An Analogue of Thyrotropin-Releasing Hormone, DN1417, Decreases Naloxone Binding in the Rat Brain (42946)

MASATOMO MORI, TOSHIO MICHIMATA, HIROFUMI ISHIHARA, MASANOBU YAMADA, TOKUJI IRIUCHIJIMA, AND SETSUO KOBAYASHI

First Department of Internal Medicine, Gunma University, School of Medicine, Maebashi 371, Japan

Abstract. **We explored whether thyrotropin-releasing hormone may affect opioid receptors in the rat brain. Adult male rats were intraperitoneally injected twice a day with varying doses of DN1417, a potent analogue of thyrotropin-releasing hormone, for 2 days, and opioid receptors of the brain (hypothalamus, striatum, hippocampus, midbrain, ponsmedulla, and cortex) homogenates were determined using [^{3}H]naloxone. Intraperitoneal injection of DN1417 in a dose of 0.3 mg/100 g body wt resulted in a significant reduction in naloxone binding of the striatum as compared with the saline-injected group, whereas naloxone binding of other brain regions was not affected by DN1417. DN1417 produced a dose-dependent decrease in naloxone binding of the striatum. The affinity constant of naloxone binding was similar between the saline- and DN1417-injected groups. *In vitro* addition of DN1417 did not interfere with the brain naloxone binding. The distribution of ^{3}H-labeled DN1417 injected peritoneally did not differ among the brain regions. The present data imply that the opioid antagonistic action of thyrotropin-releasing hormone may be due, at least in part, to the significant decrease in the striatal opioid binding in the rat brain.** [P.S.E.B.M. 1989, Vol 192]

Thyrotropin-releasing hormone (TRH), a neuropeptide concentrated in the hypothalamus but ubiquitously distributed in the brain (1), is a factor stimulating trophic hormone release from the adenohypophysis. Independent of its adenohypophyseal action, it is well known that TRH has a number of neurophysiologic functions (1). Recent observations have focused on the considerable exertion of TRH as an opioid antagonist (2, 3). However, the mechanism(s) by which TRH antagonizes opioid actions remains to be elucidated.

A biologically neuroactive substance exerts its characteristic function in the brain through binding to its specific receptors. TRH and opioid receptors are present throughout the brain (4, 5), and several groups of investigators have made attempts to clarify the specific interaction between the brain TRH and opioid receptors. It was found that addition of opioid peptides *in vitro* caused a profound interaction for TRH bound in the brain (6). In contrast, a failure of *in vitro* addition of TRH to alter the brain opioid binding has been reported (2, 7).

We scrutinized whether *in vivo* administration of DN1417, a potent analogue of TRH (8), may change the brain opioid binding which was determined employing [^{3}H]naloxone as an opioid antagonistic ligand (9). The present data herein describe that administration of DN1417 causes a profound alteration in the striatal naloxone binding.

Materials and Methods

Adult male rats of Wistar strain, each weighing 200–250 g, were fed a Purina laboratory chow diet and given tap water to drink. Animals were caged in a temperature-controlled room (22–23°C) with light going on at 6:30 AM and off 6:30 PM. DN1417 (γ-butyloractone-γ-carbonyl-histidyl-prolineamide citrate) was supplied by Takeda Chemical Ind., Osaka, Japan. Naloxone was kindly supplied by E. I. Du Pont de Nemours & Co., Garden City, NY. [^{3}H]Naloxone (specific activity = 43.9 Ci/mmol) was purchased from New England Nuclear Co., Boston, MA.

Rats were intraperitoneally injected twice a day with DN1417 in a single dose of 0.3 mg/100 g body wt or doses of 0.06–1.5 mg/100 g body wt for 2 days. Animals were decapitated, and each brain was dissected into six regions (hypothalamus, striatum, hippocampus,

Received September 19, 1988. [P.S.E.B.M. 1989, Vol 192]
Accepted April 5, 1989.

0037-9727/89/1921-0006$2.00/0

midbrain, pons-medulla, and cortex) as described previously (10). Each region of the brain was weighed, homogenized in 5 volumes of Tris-NaCl (TN) buffer (50 m*M* Tris-aminomethane, 100 m*M* NaCl, pH 7.4), and used for naloxone binding.

Naloxone binding was performed basically according to the method of Pert and Snyder (5) with some modifications. A reaction mixture consisted of 200 μl of tissue homogenates. 10 μl of 1.14 pmol of [^{3}H] naloxone, and 10 μl of TN buffer in the presence or absence of 1.14 nmol of unlabeled naloxone. After incubation at 24°C for 20 min, 0.5 ml of chilled TN buffer was added to each reaction tube, and the mixture passed by suction through a glass filter (GF/B, 2.5 cm in diameter; Whatman Inc., Clifton, NJ). The filter was washed with 5 ml of chilled TN buffer, placed on an electric heating plate, and transferred into a vial containing Aquasol-2 (New England Nuclear). The radioactivity was determined using a liquid scintillation spectrometer (Aloka model LSC-300; Aloka, Tokyo, Japan). Specific binding was calculated by subtracting the nonspecific bound (in the presence of unlabeled naloxone) from the total bound (in the absence of unlabeled naloxone). According to the method as described by Akera and Cheng (11), an affinity constant of naloxone binding was calculated by subtracting the concentration of [^{3}H]naloxone from the concentration of unlabeled naloxone to cause a 50% reduction of the saturable binding of [^{3}H]naloxone.

[*His*-^{3}H]DN1417 was prepared from DN1417 as follows. To obtain diiodo-DN1417, 20 μmol of DN1417 in 10 ml of 0.2 *M* sodium acetate buffer (pH 6.5) was incubated with 200 μmol of *N*-iodosuccinimide (Sigma Chemical Co., St. Louis, MO) in 3 ml of acetonitrile. After incubation for 2 min at room temperature, the reaction was terminated by addition of approximately 420 μmol of sodium thiosulfate in 0.4 ml of distilled water until the brown reaction mixture became colorless. The mixture was then lyophilized, dissolved in 0.5 ml of water, and centrifuged at 10,000 *g* for 30 min. Monoiodo-DN1417 was obtained according to the method of Ling et al. (12). The reaction mixture consisted of 1.8 μmol of DN1417 in 0.8 ml of phosphate buffer (0.05 *M* KH_2PO_4, pH 7.5), 1.8 μmol of NaI in 20 μl of phosphate buffer, and 1.8 μmol of chloramine T in 20 μl of phosphate buffer. After incubation for 2 min at room temperature, 1.8 μmol of sodium metabisulfite was added to terminate the reaction, and the mixture was lyophilized and dissolved in 0.5 ml of water. The supernatants of reaction mixture containing diiodo- and monoiodo-DN1417 were applied on a silica gel-60 TLC plate (5553; Merck, Darmstat, West Germany) and developed in an ascending mode (solvent system = *n*-butanol, acetic acid, and water, 4:1:1, v/v/v). Diiodo- and monoiodo-histidyl-DN1417 were visualized by Pauly's reagent. The plate corresponding to diiodo-DN1417 was scraped out and extracted in water, followed by centrifugation at 10,000 *g* for 20 min. The supernatant containing diiodo-DN1417 was lyophilized. Diiodo-DN1417 (25.5 μmol) was exposed to tritium gas in the presence of palladium catalyst (procedure performed by New England Nuclear). The radioactive product was analyzed using a TLC as described above and purified using a high-pressure liquid chromatography (HPLC) (Ultron S-C_{18} column, 4.6 × 250 mm, elution buffer = 0.02 *M* KH_2PO_4, pH 2.5, elution speed = 1.0 ml/min; Shinwa Chemical Industrial Co., Kyoto, Japan). The specific activity of this product was theoretically 3.3 Ci/mmol.

The purified [^{3}H]DN1417 (97×10^4 dpm in 10 μl of saline) was injected into the lateral ventricle of pentobarbital-anesthetized rats as described previously (13), and the radioactive analogue (87×10^5 dpm in 1 ml of saline) also injected intraperitoneally. Thirty and 60 min after injection, the rats were decapitated. The regions of each brain were dissected, weighed, and homogenized in 1 ml of chilled 99% methanol followed by centrifugation at 1000 *g* for 20 min. The radioactivity of supernatants was determined.

Statistical analysis was done by Duncan's multiple range test.

Results

Figure 1 shows effects of DN1417 on naloxone binding in the brain. DN1417 in a dose of 0.3 mg caused a significant decrease in naloxone binding of the striatum as compared with the saline-injected group (2.23 ± 0.30 vs 3.06 ± 0.16 fmol/mg wet wt, $P < 0.05$). However, naloxone binding in other brain regions was not affected by DN1417.

Table I shows effects of varying doses of DN1417 on naloxone binding in the striatum and hypothalamus. The data are results of three separate experiments and are expressed as percentage of the control values. DN1417 produced a consistent and dose-dependent

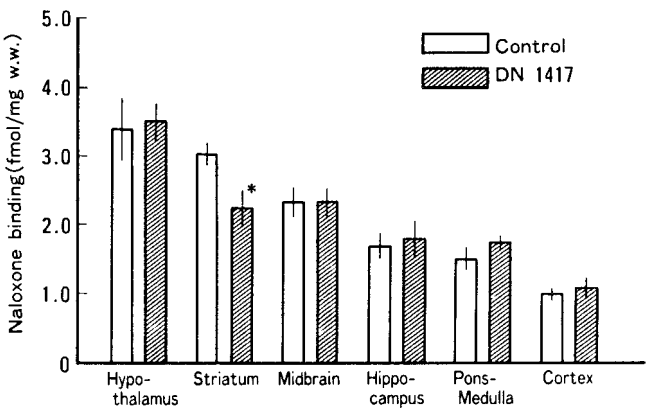

Figure 1. Effect of DN1417 on naloxone binding in the brain. Rats were intraperitoneally injected twice a day with DN1417 in a dose of 0.3 mg/100 g body wt for 2 days. Naloxone binding was determined in each brain region as described in Materials and Methods. The data are expressed as mean ± SE. Four samples were used in each group. *$P < 0.05$) differs from the saline-injected control group.

decrease in naloxone binding of the striatum. The hypothalamic naloxone binding was significantly decreased by DN1417 in an only dose of 1.5 mg.

The K_d values of both the striatal and hypothalamic naloxone binding were similar between the saline- and DN1417 (1.5 mg/100 g body wt)-injected groups (the striatum, 36.5 ± 3.2 vs 30.5 ± 4.2 nM, N = 6; the hypothalamus, 33.9 ± 6.3 vs 30.8 ± 5.3 nM, N = 6).

Figure 2 shows the *in vitro* interaction of DN1417 with naloxone binding in the brain homogenate. Unlabeled naloxone in a dose of 1.4 pmol/tube significantly decreased naloxone binding, whereas as much as 14 nmol of DN1417 did not change naloxone binding. The data showing a failure of DN1417 to interfere with naloxone binding *in vitro* are compatible with the previous report of Tache et al. (2).

Figure 3 shows the profile of radioactivity of ^{3}H-labeled DN1417 on a TLC plate and HPLC. After diiodo-histidyl-DN1417 was exposed to [^{3}H]gas, it was

Table I. Effect of Varying Doses of DN1417 on Naloxone Binding in the Striatum and Hypothalamus[a]

DN1417 (mg/100 g body wt)	Striatal naloxone binding	Hypothalamic naloxone binding
0	100.0 ± 4.3	100.0 ± 4.1
0.06	82.1 ± 5.3*	97.0 ± 2.8
0.3	73.1 ± 4.8*	89.6 ± 4.8
1.5	65.5 ± 3.8*	61.7 ± 2.3*

[a] Rats were intraperitoneally injected twice a day with varying doses of DN1417 for 2 days. Naloxone binding was determined in the striatum and hypothalamus as described in Materials and Methods. The data (percentage of the DN1417 0-injected group) resulting from three different experiments are expressed as mean ± SE. Fifteen animal samples were used in each group. * $P < 0.01$ differs from the saline (DN1417 0)-injected group.

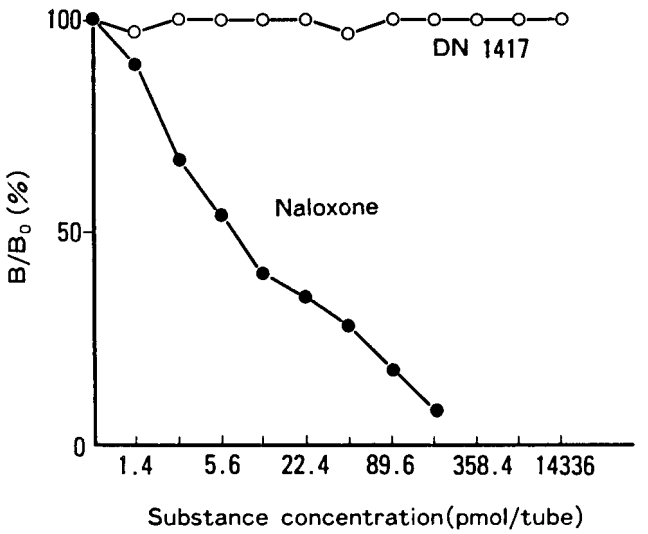

Figure 2. *In vitro* interaction of DN1417 with [^{3}H]naloxone binding in the brain. The brain homogenate was incubated with [^{3}H]naloxone in the presence of varying doses of DN1417, as described in Materials and Methods. B_0, bound naloxone in the absence of naloxone and DN1417; B, bound naloxone in the presence of substances.

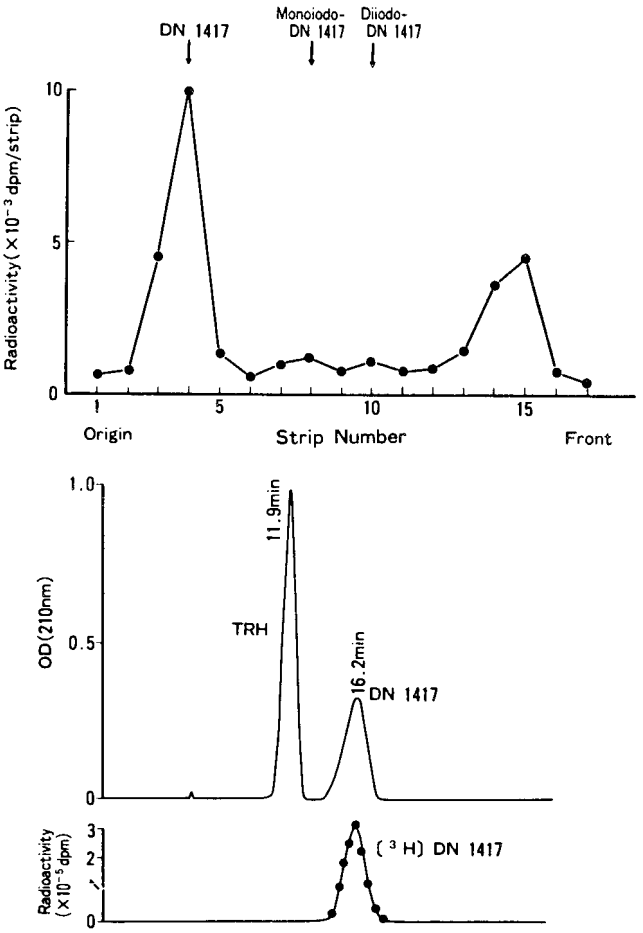

Figure 3. The profile of radioactivity of ^{3}H-labeled DN1417. The upper panel shows a radioactivity on a TLC. As described in Materials and Methods, diiodo-DN1417 was exposed to [^{3}H]gas, applied on a TLC, and developed in a solvent system of butanol: acetic acid; water (4:1:1, v/v/v). The TLC plate was cut into strips and used for count of radioactivity. The spots of unlabeled substances were visualized by Pauly's reagent. The lower panel shows an elution pattern of DN1417 and [^{3}H]DN1417 on a HPLC. [^{3}H]DN1417 was applied on a HPLC and eluted with 0.02 M KH_2PO_4 (pH 2.5) at a speed of 1.0 ml/min. Eluents were used for count of radioactivity.

analyzed that approximately 60% of the radioactivity was localized in DN1417. The radioactive DN1417 showed a single peak on a HPLC.

Figure 4 shows the distribution of purified [^{3}H] DN1417 in the brain. Sixty minutes after intraventricular injection, the highest activity was found in the hypothalamus (116.7 ± 3.7 dpm/mg wet wt). Radioactivity in the striatum (50.1 ± 3.5 dpm/mg wet wt) statistically did not differ from that in the midbrain, pons-medulla, hippocampus, or cortex. When [^{3}H] DN1417 was injected intraperitoneally, the pituitary showed the highest activity (16.6 ± 1.8 dpm/mg wet wt, 60 min after injection). However, the distribution of [^{3}H]DN1417 did not differ among the brain regions (the striatum, 3.12 ± 0.18; the hypothalamus, 3.90 ± 0.19 dpm/mg wet wt, 60 min after injection). Approximately 70% of the radioactivity which was distributed in the striatum showed the same elution time (16.2 min) on a HPLC as DN1417 did.

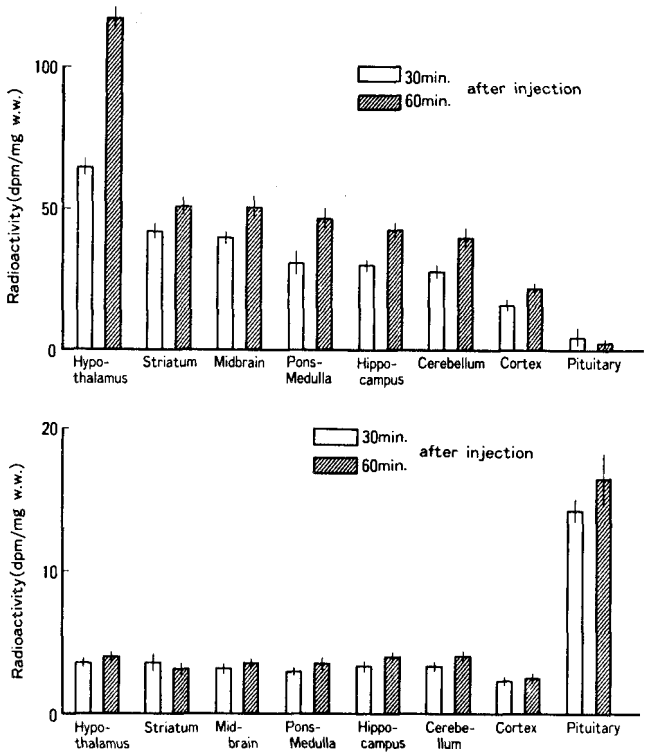

Figure 4. Distribution of [^{3}H]DN1417 in the brain regions. The upper panel shows a distribution of radioactivity of intraventricularly injected [^{3}H]DN1417 in the brain. As described in Materials and Methods, rats were injected intraventricularly with [^{3}H]DN1417, and brain regions were homogenized in methanol for count of radioactivity. The data are expressed as mean ± SE. Four samples were used in each group. The lower panel shows a distribution of [^{3}H]DN1417 in the brain after ip injection. Rats were injected intraperitoneally with [^{3}H] DN1417, and brain regions were homogenized in methanol for count of radioactivity. The data are expressed as mean ± SE. Four samples were used in each group.

Discussion

This study demonstrates that *in vivo* administration of DN1417, an analogue of TRH, caused a dose-dependent decrease in naloxone binding of the brain striatum without changing its affinity constant. In a series of the present study, DN1417 instead of TRH was used because the analogue has increased effects on the central nervous system with very low activity of thyrotropin release as compared with effects of TRH (8). The present results showed that *in vitro* simultaneous addition of DN1417 did not interfere with the brain naloxone binding. This may be interpreted to suggest that DN1417 affected naloxone binding by changing the degradation, synthesis, and/or internalization of the binding. In view of these observations, the results that the analogue did not change the affinity constant of naloxone binding have led to the concept that DN1417 may have an interaction of allosteric type with naloxone binding. The present data support the possibility that TRH may interact with brain opioid function, at least in part, by interfering with opioid binding in the striatum.

The decreased binding of naloxone was produced by DN1417 in the brain striatum, but not in other brain regions. The brain has a number of heterogeneous and integral functions, each of which corresponds to the discrete brain region (14) and is related to some degree to bioactivities of specific neuroactive substances. In addition, the heterogeneous density of TRH and opioid receptors is present throughout the brain (4, 5). Koskinen (15) reported that iv administration of TRH caused the different increase of blood flow in brain regions. Therefore, it was possible that the distribution of DN1417 varied with brain regions. However, the distribution of [^{3}H]DN1417 after peripheral injection did not differ among brain regions. The dissimilar accumulation of [^{3}H]DN1417 between the peripheral and central injection occurred, but the exact explanation for this dissimilarity has remained to be elucidated at present. The possibility has been raised that the dissimilarity may be attributed to the existence of blood-brain barrier, because the barrier has a nature featured by the slow penetration of TRH into brain (16). Butler et al. (17) observed that the distinct expression of TRH action on the brain depended on its injection routes, i.e., the peripheral versus central injection. In view of these observations, the present results have led us to the conclusion that DN1417 injected peritoneally did not accumulate exclusively in the brain striatum. Therefore, the decrease in striatal naloxone binding by DN1417 was not attributed to the different distribution of TRH analogue in the brain. Based on an efficient ratio (2.0) of DN1417 versus TRH with respect to neuropharmacologic effect (8), a dose of 0.3 mg of DN1417 which significantly decreased the striatal naloxone binding is comparable to 0.6 mg of TRH. The 0.3-mg dose of DN1417 did not change the hypothalamic naloxone binding, while its binding was apparently decreased by as much as 1.5 mg of DN1417. This was considerably higher than a dose comparable to 0.4 mg of TRH which could induce opioid antagonism (3). Therefore, the significance of changes in the hypothalamic naloxone binding remains unknown.

Naloxone is a specific antagonist for opioid action and is recognized as a preferential antagonist of opioid μ-receptor (9), but it interacts to a certain extent with other subtypes of opioid receptors. Using subtype-specific radioligands, further studies are now in progress to determine the subtype specificity of opioid receptors which could be affected by TRH administration.

1. Prasad C. Thyrotropin-releasing hormone. In: Lajtha A, ed. Handbook of Neurochemistry. New York: Plenum, Vol **8**: pp175–200, 1985.
2. Tache Y, Lis M, Collu R. Effects of thyrotropin-releasing hormone on behavioral and hormonal changes induced by β-endorphin. Life Sci **21**:841–846, 1977.
3. Bhargava HN. Dissociation of tolerance to the analgesic and hypothermic effects of morphine by using thyrotropin releasing

hormone. Life Sci **29**:1015–1020, 1981.
4. Taylor RL, Burt DR. Species differences in the brain regional distribution of receptor binding for thyrotropin-releasing hormone. J Neurochem **38**:1649–1656, 1982.
5. Pert CB, Snyder SH. Opiate receptors: Demonstration in nervous tissue. Science **179**:1011–1014, 1973.
6. Bhargava HN, Das S. Evidence for opiate action at the brain receptors for thyrotropin-releasing hormone. Brain Res **368**:262–267, 1986.
7. Martin BR, Dewey WL, Chau-Pham T, Prange AJ. Interactions of thyrotropin releasing hormone and morphine sulfate in rodents. Life Sci **20**:715–722, 1977.
8. Miyamoto M, Fukuda N, Narumi S, Nagai Y, Saji Y, Nagawa Y. γ-Butyrolactone-γ-carbonyl-histidyl-prolineamide citrate (DN1417): A novel TRH analog with potent effects on the central nervous system. Life Sci **28**:861–869, 1981.
9. Change K-J, Cuatrecasas P. Receptor and second messengers. In: Krieger DT, Brownstein MJ, Martin JB, eds. Brain Peptides. New York: Wiley, pp565–593, 1983.
10. Mori M, Prasad C, Wilber JF. Regional dissociation of histidyl-proline diketopiperazine and thyrotropin releasing hormone in the rat brain. Brain Res **231**:451–453, 1982.
11. Akera T, Cheng JK. A simple method for the determination of affinity and binding site concentration in receptor binding studies. Biochim Biophys Acta **470**:412–417, 1977.
12. Ling N, Leppaeluoto, Vale W. Chemical, biological, and immunological characterization of mono- and diiodo-thyrotropin releasing factor. Anal Biochem **76**:125–133, 1976.
13. Yamaguchi M, Mori M, Ishikawa K, Suzuki M. Antagonism by thyrotropin-releasing hormone of brain temperature in response to pentobarbital in the rat: Possible involvement of cholinergic mechanism. Proc Soc Exp Biol Med **183**:368–371, 1986.
14. Kandel ER, Schwartz JH. Principles of Neural Science. London, Edward Arnold, Ltd., 1981.
15. Koskinen L-OD. Effect of low intravenous doses of TRH, acid-TRH and cyclo(His-Pro) on cerebral and peripheral blood flows. Br J Pharmacol **87**:509–519, 1986.
16. Zlokovic BV, Lipovac MN, Begley DJ, Davson H, Rakic L. Slow penetration of thyrotropin-releasing hormone across the blood-brain barrier of an in situ perfused guinea pig brain. J Neurochem **51**:252–257, 1988.
17. Butler PD, Mann PE, Bodnar RJ. Comparison of central and peripheral thyrotropin releasing hormone administration upon stress-induced analgesia. Ann NY Acad Sci **467**:430–432, 1986.