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The Correlation Between Swim-Stress Induced Antinociception and [^{3}H] Leu-Enkephalin Binding to Brain Homogenates in Mice

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CHRISTIE, M. J., G. B. CHESHER AND K. D. BIRD. *The correlation between swim-stress induced antinociception and [^{3}H] leu-enkephalin binding to brain homogenates in mice.* PHARMAC. BIOCHEM. BEHAV. 15(6) 853–857, 1981.—Mice which had been made to swim for 3 minutes showed a tail flick latency which was significantly longer than that of unswum controls. The [^{3}H] leu-enkephalin [LE] binding to brain homogenates from swum mice was significantly reduced when compared with that from unswum controls. Scatchard analysis revealed that the reduction in binding occurred at the LE low affinity site. However, when homogenates were allowed a preincubation period of 20 min at 37°C, the difference in LE binding between swum and unswum mice was no longer apparent. These data are interpreted to suggest that the reduced LE binding may be due to the occupation of a proportion of the opiate receptor population by an endogenous ligand. A correlation between the duration of the swim induced antinociceptive response and the changes in LE binding is described which although non-significant, is consistent with the interpretation for the involvement of endogenous opiates in the observed increases in tail flick latency.

Opiate receptor Opiate binding Leucine enkephalin Stress-induced analgesia Endogenous opiates

THE production of an antinociceptive response in experimental animals by a variety of stressful procedures has been widely reported [2, 3, 4, 8, 9, 13, 17].

The sensitivity, partial or complete, of this antinociceptive response to naloxone [2, 8, 9, 17] and reports of an apparent cross tolerance to morphine [8,9] suggest the involvement of the endogenous opiates. Furthermore, stress induced analgesia has been shown to produce increases in brain levels of an opiate-like substance [4,17] and a reduction in the binding of exogenously added enkephalin to brain homogenates [5]. The latter was interpreted by the authors as suggesting an increase in receptor occupancy by an endogenous ligand.

In the present study, experiments were designed to determine if a correlation exists between the behavioural antinociceptive response and the changes in opiate receptor kinetics determined by binding techniques.

METHOD

Random bred female QS mice (18–30 g) from the University animal farm were housed in groups of 15 at 22±1° (12 hr light/12 hr dark cycle) and allowed food and water ad lib up to the time of the experiment.

Experiment 1: The Effect of Acute Stress on the Antinociceptive Response and [^{3}H] Leu-Enkephalin Binding

Mice were acutely stressed by swimming for 3 min in groups of six in water at 32±1° in a container (a standard mouse box) 29×20×15 cm, the water depth being 10 cm. Control animals were kept in groups of 6 in a mouse box of the same dimensions whilst the experimental group was being stressed.

Antinociceptive response was determined by the tail flick method [9,11] and each mouse was tested by tail flick only once. Immediately after determination of tail flick latency each mouse was killed by immersion in liquid nitrogen. The frozen brains were removed and were stored (minus the cerebellum) in liquid nitrogen, or at −70°C until assayed.

Frozen brains were homogenised (Ystral 7801, setting 5×20 sec) in 60 volumes of Tris-citrate (50 mM pH 7.4 at 4°C) and centrifuged at 50,000×G for 15 min (4°C). Pellets were resuspended and assayed immediately for [^{3}H] leu-enkephalin binding. Alternately homogenised brains were incubated for 20 min at 0°C or 37°C in the presence of 50 mM NaCl prior to centrifugation at 50,000×G for 15 min (4°C).

[^{3}H] Leu-enkephalin binding was performed as described by Lord *et al.* [15]. Homogenates (0.6–1.0 mg protein) were incubated for 120 min at 0°C in 2.0 ml Tris-citrate buffer containing 10 nM [^{3}H] leu-enkephalin (39 Ci/mmol Radiochemical Centre, Amersham, U.K.).This concentration of leu-enkephalin should saturate both low and high affinity binding sites and any changes in specific binding are likely to reflect changes in numbers of available binding sites. Specific binding was defined as the difference between values obtained in the absence and presence of 10^{-6} M levor-

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TABLE 1

THE EFFECT OF ACUTE STRESS ON THE ANTINOCICEPTIVE RESPONSE AND [^{3}H] LEU-ENKEPHALIN BINDING

	Control	Acute Stress
Tail flick latency (sec)	0.85 ± 0.10	2.01 ± 0.21*
[^{3}H] leucine enkephalin bound (fmol/mg protein)	49.0 ± 4.9	30.7 ± 3.8†

Data are means ± s.e.m. from 2 independent experiments (N=10/group). In each experiment the binding assay was performed at 0°C at a saturating concentration of [^{3}H] leu-enkephalin (Experiment 1).

*$p<0.001$ control vs acute (t-test, 18 df).

†$p<0.01$ control vs acute (t-test, 18 df).

TABLE 2

THE EFFECT OF PREINCUBATION OF HOMOGENATES AT 0°C OR 37°C ON [^{3}H] LEU-ENKEPHALIN BINDING

Preincubation Temperature	Control	Acute Stress
0°C	46.1 ± 2.2	35.2 ± 3.9*
37°C	68.2 ± 2.2	61.9 ± 3.2†

Homogenates were incubated at 0°C or 37°C prior to centrifugation. The binding assay was performed at 0°C at a saturating concentration of [^{3}H] leu-enkephalin (Experiment 1). Data are mean ± s.e.m. (N=9/group) of leucine enkephalin binding (fmol/mg protein).

*$p<0.05$ Control vs Acute ($t=2.43$, 16 df).

†$p<0.001$, 0°C vs 37°C (paired t-test, 8 df).

phanol. At the same concentration the (+) isomer, dextrorphan, had no effect on binding. Incubations were terminated by rapid filtration (Whatman GF/B) under vacuum. Filters were rinsed three times with 5 ml of ice cold buffer and radioactivity determined by liquid scintillation spectroscopy. All samples were assayed in quadruplicate. Protein was estimated by the method of Lowry *et al.* [16].

Experiment 2: The Kinetics of [^{3}H] Leu-Enkephalin Binding After Acute Stress

Groups of 6 mice were acutely swim-stressed as in Experiment 1. [^{3}H] leucine enkephalin binding was performed as described above with the exception that 10 concentrations (0.06–6.0 nM) of [^{3}H] leu-enkephalin were employed. Brains from 4 mice were pooled for each determination.

Experiment 3: The Correlation Between Acute Antinociceptive Response and [^{3}H] Leu-Enkephalin Binding

Mice were acutely swim-stressed as in Experiment 1. The duration of stress analgesia was determined by testing by tail flick separate groups each of 6 mice, tested immediately, 2, 4, 8, 16, 32 and 64 minutes after completion of the swim. In this experiment results for 84 mice (12 for each time point) were determined. Similarly, 84 mice were tested at the same time as unstressed controls.

In a similar experiment, mice were tested for tail flick latency immediately 2, 4, 8, 32 and 64 minutes (18 mice for each time point) after removal from the water bath. Immediately after the determination of tail flick latency each mouse was killed by immersion in liquid nitrogen, and [^{3}H] leu-enkephalin binding determined as in Experiment 1.

RESULTS

Experiment 1

Results shown in Table 1 indicate that a significant difference exists between the tail flick latencies of control and acutely stressed mice, $t(18)=4.98$, $p<0.001$. This difference was reflected also in the analysis of [^{3}H] leu-enkephalin binding data. Leucine enkephalin binding was significantly decreased following acute stress, $t(18)=2.95$, $p<0.01$. Within control and acute groups no correlation between tail flick latency and [^{3}H] leu-enkephalin binding was observed (control $r=0.09$; Acute stress $r=-0.21$).

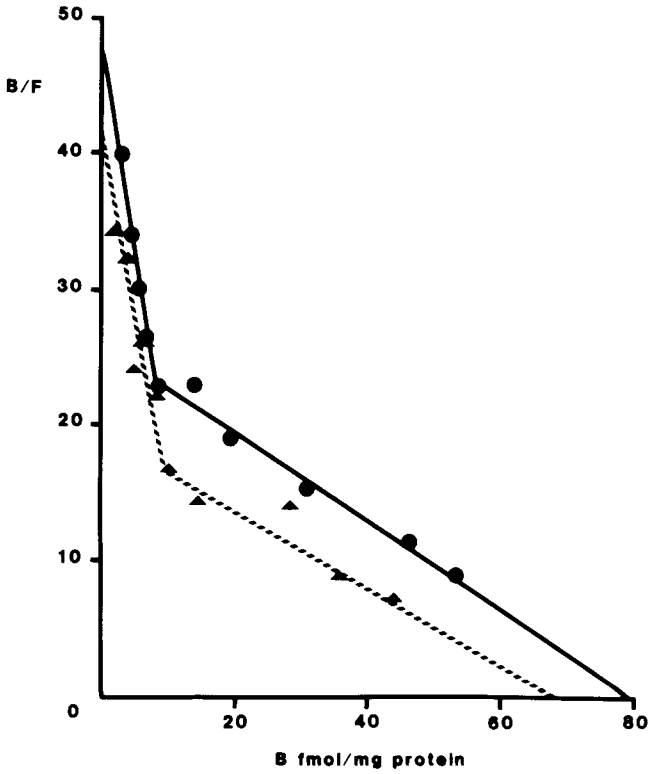

FIG. 1. Scatchard plots of [^{3}H] leu-enkephalin binding to brain homogenates from control (closed circle) and acutely stressed (closed triangle) mice (Experiment 2). Data represent analyses of pooled results from 4 separate determinations per group (4 brains per determination). Scatchard analyses of individual determinations are presented in Table 3.

Results shown in Table 2 indicate that preincubation of homogenates at 37°C for 20 min significantly elevated binding in both control and acutely stressed groups (control $t(8)=6.70$, $p<0.001$; Acute stress, $t(8)=5.54$, $p<0.001$). No

TABLE 3

APPARENT K_D AND B_{MAX} [^{3}H] LEU-ENKEPHALIN BINDING FOLLOWING ACUTE STRESS

	Control		Acute Stress	
	High Affinity	Low Affinity	High Affinity	Low Affinity
K_D (nM)	0.59 ± 0.26	4.45 ± 0.69	0.49 ± 0.08	3.63 ± 0.08
B_{max} (fmol/mg protein)	23.5 ± 8.6	97.8 ± 7.11	18.5 ± 2.1	69.4 ± 6.34*

Data represent mean ± s.e.m. from 4 independent Scatchard plots per group (total of 16 animals per group) (Experiment 2).

*$p<0.025$ control vs acute (t-test, 6 df).

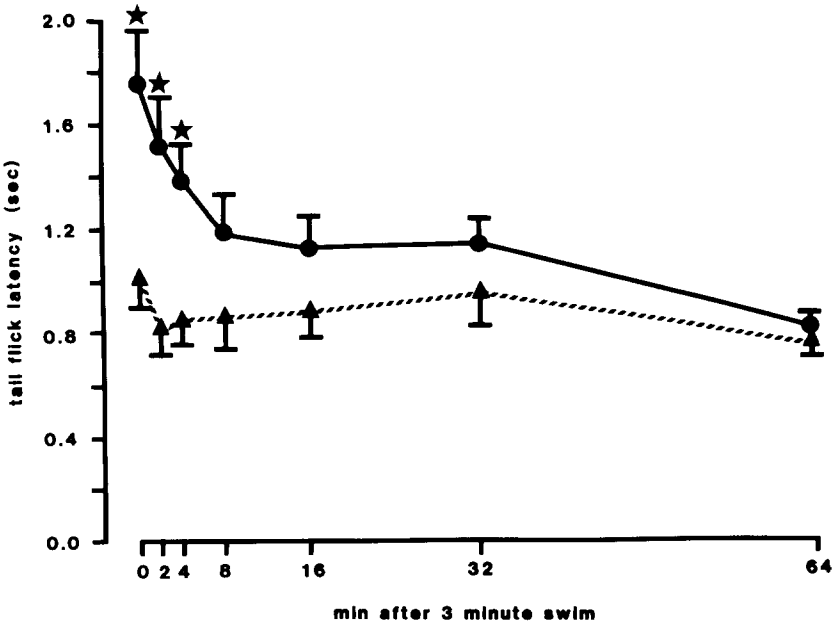

FIG. 2. The duration of stress analgesia after completion of swim (closed circle) or control (closed triangle) procedures (Experiment 3). Each data point represent mean ± S.E.M. for 12 mice. See text for statistical analysis. *$p<0.01$ (t-test, 22 df control vs. acute stress).

difference between control and acutely stressed groups was observed following this procedure, $t(16)=1.62$, $p>0.10$.

Experiment 2

Results of Scatchard analysis of saturation binding data for control and acute animals are shown in Fig. 1 and Table 3. Table 3 shows means±s.e.m. of apparent K_D and B_{max} from four independent Scatchard analyses of each group. A significant reduction in B_{max} of the low affinity site was observed in the acute group, $t(6)=2.98$, $p<0.025$. No changes were observed for other binding parameters. Pooled results of Scatchard analysis are presented in Fig. 1.

Experiment 3

A two-way analysis of variance revealed a significant difference between stressed and unstressed groups, $F(6,154)=34.54$, $p<0.001$, a significant difference with time of testing after stress, $F(6,154)=4.86$, $p<0.0001$, and a significant interaction, $F(6,154)=2.57$, $p<0.05$. One way analysis of variance of tail flick latency of the unstressed control mice showed no significant difference with time, $F(6,77)=0.69$.

The results, depicted in Fig. 2, also indicate that a significant difference (t-test, $p<0.01$) exists between tail flick latency of control and stressed mice at 0, 2 and 4 min after the completion of the 3 minute swim-stress.

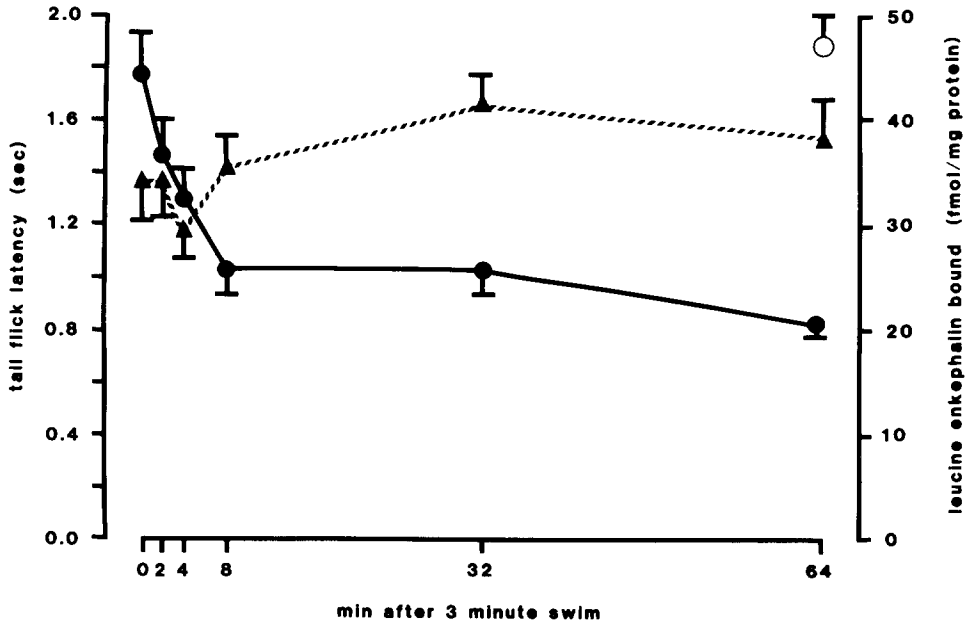

FIG. 3. The correlation between acute antinociceptive response and [^{3}H] leu-enkephalin binding (Experiment 3). Mice were tested for tail flick latency (closed circles) at various times after completion of swim and [^{3}H] leu-enkephalin binding (closed triangles) as in Experiment 1. Each data point represents mean±S.E.M. of 18 separate determinations. See text for statistical analysis.

These between group differences were reflected also in the analysis of [^{3}H] leu-enkephalin binding data (Fig. 3). A multivariate ANOVA [12] showed a significant change over time (greatest characteristic root test, $p<0.00001$) which was due primarily to a decrease over time in tail flick latency ($p<0.00001$); [^{3}H] leu-enkephalin binding means showed a slight but not significant ($0.1>p>0.05$) increase over time. The within group correlation was $r_w=0.050$ and that between groups was $r_B=-0.563$.

DISCUSSION

Our previous finding of an increase in the tail flick latency of mice after a period of warm water swimming [9] was confirmed in the present study. These results are also consistent with other reports of an antinociceptive response in experimental animals following a stressful procedure [2, 3, 8, 13, 17]. The present studies also demonstrated a significant reduction in specific [^{3}H] leu-enkephalin binding to brain homogenates from swum mice when compared with that of controls (Experiment 1). These results are consistent with those of Chance *et al.* [5].

A Scatchard analysis of saturation binding data (Experiment 2) indicated a significant reduction in the number of low affinity binding sites in homogenates from stressed mice, with no change in affinity. No differences were observed for the number or the affinity of the high affinity leu-enkephalin binding sites.

In interpreting these data several possibilities must be considered. First, one might postulate the presence in the incubation medium of free endogenous opiates and/or metabolites of [^{3}H] leu-enkephalin. In either case the result would be an effective dilution of the radioligand concentration and would be expected to yield a reduced affinity of the binding of [^{3}H] leu-enkephalin with no change in the number of available receptors. This is not in concordance with the results presented. Another interpretation of the present data is that the observed decrease in the occupation of opiate receptors by [^{3}H] leu-enkephalin in the stressed group reflects an increase in the *in vivo* occupation of these receptors by an endogenous ligand. Under the experimental conditions utilized in this study, in which animals were sacrificed by immersion in liquid nitrogen [10], and the temperature of homogenates and incubates were kept below 4° throughout [1,19] any endogenous ligand bound to opiate receptors at the time of sacrifice would be expected to remain bound. This interpretation is supported by the observation that preincubation of homogenates for 20 min at 37° produced binding data which showed no significant difference between stressed and control groups (Table 2). Under these conditions, any bound ligand would be expected to dissociate [1,19]. Nevertheless, the possibility that the reduced binding described in the stressed group may reflect some other form of masking of opiate receptors cannot be excluded at present.

If the reduced binding of [^{3}H] leu-enkephalin to low affinity sites does indeed reflect an increased occupation of opiate receptors at the time of sacrifice, *in vitro* opiate binding assays would provide an indirect method for an evaluation of the *in vivo* functional state of opiate receptor populations.

Pharmacological [15, 17, 18] and receptor binding studies [6, 7, 15] have proposed the existence of at least two classes of opiate receptor. The postulated μ receptor [18] shows low

affinity for leu-enkephalin and high affinity for morphine [7,15] and is therefore presumably involved in the antinociceptive activity of the morphine-like narcotic analgesics [14,20]. The observed reduction in low affinity [^{3}H] leu-enkephalin binding in stressed mice and the behavioural observation of an antinociceptive response in these animals pose the question as to whether the two are related.

An attempt to investigate the correlation, over time between the antinociceptive response and the leu-enkephalin binding was undertaken (Experiment 3). The results indicated that whilst the within group correlations based on individual differences between animals within each group, was $r_w = 0.050$, the between group correlation, based on the similarity between the two profiles of means over time, was $r_B = -0.563$. Although not statistically significant, the latter correlation suggests a negative relationship between changes in the two measures which, if reliable, is not predictable from the relationship based on individual differences.

The antinociceptive response which follows warm water swimming is partly, but not completely antagonized by naloxone, and shows a partial, but not complete cross tolerance with morphine [9]. These data suggest the involvement of both endogenous opiate and non-opiate mechanisms in the response. Perhaps the present finding that the between group correlation of $r_B = -0.563$, betweeen opiate receptor binding and antinociceptive responsiveness may be interpreted as being in favour of a role for endogenous opiate in the antinociceptive response. The fact that this correlation did not achieve significance might be attributed to the involvement in the behavioural response of a non-opiate mechanism which was of course not included in the present receptor binding data. Alternately brain stem or spinal endogenous opiate systems not examined in the present study might be involved in the behavioural response.

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