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# Autoradiographic localization of relaxin binding sites in rat brain

**(insulin family polypeptide/relaxin receptor/circumventricular organs/magnocellular nuclei/blood pressure)**

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ABSTRACT **Relaxin is a member of the insulin family of polypeptide hormones and exerts its best understood actions in the mammalian reproductive system. Using a biologically active $^{32}$P-labeled human relaxin, we have previously shown by *in vitro* autoradiography specific relaxin binding sites in rat uterus, cervix, and brain tissues. Using the same approach, we describe here a detailed localization of human relaxin binding sites in the rat brain. Displaceable relaxin binding sites are distributed in discrete regions of the olfactory system, neocortex, hypothalamus, hippocampus, thalamus, amygdala, midbrain, and medulla of the male and female rat brain. Characterization of the relaxin binding sites in the subfornical organ and neocortex reveals a single class of high-affinity sites ($K_d$ = 1.4 nM) in both regions. The binding of relaxin to two of the circumventricular organs (subfornical organ and organum vasculosum of the lamina terminalis) and the neurosecretory magnocellular hypothalamic nuclei (i.e., paraventricular and supraoptic nuclei) provides the anatomical and biochemical basis for emerging physiological evidence suggesting a central role for relaxin in the control of blood pressure and hormone release. We conclude that specific, high-affinity relaxin binding sites are present in discrete regions of the rat brain and that the distribution of some of these sites may be consistent with a role for relaxin in control of vascular volume and blood pressure.**

Relaxin belongs to the insulin family of polypeptide hormones and is best known for its biological activities on various parts of the mammalian reproductive system, notably elongation of the public ligament, inhibition of uterine contraction, and softening of the cervix (for reviews, see refs. 1 and 2). The effects of relaxin on nonreproductive tissues remain to be established (2).

The biological actions of relaxin are probably mediated through specific receptors located on the target cells. Receptors for relaxin have previously been partially characterized in rat and porcine uterine and porcine cervical membranes by using $^{125}$I-labeled porcine relaxin (3–5). Using a biologically active $^{32}$P-labeled human relaxin ($^{32}$P-relaxin), we have shown by ligand autoradiography specific relaxin binding sites in rat uterus, cervix, and brain (6). In this paper, we describe the localization and partial characterization of $^{32}$P-relaxin binding sites in the rat brain and discuss the physiological implications of these findings.

## MATERIALS AND METHODS

**Materials.** Synthetic human relaxin was supplied by E. Rinderknecht (Genentech). It was active in the mouse pubic symphysis assay (7) and a cAMP bioassay (6, 8). The concentration of relaxin was determined by amino acid analysis. Human insulin (Humulin) was obtained from Eli Lilly. Synthetic human angiotensin II and atrial natriuretic peptide were obtained from Sigma. Rat nerve growth factor was from Biomedical Technologies (Stoughton, MA).

**Phosphorylation of Relaxin.** Phosphorylation of relaxin with the catalytic subunit of cAMP-dependent protein kinase (from bovine heart muscle; Sigma) and [$\gamma$-$^{32}$P]ATP (specific activity, >5000 Ci/mmol; 1 Ci = 37 GBq; Amersham) and subsequent purification of the $^{32}$P-relaxin on Sep-Pak C18 cartridge (Waters) and cation-exchange HPLC (Poly CAT A, Poly LC, Columbia, MD) were as described (6).

**Binding of $^{32}$P-Relaxin to Rat Brain Tissue Sections.** Ten-week-old normal male and cyclic female Sprague–Dawley rats (Charles River Breeding Laboratories) were housed for 1 week before being sacrificed by asphyxiation with $CO_2$. The brains were frozen in powdered dry ice immediately after excision. Tissue cryosectioning and binding of $^{32}$P-relaxin to the tissue sections were performed as described (6). Sets of three consecutive 16-$\mu$m coronal sections were collected at intervals of approximately 160–350 $\mu$m throughout the entire extent of the brain. Binding regions were determined by overlaying the autoradiographs with adjacent sections counterstained with cresyl violet. For relaxin binding displacement experiments, consecutive coronal brain sections were incubated with 100 pM $^{32}$P-relaxin in the absence and presence of increasing concentrations (serial 3-fold increases) of unlabeled relaxin in the range of 0.1–100 nM. Pseudocolor reconstructions and quantitative analysis of $^{32}$P-relaxin binding autoradiographs were performed with a RAS-3000 image analysis system (Amersham). The ODs or radioactivities (cpm) of bound $^{32}$P-relaxin (maximum binding in a particular brain region) were quantified by computerized densitometry based on parallel sets of $^{32}$P-relaxin standards (ranging from 50 to 2000 cpm) blotted onto nitrocellulose membranes (Trans-Blot SF, Bio-Rad). The binding inhibition data were fit to a four-parameter equation to obtain the $ED_{50}$ (concentration of unlabeled relaxin yielding 50% displacement of the binding of $^{32}$P-relaxin). The dissociation constant for relaxin (or the inhibition constant) was calculated by the method of Cheng and Prusoff (9).

## RESULTS

**Distribution of $^{32}$P-Relaxin Binding Sites.** $^{32}$P-relaxin binding sites were found in discrete regions of the female rat brain (Fig. 1) as listed in Table 1. High concentrations of relaxin binding sites (OD > 0.2) were present in the frontal and parietal regions of neocortex (Fig. 1 *a* and *b*), in two of the circumventricular organs, organum vasculosum of the lamina terminalis (OVLT; Fig. 1*b*) and subfornical organ (Fig. 1*d*), in magnocellular paraventricular nucleus (Fig. 1*g*), basolateral amygdaloid nucleus (Fig. 1*h*), mammillary peduncle, and dorsal tegmental nucleus (Fig. 1*k*). Moderate levels of binding sites (OD > 0.1) were found in the glomerular layer and internal granular layer of the olfactory bulb, anterior olfactory nucleus (Fig. 1*a*), dentate gyrus of hippocampus (Fig.

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Abbreviation: OVLT, organum vasculosum of the lamina terminalis.

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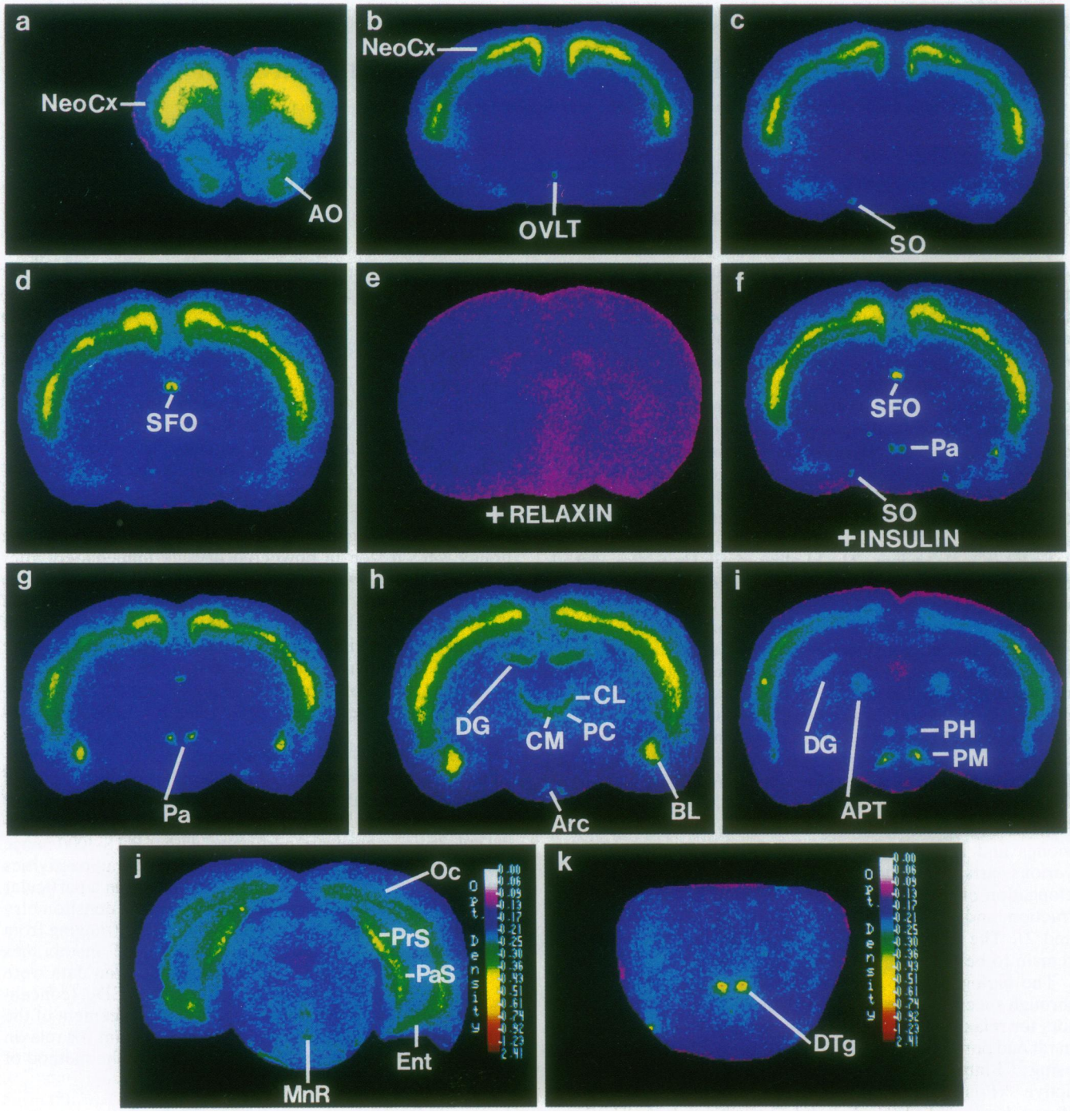


FIG. 1. Pseudocolor representation of $^{32}$P-relaxin binding sites in female rat brain. Binding of $^{32}$P-relaxin to coronal brain sections and computerized image analysis of the binding autoradiographs were performed as described. Low to high binding intensities were shown in OD units and are represented by a color spectrum from magenta to red (*i* and *j*). See Table 1 for abbreviations. The binding of 100 pM $^{32}$P-relaxin to the SO (*c*), SFO (*d*), and NeoCx (neocortex) is displaced by 100 nM unlabeled relaxin (*e*), while 100 nM insulin (*f*) does not displace the $^{32}$P-relaxin binding to SO, SFO, and Pa.

1*h*), paracentral and central medial thalamic nucleus (Fig. 1*h*), infundibular stem, premammillary nucleus (Fig. 1*i*), nucleus Darkschewitsch, interpeduncular nucleus, presubiculum and parasubiculum (Fig. 1*j*), entorhinal cortex (Fig. 1*j*), and cuneate nucleus. Low levels of binding (OD < 0.1) were found in the supraoptic nucleus (Fig. 1*c*), arcuate hypothalamic nucleus (Fig. 1*h*), centrolateral thalamic nucleus (Fig. 1*h*), anterior pretectal nucleus (Fig. 1*i*), posterior hypothalamic area (Fig. 1*i*), occipital neocortex (Fig. 1*j*), substantia nigra, medial geniculate nucleus, median raphe nucleus (Fig. 1*j*), and gracile nucleus.

Localization experiments performed on the male rat brain showed the same pattern and quantitative distribution of relaxin binding sites (data not shown).

**Characterization of Relaxin Binding Sites.** The binding of $^{32}$P-relaxin to the brain regions tested could be displaced by 100 nM unlabeled relaxin (Fig. 1*e*) but not by 100 nM insulin (Fig. 1*f*), nerve growth factor, angiotensin II, or atrial natriuretic peptide (data not shown). Relaxin binding sites in the neocortex and the subfornical organ were further characterized because of the relatively high binding intensities and the different circumventricular nature of these two regions. This

Table 1. Quantitative distribution of relaxin binding sites in the rat brain

| Area | OD |
|---|---|
| Glomerular layer (Gl) and internal granular layer (IGr) of olfactory bulb | 0.13 |
| Anterior olfactory nucleus (AO) | 0.11 |
| Frontal (Fr) and parietal (Par) cortex | 0.29 |
| Organum vasculosum of lamina terminalis (OVLT) | 0.20 |
| Subfornical organ (SFO) | 0.24 |
| Supraoptic nucleus (SO) | 0.09 |
| Paraventricular nucleus (Pa) | 0.25 |
| Basolateral amygdaloid nucleus (BL) | 0.26 |
| Arcuate hypothalamic nucleus (Arc) | 0.04 |
| Dentate gyrus (DG) | 0.11 |
| Centrolateral thalamic nucleus (CL) | 0.09 |
| Paracentral thalamic nucleus (PC) | 0.12 |
| Central medial thalamic nucleus (CM) | 0.14 |
| Infundibular stem (InfS) | 0.16 |
| Mammillary peduncle (mp) | 0.21 |
| Anterior pretectal nucleus (APT) | 0.09 |
| Posterior hypothalamic area (PH) | 0.06 |
| Premammillary nucleus (PM) | 0.16 |
| Nucleus Darkschewitsch (Dk) | 0.12 |
| Interpeduncular nucleus (IP) | 0.11 |
| Occipital neocortex (Oc) | 0.09 |
| Entorhinal cortex (Ent) | 0.15 |
| Presubiculum (PrS) | 0.17 |
| Parasubiculum (PaS) | 0.14 |
| Substantia nigra (SN) | 0.04 |
| Medial geniculate nucleus (MG) | 0.08 |
| Median raphe nucleus (MnR) | 0.07 |
| Dorsal tegmental nucleus (DTg) | 0.49 |
| Gracile nucleus (Gr) | 0.09 |
| Cuneate nucleus (Cu) | 0.13 |

Binding of $^{32}$P-relaxin (100 pM) to coronal brain sections was performed on microscope slides of three consecutive sections each from a single animal. Binding intensities were analyzed by computerized densitometry and are expressed as OD units. In detail, a square of uniform predetermined size (9 mm$^2$ on the RAS-3000 screen) was placed on the area of maximum binding in each particular region. The optical density represents the integrated intensity within that particular square, based on parallel $^{32}$P-relaxin standards ranging from 50 to 2000 cpm. Background binding was subtracted from all readings. Binding intensities corresponding to OD values of >0.04 were readily detected and resolved from background binding. Values were calculated as the mean of multiple points taken from three sections each from one to three animals, with an average coefficient of variation of 12.3% ± 1.2%. Binding areas were determined by histologic counterstaining with cresyl violet. Nomenclature and abbreviations are according to Paxinos and Watson (10).

was accomplished by measuring the inhibition of $^{32}$P-relaxin binding in the presence of increasing concentrations of unlabeled relaxin. The binding inhibition data yielded a displacement curve showing a single class of relaxin binding sites (Fig. 2). The $K_d$ for relaxin was calculated to be 1.45 ± 0.02 and 1.42 ± 0.19 nM in the neocortex and the subfornical organ, respectively.

## DISCUSSION

In view of the fact that relaxin is best known as a pregnancy-associated hormone, our previous finding of relaxin binding sites in the rat brain (6) was unpredicted. In this paper, we show that human relaxin binds to discrete regions of the male and female rat brain. These findings suggest that relaxin may exert biological actions in the central nervous system and that at least some of these activities may be unrelated to the classical actions of relaxin (1, 2).

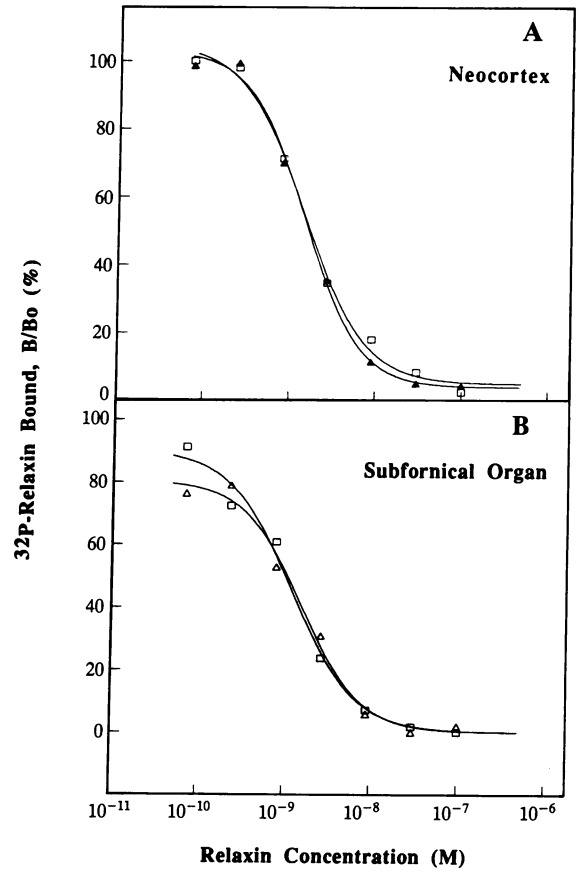


FIG. 2. Displacement of $^{32}$P-relaxin binding to the neocortex (*A*) and the subfornical organ (*B*) of male rat brain by unlabeled relaxin. Consecutive coronal sections were incubated with 100 pM $^{32}$P-relaxin in the absence (Bo) and presence (B) of unlabeled relaxin in the concentration range of 0.1 to 100 nM. The two curves in each panel (□ and ▲ in *A*; □ and △ in *B*) represent two separate determinations using different sets of serial sections from the same brain. The displacement data were fit to a four-parameter equation. The $K_d$ for relaxin was calculated to be 1.45 ± 0.02 and 1.42 ± 0.19 nM in the neocortex and the subfornical organ, respectively, according to the method of Cheng and Prusoff (9).

The specificity and affinity of the brain relaxin binding sites were very similar to those in the rat uterus (ref. 6; P.L.O., unpublished data). The binding of $^{32}$P-relaxin in numerous regions studied could not be displaced by peptides with related structures [insulin-like growth factor type 1 (6), insulin] or those with binding sites in the rat brain (nerve growth factor, angiotensin II, and atrial natriuretic peptide). The high affinity of the relaxin binding sites, determined in two brain regions of high binding intensities, suggests that at least some of the relaxin binding sites serve as high-affinity receptors for relaxin. Although rat and human relaxins share limited (44%) amino acid sequence identity, the finding that all mammalian relaxins so far isolated are biologically active in the rat suggests the presence of a specific, conserved region on the relaxin molecule that interacts with the rat relaxin receptors (1).

Relaxin binding sites were found in several brain regions known to be involved in certain physiological processes. High concentrations of relaxin binding sites were observed in two of the circumventricular organs, the subfornical organ and OVLT; both have been shown to play a physiological role in the control of blood pressure and fluid balance (11). This finding is particularly intriguing in view of the presence of relaxin binding sites in the magnocellular regions of the paraventricular and supraoptic nuclei, which are responsible for the release of vasopressin and oxytocin. The accessibility

of neurons in the subfornical organ and OVLT to blood-borne proteins suggests an anatomical basis for potential central actions of relaxin on vascular volume or blood pressure.

Several physiological studies have suggested that relaxin might affect blood pressure, with somewhat conflicting results (12–18). In studies with conscious, nonpregnant spontaneously hypertensive rats (12), chronic intravenous infusion of relaxin caused a decrease in peripheral blood pressure. In anesthetized animals, the effects of relaxin ranged from a transient decrease in blood pressure (13), to no effect (14), to an increase in blood pressure (16, 17) following peripheral relaxin injection. Mumford *et al.* (18) further demonstrated that relaxin administered into the cerebral ventricles caused a pressor response and the release of vasopressin in rats, and that lesion of the subfornical organ negated the relaxin effect. The subfornical organ and in most cases OVLT possess receptors for a number of peptides including insulin (19), insulin-like growth factor type 1 (20), angiotensin (21, 22), and atrial natriuretic peptide (23), and they have been suggested as mediators of the pressor actions of angiotensin and atrial natriuretic peptide (24). Our finding of relaxin binding sites in the subfornical organ and OVLT suggests that relaxin may have a direct action on these sites to initiate events that result in the alteration of blood pressure. However, the present study did not detect any relaxin binding in the brainstem pressor centers, such as the nucleus of the tractus solitarius and area postrema, where binding sites for angiotensin II (22) and atrial natriuretic peptide (23) are present in high densities. It is possible that the pressor effect of relaxin may be in part mediated via the central angiotensin system, since it was shown that central infusion of a specific angiotensin II receptor antagonist blocked the effect of centrally administered relaxin (25).

The presence of relaxin binding sites in the magnocellular paraventricular and supraoptic nuclei, both of which contain distinctive oxytocin and vasopressin immunoreactive cells (26), suggests a possible role for relaxin in the control of oxytocin release. Summerlee *et al.* (27) first showed that intravenous injection or central administration of porcine relaxin to lactating rats significantly inhibited the reflex milk ejection. Subsequent studies (28, 29) showed that systemic administration of relaxin suppressed oxytocin release from the posterior pituitary of lactating rats. *In vitro* studies (30) also showed that relaxin modulated the secretion of oxytocin and vasopressin from isolated neural lobe of the pituitary. The effects of relaxin on reflex milk ejection might also be mediated, at least in part, by the subfornical organ, the lesion of which abolished the relaxin effect (31). Our observation of relaxin binding to the subfornical organ provides evidence that oxytocin release may be altered by the action of relaxin at this site. However, the uneven concentrations of relaxin binding sites in the two magnocellular nuclei (lower in the supraoptic nucleus than in the paraventricular nucleus) seem to argue against a direct action of relaxin on these sites for oxytocin release.

The source of relaxin for the brain binding sites is unknown at the present time. One possibility is that peripheral relaxin acts on brain tissues in or near the circumventricular organs, which lack a blood–brain barrier to peptides. However, it is not clear whether relaxin acts directly on the magnocellular nuclei, which are the most highly vascularized parts of the hypothalamus and are more accessible to peptides in the periphery (26). Although the highest circulating levels of relaxin are found during pregnancy (2), relaxin is also secreted during the normal luteal phase of the nonpregnant female and is measurable (30–150 pg/ml) in peripheral blood (32). Serum relaxin levels have not been established in the male. However, immunoreactive and biologically active relaxin is present in human seminal plasma (average, 45 ng/ml) and the likely source is the prostate (33–35). Alternatively, relaxin is synthesized in the central nervous system. *In situ* hybridization experiments to localize relaxin mRNA synthesis will likely provide answers to this latter possibility. Recent evidence has also shown that peptides and proteins, which traditionally were thought to be excluded by the blood–brain barrier, could enter the central nervous system by saturable and nonsaturable processes (36–38). The $^{32}$P-relaxin can be used as a probe for blood–brain barrier studies.

We conclude that specific, high-affinity relaxin binding sites are present in a number of discrete regions of the male and female rat brain. The distribution of some of these sites is consistent with existing physiological evidence suggesting a role for relaxin in the regulation of blood pressure and fluid balance.

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1. Kemp, B. E. & Niall, H. D. (1984) *Vitam. Horm. (NY)* **41,** 79–115.
2. Sherwood, O. D. (1988) in *The Physiology of Reproduction,* eds. Knobil, E. & Neill, J. (Raven, New York), pp. 585–673.
3. Mercado-Simmen, R. C., Bryant-Greenwood, G. D. & Greenwood, F. C. (1980) *J. Biol. Chem.* **255,** 3617–3623.
4. Mercado-Simmen, R. C., Bryant-Greenwood, G. D. & Greenwood, F. C. (1982) *Endocrinology* **110,** 220–226.
5. Mercado-Simmen, R. C., Goodwin, B., Ueno, M. S., Yamamoto, S. Y. & Bryant-Greenwood, G. D. (1982) *Biol. Reprod.* **26,** 120–128.
6. Osheroff, P. L., Ling, V. T., Vandlen, R. L., Cronin, M. J. & Lofgren, J. A. (1990) *J. Biol. Chem.* **265,** 9396–9401.
7. Steinetz, B. G., Beach, V. L., Kroc, R. L., Stasilli, N. R., Nussbaum, R. E., Nemith, P. J. & Dun, R. K. (1960) *Endocrinology* **67,** 102–115.
8. Kramer, S. M., Gibson, U. E. M., Fendley, B. M., Mohler, M. A., Drolet, D. W. & Johnston, P. D. (1990) *In Vitro Cell. Dev. Biol.* **26,** 647–656.
9. Cheng, Y. & Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22,** 3099–3108.
10. Paxinos, G. & Watson, C. (1986) in *The Rat Brain in Stereotaxic Coordinates* (Academic, New York), 2nd Ed.
11. Phillips, M. I. (1987) *Annu. Rev. Physiol.* **49,** 413–435.
12. St-Louis, J. & Massicotte, G. (1985) *Life Sci.* **37,** 1351–1357.
13. Miller, J. W., Kisley, A. & Murray, W. J. (1957) *J. Pharmacol. Exp. Ther.* **120,** 426–437.
14. Porter, D. G., Downing, S. J. & Bradshaw, J. M. C. (1979) *J. Endocrinol.* **83,** 183–192.
15. Ahokas, R. A., Sibai, B. M. & Anderson, G. D. (1989) *Am. J. Obstet. Gynecol.* **161,** 618–622.
16. Jones, S. A. & Summerlee, A. J. S. (1986) *J. Physiol.* **381,** 37P (abstr.).
17. Parry, L. J., Poterski, R. S., Summerlee, A. J. & Jones, S. A. (1990) *J. Neuroendocrinol.* **2,** 53–58.
18. Mumford, A. D., Parry, L. J. & Summerlee, A. J. S. (1989) *J. Endocrinol.* **122,** 747–755.
19. Van Houton, M., Posner, B. I., Kopriwa, B. M. & Brawer, J. R. (1979) *Endocrinology* **105,** 666–673.
20. Matsuo, K., Niwa, M., Kurihara, M., Shigematsu, K., Yamashita, S., Ozaki, M. & Nagataki, S. (1989) *Cell. Mol. Neurobiol.* **9,** 357–367.
21. Van Houten, M., Schiffrin, E. L., Mann, J. F. E., Posner, B. I. & Boucher, R. (1980) *Brain Res.* **186,** 480–485.
22. Mendelsohn, F. A. O., Quirion, R., Saavedra, J. M., Aguilera, G. & Catt, K. J. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1575–1579.
23. Quirion, R., Dalpe, M., De Lean, A., Gutkowska, J., Cantin, M. & Genest, J. (1984) *Peptides* **5,** 1167–1172.
24. Sills, M. A., Nguyen, K. Q. & Jacobowitz, D. M. (1985) *Peptides* **6,** 1037–1042.
25. Mumford, A. D., Parry, L. J. & Summerlee, A. J. S. (1989) *Eur. J. Neurosci. Suppl.* **2,** 66.3.
26. Swanson, L. W. & Sawchenko, P. E. (1983) *Annu. Rev. Neurosci.* **6,** 269–324.

27. Summerlee, A. J. S., O'Byrne, K. T., Paisley, A. C., Breeze, M. F. & Porter, D. G. (1984) *Nature (London)* **309,** 372–374.
28. O'Byrne, K. T., Eltringham, L., Clarke, G. & Summerlee, A. J. S. (1986) *J. Endocrinol.* **109,** 393–397.
29. Jones, S. A. & Summerlee, A. J. S. (1987) *J. Endocrinol.* **114,** 241–246.
30. Dayanithi, G., Cazalis, M. & Nordmann, J. J. (1987) *Nature (London)* **325,** 813–816.
31. Summerlee, A. J. S., O'Byrne, K. T., Jones, S. A. & Eltringham, L. (1987) *J. Endocrinol.* **115,** 347–353.
32. Stewart, D. R., Celniker, A., Taylor, C., Cragun, J. R., Overstreet, J. W. & Lasley, B. L. (1990) *J. Clin. Endocrinol. Metab.* **70,** 1771–1773.
33. Loumaye, E., De Cooman, S. & Thomas, K. (1980) *J. Clin. Endocrinol. Metab.* **50,** 1142–1143.
34. Essig, M., Schoenfeld, C., D'Eletto, R., Amelar, R., Dubin, L., Steinetz, B. G., O'Byrne, E. M. & Weiss, G. (1982) *Ann. N. Y. Acad. Sci.* **380,** 224–230.
35. Weiss, G. (1989) *Biol. Reprod.* **40,** 197–220.
36. Banks, W. A. & Kastin, A. J. (1987) *Life Sci.* **41,** 1319–1338.
37. Fishman, J. B., Rubin, J. B., Handrahan, J. V., Conner, J. R. & Fine, R. E. (1987) *J. Neurosci. Res.* **18,** 299–304.
38. Banks, W. A., Kastin, A. J. & Durham, D. A. (1989) *Brain Res. Bull.* **23,** 433–437.