{3}H-LE binding to high affinity sites (5 nmol . l^{-1} ^{3}H-LE)						^{3}H-LE binding to low affinity sites (50 nmol . l^{-1} ^{3}H-LE)					
	Control		Supernatant added		% inhibition by supernatant		Control		Supernatant added		% inhibition by supernatant	
	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total
I	218.4	302.9	136.5	72.7	37.5	75.9	1098.8	1271.3	803.8	702.2	26.8	44.7
II	186.1	324.0	36.1	67.6	80.6	78.5	974.0	1360.1	570.6	542.3	41.4	60.1

Binding of ^{3}H-LE to membrane fractions was studied at two ligand concentrations — 5.0 and 50.0 nmol . l^{-1}, each in membrane fractions from 3 pooled spinal cords. Free — ^{3}H-LE binding in non-preincubated membrane fractions, Total — binding after preincubation (in fmol . mg^{-1} protein). To one half of membrane fractions the supernatant was added and the second half served as control. I and II — two independent experiments.

sites. LE or naloxone present in the incubation medium not only prevented the enhancement of ^{3}H-LE binding by preincubation, but even blocked opioid binding sites further, decreasing binding of ^{3}H-LE over the whole range of concentrations used.

In further experiments ($n = 12$) the whole isolated spinal cords were superfused *in vitro* for 30 minutes either with oxygenated Ringer solution (controls), or with Ringer solution to which enkephalin or naloxone had been added. The addition of methionine enkephalin (ME, 10 μmol . l^{-1}) into the Ringer solution significantly decreased ^{3}H-LE binding (by 20—60%) in the whole concentration range of ^{3}H-LE tested (Fig. 2*A*). After preincubation of membrane fractions from these spinal cords in a Tris buffer at 20 °C, ^{3}H-LE binding was the same as found in control frogs. On the other hand, the altered shape of the binding curve after superfusion of isolated cords with the Ringer solution with naloxone (1 μmol . l^{-1}, Fig. 2*B*) shows that naloxone affects the high affinity sites differently from the low affinity sites. At lower concentrations of ^{3}H-LE the binding was increased, while at higher concentrations of ^{3}H-LE the binding was decreased. After preincubation of membrane fractions from spinal cords treated with naloxone, ^{3}H-LE binding returned to control values only at low concentrations of labelled ligand. At higher concentrations of labelled ligand, ^{3}H-LE binding remained decreased by about 40—50%.

^{3}H-LE binding after dorsal root section or spinalization.

The ^{3}H-LE binding to non-preincubated membrane fractions and to fractions after preincubation at 20 °C, was studied 3 hours, 3 days, 7 days and 14 days after bilateral dorsal root section and 3 hours, 3 days, 7 days, 14 days and 6 weeks after spinalization. Two series of experiments were performed. In the first, one ^{3}H-LE binding was studied separately in different spinal cords at one concentration of labelled ligand (5 nmol . l^{-1}) which represents binding to the high affinity site. In the second series, membrane fractions from 6—9 spinal cords were pooled and the whole concentration range (0.5—200 nmol . l^{-1} of ^{3}H-LE) was used to clarify the reaction of both high and low affinity binding sites.

There was no change in ^{3}H-LE binding of membrane fractions from spinal cords removed 3 hours after the dorsal root section. Three days and 7 days after dorsal rhizotomy ^{3}H-LE binding to free binding sites increased by about 50%. While ^{3}H-LE binding to preincubated fractions was still not changed 3 days after rhizotomy, 7 days after rhizotomy, when Wallerian degeneration of dorsal roots in the toad (20 °C) has been shown to reach the spinal dorsal horn (Joseph and Whitlock 1972), the total ^{3}H-LE binding decreased. A futher decrease of ^{3}H-LE binding to total binding sites as well as to free sites could be observed 14 days after deafferentation (Fig. 3).

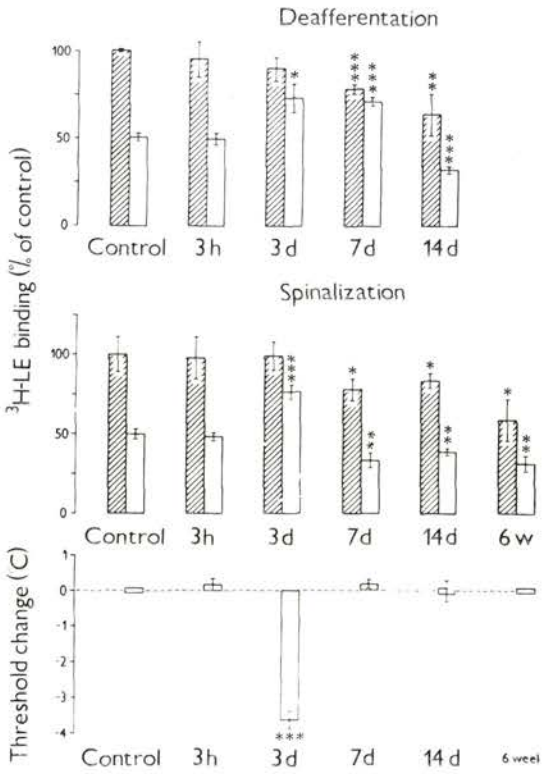

Fig. 3. The effect of deafferentation and spinalization on the ^{3}H-LE binding in spinal cords and on the threshold for flexor reflex evoked by immersion of hind limbs in hot water (after spinalization only). Upper and middle set of columns: hatched columns represent ^{3}H-LE binding after preincubation of membrane fraction (total binding), white columns — ^{3}H-LE binding to non-preincubated membranes as measured at ^{3}H-LE concentration 5 nmol . l^{-1}. The data (means ± S.E.) are expressed as % of controls, i.e. ^{3}H-LE binding in intact, but otherwise similarly treated frogs. Individual spinal cords were used in binding studies. Deafferentation: Control ($n = 11$), 3 hours ($n = 3$), 3 days ($n = 5$), 7 days ($n = 3$), and 14 days ($n = 4$) after bilateral dorsal rhizotomy. Spinalization: Control ($n = 12$), 3 hours, 3 days and 7 days ($n = 4$), 14 days ($n = 3$) and 6 weeks ($n = 2$) after spinalization. The same spinalized frogs which were tested for changes in flexor reflex threshold (lower set of columns) were used for the binding study. Significance of differences: * — $P < 0.05$; ** — $P < 0.02$; *** — $P < 0.001$.

Three hours after spinal transection, ^{3}H-LE binding to membrane fraction from spinal cord below the section was unchanged (Fig. 3). Three days after the transection ^{3}H-LE binding to free sites increased by about 50%, the total number of binding sites remained unchanged, as was also the case 3 days after dorsal rhizotomy. Seven and 14 days after spinal transection, ^{3}H-LE binding to both, free and total sites decreased significantly with respect to that found in non-transect-

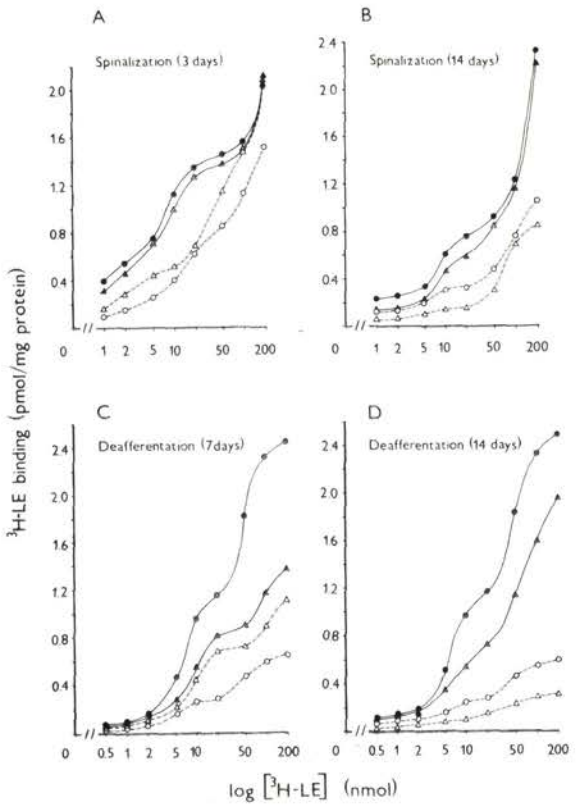

Fig. 4. The effect of spinalization (3 days, or 14 days, *A* and *B*) and deafferentation (7 days or 14 days, *C* and *D*) on specific ^{3}H-LE binding as shown by binding isotherms at ligand concentrations ranging from 0.5 to 200 nmol . l^{-1}. Open symbols — ^{3}H-LE binding in non-preincubated membrane fractions, full symbols — ^{3}H-LE binding after preincubation of membranes at 20 °C. Circles — ^{3}H-LE binding in membrane fractions from intact frogs, triangles — ^{3}H-LE binding after spinalization or deafferentation. The values shown in the four graphs are from four representative experiments performed on membrane fractions from 4—5 spinal cords in each. Each point was assayed in triplicate and the results are mean values.

ioned frogs (controls). At longer intervals after spinalization (6 weeks) a further decrease of ^{3}H-LE binding occurred.

When ^{3}H-LE binding was studied in the whole concentration range, similar results were obtained as when only one concentration of the labelled ligand was used. The most remarkable findings, namely an increased number of free sites (both of high and low affinity) 3 days after spinalization and 7 days after rhizotomy, and a decreased number of free sites 14 days after spinalization and 14 days after dorsal rhizotomy, are shown in Fig. 4 *A*—*D*. Furthermore, this figure

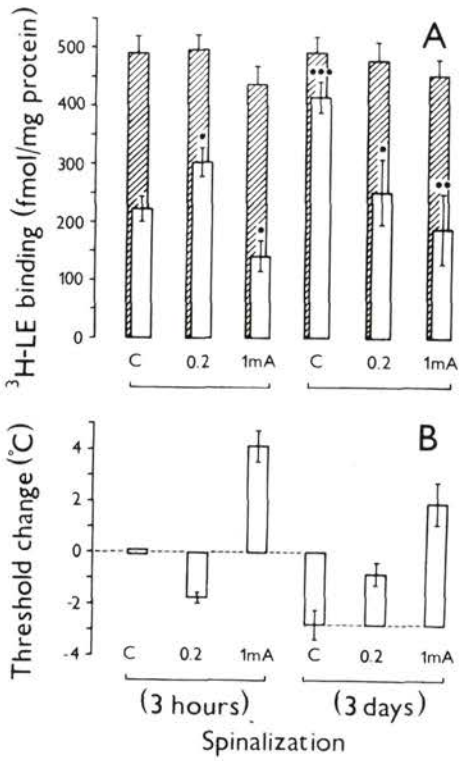

Fig. 5. The effects of transcutaneous electrical stimulation (TES) of both hind limbs on the ^{3}H-LE binding (*A*) and on the threshold for the flexor reflex evoked by hot water application (*B*) in frogs 3 hours and 3 days after spinalization. *C*: ^{3}H-LE binding and changes in threshold in unstimulated spinalized frogs (controls). 0.2 mA and 1.0 mA: TES at frequency of 30 Hz and intensity 0.2 or 1.0 mA was applied for 5 minutes. In *A*: ^{3}H-LE binding to non-preincubated membrane fractions (open columns), after preincubation at 20 °C (hatched columns), mean ± S.E., $n = 6$ in both controls and $n = 3$ in other columns. In *B*: the lowest temperature which evoked the flexor reflex in unoperated frogs has been taken as the threshold and was not changed in the same frog 3 hours after spinalization (0 — no threshold change). Each column in *B* represents the mean threshold change ± S.E., $n = 4$. Significance of differences: * — $P < 0.05$; ** — $P < 0.01$; *** — $P < 0.001$.

shows that the total number of binding sites decreased at both these time intervals after deafferentation; spinalization, however, affected total binding to a much lesser degree (i.e., decreased it only at the 14-day interval). The affinity of ^{3}H-LE sites was probably not substantially altered, because we did not find greater horizontal shifts in binding curves after spinalization or after deafferentation (Fig. 4 *A*—*D*).

The effect of spinalization on the flexor reflex evoked by nociceptive stimuli and on post-stimulation analgesia.

The increased number of free ^{3}H-LE binding sites in membrane fractions from the spinal cord of spinalized frogs (3 days) was associated with a decrease in the threshold for the flexor reflex evoked by nociceptive stimulation. The threshold for the flexor reflex evoked by immersion of the hind limb in hot water had decreased by 3.6 ±0.2 °C (Fig. 3 and Fig. 5). Seven days or more after spinalization when ^{3}H-LE binding in non-preincubated as well as in preincubated membrane fractions decreased and the ratio of free/total binding sites had returned approximately to that found in intact frogs, the threshold for the flexor reflex was also restored to the same level as that in normal frogs (Fig. 3). In a preliminary report we showed that in acutely spinalized frogs (3 hours) transcutaneous electrical stimulation (TES) of the hind limbs changed ^{3}H-LE binding to free high and low affinity binding sites in the lumbar spinal cord, while the total number of binding sites remained unchanged (Syková et al. 1982).

Frogs which were spinalized for 3 days were found to be more sensitive to TES than frogs spinalized for only 3 hours (Fig. 5). In frogs spinalized for 3 hours TES of the hind limb (30 Hz) changed both the ^{3}H-LE binding in the non-preincubated membrane fractions from lumbar spinal segments and the threshold for the flexor reflex evoked by immersion of hind limb paws in hot water. Low intensity stimulation (0.2 mA, activating solely low threshold afferents) increased the ^{3}H-LE binding in non-preincubated membrane fractions from spinal cords removed immediately after TES (i.e., during the first 1—2 minutes) by 36 ±11% when compared with the binding in the cords of spinalized frogs which were not stimulated (Fig. 5*A*). The threshold for the flexor reflex evoked by immersion of the hind limbs in hot water decreased (Fig. 5*B*). In contrast, stimulation at a higher intensity (1.0 mA, which activated also high threshold afferents), led to a decrease in ^{3}H-LE binding to free sites by 37 ±12%, this was associated with an increase in the flexor reflex threshold. Fig. 5 further shows that in frogs 3 days after spinalization (when ^{3}H-LE binding to non-preincubated membrane fractions increased and the threshold for the flexor reflex was lowered (see also Fig. 3)), TES, either at the low (0.2 mA) or at the high (1.0 mA) stimulation intensity, decreased ^{3}H-LE binding to free sites and increased the flexor reflex threshold with respect to the values in non-stimulated frogs. There was, however, no significant difference in ^{3}H-LE binding before and after TES when membrane fractions were preincubated (Fig. 5*A*).

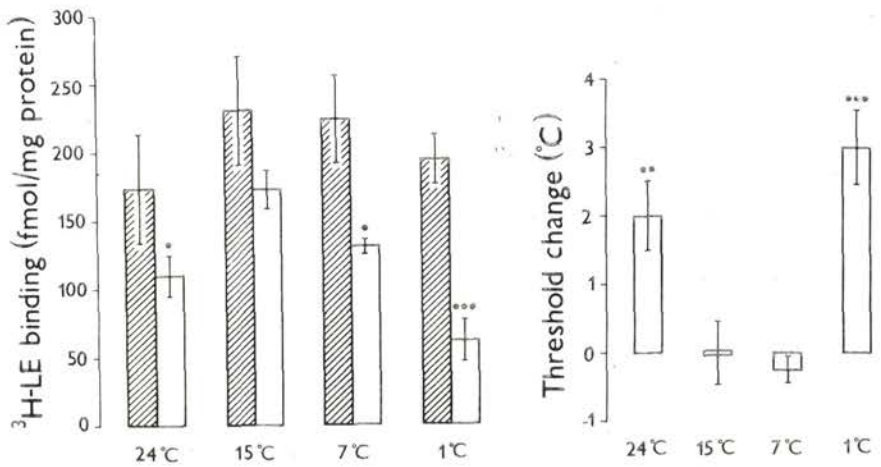

Fig. 6. The effects of various ambient temperatures on ^{3}H-LE binding at ^{3}H-LE concentration 5 nmol . l^{-1} and the threshold for the flexor reflex evoked by immersion of the hind limbs in hot water. On the left side: hatched columns represent ^{3}H-LE binding after preincubation of membrane fractions at 20 °C, white columns ^{3}H-LE binding in non-preincubated membranes. Values are expressed as fmol . mg^{-1} protein (mean ± S.E.), $n = 4$ in each column. On the right side: changes in flexor reflex threshold (mean ± S.E.), when mean threshold in frogs held in 15 °C was taken as zero change ($n = 4$ in each temperature). Significance of differences: * — $P < 0.05$; ** — $P < 0.02$; *** — $P < 0.01$.

The effects of temperature variations on ^{3}H-LE binding and flexor reflex threshold.

^{3}H-LE binding to high as well to low affinity binding sites and the flexor reflex threshold were also significantly altered by exposing the frogs for 14 days to four different ambient temperatures — 1, 7, 15 or 24 °C (Fig. 6). Using a radioactive ligand at concentrations of 5 or 50 nmol . l^{-1} (not shown) quantitatively similar results were obtained. Exposure of frogs to temperatures 24 °C, 7 °C and 1 °C significantly decreased the ^{3}H-LE binding in non-preincubated membrane fractions of the spinal cord when compared with that exposed to 15 °C. The ^{3}H-LE binding to preincubated membrane fractions did not show significant differences, although ^{3}H-LE binding tended to decrease. The threshold for the flexor reflex evoked by nociceptive stimulation (i.e., immersion of the hind limbs in hot water) was increased in frogs kept at 24 °C or 1 °C by 1—4 °C, when compared with at 15 or 7 °C (Fig. 6).

Discussion

Our data suggest that there are two binding sites for ^{3}H-LE in the frog spinal cord

(one with a higher and another with a lower affinity) similar to that found in the mammalian brain (Lord et al. 1977; Leslie et al. 1980; Fischel and Medzihradsky 1981). We did not calculate the absolute values of the maximal binding capacity (B_{max}) and affinity constant (K_d) of individual binding sites due to high scatter of data obtained in individual experiments, and the possible errors arising from all the available plotting techniques (Scatchard plot, Klotz semilogarithmic plot — see Klotz 1982; 1983a, b; Munson and Rodbard 1983), especially when a crude membrane binding system is used and more than one site is present (Munson and Rodbard 1983). Furthermore, in some of our experiments we were not able to obtain complete saturation even using a concentration of ^{3}H-LE as high as 200 nmol . l^{-1}. We therefore preferred to show the results in the form of a simple semilogarithmic graph on which differences between relevant controls and experimental approaches can be seen. We can however conclude that the affinity of ^{3}H-LE binding sites in frog spinal cord was about 5—10-times lower than that of the sites present in the mammalian brain.

The studies utilizing the ratio between the number of total and free binding sites as a measure of receptor occupancy have recently been subject of criticism. However, we suppose that this method is well-founded due to two reasons. First, the saturation of opioid receptors by ligand is slow in comparison with receptors for neurotransmitters. This prevents the fast saturation of free sites by endogenous ligand released during homogenization of the tissue. Second, the rate of degradation of the receptor-ligand complex in cold is very low (it was negligible in our experiments), this enables to see what part of the sites has been blocked by the endogenous ligand *in vivo*. Moreover, in our experiments the control tissue was treated similarly as the tissue after various experimental manipulations and differences were observed. In addition to above mentioned reasons, spinal cords were diluted 20—50-times for homogenization and again during resuspension of isolated membrane fractions. If free endogenous ligands were present their concentration would be by 2—3 orders of magnitude lower than those occurring in the spinal cord *in vivo* and they could thus hardly compete for binding with ^{3}H-LE.

In the present study, approximately one half of ^{3}H-LE binding sites in the frog spinal cord was occupied by an endogenous ligand present in the spinal cord which may be liberated and/or degraded by endogenous peptidases. The presence of a bound opioid substance which can eventually be released from its binding sites is further supported by the findings that ^{3}H-LE binding was inhibited by the supernatant obtained during the preincubation, and that the peptidase inhibitor, bacitracin, blocked the increase in ^{3}H-LE binding during preincubation of membrane fractions. Since the preincubation of membrane fractions in our experiments only changed the ^{3}H-LE binding capacity while the shape of the binding curve remained the same, we suppose that the increased binding is primarily due to liberation of ^{3}H-LE binding sites and not due to changes in affinity of binding sites.

The increase in binding may be explained by the liberation of receptor sites, previously occupied by an endogenous ligand, by the action of peptidases, rather than by any modification of the receptor molecule. Our finding that a substantial proportion of the ^{3}H-LE binding sites is blocked by the endogenous ligand therefore suggests a high degree of tonic activity of the endogenous opioidergic system in the spinal cord of the frog.

^{3}H-LE binding in the spinal cord membrane fractions was affected by the opiate antagonist naloxone. When naloxone was added to membrane fractions during preincubation, it not only did prevent the usual increase in ^{3}H-LE binding, but also decreased binding even further (it blocked nearly all high affinity binding sites and about 75% of low affinity sites, Fig. 1). However, when naloxone was added to isolated spinal cord in the superfusing Ringer solution it did not apparently become bound to sites with higher affinity, since it even increased ^{3}H-LE binding to free high affinity sites. On the other hand, naloxone decreased binding to low affinity binding sites similarly as when added to membrane fractions. We suppose that the increase in ^{3}H-LE binding evoked by superfusion with naloxone was not due to elimination of "down-regulation". Baram and Simantov (1983) observed an increased number of total opiate binding sites only after long-term incubation of neuroblastoma-glioma cells with this antagonist but not after short time incubation. Moreover, we observed only an increase in the number of free binding sites, with no change in either the total number of binding sites or any horizontal shift of the binding curves during short-term treatment with naloxone, and we can therefore assume that naloxone blocked the tonic release of the endogenous opiates from their spinal cord storage sites.

It might also be speculated that the apparent discrepancy between the binding of naloxone, added either to membrane fractions or to spinal cords *in vivo*, might be explained on the assumption that the high affinity binding sites were not accessible to naloxone. They might be localized on intracellular membranes which do not communicate with the extracellular medium, while the low affinity sites communicate with the extracellular space and naloxone added to a superfusate can reach them. The preincubation of membrane fractions from isolated cords treated with naloxone does not disclose low affinity binding sites (Fig. 2), showing that either naloxone attenuates peptidase activity directly (Hiu et al. 1981), or that the peptidases were not able to split naloxone from its complex with the receptor. This might explain some of the observed long-lasting or irreversible experimental effects of naloxone (Hill 1981).

The ^{3}H-LE binding in frog spinal cord was found to be dramatically changed by the elimination of supraspinal control and by the elimination of the input from the periphery. Since 3 days after dorsal rhizotomy or cordotomy only the number of free sites increased, and the total number of sites disclosed by preincubation and their affinity remained unaffected, we suggest that the activity of the spinal

enkephalinergic system, i.e. the amount of the endogenous opioidergic ligand released, was significantly lowered. The apparent hypersensitivity to nociceptive stimulation which developed might therefore be a result of the excitation or disinhibition of cells located in lower dorsal horns (layer IV or V cells according to Rexed 1954). We observed a decrease in the number of total ^{3}H-LE binding sites 7 days after dorsal rhizotomy or cordotomy, apparently due to Wallerian degeneration (Waller 1850). Our data are in agreement with those of Joseph and Whitlock (1972) who demonstrated in toads maintained at 20 °C that degeneration of fibers proceeded in a centrifugal manner and first reached upper dorsal horn 6 days after the dorsal root section. Opiate binding sites are apparently present on primary afferent fibres of mammals (Hiller et al. 1978) and they are lost after somatic nerve or dorsal root section (LaMotte et al. 1976; Fields et al. 1980; Nincovic et al. 1981). A decrease in the number of binding sites for ^{3}H-diprenorphine in nerve fibres has also been found in the rat spinal cord after sensory deprivation by peripheral nerve section (Csillik et al. 1982). We cannot rule out that after dorsal root section and cordotomy there may be variations not only in the number of opiate binding sites, but also in their affinity towards opiate-like ligands. For example, the dissociation rate has been shown to be accelerated by low concentrations of GTP (Pryhuber et al. 1982). However, it seems that the δ-type of opiate receptor, which is also supposed to bind ^{3}H-LE, is not affected as much by this nucleotide as the μ-type (Zukin and Ginzler 1980; Chang et al. 1981). Furthermore, significant changes in the shape of ^{3}H-LE binding curves were not observed in our experiments.

There is an ample evidence that spinal opioidergic system in spinal dorsal horn is involved in poststimulation analgesia (Sjölund and Eriksson 1979; Cervero et al. 1981; Woolf et al. 1980). We suggest, in the light of our experiments with cordotomy, TES and temperature changes, that the lower the basal enkephalinergic tone, the lower the flexor reflex threshold, i.e. spinal reflexes are enhanced and therefore the sensitivity of the animal to pain may also be increased (Fig. 3 and 5). Low or reduced activity of the spinal enkephalinergic system 3 days after spinalization might also be responsible for the increased sensitivity to TES, since endogenous opioid ligand released during TES can bind to an increased number of free opioid binding sites. From our experiments we cannot tell anything about the nature of the endogenous ligand in the frog spinal cord. Both low molecular weight peptides (enkephalins, dynorphin) and high molecular weight peptides, such as endorphins, can act as endogenous opiates in amphibians and mammals (Cone and Goldstein 1982; Przewlocki et al. 1983), and become bound to sites which bound ^{3}H-LE in our experiments.

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