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Development and Aging in the Nervous System

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PREFACE

This, the third publication of the proceedings of a series of symposia, held as part of the formal graduate level course work of the University of Miami Training Program in Cellular Aging, includes the papers presented by the participants in the Symposium on "Development and Aging in the Nervous System," held on February 19 and 20, 1973, in Miami, Florida.

The subject matter of this symposium is of special interest in view of the fact that the nervous system represents one of the two major organ systems, the cells of which are incapable of replacement, i.e., are postmitotic. Equally important is the recognition that the nervous system, both directly and indirectly, is concerned with regulation not only of motor activity, in the case of skeletal muscle, but equally so as regards regulation of the functioning of the heart and the blood vessels of the circulatory system, as well as all visceral smooth muscle, via the autonomic nervous system. Moreover, through its indirect endocrine function (i.e., in the release of epinephrine and norepinephrine), the last-mentioned represents a dramatically controlling element as regards virtually all visceral functions of the body.

In the above connection, aside from the recognized age-dependent degradatory changes which occur in the skin and in skeletal muscle with advancing age, the most striking "downhill" course with advancing time, both as regards structure and function, is seen in the sensory as well as motor functions of the human organism, which are directly related to changes in cell number with the passage of time.

Accordingly, the proceedings of this symposium have special significance in considering aging in its total sense, from embryonic development through senescence of a vital organ system of the body, both whose course as well as direction can be predicted for any species, with reasonable accuracy.

As regards the participants in this symposium, the Editor is proud to present this verbatim report of the symposium proceedings, in acknowledging the participation of speakers of the highest level of competency, representing a wide range of interests in neurobiology, including anatomy, morphology, embryology, enzymology and, including the clinical implications of age as a parameter in relation to normal and pathological functioning of the nervous system.

To Professor Eccles and Professor Richardson, as well as to the Chairman of the Department of Physiology and Biophysics, Professor Werner R. Loewenstein, go special thanks by the Editor for their valuable suggestions in the early organization of the program and in obtaining the participation of a number of the symposium speakers.

The Editor owes special thanks to Mrs. Estella Cooney for her careful attention to details in insuring the accuracy and clarity of the final published version of these proceedings and in the typing of the camera copy for its publication. To his Associate Editor, Marvin L. Sussman, go the expression of the Editor's profound appreciation for his continued, tireless efforts in the joint editing of the manuscripts from their draft form to the final camera copy from which this published work has been produced.

The Training Program must express its gratitude particularly to Mead Johnson & Company Research Center and the Pharmaceuticals Division of CIBA-GEIGY Corporation, whose contributions have helped make possible both this conference as well as the publication of its proceedings.

The symposium from which this publication has resulted was supported for the most part by funds from the National Institute of Child Health and Human Development (Training Program in Cellular Aging Grant No. HD-00142), and in part by the University of Miami Department of Physiology and Biophysics.

Miami, Florida, 1973 Morris Rockstein, Ph. D.

AGE CHANGES IN THE NEURONAL MICROENVIRONMENT

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The microenvironment of neurons in the brain is in large part composed of extracellular space formed by submicroscopic channels between the cellular elements (Schmitt and Samson, 1969). These channels, which include synaptic clefts, are believed to be sites of physiologically important reactions involving metabolites and ions, which support neuronal metabolism. They are little understood, especially in the mammalian central nervous system.

The properties of the brain extracellular space are relatively uninvestigated during the course of normal development and senescence. In the adult brain, a chloride space of about 25% has been determined (Koch and Woodbury, 1960), and experimental evidence indicates that Cl^- is largely extracellular (Van Harreveld *et al.*, 1966). An extracellular space of this magnitude, which is consistent with that estimated from impedence measurements (Van Harreveld, 1966), is considerably greater than the 5% space indicated by thiocyanate (Streicher, 1961), sulfate (Woodbury, 1958), and sucrose analyses (Reed and Woodbury, 1960) and demonstrated in electron micrographs of chemically-fixed nervous tissue (Horstmann and Meves, 1959). Chemical fixation is believed to cause a relative asphyxia with a concomitant intracellular relocation of extracellular water (Van Harreveld *et al.*, 1965). The distribution of intracellular and extracellular water in normal nervous tissue appears to be preserved accurately by rapid freezing, followed by gradual substitution of frozen tissue water with acetone (Van Harreveld, 1966; Malhotra and Van Harreveld, 1966). It is assumed that subsequent embedding of this substituted tissue in an epoxy resin does not

significantly distort the submicroscopic distribution of tissue water.

In the cerebral cortices of young adult and immature Sprague-Dawley rats, 10, 14 and 21 days of age, we have estimated the volume of tissue occupied by extracellular space and have attempted to relate sequential volume changes during normal postnatal maturation with the onset of functional maturity (Bondareff and Pysh, 1968). The cerebral cortices were rapidly frozen _in situ_ and the frozen tissue water substituted at -78° C with a 1% solution of osmium tetroxide in acetone. In random electron micrographs of each animal, each representing 42 μ^2 of cerebral cortical molecular layer, the volume of extracellular space was estimated stereologically (Weibel _et al._, 1966). In 10-day-old animals an extracellular space of 40.5% was found (Fig. 1). This diminished progressively to 31.8% at 14 days and during the third week of postnatal life, when electroencephalographic activity acquires adult-like properties (Bures, 1957; Deza and Eidelberg, 1967), the volume of extracellular space was estimated to be 26.3%, which approaches the adult value of 21.7% (Fig. 2). These data agree closely with measurements of the maturing extracellular space based on uptake studies of tracers, water and electrolyte determinations and impedence measurements. They appeared to represent accurately the changing distribution of extracellular water during the course of development of a rapidly maturing cerebral cortex (Bondareff and Pysh, 1968) (Table 1).

Although the histophysiological significance of a progressively decreasing extracellular space cannot be accessed accurately, it is tempting to relate it to the onset of electrical activity. It is probable that these spaces contain a relatively large proportion of the extracellular water and chloride ion characteristic of the immature rat brain and that these extracellular channels facilitate diffusion of ions and metabolites. Such rapid diffusion of ions and small molecules through extracellular channels has been demonstrated in leech and amphibian nervous systems (Nicholls and Kuffler, 1964; Kuffler _et al._, 1966).

It is also probable that the extracellular channels contain macromolecules which facilitate, and perhaps control, the passage of ions and small molecules through them. That this extracellular substance is polyanionic has been

suggested by several histochemical studies in which charged metallic complexes are visualized by electron microscopy, within the extracellular spaces. Such metallic substances include: ruthenium red (Bondareff, 1967a) (Fig. 3), phosphotungstic acid (Bloom and Aghajanian, 1966; Pease, 1966), bismuth iodide (Pfenninger _et al._, 1968), silver methenamine (Rambourg and Leblond, 1967) and uranyl acetate (Bondareff, 1967b). Because these substances are all positively charged, it has been assumed that their accumulation in tissues depends upon electrostatic forces binding them to anionic sites. The nature of these extracellular sites is not known precisely but it has been postulated that they may contain protein-bound mucopolysaccharide (acidic glycosaminoglycans) or glycoproteins (Pease, 1966; Bondareff, 1967 a, b; Bondareff and Sjöstrand, 1969). The possibility that these negatively charged macromolecules, which can bind water and selective ions reversibly, may influence neuronal excitability by affecting ionic fluxes at neuronal surfaces has been discussed before (Wang _et al._, 1966; Adey _et al._, 1969).

There is an apparent resemblance between brain extracellular space and the ground substance of connective tissues. Both appear to contain certain anionic substances that may be glycoproteins or mucopolysaccharides (Bondareff and Narotzky, 1972). Both appear to play prominent roles in normal histophysiology. Both undergo severe changes in water content and distribution during early development, and it is tempting to speculate that both may be profoundly changed as a consequence of normal aging. Because the ground substance of connective tissues condenses, becomes less hydrated, and is replaced by collagen as animals age (Bondareff, 1957; Joseph, 1971), we have been impelled to ask if the extracellular space of brain also changes during senescence.

We compared the volume of brain occupied by extracellular space in eight 26-month-old rats and in three 3-month-old animals (Bondareff and Narotzky, 1972). They were killed by decapitation after which the calvaria and dural membranes were rapidly removed, and the brain was exposed. A thin slice of cerebral cortex, from the parieto-occipital region near the midline, was excised and frozen rapidly. The frozen tissue water was substituted with a 1% solution of osmium tetroxide in absolute acetone at -78° C for 3 days, and the tissues were embedded in

Araldite-502. Sections, 0.5 µm thick, were cut perpendicular to the pial surface and examined with a light microscope. By this means, areas of the molecular layer of the cerebral cortex were identified in which damage due to ice crystal formation appeared minimal. These selected areas of tissue were isolated, sectioned at 500 to 700 A with an ultramicrotome and examined in an electron microscope. Five electron micrographs were prepared from each selected tissue area and the relative volume of extracellular space was estimated stereometrically in each (Weibel _et al_., 1966).

The extracellular space appeared as lakes of variable dimension, which were noticeably smaller in tissues from the senescent animals. The mean volume of cerebral cortex occupied by extracellular space was 9.6 percent (range; 4.3 to 15.0 percent) in animals 26 months old (Fig. 4). In 3-month-old animals, the mean volume of cerebral cortex occupied by extracellular space was 20.8 percent (range; 18.7 to 22.3 percent) (Fig. 5), which agrees closely with our previously reported findings of a 21.7 percent mean volume of extracellular space in the cerebral cortices of four young adult female Sprague-Dawley rats (250 g each), prepared similarly by freeze-substitution (Bondareff and Pysh, 1968). The mean volumes of extracellular space for 3-month-old and 26-month-old rats were compared with a Mann-Whitney U test and a significant difference was suggested by a result of $P < .001$. These data are summarized in Table 2.

Since the brain contains essentially no connective tissue and the extracellular space appears to perform a histophysiological role comparable to that performed by connective tissue ground substance in non-neural organs (Gersh and Catchpole, 1960), a decreased capacity to transport ions and small molecules can reasonably be anticipated in the brains of senescent animals in which the volume occupied by extracellular space is reduced to about half that characteristic of normal adults (Bondareff and Narotzky, 1972). We have attempted to examine this speculation by comparing the capacity of brain tissue in old and young animals to transport exogenous catecholamines.

It had been shown that catecholamines, introduced into different specific brain loci in freely-moving rats through chronically-implanted cannulae, spread intracerebrally several millimeters from the point of application

(Bondareff *et al.*, 1970). This can be demonstrated readily with catecholamines which can be visualized readily in brain tissue by the fluorescence histochemical method of Falck and Hillarp (Bondareff *et al.*, 1970). We injected one group of female Sprague-Dawley rats intrastriatally with 1.0 µl of a solution containing 5.0 µg/µl dopamine-HCl dissolved in 150 mM NaCl and stabilized with ascorbic acid. A second group of animals was injected intrastriatally with 0.5 µl of DL-norepinephrine-HCl, 1.0 µg/µl, similarly prepared in 150 mM NaCl. The injections were made through stainless steel cannulae which had been implanted by stereotaxic surgery, 7 days previously into the right caudate nucleus. The animals were decapitated 15 minutes after the onset of the injection and their brains rapidly removed and frozen-dried. After condensation with formaldehyde vapors, the distribution of the exogenous catecholamines was determined in serial sections of the brains by fluorescence microscopy (Falck and Hillarp, 1962; Falck and Owman, 1965). We found no significant difference in intrastriatal spread of norepinephrine in non-senescent animals (1, 3, and 9 months of age), in which the spread varied between 0.45 mm and 0.54 mm rostral, and between 0.36 mm and 0.54 mm caudal to the point of application (Table 3). Total mean spread (rostral + caudal) in 7 non-senescent animals was 0.93 mm (range; 0.81 - 1.08 mm) and the difference between 1-month-old rats and those 3 and 9 months of age was not significant ($F=1.43$; $df=1,5$; $p<0.05$). The total spread for two 26-month-old senescent animals, however, was 0.45 mm and 0.54 mm (mean total spread = 0.50 mm), or almost half that observed in non-senescent animals (Table 3). This difference in spread between senescent and non-senescent animals was significant ($F=32.21$; $df=1,7$; $p<0.01$). Similarly, the total mean intrastriatal spread of dopamine (Table 4) in three senescent 24-month-old animals was 1.26 mm (range; 1.17 - 1.35 mm) (Bondareff *et al.*, 1971), or about one half of that found in 6-month-old animals (Bondareff *et al.*, 1970).

It cannot be claimed unequivocally that the decrease in intrastriatal spread of exogenous catecholamines in senescent rats to almost half that which is characteristic of non-senescent animals is associated with change in composition or volume of the extracellular space. But several data do support our tacit assumption that intrastriatal spread represents, at least in large part, an

extracellular phenomenon. In the first place, the rate of intrastriatal spread, 0.5 mm/min is faster than that which has been ascribed to intracellular transport mechanisms (Routtenberg *et al.*, 1968); secondly, our observations that intrastriatal spread depends directly upon concentration suggests diffusion through extracellular channels (Bondareff *et al.*, 1970); and thirdly, the demonstration of reduced spread following pretreatment with dexamethosone or infusion with 20% mannitol (Bondareff *et al.*, 1970) is congruous with this assumption. Both dexamethosone pretreatment and infusion with 20% mannitol have been shown to decrease the volume of the extracellular space and to alter the normal relationship between intracellular and extracellular compartments in the brain. Both decrease intrastriatal spread by about half, suggesting further that spread is through extracellular channels.

Significant age-related decrements in both the volume of tissue occupied by extracellular space and in the capacity of brain tissue to transport exogenous catecholamines suggest an equally significant age-related change in the composition of the extracellular space. Analogy with connective tissue in non-neural organs suggests that such a change might best be sought in the constituent acidic glycosaminoglycans of extracellular proteoglycans which appear to occupy these extracellular spaces.

In preliminary studies of brains from 21-day and 24-month-old Sprague-Dawley rats, we have compared the acidic glycosaminoglycans by qualitative and quantitative methods well known to be useful in the comparison of young and old connective tissues (Breen *et al.*, 1970a, 1970b, 1973). Brains were lyophilized and defatted with chloroform-methanol, and the acidic glycosaminoglycans were isolated by ethanol precipitation after pronase digestion. Acidic glycosaminoglycan polymers were separated and identified by zone electrophoresis on cellulose acetate (Fig. 6) and quantitative measurements of uronic acid (U.A.), Hexosamine (Hex), galactosamine (Gal), and sulfate ($-SO_3^-$) were made (Table 5). Only apparently minor differences were found in the uronic acid and hexosamine contents of young and senescent brains. Similarly, only seemingly minor differences in total uronic acid and total chondroitin sulfate content were found. Yet, it may be significant that galactosamine content remained unchanged resulting in an apparent slight fall in the galactosamine per total

hexosamine mole ratio in the brains of senescent animals (Table 6).

A prominent age-related change was found in the sulfate concentration of acidic glycosaminoglycans of old rats which was about 1-1/2 times greater than that of normal young brain (Table 5). If it is assumed that all the sulfate is on the galactosamine of chondroitin sulfates, an assumption strongly supported by our electrophoretic data, then the $-SO_3^-$:galactosamine mole ratio is 0.54 in 21-day-old rat brain as compared with 0.84 in senescent brain (Table 6). The relatively high $-SO_3^-$: galactosamine mole ratio in acidic glycosaminoglycans of senescent brains may reflect age-related changes in sulfotransferase activity or changes in the availability of sulfate acceptors, both of which appear to decrease with age (Balasubramanian and Bachawat, 1964; George *et al.*, 1970). There appears to be an age-related decrease in sulfate turnover with a consequent increase in sulfated chondroitin sulfates in senescent brain extracellular space but whether such changes are related to age-related changes in the neuronal microenvironment is not known.

The extracellular compartment of brain is composed largely of intercellular channels, which contain polyanions presumed to be proteoglycans. The dynamic microenvironment which this extracellular compartment provides to nerve cells has a histophysiological significance that is not well understood. That it has been shown to change with chronological age suggests that its properties during normal development and senescence may resemble those of the connective tissue ground substance and that it may affect a functional decline in the aging nervous system.

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TABLE 1

Volume of extracellular space estimated in electron micrographs of frozen-substituted cerebral cortex of rats during early post-natal development.

Age	Number of animals	Extracellular space in %	
		Range	Mean
10 days	3	35.0-48.0	40.5
14 days	1	27.9-35.6	31.8
21 days	3	22.6-31.0	26.3
Adult	3	17.8-26.8	21.7

(From Bondareff and Pysh, 1968.)

TABLE 2

Estimated volume of extracellular space in electron micrographs of frozen-substituted cerebral cortex of rats, 26 and 3 months of age. Each estimate is based on the percentage of tissue volume occupied by extracellular space as estimated in five different electron microscopic fields. The average volumes are expressed as means ± standard deviation.

Rat (No.)	Volume per rat (%)
26 months old	
1	13.1±2.2
2	4.3±0.0
3	15.0±2.0
4	11.1±2.2
5	6.5±0.1
6	6.9±0.4
7	11.0±0.9
8	9.3±0.9
Average volume (%) per age group: 9.6±3.4	
3 months old	
9	18.7±1.5
10	22.3±1.7
11	20.6±1.1
Average volume (%) per age group: 20.8±1.5	

(Modified from Bondareff and Narotzky, 1972.)

TABLE 3

Relationship between chronological age and intrastriatal spread of 0.5 μl norepinephrine (1.0 μg/μl).

Rat #	Age (Mo.)	Spread (mm)		
		Rostral	Caudal	Total
5	1	0.54	0.36	0.90
6	1	0.45	0.54	0.99
7	1	0.54	0.54	1.08
1	1	0.45	0.54	0.99
9	3	0.45	0.36	0.81
4	3	0.45	0.36	0.81
12	9	0.54	0.45	0.99
10	9	0.18	0.27	0.45
11	26	0.27	0.27	0.54

(From Bondareff *et al.*, 1970.)

TABLE 4

Relationship between chronological age and intrastriatal spread of 1.0 μl dopamine (5.0 μg/μl).

Rat #	Age (Mo.)	Spread (mm)		
		Rostral	Caudal	Total
20	6	1.08	1.22	2.30
35	6	0.72	2.07	2.79
55	6	0.45	0.72	1.17
74	24	0.54	0.81	1.35
75	24	0.54	0.72	1.26

(From Bondareff *et al.*, 1970.)

TABLE 5

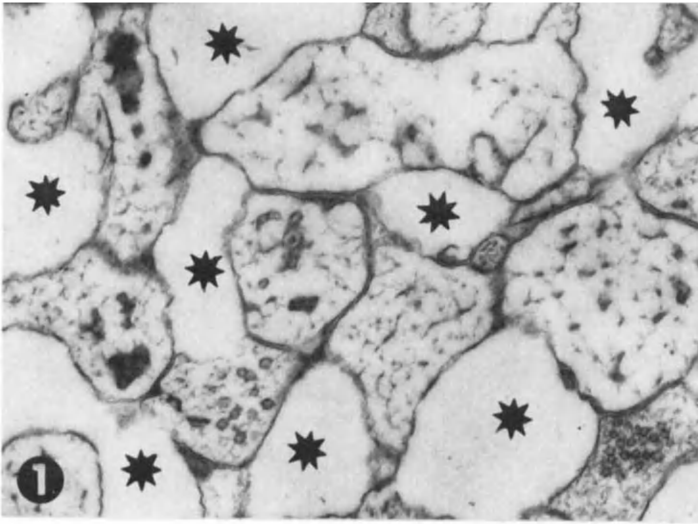
Acidic glycosaminoglycan polymers in whole brain of young and senescent rats.

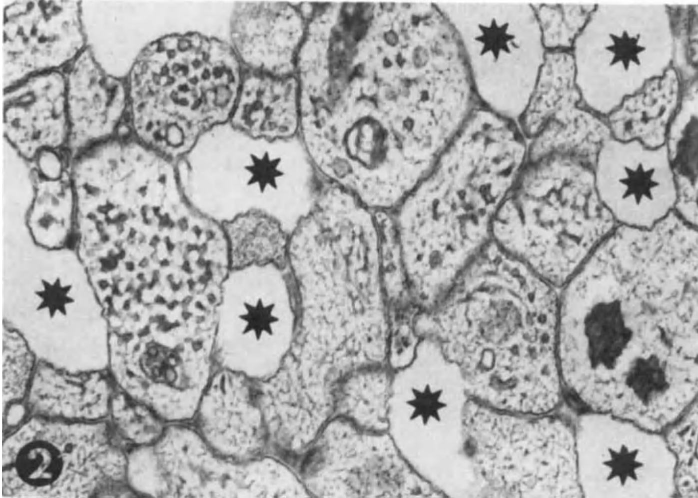
Age (Mo.)	(μmole/g lipid-free dry weight) U.A.	Hex	Gal	$-SO_3^-$
3	4.48	4.75	3.42	1.85
26	4.17	5.37	3.42	2.86

TABLE 6

Mole ratios of acidic glycosaminoglycan subunits from whole brain of young and senescent rats.

Age(Mo.)	U.A./HexN	GalN/HexN	$-SO_3^-$/HexN	$-SO_3^-$/GalN
3	0.94	0.72	0.39	0.54
26	0.78	0.64	0.53	0.84





Figs. 1 and 2 Electron micrographs of frozen-substituted rat cerebral cortical neuropil demonstrating the relative distribution of the extracellular tissue compartment (indicated by stars). X35,000 (Modified from Bondareff and Pysh, 1968.)

Fig. 1 From an immature female, 10 days old. The volume of tissue occupied by extracellular space was estimated to be approximately 40%.

Fig. 2 From a young adult female, 3 months old. The volume of tissue occupied by extracellular space was estimated to be approximately 21%.