Pharmacology Biochemistry & Behavior, Vol. 22, pp. 699–705, 1985. © Ankho International Inc. Printed in the U.S.A. 0091-3057/85 $3.00 + .00

Autoradiographic Localization of Kappa Opiate Receptors in CNS Taste and Feeding Areas

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Received 6 August 1984

LYNCH, W. C., J. WATT, S. KRALL AND C. M. PADEN. *Autoradiographic localization of kappa opiate receptors in CNS taste and feeding areas.* PHARMACOL BIOCHEM BEHAV 22(5) 699–705, 1985.—Recent evidence suggests that kappa opiate receptors may play a key role in the regulation of appetite [21]. Such evidence implies that kappa receptors might be localized within specific brain areas known to regulate ingestive behaviors. On the basis of this implication we employed an *in vitro* film autoradiographic technique using ^{3}H-ethylketocyclazozine as ligand to identify putative kappa receptors [7] within CNS "taste" nuclei [23] and surrounding areas. Coronal cryostat sections of rat brain were incubated with ligand in the presence of D-Ala^2, D-Leu^5-enkephalin (DADLE) and morphine, apposed to LKB Ultrofilm for 60 days, processed and kappa receptor densities evaluated with the aid of a hand held photometer and video image analyzer. Highest kappa receptor densities were found within various gustatory and feeding sites including the rostral pole of the nucleus of the solitary tract, parabrachial nuclei, ventral posterior and medial portions of the thalamus, medial hypothalamus, medial nuclei of the amygdala and bed nucleus of the stria terminalis. Various other midline and medial limbic areas also showed significant kappa densities.

Kappa opiate receptors Film autoradiography ^{3}H-ethylketocyclazocine Taste preference Feeding Drinking Appetite Gustatory neuroanatomy

IT is clear from numerous recent reports that opioid peptides and opiate receptors play a key role in the regulation of feeding behavior [21]. Less clear is how these effects on feeding are mediated. We have been interested in the possibility that opiate receptors may modulate palatability [16], as suggested by several recent studies. For instance, the suppression of food or water intake by opiate antagonists is enhanced by flavor [1, 12, 13, 31] and, in the case of saccharin solutions, suppression is greatest at the most highly preferred midrange of saccharin concentrations [16]. Other evidence suggesting an opiate influence on gustatory factors includes the fact that naloxone inhibits intake of sugar-water even in rats drinking with open gastric fistulas [27] and that naloxone preferentially limits the duration of drinking bouts but does not affect the latency to begin drinking [2,30]. The extremely low ED_{50} for naloxone's effect on intake of highly preferred solutions of sucrose ($\simeq$0.065 mg/kg, SC) further suggests that these antagonist effects are probably not due to generalized motor disturbances or illness [34]. That these effects are centrally rather than peripherally initiated is suggested by evidence that naloxone's quarternary analogue is relatively ineffective in suppressing saccharin or saline ingestion [3].

Although the involvement of specific opiate receptor subtypes in the modulation of feeding is not yet well established, a number of studies suggest a role for kappa (κ) opiate receptors. This evidence includes the fact that both exogenous κ agonists [19] and endogenous prodynorphin peptides [18,20] can initiate feeding and that putative κ-selective antagonists inhibit feeding and drinking at relatively low doses [10,28]. In addition, we have reported that when drinking is motivated by palatability, the putative κ antagonist Mr2266 effectively suppresses intake [15].

The present study is based on recent reports that ^{3}H-ethylketocyclazocine (^{3}H-EKC) binding to both guinea pig [7] and rat [25] brain sections exhibits pharmacological specificity for κ opiate receptors when measured in the presence of substances which displace binding to mu (μ) and delta (δ) receptors. We employed this technique in order to detect κ receptors within major nuclei of the classic gustatory pathways of the brain [23].

METHOD

Tissue Preparation

Subjects were 6 adult male Holtzman albino rats. Animals were anesthetized with ether and decapitated. Brains were carefully removed to ice-cold saline for 5 min, then were blocked fresh and transferred to dry ice. Frozen brains were wrapped in aluminum foil and stored at −80°C for 2 to 7 days. Prior to sectioning, tissue blocks were mounted on cryostat chucks and allowed to equilibrate at −15°C for several hours. Sections were cut at 32 μ through each of five preselected nuclei of the gustatory pathways outlined by Norgren [23]. Approximately six sections were taken from each area of each brain and thaw-mounted onto subbed slides as described by Rainbow *et al.* [26]. To assure firm adhesion, slides were kept at cryostat temperature for 24 hours prior to incubation.

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Sections were cut according to the flat-skull coordinate system of Paxinos and Watson [24]. The following major gustatory nuclei were examined at A-P coordinates posterior to bregma indicated in parentheses: anterior pole of the nucleus of the solitary tract (−11.8), dorsal parabrachial nucleus (−9.8), ventroposterior thalamic nuclei (−3.8), central nucleus of the amygdala (−2.8), and bed nucleus of the stria terminalis (−0.8). In addition, these sections also included other areas involved in feeding, particularly lateral, ventromedial and periventricular hypothalamic areas, locus coeruleus [17] and medial nucleus of the amygdala [5].

Tissue Incubation

Radiolabeling of κ receptors was performed at 25°C following the procedure of Goodman and Snyder [7]. Sections were preincubated for 15 min in 0.17 M tris (base) adjusted to pH 7.4, containing 120 mM NaCl and 50 μM GTP to release endogenous ligands from opiate receptors. The sections were then transferred to salt-free tris and incubated for 30 min with 1.6 nM 9-^{3}H (−)-ethylketocyclazocine (New England Nuclear; 16.4 Ci/mmole) either alone or in the presence of 30 nM morphine plus 100 nM DADLE (D-Ala^2, D-Leu^5-enkephalin) or in the presence of 1 μM levallorphan. At the end of the incubation period sections were rinsed twice in ice-cold tris (5 min each), dipped briefly in water, then dried at 60°C and apposed to LKB Ultrofilm. Films were exposed for 60 days, developed and analyzed [26].

Data Analysis

In order to identify areas of highest κ receptor binding two qualitative methods were used. The first consisted of selecting a single high contrast autoradiogram incubated in the presence of morphine and DADLE from each of the five anatomical areas and directly comparing it with the corresponding section stained with cresyl violet. This allowed precise determination of the actual nuclei showing highest κ densities. The second method consisted of enhancing the contrast of several sections from each area using a video image analyzer (Eye Com II, Spacial Data Systems, Inc., Goleta, CA). The original autoradiogram was converted by the image analyzer to a TV picture, then digitized and enhanced by various computer programs that allowed adjustment of the original range of contrasts. The enhanced images were then photographed and areas containing highest κ densities identified by comparison with the stereotaxic atlas of Paxinos and Watson [24].

Quantitative densitometry of unenhanced autoradiograms was performed by projecting them with an overhead projector (×9 magnification) onto the lens of a digital photometer similar to that used by Rainbow *et al.* [26]. The 1 mm^2 field of the photometer allowed sampling at least eight individual areas within each nucleus or area of interest.

Calibration with neutral density absorbance standards (Model 202, Gilford Instruments, Oberlin, OH) verified that the photometer voltage output was linear (r=1.00) with percent transmittance from 1 to 80%, deviating slightly (10%) from linearity between 80 and 100%. Film background transmittance was between 70 and 80%, making it reasonable to employ the inverse of the photometer voltage as a direct measure of the relative density of autoradiograms. Relative densities within the range observed in this study have been shown to be proportional to the amount of radioactive ligand bound to the tissue section [26].

RESULTS

Specificity of ^{3}H-EKC Binding

Sections incubated with ^{3}H-EKC in the presence of saturating concentrations of the μ receptor agonist, morphine, plus the δ agonist, DADLE, showed low levels of residual (presumably κ) binding. Visual comparison of these sections with those incubated with excess levallorphan (+L) to eliminate all specific binding suggested, however, that significant levels of κ binding did occur in particular areas of the rat brain. In addition, the spatial pattern of apparent κ binding appeared distinct from that of total EKC binding. In order to determine if κ binding was indeed significantly greater than nonspecific background (+L) levels, and was not merely residual binding to μ and δ receptors, quantitative densitometry was performed on several autoradiograms.

Figure 1 shows three adjacent sections at the same approximate A-P level (bregma −0.8 mm) from a single brain, developed side-by-side on a single sheet of film. All three sections are at the level of the bed nucleus of the stria terminalis (BST) and caudate/putamen (CPu). Section A was incubated with EKC alone (+O), section B with EKC plus saturating concentrations of morphine plus DADLE (M+D) and section C with EKC plus excess levallorphan (+L). It is apparent from section B that morphine plus DADLE block most of the binding of EKC in all areas. Nevertheless, slight residual binding can be seen particularly in the medial preoptic area (MPO). On the other hand, very prominent binding in receptor-dense patches within CPu (striosomal patches) in section A is almost completely blocked by addition of M+D to the incubation. In order to quantify this apparent difference in the effect of M+D on binding in CPu and MPO, we compared the optical densities in these two areas with and without M+D. Table 1 shows the results of this analysis. Specific EKC binding in the +O and M+D conditions was determined by subtracting binding in the presence of levallorphan (+L) from each of the other two conditions. The ratio of these difference values multiplied by 100 yielded the percentage of EKC binding (in excess of background) not blocked by the μ and δ receptor agonists ($\%D_\kappa$). The fact that approximately 85% of EKC binding is blocked in receptor-dense CPu patches as compared to only about 60% in MPO suggests that excess MPO binding is binding to a unique receptor subclass, presumably κ sites [6,7]. An overall ANOVA of density values (1/photometer voltage) indicated significant main effects of brain area, $F(1,90)= 5.13$, $p<0.05$, and incubation condition, $F(2,90)= 899.6$, $p<0.001$, as well as a significant area × condition interaction, $F(2,90)=27.12$, $p<0.001$. Individual comparisons among cell means by the LSD multiple range test (SPSS) indicated a highly significant difference between

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FIG. 1. Adjacent sections at the level of the bed nucleus of the stria terminalis (BST), medial preoptic area (MPO) and the caudate/putamen (CPu) incubated with: (A) ^{3}H-EKC alone, (B) ^{3}H-EKC plus unlabelled morphine (μ) and DADLE (δ), and (C) ^{3}H-EKC plus excess unlabelled levallorphan. Existence of κ receptors is indicated by the differential effect on MPO vs. CPu binding of adding μ and δ receptor ligands to the incubation, as seen by comparing sections A and B. (See Table 1 and text for further details).

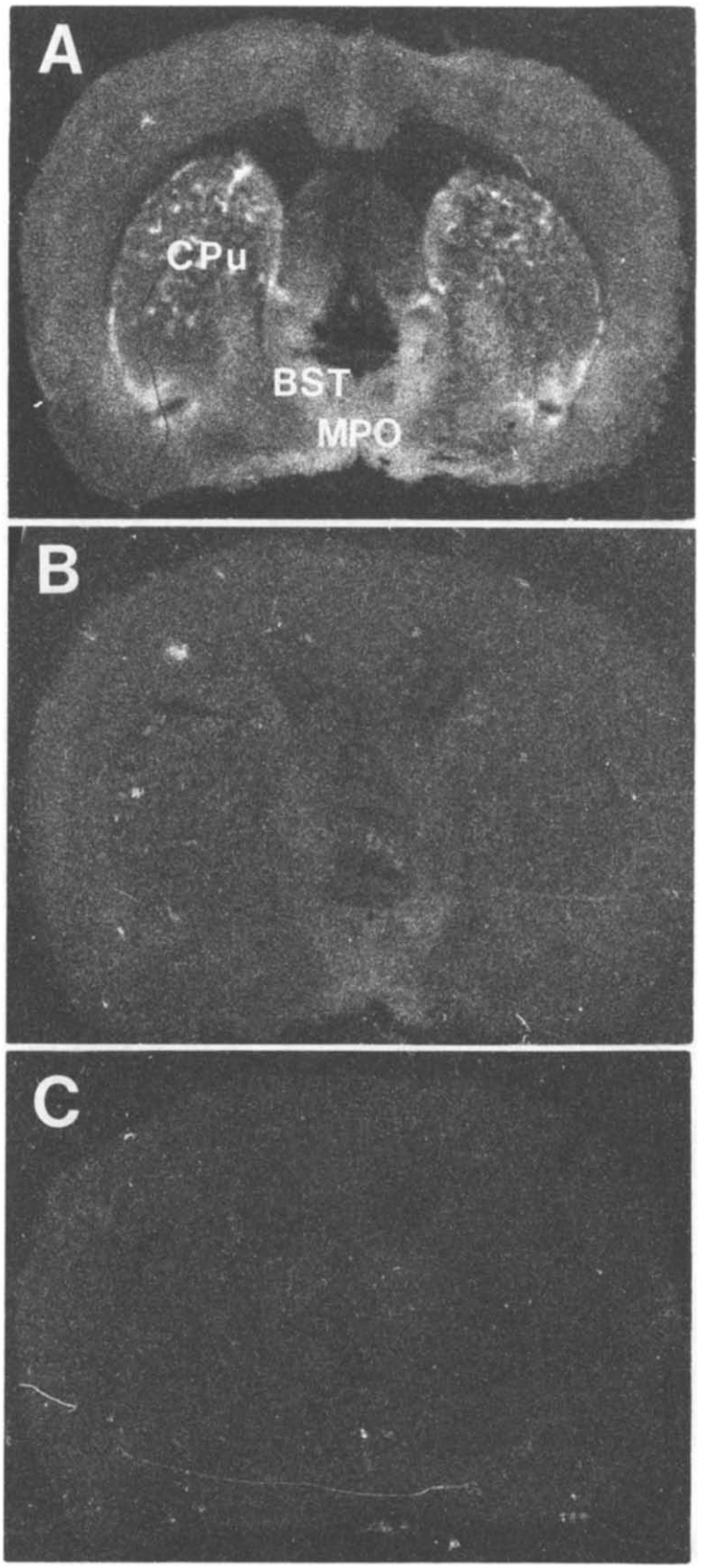
A
CPu
BST
MPO
B
C

TABLE 1

RELATIVE DENSITY* MEASUREMENTS OF ^{3}H-EKC BINDING IN RAT BRAIN SECTIONS INCUBATED WITH EKC ALONE (D_0) EKC PLUS MORPHINE AND DADLE (D_{M+D}) OR EKC PLUS EXCESS LEVALLORPHAN (D_L)

Brain Area	D_O	D_{M+D}	D_L	$\%D_\kappa$†
Left MPO	1.123 (0.034)	0.824 (0.010)	0.619 (0.006)	40.7
Right MPO	1.181 (0.039)	0.830 (0.018)	0.616 (0.005)	37.9
Left CPu ("Patches")	1.184 (0.065)	0.728 (0.013)	0.652 (0.014)	14.3
Right CPu ("Patches")	1.241 (0.027)	0.710 (0.012)	0.630 (0.009)	13.1

*Density=1/photometer output voltage (volts). Each value=mean of 8 samples (±SEM).
†$\%D_\kappa=[(D_{M+D}-D_L)/(D_O-D_L)]\times 100$.

brain areas (MPO vs. CPu) only in sections incubated with M+D ($p<0.001$).

Brain Sites of Highest κ Density

A list of kappa binding sites was compiled by matching cresyl violet stained sections to their corresponding M+D autoradiograms. Only discrete nuclei showing relatively high density contrast or general areas of moderate contrast could be identified by this method because of the low overall binding. We therefore chose several M+D autoradiograms with the clearest definition at each A-P level for further analysis using the video image analyzer. Figure 2 illustrates the appearance of digitally enhanced autoradiograms (right) and approximately corresponding atlas sections (left) at each of the five A-P levels from rostral (top) to caudal (bottom). Coordinates caudal to bregma (mm) are shown in the lower right corner of each atlas section. Areas of most dense binding are labelled and emphasized by stippling in atlas sections. The degree of correspondence between areas of relatively dense κ binding and areas implicated in taste and feeding is quite remarkable. Gustatory nuclei showing relatively dense binding include the rostral (gustatory) pole of the nucleus of the solitary tract (Sol), dorsal (gustatory) (DPB) and ventral (VPB) parts of the parabrachial nucleus, parvocellular (gustatory) portion of the ventroposterior thalamic nucleus (VPT), medial part of the central amygdaloid nucleus (Ce) and lateral (gustatory) and medial portions of the bed nucleus of the stria terminalis (BST). Other areas of relatively dense κ binding, which have been implicated in the regulation of feeding behavior included: the ventromedial nucleus of the hypothalamus (VMH), medial portions of the lateral hypothalamus (LH), the medial nucleus of the amygdala (Me) and the locus coeruleus (LC). Other areas of relatively dense binding included the medial septum (MS), medial preoptic nucleus and surrounding areas (MPO), most of the medial hypothalamus (MH), including the dorsomedial (DM) and posterior (PH) nuclei, medial portions of the thalamus (MT), medial and lateral habenular nuclei (Hb), central gray of the tegmentum (CG) and the caudal pole of the inferior colliculus (IC). More moderate binding which was apparent only in the enhanced autoradiograms include the outer layers of the cerebral cortex (unlabelled), dorsomedial tip of the caudate (CPu*), medial portions of the lateral preoptic area (LPO), dorsomedial (MT) and dorsolateral (LT) portions of the thalamus and basolateral amygdala (BLA).

DISCUSSION

A variety of behavioral evidence led us to postulate that κ receptors might be found in CNS gustatory and feeding related areas. In addition to evidence showing that flavor enhances the opiate antagonist suppression of intake [1, 12, 13, 16, 31], recent reports also suggest that when intake is stimulated primarily by taste, moderately κ-selective antagonists such as Mr2266 depress intake in a dose-dependent manner and κ-selective agonists enhance intake [14,15]. The present results provide a striking confirmation of the hypothesis that κ opiate receptors might be located in brain taste/feeding areas. We noted substantial κ binding in every major subcortical area of the brain which has been associated with classic gustatory pathways [23] and while κ binding was by no means localized exclusively in these areas, the distribution of κ sites was highly restricted.

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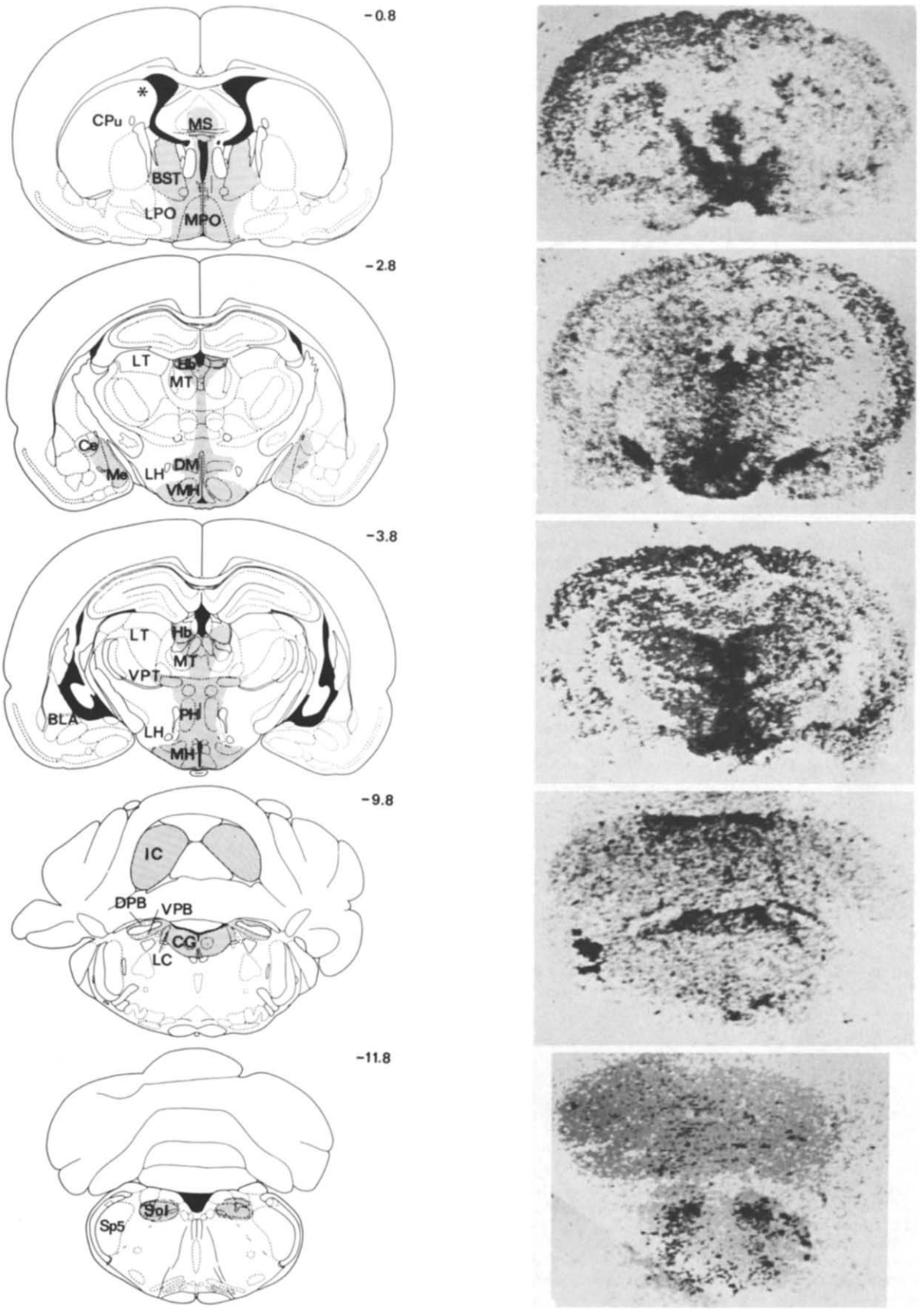
FIG. 2. Computer enhanced autoradiograms (right) and approximately corresponding stereotaxic atlas sections (left) at each of 5 A-P levels of the classic gustatory pathways of the rat brain. Flat-skull coordinates rostral to bregma (mm) are shown in the lower right corner of each atlas figure. In the enhanced images darkest areas indicate highest density κ binding. In corresponding atlas figures, stippling indicates approximate areas of highest κ density. Letters identify specific nuclei where these were apparent or general areas when specific nuclei could not be differentiated. Nuclei (or areas) of highest κ density include: bed nucleus of the stria terminalis (BST), central gray of the tegmentum (CG), central nucleus of the amygdala (Ce), dorsomedial nucleus of the hypothalamus (DM), dorsal parabrachial nucleus (DPB), medial and lateral habenular nuclei (Hb), locus coeruleus (LC), medial nucleus of the amygdala (Me), medial portions of the hypothalamus (MH), medial preoptic area (MPO), medial septal area (MS), medial portions of the thalamus (MT), posterior hypothalamus (PH), rostral pole of the nucleus of the solitary tract (Sol), ventromedial nucleus of the hypothalamus (VMH), ventral parabrachial nucleus (VPB), and the parvocellular portion of the ventroposterior nucleus of the thalamus (VPT). Other areas of moderate κ binding included: basolateral nuclei of the amygdala (BSA), the dorsomedial tip of the caudate (CPu*), medial portions of the lateral hypothalamus (LH) and lateral preoptic area (LPO), dorsolateral (LT) and dorsomedial parts of the thalamus (MT), and lateral portions of the nucleus of the spinal trigeminal tract (Sp 5). Note that tissue distortions and low levels of residual κ binding made exact specification of nuclei impossible in many cases. Also note the artifact in the left lateral part of the enhancement at bregma: −9.8 mm.


-0.8
CPu
MS
BST
LPO
MPO
-2.8
LT
Hb
MT
Ce
Me
LH
DM
VMH
-3.8
LT
Hb
MT
VPT
BLA
PH
LH
MH
-9.8
IC
DPB
VPB
CG
LC
-11.8
Sol
Sp5

Only limited data on the distribution of putative κ receptors in the rat brain have been reported to date. Quirion *et. al.* [25] noted high densities of κ sites in "various nuclei of the amygdala, thalamus, habenula and the interpeduncular nucleus" (p. 185). Somewhat lower densities were reported in the hippocampus, hypothalamus, and substantia nigra. In the present study we noted highest κ densities in medial and central nuclei of the amygdala, medial thalamus, medial and lateral habenular nuclei, medial septum and medial preoptic/anterior hypothalamic areas. We did not observe significant binding in hippocampus and our selected sections did not include either the interpeduncular nucleus or substantia nigra. Thus the present findings are generally consistent with the report of Quirion *et al.* [25]. However in our hands, ^{3}H-EKC binding to the caudate-putamen (bregma: −0.3 to −0.8mm) [24] was low in the presence of morphine and DADLE (except for slight binding at the dorsomedial tip of the caudate), while Quirion *et al.* [25] reported high levels of residual ^{3}H-EKC binding at midstriatal levels in the presence of morphiceptin and delta related peptide as μ and δ blockers. Our results are more similar to those of Goodman and Snyder [7] in the guinea pig, who noted only diffuse residual ^{3}H-EKC binding without labeling of striosomal patches in the presence of morphine and DADLE. These discrepancies may reflect differences in incubation conditions or possibly genetic differences between inbred strains of rats.

Autoradiographic studies of CNS opiate receptor distribution using ^{3}H-naloxone as ligand also show substantial localization within feeding-related areas. Herkenham and Pert [8], for example, reported localization of naloxone binding in "'olfactory' portions of the amygdala,. . . bed nucleus of the stria terminalis,. . . the 'taste' relay nucleus of the thalamus,. . . the 'thirst' detection point in the subfornical organ,. . . and visceral sensory portions of the nucleus of the solitary tract and the parabrachial nucleus" (p. 1145).

To the extent that κ receptors are specifically involved in taste and feeding, one might also predict that endogenous κ ligands would be found in feeding-related brain sites. Interestingly, several recent reports indicate that the CNS distribution of prodynorphin peptides, which appear to be endogenous κ ligands [6], also shows a marked correspondence with taste/feeding areas. Immunoreactive dynorphin has been reported throughout the nucleus of the solitary tract, particularly the rostral portion anterior to obex and lateral to the tract [22]. This area, of course, is not exclusively gustatory but also subserves other visceral functions. Using a microdissection technique, Zamir *et al.* [35] reported an abundance of prodynorphin derived peptides in various taste/feeding related areas including anterior lateral hypothalamus, bed nucleus of stria terminalis, and parabrachial nuclei. In two mapping studies, Watson, Khatchaturian and colleagues observed prodynorphin immunoreactivity in hypothalamic magnocellular nuclei, nucleus of the solitary tract, periaqueductal gray and various other scattered areas in brainstem and spinal cord [9,33].

A few attempts to localize opiate effects on feeding by direct infusion of agonists or antagonists into or near feeding-related brain sites have met with limited success. Siviy *et al.* [29] reported decreases in drinking in 24 hr water-deprived rats following bilateral injections of naloxone into lateral ventricles and LH. Czech *et al.* [4] reported decreases in drinking in water deprived rats when naloxone was infused into LH, preoptic hypothalamus (near the lateral/medial border) and zona incerta. Tepperman and Hirst [32] reported that morphine injected into VMH induced feeding in nondeprived rats. However, the putative kappa agonist ketazocine and the putative sigma agonist phencyclidine were ineffective at this site. Liebowitz and Hor [11] showed that β-endorphin injected into the paraventricular hypothalamic nucleus (norepinephrine feeding area) induced feeding in sated rats that was reversible by local injection of either naloxone or the β-adrenergic blocker phentolamine. These results offer limited support for the proposition that certain opiates may act at brain feeding/drinking sites to modulate intake.

In sum, it is clear that neither kappa receptors nor endogenous kappa ligands are found exclusively in areas with a known role in taste, feeding, or drinking but the extent to which brain areas functionally related to ingestive behaviors overlap areas containing κ receptors and prodynorphin peptides is striking. Since various treatments such as food deprivation or taste aversion conditioning are known to alter taste preference, it will be interesting in the future to determine whether deprivational enhancements of intake or other changes in consumption related to palatability may be correlated with alterations in κ receptor density in these brain areas.

ACKNOWLEDGEMENTS

We wish to express our thanks to the late Dr. Thomas Rainbow for assistance in developing the autoradiographic assay and for an original design for the digital photometer. Also thanks to the MUS Department of Earth Sciences for use of the Eye Com II image processor and to Dr. Barbara Fernandez for statistical assistance. This work was supported in part by MONTS-NSF project ISP-801149 and MSU Research Creativity Awards to WCL and CMP and NIH 5 R23N51974 to CMP.

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