Although the ability of MetRS to repress the switched mRNA leader is clearly demonstrated, we believe that the repression can still be amplified. The possibility remains that nucleotides not present in the mRNA leader participate in $tRNA^{Met}$ identity. It is also possible that nucleotides in the mRNA leader specifically prevent recognition by MetRS. The present genetic system permits an easy selection for mutants acting in cis that are more sensitive to repression by MetRS. These selections could yield nucleotide changes that would give us information about how MetRS recognizes its tRNA isoacceptors.

Our results indicate that an excess of MetRS somewhat represses wild-type *thrS* expression. This repression is seen in the decrease of expression of *thrS-lacZ* fusions (Table 1) and on protein immunoblots (Fig. 2B), where the band corresponding to the endogenous ThrRS is weaker in the presence of pNAV7 than in the presence of pUC12. Because this effect is lost with changes other than the CGU → CAU change, it appears that the wild-type *thrS* mRNA leader is weakly but specifically recognized by MetRS. Thus, the *thrS* mRNA leader may be recognized with lower affinity by MetRS and possibly other synthetases. The biological relevance of this cross talk remains to be determined.

The present data, coupled with genetic (*10*) and biochemical (*5*) analyses of the *thrS* mRNA leader–ThrRS complex, show that the synthetase recognizes both RNAs by a similar mechanism. Because the three-dimensional structure of the synthetase-tRNA interaction is under analysis, this system should provide a mechanism for studying the three-dimensional structure of an mRNA leader complexed with a translational repressor. The ability to change the regulatory specificity of *thrS* by changing the mRNA leader may make three-dimensional analysis more facile. The specificity of the mRNA leader could be changed to an *E. coli* synthetase for which the three-dimensional interaction with its cognate tRNA is available (*17*). Also, the ability to change the regulation specificity of *thrS* provides the best indication of mimicry between a regulatory site on an mRNA and another site on a stable RNA, to which a single control protein binds to both in order to perform a specific cellular activity.

REFERENCES AND NOTES

1. P. Lestienne, J. A. Plumbridge, M. Grunberg-Manago, S. Blanquet, *J. Biol. Chem.* **259**, 5232 (1984).
2. M. Springer *et al.*, *J. Mol. Biol.* **185**, 93 (1985).
3. M. Springer, M. Graffe, J. S. Butler, M. Grunberg-Manago, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4384 (1986).
4. H. Moine *et al.*, *ibid.* **85**, 7892 (1988).
5. H. Moine *et al.*, *J. Mol. Biol.* **216**, 299 (1990).
6. L. Lindahl and J. M. Zengel, *Annu. Rev. Genet.* **20**, 297 (1986).
7. J. Normanly and J. Abelson, *Annu. Rev. Biochem.* **58**, 1029 (1989).
8. M. Springer *et al.*, unpublished data.
9. Y. Komine, T. Adachi, H. Inokuchi, H. Ozeki, *J. Mol. Biol.* **212**, 579 (1990).
10. M. Springer, M. Graffe, J. Dondon, M. Grunberg-Manago, *EMBO J.* **8**, 2417 (1989).
11. L. H. Schulman and H. Pelka, *Nucleic Acids Res.* **18**, 285 (1990).
12. W. H. McClain and H. B. Nicholas, Jr., *J. Mol. Biol.* **194**, 635 (1987).
13. P. H. Hirel *et al.*, *Biochimie* **70**, 773 (1988).
14. We made the different bacteriophages (with the exception of λMΔ20-10-XII-25–ML2) used in Table 1 by cloning the Hind III–Eco RI insert of M13mp8Δ20-10-XII-25 (or its derivatives) between the left arm of λSKS107 (to its Hind III site) and the right arm of λgt4 from its Eco RI site, as described (*2*). This cloning reconstitutes the *thrS-lacZ* hybrid in its integrity. The phage λSKS107 is *imm21* and *nin5*, and we made it by cloning the Hind III–Sst I fragment of pSKS107 [S. K. Shapiro, J. Chou, F. V. Richaud, M. J. Casadaban, *Gene* **25**, 71 (1983)] that carries the 5′ terminal part of *lacZ* without a translation initiation site between the left arm of λnav8-5 (to its Sst I site) and the right arm of λNM540 (from its Hind III site) as described (*2*). M13mp8Δ20-10-XII-25 is an M13mp8 derivative carrying an insert extending from the Pst I site 1194 bp in front of *thrS* to a reconstituted Hind III site 53 bp on the 3′ side of the ATG of *thrS*. The insert also carries the XII-25 mutation (Fig. 1) that was introduced with oligonucleotide site-directed mutagenesis, as was done for all the other mutations of the *thrS* mRNA leader. The phage λMΔ20-10-XII-25–ML2 was selected from a λMΔ20-10-XII-25 lysogen of *E. coli* IBPC5421 as conferring a Lac^+ phenotype (*3, 8*). Strain IBPC5421 was lysogenized with a single copy of the recombinant phages. The F′*lacI*^{q}Tn*10* episome was introduced in the single lysogens by conjugation. The male lysogens were then transformed with pUC12, pUB4 (*2*), or pNAV7 (*13*). The strains were grown in MOPS-glucose medium, supplemented with all amino acids (*18*), tetracycline at 10 μg/ml, and isopropyl-1-thio-β-D-galactoside (IPTG) at 10^{-3} M. We added ampicillin at a concentration of 100 μg/ml at 1-hour intervals during growth to select for the presence of the plasmids.
15. High expression from the fusions mutated in the mRNA leader equivalent of the anticodon is due to the inability of the endogenous ThrRS to repress β-galactosidase synthesis from the *thrS-lacZ* fusions.
16. Low production of ThrRS by pUB4 is probably due to the repression of *thrS* expression from the *lac* promoter in the presence of glucose or the presence of the F′*lacI*^{q}Tn*10* episome. The episome somewhat represses *thrS* expression even in the presence of IPTG. The F′*lacI*^{q}Tn*10* is necessary because pNAV7 is lethal to the cells when MetRS is expressed from both the *lac* and its own promoter but not when expressed only from the latter.
17. M. A. Rould, J. J. Perona, D. Söll, T. A. Steitz, *Science* **246**, 1135 (1989).
18. F. C. Neidhardt, P. L. Bloch, S. Pedersen, S. Reeh, *J. Bacteriol.* **129**, 378 (1977).
19. J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972).
20. J. S. Butler, M. Springer, J. Dondon, M. Grunberg-Manago, *J. Bacteriol.* **165**, 198 (1986).

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Reversal of Diabetes Insipidus in Brattleboro Rats: Intrahypothalamic Injection of Vasopressin mRNA

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Messenger RNAs occur within the axons of magnocellular hypothalamic neurons known to secrete oxytocin and vasopressin. In Brattleboro rats, which have a genetic mutation that renders them incapable of vasopressin expression and secretion and thus causes diabetes insipidus, injection into the hypothalamus of purified mRNAs from normal rat hypothalami or of synthetic copies of the vasopressin mRNA leads to selective uptake, retrograde transport, and expression of vasopressin exclusively in the magnocellular neurons. Temporary reversal of their diabetes insipidus (for up to 5 days) can be observed within hours of the injection. Intra-axonal mRNAs may represent an additional category of chemical signals for neurons.

THE MRNAS ENCODING THE HORmones arginine vasopressin (AVP) and oxytocin (OT) are present in axons of the hypothalamoneurohypophysial tract (*1–3*). Oxytocin mRNA has been localized by in situ hybridization and electron microscopy in large granular vesicles of axonal varicosities in the lateral hypothalamus, the median eminence, and the posterior pituitary (*2*). Furthermore, the amounts of OT mRNA in the hypothalamoneurohypophysial tract change with functional demand on the neurons (*4*).

The presence of a neuropeptide mRNA in vesicles and evidence for its axonal transport (*2*) suggest that this mRNA could provide an intraneuronal or intercellular signal. Because of the low activity of brain ribonuclease (RNase) (*5*), we were able to investigate the metabolic fate of exogenous AVP mRNA injected into the axonal fields of these magnocellular neurons. To do so, we used the Brattleboro rat, which has a single base deletion in exon B of the propresso-

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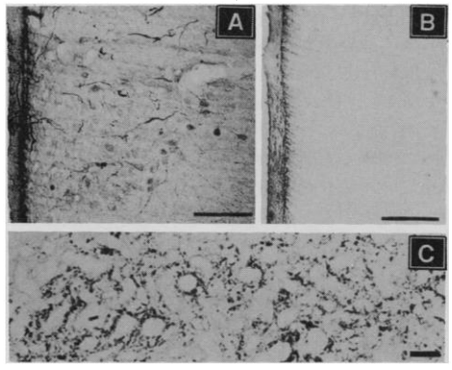

Fig. 1. (**A**) Vasopressin immunostaining of the PVN of a Brattleboro rat, 2 hours after unilateral injection of 50 ng of synthetic sense RNA coding for AVP. Rats were anesthetized with chloral hydrate (5 mg per 100 g of body weight) and killed by cardiac perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at 4°C. Hypothalami were removed and sectioned on a Vibratome (Oxford Instruments) into serial frontal sections (50 μm thick). Sections were washed in PBS and immunostained by the peroxidase-antiperoxidase method (*16*) with a rabbit anti–AVP-serum (Chemicon), which had been preabsorbed with synthetic OT (Sigma). Immunoprecipitates were made visible with diaminobenzidine and hydrogen peroxide. For control purposes, some sections were stained with antisera to ACTH and to β-endorphin (both antisera obtained from Chemicon). Scale bar = 100 μm. (**B**) Vibratome section of the PVN of a Brattleboro rat injected with isotonic saline, devoid of vasopressin immunostaining. Scale bar = 100 μm. (**C**) The posterior lobe of the pituitary of a Brattleboro rat injected with total hypothalamic RNA. Scale bar = 10 μm.

physin gene (*6*). This mutation prevents the translation of AVP in homozygotes and leads to chronic diabetes insipidus.

In preliminary experiments, Brattleboro rats received injections into the lateral hypothalamus (7) of 5 μg of total cytoplasmic RNA prepared from hypothalami of normal Sprague-Dawley rats. Northern (RNA) blot analysis of this total mRNA pool confirmed the presence of AVP mRNA as expected (*8*). We found that 18 hours after injection with this preparation Brattleboro rats exhibited cytoplasmic AVP immunoreactivity within neurons of the magnocellular hypothalamic nuclei (Fig. 1 and Table 1).

In subsequent experiments, Brattleboro rats were injected with 50 ng of a synthetic sense-strand ^{35}S-labeled AVP RNA, with a ^{35}S-labeled AVP antisense RNA (*9*), or with unlabeled AVP RNA. In confirmation of the immunoreactivity changes observed after the total mRNA injections, magnocellular perikarya of the supraoptic (SON) and paraventricular nuclei (PVN) selectively showed incorporation of radioactivity within 2 hours of injection with sense-strand RNA. In four repetitions of this experiment (each group containing three rats) about 25% (±7%) of the magnocellular neurons in the SON showed ^{35}S-labeling, as did approximately 10% (±4%) of the neurons in the PVN (Fig. 2A). The density of silver grains per cell appeared to be higher in the SON than in the PVN. After these sense-strand injections, accumulation of radioactivity was also observed in the median eminence and the posterior lobe (Fig. 2B), regions known to contain the axons of the SON and PVN neurons. Rats injected with comparable amounts of the labeled antisense AVP RNA showed radioactivity only in the ventricles and blood vessels, whereas hypothalamic perikarya did not accumulate radioactivity. The median eminence and the posterior lobe were also unlabeled in these controls (Fig. 2C).

In contrast, 2 hours after injection of synthetic unlabeled AVP RNA (the earliest time examined), immunoreactive AVP was observed in a fraction of the magnocellular neurons of the PVN (Fig. 1) and the SON; immunostaining was more pronounced in the PVN on the ipsilateral side of the injection, and AVP-immunoreactive perikarya were completely absent in the SON contralateral to the injection site. Furthermore, AVP-immunostained nerve endings in the posterior lobe appeared to be less densely distributed than structures visible in autoradiograms obtained from this organ after injection of [^{35}S]AVP RNA into the hypothalamoneurohypophysial tract. This may indicate that the fraction of nerve terminals in the posterior lobe that contained radioactive RNA was greater than the number of nerve terminals containing immunoreactive, translated AVP.

To determine whether the injection of synthetic AVP RNA or total hypothalamic RNA resulted in functional signals, we took urine samples from Brattleboro rats before and after the intracerebral injections. In normal rats urine osmolarity is well above 1200 mosmol. The urinary osmolarity of untreated homozygous Brattleboro rats, however, was in all cases below 200 mosmol throughout the time observed. Brattleboro rats treated with total cytoplasmic mRNA as well as with synthetic RNA showed increased urine osmolarity within 2 hours after the injection, declining to control levels after 5 days (Fig. 3), whereas ones injected with isotonic saline, antisense AVP RNA, or cytoplasmic mRNA taken from normal rat hindbrain did not show this effect. Moreover, the ability of microinjected hypothalamic mRNA or synthetic AVP RNA to raise urine osmolarity was lost when these materials had been digested with RNase (*10*).

Radioimmunoassays (RIAs) of homogenized hypothalamic nuclei (*11*) showed clear increases in AVP levels upon injections of synthetic AVP RNA (Table 1). Animals injected with 50 ng of synthetic full-length sense AVP RNA (*9*) had a distribution of

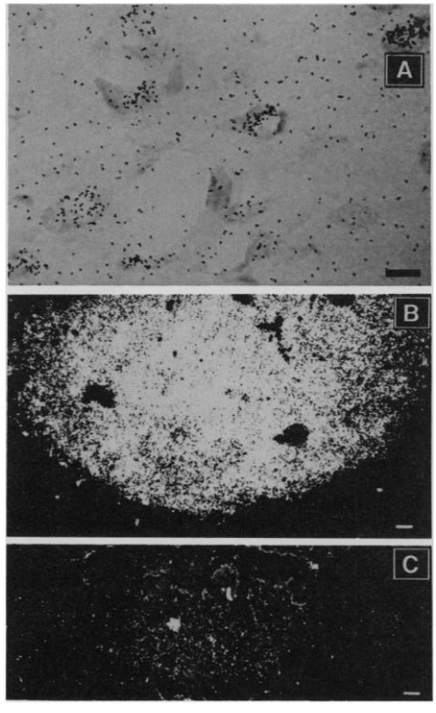

Fig. 2. Autoradiograms of PVN and posterior lobe of a Brattleboro rat injected with [^{35}S]AVP RNA. Hypothalami and pituitaries of animals, perfused with 4% paraformaldehyde 2 hours after injections with the labeled probes, were rapidly frozen and sectioned into 10-μm cryostat sections. These sections were processed for autoradiography with Ilford G5 emulsion. (**A**) Magnocellular perikarya in the PVN. (**B**) Dark-field micrograph of the posterior lobe. (**C**) Dark-field micrograph of the posterior lobe of a rat injected with the ^{35}S-labeled antisense probe. Scale bar = 10 μm.

Table 1. Amounts of immunoreactive vasopressin measured by RIA (*9*) in homogenates of hypothalamic regions from Brattleboro rats 18 hours after unilateral injection with total hypothalamic RNA. Vasopressin concentration was below detection limit in animals that had been injected with isotonic saline.

Location	AVP per milligram of protein (pg)			
	PVN	SON	Median eminence	Posterior lobe
Injected brain side	410 (± 75)	173 (± 54)	133 (± 26)	340 (± 87)
Contralateral side	120 (± 25)	67 (± 12)		

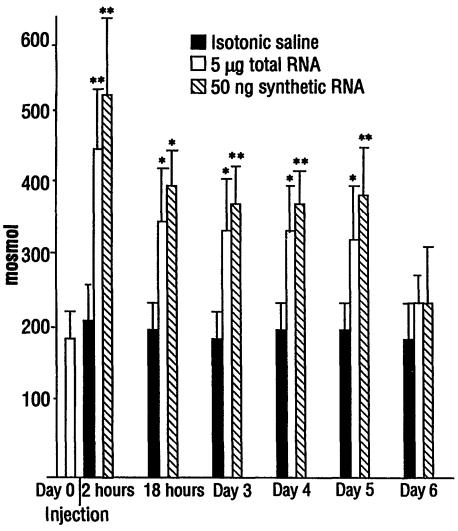

Fig. 3. Changes in urine osmolarity of Brattleboro rats after intrahypothalamic injections with total hypothalamic RNA and synthetic vasopressin RNA. Male Brattleboro rats, which were homozygous for diabetes insipidus and weighed between 250 and 260 g, were singly housed in metabolic cages. Water consumption and urine output were measured daily. Each rat received a single unilateral injection of one of the RNA preparations (dissolved in isotonic saline) into the medial portion of the hypothalamoneurohypophysial tract (7). Urine osmolarity was measured with an osmometer (Advanced Instruments, Needham Heights, Massachusetts). Statistical significance of data was evaluated by one-way analysis of variance and Scheffe *F*–post-hoc test. $^{**}P < 0.005$, $^{*}P < 0.05$.

AVP immunostaining similar to the distribution observed after injection of total RNA. In contrast to the changes seen in the SON and PVN for AVP expression, other peptides with mRNAs that would be expected to be present in the total hypothalamic mRNA were not seen in the magnocellular nuclei. Specifically, no immunostaining for adrenocorticotropic hormone (ACTH) or β-endorphin was found within the hypothalamoneurohypophysial system. The mRNAs for these two peptides are abundant in the hypothalamus but not present in magnocellular neurons (*12*). After we injected synthetic AVP RNA into brain regions other than the hypothalamoneurohypophysial tract, we could detect no AVP immunostaining in the Brattleboro rat brain and no change in urine osmolarity (*13*).

The increase in urine osmolarity and the elevated tissue AVP levels observed after injections of total cytoplasmic RNA extracts or AVP RNA indicate that functional AVP was synthesized and secreted, resulting in a partial temporary reversal of diabetes insipidus. This conclusion is supported by the detection of AVP immunoreactivity in a fraction of the magnocellular neurons and in their axons in the median eminence and the posterior lobe. These results support the interpretation that the injected mRNA was taken up by axons of the hypothalamoneurohypophysial tract and transported anterogradely to the posterior lobe, as well as retrogradely, back to the hypothalamic perikarya. This would be in accordance with other studies that have demonstrated uptake and transport of oligonucleotides and dendritic and axonal transport of RNA (*14*). Our immunocytochemical and RIA findings as well as observed physiological responses indicate that exogenous AVP mRNA was also translated by some of the magnocellular perikarya. Uptake and translation of AVP mRNA seemed to be selective processes because injections of the AVP antisense probe did not result in any labeling of these perikarya or their nerve endings in the posterior lobe. Moreover, AVP immunoreactivity or labeled RNA did not become detectable in hypothalamic neurons other than the classical magnocellular neurons in the SON and PVN. Furthermore, when we injected total RNA fractions (which certainly contained mRNA coding for peptides other than AVP), the magnocellular hypothalamic neurons did not express ACTH and β-endorphin immunoreactivities.

Our findings suggest that rat hypothalamic magnocellular neurons can accumulate and translate certain specific exogenous RNAs normally expressed in these neurons. The autoradiographic data showing concentration of ^{35}S-labeled mRNA in cell bodies as well as in the posterior lobe [most likely confined to secretory nerve terminals (*2*)] indicate that at least in Brattleboro rats RNA is accumulated and transported anterogradely as well as retrogradely upon injection into the medial portion of the hypothalamoneurohypophysial tract. We conclude that the mechanisms of uptake, transport, and translation of RNA might represent a cascade of increasing selectivity: although exogenous mRNAs may be taken up by many axons, and smaller neuronal pools may be able to provide selective transport, only a few neurons may actually translate the axonally delivered RNA. This hypothesis is supported by the finding that degradation of RNA is low in the brain (*5*). We have shown that OT mRNA is present in secretory vesicles in the hypothalamoneurohypophysial system (*2*), suggesting that these mRNAs exist in cellular compartments together with peptide signals known to be secreted. Recent studies with in situ hybridization and electron microscopy indicate a similar situation for AVP mRNA (*15*). Axonal accumulation, transport, and secretion of mRNA may offer new approaches to regulate interneuronal signaling.

REFERENCES AND NOTES

1. B. Bloch, A. F. Guitteny, E. Normand, S. Chouham, *Neurosci. Lett.* **109**, 259 (1990).
2. G. F. Jirikowski, P. P. Sanna, F. E. Bloom, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7400 (1990).
3. E. Lehmann, J. Hanze, M. Pauschinger, D. Ganten, R. E. Lang, *Neurosci. Lett.* **111**, 170 (1990); A. Levy, S. Lightman, D. A. Carter, D. Murphy, *J. Neuroendocrinol.* **2**, 329 (1990); D. Murphy, A. Levy, S. Lightman, D. Carter, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9002 (1989); D. Murphy and D. Carter, *Mol. Endocrinol.* **4**, 1051 (1990).
4. P. J. Brooks, P. K. Lund, J. D. Caldwell, G. F. Jirikowski, C. A. Pedersen, *Soc. Neurosci. Abstr.* **14**, 484.10 (1990).
5. L. Franzoni and C. A. Garcia-Argiz, *Acta Physiol. Lat. Am.* **28**, 185 (1978); B. Schulz-Harder and D. Graf von Keyserlingk, *Histochemistry* **88**, 587 (1988).
6. H. Schmale and D. Richter, *Nature* **308**, 705 (1984).
7. Injections into the hypothalamoneurohypophysial tract {coordinates: *x*: 2.2 mm right, *y*: +2 mm, *z*: + 2.2 mm from the bregma, according to G. Paxinos and C. Watson, [*The Rat Brain in Stereotaxic Coordinates* (Academic Press, New York, 1985)]} were carried out with an injection aid rather than a stereotaxic apparatus, in a modification of the method by F. R. Popick [*Life Sci.* **18**, 197 (1976)]. The injected volume was in all cases 5 µl. Seven male rats were injected either with RNA extract (*8*) or with RNA (*9*). Six rats injected with isotonic saline served as controls.
8. Total cytoplasmic RNA was isolated from the hypothalami of a total of ten Sprague-Dawley rats. Tissue was homogenized in 20 mM tris-HCl (pH 8.8), 200 mM NaCl, and 20 mM $MgCl_2$. After a 10-min centrifugation at 3000*g* at 4°C, the supernatant was digested for 20 min in proteinase K (2.5 mg/ml) in 2% SDS, 40 mM EDTA (pH 8.0), and 100 mM NaCl. Purification of RNA was then performed with a phenol:chloroform:isoamyl alcohol (50:48:2) extraction. U. Schibler, M. Tosi, A. C. Pittet, L. Fabiani, K. R. Wellauer, *J. Mol. Biol.* **142**, 93 (1980); D. Lenoir, E. Battenberg, M. Kiel, F. E. Bloom, R. J. Milner, *J. Neurosci.* 6, 522 (1986).
9. A synthetic arginine-vasopressin RNA was generated by in vitro transcription of a cDNA clone [donated by D. Richter, Eppendorf Institute, Hamburg; R. Ivell and D. Richter, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1216 (1984)] with SP6 bacterial RNA polymerase (Promega) according to the manufacturer's protocol. These synthetic RNAs were not polyadenylated; RNA was labeled with ^{35}S-labeled cytidine 5′-triphosphate (CTP) for the autoradiography experiments.
10. Control injections were carried out with an isotonic saline vehicle and synthetic antisense RNA prepared from the same cDNA clone by transcription with T7 RNA polymerase. An additional control was the injection of sense RNA that had been digested with RNase A (20 µg/ml, Boehringer Mannheim) for 1 hour at 37°C. Further controls were done with injections of total RNA extracted from rat hindbrain (a region devoid of vasopressin mRNA). Each control group contained four animals.
11. We are indebted to P. Plotsky (Salk Institute) for performing AVP RIAs. Tissue preparations and RIAs were carried out as double antibody RIAs, according to H. U. Häussler, G. F. Jirikowski, and J. D. Caldwell [*J. Chem. Neuroanat.* **3**, 271 (1990)].
12. J. J. McCabe, J. I. Morrell, D. W. Pfaff, in *In Situ Hybridization in Brain*, G. R. Uhl, Ed. (Plenum, New York, 1985), pp. 73–95.
13. For control purposes three animals were injected with the synthetic AVP RNA into the lateral ventricle and three were injected into the cerebral cortex.
14. E. J. Carazanna, K. B. Pasieki, J. A. Majzoub, *Mol. Cell. Biol.* **8**, 2267 (1988); H. H. Zingg, D. L. Lefebvre, G. Almazon, *J. Biol. Chem.* **263**, 11041 (1988); D. Carter and D. Murphy, *ibid.* **264**, 6601 (1989); S. L. Loke *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3474 (1989); L. Davis, B. Burger, G. A. Banker, O. Steward, *J. Neurosci.* **10**, 3056 (1990); E. Mohr, S. Fehr, D. Richter, *EMBO J.* **10**, 2419 (1991).
15. A. Trembleau, M. Landry, M. Fevre-Montange, A. Calas, in *Neurocytochemical Methods*, A. Calas and D. Eugene, Eds. (NATO Advanced Study Institutes Series, Springer, New York, 1991), pp. 313–318.
16. L. A. Sternberger, *Immunocytochemistry* (Wiley, New York, ed. 2, 1978).

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