Oxytocin Gene Expression in Rat Uterus

Diana L. Lefebvre, Adel Giaid, Hugh Bennett, Richard Larivière,* Hans H. Zinggt

The neurohypophyseal hormone oxytocin (OT) is the most potent uterotonic agent known and is used to induce labor. Yet, endogenous circulating OT appears not to participate in the induction of labor. As shown here, the finding of OT messenger RNA and peptide in the uterus suggests a solution for this paradox. During gestation, rat uterus OT messenger RNA increased more than 150-fold and, at term, exceeded hypothalamic OT messenger RNA by 70-fold. Thus, during parturition, OT may act primarily as a local mediator and not as a circulating hormone.

Although premature labor is a major cause of early neonatal death, the mechanisms initiating labor are poorly understood. The strongest uterotonic agent known is the hypothalamic nonapeptide oxytocin (OT), a neurohormone that is released into the circulation at the neurohypophysis (*1*, *2*). Yet, circulating OT is not essential for the initiation or maintenance of spontaneous labor (*3*–*8*). Normal parturition has been observed in rats and humans in the absence of circulating OT in cases of experimental or clinical pituitary gland dysfunction (*4*, *5*). Administration of OT antiserum suppresses lactation but fails to affect parturition (*6*). Moreover, there is no consensus whether or not an increase in the amount of circulating OT precedes the actual onset of labor (*7*, *8*). As a result, it has been difficult but seemingly unavoidable to accept the notion that OT, an endogenous product with a strong pharmacological potential, is devoid of any physiological role in parturition. We investigated the possibility that OT could exert its action by means of a pathway that differs from the classical hormonal pathway.

Whole uteri were dissected from nonpregnant, diestrous female rats and from rats at 14, 18, and 21 days of gestation and at 1, 2, and 9 days of lactation. RNA was extracted and analyzed by Northern (RNA) blot with an oligonucleotide probe complementary to OT mRNA (*9*). The amount of OT mRNA remained small in the uteri of nonpregnant rats but increased >150-fold during pregnancy (Fig. 1, A and C). The rise in OT uterine mRNA was very rapidly reversed after delivery (Fig. 1C). By contrast, hypothalamic OT mRNA increases less than threefold during pregnancy and remains elevated during lactation (*9*). With the relative intensity of the hybridization signals and the amount of total RNA per organ taken into consideration, the uterus of a 21-day-pregnant rat contains about 70 times more OT mRNA than the hypothalamus, the main site of neuronal OT gene expression (*10*).

The OT mRNA detected in pregnant rat uterus was smaller than the corresponding transcript in the rat hypothalamus. However, after removal of polyadenylate [poly(A)] tails, hypothalamic and uterine OT mRNA were of equal size (Fig. 1B). This indicates that the size difference detected between these two mRNA species is a result of differences in poly(A) tail lengths. Polymerase chain reaction (PCR) amplification was used to establish whether any other differences existed between uterine and hypothalamic OT transcripts. Three different pairs of exon-specific primers were used to amplify uterine and hypothalamic cDNAs. For each pair of primers, amplification of either uterine or hypothalamic cDNA generated products of identical size (Fig. 2). In each case, the size of the products obtained corresponded to the size predicted from the structure of the rat OT gene (*11*).

To establish whether OT peptide existed in uterine tissue, we assayed the amounts of uterine immunoreactive OT (ir-OT) by radioimmunoassay (RIA) (*12*). Concomitant with the rise in OT mRNA, there was a 35-fold increase in ir-OT during pregnancy. Uterine extracts of 21-day-pregnant rats contained 2.05 ± 0.24 ng of ir-OT per gram of wet weight (mean ± SE; $n = 4$), whereas uterine extracts of nonpregnant, diestrous rats contained only 0.06 ± 0.01 ng of ir-OT per gram of wet weight ($n = 4$). Uterine ir-OT was further characterized by high-performance liquid chromatography (HPLC) analysis, in which the ir-OT present in the uterus from pregnant rats consisted of two peaks of immunoreactivity. The first peak (54% of total immunoreactivity) co-eluted with synthetic, COOH-terminally amidated OT nonapeptide (Fig. 3). Molecular sizing of the second HPLC peak indicated that it consisted of material with an apparent relative molecular weight (M_r) of 11,000 (*13*). This material was efficiently converted to material co-eluting with the nonapeptide OT by three cycles of freezing and thawing. These data suggest that the material of larger molecular size consists of a noncovalent association of OT

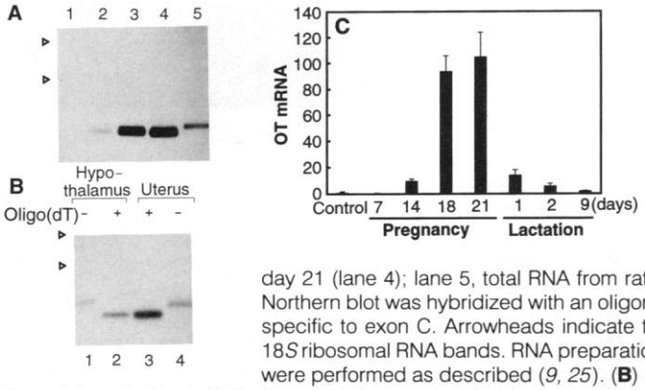

Fig. 1. (**A**) Northern blot analysis of OT mRNA in rat uterus at different stages of pregnancy. Lane 1, total uterine RNA (10 μg) from a nonpregnant, diestrus female rat; lanes 2 through 4, total uterine RNA (10 μg) at gestational day 14 (lane 2), day 18 (lane 3), and day 21 (lane 4); lane 5, total RNA from rat hypothalamus (50 μg). The Northern blot was hybridized with an oligonucleotide probe (OT-27) (*9*) specific to exon C. Arrowheads indicate the positions of the 28*S* and 18*S* ribosomal RNA bands. RNA preparation and Northern blot analysis were performed as described (*9*, *25*). (**B**) Effect of poly(A) tail removal. Lane 1, hypothalamic RNA (50 μg) with intact poly(A) tail; lane 2, deadenylated hypothalamic RNA (50 μg); lane 3, deadenylated uterine RNA (10 μg) at gestational day 18; lane 4, uterine RNA (10 μg) at gestational day 18 with intact poly(A) tail. RNA was deadenylated as described (*9*, *25*). The lanes labeled (−) contained RNA samples treated with ribonuclease H (RNase H) without prior hybridization to oligo(dT)$_{12-18}$. The lanes labeled (+) indicate RNA samples hybridized with oligo(dT)$_{12-18}$ before RNase H treatment. Oligonucleotide OT-27 was used as a probe. Arrowheads indicate the positions of the 28*S* and 18*S* ribosomal RNA bands. (**C**) Quantitative analysis of uterine OT mRNA accumulation during gestation and lactation. Northern blots were scanned densitometrically (*26*). To control for equal loading of wells, we compared total amounts of polyadenylated mRNA present in different lanes by rehybridizing the blots with labeled oligo(dT) (*25*). Maximum variability within a given blot was ±25% of the mean. Each bar represents the mean ± SE of at least three independent experiments.

D. L. Lefebvre, R. Larivière, H. H. Zingg, Laboratory of Molecular Endocrinology, Royal Victoria Hospital, McGill University, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1.
A. Giaid, Department of Pathology, Montreal General Hospital, McGill University, Montreal, Quebec, Canada H3A 1A1.
H. Bennett, Endocrine Laboratory, Royal Victoria Hospital, McGill University, Montreal, Quebec, Canada H3A 1A1.

*Present address: Institut de Recherches Cliniques de Montréal, 110 avenue des Pins ouest, Montréal, Québec, Canada H2W 1R7.
†To whom correspondence should be addressed.

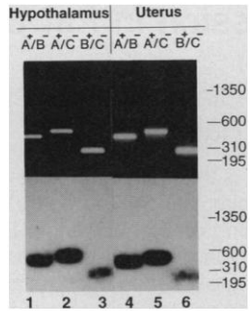

Fig. 2. Reverse transcription and PCR analysis of hypothalamic and uterine OT mRNA. After reverse transcription of RNA, cDNA was amplified by the primer pairs A^+/B^-; A^+/C^-; or B^+/C^- as described (*15*). A^+, exon A–specific sense-strand primer corresponding to sequences +3 to +26 of the rat OT gene [numbering is based on the published sequence of the rat OT gene (*11*), taking the cap site as +1]. B^-, exon B–specific antisense-strand primer (+559 to +584); B^+, exon B–specific sense-strand primer (+416 to +434); and C^-, exon C–specific antisense-strand primer (+838 to +864). Upper panel, agarose gel analysis of amplification products. First-strand cDNA was synthesized by reverse transcription of RNA derived from rat hypothalamus (lanes 1 to 3) or uterus (lanes 4 to 6). Lower panel, Southern (DNA) blot analysis of PCR-amplified products. A Southern blot of the agarose gel shown in the upper panel was hybridized with an antisense exon B–specific probe complementary to sequences +481 to +506 of the rat OT gene. Size markers are indicated in base pairs at the right of the figure.

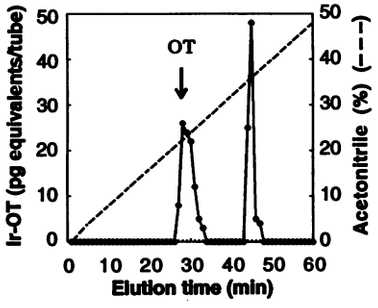

Fig. 3. HPLC analysis of immunoreactive OT extracted from the uterus of a 21-day-pregnant rat. Fractions were eluted with a linear gradient from 0 to 48% of acetonitrile in H_2O that contained 0.1% TFA (*27*). Arrow indicates the elution position of synthetic OT nonapeptide.

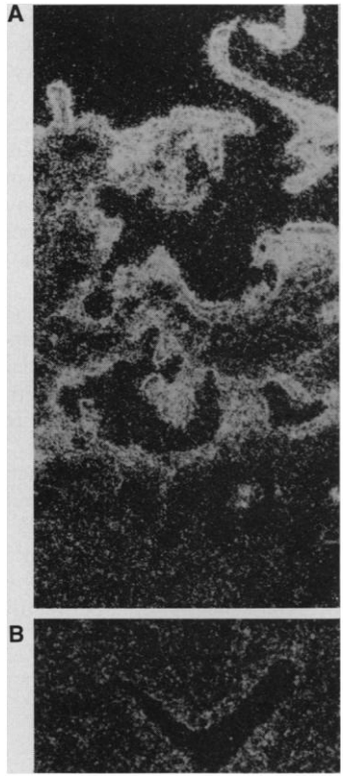

Fig. 4. In situ hybridization of a ^{35}S-labeled oligonucleotide probe (OT-27) to sections of a rat uterus. (**A**) Uterus of 21-day-pregnant rat. (**B**) Uterus of nonpregnant, diestrous female rat. Exposure time = 1 week (*28*).

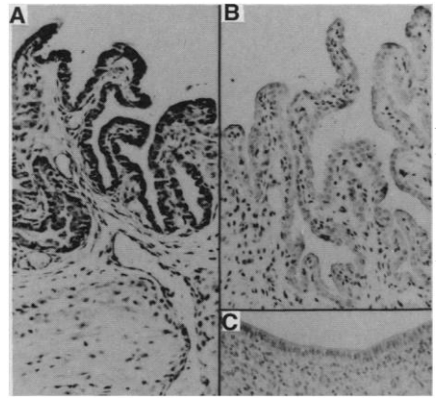

Fig. 5. Immunocytochemical staining of sections of rat uterus with an antibody to OT. (**A**) Uterus of 21-day-pregnant rat. (**B**) Same as (A), but nonimmune serum was used instead of primary antiserum. (**C**) Uterus, stained with immune serum, of nonpregnant, diestrous female rat. Sections were immunostained with an avidin-biotin complex (*29*).

and a molecule with an M_r of 10,000, most likely the OT-associated neurophysin (NpI). Similar OT-NpI complexes have been observed in extracts of the hypothalamus, ovary (*14*), and placenta (*15*).

To determine the cellular site of OT gene expression and OT peptide accumulation, we used in situ hybridization and immunocytochemistry to analyze sections of uteri from nonpregnant, diestrous rats and from rats at 21 days of gestation. The results confirmed that the OT gene is highly expressed in uteri from pregnant rats. In the uterus of a rat pregnant for 21 days, in situ hybridization revealed very dense grains over the endometrium (Fig. 4), and immunocytochemistry indicated that most of the ir-OT was present in the epithelial cell layer of the endometrium (Fig. 5A). In nonpregnant rats, these same cells displayed very little ir-OT (Fig. 5C).

The demonstration that the rat uterus itself is the major site of OT gene expression during the later stages of pregnancy expands our understanding of the role of OT in the initiation and maintenance of parturition. Our findings resolve the apparent paradox between the powerful actions of exogenously administered OT and the lack of unequivocal evidence for a role of circulating OT. Our data suggest that, with respect to parturition, OT acts as a paracrine or autocrine mediator rather than as a circulating hormone. This view is compatible with the findings that intravenous injections of OT antagonists, but not OT antibodies, suppress spontaneous uterine contractions (*6*, *16*). The identification in the endometrial epithelium of OT receptors that are linked to prostaglandin production (*17*) supports autocrine action of OT on epithelial prostaglandin synthesis. Moreover, OT produced in the endometrium may reach the myometrium via stromal veins and interact with myometrial OT receptors. Although our concept now appears as an obvious solution to this paradox, expression of the OT gene in the uterus may not have been noticed before because the gene is expressed only for a short time. Nevertheless, the presence of neurophysin-like immunoreactivity in the uterus has been noted (*18*).

Uterine OT binding sites undergo a dramatic up-regulation before parturition (*19*). Thus, it appears that the uterus contains an intrinsic OT system in which both ligand and receptor are subject to strict regulation. To what extent the two events are causally related is unclear, but several examples of ligand-induced receptor up-regulation are known (*20*). The rise in uterine OT gene expression, in concert with the rise in uterine OT receptors, may represent the trigger for parturition. Thus, dysregulation of uterine OT gene expression may be an underlying cause of premature or delayed labor.

Note added in proof: OT gene expression has also been observed in the human uterus (*30*).

REFERENCES AND NOTES

1. A. R. Fuchs and F. Fuchs, *Br. J. Obstet. Gynaecol.* **91**, 948 (1984).
2. A. J. Silverman, *Annu. Rev. Neurosci.* **6**, 357 (1983).
3. A. Giraldi, A. S. Enevoldsen, G. Wagner, *Dan. Med. Bull.* **37**, 377 (1990).
4. C. C. Gale and S. M. McCann, *J. Endocrinol.* **22**, 107 (1961).
5. J. P. Phelan, A. T. Guay, C. Newman, *Am. J. Obstet. Gynecol.* **130**, 365 (1978); T. Chard, *Am. J. Perinatol.* **6**, 145 (1989); in *Clinical Neuroendocrinology*, L. Martini and G. M. Besser, Eds. (Academic Press, New York, 1977), pp. 569–583.
6. P. Kumaresan, M. Subramanian, P. B. Anadarangan, *J. Endocrinol. Invest.* **2**, 65 (1979).
7. S. M. Sellers *et al.*, *Br. J. Obstet. Gynaecol.* **88**, 725 (1981).

8. K. DeGeest, M. Thiery, G. Piron-Possuyt, R. Driessche, *J. Perinat. Med.* **13**, 3 (1985).
9. H. H. Zingg and D. L. Lefebvre, *Mol. Brain Res.* **4**, 1 (1988).
10. To determine OT mRNA abundance in uteri and hypothalami of 21-day-pregnant rats, we densitometrically scanned autoradiograms obtained from nine independent blots as described [H. H. Zingg, G. Almazan, D. L. Lefebvre, *J. Biol. Chem.* **261**, 12956 (1986)]. On each autoradiogram, band intensities were normalized with respect to the amount of RNA loaded per lane (5 to 50 μg), and the ratio of uterine versus hypothalamic OT mRNA abundance was determined. From these measurements, the uterus of a 21-day-pregnant rat contained 2.3 ± 0.6 times more OT mRNA per microgram of total RNA than the hypothalamus of a rat at the same stage of pregnancy (mean ± SE; $n = 9$). The amount of total RNA extracted from one uterus or one hypothalamus was also determined. One whole uterus of a 21-day-pregnant rat was found to have 30 times more total RNA than a hypothalamus (6502 ± 1659 μg in the uterus versus 219.6 ± 24.1 μg in the hypothalamus; means ± SE; $n = 9$). Taken together, these results indicate that at day 21 of pregnancy, the whole rat uterus contains about 70 times more OT mRNA than the hypothalamus. We have also recently identified the presence of OT mRNA in rat placenta (*15*). However, placental OT mRNA levels are over 10 times lower than hypothalamic OT mRNA levels and over 20 times lower than uterine OT mRNA levels (*15, 21*).
11. R. Ivell and D. Richter, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2006 (1984).
12. The radioimmunoassay was performed as described (*15*) with a rabbit antibody to OT (Cal Biochem), ^{125}I-labeled OT, and synthetic standards (Peninsula Laboratories, Belmont, CA). The antibody was used at a final dilution of 1:7500. Specific binding of tracer in the absence of added hormone ranged from 20 to 25%. Assay sensitivity was 2 pg per tube. The antiserum used showed no measurable cross-reactivity with arginine vasopressin and had a greater than 100-fold lower affinity for vasotocin or lysine vasopressin [R. C. Gorewit, *Proc. Soc. Exp. Biol. Med.* **160**, 80 (1979)].
13. Molecular sizing was performed as described. HPLC fractions eluting at 44 to 48 min (Fig. 3, second peak) were pooled, frozen, dried by evaporation, and resuspended in 0.1% trifluoroacetic acid (TFA). Portions were either analyzed directly or subjected to three cycles of freezing and thawing. Samples were loaded on two I125 gel filtration columns (Waters) connected in series and eluted with 40% acetonitrile in H_2O that contained 0.1% TFA at a flow rate of 1 ml/min. Fractions were collected, lyophilized, and assayed for ir-OT by RIA.
14. L. Shukovski, J. K. Findlay, A. I. Smith, *J. Endocrinol.* **128**, 305 (1991).
15. D. L. Lefebvre, A. Giaid, H. H. Zingg, *Endocrinology* **130**, 1185 (1992).
16. M. Akerlund *et al.*, *Brit. J. Obstet. Gynaecol.* **94**, 1040 (1987); L. Wilson, Jr., M. T. Parson, G. Flouret, *Am. J. Obstet. Gynecol.* **163**, 1875 (1990).
17. V. J. Ayad, S. E. F. Guldenaar, D. C. Whates, *J. Endocrinol.* **128**, 187 (1991); A. R. Fuchs, F. Fuchs, P. Husslein, M. S. Soloff, M. J. Fernström, *Science* **215**, 1396 (1982); J. M. Wallace *et al.*, *J. Endocrinol.* **128**, 253 (1991).
18. F. F. Ciarochi, A. G. Robinson, J. G. Verbalis, S. M. Seif, E. A. Zimmerman, *Peptides (New York)* **6**, 903 (1985).
19. M. S. Soloff, M. Alexandrova, M. J. Fernstrom, *Science* **204**, 1313 (1979).
20. I. W. Barash, W. Cromlish, B. I. Posner, *Endocrinology* **122**, 1151 (1988); S. Collins *et al.*, *J. Biol. Chem.* **265**, 19330 (1990); H. de Thé, M. Vivanco-Ruiz, P. Tiollais, H. Stunnenberg, A. Dejean, *Nature* **343**, 177 (1990).
21. D. L. Lefebvre, A. Giaid, H. Bennett, R. Larivière, H. H. Zingg, unpublished results.
22. M. Goedert, *EMBO J.* **6**, 3627 (1987).
23. S. M. Hsu, L. Raine, H. Fanger, *J. Histochem. Cytochem.* **29**, 577 (1981).
24. R. C. Graham and M. J. Karnovsky, *ibid.* **14**, 291 (1966).
25. D. L. Lefebvre and H. H. Zingg, *Mol. Endocrinol.* **5**, 645 (1991).
26. H. H. Zingg, R. H. Goodman, J. F. Habener, *Endocrinology* **115**, 90 (1984).
27. Reversed-phase HPLC was performed with a model 510 liquid chromatography system and a 3.9 mm by 30 cm μBondapak C18 column (Waters). Samples were loaded onto the column in 0.1% TFA and eluted at a flow rate of 1.5 ml/min over 60 min with a linear gradient of acetonitrile and TFA. Fractions were collected for 1 min, lyophilized, and reconstituted in 1 ml of a solution that contained 0.01 N acetic acid and 0.1% bovine serum albumin. Portions (100 μl) were assayed for OT peptide content by RIA (*12*).
28. For in situ hybridization, sections were treated with proteinase K, fixed in 4% paraformaldehyde, washed with 2× sodium citrate buffer (SSC) (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate) and incubated with prehybridization buffer [50% formamide, SSC (5×), 50 mM sodium phosphate buffer (pH 7.0), salmon sperm DNA (250 μg/ml), 0.1% Ficoll (Pharmacia), 0.1% polyvinyl pyrrolidone, 0.1% bovine serum albumin, heparin (200 μg/ml), and 0.1% SDS] for 2 hours at room temperature (*22*). Sections were then incubated with the solution above containing 1×10^6 cpm per section of ^{35}S-labeled probe for 16 hours at 42°C. Washes were performed in 2× SSC, 1× SSC, and 0.5× SSC for 1 hour each at room temperature and in 0.5× SSC at 42°C for 30 min. Sections were washed in increasing concentrations of ethanol (70%, 90%, and 100%, for 10 min each), air-dried, dipped in Ilford K-5 emulsion, and exposed for 1 to 2 weeks at 4°C. Sections were counterstained in hematoxylin.
29. For immunocytochemistry, paraffin was removed from sections. After permeabilization with Triton X-100 (0.2%), sections were incubated overnight with rabbit antiserum to OT (Peninsula Laboratories.) at a dilution of 1/1000 at 4°C. The sections were immunostained by the avidin-biotin complex method (*23*) with biotinylated goat antibody–rabbit immunoglobulin G and an avidin-biotin complex (Vector Labs). The peroxidase reaction was revealed by the diaminobenzidine method (*24*). Sections were hematoxylin counterstained. As controls of specificity, some sections were incubated with nonimmune serum instead of the primary antiserum or one of the other staining procedures was omitted.
30. R. Chibbar, F. Miller, B. F. Mitchell, *Proceedings of the 39th Annual Meeting of the Society for Gynecologic Investigation*, San Antonio, TX, 18 March 1992, p. 251.
31. We thank J. Neculcea and C. Charbonneau for their expert assistance. Supported by grants from the Medical Research Council of Canada. D.L.L. is supported by a studentship from the Medical Research Council of Canada. A.G. is a recipient of a scholarship from the Heart and Stroke Foundation of Canada.

13 January 1992; accepted 17 April 1992

Early Stages of Motor Neuron Differentiation Revealed by Expression of Homeobox Gene *Islet-1*

Johan Ericson, Stefan Thor, Thomas Edlund, Thomas M. Jessell,* Toshiya Yamada

Motor neurons in the embryonic chick spinal cord express a homeobox gene, *Islet-1*, soon after their final mitotic division and before the appearance of other differentiated motor neuron properties. The expression of Islet-1 by neural cells is regulated by inductive signals from the floor plate and notochord. These results establish Islet-1 as the earliest marker of developing motor neurons. The molecular nature of the Islet-1 protein suggests that it may be involved in the establishment of motor neuron fate.

During embryonic development the vertebrate nervous system generates a diverse array of neuronal cell types, which are characterized by their position of origin, axonal projections, and synaptic connections. One of the neuronal types for which the processes of axonal pathfinding and synapse formation have been documented in most detail is the spinal motor neuron (*1*). In contrast, the events that control the generation of motor neurons remain largely obscure. In chick embryos, spinal motor neurons derive from progenitor cells in the neural tube that give rise also to other neurons and to glial cells (*2*). The commitment of neural progenitors to a motor neuron fate appears to be regulated, in part, by signals that derive from axial mesodermal cells of the notochord and floor plate cells at the ventral midline of the neural tube (*3–5*). Insight into the molecular mechanisms involved in the generation of motor neurons requires the identification of genes that are expressed at the initial stages of motor neuron differentiation. Here we report that embryonic chick motor neurons express the homeobox gene *Islet-1* (6), a member of the subfamily of homeobox genes (*7*) that contain cysteine-rich *Lin-11*, *Isl-1 Mec-3* (LIM) domains (6, *8*, 9). Other members of this family include *Lin-11* and *Mec-3*, which have been shown to regulate cell fate in *Caenorhabditis elegans* (*8*, 9).

Islet-1 was originally identified as a protein that binds to enhancer elements in the rat insulin gene (6). In the adult rat, Islet-1

J. Ericson, S. Thor, T. Edlund, Department of Microbiology, Umeå University, Umeå S-90187, Sweden.
T. M. Jessell and T. Yamada, Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Center for Neurobiology and Behavior, Hammer Health Sciences Center, Columbia University, New York, NY 10032.

*To whom correspondence should be addressed.