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AUTORADIOGRAPHIC LOCALIZATION OF (^{125}I-TYR^4)BOMBESIN-BINDING SITES IN RAT BRAIN [1]

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Abstract

The binding of (^{125}I-Tyr^4)bombesin to rat brain slices was investigated. Radiolabeled (Tyr^4)bombesin bound with high affinity (K_d = 4 nM) to a single class of sites (B_{max} = 130 fmol/mg of protein); the ratio of specific to nonspecific binding was 6/1. Also, pharmacology studies indicated that the C-terminal of bombesin was important for the high affinity binding activity. Autoradiographic studies indicated that the (^{125}I-Tyr^4)bombesin-binding sites were discretely distributed in certain gray but not white matter regions of rat brain. Highest grain densities were present in the olfactory bulb and tubercle, nucleus accumbens, suprachiasmatic and periventricular nuclei of the hypothalamus, central medial thalamic nucleus, medial amygdaloid nucleus, hippocampus, dentate gyrus, subiculum, nucleus of the solitary tract, and substantia gelatinosa. Moderate grain densities were present in the parietal cortex, deep layers of the neocortex, rhinal cortex, caudate putamen, stria terminalis, locus ceruleus, parabrachial nucleus, and facial nucleus. Low grain densities were present in the globus pallidus, lateral thalamus, and midbrain. Negligible grain densities were present in the cerebellum, corpus callosum, and all regions treated with 1 μM unlabeled bombesin. The discrete regional distribution of binding suggests that endogenous bombesin-like peptides may function as important regulatory agents in certain brain loci.

Bombesin (BN) represents one class of peptides biologically active in the mammalian brain. BN is a potent satiety agency (Gibbs et al., 1979) and hypothermic agent in cold-exposed rats (Brown et al., 1977a) after central injection. Also, BN is a potent hyperglycemic agent (Brown et al., 1977b) and causes grooming in rats (Pert et al., 1980). In addition, BN increases growth hormone and prolactin secretion from the anterior pituitary after injection into the rat (Rivier et al., 1978). These biological activities may be mediated by the endogenous BN-like peptides which have been detected in discrete brain regions by radioimmunoassay (Brown et al. 1978; Moody and Pert, 1979). Specifically, high levels of immunoreactive BN are present in the substantia gelatinosa and the nucleus of the solitary tract of the hindbrain and throughout the hypothalamus (Moody et al., 1981b). Because immunoreactive BN is released from rat hypothalamic (Moody et al., 1980) and spinal cord slices (Moody et al., 1981a) by depolarizing stimuli, it is probably stored in intracellular vesicles. When BN-like peptides are released from certain CNS neurons, they may activate specific receptors.

Central receptors for BN-like peptides have been characterized using radiolabeled (Tyr^4)BN, a potent BN analogue (Rivier and Brown, 1978). Using rat brain homogenate, filter-binding (Moody et al., 1978) and centrifugation (Pert et al., 1980) assays were developed. Recently, [^{125}I-Tyr^4]BN was observed to bind with high affinity to rat brain slices (Wolf et al., 1983). Here the kinetics, equilibrium, and pharmacology of radiolabeled (Tyr^4)BN binding to rat brain slices were investigated as well as the discrete regional distribution of binding sites using *in vitro* autoradiographic techniques.

Materials and Methods

BN-like peptides were provided by Dr. J. Rivier, Salk Institute (San Diego, CA). (Tyr^4)BN was iodinated using the chloramine T procedure and purified using gel filtration techniques (Moody et al., 1978). The specific activity of the (^{125}I-Tyr^4)BN was 400 Ci/mmol. Then receptor studies were conducted using rat brain slices. Twelve-micrometer-thick coronal sections of rat brain were cut using a cryostat and were thaw mounted onto coverslips or slides. Sections were air dried and incubated in assay buffer composed of 130 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 1 mM EGTA, 100 μg/ml of bacitracin, and 0.1% bovine serum albumin in 10 mM HEPES·NaOH, pH 7.4, plus radiolabeled peptide at 22°C in the presence or absence of unlabeled peptide. After incubation, free radiolabeled peptide was removed by two consecutive 4-min washes in buffer at 4°C. Then sections, which contained bound peptide on coverslips, were crushed and assayed for radioactivity using an LKB gamma counter, or sections on slides were apposed to emulsion-coated coverslips as described previously (Young and Kuhar, 1979). Protein was determined as described by Lowry et al. (1951).

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Results

Initial binding studies. (^{125}I-Tyr^4)BN bound to coronal rat brain slices with high affinity. Table I shows that radiolabeled (Tyr^4)BN binding was greatest in slices which contain the nucleus accumbens, intermediate in slices which contain the amygdala and hippocampus, and lowest in slices which contain the cerebellum. Because pharmacology studies indicated that (Tyr^4)BN bound with similar affinity in all regions examined, these differences may reflect alterations in receptor densities. Subsequently, binding studies were conducted using telencephalon slices derived from Konig and Klippel (1974) coordinates A7980 to 8920.

Kinetic binding studies. The kinetics of (^{125}I-Tyr^4)BN binding were investigated using coronal slices which contained the nucleus accumbens, olfactory tubercle, and striatum. Using 3 nM (^{125}I-Tyr^4)BN, specific binding increased rapidly the first 60 min, then slowly the next 30 min, and after 90 min, equilibrium was attained (Fig. 1*A*). In contrast, nonspecific binding in the presence of 1 μM unlabeled BN increased slowly throughout the time course of the experiment. If rat brain slices were incubated with (^{125}I-Tyr^4)BN for 90 min, specific binding was reversed slowly by the addition of 1 μM unlabeled BN (Fig. 2*A*). After 40 min, half of the radiolabeled (Tyr^4)BN bound specifically was reversed. Figure 2*B* shows that a replot of the dissociation binding data was linear, and a dissociation rate constant (k_{-1} = 1.7 × 10^{-2} min^{-1}) was calculated (Bennet, 1978). Also, a replot of the specific association data was linear (Fig. 1*B*), and an association rate constant (k_1 = 4.3 × 10^6 M^{-1} min^{-1}) was determined. Based on these kinetic data, an equilibrium dissociation constant ($K_d = k_{-1}/k_1$ = 4 nM) was calculated.

TABLE I

Regional distribution of (^{125}I-Tyr^4)BN to rat brain slices

Slices were derived from the indicated coordinates and binding studies performed as described under "Materials and Methods." The density of specific binding sites was determined using 1.0 nM (^{125}I-Tyr^4)BN in triplicate. The experiment was repeated three times and the mean value ± SE is indicated.

Region	Coordinates	Density (fmol/mg of protein)
Nucleus accumbens	A7980–8920	30.5 ± 0.9
Amygdala/Hypothalamus	A4380–4890	15.8 ± 1.3
Hippocampus/Midbrain	A1800–2200	10.7 ± 1.0
Cerebellum/Hindbrain	P3000	0.5 ± 0.5

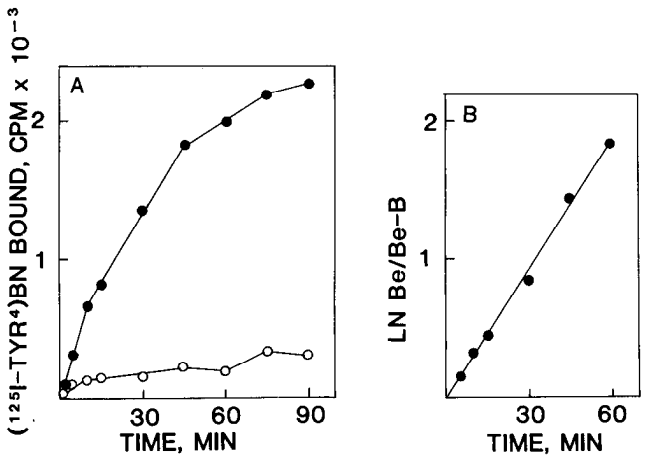

Figure 1. A, Association of (^{125}I-Tyr^4)BN to rat brain slices. Radiolabeled (Tyr^4)BN (3 nM) was incubated with coverslip-mounted 12-μm coronal slices of rat brain as described under "Materials and Methods." Specific (●) and nonspecific (○) binding were determined as a function of time after addition of (^{125}I-Tyr^4)BN. The *lines* were drawn point to point. *B*, Replot of the specific binding data where *B* represents the amount bound as a function of time and *Be* represents the amount bound at equilibrium.

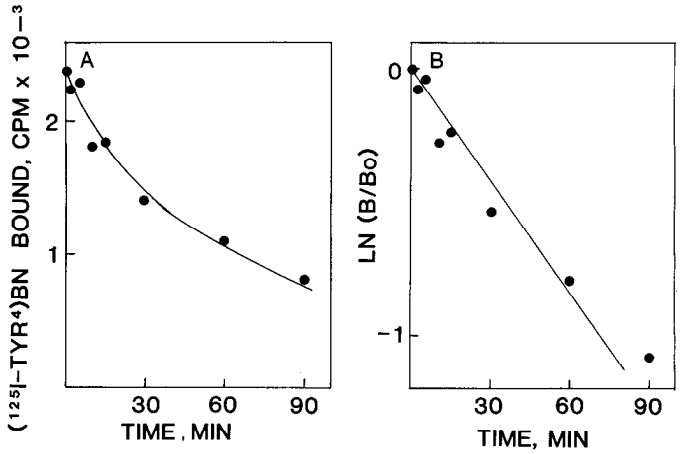

Figure 2. Dissociation of radiolabeled (Tyr^4)BN. (^{125}I-Tyr^4)BN was incubated with brain sections for 1 hr, and then 1 μM unlabeled BN was added. *A*, Amount of radiolabeled peptide bound as a function of time after addition of competitor. *B*, Replot of the specific binding data where Bo represents the amount bound prior to and B represents the amount bound as a function of time after addition of competitor. The *lines* drawn represent the best fit assuming a single class of sites.

Equilibrium binding studies. The concentration dependence of (^{125}I-Tyr^4)BN binding was ivestigated. Total binding increased greatly at low concentrations of radiolabeled (Tyr^4)BN; total as well as nonspecific binding increased slightly at high concentrations of (^{125}I-Tyr^4)BN (Fig. 3*A*). Specific binding was saturable. Using a double reciprocal plot, the specific binding data were linear (Fig. 3B). These data indicated that (^{125}I-Tyr^4)BN bound with high affinity (K_d = 6 nM) to a single class of sites (B_{max} = 130 fmol/mg of protein).

Pharmacology binding data. The ability of various peptides to inhibit (^{125}I-Tyr^4)BN binding was investigated. Figure 4 shows that BN and structurally related peptides inhibited radiolabeled (Tyr^4)BN binding in a concentration-dependent manner. The concentration of peptide required to inhibit 50% of the specific (^{125}I-Tyr^4)BN binding (IC_{50}) was 5 nM for BN and (Tyr^4)BN. In comparison, (Ac-Gly^5)BN, which lacks the N-terminal tetrapeptide, and BN-OH, in which the C-terminal is deamidated, had IC_{50} values of 50 and 2000 nM respectively. In contrast, (D-Trp^8)BN and (D-Val^{10})BN were not potent inhibitors of (^{125}I-Tyr^4)BN binding. These data indicate that amino acid residues near the N-terminal of BN may be modified or deleted with minimal loss in receptor binding activity, whereas certain amino acid residues near the C-terminal of BN are essential.

Regional distribution of binding sites. In vitro autoradiographic studies were conducted using the method of Young and Kuhar (1979). These studies indicated a widespread but varied distribution of radiolabeled (Tyr^4)BN-binding sites in certain gray matter nuclei. Control slides which were incubated with 1 μM unlabeled BN showed minimal binding, and this binding was randomly distributed throughout gray and white matter areas.

The density of radiolabeled (Tyr^4)BN-binding sites was investigated in various coronal sections derived from rat brain. In the cervical spinal cord, high grain densities were present in the substantia gelatinosa but not other regions (Fig. 5). In the medulla, high grain densities were found in the substantia

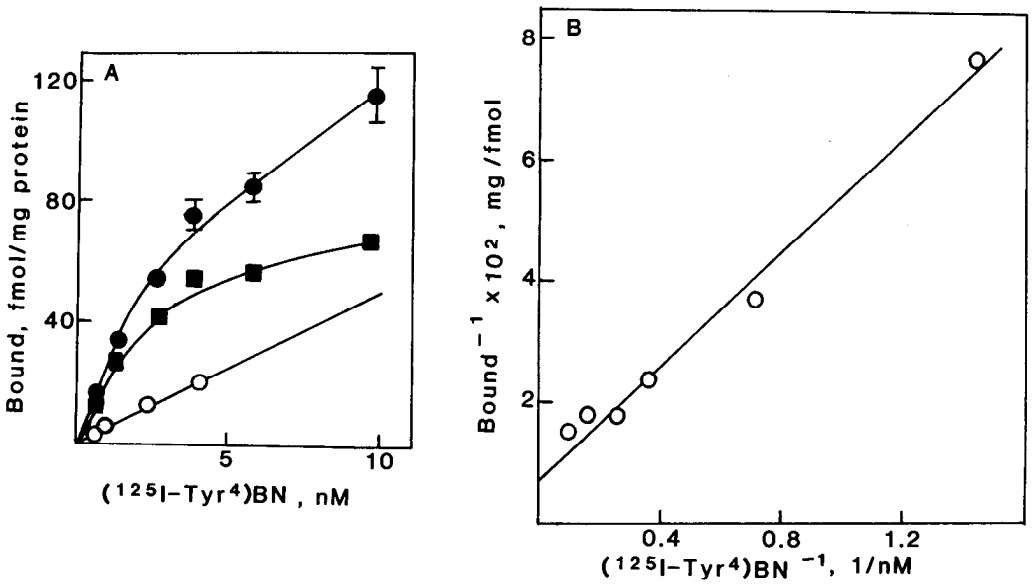

Figure 3. Binding of (^{125}I-Tyr^4)BN as a function of radiolabeled peptide concentration. *A*, Total (●) and nonspecific (○) binding were determined in triplicate at equilibrium. The difference between the two represents specific binding (■). *B*, Double reciprocal replot of the specific binding data. The *line* drawn represents the best fit assuming a single class of sites.

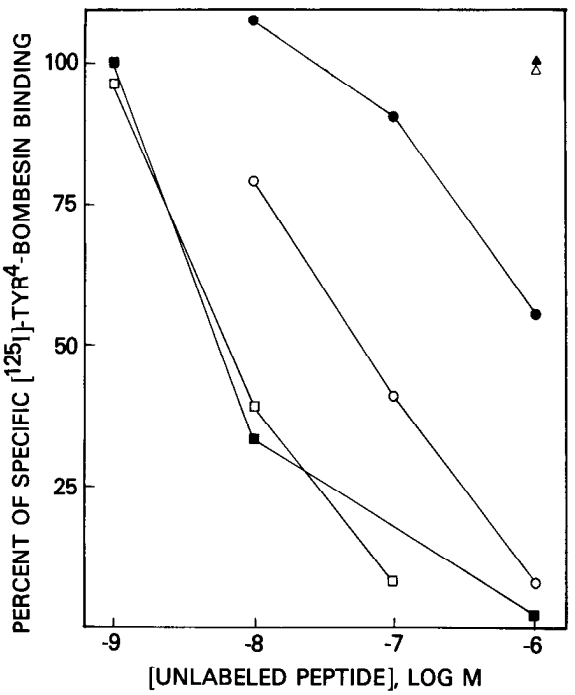

Figure 4. Pharmacology of radiolabeled (Tyr^4)BN-binding sites. The percentage of (^{125}I-Tyr^4)BN bound specifically is plotted as a function of unlabeled peptide concentration for BN (■), (Tyr^4)BN (□), (Ac-Gly^5)BN (○), BN-OH (●), (D-Trp^8)BN (▲), and (D-Val^{10})BN (△). The *lines* were drawn point to point.

gelatinosa of the trigeminal nucleus and the nucleus of the solitary tract (Fig. 6), and moderate densities were observed in the retrofacial nucleus (Fig. 7). Low densities were found in the nucleus of the spinal tract of the trigeminal nerve and the area postrema, whereas the cerebellum was virtually devoid of grains. In the nucleus of the solitary tract, highest densities were found in the caudal areas with lower densities in the rostral parts of the medulla. Low grain densities were present in the medial vestibular nucleus. In the pons, moderate grain densities were found in the floor of the fourth ventricle, locus ceruleus, and the parabrachial nucleus (Fig. 8). The nucleus of the mesencephalic tract of the trigeminal nerve, superior cerebellar peduncle, and cerebellum were devoid of grains. The dorsal tegmental nucleus had low grain densities as did the midbrain. In particular, low grain densities were found in the central gray, the substantia nigra zona reticulata, the dorsal interpeduncular nucleus and adjacent ventral tegmental area, and the superficial gray layer of the superior colliculus (Fig. 9).

In the forebrain high densities tended to be along the midline (Figs. 10 and 11). In the diencephalon the central medial thalamic nucleus had high grain densities, as did the paraventricular thalamic nucleus, the intralaminar nucleus of the thalamus, and the rhomboid thalamic nucleus. The lateral thalamus had few grains, if any. Moderate grain densities were present throughout the hypothalamus, but the periventricular nucleus and the suprachiasmatic nucleus had elevated levels (Fig. 11). In the basal ganglia, the caudate putamen had low to moderate grain densities with the exception of the ventral caudate putamen, which had high levels. The globus pallidus had few if any grains (Figs. 11 and 12). Parts of the limbic system had elevated grain densities. In particular, the nucleus accumbens, olfactory tubercle, stria terminalis, rhinal cortex, and medial and central amygdaloid nucleus had moderate to high levels (Figs. 11 and 13). Throughout the hippocampus, high densities were found, in particular in the polymorphic layer of the dentate gyrus, subiculum, the oriens layer, stratum radiatum, and the molecular layer (Figs. 9 and 10). In these regions the grain densities were highest in the dendritic regions furthest from cell bodies. In the cerebral cortex, moderate grain densities were present (Figs. 10 and 13). In particular, deep layers such as layers V and VI had higher levels than the more superficial layers. Layer IV had lower levels. In the parietal cortex (Figs. 11 and 13), variations in grain densities resulted in a columnar appearance. In the olfactory bulb the grain densities were extremely high. In particular, the granule call layer and glomerular layer were the highest, with the external plexiform layer being somewhat lower (Figs. 14 and 15).

Discussion

The discrete regional distribution of receptors for BN-like peptides has not been investigated extensively due to the low receptor densities. Using a filter-binding assay which required 30 mg of wet tissue homogenate (1.5 mg of protein), we determined that binding sites for (^{125}I-Tyr^4)BN were enriched in certain regions, such as the hippocampus, relative to other areas, such as the cerebellum (Moody et al., 1978). Using a

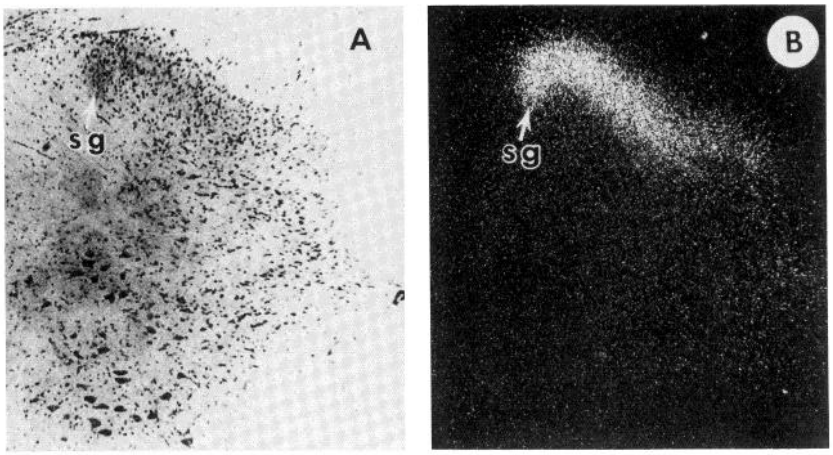

Figure 5. Distribution of (Tyr⁴)BN-binding sites in the rat cervical spinal cord. *A*, Brightfield micrograph shows one side of the spinal gray matter, with an *arrow* pointing to the substantia gelatinosa (*sg*) in the dorsal horn. The autoradiographic grains are not visible. *B*, darkfield micrograph where the tissue is not visible but the autoradiographic grains are localized exclusively to the substantia gelatinosa. Adjacent serial slices incubated with 1 μM unlabeled BN did not have elevated grain densities in the substantia gelatinosa and instead the levels were low and uniform over the entire section. *A* and *B* are micrographs of the same slide.

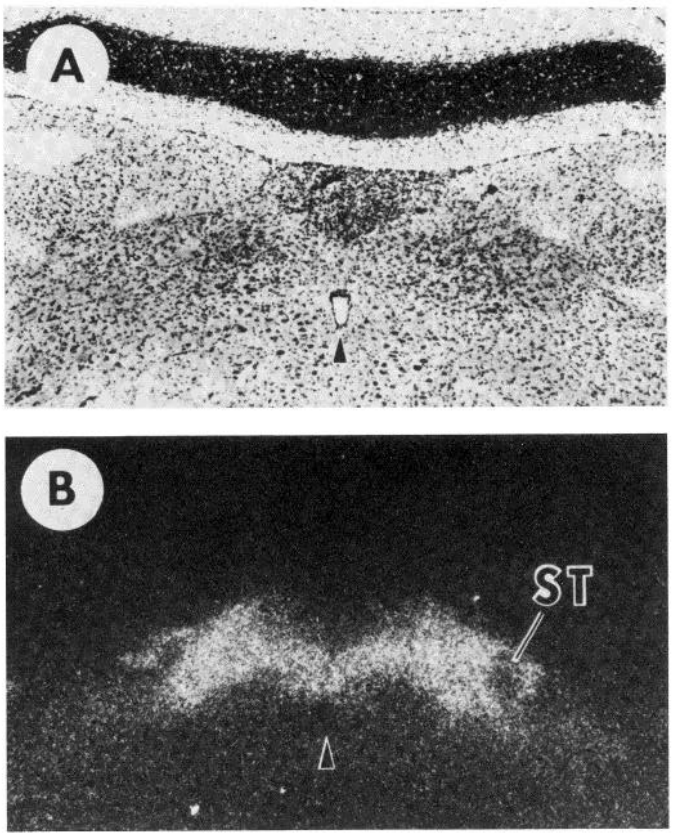

Figure 6. Binding sites in the nuclei of the solitary tract. *A*, Brightfield micrograph shows the central canal (*arrowhead*) and the nucleus of the solitary tract (*ST*) located dorsolaterally. The area postrema can be seen in the midline below the cerebellum. *B*, The darkfield micrograph shows the autoradiographic grain enrichment over the nuclei of the solitary tract. The lateral parts of the solitary tract have low grain densities.

centrifugation assay which required 10 mg of wet tissue homogenate, we found that binding sites for radiolabeled (Tyr⁴)BN were high in the amygdala, hippocampus, and hypothalamus, intermediate in the striatum as well as the thalamus, and low in the cerebellum. Subsequently, using a slice-binding assay which required 0.2 mg of protein, we observed that binding sites for BN-like peptides in the rat forebrain were abundant in certain gray matter areas such as the olfactory bulb, nucleus accumbens, hippocampus, and amygdala, relative to white matter areas such as the corpus callosum. Here the binding properties of (^{125}I-Tyr⁴)BN were investigated using rat brain slices. Also, the regional distribution of binding sites for radiolabeled (Tyr⁴)BN was investigated throughout the rat brain using *in vitro* autoradiographic techniques.

As coronal slices derived from the striatum, nucleus accumbens, and the olfactory tubercle bound (^{125}I-Tyr⁴)BN best (Table I), these slices were used for subsequent binding experiments. Kinetic studies indicated that radiolabeled (Tyr⁴)BN bound in a time-dependent manner and the ratio of specific to nonspecific binding was routinely 6/1. Equilibrium studies yielded a linear double reciprocal replot which indicated that binding of (^{125}I-Tyr⁴)BN to rat brain slices was noncooperative. The dissociation constants based on the kinetic, equilibrium, and pharmacology data (4, 6, and 5 nM) were in good agreement, suggesting that (Tyr⁴)BN binds with high affinity to a single class of sites. Previously, (Tyr⁴)BN was observed to bind with high affinity (K_d = 4 nM) to rat brain homogenate (Moody et al., 1978).

Also, the pharmacology of binding was investigated. Figure 4 shows that BN-like peptides competed for specific (^{125}I-Tyr⁴)BN-binding sites. The order of peptide potency was (Tyr⁴)BN = BN > (Ac-Gly⁵)BN > BN-OH > (D-Trp⁸)BN or (D-Val¹⁰)BN. Peptides structurally unrealted to BN did not compete for the (^{125}I-Tyr⁴)BN-binding sites. Previously, we observed that the C-terminal of BN was essential for binding to rat brain homogenate and for the ability of BN to induce hypothermia after central injection (Moody et al., 1982). Thus, conservative substitutions near the N-terminal of BN, such as Lys for Arg³, Tyr for Leu⁴, or D-Ala for Gly⁵, do not dramatically affect receptor binding or biological activity. In contrast substitution of D- for the natural L-amino acid residues at positions 8, 10, 13, or 14 reduced receptor binding and biological activity by more than two orders of magnitude. Also, the 27 amino acid peptide gastrin-releasing peptide (GRP), which has a C-terminal heptapeptide identical to that of BN (McDonald et al., 1979), inhibits specific (^{125}I-Tyr⁴)BN binding with high affinity (IC_{50} = 20 nM; S. Wolf and T. Moody, unpublished observation).

Receptors for BN-like peptides are widely distributed throughout the CNS. Here, high concentrations of (Tyr⁴)BN-binding sites were present in the substantia gelatinosa of the cervical spinal cord. Previously, high concentrations of BN-like peptides were detected in the dorsal but not the ventral horn of the spinal cord (Moody et al., 1981a; Panula et al., 1982, 1983). Thus, high concentrations of BN-like peptides and their receptors are present in laminae I and II of the spinal cord. Also, high concentrations of receptors for BN-like pep-

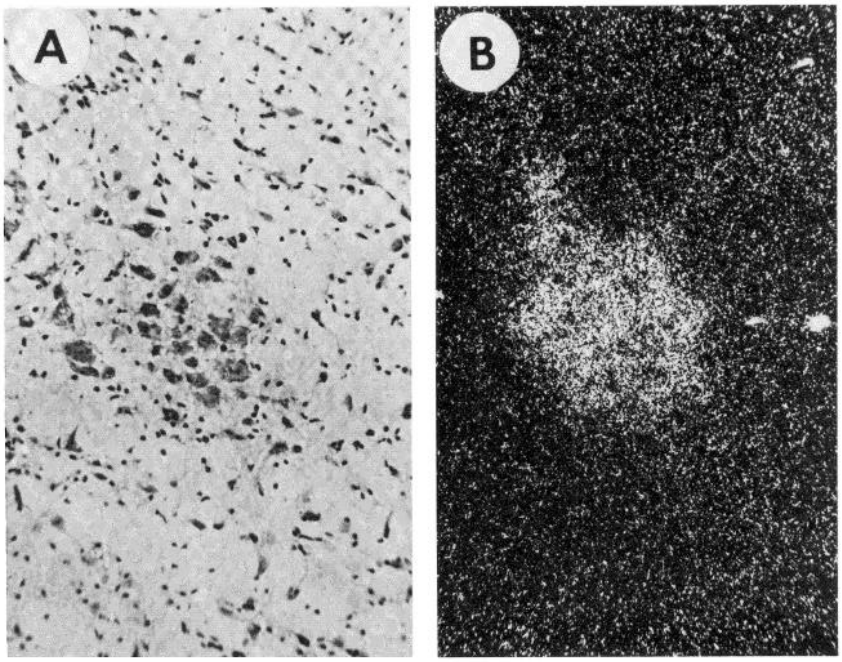

Figure 7. High density of (Tyr^4)BN-binding sites in the retrofacial nucleus. *A*, Brightfield micrograph; *B*, darkfield micrograph.

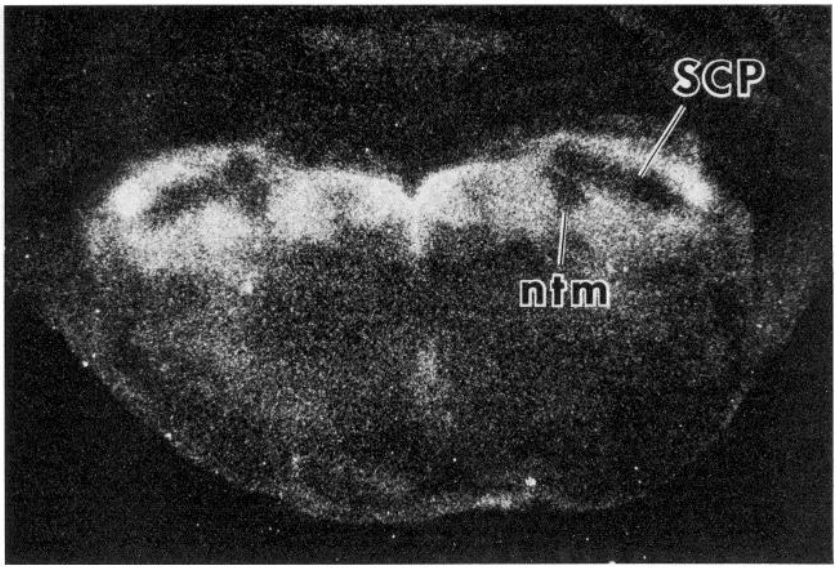

Figure 8. Binding sites in the pons. The darkfield micrograph reveals high grain densities in the floor of the fourth ventricle and the parabrachial nuclei. Grains were not elevated in the superior cerebellar peduncle (*SCP*) or in the nucleus of the mesencephalic tract of the trigeminal nerve (*ntm*).

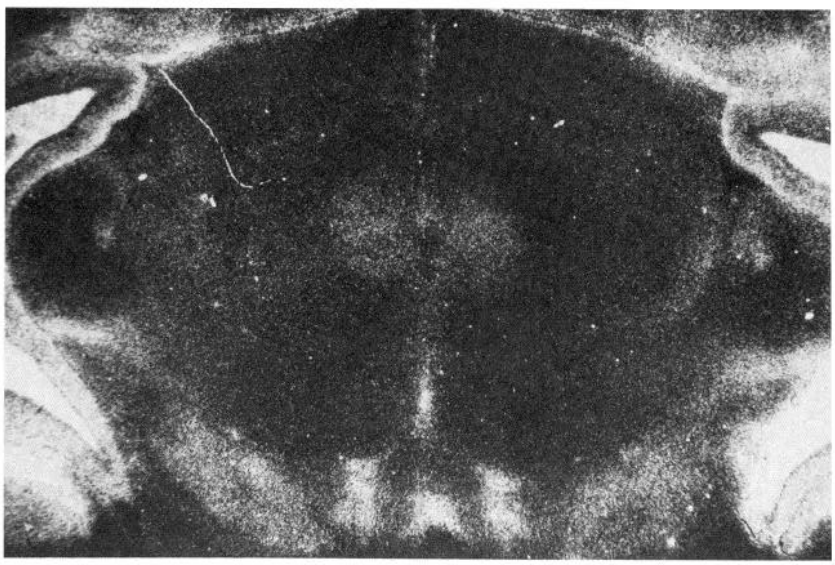

Figure 9. Binding sites in the midbrain.

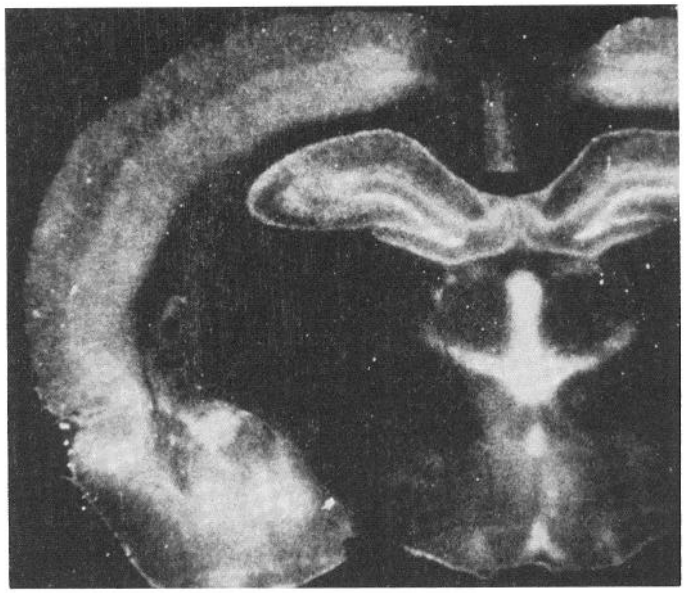

Figure 10. (Tyr^4)BN-binding sites in the diencephalon. The darkfield micrograph corresponds to Konig and Klippel (1976) coordinates A4110.

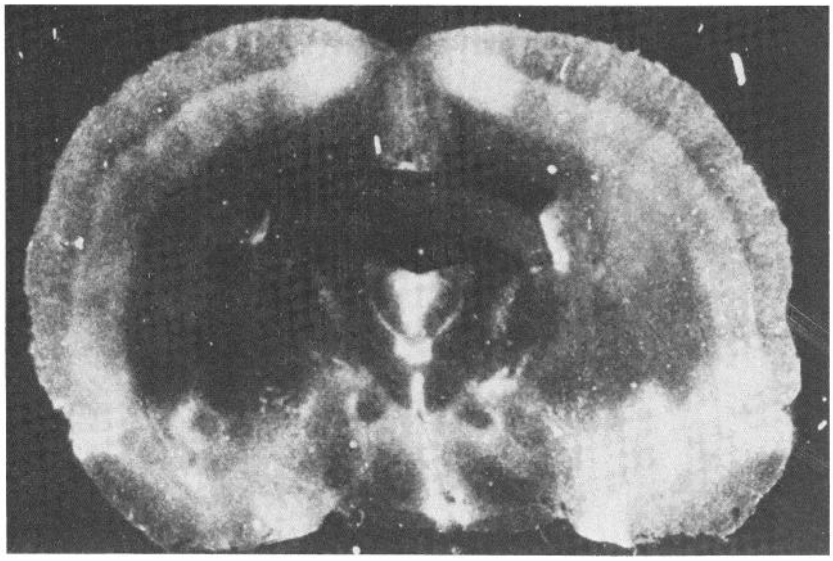

Figure 11. Distribution of binding sites. The darkfield micrograph corresponds to Konig and Klippel (1976) coordinates A5780.

tides were detected in certain hindbrain regions such as the nucleus of the solitary tract. Previously, this region was demonstrated to have high concentrations of BN-like peptides (Moody et al., 1981b, Roth et al., 1982). As BN is a hypertensive agent (Erspamer et al., 1975), BN-like peptides in the solitary tract may play a role in the central regulation of blood pressure.

Moderate grain densities were present in the retrofacial nucleus, locus ceruleus, and parabrachial nucleus, but low grain densities were present in the central gray of the midbrain. This is surprising as BN is an analgesic agent after direct injection into this brain region (Pert et al., 1980), and moderate concentations of immunoreactive BN are present in the central gray (Moody et al., 1981b).

Moderate grain densities were present in most hypothalamic regions. Previously, BN was shown to be biologically active in certain hypothalamic regions. Direct injection of BN into the anterior hypothalamic area but not other brain regions results in hypothermia (Pittman et al., 1980). Also, injection of BN reverses the gastric acid secretion induced by lesions of the lateral hypothalamus (Tache et al. 1982). Because the lateral hypothalamic area and ventromedial hypothalamic nucleus play a role in the central regulation of feeding (Morley, 1980) and BN is a potent satiety agent (Gibbs et al., 1979), some of the hypothalamic receptors for BN-like peptides may modulate appetite. Moderate densities of BN-like peptides are present in most hypothalamic regions (Moody et al., 1981b), but high levels are present in the suprachiasmatic nucleus (Roth et al., 1982). Also, high receptor densities are present in the suprachiasmatic and periventricular nuclei of the hypothalamus.

Certain parts of the limbic system were characterized by moderate to high grain densities. In particular, the nucleus accumbens, olfactory tubercle, caudate putamen, and central amygdaloid nucleus had moderate to high levels of (Tyr^4)BN-binding sites. Because BN alters dopamine turnover in the nucleus accumbens and olfactory tubercle (Widerlov et al., 1984), receptors in the striatum and olfactory tubercle may be present on dopamine-containing neurons.

In the cortex, moderate grain densities were present in the rhinal cortex, neocortex, and parietal cortex. Throughout the cortex, layers V and VI had higher grain densities than did the

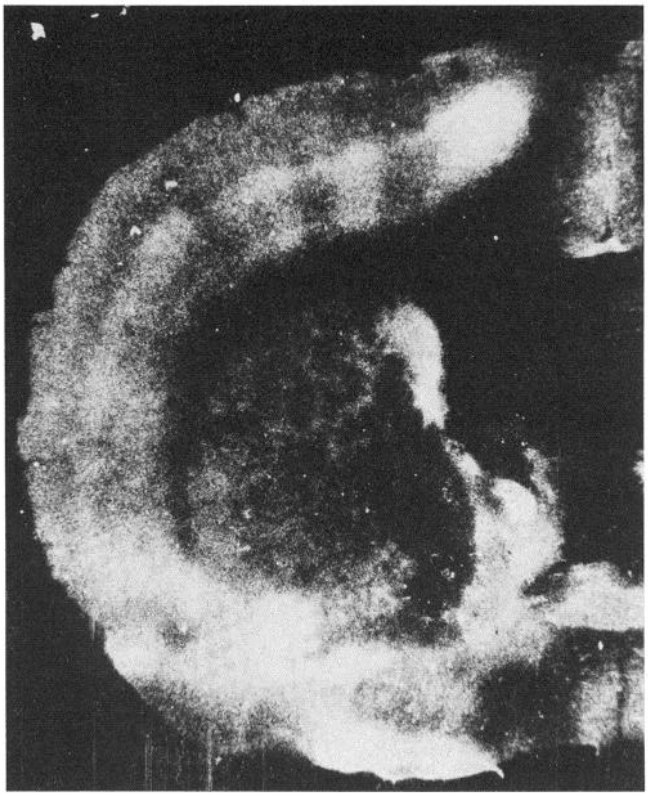

Figure 12. Distribution of binding sites. The darkfield micrograph shows the level A6860 according to Konig and Klippel (1976).

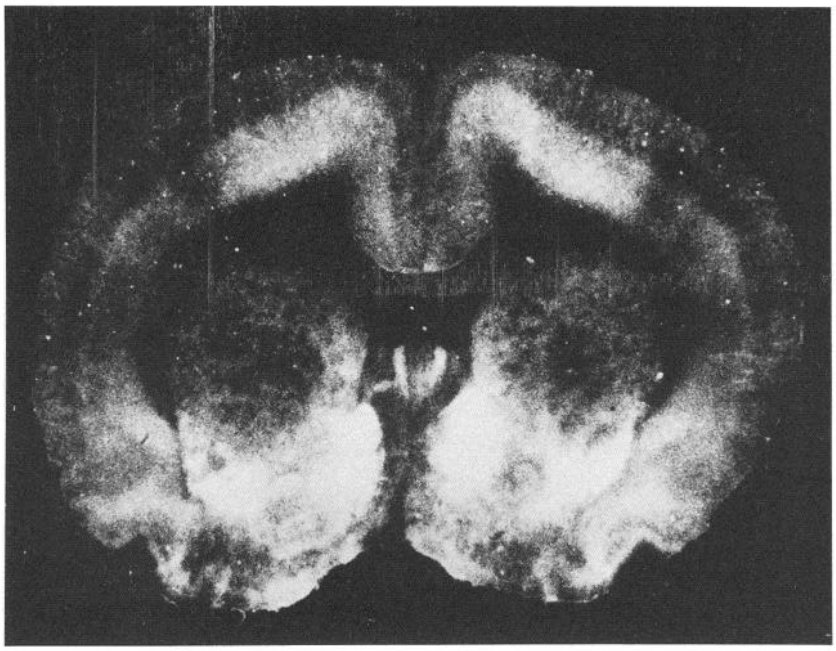

Figure 13. Distribution of (Tyr^4)BN-binding sites at A9650 according to Konig and Klippel (1976).

superficial layers. Surprisingly, the grain densities were lower in layer IV, which is enriched in neurotransmitter, drug, and neuropeptide receptors (Kuhar, 1981). The concentration of BN-like peptides, however, is low throughout the cortex (Moody et al., 1981b).

In summary, our data indicated that (^{125}I-Tyr^4)BN-binding sites are discretely distributed in certain gray but not white matter regions of the rat brain. The density of binding sites varies by approximately two orders of magnitude in high areas such as the nucleus accumbens relative to low areas such as the cerebellum. In particular, moderate to high grain densities were present in certain regions near the ventricles and central canal such as the lateral septum, bed nucleus of the stria terminalis, periventricular nucleus of the hypothalamus, locus ceruleus,

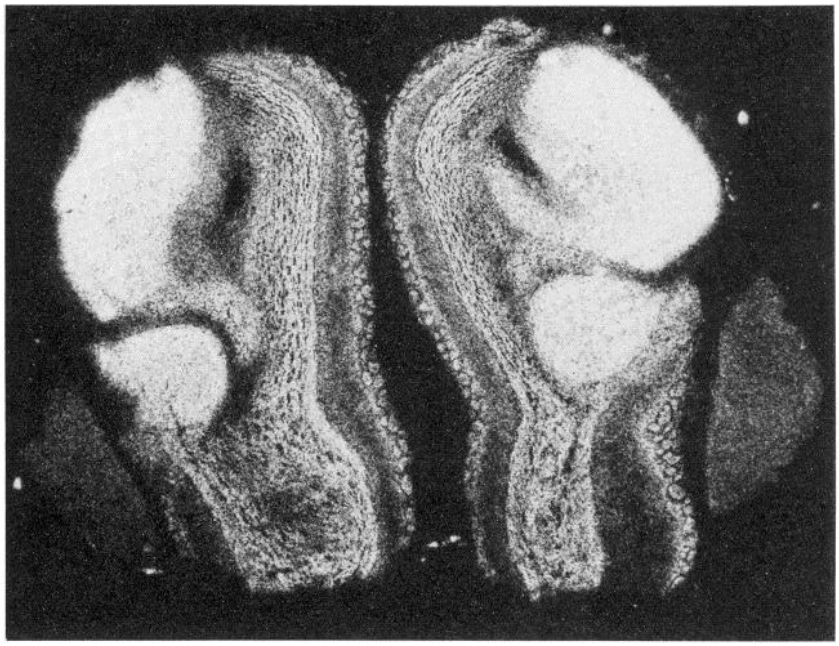

Figure 14. Distribution of binding sites at A12130.

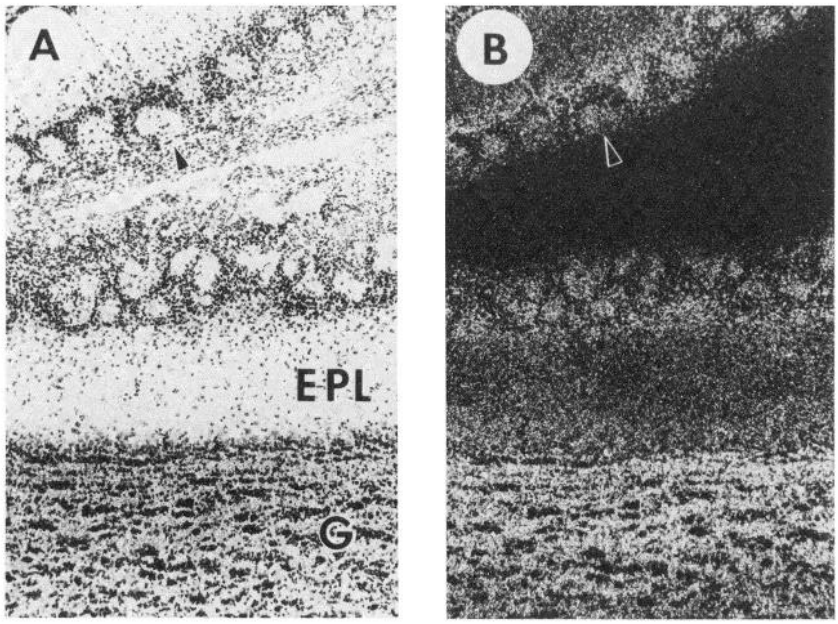

Figure 15. Distribution of (Tyr^4)BN-binding sites in the olfactory bulb. *A*, Brightfield micrograph. *EPL*, external plexiform layer; *G*, granule cell layer. *B*, darkfield micrograph where high grain densities are present in the granule cell layer, the external plexiform layer, and the glomeruli (*arrowhead*).

nucleus of the solitary tract, and substantia gelatinosa. The discrete distribution of binding sites suggests that BN-like peptides may function as regulators of neural activity.

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