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# Bombesin: Specific binding to rat brain membranes

(receptor binding/bombesin analogues/structure–activity relationships/neuropeptide/neuropharmacology)

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**ABSTRACT** The binding of a radiolabeled bombesin analogue to rat brain membranes was studied. [$^{125}$I-Tyr$^4$]Bombesin bound with high affinity ($K_D$ = 3 nM) to a single class of noninteracting sites. Binding was specific, saturable (3.8 pmol of sites/g of wet tissue), and reversible. Regional and subcellular distribution studies showed that the density of sites was 7-fold greater in the hippocampus than the medulla/pons and greater in synaptosomal fractions than in mitochondrial or nuclear fractions. The abilities of numerous bombesin analogues to induce hypothermia and to inhibit [$^{125}$I-Tyr$^4$]bombesin-binding activity correlate well. Numerous amino acid residues near the $CONH_2$-terminal are required for high-affinity binding and biological potency.

Bombesin (BN), a tetradecapeptide isolated from anuran skin (1), is biologically active in the mammalian gastronintestinal tract and the central nervous system. Upon intravenous infusion in the dog, BN induces gastrin release from the antral mucosa (2) and cholecystokinin release from the duodenal mucosa (3). Intravenous or intracisternal injection of BN in the rat induces prolactin and growth hormone secretion (4). In addition, intracisternal injection of BN in rats causes hypothermia (5), hyperglycemia (6), and inhibition of cold-induced thyrotropin secretion (7). Because endogenous BN-like immunoreactivity has been found in the brain (8) and mammalian intestine (9), BN-like peptides may function as neurotransmitters or modulators of neutral activity.

Therefore, we undertook the characterization of the BN receptor in mammalian brain. The data presented here indicate that a component present on rat brain membranes binds a BN analogue, [Tyr$^4$]BN, with high affinity. This component may be the physiologically important receptor that mediates the effects of BN-like peptides in the central nervous system.

## MATERIALS AND METHODS

**Synthesis of BN and Analogues.** Peptides were synthesized by the solid phase method as described by Rivier and Brown (10).

**Assay for Biological Activity.** The ability of peptides to induce hypothermia in rats was determined by using the protocol of Brown *et al.* (5, 6). Peptides were dissolved in artificial cerebrospinal fluid and 10 μl was injected into the cisterna magnum of rats whose light and feeding cycles were controlled. Rectal temperatures were recorded by using a Yellow Springs Instrument thermoprobe. All experiments were carried out in randomized block design with five animals per group and the data were analyzed by computer program.

**Preparation of Radiolabeled Peptide.** [Tyr$^4$]BN was radiolabeled by the chloramine-T procedure (11). Stoichiometric amounts (0.5 nmol) of Na$^{125}$I (Amersham/Searle), [Tyr$^4$]BN, and chloramine-T (Eastman) were mixed rapidly. After 30 sec the reaction was terminated by the addition of a 4-fold molar excess of sodium metabisulfite (Fisher). Radiolabeled peptide was purified by using the gel filtration techniques described by Lazarus *et al.* (12). The reaction mixture was passed through a 0.7 × 20 cm Sephadex LH-20 column. Four-tenths-milliliter fractions were collected and 2-μl aliquots were withdrawn from each fraction and assayed for radioactivity in a Searle gamma counter.

**Preparation of Membranes.** Adult male Sprague–Dawley rats (175–200 g) were decapitated and the brains were dissected. Routinely, the medulla/pons, which contained minimal receptor activity, was removed. The remaining brain was weighed, homogenized in 100 vol of 50 mM Tris·HCl, pH 7.4, at 4°C in a Brinkmann Polytron (setting 5, 15 sec) and centrifuged at 30,000 × *g* for 15 min. The resulting pellet was resuspended in 100 vol of 100 mM NaCl in 50 mM Tris·HCl, pH 7.4, incubated at 4°C for 60 min, and centrifuged at 30,000 × *g* for 15 min. The resulting pellet was resuspended in 10 vol of 50 mM Tris·HCl, pH 7.4, at 4°C. Membrane protein concentration was determined by the method of Lowry *et al.* (13).

Subcellular fractions were prepared by the method of Gray and Whittaker (14). Then, membranes were prepared from each fraction by the procedure described above.

**Binding Assay.** Routinely, 200 μl of freshly prepared homogenate (1.6 mg of protein) was incubated with 100–500 fmol of [$^{125}$I-Tyr$^4$]BN at 4°C for 24 min in the presence or absence of competitor. The buffer contained bovine serum albumin (Cohn fraction V, Sigma) at 1 mg/ml and bacitracin (Sigma) at 2 μg/ml in 50 mm Tris·HCl, pH 7.4. The total volume was 0.5 ml and each assay was performed in triplicate.

Membrane-bound [$^{125}$I-Tyr$^4$]BN was separated from free peptide by filtration under reduced pressure through GF/B filters (Whatman) that were presoaked with 1% bovine serum albumin in 50 mM Tris·HCl, pH 7.4. The filters were rinsed promptly with ice-cold 50 mM Tris·HCl, pH 7.4, containing 0.1% bovine serum albumin. Filtration time did not exceed 15 sec. The filters were assayed for radioactivity in a Searle gamma counter.

## RESULTS

**Preparation of Radiolabeled Peptide.** Fig. 1 shows the elution profile of the iodination mixture. The first peak of radioactivity eluted just behind the void volume and was adsorbed by SP-Sephadex cation-exchange resin. The second peak of radioactivity comigrated with KI and was adsorbed by QAE-Sephadex anion-exchange resin. Therefore, we designated peak I as [$^{125}$I-Tyr$^4$]BN and peak II as $^{125}$I. Homogeneity of peak I was assessed by using Brinkmann silica gel thin-layer chromatography plates (G-25) in BuOH/HOAc/$H_2O$ (4/1/5 by volume, upper phase). Because the radioactivity migrated as a single spot, the radiolabeled peptide appeared pure.

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Abbreviation: BN, bombesin.

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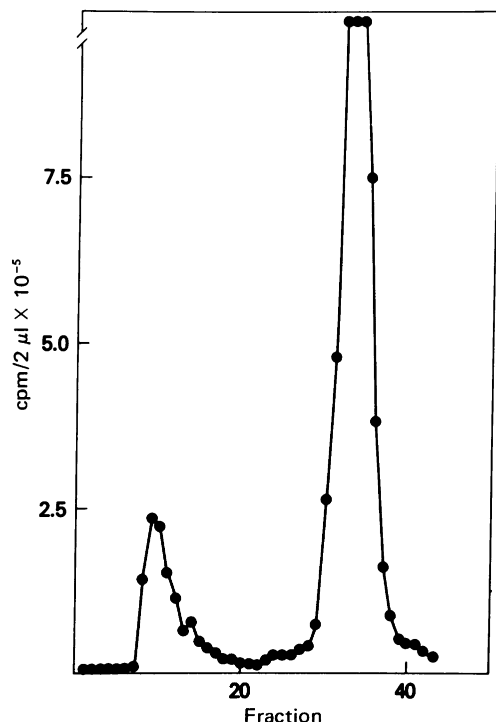


FIG. 1. Chromatography of the iodination reaction mixture on Sephadex LH-20 in methanol/acetic acid/water (10/2/1 by volume). Fraction size was 0.4 ml.

Routinely, the [$^{125}$I-Tyr$^4$]BN was pooled and titrated to pH 7.0 with Tris base. The tracer was stored at 4°C or −80°C until use. Tracer that was prepared with stoichiometric amounts, not a 10-fold molar excess, of chloramine-T had optimal specific binding. The specific activity of our [$^{125}$I-Tyr$^4$]BN preparations ranged from 100 to 400 Ci/mmol.

**Binding of Radiolabeled Peptide to Membranes.** Fig. 2 illustrates that [$^{125}$I-Tyr$^4$]BN binds to rat brain membranes. Total and nonspecific binding (binding in the presence of 1 $\mu$M [Tyr$^4$]BN) were linear functions of membrane concentration. Also, Fig. 2 shows that in the absence of membranes, 950 cpm (3% of the total cpm) were adsorbed to the filter in the presence or absence of 1 $\mu$M [Tyr$^4$]BN. Because the ratio of specific

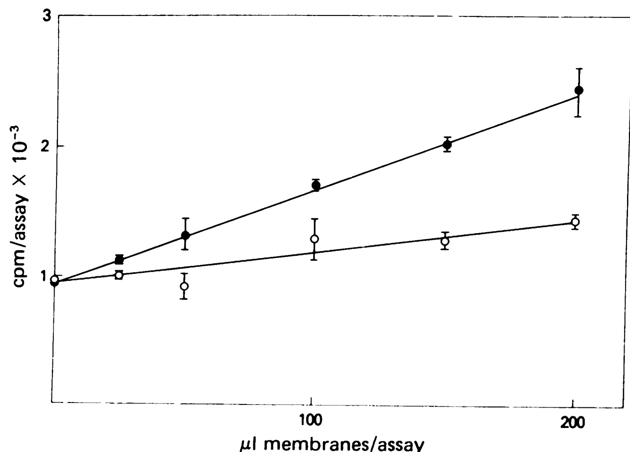


FIG. 2. Binding of [$^{125}$I-Tyr$^4$]BN as a function of membrane concentration in the presence of no additions (●) and 1 $\mu$M [Tyr$^4$]BN (○). When 200 $\mu$l of membranes are used, the specific binding is 3.2% of the total cpm added, and the nonspecific binding is 1.7% of the total cpm. Each assay was performed in triplicate. The standard error of the mean (bars) was approximately 5% of the mean.

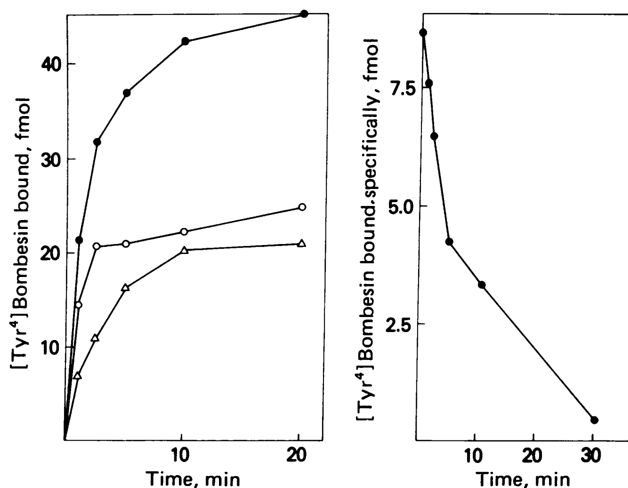


FIG. 3. Time course of [$^{125}$I-Tyr$^4$]BN association and dissociation. (*Left*) [$^{125}$I-Tyr$^4$]BN (1.7 nM) was added to 200 $\mu$l of membranes (1.6 mg of protein) and the amount of receptor–peptide complex was determined as a function of time in the presence of no additions (●) and 0.4 $\mu$M [Tyr$^4$]BN (○). The difference between the two curves represents the time course of specific BN binding (△). (*Right*) [$^{125}$I-Tyr$^4$]BN (0.5 nM) was incubated with membranes for 20 min, then 0.4 $\mu$M [Tyr$^4$]BN was added and the amount of receptor–peptide complex was determined as a function of time. Each assay was performed in triplicate and the mean value is indicated.

binding (total minus nonspecific binding) relative to filter background was enhanced by using large quantities of membranes, we routinely used 200 $\mu$l of membranes per assay. Greater membrane densities were not used because the flow rate through the filters was reduced significantly.

**Kinetic Binding Data.** The time course of [$^{125}$I-Tyr$^4$]BN association to and dissociation from rat brain membranes is shown in Fig. 3. Fig. 3 *left* shows that nonspecific binding was maximal after 2 min. In contrast, total and specific binding increased slowly, reaching a plateau value after 10 min.

Fig. 3 *right* shows that specific binding was reversed by the addition of 0.4 $\mu$M [Tyr$^4$]BN. Specific binding declined in an exponential manner and after 30 min little specific binding

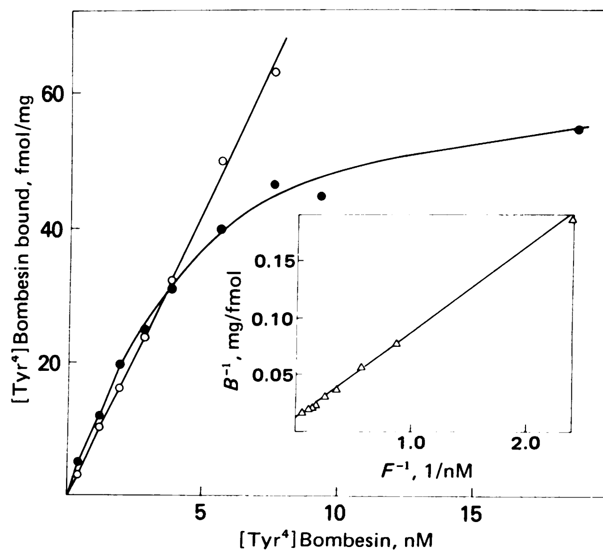


FIG. 4. Binding of [$^{125}$I-Tyr$^4$]BN as a function of radiolabeled peptide concentration. Nonspecific (○) and specific (●) binding was determined in triplicate after a 30-min incubation. (*Inset*) Double reciprocal replot of the amount of BN specifically bound ($B$) versus the amount of free BN ($F$).

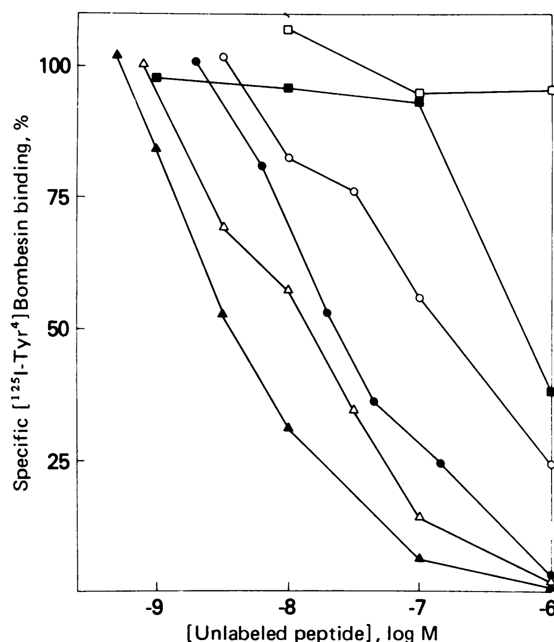


FIG. 5. Competitive inhibition of [$^{125}$I-Tyr$^4$]BN binding by various BN analogues. The percent of [$^{125}$I-Tyr$^4$]BN bound specifically is plotted as a function of unlabeled peptide concentration for [Tyr$^4$]BN (▲), [D-Ala$^5$]BN (△), BN (●), [Ac-Gly$^5$]BN (○), BN-OH (■), and des-Leu$^{13}$,Met$^{14}$-BN (□).

remained. By using the procedure of Kitabgi *et al.* (15) the association ($k_1 = 1.1 \times 10^8$ M$^{-1}$ min$^{-1}$) and dissociation rate constant ($k_{-1} = 1.0 \times 10^{-1}$ min$^{-1}$) were calculated. On the basis of these rate constants, the equilibrium dissociation constant ($K_D = k_{-1}/k_1 = 0.9$ nM) was calculated.

**Equilibrium Binding Studies.** The concentration dependence of [$^{125}$I-Tyr$^4$]BN binding to rat brain membranes was investigated. Fig. 4 shows that nonspecific binding was a linear function of peptide concentration. In contrast, specific binding appeared saturable. Because a Hill plot of the specific binding data had a slope of unity, [Tyr$^4$]BN appeared to bind to a single class of noninteracting sites. In addition, Fig. 4 *inset* shows that a double reciprocal replot of the specific binding data yields a straight line. On the basis of these data the dissociation constant ($K_D$ = 5.5 nM) and maximal binding ($B_{max}$ = 80 fmol/mg protein = 3.8 pmol/g wet tissue) were calculated.

**Competitive Inhibition of [$^{125}$I-Tyr$^4$]BN Binding.** The ability of a wide variety of neuropeptides, putative neurotransmitters, and brain receptor antagonists to inhibit specific [Tyr$^4$]BN binding was investigated. Fig. 5 shows that BN appears to inhibit [$^{125}$I-Tyr$^4$]BN binding in a competitive manner with high affinity. Fifty percent of the specific [$^{125}$I-Tyr$^4$]BN binding (IC$_{50}$) was inhibited when the BN concentration was 20 nM. [Tyr$^4$]BN inhibited with 5-fold and [D-Ala$^5$]BN with 2-fold greater affinity than BN, whereas [Ac-Gly$^5$]BN inhibited with 1/5th and BN-OH with 1/30th the affinity of BN. Des-Leu$^{13}$,Met$^{14}$-BN at 1 $\mu$M, inhibited little, if any, specific binding.

The data in Table 1 show that only those peptides that have a CONH$_2$-terminal similar to that of BN compete for the high-affinity [$^{125}$I-Tyr$^4$]BN-binding site.¶ Because substitution of D-Trp for L-Trp at position 8, D-Val for L-Val at position 10, Tyr for His at position 12, and Met-OH for Met-NH$_2$ at position 14 decreased the BN-binding affinity to at least 1/30th, Trp$^8$, Val$^{10}$, His$^{12}$, and an amidated COOH-terminal may be essential for high-affinity interaction with the receptor. Similar constraints on amino acid sequence are required for BN to induce hypothermia after intracisternal injection in rats (Table 1). These results suggest that the biological effects of BN-like peptides in the central nervous system may be mediated by the receptor that binds [$^{125}$I-Tyr$^4$]BN with high affinity. Other putative neurotransmitters, brain receptor antagonists, and neuropeptides (including neurotensin and substance P) do not compete for the high-affinity [Tyr$^4$]BN-binding site (Table 1).

**Regional Distribution of the BN Receptor.** The regional distribution of [$^{125}$I-Tyr$^4$]BN-binding sites was investigated. Table 2 shows that the highest density of sites was found in the hippocampus; the density of sites in the medulla/pons was 1/7th that in the hippocampus. Intermediate receptor densities were observed in the striatum, cortex, hypothalamus, and thalamus, followed by the midbrain and cerebellum.

**Miscellaneous Binding Data.** The subcellular distribution of [$^{125}$I-Tyr$^4$]BN-binding sites was investigated. The total number and density of specific [Tyr$^4$]BN-binding sites was

Table 1. Pharmacology of bombesin and related peptides

| Peptide | IC$_{50}$, nM | Relative potency to induce hypothermia |
|---|---|---|
| [Tyr$^4$]BN | 4 | 95 |
| [D-Ala$^5$]BN | 15 | 100 |
| [D-Ala$^{11}$]BN | 20 | 100 |
| BN | 20 | 100 |
| [D-Asn$^6$]BN | 40 | 10 |
| Litorin | 40 | 5 |
| Ranatensin | 60 | 20 |
| [Ac-Gly$^5$]BN | 100 | 100 |
| BN-OH | 600 | <1 |
| [Tyr$^{12}$]BN | 1000 | 1 |
| [D-Trp$^8$]BN | >5000 | 1 |
| [D-Val$^{10}$]BN | >5000 | 1 |
| Des-Leu$^{13}$,Met$^{14}$-BN | >5000 | <1 |

Other substances that do not compete with the BN receptor at 1 $\mu$M doses include: somatostatin, substance P, cholecystokinin-octapeptide, caerulein, eledosin, physalaemin, $\alpha$-melanotropin, [Leu$^5$]-enkephalin,. [Met$^5$]enkephalin, neurotensin, thyrotropin-releasing factor, serotonin, histamine, $\gamma$-aminobutyric acid, bicuculline, glutamate, glycine, strychnine, morphine, naloxone, phentolamine, propranolol, haloperidol, oxotremorine, atropine, diazepam, carnosine, and $\Delta^9$-tetrahydrocannabinol.

Table 2. Regional distribution of specific [$^{125}$I-Tyr$^4$]BN-binding activity in rat brain

| Region | [$^{125}$I-Tyr$^4$]BN specifically bound, fmol/mg protein |
|---|---|
| Hippocampus | 15.0 ± 2.5 |
| Striatum | 9.5 ± 3.0 |
| Cortex | 9.0 ± 2.0 |
| Hypothalamus | 8.5 ± 3.0 |
| Thalamus | 7.5 ± 2.0 |
| Midbrain | 4.0 ± 1.5 |
| Cerebellum | 3.5 ± 1.0 |
| Medulla/pons | 2.0 ± 0.5 |

Regions from fresh rat brains were dissected on ice and weighed, and membranes were prepared as described in the text. Binding assays were performed in triplicate using membranes from each brain region and 1.0 nM [$^{125}$I-Tyr$^4$]BN. The mean and standard error of the mean of four separate experiments are indicated.

¶ Bombesin (<Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$), ranatensin (<Glu-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH$_2$), and litorin (<Glu-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH$_2$) have a common CONH$_2$-terminal octapeptide except for position 13 of BN.

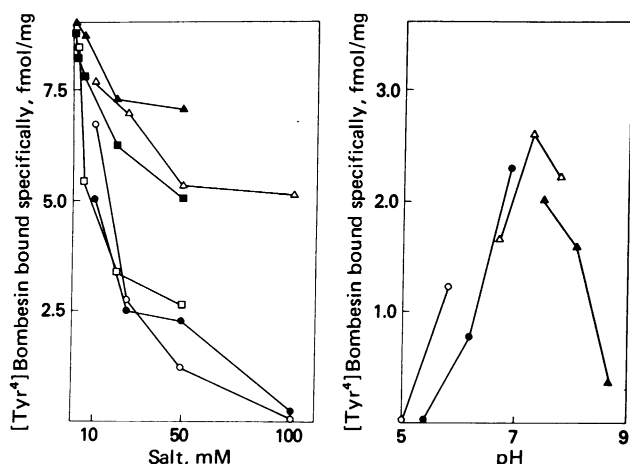


FIG. 6. Binding as a function of salt concentration and pH. (*Left*) Membranes were incubated with [$^{125}$I-Tyr$^4$]BN (0.42 nM) for 24 min in the presence of various concentrations of NaCl (○), KCl (●), $CaCl_2$ (□), $MnCl_2$ (■), LiCl (△), and $MgCl_2$ (▲). (*Right*) Unbuffered membranes were incubated with [$^{125}$I-Tyr$^4$]BN (0.2 nM) for 30 min in the presence of 25 mM acetic acid/acetate (○), Tris/acetate (●), Tris/HCl (▲), and 8.3 mM sodium phosphate (△) at the pH values indicated.

greater in the $P_2$ than $P_1$ fraction as well as the B than C fraction (data not shown). Because Gray and Whittaker (14) observed that the $P_2$ fraction contains crude microsomes and fraction B is composed of synaptosomes, whereas the $P_1$ fraction contains nuclear debris and the C fraction is composed of mitochondria, we conclude that the receptor studies here is associated with membranes derived from nerve terminals.

The effects of various salts and pH were investigated. Fig. 6 *left* shows that specific binding was reduced half maximally when 10 mM NaCl, KCl, or $CaCl_2$ was present. Little, if any, specific binding was detected in the presence of 100 mM NaCl or KCl. In contrast, 50 mM $MgCl_2$ reduced specific binding by only 25%. LiCl and $MnCl_2$ were more potent than $MgCl_2$ but less potent than NaCl, KCl, or $CaCl_2$. Fig. 6 *right* shows that specific BN binding was maximal at neutral pH values, whereas low pH values (<5.5) or high pH values (>8.6) little, if any, specific binding was detected.

Specific binding was equivalent at 4°C and 25°C; however, radiolabeled peptide was degraded more rapidly at the higher temperature (data not shown). In order to minimize proteolysis of tracer, we routinely conducted our assays at 4°C in the presence of bacitracin at 2 μg/ml.

## DISCUSSION

This study indicates that rat brain homogenate binds a radiolabeled BN analogue with high affinity. The binding is specific, saturable (3.8 pmol of sites/g wet tissue), and reversible. [$^{125}$I-Tyr$^4$]BN binds with high affinity to a single class of noninteracting sites. The dissociation constants calculated from equilibrium binding studies ($K_D$ = 5.5 nM), competition studies ($K_D$ = 4 nM), and kinetic binding studies ($K_D$ = 0.9 nM) show good agreement. Binding is maximal at physiological pH values; however, high concentrations of salts, particularly NaCl, greatly reduce the affinity of the receptor for [$^{125}$I-Tyr$^4$]BN. Such salts may impair the conformation of the receptor for radiolabeled BN or reduce electrostatic interactions between the receptor and radiolabeled peptide.

Numerous amino acid residues near the $CONH_2$-terminal may be essential for high-affinity binding. In particular, Table 1 shows that three amino acid residues near the $CONH_2$-terminal (Trp$^8$, Val$^{10}$, and His$^{12}$) may be essential for high-affinity binding and biological potency. Similar constraints on amino acid sequence are required for high-affinity binding of [Tyr$^4$]BN to pancreatic acinar cells (R. T. Jensen, T. W. Moody, C. B. Pert, M. R. Brown, J. Rivier, and J. D. Gardner, unpublished data) and high biological activity of BN in the gastrointestinal tract (16).

Also, Table 1 indicates that litorin and ranatensin, which possess the $CONH_2$-terminal octapeptide of BN with the substitution of Phe for Leu at position 13, and [D-Asn$^6$]BN inhibit the binding of [Tyr$^4$]BN with high affinity but only partially induce hypothermia. These peptides may not be as efficacious as BN (e.g., Asn$^6$ and/or Phe$^{13}$ may be required to fully induce hypothermia) or they may be more rapidly degraded *in vivo* than is BN.

Other putative brain neurotransmitters, brain receptor antagonists, and neuropeptides have low affinity for the BN receptors studied here (Table 1). Similarly, the regional distribution of the BN receptor (Table 2) differs from that of the opiate (17), muscarinic acetylcholine (18), β-adrenergic (19), diazepam (20), or neurotensin receptors (21). These data indicate that [$^{125}$I-Tyr$^4$]BN binds to a unique class of brain receptors, whose density is an order of magnitude less than putative neurotransmitter receptors previously reported.

Because Brown *et al.* (22) observed that the BN-induced hypothermia is reversed by naloxone, they postulated that BN may induce hypothermia via an opiate-dependent step. Table 1 shows that naloxone does not compete with BN on the receptor level. Therefore, low doses of BN may indirectly elicit release of endorphins, resulting in hypothermia (22) as well as analgesia (L. DeWald, J. Rivier, and A. Pert, unpublished data).

The high density of receptor sites in synaptosomes suggests that BN-like peptides may function as neurotransmitters in the central nervous system. Recent evidence indicates, however, that the endogenous peptide extracted from brain is not CNBr sensitive and, therefore, cannot be BN (8). Nonetheless, [Tyr$^4$]BN may still be valuable as a probe for the receptor that mediates the effects of endogenous BN-like peptides, just as [$^3$H]naloxone was useful to study the receptor that mediates the effects of endogenous opiate peptides (17) whose structures (and even existence) were unknown at the time of receptor characterization.

Because high concentrations of immunoreactive material (8) and [$^{125}$I-Tyr$^4$]BN-binding sites (Table 2) have been detected in the hypothalamus, BN-like peptides in this region may play an important role in regulating mammalian body temperature. The physiological significance of [Tyr$^4$]BN receptors clustered in other brain loci such as the hippocampus has yet to be investigated.

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1. Anastasi, A., Erspamer, V. & Bucci, M. (1971) *Experientia* **27**, 166–167.
2. Bertaccini, G., Erspamer, V., Melchiorri, P. & Sopranzi, N. (1974) *Br. J. Pharmacol.* **52**, 219–225.
3. Erspamer, V., Improta, G., Melchiorri, P. & Sopranzi, N. (1974) *Br. J. Pharmacol.* **52**, 227–232.
4. Rivier, C., Rivier, J. & Vale, W. (1978) *Endocrinology* **102**, 519–522.
5. Brown, M., Rivier, J. & Vale, W. (1977) *Science* **196**, 998–1000.
6. Brown, M. R., Rivier, J. & Vale, W. (1977) *Life Sci.* **21**, 1729–1734.
7. Brown, M. R., Rivier, J. E., Wolfe, A. I. & Vale, W. W. (1977) *Endocrinology* **100**, 265A.

8. Villarreal, J., Rivier, J. & Brown, M. (1978) *Endocrinology*, in press.
9. Walsh, J. H. & Holmquist, A. L. (1976) *Gastroenterology* **70**, A90/948.
10. Rivier, J. & Brown, M. (1978) *Biochemistry* **17**, 1766–1771.
11. Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123.
12. Lazarus, L. H., Perrin, M. H. & Brown, M. R. (1977) *J. Biol. Chem.* **252**, 7174–7179.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
14. Gray, E. G. & Whittaker, V. P. (1962) *J. Anat.* **96**, 79–86.
15. Kitabgi, P., Carraway, R., Van Rietschoten, J., Granier, C., Morgat, J. L., Menez, A., Leeman, S. & Freychet, P. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1846–1850.
16. Broccardo, M., Erspamer, G. F., Melchiorri, P., Negri, L. & De Castiglioni, R. (1975) *Br. J. Pharmacol.* **55**, 221–227.
17. Pert, C. B. & Snyder, S. H. (1973) *Science* **179**, 1011–1014.
18. Yamamura, H. I. & Snyder, S. H. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 1725–1729.
19. Bylund, D. B. & Snyder, S. H. (1976) *Mol. Pharmacol.* **12**, 568–580.
20. Braestrup, C. & Squires, R. F. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3805–3809.
21. Uhl, G. R., Bennett, J. P. & Snyder, S. H. (1977) *Brain Res.* **130**, 299–313.
22. Brown, M., Rivier, J. & Vale, W. (1977) *Life Sci.* **20**, 1681–1688.