Brain Research, 496 (1989) 1–13
Elsevier

BRES 14747

Research Reports

The colocalization of substance P and prodynorphin immunoreactivity in neurons of the medial preoptic area, bed nucleus of the stria terminalis and medial nucleus of the amygdala of the Syrian hamster

Charles R. Neal Jr.[1], Jennifer M. Swann[2] and Sarah Winans Newman[1]

[1]*Department of Anatomy and Cell Biology, University of Michigan Medical School, Ann Arbor, MI 48109-0616 (U.S.A.) and* [2]*Department of Biological Sciences, Rutgers University, Newark, NJ 07102 (U.S.A.)*

(Accepted 31 January 1989)

Key words: Substance P; Dynorphin; Leumorphin; Colocalization; Hamster; Mating behavior; Opiate

To determine the extent of colocalization of substance P (SP) and prodynorphin peptides within neurons of the medial nucleus of the amygdala (AMe), medial bed nucleus of the stria terminalis (BNSTm) and medial preoptic area (MPOA), we incubated colchicine-treated Syrian hamster brain tissue in an antiserum mixture containing rat anti-SP antibody combined with 1 of 3 rabbit antibodies against prodynorphin peptides: anti-dynorphin A(1–17), anti-dynorphin B(1–13) or anti-C-peptide. This was followed by incubation in a secondary antiserum mixture containing fluorescein-labelled anti-rabbit and rhodamine-labelled anti-rat antibodies. Sections were viewed with an epifluorescence microscope using blue light excitation for fluorescein and green light excitation for rhodamine. Colocalization of SP and prodynorphin labelling was observed in neurons of the caudal parts of AMe, BNSTm and MPOA, areas which are essential for male mating behavior. The colocalization was most extensive in the dorsolateral part of the caudal MPOA, the caudodorsal part of the BNSTm, and in the posterodorsal subdivision of AMe. Although all 3 dynorphin peptides coexisted with SP in these areas, dynorphin B did so less than C-peptide, and dynorphin A less than dynorphin B.

INTRODUCTION

The male Syrian hamster (*Mesocricetus auratus*) is dependent on chemosensory cues for the initiation of normal mating behavior. This behaviorally essential chemosensory information is relayed from receptors in the vomeronasal organ and olfactory mucosa[51,68] to the accessory and main olfactory bulbs[41], respectively, and thence, via the lateral olfactory and accessory olfactory tracts[10], to the medial nucleus of the amygdala (AMe), where vomeronasal and olfactory information appears to be integrated[26,27]. From the AMe, axons project to the medial preoptic area (MPOA) and the medial bed nucleus of the stria terminalis (BNSTm) through two fiber pathways, the stria terminalis[9,22,24,33], and the ventral amygdalofugal pathway[28]. Bilateral lesions of any of the areas along the chemosensory pathway described above, or the fiber tracts connecting them, can completely and permanently eliminate normal mating behavior in the male hamster[26,29,52,69].

Although this anatomical circuitry has been studied in detail, little is known about the functional interaction of structures in this system. In this study our aim was to identify neurotransmitters localized in this pathway.

Several putative peptide neurotransmitters have been identified in the MPOA, BNSTm and AMe in the rat[5–7,15,16,23,30–32,55,60,61,65,70]. While studying neurotransmitters in the hamster chemosensory path-

Correspondence: S.W. Newman, Department of Anatomy and Cell Biology, Medical Science II, University of Michigan, Ann Arbor, MI 48109-0616, U.S.A.

way, we have identified cell bodies containing substance P (SP), dynorphin A(1–17), dynorphin B(1–13) and C-peptide (the C-terminus sequence of leumorphin, dynorphin B(1–29)[14]) in these 3 regions[45,63].

Both SP and dynorphin have been implicated in the control of sexual behavior, but little is known of their respective roles in specific brain areas. There is considerable evidence that endogenous opioids may play an inhibitory role in mating behavior of the male Syrian hamster[42,43,71] and the rat[17,20,36,38,44]. Although most evidence implicates β-endorphin and μ-receptor activation as the main inhibitory system, there is also evidence for enkephalinergic[53] influences. Recently, however, evidence has been accumulating that prodynorphin products may actually play a facilitatory role in reproduction in the rat[2,21,39]. Leumorphin (dynorphin B(1–29)), a prodynorphin product, has been demonstrated to facilitate female rat sexual behavior[21,56,62]. In addition, microinjections of picomolar amounts of dynorphin A into the MPOA of a male rat facilitated copulatory behavior, whereas injections of morphine were inhibitory and saline had no effect[2]. Dynorphin B injections into the ventral tegmental area have also been shown to facilitate mounting behavior in the male rat[39]. These data clearly demonstrate that, while opiates have been generally thought of as inhibitory in their effect on sexual behavior, the dynorphins as a specific class may play a facilitating role.

The evidence that SP is involved in sexual behavior in the rat suggests that it acts as a facilitatory neurotransmitter or neuromodulator[13,66]. Injections of SP into the medial preoptic area–anterior hypothalamic area of male rats significantly reduces mounting and copulation latencies[12]. Though the role of SP with respect to male mating behavior in the hamster is not clear, we have found that SP immunoreactivity is not only present in the MPOA, BNSTm and AMe of this species, but that the amount of immunoreactivity in these structures is influenced by the level of circulating gonadal steroids[63].

Results from our laboratory indicated that the distribution of SP-containing cells overlapped that of dynorphin-containing cells in the MPOA, BNSTm and AMe of the male Syrian hamster. Therefore, in the present study we sought to determine if SP and the dynorphin peptides coexist within the same neurons. To date we are unaware of any evidence for SP colocalization with any prodynorphin peptide in the rat or hamster with the exception of their coexistence within striatal projection neurons in the rat[1]. A comparison of SP and dynorphin distribution in the substantia nigra of the rat brain showed considerable overlap[37], but actual colocalization within cell bodies was not demonstrated. Colocalization of these peptides has also been reported in the pigeon and turtle basal ganglia[54], and in the guinea pig spinal cord[67]. Preliminary findings of the work presented here have been reported previously in abstract form[47].

MATERIALS AND METHODS

Adult male Syrian hamsters (Charles River), age 75–90 days, were housed in groups in a 14:10 light:dark cycle and given food and water ad libitum. Ten hamsters were anesthetized with sodium pentobarbital (10 mg/100g b. wt.) and placed in a stereotaxic instrument. Two microliters of colchicine (160 μg/μl) were injected into the left lateral ventricle at the level of the septum (ML = +1.5 from bregma, AP = +1.5 from bregma and DV = –3.3 from dura, with bregma and lambda in the same horizontal plane). Brains of animals that did not receive colchicine were also studied. In these animals, both SP and prodynorphin cell labelling was virtually absent. This material was, therefore, not included in the present study, but has been used in separate studies to aid in fiber and terminal identification in the prodynorphin and SP systems in the hamster brain[46,63].

Following a survival period of 48 h, the hamsters were again anesthetized with pentobarbital (15 mg/100 g b. wt.) and perfused transcardially with 150 ml of a 0.1 M phosphate-buffered saline solution (PBS) with 0.1% sodium nitrite (for vasodilation), followed by 250–300 ml of fixative. The fixative was either a 4% paraformaldehyde solution in 0.1 M sodium phosphate buffer, or a 2% paraformaldehyde and 0.25% *p*-benzoquinone solution in 50 mM sodium phosphate buffer. The brains were then removed from the skull and post-fixed for 1 h (4% paraformaldehyde brains), or for 2 h (*p*-benzoquinone

brains) in the perfusion fixative, followed by soaking in a solution of 20% sucrose in PBS at 4 °C for at least 24 h. Forty-micrometer sections were cut on a freezing stage on a sliding microtome and stored at 4 °C in 0.1 M sodium phosphate buffer and 0.01% sodium azide for preservation of tissues.

The C-peptide (No. 109, bleed 6) antibody used in this study was generously provided by Dr. Stanley J. Watson Jr., Mental Health Research Institute, University of Michigan. The dynorphin A(1–17) (AK84T) and dynorphin B(1–13) (AK113T) antibodies were a generous gift of Dr. Lars Terenius, Uppsala University, Uppsala, Sweden. The SP antibody used was a monoclonal purchased from Sera-Lab (Accurate Scientific). Each of the prodynorphin antibodies were generated in rabbit while the SP antibody was generated in rat.

The indirect immunofluorescence procedure used in this study has been described elsewhere[19,58,59]. In our studies we used fluorescein isothiocyanate (FITC) for detection of prodynorphin peptides and rhodamine isothiocyanate (RITC) for detection of SP in all brains. The tissues were incubated for 30 min in 0.022 M potassium phosphate buffered saline (KPBS) and transferred to a primary antiserum diluted to the desired concentration with a solution of 0.3% triton-X in KPBS for 48–60 h. All primary antibodies were used in dilutions determined experimentally to be optimal for staining. Each primary incubation mixture contained 1:500 SP plus either 1:250 dynorphin A, 1:250 dynorphin B or 1:500 C-peptide antibodies. The mixture of anti-SP plus anti-C-peptide, anti-SP plus anti-dynorphin A or anti-SP plus anti-dynorphin B will be referred to as the 'primary antiserum' mixture in the text. After incubation in the primary antiserum, the sections were washed 3 × 5 min in KPBS and then incubated in a solution containing 1:50 goat anti-rabbit conjugated to FITC and 1:25 goat anti-rat conjugated to RITC in the 0.3% triton-X KPBS for 1 h. The sections were washed 6–10 times with water, mounted on gelatin-coated slides and coverslipped with a glycerol-phosphate buffer medium containing phenylenediamine for optimal preservation of FITC-immunofluorescence[50]. Adjacent sections were mounted and stained with Cresyl violet for cytoarchitectonic localization of immunoreactive cells.

Sections were analyzed with a Leitz Orthoplan fluorescence microscope, using an I2 filter (495 nm blue light excitation), inducing green-blue emission from FITC, and an N2 filter (525 nm green light excitation), inducing red emission from RITC.

Ten colchicine-treated hamster brains were labelled for C-peptide and SP immunoreactivity using the above technique. To determine if SP immunoreactivity is colocalized with that of other prodynorphin peptides, antisera containing anti-SP and either anti-dynorphin A(1–17) or anti-dynorphin B(1–13) antibodies were used in sections from several brains as well.

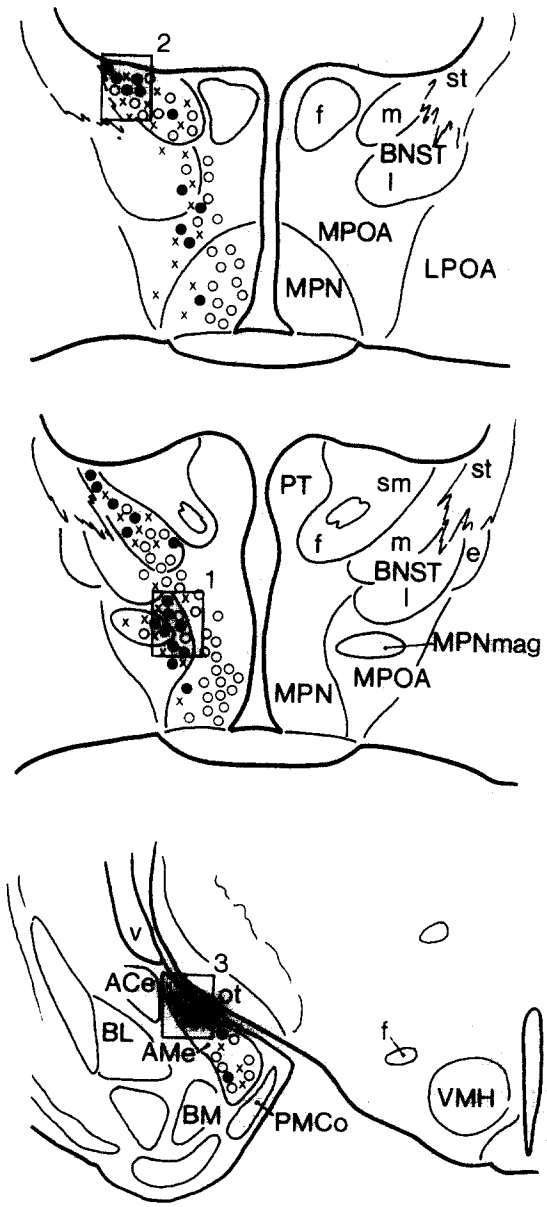
In each of the 10 brains from animals which received colchicine, all immunoreacted sections (i.e. every 120 μm) through the MPOA, BNSTm and AMe were studied, to develop a clear picture of the labelling pattern throughout the rostrocaudal extent of each nucleus. As the pattern of colocalization became clear, specific, non-overlapping, widely spaced regions were selected from each of 3 brains for closer study. Using 200× magnification, the number of single- and double-labelled cells in various microscopic fields within these regions were counted using a hand-held counter. Each field in which counts were made contained 100–200 immunolabelled cells. A cell was considered to be double-labelled when it was observed to contain both blue-green cytoplasmic labelling under the I2 filter and red cytoplasmic labelling under the N2 filter. The number of double-labelled cells within a field was divided by the total number of SP-containing or the total number of dynorphin-containing cells counted in that field to generate the values given in the results. These numerical data were generated in an attempt to convey the extent of colocalization in each area, rather than to establish statistical differences between the areas described.

To test the specificity of the antibodies used, a series of control procedures were carried out. First, the C-peptide/SP antiserum mixture was preabsorbed (i.e. incubated for 1 h at room temperature) with a 25 μM concentration of SP peptide (Peninsula Labs), a 5–10 μM concentration of rat C-terminus peptide (generously donated by Dr. Stanley J. Watson Jr.), a 50–100 μM concentration of porcine C-terminus peptide (Peninsula Labs) or a 50–100 μM concentration of human leumorphin peptide (Peninsula Labs) prior to tissue incubation, to check for primary antiserum specificity (self-blocking control).

Second, the primary C-peptide/SP antiserum was incubated with 50–100 μM dynorphin B(1–13) peptide, dynorphin A(1–17) peptide, dynorphin A(1–13) peptide or Leu-enkephalin peptide (Peninsula Labs) prior to tissue incubation, to check for cross-reactivity of the C-peptide or SP antibodies with related opioid peptides (cross-blocking control). Third, sections were incubated with only anti-SP or anti-C-peptide in the primary antiserum, followed by 1 h with both secondary antibodies, to check for non-specific background staining which might be elicited if the goat anti-rabbit-FITC or goat anti-rat-RITC recognized epitopes in the hamster brain. Fourth, sections were incubated with only anti-SP followed by only goat anti-rabbit-FITC or anti-C-peptide followed by only goat anti-rat-RITC to insure that the double labelling was not due to secondary antibodies recognizing both primaries in the tissue. Last, the dynorphin A/SP and dynorphin B/SP antisera were preabsorbed prior to immunohistochemistry with 10–25 μM concentrations of the same dynorphin A(1–17) and dynorphin B(1–13) peptides towards which they were made. The dynorphin A and SP antibodies have been characterized in detail previously[35,58,59]. All primary antisera, with or without blocking peptides added, were left at room temperature for 1 h prior to the beginning of incubation. Thus, antisera with no peptides added for blocking controls were treated identically to those in which the peptides were allowed an hour for preabsorption.

RESULTS

In all cases where rat C-peptide (5–10 μM) was added to the primary antiserum 1 h prior to tissue incubation, SP staining was unaffected but C-peptide staining was completely abolished. On the other hand, when 25 μM SP peptide was added all SP staining was abolished but C-peptide staining was unaffected (see Fig. 4). When 50–100 μM human leumorphin, 50–100 μM porcine C-peptide, 50–100



→

Fig. 1. Schematic representations of coronal sections at 3 rostrocaudal levels which showed the heaviest double-labelling (colocalization) of SP and C-peptide when using a primary antiserum containing antibodies to SP and the C-terminus of leumorphin. The boxed areas are illustrated with photographs in Figs. 2–4. ×, SP-labelled cells; ○, C-peptide-labelled cells; ●, cells staining for both SP and the C-terminus of leumorphin. Abbreviations: ACe, central nucleus of the amygdala; AMe, medial nucleus of the amygdala; BL, anterior basolateral nucleus of the amygdala; BM, basomedial nucleus of the amygdala; BNST, bed nucleus of the stria terminalis (e, external part; l, lateral part; m, medial part); f, fornix; LPOA, lateral preoptic area; MPOA, medial preoptic area; MPN, medial preoptic nucleus; MPNmag, medial preoptic nucleus, magnocellular part; ot, optic tract; PMCo, posteromedial cortical nucleus of the amygdala; PT, parataenial nucleus of the thalamus; sm, stria medullaris; st, stria terminalis; v, lateral ventricle; VMH, ventromedial nucleus of the hypothalamus.

μM dynorphin A(1–17), 50–100 μM dynorphin B(1–13) or 50–100 μM Leu-enkephalin peptides were added to the primary antiserum, neither SP nor C-peptide staining was affected. The dynorphin A peptide (10–25 μM) completely abolished dynorphin A antibody staining, while having no effect on SP staining. The same specificity was found for dynorphin B as well. When anti-SP was omitted from the primary antiserum strong opioid FITC-immunofluorescence was observed, but no RITC-immunofluorescence could be detected. When C-peptide, dynorphin A or dynorphin B peptide was omitted from the primary antiserum, strong SP RITC-immunofluorescence could be observed but no opioid FITC-immunofluorescence was detected. When the RITC-anti-rat secondary was used with the C-peptide primary, or when the FITC-anti-rabbit was used with the SP primary, no fluorescent staining was observed, demonstrating that the secondary antisera did not recognize epitopes in hamster brain tissue.

Both the benzoquinone–paraformaldehyde perfusion mixture, and the paraformaldehyde perfusion mixture gave excellent SP and prodynorphin fluorescent staining in the brain areas described in this paper.

Both FITC- and RITC-labelled cells had a diffusely labelled and brightly fluorescent cytoplasm. In

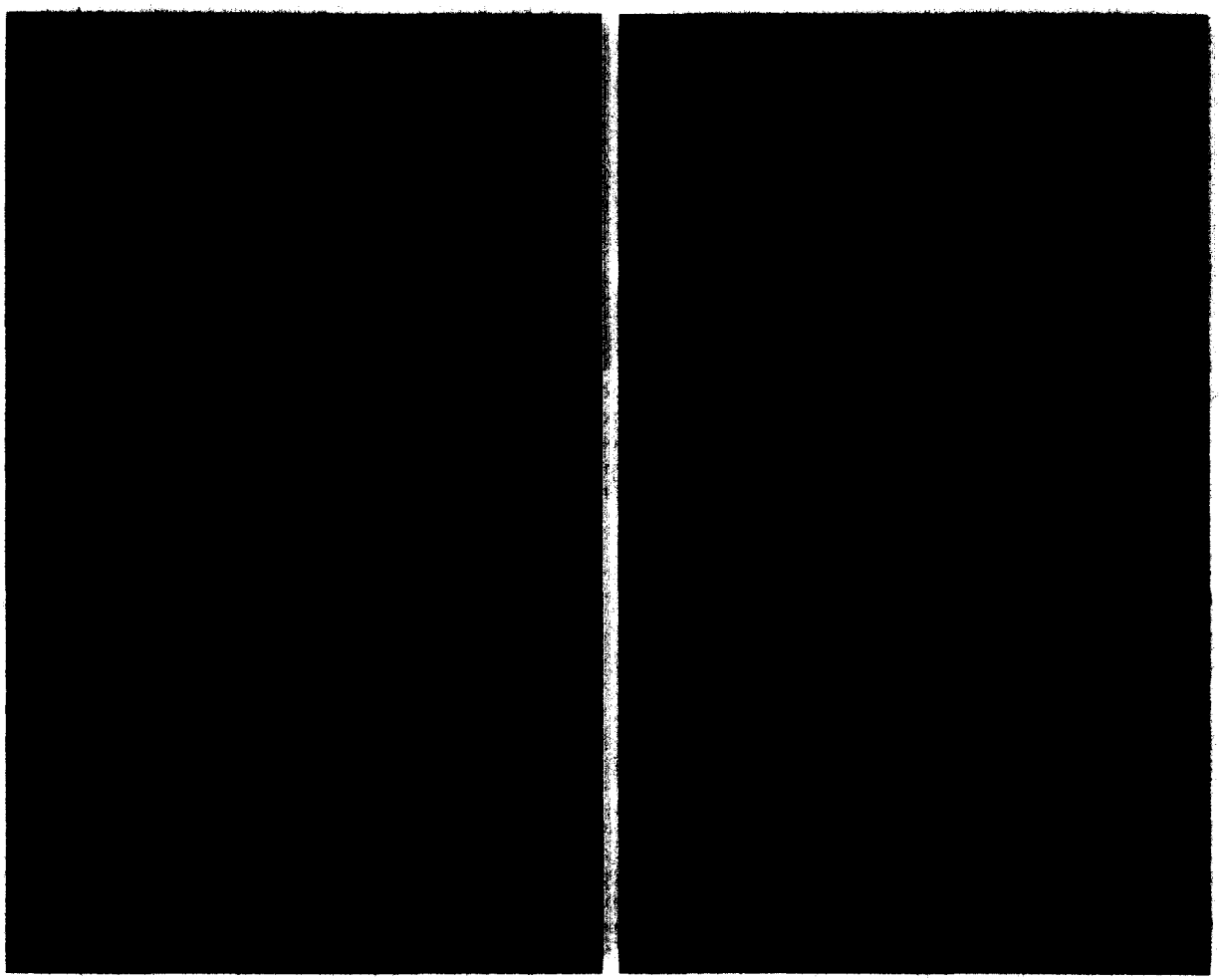


Fig. 2. Photomicrographs of SP and C-peptide immunostaining observed in the same section of the dorsolateral MPOA (Fig. 1, box 1). A: FITC-fluorescence of C-peptide immunoreactivity. B: RITC-fluorescence of SP immunoreactivity. Arrows identify representative neurons immunoreactive for both SP and the C-terminus of leumorphin, while arrowheads identify neurons labelled for only one peptide. Calibration bar = 100 μm.

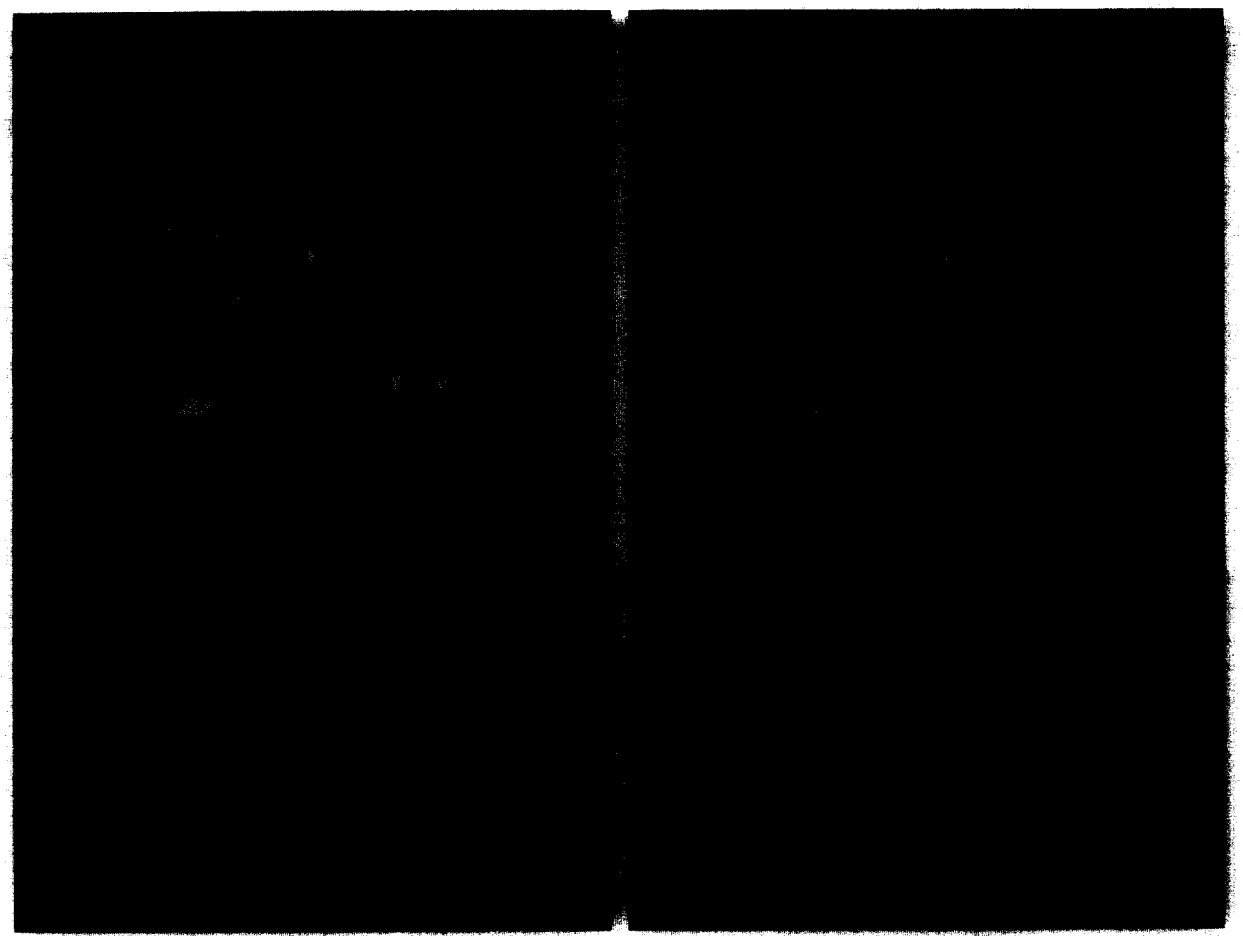


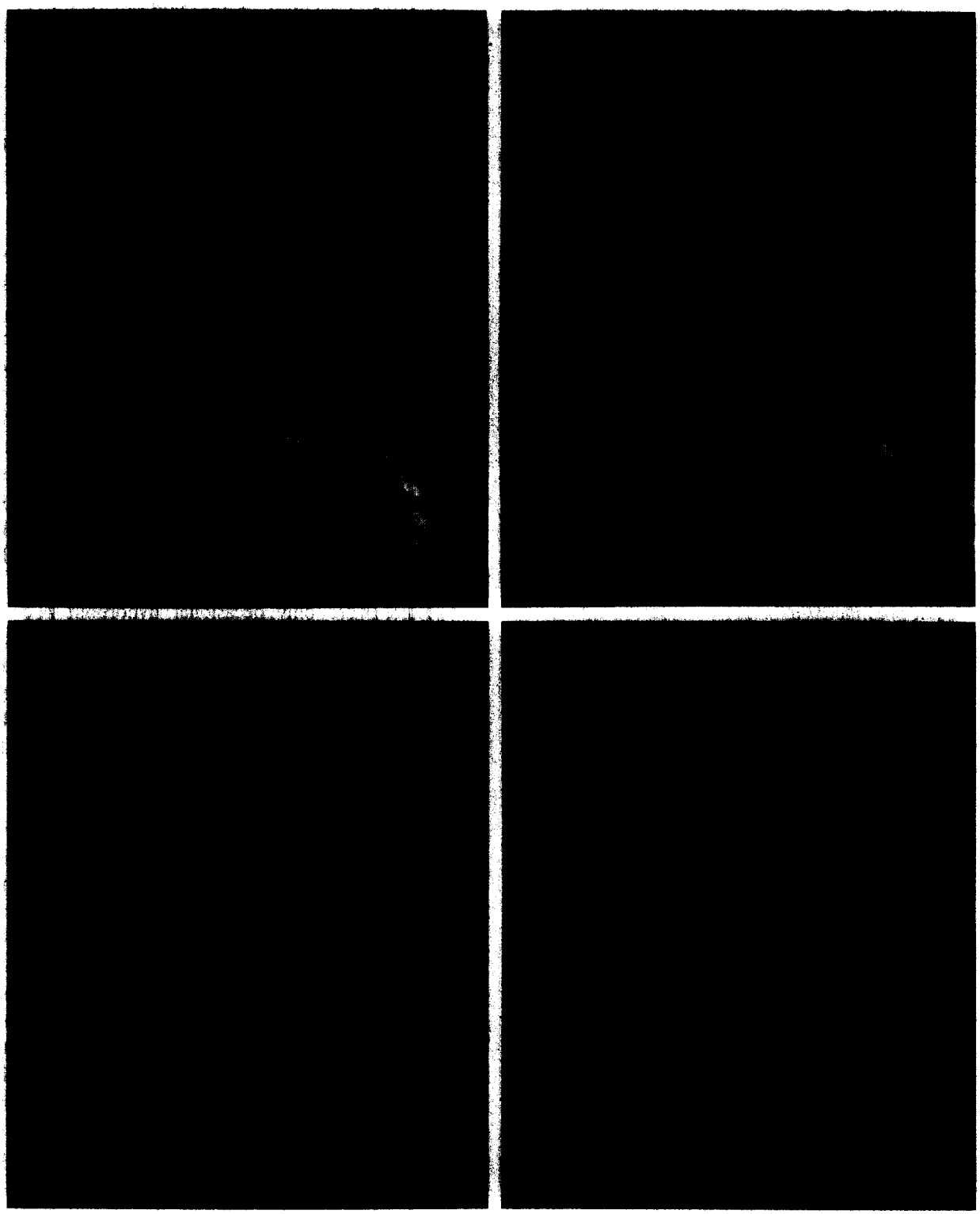
Fig. 3. Photomicrographs of SP and C-peptide immunostaining observed in the same section of the BNSTm (Fig. 1, box 2). A: FITC-fluorescence of C-peptide immunoreactivity. B: RITC-fluorescence of SP immunoreactivity. Arrows identify representative neurons immunoreactive for both SP and the C-terminus of leumorphin, while the arrowhead identifies a neuron labelled for only C-peptide. At this level, fiber and terminal labelling for both C-peptide and SP is intense, obscuring many cell bodies located within this part of the nucleus. LV, lateral ventricle. Calibration bar = 100 μm.

many cases the dendritic processes of the cells could also be visualized. In areas where fiber labelling was light (e.g. the MPOA) cells were readily distinguishable from the background by their shape. In other areas (e.g. the BNSTm) the density and intensity of fiber and terminal labelling was great enough to obscure the outlines of some labelled cells. Thus, the percentage of colocalization presented here may be a conservative estimate of the actual number of cells containing each peptide.

Numerous SP and prodynorphin cells have been demonstrated in the MPOA, BNSTm and AMe in the hamster brain using peroxidase–antiperoxidase immunohistochemistry and light microscopy[45,63].

→

Fig. 4. Photomicrographs showing FITC-fluorescence of C-peptide immunoreactivity, (A), and RITC-fluorescence of SP immunoreactivity, (B), in the same section of the AMe (Fig. 1, box 3). C: FITC-fluorescence in an adjacent section to (A) above with 10 μM of rat C-terminus peptide added to the primary antiserum 1 h prior to tissue incubation. D: RITC-fluorescence in an adjacent section to (B) above with 25 μM of SP peptide added to the primary antiserum 1 h prior to tissue incubation. These micrographs demonstrate the complete absence of immunolabelling consistently observed with preabsorption control studies in all areas observed. See text for details. Arrows identify examples of neurons immunoreactive for both SP and the C-terminus of leumorphin, while the arrowhead identifies a neuron labelled for only C-peptide. At this level, fiber and terminal labelling for both C-peptide and SP is intense, obscuring many cell bodies located within this part of the nucleus. OT, optic tract. Calibration bar = 100 μm.



The results of the present study confirmed these preliminary findings using immunofluorescence histochemistry. In all 3 areas the C-peptide antibody labelled the largest number of cell bodies while the dynorphin B antibody labelled fewer cell bodies and the dynorphin A antibody labelled the least. Since the C-peptide antibody produced the most robust labelling and showed a similar distribution to SP, we investigated the colocalization of these two peptides in detail. The SP/dynorphin B and SP/dynorphin A colocalization we observed in these same areas is also described below.

Though the degree of SP and prodynorphin colocalization was consistent from animal to animal, there were differences in the number of double-labelled cells between the BNSTm, MPOA and AMe as well as differences in the number and distribution of double-labelled cells within subdivisions of these nuclei. Lastly, there was variation in the degree of colocalization among the 3 different dynorphin antibodies.

Medial preoptic area

The most rostral level of the MPOA at which double-labelled cells were found was the mid-level of the body of the anterior commissure. At this level SP- and C-peptide-immunolabelled cells were few in number and confined to its lateral extent, where one-third of SP cells were observed to contain C-peptide and one-quarter of the C-peptide cells contained SP.

At the caudal edge of the body of the anterior commissure, the proportion of double-labelled cells increased throughout the MPOA (one-half of SP-labelled cells contained C-peptide; the same proportion of C-peptide cells contained SP), though the number of single-labelled SP and C-peptide cells had only increased slightly. Within the medial preoptic nucleus (MPN) at this level, numerous cells showed C-peptide staining, but SP-labelled cells were scarce. As few as 1 in 100 of these numerous C-peptide cells contained SP whereas 1 in 4 of the scattered SP cells were also stained for C-peptide. This staining pattern continued throughout the MPN.

Caudal to the body of the anterior commissure the number of SP- and C-peptide-immunolabelled cells remained the same but the percentage of double-labelled cells increased significantly, particularly in the dorsolateral part of the MPOA. In this area three-quarters of the SP cells were also labelled with the C-peptide antibody and two-thirds of the C-peptide cells contained SP (Fig. 1, box 1 and Fig. 2). In the ventrolateral MPOA, lateral to the MPN, slightly more than one-third of the SP cells labelled for C-peptide, while one-fourth to one-third of the C-peptide cells were double-labelled for SP.

When the primary antiserum contained antibodies to dynorphin B(1–13) combined with SP antibodies, numerous double-labelled cells were observed in the MPOA, in a pattern indistinguishable from that of C-peptide/SP double-labelling. However, since fewer dynorphin B cells were observed, the proportion of SP cells containing dynorphin B was less than that of SP cells containing C-peptide. For example, in the dorsolateral part of the caudal MPOA where about one-half of SP cells contained C-peptide, approximately two-fifths of SP cells contained dynorphin B, and the same proportion of dynorphin B cells showed SP-labelling.

There were only occasional dynorphin A-containing cells in the MPOA, and some of these also contained SP. In the dorsolateral part of this area, where C-peptide/SP and dynorphin B/SP double-labelling were heaviest, only 1 in 10 SP cells contained dynorphin A, while half of the small number of dynorphin A cells contained SP.

Bed nucleus of the stria terminalis, medial division

SP-labelled cells were found rostrally in the BNSTm, rostral to the body of the anterior commissure, whereas few C-peptide-labelled cells were observed in this area. As a result, double-labelled cells were not observed in this region. From the caudal border of the anterior commissure to the most caudal part of the BNSTm, cells containing both C-peptide and SP were observed along its dorsal border (Fig. 1, box 2 and Fig. 3). In this region one-third to one-half of all SP cells contained C-peptide. C-peptide cells containing SP also ranged from one-third to one-half at more rostral levels but decreased to one-fifth more caudally, where C-peptide cell numbers increased. At this caudal level in the BNSTm, fiber and terminal labelling for both C-peptide and SP was quite heavy (Fig. 3). The SP fiber/terminal labelling was heavier than that of

C-peptide in most brain areas. However, in the caudal dorsal part of the BNSTm the C-peptide fiber and terminal labelling was more intense.

In the ventral part of the BNSTm, the number of double-labelled cells was low, although numerous SP and C-peptide cells were present. Throughout the rostrocaudal extent of this ventral region approximately one-fourth of the cells containing SP colocalized with C-peptide while even fewer C-peptide-containing cells labelled for SP. This pattern of strong double-labelling dorsally and less double-labelling ventrally continued throughout the bed nucleus with one exception; in the preoptic part of the bed nucleus of the stria terminalis approximately half of both SP and C-peptide cells were observed to be double-labelled.

The dynorphin B staining pattern in the BNSTm was quite similar to that of C-peptide, both in quality and quantity. As a result, dynorphin B and SP double-labelling patterns were essentially identical to that described for C-peptide and SP above. There were fewer dynorphin A immunoreactive cell bodies in the BNSTm than observed with C-peptide antibody. As a result, in the dorsal part of this nucleus, where SP and C-peptide (or dynorphin B) double-labelled cells were the most numerous, only 1 in 10 cells containing SP were observed to colocalize with dynorphin A, while half of the dynorphin A-labelled cells also contained SP.

Medial nucleus of the amygdala

C-peptide-labelling was observed in the most rostral extent of the anterior dorsal subdivision of the AMe, but SP-labelled cells were confined to the caudal one-third. Throughout this caudal region, individually labelled SP and C-peptide cells filled the posterodorsal subdivision (Fig. 1, box 3 and Fig. 4).

At midcaudal levels of the AMe a moderate number of both SP and C-peptide-labelled neurons were observed. However, no double-labelled cells were found. From this level to the most caudal tip of the nucleus, double-labelled SP and C-peptide cells were numerous in the posterodorsal subdivision. SP-labelled cells containing C-peptide ranged from one-half in the rostral part of the posterodorsal AMe, to four-fifths in its caudal tip. C-peptide-labelled cells containing SP showed a similar proportion of double-labelling. C-peptide and SP colocalization was mostly confined to the dorsal part of the posterodorsal subdivision, with the ventral part of this subdivision demonstrating only 1 in 5 SP or C-peptide cells that were double-labelled.

When dynorphin B antibody was used in the primary antiserum we observed numerous dynorphin B-labelled cells in the caudal regions of the AMe. However, in the caudal tip there were fewer SP cells containing dynorphin B (two-fifths) and fewer dynorphin B cells containing SP (one-third) than were observed with C-peptide and SP, even though there was no appreciable drop in the number of labelled cells in this area. With dynorphin A antibody, we again observed the same pattern of fewer immunolabelled cells, with a lower SP ratio and higher dynorphin A ratio of double-labelling. For example, in the caudal tip of the AMe, where as much as four-fifths of the C-peptide and SP cells were double-labelled, only one-third of SP cells contained dynorphin A while two-thirds of the scattered dynorphin A cells contained SP. Again, these large variations in ratios were due to a significantly smaller number of labelled neurons when using dynorphin A, rather than C-peptide antibody, in the primary antiserum mixture.

DISCUSSION

Using indirect immunofluorescence histochemistry we have demonstrated the colocalization of SP-like and prodynorphin-like immunoreactivity in the MPOA, BNSTm and the posterodorsal subdivision of the AMe. Colocalization of SP and prodynorphin was confined to cells in the caudal parts of all these brain areas and, within these caudal regions, the double-labelled cells were more numerous in the dorsolateral than in the ventromedial territory of each nucleus.

At first glance the dorsolateral–ventromedial gradient of colocalization may appear to be related to colchicine effects due to the close proximity of the BNSTm and AMe to the lateral ventricle. However, this is not the case in the MPOA, where heaviest double-labelling was observed dorsolaterally, further from the third ventricle than the location of many single-labelled cells. The preoptic part of the bed nucleus of the stria terminalis, the part of the BNSTm located furthest from the ventricle, also

showed strong double-labelling. It should be stressed that the patterns of SP and dynorphin labelling in the present study reflect those obtained from tissue processed with individual antisera. The pattern of double-labelling is greatest in those areas in which the individual SP and dynorphin immunostaining patterns overlap. This suggests that the gradients of colocalization are related to the expression of these peptides rather than the location of colchicine placement. Other studies report similar distributions of dynorphin and SP cells with a pattern clearly not dependent on colchicine treatment[7,15].

In general, dynorphin B and dynorphin A were found within neurons containing SP with the same distribution as C-peptide, but were fewer in number. Dynorphin B cells were slightly fewer in number than those labelled with the C-peptide antibody, while dynorphin A antibody labelled the smallest number of cells. Therefore, in comparison to cells showing both SP and C-peptide immunoreactivity, fewer cells containing both SP and either dynorphin A or dynorphin B peptides were observed.

Since all 3 of these prodynorphin sequences (dynorphin A(1–17), dynorphin B(1–13) and C-peptide) are found for the same precursor molecule, there are several possible explanations for variations in antibody labelling observed within these 3 peptides. The first possibility is that the antisera have differential affinities for their respective peptides. The C-peptide antiserum may recognize the C-peptide sequence more effectively than the dynorphin B antiserum recognizes its sequence. The dynorphin B antiserum may, in turn, recognize its sequence more efficiently than does the dynorphin A antibody. Another possibility is that our perfusion techniques preserved peptide sequences differentially (C-peptide > dynorphin B > dynorphin A) and that the differences in the affinity of the antisera for their peptides are due to the effect of the perfusion rather than the nature of the antisera used. A third possibility is that a combination of the two factors is involved. These suggestions are supported by the observation that higher concentrations of dynorphin A and dynorphin B antisera than the C-peptide antiserum are required for adequate immunolabelling. However, we have observed in separate studies that, under identical conditions to those used in this study on the hamster material, all 3 prodynorphin peptides produced identical staining patterns throughout the rat brain[46]. In addition, in several areas of the hamster brain other than the 3 reported here, the dynorphin A and dynorphin B antisera produce heavier immunolabelling patterns in both cells and fibers than the C-peptide antiserum (e.g. lateral hypothalamus, arcuate nucleus)[46]. Neither differential affinities of antisera to their peptide sequences nor differential perfusion effects adequately explain these observations.

One other possible explanation is differential processing of the prodynorphin precursor peptide in various regions of the hamster brain. Differential processing of the prodynorphin precursor molecule has been demonstrated in homogenates of the rat brain and pituitary with radioimmunoassay, gel filtration and high pressure liquid chromatography[57]. In the hamster brain the precursor molecule may be cleaved to produce dynorphin A(1–8 or 1–17), dynorphin B(1–13) or leumorphin (dynorphin B(1–29)) in varying amounts in different nuclei to such an extent that immunocytochemical detection of these peptide products in nerve cell bodies differs with each prodynorphin antiserum used.

The presence of SP and prodynorphin in the MPOA, BNSTm and AMe suggests that both SP and products of the prodynorphin precursor may participate in the chemosensory control of male sexual behavior. SP has been reported to facilitate mating behavior when injected into the MPOA of the male rat by shortening mounting and ejaculation latencies[12]. Though parallel behavioral effects of leumorphin have not been studied in the male, dynorphin A injected into the MPOA has also been shown to induce copulatory behavior[2], and injections of dynorphin B into the ventral tegmental area facilitates male mounting[39].

Additional anatomical and physiological evidence further supports the notion that SP and opioid peptides play a role in mediating sexual behavior. Opiate[18] and SP[48] receptors have been localized in the MPOA, BNSTm and AMe in the rat and in the MPOA of the hamster[49], and SP has been shown to increase firing in neurons of both the MPOA[34] and the AMe[4] in the rat.

The colocalization of SP and prodynorphin opioid peptides suggests that they may interact to regulate neuronal function. SP has been shown to regulate

the secretion of proenkephalin peptides. Administration of SP induces the release of Met[5]-enkephalin from striatal and periaqueductal gray brain slices[8] and stimulates the release of Met[5]-enkephalin and Met[5]-enkephalin-Arg[6]-Phe[7] from rat spinal cord[64]. If SP has the same effect on prodynorphin products, then SP could induce release of leumorphin, dynorphin A or dynorphin B from nerve terminals which contain both SP and a prodynorphin peptide. This mechanism has been proposed for SP-induced release of thyrotropin releasing hormone in the ventral spinal cord of the rat[3].

As circulating gonadal steroids play a major role in the regulation of mating behavior, an important question is whether SP or prodynorphin production is regulated by these hormones. Neurons in the AMe, MPOA and BNSTm of the hamster actively accumulate androgens[11] and estrogens[25]. The colocalization of SP and prodynorphin peptides within neurons in these areas raises the possibility that these peptides mediate steroidal regulation of mating behavior. We have recently reported that the number of neurons displaying SP-like immunoreactivity is substantially reduced in the MPOA, BNSTm and AMe 13 weeks after castration[63], a time at which complete loss of normal mating behavior has been observed in this species[40].

Clearly further studies are warranted to determine the role of SP and prodynorphin peptides and the nature of their interaction in the regulation of mating behavior in the Syrian hamster.

ACKNOWLEDGEMENTS

We wish to thank Drs. Stanley J. Watson Jr. and Lars Terenius for generously providing us with antisera for this study. We also wish to thank Dr. James Herman, Diana Gomez and Anthony Kincaid for critical reading of the manuscript. The Morphology Core of the Center for the Study of Reproduction at the University of Michigan provided valuable technical assistance, supported by 1-P30-HD-18258. This work was supported by NIH Grant NS20629 to S.W.N. and NIGMS Grant GM10341 to C.R.N.

REFERENCES

1 Anderson, K.D. and Reiner, A., Extensive co-occurrence of substance P and dynorphin in striatal projection neurons in rats, *Soc. Neurosci. Abstr.*, 14 (1988) 76.
2 Band, L. and Hull, E., Opioids microinjected into central dopaminergic pathways: effects on copulation in male rats, *Soc. Neurosci. Abstr.*, 14 (1988) 293.
3 Bartfai, T., Iverfeldt, K. and Fisone, G., Regulation of the release of coexisting neurotransmitters. In R. George, R. Okun and A.K. Cho (Eds.), *Annual Review of Pharmacology and Toxicology, Vol. 28,* Annual Reviews, Palo Alto, 1988, pp. 285–310.
4 Ben-Ari, Y., Le Gal Le Salle, G. and Levesque, G., Effects of substance P on neurons of the medial nucleus of the amygdala. In R.W. Ryall and J.S. Kelly (Eds.), *Iontophoresis and Transmitter Mechanisms in the Mammalian Central Nervous System,* Elsevier, New York, 1978, pp. 93–95.
5 Bloom, F., Battenberg, E., Rossier, J., Ling, N. and Guillemin, R., Neurons containing β-endorphin in rat brain exist separately from those containing enkephalin: immunocytochemical studies, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 1591–1595.
6 Cuello, A.C. and Kanazawa, I., The distribution of substance P immunoreactive fibers in the rat central nervous system, *J. Comp. Neurol.*, 178 (1978) 129–156.
7 Cuello, A.C., Priestley, J.V. and Paxinos, G., Substance P and enkephalin containing pathways. In G. Paxinos (Ed.), *The Rat Central Nervous System, Vol. 1,* Academic Press, Sydney, 1985, pp. 441–469.
8 Del Rio, J., Naranjo, J.R., Yang, H.-Y.T. and Costa, E., Substance P induced release of Met[5]-enkephalin from striatal and periaqueductal gray slices, *Brain Research,* 279 (1983) 121–126.
9 De Olmos, J.S. and Ingram, W.R., The projection field of the stria terminalis in the rat brain. An experimental study, *J. Comp. Neurol.*, 146 (1972) 303–334.
10 Devor, M., Components of mating dissociated by lateral olfactory tract transection in male hamsters, *Brain Research,* 64 (1973) 437–441.
11 Doherty, P.C. and Sheridan, P.J., Uptake and retention of androgen in neurons of the brain of the Golden hamster, *Brain Research,* 219 (1981) 327–334.
12 Dornan, W.A. and Malsbury, C.W., The effects of medical preoptic area injections of substance P and cholecystokinin on male rat sexual behavior, *Soc. Neurosci. Abstr.*, 13 (1987) 613.
13 Dornan, W.A., Malsbury, C.W. and Penney, R.B., Facilitation of lordosis by injection of substance P into the midbrain central gray, *Neuroendocrinology,* 45 (1987) 498–506.
14 Evans, C.J., Barchas, J.D., Esch, F.S., Bohlen, P. and Weber, E., Isolation and characterization of an endogenous c-terminal fragment of the a-neo-endorphin/dynorphin precursor from bovine caudate nucleus, *J. Neurosci.*, 5 (1985) 1803–1807.
15 Fallon, J.H. and Leslie, F.M., Distribution of dynorphin and enkephalin peptides in the rat brain, *J. Comp. Neurol.*, 249 (1986) 293–336.
16 Finley, J.C.W., Maderdrut, J.L., Roger, L.J. and Petrusz, P., The immunocytochemical localization of somatostatin-containing neurons in the rat central nervous system, *Neuroscience,* 6 (1981) 2173–2192.
17 Gessa, G.L. and Paglietti, E., Induction of copulatory behavior in sexually inactive rats by naloxone, *Science,* 204

(1979) 203–205.
18 Herkenham, M. and Pert, C.B., In vitro autoradiography of opiate receptors in the rat brain suggests loci of opiatergic pathways, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 5532–5536.
19 Hökfelt, T., Holets, V.R., Staines, W., Meister, B., Melarder, T., Schalling, M., Schultzberg, M., Freedman, J., Björklund, H., Olson, L., Lindh, B., Elfvin, L.-G., Lundberg, J.M., Lindgren, J.A., Samuelsson, B., Pernow, B., Terenius, L., Post, C., Everitt, B. and Goldstein, M., Co-existence of neuronal messengers — an overview. In T. Hökfelt, K. Fuxe and B. Pernow (Eds.), *Progress in Brain Research, Vol. 68,* Elsevier, Amsterdam, 1986, pp. 33–70.
20 Hughes, A.M., Everitt, B.J. and Herbert, J., Selective effects of β-endorphin infused into the hypothalamus, preoptic area and bed nucleus of the stria terminalis on the sexual and ingestive behaviour of male rats, *Neuroscience,* 23 (1987) 1063–1073.
21 Imura, H., Kato, Y., Nakai, Y., Nakao, K., Tanaka, I., Jingami, H., Koh, T., Yoshimasa, Y., Tsukada, T., Suda, M., Sakamoto, M., Morii, N., Takahashi, H., Tojo, K. and Sugawara, A., Endogenous opioids and related peptides: from molecular biology to clinical medicine. The Sir Henry Dale Lecture for 1985, *J. Endocrinol.*, 107 (1985) 147–157.
22 Kevetter, G.A. and Winans, S.S., Connections of the corticomedial amygdala in the golden hamster. I. Efferents of the 'vomeronasal amygdala', *J. Comp. Neurol.*, 197 (1981) 81–98.
23 Khachaturian, H., Lewis, M.E., Schafer, M.K.-H. and Watson, S.J., Anatomy of the CNS opioid systems, *Trends Neurosci.*, 8 (1985) 111–119.
24 Krettek, J.E. and Price, J.L., Amygdaloid projections to subcortical structures within the basal forebrain and brainstem in rat and cat, *J. Comp. Neurol.*, 178 (1978) 225–254.
25 Krieger, M.S., Morrell, J.I. and Pfaff, D.W., Autoradiographic localization of estradiol concentrating cells in the female hamster brain, *Neuroendocrinology,* 22 (1976) 193–205.
26 Lehman, M.N., Winans, S.S. and Powers, B.J., Medial nucleus of the amygdala mediates chemosensory control of male hamster sexual behavior, *Science,* 210 (1980) 557–560.
27 Lehman, M.N. and Winans, S.S., Vomeronasal and olfactory pathways to the amygdala controlling male hamster sexual behavior: autoradiographic and behavioral analyses, *Brain Research,* 240 (1982) 27–41.
28 Lehman, M.N. and Winans, S.S., Evidence for a ventral non-strial pathway from the amygdala to the bed nucleus of the stria terminalis in the male golden hamster, *Brain Research,* 268 (1983) 139–146.
29 Lehman, M.N., Winans, S.S. and Powers, B.J., Stria terminalis lesions alter the temporal pattern of copulatory behavior in the male golden hamster, *Behav. Brain Res.*, 8 (1983) 109–128.
30 Ljungdahl, A., Hökfelt, T. and Nilsson, G., Distribution of substance P-like immunoreactivity in the central nervous system of the rat. I. Cell bodies and nerve terminals, *Neuroscience,* 3 (1978) 861–943.
31 Loren, I., Emson, P.C., Fahrenkrug, J., Björklund, A., Alumets, J., Hakanson, R. and Sundler, F., Distribution of vasoactive intestinal polypeptide in the rat and mouse brain, *Neuroscience,* 4 (1979) 1953–1976.
32 Malsbury, C.W. and Daood, J.T., The medial preoptic area receives a major substance P projection from the region of the ventromedial nucleus of the hypothalamus in the rat, *Soc. Neurosci. Abstr.*, 11 (1985) 681.
33 Maragos, W.F., Newman, S.W., Lehman, M.N. and Powers, J.B., The neurons of origin and fiber trajectory of amygdalo-fugal projections to the medial preoptic area in the golden hamster, *J. Comp. Neurol.*, 280 (1989) 59–71.
34 Mayer, M.L. and MacLeod, N.K., The excitatory action of substance P and stimulation of the stria terminalis bed nucleus on preoptic neurons, *Brain Research,* 166 (1979) 206–210.
35 McGinty, J.F., Hendriksen, S.J., Goldstein, A., Terenius, L. and Bloom, F.E., Dynorphin is contained within hippocampal mossy fibers: immunochemical alterations after kainic acid administration and colchicine-induced neurotoxicity, *Proc. Natl. Acad. Sci. U.S.A.*, 80 (1983) 589–593.
36 McIntosh, T.K., Vallano, M.L. and Barfield, R.J., Effects of morphine, β-endorphin and naloxone on catecholamine levels and sexual behavior in the male rat, *Pharmacol. Biochem. Behav.*, 13 (1980) 435–441.
37 McLean S., Bannon, M.J., Zamir, N. and Pert, C.B., Comparison of the substance P- and dynorphin-containing projections to the substantia nigra: a radioimmunocytochemical and biochemical study, *Brain Research,* 361 (1985) 185–192.
38 Meyerson, B.J. and Terenius, L., β-Endorphin and male sexual behavior, *Eur. J. Pharmacol.*, 42 (1977) 191–192.
39 Mitchell, J.B. and Stewart, J., The effects of intra-VTA infusions of morphine and dynorphin (1–13) on male sexual behavior, *Soc. Neurosci. Abstr.*, 14 (1988) 849.
40 Morin, L.P. and Zucker, L., Photoperiodic regulation of copulatory behaviour in the male hamster, *J. Endocrinol.*, 77 (1978) 249–258.
41 Murphy, M.R. and Schneider, G.E., Olfactory bulb removal eliminates mating behavior in the male golden hamster, *Science,* 167 (1970) 302–304.
42 Murphy, M.R., Bowie, D.L. and Pert, C.B., Copulation elevates plasma β-endorphin in the male hamster, *Soc. Neurosci. Abstr.*, 5 (1979) 470.
43 Murphy, M.R., Methadone reduces sexual performance and sexual motivation in the male Syrian hamster, *Pharmacol. Biochem. Behav.*, 14 (1981) 561–567.
44 Myers, B.M. and Baum, M.J., Facilitation by opiate antagonists of sexual performance in the male rat, *Pharmacol. Biochem. Behav.*, 10 (1979) 615–618.
45 Neal, C.R. and Newman, S.W., The anatomical distribution of peptides showing prodynorphin immunoreactivity in the central nervous system of the golden hamster, *Soc. Neurosci. Abstr.*, 13 (1987) 1701.
46 Neal, C.R. and Newman, S.W., Prodynorphin peptide distribution in the forebrain of the Syrian hamster and rat: a comparative study with antisera against dynorphin A, dynorphin B and the C-terminus of the prodynorphin precursor molecule, *J. Comp. Neurol.*, in press.
47 Newman, S.W., Neal, C.R. and Swann, J.M., The colocalization of substance P and prodynorphin immunoreactivity in the medial preoptic area, bed nucleus of the stria terminalis and medial nucleus of the amygdala of the golden hamster, *Soc. Neurosci. Abstr.*, 13 (1987) 1271.
48 O'Donohue, T.L., Helke, C.J., Burcher, E., Shults, C.W. and Buck, S.H., Autoradiographic distribution of binding sites for iodinated tachykinins in the rat central nervous system. In J.L. Henry, R. Couture, A.C. Cuello, G. Pelletier, R. Quirion and D. Regoli (Eds.), *Substance P*

and the Neurokinins, Springer, New York, 1987, pp. 93–95.
49 Ostrowski, N.L., Hill, J.M., Pert, C.B. and Pert, A., Autoradiographic visualization of sex differences in the pattern and density of opiate receptors in hamster hypothalamus, *Brain Research,* 421 (1987) 1–13.
50 Platt, J.L. and Michael, A.F., Retardation of fading and enhancement of intensity of immunofluorescence by *p*-phenylenediamine, *J. Histochem. Cytochem.,* 31 (1983) 840–842.
51 Powers, J.B. and Winans, S.S., Vomeronasal organ: critical role in mediating sexual behavior of the male hamster, *Science,* 187 (1975) 961–963.
52 Powers, B.J., Newman, S.W. and Bergondy, M.L., MPOA and BNST lesions in male Syrian hamsters: differential effects on copulatory and chemoinvestigatory behaviors, *Behav. Brain Res.,* 23 (1987) 181–195.
53 Quarantotti, B.P., Corda, M.G., Paglietti, E., Biggio, G. and Gessa, G.L., Inhibition of copulatory behavior in male rats by D-Ala^2-Met-enkephalinamide, *Life Sci.,* 23 (1978) 673–678.
54 Reiner, A., The co-occurrence of substance P-like immunoreactivity and dynorphin-like immunoreactivity in striatopallidal and striatonigral projection neurons in birds and reptiles, *Brain Research,* 371 (1986) 155–161.
55 Roberts, G.W., Woodhams, P.L., Polak, J.M. and Crow, T.J., Distribution of neuropeptides in the limbic system of the rat: the amygdaloid complex, *Neuroscience,* 7 (1982) 99–131.
56 Sakuma, Y. and Akaishi, T., Leumorphin, a novel opioid peptide, promotes lordosis in female rats, *Brain Research,* 407 (1987) 401–404.
57 Seizinger, B.R., Grimm, C., Hollt, V. and Herz, A., Evidence for a selective processing of proenkephalin B into different opioid peptide forms in particular regions of the rat brain and pituitary, *J. Neurochem.,* 42 (1984) 447–457.
58 Shimada, S., Inagaki, S., Kubota, Y., Kito, S., Shiotani, Y. and Tohyama, M., Coexistence of substance P and enkephalin-like peptides in single neurons of the rat hypothalamus, *Brain Research,* 425 (1987) 256–262.
59 Shimada, S., Inagaki, S., Kubota, Y., Kito, S., Shiotani, Y. and Tohyama, M., Coexistence of substance P and neurotensin-like peptides in single neurons of the rat hypothalamus, *Peptides,* 9 (1988) 71–76.
60 Simerly, R.B., Gorski, R.A. and Swanson, L.W., Neurotransmitter specificity of cells and fibers in the medial preoptic nucleus: an immunohistochemical study in the rat, *J. Comp. Neurol.,* 246 (1986) 343–363.
61 Sofroniew, M.V., Vascular and neural projections of hypothalamic neurons producing neurohypophyseal or ACTH-related peptides. In A.J. Baertschi and J.J. Dreifuss (Eds.), *Neuroendocrinology of Vasopressin, Corticoliberin and Opiomelanocortins,* Academic Press, New York, 1982, pp. 73–86.
62 Suda, M., Nakao, K., Sakamoto, M., Morii, N., Sugawara, A. and Imura, H., Changes in the immunoreactivities of an opioid peptide leumorphin in the hypothalamus and anterior pituitary during the estrous cycle of the rat and their relation to sexual behavior, *Brain Research,* 374 (1986) 236–243.
63 Swann, J.M. and Newman, S.W., Effect of castration and testosterone treatment on substance P levels within the vomeronasal pathway of the male golden hamster, *Soc. Neurosci. Abstr.,* 13 (1987) 1576.
64 Tang, J., Chou, J., Yang, H.-Y.T. and Costa, E., Substance P stimulates the release of Met^5-enkephalin and Met^5-enkephalin-Arg^6-Phe^7 from rat spinal cord, *Neuropharmacology,* 22 (1983) 1147–1150.
65 Uhl, G.R. and Snyder, S.H., Neurotensin: a neuronal pathway projecting from amygdala through stria terminalis, *Brain Research,* 161 (1979) 522–526.
66 Vijayan, E. and McCann, S.M., In vivo and in vitro effects of substance P and neurotensins on gonadotropin and prolactin release, *Endocrinology,* 105 (1979) 64–68.
67 Weihe, E., Leibold, A., Nohr, D., Fink, T. and Gauweiler, B., Co-existence of prodynorphin-opioid peptides and substance P in primary sensory afferents of guinea pigs. In J.W. Holaday, P. Law and A. Herz (Eds.), *Progress in Opioid Research. Proceedings of the 1986 International Narcotics Research Conference,* 1986, pp. 295–298.
68 Winans, S.S. and Powers, B.J., Olfactory and vomeronasal deafferentation of male hamsters: histological and behavioral analyses, *Brain Research,* 126 (1977) 325–344.
69 Winans, S.S., Lehman, M.N. and Powers, B.J., Vomeronasal and olfactory CNS pathways which control male hamster mating behavior. In W. Breipohl (Ed.), *Olfaction and Endocrine Regulation,* IRL Press, 1982, pp. 23–34.
70 Woodhams, P.L., Roberts, G.W., Polak, F.J.M. and Crow, T.J., Distribution of neuropeptides in the limbic system of the rat: the bed nucleus of the stria terminalis, septum and preoptic area, *Neuroscience,* 8 (1983) 677–703.
71 Wu, F.M.F. and Noble, R.G., Opiate antagonists and copulatory behavior of male hamsters, *Physiol. Behav.,* 38 (1986) 817–825.