Vol. 116, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1983 Pages 669-674

BIOCHEMICAL AND AUTORADIOGRAPHIC STUDIES OF TRH RECEPTORS IN SECTIONS OF RABBIT SPINAL CORD

NAJAM A. SHARIF, NANCY S. PILOTTE AND DAVID R. BURT*

Department of Pharmacology and Experimental Therapeutics,
University of Maryland School of Medicine, Baltimore, Maryland 21201, USA

Received September 12, 1983

SUMMARY: Receptors for thyrotropin-releasing hormone (pGlu-His-Pro-NH_2, TRH) on thaw-mounted sections of rabbit spinal cord have been identified biochemically and visualized by light microscopic autoradiography. Binding of [^{3}H][3-Me-His^2]TRH to 20 μm sections exhibited high apparent affinity and a pharmacological specificity almost identical to that previously demonstrated for spinal TRH receptors in membranes. In autoradiograms, the highest density of TRH receptors appeared in the substantia gelatinosa of the dorsal gray and around the central canal, with intermediate levels in the ventral gray.

Specific lesions coupled with high performance liquid chromatography and radioimmunoassay (1,2), as well as immunohistochemical studies (3), have demonstrated the coexistence of thyrotropin-releasing hormone (TRH) and substance P in many descending serotonergic raphé efferents to the spinal cord (SC). TRH increases the excitability of motoneurons (4-6), presumably by interacting with specific receptors. We have recently characterized such receptors in membranes of rabbit SC (7). Similar receptors in rat SC exhibit biochemical supersensitivity after codepletion of TRH and serotonin (2) and are modulated *in vitro* by substance P (8). We now report for the first time methods for studying the biochemistry of spinal TRH receptors on slide-mounted sections and describe their autoradiographic localization.

MATERIALS AND METHODS

Female white New Zealand rabbits (3-5 Kg) were anesthetized with urethane (2 g/Kg, i.p.), perfused transcardially with 100 ml of 5% dimethylsulfoxide (DMSO) in 0.9% NaCl (for biochemical studies only) and decapitated. The SC was removed and freed of pia mater. Segments of 2 cm were frozen on microtome chucks. After equilibration at -15°C for 1 h, 20 μm sections were cut, thaw-mounted onto gelatin-subbed microscope slides, and stored at -20°C for up to 2 weeks.

For binding assays, sections were thawed at room temperature, washed in cold 0.9% NaCl for 1 min with agitation and briefly (3-4 min) placed in an oven

* To whom correspondence should be addressed.

Abbreviations: TRH, thyrotropin-releasing hormone; SC, spinal cord; MeTRH, [3-methyl-His^2]TRH; i.p., intraperitoneally; DMSO, dimethylsulfoxide; K_d, equilibrium dissociation constant; B_{max}, density of binding sites; cpm, counts per min; IC_{50}, concentration inhibiting by 50%.

0006-291X/83 $1.50

at 80°C. Slides were equilibrated at 5°C for 15 min before spreading, over every 3 sections, 20 µl of 2 nM [^{3}H][3-Me-His^2]TRH ([^{3}H]MeTRH) (80 Ci/mmol, ref. 9) in sodium phosphate buffer (20 mM, pH 7.4, + 100 µM bacitracin) in the presence or absence of 10 µM TRH or test compound. Following a 1 h incubation at 5°C, the slides were washed in excess cold saline for 1 min with agitation followed by 4 min standing in the same saline. The slides were drained and the sections wiped off with damp filter discs, equilibrated overnight in 3 ml fluor, and counted by liquid scintillation spectrometry at 46% efficiency.

For autoradiographic studies, the labeled sections (on previously acid-washed and gelatinized slides) were dried at 80°C for 3 min, allowed to cool, and then exposed to tritium-sensitive sheet film (LKB Ultrofilm) for 2 months at 5°C. Autoradiograms were processed in Kodak D-19 developer for 5 min and Kodak Rapidfix for 5 min. The film was then washed under running water for 25 min and finally treated with Photo-Flo 200 hardener.

Protein concentrations in SC preparations were determined according to Lowry et al. (10).

RESULTS

No loss of [^{3}H]MeTRH binding to sections was detected up to 2 weeks of storage at -20°C. Binding was enhanced by washing of sections in cold saline before assay. Periods of exposure to 80°C (in an oven, producing surface temperatures considerably lower for short exposures) for 1, 2, 4 and 10 min increased specific [^{3}H]MeTRH binding to sections by 18 ± 8% (2), 49 ± 17% (3), 67 ± 3% (3) and 22 ± 13% (2) at 1, 2, 3.5 and 6-10 min, respectively. Equilibrium studies on thoracic SC sections revealed that the heat treatment increased the apparent binding affinity (dissociation constant, K_d, controls (5°C) = 15.4 ± 1.8 (3) nM; treated (80°C/3 min) = 5.2 ± 1 (2) nM). Inactivation of peptidases or other endogenous inhibitors of binding and/or stabilization of receptors may be responsible for this increase. Similar observations have been made in sections of rat brain (16).

Under the defined conditions (1 h at 5°C), specific binding of [^{3}H]MeTRH (2 nM, 3200 cpm) to cervical SC sections was 63-86% of the total (249 ± 9 (25) cpm/3 sections) and the blanks (with 10 µM TRH) gave 72 ± 4 (36) cpm.

An apparent steady state of saturable [^{3}H]MeTRH binding was attained between 60 and 90 min at 5°C (Fig. 1). Detailed competition experiments with unlabeled MeTRH (1-100 nM) yielded linear Scatchard plots (Fig. 2), indicating the prevalence of a single class of high affinity binding sites of K_d = 7.5 ± 0.6 nM, n = 7, and a density of sites, B_{max}, of 440 ± 36 pM per 3 sections, approximately 69 fmol/mg protein. Fresh cervical SC membrane resuspensions exhibited an apparent K_d of 2.5 ± 0.2 nM and a B_{max} of 132 ± 3 (2) fmol/mg protein (7). The higher B_{max} in resuspensions is adequately accounted for by the absence of soluble proteins, while the higher K_d in sections is probably due to a higher effective tissue concentration and incubation temperature.

Numerous TRH analogs inhibited [^{3}H]MeTRH binding in a competitive manner (Fig. 3). The drug selectivity of TRH receptors on SC sections and in SC resuspensions was almost identical (Fig. 4) and consistent with receptor identification (11,12).

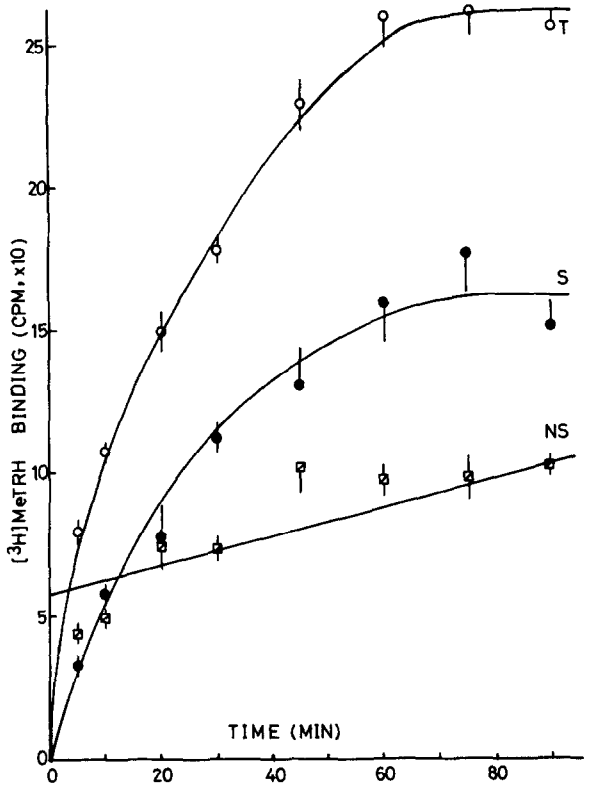


Fig. 1. Time course of [3H]MeTRH binding. Washed and preincubated rabbit SC sections were incubated with 2 nM [3H]MeTRH in the presence and absence of 10 μM TRH for various times at 5°C. Incubations were terminated by a total of 5 min rinsing of slides in cold saline (see text). Each point constitutes the meaned binding to 3 sections, and means ± S.E.M. (error bars) of 3 determinations are shown. Binding (cpm) is shown as, T = total, S = specific, and NS = nonspecific.

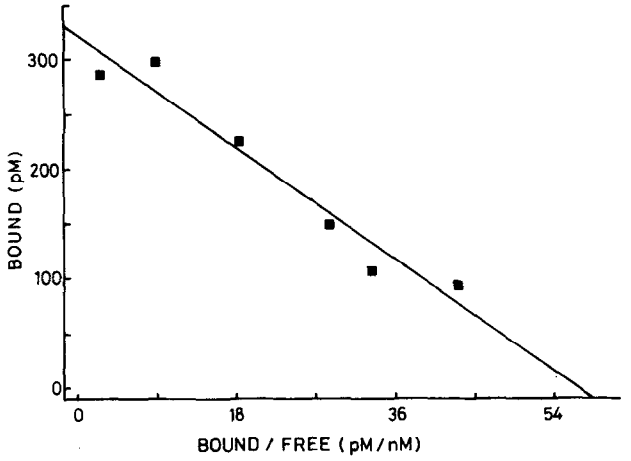
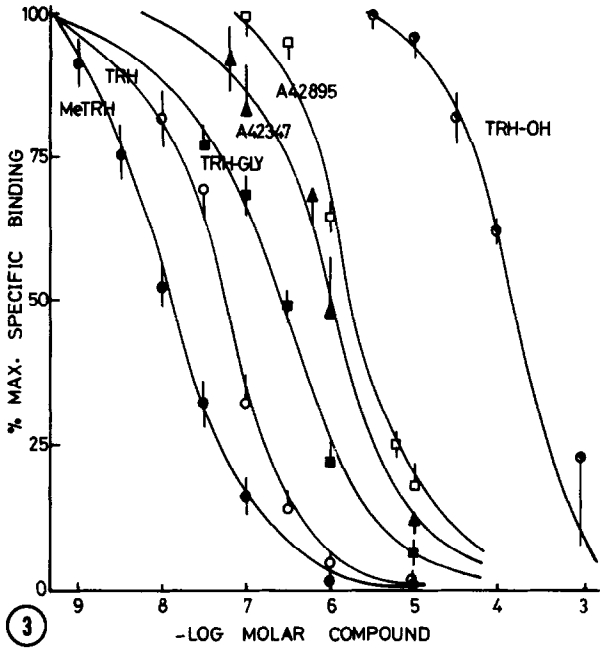


Fig. 2. Scatchard plot of specific [3H]MeTRH binding to SC sections. Data from competition experiments were analyzed to derive the dissociation constants (K_d) and receptor density (B_{max}). (For convenience in calculation, bound radioactivity was expressed in units of concentration, even though technically this has no physical meaning for a heterogeneous system.) The figure shown is a representative experiment. Separate analyses of many similar experiments yielded the values listed in Results.



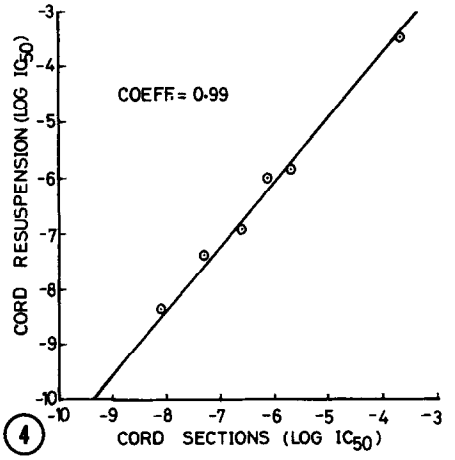


Fig. 3. Pharmacological specificity of TRH receptor binding to SC sections. TRH analogs at 5-7 concentrations competed with 2 nM [^{3}H]MeTRH during a 1 h incubation at 5°C. Results depicted are means ± S.E.M. of 4-9 separate determinations, where each concentration was tested on 3 SC sections. TRH-GLY = TRH glycinamide; A42347 = O=C<Thr-His-Pro-NH_2; A42895 = pGlu-His-Pro-NH-C_2H_5; TRH-OH = TRH-free acid.

Fig. 4. Comparison of TRH receptor specificity in sections and in whole SC membranes. A log-log correlation graph (drawn by linear regression analysis) depicting the relative drug selectivity of rabbit SC receptors in the two preparations is shown. The mean IC_{50} values for various TRH analogs for sections were derived from Fig. 3, while values for resuspensions are taken from our previous studies (7). Both axes represent molar values. Note the excellent correlation (r = 0.99).

TRH receptors appeared most concentrated in the substantia gelatinosa of the dorsal horn and around the central canal area of the cervical SC as determined by autoradiography (Fig. 5). While the rest of the dorsal gray had a very diffuse distribution of receptors, the ventral horns were uniformly labeled but somewhat less than the substantia gelatinosa and central canal area. In general, these findings corroborate our data from membrane studies (7).

DISCUSSION

Optimal conditions for studying the biochemistry of TRH receptors on slide-mounted SC sections have been determined. The relatively good correspondence between the time course (Fig. 1), the densities and dissociation constants derived from competition experiments (Fig. 2) and the pharmacological specificity of specific [^{3}H]MeTRH binding to rabbit SC sections (Fig. 3,4) and to SC membranes (7) supports the identification of TRH receptors (11,12) in

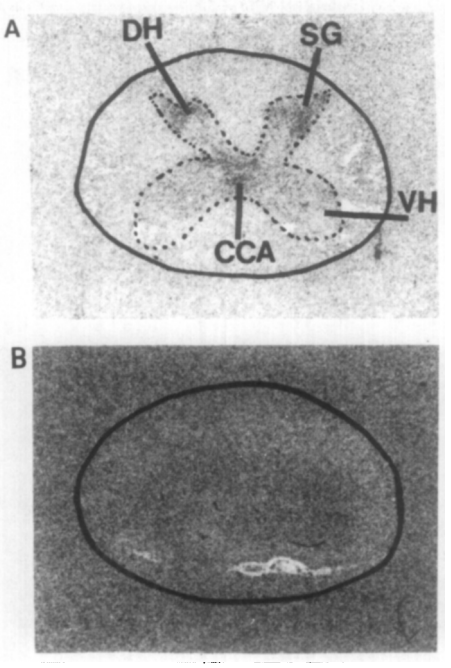


Fig. 5. Autoradiographic localization of TRH receptors in rabbit SC. Although concurrent studies in rat brain (16) suggested DMSO perfusion has a cryoprotectant effect to improve binding, the sections used here came from a rabbit killed before this effect was established. Labeled sections were exposed to LKB Ultrofilm for 2 months at 5°C and developed as described in the text. "A" was labeled with 2 nM [^{3}H]MeTRH, while "B" (blank) also contained 10 μM TRH. SG = substantia gelatinosa; DH = dorsal horn; VH = ventral horn; CCA = central canal area. The outlines of the sections (solid lines) and the gray matter (dotted lines) have been drawn in for reference. Note the dense receptor labeling in SG and CCA, and a slightly lower abundance in the VH. This profile resembles distribution data from earlier membrane studies (7) as well as a current determination in CCA membranes from rabbit SC segments: cervical = 34.7 ± 5.5, thoracic = 31 ± 0.5, lumbar = 21.7 ± 3 fmol/mg protein (5 h at 0°C binding of 1 nM [^{3}H]MeTRH; means ± S.E.M. of 2 experiments, each in triplicate). We earlier observed similar values in membranes from DH and VH (7).

both preparations. Furthermore, these properties are closely similar to those in TRH receptors of rat SC (8) and amygdala (14,15) and sheep CNS (9,13).

Autoradiographic results (Fig. 5) generally confirmed the earlier test tube experiments on dissected rabbit SC regions (7). Chemical lesions of the bulbospinal pathways in the rat (2) indicate that most TRH receptors are on postsynaptic elements, information not available from autoradiography at this resolution.

While the distribution of TRH receptors in the rabbit ventral and lateral gray and the central canal area is in good agreement with the distribution of TRH-like immunoreactivity (3,17-20), the dense labeling of the substantia gelatinosa (Fig. 5) and the previously determined high concentration of TRH receptors in dorsal gray membranes (7) represent new information that suggests TRH may be involved in modulating sensory input or relaying sensory information

to higher centers. TRH has been reported to be of benefit in several spinal disorders (21-23), but there its effects are more likely exerted at the level of ventral motoneurons.

ACKNOWLEDGMENTS

We thank Dr. F. C. Kauffman for the use of his CRYO-CUT microtome and Ms. Evelyn Elizabeth for her excellent typing. Gifts of some TRH analogs from Abbott Laboratories are gratefully acknowledged. Able technical assistance was provided in part by Mr. Tim Hartke and Ms. Ellie Zuhowski. These studies were supported by U.S. Army Medical Research and Development contract DAMD-17-81-C1279, U.S.P.H.S. grant MH-29671, and NSF grant BNS-8025469.

REFERENCES

1. Gilbert, R.F.T., Bennett, G.W., Marsden, C.A. and Emson, P.C. (1981) Eur. J. Pharmacol. 76: 203-210.
2. Sharif, N.A., Burt, D.R., Towle, A.C., Mueller, R.A. and Breese, G.R. (1983) J. Neurochem. 41: S83.
3. Johansson, O., Hökfelt, T., Pernow, B., Jeffcoate, S.L., White, N., Steinbusch, H.W., Verhofstad, A.A.J., Emson, P.C. and Spindel, E. (1981) Neuroscience 6: 1857-1881.
4. Nicoll, R.A. (1978) J. Pharmacol. Exptl. Ther. 207: 817-824.
5. Cooper, B.R. and Boyer, C.E. (1978) Neuropharmacology 17: 153-156.
6. Ono, H. and Fukada, H. (1982) Neuropharmacology 21: 739-744.
7. Sharif, N.A. and Burt, D.R. (1983) Brain Res. 270: 259-263.
8. Sharif, N.A. and Burt, D.R. (1983) Neurosci. Lett. (submitted).
9. Taylor, R.L. and Burt, D.R. (1981) Neuroendocrinology 32: 310-316.
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193: 265-275.
11. Burt, D.R. and Taylor, R.L. (1983) Thyrotropin-Releasing Hormone (Griffiths, E.C. and Bennett, G.W., Eds.) pp. 71-83, Raven Press, New York.
12. Burt, D.R. and Sharif, N.A. (1983) Handbook of Neurochemistry, 2nd Ed., Vol. 6 (Lajtha, A., Ed.), Plenum Press, New York (in press).
13. Taylor, R.L. and Burt, D.R. (1981) Brain Res. 218: 207-217.
14. Sharif, N.A. and Burt, D.R. (1983) Reg. Peptides (submitted).
15. Sharif, N.A. and Burt, D.R. (1983) J. Neurochem. (in press).
16. Pilotte, N.S., Sharif, N.A. and Burt, D.R. (1983) Brain Res. (submitted).
17. Hökfelt, T., Fuxe, K., Johansson, O., Jeffcoate, S. and White, N. (1975) Neurosci. Lett. 1: 133-139.
18. Kardon, F.C., Winokur, A. and Utiger, R.D. (1977) Brain Res. 122: 578-581.
19. Spindel, E. and Wurtman, R.J. (1980) Brain Res. 201: 279-288.
20. Jackson, I.M.D. (1980) Brain Res. 201: 245-248.
21. Sobue, I., Yamamoto, H., Konagaya, M., Lida, M. and Takayanagi, T. (1980) Lancet 1: 418-419.
22. Faden, A.I., Jacobs, T.P. and Holiday, J.W. (1981) N. Engl. J. Med. 305: 1063-1067.
23. Engel, W.K., Siddique, T., Nicoloff, J.T. and Wilbur, J.F. (1983) Neurology 33 (Suppl. 2): 176.