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Method of Sacrifice Influences Leucine Enkephalin Binding to Mouse Brain

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CHRISTIE, M. J., G. J. ZINGARELLI AND G. B. CHESHER. *Method of sacrifice influences leucine enkephalin binding to mouse brain.* PHARMAC. BIOCHEM. BEHAV. 15(6) 849–851, 1981.—The methods used for sacrifice of animals and the time taken for any dissection procedures of the brain tissue at room temperature have been shown to influence the number of available binding sites for [^{3}H] leucine enkephalin (10 nM) to mouse brain homogenates. These factors should be considered when utilizing receptor binding techniques as an indirect method for evaluating the *in vitro* functional state of opiate receptor populations.

Opiate receptors Opiate binding Enkephalins Method of killing Post-mortem changes Receptors Endorphins

SEVERAL studies have utilized *in vitro* receptor binding techniques (involving incubation at 0°C) as an indirect method for evaluating the *in vivo* functional state of opiate receptor populations [2, 3, 4, 5]. Chance *et al.* [3,4] reported reduced opiate binding to brain homogenates concomitant with conditioned fear induced antinociception. The authors interpreted these results to indicate occupancy of opiate receptors by endogenous ligands.

We have used a similar procedure to examine the possible involvement of opiate receptors in the antinociceptive response in mice which follows a period of warm water swimming [5] and have observed similar changes to those reported by Chance *et al.* [3,4]. Since dissociation rates from opiate receptors are rapid for both enkephalins [9] and for β-endorphin [1], we considered that the methods used for sacrifice and the time taken for dissection might influence results of *in vitro* receptor binding studies.

METHOD

Randomly bred QS female mice (18–30 g) from the University Animal Farm were housed in groups of 12 at 22±1° and allowed food and water ad lib until the time of sacrifice. Two experiments were conducted:

In experiment 1 animals were killed by:

(a) Immersion in liquid nitrogen, or

(b) decapitation, dissection of the brain (which took approximately 60 sec) and its immersion in liquid nitrogen, or,

(c) decapitation and immediate immersion of the head in liquid nitrogen, or

(d) stunning, followed immediately by decapitation and immersion of the head in liquid nitrogen.

Six animals were used for each group.

In experiment 2, animals were killed by:

(a) Immersion in liquid nitrogen or

(b) stunning, decapitation and immersion in liquid nitrogen, or

(c) stunning, decapitation, and the head left at room temperature for 2, 4, 8 or 16 minutes before immersion in liquid nitrogen.

Three brains per group were pooled for preparation of homogenates. Brains (minus cerebellum) were stored in liquid nitrogen, or at −70° 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 at 4°C. Pellets were resuspended and assayed immediately for [^{3}H] leucine-enkephalin binding.

[^{3}H] leucine-enkephalin binding was performed as described by Pert and Snyder [7] and Lord *et al.* [6]. 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] leucine-enkephalin (39 ci/mmol, Radiochemical Centre, Amersham, U.K.). This concentration of leucine 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 levorphanol. 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.* [7].

RESULTS

The results of experiment 1, presented in Table 1 indi-

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TABLE 1

SPECIFIC [^{3}H] LEUCINE ENKEPHALIN BINDING TO MOUSE BRAIN HOMOGENATES FOLLOWING SACRIFICE OF THE ANIMALS BY VARIOUS METHODS

	Method of Sacrifice	[^{3}H] Leucine Enkephalin Binding f mol/mg Protein (Mean ± S.E.M.)
(a)	Immersion in liquid N_2	52.8 ± 3.2
(b)	Decapitation. Brain dissected before immersion in liquid N_2	64.6 ± 2.9*
(c)	Decapitation before immersion in N_2	57.1 ± 3.8
(d)	Stunning, decapitation and then immersion in liquid N_2	43.3 ± 3.1†

Values represent mean ± S.E.M. of six separate determinations (Experiment 1).

*Significantly different from Group (a) ($p<0.025$; 10 df; $t=2.73$).

†Significantly different from Group (c) ($p<0.02$; 10 df; $t=2.81$).

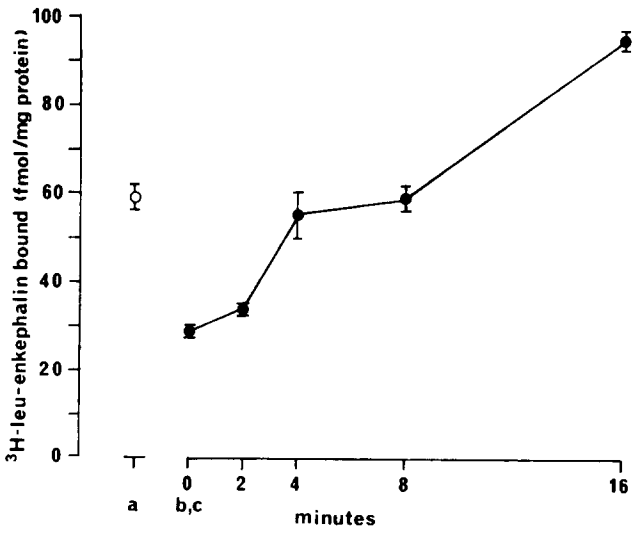

FIG. 1. Effect of leaving decapitated mouse heads at room temperature (22°±1) for various times prior to their immersion in liquid nitrogen on specific [^{3}H]-leucine-enkephalin binding. Each data point represents mean ± s.e.m. of quadruplicate determinations for 3 pooled brains. Open Circle = group (a), killed by immersion in liquid nitrogen. Closed Circle = group (b) and (c), decapitated followed by immersion in liquid nitrogen after various times standing at room temperature.

cated that the method of killing influences [^{3}H]-leucine enkephalin binding to mouse brain homogenates. Decapitation and the removal of the brain (Group 1(b)) (a procedure taking about 60 sec) prior to immersion in liquid nitrogen resulted in a 22% increase in receptor binding over that recorded when the mice were plunged immediately into liquid nitrogen (Group 1 (a)) ($t(10)=2.73, p<0.025$). Animals which had been stunned prior to decapitation (Group 1(d)) had 24% fewer available binding sites than those which had been killed by decapitation (Group 1(c)) ($t(10)=2.81, p<0.02$).

The results of experiment 2 (Fig. 1) provide evidence for post-mortem changes that occur if any delay takes place between killing of the animal and the freezing of brain tissue. The brains of animals which had been stunned and decapitated prior to immersion in liquid nitrogen bound 53% less [^{3}H]-leucine enkephalin than did the brains of animals which had been sacrificed by immersion in liquid nitrogen without stunning. These results are consistent with those in experiment 1. The results for groups 2(b) and (c) of experiment 2 show that a direct correlation exists between the elapsed time between sacrifice and the freezing of the brain and binding of [^{3}H]-leucine enkephalin. The receptor binding for brains which had been allowed to stand at room temperature for 16 mins was 241% greater than that recorded for brains which had been immersed in liquid nitrogen immediately after the animals had been stunned and decapitated. This experiment has been replicated twice with similar results (data not shown).

DISCUSSION

The results presented here suggest that opiate binding data might not be an accurate indication of the functional opiate receptor state at the time of sacrifice, unless due consideration is given to the post-mortem changes that may occur. The dissection procedure which preceded the immersion of the brain in liquid nitrogen elevated leucine enkephalin binding above that of animals which had been killed by immersion in liquid nitrogen. It is conceivable that dissociation of endogenous ligands occurs during the 60 sec of the dissection procedure (see [9]). The elevation of binding observed over several minutes in experiment 2, groups (b) and (c) (Fig. 1) is also consistent with this interpretation. Animals stunned with a blow prior to decapitation showed significantly less leucine-enkephalin binding than animals killed by decaptiation (Table 1). Perhaps this procedure results in release of endogenous opiates. Whether changes observed relate to the high or low affinity binding site cannot be determined from the present studies as a saturating concentration (10 nM) was employed throughout. The present results are consistent with the interpretation that the reduction in opiate binding following stress [3, 4, 5] is due to occupation of opiate receptors by endogenous ligands.

Similar observations in rats relating to method of killing and the levels of brain enkephalin determined by radioimmune assay have been reported [10]. These phenomena may be related to the findings in the present study.

The present results suggest that the method of sacrifice must be considered when utilizing opiate receptor binding techniques as an indirect method for evaluating the *in vivo* functional state of opiate receptor populations.

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