ules soon after fertilization[3,4,7,8]. Judging from the molecular weight, the 35 kD trypsin-like protease found in the present study could correspond with the enzyme purified by Alliegro and Schuel[7].

The activity of the 40 kD chymotrypsin-like protease was almost completely lost when the eggs were fertilized. The fate of this protease after insemination is unclear. Its disappearance might result from: (1) release from the egg to the surrounding seawater (2) transfer to a different compartment within the egg (3) autodigestion or digestion by other proteases. Studies on the role which this protease may play in sea urchin fertilization are now in progress.

Acknowledgments. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

* T.Y.'s present address.

1 Sawada, M. T., Someno, T., Hoshi, M., and Sawada, H., Devl Biol. *133* (1989) 609.
2 Hashimoto, N., Kishimoto, T., and Nagahama, Y., J. exp. Zool. *247* (1988) 177.
3 Vacquier, V. D., Epel, D., and Douglas, L. A., Nature (London) *237* (1972) 34.
4 Vacquier, V. D., Tegner, M. J., and Epel, D., Nature (London) *240* (1972) 352.
5 Vacquier, V. D., Tegner, M. J., and Epel, D., Exp. Cell Res. *80* (1973) 111.
6 Sawada, H., Miura, M., Yokosawa, H., and Ishii, S., Biochem. biophys. Res. Comm. *121* (1984) 598.
7 Alliegro, M. C., and Schuel, H., Biochemistry *24* (1985) 3926.
8 Alliegro, M. C., and Schuel, H., Devl Biol. *125* (1988) 168.
9 Heussen, C., and Dowdle, E. B., Analyt. Biochem. *93* (1980) 257.
10 Laemmli, U. K., Nature (London) *227* (1970) 680.
11 Aoyagi, T., Takeuchi, T., Matsuzaki, A., Kawamura, K., Kondo, S., Hamada, M., and Umezawa, H., J. Antibiot. *22* (1969) 558.
12 Umezawa, H., Aoyagi, T., Morishima, H., Kunimoto, S., Matsuzaki, M., Hamada, M., and Takeuchi, T., J. Antibiot. *23* (1970) 425.

0014-4754/92/030287-04$1.50 + 0.20/0

Pre- and post-natal ontogeny of neutral endopeptidase 24-11 ('enkephalinase') studied by in vitro autoradiography in the rat

I. Dutriez, N. Salès, M.-C. Fournié-Zaluski and B. P. Roques

INSERM U 334, DRIPP, Service hospitalier Frédéric Joliot, place du Général Leclerc, F-91406 Orsay, and INSERM U 266 CNRS UA 498, Département de Chimie Organique, 4 avenue de l'Observatoire, F-75270 Paris Cedex 06 (France)
Received 2 April 1991; accepted 30 September 1991

Abstract. Neutral endopeptidase (NEP, enkephalinase, CALLA) which is present in various neural and non-neural tissues, is able to cleave a variety of regulatory peptides. The distribution of NEP has been studied during rat pre- and post-natal development by autoradiography after in vitro binding of the tritiated inhibitor [^{3}H]HACBO-Gly to whole-body and organ sections. In the central nervous system (CNS), where the presence of NEP has been related to the termination of the action of enkephalins, the external layer of the olfactory bulbs is the only structure prominently labeled before birth. Other CNS structures rich in NEP in the adult, such as the nigrostriatal tract, are progressively labeled after birth. Outside the CNS, the progressive appearance of NEP in the kidney, the lungs and the salivary glands suggests its concomitant involvement in adult physiological functions, including fluid balance control, possibly by cleaving the atrial natriuretic peptide (ANP) and other peptides. On the other hand, transient or enhanced expression of NEP is observed during the development of several organs such as the sensory organs, the heart and the major blood vessels, the intestine, the bones and the genital tubercle. In addition to the still incompletely known physiological functions of the enzyme, the developmental pattern of its expression in several tissues strongly suggests a modulatory role for NEP in the ontogeny of a large number of organs.
Key words. Neutral endopeptidase (NEP); enkephalinase; CALLA; NEP inhibitor; [^{3}H]HACBO-Gly; autoradiography; atrial natriuretic peptide.

Neutral endopeptidase 24.11 (NEP, enkephalinase) is a membrane-bound zinc metallo-endopeptidase of 749 amino acids. This ectoenzyme was first isolated and purified from kidney brush border, and was then found in the brain, where it inactivates enkephalins. NEP has recently been shown to be identical to the common acute lymphoid leukemia antigen CALLA[1].

NEP cleaves the bond at the amino-side of a hydrophobic amino acid and its preferred substrates are small peptides such as the enkephalins, substance P and neurotensin[2]. Several other peptides of biological interest have also recently been shown to be cleaved by NEP: these include atrial natriuretic peptide[3], somatostatin[4], endothelins[5], and even small proteins such as interleukin IL-α1[6].

The diversity of potential substrates for NEP, and its wide distribution in neural and non-neural tissues[7-9], suggest that it has several physiological functions. These can be investigated using inhibitors[1,10] such as thiorphan[11] and its prodrug acetorphan, which have been

tested in clinical trials as pain-releaving and antidiarrheal agents [12]. Inhibitors of the enkephalin-degrading enzyme have also been suggested as possible new antidepressants, since the opioidergic system plays a critical role in the control of mood [12]. Moreover, because peripheral NEP is involved in the degradation of ANP [13, 14], NEP inhibitors have recently been introduced in preliminary clinical studies as anti-hypertensives [12].

When radiolabeled, NEP inhibitors can also be used in vivo and in vitro as powerful and selective tools for detecting the localization of the enzyme, as was initially shown with the tritiated inhibitor [^{3}H]HACBO-Gly, which was used to visualize NEP in the brain and spinal cord of rats [7, 15, 16] and humans [17, 18], and in various other rat tissues, by in vitro and ex vivo [9] autoradiography. [^{3}H]HACBO-Gly has also been used to study the ontogeny of the enzyme in the spinal cord of the human fetus [8].

This paper describes a detailed in vitro study of NEP distribution during the pre-natal and post-natal development of the rat. The first goal of this work was to compare the localization of the enzyme with the appearance of morphologically differentiated structures and with the onset of physiological functions. Such comparisons are expected to shed some light on the physiological role of NEP and on the pharmacology and possible side effects resulting from the therapeutic use of its inhibitors in man.

Moreover, peptides such as Met-Enkephalin, Leu-Enkephalin, bombesin and the related peptides, gastrin and CCK, which can be degraded by NEP, have been demonstrated to play a role in normal and tumoral proliferation and/or in cellular differentiation [19]. NEP could therefore be implicated in the regulation of these mechanisms, and its transient expression during normal development could be indicative of such a role.

Materials and methods

Chemicals. [^{3}H]-N-(2RS)-3-hydroxylaminocarbonyl-2-benzyl-1-oxopropyl-glycine, [^{3}H]HACBO-Gly, 40 Ci/mmol) was synthesized in our laboratory and tritiated by reduction of the benzylidene precursor [20] at the Commissariat à l'Energie Atomique (CEA), Saclay, France. Thiorphan was synthesized in our laboratory as described [11].

Animals. Female Sprague-Dawley rats were from IFFA-CREDO, Les Oncins, France. Ten females, sperm-positive on a specific day, were used for prenatal studies. Gestational day E-1 designates the day following a successful insemination. Pre-natal stages E-12, E-14, E-16, E-18 and E-21 were studied.

Three litters of animals born in our laboratory were used for post-natal studies (post-natal days P-2, P-7, P-14, P-21 and P-28).

Preparation of tissues. For each gestational stage studied, two pregnant females were anesthetized with pentobarbital (40 mg/kg i.p.), the fetuses were removed, the crown-rump length (CRL) was measured, and the mothers were thereafter killed by decapitation. Four to six young rats were randomly chosen on each post-natal day considered and were killed with ether. Whole fetuses and young rats up to P-2 were then frozen by immersion (5 min) in isopentane cooled with liquid nitrogen. Animals aged P-14, P-21 or P-28 were previously quickly dissected to separate head and neck, trunk and limb regions, and the parts frozen as above.

Sagittal 20 µ-thick sections were cut on a cryostat at −15 to −20 °C, thaw-mounted on polylysine-coated slides, and stored at −20 °C until needed.

Autoradiographic procedures. Every fifth section was incubated with 3 nM [^{3}H]HACBO-Gly in 50 mM TRIS buffer (pH 7.4). The incubation time was 60 min at room temperature and was followed by six 10-s rinses in TRIS buffer alone, at +4 °C. The sections were, thereafter immediately dried with cold air. Non-specific binding was evaluated on adjacent sections, incubated as above but in the presence of 1 µM thiorphan or 1 µM unlabeled HACBO-Gly.

The sections and slide-mounted calibrated tritium standards (Amersham) were closely apposed to tritium-sensitive Hyperfilms (Amersham) inside X-ray cassettes. The exposure time was 7 weeks, after which the films were developed in Kodak D-19b developer for 5 min at 20 °C, fixed in Ilford Hypam fixative for another 5 min, rinsed for 20 min under running tap water and finally dried.

The identification of the labeled structures was performed by comparison of negative enlargements of the autoradiograms with positive prints of the corresponding histological sections stained with periodic acid-Schiff and hematoxylin.

The optical densities (O.D.) of parts of the autoradiographic prints were measured for a few selected structures, i.e. the placenta, the kidneys and the lungs, which were sufficiently developed to allow the comparison of their relative densities during the period studied. Tritium scales (Amersham) from the same batch were used to ascertain that there were no important differences in the darkening of the tritium films used in this experiment. No attempt was made to convert the O.D. into moles per weight as variations in the quenching of various organs at different stages of development are largely unknown and may be important.

Results

The tritiated inhibitor [^{3}H]HACBO-Gly has previously been shown to be a convenient ligand for autoradiographic studies of NEP localization in nervous and fetal tissues of rat [5] and humans [7–9, 17].

In a pilot experiment, we verified that the labeling of the central nervous system and of peripheral organs during rat gestational and early pre-natal development could be prevented by adding 1 µM unlabeled thiorphan or HACBO-Gly to the incubating bath. Autoradiograms obtained in such conditions exhibited a very faint and

Figures 1–9. Autoradiograms showing NEP localization in cryostat sections of rats at various stages of prenatal development (E-12 to E-21) and of post-natal development (P-2 to P-28).

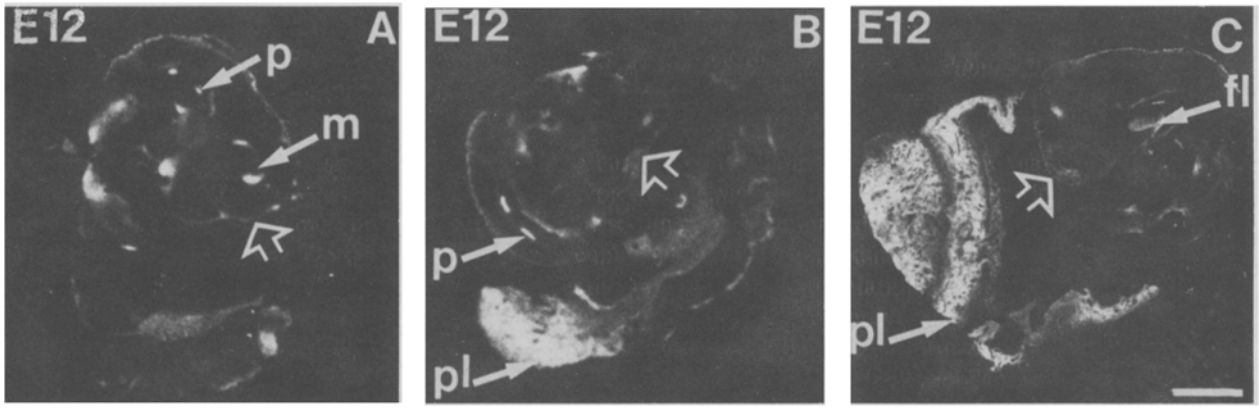

Figure 1. Sagittal sections of rat embryos with placenta and annexes at E-12. Open arrow designates the head of the embryo. fl, forelimb anlage; m, mesencephalic flexure; p, prevertebral column; pl, placenta. Bar 2 mm.

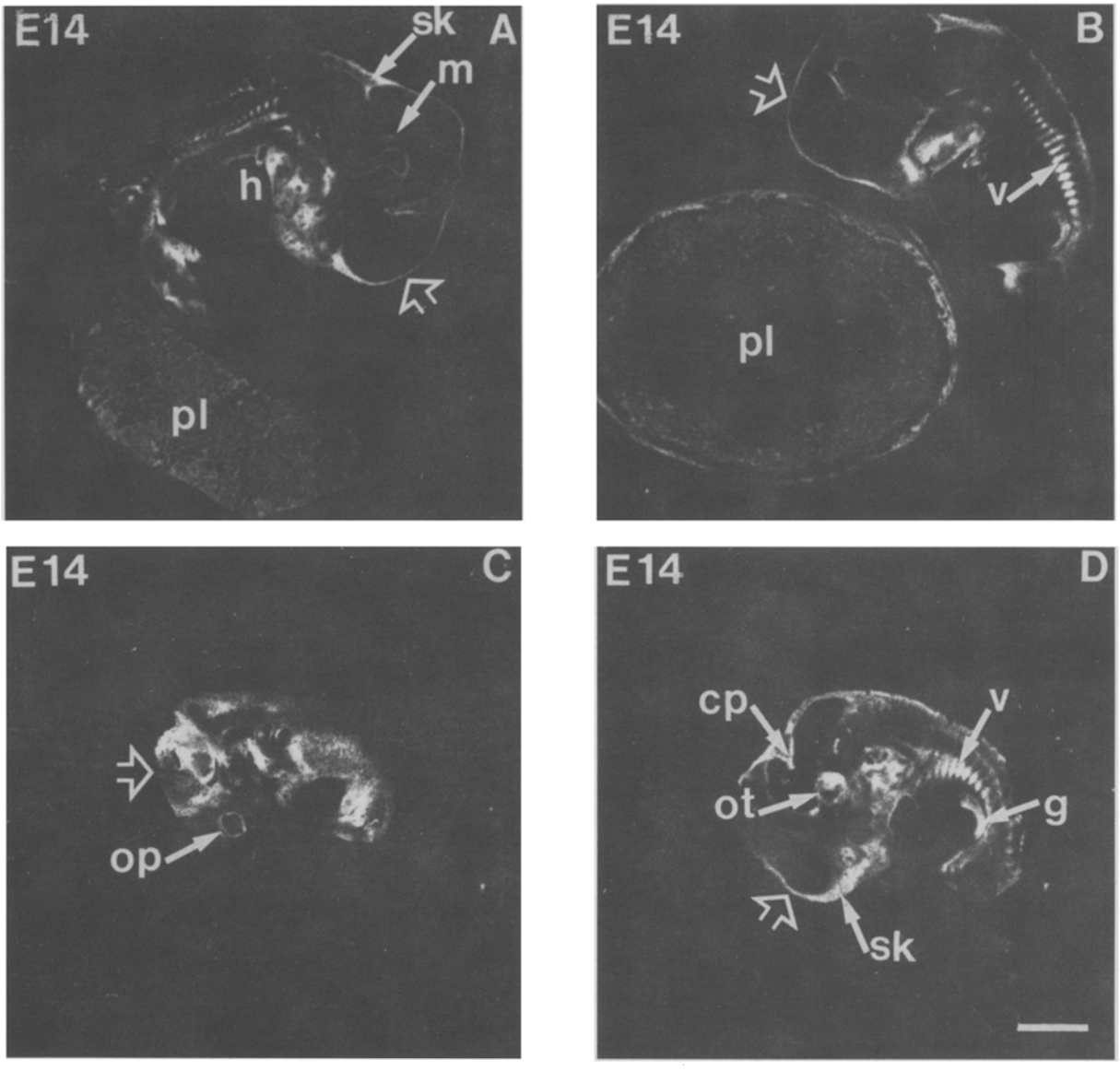

Figure 2. Sagittal medial (*A, B*) or sagittal lateral (*C, D*) sections from rat embryos at E-14. Open arrow designates the head of the embryo. cp, choroid plexuses; g, gut; h, heart; m, mesencephalic flexure; op, optic vesicle; ot, otic vesicle; pl, placenta; sk, skull; v, vertebrae. Bar 2 mm.

homogeneous labeling, and non-specific binding measured by liquid scintillation counting of labeled sections or by densitometry of autoradiograms accounted for less than 15% of the total binding (data not shown), which was in good agreement with previous studies [7, 9].

Prenatal development. E-12 (CRL 6.5 mm) fig. 1: The dorsal part of the embryo is outlined by a medium-density darkening of the autoradiogram in the tissues surrounding the cephalic and spinal neural tube (fig. 1 A). This labeling is more pronounced in the regions caudal and dorsal to the rhombencephalic vesicle (fig. 1 B). Within the central nervous system itself there is prominent labeling in the floor of the mesencephalic flexure (figs 1 A, 1 C). Within peripheral organs, the caudal end

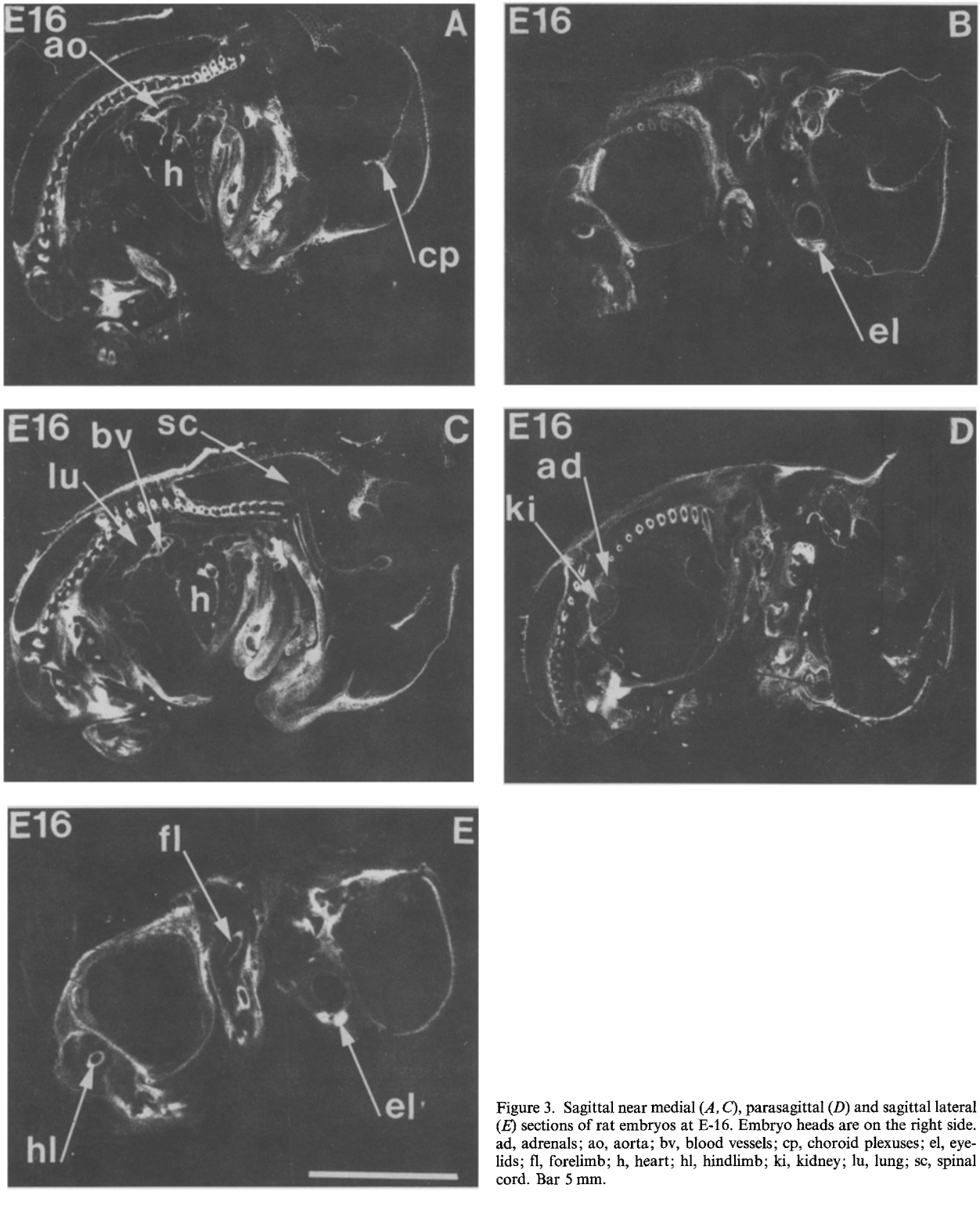

Figure 3. Sagittal near medial (*A, C*), parasagittal (*D*) and sagittal lateral (*E*) sections of rat embryos at E-16. Embryo heads are on the right side. ad, adrenals; ao, aorta; bv, blood vessels; cp, choroid plexuses; el, eyelids; fl, forelimb; h, heart; hl, hindlimb; ki, kidney; lu, lung; sc, spinal cord. Bar 5 mm.

of the hindgut exhibits some degree of labeling. Prominent labeling is also noticeable in the prevertebral column (figs 1A, 1B) and in the forelimb anlage (fig. 1C).

At this early stage of development, the myometrium and the maternal part of the placenta are clearly labeled.

E-14 (CRL 11 mm) fig. 2: Within the cerebral vesicles and the spinal cord, no labeling can be observed in the cerebral tissue except around the mesencephalic flexure, where it is now limited to a caudally located structure (fig. 2A). On the other hand, all the structures surrounding the central nervous system are clearly labeled: these include the newly formed choroid plexuses, the mesenchymatous condensations from which the bones of the skull roof originate, and the cartilaginous anlagen of the base of the skull. Slightly more lateral sections through the cephalic region show prominent labeling of the vibrissae and of the optic (fig. 2C) and otic (fig. 2D) vesicles. In the coelomic cavity, the heart is evidenced by a thin rim of labeling (fig. 2A) while the caudal portion of the

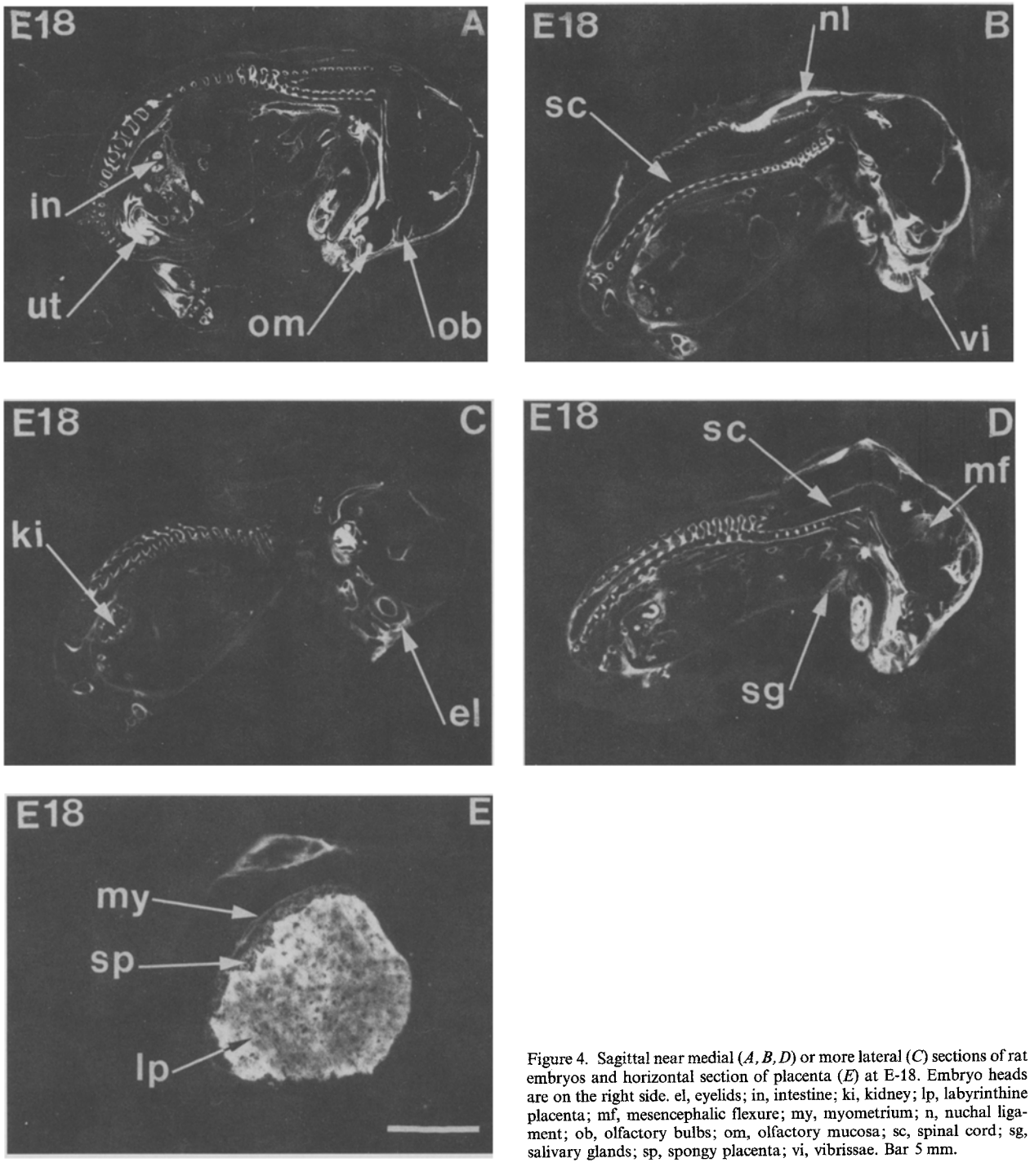

Figure 4. Sagittal near medial (*A, B, D*) or more lateral (*C*) sections of rat embryos and horizontal section of placenta (*E*) at E-18. Embryo heads are on the right side. el, eyelids; in, intestine; ki, kidney; lp, labyrinthine placenta; mf, mesencephalic flexure; my, myometrium; n, nuchal ligament; ob, olfactory bulbs; om, olfactory mucosa; sc, spinal cord; sg, salivary glands; sp, spongy placenta; vi, vibrissae. Bar 5 mm.

gut (figs 2B, 2D) is conspicuously labeled. Not only the ventral corpus of the cartilaginous vertebrae but also their lateral and dorsal arches are labeled at this stage. Placental structures exhibit a lower and more diffuse pattern of labeling than at E-12 (fig. 2B).

E-16 (CRL 16.5 mm) fig. 3: Few changes take place in the central nervous system: some weakening of the labeling around the mesencephalic flexure, and the appearance of a slightly labeled area in the region where the cerebellum develops, are the only noticeable events. The spinal cord remains unlabeled except for its meningeal envelopes (figs 3A, 3C).

The developing eyelids are prominently labeled (fig. 3B). In the thoracic and abdominal cavities several structures exhibit various degrees of labeling intensity:

- the heart wall, in a region restricted to the auriculoventricular groove, and major blood vessels such as the aorta (fig. 3A) and the pulmonary arteries penetrating the faintly labeled lung rudiment (fig. 3C, table).
- the first labeling of the metanephros and of the adrenal gland is observed at this stage (fig. 3D).

In the skeleton, as successive segments of appendal bones differentiate, it becomes apparent that the tritiated inhibitor binds to a restricted peripheral zone of the cartilaginous matrix (fig. 3E).

E-18 (CRL 25 mm) fig. 4: In the brains of E-18 embryos, the outer layer of the olfactory bulbs is conspicuously labeled (fig. 4A). Several sensory organs, such as parts of the olfactory mucosa (fig. 4A), the vibrissae (fig. 4B), the optic cup and the eyelids (fig. 4C) also exhibit an intense labeling.

In the region of the neck and of the chest, a moderate labeling appears in the salivary glands (fig. 4D), whereas other structures, clearly differentiated at E-18, such as the thyroid and the thymus, are not labeled. The labeling of the lungs is lower than at E-16 and almost undistinguishable from the background (table). This may be due to a growth rate of the organ higher than the increase in its enzyme content.

The upper part of the abdominal cavity contains very few NEP-positive structures: in particular, no darkening of the autoradiogram can be distinguished at the level of the duodenum and of the pancreas. On the other hand, in the lower abdominal region, portions of the terminal intestinal loops and the urogenital apparatus (fig. 4A) are intensely labeled.

As regards skeleton-related structures, the most striking event is the appearance of a prominent labeling of the nuchal ligament (fig. 4B). This ligament separates two brown fat pads, which do not exhibit binding sites for the tritiated inhibitor at this stage.

Finally, the labyrinthine portion of the placenta is intensely labeled whereas the myometrium and the basal spongy zone exhibit a medium-density labeling (fig. 4E, table).

E-21 (CRL 40 mm) fig. 5A: In the head, few changes take place except for some weakening of the overall labeling of the eye (fig. 5A). In the trunk, the lungs (fig. 5A) as well as the kidneys (fig. 5B) and the terminal portions of the intestine (fig. 5C) are clearly labeled. The epiphyses of the appendal bones are surrounded by a thin rim of binding sites, but now the whole diaphysis is quite homogeneously labeled (fig. 5B). The well-differentiated cervical brown fat pads are lightly labeled (figs 5A, B, C). The labyrinthine portion of the placenta is still the most heavily labeled structure in this organ (fig. 5A, table).

Post-natal development. P-2, fig. 6: In the central nervous system, the dorsal part of the cervical spinal cord exhibits a faint labeling. Regarding peripheral non-nervous structures, the most striking change is the marked increase in the density of binding sites in the cervical brown fat pads; this density approximately equals that in the nuchal ligament.

On the other hand an apparent lowering of the intensity in the labeling of the heart and of the major blood vessels has taken place, so that these structures can hardly be identified on the autoradiograms.

In the salivary glands, two regions with different densities of binding sites can be observed, whereas the well-developed thymus remains unlabeled.

P-7, fig. 7: Only subtle changes can be observed at the end of the first post-natal week with respect to the perinatal status. In the sensory organs, there has been some decrease in the labeling of the eyes, which contrasts with the conspicuous labeling of the vibrissae.

P-14, fig. 8: Within the brain a faint and diffuse labeling appears as a continuous zone extending from the mesencephalic nigral to the diencephalic striatal areas (fig. 8A). The apparent decrease in the labeling of the kidney (table) can tentatively be related to the development of a zone of lower density in the outer cortex. This zone is clearly evidenced at P-14. Another striking modification concerns the apppendal bones, where well-delimited zones of high-density binding are located in the epiphyseal plates and the secondary ossification centers (fig. 8B).

Semi-quantitative measurements of NEP 24.11 during ontogeny in three selected rat tissues.

	Placenta	Kidney	Lungs
E-12	119 ± 38	N.D.	N.D.
E-16	C.Z. 69 ± 15 P.Z. 52 ± 12	39 ± 9	29 ± 15
E-18	C.Z. 115 ± 37 P.Z. 55 ± 26	61 ± 13	8 ± 4
E-21	C.Z. 175 ± 41 P.Z.N.D.	278 ± 16	99 ± 30
P-2	–	224 ± 56	59 ± 31
P-7	–	122 ± 45	28 ± 12
P-14	–	O.C. 92 ± 39 I.C. 226 ± 20	139 ± 36

(O.D. 10^3) of autoradiograms from rats at various stages of development. Values are the means of 15 measures in 3 different rats. Individual variations in the darkening of the films do not exceed 8%,
C.Z.: central zone, P.Z.: peripheral zone of the placenta, O.C.: outer cortex, I.C.: inner cortex of the kidney, N.D.: not determined.

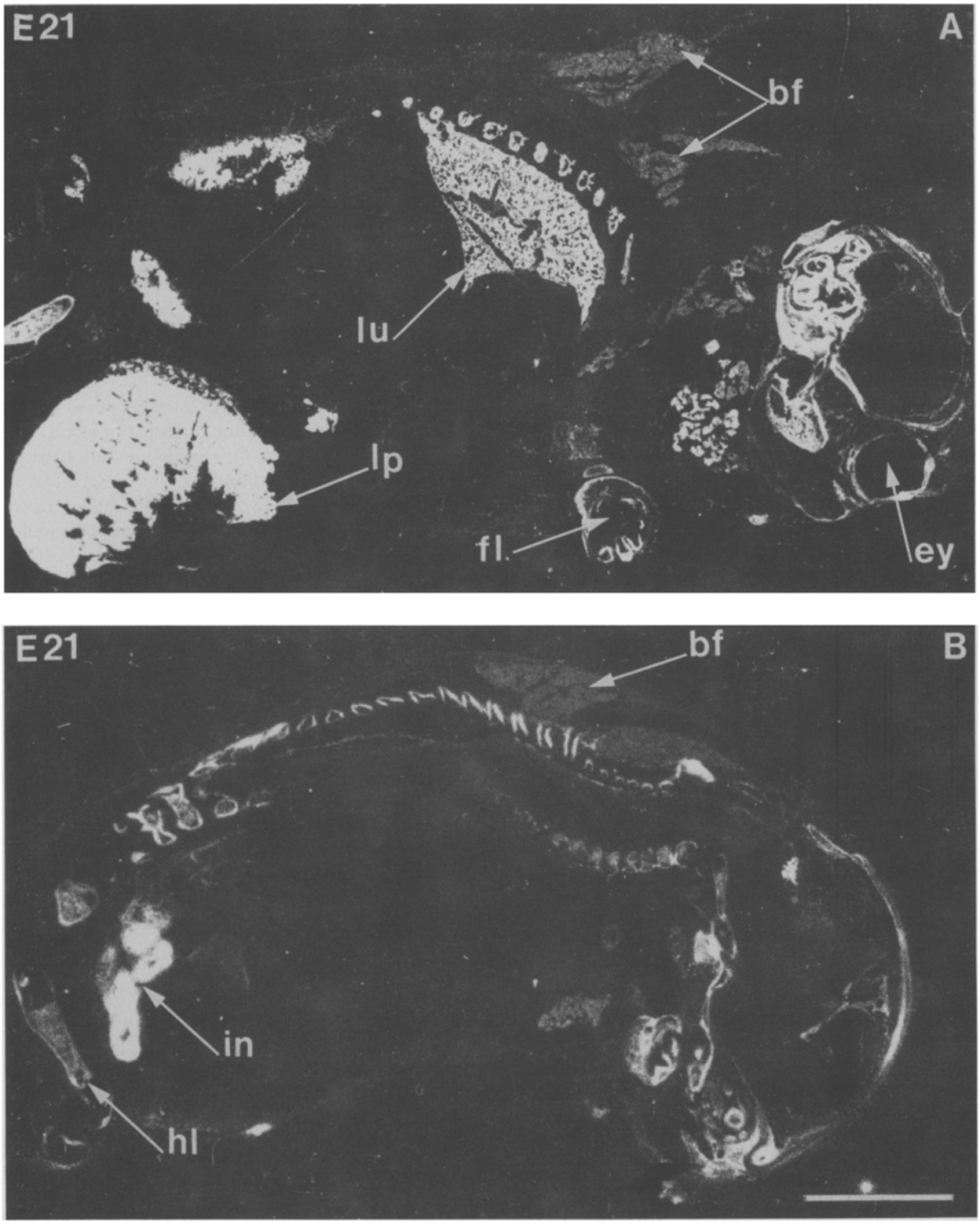

Figure 5. Sagittal near medial (*B*) or more lateral (*A, C*) sections of rat embryos and horizontal section (*A*) of placenta at E-18. bf, brown fat pads; ey, eye; fl, forelimb; hl, hindlimb; in, intestine; ki, kidney; lp, labyrinthine placenta; lu, lung; nl, nuchal ligament. Bar 5 mm.

P-21, fig. 9: At the end of the third post-natal week, the brain labeling is more clearly delimited to the nigro-striatal tract and also extends to the globus pallidus.

P-28, not shown: The structures examined, i.e. the head and the cervical and abdominal regions, do not demonstrate further changes with respect to earlier post-natal stages.

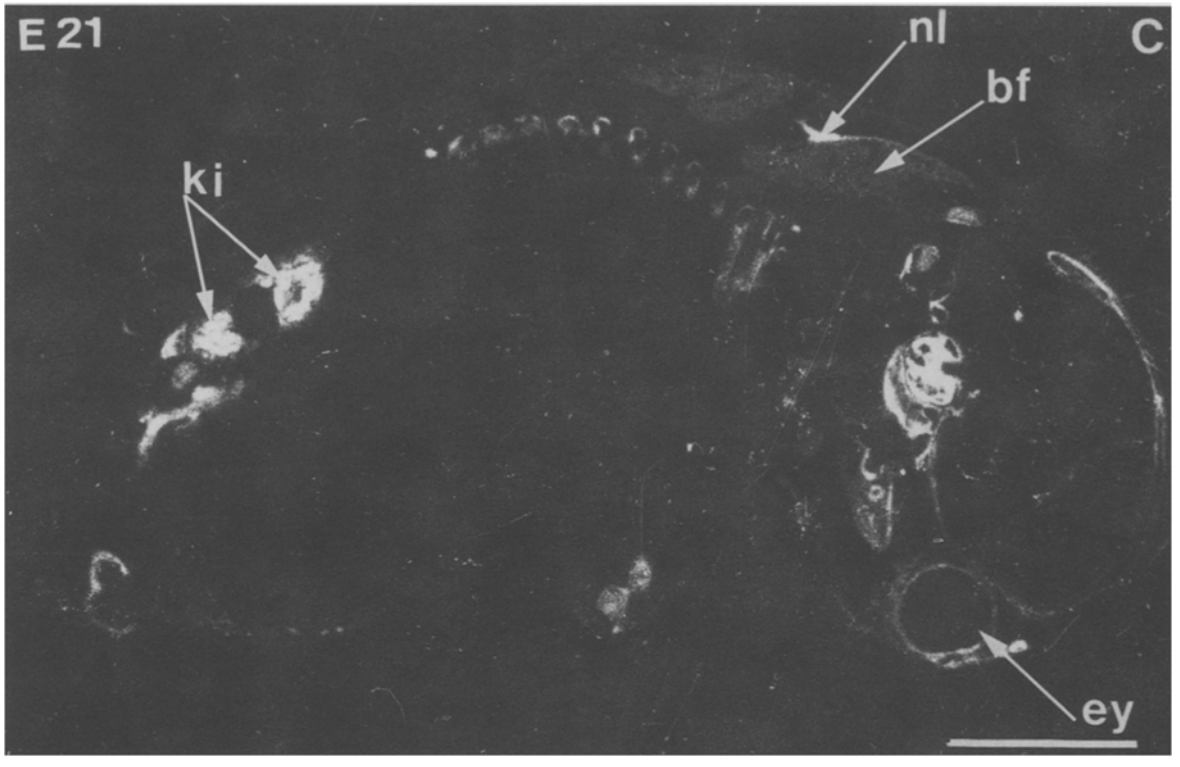

Figure 5.

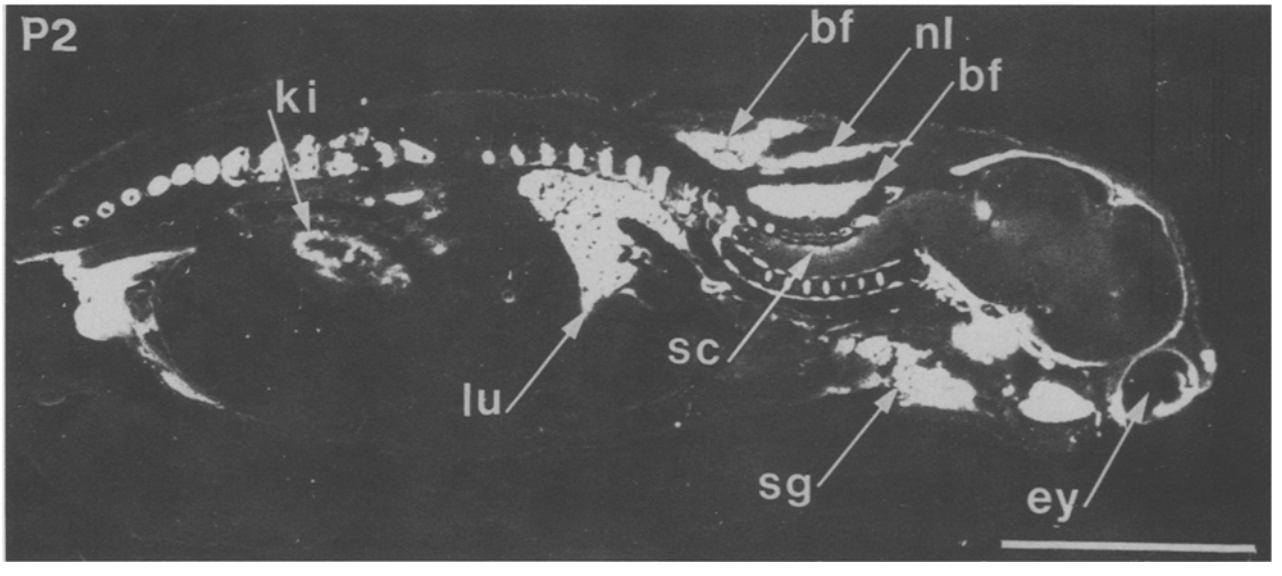

Figure 6. Parasagittal section of a rat pup at P-2. bf, brown fat pads; ey, eye; nl, nuchal ligament; ki, kidney; lu, lung; sc, spinal cord; sg, salivary gland. Bar 1 cm.

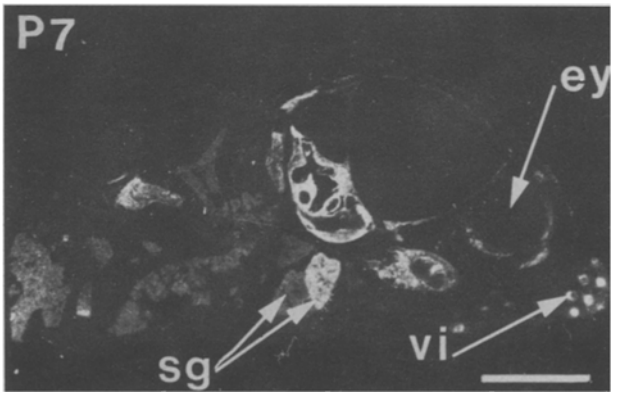

Figure 7. Parasagittal section of the head and neck of a rat at P-7. ey, eye; sg, salivary gland; vi, vibrissae. Bar 5 mm.

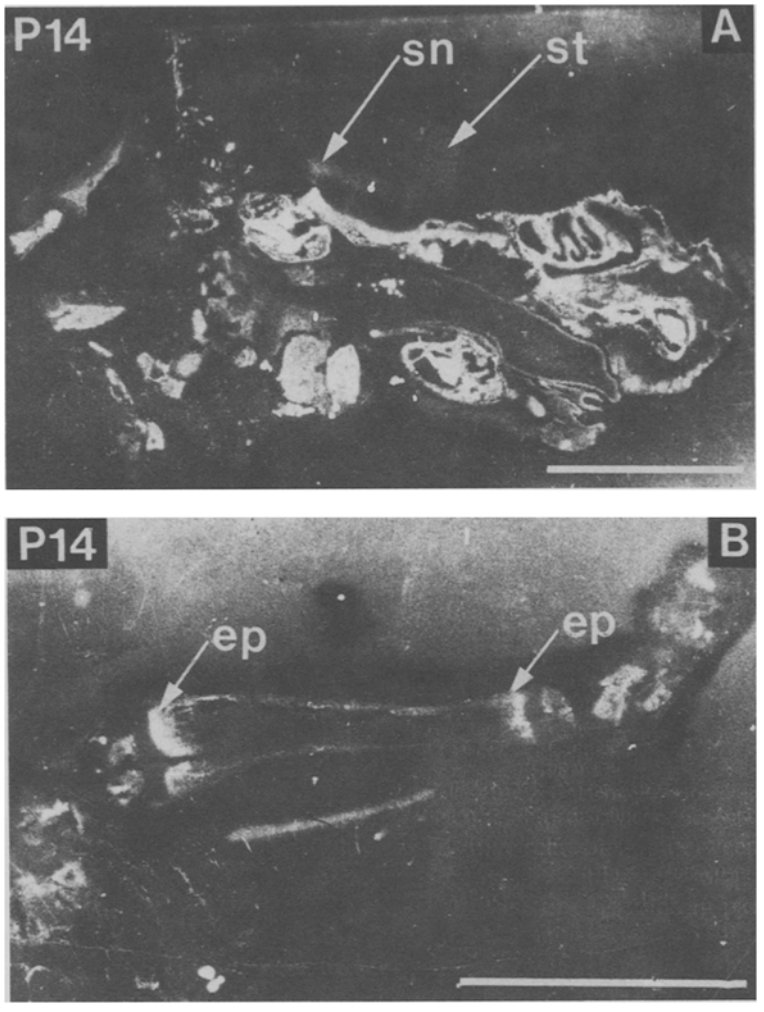

Figure 8. Parasagittal section of the head and neck (*A*) and longitudinal section of a femoral bone (*B*) of a rat at P-14. st, striatum; ep, epiphyseal plate; sn, substantia nigra. Bar 1 cm.

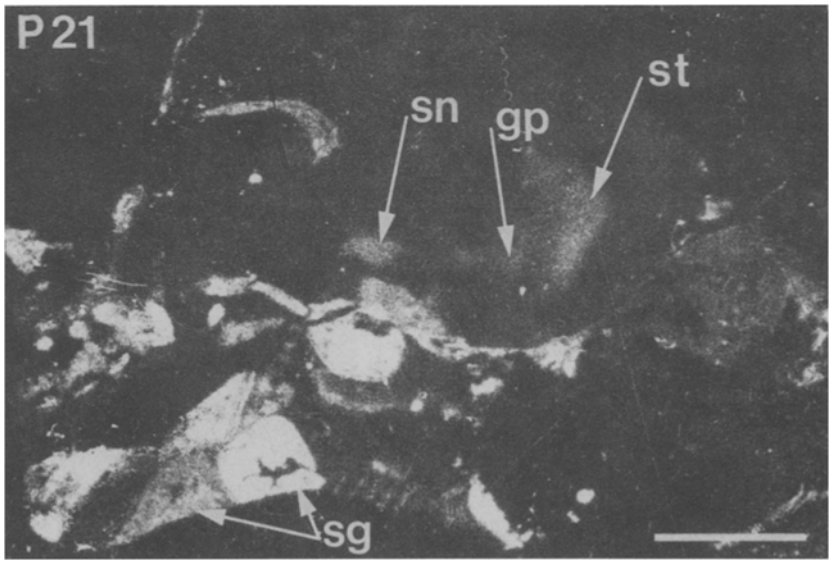

Figure 9. Parasagittal section of the head and neck of a rat at P-21. gp, globus pallidus; sg, salivary glands, sn, substantia nigra; st, striatum. Bar 5 mm.

Discussion

The present study confirms previous observations of the diversity of NEP localizations in the CNS [7, 18] and in peripheral nervous and non-nervous organs of adult animals [9] using autoradiographic [7], immunocytochemical [21, 22], and immunoradiometric [23] methods and extends them to prenatal stages of development. Our results are in good agreement with ontogenetic studies on the localization of NEP in some peripheral tissues from other mammalian species such as fetal calves [24] or rabbit embryos [25], and show that NEP expression takes place prenatally in mesoblastic and entoblastic as well as ectoblastic structures.

In spite of the widespread distribution of NEP, modulation of pain, and behavioral changes, which mainly result from the degradation of the opioid peptides enkephalins at central and spinal levels, are as yet the best-documented effects of NEP inhibitors [1].

NEP also plays a role in the regulation of fluid homeostasis, by inactivating the circulating atrial natriuretic peptide ANP, partly in the kidney [13, 14]. Accordingly, NEP inhibitors such as thiorphan and the carboxyl-containing NEP blocker UK69,578 have been shown to exhibit diuretic and natriuretic effects in humans [12]. On the other hand, the mechanisms by which NEP controls bowel fluid movements has been suggested to be due to its involvement in the inactivation of the opioid peptides at the level of the nerve endings, since the antidiarrheal effects of NEP inhibitors were shown to be reversible by naloxone [26]. However, the presence of NEP in several other organs has not yet been associated with any functional effect. The timing of NEP expression during development could therefore be compared with that of putative substrates and then tentatively correlated with the appearance of their physiological functions.

In the prenatal and developing central nervous system of the rat, the appearance of NEP was noted in three main structures, where the enzyme is likely to terminate the action of enkephalins. The olfactory bulbs are the only structures strongly and permanently labeled before and, to a lesser extent, after birth. This is in good agreement with the possibility of a NEP-regulated role for peptides in olfaction at birth-time. NEP was not detected in the spinal cord before the early post-natal stages of development. This contrasts with its ante-natal presence in the human fetus [8] and may reflect species differences, with possible implications for pain perception.

Surprisingly, it was only at the end of the second post-natal week that NEP was demonstrated in the nigrostriatal tract, the most conspicuously labeled nervous structure in the adult rat [7]. As mu opioid receptors appear early in prenatal development [27], the appearance of NEP seems, in this case, to be more closely related to that of delta opioid receptors [28]. Other NEP-rich brain structures in the adult rat, such as the neo-cortex and the cerebellum [7], were not labeled during the first post-natal month, demonstrating a late or very progressive appearance of the endopeptidase, unrelated to the development and differentiation of these brain areas. Recently, the developmentally regulated presence of ANP receptors has been demonstrated in the cortex during the embryonic period [29], which suggests a role for ANP and its receptors in neuronal differentiation and migration. Our findings apparently preclude a modulatory role for NEP in such effects, and the only brain region where NEP is transiently expressed is the mesencephalic flexure at approximately the time when nigral neurons extend processes towards their striatal targets.

On the other hand, NEP expression is strongly enhanced during the development of sensory organs such as the eyes or the vibrissae. It would therefore be important to study the expression of putative NEP-substrate(s) during the same periods. The presence of substance P [30, 31] in the adult retina and ocular nerve endings is well documented, although other peptides such as VIP, NPY, CGRP and enkephalins have also been found in eye tissues [32]. More recently, immunoreactive ANP has also been found in rat and rabbit retina [33] and in the lacrymal gland of the pig [34]. One or several of these peptides could play a role not only in developmental events but also in adult physiological functions, as already proposed for substance P [35].

In peripheral non-nervous organs, such as the kidney, the lungs and the salivary glands, the appearance and the tissue localization of NEP closely parallels the morphological differentiation of the structures, suggesting a progressive involvement of the enzyme in adult physiological functions. This could include cleavage of ANP in the kidney [13] and SP in the lung [36]. In the placenta, NEP is more abundant in maternal structures at early stages and in fetal structures at late stages of gestation. It is therefore possible that NEP fulfills a protective role against foreign peptides, first for the mother and then for the fetus. In addition, the heavy labeling of the terminal portion of the intestine may be related to the inactivation of peptides in the amniotic fluid. Alternatively, as – at least in the adult – the terminal portion of the intestine is mainly concerned with absorption processes, a function in fetal fluid homeostasis might be possible. There is also considerable evidence implicating several putative NEP substrates such as gastrin, PYY, CGRP and CCK in the control of gastrointestinal epithelial cell proliferation [37]. Interestingly, the CGRP-like peptide bombesin accelerates the growth of the entire gastrointestinal tract in suckling rats [38].

The rather weak and stable labeling of bone marrow could be mainly due to CALLA-positive lymphoid progenitor cells in the course of their differentiation [39] and/or to neutrophils. ANP receptors have also been found in this tissue and in the growing bone; the distribution of NEP also closely resembles that of somatostatin [45].

In the heart and major blood vessels, in the genital tubercle and in the cervical region including the nuchal ligament and the brown fat pads, the presence of ANP or of

ANP receptors has been shown in the adult, but often in more limited amounts, suggesting the involvement of NEP in developmental events[40]. In several tissues where NEP has been localized, such as the testis[41], adipocytes[42] interscapular brown fat pads[43] and bone[44], ANP receptors are physiologically coupled to cGMP. NPY, a 36 amino acid tyrosine-rich peptide, also cleaved in vitro by NEP, was found to be colocalized with the metallopeptidase in the brown fat where the peptide could exert antilipolytic effects[46]. The distribution of NEP closely parallels that of ANP during heart development[47], both being present in the ventricle at early stages but in the atrial region only later. Likewise, it is interesting to observe that, contrasting with the rather high concentration of NEP in heart and aorta in the fetus, the enzyme is hardly detected in blood vessels in the adult rat[48] although it has recently been formally characterized by using an ^{125}I radiolabeled inhibitor[49]. It would be interesting to know whether this hyperexpression of NEP in the cardiovascular system is related to a concomitant increase in released ANP, which could be transiently involved in trophic processes.

In conclusion, the distribution of NEP during ontogeny strongly suggests its participation in the development of several peripheral organs as well as its implication in physiological functions in the CNS and in the periphery during adulthood. A study of substrate candidates in ontogeny and the effects of NEP inhibitors in the adult should help clarify these two aspects.

Acknowledgments. The authors thank Dr Ann Beaumont for critical reading of the manuscript. The secretarial assistance of M. H. Badoureaux and of C. Dupuis, is gratefully acknowledged.

1 Roques, B. P., Beaumont, A., Dauge, V., and Fournié-Zaluski, M. C., in: Handbook of Experimental Pharmacology. Eds G. V. R. Born, P. Cuatrecasas, H. Herken and A. Schwartz. Springer Verlag, Heidelberg (1991) in press.
2 Turner, A. J., in: Neuropeptides and Their Peptidases, pp. 183–198. Ed. A. J. Turner. Ellis Horwood series in Biomedicine, VCH Publishers 1987.
3 Stephenson, S. L., and Kenny, A. J., Biochem. J. *243* (1987) 183.
4 Sakurada, C., Yokosawa, H., and Ishii, S. I., Peptides *11* (1990) 287.
5 Sokolovsky, M., Galron, R., Kloog, Y., Bdolah, A., Indig, F. E., Blumberg, S., and Fleminger, G., Proc. natl Acad. Sci. USA *87* (1990) 4702.
6 Pierart, M. E., Najidovski, T., Appelboom, T. E., and Deschodt-Lanckman, M. M., J. Immun. *140* (1988) 3808.
7 Waksman, G., Hamel, E., Fournié-Zaluski, M. C., and Roques, B. P., Proc. natl Acad. Sci. USA *83* (1986) 1523.
8 Salès, N., Charnay, Y., Zajac, J. M., Dubois, P. M., and Roques, B. P., J. chem. Neuroanat. *2* (1989) 179.
9 Salès, N., Dutriez, I., Mazière, B., Ottaviani, M., and Roques, B. P., Reg. Pept. *33* (1991) 209.
10 Thorsett, E. D., and Wyvratt, M. T. in: Neuropeptides and Their Peptidases, p. 229. Ed. A. J. Turner. Ellis Horwood series in Biomedicine, VCH Publishers 1987.
11 Roques, B. P., Fournié-Zaluski, M. C., Soroca, E., Lecomte, J. M., Malfroy, B., Llorens, C., and Schwartz, J. C., Nature (London) *288* (1980) 286.
12 Roques, B. P., and Beaumont, A., Trends pharmac. Sci. *11* (1990) 245.
13 Koehn, J. A., Norman, J. A., Jones, B. N., Le Sueur, L., Sakane, Y., and Ghai, R. D., J. biol. Chem. *262* (1987) 11623.
14 Kenny, A. J., Stephenson, S. L., and Turner, A. J., in: Mammalian Ectoenzymes, p. 169. Eds A. J. Kenny and A. J. Turner. Elsevier, London 1987.
15 Waksman, G., Hamel, E., Delay-Goyet, P., and Roques, B. P., Brain Res. *436* (1987) 205.
16 Zajac, J. M., Lombard, M. C., Pechanski, M., Besson, J. M., and Roques, B. P., Brain Res. *477* (1989) 400.
17 Zajac, J. M., Charnay, Y., Soleilhac, J. M., Salès, N., and Roques, B. P., FEBS Lett. *216* (1987) 118.
18 Zajac, J. M., and Roques, B. P., in: Brain Imaging. Techniques and Applications, p. 18. Eds N. A. Sharif and M. E. Lewis. Ellis Horwood Limited, John Wiley & Sons, New York 1989.
19 Davila-Garcia, M. I., and Azmitia, E. C., Dev. Brain Res. *49* (1989) 97.
20 Waksman, G., Bouboutou, R., Devin, G., Besselievre, R., Fournié-Zaluski, M. C., and Roques, B. P., Biochem. biophys. Res. Commun. *131* (1985) 262.
21 Ronco, P., Pollard, H., Galceran, M., Delauche, M., and Schwartz, J. C., Lab. Invest. *58* (1988) 210.
22 Matsas, R., Kenny, A. J., and Turner, A. J., Neuroscience *18* (1986) 991.
23 Gee, N. S., Bowes, M. A., Buck, P., and Kenny, A. J., Biochem. J. *228* (1985) 119.
24 Johnson, A. R., Gray, L. D., Youngblood, E., and Sullivan, J., J. cell. Biochem. *43* (1990) 243.
25 Lecavalier, H., Crine, P., and Malo, C., Biol. Neonate *56* (1989) 342.
26 Marcais-Collado, H., Uchida, G., Costentin, J., Schwartz, J. C., and Lecomte, J. M., Eur. J. Pharmac. *144* (1987) 125.
27 Kent, J. L., Pert, C. B., and Herkhenham, M., Devl. Brain Res. *2* (1982) 487.
28 Spain, J. W., Roth, B. L., and Coscia, C. J., J. Neurosci. *5* (1985) 584.
29 Tong, Y., and Pelletier, G., Neuropeptides *16* (1990) 63.
30 Tervo, K., Tervo, T., Eranko, L., Eranko, O., and Cuello, A. C., Histochem. J. *13* (1981) 435.
31 Stone, R. A., and Kuwayama, Y., Archs Ophtal. *103* (1985) 1207.
32 Stone, R. A., Kuwayama, Y., and Laties, A. M., Experientia *43* (1987) 790.
33 Palm, D. E., Keil, L. C., Sassani, J. W., and Severs, W. B., Brain Res. *504* (1989) 142.
34 Lange, W., Lang, R., Basting, C., and Unger, J. W., Exp. Eye Res. *50* (1990) 313.
35 Anderson, J. A., Malfroy, B., Richard, N. R., Kullerstrand, R., Lucas, C., and Binder, P. S., Regulatory Peptides *29* (1990) 49.
36 Barnes, P. L., Experientia *43* (1987) 832.
37 Goodlats, R. A., and Wright, N. A., Experientia *43* (1987) 780.
38 Lehy, J., Puccio, F., Chariot, J., and Labeille, D., Gastroenterology *90* (1986) 1942.
39 LeBien, T. W., and McCormack, R. T., Blood *73* (1989) 625.
40 Vollmar, A. M., Klin. Wochenschr. *68* (1990) 699.
41 Marala, R. B., and Sharma, R. K., Biochem. J. *251* (1988) 301.
42 Göke, R., Göke, P., Noll, B., Richter, G., Fehmann, H. C., and Arnold, R., Biomedical Res. *10* (1989) 463.
43 Okamura, H., Kelly, P. A., Chabot, J. G., Morel, G., Belles-Isles, M., and Heisler, S., Biochem. biophys. Res. Commun. *156* (1988) 1000.
44 Fletcher, A. E., Allan, E. H., Casley, D. J., and Martin, T. J., FEBS Lett. *208* (1986) 263.
45 Bruns, C., Dietl, M. M., Palacios, J. M., and Pless, J., Biochem. J. *265* (1990) 39.
46 Valet, M., Berlan, M., Beauville, M., Crampes, F., Montastruc, J. L., and Lafontan, M., J. clin. Invest. *85* (1990) 291.
47 Smith, F. G., Sato, T., Varille, V. A., and Robillard, J. E., J. devl Biol. *12* (1989) 55.
48 Tamburini, P. P., Koehn, J. A., Gilligan, J. P., Charles, D., Palmesino, R. A., Sharif, R., McMartin, C., Erion, M. D., and Miller, M. J. S., Pharm. exp. Ther. *251* (1989) 956.
49 Soleilhac, J. M., Lucas, E., Beaumont, A., Turcaud, S., Michel, J. B., Crine, P., Fournié-Zaluski, M. C., and Roques B. P., Molec. Pharmac. (1991), submitted.

0014-4754/92/030290-11$1.50 + 0.20/0