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Abstract

DISTRIBUTION AND PROPORTION OF IMMUNOREACTIVE NEURON SPECIFIC ENOLASE CONTAINING CELLS IN THE RAT PINEAL GLAND

by

Alfonso Miranda C.

The distribution and proportion of cytoplasm containing immunoreactive neuron specific enolase (NSE) in the rat pineal have been investigated. Step serial sections of the pineal glands of 7 male Sprague-Dawley rats were stained for NSE using an indirect immunoperoxidase method. The volumes of the pineals and of the regions containing reactive cells were derived from the distance between sections and the sum of the corresponding sectional areas of camera lucida drawings. The proportion of the NSE areas actually consisting of NSE reactive cytoplasm was measured using an image array processor.

Cytoplasm reactive for NSE was consistently localized to the posterior and dorsal aspects of the pineal. To express this in quantitative terms the volume of reactive cytoplasm in the anterior and posterior halves of the glands were determined separately. It was found that on the average 79% of the total NSE volume was found in the posterior 46% of the pineal volume.

The volume occupied by NSE positive cells ranged from 0.02×10^{-3} mm^3 to $42.7 \times 10^{-3} mm^3$ per pineal. The average volume was 2.1% of the pineal volume, which was 0.334 mm^3 (S.D. 0.21). Pineal volume also exhibited a

wide range from 0.151 to 0.727 mm^3. This great variability in both the volume of pineal and of NSE reactive cytoplasm is discussed, and it is suggested that when the factors that control this variability are identified, pineal functions will be more readily understood.

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DISTRIBUTION AND PROPORTION OF IMMUNOREACTIVE NEURON SPECIFIC ENOLASE CONTAINING CELLS IN THE RAT PINEAL GLAND

By

Alfonso Miranda C.

A Thesis in Partial Fulfillment

of the Requirements for the Degree

Master of Science in Anatomy

December 1983

Each person whose signature appears below certifies that this thesis in his opinion is adequate, in scope and quality, as a thesis for the degree of Master of Science.

Paul J. McMillan, Professor of Anatomy, Chairman

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INTRODUCTION

The pineal gland, or epiphysis cerebri, forms part of the diencephalic region, the epithalamus. Since a hormone-like indoleamine, melatonin (5-methoxy-N-acetyltryptamine), was identified and isolated from 200,000 bovine pineals by Lerner et al. (1960), pineal research has flourished.

It was demonstrated by Snell (1965) that the melatonin effect on epidermal melanocytes of guinea pigs is very small in comparison with amphybian melanocytes. Subsequent studies by Wurtman et al. (1965) showed that melatonin has an antigonadal effect in rats. Soon after melatonin was discovered in the pineal, it was found that the light:dark cycle was an important factor in determining the biochemical activity of the gland (Wurtman et al., 1963). Characteristically, periods of light are associated with low levels of melatonin with high levels being found during the dark phase. The influence of the light and dark cycle was found to be mediated via the sympathetic nervous system (Wurtman et al. 1964).

In this literature review the present knowledge of the structure, development, cytology and innervation of the rat pineal gland will be summarized as a basis for investigating the distribution and proportion of pinealocytes containing neuron specific enolase.

Gross Anatomy:

Pineal bodies of different mammals are considerably variable with respect to form and position. The rat pineal body, as pointed out by Gregoreck et al. (1977) and Vollrath (1981), is basically an elongated organ

with a club-shaped distal thickening. The intermediate and proximal parts sometimes show signs of regression.

A classification that adequately covers the topographical relationship between the pineal body and the third ventricle of the brain, as well as the form and arrangement of the pineal tissue, has been recommended by Vollrath (1981). In this scheme pineal glands, proximally located, i.e. resting on the posterior aspect of the diencephalon, are designated type A. When the gland is elongated it is a proximo-intermediate type, classified AB. If the parenchyma of the gland extends from the diencephalon to the vicinity of the cerebellum, it is a proximo-intermedio-distal gland, designated ABC. If there are parts of the gland that are reduced in size, the capital letters are replaced by small letters of the Greek alphabet. For example, type αßC gland is an elongated structure with the bulk of the pineal tissue lying in a superficial (distal) position, and proximal and intermediate portions are present but reduced in size.

Close inspection of the pineal complex (Boeckmann, 1980) shows that, in the rat, it consists basically of a proximal, intermediate and a distal part. The distal part or superficial pineal, lying in the immediate vicinity of the skull near the confluence of sinuses, represents the major part of the pineal complex. The proximal part, or deep pineal, lies between the habenular and posterior commissures. Anteriorly, where the deep pineal borders the shallow intercommissural recess, the deep pineal is covered by a virtually continuous ependyma consisting of a single layer of cuboidal cells lacking cilia.

Posteriorly, the deep pineal extends into the variable intermediate part, also referred to as the pineal stalk. The deep pineal may come into

contact with cerebrospinal fluid (CSF) in the region of the suprahabenular recess dorsally and the subarachnoid space ventrally. Following the classification proposed by Vollrath (1981), the pineal of the rat is of αßC type.

Boeckmann (1980) showed that the stalk region of the rat pineal complex exhibits great intraspecies variation. Depending on the amount and location of parenchymal tissue in the stalk region, three basic types of pineal complexes can be distinguished. In 96 male Sprague-Dawley rats only 18% showed a pineal complex as described above, namely with a continuous parenchymal stalk linking the deep and superficial pineals. In 6% of the rats investigated, parenchymal cells were not present in the pineal stalk. Here the pineal complex consisted of only two parenchymal parts, the superficial and the deep pineals. In 76% of the animals investigated a slender parenchymal stalk extended from the deep pineal toward the superficial pineal without reaching it.

There are no statistically significant differences in the size of the deep pineal in male and female Wistar rats (Boeckmann, 1980). However, great intraspecies variation is found in the topographical relationship between the deeply located pineal tissue and the third ventricle, being influenced on the one hand by the width of the intercommissural region and on the other hand by the width of the dorsal extension of both the habenular commissure and the pineal stalk.

In female rats weighing 160-180 gms pineal weights range from 0.4 to 1.8 mg (Axelrod et al., 1965). Diurnal changes were reported in which the lowest values were seen at 18:00 h. Fiske (1960) reported the reduction in weight of the pineal glands of Sprague-Dawley rats placed in continuous light

for six to twenty-five weeks of continuous light exposure, but the difference was not significant when compared to the controls.

The pineal volume of Rattus albino measured by Legait (1976) quoted by Vollrath (1981) is 0.6149 mm^3 , a correlation coefficient between pineal volume and body weight was found to be 0.8731, and the allometry coefficient 0.60 in Wistar rats weighing 6 to 411 g. Becker and Vollrath (1983) measured in 345 gm Sprague-Dawley rats an average pineal volume of 0.461 mm^3. In this study no correlation with body weight was found. The coefficient of variation of the pineal volume was about 40% within groups of 8 to 9 rats. Quay (1956b) also observed a great interindividual variation in pineal volume in a group of 33 mice (Peromyscus neucopus) with a coefficient of variation of 43%. From Boeckmann (1980) it is known that the superficial pineal measures between 1,600 and 1,950 µm in length with a maximal average diameter of 820 µm. The volume of the deep pineal exhibited great interindividual variation in Sprague-Dawley rats, but it amounted to 127 $\pm$ 39 $x10^5 \mu m^3$ in females. The difference between males and females was not statistically significant ($p>0.05$).

Development of the pineal gland in rats:

Generally three major developmental or maturational phases have been distinguished (Vollrath, 1981). These overlap to a significant extent. The morphogenetic phase is considered to begin at about the 12th embryonic day and to extend until the young are delivered. The cellular proliferation phase commences on the 16th embryonic day and terminates within several days after birth. The cellular hypertrophy and differentiation phase begins roughly

at birth and ends 9 to 12 weeks postpartum.

In mammals the primordium of the pineal diverticulum lies in the dorsal median area of the neural tube at the level of the neuraxis, which becomes the diencephalon. The diverticulum originates in the area between the posterior and habenular commissures, i.e., in an intercommissural position (Calvo and Boya, 1981b).

It has been established previously that the epiphyseal primordium in the Albino rat makes its appearance between days 14 and 14.5 of intrauterine life and constitutes a distinct evagination by day 15 (Calbough, 1973). Calvo and Boya (1981b), however, show that the pineal analage may be recognized in rat embryos by 13.5 days of development.

The presumptive pineal epithelium shows no detectable morophological differences with the adjacent neuroepithelium. This primordium can be identified only on a topographical basis. The simultaneous appearance of the pineal evagination with the initial differentiation of the posterior commissure permits a definitive identification of the pineal evagination (Calvo and Boya, 1981b).

From its initial appearance at 13.5 days to 17 days the pineal analage grows as a tubular evagination. The apices of the pseudostratified epithelial cells are held together by junctional complexes (Calbough, 1973). Its wall thickens by intense periluminal mitotic activity. At 17 days of development important changes take place in the configuration of the pineal analage, which will soon become a solid organ (Calbough, 1973; Calvo and Boya, 1981b). The pineal assumes its characteristic compact appearance as a result of infolding and thickening of the epithelium, resulting in the obliteration of

the pineal recess. Beginning in the distal part of the gland and advancing proximally, the walls fold inward while the cells migrate into the recess through gaps where the terminal bars have disappeared (Calvo and Boya, 1981b).

Kappers (1960), as quoted by Calvo and Boya (1981b), interpreted isolated cavities as seen in sagittal section to be the formation of follicles in the dorsal wall of the pineal. However, Calvo and Boya (1981a, 1981b) showed that these cavities originate by obliteration of the pineal recess.

Until 18 days development the increase in volume of the gland is due to periluminal mitosis very much like the neuroepithelium generally. Thereafter, interstitial mitosis takes place. The thickening of the epithelium takes place at the expense of the already reduced lumen, resulting in the rapid closure of the pineal recess. After 18.5-19 days, the recess is reduced to a narrow proximal fissure. The cavities formed by the fusion of the recess walls also continue to disappear.

Turning now to a review of rat pineal differentiation at the cellular level, microscopic and ultramicroscopic studies have been performed both pre- and postnatally. At 15.5 days the pineal recess is large, and its epithelium is composed of tall cells placed perpendicularly to the basal lamina (Calbough, 1973; Calvo and Boya, 1981a). The pinealoblast nuclei tend to be located in the central zone of the epithelium, leaving a basal and apical zone composed of cellular processes.

The pinealoblasts appear undifferentiated, and no cellular subtypes may be identified. They are very rich in free polyribosomes throughout their cytoplasm, and their mitochondria are usually small. In the vicinity of the

pineal recess the pinealoblasts display junctional mechanisms, especially zonula adherentes. The apical cytoplasm, located above the junctional complexes, adopts a club-like form. Occasional basal bodies and cilia may be found, just as they can froom the first stages of pineal development (Calvo and Boya, 1981a).

Calbough (1973) described the presence of cilia and junctional complexes in the apical zone of the pinal epithelium in rat embryos of 18 days. Calvo and Boya (1981), on the other hand, observed these structures from the first stages of pineal development.

At 16-16.5 days of development the apical zone displays abundant mitotic figures. In comparison with the earlier stages the number and size of the nucleoli was increased, and basal bodies and cilia are more frequent. The cilia lack the central pair of microtubules, and longitudinal sections of cilia frequently show a sudden narrowing near the base. Groups of pinealoblast processes partly devoid of a basal lamina have been found projecting into the underlying mesenchyme, where they contrast directly with the mesenchymal cells. In later stages complete cells may be observed among the mesenchymal elements outside the basal lamina.

At 17 days the obliteration of the pineal recess takes place. The discontinuity of the basal lamina separating the parenchyma from the stroma has been noted in the adult rat pineal gland (Wartenberg, 1968) as quoted by Calvo and Boya (1981a). The invasion of the pineal analage by connective tissue begins at 17.5 days, and continues in later stages. Capillaries of immature appearance begin to be found at 17 days of development. A narrow space may be seen separating the endothelial cells from the pinealoblasts and

their incomplete basal lamina.

From 18 days of development onward luminal spaces have been obliterated. There are, however, rosette-forming pinealoblasts joined at their apices by junctional complexes. Pinealoblasts become smaller, having processes which are distributed throughout the parenchyma. The numbers of intrapineal vessels increases progressively.

After 19.5-20 days of development, differences begin to be observed in pinealoblast cytoplasmic density. At birth two different pinealoblast types, clear and dark, may be differentiated. The clear pinealoblast displays an ovoid nucleus of dispersed chromatin and clear nucleoplasm. The perinuclear cytoplasm contains free ribosomes, rough endoplasmic reticulum, small mitochondria, a well-developed Golgi system, and coated vesicles throughout the cytoplasm. Occasional lipid droplets, microfilaments and some microtubules are also present.

The dense pinealoblast is more scarce and appeared isolated or in the form of bands. Their nuclei are smaller, having chromatin, which is disposed in clumps in a dense nucleoplasm. The processes tend to be thinner than in the clear pinealoblast, and some organelles, such as rough endoplasmic reticulum and free ribosomes, are less numerous. Other features seen in the dense pinealoblast are centrioles, cilia, and small dense granules of various shapes (Calbough, 1973; Calvo and Boya, 1981a).

The innervation of the pineal gland begins to develop during the prenatal period. By day 18 of gestation nerve fibers derived from the posterior commissure penetrate three quarters of the length of the developing pineal (Gardner, 1953). The postganglionic sympathetic fibers penetrate the

gland postnatally (Gardner, 1953). Very soon after birth the sympathetic fibers are found primarily in the capsule of the gland with a few fibers terminating in the vicinity of the parenchymal cells. By two days after delivery sympathetic nerve fibers are abundant in the apical portion of the gland. A day later the complete gland is innervated. At first the axons of the nervi conarii are in large bundles surrounded by Schwann cells, but later more Schwann cells develop so that the axons are in smaller bundles or are individually enclosed (Machado, 1971) as quoted by Altar (1982).

	Development of pineal morphology	Pre-or-post natal age (days)
		0 (conception)
Vollrath (1981)	Start of morphogenetic phase	12
Calvo et al. (1981)	Evagination of diencephalon	13.5
Vollrath (1981)	Start of cellular proliferation phase	16
Calvo et al. (1981)	Intensive periluminal mitotic activity (growth into tubular) evagination)	17
Calbough (1973)	Penetration of nerve fibers from posterior commissure	18
Calvo et al. (1981)	Closure of pineal recess	18.5-19
Calvo et al. (1981) Calbough (1973)	Appearance of endoplasmic reticulum, mitochondria, Golgi apparatus, vesicles and microtubules. 2 types of cells.	20
Gardner (1953) Vollrath (1981)	Pineal receives limited sympathetic innervation, end of morphogenetic phase, beginning of hypertrophy phase	Birth
Gardner (1953)	Complete gland is innervated by sympathetics	3
Machado (1971)	Maturation of Schwann cells in nervi conarii	After the first week
Vollrath (1981)	End of cell proliferation phase several days after birth	

Table 1

Morphological features of the Developing Rat Pineal

* * * * * * * * * * * * * * * *

Histology:

The first comprehensive account of the growth and histological appearances of the pineal gland in the albino rat was given by Izawa (1925). He observed that when it was stained with iron hematoxylin, the pineal is composed of two kinds of cells: parenchymal and neuroglial cells. The latter nuclei stain darker, its granular chromatin having a strong affinity for the hematoxylin. Parenchymal cells are of different forms (oval, flattened, spindle-shaped, and polygonal). In general the cytoplasm is not abundant, and the nucleus is placed eccentrically. The nuclei vary greatly in size and shape; some are round or oval, while others are irregular in form. The nucleus has one or two small round nucleoli, which are situated excentrically or along the nuclear membrane and have a great affinity for the iron-hematoxylin dye. Concretions or brain sand were not present. Wallace et al. (1969) describes the parenchymal cell as having long processes which end in bulb- like swellings on the pericapillary space.

Wallace et al. (1969) did studies on the proportion of different cell types in the adult rat, showing that the pineal gland is primarily a cellular organ with the main unit being the pinealocyte. He found the gland to consist of 82% parenchymal cells, 12% neuroglial cells, 5% endothelial cells, and 2% were unidentified.

In the rat various histological patterns have been found. Structural differences between central and peripheral regions of the pineal gland have been reported. Cortical regions have more lipid (Quay, 1957), greater blood content (Quay, 1958), and show cytologically less response to continuous light than medulla (Quay, 1963) quoted by Vollrath (1981). This subdivision of

the pineal organ into cortical and medullary regions is also made by Blumfield and Tapp (1970), in which nuclear size measurements yielded two groups of pinealocytes. In the periphery the pinealocyte nuclei were found to be smaller than in the center. In other studies the reverse was found (Quay, 1963) quoted by Vollrath (1981). Freund et al. (1977) demonstrated by immunohistochemical studies melatonin or a melatonin-like substance in the cortex only. The physiological meaning of this subdivision is not yet known.

A distinction has been made, depending on the tinctorial differences between chromophobic pinealocytes and chromophilic pinealocytes. Orange G-, aniline blue and chrome alum haematoxylin phloxine provide this differentiation in Sprague-Dawley rat pineals (Quay, 1956a; Tapp and Blumfield, 1970). The latter authors designated lightly stained granular cells as type I. Type II cells were distinctly granular and eosinophilic, and the type III were small, dense, irregularly-shaped cells. Blumfield and Tapp (1970) did a study of the nuclear size of these three cell types. Type I cells had nuclei measuring 8.3 µm in diameter, and type II cell nuclei measured 6.7 µm in diameter, and type III measured 5.4 µm in diameter. These measurements of nuclear size are said to be useful because nuclear and nucleolar size undergo circadian changes, showing a peak at midday, followed by a gradual decline to a minimum at the end of the light period (Vollrath, 1981).

In addition to pinealocytes, glial cells are present. In the rat they amount to 12% (Wallace et al. 1969). A great step forward in the classification of the glial cells in the rat pineal has recently been made by means of immunohistochemical studies. Møller et al. (1978), using antibodies against the glial marker protein (S-100 protein) and glial fibrillary protein (GFA),

showed that the pineal glial cells are indeed similar to astrocytes.

Ultrastructure of the rat pinealocyte:

There is still need for more research to understand the functional significance of the differences that have been reported in the ultrastructural morphology of pinealocytes of different species. In this section the findings in four mammals belonging to the order of Rodentia will be discussed.

The ultrastructure of the albino rat pineal was studied in detail by Wolf (1965). Pinealocyte nuclei were found to exhibit a finely granular nucleoplasm with condensations of chromatin localized peripherally and around the prominent nucleolus. Perichromatin and interchromatin granules were observed in nucleoplasm. Nuclei varied in shape from round to various folded configurations. Their nuclear envelope had pores, a distinct perinuclear cistern, and only scattered ribosomes on the outer membrane. Similarly the rough endoplasmic reticulum (RER) was not heavily populated with ribosomes, although free ribosomes and polysomes were considered abundant. Occasionally free ribosomes were associated with RER in arrays resembling Nissl bodies.

Smooth endoplasmic reticulum (SER) was present as an interanastomosing labyrinth in both the perikaryon and cell processes. Intergrades with RER were found. Numerous lipid droplets often closely associated with the endoplasmic reticulum were a notable characteristic of rat pinealocytes.

The Golgi apparatus appeared to be randomly dispersed in the perikaryon. Coated vesicles, multivesicular bodies and "grumose" inclusions were

frequently associated with the Golgi apparatus. The "grumose" (pile of dirt) inclusions were irregularly-shaped membrane-limited vesicles containing a matrix of fine electron dense granules of irregular size and shape. These were assumed to be the characteristic secretory granules of the rat pinealocyte.

Mitochondria were abundant in a variety of sizes and often with irregularly arranged cristae. Wolf (1965) also observed "synaptic ribbons" and correctly concluded that they were in reality rodlets. He described them as being numerous in perikarya and cellular processes but never associated with nerve terminals.

Matsushima et al. (1979) made electron microscopic observations on the cotton rat (Sigmodon hispidius), which is a seasonal breeder. The pinealocyte nucleus is predominantly round or oval in shape, but irregular ones are also present exhibiting invaginations of the nuclear envelope. A slight concentration of chromatin near the nuclear envelope was found. The nucleoli usually lie in the peripheral region of the nucleus and are composed of irregular strands of electron-opaque material, the nucleolonema. Many nongranulated vesicles (40 to 80 nm diameter) and a few granulated vesicles (about 100 nm in diameter) are associated with the Golgi cisternae.

Some pinealocyte processes in the parenchyma and a few in perivascular spaces contain large numbers of these vesicles. Matsushima et al. (1979) did not identify subclasses of pinealocytes, but if classification is based on the type of secretory processes, as suggested by Pévet and Kappers (1977), then the Cotton rat pineal consists of a single population of pinealocytes (type I) with processes containing granulated vesicles and non-

granulated vesicles. Matsushima et al. (1979) postulate that granular vesicles as well as non-granulated vesicles, have their origin in the Golgi complex. An interesting finding obtained in this study is the occurrence of peculiar inclusion bodies. These inclusions usually occur singly in the juxtanuclear region of the pinealocyte cytoplasm and occasionally in their processes. These are round in shape, not bounded by a limiting membrane, and composed of fine granular or filamentous material of high electron-opacity, which are similar in appearance to the substance seen in the nucleolonema.

The observations made on the mole-rat (Spalax ehrenbergi) by Pévet et al. (1976) are of special importance, since this mammal is a blind subterranean species. According to Pévet et al. (1976), the mole rat has non-functional eyes, which are covered by skin. They do not say whether or not the eyes or the optic nerve are atrophied. Two different types of populations of pinealocytes (I and II) were observed. They differed in location and especially in their content of cell organelles involved in synthetic processes. Table 2 summarizes their findings.

	Population I	Population II
Location	Homogeneously distributed throughout the parenchyma.	Always located near a perivascular space.
Nucleus	Oval or polygonal chromatin finely dispersed.	Lobular numerous chromatin aggregations in a zone adjoining the nuclear envelope.
Perikaryon	Granular vesicles. Concentration of ribosomes and cistarnae of RER. Numerous glycogen granules.	Accumulation of proteinaceous material in some cistarnae of the RER and between the two layers of the nuclear envelope. Some glycogen granules.

Table 2

Characteristics of two different populations
of pinealocytes of the mole-rat
* * * * * * * * * * * * * * * * *

Mitochondria, ribosomes, granular endoplasmic reticulum, lysosomes, lipid inclusions and glycogen were present in the perikarya of both types of pinealocytes (I and II).

Pévet et al. (1976) suggested that these ultrastructure charcteristics are of cells engaged in intense synthetic activity and that pineal function is enhanced by excessive darkness or blinding. Thus, it can be suggested that the intense synthetic activity observed in mole-rat pinealocytes is due to the lack of light stimulation of this animal which lives in permanent darkness.

Another report by Pévet and Yadav (1980) describes the fine structure of the pinealocyte of the Malaysian rat (Rattus sabanus). Rattus sabanus is geographically distributed 15° N and S of the equator in Southeast Asia. In

Malaysia the animal is commonly endemic to tall forests of the interior lowlands and foothills away from human settlements. The animal breeds throughout the year, but most frequently from July to September and least frequently from January to March.

The parenchyma of the pineal of the Malaysian rat was found to consist of light and dark pinealocytes. The ultrastructure of these two types of cells is not basically different, the granular vesicles being present in both types. Their differentiation is based only on a difference in cytoplasmic density. The pinealocytes are homogeneously scattered in the pineal tissue. All the cells are irregular in shape, showing cytoplasmic processes emerging from the cell bodies. The nuclei are large, oval and sometimes lobulated. Many Golgi complexes are widely dispersed around the nucleus. Each consists of a system of flattened sacs associated with populations of vesicles of varying diameter. The granular vesicles measure 50 to 300 nm in diameter and are very numerous. They are especially concentrated at the endings of pinealocyte processes near the pericapillary space. Numerous small clear vesicles are present in the same area, suggesting, according to Pévet and Yadav (1980), that their origin is from membrane-bound granular vesicles after dissolution of the dense core. Images suggesting the release of the content of the clear vesicles and that of the dense core directly into the perivascular space were presented by Pévet and Yadav (1980).

Numerous mitochondria, cisternae of the granular endoplasmic reticulum and polyribosomes are present within the cytoplasm. Lipid droplets are very scarce. Ciliated structures were rarely found. Centrioles were also present in association with ciliated structures or isolated elements. Lyso-

somal structures were scarce; however, some pinealocytes appeared to be filled with them.

Vesicle-crowned rodlets were found by Pévet & Yadav (1980) to be very numerous in the pinealocytes of the Malaysian rat. The vesicle-crowned rodlets (synaptic ribbons) consist of an electron-dense rod surrounded by electron-lucent vesicles, the length of the rod being variable. These aggregates occur singly, in parallel groups or in randomly dispersed arrays. Their orientation in the perikaryon was not uniform but could be found either perpendicular or parallel to the cell membranes. The rodlets are more frequently found in the endings of the pinealocyte processes. In the endings some of the rodlets lie free in the cytoplasm, but most of them are located perpendicular to the membrane in a direct topographical relation to the perivascular space.

Innervation:

The functional innervation of the mammalian pineal gland has been of interest to scientists. Gardner (1953) demonstrated in the hooded rat a relatively rich nerve supply, which includes central fibers coming from the habenular and posterior commissures and peripheral, autonomic fibers that make their entry to the gland along with the dorsal pineal artery.

Three types of granulated vesicles have been described in autonomic nerve endings in the pineal of albino rats (Duffy et al., 1970). Type IA were the smallest (40-50 nm) and have an eccentrically placed dense granule. The larger granules have a diameter of about 100 nm and are subdivided on the basis of the position of the electron dense granule being eccentric in type IB

and central in type II. The content of these vesicles was investigated using reserpine treatment, which caused the type IA and type IB granules to be reduced in size and lose their dense granule. This is taken to indicate that these granules contain norepinephrine and/or serotonin. Type II granules were unaffected.

Etcheverry et al. (1968) studied the effect of dichromate with and following formaldehyde fixation. In the latter case norepinephrine is not retained so that their observation of an electron dense product indicates that an indoleamine is contained in the granules. They suggested that serotonin as well as norepinephrine may be found in these pineal nerve endings. Additional evidence was presented by Bak et al. (1970), who studied the localization of serotonin in rat pineal by means of electron microscopic autoradiography. Following treatment with the serotonin precursor tritiated 5-hydroxytrytophan, the nerve endings were heavily labeled. Since these nerve endings degenerate following superior cervical ganglionectomy (Etcheverry et al., 1968), it can be assumed that their primary neurotransmitter is norepinephrine. It appears that the presence of serotonin in these endings has been proven, but to what extent it has replaced norepinephrine is not clear.

Wurtman et al. (1964) demonstrated that the light induced inhibition of pineal indole synthesis was mediated via the sympathetics. Therefore various studies have been undertaken to determine the path by which retinal influence is carried to the pineal. Pickard et al. (1981) demonstrated anterograde transport of horseradish peroxidase (HRP) from the retina to the suprachiasmatic nucleus of Syrian hamsters. Recent studies by Klein et al. (1983) support the view that the pathway of retinal influence from the retina

to the preganglionic sympathetic cells in the spinal cord involves the suprachiasmatic and paraventricular nuclei. Bilateral destruction of the latter nuclei, known to project to the spinal cord, prevents the influence of light on the pineal.

Although many studies have shown the importance of the innervation of the pineal which arises in the superior cervical ganglia, it is becoming increasingly apparent that there may be other fibers innervating the pineal whose cell bodies are not located in these ganglia. Neuroanatomic evidence for central connections between the stria medullaris-habenular complex and the rat pineal was reported by Dafny (1983). He observed retrograde movement of HRP from the superficial pineal to small neurons of the habenula and stria medullaris. In electrophysiological studies of the rat by Ronnekliev et al. (1980) stimulating electrodes were placed stereotaxically in the medial habenular nucleus, and a recording electrode was placed in the pineal gland. Two distinct populations of electrically active pineal cells were found, the silent cells, which were activated by habenular stimulation, and spontaneously spiking cells which show a very regular pattern of firing. None of these spontaneously active cells responded to habenular stimulation. The authors reasoned that the fibers passing from the habenula to the pineal gland are probably of the myelinated variety, based on the conduction velocity of the fiber (5.0 m/s).

Semm et al. (1981) demonstrated that electrical activity of the spontaneously spiking pinealocytes can be altered by exogenous stimuli such as the neurotransmitters norepinephrine (NOR) and acetylcholine (ACH) and pineal-related substances (melatonin). Melatonin was applied by a pressure

microejection technique onto the pineal cells. During the daytime most cells responded with an increased impulse frequency, but the majority of the responses recorded at 19:00h were inhibitory. Also, microelectrophoretic application of NOR exhibited excitatory responses during the day, but at night, when sensitivity of the ß receptors decline, more inhibitory responses were recorded. The author's results indicate that the pineal cells, which are excited by electrical stimulation of the lateral habenular nucleus, also respond in a similar manner to the microelectrophoresis of acetylcholine. Therefore, the habenular innervation of the rat pineal described by Ronnekliev et al. (1980) may be cholinergic in nature.

Neuron specific enolase - a marker for APUD cells:

Neurons and endocrine cells in the past have been regarded as developmentally and functionally distinct. Neurons are of ectodermal origin and function primarily as elongated conducting structures that directly control the behavior of other cells by transmission of electrical impulses and localized release of transmitter substances. Endocrine cells have been thought to be mostly of nonectodermal origin and to control the behavior of most other cells indirectly by releasing hormones into the blood.

Increasing evidence now suggests the existence of a diffuse system of endocrine cells that are closely related to neurons in terms of development or function or both. Among these cells are medullary chromaffin cells of the adrenal gland, C cells of the thyroid, chief cells of the carotid body, pancreatic islet ß (insulin), and α (glucagon) cells, and anterior pituitary cells (corticotrophs, ACTH and melanotrophs, MSH). The properties these cells

exhibit in common include: 1) the ability to synthesize aromatic amines or polypeptide secretory products or both, 2) the ability to take up, decarboxylate and store aromatic amine precursors, such as 5-hydroxytryptophan and dopa, and 3) the presence of high levels of esterases, cholinesterases and ß-glyceryl phosphate dehydrogenase. These cytological characteristics are termed APUD (Amine Precursor Uptake and Decarboxylation) (Pearse, 1969). The list of APUD cells was later expanded to include other monoamine-metabolizing and polypeptide-containing cells, such as the pinealocyte, bronchial Kulchitsky cell, melanocyte and Merkel cells (Pearse, 1979; Marangos et al., 1982; Jiang et al., 1981).

A soluble, acidic protein specific for nerve cells has recently been isolated from rat whole brain extract by Marangos et al. (1975a). This nerve-specific protein has been isolated from other species including cat, human and bovine (14-3-2 protein) (Marangos et al., 1975a, 1977). The methods of purification described for the rat (Marangos et al., 1975a) were utilized for the isolation of nerve-specific protein from both cat and human. The comparison of feline and human nerve-specific protein (cat and human) with that of rat by electrophoretic and immunological criteria revealed that these proteins are very similar (Marangos et al., 1975b, 1977).

Recently, it has been determined that nerve-specific protein represents a neuronal form of the glycolytic enzyme enolase, 2-phospho-D-glycerate hydrolase (E.C. No. 4.2.1.11) (Pickel et al., 1976). So a more appropriate designation as proposed by Marangos et al. (1975b) is neuron-specific enolase (NSE).

At present three forms of the glycolytic enzyme, enolase, have been

isolated from rat whole brain extract:

The first NNE (non-neuronal enolase) ($\alpha\alpha$) is the least acidic and appears to be immunologically similar to the liver enolase ($\alpha'\alpha'$), but the identity has not been proven. Fraction NNE has been purified, characterized, and shown to be structurally and immunologically distinct from NSE. They are therefore products of separate genes (Schmechel et al., 1978b). By sedimentation velocity NNE was shown to have a M.W. of 87,000 and is composed of two subunits with a M.W. of 43,500 (Marangos et al., 1978a, 1978b; Pickel et al., 1976; Schmechel et al., 1978b).

The second is a hybrid form ($\alpha\gamma$). At present the localization and properties of the hybrid form of enolase are not clear. Structural and immunological evidence indicate that the molecule consists of one NNE subunit and one NSE subunit (Schmechel et al., 1978b).

The third form, NSE ($\gamma\gamma$), is the most acidic fraction. The methodology used for the purification of NSE are: salt precipitation, DEAE-cellulose ion exchange chromatography, sephadex G-150 gel filtration, and the final step is column isoelectric focusing. Immunological evidence (Ouchterlony diffusion plates) supports the conclusion that a pure NSE was obtained because only one precipitation arc was seen when NSE reacted against antiserum. It has an isoelectric point of 4.7 in the absence of denaturing agents and 5.0 in the presence of 2.0M urea. Sedimentation velocity and equilibrium data indicate a homogenous material with a molecular weight of 78,000. Sedimentation in 6M guanidine HCl containing 0.02% glutathione yielded a molecular weight of 39,000, indicating that the native molecule is a dimer (Marangos et al., 1975a).

By modern techniques, such as radioimmunoassay (RIA) and immunocytochemical staining, it is possible to measure and localize specific proteins of cells and organs. Recent RIA measurements of NSE have proven especially valuable in the study of this glycolytic enzyme and its distribution. High levels of NSE have been found in the central nervous system (CNS). Significant levels are found in the peripheral nervous system but at much lower concentrations than in the CNS. Significant levels have also been found in the pineal gland and other organs that contain diffuse endocrine (APUD) cells, such as parafollicular cells of thyroid, adrenal medullary chromaffin cells, glandular cells of pituitary, and islet cells of the pancreas (Marangos et al., 1979).

The immunocytochemical localization of NSE to neurons and APUD cells has shown that NSE is a very useful marker for peripheral and central neuroendocrine cells (Pickel et al., 1976; Schmechel et al., 1978a; Tapia et al., 1981). The physiologic role for different enolase isoenzymes is not clear. Nevertheless, they provide a useful marker for nerve cells and neuroendocrine or neuron-like cells.

Not only do cells of the APUD classification have secretory properties similar to those of a neuron, they also exhibit excitable membrane properties in tissue culture (Tischler et al., 1977). Moreover, as previously discussed, Ronnekliev et al. (1980) showed that there are two distinct excitable neuron-like elements in the pineal gland, the silent cells, and the spontaneously active ones. Recent electrophysiological data obtained from neurons in culture showed that electrical activity is correlated with high NSE concentration (Schmechel et al., 1980a). Intracellular recordings of cultured spinal

cord neurons, dorsal root ganglion cells and epithelial cells were made. The spinal cord neurons were all immunoreactive for NSE and exhibited spontaneous electric activity. Epithelial cells had neither electrical activity nor NSE. By correlating electrical with NSE of individual cells, it was found that the appearance of electrical activity was correlated with the appearance of NSE.

A similar finding has been made in developing brain. Neuron precursor cells, which are dividing and migrating to their final destination, contain NNE. Neuron specific enolase appears late in differentiation, presumably after onset of synaptic activity. Migrating cells may contain hybrid enolase ($\alpha\gamma$), and some nerve cells may not completely switch over to use NSE even in the adult (cerebellar stellate and basket cells). So neuron specific enolase is not only a marker for nerve cells but may also be indicative of the degree of differentiation and/or the activity state of the cell (Schmechel et al., 1980b).

Conclusions from the literature review

From the literature reviewed it is clear that the rat pineal, best known for its production of melatonin and its antigonadal activity, is morphologically characterized in the following ways.

1. It develops from a neuroepithelial evagination from the presumptive third ventricle.

2. In addition to the well-known sympathetic innervation, parasympathetic fibers as well as direct connections with the central nervous system (CNS) are now recognized.

3. Some descriptors (fat, vascularization, nuclear size and melatonin) have suggested a division into a cortex and medulla.

4. Pinealocytes have been divided into two types using histological stains, ultrastructurally (dark and light or by mode of secretion) and electrophysiologically (the spontaneously spiking on the silent cells). No correlations between these types have been made.

5. Pinealocytes have been shown to possess properties that group them with the APUD system of neuroendocrine cells, and in agreement with this, at least some of them contain NSE.

Since functional relationships have not been clearly established for the different regions of the pineal or for the different morphological types of pinealocytes, other descriptors are needed to help establish these relationships. Neuron specific enolase could be such a descriptor. Its association with fully differentiated and active neurons suggests that it would identify pinealocytes most closely related to nerve cells. Therefore the present study was undertaken to determine whether or not pinealocytes could also be subdivided into different populations based on their NSE content. To further characterize those cells reactive for NSE their distribution and proportion in the pineal have been quantitatively studied.

PROCEDURE

Animals:

White male rats from 136-154 gm of the Sprague-Dawley strain obtained from Simonsen Laboratories were used. One rat (G1) was maintained at a light/dark ratio of 12:12, six rats (I,II,III,IV,V,VI) were sacrificed on the day they arrived. They were perfused for 15 min via the ascending aorta under ether anesthesia. The brains were sliced to obtain a 2:3 mm thick block containing cerebellar cortex, pineal gland and cerebral visual cortex. They were rinsed with 0.1M cacodylate buffer for 12 hrs., then dehydrated and blocked in paraffin.

The fixative used was glutaraldehyde at a 1% concentration in 0.05M cacodylate buffer pH 7.4. It was prepared as follows:

25% glutaraldehyde solution	12 cc
0.1M cacodylate buffer pH 7.4	150 cc
0.4M sucrose	60 cc
Distilled water	78 cc
Total	300 cc/animal

The blocks were dehydrated with isopropyl alcohol in increasing concentrations according to the following schedule:

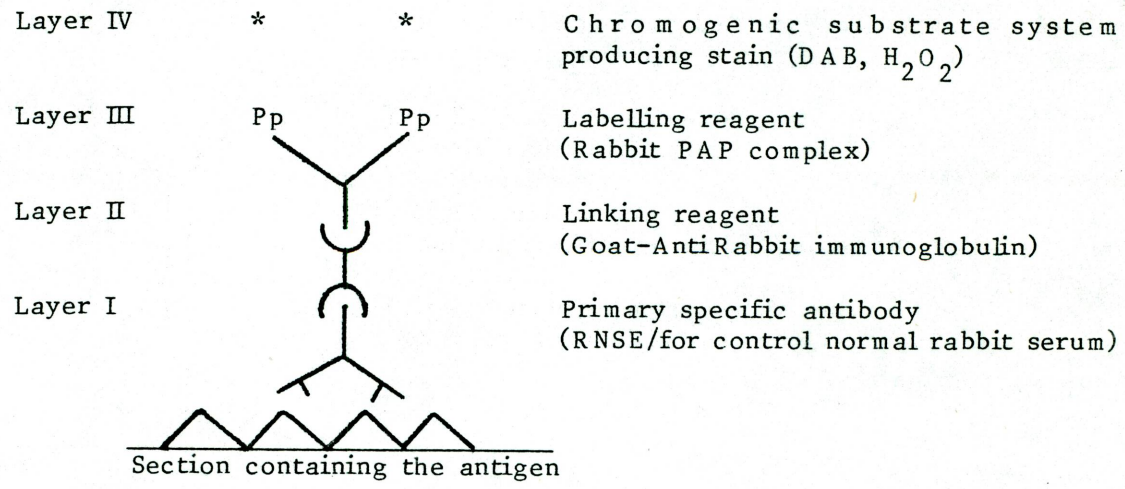
isopropyl alcohol 75%	2 hrs.
isopropyl alcohol 85%	2 hrs.
isopropyl alcohol 95%	2 hrs.
isopropyl alcohol 100%	3 hrs. (3 changes, 2 hrs. each)

From absolute alcohol the tissues were transferred to a 50-50 xylene-

alcohol mixture (1-2 hrs), then into pure xylene for 1-2 hrs and 50-50 xylene-paraffin for 1-2 hrs in the oven at 60°C. After saturation with the paraffin-solvent mixture, the tissues were infiltrated with melted paraffin in a constant temperature oven at 60°C. Four changes of paraffin were used to remove the clearing fluid. The tissue blocks were then blocked in paraffin for sectioning. Sections were cut at 7 microns thickness, placed on glass slides, and allowed to dry at room temperature.

Immunohistochemical staining:

The NSE localization procedure used was the unlabeled-antibody-enzyme indirect method of Sternberger et al. (1970). The layers on the stained section may be illustrated as follows:



The procedure consisted of the following steps in which sections were:

1. Deparaffinized and hydrated to 0.01 M phosphate buffered saline (PBS) pH 7.3 containing 0.85% NaCl.

2. Treated with normal goat serum (1:10 solution) for 15 minutes in order to block nonspecific binding.

3. Reacted with antiserum to neuron specific enolase (1:1600 dilution)

for 12 hrs.

4. Washed three times in cold PBS to remove excess anti-neuron specific enolase (three minutes each).

5. Reacted with goat anitserum to rabbit immunoglobulin (1:40 dilution).

6. Removal of excess antiserum with three changes of PBS.

7. Applied horseradish peroxidase-anti-peroxidase complex (PAP) (1:10 dilution) for 1 1/2 hr.

8. Washed off excess PAP three times in cold PBS.

9. Develop peroxidase reaction with freshly made 0.03% diaminobenzidine and 0.01% hydrogen peroxide in 0.05M acetate-citrate buffer pH 5.6 for 3-5 minutes.

10. Stop reaction with PBS for 5 minutes.

11. Stained, dehydrated and mounted:

1. Haematoxylin	2 minutes
2. Rinsed in tap water	2 minutes
3. 35% isopropyl alcohol	2 minutes
4. 70% isopropyl alcohol	2 minutes
5. 100% isopropyl alcohol	2 minutes
6. 100% isopropyl alcohol	2 minutes
7. Xylene	2 minutes
8. Xylene	2 minutes
9. Mounted in permount	

Note: In those steps where sera are used, the sections were covered by a polyethylene film and put on a rocking apparatus in a humid chamber (McMillan, 1982).

For the control sections the only change was that in step 3 of the procedure normal rabbit serum (1:1600) was applied instead of the antiserum to neuron-specific enolase. The other steps were followed in the routine way. No brown reaction product on either nerve cells or pineal cells was observed in the control sections. Therefore, the obviously brown reactive cells in the specifically stained sections were the neuron-specific enolase containing pinealocytes.

Volumetric Measurements:

The volume measurements of the pineal gland were computed from the outline camera lucida drawings of every fifth section in the following manner. The area of each outline drawing was determined by planimetry. The sum of the areas was multiplied by the center to center distance between sections to give the estimated volume. In a similar way the volume of the regions of the pineal containing immunoreactive NSE were estimated. These regions were only partly composed of reactive cells.

An image array processor was used to determine the proportion of the reactive regions, which were stained for NSE. To do this the regions were scanned at 400X magnification and the images obtained in terms of absorbancies. These images were then analyzed so that the proportion of picture points (pixels) having absorbancies greater than a selected threshold could be determined. This threshold was chosen so that only the stained elements were accepted for the analysis. To determine the volume occupied by stained cells the volume of the regions determined by planimetry was multiplied by the proportion as determined with the image processor.

In the analysis of the anterior and posterior halves the same methods

were used. These were applied to the camera lucida drawings, which were divided by a line halfway between anterior and posterior poles. Since it was apparent that the regions containing NSE were more concentrated posteriorly, the proportion of the regions stained for NSE was determined separately for the anterior and posterior halves. In two glands it was not possible to obtain accurate measurements from both anterior and posterior halves, therefore a single value was used for both halves.

RESULTS

I. General histological appearance:

The general histological characteristics of the rat pineal gland observed in this study agree with the work of Izawa (1925). The superficial part of the epiphysis cerebri is pear-shaped. Its capsule consists of a few layers of irregularly-shaped flattened cells. The pineal parenchyma has a very rich blood supply. In almost all specimens studied prominent pericapillary spaces were found which are surrounded by adjacent pinealocytes.

The pineal parenchymal cells have a random arrangement throughout the gland, occasionally forming small rosette-like structures. Pinealocytes have various shapes with nuclei which may be round or oval. The one or two nucleoli are situated eccentrically or along the nuclear membrane.

The pinealocyte cytoplasm has inconspicuous borders with the normal H&E stain, but on the average these cells are about twice the width of the nucleus. Interstitial cells are few. They are recognizable by their nuclei, which are ovoid and more flattened than those of the pinealocytes. Often two or more are seen close together, usually situated near pericapillary spaces but also scattered throughout the gland.

A division of the gland into a cortex and medulla was not found, nor were calcarious secretions seen in the entire study.

Table 3 shows the pineal volumes in mm^3 in comparison with the body weight in grams. The range of pineal volumes was from 0.15 to 0.73 mm^3, a 4.8 fold difference with a mean and S.D. of 0.334 ± 0.21 mm^3. This compares with 0.461 mm^3 reported by Becker and Vollrath (1983) for 345 gm rats. The

pineal volume does not appear to be correlated with body weight either in the present study or that of Becker and Vollrath (1983).

Animal LD	Body Weight (g)	Pineal volume (mm^3)
VI	154	0.284
V	150	0.151
G1*	150	0.519
IV	148	0.230
I	147	0.727
III	136	0.178
II	130	0.258

Table 3

This specimen represents a laboratory rat exposed to 12:12 light/dark condition over a period of 15 days. The specimens (I,II,III,IV,V,VI) were sacrificed as soon as they arrived from the supplier.

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II. Demonstration of NSE containing cells:

Pineal gland. The brown peroxidase reaction product clearly identified cells which bound the NSE antibodies. Control sections treated with normal rabbit serum in place of the primary antiserum were negative. From a study of counterstained sections (Fig. 1 and 2) it can be seen that many pinealocytes as well as interstitial cells and endothelial cells were unreactive. That

the reaction product is confined to the cytoplasm is demonstrated in Figures 3 and 4 where the nuclei appear as voids. Fat droplets are also not stained (Fig. 4). Processes of reactive pinealocytes are filled with the reaction product (Figs. 4 and 5). A tendency for the reactive cells and their processes to surround perivascular spaces is evident (Figs. 3,5 and 7), although not all capillaries are surrounded by NSE. Interestingly, one pinealocyte reactive for NSE was found in mitosis (anaphase) (Fig. 6).

Nerve tissue:

As a control of the specificity of the antiserum the reactions observed in the brainstem and cerebellum were recorded. It can be seen in Figures 8 and 9 that reaction product is found in both the perikarya and processes of nerve cells. Glial cells did not stain. The reaction observed in the cerebellum corresponds to the reported cellular distribution of NSE (Schmechel et al., 1978b). Purkinje cells are variably reactive (Fig. 10).

III. Distribution of NSE immunoreactive areas:

At low power the most prominent feature of the neuron-specific enolase antibody localization was its distribution within the parenchymal substance of the rat superficial pineal gland (Figs. 11, 12 and 13). In some of the cases (numbers VI,G1,II) it was generally distributed with a greater concentration posteriorly, but in other cases it was largely limited to the postero-dorsal region (numbers III,IV,I and V). See Table 4.

Specimen #	NSE Vol $\mu m^3/10^6$ Ant	Post	% of Total Enolase Vol. in Post half
III	0	0.02	100
IV	0.39	8.4	96
I	0.7	6.9	90
V	0.01	0.04	80
II	0.4	0.8	67
G1	15.4	27.3	64
VI	2.0	2.5	56

Table 4

Volume of NSE positive cells in anterior and posterior halves of the pineal.

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It is evident from Table 4 that the pineal volume occupied by NSE positive cells is consistently greater in the posterior half of the gland. An average of 78% of NSE reactive cytoplasm was found posteriorly. In addition, as can be seen in Figures 11, 12 and 13, there is a preferential localization peripherally and dorsally.

IV. Volumetric proportion of NSE Immunoreactivity:

The data summarized in Table 5 documents a great interindividual variation in both the volume and the proportion of the pineal reactive to the NSE antiserum. The correlation between pineal volume and total NSE reactive volume is low (corr. coeff. = 0.50), even though both show a large variation between individuals. The average proportion of the pineal occupied

by NSE reactive cells was 2.1%.

Specimen	Total pineal vol. ($X10^{-3} mm^3$)	NSE total vol. $X10^{-3} mm^3$)	Volumetric proportion of NSE (%)
G1	519	42.7	8.2
IV	230	8.79	3.8
I	727	7.7	1.0
VI	284	4.5	1.6
II	258	1.2	0.5
V	151	0.05	0.04
III	178	0.02	0.01

Table 5

The volumetric proportions of NSE volume
per gland as a percentage of the total pineal volume)

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DISCUSSION

In the present study we have sought to directly establish the cellular localization of neuron specific enolase (NSE) in the rat pineal gland by immunohistochemistry. The results presented demonstrate the utility of the immunoperoxidase technique using the unlabeled antibody enzyme method of Sternberger et al. (1970).

The detection of antigens in tissue cells depends on the use of methods of preparation which will not destroy the immunological activity of the antigen. Neuron specific enolase (NSE) is a freely diffusible, cytoplasmic protein. Excessive fixation can abolish its immunoreactivity (e.g antigen is preserved but masked by extensive formation of crosslinking or the antigenicity is destroyed). The other extreme is also undesirable; that is, the antigen may be lost or easily translocated during fixation (G.R. Bullock and P. Petrusz, 1982).

Previous neuron specific enolase (NSE) localization studies have detected the presence of artifactual intranuclear staining (Schmechel et al., 1978a) as well as weak or even the absence of staining (Pickel et al., 1977) of brain stem neurons using Zamboni's (picric acid-paraformaldehyde) fixative. This represents diffusion artifact, loss of antigen, or partial denaturation.

These earlier data as well as the results of preliminary experiments led to a methodology which incorporated the following precautionary measures:

1. A saline prewash was not used since enolase is readily solubilized

and diffusible.

2. One percent glutaraldehyde was the fixative of choice. Its low concentration provided adequate fixation without masking the antigen. The even and specific staining of nerve cells in the brain stem and cerebellum along side the pineal confirmed this choice of fixation.

3. A preincubation of the sections with normal goat serum and the use of carefully flattened sections were the precautions taken to reduce nonspecific staining at the edges of the tissue sections and on any protruding parts of the tissue (Moller et al., 1978).

4. To insure even distribution and availability of the antibodies to the sections the antisera were covered with a film of polyethylene, and the slides were rocked during incubation (McMillan, 1982).

I. Distribution of immunoreactive neuron specific enolase in the pineal:

In this study no regional differences in the histological organization of the superficial pineal were observed. Therefore, the finding of a preferential distribution of NSE was unexpected. This is not to say that regional differences have not been reported, but rather that there has been some question about their significance. Quay (1957) reported a cortical region having more lipid and a greater blood content (Quay, 1958). Freund et al. (1977) observed melatonin immunostaining fluorescence only at the marginal zone of the pineal gland. The observation in the present study was that the highest immunostaining for NSE was found in the posterior half of the gland, being most concentrated along the dorsal edge. Quantitative analysis revealed that on the average in the posterior 46% of the pineal volume 79%

of the total neuron specific enolase (NSE) immunoreactivity was found.

The previous report of Schmechel et al. (1978a) of the immunohistological localization of neuron specific enolase (NSE) in the pineal gland did not suggest the presence of any characteristic distribution. It should be noted that in spite of the great interindividual differences in the quantity of immunoreactive NSE, the preferential localization to the posterior part of the gland was consistently observed.

The characteristic localization of NSE may suggest a functional activity which is unique to those regions containing NSE reactive cells. It has been demonstrated that NSE immunostaining in cultured neurons can be correlated with the onset of electrical activity (Schmechel et al., 1980a). In a study of pinealocytes, in vivo spontaneous spike activity could only be obtained within the first 200 µm of the surface (Dafny and McClung, 1975). Freund et al. (1977) reported the immunofluorescent localization of melatonin to be restricted to the marginal zone. These data suggest that the NSE immunoreactive cells may represent a subpopulation of pinealocytes with unique functional activity. That they are no nerve cells is indicated by the mitotic cell which was NSE positive.

An explanation for this distribution of NSE in the superficial pineal is not yet available, but in this context the possibility that innervation is a factor should be considered. The sympathetic and parasympathetic fibers of the nervi conarii penetrate the epiphysis cerebri symmetrically at its posterior pole (Krstić, 1979); Vollrath, 1981). The spontaneously spiking cells in the pineal gland are believed to be controlled by sympathetics (Ronnekliev et al., 1980; Semm et al. 1981). There are, however, no data on the

distribution of these cells in the gland. The parasympathetic innervation does not seem to supply an explanation since it is not distributed to the perivascular spaces as are the sympathetics around which much of the enolase is localized. In addition, there is little experimental data to support the idea that the parasympathetics have a significant influence on pinealocyte function.

Although the silent cells decribed by Ronnekliev et al. (1980) are thought to be driven by cholinergic fibers, these probably are from central connections. The central and commissural fibers are restricted to the proximal part of the superficial pineal. Therefore the silent cells would not appear to be candidates for the NSE reactive cells.

In summary, evidence to date would suggest that the cells which exhibit spontaneous electrical activity would be the most likely ones to exhibit NSE. This possibility should be further explored.

II. Volume of NSE immunoreactivity in the pineal

Radioimmunoassay of NSE in the pineal of rats has indicated that the concentration is about one fourth that of the brain (Schmechel et al., 1978a). In the present study 2% of the pineal volume was found to contain NSE reactive cytoplasm. It is probable that this is considerably less than would be expected from the RIA data. Therefore until a figure for the proportion of brain occupied by cytoplasm containing NSE is available, so that pineal-brain comparisons can be made, it should be assumed that the preparative methods have blocked the staining of a large part of the pineal NSE.

The great range in the volume of NSE-containing cytoplasm observed

in this study of the pineal indicates a lack of homogeneity in the population of rats studied. Presently there is no explanation for this heterogeneity. The animals were all killed during the day so that circadian changes would not be a major factor. As was found in previous studies (Becker & Vollrath, 1983) there was also a large variation in pineal volume. The correlation coefficient between pineal and NSE volume was 0.50. Although this is not a strong correlation, a trend is suggested and with larger numbers of animals may be established.

Previous investigators have observed large variations in pineal measurements. As already noted, Becker and Vollrath (1982) observed a variation of pineal volume within groups of 8-9 rats of about 40%. Quay (1956b) observed in a group of 33 mice (Peromyscus leucopus) a coefficient of variation in pineal volume of 43%. The coefficient of variation of pineal volume in the present study was 63%. In the study of NSE concentration (ng/mg protein) Schmechel et al. (1978a) pooled pineals of 5-20 rats for each assay. They reported the mean value of 4 or more groups to be 2,650 ng/mg with a S.E. of 400. In comparison with the brain NSE (12,500 ng/mg, S.E. 105) this is a very high variation, especially since it represents pools of animals. It is clear that the variation in NSE volume observed in the pineal in the present study does not disagree with this observation.

As large variations in measurements of various pineal parameters continue to be observed it becomes increasingly important that the reasons for the variations be explored. When conditions which influence these variations can be controlled, it will be possible to say much more concerning the functions of the pineal.

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APPENDIX

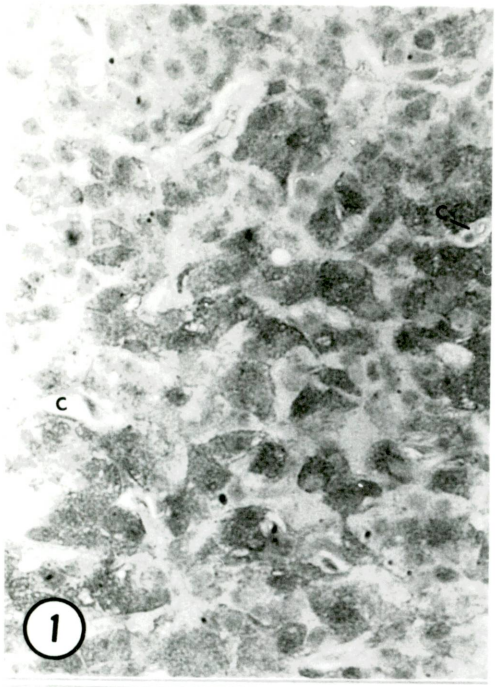
PLATE 1

EXPLANATION OF FIGURES

1. Low-power photomicrograph of a section of rat pineal gland stained for NSE and counterstained with haematoxylin. Stained cells have angular projections between unstained pinealocytes. Note the distribution of reaction product around capillaries (c). 300 X

2. High power micrograph of several of the NSE cells in the rat pineal gland. Note that the reaction product (NSE) is restricted to the cytoplasm of the reactive cells. Counterstained with hematoxylin. 608 X

3. Immunostaining (NSE) in pinealocytes which have not been counterstained. Reaction product is often found around perivascular spaces (*); see the capillary (c) at lower left. 600 X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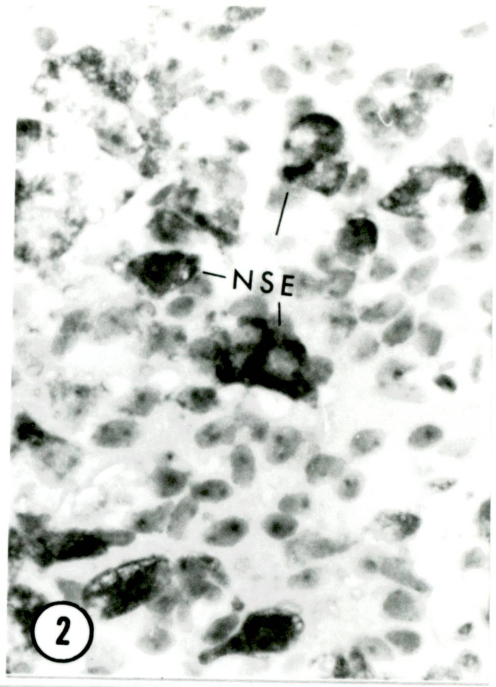
C
1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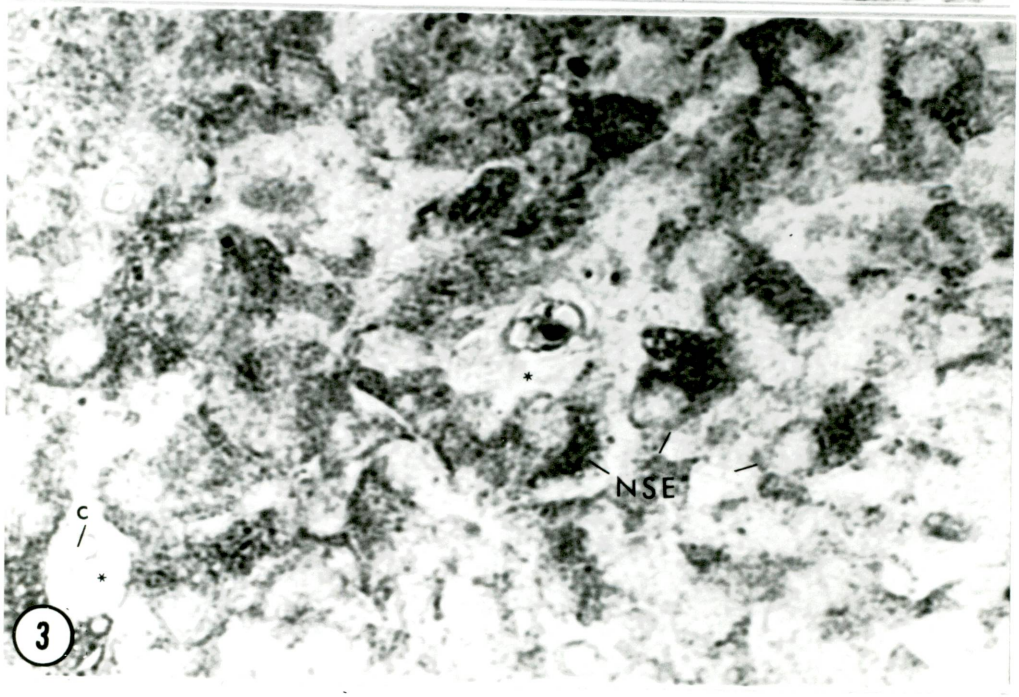

NSE
C
3

PLATE 2

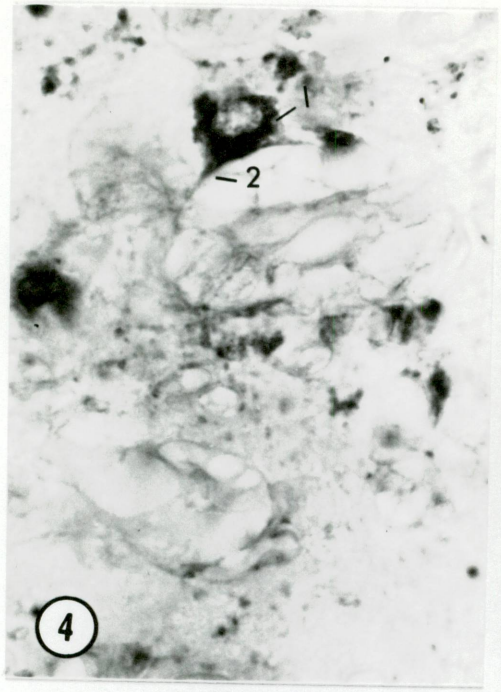
EXPLANATION OF FIGURES

4. Single NSE containing pinealocyte. Lipid droplets (1) and cytoplsmic processes (2) are demonstrated. 1512X

5. Stain for NSE along the postero-dorsal boarder of the pineal. The upper arrowhead points to a very darkly stained pinealocyte with its process extending toward a blood vessel (Bv). Note the thickness of the stained marginal zone (arrow 3). Immunoperoxidase stain. Counterstained with hematoxylin. 640X

6. NSE-cell of rat pineal gland in mitosis (Anaphase) (2). Immunoperoxidase stain. Counterstained with hematoxylin. 1071X

7. Relations of NSE of perivascular areas. Note the lack of immunoreactive material around the upper capillary (c) in contrast to the intense immunoreaction visible around the lower one. Pericapillary spaces (*) and an unstained pinealocyte (5). Counterstained preparation 896X.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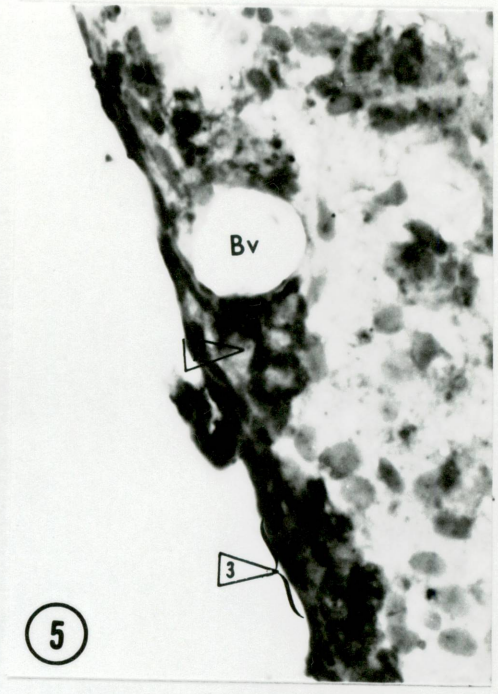
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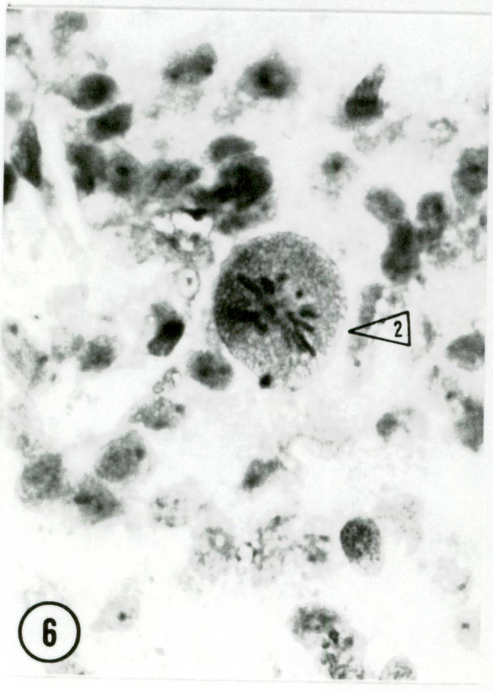
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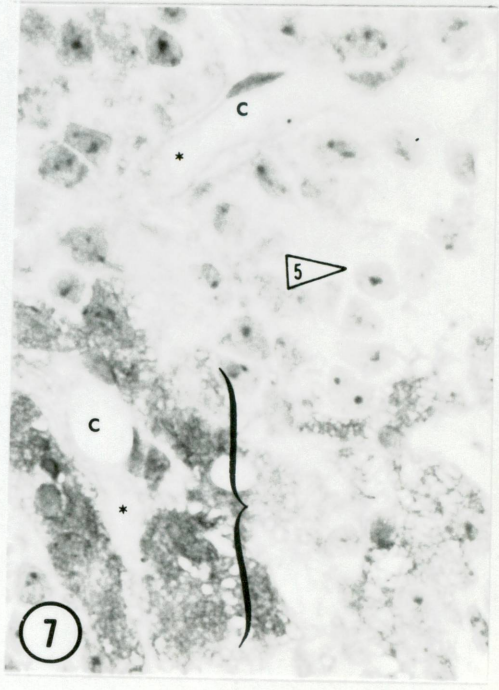
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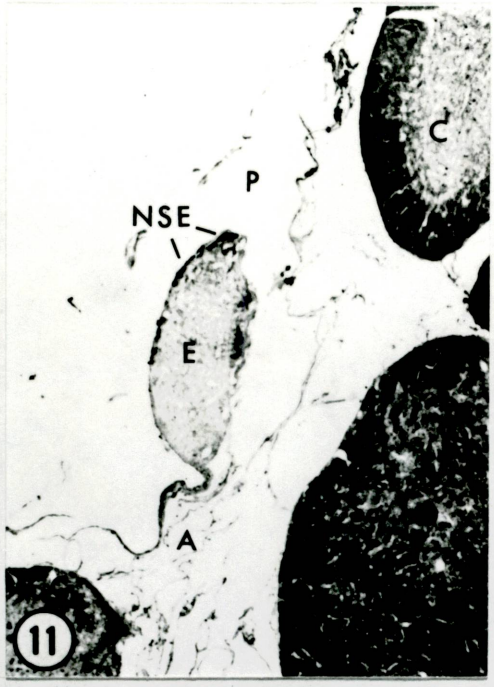
PLATE 3

EXPLANATION OF FIGURES

8 & 9. Immunohistochemical localization of NSE in neurons of the reticular formation of the medulla of the rat. Many stained axons are seen in cross section in Figure 8. Figure 8 640X. Figure 9 560X.

10. Rat cerebellum showing Purkinje cells (P). One is unstained for NSE (5) and 3 of the reactive Purkinje cells are labeled (6). The molecular layer (M) in the in the upper left is strongly labeled, as is the cytoplasm of the granule cells in the granular layer (G). Immunoperoxidase stain. 690X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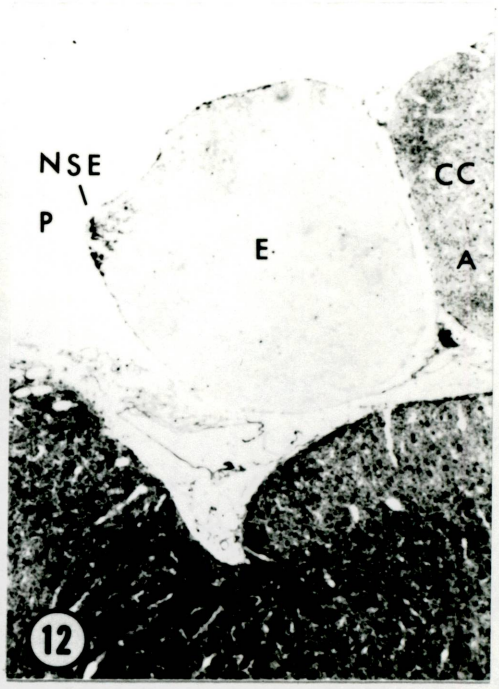
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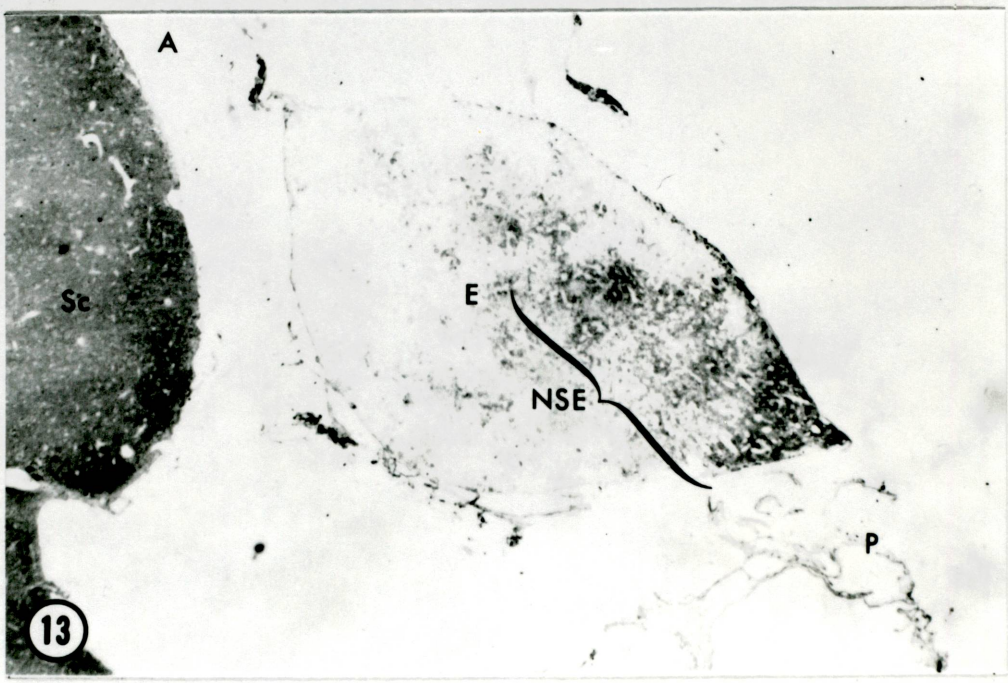
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PLATE 4

EXPLANATION OF FIGURES

11,12, & 13. Three pineal glands showing the distribution of NSE. The staining (NSE) is localized to the posterior region of the glands. Epiphysis cerebri (E), anterior pole (A), posterior pole (P), cerebral cortex (C C), cerebellum (C), superior colliculus (S C or SC/L Q). Immunoperoxidase stain. Figure 11 and 12 40 X. Figure 13 50 X.

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