

# Immunohistochemical and Biochemical Studies on Lys<sup>8</sup>-Asn<sup>9</sup>-Neurotensin<sup>8-13</sup> (LANT6)-Related Peptides in the Basal Ganglia of Pigeons, Turtles, and Hamsters

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## ABSTRACT

The distribution of the neurotensin-related hexapeptide LANT6 within the basal ganglia and its projection targets was studied in turtles, pigeons, and hamsters by using immunohistochemical techniques, radioimmunoassay (RIA), gel chromatography, and high performance liquid chromatography (HPLC).

The results in turtles and pigeons were fundamentally similar. Within the basal ganglia, LANT6-like immunoreactivity (LLI) was observed in a population of large striatal neurons (comprising 1-5% of the total number of striatal neurons) and in essentially all of the medium-large pallidal neurons. In addition, LLI was observed in neurons of such other "striatal" and "pallidal" cell groups as the olfactory tubercle and ventral pallidum, respectively. Within the dopaminergic cell fields of the tegmentum, to which the pallidal cell groups project, LLI-containing fibers were abundant. Knife-cut studies confirmed that the majority of these LLI-containing fibers arise from telencephalic levels. Biochemical studies with RIA and HPLC showed large amounts of immunoreactive LANT6 (iLANT6) in the basal telencephalon (477 pmol/g) and tegmentum of pigeons (259 pmol/g), and this material was indistinguishable from the synthetic peptide. Lower levels of iLANT6 were demonstrated in the basal telencephalon (82 pmol/g) and tegmentum (156 pmol/g) of turtles, and the majority of this activity appeared to be associated with larger molecular forms of LANT6 or a peptide related to LANT6. In addition, one or more substances resembling Neuromedin N (NMN), a mammalian counterpart to LANT6, were detected in the turtle nervous system.

The labeling patterns in hamsters were similar to those in pigeons and turtles, except that in hamsters fewer neurons were labeled and the labeling was generally lighter. The lighter level of labeling may reflect a difference between the LANT6-like material present in hamster nervous system and authentic LANT6. Biochemical studies revealed that a Neuromedin N-like substance, as well as high molecular weight forms of a LANT6-like substance, are present in hamster brain. In hamsters, neurons within globus pallidus, the entopeduncular nucleus, the ventral pallidum, and the polymorph layer of the olfactory tubercle were labeled for the presence of LANT6. Fiber labeling for LANT6 in the dopaminergic tegmental cell groups that receive pallidal input was, however, light.

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Thus, the present results establish that LANT6 in pigeons and LANT6-related peptides in turtles and hamsters are present within many pallidal neurons. In pigeons and turtles, these pallidal neurons give rise to a major LLI-containing projection to the dopaminergic cell groups of the tegmentum. Thus, LANT6 in birds and related peptides in reptiles and mammals may be used as a neurotransmitter/neuromodulator by pallidal neurons of the basal ganglia, particularly in their projections to the dopaminergic cell groups of the tegmentum. This suggestion is consistent with recent studies that show neurotensin and neurotensin-related substances, such as Neuro-medin N, activate dopaminergic tegmental neurons (Kalivas, '85; Kalivas and Richardson-Carlson, '85; Kalivas et al., '86).

**Key words:** globus pallidus, substantia nigra, basal ganglia striatum, radioimmunoassay

In a previous immunohistochemical study (Reiner and Carraway, '85), we reported that neurons of the globus pallidus and the ventral pallidum in birds, reptiles, and mammals label positively for the presence of the neurotensin (NT)-related hexapeptide, LANT6. In birds and reptiles, positive labeling for LANT6 was also prominent in fibers and terminals in the midbrain projection targets of pallidal neurons. In addition, we reported that LANT6-related material is present in neurons that may be pallidal neurons in sharks, lungfish, and frogs (Reiner and Carraway, '85; Reiner and Northcutt, '86). The presence of LANT6 or a similar peptide in pallidal neurons in members of such disparate vertebrate groups suggests that this peptide plays an important and phylogenetically conserved role in pallidal functions. Since LANT6 has been found in synaptosomes of the avian brain and has been shown to possess pharmacological activity at low concentrations (Carraway et al., '83), it appears likely that LANT6 may be used as a neurotransmitter/neuromodulator. In light of the demonstrated influence of NT on tegmental dopaminergic neurons (Andrade and Aghajanian, '81; Kalivas et al., '83; Kalivas, '85), the possibility that pallidal neurons may use LANT6 or related peptides to influence tegmental dopaminergic neurons is of particular interest. Kalivas and coworkers (Kalivas, '85; Kalivas and Richardson-Carlson, '85; Kalivas et al., '86) have recently shown that Neuromedin N, a peptide that differs from LANT6 only in the substitution of isoleucine for asparagine and that may be the equivalent of LANT6 in some mammalian species, increases the activity of dopaminergic neurons, apparently acting on a different receptor than acted upon by NT.

In the present study, the distribution of LANT6-positive neurons in the pallidal cell fields of birds, reptiles, and mammals was examined in detail. In addition, the distribution of LANT6-positive neurons and fibers within the tegmental projection targets of the pallidum was examined, and knife-cut studies were carried out in birds and reptiles to determine whether tegmental LANT6-positive fibers originated from telencephalic sources. Finally, biochemical studies were carried out to clarify the identity of the LANT6-related substances in the tegmentum and ventral telencephalon of birds, reptiles, and mammals.

## MATERIALS AND METHODS

White Carneaux pigeons (*Columba livia*), red-eared turtles (*Pseudemys scripta*), and Djungarian hamsters (*Phodopus sungorus*) were used in the present study. All animals were deeply anesthetized with ketamine and perfused

through the left ventricle with 6% dextran in phosphate buffer (pH7.2) followed by 4% paraformaldehyde in phosphate buffer (pH7.2), except pigeons, for which the paraformaldehyde-lysine-periodate fixative was used (McLean and Nakane, '74). Brains were subsequently sectioned frozen at 40 microns on a sliding microtome, and sections through the forebrain and midbrain were processed according to the peroxidase-antiperoxidase (PAP) immunohistochemical procedure, as described previously (Sternberger, '79; Reiner et al., '82b, '83, '84b, c; Reiner and Carraway, '85; Brauth et al., '83), using an antiserum toward LANT6 (TG-22) that has been shown not to crossreact with NT (Carraway et al., '83; Reiner and Carraway, '85). A 1:1,000 primary antiserum dilution was used in the present study and the staining described in this paper could be blocked with 10–50  $\mu$ M synthetic LANT6, but not with 10–100  $\mu$ M NT. An antiserum specific for the last 8 amino acids of NT (HC-8) was also used in the present study in order to compare the LANT6 distribution observed to the distribution of NT. This antiserum is specific for NT and does not crossreact with LANT6 (Carraway et al., '82). Labeling with the NT antiserum was not blocked by 10–100  $\mu$ M LANT6, but was blocked by 10–50  $\mu$ M NT.

For the biochemical studies, the basal telencephalon and tegmentum were dissected free on ice from three turtles and two pigeons. Whole brain minus cerebellum was obtained from hamsters by decapitation after ether anesthesia. These tissues were extracted into 10 volumes of 0.1N HCl, boiled for 5 minutes, and lyophilized. The extract was then reconstituted in an assay buffer and subjected to RIA for LANT6 and NT, using previously described procedures (Carraway et al., '82, '83). Antiserum HC-8, which is directed toward the biologically active C-terminal octapeptide of NT, was used to measure NT (Carraway and Lee-man, '76). Antiserum TG-22, raised toward LANT6 and shown to crossreact less than 0.01% with NT, was used to measure LANT6 (Carraway et al., '83). The assays were allowed to come to equilibrium, bound and free radioactivity were separated by addition of dextran-coated charcoal, and bound radioactivity was quantitated using a 16-well gammacounter. Online computerized data analysis was performed using a log-logit linearization program (IN/US Service Corp., Fairfield, NJ). Each sample was assayed over a 20–30-fold range and concentrations were measured near the ED50 for each assay. HPLC was performed using a dual pump system (Model 204, Waters Associates, Milford, MA) and a column (3.5 mm  $\times$  30 cm) of a u-Bondapak C-18. The column was equilibrated at 1.5 ml/min with buffer as indi-

TABLE 1. Terms for Homologous Structures of Basal Ganglia and Related Cell Groups in Pigeons, Turtles, and Hamsters\*

Pigeons	Turtles	Hamsters
Lobus parolfactorius (PP)	Area d (d)	Medial Striatum
Paleostriatum augmentatum (PA)	Paleostriatum augmentatum (PA)	Lateral Striatum
Paleostriatum primitivum (PP)	Globus Pallidus (GP)	Globus Pallidus (GP) + Entopeduncular Nucleus (EP)
Nucleus intrapeduncularis† (INP)	?	?
Ventral paleostriatum (VP)	Ventral Paleostriatum (VP)	Ventral Pallidum (VP)
Dorsointermediate thalamic nucleus‡ (DIP)	?	Ventral-anterior/ventral-lateral thalamic nuclei (VA-VL)
Posterior nucleus of the ansa lenticularis (ALp)	Entopeduncular nucleus (EP)	Subthalamic nucleus
Lateral spiriform nucleus (SpL)	Dorsal nucleus of the posterior commissure (nDCP)	nucleus of the posterior commissure ?
Ventral tegmental area (AVT)	Ventral tegmental area (AVT)	Ventral tegmental area (AVT)
Nucleus tegmentopedunculopontinus, pars compacta (TPc)	Substantia nigra, pars compacta (SNc)	Substantia nigra, pars compacta (SNc)
Nucleus tegmentopedunculopontinus, Pars dorsolateralis (TPd)	Substantia nigra, pars ventralis (SNv) + nucleus profundus mesencephali (nPM)	Substantia nigra, pars reticulata (SNr)
Nucleus tegmentopedunculopontinus, pars posterior (TPp)	**	Nucleus tegmentopedunculopontinus (TP)

\*See text for references or evidence supporting proposed homologies.

† Although at one time thought to be comparable to the internal subdivision of the mammalian globus pallidus, recent histochemical data indicate that this is not the case (Reiner et al., '83, '84). The avian INP does not clearly resemble any single structure in reptiles or mammals.

‡ It is currently uncertain whether the avian DIP and hamster VA-VL are independently evolved or represent evolutionary derivations from the same single structure of the common ancestor.

\*\* Although turtles appear to possess a correspondent of the avian TPp and mammalian TP, this structure has not been assigned a name and is not present at the levels illustrated in this paper.

cated, and after injection of the sample, a 30-minute linear gradient of 0–30% acetonitrile was applied, while collecting fractions of 0.75 ml (see figure legends). Synthetic LANT6 (H-Lys-Asn-Pro-Tyr-Ile-Leu-OH) and native chicken NT (< Glu-Leu-His-Val-Asn-Lys-Ala-Arg-Arg-Pro-Tyr-Ile-Leu-OH) served as standards. Gel chromatography was performed on a Sephadex G-25 column (1.5 × 100 cm) using 0.2 M acetic acid and a flow rate of 15 ml/hr. After the sample (2 ml) was applied, the first 46 ml effluent was discarded and fractions of 2.1 ml were collected. HPLC fractions and gel chromatography fractions were examined using radioimmunoassay for the presence of immunoreactive LANT6 (iLANT6) and immunoreactive NT (iNT) using the antisera described above. In the case of hamsters and turtles, some fractions were also examined for the presence of immunoreactive Neuromedin N (iNMN) using a rabbit antiserum against NMN-thyroglobulin conjugates (Carraway and Mitra, unpub. obs.). This antiserum, which is primarily directed toward the C-terminal region of NMN, crossreacts only about 3% with NT. It was found to be useful for radioimmunoassay but not for immunohistochemistry, due to this partial crossreaction with NT.

## RESULTS

The anti-LANT6 antiserum was used to study the distribution of LANT6-positive neurons in the basal ganglia and

in neurons and fibers of the ventral thalamus and tegmentum in pigeons, turtles, and hamsters. The results for each animal group are presented separately. To facilitate comparisons among the species under investigation, Table 1 presents the names for the homologous structures of the basal ganglia and related cell groups in pigeons, turtles and hamsters.

### Pigeons

**Biochemistry.** Biochemical studies on extracts of pigeon brain established the presence of a single form of immunoreactive LANT6 (iLANT6), which was indistinguishable from the synthetic peptide. When subjected to RIA, extracts of pigeon brain diluted in parallel to standard LANT6 (not shown), and particularly high concentrations were measured in basal ganglia and tegmentum (Table 2). During HPLC on u-Bondapak C-18, iLANT6 from pigeon brain eluted as a single peak with the same retention time as synthetic LANT6 (Fig. 1A). Similar studies confirmed the presence of immunoreactive NT (iNT) in these preparations (Table 2) and established that this material separated from iLANT during HPLC, eluting at the position of chicken NT (Fig. 1A).

**Immunohistochemistry: Basal ganglia.** In the basal ganglia of pigeons, numerous LANT6-like immunoreactivity (LLI)-containing neurons were observed in both the striatal

TABLE 2. Levels of iLANT6, iNT, and iNMN in Various Regions of Pigeon, Turtle, and Hamster Brain

Animal	Region	iLANT6 (pmol/g)	iNMN (pmol/g)	iNT (pmol/g)
Pigeon	basal telencephalon	385-570	ND*	243-322
Pigeon	tegmentum	249-369	ND	100-129
Pigeon	tectum	134-164	ND	10-25
Turtle	basal telencephalon	69-94	ND	380-477
Turtle	tegmentum	141-173	ND	167-238
Turtle	rest of brain	62-77	21-25	111-131
Hamster	whole brain	2.8	9.6	14.4

\*ND = not done.

and pallidal subdivisions of the basal ganglia (Fig. 2). Within the striatal subdivision of the basal ganglia, LLI-containing neurons were predominantly found in the lateral striatal subdivision, the paleostriatum augmentatum (PA) (Figs. 2,3). LLI-containing neurons constituted approximately 5% of the total number of neurons in PA, based on counts of the LLI-containing neurons in PAP-labeled tissue in comparison to counts of cresyl violet stained neurons in adjacent Nissl-stained sections. The density of the LLI-containing neurons was much lower in the medial striatum, the so-called lobus parolfactorius (LPO). Fewer than 1% of the total number of neurons were LLI-containing in lateral LPO, and LLI-containing neurons were rarely observed in medial LPO. The LLI-containing neurons of PA-LPO appeared to represent a single cell type, with round perikarya ranging from 15-20 microns in size. Although the dendrites of these neurons were not heavily labeled, the neurons appeared to be multipolar with thin smooth dendrites that did not branch profusely. In Nissl-stained sections through PA-LPO, neurons in the size range of the LLI-containing neurons were found to make up 5-8% of the total number of striatal neurons. Few, if any, of the neurons within PA-LPO in the Nissl-stained material were larger than the LLI-containing neurons. Thus, the LLI-containing neurons appear to represent the majority, if not all, of the largest neurons within the avian striatum.

LLI-containing neurons were extremely abundant throughout the entire extent of the pallidal subdivision of the avian basal ganglia, termed the paleostriatum primitivum (PP) (Figs. 2,3). The LLI-containing perikarya within PP, as well as their dendrites, were very well labeled (Fig. 3). PP also contained a relatively dense plexus of LLI-containing processes that appeared to represent the dendrites of PP neurons whose perikarya were situated in adjacent planes of section. LLI-containing neurons in PP typically ranged from 20-40 microns in size, although a few labeled cells were observed in the 15-20 microns size range. Counts of Nissl-stained cells in PP indicated that essentially all cells in this size range (15-40 microns) were labeled for LANT6. In Nissl-stained material, PP was also observed to contain a number of smaller cells (10-15 microns). Neurons within PP in this size range did not label for LLI. At least some of these cells have been found to contain substance P in a previous study (Reiner et al., '83), and recent results (Reiner, unpub. obs.) indicate that choline acetyltransferase-containing cells in this size range are also present in PP.

In addition to their localization within the basal ganglia, LLI-containing neurons were also observed in the bed nucleus of the stria terminalis, the olfactory tubercle, the nucleus of the diagonal band, and the ventral paleostriatum (Figs. 2,3). The LLI-containing neurons in these struc-

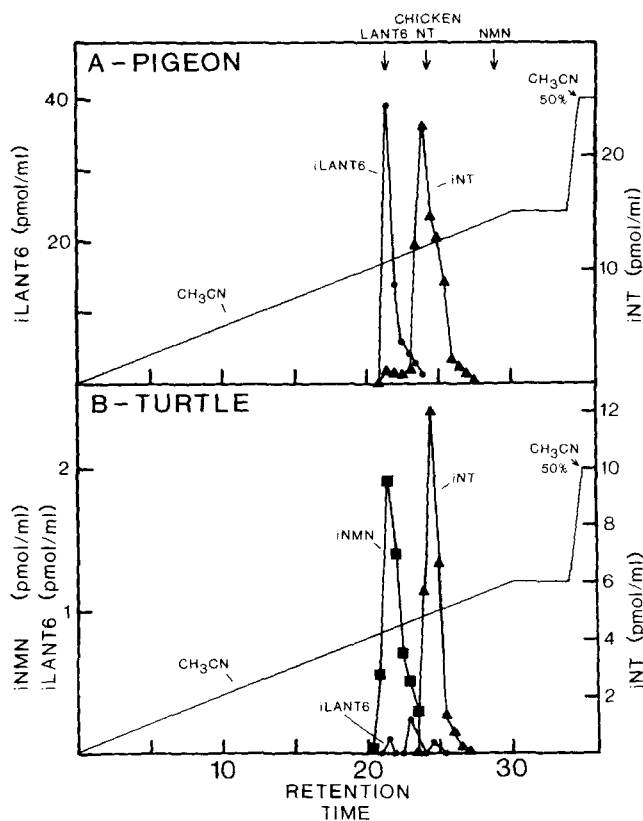


Fig. 1. HPLC of the extracts from pigeon basal telencephalon (A) and turtle brain (B). Buffer A was a solution containing 10 mM  $\text{KH}_2\text{PO}_4$  and 150 mM NaCl in distilled water; buffer B was this same solution made in 50% acetonitrile. As shown in both A and B, a linear gradient of acetonitrile was applied (0-30% in 30 minutes), held for four minutes, and then a 30-50% acetonitrile gradient applied over 1 minute. A flow rate of 1.5 ml/min was used and 0.75 ml fractions were collected. These fractions were examined for the presence of iLANT6, iNT, and iNMN using radioimmunoassay. As shown in A, large amounts of iLANT6 (solid circles) and iNT (triangles) were observed in pigeon basal telencephalon (with recoveries greater than 90%) and these were found to elute in the expected positions for LANT6 and chicken NT. Similar results (not shown) were obtained for whole pigeon brain, thereby suggesting that both LANT6 and chicken NT are present in the pigeon basal telencephalon and tegmentum. In turtle brain, by contrast, only a small amount (approximately 5%) of the total iLANT6 (circles) was recovered during HPLC and only part of this eluted in the position of LANT6. The LANT6-like substance eluting in the position of LANT6 (or possibly a different substance eluting in the same position) was much more readily detected with the anti-NMN antiserum (squares). This substance cannot be NMN itself, however, because NMN elutes at 29 minutes in this HPLC system. Thus, these results suggest that LANT6-like as well as NMN-like substances are present in turtle brain. The iNT (triangles) detected in turtle brain was found to elute in the position of chicken NT, thereby suggesting that turtle brain iNT is identical or highly similar to chicken NT.

## Abbreviations

A	cornu ammonis	MLd	nucleus mesencephali lateralis, pars dorsalis
Aa	anterior archistriatum	N	neostriatum
AC	anterior commissure	nBOR	nucleus of the basal optic root
AH	anterior hypothalamic nucleus	nDB	nucleus of the diagonal band
ALp	posterior nucleus of the ansa lenticularis	nDCP	dorsal nucleus of the posterior commissure
AVT	ventral tegmental area	NI	neostriatum intermedium
BLA	basolateral amygdaloid nucleus	N III	third cranial nerve
CA	central amygdaloid nucleus	n III	nucleus of the third cranial nerve
CC	corpus callosum	n IV	nucleus of the fourth cranial nerve
CN	core nucleus of the dorsal ventricular ridge	nPH	periventricular hypothalamic nucleus
CO	optic chiasm	nPM	nucleus profundus mesencephali
cp	pyriform cortex	nSL	lateral septal nucleus
D	dentate gyrus	nSM	medial septal nucleus
d	area d	nSO	supraoptic nucleus
DVR	dorsal ventricular ridge	nST	bed nucleus of the stria terminalis
E	ectostriatum	PA	paleostriatum augmentatum
EP	entopeduncular nucleus	PG	periaqueductal gray
EW	nucleus of Edinger-Westphal	PP	paleostriatum primitivum
Fi	fimbria	PT	pallial thickening
FLM	fasciculus longitudinalis medialis	Re	nucleus reuniens
FPL	fasciculus prosencephali lateralis	Ru	nucleus ruber
FRL	lateral reticular formation	SC	superior colliculus
Fx	fornix	SI	substantia innominata
GCT	central gray	SN	substantia nigra
GP	globus pallidus	SNc	substantia nigra, pars compacta
HA	hyperstriatum accessorium	SNr	substantia nigra, pars reticulata
Hb	habenula	SNv	substantia nigra, pars ventralis
HD	hyperstriatum dorsale	SpL	nucleus spiriformis lateralis
HV	hyperstriatum ventrale	St	striatum
HYPO-	hypothalamus	TeO	optic tectum
THAL		THAL	thalamus
ICo	nucleus intercollicularis	TO	optic tract
IHA	intercalated nucleus of the hyperstriatum accessorium	TP	nucleus tegmentipedunculopontinus
Imc	nucleus isthmi, pars magno-cellularis	TPd	nucleus tegmentipedunculopontinus, pars dorsolateralis
Imr	nucleus isthmi, pars rostralis	TPp	nucleus tegmentipedunculopontinus, pars posterior
INP	nucleus intrapeduncularis	TSC	torus semicircularis
IP	nucleus interpeduncularis	TSM	tractus septomesencephalicus
Ipc	nucleus isthmi, pars parvocellularis	Tu01	olfactory tubercle
La	nucleus laminaris	VMH	ventromedial hypothalamic nucleus
LPO	lobus parolfactorius	VP	ventral paleostriatum/ventral pallidum
MG	medial geniculate nucleus	ZI	zona incerta

tures did not constitute as great a percentage of the total population as in the case of PP. For example, in the ventral paleostriatum (VP), LLI-containing neurons made up only approximately 20% of the total population (Fig. 3). In addition, LLI-containing neurons were also observed in the portions of the avian telencephalon dorsal to the lamina medularis dorsalis (LMD), the boundary line between the basal ganglia and the dorsal ventricular ridge. Although LLI-containing neurons were scattered throughout this region (which includes both the dorsal ventricular ridge and the Wulst), LLI-containing neurons were particularly abundant or well labeled in the hyperstriatum accessorium, the medial hyperstriatum ventrale, the medial neostriatum, and the ectostriatum.

**Immunohistochemistry: Projection targets of the basal ganglia.** Since neurons of PP in birds are known to give rise to the major efferent projections of the avian basal ganglia (Karten and Dubbeldam, '73; Kitt and Brauth, '81; Reiner et al., '82a), the distribution of LLI-containing fibers in the projection targets of the PP was examined. At diencephalic and mesencephalic levels, LLI-containing fibers were observed in the ansa lenticularis (AL), the outflow bundle of PP. Surprisingly, however, LLI-containing fibers, although present, were not prominent in the major diencephalic and pretectal projection targets of the PP, namely, the posterior nucleus of the ansa lenticularis (ALp), the

dorsointermediate nucleus (DIP) of the thalamus, and the lateral spiriform nucleus (SpL) of the pretectum (Karten and Dubbeldam, '73; Kitt and Brauth, '82; Reiner et al., '82a). The neurons of ALp and SpL, however, were observed to contain LLI (Fig. 4).

Numerous well-labeled LLI-containing fibers and terminals were observed in the ventral tegmental area (AVT), the nucleus tegmentipedunculopontinus (TP), and the posterior subdivision of TP (TPp, which was previously referred to by one of us as the rostral subdivision of the locus coeruleus<sup>1</sup>) (Figs. 5,6). These latter three cell groups contain dopaminergic neurons in birds and correspond to the AVT, substantia nigra, and nucleus tegmentipedunculopontinus of mammals, respectively (Dube and Parent, '81; Reiner et al., '83). Previous studies in birds have shown that PP projects to TP and TPp (Karten and Dubbeldam, '73), while VP projects to the AVT (Kitt and Brauth, '81), which is

<sup>1</sup>The region indicated as TPp in the present study was identified as a rostral portion of locus coeruleus in the Karten and Hodos ('67) atlas, and also by Reiner et al. ('83), who noted that the region actually contains dopaminergic, not adrenergic neurons. The term TPp is adopted for this region in the present study because 1) the region appears to be a caudal continuation of TP, and 2) continued use of the term locus coeruleus, pars rostralis for this region might engender unnecessary confusion.

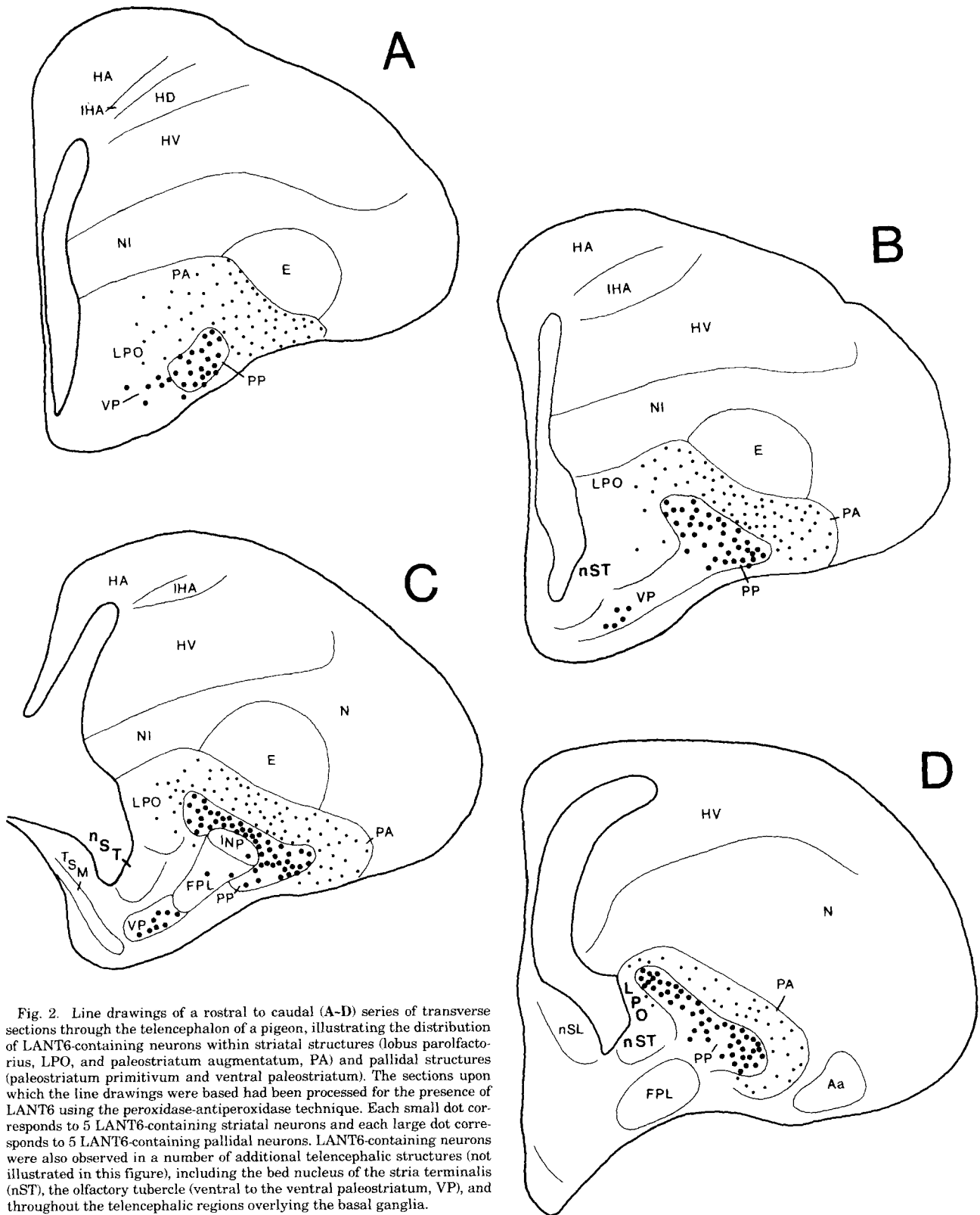


Fig. 2. Line drawings of a rostral to caudal (A-D) series of transverse sections through the telencephalon of a pigeon, illustrating the distribution of LANT6-containing neurons within striatal structures (lobus parolfactorius, LPO, and paleostriatum augmentatum, PA) and pallidal structures (paleostriatum primitivum and ventral paleostriatum). The sections upon which the line drawings were based had been processed for the presence of LANT6 using the peroxidase-antiperoxidase technique. Each small dot corresponds to 5 LANT6-containing striatal neurons and each large dot corresponds to 5 LANT6-containing pallidal neurons. LANT6-containing neurons were also observed in a number of additional telencephalic structures (not illustrated in this figure), including the bed nucleus of the stria terminalis (nST), the olfactory tubercle (ventral to the ventral paleostriatum, VP), and throughout the telencephalic regions overlying the basal ganglia.

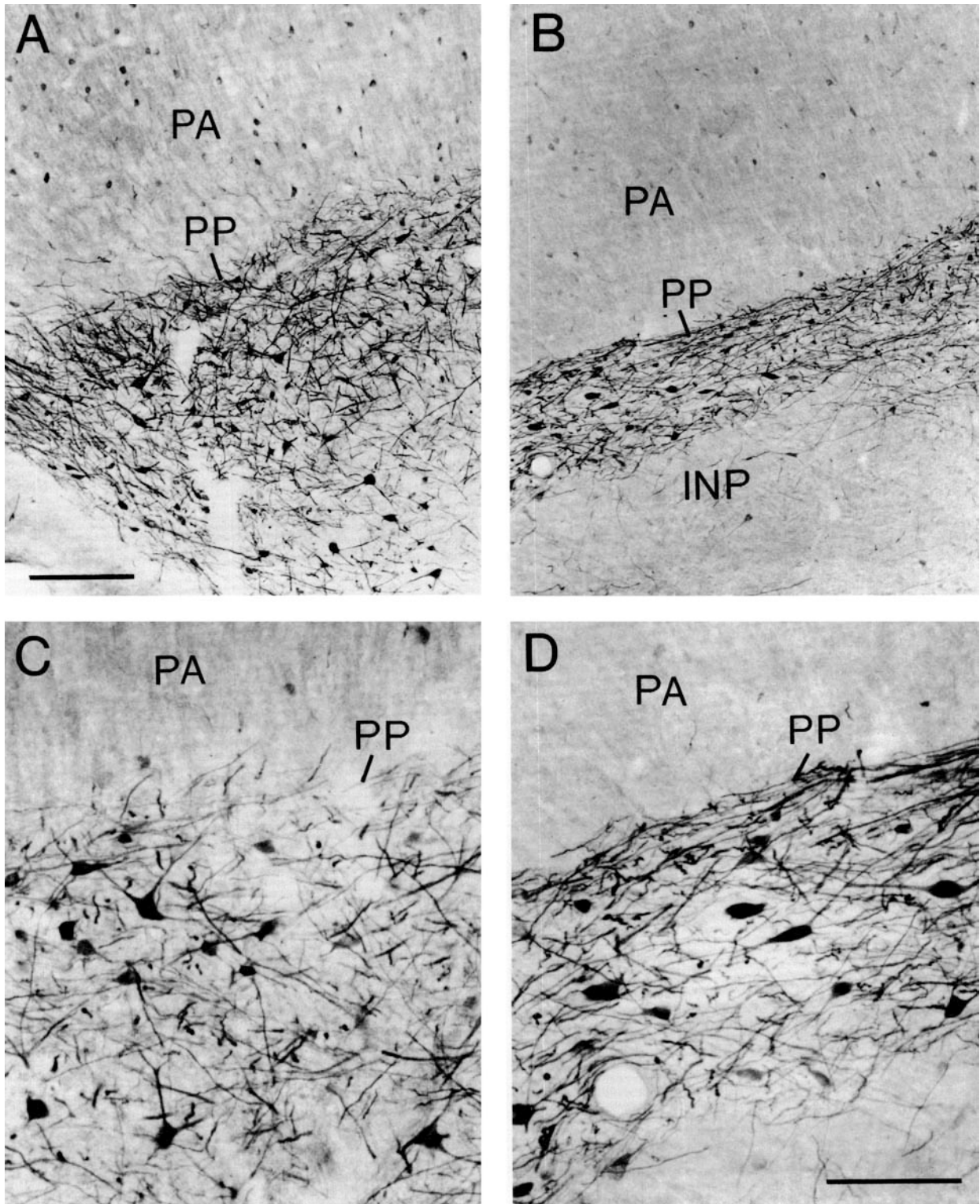


Fig. 3. Photomicrographs of LANT6-positive labeling in transverse telencephalic sections processed according to the PAP procedure. A low power view of PP and PA at a rostral (A) level corresponding to that shown in Figure 1B and a slightly more caudal view (B) corresponding to that shown in Figure 1C, as well as higher power views of PP at these same levels (C, rostral, D, caudal) are shown. Scale bars: A = 200 microns; B = same magnification as A; C = same magnification as D; D = 100 microns.



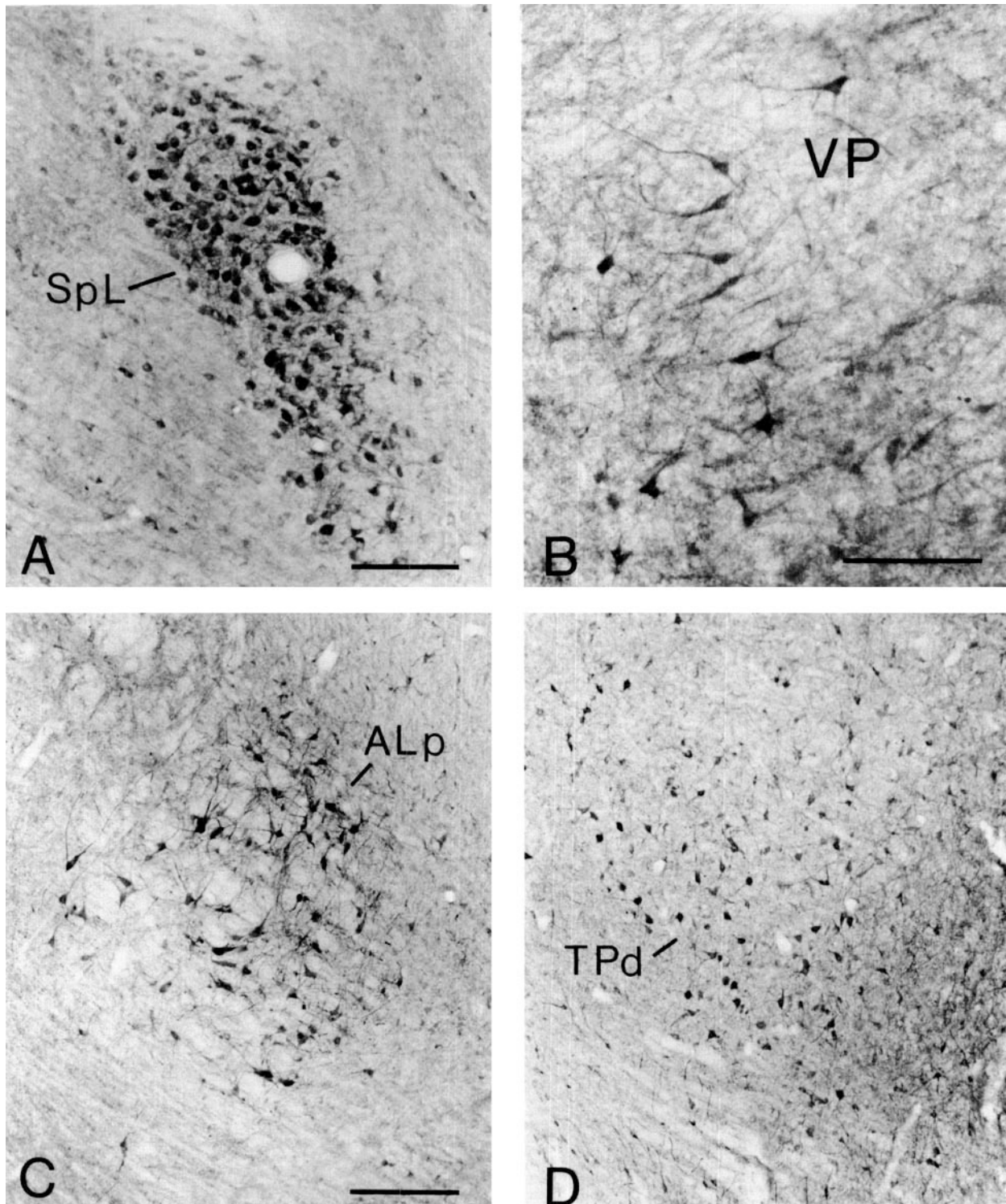


Fig. 4. Photomicrographs of LANT6-containing neurons labeled according to the PAP procedure in nucleus spiriformis lateralis (SpL) (A); the ventral paleostriatum (VP) (B); the posterior nucleus of the ansa lenticularis (ALp) (C); and the dorsolateral portion of the nucleus tegmentipedunclopontinus (TPd) (D). Scale bars: A = 200 microns; B = 100 microns; C = 200 microns; D = same magnification as C.



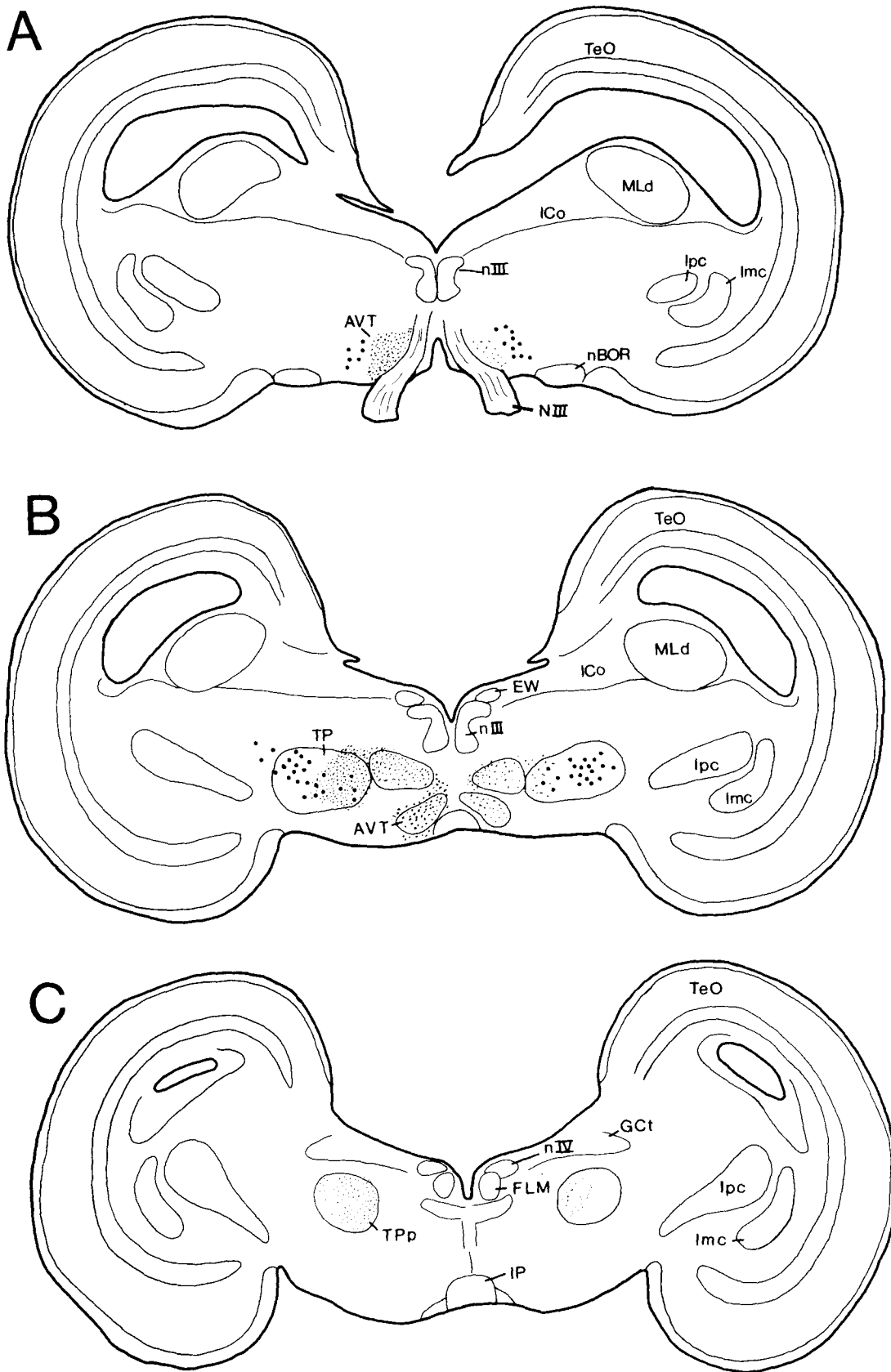


Fig. 5. Line drawings of a rostral to caudal series (A-C) of transverse sections through the midbrain of a pigeon whose outflow from the right basal ganglia had been severed at midiencephalic levels. The line drawings, which are based on sections processed according to the PAP procedure, illustrate the distribution of LANT6-containing neurons (each dot represents 5 neurons) and fibers (shown by the fine dots) in the fields of tegmental

catecholaminergic neurons. LANT6-containing neurons and fibers were also present in other portions of the midbrain, but are not illustrated. Comparison of the right side of the midbrain to the left in the above drawings shows the diminution of LANT6-containing fibers observed following the knife cuts of the basal ganglia outflow to the midbrain.

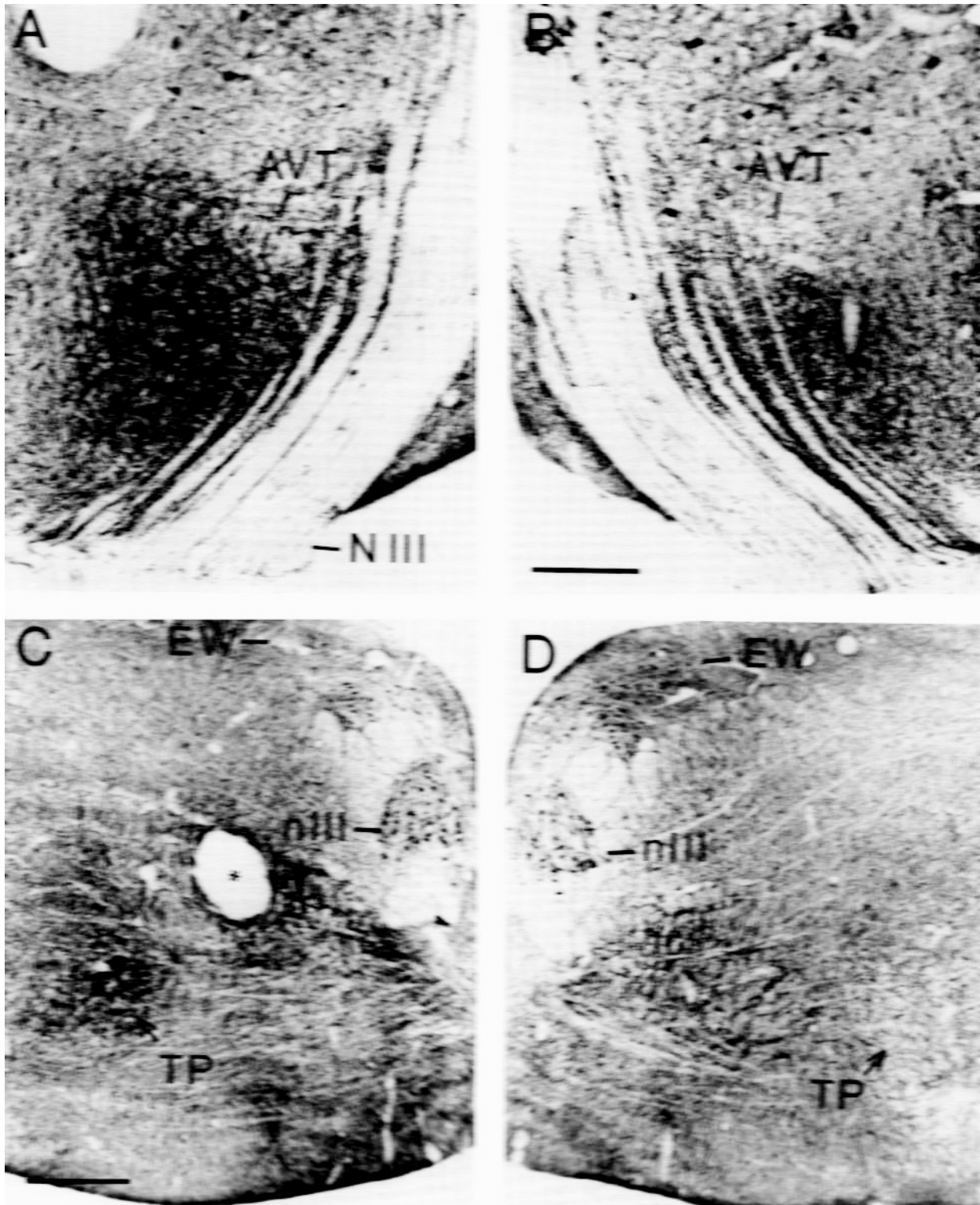


Fig. 6. Photomicrographs of transverse sections through the ventral tegmental area (A and B) and the TP region (C and D) of the same bird as illustrated in Figure 5. The sections were processed according to the PAP technique for the presence of LANT6; photomicrographs on the left are of the normal side of the brain, whereas those on the right are of the side of

the brain that received the knife cut. LANT6-containing fibers are clearly reduced in AVT and TP on the side of the brain ipsilateral to the knife cut. Scale bars: A = same magnification as B; B = 200 microns; C = 500 microns; D = same magnification as C.

consistent with the possibility that at least some of the LLI-containing fibers in these regions arise from LLI-containing pallidal neurons of the telencephalon. In contrast, the portion of the avian striatum containing the majority of the LLI-containing striatal neurons (i.e., PA) has not been found to have substantial projections outside of the telencephalon, and LLI-containing neurons were sparse in the portion of the striatum that projects to the tegmentum (i.e., LPO) (Karten and Dubbeldam, '73; Kitt and Brauth, '81). Thus, although LLI-containing neurons of PA and lateral LPO may project to the tegmentum, it appears unlikely that the striatum is a major source of the LLI-containing tegmental fibers. In addition to the LLI-containing fibers in the tegmentum, LLI-containing neurons were abundant in the lateral portions of AVT and TP (Figs. 4,5).

In order to examine the possibility that the LLI-containing fibers in the tegmentum arise from the LLI-containing PP and VP neurons, the outflow bundle of the basal telencephalon, the ansa lenticularis (AL), was surgically disconnected from the tegmentum by means of a stereotaxic knife cut (Karten and Hodos, '67) of the AL at mid-diencephalic levels. Following a 10–14 days postsurgical survival period, the numbers of LLI-containing fibers ipsilateral to the knife cut were dramatically reduced in AVT, TP, and TPp (Figs. 5,6). The location of the knife cut was confirmed in Nissl-stained material. The accuracy of the knife cut was further confirmed by the observations of a dramatic loss in substance P-containing fibers in the ipsilateral AVT, TP, and TPp, indicating that the striatonigral tract had been severed (Reiner et al., '83; Reiner, '86), which would occur unavoidably upon severing of the contiguous AL. Thus, these knife cuts surgically disconnected both the striatal and pallidal subdivisions of the basal ganglia from the tegmentum.

**Immunohistochemistry: Neurotensin vs. LANT6.** In contrast to the labeling pattern obtained with the anti-LANT6 antiserum, no labeled neurons were observed in any of the basal telencephalic cell groups with the anti-NT antiserum, except in the bed nucleus of the stria terminalis. Within portions of the telencephalon overlying the basal ganglia, NT-containing neurons were abundant in medial hyperstriatum ventrale and medial neostriatum. Within the diencephalon, NT-containing neurons were abundant in the preoptic area and in the dorsomedial and dorsolateral thalamic nuclei. LLI-containing neurons had also been observed to be abundant in these thalamic nuclei. NT-containing neurons were not observed in ALp, TP, or Spl. NT-containing fibers matching the distribution of LANT6-containing fibers were, however, observed in AVT, TP, and TPp. Although NT-containing neurons were not observed in PP or VP, knife cuts of the AL that reduced the number of LANT6-containing fibers in these tegmental fields equally reduced the number of NT-containing fibers in these fields (Fig. 7). The significance of this surprising result, which appears to suggest that NT and LANT6 may be in the same terminals in the tegmentum, will be evaluated further in the Discussion section. The overall results of the studies using the anti-NT antiserum suggest that LANT6 clearly seems to be present in neurons of many regions that appear not to contain NT-containing neurons. This result further corroborates that the anti-LANT6 antiserum and the anti-NT antiserum are specific for different antigens. In the few regions where both LANT6-containing and NT-

containing neurons were observed, it is presently uncertain whether LANT6 and NT co-occur in individual neurons.

### Turtles

**Biochemistry.** Biochemical studies on extracts of turtle brain established the presence of multiple forms of iLANT6, the majority of which behaved differently than the synthetic peptide during HPLC and gel chromatography. RIA of crude extracts indicated that, whereas the levels of iNT were comparable in turtle and pigeon brain, the measured levels of iLANT6 were generally much lower (2–5-fold) in turtle brain than in pigeon brain (Table 2). During HPLC on u-Bondapak C-18, only 5% of the iLANT6 from turtle brain was recovered from the column, 1% eluting at the position of synthetic LANT6 (retention time, 21.5) and 4% as other substances (Fig. 1B). Using the radioimmunoassay toward NMN, much larger measurements were obtained (Fig. 6B) for the material coeluting with LANT6 (retention time 21.5 min). For the peak fraction, the ratio of iNMN to iLANT6 was approximately 20 to 1. The results for iNT (Fig. 1B) indicated that 95% of the activity applied to the column was retrieved, eluting at the position for chicken NT (retention time, 24.5 min).

To account for the poor recovery of iLANT6 during HPLC, we hypothesized that iLANT6 is largely unprocessed in turtle brain and, thus, is associated with large molecular forms of a LANT6-related peptide. In order to test this possibility, extracts of brain were subjected to chromatography on Sephadex G-25 (Fig. 8). Six peaks of iLANT6 activity were obtained, the largest of which eluted at the void volume of the column (Fig. 8A). The overall recovery for iLANT6 was about 90%, and about 20% of the activity eluted in the region of synthetic LANT6. The antiserum toward NMN gave three peaks, the largest of which eluted in the region common to LANT6 and NMN (Fig. 8B). The antiserum toward NT gave a single peak eluting in the region of chicken NT (Fig. 8C).

In order to further test the idea that iLANT6 in the void volume of Figure 8 represented large molecular forms of a LANT6-related peptide, this material was treated with pepsin, an enzyme that has been shown to mimic the *in vivo* processing for NT and related peptides (Carraway et al., '86). As shown by the triangles (Fig. 8A), pepsin treatment increased the measured activity 2–3-fold, which is consistent with the idea that pepsin treatment generated smaller and more immunoreactive peptides than typically present in turtle nervous system.

A sample of the pepsin-treated material was examined with HPLC to identify these smaller peptides. As shown in Figure 9B, a number of different LANT6-related and NMN-related substances were generated by pepsin treatment of the void volume shown in Figure 8. The major peak of iLANT6 was found to show a very similar retention time (20.5–22 min) to synthetic LANT6. This peak coincides with one observed for iLANT6 in HPLC of turtle brain extract (Fig. 1B). Smaller peaks of iLANT6 were observed preceding (18 min) and following (23 min) the major peak (Fig. 9B). It seems possible that some of these peaks of iLANT6 represent slightly different sized versions of LANT6 or a substance closely resembling LANT6. Two major peaks (eluting at 21 min and 25.5 min) and one minor peak (eluting at 23 min) of iNMN were observed in the pepsin-treated material (Fig. 9B). None of these peaks, however, coeluted

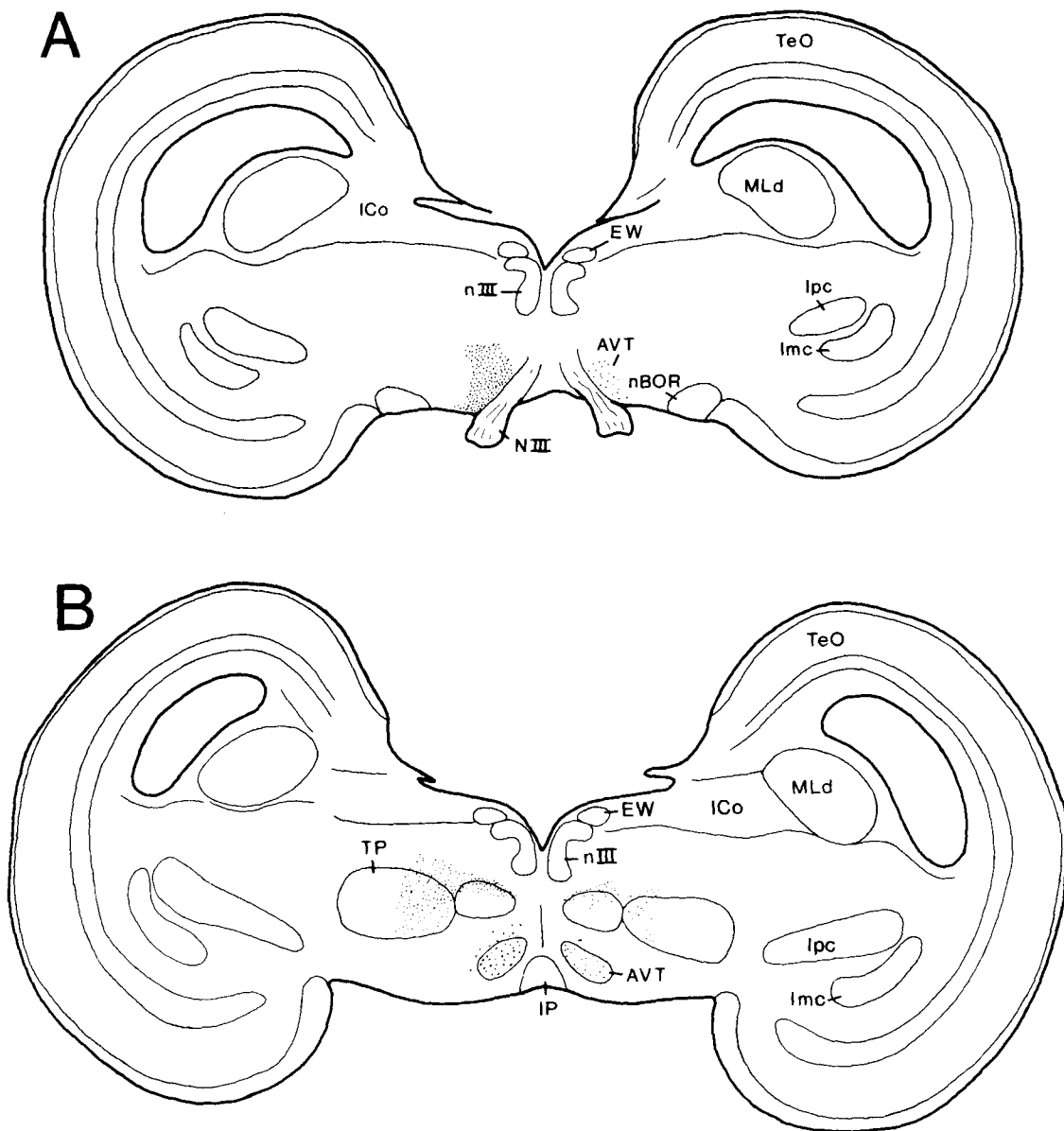


Fig. 7. Line drawings of a rostral to caudal (A to B) pair of transverse sections through the midbrain of the same pigeon as illustrated in Figures 4 and 5. The sections on which these drawings are based had been processed for the presence of neurotensin according to the PAP procedure. Neurotensin-containing fibers were clearly reduced in AVT and TP on the side of the brain ipsilateral to the knife cut (right side). Neurotensin-containing neurons and fiber were present in several cell groups of the midbrain, but are not illustrated above.

with synthetic NMN. Some of the peaks of iNMN may represent slightly different sized versions of the same NMN-like peptide. Interestingly, we showed above that turtle brain extracts contain an NMN-like peptide that elutes at 21 min (Fig. 1B). Thus, pepsin treatment of the large molecular weight LANT6-related peptide(s) in turtle brain can generate some of the same smaller NMN-related peptides (the iNMN at 21 min) and LANT6-related peptides (the iLANT6 at 20.5–22 min) as naturally generated in turtle brain.

The iNMN-rich fraction eluting at 25.5 min contained little iLANT6, but the other two iNMN-rich fractions also yielded peaks of iLANT6. This result may mean that the substances eluting at 21 min and 23 min possess both LANT6-like and NMN-like properties, or it may mean that distinct NMN-like and LANT6-like substances eluted at both 21 min and 23 min. If the former were true, however, then the ratio of iNMN to iLANT6 in HPLC fraction 21 of turtle brain extract (Fig. 1B) should be the same as in HPLC fraction 21 of the pepsin-treated material (Fig. 9B).

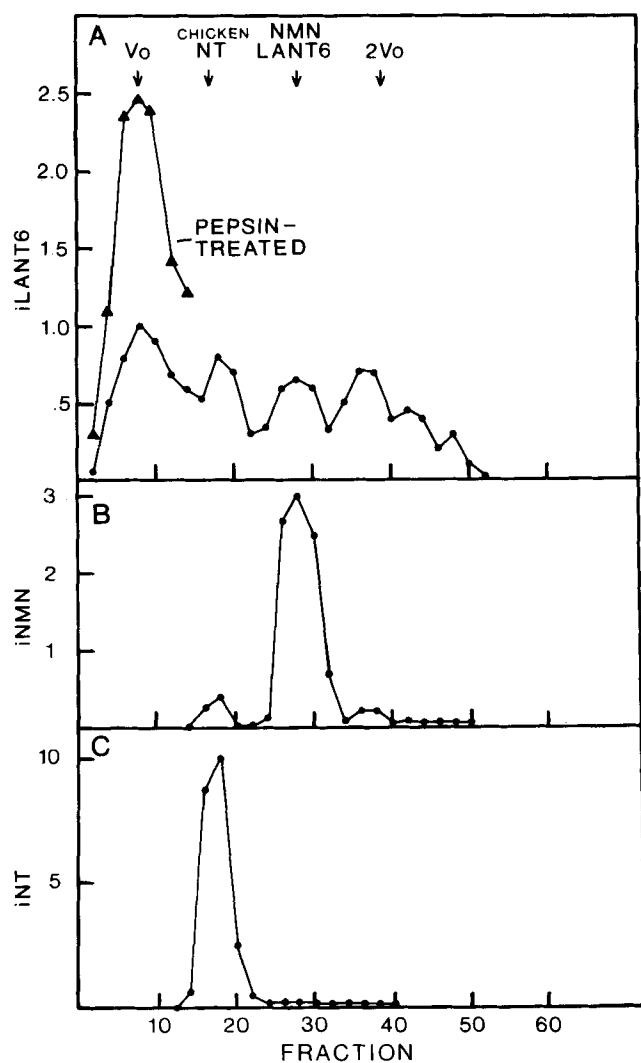


Fig. 8. Gel permeation chromatography of an extract of turtle brain on a Sephadex G-25 column ( $1.5 \times 100$  cm). The sample, an HCl extract of a 0.4 g turtle brain in 2 ml, was applied, and after 46 effluent was discarded, fractions of 2.1 ml were collected. Aliquots were lyophilized and subjected to RIA and the concentrations determined in each assay are given. Note that 6 different peaks of iLANT6 were obtained (A) and only 20% of the iLANT6 eluted in the expected position for LANT6. Note also that a large amount of the iLANT6 eluted in the void volume (Vo). In contrast, the bulk of the iNMN eluted in the position at which both LANT6 and NMN elute (B), whereas all of the iNT eluted in the position of chicken NT. These results are consistent with the results of the HPLC studies for iNMN and iNT, indicating that chicken NT and a peptide similar in structure to NMN are present in turtle brain. The results for iLANT6 suggest that iLANT6 in turtle brain is present in peptides of varying size, including some of high molecular weight. Consistent with this possibility, pepsin treatment of the early fractions (A) increased the levels of iLANT6 detected 2–3-fold.

Since the ratios differ, it seems more likely that distinct LANT6-like and NMN-like substances are eluting in HPLC fraction 21.

Finally, it is of interest to note that pepsin-treatment generated 50–100 times as much iLANT6 as iNMN. The gel chromatographic studies indicate that much of the iNMN stored in turtle nervous system is in low molecular weight form. Together, the HPLC data on the pepsin-treated

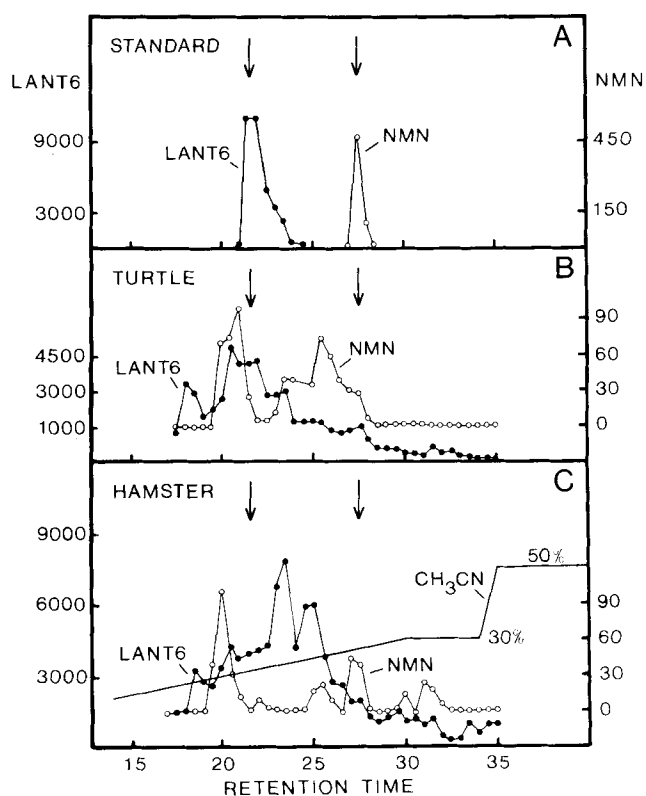


Fig. 9. HPLC of the pepsin-treated high molecular weight iLANT6 from turtle brain (B) and hamster brain (C). The HPLC profile for the standards is presented in A. The HPLC was carried out as described previously. The scale for the amount of iLANT6 per fraction is shown at the left; the scale for the amount of iNMN per fraction is shown to the right. Both scales represent fmol/fraction. The results indicate that in both turtle and hamster a number of different LANT6-like and NMN-like peptides were generated by the pepsin treatment. See text for further details.

material and the gel chromatographic data suggest that most of the iLANT6 in turtle brain is stored in high molecular weight form, while most of the iNMN is processed to a low molecular weight form.

**Immunohistochemistry: Basal ganglia.** The results in turtles were very similar to those in birds. LLI-containing neurons were abundant and widespread in the telencephalon, both in the basal ganglia and in the dorsal ventricular ridge (DVR) and cortex. Within the basal ganglia, LLI-containing neurons were extremely abundant in globus pallidus and considerably less abundant in the striatal subdivision of the basal ganglia (Figs. 10,11). In lateral striatum, termed the paleostriatum augmentatum (or PA, as in birds), LLI-containing neurons were large (15–20 microns) and made up about 1% of the total number of PA neurons. In Nissl-stained material, neurons that were the size of LLI-containing neurons were observed to represent about 1% of the total population. In contrast, the remaining 99% of the PA neurons were 10–15 microns in size. Thus, LLI-containing neurons appear to make up a distinct population of neurons that represents the predominant, if not the only, type of large neuron in PA. The density of LLI-containing neurons in the medial striatum of turtle, termed area d, was much less than in PA, and LLI-containing neurons

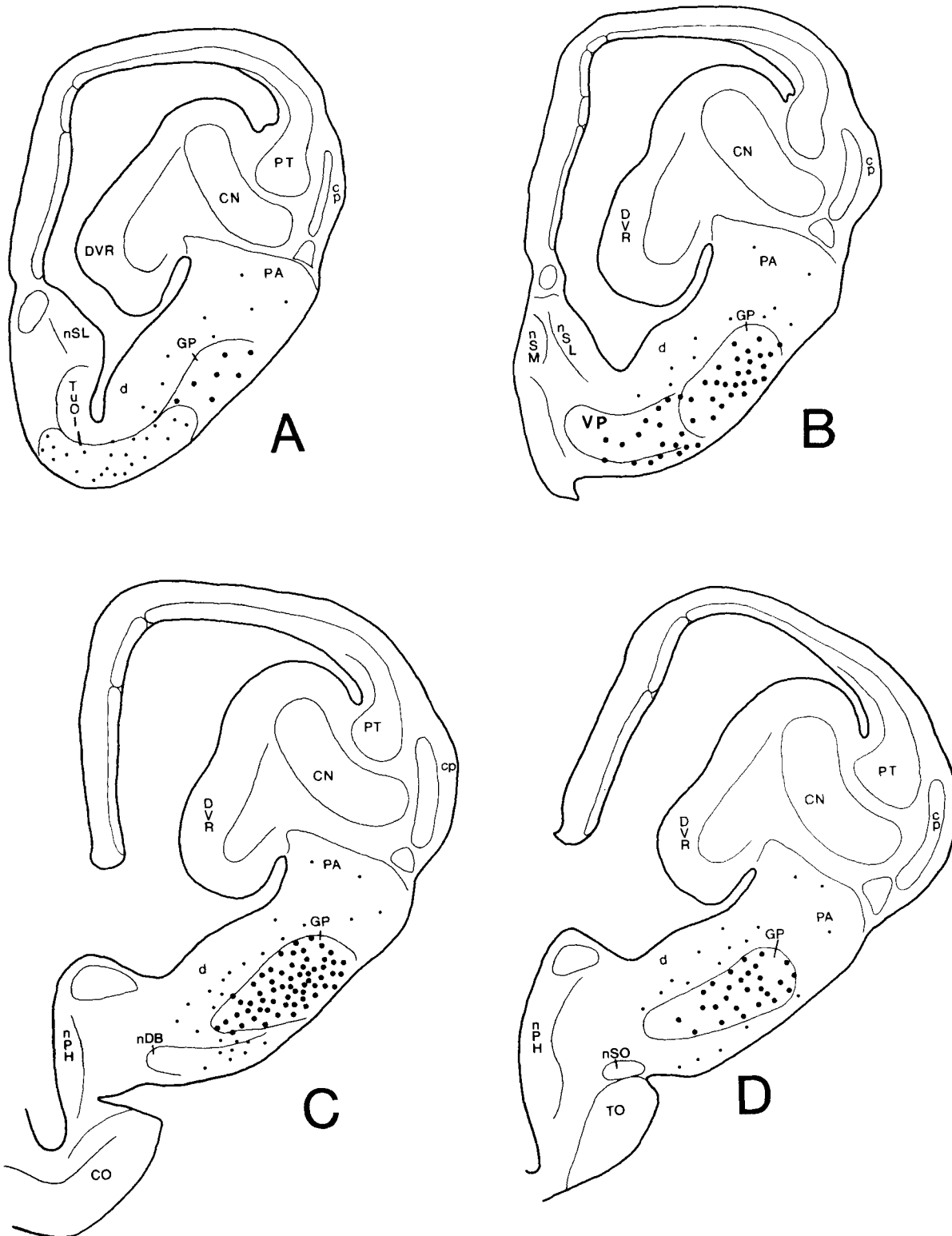


Fig. 10. Line drawings of a rostral to caudal series (A-D) of transverse sections through the turtle telencephalon that had been processed for the presence of LANT6 according to the PAP procedure. The drawings illustrate the distribution of LLI-containing neurons observed in striatal (the paleostriatum augmentatum, PA, area d and the olfactory tubercle, Tu01) and

pallidal (the globus pallidus, GP, and the ventral paleostriatum, VP) structures of the telencephalon. Each large dot represents 5 pallidal neurons and each small dot represents 5 striatal neurons. LLI-containing neurons were also widespread in the portions of the telencephalon overlying the basal ganglia, but these labeled neurons are not illustrated above.

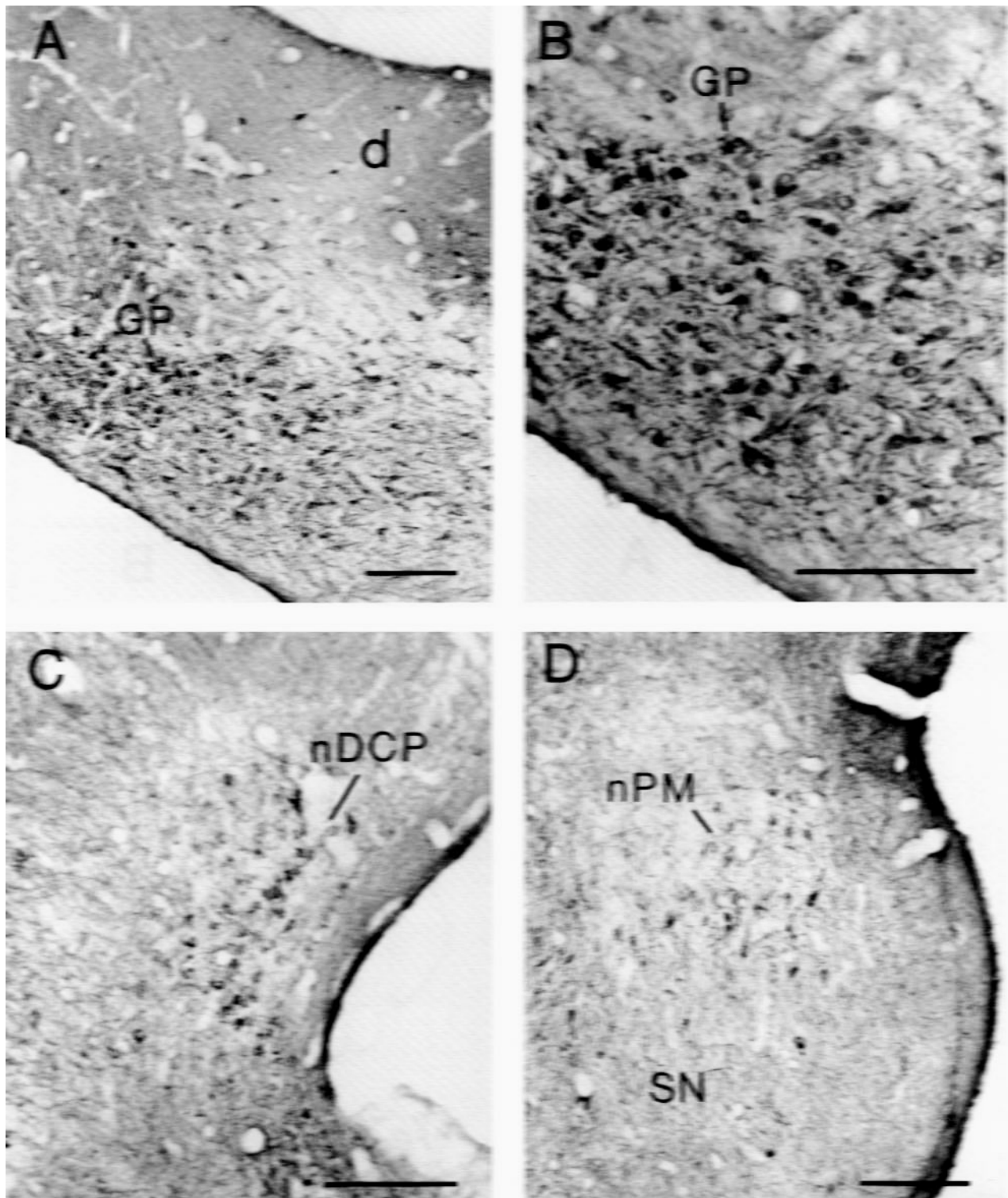


Fig. 11. Photomicrographs of transverse section through the turtle forebrain and midbrain showing LLI-containing neurons in a low power view of the globus pallidus and area d (A), a higher power view of globus pallidus (B), the dorsal nucleus of the posterior commissure (C), and the so-called nucleus profundus mesencephali (nPM) (D). Scale bars: A = 100 microns; B = 100 microns; C = 200 microns; D = 200 microns.



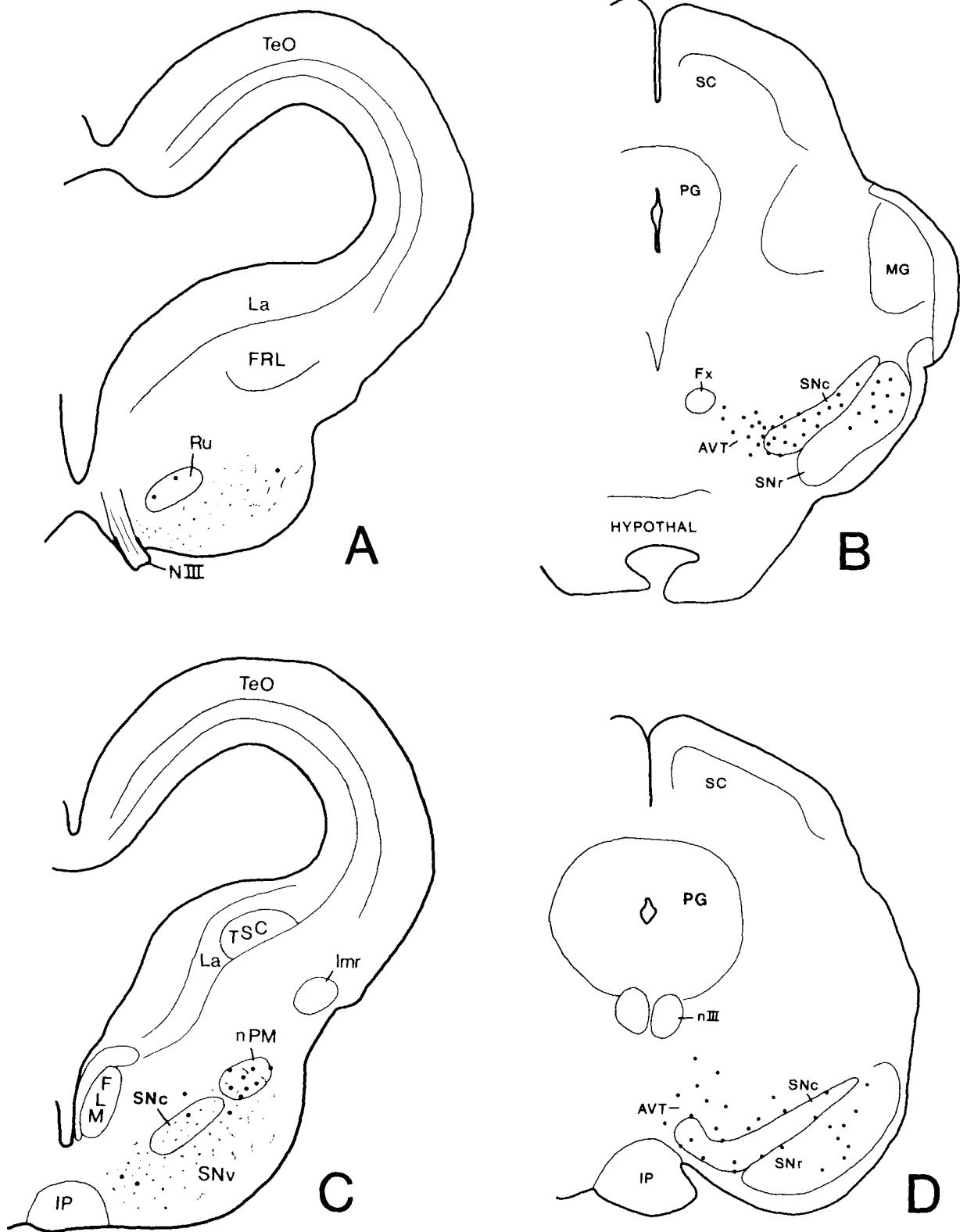


Fig. 12. Line drawings of a rostral to caudal pair of transverse sections through the midbrain of a turtle (A and C) and a hamster (B and D). Dots illustrate the location of LLI-containing neurons; fine dots illustrate the location of LLI-containing fibers. Although LLI-containing neurons and fibers were observed throughout the midbrain, only LLI-containing neurons and fibers in the tegmentum are illustrated.

were rarely observed within 100 microns of the ventricular wall in medialmost area d. Within the globus pallidus (GP), LLI-containing neurons were very densely packed and appeared to include essentially all GP neurons (Figs. 10,11). LLI-containing neurons in GP ranged from 15–30 microns in size. Numerous LLI-containing processes were present in GP. These processes appeared to be the dendrites of LLI-containing GP neurons. Neurons containing LLI were also observed in the olfactory tubercle, the ventral paleostriatum, and the nucleus of the diagonal band. Within these structures, LLI-containing neurons made up a much lesser percentage of the total number of neurons than was the case in GP. For example, in VP, LLI-containing neurons made up only approximately 15% of the total number of neurons.

**Immunohistochemistry: Projection targets of the basal ganglia.** Since LANT6-positive labeling was observed in pallidal neurons, the projection targets of GP were examined for the presence of LLI-containing fibers and terminals. Within the midbrain, LLI-containing fibers were sparse in the dorsal nucleus of the posterior commissure (nDCP) and the so-called entopeduncular nucleus, the homologues of the avian SpL and ALp, respectively. As true of the avian ALp, neurons of the entopeduncular nucleus were found to contain LLI, and as true of the avian SpL, neurons of nDCP were found to contain LLI (Fig. 11). Within the tegmentum, LLI-containing fibers were observed in AVT, the substantia nigra, and the nucleus profundus mesencephali (nPM) (Fig. 12). Although intensely labeled, these fibers were sparser than in the avian tegmentum. Numerous LLI-containing neurons were observed in nPM (Figs. 11,12), which suggests that this cell group corresponds to the lateral portion of TP, previously referred to as the dorsal subdivision of TP, or TPd (Reiner et al., '83; Kitt and Brauth, '81). Four to 6 weeks after aspiration of the basal ganglia or stereotaxic knife cuts of the basal ganglia outflow (Powers and Reiner, '80), the LLI-containing tegmental fibers ipsilateral to the knife cut were greatly reduced in number.

**Immunohistochemistry: Neurotensin vs. LANT6.** Immunohistochemical studies, using the HC-8 antiserum, of the distribution of NT in turtles yielded results similar to those in pigeons. NT+ labeling was absent from neurons of the basal telencephalon, except for a small region at the medial edge of area d that may correspond to the bed nucleus of the stria terminalis. NT+ labeling was also absent from neurons of the DVR and cortex of the telencephalon. NT-containing neurons were not observed in the projection targets of pallidal neurons. Although NT+ labeling was absent from pallidal neurons, NT+ labeling was observed in fibers in the tegmental targets of the pallidal neurons. As in pigeons, the lesions or knife cuts that reduced the LLI-containing fibers in the ipsilateral tegmentum also reduced the NT+ fibers in the tegmentum. Also as in pigeons, NT-containing neurons were present in several of the same diencephalic cell groups as LLI-containing neurons, including the preoptic area, the dorsomedial and dorsolateral thalamic nuclei, and the dorsal lateral geniculate nucleus (in which nearly all neurons appeared to be labeled for both NT and LANT6).

### Hamsters

**Biochemistry.** Since previous studies (Carraway et al., '83) had suggested that LANT6 itself is not present in rat brain, gel chromatography was used to characterize the

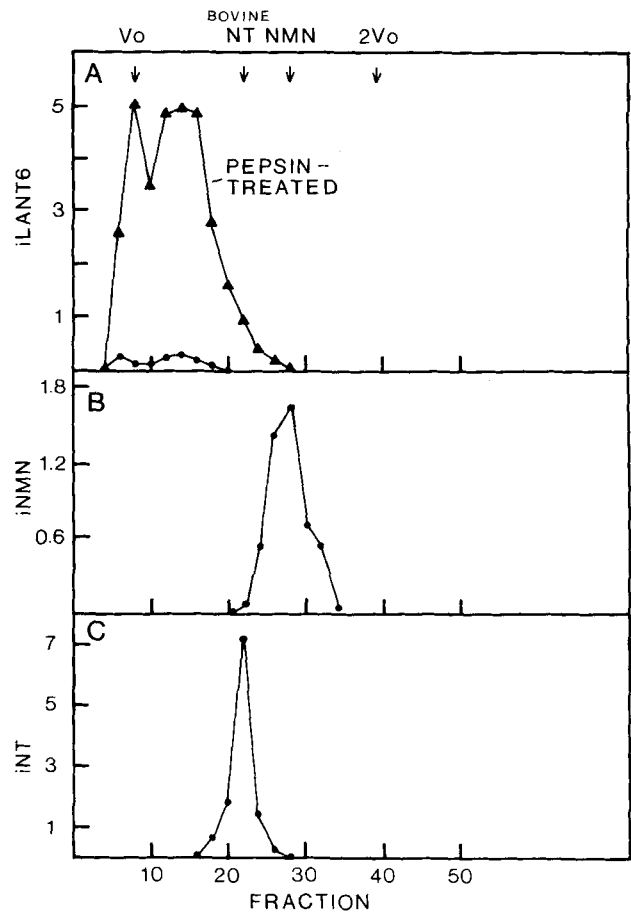


Fig. 13. Gel permeation chromatography of an extract of hamster brain on Sephadex G-25, showing the amounts and retention time of the iLANT6, iNMN, and iNT eluted. The results show that all of the iLANT6 eluted in the early fractions (including the void volume), whereas iNMN and iNT eluted in the positions expected for NMN and NT, respectively. These results suggest that iLANT6 in hamster brain resides in high molecular weight forms of a peptide related in structure to LANT6; peptides similar or identical to porcine NMN and bovine NT are also present. Consistent with the hypothesized presence of high molecular weight peptides related to LANT6, pepsin treatment of the early fractions increased the levels of detectable iLANT6 20-fold.

LANT6-like immunoreactive substances present in hamster brain (Fig. 13). As in rats, the levels of immunoreactive iLANT6 in hamster brain were very low (Table 2) and the bulk of this eluted as larger molecular weight forms of iLANT6. In contrast, substantial amounts of iNMN and iNT were found in hamster brain (Table 2) and these eluted in the expected positions for NMN and NT, respectively. Pepsin treatment of the LANT6-like material in fractions 4–29 increased the levels of iLANT6 about 20-fold, thus suggesting the presence of large molecular forms of a LANT6-like peptide in hamster brain.

In order to clarify the identity of the smaller peptides generated by pepsin treatment from these large molecular weight forms of a LANT6-like peptide, a sample of the pepsin-treated material was examined with HPLC (Fig. 9C). A number of different peaks of iLANT6 and iNMN were observed. The major peak of iLANT6 eluted later (23

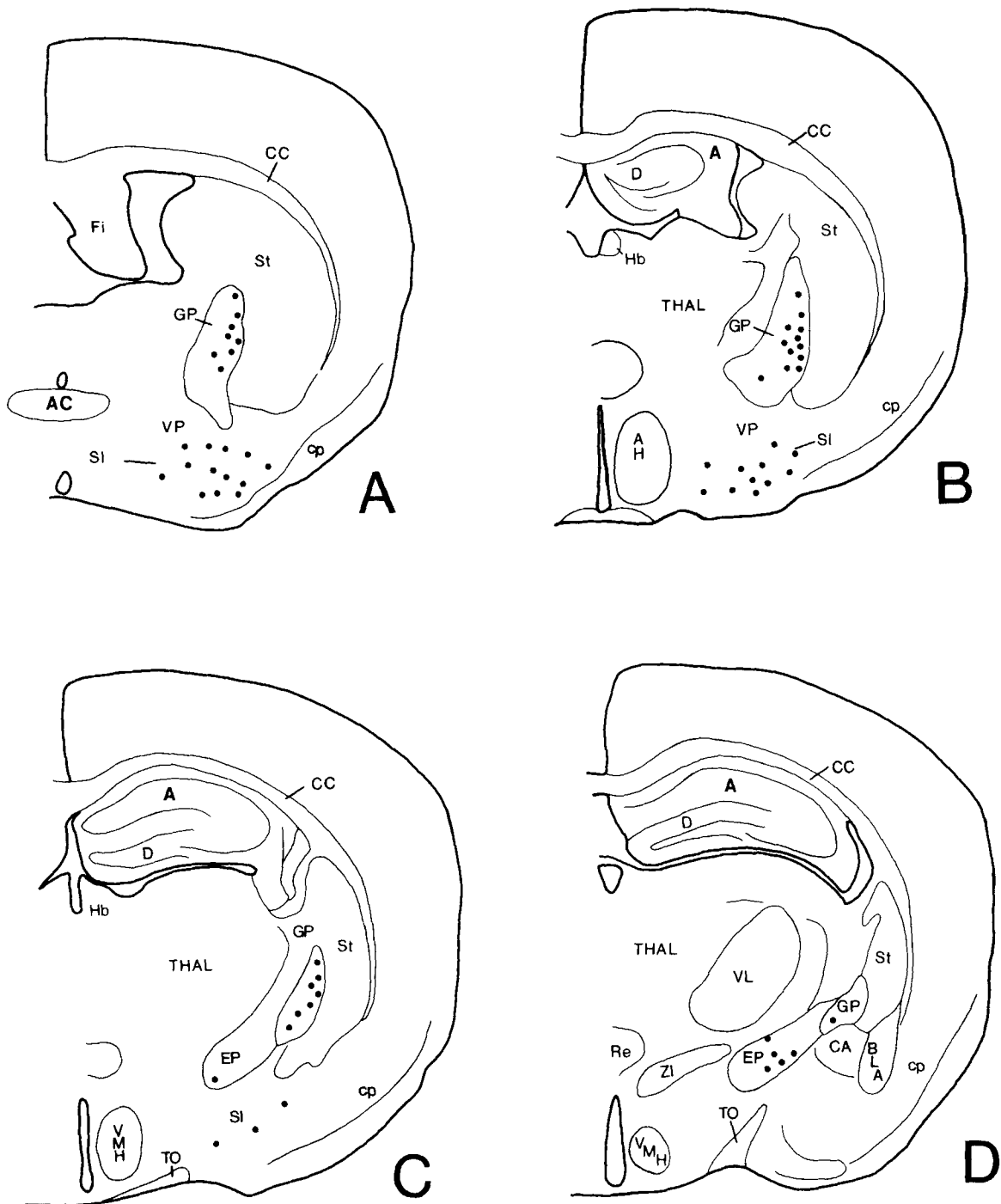


Fig. 14. Line drawings of a rostral to caudal (A-D) series of transverse section through the telencephalon of a Djungarian hamster, illustrating the location of LLI-containing neurons in pallidal portions of the telencephalon. Each dot represents 5 neurons. LLI-containing neurons were not observed in striatal portions of the telencephalon.

min) than synthetic LANT6 (21.5 min). Increasingly smaller peaks of iLANT6 were observed at 25 min, 20.5 min, and 18.5 min. Thus, although the gel chromatographic data indicate that large forms of iLANT6 predominate in the hamster nervous system, the pepsin treatment shows that these large peptides yield smaller peptides that elute very close to LANT6, but are not identical to LANT6. It is possible that some of these LANT6-like substances represent different-sized versions of the same LANT6-like substance. Two peaks of iNMN were observed, one at 27.5 min that coincides with synthetic NMN and one at 20 min. The peak at 20 min may represent an extended version of NMN. Neither of these peaks coincided with any of the iLANT6 peaks. It should be noted that, as in turtles, pepsin treatment generated 50–100 times more iLANT6 than iNMN. This result and the gel chromatographic data indicate that much of the iNMN in hamster brain is processed to a low molecular weight form, while nearly all of the iLANT6 is stored in high molecular weight form.

**Immunohistochemistry: Basal ganglia.** The labeling patterns observed in hamsters were similar to those observed in pigeons and turtles, except that fewer LANT6-positive neurons were observed and the labeling was, in general, lighter. Despite the lighter level of labeling, LLI-containing neurons were observed in globus pallidus and the entopeduncular nucleus, the ventral pallidum, and the polymorph layer of the olfactory tubercle (Figs. 14,15). LLI-containing neurons were also observed in all layers of the cortex, in the bed nucleus of the stria terminalis, and the hippocampus. Unlike in birds, and reptiles, LLI-containing neurons were not, however, observed in the striatum. Within globus pallidus, LLI-containing neurons made up about 20% of the total number of neurons (Fig. 14). The labeled globus pallidus neurons did not differ in size from unlabeled globus pallidus neurons, both being 15–25 microns in size. In the ventral pallidum, LLI-containing neurons made up only 5% of the total population (Fig. 14). The LANT6-positive labeling observed in hamsters could be blocked with 50 micromolar LANT6, but not with 50 micromolar NMN or 50 micromolar NT. This result suggests that a LANT6-like peptide other than or in addition to Neuromedin N may be present in the hamster nervous system. The latter interpretation is supported by the biochemical studies on hamster brain described above.

**Immunohistochemistry: Projection targets of the basal ganglia.** Within the diencephalic and tegmental targets of globus pallidus, the entopeduncular nucleus and the ventral pallidum (Carter and Fibiger, '78; Troiano and Siegel, '78; Haber et al., '85; Nauta, '79; Parent and De Bellefeuille, '83), LLI-containing fibers were sparse and lightly labeled, which may relate to the comparatively light level of labeling observed in the pallidal neurons of these basal telencephalic cell groups. Within the tegmentum, LLI-containing neurons were observed in the pars reticulata and the pars compacta of the substantia nigra and in the perinterpeduncular region and AVT (Fig. 12). Within the nigra, LLI-containing neurons were more abundant in the pars reticulata than in the pars compacta.

## DISCUSSION

The present results demonstrate that LLI-containing neurons are abundant in the basal telencephalon of pigeons, turtles, and hamsters. Within the basal telencephalon, LLI-containing neurons are particularly abundant in the palli-

dal subdivision of the basal ganglia. In pigeons and turtles, all or nearly all of the pallidal neurons in the reported size range of pallidal projection neurons (Kitt and Brauth, '81,'82; Reiner et al., '82a) contained LLI. In hamsters, LLI-containing neurons were abundant in both pallidal subdivisions (the globus pallidus and the entopeduncular nucleus) and in the ventral pallidum and polymorph layer of the olfactory tubercle (both of which are thought to contain pallidal neurons, Switzer et al., '82; Heimer et al., '85).

## Biochemistry

The present biochemical studies suggest that these pallidal neurons in pigeons contain a peptide identical to LANT6, as identified in chicken GI tract (Carraway et al., '83), while those in turtles contain primarily larger molecular weight forms of LANT6 or a LANT6-like peptide. As has been shown for other larger forms of NT-related peptides, pepsin treatment increased the ability of the larger forms to compete with LANT6 in the RIA by generating low molecular weight forms of iLANT6 (Carraway et al., '86). These results suggest that in turtles the precursor(s) containing LANT6 (or a LANT6-like peptide) is incompletely processed, thereby resulting in higher levels of large molecular weight forms of iLANT6 than seen in pigeons. The present results suggest that an NMN-like peptide(s) is also present in turtle brain. The localization of the NMN-like substance(s) in turtle brain is, however, presently uncertain.

Hamster basal ganglia stained relatively weakly for the presence of LANT6-related material and also gave very low levels of iLANT6 by RIA. This is likely to reflect the presence in hamsters of a phylogenetic variant of LANT6 rather than LANT6 itself. One variant, demonstrated first in porcine spinal cord (Minamino et al., '84) and also present in feline tissues (Carraway and Mitra, unpub. obs.), is NMN. This peptide, however, did not block the staining with the anti-LANT6 antiserum used here and also did not cross-react significantly in the RIA for LANT6. In addition, the chromatographic results indicated that the LANT6-related peptide(s) in hamster brain could be separated from the NMN-like material present in hamster brain. The LANT6-related material in hamster brain, however, appeared to be primarily associated with high molecular weight substances. Pepsin treatment of these large molecular weight forms of iLANT6 generated high levels of several smaller iLANT6 peptides and lower levels of NMN and a related peptide. Thus, it would seem that both LANT6-related and NMN-related substances exist in hamsters. Much of the iLANT6 in hamster brain appears to be stored in a large molecular weight peptide(s), as was also observed in turtles.

## Immunohistochemistry

Although LLI-containing striatal neurons were observed in pigeons and turtles, LLI-containing striatal neurons were not observed in hamsters. It is possible that such striatal neurons were not detected with the TG-22 antiserum because they contain a LANT6-like peptide in amounts that are below the threshold of detectability with the TG-22 antiserum. In this case, these neurons may be more readily revealed by an antiserum that is specific for the LANT6-like peptide present in hamsters. The identity of this peptide is, however, currently unknown. In contrast to hamsters, LANT6+ neurons are present in both the striatum and pallidum of rhesus monkey (Reiner, unpub. obs.).

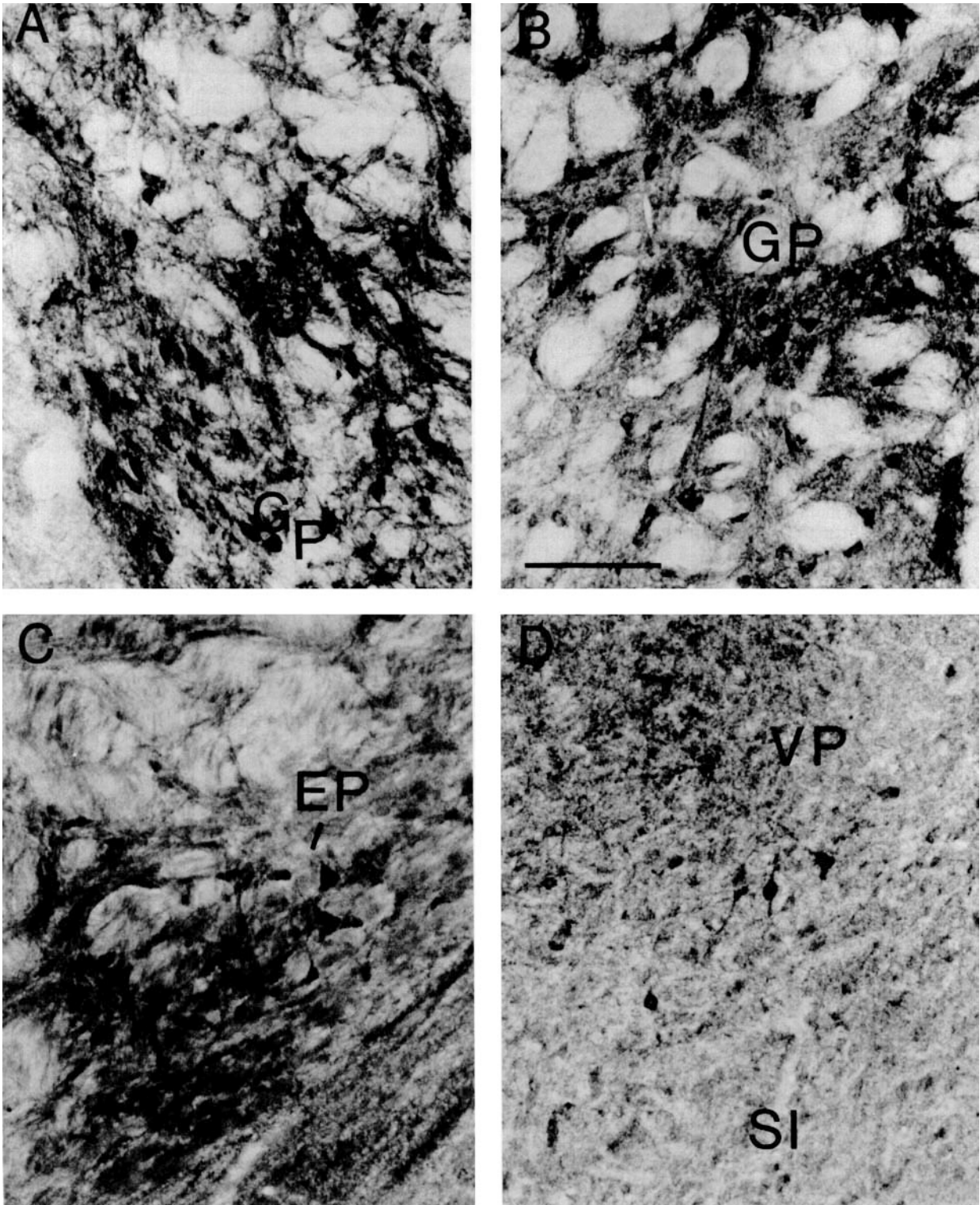


Fig. 15. Photomicrographs of transverse sections through the telencephalon of a hamster, illustrating LLI-containing neurons in globus pallidus (A and B), the entopeduncular nucleus (C), and the ventral paleostriatum (D). The sections were processed according to the PAP procedure. Scale bars: A = same magnification as B; B = 100 microns; C = same magnification as B; D = same magnification as B.

Within the basal telencephalon of mammals, birds, and reptiles, LLI-containing neurons were also observed in the ventral pallidum (termed ventral paleostriatum in sauropsids), the nucleus of the diagonal band, and the olfactory tubercle.

Consistent with the presence of LLI in pallidal neurons, LLI-containing fibers were prominent in the fields of tegmental dopaminergic neurons, which receive pallidal input. In mammals these fields are termed the ventral tegmental area, the substantia nigra, and the nucleus tegmentopedunculo-pontinus. These fibers were much more abundant and heavily labeled for LANT6 in birds and reptiles than in hamsters, which again may reflect the difference in structure between LANT6 (and the LANT6-like substance in reptiles) and its mammalian correspondent. Further, the LLI-containing tegmental fibers were more abundant and heavily labeled in pigeons than in turtles, which may be accounted for by the apparent differences between birds and turtles in terms of either the structure or processing of LANT6. These dopaminergic cell fields have been shown to receive input from globus pallidus (or its avian and reptilian homologues) and from the ventral pallidum (or the ventral paleostriatum, as this region has been termed in birds and reptiles) (Kitt and Brauth, '81; Reiner et al., '80; Reiner, '79; Parent and De Bellefeuille, '83; Carter and Fibiger, '78; Troiano and Siegel, '78; Nauta, '79; Haber et al., '85). In pigeons, knife cuts of the outflow tract of PP and VP dramatically reduced LLI-containing fibers in the ipsilateral AVT, TP, and Tpp. Similar results were obtained in turtles following aspiration of the basal ganglia or unilateral knife cuts of the basal ganglia outflow. Thus, it appears likely that LLI is present in the terminals of pallidal neurons in these dopaminergic tegmental cell fields. It appears unlikely that the LANT6+ neurons of the lateral striatum in pigeons and turtles make a significant contribution to these tegmental LLI-containing fibers since the lateral striatum of birds and reptiles has only meager projections outside of the telencephalon (Karten and Dubbeldam, '73; Reiner, '79; Brauth and Kitt, '80; Kitt and Brauth, '81). The sparse LLI-containing neurons of the medial striatum may, however, make a contribution to the tegmental LLI-containing fibers. It is also possible that the LLI-containing neurons of the bed nucleus of the stria terminalis contribute to tegmental LLI-containing fibers since this region has been reported to project to or through the tegmentum (Kitt and Brauth, '81). Based on the present results in birds and reptiles and based on the presence of LLI-containing pallidal neurons in hamsters, it seems likely that some tegmental fibers containing LLI in hamsters also originate from pallidal neurons, which includes neurons of globus pallidus and the entopeduncular nucleus, as well as neurons of the ventral pallidum and the polymorph layer of the olfactory tubercle (Switzer et al., '82; Heimer et al., '85). In preliminary studies, neurons of PP and the ventral paleostriatum of pigeons have been simultaneously immunofluorescently labeled for the presence of LANT6 and retrogradely labeled by a tegmental injection of fluorogold, thereby confirming that LANT6-positive pallidal neurons project to the tegmentum (Reiner, unpub. obs.). Thus, the results suggest that pallidal neurons may use LANT6 or a LANT6-like substance in influencing tegmental dopaminergic neurons in birds, reptiles, and mammals. Since, however, residual LLI-containing fibers were observed in the tegmental dopaminergic cell fields of birds and reptiles following disrup-

tion of the basal ganglia input, the possibility exists that some of the LLI-containing tegmental fibers do not arise from pallidal neurons.

Despite the abundance of LLI-containing neurons in globus pallidus (or its homologue in pigeons and turtles), LLI-containing fibers were surprisingly sparse in nontegmental projection targets of pallidal neurons. In hamsters, the ventral anterior and ventral lateral thalamic nuclei (VA-VL) were sparsely labeled for fibers. Light fiber labeling in VA-VL is not surprising, however, since LLI-containing neurons were more lightly labeled and less frequent in the entopeduncular nucleus, the source of pallidal input to VA-VL (Carter and Fibiger, '78), than in globus pallidus. As noted above, however, LANT6-positive fiber labeling in the tegmental projection targets of globus pallidus in hamsters was also light. The overall lightness of fiber labeling in the various pallidal projection targets in hamsters may be a reflection of the apparent difference in structure between the LANT6-like peptides found in these regions in hamsters and LANT6 in birds. In both pigeons and turtles, LLI-containing fibers were prominent in the tegmentum. Thus, the paucity of LLI-containing fibers in the avian DIP and SpL (which receive pallidal input from the avian paleostriatum primitivum, Karten and Dubbeldam, '73; Kitt and Brauth, '82; Reiner et al., '82a) and the chelonian nDCP (which is homologous to SpL, Reiner et al., '80) is surprising. Possibly, the pallidal neurons projecting to these structures do not contain LANT6 or contain lesser amounts of LANT6 than is the case for pallidotegmental projection neurons. An additional possibility is that individual pallidal neurons project to both the tegmentum and to the pretectum or diencephalon, but send little or no LANT6 along the collaterals to the pretectal or diencephalic targets.

### LANT6 and GABA

Pallidal neurons in mammals are generally thought to use GABA as their transmitter (McGeer et al., '78). The synthetic enzyme for GABA, i.e., glutamic acid decarboxylase (GAD), is present in pallidal neurons in birds and reptiles, thus suggesting that these neurons are GABAergic (Reiner, '86; Reiner, unpub. obs.). In light of the large numbers of pallidal neurons found to contain LANT6 in the present study, it seems likely a priori that LANT6 and GABA co-occur in pallidal neurons in birds, reptiles, and mammals. Further, the results of the present study and unpublished studies using an antiserum against GAD (Reiner, unpub. obs.) show that GABAergic neurons are present in many of the same cell groups that contain LLI-containing neurons in birds and reptiles. In birds, GAD+ neurons are present in lateral portions of TP, in ALp, in SpL, in the ventral paleostriatum, and in the striatum, all of which contain LANT6+ neurons of similar morphology and abundance to the GAD+ neurons. The corresponding cell groups in turtle (nPM, the entopeduncular nucleus, nDCP, VP, and the striatum) also contain GAD+ neurons that are of a similar morphology to the presently reported LLI-containing neurons in these structures. Using double-labeling procedures, it was recently confirmed that GAD and LANT6 co-occur in the neurons of the above noted cell groups of the avian forebrain and midbrain, including the pallidum (Reiner, '86). It thus seems likely that GABA and a LANT6-like peptide also co-occur in neurons of the homologous regions of the turtle brain. These findings are of particular interest with respect to the lateral spiriform

nucleus of birds and its reptilian homologue, since the neurons of these cell groups have previously been found to be enkephalinergic (Reiner et al., '82b; Brauth and Reiner, '82). Thus, the above results indicate that LANT6, GABA, and enkephalin all co-occur in individual neurons of SpL and nDCP. Since SpL and nDCP are known to project to the deep tectal layers (Reiner et al., '80, '82a,b), SpL and nDCP terminals in the tectum must use at least two different neuropeptides and a neurotransmitter in influencing tectal neurons. In hamsters, in addition to the LLI-containing telencephalic pallidal neurons, LLI-containing neurons were observed in the substantia nigra, particularly in the pars reticulata, and ventral tegmental regions. Since neurons in these telencephalic and tegmental regions have been reported to contain GABA in mammals (Heimer et al., '85; Oertel et al., '82), it is possible that GABA and LLI co-occur in some of these neurons.

### Functional considerations

The present findings suggest that LANT6 may be released from pallidal terminals and have an important influence on tegmental dopaminergic neurons. Although little information is available on the physiological actions of LANT6 (Carraway et al., '83; Kitabgi et al., '84), NT is known to excite midbrain dopaminergic neurons and promote increased dopamine (DA) turnover (Andrade and Aghajanian, '81; Nemeroff and Prange, '82; Kalivas, '85; Kalivas et al., '83). Consistent with such an effect on dopaminergic neurons, high levels of NT receptors have been reported in the mammalian AVT and substantia nigra, pars compacta (Quirion et al., '82). Similarly, high levels of NT receptors have also been found in the avian AVT and TP (the avian SN homologue) (Brauth et al., '85, '86). To further examine the possibility that LANT6 influences dopaminergic neurons, it will be important to determine whether distinct LANT6 receptors as well as distinct NT receptors are present on midbrain dopaminergic neurons. Kalivas et al. ('86) has recently suggested, based on physiological differences between the effects of NT and NMN, that distinct NMN receptors are present on midbrain dopaminergic neurons in rats and that Neuromedin N increases dopamine turnover in the rat ventral tegmental area and substantia nigra. It seems likely that LANT6 (or a LANT6-like peptide) has similar effects on tegmental dopaminergic neurons in birds, reptiles, and mammals.

### LANT6 and Neurotensin

Although LANT6 is structurally related to NT, neurotensin has not previously been observed in neurons of the GP or VP in mammals (Jennes et al., '82; Uhl et al., '79). Carraway and coworkers (Carraway and Bhatnagar, '80a, '80b; Carraway and Ferris, '83; Carraway et al., '82) have shown that avian NT is identical to mammalian NT, except for three amino acid substitutions in its N-terminal portion, and recent work indicates that turtle NT and chicken NT are identical (Carraway, unpub. obs.). In the present study, LLI-containing neurons and NT-immunoreactive neurons were observed in some of the same cell groups of the avian and reptilian forebrain. In birds these cell groups include the bed nucleus of the stria terminalis, the medial neostriatum, the medial hyperstriatum ventrale, the dorsomedial thalamic nuclei, and the preoptic region; in turtles they include the bed nucleus of the stria

terminalis, the dorsomedial and dorsolateral thalamic nuclei, the dorsal lateral geniculate nucleus, and the preoptic region. The LLI-containing neurons and the NT-containing neurons in these cell groups are of a similar morphology and abundance. Thus, LLI and NT may co-occur in at least some cell bodies of the nervous system of birds and reptiles, although this possibility has yet to be established experimentally. In rats, NT-containing neurons have been observed in the bed nucleus of the stria terminalis, in the ventral tegmental area, and, more sparsely, in the substantia nigra, pars compacta (Jennes et al., '82; Hokfelt et al., '84). Some of the ventral tegmental and nigral NT+ neurons have been reported to contain dopamine also (Hokfelt et al., '84). LLI-containing neurons were observed in these same telencephalic and tegmental regions in hamsters in the present study. As in pigeons and turtles, this raises the possibility that NT and a LANT6-like peptide co-occur in some regions of the mammalian brain. Nonetheless, in pigeons, turtles, and hamsters, NT-containing neurons have not been observed in most of the brain regions in which LLI-containing neurons are present.

In pigeons and turtles, NT-containing fibers showed a similar distribution to the LLI-containing fibers within the tegmental targets of pallidal neurons. Since the anti-NT antiserum used in the present study is specific for NT and crossreacts only negligibly with LANT6, it appears likely that the labeling of these fibers by the anti-NT antiserum does not represent crossreactive labeling of LLI-containing fibers. Further, knife cuts that reduced the number of LLI-containing fibers in the tegmentum seemingly equally reduced the number of NT-containing fibers in the same regions. There are several possible explanations for this result. First, LLI and NT may be present in the same tegmental fibers, which arise from pallidal neurons. If this is the case, it is necessary to account for the failure to observe NT in pallidal cell bodies. A possible explanation for such a failure is that both LANT6 and NT may be synthesized in pallidal cell bodies, but they may be processed differently in perikarya than in terminals. For example, LANT6 and NT may not be cleaved from their precursor molecules (or possibly they are present in the same precursor) within pallidal cell bodies and because of their positions within the precursors only LANT6 may present its antigenic sites for immunohistochemical detection within the cell body. Neurotensin may not be detectable until it is cleaved from the precursor after the precursor is shipped out of these cell bodies. A second explanation of the effects of knife cuts on tegmental NT-containing fibers is that these cuts severed two separate descending tracts, a LLI-containing pallidotegmental tract and an NT-containing fiber bundle from an unknown source within the telencephalon or rostral diencephalon. The likelihood of this possibility is currently unclear. The bed nucleus of the stria terminalis is the only forebrain region containing NT+ neuronal cell bodies that is known to project to the tegmental dopaminergic neurons (Kitt and Brauth, '81), but this cell group also contains LANT6+ neurons. Thus, if the NT-containing neurons of the bed nucleus of the stria terminalis do project to the tegmentum, which has not been demonstrated experimentally, it is possible that these terminals contain both NT and LANT6. A third possibility is that the LLI-containing tegmental fibers are a separate population from the NT-containing fibers and that the knife cuts did



not disconnect the NT-containing fibers from their source. Under these circumstances, the loss of NT-containing fibers would presumably be secondary to the loss of the descending input from the forebrain. Since Kalivas ('85) has noted that some of the NT-containing fibers in the AVT of rats arise from a region ventral to locus coeruleus, it is possible that the NT-containing fibers in the avian and reptilian tegmentum do not arise from the forebrain. This line of reasoning raises the possibility that the loss of LLI-containing fibers consequent to the knife cut may also not be a direct effect of the knife cut. It is unclear, however, why tegmental fibers should be secondarily reduced so greatly by loss of the descending basal ganglia input. In summary, the basis for the loss of the NT-containing fibers from the fields of tegmental dopaminergic neurons following the knife cuts is uncertain. A number of lines of evidence (summarized above) make it likely that the observed loss of the LLI-containing fibers from these regions is largely consequent to the transection of a descending LLI-containing pallidotegmental projection.

### Evolutionary considerations

Previously we noted that LANT6 or a LANT6-like peptide has been conserved in pallidal neurons since the divergence of bony and cartilaginous fish, thus suggesting that this peptide plays an important and evolutionarily conserved role in basal ganglia functions. The present results further show that LANT6 or a similar peptide may play a role in several additional cell groups involved in basal ganglia circuitry. For example, the posterior nucleus of the ansa lenticularis and the entopeduncular nucleus of turtles are LANT6+ (and GABA+), thus further supporting the previously suggested homology between these two structures (Baker-Cohen, '68; Brauth and Kitt, '80). The purported mammalian homologue of this cell group, the subthalamic nucleus (Baker-Cohen, '68; Brauth and Kitt, '80; Karten and Dubbeldam, '73), was not, however, observed to contain unequivocally LANT6-positive neurons. An antiserum against the LANT6-like peptide present in hamsters may be required to visualize any LANT6-like peptide that may be present in these neurons. On the other hand, the subthalamic nucleus reportedly may use glutamate (but not GABA) as a transmitter (Heimer et al., '85). This raises the possibility that either the subthalamic nucleus is not homologous to ALp of birds and the entopeduncular nucleus of reptiles, or it is homologous, but its neurons use a different transmitter(s). The dorsolateral edge of TP, or TPd (Karten and Dubbeldam, '73) in birds and the nPM of turtles also contains LANT6+ neurons, and these two structures are presumably homologous. The presence of many LANT6-positive neurons in the substantia nigra, pars reticulata of hamsters suggests that this region may be comparable to the turtle nPM and the avian TPd, as previously suggested on other histochemical grounds (Baker-Cohen, '68).

Thus, LANT6 may be a useful marker for identifying components of the basal ganglia system in vertebrate species that have not been well studied previously. Recent studies have shown that LANT6 or a LANT6-like peptide is also phylogenetically conserved within retinal ganglion cells and their central projections (Eldred et al., '84; Li et al., '84). The apparent conservatism in the presence of LANT6-positive labeling in a number of components of the basal ganglia system suggests that LANT6 or a similar

peptide may play a role of fundamental, and hence evolutionarily conserved, importance in basal ganglia functions.

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