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A ROLE FOR BOMBESIN IN SENSORY PROCESSING IN THE SPINAL CORD[1]

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Abstract

Bombesin (BN)-containing neuronal processes were demonstrated in laminae I and II of the dorsal horn of the cat, rat, and mouse spinal cord by immunocytochemistry and radioimmunoassay. Dorsal rhizotomy in the cat resulted in a marked decrease in BN immunoreactivity in the dorsal horn indicating that BN is contained in primary sensory afferents. BN-binding sites were also localized in superficial laminae of the dorsal horn. The presence of both BN and BN-binding sites in the dorsal horn suggested that BN may be involved in sensory processing in the spinal cord. Consistent with this hypothesis, it was demonstrated that an injection of BN into the spinal cord caused a biting and scratching response indicative of sensory stimulation. The effect was similar to that observed after injection of substance P into the cord with the exception that the BN effect lasted about 100 times longer than that induced by substance P. Taken together, these data indicate that BN may be a neurotransmitter of primary sensory afferents to the spinal cord.

Bombesin (BN) is a tetradecapeptide amide originally isolated and sequenced from the skin of the frog *Bombina bombina*. More recently, however, BN immunoreactivity has been identified in the mammalian central nervous system (Brown et al., 1978; Moody and Pert, 1979; Walsh et al., 1979; Moody et al., 1980, 1981a, c; Roth et al., 1982), sympathetic nervous system (Schultzberg, 1983), gastrointestinal tract (Polak et al., 1976; Dockray et al., 1979; Walsh et al., 1979; Buffa et al., 1982; Furness et al., 1982), human fetal lung (Wharton et al., 1978; Track and Cutz, 1982; Tsutsumi et al., 1983), adult lung (Cutz et al., 1981; Polak and Bloom, 1982; Track and Cutz, 1982; Tsutsumi et al., 1983), and oat cell carcinoma of the lung (Wood et al., 1981, Erisman et al., 1982; Sorensen et al., 1982; Moody et al., 1981b, 1983; Tsutsumi et al., 1983). The precise structure of mammalian BN is currently unknown, but it has been suggested that the mammalian form of BN may be gastrin-releasing peptide (GRP), a recently identified peptide that has structural and biological similarities to BN (McDonald et al., 1979, 1983; Brown et al., 1980; Erspamer et al., 1981; Yanaihara et al., 1981), or neuromedin B, a recently identified BN-like peptide (Minamino et al., 1983).

Although the function of BN in anurans is largely unknown, a number of pharmacological actions have been identified in mammals. Peripheral actions of BN include stimulatory effects on gut and uterine smooth muscle contraction (Bertaccini et al., 1974a, b; Caprilli et al., 1975; Barthio et al., 1982; Davison, 1983), stimulation of release of gastrin and cholecystokinin from gut (Bertacinni et al., 1974a, b; Erspamer et al., 1974; Hirschowitz and Molina, 1983), growth hormone from the pituitary gland (Bicknell and Chapman, 1983), amylase and trypsinogen from the pancreas (Jensen et al., 1978; Andriulli et al., 1983), and also induction of tumor growth (Rosengurt and Sinnett-Smith, 1983). Central pharmacological actions of BN which have been reported are modulation of anterior pituitary hormone release (Rivier et al., 1978; Tache et al., 1979; Westendorf and Schonbrunn, 1982); inhibition of gastric acid release and stimulation of gastric mucus secretion (Tache et al., 1981; Tache, 1982; Tache and Collu, 1982); stimulation of neuronal electrical activity (Phillis and Limacher, 1974; Phillis and Kirkpatrick, 1979; Suzue et al., 1981; Tartara et al., 1982); stimulation of sympathetic outflow (Tache and Brown, 1982); and induction of analgesia (Pert et al., 1980), hyperglycemia (Brown and Vale, 1976; Brown et al., 1979), poikilothermia (Brown et al., 1977; Pittman et al., 1980; Tache et al., 1980), and a stereotypic scratching behavior (Brown et al., 1977). BN may also induce a number of pharmacological effects by both peripheral and central actions. The best examples of this are the central and peripheral actions of BN on induction of satiety (Gibbs et al., 1979; Martin and Gibbs, 1980; Baile and Della-

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Ferra, 1981; Gibbs and Fauser, 1981; Morley and Levine, 1981; Smith et al., 1981; Stein and Woods, 1981; Kulkosky et al., 1982; West et al., 1982; Hsaio and Spencer, 1983) and gastric emptying (Tepperman and Evered, 1980; Tache and Brown, 1982; Hsaio and Spencer, 1983).

Recent results have demonstrated that, within the central nervous system, the dorsal horn of the spinal cord has relatively high concentrations of BN-immunoreactive peptides (Moody et al., 1981c; Massari et al., 1983). The fact that some of the BN in the dorsal horn is derived from primary sensory afferents (Fuxe et al., 1983; Massari et al., 1983; Panula et al., 1983) suggested the possibility that BN may play a role in the transmission of sensory information to the central nervous system. Alternatively, other sources of BN to the dorsal horn might modulate neural transmission of primary sensory afferents. In the present study, we investigated the physiology and anatomy of BN and BN receptors in the spinal cord. It was found that BN and BN receptors were localized in superficial laminae of the dorsal horn of the spinal cord and that microinjection of BN into the cord induced a vigorous scratching response indicative of sensory stimulation.

Materials and Methods

Dorsal rhizotomy. Dorsal rhizotomy was performed 10 days before cats were sacrificed, essentially as described previously (Massari et al., 1983). Three cats were anesthetized with pentobarbital (35 mg/kg) and operated on using sterile procedures. The dorsal roots of L1 through S3 were exposed by performing a hemilaminectomy on the right lumbosacral vertebrae. The dorsal roots were cut extradurally using a small pair of dissecting scissors under a dissecting microscope, taking care to leave the dorsal radicular arteries and ventral root intact. The area was then gently packed in Gelfoam and sutured in layers. All cats received 600,000 units of Bicillin, injected intramuscularly, postoperatively.

Radioimmunoassay. Spinal cords from unoperated cats, rats, and mice were removed and frozen on dry ice and stored at −70°C. Cervical spinal cord slices of 300 to 500 μm in thickness were cut in a cryostat at −10°C. Sections were then microdissected as described previously (Moody et al., 1981a, c; Massari et al., 1983). Samples were dissected into 2 N acetic acid on ice. The acidified tissues were boiled for 10 min and homogenized by sonication using a Kontes cell disrupter. An aliquot was removed for protein determination (Lowry et al., 1951).

BN immunoreactivity was measured as described previously (Moody et al., 1983). Homogenates were centrifuged at 10,000 × *g* for 10 min, and the supernatants were removed and dried by vacuum centrifugation (Savant Inc, Hicksville, NY). The samples were resuspended in radioimmunoassay (RIA) buffer which consisted of phosphate-buffered saline (PBS), pH 7.4, containing 0.25% bovine serum albumin. A rabbit anti-BN serum at 1:100,000 dilution was incubated with sample or standard for 1 hr at 4°C. Then 5,000 cpm (about 4 fmol) of [^{125}I-Tyr^4] BN were added, and the solution was incubated for 16 hr at 4°C; the total volume was 0.4 ml. After incubation, 200 μl of normal rabbit serum were added at 1:200 dilution followed by 100 μl of goat anti-rabbit serum at 1:20 dilution. After 30 min, 200 μl of 12% polyethylene glycol were added to enhance precipitation, and the samples were centrifuged at 1,000 × *g* for 20 min. The supernatant was removed and the pellet was counted for radioactivity in an LKB gamma counter (LKB Instruments, Inc., Gaithersburg, MD).

Immunocytochemistry. Rats and mice were anesthetized and perfused through the ascending aorta with ice-cold PBS containing 0.5% sodium nitrite followed by 200 to 300 ml of ice cold 4% formaldehyde in PBS, pH 7.4. Normal and operated cats were anesthetized and perfused through the abdominal aorta according to the method of Loren et al. (1980) using 2% glyoxylic acid in the preperfusion buffer and 2% paraformaldehyde in the perfusion buffer. The spinal cords were rapidly removed, cut into 3.0-mm sections, and postfixed for 30 min in the same fixative. The tissue slices were rinsed for 48hr in PBS containg 20% sucrose (w/v), frozen on dry ice, cut into 20-μm coronal sections in a cryostat, mounted on chrome-alum-coated slides, and processed for the indirect immunohistochemical procedure of Coons (1958). The antibody used was a monoclonal antibody generated in mice. Sections were incubated overnight in the BN antibody diluted 1:1000 in PBS containing 0.3% Triton X-100 (v/v). The sections were washed three times for 5 min each in PBS with 0.3% Triton X-100 and then incubated for 30 min in fluorescein isothiocyanate-conjugated goat anti-mouse IgG (N. L. Cappel Laboratories, Cochranville, PA), diluted 1:300 in PBS with 0.3% Triton X-100. The sections were washed three times for 5 min each, rinsed in PBS, and mounted in Gelvatol (Monsanto Chemical Co., Indian Orchard, MA). Sections were examined under a Leitz Orthoplan fluorescence microscope equipped with a Ploem illuminator. Preabsorption of BN antisera overnight with synthetic BN eliminated all staining, whereas preabsorption with substance P (SP) had no effect (peptides from Peninsula Laboratories, San Carlos, CA).

Autoradiography. Frozen, unfixed spinal cords from rats, cats, and mice were cut into 20-μm sections in a cryostat at −20°C and mounted on chrome-alum-coated slides. BN autoradiography was performed as described prevously (Wolf et al., 1983). Briefly, slides were incubated in a buffer composed of 10 mM HEPES, pH 7.4, 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.1% bovine serum albumin, 0.07 mM bacitracin, and 3 nM [^{125}I-Tyr^4]BN in the presence or absence of 1 μM unlabeled BN. Incubations were performed for 60 min at 22°C, followed by two consecutive 4-min washes in buffer at 4°C. Slices then were either counted by scraping into test tubes and assaying radioactivity in a Micromedic gamma counter or prepared for autoradiography using tritium-sensitive film (Ultrofilm, LKB) as described previously (Quirion et al., 1983). The exposure time was 48 hr. After this exposure, films were processed in Kodak D19 at 22°C for 4 min and then fixed for 5 min.

Intrathecal injections and behavioral analysis. Intrathecal injections (5 μl) into the lumbar region of unanesthetized male CD1 mice (20 to 25 gm) and analysis of the biting and scratching response were performed as described previously (Hylden and Wilcox, 1981). BN, GRP, or SP (100 pmol; Peninsula Laboratories) or vehicle (0.01 N acetic acid in saline, pH 3.5) at a concentration of 100 pmol/5 μl was injected and the number of biting or scratching responses per period was counted. A response was defined as either the nose touching the abdomen or the hindlimb touching the body. The behavioral response was analyzed both quantitatively and qualitatively.

Results

As shown in Table I, the highest concentrations of BN in all three species were found in the dorsal horn of the cervical spinal cord. In the cat, the dorsal horn dissection corresponds to laminae I to III as described previously (Massari et al., 1983), while in the rat and mouse, some of lamina IV may be included in the dissection. BN concentrations were about 25 times higher in the dorsal horn than in the ventral horn. In the mouse dorsal horn, concentrations of BN were similar to that in the cat and rat, whereas in the ventral horn, levels were somewhat higher compared to the rat or cat, although these ventral horn concentrations were at the minimal detectable level of the BN RIA.

The results of immunocytochemical studies were consistent with the RIA findings. Immunoreactive fibers were observed in laminae I and II in the cat, rat, and mouse spinal cords (Fig. 1). Few, if any, fibers were found in other regions of the rat and mouse spinal cord. In the cat cord, a number of immunoreactive fibers were observed in lamina X adjacent to the central canal. In general, the staining of spinal cord with BN antisera was sparse and was much less intense than staining of SP (personal observations). This is consistent with the fact that BN is in much lower concentrations in the spinal cord than in SP. After dorsal root transection, very few BN-immunoreactive fibers were observed in the superficial lamina of the dorsal horn of the spinal cord; however, BN-immunoreactive fibers could still be observed in lamina X of the cord. Alternate

TABLE I

Distribution of bombesin in the cervical spinal cord[a]

	Dorsal Horn	Ventral Horn
	fmol/μg of protein	
Cat	0.46 ± 0.05	0.02 ± 0.001
Rat	1.04 ± 0.18	0.04 ± 0.005
Mouse	0.47 ± 0.13	0.21 ± 0.03

[a] Values are mean ± SEM.

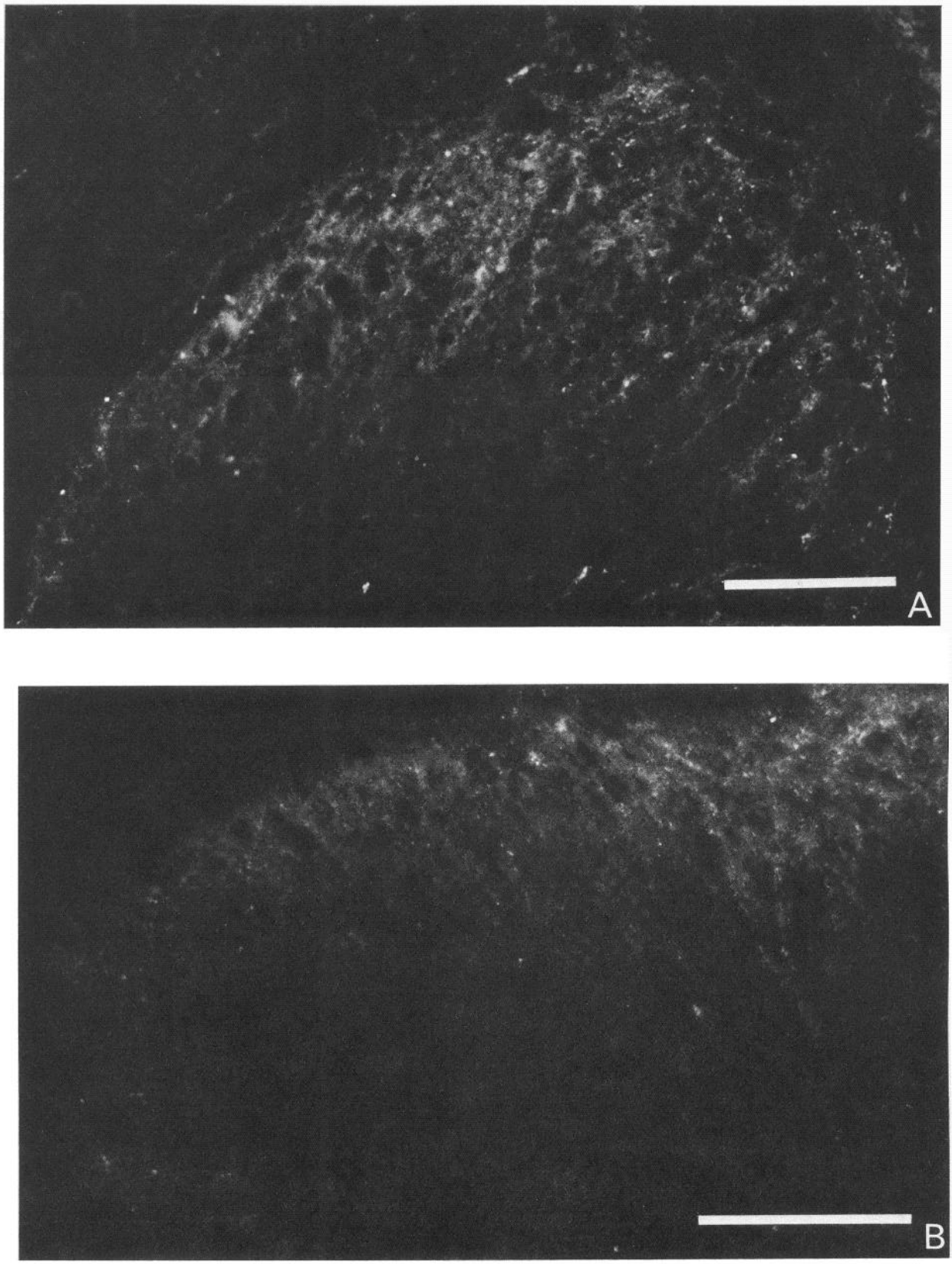

Figure 1. Immunocytochemical localization of BN-immunoreactive fibers in the dorsal horn of the cat thoracic (*A*) and mouse cervical (*B*) spinal cord. *Bar* = μm.

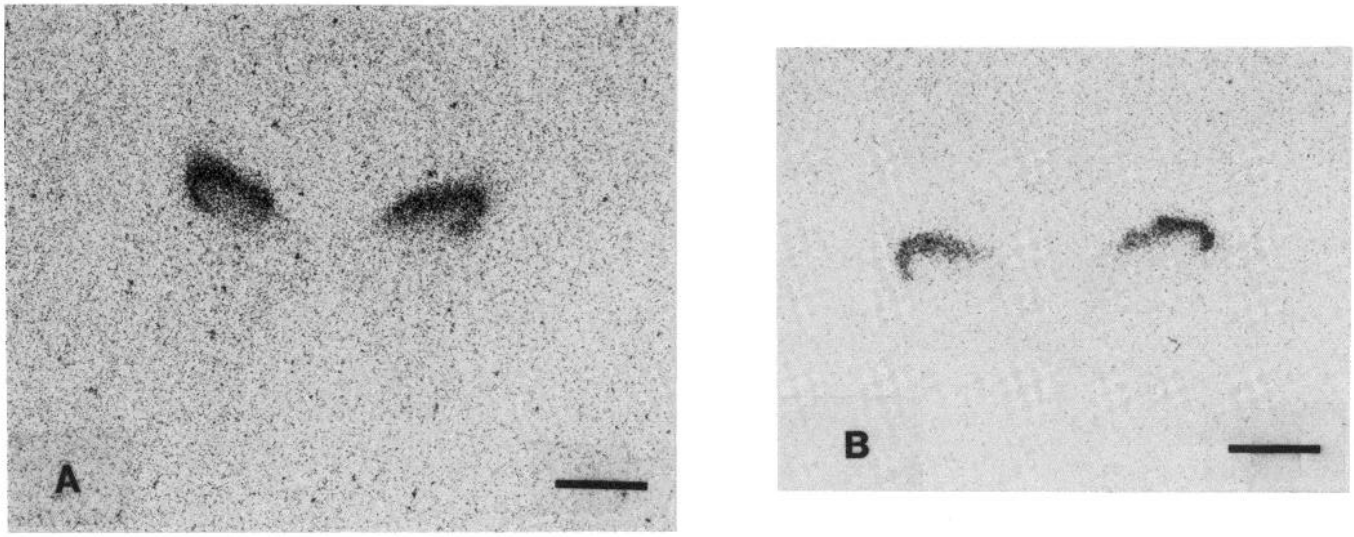

Figure 2. Autoradiographic localization of BN-binding sites in the cat lumbar (*A*) and rat cervical (*B*) spinal cord. *Bar* = 1 mm.

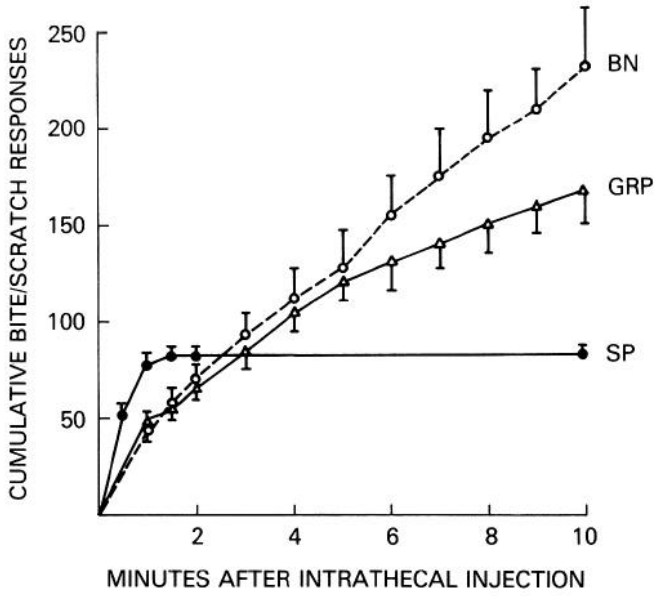

Figure 3. Quantitative effects of 100 pmol of BN, GRP, and SP on the bite/scratch response after intrathecal injection.

sections stained for SP also showed the expected decrease after rhizotomy.

The results of the autoradiographic localization of BN-binding sites in the cat and rat are shown in Figure 2. The distribution of BN-binding sites is quite striking. Binding sites can be observed in the superficial laminae of the dorsal horn—laminae I to III and perhaps IV of the dorsal horn; no autoradiographic grains could be observed in the white matter or in the ventral horn. A similar distribution was observed throughout the length of the cat spinal cord and from the cervical to the lumbar cord of the rat and mouse. Neither the pattern nor the intensity of the BN-binding site autoradiography was altered in spinal cord sections from cats that received dorsal rhizotomies 7 days prior to sacrifice.

Intrathecal administration of BN, GRP, or SP into the lumbar region rapidly induced a vigorous bite/scratch response, as shown quantitatively in Figure 3. Vehicle-injected mice exhibited fewer than 10 responses over the entire 10-min observation period. The major difference between the effects of the peptides was temporal in nature. The durations of action (the time at which half of the injected mice were still responding) of SP, GRP, and BN were 1, 14, and 120 min, respectively (Fig. 4). There was also a qualitative difference between the actions of the peptides. Although the bite/scratch actions of the peptides appeared similar initially, being directed to the abdominal region, the response to BN and GRP soon generalized to other parts of the body and came to resemble a vigorous grooming behavior.

Discussion

The results of immunocytochemical and RIA studies demonstrated that BN is located in superficial laminae of the spinal cord of the rat, cat, and mouse. Quite low concentrations of BN were detected in other regions of spinal cord. Consistently, the BN-binding sites were also localized only in superficial laminae of the cord. The presence of both BN and BN receptors in the dorsal horn of the cord suggested the possibility that BN might be involved in processing of sensory information. Furthermore, the fact that some of the BN in the dorsal horn is derived from primary sensory afferents (this study and Fuxe et al., 1983; Massari et al., 1983; Panula et al., 1983) indicates that, in some cases, BN may be a sensory neurotransmitter.

The results of studies injecting BN intrathecally into lumbar spinal cord support the hypothesis that BN may play a role in sensory processing. The peptide dramatically elicited a stereotypic scratching and grooming response directed at the abdominal region. The fact that the bite/scratch response was directed at the abdominal region is consistent with a lumbar spinal cord innervation from this region. The response was similar to that elicited with SP administration with the exception that the BN effect lasted about 100 times longer than that induced by SP and generalized to other body regions. GRP also induced a similar effect that lasted about 10 times longer than the SP effect. This temporal difference may result from different rates of degradation by peptidases or by different postsynaptic events after different receptor stimulation. It has been demonstrated that BN and SP bind to different receptors (Jensen et al., 1978; Moody et al., 1978; Quirion et al., 1983). The long-term qualitative differences between SP and BN or GRP may result from diffusion to other spinal and, perhaps, brain sites.

Previous investigations had demonstrated that, when either BN or GRP was administered intraventricularly to rats and mice, a stereotypic behavior characterized by grooming and scratching of the face and neck was elicited (Brown et al., 1977, 1980; Katz, 1980; Gmerek and Cowan, 1981; Kulkosky et al.,

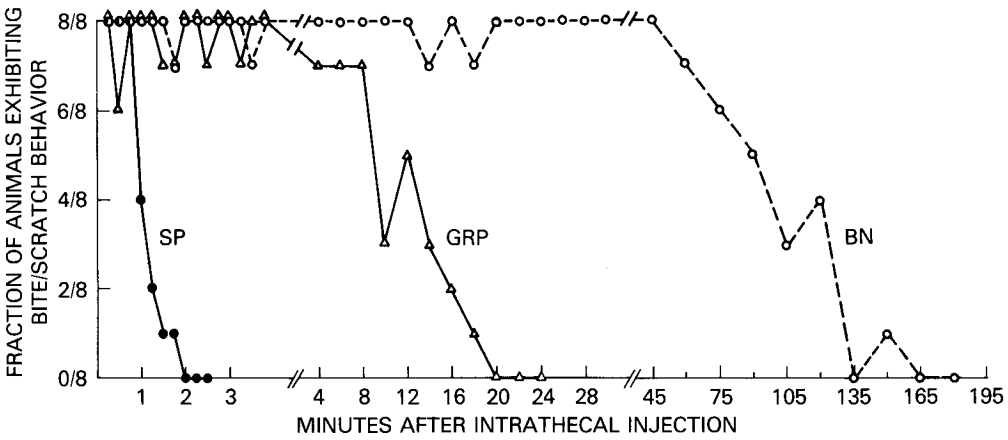

Figure 4. A comparison of the duration of action of 100 pmol of BN, GRP, and SP on the bite/scratch response after intrathecal injection.

1982). Induction of grooming has been reported after the administration of a number of other peptides such as litorin (Kulkosky et al., 1981), eledoisin (Katz, 1980), SP (Katz, 1979), ACTH (Ferrari et al., 1963; Gispen et al., 1975; Gispen and Isaacson, 1981), α-melanocyte stimulating hormone (Gispen et al., 1975; O'Donohue et al., 1981), β-endorphin (Gispen et al., 1976), dynorphin (Zwiers et al., 1981), and caerulein (Jerome et al., 1981). It has been proposed that grooming in rodents is comparable to stress-induced displacement behaviors in humans such as "nail-biting" (Bolles, 1960; Barnett, 1963; Fentress, 1968; Hinde, 1970; Delius et al., 1976; Wiegant and Gispen, 1977; Cohen and Price, 1979; Jolles et al., 1979) or may be induced by a shift in sensitivity to normal stimuli which induce grooming. As intraventricular administration of BN causes alteration in body temperature (Brown et al., 1977; Tache et al., 1980), the possibility exists that rats increase grooming rates more for thermoregulatory purposes (cf. Kulkosky et al., 1982). An alternative hypothesis is that BN is involved in primary sensory transmission in the brain as in the spinal cord. Intraventricularly administered BN may cause grooming and scratching by stimulating second-order cranial sensory neurons which mimic a painful or irritating stimulation of the skin of the face and neck region. Consistent with this hypothesis are the recent findings that BN-immunoreactive peptides (Moody et al., 1981a, c; Panula et al., 1982; Fuxe et al., 1983; Chronwall et al., 1984) and receptors (Zarbin et al., 1984) are located in the substantia gelatinosa of the spinal trigeminal nucleus. The spinal trigeminal nucleus also contains SP (Hokfelt et al., 1983) and SP receptors (Quirion et al., 1983; Shults et al., 1984) which also appear to be involved in the processing of primary sensory input from the face and neck (cf. Hokfelt et al., 1983). It is likely that SP and eledoisin may interact on SP receptors in this region to induce scratching and that BN, GRP, and litorin may interact with trigeminal BN receptors, as each of these latter peptides are agonists for the BN receptors (Broccardo et al., 1975; Jensen et al., 1978; Moody et al., 1978).

The mechanism of action of BN modulation of sensory input is unclear, but a number of possibilities are possible. The most likely alternative is that BN, like SP, is a neurotransmitter of primary sensory afferents. This is probable since dorsal rhizotomy decreases the concentration of BN-like peptides in the dorsal horn as determined by immunohistochemistry and RIA in the rat (Panula et al., 1983) and the cat (this study and Massari et al., 1983), respectively. Opioid receptors have been localized on primary sensory afferents to the spinal cord, and it is thought that opiates presynaptically inhibit sensory impulses via axoaxonic synapses (cf. Jessel, 1983). Whether opioid receptors are localized on BN afferents and can mediate the release of BN is not known at present. Recent findings (Fuxe et al., 1983) support the suggestion that BN is contained in the dorsal root ganglion and indicate that BN and SP are, in fact, often co-localized in primary sensory afferents. Evidence suggesting that BN and SP are co-localized in the dorsal horn is surprising in light of the previous findings that the release of SP but not BN is induced by capsaicin stimulation of the rat spinal cord (Moody et al., 1981c). In this regard, it is important to note the recent results of Panula et al. (1983) which indicate that BN and SP are contained in different primary sensory neurons. In addition to the BN contained in primary sensory afferents, there is a proportion of BN in the dorsal horn that is derived from central neurons (Massari et al., 1983). These central BN-containing neurons could innervate opiate or nonopiate interneurons or secondary sensory neurons. The fact that dorsal rhizotomy does not eliminate BN binding to the dorsal horn indicates that BN receptors are not primarily localized on sensory afferents.

In summary, it appears that BN is contained in both primary sensory afferents and central neurons in laminae I and II of the dorsal horn of the spinal cord. The data also indicate that the dorsal horn contains BN receptors, since BN-binding sites were located there and BN pharmacological actions indicative of sensory stimulation could be demonstrated. Further investigation will be required to determine the precise anatomical and physiological relationships between BN-, SP-, and opiate-containing neurons of the dorsal horn and their roles in mediating the neurotransmission of sensory and nociceptive stimuli.

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