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Quantitative investigations into the histostructural nature of the human putamen

I. Staining, cell classification and morphometry

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Summary. Combined staining with aldehyde and cresyl violet allows a reliable morphological distinction to be made between seven different types of neurons in the human putamen. We examined the age distribution of nearly 42,000 neurons in 27 normal putamina, using a semiautomatic morphometric procedure on defined tissue blocks. For morphometric evaluation and stereological calculations a section thickness of 20 µm is recommended. We modified routine aldehyde fuchsin cresyl violet combination staining for nervous tissue, since Braak's original method (Braak 1978, 1980) was developed for thick sections. The results show that neuronal density varies with age for the different types of neurons.

Key words: Putamen – Human – Aldehyde fuchsin – Lipofuscin – Morphometry – Stereology – Neuron classification – Ageing

Introduction

Morphometric investigations into the human putamen have been carried out since 1950 (Yano 1950). Most of these studies were, however, handicapped by the inadequate methods used, or by the examination of insufficiently large samples (Yano 1950; Carman 1966; Tabuchi 1969; Schröder 1970; Dom et al. 1973; Bugiani et al. 1978; Oyanagi et al. 1987; Pesce et al. 1987; Goto et al. 1989; Nakae et al. 1990). Age changes in the neuron density, the area occupied by neurons and the absolute number of cells have only been investigated by Lange et al (1974); Schröder et al. (1975); Lange et al. (1976) and Eggers (1989) for the whole neuron population.

During the last two decades, Haug et. al. (1967a, 1967b, 1979, 1980a, 1980b, 1984) have developed a more robust stereological technique and more reliable morphometric methods for the quantification of histological structures, in order to investigate age changes in neuron populations.

Braak (1971, 1978, 1983) also introduced a powerful pigment architectonic method for explorating parts of the central nervous system. His combination of aldehyde fuchsin with cresyl violet (AFC) stains the intracellular neurolipofuscins and the Nissl substance. We have modified this procedure, because we use 10 to 30 µm sections for morphometry instead of the 800 µm sections employed in Braak's pigment architectonic investigations. The thin sections are more vulnerable to the oxidation step of staining than the thick ones.

Braak and Braak (1982) introduced the classification of five different AFC-staining types of neurons. This classification has been extended by us to include two additional neuronal cell types (Figs. 1 and 2).

In Part 2 (The structural distribution of different cell types) we present a mathematical approach based on morphometry and designed to identify otherwise unrecognisable arrangements of cells.

Material and methods

We investigated 27 neurologically normal human brains with a uniform age distribution between 25 and 100 years. Because two new types of neurons were recognized after the first examinations, the number of types of neurons is different (13 brains containing 5 types of neurons and 14 containing 7 types).

The brains, including the neostriatum, were removed and dissected at a time between 12 hours and a few days after death, and fixed in 4% formalin diluted with a 0.9% sodium solution. Embedding was carried out as described by Haug (1979) and Cronjäger (1986).

20 µm histological sections were cut and mounted on gelatinized slides (modified heat-degraded 1% gelatin in aqua bidest).

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The stock aldehyde fuchsin solution contains 0.5 g pararosaniline, 100 ml 70% ethyl alcohol, 1 ml 37% HCl, 1 ml croton aldehyde and 100 ml aqua bidest. It must be allowed to stand for about 7 days before use.

After maturation of the stock solution, the final incubation medium was made up: 400 ml 60% ethyl alcohol, 50 ml 98–100% formic acid, 5 ml 30% hydrogen peroxide (H_2O_2) and 100 ml aqua bidest. This is then added to an equal volume of the stock solution (Schmitt 1991).

After deparaffination and 15 min. oxidation in 40% hydrogen peroxide, the slides were incubated in the aldehyde fuchsin working solution for 24 hours. The sections were then carefully differentiated in descending concentrations of ethyl alcohol and counterstained with cresyl violet. Finally, the sections were mounted as usual (Schmitt 1991).

The morphometric and stereological methods used have been described by Haug 1972, 1983, 1984a; Kühl 1981; Barmwarter 1981; Sass 1982a, 1982b; Schmitt 1991. The measuring itself was carried out with a MOP-Videoplan system (Schmitt 1991).

In each putamen we estimated in an area of 39825 µm² consisting of 675 optical fields of 59 µm². Each neuron was measured planimetricaly and the according values including coordinates of the gravity points stored.

After morphometric evaluation of the 27 putamina was complete, we had examined a total of 42000 neurons. We used N_v for numerical density [1], A_A for the projection area after correction for shrinkage [5], N for the total number of neurons [4]: V is the age-dependent volume of the left and right putamina [3], based on the data of Eggers (1989b).

$$N_v = \frac{\dot{n} \times R_T \times 10^9}{A \times P \times (t + 2d - 2k)} \quad [1]$$

(Haug 1967b)

$$R_T = 0{,}4479 + 0{,}00107x \quad [2]$$

(Eggers 1989b)

$$V = 11{,}873 - 0{,}02931x \quad [3]$$

(Eggers 1989b)

$$N = N_V \times 1000 \times V \quad [4]$$

(Eggers 1989b)[1])

$$A_A = \frac{A_M}{R_T^{2/3}} \quad [5]$$

(Haug 1979)

$$N_Q = \frac{\dot{n}[1; 2; 5; 6; 7]}{\dot{n}[3; 4]} \quad [6]$$

(Schmitt 1991)

Abbreviations are as follows:

A	= measuring grid area [µm²]
A_A	= area remaining after correction for shrinkage [µm²]
A_M	= measured projection area [µm²]
AFC	= combined aldehyde fuchsin and cresyl violet staining
d	= average diameter of nucleus
k	= lost caps of neurone nuclei
n	= sample size (27 putamina)
N	= total number of neurons in putamina of both hemispheres
$\dot{n}$	= number of neurones counted ($\Sigma \dot{n}\{i, j, k \ldots\}$)
$\dot{n}\{i, j, k, \ldots\}$	= number of neurones of each type (i, j, k)
N_Q	= ratio of small to large neurones
N_v	= number of particles per unit volume [1 mm³] (= numerical density)
P	= number of area units evaluated
R_T	= age-dependent regression due to correction for shrinkage
t	= thickness of the section
V	= bilateral putamen volume [cm³]
x	= age [years]

R_T is used as in the Eggers' formula (1989b).

We calculated (N_Q) [6], the ratio between small (nerve cell types 1, 2, 5, 6 and 7) and large neurones (nerve cell types 3 and 4) (Foix et al. 1925; Namba 1957; Peele 1961; Treff 1964; Tabuchi 1969; Dom et al. 1973; Lange et al. 1974; Bøttcher 1975; Bugiani et al. 1978). These parameters were determined for each evaluated putamen and for each neurone type (type groups as well as all types together). The age changes were analysed by linear regression.

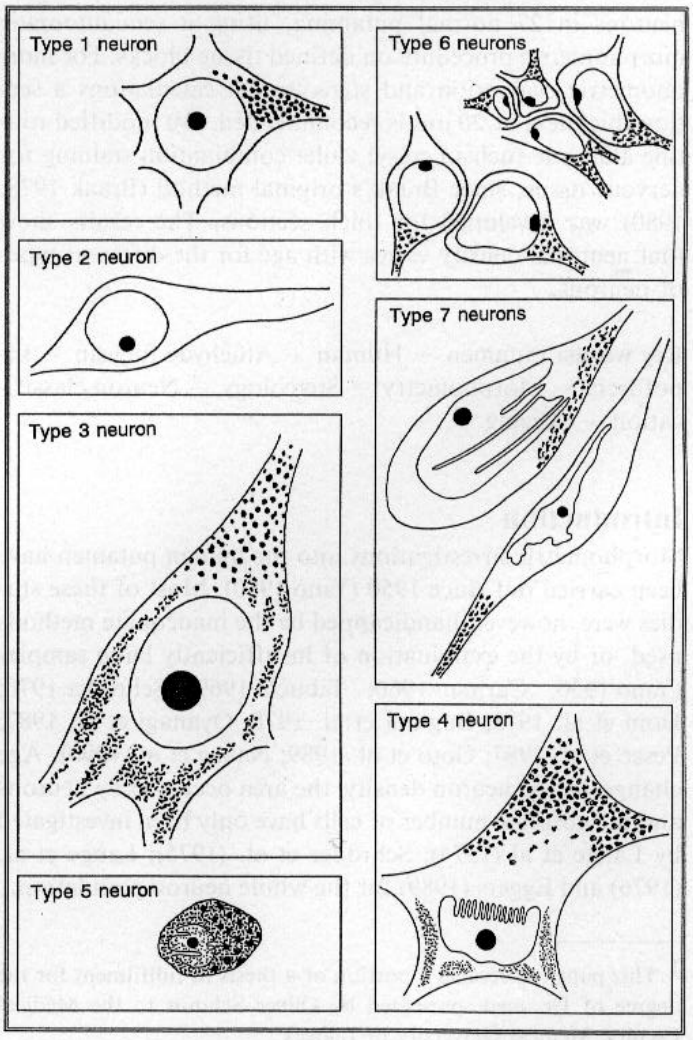


Fig. 1. Semischematic drawings of the seven neuron types found in the human putamen after the AFC procedure (modified after Braak (1982) and broaden after Schmitt (1991).

[1]) The constant (1000) was used because N_v is expressed in mm³ and V in cm³.

Results

During the initial measurements we found 2 new types of neurons in addition to the 5 types described by Braak (1982). These are types 6 and 7 (Fig. 1 and 2).

The small type 6 neurones possess a special feature, in that they lie close together in groups of 2 or 3 cells. They show the typical configuration of neurons, having a nucleolus within the nucleus, and a small perinuclear region of cytoplasm which contains a small number of neurolipofuscin granules (Figs. 2f and 2h).

The type 7 cells were sparsely distributed. They are as large as the type 3 or 4 neurons, and have an extensively lobulated nucleus. They are mostly spindle-shaped and possess a richly pigmented cytoplasm (Fig. 2g)

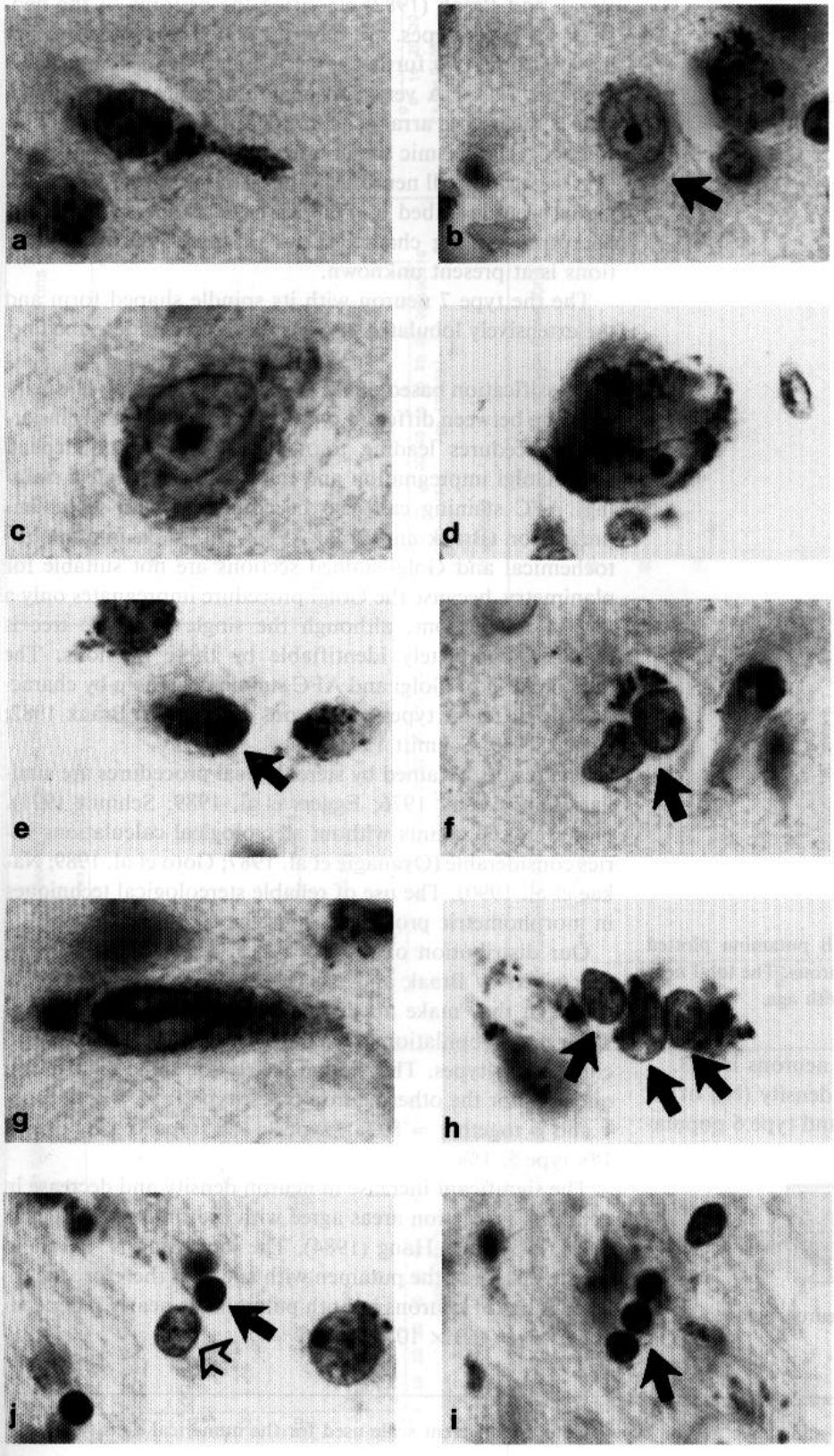


Fig. 2. Microphotographs of the seven neuron types, the cluster phenomenon of type 6 neurons and glia cells for comparation with type 6 neurons in the human putamen after the AFC procedure (× 1260). a) Type 1 neuron, b) Type 2 neuron, c) Type 3 neuron, d) Type 4 neuron, e) Type 5 neuron, f) two clustered type 6 neurons, g) Type 7 neuron, h) three clustered type 6 neurons, i) three clustered oligodendroglia cells, j) a oligodendroglia cell (black arrow) and a astroglia cell (open arrow). Glia cells were not evaluated. They are presented here in order to compare them morphologically with the new type 6 neuron.

We calculated the total number (N) of all neuron types and type groups in the putamina of both hemispheres. Fig. 3 shows the total number (N) of all neuron types and of type 6 cells. The total number (N) consists mainly of type 1 cells (61%), while type 6 (28%) constitutes the next biggest population (Fig. 4). The mean total number of neurons (160×10^6) remains nearly constant as age increases.

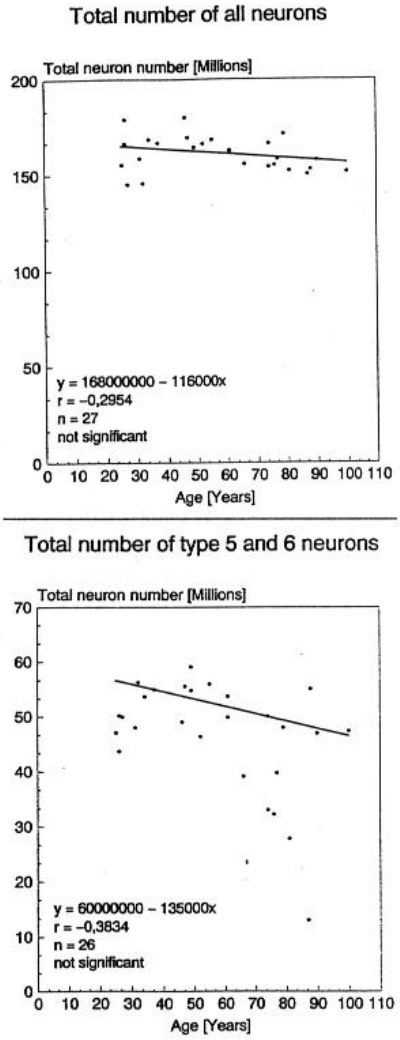


Fig. 3. Total number of neurons (N) in both putamina plotted against age. a) All neuron types. b) Type 6 neurons. The total neuron number does not correlate significantly with age.

The mean ratio (N_Q) of small to large neurons is 143.

We found a significant increase in the density (N_v) of *all neurons* with age, and also in the type 1 and type 6 populations (Fig. 5). The mean projection areas of the 1, 2, 4 and 5 neuron populations decrease significantly with age (Fig. 6).

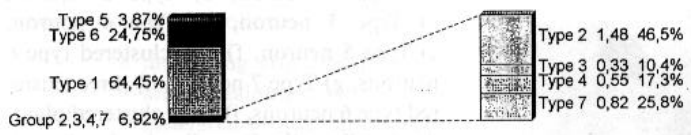


Fig. 4. The relative frequencies of different neuron types (c) in 13 brains.

Haug (1984) found as well a increasing density and decreasing area of certain neuron types in the cerebral cortex.

Discussion

Braak and Braak (1982) classified the neurons of the neostriatum into 5 types. We have expanded this classification by describing two further types of neurons in the putamen (Schmitt 1991). A very conspicuous feature of the type 6 cells is their close arrangement in groups of 2 to 3 cells with frequent cytoplasmic membrane contacts. A close association between small neurons in the striatum of the rat has already been described by Dimova (1980). The synaptic and neurotransmitting characteristics of these neuron populations is at present unknown.

The the type 7 neuron with its spindle shaped form and the extensively lobulated nucleus has not been described before.

Classification based on AFC staining is one way of distinguishing between different types of neurons. Further important procedures leading to neuron classification depend upon Golgi impregnation and immunohistochemical staining. AFC staining can also be combined with Golgi impregnation (Braak and Braak 1982). However, immunohistochemical and Golgi-stained sections are not suitable for planimetry, because the Golgi procedure impregnates only a few of all neurons, although the single dendritic tree is rendered completely identifiable by these methods. The combination of Golgi and AFC staining can help by characterising different types of neurons (Braak and Braak 1982; Eggers 1989; Schmitt 1991).

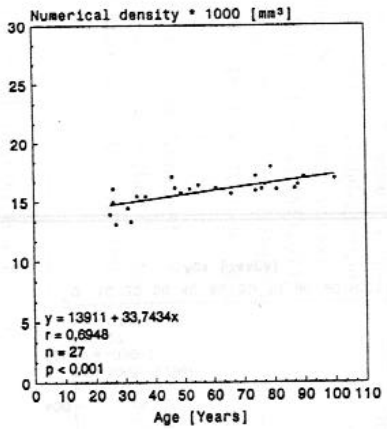
The results obtained by stereological procedures are similiar (Lange et al. 1976; Eggers et al. 1989; Schmitt 1991). However, cell counts without stereological calculations varies considerable (Oyanagie et al. 1987; Goto et al. 1989; Nakae et al. 1990). The use of reliable stereological techniques in morphometric procedures must be recommanded.

Our distribution of the cell types is nearly the same as that given by Braak and Braak (1982) and Groves (1983), although they make no distinction between types 1 and 6. Their main population of small to medium sized neurons includes both types. They found nearly the same relative frequencies for the other neuron types; type 1: 95% (our types 1 and 6 together ≈ 89%), type 2: 1%, type 3: 1%, type 4: 1% type 5: 1%.

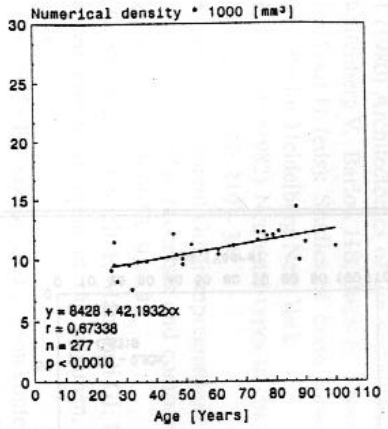
The significant increase in neuron density and decrease in neuronal projection areas agree with the findings of Eggers et al. (1987) and Haug (1984). The increasing N_v is due to the shrinkage of the putamen with age, and therefore the total number of neurons in both putamina is nearly independent of age (160×10^6).

Fig. 5. Numerical density (N_V) of all neurons and single neuron types. Note the different scale used for the numerical density. ▶

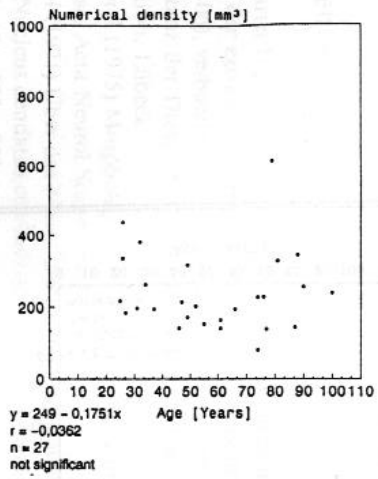
Numerical density of all neuron types



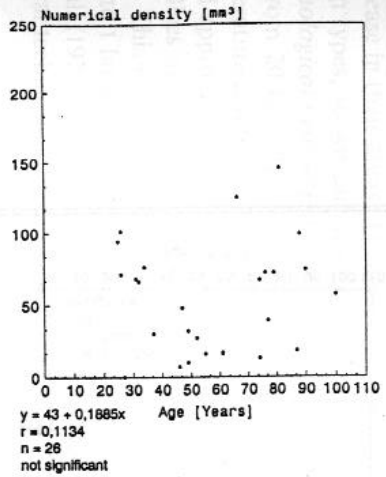
Numerical density of type 1 neurons



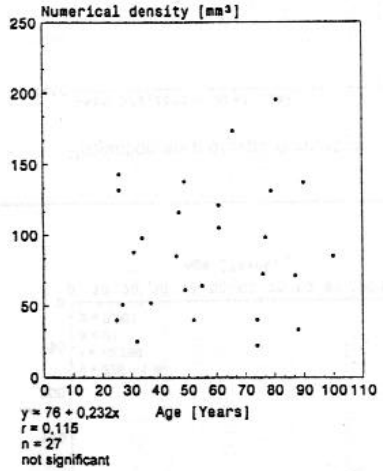
Numerical density of type 2 neurons



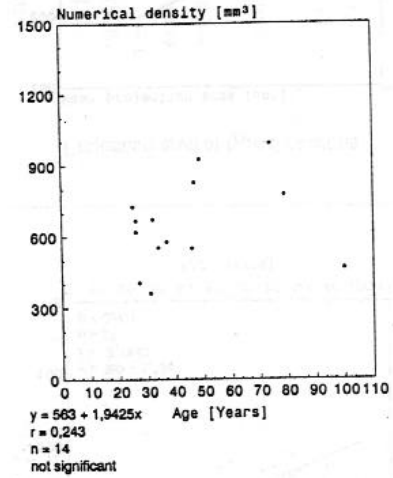
Numerical density of type 3 neurons



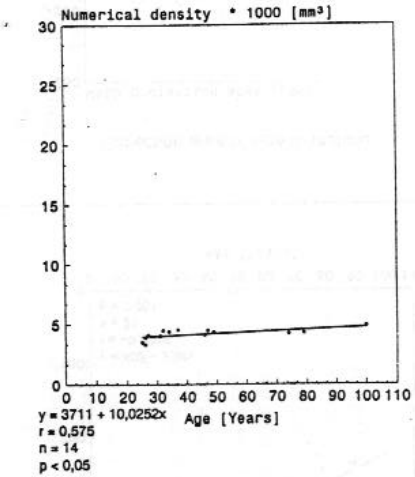
Numerical density of type 4 neurons



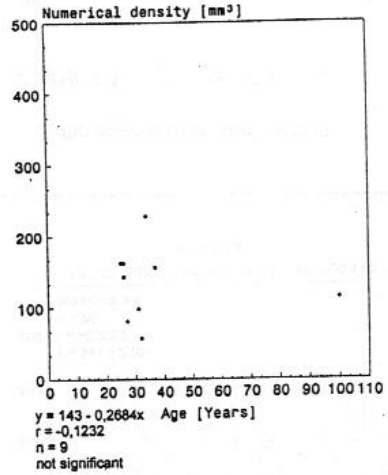
Numerical density of type 5 neurons



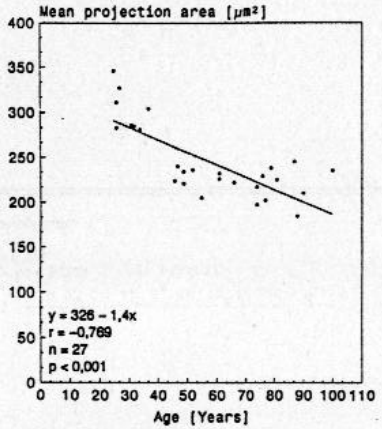
Numerical density of type 6 neurons



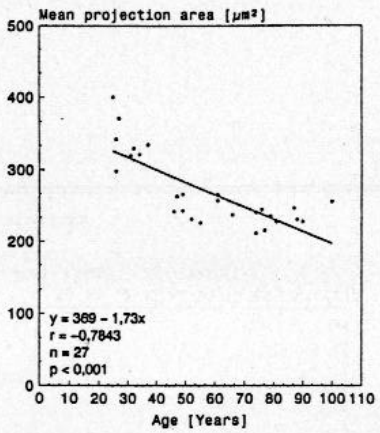
Numerical density of type 7 neurons



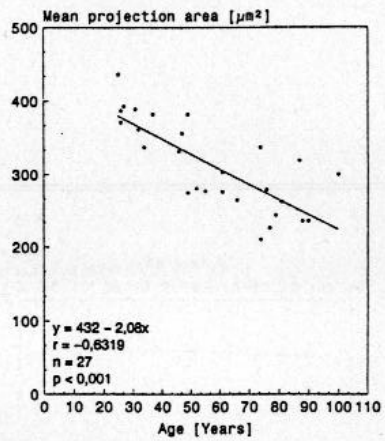
Projection area of all neuron types



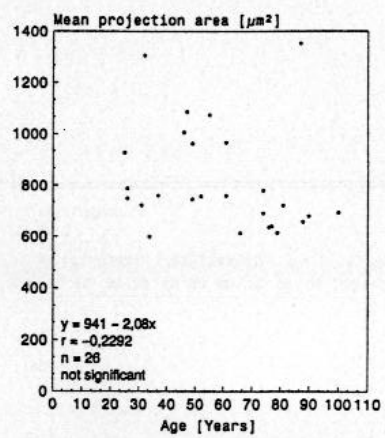
Projection area of type 1 neurons



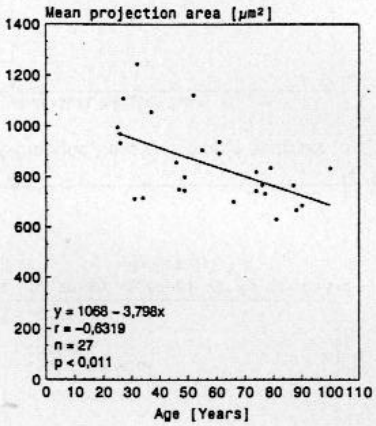
Projection area of type 2 neurons



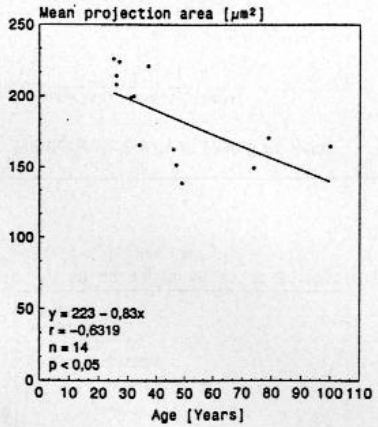
Projection area of type 3 neurons



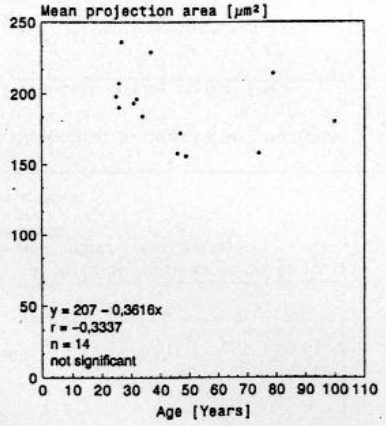
Projection area of type 4 neurons



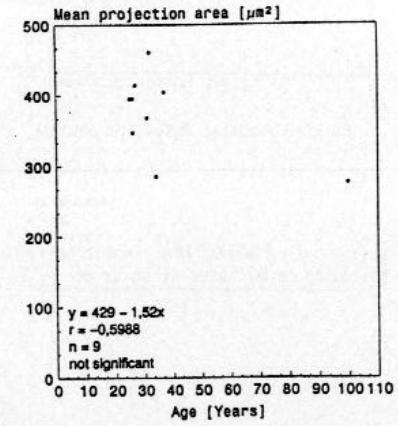
Projection area of type 5 neurons



Projection area of type 6 neurons



Projection area of type 7 neurons



In addition to the significant density increase with age of types 1 and 6 (90% of all neurons), types 1, 2, 4 and 5 show a decrease in their areas. This response was not found in all neuron types, so we can describe it as a normal *"selective morphological neuron population aging"*. The high variability (from 20 to 270) of the neuron ratio (N_Q) reported in the literature can be attributed to the failure to use a stereological approach. It should be mentioned that large cells in thin slices are sectioned many times, but small ones only rarely. This leads to an error in counting. Our value of 143 small to 1 large neuron lies close to the values given by Lange et al. (1974) and Dom et al. (1973), who also used a stereological technique.

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◀ Fig. 6. Mean projection areas (A_A) of all neurons and certain neuron types.

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