References and Notes

1. N. Sharon and H. Lis, *Chem. Eng. News* **59** (No. 13), 21 (1981); K. Olden *et al.*, *J. Cell. Biochem.* **18**, 313 (1982); G. Ashwell and A. G. Morell, *Adv. Enzymol.* **41**, 99 (1974).
2. M. R. Sairam, in *Hormonal Proteins and Peptides*. C. H. Li, Ed. (Academic Press, New York, 1983), vol. 11, pp. 1–79; J. G. Pierce and T. F. Parsons, *Annu. Rev. Biochem.* **50**, 465 (1981).
3. E. V. Van Hall *et al.*, *Endocrinology* **88**, 456 (1971); H. Papkoff, *Acta Endocrinol. (Copenhagen)* **48**, 439 (1965); G. D. Braunstein *et al.*, *Biochem. Biophys. Res. Commun.* **42**, 962 (1971).
4. M. L. Dufau, K. J. Catt, T. Tsuruhara, *Biochem. Biophys. Res. Commun.* **44**, 1022 (1971); M. Ryle *et al.*, in *Gonadotropins and Ovarian Development*. W. R. Butt *et al.*, Eds. (Livingstone, London, 1972), pp. 144–158.
5. W. R. Moyle, O. P. Bahl, L. Marz, *J. Biol. Chem.* **250**, 9163 (1975); J. M. Goverman, T. F. Parsons, J. G. Pierce, *ibid.* **257**, 15059 (1982).
6. The hormone was chemically deglycosylated by treatment with anhydrous HF, which results in the loss of 75 to 80 percent of the total carbohydrate moiety without any effect on the peptide structure. See P. Manjunath and M. R. Sairam, *Methods Enzymol.* **109**, 725 (1985).
7. M. R. Sairam and P. W. Schiller, *Arch. Biochem. Biophys.* **197**, 291 (1979); M. R. Sairam and P. Fleshner, *Mol. Cell. Endocr.* **22**, 41 (1981); P. Manjunath and M. R. Sairam, *J. Biol. Chem.* **257**, 7109 (1982); _____, J. Sairam, *Mol. Cell. Endocr.* **28**, 125 (1982); N. K. Kalyan and O. P. Bahl, *Biochem. Biophys. Res. Commun.* **102**, 1246 (1981).
8. The subunits are designated LH-α, LH-β, and so on. The structure of the α subunit in the three pituitary glycoprotein hormone is identical. The β subunit, which is different in each hormone, is regarded as being hormone-specific.
9. M. R. Sairam, *Arch. Biochem. Biophys.* **204**, 199 (1980).
10. _____, *ibid.* **194**, 71 (1979); *Biochem. J.* **197**, 535 (1982).
11. The protein-binding assay was performed as described (*7*).
12. Initial studies with deglycosylated hCG α support the conclusions of the present studies. The extremely rapid reassociation kinetics of acid or thermally dissociated deglycosylated hCG subunits has been reported [see P. Manjunath and M. R. Sairam, *J. Biol. Chem.* **258**, 3554 (1983)].

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Thyrotropin-Releasing Hormone Induces Rhythmic Bursting in Neurons of the Nucleus Tractus Solitarius

Abstract. *The nucleus tractus solitarius (NTS) contains neurons that are part of the central neuronal network controlling rhythmic breathing movements in mammals. Nerve terminals within the NTS show immunoreactivity to thyrotropin-releasing hormone (TRH), a neuropeptide that has potent stimulatory effects on respiration. By means of a brainstem slice preparation in vitro, TRH induced rhythmic bursting in neurons in the respiratory division of the NTS. The frequency of bursting was voltage-dependent and could be reset by short depolarizing current pulses. In the presence of tetrodotoxin, TRH produced rhythmic oscillations in membrane potential whose frequency was also voltage-dependent. These observations suggest that TRH modulates the membrane excitability of NTS neurons and allows them to express endogenous bursting activity.*

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In mammals, rhythmic breathing movements are controlled by a central neuronal network composed of several groups of neurons found in the brainstem (*1*). Most of the neurons in the dorsal respiratory group (DRG), which is located within the nucleus tractus solitarius (NTS), are active during inspiration (*2*). Several neuropeptides have been found within nerve terminals in the NTS by means of immunohistochemical techniques; one of these peptides is thyrotropin-releasing hormone (TRH) (*3*). The application of exogenous TRH to the brainstem increases respiratory rate and minute ventilation (*4*). In addition, respiratory depression caused by barbiturates is diminished by TRH (*5*). These observations suggest that TRH may play a central role in the neural control of respiration; however, the effects of TRH have not been studied at the cellular level. We used a brainstem slice preparation that allows stable intracellular recordings to be made in vitro from NTS neurons and found that TRH produces rhythmic bursting activity in neurons in the respiratory part of the NTS.

Brainstem slices were obtained from adult guinea pigs (350 to 500 g in body weight) by means of standard tissue slicing techniques (*6*). Three to four slices (400 μm thick) were cut in transverse section beginning at the level of the obex and continuing toward the rostrum. The use of transverse slices facilitated the identification of the tractus solitarius and its surrounding nuclear areas. In several experiments, parasagittal and longitudinal slices were studied to ensure that our observations did not depend on the presence or absence of a particular part of a neuron's dendritic field. No differences in neuron properties or responses were observed for the different slice orientations. After being cut, the slices were transferred immediately to a recording chamber and were subfused with oxygenated Ringer solution (*6*). All drugs were applied to the bath from separate reservoirs of Ringer solution. A mixture of 95 percent O_2 and 5 percent CO_2, warmed and humidified, was blown over the slices. All experiments were performed at 37°C.

Three criteria were used to determine whether or not a neuron was healthy: (i) a stable resting potential of at least −50 mV, (ii) a spike overshoot of at least +35 mV (spike amplitude, >85 mV), and (iii) an input resistance of at least 50 megohms. Only neurons meeting all three criteria were used. In general, we obtained stable recordings from NTS neurons for periods of 1 to 4 hours without deterioration of their membrane properties. Observations were based on recordings from 23 neurons in 22 slices.

The distribution of respiratory neurons in the brainstem of guinea pigs has been mapped in situ by means of extracellular recording techniques (*7*) (Fig. 1A). Because respiratory neurons as such could not be identified in the slice, we have referred to these cells simply as NTS neurons. However, these neurons could be distinguished from those in adjacent parts of the NTS and reticular formation by both their morphology and their membrane properties (*8*).

In the slice preparation, NTS neurons in the DRG did not show a pattern of activity suggestive of a respiratory rhythm. They did, however, show nonrhythmic activity at a rate of 1 to 3 spikes per second (Fig. 1B, upper trace). After TRH was added to the bath, a rhythmic bursting pattern was established in 70 percent of the neurons studied (Fig. 1B, lower trace) and was observed for concentrations of TRH between 0.1 and 10.0 μ*M* (*9*). The development of rhythmic bursting activity followed a stereotyped time course (Fig. 1C). Five to 10 minutes after the slice was exposed to TRH, the action potential of NTS neurons developed a depolarizing afterpotential (DAP). The size of the DAP increased with time and was associated with the appearance of rhythmic bursting activity. Exposure to TRH for 1 to 2 hours led to a decline in bursting activity that may have been due to a desensitization phenomenon (*9*). The action potential of NTS neurons, however, still showed a DAP.

Depolarizing afterpotentials are characteristic of endogenous pacemaker and bursting neurons in both invertebrates and mammals (*10*). The development of a DAP after exposure to TRH therefore suggests that rhythmic bursting activity

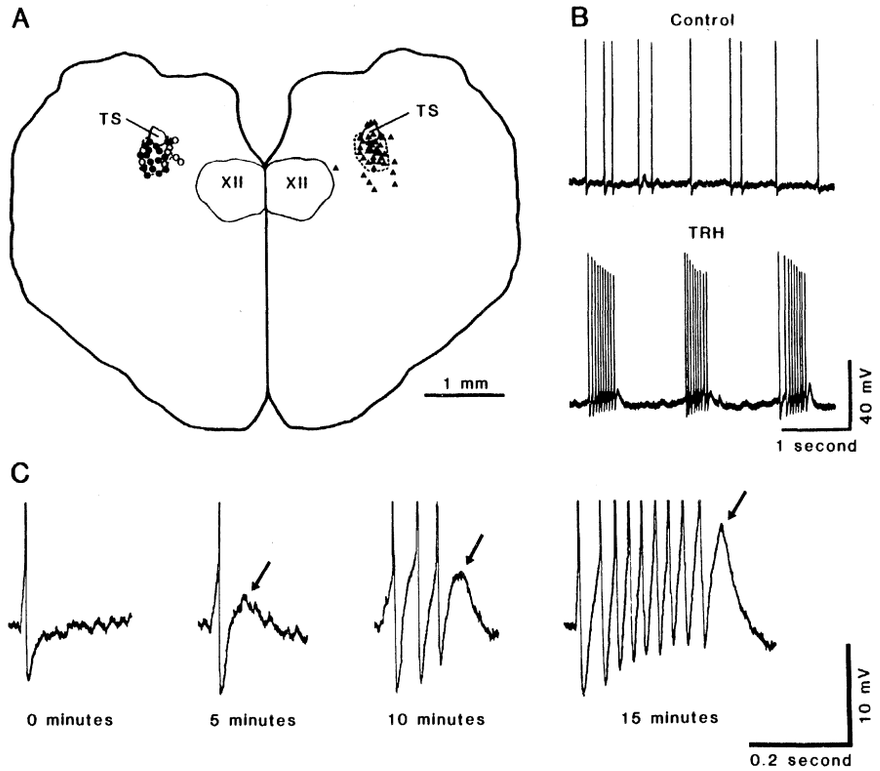

Fig. 1. (A) Cross section of the brainstem approximately 1.0 mm rostral to the obex. (Right) Map of the DRG in the guinea pig. Triangles show the distribution of identified respiratory neurons, including inspiratory and expiratory types. The dashed line indicates the region where the highest density of respiratory neurons was located and corresponds to the DRG in the guinea pig. This region was below the tractus solitarius (TS) in the ventral NTS, approximately 0.25 to 1.5 mm rostral to the obex. (Left) Locations of neurons studied in the slice preparation (0 to 1.6 mm rostral to the obex). These sites were measured relative to the tractus solitarius. Closed circles represent neurons that responded to TRH, and open circles represent neurons that did not. (B) Effect of TRH on NTS neurons: activity in an NTS neuron before (top trace) and after (bottom trace) exposure to 0.4 μM TRH for 30 minutes. This neuron was current clamped to −45 mV. (C) Development of the DAP and bursting activity: high-gain records of spike activity at various times after exposure to TRH (same neuron as in B). The tops of the spikes are truncated. Before the slice was exposed to TRH (0 minutes), the spike afterpotential showed a gradual return to baseline in less than 0.1 seconds. At 5 minutes, a prominent DAP appeared (arrow). The size of the DAP increased with time and was associated with the development of rhythmic bursting activity.

may have been due to an endogenous rather than a synaptic mechanism. This hypothesis was tested in three types of experiments. Because one property of an endogenous rhythm is that the frequency of bursting is voltage-dependent (*11*), the effect of membrane polarization on the TRH-induced bursting rhythm was examined (Fig. 2A). As the membrane potential was made more negative, the interval between bursts lengthened. At very negative membrane potentials (−80 mV), neither bursting activity nor rhythmic oscillations in membrane potential occurred. The absence of membrane potential oscillations at −80 mV suggests that these neurons did not receive phasic excitatory synaptic input.

Endogenously generated rhythms also display a characteristic response to short perturbations in membrane potential (*11*) that affect both the duration of the interval between bursts and the times when subsequent bursts occur. Short perturbations in membrane potential were induced by applying a depolarizing current pulse after three unperturbed bursts (Fig. 2B). The duration of the interval between bursts increased by 66 percent. A second perturbation after the fourth burst produced a similar effect. A normal interval (1.12 seconds) was established between bursts five and six, but the rhythm had been delayed by 1.4 seconds. These results are consistent with the hypothesis that bursting activity is produced by an endogenous mechanism.

Neurons with endogenous bursting capabilities show voltage-dependent oscillations in membrane potential that are resistant to tetrodotoxin (TTX) (*12*).

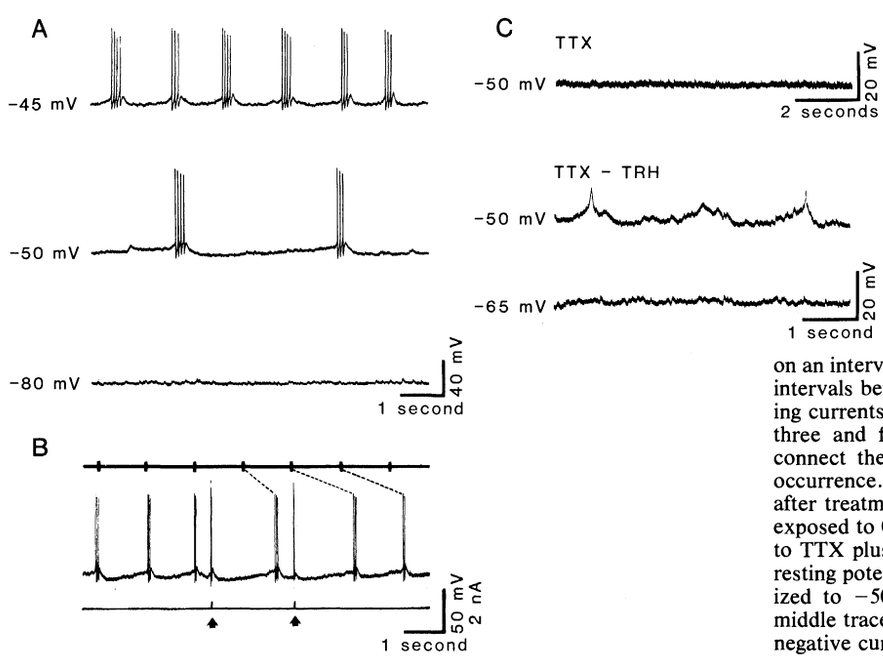

Fig. 2. (A) Voltage dependence of the interval between bursts in a neuron exposed to 0.5 μM TRH for 45 minutes. (Top trace) The neuron was depolarized to −45 mV by applying a positive current. The average interval between bursts was 1.4 seconds. At a resting membrane potential of −50 mV, the average interval was 3.7 seconds (middle trace). Hyperpolarization to −80 mV, induced by applying a negative current, resulted in the disappearance of rhythmic bursting activity (bottom trace). (B) Effects of perturbations in membrane potential on rhythmic bursting activity. The scale (top trace) indicates the predicted occurrence of a burst cycle based on an interval of 1.1 seconds. This value is the average of the intervals between the preceding ten bursts. Short depolarizing currents (0.25 nA for 50 msec) were applied after cycles three and four (bottom trace, arrows). The dashed lines connect the last three bursts with their predicted time of occurrence. (C) Rhythmic oscillations in membrane potential after treatment of neurons with TTX. An NTS neuron was exposed to 0.5 μg of TTX per milliliter alone (top trace) and to TTX plus 0.6 μM TRH (middle and bottom traces). The resting potential of this neuron was −57 mV. It was depolarized to −50 mV by applying a positive current (top and middle traces) and hyperpolarized to −65 mV by applying a negative current (bottom trace).

These oscillations are thought to be the basis of bursting activity. In the presence of TTX, TRH also induced oscillations in membrane potential (Fig. 2C). The frequency of these oscillations was voltage-dependent, and the oscillations disappeared when the membrane was hyperpolarized. The voltage dependence of the oscillations was similar to that observed for rhythmic bursting (Fig. 2A), which suggests that the oscillations were responsible for the development of bursting activity. This experiment also showed that TRH affected NTS neurons in the absence of spike-induced transmitter release.

Thyrotropin-releasing hormone thus alters the activity in some NTS neurons from a nonrhythmic to a rhythmic pattern. The most straightforward explanation for our observations is that TRH acts directly on the membrane properties of NTS neurons, transforming them into endogenous burster neurons (*13*). Neurons that exhibit endogenous bursting behavior in the presence of neuroactive substances have been observed in the nervous systems of invertebrates (*14*) and are referred to as conditional bursters. The action of TRH on NTS neurons within the DRG supports the hypothesis that this neuropeptide takes part in the control of rhythmic breathing in mammals. Further experiments are required to assess the role of conditional bursting activity in this system and to determine the mechanism by which TRH modulates the membrane excitability of NTS neurons.

References and Notes

1. M. I. Cohen, *Physiol. Rev.* **59**, 2367 (1979); D. W. Richter, *J. Exp. Biol.* **100**, 93 (1982); C. von Euler, *J. Appl. Physiol.* **55** (6), 1647 (1983).
2. Although most respiratory neurons in the DRG fire during inspiration, a limited number fire during expiration. In cats, about 5 percent of the respiratory neurons in the DRG are classified as expiratory [see Cohen in (*1*)]. In the DRG of guinea pigs, the number of expiratory neurons is about 20 percent (*7*). In addition, the DRG of both cats and guinea pigs contains second-order sensory neurons called pump units. These neurons convey information from lung stretch receptors.
3. T. Hokfelt *et al.*, *Neurosci. Lett.* **1**, 133 (1975); T. J. McCown, *Soc. Neurosci. Abst.* **10**, 825 (1984).
4. R. D. Myers *et al.*, *Brain Res.* **126**, 105 (1977); Y. Yamamoto *et al.*, *Acta Physiol. Scand.* **113**, 541 (1981); J. Hedner *et al.*, *Neurosci. Lett.* **25**, 317 (1981); *Pediatr. Res.* **16**, 543 (1982); *Acta Physiol. Scand.* **117**, 427 (1983); D. Niewoehner, *Peptides* **4**, 277 (1983); J. W. Holaday and A. I. Faden, *Regulatory Peptides* **7**, 111 (1983).
5. G. W. Kraemer *et al.*, *Pharmacol. Biochem. Behav.* **4**, 709 (1976); D. K. Andry and A. Horita, *ibid.* **6**, 55 (1977).
6. Our slicing procedure was based on that described by R. Llinas and M. Sugimori [*J. Physiol.* **305**, 171 (1980)]. Briefly, guinea pigs were anesthetized with methoxyflurane and decapitated. The brainstem, with the cerebellum attached, was quickly removed and placed for 30 to 45 seconds in Ringer solution cooled to 0°C. After being cooled, the cerebellum was removed. The brainstem was cut into a block of tissue and glued with cyanoacrylate to a cooled Teflon block. Slices were cut with a Vibratome (Camden Instruments) while the tissue was submerged in cold Ringer solution (125 m*M* NaCl, 26 m*M* $NaHCO_3$, 6.2 m*M* KCl, 2.4 m*M* $CaCl_2$, 1.3 m*M* $MgSO_4$, 1.25 m*M* $NaHPO_4$, and 10 m*M* glucose). All solutions were aerated with a gas mixture of 95 percent O_2 and 5 percent CO_2. Slices were viewed under a dissecting microscope. Glass microelectrodes were filled with 3*M* KCl and had resistances between 50 and 80 megohms.
7. G. B. Richerson and P. A. Getting, *Soc. Neurosci. Abstr.* **9**, 1163 (1984). Guinea pigs were anesthetized with methoxyflurane and nitrous oxide, paralyzed with gallamine triethiodide, and artifically ventilated. The firing of NTS neurons was recorded extracellularly by means of glass microelectrodes. Fast green was ejected from the microelectrode to mark the recording site for later histological analysis. A cuff electrode was used to monitor phrenic nerve activity. Cycle-triggered histograms [see Cohen in (*1*)] were used to correlate the activity of respiratory neurons in the NTS with the phrenic nerve discharge. The types of respiratory neurons in the guinea pig were similar to those in the cat. The location of the DRG, however, was slightly more medial.
8. M. S. Dekin and P. A. Getting, *Brain Res.* **324**, 180 (1984); *Fed. Proc. Fed. Am. Soc. Exp. Biol. Abstr.* **44**, 1584 (1985).
9. After the brain slices had been exposed to TRH for a long period, a wash with normal Ringer solution for about 1 hour was required before another bursting response could be elicited. During the wash, individual action potentials gradually lost their DAP. The mechanism underlying the apparent desensitization and its removal was not studied in detail. The long periods of time required both to elicit a TRH response and to recover from desensitization prevented the determination of a dose-response relation for TRH in a single neuron.
10. S. H. Thompson and S. J. Smith, *J. Neurophysiol.* **39**, 153 (1976); R. D. Andrew and F. E. Dudek, *Science* **221**, 1050 (1983).
11. H. M. Pinsker, *J. Neurophysiol.* **40**, 527 (1977).
12. F. Strumwasser, *J. Psychiatr. Res.* **8**, 237 (1971).
13. Although graded release of transmitter cannot be ruled out as a possible mechanism for rhythmic bursting, we feel that this is unlikely. A synaptic mechanism would be difficult to reconcile with the polarization (Fig. 2, A and C) and resetting (Fig. 2B) experiments.
14. I. B. Levitan, A. J. Harmon, W. B. Adams, *J. Exp. Biol.* **81**, 131 (1979); W. W. Anderson and D. L. Barker, *J. Exp. Zool.* **216**, 187 (1981).

15. We thank D. Gallagher and J. Richerson for technical assistance and C. Edwards, J. L. Feldman, and D. R. McCrimmon for critical reading of the manuscript. Supported in part by NIH grants HL32336 and NS15350 (to P.A.G.), postdoctoral fellowship MH15172 (to M.S.D.), and predoctoral fellowship GM07337 (to G.B.R.).

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Trans-Activator Gene of Human T-Lymphotropic Virus Type III (HTLV-III)

Abstract. *Human T-lymphotropic virus type III (HTLV-III) encodes a* trans*-acting factor that activates the expression of genes linked to the HTLV-III long terminal repeat. By functional mapping of complementary DNA transcripts of viral messenger RNA's the major functional domain of the gene encoding this factor was localized to a region immediately before the* env *gene of the virus, a region previously thought to be noncoding. This newly identified gene consists of three exons, and its transcription into messenger RNA involves two splicing events bringing together sequences from the 5' part (287 base pairs), middle (268 base pairs), and 3' part (1258 base pairs) of the HTLV-III genome. A similar messenger RNA with a truncated second exon (70 base pairs) does not encode a* trans*-acting function. It is proposed that this second messenger RNA is the transcript of a gene (3'-*orf*) located after the* env *gene. Messenger RNA's were also identified for the* env *and* gag-pol *genes of HTLV-III.*

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Human T-lymphotropic virus type-III (HTLV-III) is etiologically associated with the acquired immune deficiency syndrome (AIDS) (*1, 2*). It belongs to the group of exogenous retroviruses called HTLV whose other members include HTLV-I and HTLV-II. HTLV-I has been etiologically linked to human adult T-cell leukemia-lymphoma (ATLL) (*3, 4*), and HTLV-II, isolated originally from a patient with hairy cell leukemia (*5*), has not yet been linked to any human disease. These viruses share a number of biological and structural properties which include a tropism for $OKT4^+$ lymphocytes (*2, 6*), the ability to induce giant multinucleated cells in vivo and in vitro (*2, 7*), weak immunologic cross-reactivity of some virally encoded proteins (*8*), and distant nucleic acid sequence homologies (*9, 10*). Despite these similarities, HTLV-III differs from HTLV-I and HTLV-II in many aspects of its structure and biology. For example, while infection of human T lymphocytes with HTLV-I or HTLV-II often results in transformation and immortalization (*3, 4*), infection with HTLV-III generally leads to cell death (*1, 2*).

The genomes of HTLV-III and related viruses have been molecularly cloned and sequenced (*10–14*), and five open reading frames (ORF's) have been identified (*11–15*) (Fig. 1). On the basis of the predicted amino acid sequence and alignment with known proteins of other retroviruses, it was postulated that the first, second, and fourth reading frames from the 5' end of the genome constituted the *gag*, *pol*, and *env* genes of HTLV-III. The third open reading frame, termed *sor*, has no correspondence in the