Scanning Microscopy

Volume 2 | Number 2 Article 36

11-23-1987

Quantitative Morphology of Synaptic Plasticity in the Aging Brain

Carlo Bertoni-Freddari
Gerontological Research Department of the INRCA

William Meier-Ruge
University of Basel

Jurg Ulrich
University of Basel

canning Microscopy, Vol. 2, No. 2, 1988 (Pages 1027-1034) 0891-7035/88$3.00+.00
canning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

QUANTITATIVE MORPHOLOGY OF SYNAPTIC PLASTICITY IN THE AGING BRAIN.

Carlo Bertoni-Freddari*, William Meier-Ruge[1] and Jurg Ulrich[1].

Center of Cytology, Gerontological Research Department of the INRCA, Via Birarelli, 8 60121 Ancona, Italy; [1]Division of Gerontological Brain Research, Department of Pathology, University of Basel, CH-4003 Basel, Switzerland.

(Received for publication April 10, 1987, and in revised form November 23, 1987)

ABSTRACT

Quantitation of synaptic ultrastructural changes is of great importance in neurobiology, since merely qualitative alterations, if not extreme, are not readily detectable. In the present paper we discuss our previous and present findings on the number (numerical density: Nv), size (average length of the synaptic profiles: L) and surface contact area (surface density: Sv) of the synaptic junctions in aging rodent and human brains. We found that number and size of the synapses are in a close inverse relationship so as to maintain the total surface contact area among the nerve cells constant. These three parameters are closely related to each other, their quantitation may thus represent a reliable index of the morphological aspects of synaptic plasticity, i.e. the modification of ultrastructure occurring at synaptic membranes after transient changes in synaptic activity. During aging, the morphological plasticity of synapses appears to be seriously impaired: the number of synapses and the total surface contact area among the nerve cells are markedly reduced. However, old nerve cells seem to retain the ability to modify their synaptic endings and to partially compensate for the reduced surface density of the contact zones by expanding the average size of the persisting junctions. Our recent studies on synaptic plasticity in human brains from old and demented subjects showed that while the size of the synaptic contacts remains constant, the numerical and surface densities undergo a further decrease in demented brains relative to that in normal aging.

Key words: Morphometry of synaptic junctions, E-PTA staining, Aging of human hippocampus, Senile dementia, Synaptic plasticity, Aging of synaptic membranes, Cerebellar glomerulus, Aging of the cholinergic system.

*Address for correspondence:
C. Bertoni-Freddari, Center of Cytology, Gerontological Research Department I.N.R.C.A., Via Birarelli 8, 60121 ANCONA, Italy.

Phone No. 71/589991

Introduction

The study of synaptic ultrastructure is of great importance in neurobiology, since modification of synaptic morphology has been reported to be involved in learning, memory, sensitization and other CNS functions (Lynch and Baudry, 1984; Cotman et al., 1981). Qualitative descriptions of the ultrastructural features of synapses are, however, not sufficient to evidence changes, if any, between different groups of biological samples and, moreover, statistical comparisons cannot be performed.

Quantitative morphological methods and E.M. histochemistry of selected macromolecules offer the possibility to quantitate several defined biological parameters at synaptic regions (Bloom and Aghajanian, 1968; Vrensen and De Groot, 1974). The number of synaptic contacts per unit area (Na) or per unit volume, referred to as numerical density (Nv), together with the length of synaptic appositions (L) are frequently reported in the current literature (Nieto-Sampedro et al, 1982; Carlin and Siekevitz, 1983; Dyson and Jones, 1984; Greenough et al., 1985; Siekevitz, 1985; Bertoni-Freddari et al., 1986a;Geinisman et al.1986). With reference to quantitative cytochemistry, conventional OsO_4 staining or ethanol phosphotungstic acid (E-PTA) staining (Bloom and Aghajanian, 1968) are currently employed, depending on the morphological aspect of the synaptic endings to be investigated (Dyson and Jones, 1984).

Environmental factors, diet, pharmacological treatments, experimental lesions and time have been shown to induce significant and measurable changes of synaptic physiology, biochemistry and ultrastructure supporting the contention that synaptic junctions, although well differentiated areas of the neuronal membrane, are very plastic structures even in the fully developed adult nervous system (Cotman et al., 1981). The term synaptic plasticity serves to define such a dynamic condition of synapses and is used to indicate any persistent change (e.g. in potency, firing rate, efficacy, ultrastructure, etc.) resulting from transient changes in synaptic activity.

In the present paper we will discuss the morphological aspects of synaptic plasticity in the aging brain and cerebellum of rodents and of human beings, taking into account the data available in the literature and our own results on E-PTA stained synaptic junctions.

Materials and Methods

Animal studies.

Five female Wistar and five Fisher 344 rats of 3 (young), 12 (adult) and 28-30 (old) months of age were used.

Tissue pieces from brains and cerebella of decapitated animals were fixed for 24 hours at 4 °C in 2.5% phosphate buffered (pH 7.4) glutaraldehyde. The anatomical models used in our investigations were: (a) the cerebellar glomerulus (Wistar rats), a very well defined area in the granular layer of the cerebellar cortex receiving fibers using different types of neurotransmitter (Balasz et al., 1975) and (b) the supragranular layer of the right hippocampal dentate gyrus (Fisher 344 rats) which is reported to be innervated by cholinergic afferents arising in the septum (Mosko et al., 1973). Synaptic junctions were stained by means of the E-PTA preferential technique in slices (1mm thickness) of fixed tissue. Briefly, after rinsing in phosphate buffer, the tissue slices were dehydrated up to 95% alcohol. Subsequently they were incubated at 60 °C for 1 hour in 1% phosphotungstic acid in absolute ethanol (E-PTA) containing 5 drops of 95% ethanol/25 ml of staining solution. Embedding in Araldite was performed according to the conventional E.M. procedure.

Morphometry. Twenty one E.M. pictures were taken from 3 tissue samples of the same animal. The area of the tissue photographed at 8,300x and 6,700x, was 54,4 μm^2 and 89,7 μm^2 for the cerebellum and hippocampus, respectively. A quadratic lattice of 1 cm rule was printed on each photo (Fig. 1) and the number of intersection points of the synapses with the lines of the lattice as well as the overall number of synaptic contacts per picture were scored. The following synaptic parameters were calculated on each picture:

Sv = surface density (total surface area of the synaptic contact zones in a unit volume of tissue) obtained from: 2Ni/Lt; where Ni is the number of intersection points of the synapses with the lines of the quadratic lattice of total length Lt.

Nv = numerical density (number of synapses in a unit volume of tissue); the formula we used was: $(8/\pi^2)*(Na/L)$ where Na is the number of synapses per unit area and L is the average length of the synaptic profile.

(This formula appears now to be superseded by a new one for the dissector technique (Braengaard and Gundersen, 1986), nevertheless the results we got in our present and previous studies are in agreement with literature data reporting on this parameters in normal and pathological aging (Dyson and Jones, 1984; Davis et al., 1987).

L = average length of the single synapse, given from: La/Na, where La is the total length of the synaptic profiles per unit area calculated from: $(\pi/2)*(Ni/Lt)$.

The rough data obtained from these formulas were corrected for the overestimation due to the section thickness (Holmes'effect) by means of the following factor K = 1 + 3T/2L; where T is the section thickness of 0.06 μm. (For further details see Bertoni-Freddari and Giuli, 1980).

Human studies.

The supragranular layer of the hippocampal dentate gyrus from autoptic samples of five adult (mean age: 55.8 years), five old (mean age: 81.4 years) and five mentally impaired (mean age: 83.6 years) subjects was chosen for our studies. All the autoptic samples were cytologically examined to control both the preservation of the tissue and to confirm the diagnosis of senile dementia or normal aging on the basis of histopathological alterations such as number of senile plaques and neurofibrillary tangles.For a better preservation, the tissue pieces were fixed in a 2.5% glutaraldehyde + 1% formaldehyde phosphate buffered (pH 7.4) solution and processed for E-PTA staining and EM embedding according to the procedure described above for animal studies.

Morphometry. As above, 3 slices were obtained from each tissue sample and 7 E.M. pictures at 6,750x were taken from each slice. The image analyser ASBA (Wild + Leitz AG) was used to measure Sv, Nv and L on negatives of 39.76 μm^2 per picture. L was a direct measurement performed by the ASBA system on the negative, whereas Nv and Sv were calculated by the ASBA computer using the following formulas:

Nv = Na/L+T; where Na is the number of synapses per area and T is the section thickness.

Sv = Nv*S; where S is the average area of the single synapse and is calculated assuming the single contact a circular disc of mean radius $r = (\pi/4)*L$ (Bertoni-Freddari and Giuli, 1980).

Statistical comparisons,both for animal and human studies, were performed using the Student's t-test.

Results

The E-PTA technique, employed in our studies, is a preferential staining procedure for synaptic paramembranous material and allows visualization of pre- and postsynaptic appositions even in the developing nervous system. The morphological categorization of E-PTA stained synapses, proposed by Dyson and Jones (1976), states that the functioning contacts are those appearing either as two parallel black lines or as a dotted and a full line against a pale background (Figs. 1 and 2). For our quantitative investigations we took into account only these two morphological features of the synapses, i.e., those contacts displaying both pre- and post- synaptic appositions separated by a cleft.

Fig. 1 shows a rat cerebellar glomerulus stained by means of this technique. The quadratic lattice, printed above each photo, was used to calculate the synaptic morphometric parameters as explained in Material and Methods.

Tables 1 and 2 show the results we found in rat cerebellum and hippocampus during aging. In both these areas of the CNS we found very similar alterations. The number of synapses per unit volume of tissue (Nv) increased 12 and 20% between 3 and 12 months of age in the cerebellum was reduced by 25 and 32%. The average area of the single contact (L) changed in opposite direction to that of Nv: it decreased 7 and 10% in the cerebellum and hippocampus, respectively, in young rats relative to the values from the adult group and increased 7 and 17% in old animals. With regard to the surface density of the synaptic contact zones (Sv), no difference was found between young and adult animals in both the CNS areas investigated; however, in old rats significant decrease of this parameter was observed both in the cerebellum and hippocampus.

Fig. 2 shows an EM picture taken from a human dentate gyrus stained by means of the E-PTA technique. Although human brains were fixed both with 1% formaldehyde and 2.5% glutaraldehyde, no qualitative differences in E-PTA positive junctions were seen between human and rodent synapses.

In Table 3 the results on synaptic parameters in humans are presented. Numerical and surface density in adults were significantly higher than in old and demented subjects in which the average length of the single contact was higher than in adults. Due to the small number of brains used (5 per group), a statistically significant difference regarding the measured synaptic parameters was not evident between old and demented subjects; however, it is interesting to note the 16 and 17% decrease of Nv and Sv, respectively, in the demented relative to old subjects.

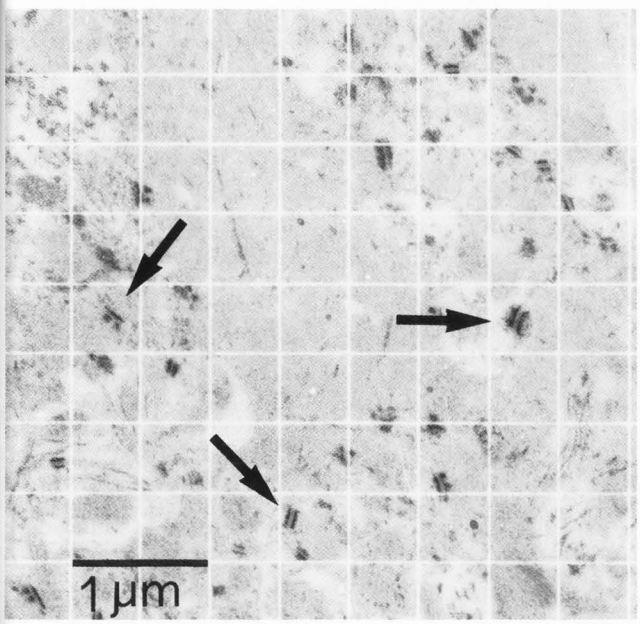

Fig. 1. Rat cerebellar glomerulus stained by means of the E-PTA preferential technique. Arrows: intersection points of the synapses with the l ines of the quadratic lattice used for our morphometric countings.

TABLE 1. Synaptic morphometric parameters from rat cerebellum. Mean ± S.E.M.

Age	Nv (Syn/µm^3)	L (µm)
3	1.6550 ± 0.0595	0.2241 ± 0.0058
12	1.8542 ± 0.0460	0.2090 ± 0.0035
28	1.3896 ± 0.0577	0.2229 ± 0.0051

Age	Sv (µm^2/µm^3)
3	0.1199 ± 0.0038
12	0.1213 ± 0.0021
28	0.0988 ± 0.0031

Statistical comparisons (Student's test):

Nv:	3/12	p<0.05	3/28	p<0.01
	12/28	p<0.001		
L :	3/12	p<0.02	3/28	N.S.
	12/28	p<0.02		
Sv:	3/12	N.S.	3/28	p<0.001
	12/28	p<0.001		

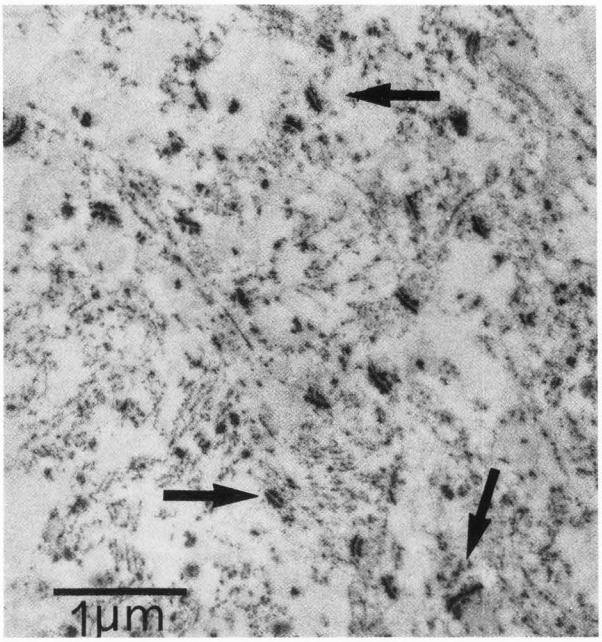

Fig. 2. Human dentate gyrus: E-PTA staining after double fixation with formaldehyde 1% + glutaraldehyde 2.5%. Arrows: synaptic endings.

TABLE 2. Synaptic morphometric parameters from rat hippocampus. Mean ± S.E.M.

Age	Nv (Syn/µm^3)	L (µm)
3	0.4723 ± 0.0091	0.3146 ± 0.0030
12	0.5637 ± 0.0094	0.2825 ± 0.0026
30	0.3810 ± 0.0073	0.3309 ± 0.0028

Age	Sv (µm^2 /µm^3)
3	0.0720 ± 0.0012
12	0.0695 ± 0.0011
30	0.0650 ± 0.0012

Statistical comparisons: (Student's t-test):

Nv:	3/12	p<0.001	3/30	p<0.001
	12/30	p<0.001		
L :	3/12	p<0.001	3/30	p<0.01
	12/30	p<0.001		
Sv:	3/12	N.S.	3/30	p<0.001
	12/30	p<0.001		

TABLE 3. Synaptic morphometric parameters from human dentate gyrus. Mean ± S.E.M.

	Nv (Syn/μm^3)	L (μm)
Adult	1.5991 ± 0.1331	0.2425 ± 0.0043
Old	0.7457 ± 0.0773	0.3089 ± 0.0120
Dement	0.6270 ± 0.0270	0.3002 ± 0.0084

	Sv ($\mu m^2/\mu m^3$)
Adult	0.1798 ± 0.0097
Old	0.1319 ± 0.0064
Dement	0.1083 ± 0.0081

Statistical comparisons (Student's t-test):

Nv: Adult/Old	$p<0.001$
Adult/Dement	$p<0.001$
Old/Dement	N.S.
L : Adult/Old	$p<0.001$
Adult/Dement	$p<0.001$
Old/Dement	N.S.
Sv: Adult/Old	$p<0.01$
Adult/Dement	$p<0.01$
Old/Dement	N.S.

Discussion

Quantitation of synaptic ultrastructural features is of growing and relevant interest in the field of neurobiology since qualitative changes, if not extreme, are difficult to detect and compare. In performing quantitative studies of structural dynamics at synaptic regions, methodological problems deserve consideration.

Shrinkage of the tissue during embedding process is well known and has been recently quantitated by Haug et al. (1984), therefore careful attention must be paid to this step. Usually fixation is carried out employing glutaraldehyde alone at concentrations varying from 1 to 5% for different times and temperatures. Glutaraldehyde is a good fixing means because of its 2 aldeidic groups, but it is a big molecule and , as a consequence, it does not penetrate easily into the biological samples. Moreover, while the surface of the tissue is fixed in a few minutes, to fix the core of the sample it takes hours or days depending on the thickness of the biological material. In our present investigation, to overcome the shrinkage problems due to embedding procedures, we fixed our human samples by means of a double fixing solution of 2.5% glutaraldehyde + 1% formaldehyde dissolved in phosphate buffer at physiological pH 7.4. Formaldehyde, has only one aldeidic group, but it is a small molecule and can easily penetrate and fix the empty places left by glutaraldehyde. In addition, slices of one mm thickness were cut after a few hours the tissue was immersed in the fixing solution, in order to facilitate the penetration of the aldehydes. At EM level, looking at nuclear bodies which are E-PTA positive, no shrinkage could be detected in our samples.

Staining of synapses by means of conventional osmium tetroxide methods offers the possibility to quantitate several parameters at the nerve endings, as number of synapses, number of vesicles, number and surface density of the mitochondria, area of the single terminal, etc.. However, the method is unspecific mainly due to the binding of OsO_4 to unsaturated fatty acids of the membrane. The evident drawback of this procedure is that the general increase in contrast of all the cellular organelles does not facilitate the identification of specific structures and to perform selective countings. In 1968 Bloom and Aghajanian introduced the selective E-PTA staining for the synapses: this procedure, although unable to evidence all the morphological features seen in OsO_4 stained tissue, has the advantage of being selective for the synaptic paramembranous material (Figs. 1 and 2). Ultrastructural features of E-PTA stained junctions are qualitatively different from those seen in osmium tetroxide treated tissue, but, in fact they represent the same active zones and, in addition, can be clearly visualized against the background. The specific sites of reaction of the E-PTA staining are reported to be basic proteins and the fact that the pre- and postsynaptic densities are highly positive to this compound, has suggested that synaptic junctions are very rich in basic amino acids (Pfenninger, 1971).

With regard to the methods of investigation in quantitative synaptology, the morphometric procedures introduced by Weibel (1979) are currently employed both for quantitation

of OsO_4 and E-PTA stained junctions to measure those parameters which can undergo changes under different experimental or environmental conditions: synaptic size and number being the most investigated ones (Bondareff and Geinisman, 1976; Glick and Bondareff, 1979; Greenough et al., 1978; Fisher et al., 1979; Geinisman et al. 1986). A major criticism which may be raised against such studies is that generally they consider single parameters of synapses (Curcio and Hinds, 1983; Scheff et al., 1985) and even when several data are determined a proper discussion correlating one parameter with another is lacking. Such correlations are important in studies on synaptic plasticity, since changes of synaptic morphology are not independent, but may influence each other as has been demonstrated in hypoxia, malnutrition, and aging (Fisher et al., 1979; Chen and Hillman, 1980; Hillman and Chen, 1981; Bertoni-Freddari et al., 1986a,b). As a matter of fact, the morphological aspect of synaptic plasticity involves so many ultrastructural features of the junctional zones that it should be tested by measuring closely related parameters. We (Bertoni-Freddari and Giuli,1980) and Hillman and Chen (1981) have found that number, size and total surface area of the synaptic contact zones, i.e. Sv, are in a close relationship and, taken together as a group, may represent a reliable measure of the dynamic morphology of synaptic endings. In particular, in our studies on aging of the animal brain we demonstrated time-related morphological rearrangements of the synaptic Nv, L and Sv which support the contention that different periods during the life of a given organism are characterized by specific patterns of synaptic connectivity (Bertoni-Freddari et al., 1986b). As summarized in Table 1 and 2, in the young CNS (3 months) there is a high number of large contacts which provide a high total contact area both in the cerebellum and hippocampus. During adulthood (12 months) nerve cell contacts are higher in number, and although their size is significantly reduced, the resulting Sv is constant as compared to young rats. We think that this constancy in total synaptic contact area indicate that the overall synaptic connectivity is well established in the first months of life and is maintained constant throughout adulthood. Aging appears to be characterized by a marked reduction in Sv which is due to a decreased number of contacts although the synaptic size is increased. We interpret these data as a partially impaired, time-related adaptive capacity of old CNS which becomes evident when compensatory phenomena are envisaged in the increase of the average length of the single contact.

Our data on old human hippocampi (Table 3) appear to confirm the results in rodents: number and total contact area of the synapses are decreased whereas their length is increased as compared to adult brains. Furthermore, relative to adult values, reductions in the demented group regarding Nv and Sv are markedly higher than in age-matched hippocampi; however, these alterations did not attain statistical significance. The present findings are compatible with biochemical results on human brains indicating a marked (60-80%) decrease in cholineactyltransferase (CAT) activity in old vs. adult hippocampi and no difference between old and demented samples (Davies, 1979). A possible explanation for the similarity of Davies' and our data lies in the fact that the area we sampled is known to be cholinergic, and therefore the 60% reduction we found in the number of synapses may well represent the morphological aspect of age as well as pathology-dependent decrease in CAT activity. Our present results must however be taken as preliminary, since the number of samples was too small (5 brains/group) to perform statistical comparisons.

Turnover of the synaptic junctions (rearrangement and/or replacement of the contacts) is an ongoing process which takes place throughout the organism's lifespan (Cotman and Nieto-Sampedro, 1984). Consistent ultrastructural findings, complemented by biochemical data on the composition of synaptic plasma membranes, have been reported in different conditions to support this plastic state of the synaptic contacts (Dyson and Jones, 1976; Adams and Jones, 1982; Guldner and Phillips, 1986). In particular, it has been clearly demonstrated that the rate of modification of synaptic ultrastructure is dependent both on the duration and the intensity of the stimulation (Greenough et al., 1978; Cotman et al., 1981; Dyson and Jones, 1984). Moreover, perforated postsynaptic densities have been shown to play a role in synaptic plasticity (Nieto-Sampedro et al., 1982; Siekevitz, 1985; Geinisman et al., 1986) supporting the contention that synaptic connectivity (mainly the number of contacts) can be modified by splitting mechanisms taking place at the larger contact zones (Carlin and Siekevitz, 1983; Dyson and Jones, 1984).

Our recent data (Bertoni-Freddari et al., 1986a) on synaptic plasticity in rat dentate gyrus at 3, 12 and 30 months of age seem to be in agreement with the above statement, since, while the total area of synaptic contact zones is the same in young and adult rats, in the latter group this area is fragmented into junctional zones of smaller size. With regard to human studies during normal and pathological aging, the present results on synapses confirm and further extend the data from Davies et al. (1987) reporting a 55% decrease in the number of contacts in the temporal cortex of demented patients. The consistent enlargement of the synaptic average area found by us both during aging and dementia, on one side appears to represent the 'synaptic aspect' of Buell and Coleman's data (1981) showing an increased axonal sprouting in the old vs. adult human brain; on the other side supports the idea that, although impaired, synaptic plasticity is retained by nerve cells also in pathological conditions (Geddes et al., 1985). To explain the pathogenesis and progression of Alzheimer's disease and senile dementias, a hypothesis has been recently formulated regarding the role played by the increased deterioration of neuronal membranes in the development of such diseases (Bertoni-Freddari, 1987). The main statement of this hypothesis is that if the membranes of the nerve cells are damaged beyond a defined individual threshold, dementia is clinically evident.We are aware that such a concept appears, at present, speculative since a lot of heterogenous histopathological cellular alterations (neuronal loss, neurotransmitters deficits, changes in receptor binding properties, etc...) have been found in the demented brains. Nevertheless a rationale has been proposed for the involvement of neuronal membrane deterioration in such apparently membrane independent changes (see for details Bertoni-Freddari, 1987). In this respect, keeping in mind that synaptic junctions are very sensitive and vulnerable sites, our present data lend further support to the crucial role played by the plasma membrane structure integrity in normal and pathological aging.

The data on quantitative morphology of synaptic junctions, mainly the number of synapses per area or volume, in the aging brain, both in animal (Klein, 1983; McWilliams and Lynch, 1984; Rogers et al., 1984; Bertoni-Freddari et al., 1986a, 1986b; Hinds and MacNelly, 1979; Hoff et al., 1982) and human samples (Cragg, 1975; Huttenlocker, 1979) are controversial, and this discrepancy has been discussed in recent reports (Curcio and Hinds, 1983; Scheff et al., 1985). We feel that several factors can influence synaptic countings. Therefore, in such studies, it is essential to present a proper protocol and strictly follow it. First, according to Weibel's morphometric criteria, the area to be investigated must be

readily identifiable in all biological samples. Such an area besides being devoid of any structural anisotropy, should be physiologically well characterized in order to correlate the data to specific biological functions. Second, the ultrastructure of synapses should be clearly visualized using specific staining procedures for the active zones. Third, in order to get a reliable measure of changes occurring at the ultrastructure level, other parameters in addition to the number and/or length of contacts should be quantified. A comparison of data from different studies should take into account the differences in the methodology employed in these investigations. In our studies we have always fulfilled the criteria listed above and consistently found an age-dependent decrease of number of contacts in different areas of rodent and human brain, thus supporting the idea that synaptic morphological alterations, including the number of junctions, are generally characteristic phenomena of the aging CNS. Synaptic plasticity is itself a generalized phenomenon expressed at biochemical, physiological and behavioural levels (Cotman et al., 1981). Our previous and present findings represent the morphological aspect of synaptic plasticity during aging.

References

Adams I, Jones DG. (1982). Quantitative ultrastructural changes in rat cortical synapsed during early-, mid- and late-adulthood. Brain Res. 239, 349-363.

Balasz R, Hajos F, Johnson AL, Reynierse GLA, Tapia G, Wilkin G. (1975). Subcellular fractionation of rat cerebellum: an electron microscopic and biochemical investigation. III. Isolation of large fragments of cerebellar glomeruli. Brain Res. 86, 17-30.

Bertoni-Freddari C, Giuli C. (1980). A quantitative morphometric study of synapses of rat cerebellar glomeruli during aging. Mech. Dev. 12, 127-136.

Bertoni-Freddari C, Giuli C, Pieri C, Paci D. (1986a). Quantitative investigation on the morphological plasticity of synaptic junctions in rat dentate gyrus during aging. Brain Res. 366, 187-192.

Bertoni-Freddari C, Giuli C, Pieri C, Paci D. (1986b). Age-related morphological rearrangements of synaptic junctions in the rat cerebellum and hippocampus. Arch. Geront. Geriatr. 5, 297-304.

Bertoni- Freddari C. (1987). Age-dependent deterioration of neuronal membranes and the pathogenesis of Alzheimer's disease: a hypothesis. Medical Hypotheses, In Press.

Bloom FE, Aghajanian GK. (1968).Fine structural and cytochemical analysis of the staining of synaptic junctions with phosphotungstic acid. J. Ultrastruct. Res. 22, 361-375.

Bondareff W, Geinisman Y. (1976). Loss of synapses in the dentate gyrus of the senescent rat. Am. J. Anat. 145, 129-136.

Braendgaard H, Gundersen HJG. (1986). The impact of recent stereological advances on quantitative studies of the nervous system. J. Neurosci. Meth. 18, 39-78.

Buell SJ, Coleman, PD. (1981). Quantitative evidence for selective dendritic growth in normal human aging but not in senile dementia. Brain Res. 214, 23-41.

Carlin RK, Siekevitz P. (1983). Plasticity in the central nervous system: do synapses divide? Proc. Natl. Acad. Sci. U.S.A. 80, 3517-3521.

Chen S, Hillman DE. (1980). Giant spines and enlarged synapses induced in Purkinje cells by malnutrition. Brain Res. 187, 487-493.

Cotman CW, Nieto-Sampedro M, Harris EW. (1981). Synapse replacement in the adult system of adult vertebrates. Physiol. Rev. 61, 684-784.

Cotman CW, Nieto-Sampedro M. (1984). Cell biology of synaptic plasticity. Science 225, 1287-1294.

Cragg BG. (1975). The density of synapses and neurons in normal, mentally defective and aging human brains. Brain 98, 81-90.

Curcio CA, Hinds JW. (1983). Stability of synaptic density and spine volume in dentate gyrus of aged rats. Neurobiol. Aging 4, 77-87.

Davies P. (1979). Neurotransmitter-related enzymes in senile dementia of the Alzheimer type. Brain Res. 171, 319-327.

Davies CA, Mann DMA, Sumpter PQ, Yates PO. (1987). A quantitative morphometric analysis of the neuronal and synaptic content of frontal and temporal cortex in patients with Alzheimer's disease. J. Nerol. Sci. In Press.

Dyson SE, Jones DG. (1984). Synaptic remodelling during development and maturation: junction differentiation and splitting as a mechanism for modifying connectivity. Brain Res. Dev. 13, 125-137.

Dyson SE, Jones DG. (1976). The morphological categorization of developing synaptic junctions. Cell Tiss. Res. 167, 363-371.

Fisher J, Langmeier M, Trojan J. (1979). Shortening of presynaptic dense projections produced in the synapses of rat cerebral cortex by prolonged repeated hypoxia in early ontogenesis. Physiol. Bohemoslov. 29, 93-98.

Geddes JW, Monaghan DT, Cotman CW, Lott IT, Kim RC, Chang Chu H. (1985). Plasticity of hippocampal circuitry in Alzheimer's disease. Science 230, 1179-1181.

Geinisman Y, De Toledo-Morrel L, Morrell F. (1986). Loss of perforated synapses in the dentate gyrus: morphological substrate of memory deficit in aged rats. Proc. Natl. Acad. Sci. U.S.A. 83, 3027-3031.

Glick R, Bondareff W. (1979). Loss of synapses in the cerebellar cortex of the senescent rat. J. Geront. 34, 818-822.

Greenough WT, West RW, DeVoogt TJ. (1978). Subsynaptic plate perforations: changes with age and experience in the rat. Science 202, 1096-1098.

Greenough WT, Hwang HMF, Gorman C. (1985). Evidence for active synapse formation or altered postsynaptic metabolism in visual cortex of rats reared in complex environments. Proc. Natl. Acad. Sci. U.S.A. 82, 4549-4552.

Guldner FH, Phillips SC. (1986). Structural plasticity of synaptic contacts in the central nervous system. In: Current Topics in Research on Synapses, Vol. 3 Alan R. Liss, Inc. New York, 147-169.

Haug H, Khl S, Mecke E, Sass NI, Wasner K. (1984). The significance of morphometric procedures in the investigation of age changes in cytoarchitectonic structures of human brain. J. Hirnforsch. 25, 353-374.

Hillman DE, Chen S. (1981). Vulnerability of cerebellar development in malnutrition-II. Intrinsic determination of total synaptic area on Purkinje cell spines. Neurosci. 6, 1263-1275.

Hinds JW, MacNelly NA. (1979). Aging in the rat olfactory bulb: quantitative changes in mitral cell organelles and somatodendritic synapses. J. Comp. Neurol. 184, 811-820.

Hoff SF, Scheff SW, Benardo LS, Cotman CW. (1982). Lesion-induced synaptogenesis in the dentate gyrus of aged rats: I. Loss and reaquisition of normal synaptic density. J. Comp. Neurol. 205, 246-252.

Huttenlocker (1979). Synaptic density in human frontal cortex-Developmental changes and effects of aging. Brain Res. 163, 195-205.

Klein AW. (1983). Synaptic density correlated with maze performance in young and aged rats. A preliminary study. Mech. Age. Dev. 21, 245-255.

Lynch G, Baudry M. (1984). The biochemistry of memory: a new and specific hypothesis. Science. 224, 1057-1063.

McWilliams JR, Lynch G. (1984). Synaptic density and axonal sprouting in rat hippocampus: stability in adulthood and decline in late adulthood. Brain Res. 294, 152-156.

Mosko S, Lynch G, Cotman CW. (1973). The distribution of septal projections to the hippocampus of the rat. J. Comp. Neurol. 152, 163-174.

Nieto-Sampedro M, Hoff SF, Cotman CW. (1982). Perforated postsynaptic densities: probable intermediates in synapses turnover. Proc. Natl. Acad. Sci. U.S.A. 79, 5718-5722.

Pfenninger K. (1971). The cytochemistry of synaptic densities. J. Ultrastruct. Res. 34, 103-122.

Rogers J, Zornester SF, Bloom FE, Mervis RE. (1984). Senescent microstructural changes in rat cerebellum. Brain Res. 292, 23-32.

Scheff SW, Anderson KJ, DeKosky TS. (1985). Strain comparison of synaptic density in hippocampal CA1 of aged rats. Neurobiol. Aging 6, 29-34.

Siekevitz P. (1985). The postsynaptic density: a possible role in long-lasting effects in the central nervous system. Proc. Natl. Acad. Sci. U.S.A. 82, 3494-3498.

Vrensen G, De Groot D. (1974). Quantitative stereology of synapses: a critical investigation. Brain Res. 58, 25-35.

Weibel ER. (1979). Stereological Methods. Vol. 1. Practical methods for biological morphometry. ER Weibel (ed). Academic Press. New York. 100-120.

Discussion with Reviewers

T. M.B. Bushmann and R.J. Bushmann: The p values that you report are not correct because you are using multiple t-tests. Bonferroni probabilities would be more acceptable. Better yet, why don't you use analysis of variance, which is the appropriate statistical test for the multiple groups that you are comparing?
Authors: We report the p values for the Student's t-test. We agree with the fact that ANOVA is a good statistical test, nevertheless we are mainly interested in showing differences between paired samples, as for instance adult vs. old and old vs. dement therefore we preferred the Student's test.

O.J. Castejon: Could you differentiate between synaptic active zones and dendro-dendritic attachment plaques in the cerebellar glomerulus using E-PTA staining? The lower arrow in Fig. 1 seems to indicate the short symmetrical contact between granule cell dendrites and not the axodentritic contact between the mossy fiber and granule cell dendrite.
Authors: No, we cannot differentiate between synaptic active zones and dendro-dendritic attachment plaques since E-PTA staining does not allow any identification of the type of transmitter used or of the kind of synaptic contact.

O.J. Castejon: The synaptic parameters in demented subjects are really interesting. What do you mean by demented subjects? Which clinical diagnoses are involved? What specific tests were involved in these patients?
Authors: The demented state was diagnosed by physicians during the life of the individual, by means of neuropsychologic testing. However in all the cases we investigated at EM level the demented state was ascertained at histopathological level (No. of plaques and NFT containing neurons) after death.

Reviewer III: For animal study $Nv=(8/\pi^2)*(Na/L)$. For human study $Nv=Na/(L+T)$. Shouldn't in the second equation be L? We do not see how the Nv calculated in the animal studies can be the same as the Nv calculated in the human studies? The equation appears to use the same terms but in different relationship such that they cannot be calculating the same Nv.

Authors: With regard to the formula used to calculate Nv in animal and human studies,they are equivalent,as can be easily proved giving Na=10; T=0.06 and L=0.025.
If $Nv = (8/\pi^2)*(Na/L)$ then is 32.455
If $Nv = Na/(L+T)$ then is 32.256 (0.6% less)
The only reason we used a different formula in human studies in that it is simpler than the previous one and can be easily introduced in the ASBA program.

Reviewer III: A major problem exists when interpreting the values of parameters like Nv and Sv as changes in number or area of synaptic contact. The problem arises from the relative nature of these parameters, that is, these are concentrations or densities, and their values are based not only on the absolute number and surface areas of synapses in the brain but also on the volume of the reference space chosen. Statistically significant differences in Sv and Nv will be detected when the reference volume alone changes (shrinks or swells) differentially from age to age. An age-dependent, differential

shrinkage during tissue processing has already been documented (see Haug et al. J. Hirnforschung 25: 353-374, 1984). Our question, then, is how can plasticity be studied from data that are only expressions of concentration changes?
Authors: To avoid shrinkage of the tissue in the samples studied, we used two fixing media: 2.5% glutaraldehyde and 1% formaldehyde, at variance with the E-PTA method from Bloom and Aghajanian (1968). a full explanation of the reason we employed this double fixation is given in the discussion. However, if we consider that an age-dependent shrinkage in the dentate gyrus molecular layer has been demonstrated (Geddes et al. Science Vol. 230, 1179-1181, 1985) our age-dependent decrease in the number of synapses and related alterations is to be considered an underestimation of the real situation in the living tissue.

Reviewer III: In your studies, the equation Sv = Nv*S is used. S is the average surface area of assumably circular, synaptic contacts and is derived from L. L is the average of the synaptic lengths which are measured directly on micrographs. Doesn't section thickness cause a significant overestimation of L and therefore Sv?
Authors: In the text when we say that L is measured directly on micrographs we mean that the ASBA system has been programmed to take into account the section thickness and the factor for Holmes' correction in the calculation of L.

A. Thureson-Klein: Regarding the hypothesis that a "neuronal membrane deterioration" underlies the pathogenesis of Alzheimer's disease and other forms of dementia, some alternate possibilities should be discussed. Is it possible to rule out that a considerable loss of neurons has occurred in the autopsy specimens, i.e. can the authors dismiss the "cholinergic hypothesis" as an important factor in Alzheimer's disease (Appel. Ann. Neurol. 10: 499-505, 1981)? The neuronal loss in severe dementia may be a primary event in various types (Arendt and Bigl, Neuroscience 17: 277-279, 1986) whether hereditary or induced.
Authors: We acknowledge that the cholinergic system may be an important factor in senile dementia, nevertheless, as reported by Bluszstain and Wurtman (Science 221: 614, 1983), the increased vulnerability of cholinergic neurons during aging and dementia appears to be due just to a self-deterioration process which seems to take place at neuronal membrane level.

A. Thureson-Klein: Microtubuli changes (Heston, 1981 in The Epidemiology of Dementia, Oxford Univ. Press), loss of somatostatin in the hippocampus, plaque formation in certain portions of the brain are other changes observed in patients with Alzheimer's disease. Do the authors have evidence for changes in the staining of these densities, particularly in old demented specimens?
Authors: From this question it is evident that a lot of systems and cellular organelles are involved in the pathogenesis and progression of the demented state. We are of the opinion that to explain the widespread diffusion in the CNS of cellular and tissue alterations, it is necessary to find out a ubiquitous factor which may be involved in such alterations: we believe that such a factor may well be the neuronal membrane.

A. Thureson-Klein: Since the staining by the authors is specific for the synaptic contact zones, it would be of interest to have a more detailed discussion of the various protein(s) and other components of the stained material. It has been suggested that the efficiency of synaptic transmission is reflected morphologically by the size of postsynaptic densities (Petukov and Popov, Neuroscience 18: 823, 1986). Do the authors have evidence for changes in the staining of these densities, particularly in old demented specimens?
Authors: Bloom and Aghajanian (1968), the researchers who introduced the E-PTA procedure, described in detail the staining properties of their method. No specific component of the synaptic functions is stained by the phosphotungstic acid which is believed to link better to proteins rich in basic amino acids. We have no evidence of changes in staining properties of the E-PTA solution in the different specimens we sampled.