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Brain Opioid Receptors in the Hibernating Bat, *Myotis lucifugus*: Modification by Low Temperature and Comparison with Rat, Mouse and Hamster

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WILKINSON, M., G. D. BUCHANAN, W. JACOBSON AND E. V. YOUNGLAI. *Brain opioid receptors in the hibernating bat,* Myotis lucifugus: *Modification by low temperature and comparison with rat, mouse and hamster.* PHARMACOL BIOCHEM BEHAV **25**(3) 527–532, 1986.—Several studies have provided evidence that brain opioid peptides may be involved in the control of the hibernation cycle. We have now examined the influence of hibernation on central opioid receptors. We have characterized the receptor binding properties of [^{3}H]-naloxone in slices of the cerebral cortex and hypothalamus of the bat *Myotis lucifugus*. These receptors possess those characteristics expected of an opioid site namely high affintiy, stereospecificity and saturability. Under normal incubation conditions (30°C) we observed no effect of hibernation on [^{3}H]-naloxone binding. However, when binding assays were performed at temperatures corresponding to the appropriate body temperature (e.g., 4°C for hibernation) we detected a significant low temperature-induced increase in hypothalamic binding. Similar experiments in rat, mouse and hamster revealed that [^{3}H]-naloxone binding was also increased at 4°C when compared to 30°C. This was true in hypothalamus and cortex. Additional studies in the rat demonstrated that the opioid receptor is of higher affinity at low temperatures. The behavioural and neurochemical consequences of this change in the opioid receptor, and whether this might be involved in the regulation of hibernation, remain to be studied.

Hibernation Bat Brain opioid receptors Hypothalamus

PHARMACOLOGICAL studies on hibernating mammals have been limited to readily available and easily maintained species [16]. Bats are poorly studied, since they require special facilities (e.g., cold, draft-free cages, high humidity) and collection in winter is often difficult. Our own studies of the bat *Myotis lucifugus* (see [4,5]) were facilitated by the discovery of a large hibernaculum, accessible in winter, located 360 kilometers from the laboratory. Bats are easily obtained, and after a brief arousal remain hibernating if transported in refrigerated containers. This paper describes our studies on hibernation-induced modification of opiate receptors in the brain of hibernating *Myotis lucifugus*.

Several groups have provided experimental evidence that opioid peptides may be involved in the control of hibernation. Blockade of opioid receptors with opioid antagonists results in some degree of arousal in the hibernating Turkish hamster [18], garden dormouse [15] and golden-mantled ground squirrel [2]. Oeltgen *et al.* [19] have isolated an opiate-like hibernation 'trigger' factor from the blood of the hibernating woodchuck. Further evidence that the opioid system may be altered during hibernation comes from the observation that physical dependence on morphine cannot be induced in the hibernating golden-mantled ground squirrel *(Citellus lateralis)* at doses which induce strong dependence during euthermia [1]. Kramarova *et al.* [14] have reported that the brain of the ground squirrel *Citellus suslicus* contains high levels of methionine enkephalin during deep hibernation. Our own preliminary observations (Buchanan and Wilkinson, in preparation) have revealed that a single injection of naltrexone (50 mg/kg; SC) rapidly induced arousal in most, but not all, hibernating bats. Some bats left their perch and moved around the cage for 15 to 45 minutes before returning to roost. Others, although not leaving their perch, showed signs of arousal such as an increase in respiration rate, head movements and erect ears. Saline-injected bats showed few signs of movement or arousal. These observations led us to compare opioid ([^{3}H]-NAL) binding sites (receptors) in the brains of active and hibernating bats.

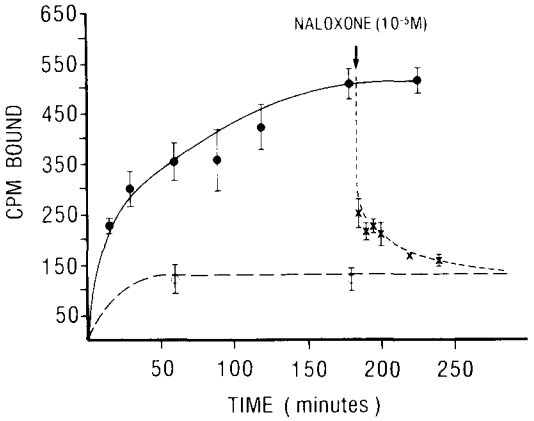

FIG. 1. Time-course and reversibility of total (solid circles) and non-specific (open circles) binding of [^{3}H]-NAL to cortical slices of bat brain. Values are means±S.E.M. (n=6). The arrow indicates the addition of unlabelled naloxone (10^{-5} M) to displace the label.

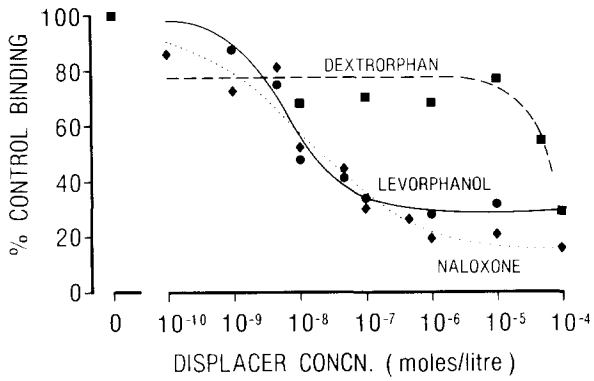

FIG. 2. Competition curves for inhibition of [^{3}H]-NAL binding (1 nM) to cortical slices of bat brain. IC_{50} values are given in the text.

METHOD

Bats

Hibernating *Myotis lucifugus* of both sexes were collected from an abandoned mine in southwest Renfrew County (lat. 45°18'N) Ontario. The bats were briefly aroused (<2 hr) during collection but quickly returned to a state of hibernation when placed in the chilled container used for transport. They were kept in Hamilton in an artificial hibernaculum at 4°C until used. Some bats were aroused from hibernation by placing them in a small wooden cage in the laboratory and providing them with drinking water ad lib. Active bats kept for more than 24 hours were fed 10–15 mealworms (*Tenebrio molitor* larvae) per day. At autopsy, bats were weighed, then killed by cervical dislocation.

Rats, Hamsters and Mice

Male Sprague-Dawley rats (150–200 g) and male Golden Hamsters (100–120 g) were obtained from Canadian Hybrid Farms, Halls Harbor, Nova Scotia. Male Swiss-Webster mice (25–30 g) were purchased from High Oak Ranch, Goodwood, Ontario. They were housed under controlled lighting conditions (lights on 0700–1900 hr) and given food and water ad lib.

Brain Tissue

Animals were sacrificed by decapitation, usually before 1100 hr. The whole brain was removed rapidly and placed in ice-cold Dulbecco's phosphate buffered saline (DPBS, pH 7.4 Gibco). Blocks of cortical tissue were removed from either side of the midline and placed in fresh, cold DPBS. The hypothalamus was dissected by two coronal cuts made just rostral to the mamillary bodies and just caudal to the optic chiasm. Lateral cuts were made at a point approximately $^2/_3$ of the distance from the pituitary stalk to the lateral fissures. A final horizontal cut was made at approximately 2 mm dorsally for rat and hamster and approximately 1 mm for bat hypothalamus.

Binding Assay

[^{3}H]-NAL binding sites were labelled in slices of brain tissue using a technique recently developed in our laboratory [3, 13, 22].

The isolated tissue blocks (see above) were cut into 350 μm coronal slices on a McIlwain tissue chopper (Brinkmann Instruments) and then gently transferred to fresh, cold DPBS. They were carefully separated into individual slices with fine forceps under binocular magnification. Hypothalamic slices were further divided at the midline (third ventricle) to yield hemicoronal slices (hamster and rat). The bat hypothalami were too small to be sliced and were merely divided at the midline (third ventricle) into two equal pieces.

Single brain fragments were placed into individual wells of a Linbro multi-well tissue culture plate (24 wells; 3.5 ml capacity). Each well contained 500 μl of cold DPBS.

NAL (20 μl) was added to some of the wells, at a final concentration of 10^{-5} M, for the determination of non-specific binding. After 5 min, [^{3}H]-NAL (20 μl) was added to all wells at various concentrations (nM) depending upon the particular experiment. Incubation was performed at 30° or 4°C for varying lengths of time (see below).

At the end of the incubation an aliquot of the medium (50 μl) was removed for the estimation of the "free" concentration of [^{3}H]-NAL at equilibrium. The remainder of the buffer was aspirated with a pasteur pipette, the slices were washed twice with ice-cold buffer (0.5 ml; ×5 min) and then placed, singly, in scintillation vials which contained Aquasol (New England Nuclear; 2 ml). The vials were shaken vigorously and allowed to stand a minimum of 2 hr before they were counted in a Beckman LS 5801 liquid scintillation counter. Counting efficiency was 48%.

Results are expressed as dpm bound per mg of tissue (wet weight). Normally, 5 or 6 slices were used for each assay point (3–4 for NSB). A separate, representative group of fresh slices (8–10) were weighed at the start of the assay to obtain a mean slice weight.

Drugs

[^{3}H]-NAL (51 Ci/mmole) was obtained from Amersham Corp. The following drugs were generously donated: NAL (E. I. du Pont de Nemours), levorphanol and dextrorphan (Hoffmann-LaRoche). Other drugs and supplies were obtained commercially.

RESULTS

Characterization of [³H]-NAL Binding Sites in Bat Brain

Opioid binding sites have previously been characterized in slices of rat and mouse brain [3,13]. Our unpublished data on hamster brain slices are entirely consistent with those for rat and mouse, i.e., the binding of [³H]-NAL is reversible, stereospecific, saturable and of high affinity. The characterization steps for opioid binding in bat brain are included in the present study.

Time-course and reversibility (Fig. 1). Unless otherwise indicated these experiments were performed with slices of cerebral cortex. Non-specific binding (NSB) quickly reached a maximum value whereas total and specific binding reached equilibrium by 180 min of incubation (30°C). Figure 1 also illustrates that binding of [³H]-NAL (1 nM) is rapidly reversible following the addition of excess (10^{-5} M) unlabelled NAL. All subsequent assays were incubated at 30°C for 180 min.

The optimum number of washes was determined by comparing total and NSB in unwashed slices (i.e., slices removed from wells directly to vials) with binding in variously washed slices (×1 to ×4). Specific binding remained constant for 2 to 4 washes (data not shown). Accordingly, two washes were used routinely in all assays.

Effect of number of slices on [³H]-NAL binding. The absence of binding artifacts, such as receptor or ligand degradation, can be confirmed through the construction of a tissue quantity versus binding plot [6]. Incubation of 1, 2, 3 or 4 slices per well with [³H]-NAL (1.5 nM) yielded a linear increase in total and NSB. Values of the correlation coefficients, obtained from linear regression analysis, were 0.99 and 0.99, respectively.

Thermolability of the binding site. The complex structure of cell membrane proteins, including receptor sites, should be denatured at elevated temperatures [6]. Specific binding was completely eliminated by incubation at 100°C for 5 min.

Drug specificity of [³H]-NAL binding. Competition curves for naloxone, levorphanol and the inactive enantiomer dextrorphan are shown in Fig. 2. Curves were plotted using the ALLFIT program of de Lean, Munson and Rodbard as obtained from the Biomedical Computing Technology Information Center, Vanderbilt University (adapted for an Apple IIe microcomputer). The IC_{50} values are as follows: naloxone, 5.9×10^{-9} M; levorphanol, 1.3×10^{-8} M; dextrorphan, 3.0×10^{-4} M. The data for levorphanol and dextrorphan confirm that [³H]-NAL is bound stereospecifically since dextrorphan is several orders of magnitude less potent in its ability to compete with [³H]-NAL binding.

Binding of [³H]-NAL to Cortex and Hypothalamus of Active and Hibernating Bats

A comparison was made between opioid binding in brain tissue from active and hibernating bats. These assays were performed under standard incubation conditions (30°C for 180 min). Figure 3 illustrates that in cerebral cortex and hypothalamus hibernation makes no significant difference to [³H]-NAL binding. For cortex the binding curves are superimposable, whereas for hypothalamus the values were not different (by Student's *t*-test; values are, active: 3966±437 and 5400±400 at 1.8 and 4.1 nM, respectively; hibernating: 3395±750 and 4350±360, respectively). Note that the hypothalamic fragments were assayed at only two concentrations of the ligand because of the very limited amount of tissue available.

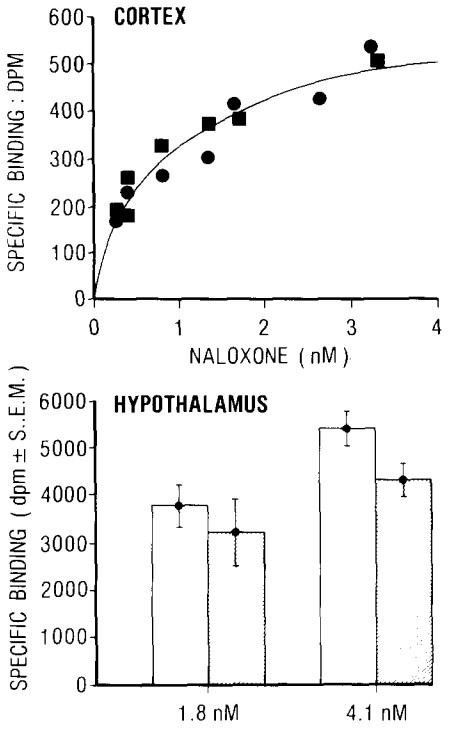

FIG. 3. Effect of hibernation (solid circles and shaded bars) on specific [³H]-NAL binding to cortical slices and hypothalamic fragments (n=6 per groups) from bat brain. Assays were performed at 30°C. Note that the hypothalamus was assayed at two concentrations only (1.8 and 4.1 nM). Control (active) values are solid squares, cortex; open squares, hypothalamus, respectively.

We next determined the binding of [³H]-NAL to cortical and hypothalamic tissue from *hibernating* bats at the normal assay temperature (30°C) and compared it with binding at 4°C, which is the approximate body temperature during hibernation. A preliminary experiment showed that equilibrium binding of [³H]-NAL was achieved by 16 hr at 4°C and remained constant for at least 20 hr (cold room; results not shown). Assays were therefore run for 18–20 hr.

Figure 4 (upper) reveals that cooling of cortical tissue from hibernating bats does not increase the maximal binding of [³H]-NAL when compared to binding at 30°C, i.e., B_{max} at 30°C: 657±85 dpm/mg; B_{max} at 0°C: 531±63 dpm/mg. However, the affinity of the binding (K_D) does appear to increase with cooling (values are 0.55±0.14 nM (30°C) vs. 0.15±0.06 nM (0°C)). Sufficient tissue was not available to statistically analyze more extensive binding curves. This phenomenon was examined in more detail with the larger brains from mouse, rat and hamster (see below). Figure 4 also shows (lower picture) that hypothalamic binding, at 4°C, increased by 106%. This cold-induced increase in hypothalamic binding was confirmed in two subsequent experiments (78% and 60% increases; [³H]-NAL at approximately 1 nM).

These observations raise the interesting possibility that low temperature induces an elevation in hypothalamic opioid binding during hibernation. Indeed, when hypothalamic fragments from *active* (i.e., non-hibernating) bats were cooled and assayed at 4°C we observed an increase in [³H]-NAL (1 nM) binding (at 30°C: 2784±164 dpm; at 4°C: 5114±120 dpm; +83%; n=6 per group).

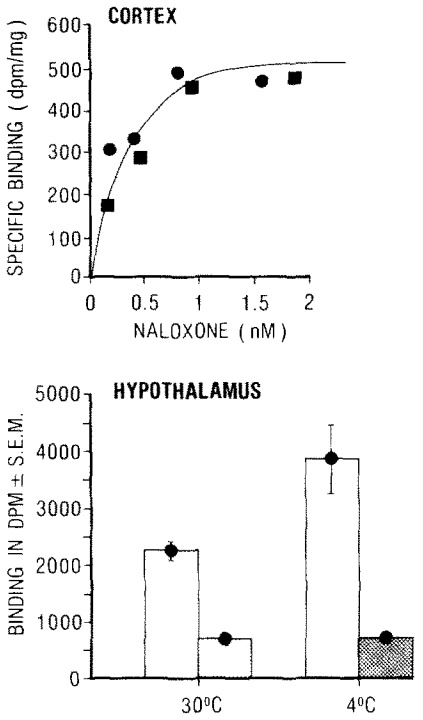

FIG. 4. Effect of low temperature (4°C) assay on [^{3}H]-NAL binding to cortical slices (upper) and hypothalamic fragments (lower) obtained from *hibernating* bats. In the upper portion the symbols are: solid circles—assayed at 4°C; solid squares—assayed at 30°C. In the lower part of the figure open bars represent total binding and shaded bars non-specific binding in hypothalamus ([^{3}H]-NAL is 1 nM).

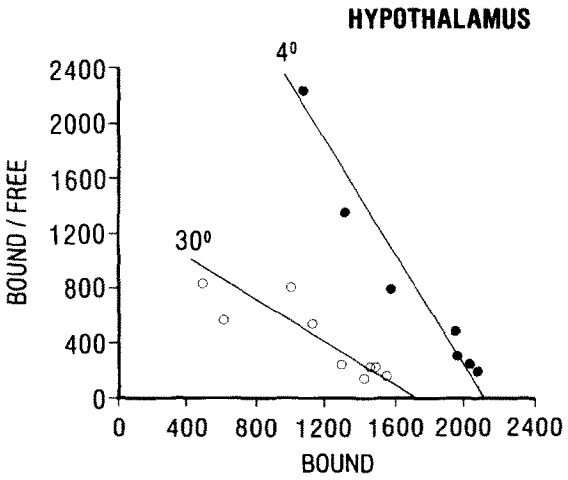

FIG. 6. Scatchard plot (bound/free vs. bound) of [^{3}H]-NAL binding to slices of rat hypothalamus at 4°C and 30°C. B_{max} and K_D values are, 30°: 1705±128 cpm/mg (1.26±0.26 nM) and 4°: 2127±70 cpm/mg (0.51±0.06 nM), respectively.

Effects of Cooling on [^{3}H]-NAL Binding in Brain Tissue From Rat, Hamster and Mouse

The results of the following experiments answered the question: is the bat hypothalamus unusually susceptible in terms of low temperature induction of opioid binding? We examined the binding of [^{3}H]-NAL to slices of cortex and hypothalamus obtained from normal, active male rats, mice and hamsters. Figure 5 shows that in these species also there is a cold-induced elevation of hypothalamic [^{3}H]-NAL receptors. A small effect is also seen in cortical slices.

These data were obtained at a single point on the binding curve (approximately 1 nM). To determine the nature of the binding increase (i.e., a change in B_{max} and/or K_D) we next performed binding assays over a range of [^{3}H]-NAL concentrations. These experiments were conducted using male rats. Figure 6 clearly shows that the low temperature-induced elevation in [^{3}H]-NAL binding in rat hypothalamus occurs via a marked change in affinity. Figure 7 illustrates a similar effect of low temperature on cortical [^{3}H]-NAL binding.

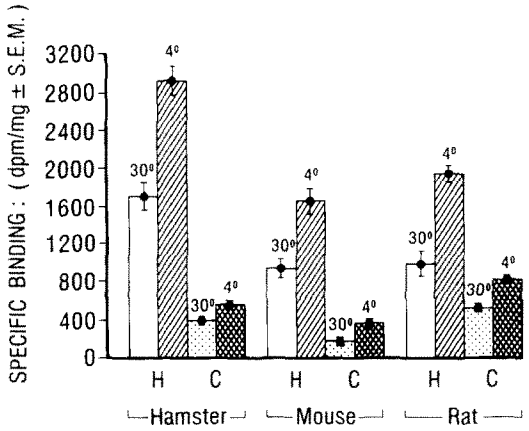

FIG. 5. Effect of low temperature incubation on specific [^{3}H]-NAL binding (1 nM) to slices of hypothalamus and cortex from active hamster, mouse and rat (n=8 to 10 slices). H=hypothalamus; C=cortex.

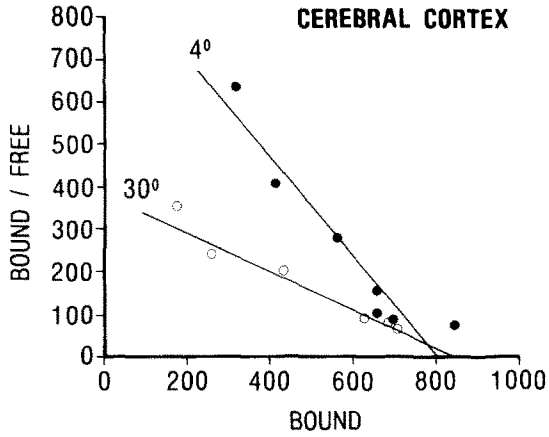

FIG. 7. Scatchard plot of [^{3}H]-NAL binding to slices of rat cerebral cortex at 4° and 30°C. B_{max} and K_D values are as follows: 30°: 824±39 cpm/mg (1.96±0.19 nM) and 4°: 802±40 (0.82±0.12 nM), respectively.

DISCUSSION

Our preliminary characterization of [^{3}H]-NAL binding in the brain of *Myotis lucifugus* revealed that these opioid receptors have the appropriate characteristics previously described in other mammals. Namely, the binding of [^{3}H]-NAL is reversible, stereospecific, saturable and of high affinity Strictly speaking, this is true only for cerebral cortical tissue since we were unable to obtain sufficient hypothalamus for a full characterization.

We observed no major differences in the levels of [^{3}H]-NAL binding in cortex and hypothalamus of hibernating and non-hibernating bats. This contrasts with the interesting report (in Abstract form) by Dean *et al.* [9] which describes a hibernation-induced down-regulation of hippocampal dihydromorphine binding sites in the California ground squirrel *(Citellus lateralis)*. Note that in our study subtle differences in other brain areas may be present which were not obvious because of shortage of tissue. However, we noted a striking difference in binding when the assays were performed at temperatures which corresponded to the appropriate body temperature (i.e., 4°C) for hibernating bats. The effect of cooling appeared to be selective for the hypothalamus and resulted in a large increase in specific binding (Fig. 4). This increase was also seen when hypothalamic fragments were obtained from active bats and then assayed at 4°C. Thus, hibernation seems to be associated with an increase in hypothalamic [^{3}H]-NAL binding probably as a result of cooling.

Further, comparative experiments on hamster, mouse and rat brain revealed that in these species [^{3}H]-NAL binding is also elevated at 4°C when compared to assays performed at 30°C. Some effect was also noted in cortical tissue. A closer examination of the binding in rat hypothalamus and cortex provided evidence that cooling of the tissue induces a high affinity state of the [^{3}H]-NAL site (Figs. 6 and 7). B_{max} values were not significantly affected although there is a tendency to lower values in hypothalamus. Previous studies in homogenate of whole brain also noted that [^{3}H]-NAL binding is elevated at low temperature via a change in the K_D value [8, 16, 21]. The mechanism to account for this remains unknown although a change in membrane viscosity may be partially responsible. Heron *et al.* [11] have demonstrated that naloxone receptor binding increases, via modification of B_{max} and K_D, when mouse brain membranes are treated with cholesteryl hemisuccinate. It appears likely that a similar increase in membrane viscosity would occur at low temperatures. Viscosity related changes in receptor binding may be a common phenomenon. Heron *et al.* [10] describe elevations in serotonin receptor binding and refer to other work on β-adrenergic receptors (β-R) (see also [20]). Our own studies on β-R in brain slices did not reveal an effect of temperature [23] but Hertel and Staehelin [12] describe a low temperature induction of high affinity β-R in C6 glioma cells. As suggested by Heron *et al.* [10], this effect on β-R is clearly dependent upon tissue type.

Our results suggest that opioid receptors in the hypothalamus of the cold, hibernating bat exist in a high affinity state. Whether other neurotransmitter/neuromodulator sites are affected by body temperature remains to be studied. It would be particularly worthwhile to investigate the Turkish hamster which also enters hibernation when exposed to reduced temperature [18]. A further intriguing question relates to the possibility that changes in opioid receptor affinity are instrumental in the maintenance of cold-induced hibernation or torpor. Our own results (this report) and those of others [2, 15, 18] indicate that blockade of opioid receptors with antagonists such as naloxone or naltrexone can induce arousal, albeit in a limited fashion. Our observations suggest that the synaptic effect of endogenous opioid peptides is amplified by binding to a low temperature-induced high affinity receptor. In a situation where opioid peptide levels actually increase during hibernation, either via Met-enkephalin [14] or the so-called 'hibernation trigger' [19], the opioidergic influence would be maximized. Our demonstration that hypothalamic opioid receptors are transformed to a high-affinity state at reduced temperature would be consistent with the observation that physical dependence to morphine does not develop during hibernation (at 5°C) of *Citellus lateralis* [1]. Crain *et al.* [7] have reported that tolerance to opioid peptides does not develop in mouse spinal cord neurons when maintained below 35°C.

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