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The Distribution of Proenkephalin-Derived Peptides in the Central Nervous System of Turtles

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ABSTRACT

The present study was carried out to examine if peptides similar to the various opioid peptide products of mammalian proenkephalin are present in the turtle central nervous system and to determine their distribution. Antisera against several enkephalin peptides were used: 1) leucine-enkephalin (LENK), 2) methionine-enkephalin (MENK), 3) methionine-enkephalin-arg^6-phe^7 (MERF), 4) methionine-enkephalin-arg^6-gly^7-leu^8 (MERGL), 5) Peptide E (PEPE), and 6) BAM22P. Their specificity and cross-reactivity were carefully examined. The results indicated that LENK, MENK, and MERF (or highly similar peptides) are present in the turtle central nervous system, and that a peptide showing immunological similarity to BAM22P and PEPE also appeared to be present. In contrast, MERGL did not appear to be present. The distributions of the immunoreactive labeling for LENK, MENK, MERF, BAM22P, and PEPE were indistinguishable, and double-label studies showed that LENK, MERF, and BAM22P were colocalized within individual neurons and fibers. Although all of the above substances were observed in the same cell groups, there was some regional variation, in terms of which enkephalin peptide appeared to be most abundant.

The distributions of these enkephalin peptides were very similar to those previously described in mammals and birds. Enkephalin was more abundant in the basal ganglia than in overlying telencephalic regions. Within the basal ganglia, enkephalin was present in striatal neurons and fibers and in pallidal fibers, thereby suggesting the existence of an enkephalinergic striatopallidal projection. Sensory relay nuclei of the thalamus were generally poor in enkephalinergic fibers, whereas the hypothalamus was rich in enkephalinergic neurons and fibers. Enkephalinergic neurons and fibers were present in the midbrain central gray. As is true of neurons of the nucleus spiriformis lateralis of the avian pretectum, the neurons of the homologous cell group in turtles, the dorsal nucleus of the posterior commissure of the pretectum, were found to contain enkephalin and have an enkephalinergic projection to the deep layers of the ipsilateral tectum. Enkephalinergic neurons and fibers were also abundant in the entry zones of the trigeminal nerve and dorsal root fibers of the spinal cord.

The present results indicate that: 1) consistent with previously published biochemical studies (Lindberg and White, '86), proenkephalin in reptiles is similar in structure to that of mammals and, with the exception of MERGL, gives rise to similar or identical enkephalin peptides, and 2) the enkephalin peptides are found in many of the same systems of reptilian brain as mammalian and avian brain, and, therefore, may play a role in similar functions (e.g., basal ganglia motor functions) as in mammals and birds.

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Three distinct families of opioid peptides—the enkephalin peptides, the dynorphin peptides, and the endorphin peptides—have been identified in the nervous system in mammals (Hughes et al., '75; Akil et al., '84; Weber et al., '83; Khachaturian et al., '85). The peptides within each opioid peptide family are related in that they all derive from a common large precursor molecule—proenkephalin in the case of enkephalin peptides, prodynorphin in the case of the dynorphin peptides, and POMC in the case of the endorphin peptides (Lewis et al., '79; Stern et al., '79; Mizuno et al., '80; Rossier et al., '80; Kilpatrick et al., '81; Comb et al., '82; Jones et al., '82a,b; Kakidani et al., '82; Noda et al., '82; Bloch et al., '83; Gubler et al., '84). Since the members of a given opioid family have a common precursor, those members necessarily co-occur within individual neurons of the nervous system (Rossier et al., '80; Bloch et al., '83; Khachaturian et al., '83b, '85; Watson et al., '83; Weber et al., '83; Akil et al., '84). Although the CNS distribution of the three families of opioid peptides shows some overlap, the families: 1) are almost exclusively found in different neurons, 2) have different distributions, and 3) presumably play different (although possibly somewhat overlapping) roles in CNS function (Bloom et al., '78; Watson et al., '78, '82b; Weber et al., '83; Akil et al., '84; Khachaturian et al., '85).

Opioid peptides have also been found in nervous tissue in members of a wide variety of nonmammalian animal taxa, ranging from planarians to birds (Simantov et al., '76; Dubois et al., '79; Bayon et al., '80; Blahser and Dubois, '80; Jackson et al., '80; Reaves and Hayward, '80; Zipser, '80; De Lanerolle et al., '81; Doerr-Schott et al., '81; Finger, '81; Ryan et al., '81; Schulman et al., '81; Brauth and Reiner, '82; Dores, '82a; Gold and Finger, '82; Kuljis and Karten, '82; Reiner et al., '82b, '84b; LaValley and Ho, '83; Northcutt et al., '83, '86; Reiner, '83, '87; Brauth, '84; Georges and Durois, '84; Khachaturian et al., '84; Nozaki and Gorbman, '84, '85; Rzasa et al., '84; Reiner and Northcutt, '87). Several biochemical studies using RIA and/or HPLC have shown that members of each of the three families of opioid peptides are present in the nervous sytems of modern amphibians, thereby suggesting that the three distinct opioid peptide families had evolved as of early amphibians (Loh, '79; Cone and Goldstein, '82; King and Millar, '80; Kilpatrick et al., '83; Martens and Herbert, '84). The pentapeptide members of the enkephalin family have, in fact, been found in earthworm nervous tissue (Rzasa et al., '84), suggesting that the enkephalin peptides might have appeared in the nervous system prior to the vertebrate-invertebrate divergence.

Since the three distinct opioid peptide families appear to have evolved as of the early amphibians (if not earlier), it is not surprising that the available data strongly support the conclusion that endorphin peptides, enkephalin peptides, and dynorphin peptides are all present in the CNS of reptiles. The presence of endorphin peptides has been demonstrated in the reptilian nervous system and pituitary in a series of biochemical and immunohistochemical studies on *Anolis* by Dores and coworkers (Dores, '82a,b, '83; Dores and Surprenant, '83, '84; Dores et al., '84; Khachaturian et al., 84). The presence of several enkephalin peptides in the reptilian nervous system has been suggested by immunohistochemical studies (Naik et al., '81; Brauth and Reiner, '82; Brauth, '84; Wolters et al., '86) and confirmed by a recent biochemical study (Lindberg and White, '86). The presence of dynorphin peptides in the nervous system of reptiles has been demonstrated immunohistochemically with antisera that do not cross-react with enkephalin peptides (Reiner, '83, '86). In previous immunohistochemical studies, however, the distribution of enkephalin peptides in the reptilian nervous system has been examined using only antisera against leucine-enkephalin (LENK) and methionine-enkephalin (MENK) (Naik et al., '81; Brauth and Reiner, '82; Brauth, '84; Wolters et al., '86). In light of the potential cross-reactivity of anti-LENK or anti-MENK antisera with dynorphin peptides (which all contain the LENK sequence at their N-terminus), it is possible that previous studies on the distributions of LENK+ (or MENK+) cell bodies and fibers have not distinguished between enkephalinergic and dynorphinergic neurons (McGinty et al., '83; Reiner, '83; Reiner et al., '84b). In the present study, the distributions of several members of the enkephalin family of peptides were examined using immunohistochemistry. These enkephalin peptides included LENK, MENK, methionine-enkephalin-Arg^6-Phe^7 (MERF), methionine-enkephalin-Arg^6-Gly^7-Leu^8 (MERGL), BAM22P, and Peptide E (PEPE). In the case of LENK, MENK, and MERF, several different antisera against each were used. Since the antisera against the larger enkephalin peptides were found not to cross-react with dynorphin, their use allowed the unequivocal determination of the distribution of enkephalinergic perikarya and fibers in the reptilian CNS. Further, it was thought that the present studies, in conjunction with previous biochemical studies, might shed further light on the identities of the various enkephalin peptides present in the turtle nervous system and, therefore, on the similarity of turtle proenkephalin to mammalian proenkephalin.

MATERIALS AND METHODS

Both red-eared (*Pseudemys scripta*) and painted turtles (*Chryemys picta*) were used in the present study. Turtles were housed as described previously; some turtles received an intraventricular injection of colchicine (60 μg/3 μl distilled H_2O) to enhance perikaryal immunoreactivity, as described previously (Reiner and Beinfeld, '85). Turtles were deeply anesthetized with ketamine and perfused transcardially with 6% dextran in phosphate buffer (pH 7.2), followed by 4% paraformaldehyde in phosphate buffer (pH 7.2). Colchicine-treated turtles were perfused 72–120 hours after the colchicine injection. After perfusion, the brains were removed and postfixed in 4% paraformaldehyde in phosphate buffer (pH 10.4) for 2–16 hours. The brains were then immersed in sucrose-phosphate buffer for 24–48 hours and sectioned frozen at 40 microns on a sliding microtome. Sections were processed for immunohistochemistry according to either the immunofluorescence technique (Coons, '58) or the PAP technique (Sternberger, '79). The details of our use of these procedures has been described previously (Reiner et al., '84b,c; Reiner and Beinfeld, '85). In brief, tissue was washed 3 times in 0.1 M phosphate buffer (pH 7.2) (PB) and incubated at 4°C in the primary antiserum for 48–72 hours. For the immunofluorescence procedure, after wash-

ing 3 times in PB, tissue was incubated for 1 hour at room temperature in FITC-labeled secondary antiserum directed against the IgG of the animal species in whom the primary antiserum was raised. After washing 3 times in PB, tissue was then mounted and examined by means of a Leitz epi-illumination fluorescence microscopy system. For the PAP technique, after incubation in the primary antiserum and 3 washes in PB, tissue was incubated for 1 hour at room temperature in an unlabeled secondary antiserum directed against the IgG of the animal species in whom the primary antiserum was raised. Tissue was then washed 3 times in PB and incubated for 1 hour at room temperature in peroxidase-antiperoxidase (with antiperoxidase raised in the same animal species as the primary antiserum). After 3 washes in PB, the tissue was processed for HRP visualization with diaminobenzidine tetrahydrochloride (DAB). Tissue was preincubated in a solution of DAB (100 mg DAB.4HCl in 100 ml .05 M cacodylate-.05 M imidazole buffer, pH 7.0) for 10 minutes, followed by an additional 10 minutes' incubation in this solution following addition of 300 μl of 3% H_2O_2 per 100 ml incubating medium. Tissue was then washed, mounted on gelatin-coated slides, and coverslipped. Cell groups of the turtle brain and terminology for these cell groups were as used previously (Powers and Reiner, '80; Reiner et al., '84c; Reiner and Beinfeld, '85).

Antisera against several different enkephalin peptides were used in the present study (see Table 1): anti-LENK (A206, courtesy of K.J. Chang), anti-LENK (ImmunoNuclear Corp.), anti-LENK (mouse monoclonal antibody obtained from Sera-Labs, Accurate Chemical Co.), anti-MENK (A900, courtesy of K.J. Chang), anti-MENK (ImmunoNuclear Corp.), anti-BAM22P (RB334B6, courtesy of A. Baird), anti-MERF (L150, courtesy of G.J. Dockray), anti-MERF (courtesy of H.Y.T. Yang), anti-MERGL (L189, courtesy of G.J. Dockray), and anti-PEPE (courtesy of S.J. Watson). The specificities and cross-reactivities of the above antisera have been described in previous studies (Miller et al., '78; Williams and Dockray, '82, '83; Bloch et al., '83; Majane et al., '83; Cuello et al., '84; Giraud et al., '84; Reiner et al., '84b) and were examined in detail during the present study. The present findings on the specificity of the above antisera are described in the Results section. In brief, the antisera against BAM22P, PEPE, MERGL, and MERF showed little or no cross-reactivity with dynorphin peptides. Both the MENK antisera and the ImmunoNuclear anti-LENK antiserum showed negligible cross-reactivity with longer dynorphin peptides, but they did show some cross-reactivity with a shorter dynorphin peptide, Dynorphin A(1–8). The Chang A206 anti-LENK antiserum has been previously reported to show considerable cross-reactivity with Dynorphin A(1–8) (Reiner et al., '84b; McGinty et al., '83). The monoclonal anti-LENK antibody reportedly shows no cross-reactivity with Dynorphin A(1–13) (Cuello et al., '84). The above antisera were generally found to show little cross-reactivity with enkephalin peptides other than their target peptide, except that the LENK and MENK antisera recognized each other's antigens to a significant extent and the BAM22P and PEPE antisera recognized each other's antigens. To further distinguish enkephalin-containing cells and fibers from dynorphin-containing cells and fibers, the distribution of the labeling obtained with the various enkephalin antisera was compared to that obtained with an antiserum highly specific for the C-terminus of Dynorphin A(1–17) (obtained from L. Terenius). It was found that none of the above enkephalin antisera, except the A206, produced even light labeling of the dynorphinergic striatonigral tract of turtles (Reiner, '86a) and the A206 produced only light labeling of the striatonigral tract.

To determine whether LENK is present in the same enkephalinergic neurons as the other enkephalin peptides, a simultaneous immunofluorescence procedure was used, as described previously (Erichsen et al., '82; Reiner et al., '85; Wessendorf and Elde, '85), to colocalize LENK (with the monoclonal antibody) with each of the following: BAM22P (with the anti-BAM22P antiserum) and MERF (with Dockray's anti-MERF antiserum). As noted below, the monoclonal antibody shows no cross-reactivity with either BAM22P or MERF. Further, neither the anti-BAM22P antiserum nor the anti-MERF antiserum cross-reacts with LENK. Tissue was incubated in a primary antiserum mixture containing 1:10 monoclonal anti-LENK and one of the two other primary antisera noted above (both raised in Rb) at a 1:500 dilution for 48–72 hours. Tissue was washed 3 times in PB and incubated in a secondary antisera mixture containing 1:50 goat antimouse IgG conjugated to FITC and 1:50 goat antirabbit IgG conjugated to TRITC for 1 hour at room temperature. Tissue was washed, mounted on gelatin-coated slides, and coverslipped with 9:1 glycerol-carbonate buffer (pH 9.0) and examined with a Leitz epi-illumination fluorescence microscopy system. Double-labeled cells and fibers were identified by successively viewing FITC-labeling (with the I2 cube) and TRITC-labeling (with the N2 cube) of individual neurons and fibers.

RESULTS

Antibody specificity

The results of the blocking and cross-blocking studies performed are presented in Table 1. All of the antisera examined could be blocked with 25–100 microMolar amounts of their target peptide. Cross-blocking data are based on the use of 50–100 microMolar concentrations of the peptide used for cross-blocking studies. Data for the Chang A206 anti-LENK are based on previous studies (Miller et al., '78; McGinty et al., '83; Cuello et al., '84; Reiner et al., '84b). As can be seen, the Chang anti-LENK antiserum shows significant cross-reactivity with MENK and Dynorphin A(1–8). Thus, the Chang A206 antiserum appears to be largely directed at the N-terminus of LENK, which would account for the extensive cross-reactivity of the antiserum with peptides that possess the same N-terminal amino acid sequence as LENK. In comparison, the ImmunoNuclear anti-LENK antiserum shows much less cross-reactivity with Dynorphin A(1–8), and negligible cross-reactivity with Dynorphin A(1–17) and Dynorphin A(1–13). Among the enkephalin peptides, the ImmunoNuclear anti-LENK antiserum cross-reacts considerably with MENK and PEPE, but only slightly with MERF, BAM12P, and BAM22P. These results suggest that the ImmunoNuclear anti-LENK antiserum is largely specific for the C-terminus of LENK, with some cross-reactivity for small peptides (i.e., MENK and Dynorphin A 1–8) possessing the same N-terminus as LENK. The greater cross-reactivity of the ImmunoNuclear anti-LENK antiserum with PEPE than with BAM22P presumably reflects the fact that both the N-terminus and the C-terminus of PEPE are identical to those of LENK, whereas only the N-terminus of BAM22P is identical to that of LENK. The monoclonal anti-LENK antibody is reported to show considerable cross-reactivity with MENK, and no cross-reactivity with Dynorphin A(1–13), or

Abbreviations

a area a (Riss et al., '69)
AP area pretectalis
AT area triangularis
BA nucleus basalis amygdalae
BOR basal optic root
CA nucleus centralis amygdalae
Cb cerebellum
CbL nucleus cerebellaris lateralis
CbM nucleus cerebellaris medialis
cd cortex dorsalis
cdm cortex dorsomedialis
cm cortex medialis
CN core nucleus of the DVR
CO chiasma opticum
Co cochlear nuclei
CP commissura posterior
cp cortex pyriformis
cpv cortex pyriformis, pars ventralis
d area d (Riss et al., '69)
d IV decussatio nervi trochlearis
DLA nucleus dorsolateralis anterior
DMA nucleus dorsomedialis anterior
DSOD dorsal nucleus of the supraoptic decussation
DVR dorsal ventricular ridge of the telencephalon
FD funiculus dorsalis
FL funiculus lateralis
FLM fasciculus longitudinalis medialis
FRL formatio reticularis lateralis mesencephali
FV funiculus ventralis
GC griseum centrale
GCL granule cell layer
GLd nucleus geniculatus lateralis pars dorsalis
GLv nucleus geniculatus lateralis pars ventralis
GP globus pallidus
HL nucleus habenularis lateralis
HM nucleus habenularis medialis
IP nucleus interpeduncularis
Imc nucleus isthmi magnocellularis, pars caudalis
Imr nucleus isthmi magnocellularis, pars rostralis
Ipc nucleus isthmi parvocellularis
LA nucleus laminaris of the torus semicircularis
LGE laminaris granularis externa
LGI laminaris granularis interna
LL lateral lemniscus
LM nucleus lentiformis mesencephali
LoC locus coeruleus
M nucleus mamillaris
MA nucleus medialis amygdalae
MC mitral cell layer
ME median eminence
ML molecular layer
M V nucleus mesencephalicus nervi trigemini
m V nucleus motorius nervi trigemini
NHy neurohypophysis
NPd nucleus pretectalis dorsalis
NPv nucleus pretectalis ventralis
N V nervus trigemini
N VIII nervus octavus
nAmb nucleus ambiguus
nBOR nucleus of the basal optic root
nDB nucleus fasciculus diagonalis Brocae
nDCP nucleus dorsalis commissuralae posterioris
nFLM nucleus fasciculi longitudinalis medialis
nMH nucleus medialis hypothalami
nPH nucleus periventricularis hypothalami
nPM nucleus profundus mesencephali
nS nucleus septalis
nSL nucleus septalis lateralis
nSM nucleus septalis medialis
nSO nucleus supraopticus
nSP nucleus suprapeduncularis
nSPM nucleus suprapeduncularis medialis
nTOL nucleus tracti olfactorii lateralis
nTS nucleus tracti solitarii
nV nucleus ventralis
nVH nucleus ventromedialis hypothalami
n III nucleus nervi oculomotorius
n IV nucleus nervi trochlearis
n VI nucleus nervi abducens
n VII nucleus nervi facialis
n X nucleus motorius dorsalis nervi vagi
n XII nucleus nervi hypoglossi
OI oliva inferior
OS oliva superior
PA paleostriatum augmentatum
Pb nucleus parabrachialis
PD peduncularis dorsalis fasciculi prosencephali lateralis
PH primordium hippocampi
Pr V nucleus princeps nervi trigemini
PT pallial thickening
PV peduncularis ventralis fasciculi prosencephali lateralis
R nucleus rotundus
Rai nucleus raphes inferior
Ras nucleus raphes superior
Re nucleus reuniens
Ri nucleus reticularis inferior
Ris nucleus reticularis isthmi
Rm nucleus reticularis medialis
Rs nucleus reticularis superior
Ru nucleus ruber
SGC stratum griseum centrale
SGD nucleus substantiae griseae dorsalis
SGF stratum griseum et fibrosum superficiale
SGP stratum griseum periventriculare
SGV substantiae griseae ventralis
SM stria medullaris
Sm supramamillary region
SN substantia nigra
SO stratum opticum
ST stria terminalis
Ta nucleus tangentialis
Tel telencephalon
TeO tectum opticum
TO tractus opticus
TOl tractus opticus, pars lateralis
TOm tractus opticus, pars medialis
TrL tract of Lissauer
TSC torus semicircularis
TTd nucleus descendens nervi trigemini
TuOl tuberculum olfactorium
TT tractus tectothalamicus
V ventricle
VeD nucleus vestibularis descendens
VeL nucleus vestibularis lateralis
VeS nucleus vestibularis superior
VP ventral paleostriatum

with MERF (Cuello et al., '84). In the present study, this antiserum was additionally found to show no cross-reactivity with BAM22P, and the absence of cross-reactivity with MERF was confirmed. Thus, the monoclonal anti-LENK antibody appears to be specific for the enkephalin pentapeptides. The Chang anti-MENK antiserum showed considerable cross-reactivity with LENK (Miller et al., '78; Gall et al., '81; Reiner et al., '82b). The cross-reactivity of this antiserum with dynorphin peptides or the larger enkephalin peptides was not examined. The ImmunoNuclear anti-MENK antiserum showed no or negligible cross-reactivity with the dynorphin peptides, but some slight cross-reactivity with LENK, MERF, BAM22P, and PEPE. Thus, the ImmunoNuclear anti-MENK antiserum appears to be largely specific for MENK. Both of the anti-MERF antisera were highly specific for MERF, with negligible or no cross-reactivity with the dynorphin peptides or with the various other enkephalin peptides. Dockray's anti-MERF antiserum appeared to be more specific for MERF-OH, since it was blocked by MERF-OH but not by MERF-NH_2, as previously reported (Williams and Dockray, '83). Yang's anti-MERF antiserum appeared to be more specific for MERF-NH_2 since it was blocked by MERF-NH_2, but only partially blocked by MERF-OH. Thus, although Yang's antiserum may have some cross-reactivity with FMRFamide (Dockray et al., '81), which possesses the same C-terminus tetrapep-

tide sequence as MERF-NH, it produced the same labeling pattern in the turtle brain as Dockray's anti-MERF antiserum. So, it appears unlikely that labeling observed with Yang's antiserum was due, to any great extent, to cross-reactive labeling of FMRFamide-containing neurons and fibers. The anti-MERGL antiserum is highly specific for MERGL, but does show some very slight cross-reactivity with LENK, MENK, MERF, and BAM12P. Giraud et al. ('84) have reported that in RIA this antiserum shows negligible cross-reactivity with other enkephalin peptides. It seems likely that the very slight cross-reactivity of the anti-MERGL antiserum with the other enkephalin peptides was immunohistochemically apparent in the present study because the overall levels of labeling for MERGL in the turtle central nervous system were very low. The anti-MERGL antiserum did label neurons and fibers of the rat nervous system intensely, thereby suggesting that the weak labeling of turtle nervous tissue with this antiserum reflects the apparent absence of MERGL in turtle nervous tissue. The anti-BAM22P antiserum showed complete cross-reactivity with Peptide E, but no or little cross-reactivity with dynorphin peptides or the other enkephalin peptides. Thus, the anti-BAM22P antiserum appears to be specific for a portion of the C-terminus of BAM22P common to both BAM22P and PEPE. The anti-PEPE antiserum showed no cross-reactivity with any of the dynorphin peptides, but did show cross-reactivity with enkephalin peptides, particularly the longer ones, such as BAM22P (with which it showed nearly complete cross-reactivity) and BAM12P. In addition, the anti-PEPE antiserum showed some slight cross-reactivity with the shorter enkephalin peptides, including LENK, MENK, and MERF. Thus, the anti-PEPE antiserum appeared to be specific for enkephalin peptides, with its greatest binding affinity for the midportion and C-terminus characteristic of PEPE (i.e., PEPE, BAM22P, and BAM12P).

Therefore, of the antisera against the enkephalin peptides used, only Chang's anti-LENK antiserum and the ImmunoNuclear anti-LENK antiserum showed significant cross-reactivity with dynorphin peptides, and in the case of the ImmunoNuclear antiserum, this cross-reactivity was with Dynorphin A(1–8) but not the longer forms of Dynorphin A. Despite the slight cross-reactivity of the ImmunoNuclear antiserum with Dynorphin A(1–8), the labeling pattern obtained with this antiserum was indistinguishable from that obtained with the other antisera against enkephalin peptides. Since the ImmunoNuclear antiserum produced the heaviest labeling of all the antisera used (presumably because of its greater cross-reactivity with other enkephalin peptides), in the presentation of the results below and accompanying line drawings, the distribution of enkephalin is largely based on the ImmunoNuclear anti-LENK antiserum. The cell body distribution is based on the results from colchicine-treated turtles. Colchicine treatment was found to greatly increase perikaryal labeling. It is recognized that the demonstration of labeling with the various anti-LENK antisera used does not necessarily demonstrate the specific presence of LENK in the turtle CNS, since even the highly specific anti-LENK monoclonal antibody cross-reacts with MENK. Nonetheless (as considered further in the Discussion section), intense labeling was observed in the turtle CNS with the ImmunoNuclear anti-LENK antiserum even when the antiserum was blocked with MENK. Such results favor the conclusion that LENK is present in the turtle CNS.

The distributions obtained for the various enkephalin peptides were the same, the major differences being in the

TABLE 1. Results of Blocking and Cross-blocking Studies of the Different Enkephalin Antisera Used in This Study[1]

Antiserum	Normal	LENK	MENK	MERF	MERGL	BAM12P	BAM22P	PEPE	DYNA(1–8)	DYNA(1–13)	DYNA(1–17)
LENK (Chang)	*****	**	**	ND	ND	ND	ND	ND	**	ND	ND
LENK (IN)	****	—	**½*	***	ND	***	***½*	**	**½*	****	****
LENK (AC)	***	—	—	***	ND	ND	***	ND	ND	***	ND
MENK (Chang)	***	**	—	ND	ND	ND	ND	ND	ND	ND	ND
MENK (IN)	***	**½*	—	**½*	ND	ND	**	**½*	***	***	***
MERF (Dockray)	***	***	***	—	ND	ND	***	***	***	***	***
MERF (Yang)	***	***	***	—	ND	ND	***	***	***	***	***
MERGL (Dockray)	*	—	—	½*	—	½*	ND	ND	*	ND	ND
BAM22P (Baird)	***	***	***	***	ND	***	—	—	***	***	ND
PEPE (Watson)	***	**½*	**½*	**	ND	*½*	*	—	***	***	***

[1]The left hand column identifies the antiserum. The second column from the left indicates the staining intensity obtained when a 1:500 to 1:1000 dilution of that antiserum was used, with a larger number of asterisks indicating a higher staining intensity. The succeeding columns indicate the attenuation of staining intensity obtained with a 50–100 micromolar concentration of the opioid peptide at the head of the column. If the staining intensity was unaffected by blocking with the opioid peptide at the head of the column, then the number of asterisks is the same as in the second column from the left for that antiserum. A reduction in the number of asterisks indicates the relative amount of attenuation in staining intensity obtained in the blocking study. A dash means staining was completely blocked. ND means not done. Abbreviations are defined in the text.

relative intensity with which neurons and fibers in specific regions were labeled by the different antisera. The major characteristics of the labeling obtained with the anti-MENK, anti-MERF, anti-MERGL, anti-PEPE, and anti-BAM22P antisera are presented subsequent to the description of the anti-LENK labeling pattern. Since the anti-MENK, anti-MERF, and anti-MERGL antisera used were largely specific for their target antigens, the labeling patterns observed presumably reflect the distribution of these peptides in the turtle CNS. The anti-BAM22P and anti-PEPE antisera are largely specific for a large enkephalin peptide resembling BAM22P and PEPE. The labeling pattern obtained with these antisera presumably reflects the distribution of this peptide (or peptides) in the turtle CNS. Although the anti-MERGL antiserum labeled enkephalinergic neurons well in rats, it produced very little labeling in turtle CNS, and what was observed appeared to be largely cross-reactive labeling of other enkephalin peptides. Thus, MERGL appears to be absent from turtle CNS.

LENK distribution

Telencephalon. LENK+ perikarya were widespread and abundant in the telencephalon of the turtle. The most prominent accumulation of LENK+ neurons was observed in the striatal portion of the basal ganglia (Figs. 1–3, see also Fig. 10), which in turtles comprises area d and the paleostriatum augmentatum (PA). Numerous LENK+ neurons were also observed in the "striatallike" cell group, the olfactory tubercle (TuOl) (Fig. 1). Consistent with the abundance of LENK+ neurons in the striatum, abundant LENK+ fibers were present in the pallidal cell fields of the basal telencephalon (Figs. 2, 3, see also Fig. 10), the globus pallidus (GP) and the ventral paleostriatum (VP), which are comparable to the mammalian globus pallidus and ventral pallidum, respectively (Brauth et al., '83; Reiner et al., '84a). Prominent accumulations of LENK+ neurons were also observed in area a (the anterior olfactory nucleus), the septal nuclei (particularly the medial septal nucleus), and a region lateral to the medial amygdaloid nucleus (MA) and dorsal to the nucleus of the lateral olfactory tract (nTOL), which appears comparable to the central amygdaloid nucleus (CA) of mammals (Figs. 1–5). In addition, scattered LENK+ neurons were observed in the periventricular portions of the dorsal ventricular ridge (DVR), in the primordium hippocampi (PH), throughout the rostrocaudal extent of all four of the cortical regions of the turtle telencephalon, in the pallial thickening (PT), in the ventral portion of pyriform cortex (cpv), in nTOL, in the basal amygdaloid nucleus (BA), and in MA (Figs. 1–6). Within the olfactory bulb, a few LENK+ neurons were present in the mitral cell layer.

LENK+ fiber labeling within the telencephalon was most prominent in the GP and VP (Figs. 2, 3, see also Fig. 10). Labeling in the VP, in particular, was denser than in any other brain region. Fiber labeling in the VP and GP appeared to consist of numerous labeled terminals (presumably arising from LENK+ striatal neurons) that coated the thick (presumably dendritic) processes of GP and VP neurons. Fiber and terminal labeling was also prominent in the striatum, where the density of the labeled fibers served to demarcate the turtle striatum from the overlying DVR, which contained only scattered LENK+ fibers. LENK+ fibers were largely absent from PT and the lateral portion of the dorsal cortex (cd). A dense but lightly labeled accumulation of LENK+ fibers that spanned the periventricular, cellular, and deeper two-thirds of the molecular layer of the cortex was present in the medial half of cd as well as in the dorsomedial cortex (cdm) and the medial cortex (cm) (Figs. 2–6, see also Fig. 11). These fibers were more heavily labeled posterior to the interventricular foramen. At all levels, fibers in cm were more heavily labeled than in cdm and cd. In addition, a thin band of LENK+ fibers was present at the pial surface of cdm, cm, and medial cd, and also at the pial surface of pyriform cortex (cp), the olfactorecipient cortex (Reiner and Karten, '85). Scattered LENK+ fibers were present in the cell body layer of cp; LENK+ fibers were particularly prominent in the ventral subdivision of cp, and were also present in the septal region, BA, MA, CA, and nTOL (Figs. 3–5, see also Fig. 10). Within the olfactory bulb, sparse LENK+ fibers were present in the internal granular layer (LGI) and in the layer superficial to the mitral cells. LENK+ fibers were also present in area a and nTOL.

Diencephalon. LENK+ neurons and fibers were more abundant and more heavily labeled in the hypothalamus than in other portions of the diencephalon. Within the hypothalamus, prominent accumulations of LENK+ neurons were present in the preoptic region, the periventricular hypothalamus (nPH), the ventromedial hypothalamus (nVH), the medial hypothalamus (nMH), and the lateral hypothalamus (Figs. 2–5). Within the preoptic region, LENK+ cells gave rise to processes that penetrated the ependymal wall of the ventricle and appeared to be in contact with the ventricular cavity (see Fig. 11). LENK+ cells were found in nPH throughout the entire length of the hypothalamus. LENK+ neurons were also present in the mamillary region, tuberal region, and in the neurohypophysis (NHy). LENK + fibers were present throughout the hypothalamus, but more highly concentrated medially than laterally. The nVH contained a dense accumulation of fine LENK+ fibers that defined the extent of nVH (Fig. 12). LENK+ neurons of nVH tended to be located at the peripheral edges of this cell group. LENK+ fibers were also present in the median eminence (ME) and the neurohypophysis.

Within the epithalamus, LENK+ neurons and fibers were present in more lateral portions of the medial habenular nucleus (HM), and LENK+ fibers were present in the lateral habenular nucleus (HL) (Figs. 4, 5, see also Fig. 12). Within the thalamus, nucleus rotundus (R) and the core of nucleus reuniens (Re) were devoid of LENK+ fibers and neurons (Figs. 4, 5, see also Fig. 12). These cell groups are involved in the processing of visual and auditory input and project to separate regions of the DVR (Hall and Ebner, '70; Reiner and Powers, '78, '80, '83; Balaban and Ulinski, '81a,b). The retinorecipient cell group, the dorsal lateral geniculate (GLd), which projects to cd and PT, is also largely devoid of LENK+ fibers and contains only a few LENK+ neurons. Nucleus ventralis (nV), an apparent somatosensory relay nucleus of the thalamus (Hall et al., '77; Kunzle and Woodson, '82), is largely devoid of LENK+ fibers and contains no LENK+ neurons. In contrast, abundant LENK+ fibers were observed in the nonspecific projection nuclei of the turtle thalamus, the dorsolateral (DLA), and the dorsomedial anterior nuclei (DMA) (Figs. 4, 5, see also Fig. 12). These cell groups, however, were rarely observed to contain LENK+ neurons. Within the ventral thalamus, the diencephalic region interposed between thalamus and hypothalamus, scattered LENK+ neurons and fibers were observed.

Midbrain. Prominent numbers of LENK+ neurons were observed in several regions of the midbrain, including the

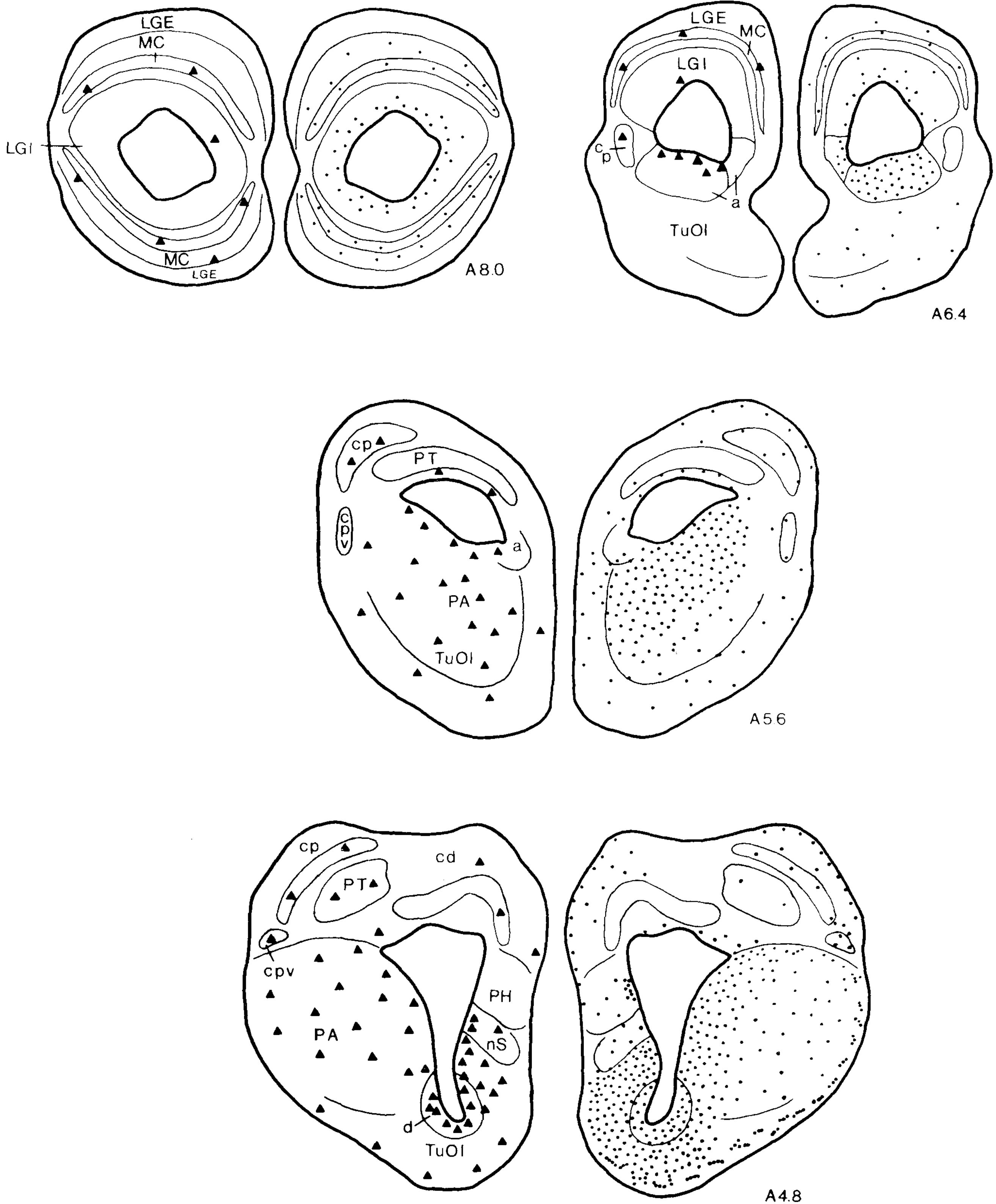


Fig. 1. Line drawings of transverse sections through turtle olfactory bulb and rostral telencephalon illustrating the location and relative density of LENK+ fibers and terminals (dots), as shown on the right side of each drawing, and perikarya (triangles), as shown on the left side of each drawing, observed with immunohistochemical procedures. Each triangle corresponds to 1–5 LENK+ neurons. The numbers to the lower right indicate the anterior-posterior level of the section in stereotaxic coordinates.

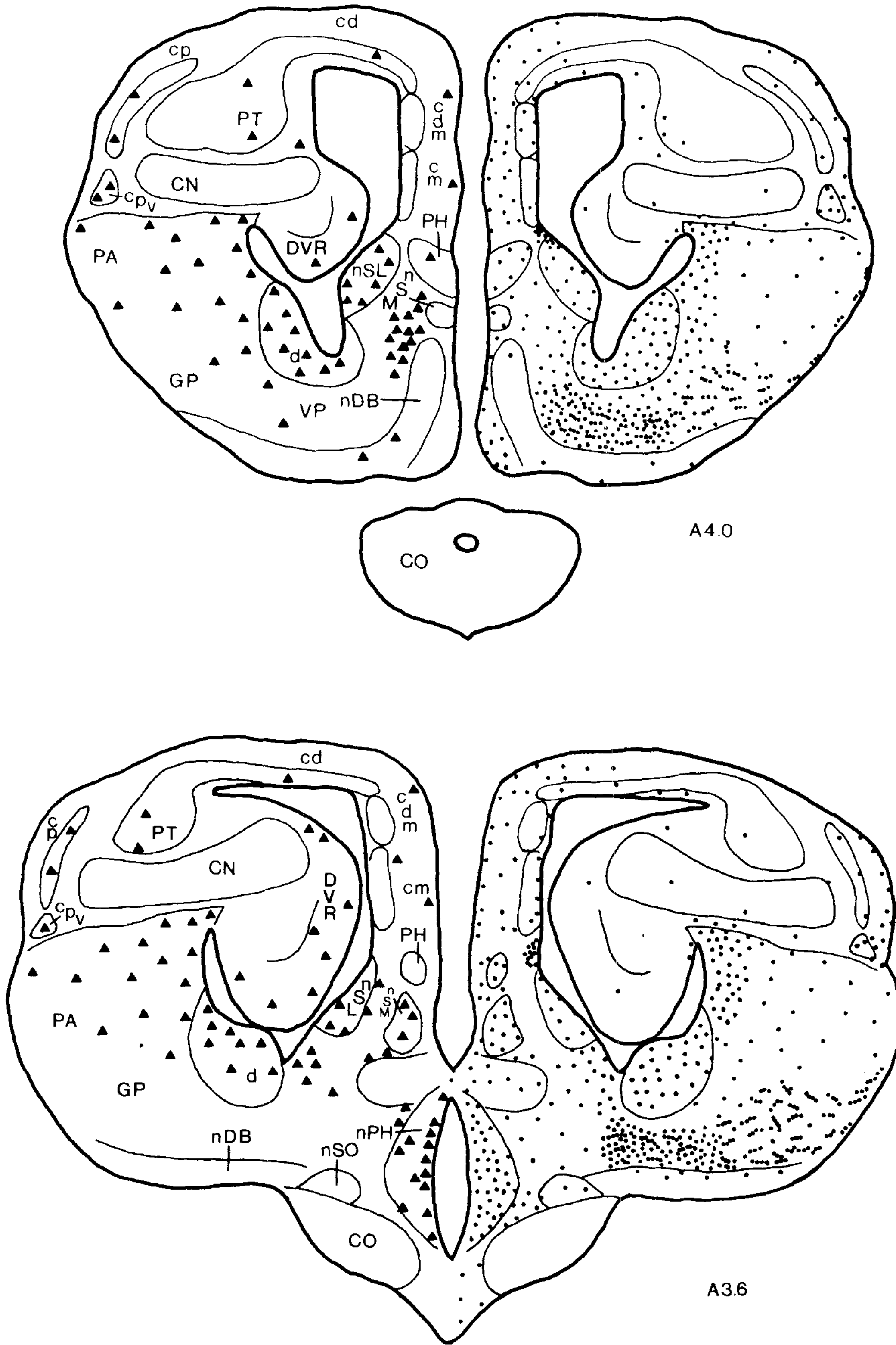


Fig. 2. Line drawings of transverse sections through slightly more caudal telencephalic levels of turtle brain than shown in Figure 1. Dots and triangles in Figures 2–9 indicate the relative density of LENK+ fibers and perikarya, respectively, observed with immunohistochemical methods.

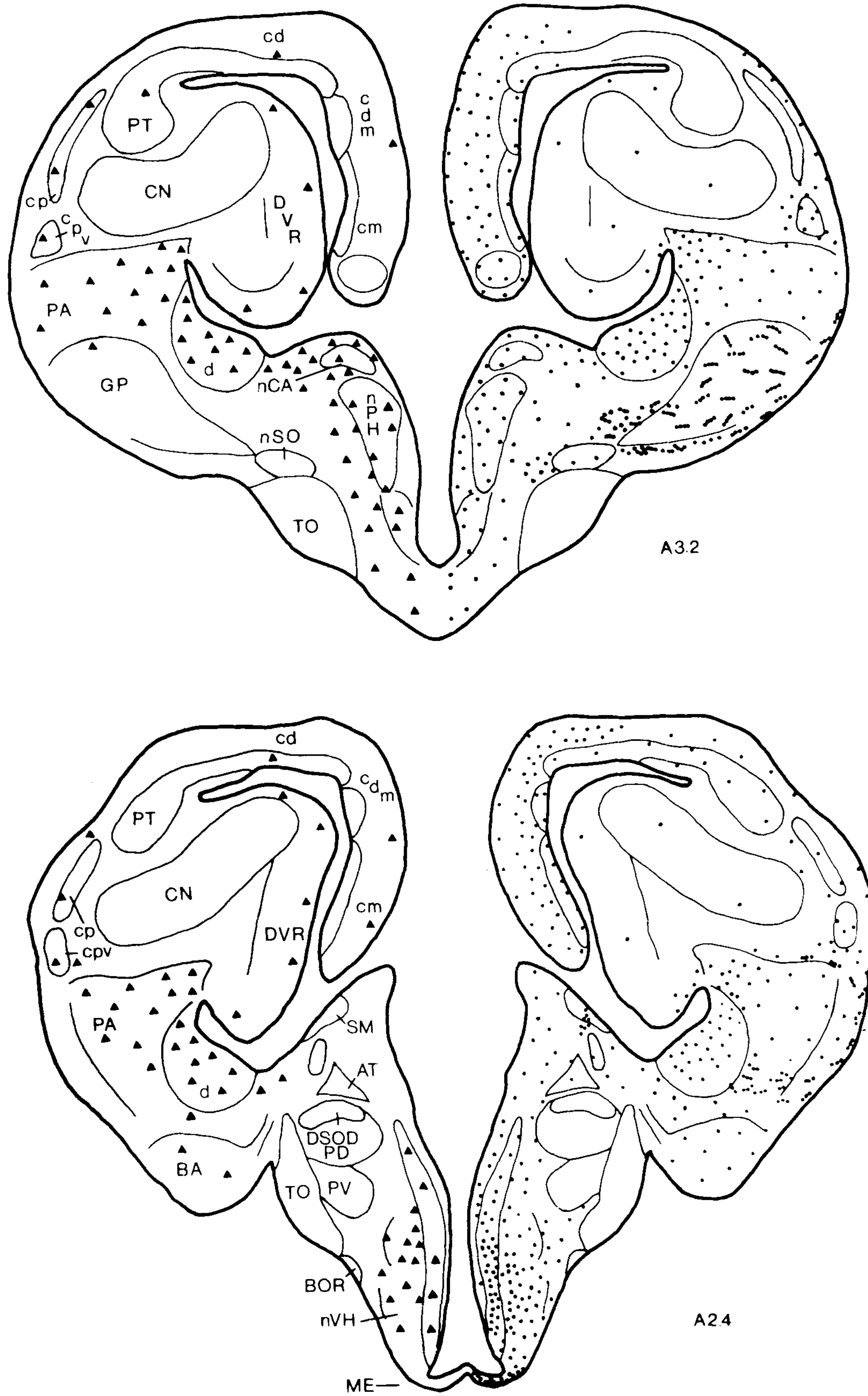
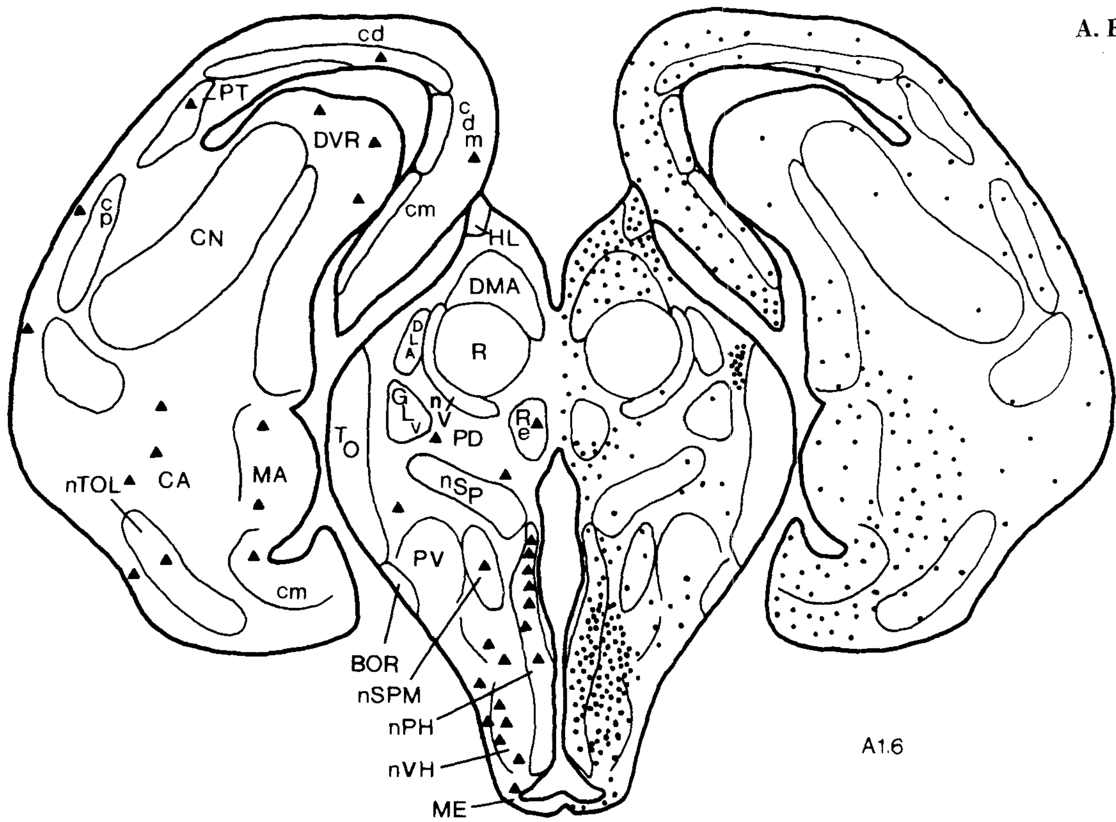


Fig. 3. Line drawings of transverse sections through the midtelencephalon and rostral diencephalon of turtle showing the distribution of LENK+ fibers, terminals, and perikarya.



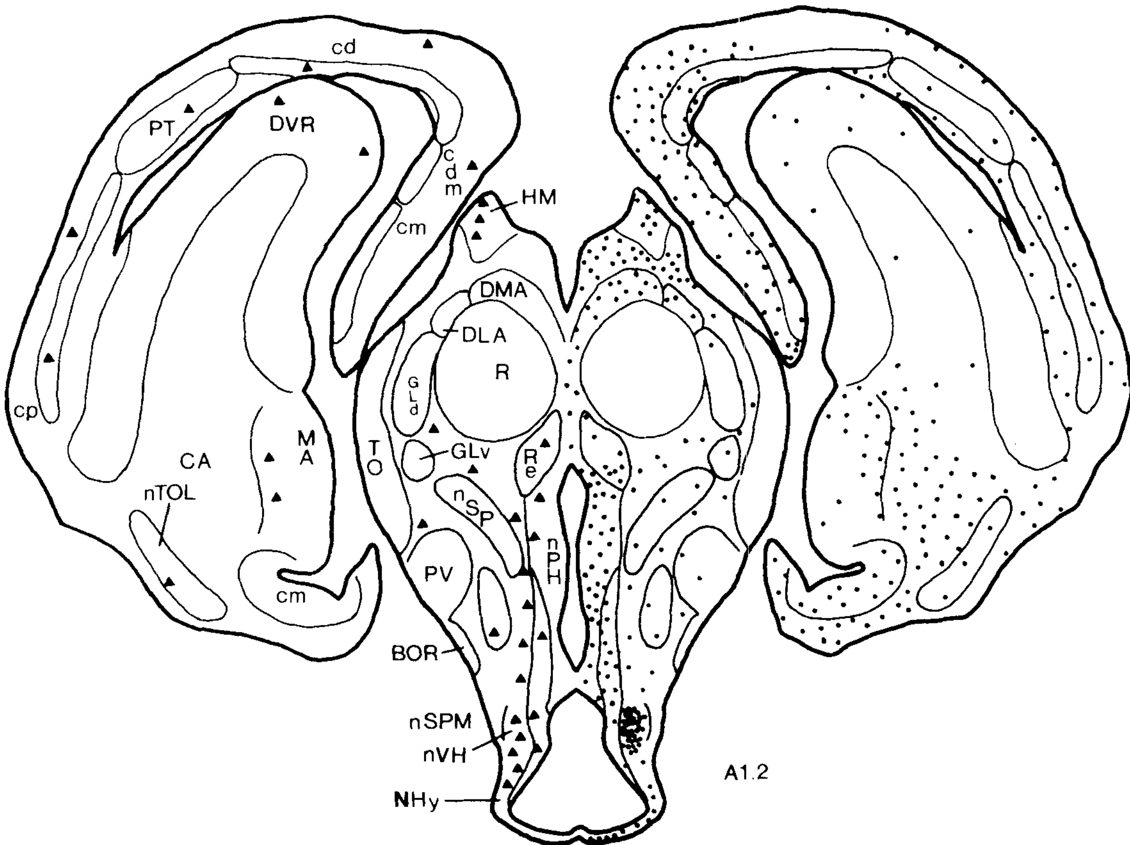


Fig. 4. Line drawings of transverse sections through the caudal telencephalon and middiencephalon of turtle showing the distribution of LENK+ fibers, terminals, and perikarya.

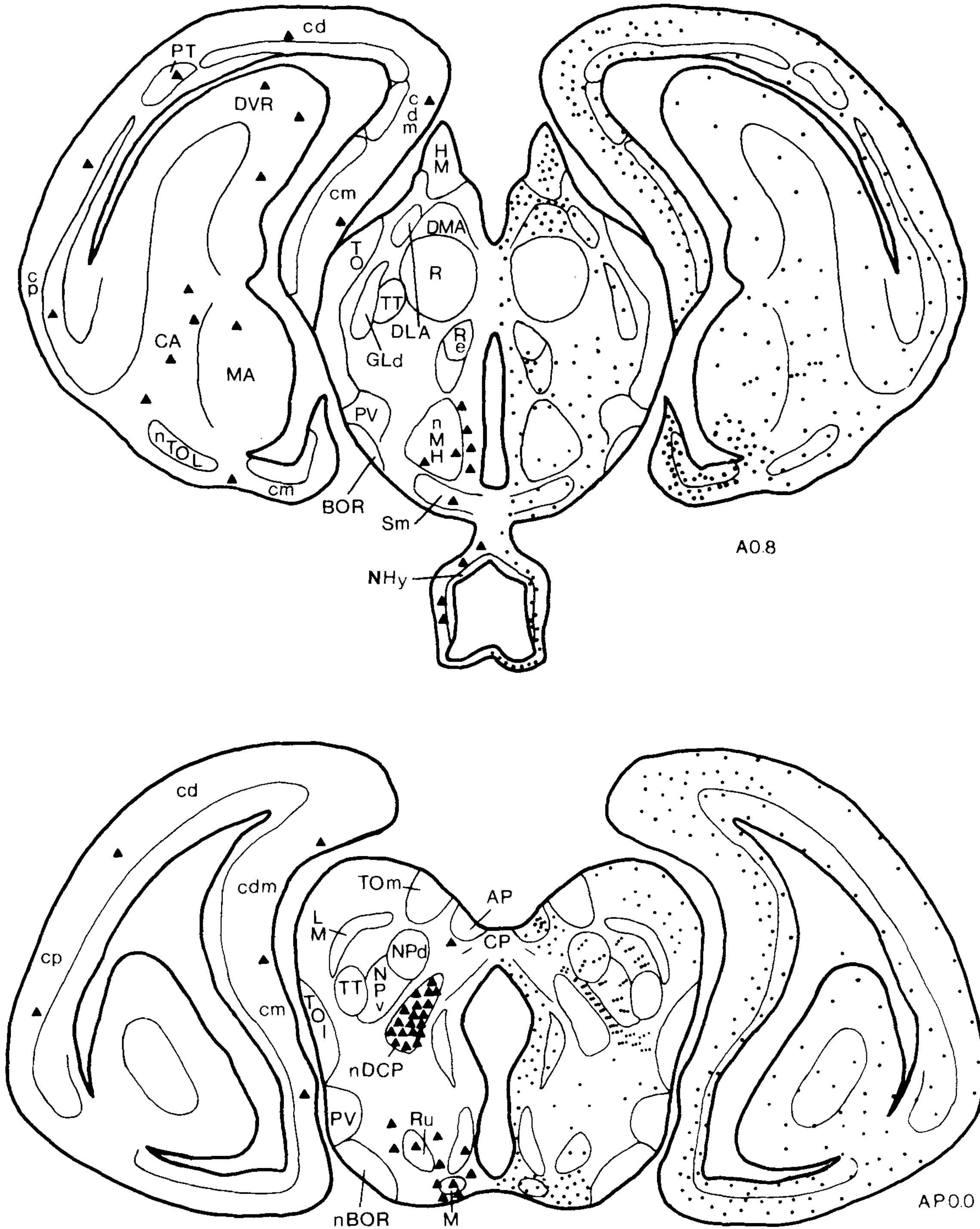


Fig. 5. Line drawings of transverse sections through the caudal telencephalon and mesodiencephalic junctional region of turtle showing the distribution of LENK+ fibers, terminals, and perikarya.

dorsal nucleus of the posterior commissure (nDCP) of the pretectum, the medial tegmentum, the laminar nucleus (La) of the torus semicircularis (TSC), and the stratum griseum periventriculare (SGP) of the tectum (Figs. 5, 6, see also Fig. 12). Fiber labeling in the tegmentum was generally light, consisting of scattered LENK+ fibers—with a somewhat greater accumulation of fibers in the medial tegmental (including AVT and the peri-interpeduncular region). LENK+ fibers were abundant in La and in the periventricular portions of the midbrain. Within area pretectalis (AP), a dense patch of coarse LENK+ fibers and a few LENK+ cells were observed (Fig. 5). Thick processes that appeared to be the processes of the LENK+ nDCP cells were observed to fan out from nDCP, pass through and

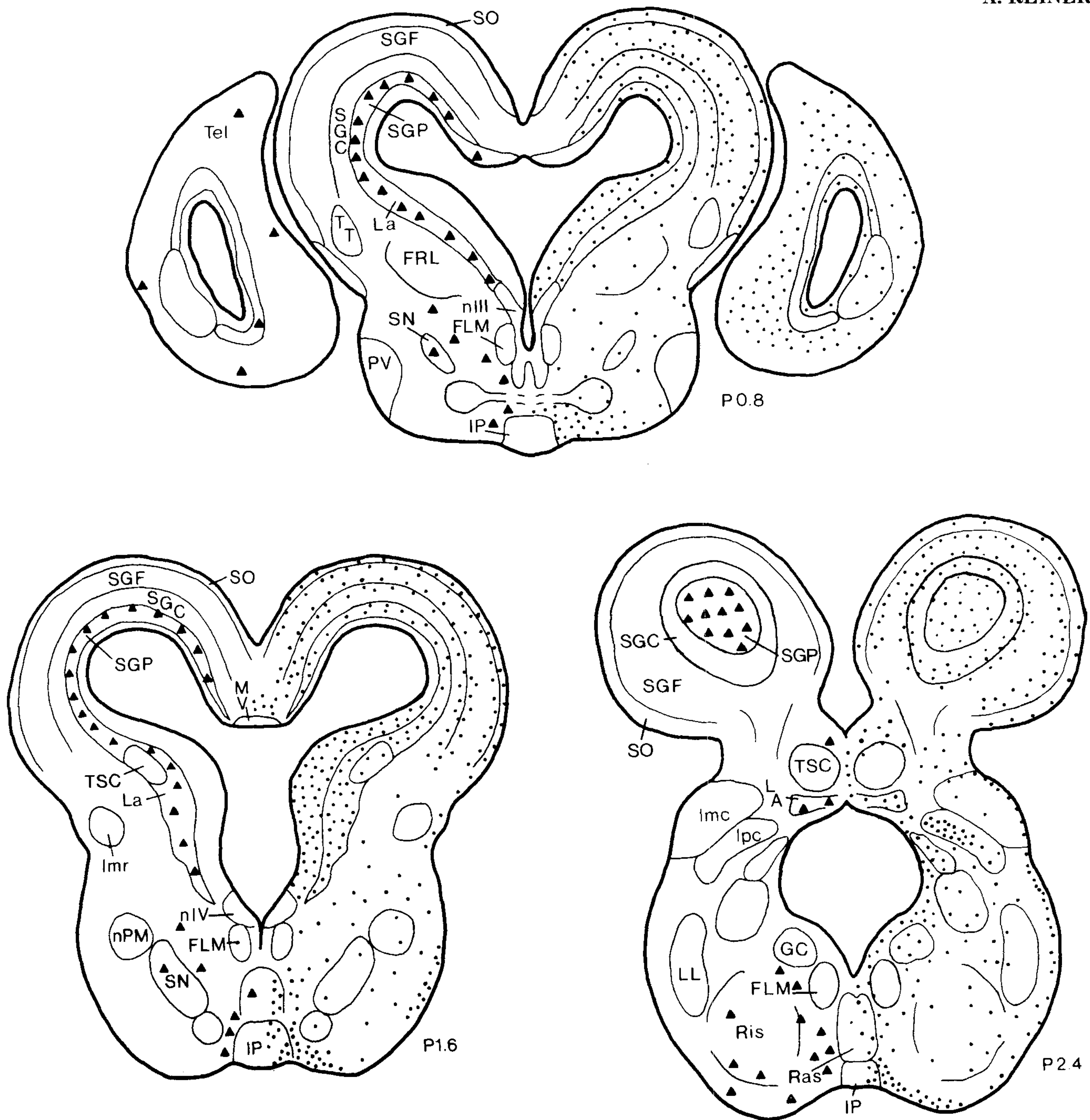


Fig. 6. Line drawings of transverse sections through the midbrain of turtle showing the distribution of LENK+ fibers, terminals, and perikarya.

around the adjacent pretectal nuclei, and enter the tectum from its rostral, medial, and lateral edges (Fig. 5, see also Fig. 12). Within the tectum, these fibers were observed to course in the stratum album centrale (SAC) and ramify into more superficial and deeper tectal layers. Within the optic tectum (TeO), LENK+ fibers were abundant and continuously distributed throughout the deep fiber (SAP) and gray (SGP) layers, in the central fiber (SAC) and gray (SGC) layers, and in the deeper (nonretinorecipient) half of the superficial gray and fiber layer (SGF) (Fig. 6, see also Fig. 12). LENK+ fibers were also evident in two separate bands in the superficial (retinorecipient) half of SGF. The more superficial of these was located immediately deep to the stratum opticum (SO) and appeared to consist of coarse LENK+ fibers. This superficial band is separated from the deeper LENK+ band of the retinorecipient tectum by a zone that was relatively free of LENK+ fibers. The deeper retinorecipient band consists of numerous thin radially oriented LENK+ fibers that give rise to ramifications within the band parallel to the pial surface. This band of fibers, which coincides with the densest retinorecipient zone of the turtle tectum (Bass and Northcutt, '81), is separated from

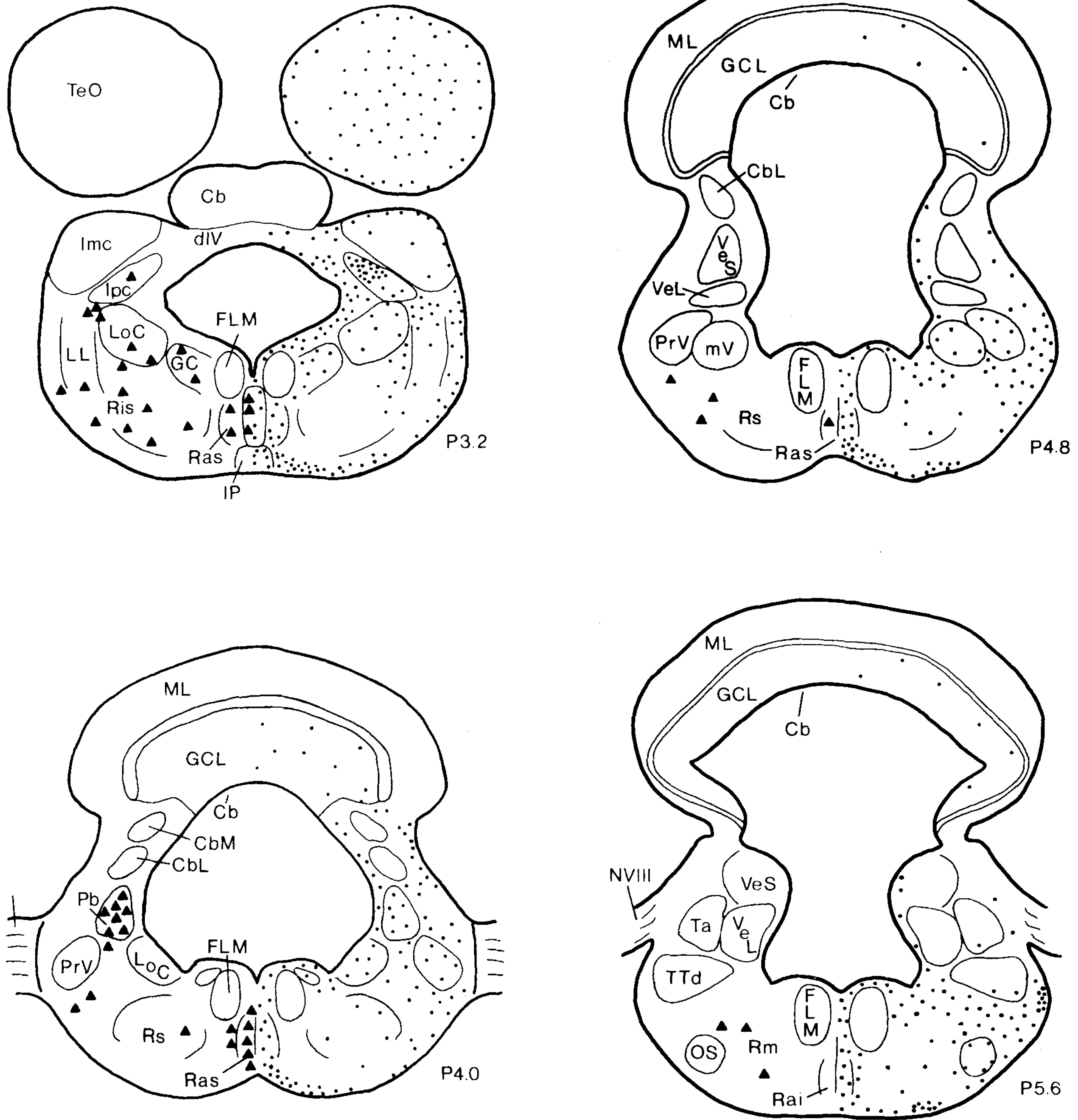


Fig. 7. Line drawings of transverse sections through the rostral rhombencephalon of turtle illustrating the distribution of LENK+ fibers, terminals, and perikarya.

deeper LENK+ fibers by a zone that is relatively poor in LENK+ fibers.

Rhombencephalon. Within the rostral (or isthmic) portions of the hindbrain, several cell groups containing prominent populations of LENK+ neurons were observed, including the parabrachial region (Pb), the superior raphe (Ras), the nucleus of the lateral lemniscus, the lateral isthmic reticular formation (Ris), and a cell group along the lateral edge of the parvocellular isthmic nucleus (Ipc) (Fig. 7). The latter appears to be the enkephalinergic cell group reported to give rise to centrifugal projections to the retina of the contralateral eye, as recently described by Weiler ('85). LENK+ fiber labeling within the isthmus was prominent in periventricular regions, lateral to and within Ras, within medial Ipc, in the peri-interpeduncular region, and along the ventromedial floor of the brainstem. LENK+ fibers were also evident in the central gray (GC), the locus coeruleus (LoC), and surrounding the lateral lemniscus (LL). LENK+ fibers were scarce in Ris and in both subdivisions (rostral and caudal) of the magnocellular isthmic nucleus, although the dorsolateral rim of the magnocellular isthmic nucleus did contain prominent LENK+ fibers.

Within the metencephalon, scattered LENK+ fibers, which appeared to be mossy fibers, were present in the GCL of the Cb (see Fig. 13). LENK+ fibers in GCL were more abundant in caudal Cb than rostral (Figs. 7, 8). Within the pons, LENK+ cells were observed in the lateral portions of the superior (Rs) and medial reticular formation (Rm). LENK+ fibers were present in Ras, in the ventromedial pons, and in the motor and sensory cell groups of the trigeminal nerve. Within the lateral pontine regions, heavily labeled LENK+ terminals and fibers coated neurons of the motor nucleus of the trigeminal nerve (MV) (Fig. 7, see also Fig. 13), and a dense accumulation of LENK+ fibers was present along the pial surface lateral to the descending tract of the trigeminal nerve (TTd) (Figs. 7, 8, 9). At caudal pontine levels, LENK+ neurons were present between TTd and the motor nucleus of the facial nerve (nVII), and numerous neurons were present in lateral Rm (Figs. 8, 9). Sparse LENK+ fibers were present in the cochlear nuclei.

Within the medulla, LENK+ neurons were present in the nucleus of the solitary tract (nTS), in the descending vestibular nucleus (VeD), in lateral intermediate reticular formation (Ri), in the motor nucleus of the vagus nerve (nX), in the motor nucleus of the hypoglossal nerve (nXII), and in the area lateral to caudal TTd (Figs. 8, 9). Within the spinal cord, LENK+ neurons were present in Lissaur's zone (along the pial surface of the dorsal root entry zone), in the dorsal horn, and in the intermediate gray of the cord (Figs. 9, 13). LENK+ neurons were also observed—but only rarely—in the ventral horn. LENK+ fiber labeling in the medulla was most prominent in the ventrolateral quadrant of the brainstem. A region of dense LENK+ fibers was present along the pial surface lateral to TTd. This zone of labeling was continuous with the similar zone in the dorsal root entry zone of the spinal cord (Figs. 8, 9). Within the medulla, LENK+ fibers were also present in nTS, the intermediate raphe (Rai), nVII, nX, and nXII. Within nVII, LENK+ fibers and terminals appeared to coat large unlabeled neurons (Fig. 8). LENK+ fibers were also observed along the ventromedial floor of the medulla; the labeled fibers were continuous with fibers of a similar appearance and location in the pons. Within the spinal cord, LENK+ fiber labeling was most prominent in Lissauer's zone, and less dense accumulations of fibers were present in the dorsal horn and dorsal portions of the lateral funiculus. LENK+ fibers were also present in the ventral horn (Figs. 9, 13).

MENK distribution

The labeling pattern with each of the two MENK antisera was virtually indistinguishable from that observed with the LENK antisera. Both LENK and MENK antisera labeled neurons in the same cell groups, although the MENK antisera consistently labeled fewer neurons. Similarly, although the fiber labeling patterns were indistinguishable, MENK+ fibers tended to be more lightly labeled, even when higher concentrations of the MENK antisera than the LENK antisera were used.

MERF distribution

The MERF labeling pattern was the same for both anti-MERF antisera and was indistinguishable from the LENK+ pattern, except that the labeled neurons and fibers tended to be more lightly labeled and fewer in number than for the anti-LENK antisera. Nonetheless, labeled neurons and fibers were observed in the same cell groups with both the anti-MERF and anti-LENK antisera. The most conspicuous regions where MERF+ neurons and fibers were fewer in number and more lightly labeled were PA, cp, cpv, cdm, and cm.

PEPE/BAM22P distribution

Since the anti-PEPE and anti-BAM22P antisera showed nearly complete (anti-PEPE) or complete (anti-BAM22P) cross-reactivity with each other's antigens, the results for the two antisera are presented together. PEPE/BAM22P+ neurons and fibers were observed in the same cell groups as were LENK+ neurons and fibers. Although PEPE/BAM22P+ labeling of neurons and fibers in many of these cell groups was less intense than for LENK, many other groups contained neurons and fibers that labeled more heavily for PEPE/BAM22P. In particular, neurons of ventromedial area d, the nucleus of the anterior commissure (nCA), and regions near nCA were very PEPE/BAM22P+. Both the cell bodies and dendrites were heavily labeled. LENK+ neurons in these regions showed lighter perikaryal labeling and little dendritic labeling. In contrast, neurons of PA were more heavily labeled for LENK than for PEPE/BAM22P; similarly, within the hypothalamus and in nDCP, neurons were more heavily labeled for LENK. Fiber labeling for PEPE/BAM22P was heavy in the VP, but much lighter in GP. This is consistent with the finding that medial striatal cells (i.e., area d and medial PA), which presumably project to VP, are more heavily labeled for PEPE/BAM22P than lateral striatal cells (i.e., lateral PA), which project to GP. Although the MENK, MERF, and PEPE/BAM22P labeling patterns were highly similar to that obtained for LENK, the overall number of neurons labeled with PEPE/BAM22P was much greater than for MENK and MERF.

MERGL distribution

Only extremely light labeling was obtained with the anti-MERGL antiserum, and it was restricted to the regions most heavily labeled by the other enkephalin antisera. In addition, the low level of labeling could be blocked or greatly attenuated with other enkephalin peptides (see Table 1). Thus, MERGL appears to be absent from the turtle central nervous system.

Double-label studies

The simultaneous immunofluorescence technique was used to study the colocalization of LENK and MERF (using Dockray's antiserum) and the colocalization of LENK and BAM22P. LENK and MENK colocalization was not examined because the complete cross-reactivity of the monoclonal anti-LENK antibody with MENK rendered LENK-MENK colocalization studies with these antisera meaningless. LENK and PEPE colocalization studies were not carried out because the anti-PEPE and anti-BAM22P antisera have similar specificities. The anti-BAM22P, rather than the anti-PEPE, antiserum was used because it cross-reacts much less with other enkephalin peptides.

In the cell groups examined (area d, nCA, nPH, nDCP, the preoptic area, the mamillary region, and Lissauer's tract), neurons that contained LENK also contained BAM22P and MERF (Figs. 14, 15). In the regions examined, essentially all fibers examined that contained LENK also contained BAM22P and MERF, although these fibers tended to be much more lightly labeled for the latter two.

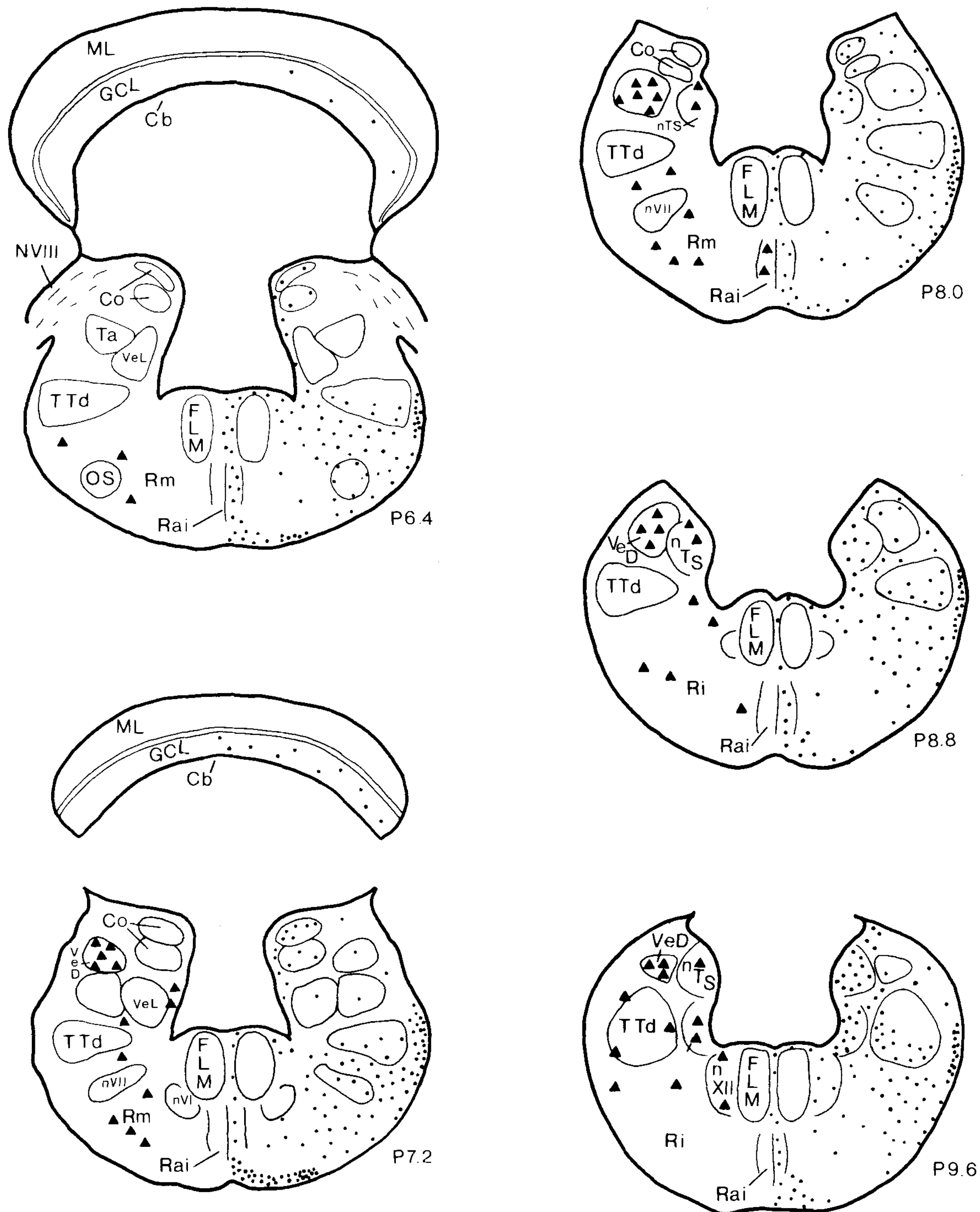


Fig. 8. Line drawings of transverse sections through the caudal rhombencephalon of turtle illustrating the distribution of LENK+ fibers, terminals, and perikarya.

DISCUSSION

The present studies demonstrate that peptides highly similar or identical to several of the major opioid peptide products derived from mammalian proenkephalin are present in the turtle central nervous system. These peptides include LENK, MENK, MERF, and PEPE/BAM22. All four (or similar peptides) are widely distributed and present in many neurons and fibers of the turtle CNS. Further, the distributions for these four substances are indistinguishable, and colocalization studies revealed that LENK, MERF, and PEPE/BAM22P are found in the same neurons. Although colocalization studies were were not carried out using the anti-MENK antisera, present results strongly suggest that MENK is also present in neurons that contain LENK, MERF, and PEPE/BAM22P. This argument is based on the similarities in distribution between MENK and the other enkephalin peptides and on the observation that all

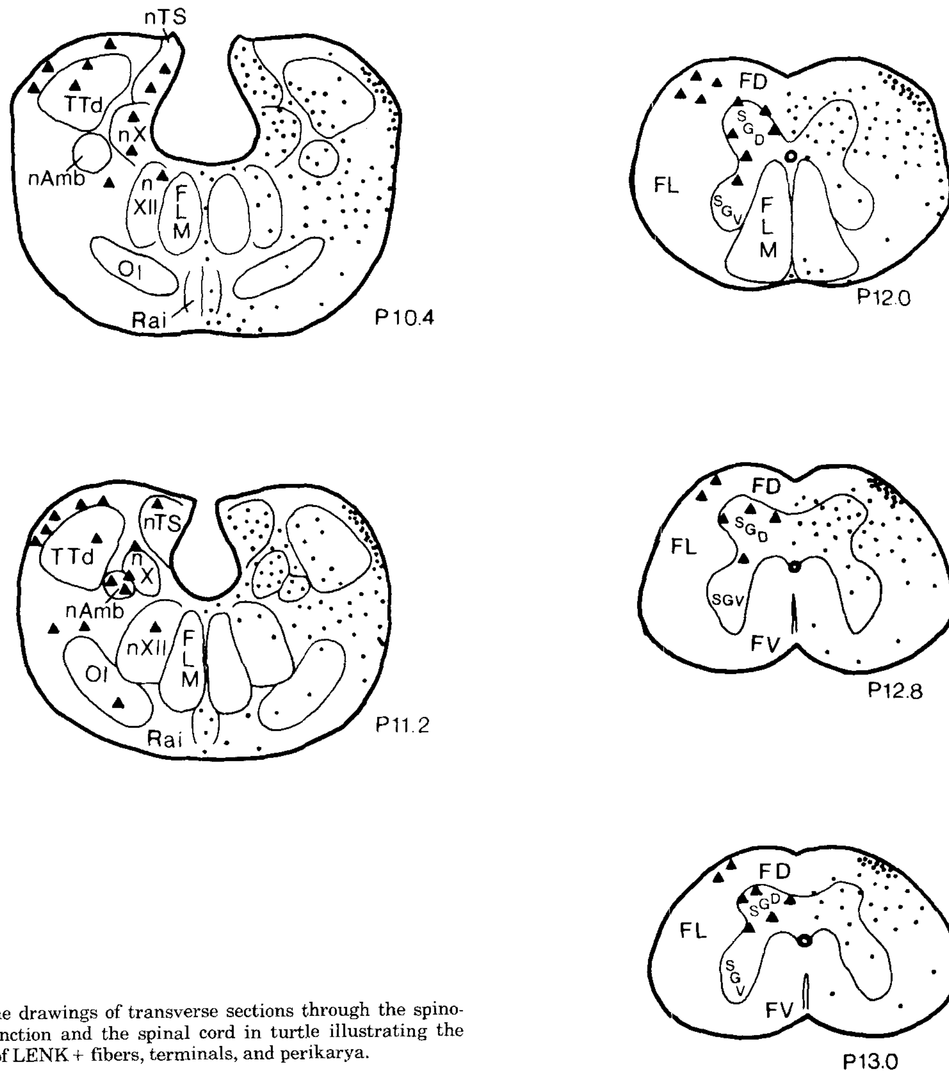


Fig. 9. Line drawings of transverse sections through the spinomedullary junction and the spinal cord in turtle illustrating the distribution of LENK + fibers, terminals, and perikarya.

neurons of nDCP could be labeled for MENK (even when the anti-MENK antisera were blocked with LENK), as well as for LENK, MERF, and PEPE/BAM22P. Therefore, it seems likely that a proenkephalinlike peptide is synthesized by neurons in turtles, and this precursor contains regions whose amino acid sequences are highly similar or identical to LENK, MENK, PEPE/BAM22P, and MERF. The labeling obtained with anti-MERGL in the present study was extremely light and blockable by various enkephalin peptides, implying that turtle proenkephalin does not contain a region resembling MERGL, or that a MERGL-like region is present but sufficiently different in amino acid sequence as to render it noncross-reactive with the antiserum used here. A recent radioimmunoassay and high performance liquid chromatography (HPLC) study on the reptilian nervous system (Lindberg and White, '86) supports the conclusion that the LENK-like, MENK-like, and MERF-like substances detected in the turtle central nervous system in the present study are, in fact, LENK, MENK, and MERF. This study also found no evidence for the presence of MERGL in the turtle nervous system (Lindberg and White, '86).

Structure of turtle proenkephalin and the evolution of proenkephalin

Recently, Herbert and coworkers (Martens and Herbert, '84; Herbert et al., '85) determined the structure of proenkephalin in *Xenopus* using cDNA cloning technology. They found that *Xenopus* proenkephalin possessed no copies of LENK and five copies of MENK, instead of one copy of LENK and four of MENK as in proenkephalin in mammals. In addition, the MERF sequence was present in *Xenopus* proenkephalin, but the MERGL sequence was not. A MERGL-like sequence, in which the last amino acid has been replaced by tyrosine and which does not cross-react with antisera specific for MERGL, however, is present in *Xenopus* (Martens and Herbert, '84). Kilpatrick et al. ('83) used HPLC and RIA (using highly specific antisera) to study *Rana pipiens* central nervous system and confirmed the findings of Herbert and coworkers (Martens and Herbert, '84; Herbert et al., '85) that MERGL appeared to be absent in frogs, but reported that LENK, as well as MENK and MERF, were present. Although the ratio of MENK to MERF levels was about 4:1 (as in mammals), the ratio of

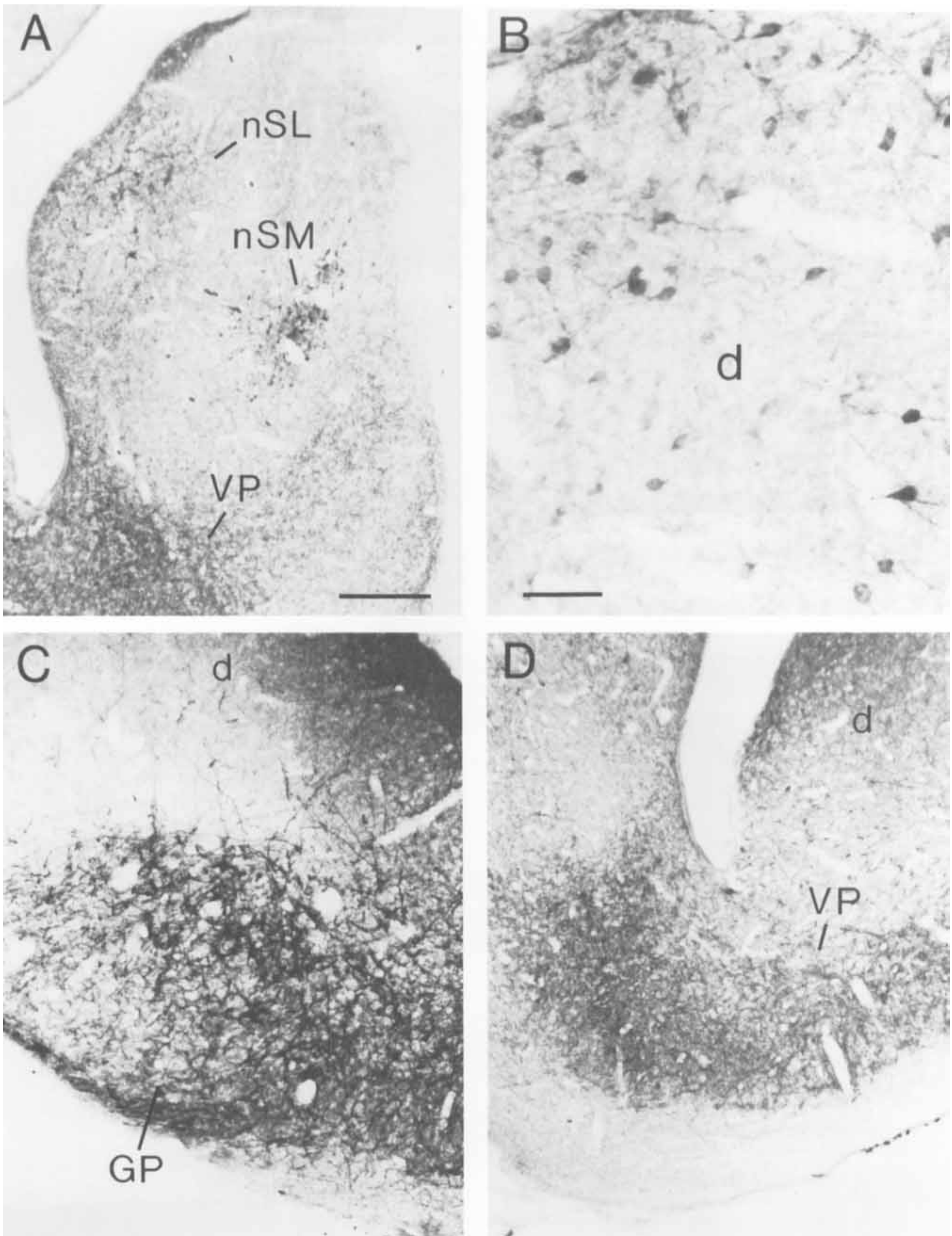


Fig. 10. Photomicrographs of labeling in PAP-stained transverse sections through the turtle brain. **A.** LENK+ fiber and terminal labeling in the septal region (medial to the right). **B.** PEPE+ perikaryal labeling in area d of the striatum (medial to the left). **C.** LENK+ fiber labeling in the globus pallidus of the basal ganglia (medial to the right). **D.** LENK+ fiber labeling in the ventral paleostriatum, a pallidal portion of the basal telencephalon (medial to the left). Scale bars: A,C,D (same magnification) = 250 microns, B = 50 microns.

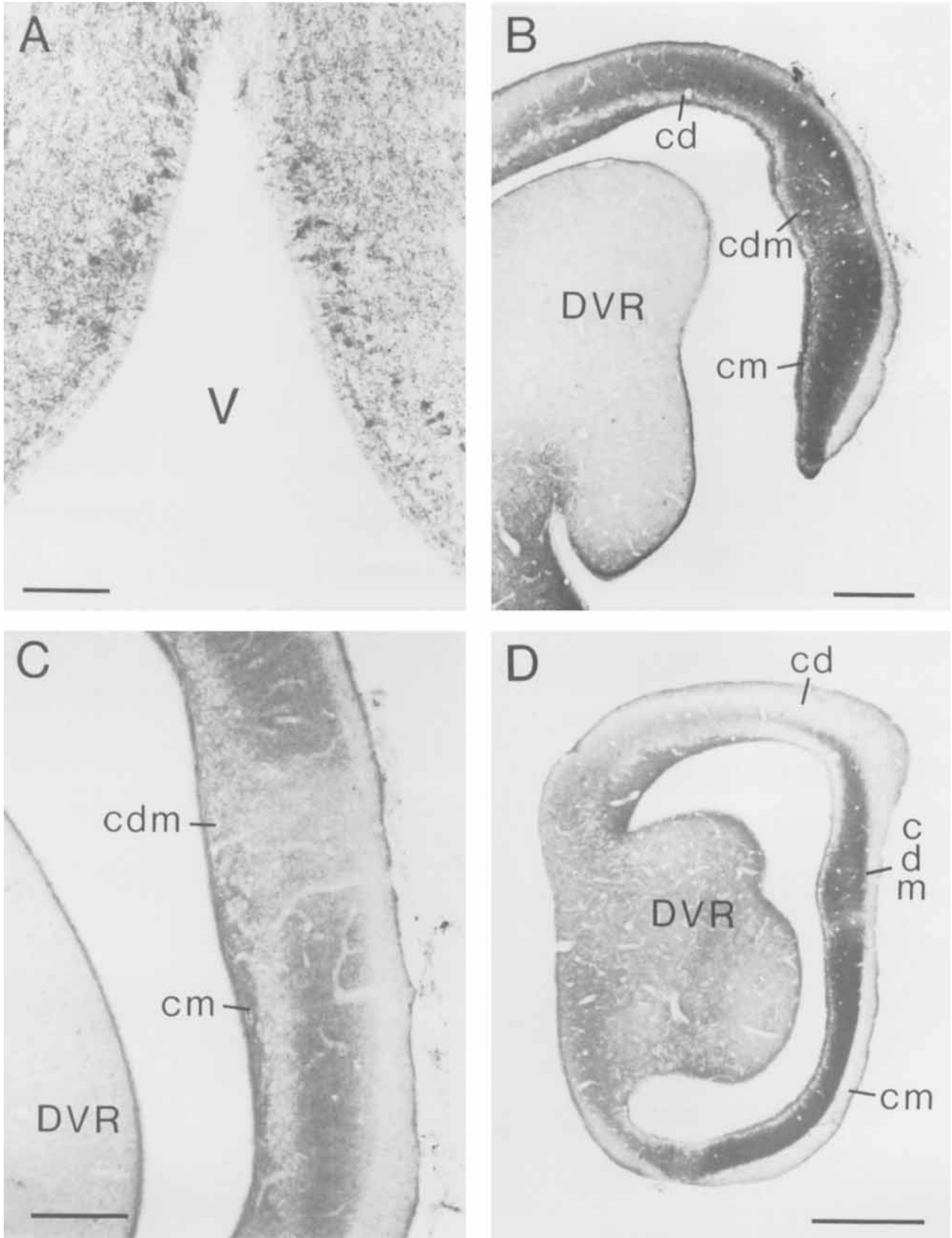


Fig. 11. Photomicrographs of LENK+ labeling in PAP-stained transverse sections through the turtle brain. **A.** LENK+ perikarya with labeled processes extending through the ependymal lining of the ventricle. **B.** LENK+ fiber labeling in the medial half of the cortex (medial to the right). **C.** A more high-power view of the LENK+ fiber labeling in the medial half of the cortex comparing the labeling pattern in cm and cdm (medial to the right). **D.** The fiber labeling pattern in the medial half of the cortex at caudal telencephalic levels (medial is to the right). Scale bars: A = 100 microns, B = 500 microns, C = 250 microns, D = 1,000 microns.

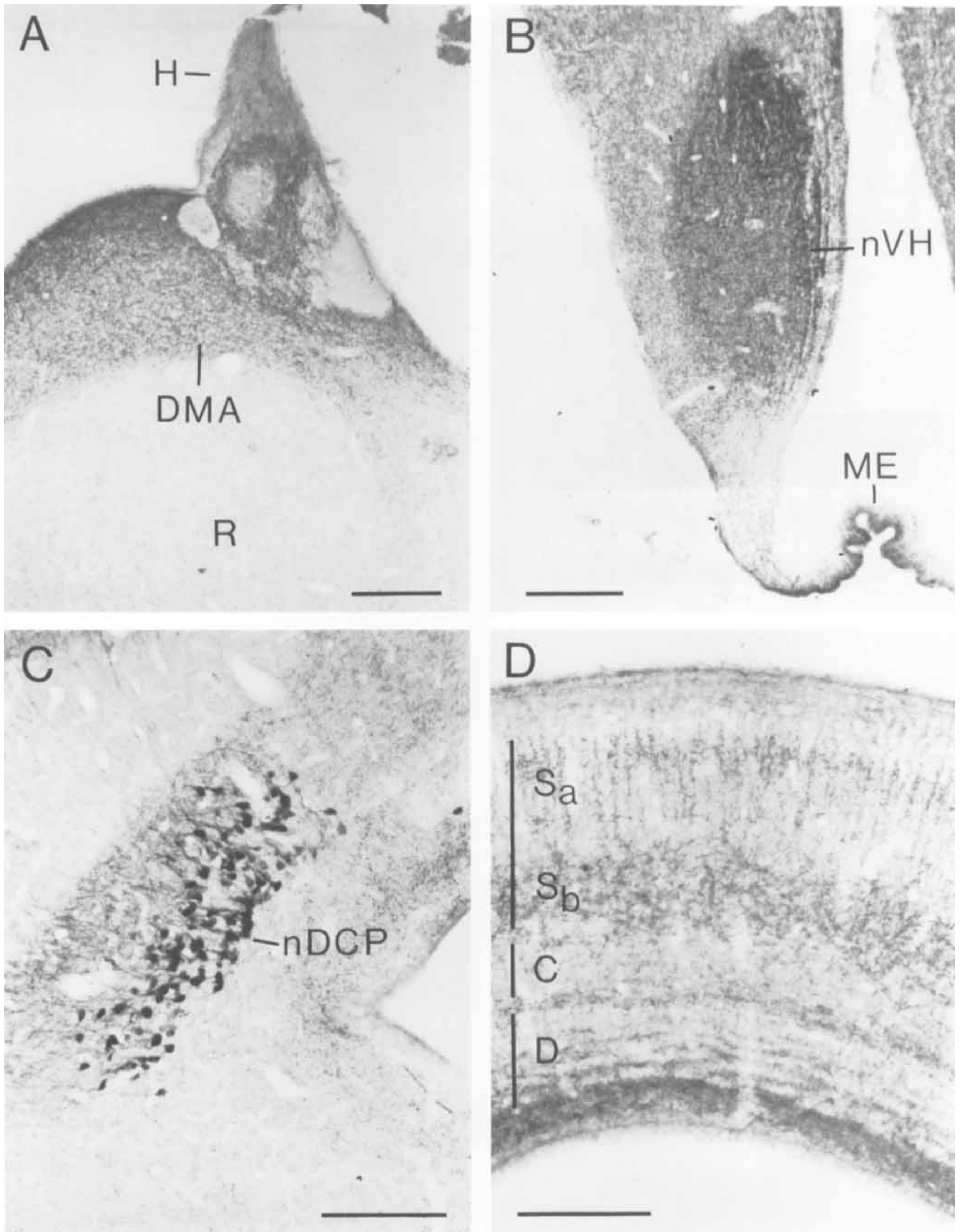


Fig. 12. Photomicrographs of LENK+ labeling in PAP-stained transverse sections through the turtle brain. **A.** LENK+ fiber labeling in the epithalamus and dorsal portions of the thalamus (medial to the left). **B.** LENK+ fiber labeling in the hypothalamus at the level of nVH (medial to the right). **C.** LENK+ perikaryal labeling in nDCP of the pretectum (medial to the right). **D.** LENK+ fiber labeling in the tectum (Sa, retinorecipient portion of SGF; Sb, nonretinorecipient portion of SGF; C, SGC; D, SGP). Scale bars: A,B = 250 microns, C,D = 200 microns.

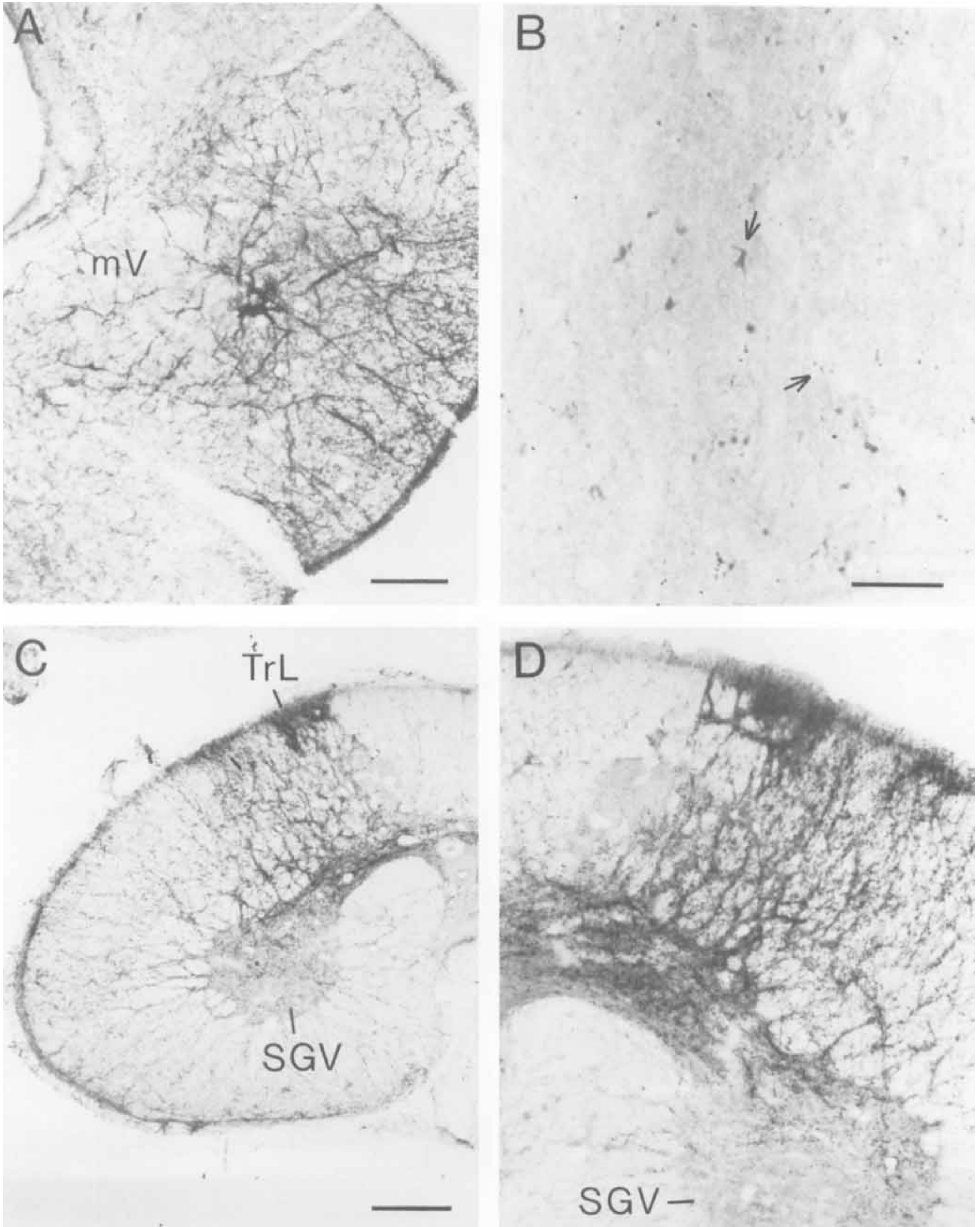


Fig. 13. Photomicrographs of LENK+ labeling in PAP-stained transverse sections through the turtle brain and spinal cord. **A.** LENK+ fiber labeling in the ventrolateral rhombencephalon at the level of the motor nucleus of the trigeminal nerve (mV). **B.** LENK+ fibers in the cerebellum. **C.** LENK+ fiber labeling in the cervical spinal cord. **D.** A more high-power view of LENK+ fiber labeling in the dorsal horn of the spinal cord. Scale bars: A = 100 microns, B = 50 microns, C = 200 microns.

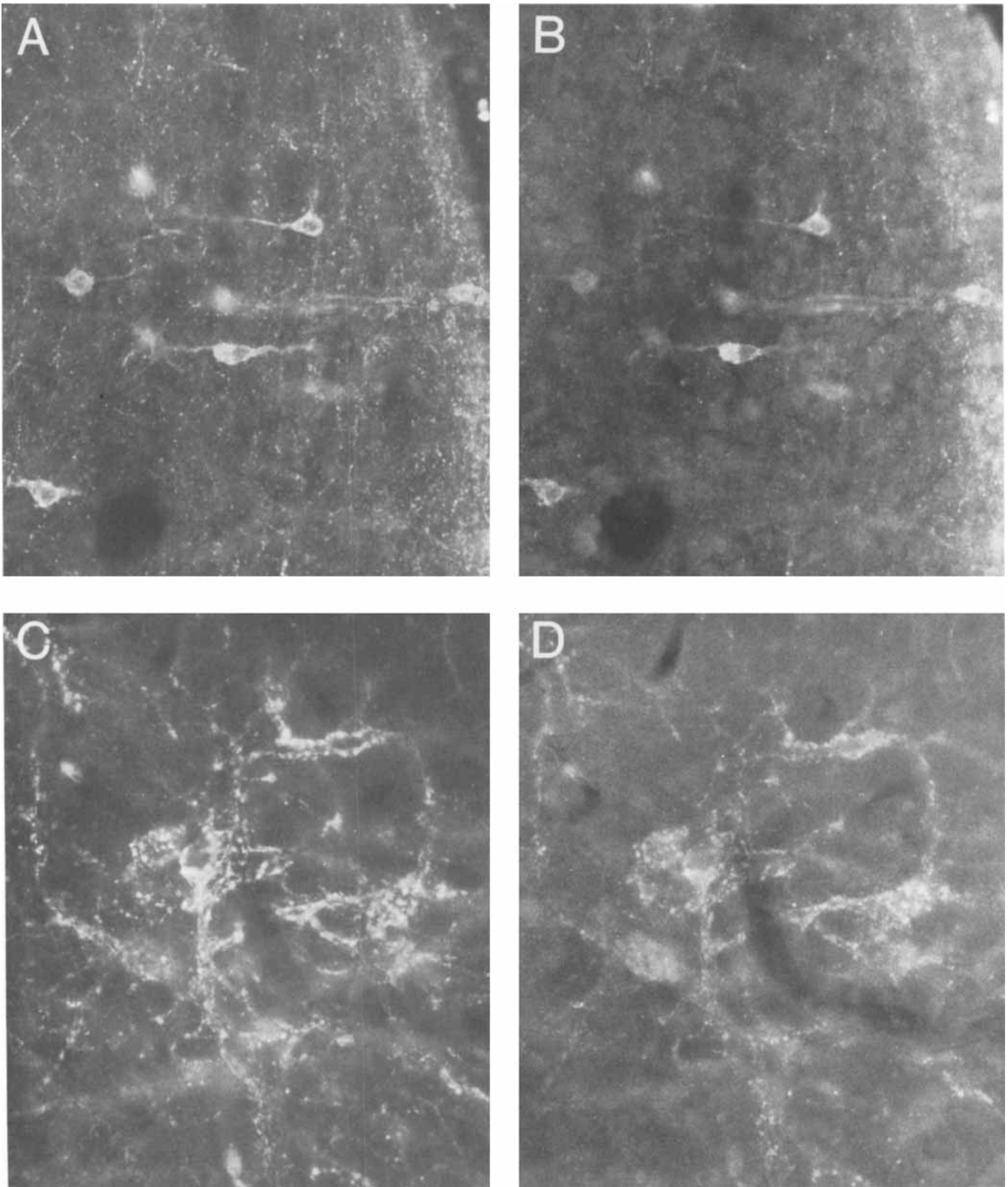


Fig. 14. Pairs of photomicrographs of two transverse sections through the turtle brain that had been processed according to the simultaneous immunofluorescence procedure for the colocalization of LENK and MERF. **A.** LENK+ FITC-labeled perikarya and fibers in a single field of view through the rostral periventricular hypothalamic region. **B.** MERF+ TRITC-labeled perikarya and fibers present in this same field of view. Note that the same perikarya are labeled for both LENK and MERF. Medial is to the right in A and B. **C.** LENK+ FITC-labeled fibers and terminals in a single field of view through the ventrolateral rhombencephalon near the facial nucleus. **D.** MERF+ TRITC-labeled fibers and terminals present in this same field of view. Note that the same fibers and terminals are labeled for both LENK and MERF. Medial is to the left in C and D.

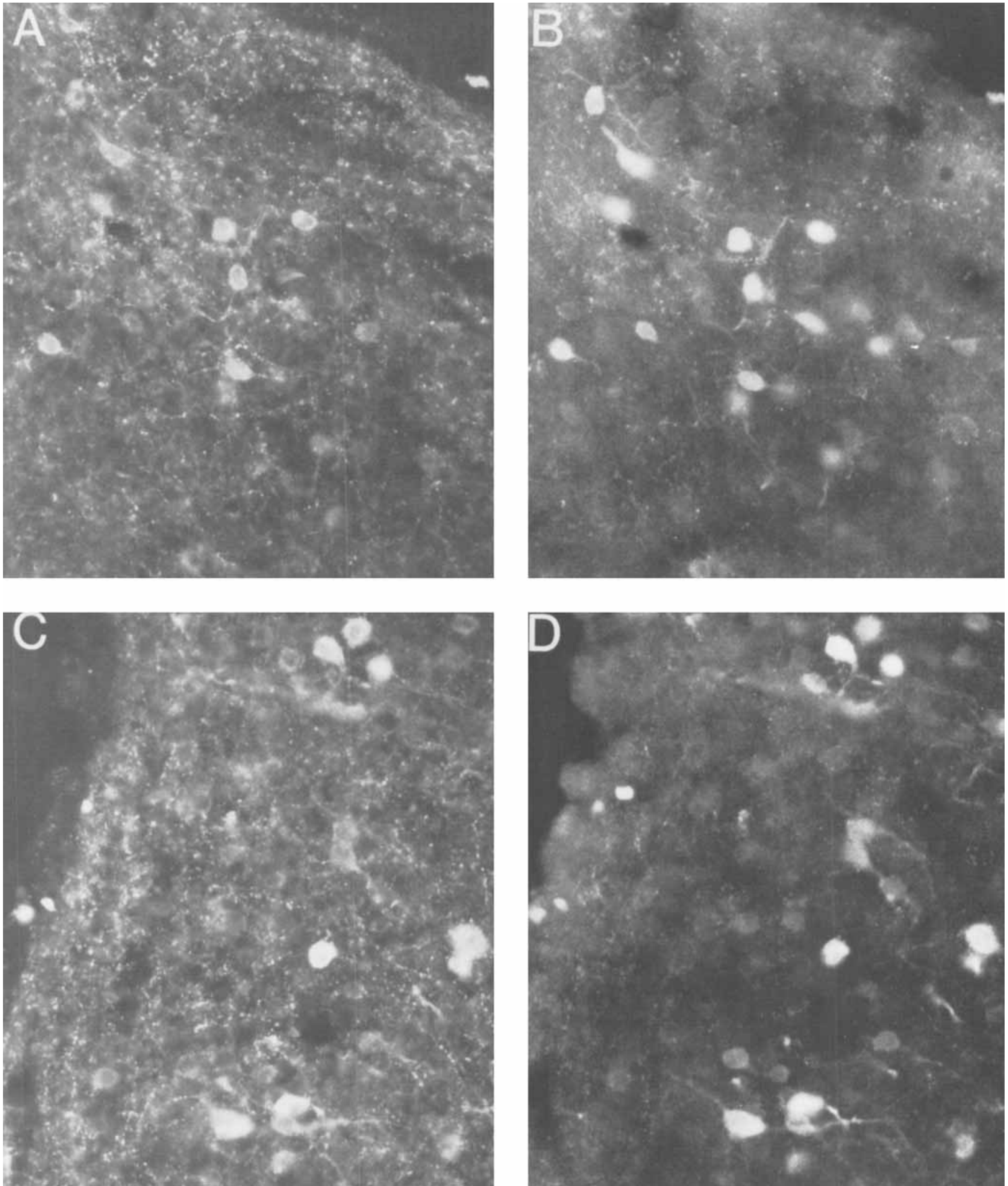


Fig. 15. Pairs of photomicrographs of two transverse sections through the turtle brain that had been processed acccording to the simultaneous immunofluorescence procedure for the dual localization of LENK and BAM22P. **A.** LENK+ FITC-labeled perikarya and fibers in a single field of view through area d of the striatum. **B.** BAM22P+ TRITC-labeled perikarya and fibers present in this same field of view. Note that the same perikarya are labeled in both and that some of the perikarya appear to be more heavily labeled for BAM22P than for LENK. Medial is to the right in A and B. **C.** LENK+ FITC-labeled perikarya and fibers in a single field of view through the preoptic region. **D.** BAM22P+ TRITC-labeled perikarya and fibers present in this same field of view. Note that the same perikarya are labeled for both LENK and BAM22P. Medial is to the left in C and D.

MENK to LENK levels was about 25:1, which differs markedly from the typical ratio in mammals. Since Kilpatrick et al. ('83) enzymatically treated their tissue extracts, it is possible that the small amounts of LENK detected in their procedures had been enzymatically cleaved from the N-terminus of the dynorphin peptides present in frog CNS (Cone and Goldstein, '82). Thus, although the LENK sequence may be present in proenkephalin in some amphibians, in the amphibian species that have been studied it seems likely that the LENK sequence is not present in proenkephalin. Whether the absence of LENK from proenkephalin in frogs represents the primitive amphibian condition, one that may have been passed on to reptiles, or whether it is a derived condition that evolved in the frog lineage is uncertain. Studies of members of the other extant orders of amphibians (urodeles and apodans) will be required to resolve this issue.

Even if LENK were absent from proenkephalin in ancestral amphibians, this does not rule out the possibility that the LENK sequence became part of proenkephalin during the early evolution of reptiles. The data presented here, the work of Lindberg and White ('86), and previous studies on the avian nervous system, in fact, strongly favor the conclusion that the LENK sequence is contained within proenkephalin of birds and reptiles. First, in the present study the ImmunoNuclear anti-LENK antiserum was specific for LENK and produced substantial LENK+ labeling even when blocked with 100 μM Dynorphin A(1–8) or 200 μM MENK. In contrast, labeling with this antiserum in *Xenopus* was essentially completely blocked by 50 μM Dynorphin A(1–8) or MENK (Reiner, unpub. obs.). Further, in an unpublished RIA study of the relative levels of MENK and LENK in different regions of the turtle central nervous system, the ratio of MENK to LENK ranged from about 4:1 to 2:1, comparable to that found in mammals (J.D. White, A. Reiner and J.F. McKelvy, cited in Reiner, '87). Similarly, Lindberg and White ('86) report a MENK:MERF:LENK ratio for whole turtle brain of approximately 4:1:1. Further, in pigeon brain the MENK to LENK ratio has been found to range from 10:1 to 3:1 on a regional basis (Bayon et al., '80). In chicken spinal cord a 2.5:1 ratio has been reported (Maderdrut et al., '86). It seems unlikely that the substantial levels of LENK immunoreactivity detected in these studies could represent LENK enzymatically cleaved from dynorphin peptides or cross-reactive recognition of dynorphin peptides. Dynorphin peptide levels found in the mammalian nervous system are only about 1/10 of those found for enkephalin peptides (Zamir et al., '84). Finally, White et al. ('85) have recently reported, based on HPLC studies, that LENK and MENK are both present in avian neural tissue. Thus, the currently available data favor the view that LENK is part of proenkephalin in birds and reptiles, as well as in mammals.

It seems very likely that MERF and MENK are also part of this precursor in reptiles, based on the present data, on the work of Lindberg and White ('86), and on the HPLC data demonstrating that MERF and MENK appear to be present in the avian nervous system (Kilpatrick et al., '83, White et al., '85; Maderdrut et al., '86). In light of the radioimmunoassay data cited above, therefore, it appears likely that turtle proenkephalin contains four copies of the MENK sequence, one copy of the LENK sequence, and one of the MERF sequence. The work of Lindberg and White ('86) indicates that the MENK:LENK:MERF ratio may not be 4:1:1 in proenkephalin of lizards and crocodilians, or if it is, proenkephalin is apparently processed differently in lizards and crocodilians than in turtles. Finally, the present data also indicate that a high molecular weight peptide(s) similar to BAM22P and/or PEPE is present in turtle proenkephalin. This substance presumably occupies the same location in turtle proenkephalin as do Peptide E and BAM22P in mammalian proenkephalin. The results suggest that such a high molecular weight enkephalin peptide(s) may be a major product of proenkephalin in at least some portions of the turtle central nervous system. The results of Lindberg and White ('86) indicate, however, that in turtle brain on the whole, proenkephalin is largely processed to the low molecular weight enkephalin peptides. Finally, the MERGL sequence, as such, appears to be absent from turtle proenkephalin. It is possible, however, that turtle proenkephalin contains a sequence resembling MERGL, as appears true in frogs, that does not cross-react with the anti-MERGL antiserum used in this study or that of Lindberg and White ('86). The MERGL sequence is also reportedly absent from proenkephalin in frogs (as noted above), lizards, crocodilians, and birds (Kilpatrick et al., '83; Martens and Herbert, '84; Lindberg and White, '86). Thus, MERGL as such may be a uniquely mammalian enkephalin peptide. Only placental mammals have been studied, however, and it is possible that MERGL may not have evolved until a relatively late point in mammalian evolution.

Comparison to previous studies in reptiles

Several previous studies have reported the distribution of enkephalin labeling in the CNS in reptiles. Two of these studies used Chang's anti-LENK antiserum (Naik et al., '81; Brauth, '84), and a third used anti-MENK and anti-LENK from a different source (Wolters et al., '86). In light of the potential cross-reactivity of antisera against the enkephalin pentapeptides with dynorphin peptides, it is possible that some of the enkephalin-positive labeling described in the previous studies represented cross-reactive labeling of dynorphin peptides. As discussed, the use of multiple specific antienkephalin antisera in the present study ensures that the pattern described here represents the pattern of enkephalin distribution. Despite the possibility of cross-reactive dynorphin labeling in previous studies on reptiles, the labeling patterns described here are largely similar to those in previous studies. This is not surprising since enkephalin and dynorphin are very similarly distributed in the nervous system, as reported in mammals (Khachaturian et al., '85) and as seen in birds and reptiles (Reiner et al., '84a,b; Reiner, '86a). Major similarities were observed between the present study and previous studies on the distribution of enkephalin in reptiles. At the telencephalic level, all reptiles possess much higher levels of enkephalinergic fibers and cell bodies in the basal ganglia than in the DVR and cortex, with essentially all basal ganglia enkephalinergic neurons being localized in the striatum (area d and PA in turtles) and fiber densities being higher in the GP. In addition, enkephalinergic neurons are present in such "striatal" regions as the TuOl, and enkephalinergic fibers are prominent in such "pallidal" regions as the ventral paleostriatum. Within the DVR, enkephalinergic neurons are sparse in all reptiles studied, whereas within cortex, enkephalinergic neurons and fibers are most abundant in the medial portions of cortex. The septal region of all reptiles also shows an abundance of enkephalinergic fibers and neurons. Within the diencephalon, enkephalinergic fibers and neurons are abundant in the hypothalamus, but much sparser in more dorsal parts of the diencephalon. Within the thalamus, enkephalinergic fibers are

present in cell groups with nonspecific telencephalic projections, but are entirely absent or extremely sparse in sensory thalamic projection nuclei. In the midbrain, enkephalinergic neurons are prominent in nDCP in all reptiles, and this cell group gives rise to an enkephalinergic input to the tectum that presumably terminates in the deeper tectal layers (Brauth and Kitt, '80; Reiner et al., '80; Brauth and Reiner, '82; ten Donkelaar and de Boer-van Huizan, '81). Additional enkephalinergic neurons that innervate retinorecipient tectal layers are present in deep or intermediate tectum in all reptiles. Tegmental levels of enkephalin are low, but some enkephalinergic fibers and neurons are present in the medial tegmentum. Although dynorphinergic fibers and terminals that arise from striatal neurons are prominent in the substantia nigra, enkephalinergic fibers were largely absent from the substantia nigra (Reiner, '83, '86a). Within the isthmic region, many enkephalinergic neurons have been reported in Pb and the nucleus of LL in turtles and lizards. Enkephalinergic fibers are prominent in Pb, LoC, and the central gray. Within the rhombencephalon, noteworthy accumulations of enkephalinergic neurons are present in the raphe, near nVII and within and lateral to caudal TTd. The enkephalinergic cells observed near nVII in turtles are present in the same region as centrifugal neurons projecting to the cochlea (Strutz, '82), which in mammals have been shown to be enkephalinergic (Altschuler et al., '84). In the spinal cord, enkephalinergic neurons and fibers are most prominent in the dorsal root entry zone and the dorsal horn.

Some differences in enkephalin distribution are present in the published studies. Enkephalinergic neurons were not observed in the supraoptic nucleus in the present study, but they were by Naik et al. ('81) in lizards. In rats, dynorphinergic neurons, but not enkephalinergic neurons, have been observed in the supraoptic nucleus (nSO) (Watson et al., '82a). In other mammals, however, enkephalinergic as well as dynorphinergic neurons appear to be present in nSO (Vanderhaeghen et al., '83; Khachaturian et al., '85). Thus, it is uncertain if the reported difference between turtles and lizards regarding enkephalinergic neurons in nSO is a real species difference or whether the reported enkephalin staining in nSO neurons in lizards stems from cross-reactive labeling of dynorphinergic neurons (Chang's anti-LENK antiserum was used in the lizard study). Other minor differences were also observed. Naik et al. ('81) did not observe enkephalinergic neurons in Ra, medial tegmentum, and La. These authors did not pretreat their lizards with colchicine, however, and it is possible that such treatment would have made enkephalinergic neurons evident in these cell groups.

Comparison to previous studies in birds and mammals

The enkephalin labeling patterns in the present study bear a great resemblance to those in mammals and birds (Cuello and Paxinos, '78; Sar et al., '78; Wamsley et al., '80; Bayon et al., '80; Del Fiacco et al., '80, '82; De Lanerolle et al., '81; Reiner et al., '82b, '84a,b; Khachaturian et al., '83a,c, '85). The resemblance to birds is greater than to mammals, presumably owing to the greater overall similarity between the brains of reptiles and the brains of birds than between the brains of reptiles and mammals. A few of the major characteristics of the enkephalinergic labeling pattern common to mammals, birds, and reptiles are: 1) high levels of enkephalin in a population of striatal neurons and of pallidal fibers (indicating the presence of a population of enkephalinergic striatopallidal projection neurons), 2) low numbers of enkephalin neurons and fibers in telencephalic cortex or its reptilian and avian equivalents (i.e., cortex and DVR in reptiles, Wulst and DVR in birds), 3) high levels of hypothalamic enkephalin, notably in preoptic neurons and nVH fibers, 4) high levels of enkephalinergic fibers in the central and periventricular gray of the midbrain, and 5) high levels of enkephalinergic fibers associated with TTd of the medulla and the dorsal horn of the spinal cord. It seems very likely that these enkephalinergic systems have been inherited by reptiles, birds, and mammals from their common reptilian ancestors. The particular significance of the similarities observed at telencephalic levels has been considered, together with other shared features of telencephalic organization, in previous discussions of the evolution of the basal ganglia (Reiner et al., '84a).

Major features of the enkephalinergic labeling pattern that reptiles share in common with birds but not with mammals include the high levels of enkephalin in the pretectal cell group, nDCP (which is homologous to the avian lateral spiriform nucleus, or SpL), and its projection to the tectum (Reiner et al., '80, '82a,b), the high levels of enkephalin in neurons with radially ascending dendrites that enter the retinorecipient tectum neuropil, and the high levels of enkephalin in neurons of nucleus laminaris of the midbrain (which appears to be comparable to the avian nucleus intercollicularis). The apparent absence in mammals of an enkephalinergic correspondent of the reptilian nDCP-tectal pathway is of particular interest. In both reptiles and birds, this enkephalinergic cell group is the major target of the pallidal portion of the basal ganglia (Brauth and Kitt, '80; Reiner et al., '80, '82a,b). The neurons of nDCP and SpL also contain GABA and the neurotensin-related hexapeptide LANT6 (Reiner, '86b; Reiner and Carraway, '87) and may influence neurons of the deep tectal layers that project to hindbrain premotor cell groups. This basal ganglia-tectal pathway may be the major circuit by which the reptilian and avian basal ganglia influence motor functions. Despite the prominence of this pretectal cell group in reptiles and birds, a comparable enkephalinergic cell group has not been observed in the pretectum of mammals, although the nucleus of the posterior commissure shows some connectional similarity to nDCP and SpL (Reiner et al., '84a). Overall, the differences noted between birds and reptiles on one hand and mammals on the other appear to relate mainly to the larger size and somewhat different cytoarchitectural organization of the midbrain roof in birds and reptiles compared to that in mammals (Reiner and Karten, '82), and to an attendant hypertrophy of cell groups related to the midbrain roof in birds and reptiles compared to mammals.

Conclusions

In general, the present results in conjunction with previous studies on the molecular structure and distribution of other neuropeptides (e.g., substance P, CCK8, somatostatin, and neurotensin) in the turtle central nervous system (King and Millar, '79; Carraway et al., '82; Reiner et al., '84c; Weindl et al., '84; Reiner and Beinfeld, '85) suggest that neuropeptide evolution among amniotes has been very conservative. The present results show that a proenkephalinlike peptide that contains LENK, MENK, MERF, PEPE/BAM22P, or highly similar peptides appears to be present in neurons of the turtle central nervous system. These

neurons and their fibers are widespread and abundant in the turtle central nervous system and their distribution shows many similarities with that in birds and mammals. The present results thus suggest that many of the major features of enkephalin distribution in birds and mammals had already arisen in the reptilian ancestors of living birds, reptiles, and mammals. Since opiate receptors of the mu and delta types have been reported in reptiles (Pert et al., '74; Buatti and Pasternak, '81; Moon-Edley et al., '82), it seems likely that the enkephalin peptides exert their physiological effects in turtles by means of the same receptor types as in birds and mammals. Thus, the enkephalins appear to play a functional role in many of the same systems in reptiles as in birds and mammals, and it seems likely that this role is mediated by similar synaptic events.

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