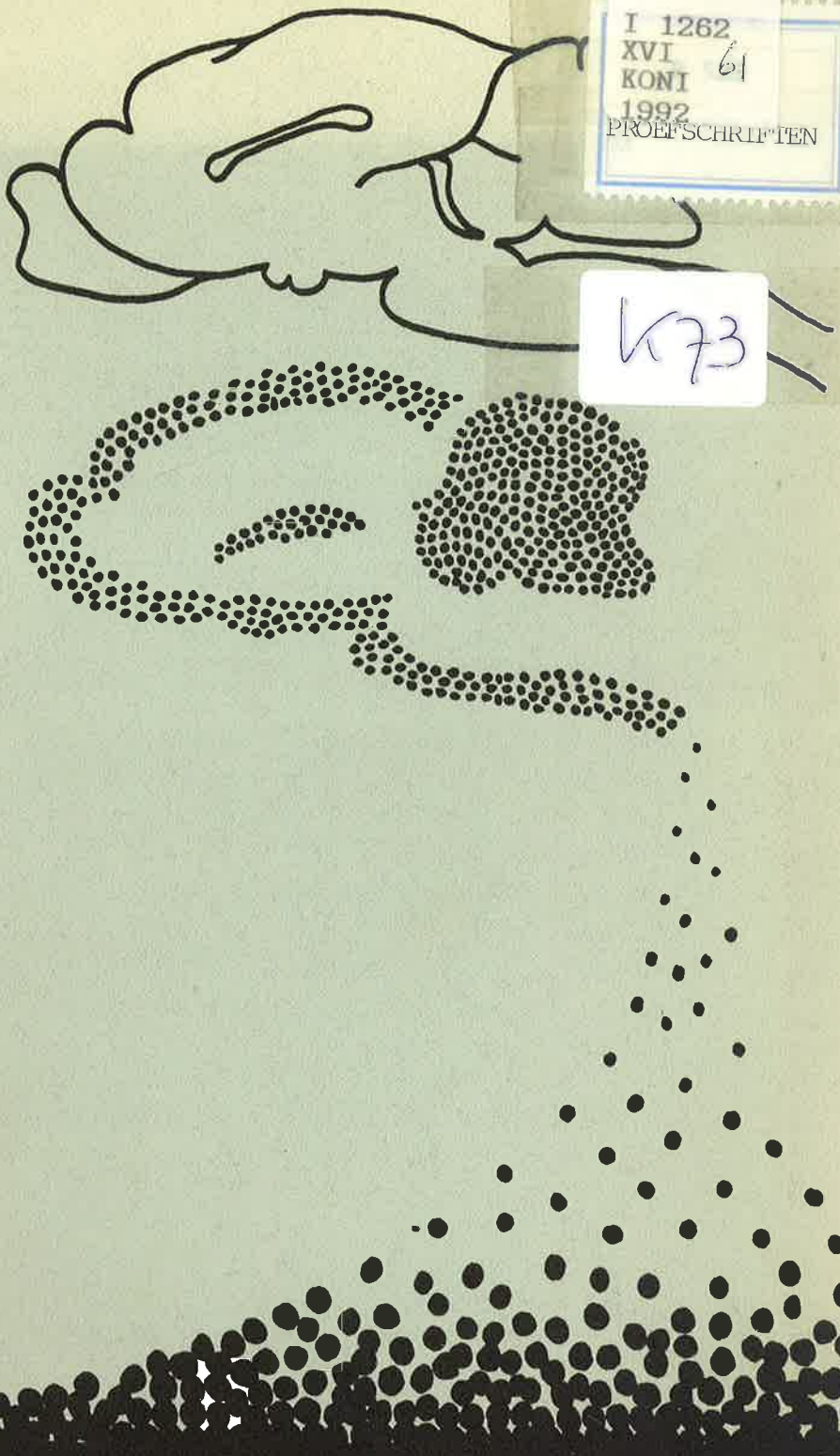

# OBSERVATIONS ON THE CENTRAL NERVOUS SYSTEM OF THE RAT: FUNCTIONAL CHANGES IN RELATION TO AGEING

I.F. DE KONING-VEREST

# OBSERVATIONS ON THE CENTRAL NERVOUS SYSTEM OF THE RAT: FUNCTIONAL CHANGES IN RELATION TO AGEING

Proefschrift

ter verkrijging van de graad van doctor in de geneeskunde
aan de Rijksuniversiteit te Utrecht,
op gezag van de Rector Magnificus Prof. Dr. M.A. Bouman,
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op dinsdag 19 mei 1981 des namiddags te 4.15 uur

door

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geboren op 24 januari 1950 te Breda

1981

Drukkerij Uitgeverij H. Gianotten B.V., Tilburg

Promotor: Prof. Dr. C. F. Hollander

Dit proefschrift kwam tot stand in het Medisch Biologisch Laboratorium TNO te Rijswijk onder leiding van Dr. O. L. Wolthuis

1.

In veel studies naar veranderingen ten gevolge van het verouderingsproces in proefdieren zijn de conclusies helaas gebaseerd op resultaten verkregen met proefdieren die wat leeftijd betreft hoogstens volwassen genoemd kunnen worden.

2.

Maatregelen om de niet aflatende stroom van "oncontroleerbare geneesmiddelen" in te dammen zijn dringend gewenst.

3.

Het verdient aanbeveling om bij het onderzoek naar de beinvloeding van het ouderdomsproces bij de mens ook het effect van langdurige beperking van de hoeveelheid voedsel te betrekken.

W.B. Forbes.
In: The psychobiology of aging: problems and perspectives. Ed.: D.G. Stein. Elsevier, New York, 1980.

4.

Verminderde taboesfeer rond stervensbegeleiding en stervenseffectuering werkt bij ondeskundige begeleiding en behandeling drempelverlagend. Een systematische bestudering en evaluatie van de vele vormen van hulpverlening is derhalve dringend gewenst.

5.

De opvatting dat de stapeling van het ouderdomspigment lipofuscine een van de meest consistente verandering in het organisme, ten gevolge van het ouderdomsproces is, kan op grond van de resultaten van het onderzoek beschreven in dit proefschrift niet gedeeld worden.

6.

De meest effectieve werking van zogenaamde snelheidsdrempels wordt helaas uitgeoefend op het dienst- en hulpverlenend verkeer.

7.
Uit het feit dat apotheekhoudende huisartsen, in tegenstelling tot apothekers, niet verplicht zijn tot het verstrekken van bijsluiters bij niet-narcotische analgetica blijkt ook de overheid bij te dragen tot een verschil in kwaliteit van de geneesmiddelenvoorziening.

8.
De resultaten van metingen van de motorische aktiviteit in proefdieren hangen in hoge mate af van de gebruikte technieken.

9.
Het is de vraag in hoeverre afwijkingen gevonden in verschillende proefdieren als model kunnen dienen voor de veel voorkomende seniele dementie bij ouderen.

National Academy of Sciences,
Mammalian models for ressearch on aging.
National Academy Press, Washington D.C., 1981

10.
Het is opvallend dat mensen die op fanatieke wijze tegen elke vorm van discriminatie zijn, wel haast op religieuze wijze de meest onsmakelijke grappen over onze zuiderburen maken.

11.
Gezien het snel toenemende aantal doe-het-zelf supermarkten is te veronderstellen dat het stijgende werkeloosheidspercentage deels te wijten is aan degenen die menen dat alleen de eigen arbeid adelt.

12.
Als op de huidige voet doorgegaan wordt met de verstedelijking van het landschap en vervuiling van het milieu zullen de kleuren gevoerd door de strijdkrachten veranderen van schutkleuren in signaalkleuren.

13.

Het idee om het woord mens te gebruiken in plaats van man of vrouw verdient nadere studie. Afgezien van regionale gebruiken pleegt men namelijk in het algemeen met de uitdrukking "dat mens" op degenererende wijze een vrouwspersoon aan te duiden.

Stellingen bij het proefschrift:
Observations on the central nervous system of the rat: functional changes in relation to ageing

I.F. de Koning-Verest
Utrecht, 19 mei 1981

aan mijn ouders

aan Fred

Dit onderzoek werd gesteund door de Stichting voor Medisch Wetenschappelijk Onderzoek FUNGO die wordt gesubsidieerd door de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO).

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# CHAPTER 1
# INTRODUCTION

## 1.0. GENERAL

The decrease of the number of births and the increase in the average life-span result in an increased "greying" of the population. Since the ageing individual generally shows deficits in adaptive behaviour as well as in learning and memory functions, such a greying of the population may have consequences for the "average" attitude of society towards the elderly, and more knowledge of and interest in the process of mental ageing of man appears appropriate.

Little is known about mechanisms behind the decline of learning and memory processes, about the underlying physiological and biochemical changes, and about the possibilities to influence these processes in a positive way by pharmacological means. Attempts to investigate these biological processes or the pharmacological influence on these processes are usually carried out in animal model experiments, which make it possible to study them in relation to the changed behaviour of the animal.

In the present investigation it was attempted to obtain answers to such questions as: 1) do aged animals show acquisition deficits 2) if so, which phase(s) of the learning and memory processes are particularly affected 3) which physiological or biochemical processes might be contributing to or be responsible for these deficits and 4) is it possible to reduce the observed deficits by pharmacological means. For a number of reasons (see i.e. Burek and Hollander, 1980) the rat was selected as the experimental animal for the present study.

## 1.1. LEARNING AND MEMORY IN RELATION TO OLD AGE

Learning is thought to represent an interaction of the natural and individual histories of an organism which allows it to adapt behaviourally to certain environmental changes (Gold and McGaugh, 1975). Evidence is available which suggests that in animals, as in man, there are

age-related changes in learning and memory processes. Upon a closer look, however, much of this evidence is based on experiments with young-adult or adult rodents. For example, Thompson et al. (1965) used young rats of about 40 days of age and "old" ones of 100 days in an active avoidance test. The oldest rats in learning experiments of Ray and Barrett (1973) were 350 days. In the experiments of Campbell and Campbell (1962) on retention of a passive avoidance response the young animals were 18 and 23 days old, whereas the older animals - which performed better than the younger ones - were 54 and 100 days old. As a last example, there is the study on maze learning with rats of 30 and 150 days old (McGaugh and Cole, 1965), which were interpreted in a later publication (Gold and McGaugh, 1975) as an indication of age-related deficiency of memory storage processes in the old rats. To extrapolate such findings to real old age is unjustified. It will be clear that an animal of a species with an average life-span of more than 24 months cannot be called "aged" when it has lived 12 months. For this study, therefore, rats are only considered as old when the animals have past the 50% survival age (Burek, 1978; Burek and Hollander, 1980); for the rats used in this study this age is 30 months.

Studies dealing with the effects of ageing on learning and memory processes have produced conflicting results. Some authors saw no clear age differences in learning (Birren, 1962; Botwinick et al., 1962, 1963; Kay and Sime, 1962), whereas others (Doty, 1966[a+b]; Goodrick, 1968, 1972, 1973; McNamara et al., 1977; Michel and Klein, 1978; Ordy et al., 1978; Wolthuis et al., 1976) provided evidence for the decline of learning capacity with age. One possible explanation for these discrepancies might be the differences in the task complexity used by these authors. There is evidence that aged animals acquire simple tasks just as rapid as young animals, whereas, if the task gets more complex, age differences in acquisition appear (Doty, 1966[a+b]; Goodrick, 1972). On the other hand, Wolthuis et al. (1976) found clearcut acquisition deficits in aged rats using a relatively simple test.

A fundamental problem in all studies on age-dependent changes in learning and memory is, that there are so many other factors - known to influence acquisition - which also may change at old age. These factors form variables which are difficult to control or to compensate for. Such

variables include motivation, speed of performance (Birren et al., 1962), motor activity (Goodrick, 1966; Hofecker et al., 1978; Verzár, 1965) and susceptibility to interference (Dye, 1969). In food reinforced learning situations (Goodrick, 1968, 1972, 1973; Michel and Klein, 1978; Verzár-McDougall, 1957) the age-related learning deficits found might have been caused by differences in motivation levels between the different age groups due to differences in effect of food deprivation. Although it will be clear that because of the above mentioned age-dependent variables such motivational levels are hard to measure, these authors could at least have measured the average daily food consumption of their young and old animals. If no differences had been detected, there would at least have been more reason to believe that learning and not motivational levels were responsible for the differences observed. Similarly it will be clear that age-dependent learning differences found in tests on transfer of handedness (Rapaport and Bourlière, 1963), active avoidance in a "jump box" (Gordon et al., 1968) or passive avoidance in a runway (Ordy et al., 1978) may reflect differences in motor skill and activity rather than in learning ability. It would be more appropriate in research of this kind to investigate age-dependent changes in learning ability by the use of tasks which are not primarily based on speed but rather based on choice variables (Sprott and Stavness, 1975). The mechanical simplicity of the discrimination test chosen by Wolthuis et al. (1976), based on conditioned suppression of drinking behaviour, made it unlikely that age differences in motor skill or susceptibility to interference affected the results obtained with that test. Such a type of test, therefore, is suitable for the study of the relation between ageing, learning and memory function in the rat.

When one reads the literature on experiments with animal models in this field, the question arises whether the results have any relevance for humans. At the present stage of research this question cannot be answered. Apart from the fact that in humans many parameters cannot be tested for ethical reasons, also the extrapolation of changes in animal behaviour to man or vice versa meets considerable difficulties. Yet, a small number of findings do suggest that these views may be too pessimistic. Experiments with normal human subjects have shown an age-related decline in learning and short-term memory (Botwinick, 1975; Welford,

1958); especially performance on the basis of visually presented material decreases rapidly with age (Adamowicz, 1976; McGhie et al., 1965; Walsh and Thompson, 1978). In connection with the age differences in short-term memory in humans it is interesting to know that a number of recent studies similar age differences in short-term memory have been reported in monkeys (Bartus et al., 1978) and in rats (Gold and McGaugh, 1975; Jakubczak, 1973; Ordy et al., 1978). There are thus resemblances between learning deficits found in humans and in animals, suggesting that an animal model of the age-related decrements in learning and memory processes is useful. It is possible, therefore, to test in animal models hypotheses based on the current theories concerning the age-related acquisition decrements in humans. The relevance of the results for humans can only be evaluated in the future, when hopefully the present scarcity of data will be relieved.

## 1.2. POSSIBLE CAUSES OF THE AGE-RELATED ACQUISITION DEFICITS

At the present state of our knowledge and for the purpose of this study ageing is taken to be the result of multi- focal lesions. If this is accepted, it is clearly impossible to single out one factor as "the cause" of the age- related acquisition deficits. It will also be clear that for an investigation a selection from the many possible mechanisms has to be made, not only for practical reasons but also because it seems unlikely that every little change in the CNS during the process of ageing will have consequences for its functions in learning and memory. It is conceivable, however, that changes in morphology, neurochemistry or electro- physiology of the CNS do affect functions of the brain which are involved in the learning and memory processes. Such changes are, therefore, worth considering.

### 1.2.1. Morphological changes

The known major changes in the brain associated with ageing are gross changes such as reduction of brain weight and volume, increase in the size of the ventricles and microscopic changes such as a loss of neurons, deposition of age pigment, loss of dentritic spines, loss of synap-

tic contacts, amyloid deposits, senile plaques and formation of neurofibrillary tangles (Brizzee, 1975; Nandy, 1978). Of all these age-related morphological alterations none have excited greater interest than the loss of neurons and the accumulation of the age pigment lipofuscin (Brizzee, 1975). Numerically, the neurons represent a minor constituent of the central nervous system. Because these cells do not reproduce after early postnatal life, however, they may be regarded as a prototype cell paralleling the life-span of the organism. Brody (1955) found an age-related loss of neurons in the cerebral cortex of human brains. His counts in various cortical areas show that the number of neurons does not decrease uniformly in the various cortex regions. There is evidence that neuronal loss in certain cortical areas is greater than in others. Probably, certain functions will suffer more than others (Brody, 1973). To what degree plasticity, e.g. functional take-over by other neurons, occurs in aged brains is uncertain. Ball (1977) demonstrated a gradual loss of neurons in the hippocampus in aged humans. A sizable loss of neurons in the hippocampus of aged rats was found by Landfield et al. (1977) and by Brizzee and Ordy (1979). A decreased number of neurons was also found in rat frontal cortex by Stein and Firl (1976) and in rat cerebellum by Peng and Lee (1979). Other anatomical studies on ageing, however, have suggested stability in the neuronal population of the cerebral cortex in rats (Brizzee et al., 1968; Diamond et al., 1977; Klein and Michel, 1977) and in brain tissue of humans (Cragg, 1975). The discrepancy in published results may be attributable in part to the following: 1) difficulty in distinguishing small neurons from glia and other small cells on the basis of the presence of nucleoli or by differential staining in lightmicroscopic preparations, 2) use of different staining procedures or histological methods of tissue preparations by different authors, and 3) differences in the so-called quantitative histological techniques that have been used for the enumeration of cell populations (Ordy et al., 1978).

The results obtained with glial cell counting show similar discrepancies. Some results point to an increasing number of neuroglia cells with age (Brizzee et al., 1968; Landfield et al., 1977); others, however, did found no differences in glia number in ageing rat brains when compared with brains of young rats (Diamond et al., 1977; Klein and Michel, 1977; Ordy et al., 1978). At this stage of research, therefore, it is not

at all certain that a reduction of the number of neuronal cells and an increase of glial cells in aged brains occur.

The most consistent age-related intracellular change of both neurons and glial cells is the accumulation of the age pigment lipofuscin (Bondareff, 1957; Brizzee et al., 1969; Brizzee et al., 1974; Brody, 1960; Nandy, 1978). Lipofuscin is generally regarded as a mixture of indisposable metabolites and may be considered as intracellularly deposited "garbage" (Björkerud, 1964). Until recently, cell loss and lipofuscin accumulation in the brain have been studied separate from learning and memory functions. In the past few years, however, two brain regions have received increasing attention in animal studies of age-related differences in short-term memory in relation to cell activity, cell number and lipofuscin accumulation. These regions are the cerebral cortex and the limbic system, particularly the hippocampus. Landfield et al. (1977) suggested that the hippocampal pathology is related to impaired memory found in aged rats. In a preliminary report Ordy et al. (1978) published age differences in short-term memory and cell loss from the visual cortex of the rat and in a further study Brizzee and Ordy (1979) suggested that the significant age differences found in short-term passive avoidance memory might be related - at least in part - to significant cell loss and/or increased lipofuscin accumulation in the hippocampus and the visual cortex of the rat brain. It must be clear, however, that the validity of these suggestions is somewhat doubtful in view of the observed discrepancies in the results of neuronal and glial cell counts.

### 1.2.2. Neurochemical changes

Although there is great interest in the neurochemistry of the brain, literature on changes in composition and metabolism during the whole life-span is scarce and fragmentary. The few data on age-related changes in important constituents of the brain can be summarized as follows.

In the human brain the percentage of water decreases from approximately 90% at birth to about 77% at an age of 30 years. From then on there is a slight increase to about 79% at an age of 80 years (Himwich

and Himwich, 1959). In view of the fact that most of this water is located intracellularly, changes in the percentage of water may reflect alterations in both cell number and cell function (Timiras and Vernadakis, 1972).

The greatest changes in lipid content, composition and turnover in the human brain appear to take place during the years of development. As a percentage of the brain weight the total lipids increase from 3.5% at birth to 10.5% at 33 years and subsequently decline to 7.5% by 98 years of age (Rouser and Yamamoto, 1969). The increase of the amount of myelin during development accounts for a great deal of the increase of lipid during that period. It seems likely that also the decrease in the amount of myelin contributes to the decreased lipid concentration in the brain during ageing (Horrocks et al., 1975).

According to most sources the DNA content of the human brain remains relatively constant from the age of 40 onwards and may even be increased at the age of 90 (Himwich and Himwich, 1959). This alleged increase might be attributed to the proliferation of glial cells and to an increase in pyknosis of neurons (Timiras and Vernadakis, 1972). The RNA content in human and the rat brain increases from birth to adulthood, where it levels off and then falls rapidly at senescence.

Age-dependent decreases have been noted for several amino acids (e.g. glutamate and aspartate) in the rat brain during senescence, whereas on the other hand the levels of other amino acids such as alanine and glycine seem to increase with age (Timiras et al., 1973). The changes in protein turnover, measured at several points during the whole life-span, tend to follow the above mentioned patern for RNA, i.e. an increase towards adulthood, when it reaches a plateau, and a decrease at senescence (Ordy and Kaack, 1975).

In general, neurotransmitter levels, and the activities of the synthesis and degrading enzymes decline significantly in many regions of the human and the animal brain during ageing (Ordy et al., 1975; Samorajski, 1977).

In addition to the changes in composition of important components of the brain, age-related changes in cerebral circulation and metabolism also appear to take place. There is a gradual age-related decline of cerebral blood flow in normal subjects; in arteriosclerotic and senile

psychotic patients such a decline is even more pronounced (Sokoloff, 1972). The oxygen consumption of the entire brain decreases both in humans and in animals (Peng et al., 1977; Sokoloff, 1972; Timiras and Vernadakis, 1972). However, the oxygen uptake of brain mitochondria remains unchanged throughout the life-span (Sylvia and Rosenthal, 1978; Timiras and Vernadakis, 1972). In the glycolytic pathway, on which the energy formation in the brain almost exclusively depends, most enzymes remain unchanged with age; only the activities of two key enzymes, hexokinase and phosphofructokinase increase and decrease, respectively (Meier-Ruge et al., 1978), which could explain the reduced cerebral glucose consumption in aged subjects (Sokoloff, 1975).

It will be clear that many changes in the brain occur during ageing and therefore many approaches are possible when it is attempted to study ageing in relation with learning and memory functions. In view of the significance that has been attributed to RNA in learning and memory, changes in RNA per cell may account for the characteristic changes that take place in memory with senescence (Hydén, 1967). Since the cholinergic system has been implicated in memory processes (Deutsch, 1971) the age-related changes shown in this system (Domino, 1978; Frolkis et al., 1973; Perry et al., 1977) could also attribute to the deficits in learning and memory function with age. Wang et al. (1974) reported a significant correlation between the reduced cerebral blood flow, changes in the EEG and impairment in intellectual performance in groups of healthy elderly volunteers over 60 years of age. These are all examples of the neurochemical alterations that have been correlated with losses in learning and memory. It is conceivable, however, that other age-related neurochemical changes have an even greater impact or are interfering in a more selective way in the learning and memory functions of the ageing brain. Unfortunately, studies which directly relate age deficits of these functions with neurochemical changes are exceedingly rare.

### 1.2.3. Electrophysiological changes

Cerebral changes during life, such as growth, development, ageing and eventual degeneration have long been known to be accompanied by changes in the electrical activity of the brain. Numerous investigations have

reliably shown progressive changes in the human electroencephalogram (EEG) with age. It appears that with the onset of senescence a slowing of the alpha frequency, a decrease in the alpha activity and an increase in theta activity occured, accompanied by a general decrease in EEG responsivity (for review: Marsh and Tompson, 1977). Recently, the technique of registration of evoked responses (ER) - i.e. electrical changes recorded from the scalp, initiated by brief stimuli such as click or light pulses - has been used in human ageing studies. In contrast to the EEG, such responses are controlled by the stimulus and thereby provide additional information about different sensory systems, functional pathways and brain areas involved in the response (Beck et al., 1975). The data from the ER studies in aged humans have generally demonstrated increased amplitudes of early components and substantially decreased amplitudes of later components when passive stimulation is used: i.e. when the individual is not expected to react to the stimulus. This trend is apparent when visual (Beck et al., 1975; Dustman and Beck, 1969; Straumanis et al., 1965) or somatosensory stimulation (Beck et al., 1975; Lüders, 1970) is applied. The amplitudes of the auditory ER does not seem to change with age (Beck et al., 1975). Latencies of both early and late components are longer in old than in young subjects both following visual and somatosensory stimulation (Drechsler, 1978). When active stimulation is applied, i.e. when the individual is expected to react to the stimulus (e.g. by pressing a bar or pushing a button), different results are obtained in different laboratories (see: Snyder and Hillyard, 1979). Although technical and methodological differences among studies certainly contribute to the confusion in this area, it is also possible that differences in motivation of the subjects may be wholly or in part responsible for the reported discrepancies (Marsh and Thompson, 1973).

The question of a relationship between age-related EEG and/or ER changes, and cognitive measures has frequently been raised. Such relationships, however, have only been demonstrated in patients which suffer from considerable mental deterioration or other types of pathology, so that the conclusions drawn have only limited value (Thompson and Michalewski, 1978; Timiras and Vernadakis, 1972). Some authors have tried to correlate the changed evoked responses in elderly people with their decreased performance abilities (Marsh, 1975; Snyder and Hillyard, 1979).

The rationale for such a correlative study may be that the first components of the ER are generally thought to represent the direct transmission of information, whereas the later components are considered to be related to information processing or perhaps early storage phenomena (Straumanis et al., 1965). Since Snyder and Hillyard (1979) have also pointed out that differences in attention levels may affect these late components, it is difficult to draw clear conclusions from these studies. Especially so since attention levels and attention spans may exhibit age-related changes of their own.

It appears from the literature that age-related EEG or ER changes have not been extensively studied in experimental animals. According to Eleftheriou et al. (1975) the EEG's of aged mice exhibit the same phenomena as have been observed in humans. On the other hand, Barnes (1979) did not find any differences in the EEG's of young and aged rats; she suggested that the slowing down of EEG phenomena observable in human EEG's may reflect pathological changes rather than normal changes during ageing. In neurophysiological studies of the hippocampi of old rats in vivo (Landfield et al., 1978) as well as in vitro (Landfield and Lynch, 1977) synaptic potentiation during repetitive stimulation was found to be impaired. This suggests that aged synapses may be more sensitive to depressive effects of repetitive stimulation. These neurophysiological data seem consistent with neurochemical and morphological findings and point at an impairment of synaptic functions in the hippocampi of aged animals. It is conceivable that the impaired synaptic potentiation in hippocampi of aged rats (Landfield et al., 1978) could influence the efficiency of information processing or storage. Landfield and Lynch (1977) suggested that such an impaired hippocampal synaptic potentiation may be responsible for the short-term-memory deficits observed in ageing rats. Tests of synaptic physiology in other structures have to be conducted in aged and young animals, however, to investigate whether these changes are restricted to the hippocampus and not part of a general change in synaptic efficacy at old age.

## 1.3. NEUROPHARMACOLOGY AND AGEING

The current psychopharmacological approaches in the treatment of the CNS dysfunction which so frequently accompanies ageing, may be divided into unspecific and specific pharmacotherapies. Unspecific pharmacotherapy is aimed at treating pathology which occurs in aged people, whereas specific pharmacotherapy is oriented towards counteracting the "physiological" changes of ageing per se. It will be clear that the borderline between the two is vague, since "physiological" ageing is hard to define. It should be pointed out, however, that the aim of specific therapy is not primarily the prolongation of life but the improvement of the quality of life and the relief of discomforts which usually accompany old age.

Both the specific and the unspecific pharmacotherapies have their problems since the pharmacodynamics - i.e. the effects of the drugs at their site of action - as well as the pharmacokinetics - i.e. absorption, distribution, metabolism and excretion of drugs - may be considerably altered at old age. While less is known about the pharmacodynamics of drugs in the aged, age-related pharmacokinetic changes (for review see: Triggs and Nation, 1975) are well known to influence the drug responses in the aged. Certainly both processes have to be kept in mind in geriatric pharmacotherapy.

A well known, but still controversial geriatric agent is procaine (KH3®, Gerovital®). Although Aslan and coworkers (1956, 1959, 1960[a+b], 1965) have published several papers on the rejuvenating effects of this agent, the design of the studies has met scepticism in the medical community. Many investigators have undertaken studies of procaine therapy with more objective measures of its effectiveness (for review: Bender et al., 1977; Ostfeld et al., 1977). None of these investigators, however, was able to substantiate any of the claims on the treatment of the ageing process made by Aslan and her coworkers. Procaine seems to be either completely ineffective in this respect or is certainly inferior to other available drugs in the treatment of the ailments.

As examples of substances which might affect the decline of cognitive functions caused by the ageing process per se piracetam, etirace-

tam, meclofenoxate, Hydergine® and desglycinamide-arginine-vasopressin (DG-AVP, Org. 5667) have been studied. The effects of these drugs have recently been reviewed, among others, by Reisberg et al. (1979) and will be further discussed in chapter 6. These drugs show promise for use in geriatry, but need much further intensive basic and clinical testing.

A very difficult point in the study of the typical effects of these drugs in geriatry is formed by the high costs of this kind of research on account of the necessary large numbers of expensive old animals; the geriatric effects of these drugs of course cannot be properly tested in young animals.

## 1.4. AIMS OF THE PRESENT STUDY

In the present study the effects of ageing on learning and memory functions of the rat is investigated in detail to gain more insight into the nature of the decline in acquisition with age.

Chapter 2 describes experiments on the age-related acquisition deficits in two strains of rats. Attempts were made to relate these findings to possible age differences in activity levels and to reported changes in sensitivity to footshock. Since acquisition may be considered as a special form of adaptive behaviour, another form of adaptive behaviour, i.e. changes in diurnal drinking rhythms, was also studied in relation to the age of the animals.

Chapter 3 is devoted to the question whether a particular phase of the learning and memory processes is affected by the ageing process. Experiments focussed on sensory transduction, registration, short-term memory formation and retention are described.

In chapter 4 a study of glutamate metabolism, taken as an index of the metabolic state of the brain, in three age groups of rats is described. The aim was to investigate whether the acquisition deficits found might be due to gross changes in CNS metabolism due to ageing.

In chapter 5 experiments are described which were performed to investigate in more detail the reported meclofenoxate-induced decreases of the lipofuscin content of several brain structures of aged rats, in relation to the effects of this decrease on acquisition. These kinds of effects of meclofenoxate were reported in the literature. The experiments

are performed for testing hypothesis concerning the lipofuscin accumulation as an etiological factor for the CNS dysfunction at old age.

Chapter 6 presents the results of experiments in which it was attempted to improve acquisition of aged rats by the administration of several drugs.

In chapter 7 an experiment is described in which the activity of choline-acetyltransferase (CAT) was determined in the hippocampus of rats of different ages. In the literature it was reported that the activity of this enzyme was decreased in some brain regions of normal old humans and in those of humans suffering from senile dementia.

Finally, chapter 8 contains a general discussion of the results obtained in the above mentioned studies.

# CHAPTER 2

# BEHAVIOURAL OBSERVATIONS IN OLD RATS

## 2.1. INTRODUCTION

Conflicting observations have been reported with regarding to age-related changes of learning and memory in rats. Some authors (Birren, 1962; Botwinick et al., 1963; Kay and Sime, 1962) did not observe age differences in learning, whereas others, who used more complex tasks (Doty, 1966[a+b]; Goodrick, 1972; McNamara et al., 1977; Michel and Klein, 1978) provided evidence for the decline of learning capacity with age. In the selection of a method for studying possible deficits in old animals it has to be taken into account that aged animals are more susceptible to interference (Dye, 1969), have a lower response speed (Birren et al., 1962) and in general show reduced motor activity (Goodrick, 1966; Hofecker et al., 1978; Verzàr, 1965). In a relatively simple test based on conditioned suppression of drinking Wolthuis et al. (1976) found age-related acquisition deficits in rats. The simplicity of this test made it unlikely that the deficits could be attributed to decreased motor skill or higher susceptibility to interference of the aged animals.

It is possible that the performance deficits seen in old rats are attributable to secondary factors such as differences in motor activity or differences in sensitivity to footshock. From the findings of Wolthuis et al. (1976) the question whether decreased activity with age contributed to the acquisition deficits found cannot be answered conclusively. To get a better insight whether acquisition of the different age groups and their activity levels are related a device was constructed in which vertical (Wolthuis et al., 1976) and horizontal movements in an open field situation (Tanger et al., 1978) could be measured and registered simultaneously (van den Steen and Vanwersch, in press).

In the drinktest used in the present experiments the training of the animals was based on punishment by electric footshocks. A decreased sensitivity to footshock would lead to a reduction in punishment level resulting in a decreased motivation. This subject is a matter of controversy since in two studies diametrically opposed results have been

reported. Parê (1969) reported an increase in footshock thresholds with age, whereas Gordon et al. (1978) found a progressive decrease in shock thresholds. These discordant results may be due to differences between the two experiments as differences in the shock delivery systems used, age range of the animals tested, the range of current intensities presented and the threshold measure used. In addition other factors, such as the smoothness of the cage floor surface, resulting in differences of cornification, may also be important. Because of this lack of agreement on the matter of age-related shock sensitivity, it was decided to measure the footshock sensitivity of the rats used in the acquisition experiments.

It was considered necessary to determine whether the acquisition deficits found are limited to only one, perhaps peculiar strain of rats or whether these deficits can also be found in a different strain. Therefore, pigmented BN/BiRij rats were subjected to the same training procedure as used for the albino small Wistar WAG/Rij rats.

To examine whether age-related differences could also be demonstrated in more basic, adaptive functions of the central nervous system (CNS), the adaptation of drinking activity in reaction to reversed light-dark cycles was chosen as a paradigm. The normal drinking activity of the rat is known to reach a maximum during the night and a minimum during the day (Siegel and Stuckey, 1947). Since this basic rhythm is profoundly affected by changes in illumination schedule (Bunning, 1967), it was decided to investigate whether the speed whereby adaptation of drinking patterns occurs in reaction to a reversal of the light-dark cycles is age-dependent. It was thought that the aged animals might adapt slower to the changed environmental stimuli and would show their "behavioural rigidity" in the way this term was used by Goodrick (1975).

## 2.2. MATERIALS AND METHODS

### Animals

Virgin female albino WAG/Rij or pigmented BN/BiRij rats were used in the experiments. The rats were bred under specific pathogen free (SPF) conditions and maintained under clean conventional conditions (Hollander, 1976; Burek, 1978) at the Institute for Experimental Gerontology TNO or

the Medical Biological Laboratory TNO, Rijswijk, The Netherlands. Detailed information on husbandry and age-associated pathology of these rats is provided by Burek (1978). The animals entering the studies were experimentally naive unless otherwise specified and were all in good health.

2.2.1. Experiments in the drinktest

The WAG/Rij rats used were 12, 18, 24 and 30 months old at the beginning of the experiment. Mean (± S.E.M.) body weights (g) at the start of the experiment were 198 ± 2.4 (12 months), 207 ± 3.7 (18 months), 211 ± 7.1 (24 months) and 201 ± 6.4 (30 months). In the experiment with the BN/BiRij rats rats of 3 and 30 months were used. Their mean (± S.E.M.) body weights (g) at the start of the experiment were 153 ± 4.1 (3 months) and 227 ± 3.7 (30 months).

The rats were tested individually in polycarbonate cages (32x25x 15 cm) and remained there for the entire duration of the experiment. The test cages were placed in rooms maintained at 22 ± 2 °C, with a humidity between 50 and 60% and a light-dark cycle changing at 7 a.m. and 7 p.m. Testing was fully automated, humans entered the rooms only twice weekly for maintainance and cleaning. The drinktest, previously described in detail (Wolthuis et al., 1976), comprised the following task for the animals. The animals were exposed to four sets of signals: (1) light only, (2) sound only, (3) neither light nor sound, (4)both light and sound. Licking the water tube when both light and sound were "on" was registered as a "correct" response and was followed by the delivery of 0. 25 ml water in 5 seconds. Licking during any of the other signal periods was registered as an "incorrect" response, was not rewarded with water and was punished by shocks delivered through a grid floor under the water tube for 5 seconds. The signals were provided by a small light bulb (0.1 W) 15 cm above the cage floor and a hearing-aid earphone delivering a pure tone of 1600 Hz. with an intensity of 15 dB above background. Signals lasted 15 seconds and were presented in a random order 24 hours a day. The animal could escape the shock by retreating to a nearby non-electrified section of the cage which was out of reach of the water tube. During the 5 seconds periods of reward or punishment further lick responses were not counted in order to prevent the recording of mere rates of licking.

Before the training of the rats started the animals were habituated to the cages during 3 days. During the training period the constant-current footshock was increased stepwise by 100 μA per two days starting with 100 μA on day 1 to 300 μA on day 5. On day 14 the current was increased to 400 μA in order to determine whether still higher shock levels resulted in a further improvement in performance. Three successive experiments were performed with WAG/Rij rats of 12, 18, 24 and 30 months old. Since the procedure in all three experiments was identical and the results of the experiments were virtually the same, all results from the same age groups were pooled. Two successive experiments were performed with the BN/BiRij rats. Since the procedures in these experiments were also identical and the results were virtually the same, the results were pooled.

### 2.2.2. Measurements of spontaneous motor activity and open field behaviour

The WAG/Rij rats used were approximately 3, 12, 24 and 30 months old. Although these rats received no drug treatment they were once daily injected intraperitoneally (i.p.) with a saline solution (7.5 ml per kg body weight) in order to serve as control animals for rats treated with meclofenoxate (see chapter 5). Mean (± S.E.M.) body weights (g) at the start of the experiment were 144 ± 5.1 (3 months), 216 ± 5.2 (12 months), 256 ± 5.0 (24 months) and 262 ± 6.2 (30 months).

Movements in the horizontal plane in the open field were registered by an automated TV-based system that followed a white rat on a black floor of 1x1 meter (Tanger et al., 1978). Simultaneously movements in the vertical direction were registered by means of a capacitive movement transducer system (van den Steen and Vanwersch, in press; Wolthuis et al., 1975) based on the placing of the animal between two 25 cm spaced plates of a capacitor, each larger than 1x1 meter, and recording the disturbances of the dielectricum by the movements (changes in shape) of the rat. To enable the video camera to view the animal the upper plate consisted of a metal grid. Computer processing of the punched tape output results in two sets of graphs showing various characteristics of the behaviour of the animals. Computed were the distance run, the distribution of speeds during a specific period, the time spent in the inner

field, i.e. a 60x60 cm area in the center of the field (the remaining part of the whole field is defined as outer field), the number of crossings from outer to inner field and vice versa and the number of times the rat enters corners (corners are defined as squares of 20x20 cm in each corner of the field). The graphs dealing with vertical activity show the changes in the number of rearings as a function of time, the percentage of time spent in an upright position and the number of movements of two categories of increasing amplitudes. The rats were injected 30 minutes before they were placed into the device and were tested for 1 hour. The order of testing of individuals in the four groups was balanced in order to compensate for the time of the day. The temperature in the experimentation room was 21-23°, the ambient noise level 72 dB and the light level was 360 lux.

### 2.2.3. Measurements of the footshock sensitivity

The WAG/Rij rats used were 3, 12 and 30 months old at the time of the measurements. Mean (± S.E.M.) body weights (g) were 169 ± 3.5 (3 months), 211 ± 4.6 (12 months) and 258 ± 7.7 (30 months).

For the measurements of the thresholds to inescapable footshocks the rats were placed into a transparant Makrolon cage (27x13x16.5 cm) with a grid floor bottom. Grid rods were 3 mm in diameter and interrod distance was 12.5 mm. The grid floor was connected to a scrambling stimulator with a constant current output, which could be increased or decreased by steps of 10 μA. Only "flinch" reactions (Gispen et al., 1970) were noted. After the rats had been placed on the grid floor, they received an initial footshock of 180 μA; the footshock intensity was then decreased in steps of 10 μA until no flinch reaction was seen. From there on, an up and down method was used, i.e. footshock intensity was decreased or increased in steps of 10 μA depending on whether a positive or negative response was seen, respectively. Since a footshock was only delivered when all four paws were in contact with the grid floor, intertrial intervals varied. After 4 positive and 4 negative responses had been obtained, the animals were returned to their home cages. The results were calculated according to the method described by Wetherill (1966).

2.2.4. Measurements of the drinking activity in reaction to reversal of light-dark cycles

Three groups of 8 WAG/Rij rats of 3, 12 and 30 months old were used. Mean (± S.E.M.) body weights (g) were 147 ± 3.3 (3 months), 192 ± 5.2 (12 months) and 219 ± 7.2 (30 months).

The drinktest apparatus used has briefly been described in section 2.2.1. of this chapter. The animals were placed individually in the cages of the drinktest apparatus and their number of drinking responses per 3 hour-periods were accumulated, 24 hours per day, during 6 days. No reinforcement was used, each lick at the water tube was recorded and resulted in the delivery of 0.25 ml of water during the following 5 seconds. During these 5 seconds registration of further licks was blocked, thereby avoiding registration of mere licking frequencies. After these 6 days of a 12/12 hour lighting schedule switched at 7 a.m. and 7 p.m. , light-dark cycles were reversed for 18 days, starting with two light periods of 12 hours in succession. At the end of these 18 days, i.e. on day 24 of the experiment, the normal 12/12 cycle (light "on" during day time) was reinstated. At the beginning of this return to the normal rhythm, two dark periods of 12 hours followed one another. After the last reversal the drinking activity was followed for another 10 days.

2.3. RESULTS

2.3.1. Experiments in the drinktest

To investigate whether the acquisition deficits develop suddenly or gradually, four groups of WAG/Rij rats of 12, 18, 24 and 30 months, respectively, were trained in the drinktest apparatus. The results of this experiment can be seen in figure 1. The following differences between the age groups were found to be significant on day 20, when the results on that last day of training are analysed according to Welch (1947) using the multiple interference method due to Newman-Keuls (Miller, 1966). The percentage correct responses of the 12 months old animals is significantly ($p < 0.05$, two tailed) higher than that of the 18, 24 and 30 months old rats. The results of the 18 and 24 months and those of the

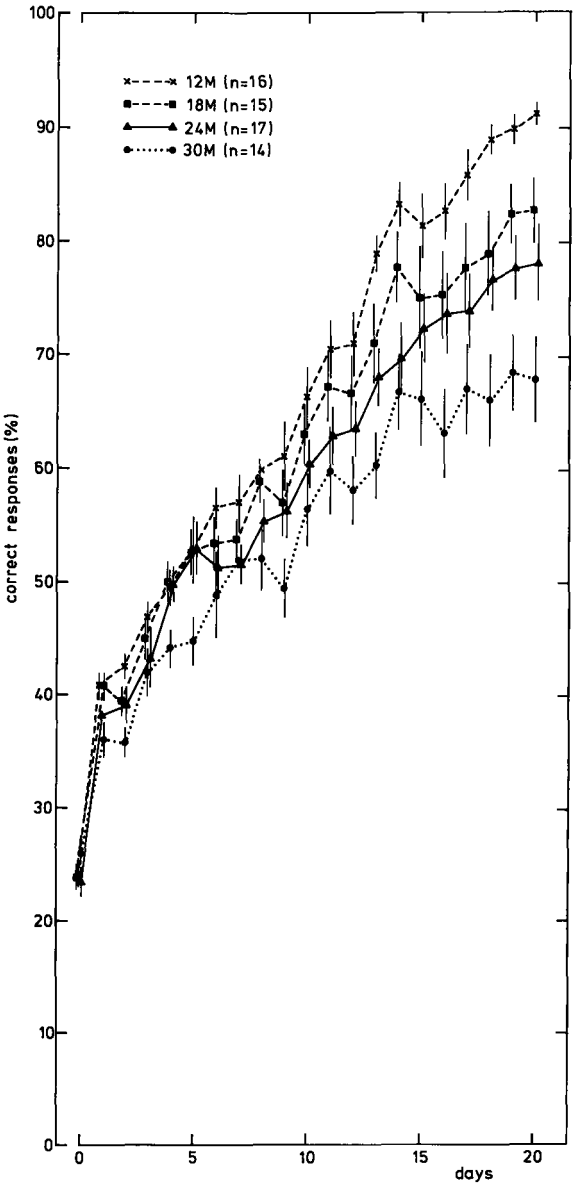



Fig. 1. A comparison of acquisition of 12, 18, 24 and 30 months (M) old WAG/Rij rats trained in the drinktest apparatus. The results are expressed as the means ± S.E.M. of the percentages of correct responses (correct/total).

24 and 30 months old animals do not differ significantly ($p > 0.05$, two tailed) between each other. The results of the 18 months old animals, however, are significantly ($p < 0.05$, two tailed) different from those of the 30 months old animals. From the learning curves and the data of the last day of the training it can be concluded that the deficits in acquisition develop gradually.

To determine whether the acquisition deficits found are limited to only one strain of rats, BN/BiRij rats were subjected to the same training procedure as used for the WAG/Rij rats. Figure 2 demonstrates that - similar to the WAG/Rij rats - 30 months old BN/BiRij rats show a deficit in acquisition when compared with 3 months old rats. Comparison of the performances of the BN/BiRij rats with those of the WAG/Rij rats of the same age under identical conditions (Wolthuis et al., 1976) (figure 3) shows that the learning curves of the BN/BiRij rats are quite different from those of the WAG/Rij rats. The 3 months BN/BiRij rats acquired the task much more slowly than the WAG/Rij rats of the same age. The BN/BiRij rats did finally reach nearly the same level of performance but only after 25 days of training (see figure 3). The 3 months WAG/Rij rats reach this level already after approximately 17 days. A similar trend is visible when the 30 months old rats of both strains are compared. Also in this case the performance of the 30 months BN/BiRij rats finally reached a plateau at a level of 65-70% correct responses, very much like the 30 months old WAG/Rij rats.

### 2.3.2. Measurements of spontaneous motor activity and open field behaviour

The results of measurements of open field behaviour during 60 minutes of 3, 12, 24 and 30 months old WAG/Rij rats can be seen in figure 4. The top left graph shows the cumulative distance run in meters as a measure of motor activity; the middle one of the upper 3 graphs shows the cumulative number of changes in corners as a measure of exploratory behaviour (see Bureš et al., 1976); the top right and the bottom left graphs show the number of entries into the inner field and the time spent there respectively as one of the parameters of emotionality (see Bureš et al., 1976); the middle and the right graphs of the bottom line show the cumu-

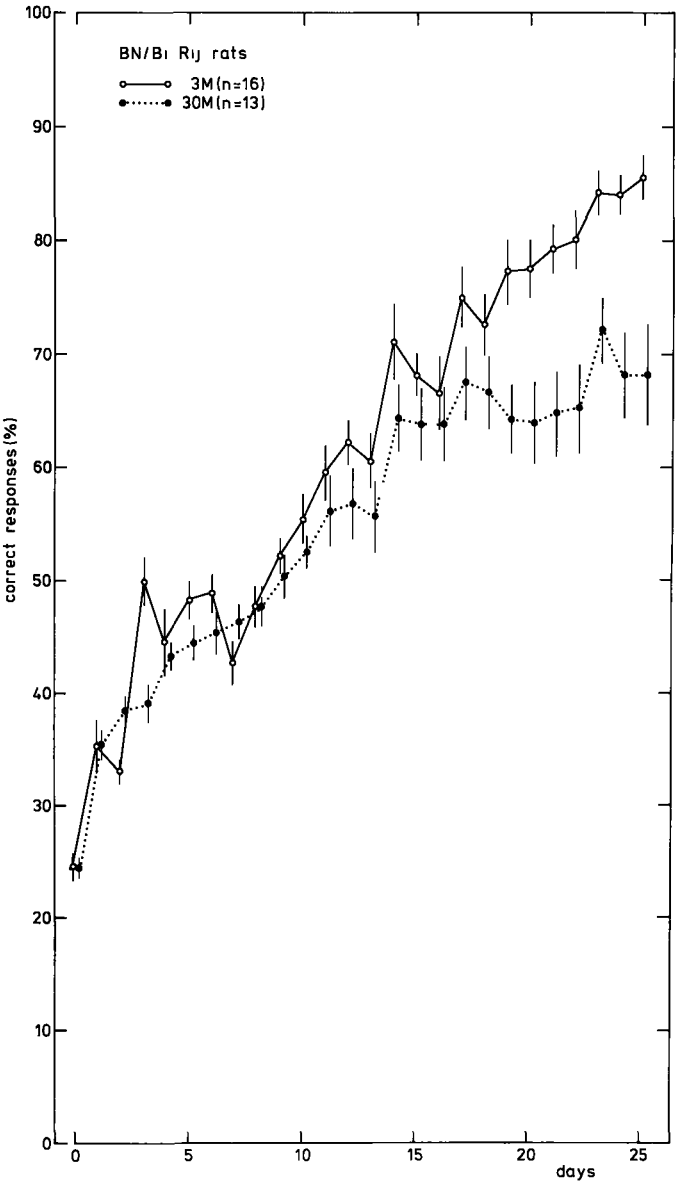



Fig. 2. A comparison of the rate of acquisition of 3 and 30 months (M) old BN/BiRij rats trained in the drinktest. The results are expressed as in fig. 1.

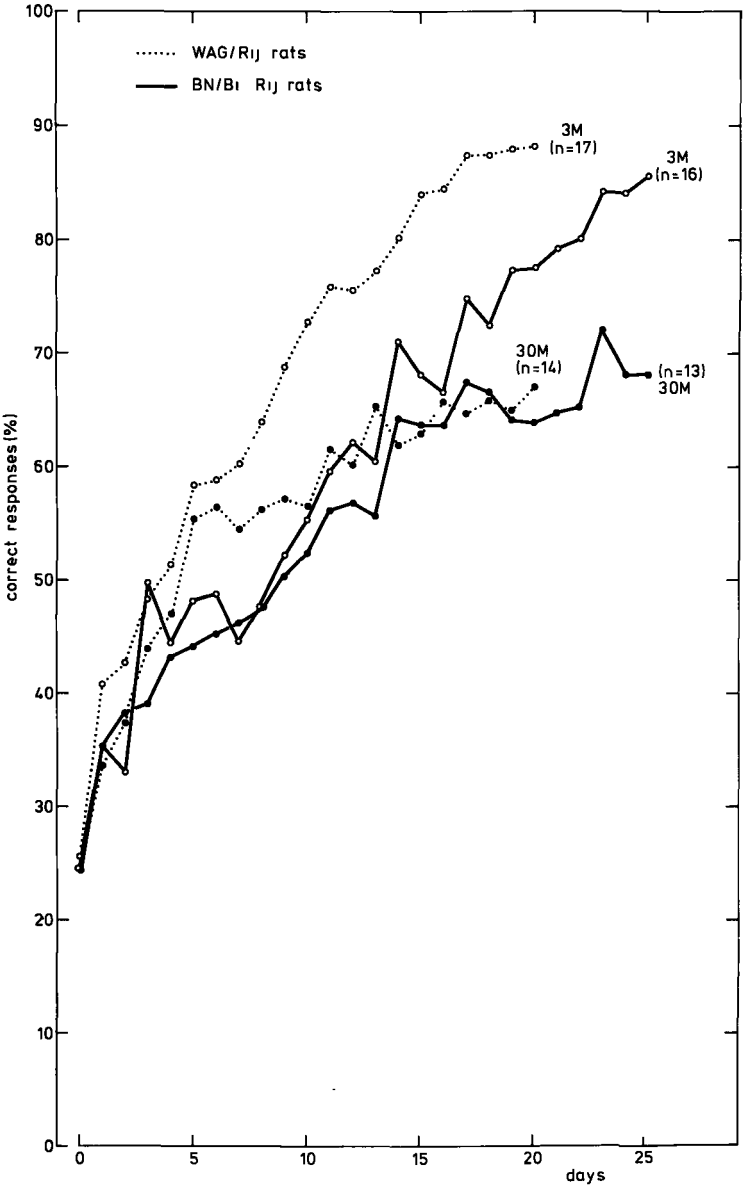



Fig. 3. A comparison of the rate of acquisition of 3 and 30 months (M) old WAG/Rij rats (taken from Wolthuis et al., 1976) and 3 and 30 months (M) old BN/BiRij rats trained in the drinktest. The results are expressed as mean percentages correct responses. For the sake of clarity the S.E.M's have been omitted in this figure.

lative time that movements of the indicated speed classes were detected. The values of the distance run in 60 minutes of the 3, 12, 24 and 30 months old rats are all significantly ($p < 0.05$, two tailed) different from one another when analysed according to Welch (1947) using the multiple interference method according to Newman-Keuls (Miller, 1966). The gradual decrease in distance run with age causes also a gradual decrease in entries into corners, entries into inner field and time spent in inner field because the fact that these parameters are directly dependent of the hight of the motor activity. From the graphs on speed distribution it can be deduced that the speed at which the animal moves decreases with age.

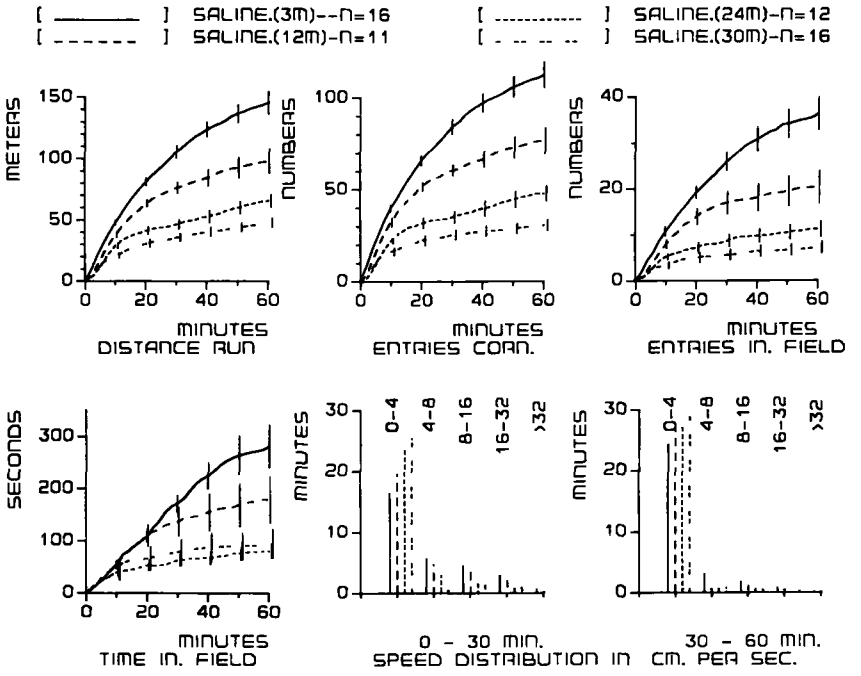



**Fig. 4.** The results of measurements of open field behaviour during 60 minutes of 3, 12, 24 and 30 months (m) old WAG/Rij rats with the automated TV-based system. All values are expressed as means ± S.E.M.

The results of the measurements of the vertical movements during 60 minutes of the rats of the four age groups are demonstrated in figure 5. For the statistical evaluation of the results the values of the different parameters during the 60 minutes of measurement are summed for

each animal for the different age groups. The values are analysed according to Welch (1947) using the multiple interference method due to Newman-Keuls (Miller, 1966). In the small vertical movements of amplitude class 2 the activity of the 12 months old animals is significantly ($p < 0.05$, two tailed) lower than that of the 3, 24 and 30 months old rats. In the large vertical movements of amplitude class 3 the activity of 3, 12, 24 and 30 months old animals are all significantly ($p < 0.05$, two tailed) different from one another, the activity of the young rats being the highest and that of the 30 months old animals being the lowest. Both "rearing count" and "rearing time" of the animals of the four age groups differ significantly ($p < 0.05$, two tailed) between one another; the values of these parameters decrease with age. These results indicate that the vertical activity of large movements decreases gradually with age.

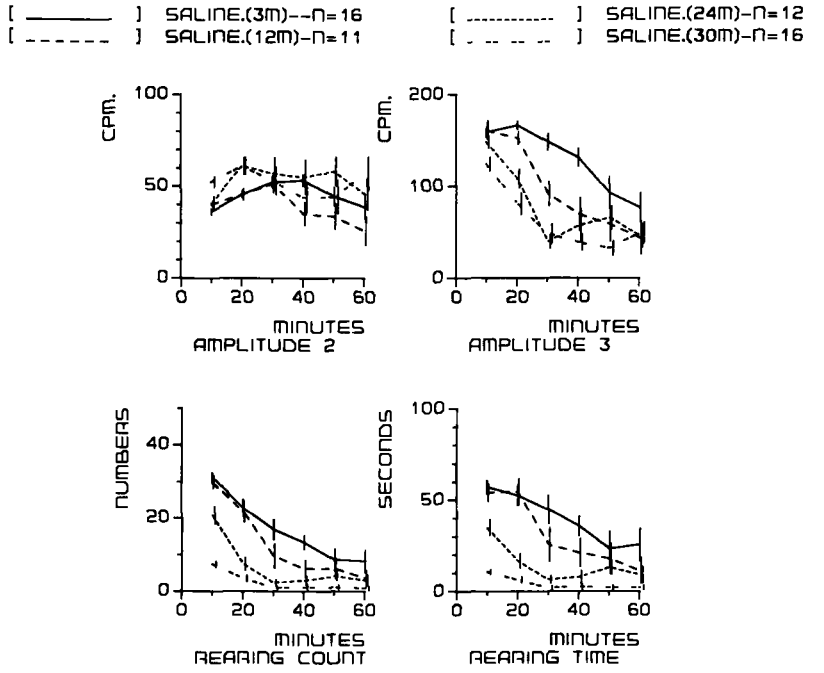



**Fig. 5. The results of the measurements of vertical movements during 60 minutes of 3, 12, 24 and 30 months (m) old WAG/Rij rats. The upper graphs represent vertical movements of increasing amplitudes, movement amplitude 3 being the largest. The results are averaged over 10 minute periods. The lower left graph shows the number of rearings per 2 minutes averaged over 10 minute periods and the lower right graph the time spent in an upright position per 2 minutes averaged over 10 minute periods. All values are expressed as means ± S.E.M.**

2.3.3. Measurements of the footshock sensitivity

The results of the measurements of the footshock thresholds are shown in table 1.

TABLE 1

FOOTSHOCK THRESHOLDS OF FEMALE WAG/Rij RATS

| Age | 3 months (n = 10) | 12 months (n = 10) | 30 months (n = 10) |
|---|---|---|---|
| "Flinch" thresholds in μA ($\bar{x} \pm$ S.E.M.) | 62.0 ± 5.1 | 56.9 ± 6.3 | 61.2 ± 3.8 |

No significant ($p > 0.05$, two tailed) differences in flinch thresholds were found between 3, 12 and 30 months olds rats. Histological observations of the paws of several of these animals did not reveal any differences in the thickness of the stratum corneum between 3 and 30 months old rats.

2.3.4. Measurements of the drinking activity in reaction to reversal of light-dark cycles

To examine whether age-related differences could also be demonstrated in more basic, adaptive functions of the CNS, the adaptation of drinking activity in reaction to reversed light-dark cycles was studied. Although the old animals (30 months) slightly lagged behind the adult and the young (12 and 3 months) rats in adapting their drinking pattern to reversal of the lighting conditions, large age differences in speed of phase-shifting were not observed (figure 6). Similarly, return to normal lighting conditions did not result in appreciable differences between the age groups.

Under the non-reinforced conditions of the present experiment the daily number of drink responses of the 30 months old animals was signifi-

cantly ($p < 0.05$, two tailed) greater than that of the young and adult rats. This increased number of responses seems to be due to a greater waste of water and to an increased urine production in these old rats.

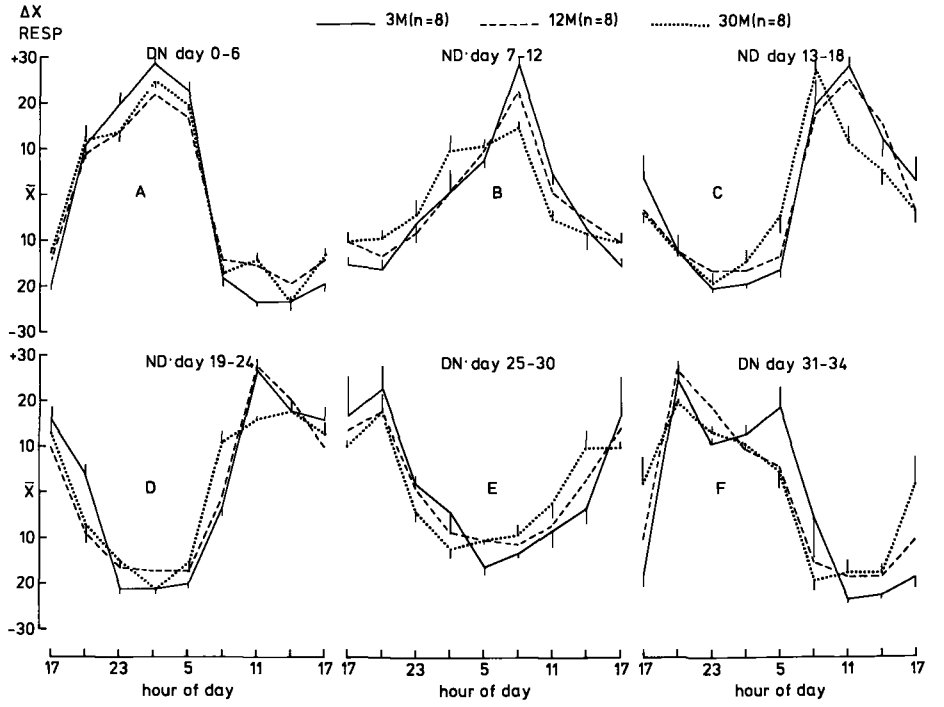



**Fig. 6.** **The distribution of drinkresponses of *WAG/Rij* rats of different ages during the hours of the days as affected by reversal of day- night illumination. The first 6 days of standard day-night (DN) lighting (curves A) were followed by 18 days of reversed (ND) lighting (curves B, C and D) and subsequently standard lighting (DN) was restored for 10 days (curves E and F). The results are expressed as the mean (± S.E.M.) differences between the actual number of responses per 3 hours and the number of responses per 3 hours when the total number of responses are averaged over 24 hours (ΔX). First the mean daily changes per group were calculated and thereafter the results of 6 days were averaged. For the sake of clarity the S.E.M. of the 12 months old rats have been omitted.**

It should be noted that all animals, including the old rats, could easily fulfil their daily need of water within a period of 30 minutes; i.e. only a fraction of the day had to be spent on drinking. There-

fore it is noteworthy (see figure 7) that the drinking activity of the 30 months old rats between the peaks does not return to near-zero as that of the 12 and 3 months old animals. This suggests that the old rats have much shorter periods of sleep or rest.

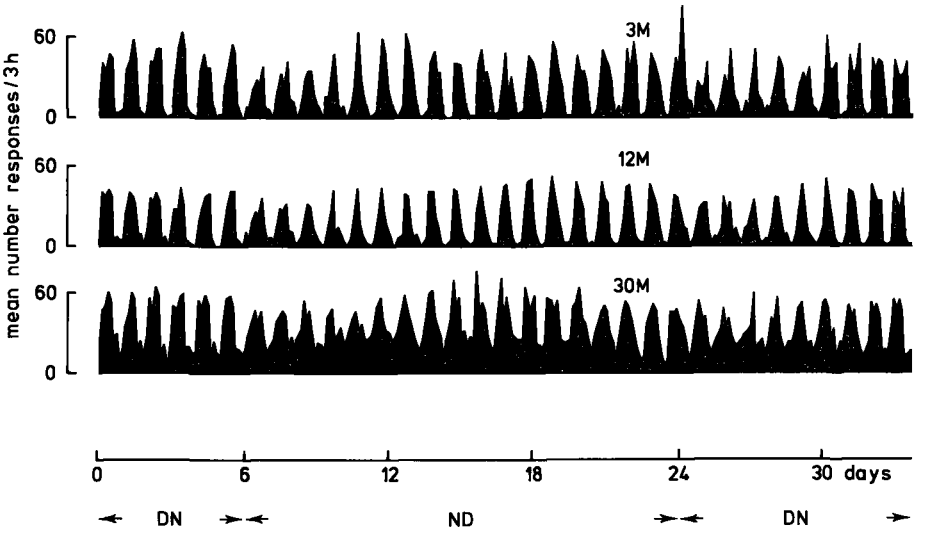



**Fig. 7. The changes in drinking activity of 3, 12 and 30 months (M) old WAG/Rij rats sampled per 3 hours. The first 6 days of standard day-night (DN) lighting were followed by 18 days of reversed (ND) lighting and subsequently standard lighting (DN) was restored for 10 days.**

## 2.4. DISCUSSION

The results obtained with four groups of rats of various ages (12, 18, 24 and 30 months) showed that deficits in acquisition develop gradually with increasing age (figure 1). These results are compatible with those of Verzár-McDougall (1957) who found with groups of rats of increasing age, that the number of animals which learn a maze task very slowly or not at all, increases considerably with age. It is interesting to note, that also in their study the deficits became clearly visible when the rats had reached the age of 18 months. In the present study this was also the case; acquisition of the 12 months old rats was as fast as that of the 3 and 12 months old rats in the experiments of Wolthuis et

al. (1976) in which no differences in acquisition rate of these two age groups have been found.

The age-dependent acquisition deficits observed in the present experiments might be attributable to changes in activity levels with age. Measurements of spontaneous motor behaviour and behaviour in an open field situation showed primarily an age dependent decrease in motor activity in the horizontal dimensions (figure 4), so that no conclusions could be drawn about the entries into inner field and about the time spent in the inner field, which are generally considered as one of the indices of emotionality (Bureš et al., 1976). As to movements in the vertical dimension, there are similarities and differences between the present results and those obtained earlier by Wolthuis et al. (1976). They also found a significant greater motor activity in 3 months old rats than in 12 and 30 months old rats. On the other hand they found no age related differences in "rearing counts" and "rearing time", except during the first 16 minutes. These differences might be explained by differences in the devices used; in the present study the area in which the animal moved was 100x100 cm, whereas in the previous experiments of Wolthuis et al. (1976) this was only 30x20 cm (bottom of a glass cage). Indeed, results of earlier studies of Hofecker et al. (1974) on age-related activity patterns differed from those of later ones (Hofecker et al., 1978) in which a different device was used. The results of activity measurements, therefore, depend to a large extention of the technique used. Nevertheless, there is a large difference in motor activity between 3 and 12 months old rats and since there are no differences in acquisition rate between rats of these two age groups, it seems very unlikely that the differences in motor activity between these age groups by themselves are responsible for the differences in acquisition.

Another factor which could contribute to the age-related differences in performance might be a difference in sensitivity to footshock. In the acquisition experiments in the drinktest animals were punished by electric footshocks. A decreased sensitivity to footshock would lead to a reduction in punishment level resulting in a decreased motivation. It is not very likely that a difference in footshock sensitivity can be held responsible for the acquisition decrements observed because in the first place similar decrements are found when no footshock is used (Wolthuis et

al., 1976) and in the second place, because performance during the first week was comparable for all age groups; differences began to appear only several days after day 4, when maximum footshock levels had been reached. Furthermore, when the shock level was increased on day 14, this had no noticeable effect on performance. Because in the literature the results of the measurements of footshock sensitivity in rats of different ages were not uniform (Gordon et al., 1978; Paré, 1969) footshock sensitivity in rats of three age groups was measured by determining the threshold current for a flinch reaction. No significant differences were found between the 3, 12 and 30 months old rats. In addition, it was found that the thickness of the epidermis (including the horny layer) of the paws was approximately the same in the 3 and 30 months old rats. Apparently, the rats used in these experiments did not develop thickened layers of the stratum corneum on the paws. Apart from differences in experimental procedures the discordant results of Gordon et al. (1978) and Paré (1969) may have been due to differences in cage floors or shavings, which might influence the thickness of the epidermis of the paws or lead to differences in hydration state of the skin of the paws. Whatever this may be, in the present experiments a decreased footshock sensitivity cannot explain the observed acquisition deficits of the old rats.

Similar to the WAG/Rij rats of the same age, pigmented BN/BiRij rats of 30 months old showed a deficit in acquisition in the drinktest when compared with the 3 months old rats of the same strain (figure 2). When compared with the WAG/Rij rats (figure 3) the BN/BiRij rats acquired the task much more slowly but ultimately reached the same level of performance. Clearly, the performance of the 30 months BN/BiRij rats also reached a plateau at a level of 65-70% correct responses, very much like the 30 months old WAG/Rij rats. Independent of the speed of acquisition, therefore, the acquisition deficits found in rats are obviously not limited to one strain.

When two cages, one with BN/BiRij rats and the other with WAG/Rij rats are placed side by side, only brief observation is required to note that the BN/BiRij rats are livelier and more active than the WAG/Rij rats. However, when the spontaneous motor behaviour of each animal was measured individually in an automated device (Wolthuis, 1971), the activity of both the 3 and 30 months old

BN/BiRij rats was equivalent to that of WAG/Rij rats of the same age. Possibly, the visually observed higher activity of the BN/BiRij rats does not represent a higher level of spontaneous activity per se, but a higher stimulus induced re-activity, when an observer is watching them.

After the light-dark reversal the old (30 months) animals only slightly lagged behind the adult and the young (12 and 3 months) rats in adapting their drinking pattern (figure 6). Similarly, return to normal lighting conditions did not result in appreciable differences between the age groups. This absence of a difference is in keeping with results on food intake obtained by Jakubczak (1975). He found that the pattern of food intake of 785 days old rats was as adaptable to reversed light-dark illumination as food intake patterns of 220 days old rats. It is known that water and food intake in the rat follow the same pattern (Siegel and Stuckey, 1947), although under certain experimental conditions illumination may affect fluid intake and food intake independently (Rusak and Zucker, 1974). Under the non-reinforced conditions of the present experiment the daily number of drink responses of old rats was significantly greater than that of young and adult animals. This increased number of responses of the old animals is most likely due to what might be called "behavioural negligence". In fact, the shavings around the watertubes in the cages of the old rats were usually soaked with spilled water, this was not so in the cages of the younger rats. Observations suggest that this spill of water is the result of briefly sucking the watertube, thereby triggering the pump and then turning away before the 5 seconds period of water delivery has ended. It should be noted that all animals, including the old ones, could easily fulfil their daily need of water within a period of 30 minutes; i.e. only a small fraction of the day had to be spent on drinking.

It is noteworthy (see figure 7) that in contrast to the young and adult rats drinking patterns of the old rats under the normal lighting conditions of the first 6 days did not show a prolonged daily period of near-zero drinking activity as was observable in 3 and 12 months old rats. This suggests that rest periods or sleepwake cycles in these aged rats are altered. Changes in sleep patterns with age are well known in humans (Timiras and Vernadakis, 1972). In rats it has been observed that

animals of advanced age have a reduced sleep-time and shortened sleep-bouts (Rosenberg et al., 1979). The present observations are in agreement with these findings and suggest that the old animals take frequent brief naps and take short sucks at the watertube in between. In view of the interest in recent years in the possible relation between learning and the quality of sleep, especially Rapid Eye Movement (REM) sleep, it might be worthwhile to study the relationship between ageing, sleep patterns and learning in more detail. It might turn out, that changed sleep patterns are one of the causes for the acquisition deficits in aged rats.

Summarizing, the data obtained so far do not point at a factor or factors which can be held responsible for the acquisition deficits found in the ageing rats. One of the puzzling questions is why the acquisition curves of 3 and 30 months old rats are so similar during the first one or two weeks, whereas in the later stages of acquisition they diverge and in the case of the 30 months old rats seem to reach a plateau at a much lower level than at a younger age (figure 3). One of the most important factors which is involved in acquisition is motivation. It must be considered whether differences in motivation between old and young rats play a role in the different rates of acquisition. The two factors in the drink-test which can motivate the animals are the degree in punishment and the need for water. Motivational differences on the basis of differences in the degree in punishment, i.e. in footshock thresholds were excluded in this study. Since the rats are continuously in the apparatus and the total number of correct - i.e. water-rewarded - responses per day was found to be fairly constant throughout the whole training period, the animals are never deprived in water. What happens is that the number of incorrect - i.e. unrewarded - responses decreases when the acquisition progresses. Since the number of correct responses per day in young and aged animals is roughly the same, it seems reasonable to assume that the need for water and the motivational levels in this respect are approximately equal. It is very remarkable, however, that in the training situation the behavioural negligence - observed in the non-reinforced situation - does not appear in the old animals. In addition, if motivational factors were involved one has to assume that the motivation levels change in the second half of the training period during which the acquisition curves start to diverge. Taken together, the age-related differences

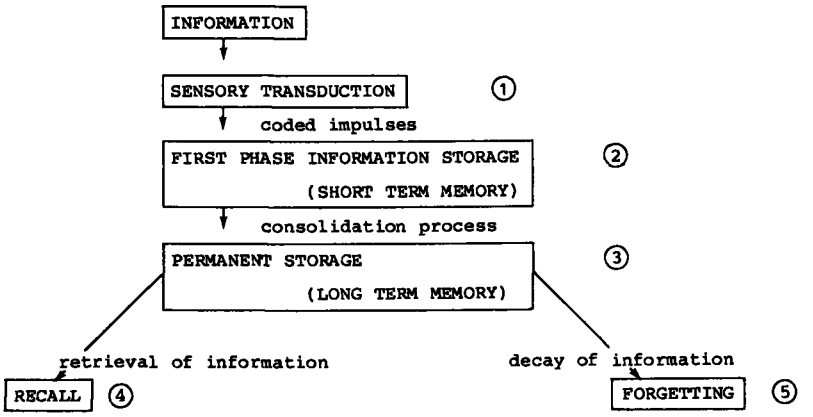
seems not to be due to the rather basic motivational factors mentioned. Other causes for the age-related acquisition differences found must be involved.

# CHAPTER 3

# THE EFFECTS OF AGEING ON SOME PHASES OF THE LEARNING AND MEMORY PROCESSES

## 3.1. INTRODUCTION

Learning and memory are often mentioned together, mainly because they are operationally indistinguishable. According to Melton (1963) learning is defined in terms of trials or experiences, whereas memory is defined in terms of time intervals between trials. It is generally believed that the learning and memory processes consist of several different phases which are usually presented (see e.g. Glassman, 1969) in the following hypothetical scheme:




The phases ① and ② together are also called the registration phase.

According to some authors an intermediate or recent memory can be distinguished between the short and the long-term memory (McGaugh, 1969). In this study, however, the phases as given in the scheme has been followed.

It is generally assumed that upon reaching the brain, the information contained in the impulscodes is stored in the short-term memory phase. Speculations about the form in which this storage occurs are abundant and vary from "reverberating" neuronal circuits to "regenerating"

neuronal circuits. For the information storage in this phase, so it seems, coherent electrical activity is necessary because any disturbances of this activity by electro-convulsive shock, convulsive drug administration or deep anaesthesia leads to disruption of storage (Jarvik, 1964; McGaugh, 1966). Perhaps this electrical activity "triggers" a number of metabolic processes required for long-term storage. There is a wealth of experimental data which indicates that if protein synthesis in the brain is sufficiently blocked animals still acquire a task reasonably well (Barondes, 1970). Under those conditions retention during approximately the first hour is good, but from then on starts to decrease. It is generally accepted that these results indicate that for long-term memory formation protein synthesis is required and also that short-term memory, as a nonpermanent form of information storage, has come to an end (Glassman, 1969). In the long-term memory the information seems to be stored in a different way; all sorts of measures which disturb the coherent electrical activity of the brain are ineffective in disrupting long-term memory. Recall of stored information occurs through a retrieval process about which very little is known.

In animals and humans detailed studies on the different phases of learning and memory processes in relation to ageing are scarce and the interpretations of the results are often controversial. Since the registration process consists of sensory transduction and the first phase information storage processing of the nerve impulses in the central nervous system (CNS) it is possible to gain some insight into this process by studying the eye as a sensory organ and the processing of visual information in the CNS. The first approach is usually to record visual evoked responses (VER's) from the brain cortex following visual stimulation and to measure the amplitudes of the various components of the responses as well as the time interval (latency following the stimulus) after which they reach their maximum.

Considerable controversy exists in the literature regarding the effect of age on the amplitude of the VER in humans. Perry and Childers (1969) reported a decrease in amplitude with increasing age, while Dustman and Beck (1969) and Straumanis et al. (1965) stated that higher amplitudes were seen in elderly individuals. The results of Celesia and Daly (1977) and Drechsler (1978) show no statistically significant corre-

lation between age and hight of the amplitudes. The latencies of the early components of the VER, however, are uniformly longer in all studies in aged humans (Celesia and Daly, 1977; Drechsler, 1978; Staumanis et al., 1965). The increased latency of the early components of the VER probably reflects a slower conduction velocity in the optic nerve, the optic pathways or both which may be caused by segmental demyelination of the optic pathways or by a decrease in effectiveness of myelination as ageing progresses (Celesia and Daly, 1977).

Experiments with normal human subjects have shown an age-related decline in short-term memory (Botwinick, 1975; Welford, 1958); especially performance on the basis of visually presented material decreases rapidly with age (Adamowicz, 1976; McGhie et al., 1965; Walsh and Thompson, 1978). In connection with the age differences in short-term memory in humans it is interesting that in a number of recent studies similar age differences in short-term memory have been reported in monkeys (Bartus et al., 1978) and in rats (Goodrick, 1968, 1973; Jakubczak, 1973; Ordy et al., 1978). Age-related changes in this stage of the learning and memory process seem to occur in many species.

Only few studies on possible changes of the long-term retention of learned information with age have been found. In humans long-term retention is usually assumed to be little affected by age. Indeed, Botwinick and Storandt (1974) found that increased age was not associated with a decreased function of the long-term memory. On the other hand, however, significant age differences in recall and recognition have been interpreted as loss of long-term memory with age (Craig, 1977; Walsh, 1975). The results obtained in rats did not point to age differences in long-term retention (Dye, 1969; Goodrick, 1968).

On possible age-related changes of the retrieval and the decay process only very few reports exist; the results reported are inconclusive and will not be discussed here.

In the present study, attempts were made to investigate whether deficits in the processing of visual information, in short-term memory or in retention might be held responsible for the acquisition deficits found in aged rats. It has been tried to obtain more insight in these mechanisms by recording visual evoked responses, testing alternation behaviour and by carrying out passive avoidance tests in rats of various ages.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Effects of age on the electroretinogram and the visual evoked responses

The female WAG/Rij rats used were approximately 3, 12, 24 and 30 months old. They were injected intraperitoneally (i.p.) once daily during ten weeks with a saline solution (7.5 ml/kg). After a shortlasting open field test all animals were trained in the drinktest. In fact these animals also served as control animals for experiments which will be reported later. Since the same rats were tested on rate of acquisition, this enabled us to correlate possible age-related changes in visual evoked responses (VER's) with alterations in the rate of acquisition.

Mean (±S.E.M.) body weights (g) at the start of these experiments were 144 ± 5.1 (3 months, 216 ± 5.2 (12 months), 256 ± 5.0 (24 months) and 262 ± 6.2 (30 months).

One day prior to the measurements, cortical electrodes were inserted under sodium hexobarbital (185 mg/kg) anaesthesia. A hole was drilled in the skull, its center 3 mm to the left of the midline and 2 mm in front of the suture between parietal and interparietal bones, i.e. in the area overlying area 17 according to the atlas of Krieg (1946). The dura mater was left intact. One electrode was inserted in the hole and an indifferent electrode was secured on the nasal bone. Both were Ag-AgCl electrodes with a diameter of 0.75 mm. They were insulated and attached to the skull with Sevriton® dental cement (De Trey Frères, Zürich, Switzerland); only the tips of the electrodes remained free. The following day, when the animals had been adapted to total darkness for at least 12 hours, the rats were i.p. injected with 100 mg/kg gallamine triiodide (Flaxedil®, Specia, Paris, France). When paralysis set in, the animals were intubated with a polythene cannula and connected to a respiration pump. The head was gently fixed in a head holder. The pupil of the left eye was dilated with 1% atropine sulphate in 0.9% NaCl solution. The right eye was covered. All procedures were carried out in dark red light (Kodak Wratten filters No 2). To measure the electroretinogram (ERG) an electrode was connected by a fluid bridge of 0.9% NaCl solution with the left eye.

The light source was a light emitting diode (LED) (GaP-Lumineszenzdiode, Siemens, Type CQX 131) emitting monochromatic light at 560 nm, i.e. well within the spectral sensitivity of the eye of the albino rat (Silver, 1967). The advantage of this way of light flash administration is that the amount of light emitted is linearly related to the current through the LED; in this case the amount of light reaching the eye was $133 \times 10^{-9}$ lumen per mA. The LED was placed at a distance of approximately 3/4 cm from the left eye. The axis through the light source pointed downwards at an angle of $10^{o}$ with the horizontal plane through the eye and at an angle of $45^{o}$ with the sagittal plane.

After proper amplification and filtering (high and low frequency cut off 120 c/s and 0.35 c/s, respectively), the signals were visualized on a Tektronix 5103 N storage oscilloscope with 5 A 22 N plug-in amplifiers and stored by a Hewlett Packard 3960 instrumentation tape recorder. All cortical events following the flash during 500 msec were recorded. Averaging of 100 cortical recordings per animal at each light intensity took place by a Focal program on a PDP8/I computer.

Thirty minutes after the moment that the animal was fixed into its position, light flashes were administered of 2 msec duration. The interflash interval was 2 sec $\pm$ 20% random. In 13 blocks, each of 100 flashes, the light intensity corresponding with 0.05 mA was systematically increased from one block to another by a factor 2. The ERG's and VER's are performed at four light intensities, corresponding with 0.4, 3.2, 25 and 200 mA, i.e. $53.2 \times 10^{-9}$, $425.6 \times 10^{-9}$, $3325 \times 10^{-9}$ and $26600 \times 10^{-9}$ lumen respectively.

The procedure followed has been described by Wolthuis and de Wied (1976).

### 3.2.2. Effects of age on learning in a T-maze

The female WAG/Rij rats used were 3 and 30 months old at the beginning of the experiment; the experiment took 3 weeks. All rats used were experimentally naive.

The apparatus used was an enclosed T-maze, made of aluminium with roofs made of clear perspex (figure 8). The T-maze consists of a start box (20x15x20 cm), a leg and two arms (50x15x20 cm), a choice compartment

(15x15x20 cm) and two goal boxes (20x15x20 cm). The startbox and the entrance of both arms could be blocked by means of sliding doors to prevent retracing. An indirectly shining 40 W light bulb provided the illumination of the experimental room. Above each goalbox a small 0.3 W light bulb was installed; the light was flashing at 3 Hz and its intensity could be regulated in a stepwise fashion. The bottom of the T-maze was covered with polythene. The goalboxes contained Petri dishes, which were used to deliver a few small grains of chocolate as a reward.

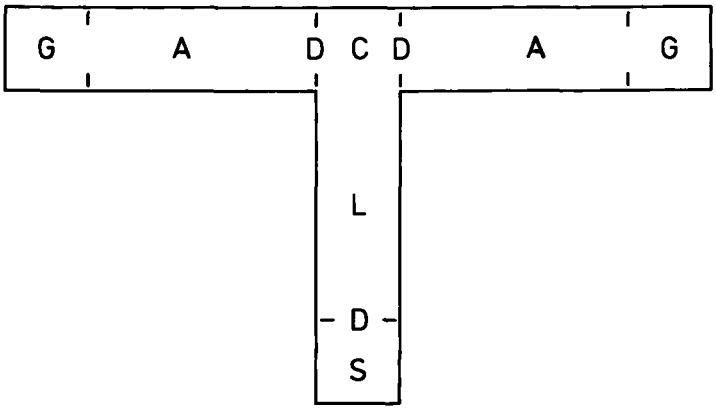



Fig. 8. Floorplane of the T-maze used. S, startbox; D, sliding door; L, leg; C, choice compartment; A, arms; G, goalbox. Leg and arms 50 cm long.

During the week prior to the training experiment the animals were gentled by hand and were adapted to the T-maze, one by one, for 15 minutes during the last consecutive two days. The animals were given a chance to acquire a taste for the chocolate grains. In the first week of training the animals were trained to alternate their choices between the left and the right arm of the maze during eight successive trials, twice daily for five days. The correct arm of the T-maze was indicated by a flashing light above the correct goalbox; a correct alternation response was rewarded with a few chocolate grains in the Petri dish in the goalbox. To prevent olfactory stimuli from interfering, the "wrong" goalbox also contained a Petri dish with chocolate grains. This Petri dish, however, was covered with wire mesh which prevented that the animal consumed the

reward. The first three days of the second week of training the same procedure was followed except that the intensity of the light above the "correct" goalbox was decreased day by day until on day 4 of the second week the rats were trained to alternate without the light cue. On the fifth day of the second week the rats were trained to alternate in 11 successive runs, i.e. with 10 possibilities to alternate, without an interval between runs. In the third week the animals were tested. On day 1 of this week they received a last training session, i.e. without time interval. On day 2 they were tested with 1 minute intervals between the runs, on day 3 they received again a training session and on day 4 they were tested with 2 minute intervals between the runs. During these train ing and testing sessions the animals had to alternate from left to right and back 10 times out of 10 possible alternations to reach a perfect score. On day 2 and 4, during the intervals of 1 and 2 minutes respectively between the runs, the animals were placed into a circular box to isolate the animals from any directional cue. No time limit was imposed upon the animals, the only criterion was the correct or incorrect choice in the T-maze.

Two successive experiments were performed. Since the procedure in both experiments was identical and the results were virtually the same, all results were pooled.

### 3.2.3. Effects of age on passive avoidance learning

The female WAG/Rij rats used were 3, 12 and 30 months old and experimentally naive. Mean ($\pm$ S.E.M.) body weights (g) were 151 $\pm$ 3.2 (3 months), 197 $\pm$ 4.7 (12 months and 213 $\pm$ 6.8 (30 months).

The principle of the test is well known. When given a choice between a light and a dark compartment, rats exhibit a preference to stay in the dark compartment, however, they tend to avoid this compartment initially but after a long latency period they enter it again.

A two compartment light-dark passive avoidance apparatus was employed consisting of two symmetrical plexiglass cages (30x30x30 cm) standing on a shock grid and connected by a pneumatically operated guillotine door guarded by four infrared beams. One compartment was dark and the other one illuminated by a small fluorescent tube. The four light

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Page 40: the second paragraph from the bottom, should read as follows:
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The principle of the test is well known. When given a choice between a light and a dark compartment, rats exhibit a preference to stay in the dark compartment. Once they have received a footshock in the dark compartment, however, they tend to avoid this compartment initially but after a long latency period they enter it again.

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Another method to quantify the reluctance of the rats was also used, i.e. to count the number of rats from a group that did not enter the dark compartment during the allowed 30 minutes.

emitting diodes which generated the infrared light beams were mounted two by two on the light and the dark side of the door. On each side the distance between the upper and the lower light was 1 cm. Both beams were interrupted by the animal's head or body when it passed the door-opening, but tail movements in the door opening could only interrupt one beam at a time. By electronically recording and interpreting the sequential interruption and reinstatement of beam continuity on both sides of the door, the direction of movements of the animal could be registered without interference or false detections from tail flicks. The equipment, built in the laboratory, registers the latency to enter the dark compartment, the time spent in the dark compartment per period of 3 minutes, the number of times the animal changed compartments and the number of approaches into the dark compartment. These approaches are defined by the order in which beam continuity is broken and restored. When an animal sticks its head into the dark compartment it either retreats or it advances. The first is registered as an approach, the second as a change of compartment. Registration of all parameters occurred automatically on paper tape.

Three experiments were carried out, each with 6 groups of 4 rats. The results of the experiments were pooled. The procedure was as follows: an animal was placed into the illuminated box with its head facing away from the guillotine door. When the animal had entered the dark compartment, the guillotine door was closed automatically by a pneumatic device constructed in a way which prevented damage to the tail. The door remained closed for 15 seconds during which period the animal received a scrambled footshock of 250 μA (constant current). Only the three experimental groups received footshock, the three control groups did not. After 15 seconds the door opened again, the rat escaped to the illuminated compartment and was returned to its home cage after it had calmed down (15-30 seconds). The next four days each animal was tested during 30 minutes.

## 3.3. RESULTS

### 3.3.1. Effects of age on the electroretinogram and the visual evoked responses

As an example of the ERG figure 9 gives the averaged ERG's of the rats of the four age groups obtained with lightflashes of the highest lightintensity (200 mA LED current, i.e. 26,600x$10^{-9}$ lumen).

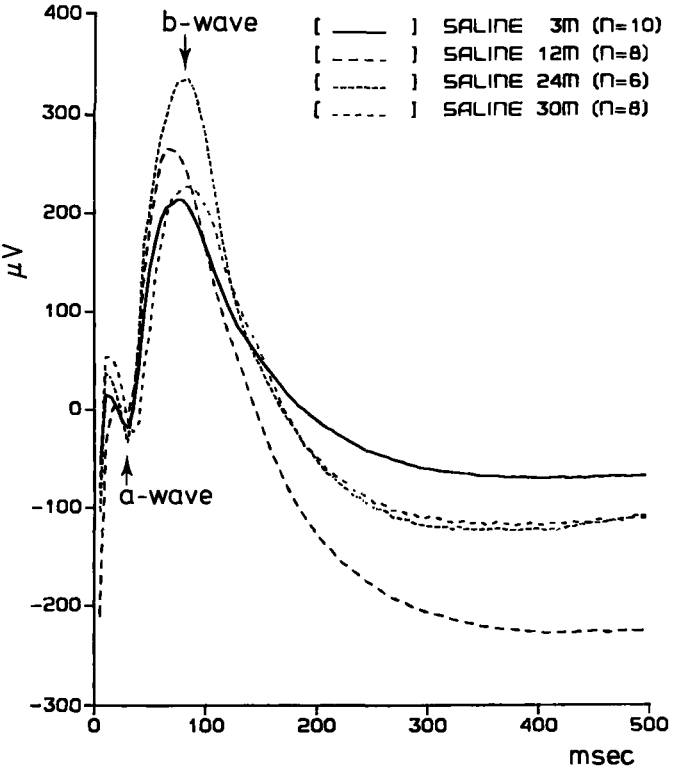


Fig. 9. Computer averaged ERG's at the highest lightintensity (200 mA LED current, i.e. 26,600x$10^{-9}$ lumen) of the 3, 12, 24 and 30 months (m) old rats. At this lightintensity the a-wave is clearly detectable. Abscissa milliseconds (msec), ordinate μVolts.

The amplitudes of the b-waves of the ERG of the rats of different ages at four lightintensities are plotted in figure 10. Figure 11 shows the latencies to the peak values of the b-waves at four lightintensities for the different age groups.

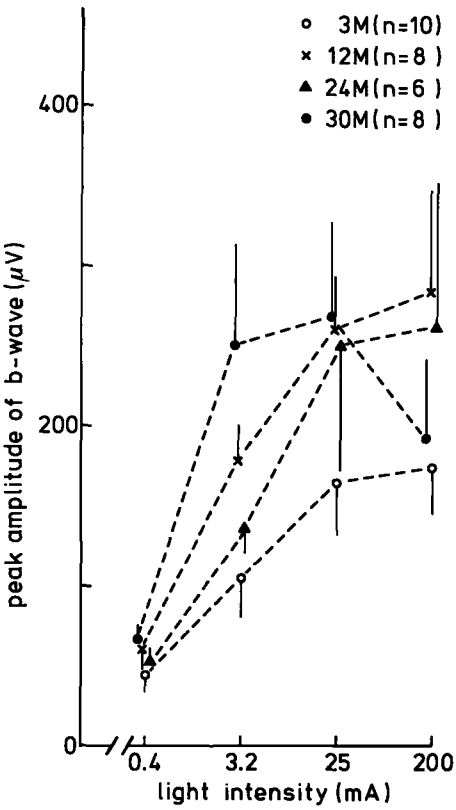


Fig. 10.

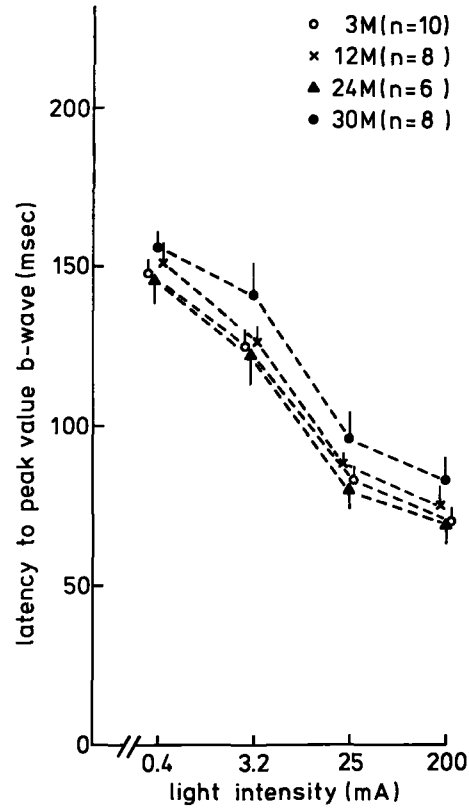


Fig. 11.

Fig. 10. The mean (± S.E.M.) amplitudes of the b-waves of the ERG of the 3, 12, 24 and 30 months (M) old rats. The amplitudes are measured at four lightintensities: 0.4, 3.2, 25 and 200 mA LED current corresponding with $53.2 \times 10^{-9}$, $425.6 \times 10^{-9}$, $3,325 \times 10^{-9}$ and $26,600 \times 10^{-9}$ lumen respectively.

Fig. 11. The mean (± S.E.M.) latency periods (flash to peak of b-wave of the ERG) in msec of the 3, 12, 24 and 30 months (M) old animals. The latencies are measured at four different lightintensities. 1 mA current through the LED corresponds with $133 \times 10^{-9}$ lumen on the eye of the rat.

Figure 12 shows the averaged VER of the animals of the four age groups obtained with lightflashes of the highest lightintensity (200 mA LED current, i.e. $26{,}600 \times 10^{-9}$ lumen). The VER components were identified as indicated by Creel et al. (1974), which is shown in figure 12. The designations $P_2$ and $N_2$ were omitted; these small peaks were not present in the averaged traces.

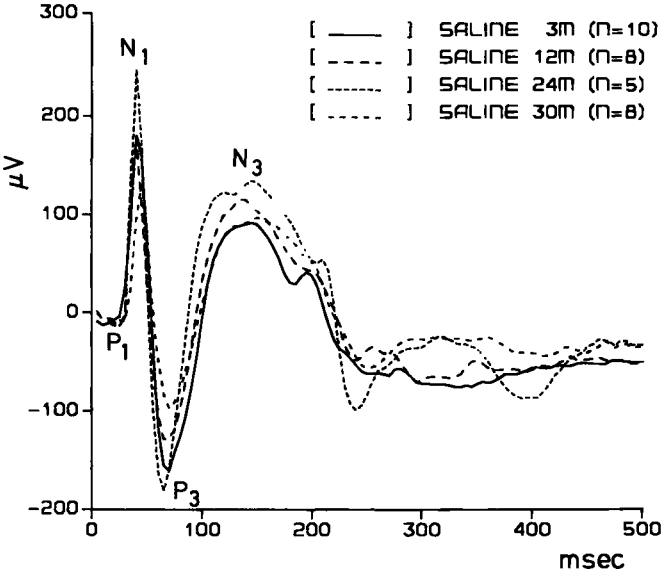


Fig. 12. The averaged VER at the highest lightintensity (200 mA LED current, i.e. $26{,}600 \times 10^{-9}$ lumen) of the 3, 12, 24 and 30 months (m) old rats. Abscissa milliseconds (msec), ordinate μVolts.

The peak to peak amplitudes of the various VER components measured at four lightintensities are shown in figure 13. The latency periods between the flash and the peak value $N_1$ of the VER measured at four lightintensities of the different age groups are given in figure 14. Since the ERG as well as the VER in each animal are dependent on the lightintensity and since the VER is dependent on the ERG response, the values of the ERG and VER responses were combined for the statistical evaluation of the results. For each of the four lightintensities six parameters have been measured, i.e. of the ERG the heights of the b-wave and the latencies to the peak value of the b-wave and of the VER the peak to peak amplitudes $N_1$-$P_1$, $N_1$-$P_3$, $N_3$-$P_3$ and the latencies to the peak

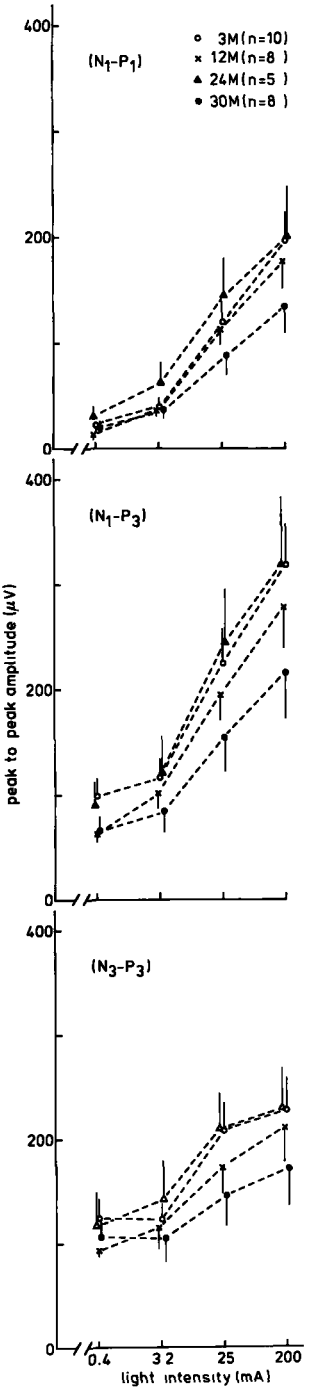


Fig. 13. Peak to peak amplitudes (mean ± S.E.M.) in μVolts of the various VER components measured at four lightintensities of the 3, 12, 24 and 30 months (M) old rats. 1 mA current through the LED corresponds with $133 \times 10^{-9}$ lumen on the eye of the rat.

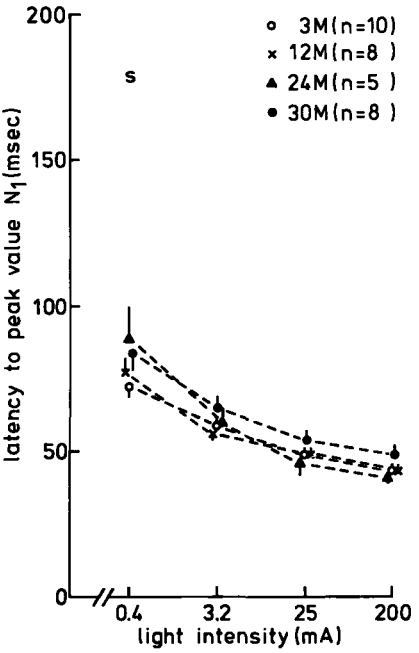


Fig. 14. The latency periods (means ± S.E.M.) in msec between the flash and the peak value $N_1$ of the VER measured at four lightintensities of the 3, 12, 24 and 30 months (M) old animals. 1 mA current through the LED corresponds with $133 \times 10^{-9}$ lumen on the eye of the rat.

value of $N_1$. The values obtained were ranged according to the first principle component of the ERG and VER (Kendall, 1965). For any of the variates the animals were ranged in order of increasing peak or peak to peak values and decreasing latencies. For each animal these ranges were summed and these sums were ranged again. Possible differences between the age groups were tested according to Kruskal-Wallis (1952) on the 5% level. As also appeared from the figures no significant ($p > 0.05$, two tailed) age-related differences were found.

### 3.3.2. Effects of age on learning in a T-maze

In order to test the various age groups on their short-term memory the following procedure was employed. In two weeks the animals were trained to alternate in a T-maze until they reached a high percentage (approximately 85%) of alternation. They were then tested by increasing

the time-interval between the runs to see whether they still remembered the previous direction. The results are shown in figure 15 and are statistically analysed according to Wilcoxon (de Jonge, 1963) at the 5% level. The training results on day 1 and 3 did not differ significantly between the young and old animals. When the animals were tested in the T-maze both with one minute (day 2) and two minutes (day 4) between the runs the percentages of alternation slightly decreased but again did not differ significantly between the 3 and 30 months old animals.

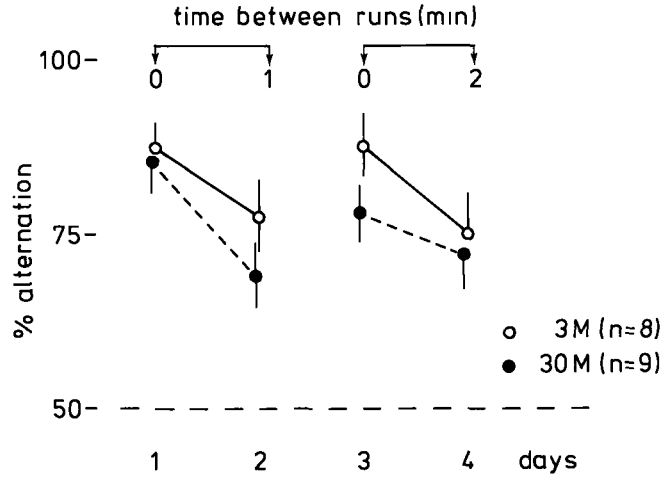


Fig. 15. The percentages of alternation of trained rats of 3 and 30 months (M) old. On day 1 and day 3 the alternation was tested in 11 successive runs. On day 2 the alternation was tested with a one minute interval between the runs; on day 4 the alternation was tested again with two minutes between the runs.

### 3.3.3. Effects of age on passive avoidance learning

Rats that have received a footshock in the dark compartment during session I of a series of daily sessions, are reluctant to enter that compartment during subsequent sessions, but this reluctance gradually wanes as the days pass. In order to quantify this reluctance the latency periods were measured between the moment that the rat was placed into the light compartment and the moment it entered the dark one (figure 16). The rats were given only 30 minutes time to respond at all. A possible effect of age on the "latency to enter" in session II was tested

according to Kruskal-Wallis (1952). It appeared that age had a significant effect ($p < 0.05$, two tailed) on the latency time to enter the dark compartment. To compare the different age groups the results were tested in pairs according to Dunn (1964) with multiple interference according to Newman-Keuls (Miller, 1966). The latency time was significantly ($p < 0.05$, two tailed) longer in the 12 months old animals than in the 3 months old rats. No significant differences can be detected between the results of the 12 and 30 and those of the 3 and 30 months old rats. The latency times in session III, IV and V did not show age-related differences.

Another method to quantify the reluctance of the rats was also used, i.e. to count the number of rats from a group that entered the dark compartment during the allowed 30 minutes. In session II these values are 0 for the 3 months, 8 for the 12 months and 6 for the 30 months old animals. When these values are tested according to the two times three contingency table of Woolff (1967), a significant ($p < 0.05$, two tailed) difference has been found between the results of the 3 months old animals and those of the 12 and 30 months old rats.

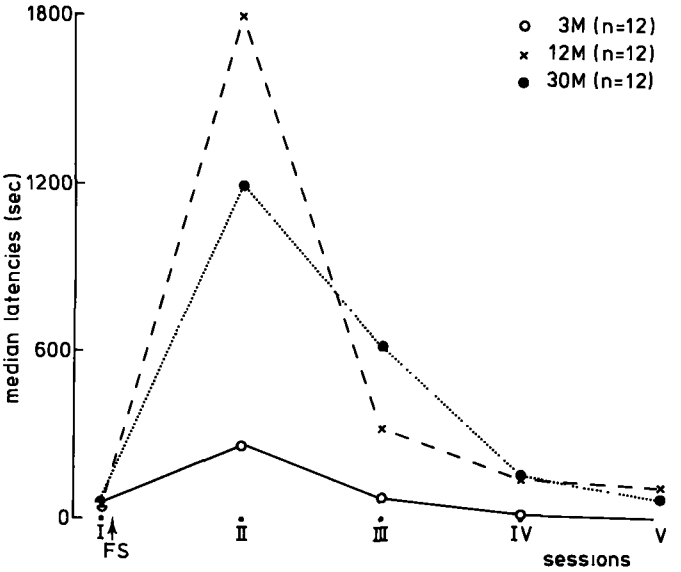


Fig. 16. The median latency times to enter the dark compartment of 3, 12 and 30 months (M) old animals, which received a footshock (FS) in the dark compartment in session I and were tested once daily during 30 minutes on the subsequent four days.

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I.F. de Koning-Verest

# E R R A T U M

Page 40: the second paragraph from the bottom, should read as follows:
de op één na laatste alinea van onder moet worden:

The principle of the test is well known. When given a choice between a light and a dark compartment, rats exhibit a preference to stay in the dark compartment. Once they have received a footshock in the dark compartment, however, they tend to avoid this compartment initially but after a long latency period they enter it again.

Page 48: the first sentence of the second paragraph should read as follows:
de eerste zin van de tweede alinea moet worden:

Another method to quantify the reluctance of the rats was also used, i.e. to count the number of rats from a group that did not enter the dark compartment during the allowed 30 minutes.

After the first entry in session II, the 3 months old animals made the highest number of crossings, followed by the 12 and 30 months old rats; the mean values were 12, 1 and 2 respectively.

## 3.4. DISCUSSION

The ERG represents the graded electrical response of the cells (mainly the photoreceptors and the bipolar cells) in the outer layers of the retina. In the rats tested no age-related differences were found in the amplitude nor in the latency of the ERG. A prerequisite for a normal ERG is that the rods and cones, the pigmentation, the synaptic transmission in the outer and the inner retinal layers and the blood circulation in the retina are normal (see: Ikeda et al., 1978). Since the ERG's of the animals of all age groups appeared to be quite normal and no significant differences have been found between the ERG's of young, adult and old animals, the retinal function of the rat does not seem to be affected by age. Unfortunately, no indications about possible changes in the retinal function with age have been found in the literature, therefore a comparison with other findings is not possible.

The finding that the amplitudes of the VER components did not show age-related differences is in agreement with some results obtained in humans. Celesia and Daly (1977) as well as Drechsler (1978) did not find age-related differences in the amplitudes of the VER. In contrast, other authors found increased amplitudes of early components and decreased amplitudes of the later components of the VER (Beck et al., 1975; Dustman and Beck, 1969; Straumanis et al., 1965). Perry and Childers (1969) reported a decrease only in the amplitude of the VER with increasing age. The latencies of the early components of the VER are uniformly longer in all studies in humans (Celesia and Daly, 1977; Drechsler, 1978; Shaw and Cant, 1980; Straumanis et al., 1965). This is at variance with the present results in rats, where no significant age-related changes in the latencies of the VER were found. It is generally accepted that the early components of the VER reflect processes involved in the transmission of visual information in the central nervous system. Taken together, the absence of significant age-related changes in the shapes, the amplitudes and the latencies of the ERG and the VER makes it unlikely that the

registration of visual information is seriously hampered in old rats. This does not necessarily imply that more complex visual information processing also remains unaffected by age. In the present study the visual stimuli used have been rather simple and more complex light patterns might have given different results. Still, the acquisition deficits found, cannot be explained by deficits in visual registration in the senescent rats.

Under appetitive and non-appetitive test conditions Pond and Schwartzbaum (1972) and Schwartzbaum and Kreinick (1973) showed that increases in behavioural reactivity are associated with suppression of late VER components. Moreover, drugs which pharmacologically induce arousal, e.g. amphetamines also suppress late components or afterdischarges of VER (Fleming et al., 1974). Accordingly, late VER components are suppressed when arousal is induced behaviourally (Rhodes and Fleming, 1970). The present finding that no significant differences in the late VER components $N_3$-$P_3$ existed between the different age groups, may suggest that there were no differences in arousal under the present test conditions. However, a decrease in the late components (as generally seen in aged humans) may have been fortuitously cancelled out by an increase as a result of a decreased arousal in the aged rats.

It is surprising that old animals learn alternation behaviour in the T-maze as well as young animals. It is possible that the task is too simple to find age differences in acquisition (Goodrick, 1972). The absence of age-related differences with respect to the training results in the T-maze was rather fortunate in that it gave the young and the old rats equal starting points for the test on short-term memory (the increase of run-interval). Both when 1 and 2 minutes intervals were added between the runs there was a reduction of the percentages of alternation (figure 15) of both young and old animals; no significant differences in alternation behaviour could be detected between the 3 and 30 months old animals. From these results it seems rather unlikely that the short-term memory of the rats was affected by age. This conclusion is in agreement with the findings of Wallace et al. (1980) who found no decline in short-term memory as measured by retention of previous choices in a spatial maze after short intervals and in performance in a modified form of Konorski's discriminated delayed response task. The results of this study

and the results of Wallace et al. (1980) seem to be contradictory to those obtained by Doty (1966), Goodrick (1968, 1973) and Ordy et al. (1978) in rats and those of Adamowicz (1976), Botwinick (1975), McGhie et al. (1965), Walsh and Thompson (1978) and Welford (1958) in humans. However, in Goodrick's studies (1968, 1973) short-term retention was hypothesized to be inferior because older rats needed far more trials to acquire a complex maze than young animals. There does not seem to be a firm basis in the literature suggesting that massing trials necessarily implicate short-term memory functions. In addition, the rats used by Goodrick (1968) had previously been given training with other daily trials distributions. It is difficult to determine, therefore, if massing of trials was the only variable which produced the observed facilitation of acquisition in aged rats. In the experiments of Ordy et al. (1978) age differences in 2- and 6-hour short-term passive avoidance retention have been found., These time limits far exceed those which are generally believed to encompass the short-term memory phase (Watkins, 1974). Therefore, it is more likely that Ordy and his colleagues have been measuring age differences in consolidation mechanisms, retrieval processes or even in levels of motor activity, which might profoundly affect the results of passive avoidance testing. The interpretation of the studies on human subjects is difficult and the analysis and identification of the changes in learning and memory processes rather hazardous because of the interaction with cultural, emotional and motivational factors. It is very difficult, therefore, to state that only short-term memory is affected and that other factors are definitely and adequately excluded.

Since in the present experiments no time limit has been imposed on the animals during the T-maze training and testing sessions, it is unlikely that motor activity, speed of reaction or motivational levels have affected the results. The animals did not have to make a response within a given time period; the choice of the correct goal box was the only criterion. The present results turned out to be reproducable and, together with the results of Wallace et al. (1980) indicate that in rats short-term memory is not significantly affected by ageing.

In the passive avoidance learning task the latency to enter the dark compartment in which the rats had received a footshock 24 hours earlier, was longest in the 12 months old rats, the median latency being

1800 seconds versus 1200 seconds for the 30 months old rats and 276 seconds for the 3 months old animals (figure 16). However, the number of crossings was found to be inversely related with age. This could well mean that the young rats simply had a higher motor activity than the old ones. Interpreted along this line, this finding confounds the data on "latency to enter" and made an interpretation impossible. McNamara et al. (1977) also tested rats of different age groups in a similar passive avoidance learning task. The animals were tested 2 minutes or 30 days after they had been trained. According to these authors the first test provided information about acquisition and the test after 30 days about retention. Both appeared to be impaired in very young (30 days) and old (547 days) rats, compared to young (180 days) and adult (365 days) rats. They suggested that incomplete brain maturation and senescent brain changes may have been responsible for these effects, respectively. Unfortunately these authors did not take the age-related differences in motor activity into account. As shown by Seliger (1975), who injected amphetamine in moderate doses, different levels of motor activity have a profound influence on the results of retention testing in a passive avoidance task. Therefore, the present results confirm the data in the literature, but are inconclusive since no method is known to correct for age differences in motor activity.

With the experiments described in the processing of visual information, in short-term memory or in retention might be held responsible for the acquisition deficits found in the aged rats. The results of the experiments indicate that it is unlikely that the acquisition deficits found can simply be explained by deficits in visual registration or in short-term memory in the senescent rats. Possible retention deficits cannot be eliminated by the techniques employed. Further investigations on possible age-related effects on retrieval, decay and on the permanent storage of information will be necessary for an insight in the age affected phases of the learning and memory processes.

# CHAPTER 4

# GLUTAMATE METABOLISM IN AGEING RAT BRAIN*

## 4.1. INTRODUCTION

One of the characteristics of old age is the change in function of the central nervous system (CNS), amongst others noticeable as deficits in adaptive behaviour, learning and memory. In animal model experiments, such deficits have been demonstrated by several investigators (Goodrick, 1968 and 1973; McNamara et al., 1977; Wolthuis et al., 1976). Wolthuis et al. (1976) reported deficient acquisition in 30 months old rats, when compared with acquisition in 12 or 3 months old rats. In the course of those studies the question arose of whether the learning and memory deficits might be a consequence of possible gross and overall metabolic changes in the brain during ageing. Since it was impossible to investigate brain metabolism in its entirety, it was attempted to study a representative part. A study of glutamic acid turnover was thought to offer some insight into the overall metabolic state of the brain. At least five arguments support the notion that glutamate occupies key positions in cerebral metabolism. It is a neurotransmitter (Johnson, 1972), it is a precursor for gamma-aminobutyrate (GABA) (Berl et al., 1961, 1970[a], 1970[b]; Seiler and Wagner, 1976), and it is important for cerebral energy metabolism via α-ketoglutarate and the tricarboxylic acid cycle (van den Berg et al., 1966, 1969). Moreover, the glutamate-glutamine system regulates the ammonia levels in the brain (Berl et al., 1962; Weil-Malherbe, 1962) and, last but not least, glutamate is incorporated into proteins (Blomstrand and Hamberger, 1970) and peptides (Reichelt and Kvamme, 1967).

The choice of glutamate turnover for investigation was encouraged by results of other investigators suggesting age-related changes in glutamate metabolism. Davis and Himwich (1975) found a marked decrease of

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glutamic acid content in aged rat brains. Kanungo and Kaur (1969) reported low activity of glutamate dehydrogenase in the brains of aged rats. Moreover, Fonda et al. (1973) found a lower activity of glutamate decarboxylase and a higher activity of GABA transaminase in the brains of ageing mice and concluded that there is an age-related change in the glutamate system in mouse brain (Kirzinger and Fonda, 1978). It was therefore decided to study the overall brain glutamate metabolism in brains of rats of various ages.

Much evidence is available that glutamate metabolism in the brain is compartmentalised (Berl et al., 1961, 1962, 1970[a], 1970[b]; van den Berg, 1970; van den Berg and Ronda, 1976[a], 1976[b]). At least two glutamate pools - a "large" and a "small" one - appear to exist in rodent brain (van den Berg et al., 1969). Acetate is a precursor for glutamate in the small pool. When radioactive acetate is injected, the specific activity of glutamine, a metabolite of glutamate, is unexpectedly found to be higher than that of glutamate (van den Berg et al., 1966; O'Neal and Koeppe, 1966). Glucose can be used as a precursor for the large pool, although it is also a (minor) precursor for the small one. The large pool differs from the small one in that, when labelled glucose is injected, the specific activity of glutamate is found to be higher than that of glutamine ( Gaitonde et al., 1965; O'Neal and Koeppe, 1966). In the present experiments the kinetics of the small and the large pool in young, adult and aged rat brains were studied following the labelling of glutamate, glutamine and aspartate after injection of $[^{3}H]$acetate and d-$[2-^{14}C]$glucose.

## 4.2. MATERIALS AND METHODS

The female WAG/Rij rats used, 3, 12 and 30 months old, were virgins and experimentally naive. Their body weights were 162 $\pm$ 2 g (n=14), 196 $\pm$ 4 g (n=15) and 216 $\pm$ 8 g (n=16), respectively. Each rat was injected intraperitoneally between 9 and 12 a.m. with a mixture of 300 µCi of $[^{3}H]$acetate and 30 µCi of d-$[2-^{14}C]$glucose. The animals were killed by immersion in liquid nitrogen 10 or 20 minutes after the injection.

The brains were chiselled out, pulverised in dry ice and extracted with 5 ml of 5% trichloroacetic acid (TCA) solution. The samples were

kept overnight in a refrigerator; 5 ml of cold water were added to each and the tubes were centrifuged (10 min at 1000 g). The precipitate was then washed twice with 5 ml of water. The precipitates were used for protein determination (Lowry et al., 1951) after washing successively with acetone, ethanol-ether (3:1, v/v), chloroform-methanol (2:1, v/v), and ether to remove the lipids. The supernatants were neutralized with NaOH and diluted to 50 ml. Aliquots of these solutions were added to a Dowex 1 column (7 mm I.D., 10 cm high). The Dowex 1 (Agl-X4, 200-400 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) was eluted successively with 10 ml of $H_2O$ and 10 ml of 0.035 N acetic acid. The combined eluents included glutamine. The column was subsequently eluted with 70 ml of 0.1 N acetic acid and 45 ml of 0.25 N acetic acid to obtain glutamic acid and aspartic acid, respectively. The solution containing glutamine was hydrolysed with 2 N HCl (1 h at 100 $^{o}$C) and the glutamate formed from glutamine was isolated on a Dowex 1 column as described. The amino acids were quantified according to the ninhydrin method (Moore and Stein, 1954). The radioactivity was measured by liquid scintillation counting in the Isocap/300 (Searle). The scintillation fluid used was Tritosol as described by Fricke (1975). Before counting, all samples were freeze-dried to remove the tritium present in water or other volatile compounds; this was not done when the total activity in the acid-soluble fraction was measured. The whole procedure was originally described by Cheng and Waelsch (1963) and van den Berg et al. (1969). Two successive experiments were performed. Since the procedure in both experiments was identical and the results of the two experiments were virtually the same, all results were pooled. The specific radioactivities of the amino acids have been corrected for injected doses of 300 µCi of [$^{3}$H] acetate and 30 µCi of d-[2-$^{14}$C] glucose per 200 g body weight. All results were statistically analysed by the Bonferoni multiple t-test (Miller, 1966) modified according to Welch (1947). All testing was two tailed as indicated by $p_2$. [$^{3}$H] acetic acid (sodium salt; specific activity 250 mCi/mmol) and d-[2-$^{14}$C] glucose (specific activity 3.0 mCi/mmol) were obtained from the Radiochemical Centre (Amersham, Buckinghamshire, U.K.).

## 4.3. RESULTS

### Amino acids and protein levels

The concentrations of glutamate, aspartate and glutamine in brain are reported in Table I. The level of glutamate decreased significantly ($p_2 < 0.05$) in the brains of the 30 months old rats when compared to 3 months old rats. The amount of aspartate decreased significantly ($p_2 < 0.05$) in brains of 30 months old rats when compared to those of 3 and 12 months old. The glutamine concentration did not differ between the age groups. The protein levels in the brains of the 3, 12 and 30 months old rats were, respectively, 6.99 ± 0.17 (n=14), 7.04 ± 0.36 (n=15) and 6.97 ± 0.18 (n=15) (mean ± S.E.M.) mg per 100 mg frozen brain tissue; no age-related significant differences were found.

TABLE I

AMINO ACID LEVELS IN FROZEN RAT BRAIN TISSUE

The results (in µmol/g) are the means ± S.E.M.

| | Age | | |
|---|---|---|---|
| | 3 months (n=14) | 12 months (n=15) | 30 months (n=16) |
| Glutamate | 9.75 ± 0.30 | 9.23 ± 0.34 | 8.51 ± 0.22* |
| Aspartate | 2.52 ± 0.09 | 2.52 ± 0.11 | 2.19 ± 0.05** |
| Glutamine | 3.63 ± 0.19 | 3.59 ± 0.27 | 3.91 ± 0.53 |

* Significantly different from values of 3 months ($p_2 < 0.05$).

** Significantly different from values of 3 and 12 months ($p_2 < 0.05$).

### Incorporation of $[^3H]$acetate

The incorporation of tritium from $[^3H]$acetate into the acid-soluble fraction of the brain did not differ significantly between the three age groups, either at 10 or at 20 minutes after injection of the precursors (Table II). Most of the tritium in the acid-soluble fraction was present in water. Similarly, the incorporation of $[^3H]$acetate into

TABLE II

RADIOACTIVITY OF THE ACID-SOLUBLE FRACTION IN RAT BRAIN, 10 AND 20 MINUTES AFTER INJECTION OF 300 µCi OF [$^{3}$H]ACETATE AND 30 µCi OF d-[2-$^{14}$C]GLUCOSE

The results ($10^{-6}$ x dpm per g of frozen brain tissue) are the means ± S.E.M.

| Incorporation of | Time (min) | Age: 3 months | (n) | 12 months | (n) | 30 months | (n) |
|---|---|---|---|---|---|---|---|
| [$^{3}$H] | 10 | 3.43 ± 0.18 | (7) | 3.26 ± 0.11 | (7) | 3.16 ± 0.12 | (8) |
| | 20 | 3.57 ± 0.25 | (7) | 4.17 ± 0.39 | (8) | 3.76 ± 0.10 | (8) |
| [$^{3}$H] (fraction minus water) | 10 | 0.16 ± 0.02 | (7) | 0.15 ± 0.02 | (7) | 0.15 ± 0.02 | (8) |
| | 20 | 0.18 ± 0.03 | (7) | 0.14 ± 0.02 | (8) | 0.12 ± 0.01 | (8) |
| [$^{14}$C] | 10 | 0.51 ± 0.02 | (7) | 0.53 ± 0.03 | (7) | 0.42 ± 0.03 | (8) |
| | 20 | 0.61 ± 0.05 | (7) | 0.82 ± 0.07 | (8) | 0.66 ± 0.01 | (8) |

the protein fraction of the brain did not show a significant difference between the 3, 12 and 30 months old rats (Table III). The incorporation of $^{3}$H-label into glutamate did not differ between the age groups, either 10 or 20 minutes after injection of the precursors (Table IV). The incorporation of the radioactivity into aspartate and glutamine is expressed as the relative specific activity (RSA), i.e. the specific activity of the amino acid in relation to the specific activity of glutamate. The RSA of $^{3}$H-labelled aspartate did not differ significantly between the three age groups, either 10 or 20 minutes after injection of the precursors (Table IV). However, 10 minutes after injection of the precursors, the RSA of tritium-labelled glutamine in the 30 months old rats was significantly ($p_2 < 0.05$) lower than that of the 3 months old rats. This difference had disappeared 20 minutes after injection of the precursors (Table IV).

TABLE III

RADIOACTIVITY OF THE PROTEIN FRACTION IN RAT BRAIN, 10 AND 20 MINUTES AFTER INJECTION OF 300 µCi OF [$^{3}$H]ACETATE AND 30 µCi OF d-[2-$^{14}$C]GLUCOSE

The results ($10^{-5}$ x dpm per g of frozen brain tissue) are the means ± S.E.M.

| Incorporation of | Time (min) | Age: 3 months | (n) | 12 months | (n) | 30 months | (n) |
|---|---|---|---|---|---|---|---|
| [$^{3}$H] | 10 | 0.24 ± 0.01 | (7) | 0.30 ± 0.02 | (7) | 0.29 ± 0.02 | (8) |
| | 20 | 0.29 ± 0.01 | (7) | 0.33 ± 0.04 | (8) | 0.32 ± 0.02 | (7) |
| [$^{14}$C] | 10 | 0.10 ± 0.01 | (7) | 0.10 ± 0.00 | (7) | 0.10 ± 0.01 | (8) |
| | 20 | 0.17 ± 0.01 | (7) | 0.19 ± 0.01 | (8) | 0.17 ± 0.01 | (7) |

TABLE IV

THE INCORPORATION OF [$^{3}$H]ACETATE INTO AMINO ACIDS IN RAT BRAIN, 10 AND 20 MINUTES AFTER INJECTION OF 300 µCi OF [$^{3}$H]ACETATE AND 30 µCi OF d-[2-$^{14}$C] GLUCOSE

The specific activity (SA) of glutamate is expressed as dpm/µmol x $10^{-3}$. The results for aspartate and glutamine are expressed as RSA; i.e. the specific activity of the amino acid in relation to that of glutamate. The results are the means ± S.E.M.

| | Time (min) | Age: 3 months | (n) | 12 months | (n) | 30 months | (n) |
|---|---|---|---|---|---|---|---|
| SA | | | | | | | |
| Glutamate | 10 | 3.69 ± 0.42 | (7) | 3.82 ± 0.24 | (7) | 4.12 ± 0.38 | (8) |
| | 20 | 3.49 ± 0.41 | (7) | 3.85 ± 0.38 | (8) | 4.07 ± 0.43 | (8) |
| RSA | | | | | | | |
| Aspartate | 10 | 0.62 ± 0.07 | (7) | 0.67 ± 0.08 | (7) | 0.67 ± 0.07 | (8) |
| | 20 | 0.55 ± 0.09 | (7) | 0.64 ± 0.06 | (8) | 0.77 ± 0.08 | (8) |
| Glutamine | 10 | 3.97 ± 0.18 | (7)* | 3.36 ± 0.19 | (7) | 2.69 ± 0.22 | (8)* |
| | 20 | 2.78 ± 0.23 | (7) | 2.97 ± 0.18 | (8) | 2.58 ± 0.19 | (8) |

* Significantly different ($p_2 < 0.05$).

Incorporation of $[2\text{-}^{14}C]$glucose

The incorporation of $^{14}C$-label into the acid soluble fraction of the brains of rats of 3, 12 and 30 months old did not change significantly with age (Table II). Neither did the incorporation of $^{14}C$-label into the brain protein fraction in the three age groups (Table III). The incorporation of $^{14}C$-label into glutamate did not differ significantly between the three age groups, either 10 or 20 minutes after injection of the precursors (Table V). Twenty minutes after injection of the radioactivity the incorporation of $^{14}C$-label into aspartate and glutamine in the 12 months old rats, however, was significantly increased in comparison with the incorporation in these amino acids in the 3 months old rats. The specific activity of the amino acids in the brains of the 30 months old rats did not differ significantly with those of the 3 and 12 months

TABLE V

THE INCORPORATION OF $[2\text{-}^{14}C]$GLUCOSE INTO AMINO ACIDS IN RAT BRAIN, 10 AND 20 MINUTES AFTER INJECTION OF 300 µCi OF $[^{3}H]$ACETATE AND 30 µCi OF $[d\text{-}2\text{-}^{14}C]$ GLUCOSE

The specific activity (SA) of glutamate is expressed as dpm/µmol x $10^{-3}$. The results for aspartate and glutamine are expressed as RSA; i.e. the specific activity of the amino acid in relation to that of glutamate. The results are the means ± S.E.M.

| | Time | Age | | | | | |
|---|---|---|---|---|---|---|---|
| | (min) | 3 months | (n) | 12 months | (n) | 30 months | (n) |
| SA | | | | | | | |
| Glutamate | 10 | 16.15 ± 1.00 | (7) | 15.50 ± 0.92 | (7) | 14.54 ± 1.14 | (8) |
| | 20 | 25.39 ± 2.89 | (7) | 30.88 ± 3.10 | (8) | 26.54 ± 0.90 | (8) |
| RSA | | | | | | | |
| Aspartate | 10 | 0.79 ± 0.06 | (7) | 0.79 ± 0.03 | (7) | 0.73 ± 0.05 | (8) |
| | 20 | 0.69 ± 0.06 | (7) | 0.80 ± 0.06 | (8) | 0.83 ± 0.03 | (8) |
| Glutamine | 10 | 0.61 ± 0.06 | (7) | 0.66 ± 0.05 | (7) | 0.61 ± 0.06 | (8) |
| | 20 | 0.60 ± 0.07 | (7) | 0.76 ± 0.04 | (8) | 0.74 ± 0.06 | (8) |

old rats. The RSA of $^{14}$C-labelled aspartate and $^{14}$C-labelled glutamine did not show significant age-related differences, either 10 or 20 minutes after injection (Table V).

## 4.4. DISCUSSION

The decreased levels of glutamate and aspartate found in the brains of 30 months old rats, compared to those of 3 and 12 months old rats, are completely in accordance with the results of Davis and Himwich (1975) and of Timiras et al. (1973). No significant age-related differences between the glutamine levels in the brains were found; a finding which is also in accordance with the results of Timiras et al. (1973) and Davis and Himwich (1975). In contrast, Kirzinger and Fonda (1978) observed an age-related increase in the glutamine level with age, but no differences in glutamate and aspartate concentrations in brains of 12 and 30 months old mice. Possibly this discrepancy between the findings in rats and mice is due to species differences. Changes in levels of cerebral amino acids are not easily interpretable. To get an insight into the possible importance of the decrease in glutamate and aspartate levels in aged rat brains, the metabolism of glutamate was investigated in the present experiments by means of isotopic techniques. The results of this study indicate that there are no age-related changes in the synthesis of glutamate from either glucose or acetate and that the conversion of glutamate into glutamine is only slightly affected by ageing.

The RSA of [$^{3}$H]aspartate and [$^{14}$C]aspartate does not change with age. Aspartate is formed from oxaloacetate, an intermediate of the tricarboxylic acid cycle. The degree of labelling of aspartate, therefore, can be considered as an indicator for the rate of turnover in the tricarboxylic acid cycle. Since no differences in the RSA of aspartate were found between the three age groups it can be concluded that the rate of turnover in this cycle is unaffected by age. Because the tricarboxylic acid cycle is directly related to energy production, it is likely that no great age-related changes in energy metabolism in rat brain occur. Another argument for this conclusion is the fact that, although the metabolism of glucose itself was not measured directly, no age-related changes in glucose incorporation were found in these experiments. The conclusion

is in accordance with the results of Sylvia and Rosenthal (1978), who demonstrated in intact, normally circulated rat brains that ageing per se had no effect on the mitochondrial energy producing system at "rest". The conclusion, however, is contrary to that reached by Patel (1977), who found a reduction in the oxidation of labelled glucose in cortex slices from senescent rats, which pointed to a decreased energy metabolism.

In the present experiments no differences in the protein levels were observed between the three age groups. These results correspond with those of Hollander and Barrows (1968) who found no age-related differences in the protein levels in rat and mice brain and with those of Fonda et al. (1973), who similarly found no age-related differences in the amounts of protein in mouse brain. That the incorporation of the radioactivity into brain proteins of 3, 12 and 30 months old rats did not differ in the present experiments suggests that there are no gross age-related changes in over-all cerebral protein synthesis; specific changes cannot be detected, however, by the technique used.

Hindfelt et al. (1977) showed that there is an increased synthesis of glutamine in brains of experimental animals given high doses of ammonia, and that a direct relation between ammonia levels and glutamine synthesis appears to exist. In the present experiments the incorporation of the radioactivity into glutamine was not increased in aged rat brain. This, in addition to the unchanged glutamine levels, suggests that the ammonia levels in the aged rat brain were not increased. Here our results are again in contrast with those of Kirzinger and Fonda (1978), who found an increased ammonia level in the brains of senescent mice. The differences in glutamine/glutamate ratio of aged brain ($0.46 \pm 0.04$, n=16) in comparison with that of young brain ($0.38 \pm 0.02$, n=14) were not significant and therefore do not support the results of Kirzinger and Fonda (1978) in mice. These authors found that the glutamine/glutamate ratio increased from 0.55 in young brains to 0.96 in aged brains.

The RSA of $^{14}$C-labelled aspartate and glutamine did not differ between the three age groups. Since glucose is the precursor of the large glutamate pool in brain it can be concluded that no age related changes in the metabolism of glutamate in the large compartment take place.

The RSA of $^{3}$H-labelled aspartate did not change with age. The RSA of $^{3}$H-labelled glutamine in brains of 30 months old rats, however,

was decreased 10 minutes after injection of the mixture containing [$^3$H]acetate, when compared with 3 months old rats. This difference had disappeared 20 minutes after injection. The presence of a difference in incorporation after 10 minutes and the absence of such a difference after 20 minutes points to a slowing of the rate of incorporation of [$^3$H]acetate into glutamine in 30 months old rats. Since acetate is a precursor of the small glutamate pool, these results suggest an altered metabolism of glutamate in the small pool in brains of aged rats. To substantiate this finding it would be useful to measure [$^3$H]acetate incorporation into glutamate and glutamine after periods shorter than 10 minutes. It would then also be clarified whether the initial specific activity of glutamate in 30 months old rat brain is higher than that in adult or young rat brains, which might be expected in view of the lower level of cerebral glutamate in aged animals.

Since glutamate turnover remains unchanged in the large pool and since it has been suggested that the large glutamate pools is preferentially associated with neuronal structures and the small pool with glial cells (Balázs et al., 1973; Norenberg and Martinez-Hernandez, 1979; Quastel, 1975), it would be tempting to suggest that metabolism of transmitter glutamate remains unchanged in old age. In our opinion such a conclusion should not be drawn from the present results. Perhaps experiments with fractions rich in glial cells or nerve terminals will further help to elucidate these problems.

Although the present results show that in the small glutamate pool the metabolism of glutamate is somewhat slower - viz. the initially delayed [$^3$H]acetate incorporation into glutamine - all other parameters measured indicate that over-all and large age-related metabolic changes in rat brain do not occur. The deficits in acquisition of old rats (Goodrick, 1968, 1973; Wolthuis et al., 1976), therefore, cannot be simply explained by gross changes in cerebral metabolism.

# CHAPTER 5

# EFFECTS OF MECLOFENOXATE ON ACQUISITION AND AGE-PIGMENT ACCUMULATION IN THE CENTRAL NERVOUS SYSTEM OF THE RAT

## 5.1. INTRODUCTION

In the previous experiments (see chapter 4), clear indications of gross age-related metabolic changes were not found. Nevertheless, the possibility remains that neuronal function in aged rats is impaired by the accumulation of indisposable metabolites. Therefore, it was decided to investigate the "age-pigment" lipofuscin because it is generally accepted that this is some sort of intracellularly deposited "garbage" (Björkerud, 1964). The increasing intracellular accumulation of this age-pigment is one of the consistent cytological changes correlated with ageing of all animal species (Bondareff, 1957; Brizzee et al., 1969, 1974; Brody, 1960; Strehler et al., 1959; Timiras, 1972). The origin of lipofuscin is not clearly understood. Many arguments are in favour of a mitochondrial or a lysosomal origin (for review see: Glees and Hasan, 1976). In a quantitative microscopical study Reichel et al. (1968) demonstrated that in aged rodent brain the average volume occupied by lipofuscin was largest (about 5%) in the hippocampus. Although the hippocampus is the most heavily pigmented area, the Purkinje cell layer, however, because of the relative scarcity of cells, is the area of maximum pigmentation per cell (about 17.5% of the cell volume). In the hippocampus about 8% of the cell volume is occupied by the pigment. Because of the large volume occupied by lipofuscin, it is conceivable that in aged animals neuronal function is disturbed and this may, in turn, lead to acquisition deficits.

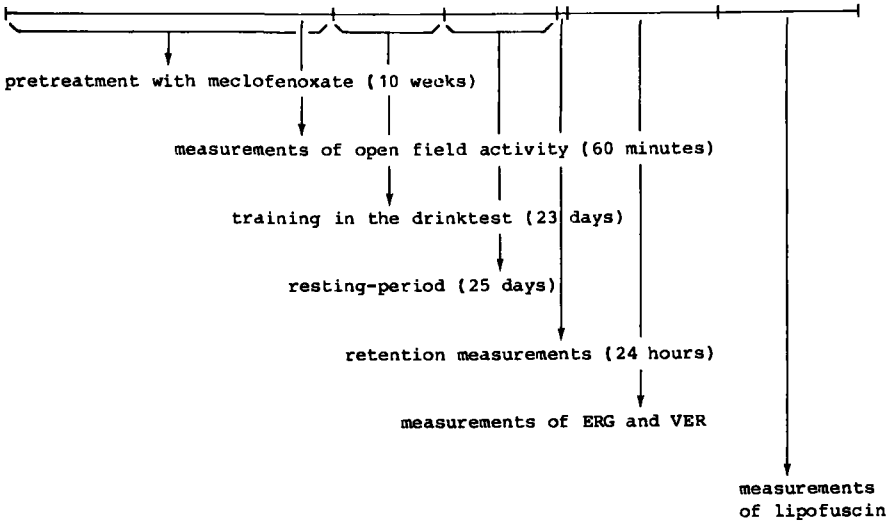
To investigate the possible effects of the accumulation of lipofuscin in the neurons, it was decided to determine if reduction of the amount of lipofuscin in the brain is paralleled by changes in learning and memory processes. A pharmacologic agent which according to the literature should reduce the amount of lipofuscin in neurons of rodents is meclofenoxate (centrophenoxine, Lucidril®, Helfergin®) (Glees, 1975; Meier and Glees, 1971; Nandy, 1968, 1978[b]; Nandy and Bourne, 1966; Riga and Riga, 1974). In an animal study Nandy (1978[a]) found a significant

improvement of learning and memory in old mice treated with meclofenoxate, associated with a marked reduction of lipofuscin pigment in the neurons. Clinical trials with meclofenoxate in geriatric patients with such symptoms as confusion, psychosomatic asthenia, disturbances of memory or of intellectual concentration revealed improvement after several weeks of treatment (Gedye et al., 1972; Schmidt and Broicher, 1970). Other studies, however, demonstrated no alleviation of such symptoms in geriatric patients (Oliver and Restell, 1967; Schilter, 1974). Other effects of treatment with meclofenoxate are an increase in the glucose metabolism of the brain (Nickel et al., 1963), an increase in activity (Wietek, 1963) and an increase in median, mean and maximum survival time of mice (Hochschild, 1973). Meclofenoxate is the hydrochloride of the dimethyl-amino-ethylester of p-chlorophenoxyacetic acid. Dimethylaminoethanol is a natural body component; it is a precursor of acetylcholine. p-chlorophenoxyacetic acid is a synthetic substance chemically related to the plant growth hormone "auxin". The toxicity of the drug is practically nihil in doses found effective by the above mentioned authors.

In the present study the animals were treated one time daily during 10 weeks with meclofenoxate. Only after such a prolonged treatment the effects of meclofenoxate on the amount of lipofuscin are observed (Meier and Glees, 1971; Nandy, 1968). It was determined whether this treatment resulted in a change of the lipofuscin content of the brain and also whether in aged rats this was accompanied by changes in behaviour. Lipofuscin was quantitatively measured in three substructures of the central nervous system (CNS). The hippocampus was selected for its role in learning and memory processes (Isaacson, 1975), the cerebellum because of its involvement in motor coordination and the cerebral cortex for its integrative function. Attempts to quantitatively determine lipofuscin on a morphological base, under the microscope gave unsatisfactory results due to lack of accuracy of the method. A quantitative method described by Fletcher et al. (1973) and later used by Csallany and Ayaz (1976) was tried and appeared insufficiently reproducible in our hands. The extraction procedure was improved and the method then appeared to be sensitive enough to determine lipofuscin in the selected substructures of the brain.

## 5.2. MATERIALS AND METHODS

The experimental schedule for these experiments was as follows:




The age of the female, virgin WAG/Rij rats used was at the start of the drinktest experiment 3, 12, 24 and 30 months. At the start of the drinktest experiment their mean (± S.E.M.) body weights (g) were 144 ± 5.1 (3 months), 216 ± 6.2 (12 months), 256 ± 5.0 (24 months) and 262 ± 6.2 (30 months). The experimental animals of the four age groups were injected intraperitoneally (i.p.) once daily with meclofenoxate at a dosage level of 80 mg/kg for 10 weeks preceding the start of the drinktest. The control animals received corresponding doses of saline. The volume of the injections was 7.5 ml/kg. In the tenth week of the injection-period the open field behaviour was measured with the automated TV-based system, described in chapter 2. After the injection period of 10 weeks the animals were trained in the drinktest. During 25 days following the end of the drinktest experiment the animals were placed in their home cages. On the 26th day retention was measured during 24 hours in the drinktest. After these behavioural experiments, the electroretinograms (ERG's) and the visual evoked responses (VER's) were recorded to get an impression of the functioning of the visual perception. Finally, after these electro-

fysiological measurements the animals were sacrified; cerebellum, cerebral cortex and hippocampus were dissected and frozen at -20 $^{o}$C for the quantitative analysis of lipofuscin.

### 5.2.1. Effects of meclofenoxate on spontaneous motor activity and open field behaviour

The automated TV-based system for the measurements of horizontal and vertical movements has been described in section 2.2.2. of chapter 2. On the day of the measurements, each rat received its daily dose of meclofenoxate or saline 30 minutes before the test and was tested for 1 hour. The order of testing of individual animals of the eight groups was balanced in order to compensate for the time of the day. Movements measured in the horizontal plane were: the distance run, the distribution of speeds during a specific period, the time spent in the inner field, the number of crossings from outer to inner field and vice versa and the number of times the animals entered corners. Movements measured in the vertical direction were the changes as well as the number of rearings as a function of time, the percentage of time spent in an upright position and the number of movements of two categories of amplitudes. Five successive experiments were performed. Since the procedures in all experiments were identical and the results of the five experiments were virtually the same, the results were pooled. The significances were calculated according to Welch (1947), using the multiple interference method according to Newman-Keuls (Miller, 1966).

### 5.2.2. Effects of meclofenoxate on "drinktest learning"

The apparatus and the task the animals had to perform have been described in section 2.2.1. of chapter 2. The drinktest is based on conditioned suppression of drinking behaviour, i.e. rats are trained to avoid licking a water tube when a set of signals indicated that no water will be delivered. An incorrect response is not rewarded with water and is punished by shocks delivered through a grid floor under the water tube for 5 seconds. After 3 days of habituation and 20 days of training the animals were placed in their home cages for 25 days. After those 25 days

retention of the acquired behaviour was tested for 24 hours in the drinktest apparatus. During these retention measurements the same signals were presented as during the training period; the only difference being that upon an incorrect response no punishment followed. Earlier it had been found that extinction of the acquired behaviour in the drinktest occurs very rapidly when every response is followed by delivery of water; consequently possible age-related differences in extinction speed would be hard to detect. Therefore during these retention measurements a procedure was chosen in which the animals received water only after a correct response. Five successive experiments were performed and the results were pooled.

### 5.2.3. Effects of meclofenoxate on the electroretinogram and the visual evoked response

The measurement procedures for the ERG and the VER are described in section 3.2.1. of chapter 3. In short, one day prior to the measurements cortical electrodes were inserted under sodium hexobarbital anaesthesia. The following day, when the animals had been adapted to total darkness for at least 12 hours, the animals were paralysed with 100 mg/kg gallamine triiodide (Flaxédil®), intubated and artificially ventilated. The head was gently fixed in a head holder and the left pupil was dilated with 1% atropine sulphate solution in 0.9% NaCl. This solution also formed an electrical bridge between the left eye and the electrode for measuring the ERG. All procedures were carried out in dark red light (Kodak Wratten no 2 filters). The light source to evoke the response was a light emitting diode (LED); the ERG's and the VER's were measured at four light intensities, i.e. 0.4, 3.2, 25 and 200 mA LED current. Flashes of 2 msec duration were administered; the interflash interval was 2 sec $\pm$ 20% random. Per light intensity 100 cortical events were recorded. After each flash 500 msec was recorded. To obtain the average VER, 100 cortical events were averaged by computer. The results were statistically analysed as described in section 3.3.1.

5.2.4 The effect of meclofenoxate on lipofuscin accumulation in the central nervous system

Directly after the measurements of the ERG and the VER the animals were sacrified; cerebellum, cerebral cortex and hippocampus were dissected and frozen at -20 °C for the quantitative analysis of lipofuscin. The extraction method for the age-pigment described earlier by Fletcher et al. (1973) and by Csallany and Ayaz (1976) was modified. The brain structures were defrosted and weighed. Chloroform-methanol, 2:1 (v/v) was added in a volume-to-weight ratio of 20:1. The tissues were homogenised in a Braun (Melsungen) homogenisator at 1500 rpm. The homogenate was sonnicated for 15 minutes. After complete homogenization the homogenate was stirred with 50 ml water for 1 hour at 50 °C two times in succession. The combined water layers were stirred four times with 25 ml chloroform-methanol, 2:1 (v/v) for 1 hour at 50 °C. The solvent of the combined organic layers was removed on a rotary evaporator to ca. 2 ml and thereafter the extract was evaporated to dryness under nitrogen. The dried extract was taken up in 0.5 ml chloroform-methanol, 1:9 (v/v); this mixture was sonnicated during 15 minutes. Subsequent purification of the extract was performed by chromatography on a 1.0x50 cm Sephadex LH 20 column with chloroform-methanol, 1:9 (v/v) as the mobile phase. The fluorescence of 2 ml fractions of the column eluant was determined at maximum excitation and emission wavelengths. The fluorescence measurements were made on a Band Atomic fluorescence spectrophotometer, Model SF1. The instrument was calibrated to read 500 relative fluorescence units against a quinine sulphate solution (1 µg/ml 0.1 N $H_2SO_4$). The statistical evaluation of the results will be described in section 5.3.4.

5.3. RESULTS

5.3.1. Effects of meclofenoxate on spontaneous motor activity and open field behaviour

The results of open field behaviour of 3, 12, 24 and 30 months old rats treated with meclofenoxate and those of the saline treated animals of the same ages can be seen in figure 17. The results of 60

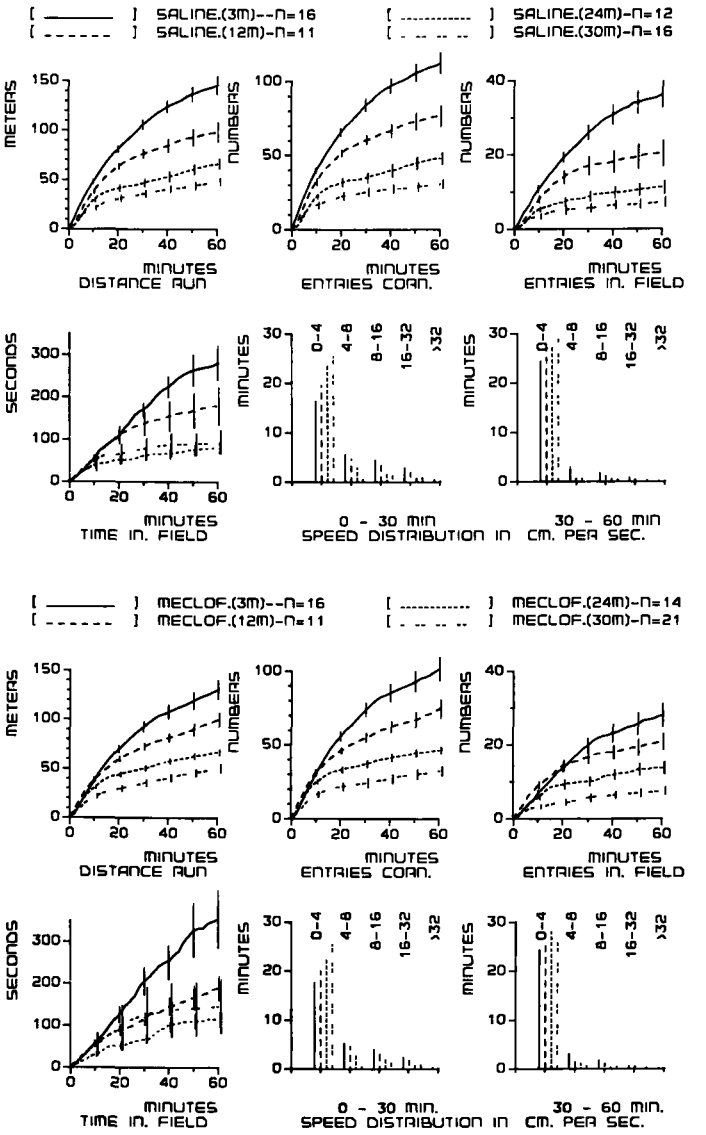



Fig. 17. The results of measurements of open field behaviour with the automated TV-based system during 60 minutes of 3, 12, 24 and 30 months (m) old rats treated with saline or with meclofenoxate. All values are expressed as means ± S.E.M. For explanation of the graphs see text.

minutes of behaviour recording are presented in six graphs of each treatment group. The top left graph shows the cumulative distance run in meters as a measure of motor activity; the middle one of the upper 3 graphs shows the cumulative number of changes in corners as a measure of exploratory behaviour (see: Bureŝ et al., 1976); the top right and the bottom left graphs show the number of entries into the inner field and the time spent there, respectively as one of the parameters of emotionality (see: Bureŝ et al., 1976); the middle and the right graphs of the bottom line show the cumulative time that movements of the indicated speed classes were detected. The values of the distance run in 60 minutes by the 3, 12, 24 and 30 months meclofenoxate treated and control animals were all significantly ($p < 0.05$, two tailed) different from one another. Treatment with meclofenoxate causes no significant differences in the distance run in any of the age groups. The gradual decrease in distance run with age causes also a gradual decrease in entries into corners, entries into inner field and time spent in inner field because of the fact that these parameters are directly dependent on the motor activity, e.g. expressed as the distance run. From the graphs on speed distribution it can be deduced that the speed at which the animal moves decreases with age in both control and treated animals. When the upper and the lower graphs are compared one by one, meclofenoxate induces not an increase in activity as mentioned in the literature (Wietek, 1963).

The results of 60 minutes measurement of the vertical movements of the rats of the four age groups, treated with saline or with meclofenoxate, are demonstrated in figure 18. For the statistical evaluation of the results the values for the different parameters during the 60 minutes of measurement are taken together per age group. In the small vertical movements of amplitude class 2 the activity of the 12 months old control animals is significantly ($p < 0.05$, two tailed) lower than that of the 3, 24 and 30 months old control animals. Of the rats treated with meclofenoxate the activity of the 30 months old rats in this amplitude class is significantly ($p < 0.05$, two tailed) higher than that of the 3, 12 and 24 months old animals. In the large vertical movements of amplitude class 3 the activity of 3, 12, 24 and 30 months old control rats ar all significantly ($p < 0.05$, two tailed) different from one another, the activity of the young rats being the highest and that of the 30 months old rats being

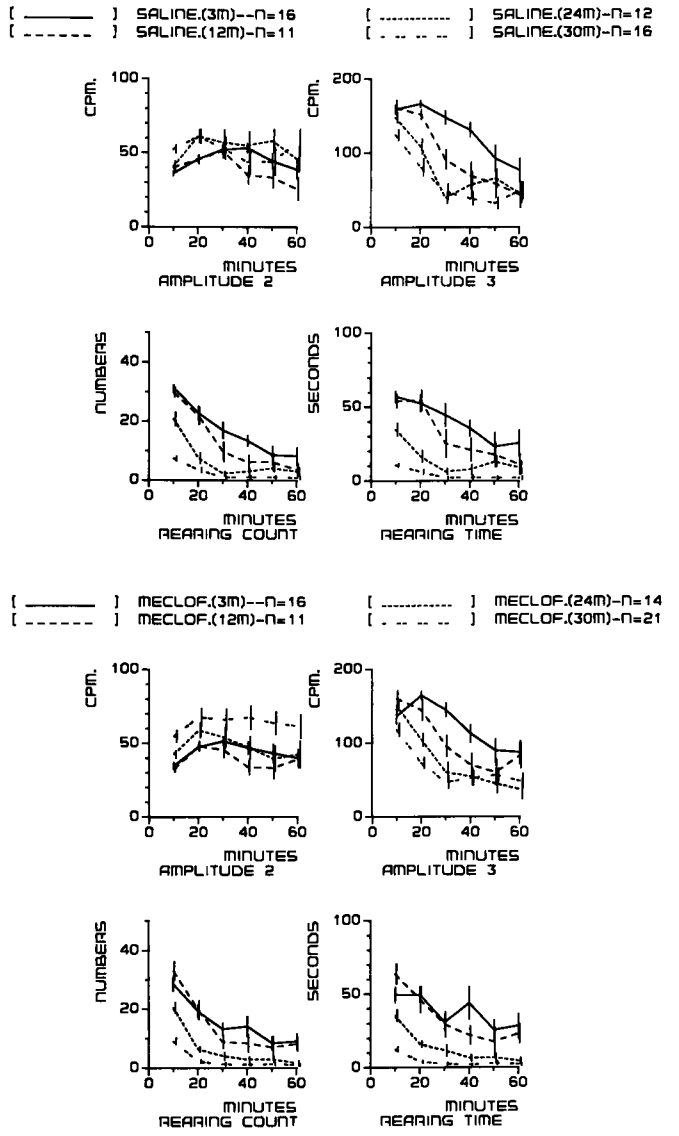



Fig. 18. The results of the measurements during 60 minutes of vertical movements of 3, 12, 24 and 30 months (m) old rats treated with saline or with meclofenoxate. The upper graphs represent vertical movements of increasing amplitudes, amplitude 3 being the largest. The results are averaged over 10 minute periods. The lower left graph shows the number of rearings per 2 minutes averaged over 10 minute periods and the lower right graph the time spent in an upright position per 2 minutes averaged over 10 minute periods. All values are expressed as means $\pm$ S.E.M.

the lowest. In the meclofenoxate treated animals the activity of the 3 months old animals in this amplitude class is significantly ($p < 0.05$, two tailed) higher than that of the 12, 24 and 30 months old animals, the activity of the 12 months old rats is significantly ($p < 0.05$, two tailed) higher than that of the 24 and 30 months old animals, while the activity of the 24 and 30 months old animals do not significantly differ from each other. Both "rearing count" and "rearing time" of the control animals of the four age groups differ significantly ($p < 0.05$, two tailed) from one another; in the meclofenoxate treated animals the values for these parameters of the 3 and 12 months old animals do not differ significantly from each other, but are significantly ($p < 0.05$, two tailed) higher than those of the 24 and 30 months old rats, which also differ significantly ($p < 0.05$, two tailed) from each other. The values of the "rearing count" and "rearing time" decrease with age in both treatment groups. Treatment with meclofenoxate does not cause significant differences in activity in the large vertical movements nor in the values of "rearing count" and "rearing time" in all age groups. In the small vertical movements meclofenoxate only causes a significantly ($p < 0.05$, two tailed) higher activity in the 30 months old animals. Taken together, meclofenoxate induces no increase in the vertical activity in the rats of all age groups.

### 5.3.2. Effects of meclofenoxate on drinktest learning

To investigate whether a possible decrease in lipofuscin in brains of rats treated with meclofenoxate influences learning in the drinktest apparatus, rats of 3, 24 and 30 months old treated with saline or with meclofenoxate - see section 5.2 - were trained. The results of the 12 months old animals have been omitted due to procedural failures. The results of this experiment are shown in figure 19. Statistical analysis of the results on day 20 of the experiment according to Welch (1947) using the multiple interference method according to Newman-Keuls (Miller, 1966) shows that the percentage correct responses of the 30 months old rats are significantly ($p < 0.05$, two tailed) fewer than those of the 3 and 24 months old rats. Treatment with meclofenoxate had no significant effect on the rate of acquisition in this test, neither in the young nor in the old animals.

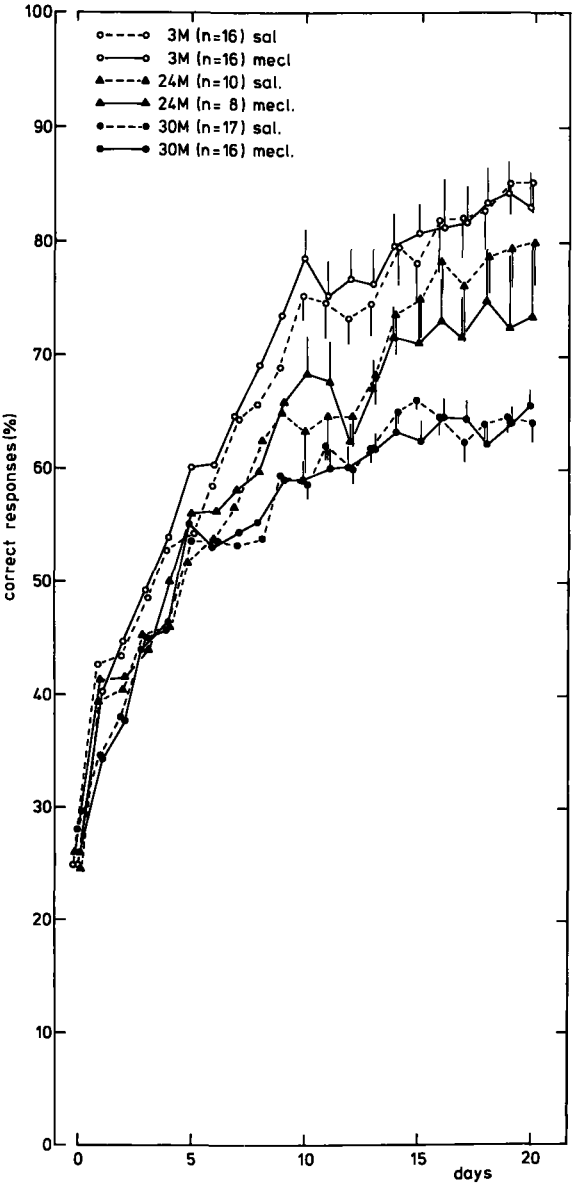



Fig. 19. A comparison of acquisition of 3, 24 and 30 months (M) old WAG/Rij rats, treated with saline or with meclofenoxate, trained in the drinktest apparatus. The results are expressed as the means of the percentage correct responses (correct/total). For the sake of clarity the S.E.M.'s have been omitted in this figure for the first 10 days of the experiment; for the last 10 days the S.E.M.'s have been only drawn to one side.

During 25 days following the end of the drinktest experiment the animals were placed in their home cages. After those 25 days retention was measured during 24 hours in the drinktest apparatus. The results of the retention measurements are represented in figure 20. The results have been averaged over periods of 6 hours. Since in this experiment a procedure has been chosen in which the animals remained rewarded with water following a correct response but receive no punishment after an incorrect response, the possibility exists that within the period of 24 hours the animals relearn the task of drinking upon presentation of the correct combination of signals. In fact, this can be seen in figure 20, where the 3 months old animals obviously relearned the task after the first period of 6 hours. In this experiment, therefore, the process of relearning confounds the measurement of retention. It seems reasonable to accept, however, that the results of the first period of 6 hours are for the greater part determined by the retention of previously acquired behaviour. When the results are analysed according to Welch (1947), using Bonferoni's statistics (Miller, 1966) for simultaneous interference it appears that the retention of the 3 months old animals is significantly ($p < 0.05$, two tailed) better than that of the 24 and 30 months old animals in the first 6 hours period. Treatment with meclofenoxate has no significant effect on the retention of the animals in any of the age groups during this period.

### 5.3.3. Effects of meclofenoxate on the electroretinogram and the visual evoked repsonse

To investigate whether a possible decrease in lipofuscin in the brains of animals treated with meclofenoxate influences the ERG and VER, these phenomena were measured in rats of different ages. Examples of ERG and VER can be seen in figures 9 and 12 of chapter 3, respectively. The VER components were identified as proposed by Creel et al. (1974) and are shown in figure 12.

The heights of the b-waves of the ERG's of the rats of different ages treated with saline or with meclofenoxate at four lightintensities can be seen in figure 21. Figure 22 shows the latencies to the peak of the b-wave at four lightintensities for the different groups of rats.

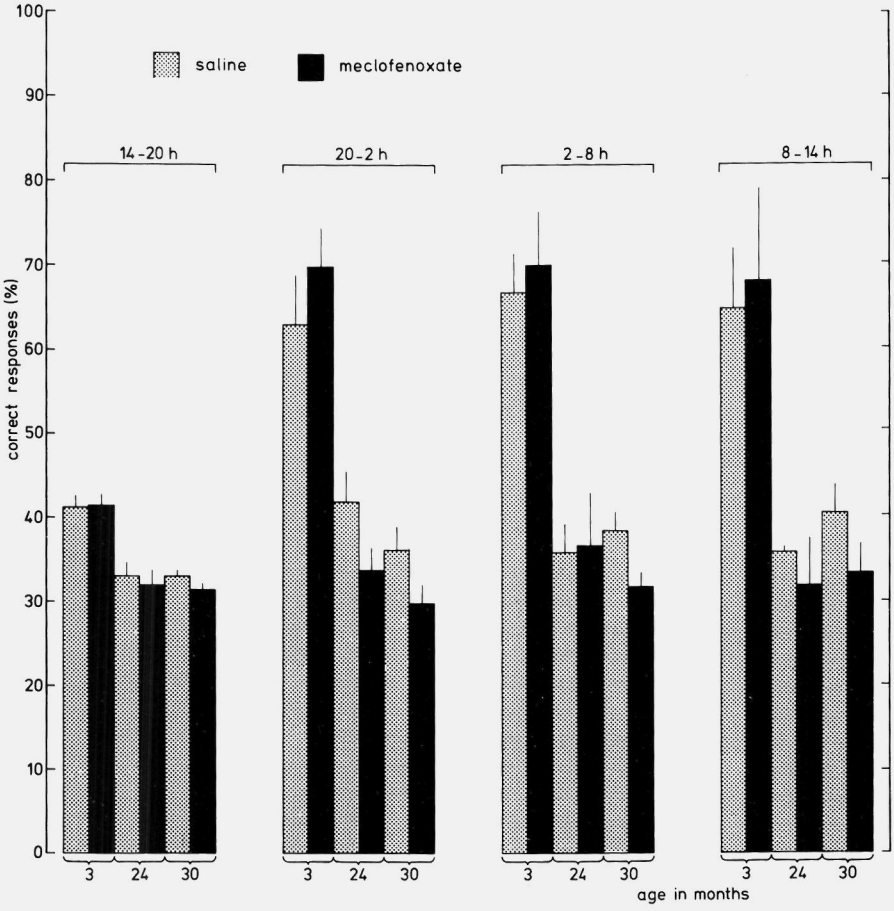



Fig. 20. The results of measurements during 24 hours of retention in the drink-test apparatus of rats of 3, 24 and 30 months treated with saline or with meclofenoxate. The number of animals per group is the same as in figure 19. The results are averaged over periods of 6 hours and are expressed as means of the percentage correct responses. The S.E.M.'s only have been drawn to one side. As indicated in the text, only the results of the first period of 6 hours can be considered as a reasonable indicator for the degree of retention.

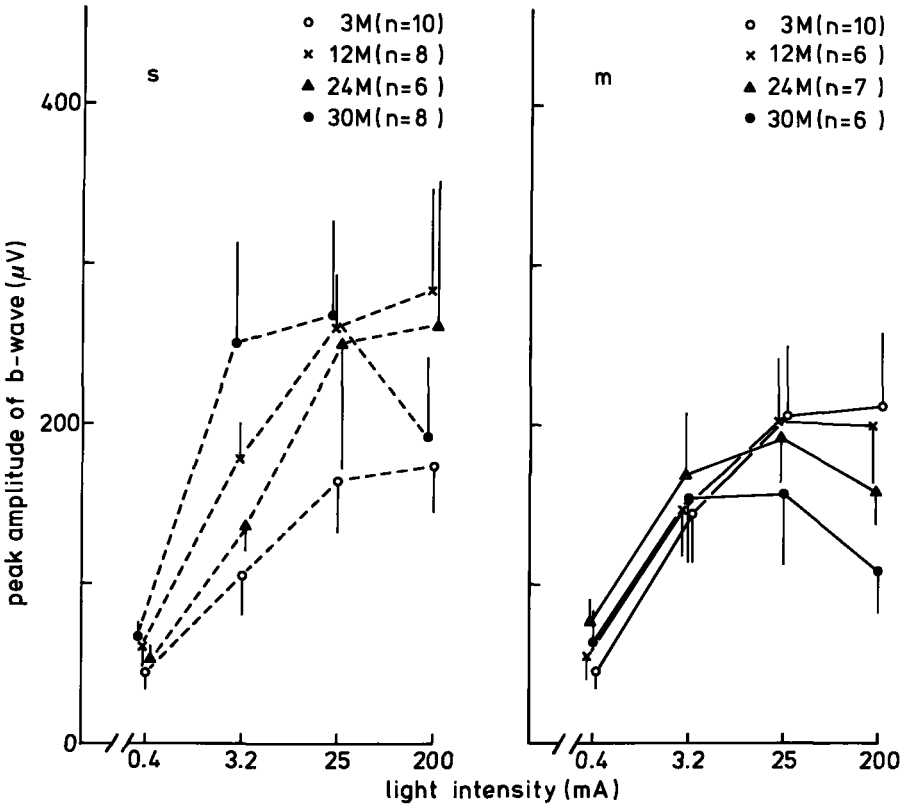



Fig. 21. The amplitudes (mean ± S.E.M.) of the b-waves of the ERG's in μV of saline treated (s) and meclofenoxate (m) treated rats of 3, 12, 24 and 30 months (M) old rats are shown in the left and the right graph, respectively. The heights have been measured at four different lightintensities; 1 mA current through the LED corresponds with 133 x $10^{-9}$ lumen on the eye of the rat.

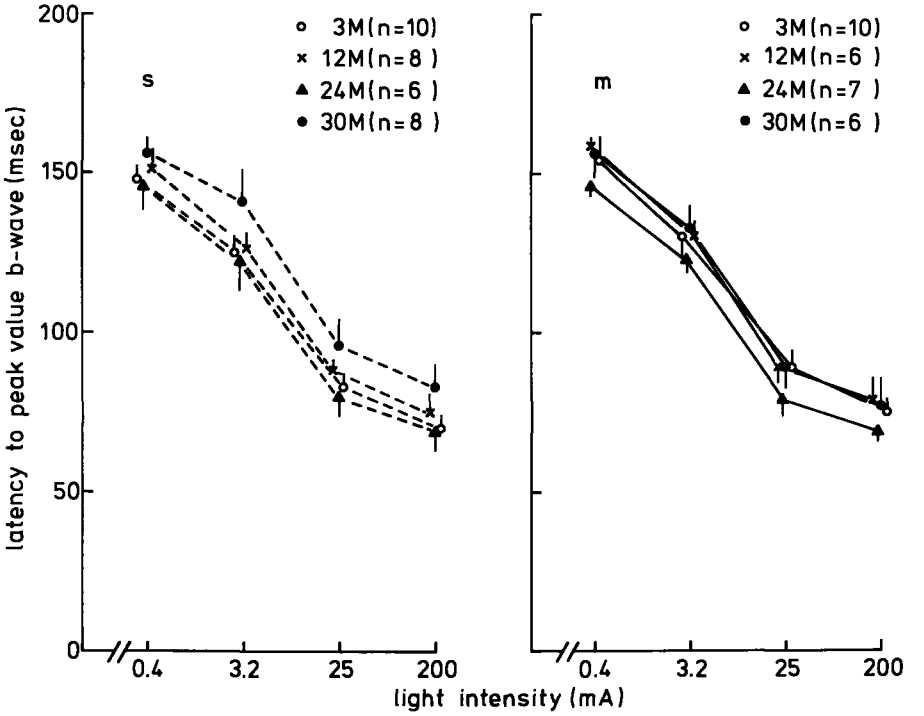



Fig. 22. The latency periods (mean ± S.E.M.) from flash to peak of the b-wave of the ERG in msec of rats of different age groups treated with saline (s) or with meclofenoxate (m). For details see figure 21 which shows results from the same experiment.

The peak to peak amplitudes of the various VER components measured at four lightintensities can be seen in figure 23. The latency periods between the flash and the peak value $N_1$ of the VER measured at four lightintensities in the different age groups are shown in figure 24.

Since the ERG as well as the VER are dependent on the lightintensity and since the VER is dependent on the ERG response, the values of

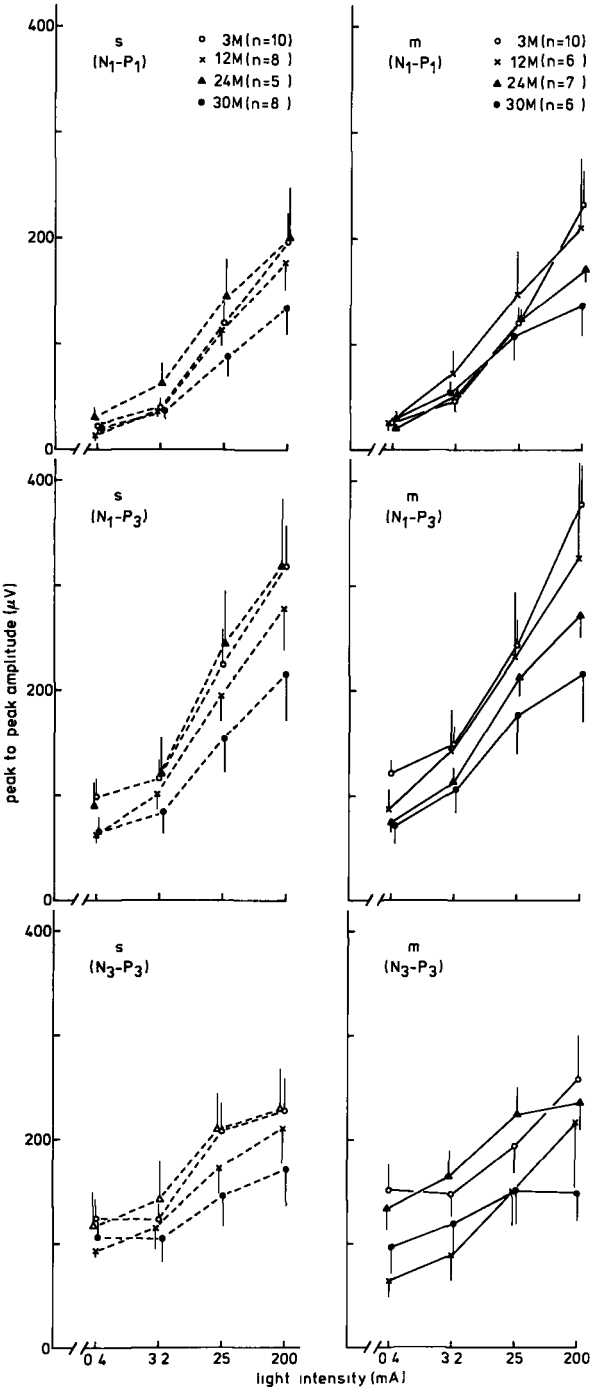

s
(N1-P1)
3M(n=10)
12M(n=8)
24M(n=5)
30M(n=8)
m
(N1-P1)
3M(n=10)
12M(n=6)
24M(n=7)
30M(n=6)
s
(N1-P3)
m
(N1-P3)
s
(N3-P3)
m
(N3-P3)
peak to peak amplitude (μV)
400
200
0
0 4
3 2
25
200
light intensity (mA)

**Fig. 23.**

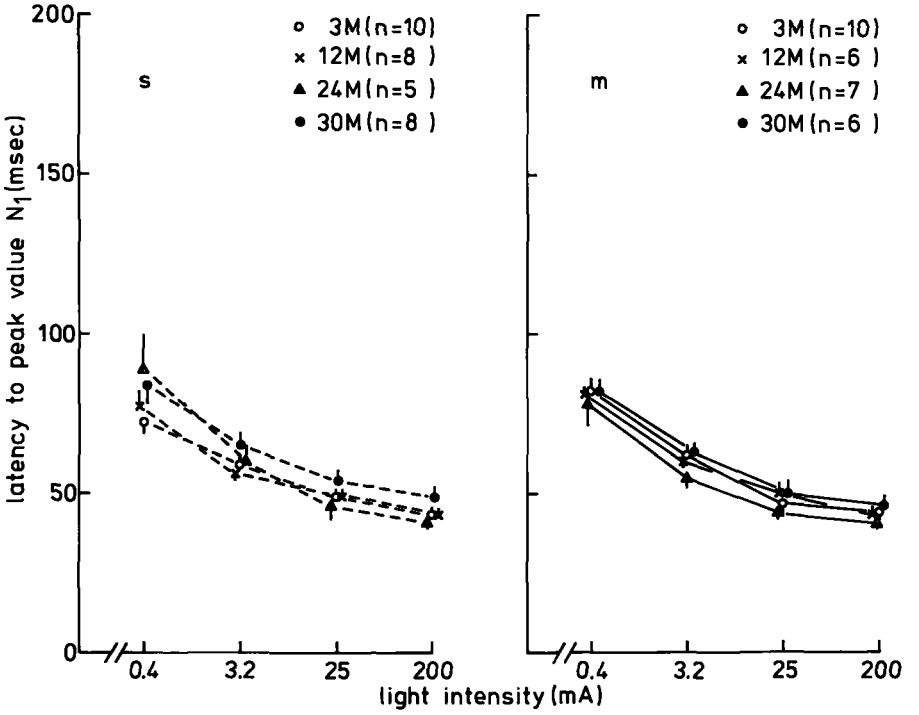



Fig. 24. The latency periods (mean ± S.E.M.) between the flash and the peak value $N_1$ of the VER in msec of rats of different age groups treated with saline (s: left graph) or with meclofenoxate (m: right graph). For details see figure 21 which shows the results from the same experiment.

Fig. 23. Peak to peak amplitudes (mean ± S.E.M.) of the various VER components in μV. Rats of different age groups treated with saline (s: left graphs), or with meclofenoxate(m: right graphs). For details see figure 21 which shows results from the same experiment.

the ERG and the VER have to be combined for the statistical evaluation of the results. Six parameters have been measured at four lightintensities, i.e. of the ERG's the amplitudes of the b-waves and the latencies to the peak of the b-wave and of the VER's the peak to peak amplitudes $N_1$-$P_1$, $N_1$-$P_3$, $N_3$-$P_3$ and the latencies to the peak of $N_1$. The values obtained from the animals were ranged according to the first principal component of the ERG and the VER (Kendall, 1965). For any of the variates the animals were ranged in order of increasing peak to peak values and decreasing latencies. For each animal these ranges were summed and these summes were ranged again. Possible differences between the age groups were tested according to Kruskall-Wallis (1952) at the 5% level. As described in chapter 3 no age-related differences have been found either of the ERG or the VER. Treatment with meclofenoxate did not cause significant ($p > 0.05$, two tailed) differences in the measured parameters in the four age groups when compared with saline treated animals.

### 5.3.4. The effect of meclofenoxate on lipofuscin accumulation in the central nervous system

To investigate the effect of treatment with meclofenoxate on the accumulation of lipofuscin in the CNS the amount of lipofuscin is determined in the cerebellum, the cerebral cortex and the hippocampus of rats of different ages treated with saline or with meclofenoxate. The relative fluorescence of 2 ml fractions of the column eluant of the extracts (see section 5.2.4.) were determined. From each extract an elution pattern was made and in all patterns one peak of the lipofuscin was observed. The area under the peak was calculated to obtain the relative fluorescence per extract. The extinction maxima of all extracts were 380-385 nm and the emission maxima 435-440 nm. The results of these measurements are shown in figure 25.

A significant ($p < 0.05$, two tailed) correlation was found between the amounts of lipofuscin in the three regions of the brain, both in the control as well as in the treated animals at all age groups, when the results are analysed according to Friedman (de Jonge, 1963). Because of this significant correlation the values of the three brain regions are summed per rat. The summed values were ranged and tested at the 5% level

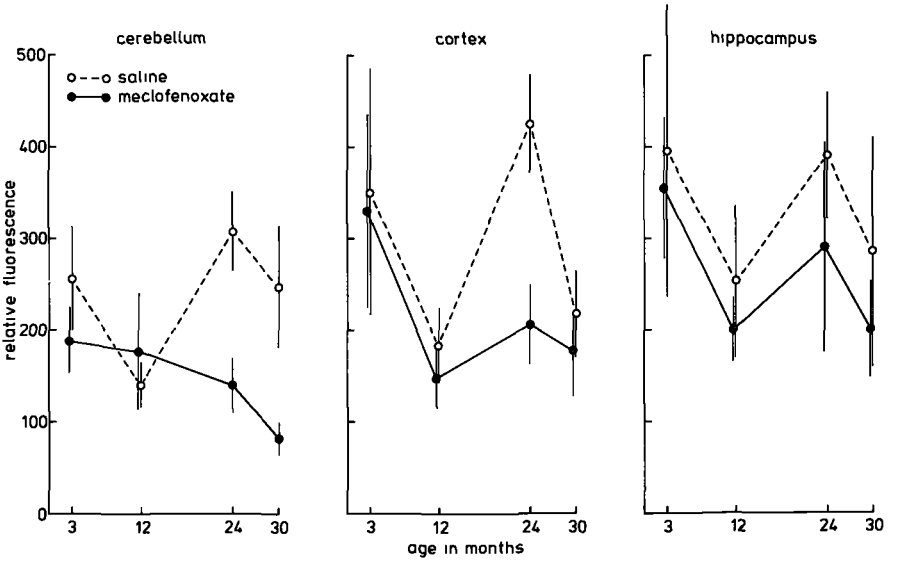



Fig. 25. The relative fluorescence (mean ± S.E.M.) of lipofuscin extracted from the cerebellum, cerebral cortex and the hippocampus of rats of 3, 12, 24 and 30 months old treated with saline or with meclofenoxate.

according to Kruskal-Wallis (1952). No significant ($p > 0.05$, two tailed) age-related differences were found in the amount of lipofuscin present in the brains of the animals. To determine if treatment with meclofenoxate has an effect on the accumulation or lipofuscin the summed values of the control groups and those of the treated groups were tested according to Wilcoxon (de Jonge, 1963). Although it is difficult to determine from the separate figures, especially in the case of the hippocampus, it appeared from the statistical evaluation that treatment with meclofenoxate significantly ($p < 0.05$, two tailed) decreased the amount of lipofuscin in the brains of the rats of the four age groups.

## 5.4. DISCUSSION

In this study it was attempted to correlate changes in the amount of lipofuscin in the brain with behavioural functions. No significant

age-related differences were found in the amount of lipofuscin present in the brains of the animals. This finding is contrary to the conclusions reached by e.g. Bondareff (1957), Brizzee and Ordy (1979), Brody (1960) and Reichel et al. (1968) who found an age-related increase. These authors used histological methods, which in our own experience are rather inaccurate. When observed through a fluorescence microscope the appearance of lipofuscin in the brains of the young rats is quite different from that in old animals; "young" lipofuscin is highly scattered throughout the cells, whereas "old" lipofuscin appears in large clumps in the majority of cells. Nandy (1971) also found such differences between young and old lipofuscin. These differences in appearance make it very difficult to quantify the amount of lipofuscin on a morphological basis.

The findings presented here, that no age-related differences exist in the amount of lipofuscin are in agreement with the results of Siakotos et al. (1977), obtained in human brains. With a subcellular fractionation technique these authors found that the amount of lipofuscin per unit dry weight is at its maximum during the first three months of life then decreases to reach a minimum at about 17 years and increases thereafter. The amounts, however, never reach the values of the first three months, not even at 80 years. These authors assumed that the presence of lipofuscin-like organelles as lipofuscin precursors were responsible for those large amounts of measured lipofuscin at a very early age. It is possible that with the quantification method used in the present study lipofuscin precursors are also measured together with "real" lipofuscin, resulting in the absence of age-related differences.

The present finding that 10 weeks treatment with meclofenoxate, a substance reported to reduce the amount of lipofuscin in the CNS (Glees, 1975; Meier and Glees, 1971; Nandy, 1968, 1978[b]; Nandy and Bourne, 1966; Riga and Riga, 1974), significantly decreased the amount of lipofuscin is in agreement with the results of these studies. The mechanism whereby meclofenoxate causes a reduction in the lipofuscin content of the brain is unknown. Nandy (1968) found that treatment with meclofenoxate caused a reduction of the activity of succinic and lactic dehydrogenase and cytochrome oxidase and an increased activity of glucose-6-phosphate dehydrogenase. He suggested that the drug might help to eliminate the lipofuscin pigment by a diversion of the glucose metabolism via the pen-

tose cycle. The reduction of the activities of simple esterase and acid phosphatase also found by this author might be due to the effect of meclofenoxate on the lysosomes which are intimately related to the genesis of this pigment (see: Nandy, 1978[c]).

To investigate the effect of treatment with meclofenoxate on the activity of animals of different age groups, horizontal and vertical movements were measured in an open field situation. Both in the control and in the treated animals the activity recorded as horizontal movements decreased gradually with age (figure 17). Treatment with meclofenoxate caused no significant differences in the distance run in any of the age groups. In the four parameters of the vertical activity measured, treatment with meclofenoxate caused a significant higher activity in the small movements of amplitude class 2 only in the 30 months old animals. In the other parameters measured no significant effect of treatment was found in any of the age groups. Taken together, treatment with meclofenoxate did not cause an increase in activity in any of the age groups. Wietek (1963) and Thuillier et al. (1960) reported a significant increase of spontaneous activity in mice treated with 150 mg/kg meclofenoxate for ten days. Since the results of activity measurements depend to a large extent on the technique used and since it is not known how the activity has been measured by both authors, it is very difficult to compare their results with those of the present study. In addition, the shorter duration of treatment could be the reason of the different results of the studies. The results of the present study are in agreement with those of Schilter (1974) who found no significant increase in activity in humans treated during 6 weeks with meclofenoxate. The increase of lucidity and vigilance after treatment with meclofenoxate claimed by Wietek (1963) are very difficult to determine in animals.

To investigate if a reduction of the amount of lipofuscin is parallelled by an improvement of acquisition in animals of different age groups were tested in the drinktest apparatus after treatment for 10 weeks either with meclofenoxate or with saline. The acquisition of the meclofenoxate treated animals did not differ significantly from that of the control animals in any of the age groups tested (figure 19). Similarly, treatment with meclofenoxate had no significant effect on the retention of the learned information after 25 days (figure 20). Since the

amount of lipofuscin was significantly decreased after treatment with meclofenoxate in the brains of the animals tested it appears that a reduction of the amount of lipofuscin in the brains of the rats is not parallelled by improvement of acquisition in the drinktest. This finding seems to be in contrast with that of Nandy (1978[a]), who found that meclofenoxate treated mice learned a task in significantly fewer trials. He also found a reduction of neuronal lipofuscin pigment in certain brain structures. To what extent the observed improvement of learning was a direct effect of meclofenoxate cannot be verified in the procedure followed by Nandy. In the present study, however, no direct effect of meclofenoxate on drinktest learning could have been involved because the animals were not treated with the drug during the actual drinktest experiment.

The finding that reduction of brain lipofuscin content had no effect on the impaired acquisition of the old animals is in agreement with the results of Freund (1979). He determined the effect of vitamin E consumption on the accumulation of lipofuscin in brains of mice. The addition of extra vitamin E to diets caused a decrease of the brain lipofuscin content in old mice but this decrease had no significant effect on the shuttle-box learning deficits of these old animals. Although Brizzee and Ordy (1979) stated that significant age-related differences in retention in rats were correlated significantly with increases in intraneuronal lipofuscin in the hippocampus and the visual cortex, they did not investigate the effect of reduction of lipofuscin on learning. Their statement, therefore, is not based on results they reported.

The present study and that of Freund (1979) leads to the conclusion that the accumulation of lipofuscin is probably not the cause of the behavioural decline with ageing. This conclusion lends experimental support to the assumption of Mann and Sinclair (1978), that it is unlikely that removal of lipofuscin, per se, has any bearing on the reported improvement in intellectual capability in demented humans after treatment with meclofenoxate (Gedye et al., 1979; Schmidt and Broicher,. 1970). In their opinion this improvement might result from an increase in cellular efficiency, possible brought about by stimulation of metabolic pathways involved in these processes (Nandy, 1968).

As has been discussed in chapter 2 no significant age-related changes have been found in the shapes, the amplitudes and the latencies of the ERG and the VER. The reduction of the amount of lipofuscin in the brain after treatment with meclofenoxate has no significant effects on various parameters of the ERG and the VER.

The findings of the present experiments make it very likely that the amount of lipofuscin is irrelevant for CNS function. On the one hand, no differences in amounts of lipofuscin could be detected in young and old animals, whereas the acquisition rate of old animals was considerable decreased. On the other hand, treatment with meclofenoxate caused a reduction of lipofuscin in aged animals without affecting in any way the existing age dependent behavioural differences. In addition, the parameters of the ERG and VER which show no age dependent differences are also unaffected by meclofenoxate treatment and the resulting lipofuscin reduction in old animals. It is theoretically possible that functionally it makes a great difference whether lipofuscin is present in a diffuse form as in young animals or in the form of intracellular aggregates as in old animals. Therefore, it was interesting to know whether treatment with meclofenoxate affected the appearance of the lipofuscin aggregates left behind in the old animals. Observed through a fluorescence microscope, the lipofuscin aggregates appeared to be unchanged after meclofenoxate treatment. Whether they were reduced in quantity was very hard to determine. On the basis of these results it is difficult to gain evidence in favour or against the ideas of Siakotos et al. (1977), i.e. the fluorescence material which is extracted from brains of young subjects may in fact be lipofuscin precursors.

In conclusion, the present results do not confirm the idea that the amount of lipofuscin in the brain is directly correlated with age-related changes in behaviour. In fact, the question can be asked whether any substantial age-related changes in the amount of lipofuscin in the brain take place at all. The changes observed may simply be caused by differences in the aggregation of the pigment.

# CHAPTER 6

# EFFECTS OF TREATMENT WITH PIRACETAM, ETIRACETAM, DESGLYCINAMIDE-ARGININE-VASOPRESSIN AND HYDERGINE® ON ACQUISITION OF OLD RATS

## 6.1. INTRODUCTION

Interest in the pharmacological influence of ageing has grown in recent years. In attempts to exert a favourable influence on cerebral function in the aged various kinds of drugs are used, varying from "vaso-active drugs", "haemokinators", "cerebral metabolic improvers" to "cerebral activators" (Hyams, 1978). The beneficial effects of treatment with these drugs, however, are not always convincingly demonstrated (see: Hyams, 1978). Since the acquisition deficit of old rats in the drinktest is a consistent phenomenon it seems useful to test the effect of "geriatric agents" on acquisition of old animals in the drinktest. Piracetam (Nootropil®), etiracetam, Hydergine® and desglycinamide-arginine-vasopressin (DG-AVP, Org 5667) have been chosen for the present investigation.

Piracetam (2-pyrrolidone-acetamide) is a representative of the nootropic substances defined by Giurgea (1978) as CNS-acting drugs which act directly on the higher intergrative brain mechanisms by enhancing their efficacy, resulting in a positive, direct impact on mental functions. Piracetam is a virtually nontoxic drug with positive effects on learning and memory processes in both animals (Wolthuis, 1971) and humans (Dimond and Brouwers, 1976). The attempts to analyse the acquisition enhancing effects of piracetam so far have shown that the compound protects the brain against the effects of hypoxia (Giurgea et al., 1970), enhances transcallosal potentials (Giurgea and Moyersoons, 1972), evoked potentials following tooth pulp stimulation (Krug et al., 1977) and also facilitates interhemispheric visual informtion transfer (Bureŝova and Bureŝ, 1976). Interestingly, even in large doses, the drug has no effects on autonomic functions, arousal level, limbic lobe activity and psychomotor activity. In some controlled clinical studies with patients suffering from organic brain syndrome, the investigators found that piracetam produced significantly greater improvement in their patient's gene-

ral mental condition and behaviour than a placebo (Macchione et al., 1976; Stegink, 1972). Other studies, however, do not show statistically significant positive effects of piracetam (Abuzzahab et al., 1978; Diesfeldt et al., 1978).

Etiracetam is the ethylated derivative of piracetam. Etiracetam enhances acquisition in the rat more effectively than piracetam (Wolthuis, 1980). As is the case with piracetam, it is very unlikely that the etiracetam enhanced acquisition is due to aspecific factors such as changes in footshock sensitivity, increased activity levels, tranquillizing or sedative effects. The effects of etiracetam in aged subjects have not been tested up till now.

The most extensively studied and most widely prescribed drug in this field is Hydergine®; it consists of the mesylates of dihydrogenated derivatives of the alkaloids of ergotoxine - i.e. dihydroergocornine, dihydroergocristine and dihydroergokryptine. The major effects of Hydergine® are now thought to be (for review see: Hyams, 1978): improved neuronal metabolism, improved astrocyte metabolism, favourable influence on cellular ATP balance, stimulation of protein synthesis in the brain, increase in electrical activity of the brain and improvement in brain microcirculation. Hughes et al. (1976) presented a critical review of 12 clinical trials of Hydergine® in patients with symptoms associated with dementia. Qualitative and quantitative comparisons of improvement in symptoms showed that Hydergine® consistently produced statistically significant improvements in many clinical features associated with dementia. The degree of improvement, however, was small and there was little or no evidence of long term benefit. Hughes et al. (1976) concluded that Hydergine® is of minor value in the treatment of dementia. The authors stressed the need for further research with improved methodology and trial design.

The number of pharmacological and clinical investigations of the effects of treatment with neuropeptides has been growing rapidly in recent years. The vasopressin analogs desglycinamide-arginine-vasopressin (DG-AVP, Org 5667) and desglycinamide-lysine-vasopressin (DG-LVP) have approximately the same type of effect on consolidation of information and on retrieval of the consolidated information in rats (Greven and de Wied, 1980; Rigter et al., 1974) and almost completely lack the endocrine

effects of vasopressin (de Wied et al., 1972). Legros et al. (1978) studied the effects of lysine$^{8}$-vasopressin in elderly men (approximately 60 years). They concluded that patients given vasopressin performed better in tests involving attention, concentration and motor activity. The treated patients were superior in the retention of visual graphic material, digit span, word retention and word recall. It should be noted that the subjects were an elderly group which was not specifically screened for the presence of pre-existing cognitive decline. Oliveros et al. (1978) treated 4 amnesia patients in an open pilot trial and obtained very encouraging results.

The effects of the above mentioned agents on the acquisition deficits of the aged rats in the drinktest were investigated.

## 6.2. MATERIALS AND METHODS

The female WAG/Rij rats used were 30 months old at the beginning of the experiment and were experimentally naive.

### 6.2.1. Effects of piracetam and etiracetam on acquisition of old rats in the drinktest

The mean ($\pm$ S.E.M.) body weights (g) of the animals at the start of the experiment was 277 $\pm$ 5.6. Two experiments were carried out each with 3 groups of 12, 14 and 13 rats respectively. The results obtained with identically treated animals were pooled. The animals were injected intraperitoneally (i.p.) once daily at about 2.00 p.m. with piracetam (150 mg/kg), etiracetam (30 mg/kg) or with a saline solution, for two weeks preceeding the start of the drinktest. The volume of the injected drugs and the saline solution was 1 ml/kg. After the injection period of 2 weeks the animals were trained in the drinktest. During training in the drinktest the animals were also injected i.p. once daily at 2.00 p.m. with the drugs or saline solution. The apparatus and the task the animals had to perform have been described in section 2.2.1. of chapter 2. The drinktest is based on conditioned suppression of drinking behaviour, i.e. rats are trained to avoid licking a water tube when a set of signals indicate that no water will be delivered. An incorrect response is not

rewarded with water and is punished by shocks delivered through a grid floor under the water tube for 5 seconds. After 3 days of habituation the animals were trained during 20 days.

6.2.2. Effects of Hydergine® on acquisition of old rats in the drinktest

The mean (± S.E.M.) body weights (g) of the animals at the start of the experiment was 257 ± 3.9. Two experiments were carried out, each with 4 groups of 6 rats. The results obtained with identically treated animals were pooled. The apparatus and the task the animals had to perform were the same as in the previous series (6.2.1.). After 3 days of habituation the animals were trained during 24 days. On the 25th day extinction of the acquired behaviour was tested for 24 hours. During these extinction measurements the same signals were presented as during the training period; the only difference being that upon an incorrect response no punishment followed (see section 5.2.2. of chapter 5). During the period in which the animals were kept in the drinktest apparatus they received each day at 2.00 p.m. a subcutaneous injection of Hydergine® at a dose level of either 0.5 mg/kg or 1 mg/kg or 3 mg/kg. The control group received a control solution. The volume of the injections was always 3 ml/kg.

6.2.3. Effects of desglycinamide-arginine-vasopressin (DG-AVP, Org 5667) on acquisition of old rats in the drinktest

The mean (± S.E.M.) body weights (g) of the animals at the start of the experiment was 254 ± 3.7. One experiment was carried out with 4 groups of 6 rats. The apparatus and the task the animals had to perform were the same as in the two previous series (6.2.1. and 6.2.2.). After 3 days of habituation the animals were trained during 20 days. On the 21th day extinction of the acquired behaviour was tested for 24 hours as described in section 6.2.2. During the period in which the animals were kept in the drinktest apparatus they received each day at 2.00 p.m. a subcutaneous injection of DG-AVP at a dose level of 3 µg/kg or 30 µg/kg or 300 µg/kg. The control group received saline. The injection volume was

always 1 ml/kg.

## 6.3. RESULTS

### 6.3.1. Effects of piracetam and etiracetam on acquisition of old rats in the drinktest

To investigate whether treatment with piracetam or etiracetam influences acquisition of old animals in the drinktest, rats treated with 150 mg/kg piracetam or with 30 mg/kg etiracetam were tested in the drinktest apparatus. The results of this experiment are shown in figure 26. Statistical analysis of the results on day 20 of the experiment according to Welch (1947) using the multiple interference method according to Newman-Keuls (Miller, 1966) shows no significant ($p > 0.05$, two tailed) differences between the three experimental groups. From the learning curves and the data of the last day it can be concluded that neither a treatment with piracetam nor one with etiracetam had an effect on acquisition of old rats in the drinktest.

### 6.3.2. Effects of Hydergine® on acquisition of old rats in the drinktest

To investigate whether treatment with Hydergine® influences acquisition of old animals in the drinktest, rats treated with 0.5 mg/kg, 1 mg/kg, 3 mg/kg Hydergine® or with a control solution were trained in the drinktest. The extinction of the required behaviour was measured after the training period on day 25. The results of this experiment are shown in figure 27. Statistical analysis on a 5% level of the results on day 24 of the experiment according to Welch (1947) using the multiple interference method according to Newman-Keuls (Miller, 1966) shows that no significant differences existed between the four experimental groups. From the learning curves and the data of the last day it can be concluded that treatment with Hydergine® in the doses used has no effect on acquisition of old rats in the drinktest. Statistical analysis on a 5% level of the results of the first 6 hours of the extinction measurements - this period can be considered as a reasonable indicator for the degree

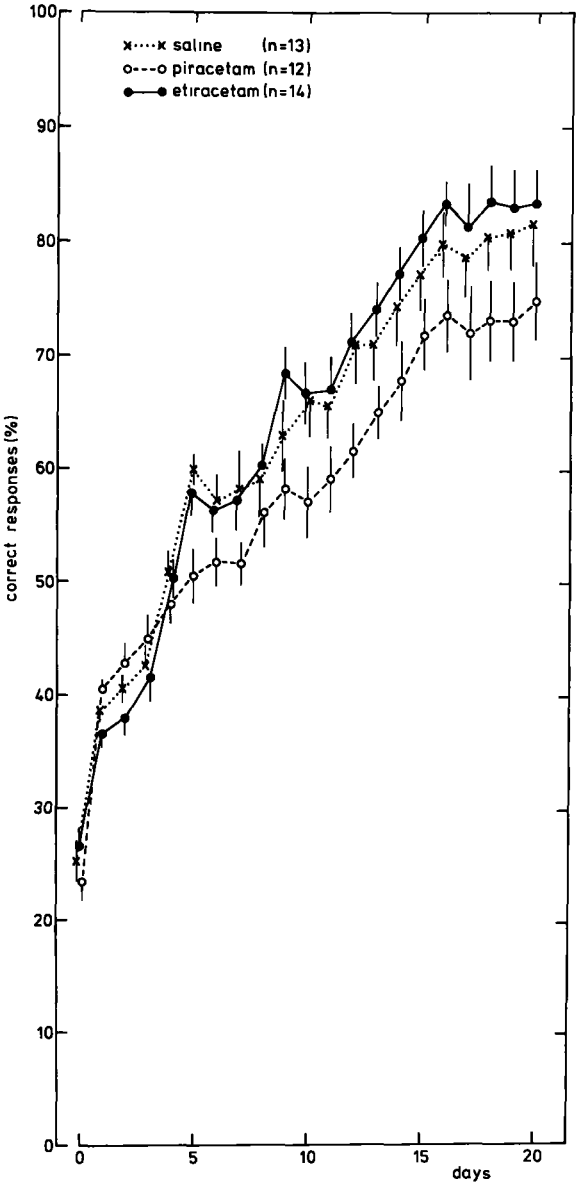



Fig. 26. A comparison of drinktest acquisition of 30 months old rats daily injected i.p. with piracetam (150 mg/kg), etiracetam (30 mg/kg) or saline. The results are expressed as the means ± S.E.M. of the percentage correct responses (correct/total).

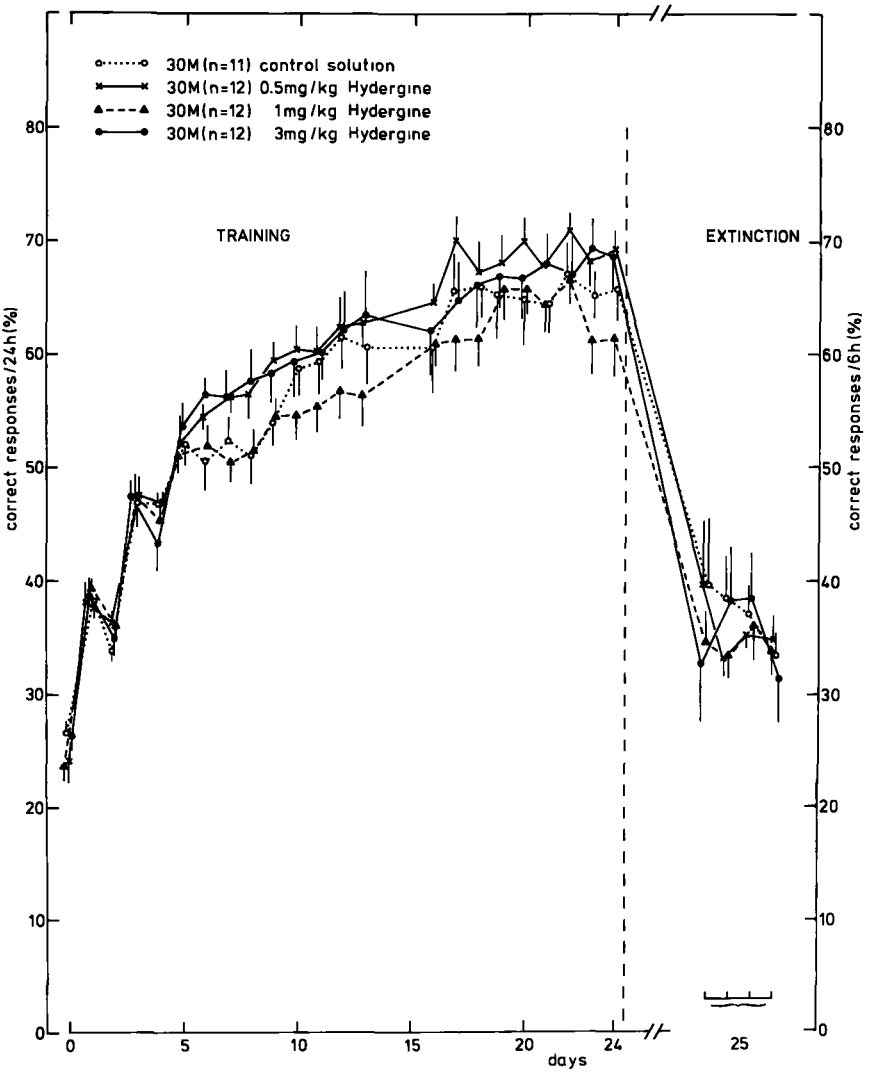



Fig. 27. A comparison of acquisition of 30 months (M) old rats treated with Hydergine® at dose levels of 0.5 mg/kg, 1 mg/kg or 3 mg/kg or with control solution. The results of the extinction measurements on day 25 are averaged over periods of 6 hours. All results are expressed as the means ± S.E.M. of the percentage correct responses (correct/total).

of extinction as indicated in section 5.3.2. - do not point to significant differences in extinction between the treated and the control animals.

### 6.3.3. Effects of desglycinamide-arginine-vasopressin (DG AVP, Org 5667) on acquisition of old rats in the drinktest

To investigate whether treatment with DG-AVP influences acquisition and extinction of old animals in the drinktest, rats treated with 3 µg/kg, 30 µg/kg or 300 µg/kg DG-AVP or with saline were trained in the drinktest. The extinction of the acquired behaviour was measured after the training period on day 21. The results of this experiment are shown in figure 28. Statistical analysis on a 5% level according to Welch (1947) using the multiple interference method according to Newman-Keuls (Miller, 1966) of the results on day 20 and of the results of the first 6 hours of the extinction measurements shows that no significant differences exist between the four experimental groups. From these data and the learning curves it can be concluded that the treatment with DG-AVP in the doses used has no effect on acquisition and extinction of old rats in the drinktest.

## 6.4. DISCUSSION

The acquisition of 30 months old animals in the drinktest is impaired when compared with that of young animals (Wolthuis et al., 1976; this study). To investigate whether it is possible to improve acquisition of the old animals in the drinktest by pharmacological means, 30 months old rats were treated with agents which are already applied as geriatric drugs or which could have such an activity.

Treatment of old animals either with piracetam or with etiracetam had no significant effect on acquisition in the drinktest. Pretreatment of the animals with piracetam seemed necessary because the delayed effects of this agent. Wolthuis (1971) showed that differences in acquisition between control and experimental groups became significant after about 8 days of training and Dimond (1975) found that positive effects of this drug on the performance of certain tasks in human volunteers were

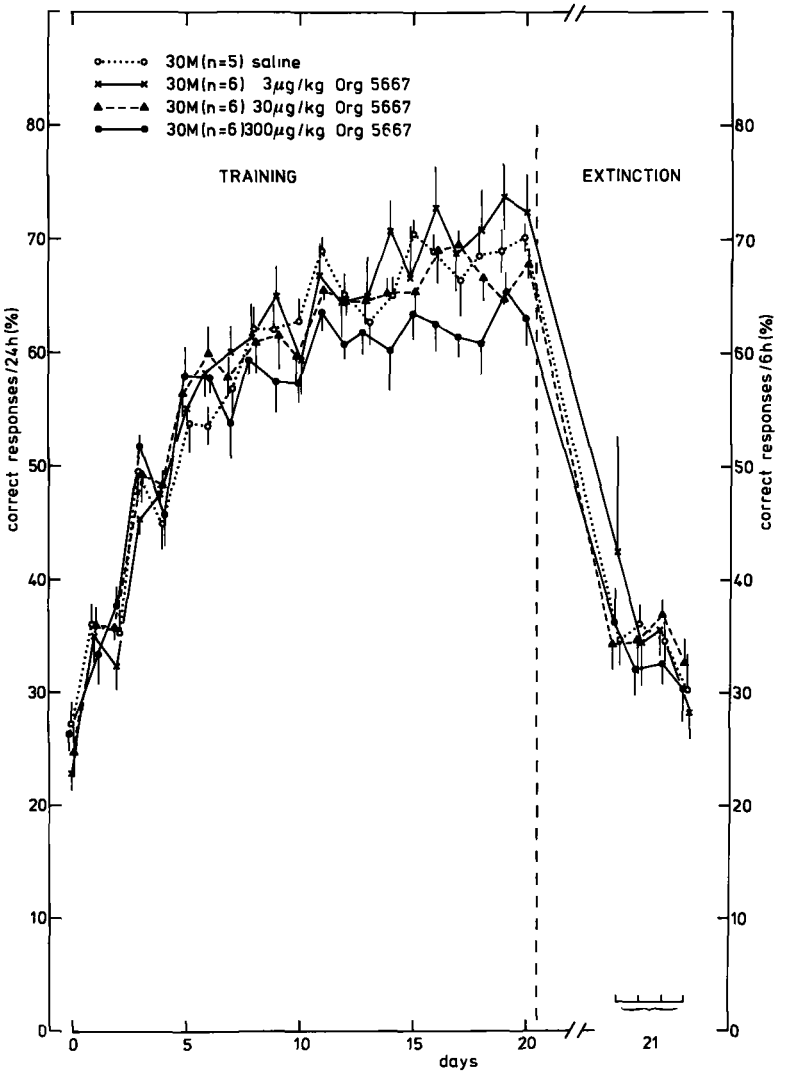



Fig. 28. A comparison of acquisition of 30 months (M) old rats treated with DG-AVP (Org 5667) at dosage levels of 3 µg/kg, 30 µg/kg or 300 µg/kg or with saline. The results of the extinction measurements on day 21 are averaged over periods of 6 hours. All results are expressed as the means ± S.E.M. of the percentage correct responses (correct/total).

detectable after two weeks but not after one week of treatment. It appeared (Wolthuis, 1980) that etiracetam also exhibits these delayed effects; pretreatment with this drug appeared to be more effective.

The influence of piracetam and etiracetam on extinction of acquired behaviour was not investigated since at the time of the experiments it was only known that piracetam has no effect on extinction. After the present experiments had been finished, Wolthuis (1980) found that administration of etiracetam during acquisition renders the acquired behaviour more resistant to extinction. It could be possible that etiracetam, without having a significant effect on acquisition, might have an effect on the extinction of old animals.

The finding that etiracetam and especially piracetam has no significant effect on acquisition of old rats is in contrast with most findings in literature. Giurgea and Mouravieff-Lesuisse (1972) found a benificial effect of piracetam on performance of 1 year old rats in a water-filled maze. In view of the difference in tasks a definite conclusion is difficult to make. Since no known results of an investigation of the effects of etiracetam on acquisition of old animals exists the data of the present experiments cannot be compared with others. It might be that piracetam and etiracetam have no effect on this type of learning in the drinktest; Wolthuis (1971), however, found in young rats an enhancing effect of piracetam on acquisition in a drinktest system with a different procedure of testing. In line with the present results on aged rats are the results of Means et al. (1980), who found no significant changes in both acquisition and retention of active and passive avoidance conditioning in young and older (450 days) rats (the older rats were deficient in these tasks) whereas piracetam has been shown to improve both active and passive avoidance learning in young rats affected by brain hypoxia (Giurgea et al., 1970; Sara and Lefevre, 1972).

Treatment with Hydergine® has no effect on acquisition and extinction of old animals in the drinktest. This finding is in contrast with the results in humans, i.e. a small but significant improvement of symptoms of dementia (Hughes et al., 1976). Since no known results of an investigation of the effects of Hydergine® on acquisition of old animals exist the data of the present experiments cannot be compared with others. The absence of an effect of Hydergine® on acquisition in aged

rats is perhaps not surprising, since Emmenegger and Meier-Ruge (1968) reported that the effects of Hydergine® are among other things due to improvement of neuronal metabolism. The data on glutamate metabolism in the aged rats presented in chapter 4 suggest that in the strain of rats investigated here brain metabolism may be hardly affected by age. If this is true, then there is little room for improvement by Hydergine®.

Finally, it appeared that treatment with DG-AVP has no effect on acquisition and extinction of old animals in the drinktest. Again this finding is in contrast with results of treatment with lysine-vasopressin in humans (Legros et al., 1978). It was surprising that this peptide had no effect on extinction in the drinktest since de Wied (1971) found that lysine-vasopressin inhibited extinction of an avoidance response not only at 2 and 4 hours but also 24 and 72 hours after injection. As described in the introduction of this chapter all vasopressin analogues mentioned have qualitatively the same kind of effects on learning and memory process.

Data in the literature suggest that the agents used in the present experiments - except etiracetam- cause an improvement in many types of performances of patients with memory deficits. These agents have, however, no effect on acquisition of old rats in the drinktest. It is possible that the acquisition deficits of old animals in the drinktest as such have nothing to do with the memory deficits as observed in humans and that, therefore, the agents used are not active in this respect in the old animals. The question can be raised if the drinktest procedure is the correct method to test possible effects of these agents. In this regard the following may be said. The rats were injected once daily at 2.00 p.m. Since lighting was not reversed, this means that they were injected in their inactive period. It may be assumed that the concentrations of these compounds will drop after few hours. For instance, it has been shown that piracetam reaches a maximum concentration in the rat brain 2 hours after injection (Ostrowski, 1975). If this is approximately true for all agents tested then the concentrations of potentially active drugs will have dropped below an effective level by the time that the rats become active and make the majority of their drinking responses. When the drugs exhibit delayed effects, however, which appeared for piracetam (Wolthuis, 1971) and etiracetam (Wolthuis, 1980) and is also reported for

Hydergine® in humans (Hyams, 1978), the time of administration of the drugs could be of less importance. It would, therefore, be worthwhile to repeat the tests with reversed lighting conditions, where the drugs will be administered closely before the daily peak of performance occurs. For the moment, it can be only be concluded that none of the drugs applied show a benificial effect on the acquisition deficits of aged rats when these drugs are administered at a time of the day when the rats are relatively inactive.

# CHAPTER 7

# CHOLINE-ACETYLTRANSFERASE ACTIVITY IN HIPPOCAMPI OF YOUNG AND OLD RATS

## 7.1. INTRODUCTION

Alzheimer's disease (senile dementia of the Alzheimer's type) is a well known and tragic organic brain process in humans characterized by early intellectual deterioration. There is debate about whether the disease is an accelerated form of normal ageing. Bowen et al. (1979), however, after extensive biochemical analysis of whole temporal lobe from cases of dementia and controls suggest that Alzheimer's disease is a primary degenerative nerve-cell disorder rather than the result of accelerated ageing. Morphologically the disease is amongst others characterized by the formation of large numbers of "senile" plaques and neurofibrillary tangles in the neurons of the frontotemporal cortex and the hippocampus (Constantinidis, 1978; NAS, 1981). Neurochemically, studies in recent years have demonstrated a wide spread deficiency in choline-acetyltransferase (CAT) activity in postmortem samples from cortical regions - notably from the hippocampus - of patients (Bowen et al., 1979; Davies and Maloney, 1976; Perry et al., 1977[a]; Reisine et al., 1978; White et al., 1977). In normal old people also a decreased activity of hippocampal CAT has been found when compared with young subjects (Perry et al., 1977[b]).

In ageing laboratory animals deficits in learning and memory processes have been demonstrated; it was thought possible that the acquisition deficits might be accompanied by a decrease in hippocampal CAT activity with age. Vyayan (1977) found that in mice the hippocampal CAT activity declines significantly with age after 8 months; the CAT activity in the cerebellum of these mice, however, remained unchanged.

It was decided to measure the hippocampal activity of CAT, an enzyme which is essential for the synthesis of acetylcholine, in rats of different ages.

## 7.2. MATERIALS AND METHODS

The female WAG/Rij rats used were 3, 14 and 30 months old and experimentally naive. The animals were decapitated and the hippocampus was dissected. Homogenates (0.5% w/v) were prepared in 0.1 % Triton X-100 in 0.1 mM EDTA and 150 mM $KH_2PO_4$, pH=7.9. These samples were homogenised in a Braun (Melsungen) homogenisator at 1500 rpm.

For the CAT assay the radiochemical method of Fonnum (1975) modified according to Molenaar (personal communication) was used. The incubation mixture contained (final concentration): [1-$^{14}$C]acetyl-CoA (specific activity 72.7 mCi/mmol; Radiochemical Centre, Amersham, Buckinghamshire, U.K.) diluted with unlabeled acetyl-CoA (Boehringer, Mannheim, Germany) at a final concentration of 0.212 mM, 52. 5 mM NaCl, 50 mM $KH_2PO_4$, 2 mM choline chloride, 0.033 mM EDTA and 0.33 mM eserin salicylate. The enzyme solution (40 µl) was placed in a microtube and 20 µl homogenate mixture was added. The solution was mixed by hand and incubated for 30 minutes at 37°. The reaction was stopped by adding 0.1 ml 14% trichloroacetic acid (TCA) solution to the reaction medium; the microtubes were kept at 0° for 5 minutes. Thereafter the contents of the microtubes were transfered to scintillation vials; 5 ml of 0.05 M $NaH_2PO_4$ (pH=7.0), 2 ml of acetonitrile containing 10 mg of tetrafenylboron (Kalignost) and 5 ml of toluene scintillation mixture was added to the vials. The vials were shaken lightly for one minute by hand and the contents were allowed to separate into two layers for 5 minutes. By this procedure the acetylcholine (ACh) is extracted into the toluene phase whereas the acetyl-CoA is left in the aquous phase. The radioactivity was measured by liquids scintillation counting in a Searle Mark III Liquid Scintillation System. The counting efficiency was 90%. The activity of CAT was expressed as µM ACh synthesized/gram of hippocampus tissue/hour.

## 7.3. RESULTS

The results of the measurements of the CAT activity in the hippocampus of rats of different ages is shown in figure 29. When these results are analysed according to Welch (1947) using Bonferoni's statis-

tics (Miller, 1966) for simultaneous interference it appears that the CAT activity in the hippocampi of the 3 months old rats is significantly ($p < 0.05$, two tailed) lower than that in the 14 and 30 months old animals. The CAT activity in the hippocampi of the 14 months old rats does not differ significantly ($p > 0.05$, two tailed) of that in the 30 months old animals.

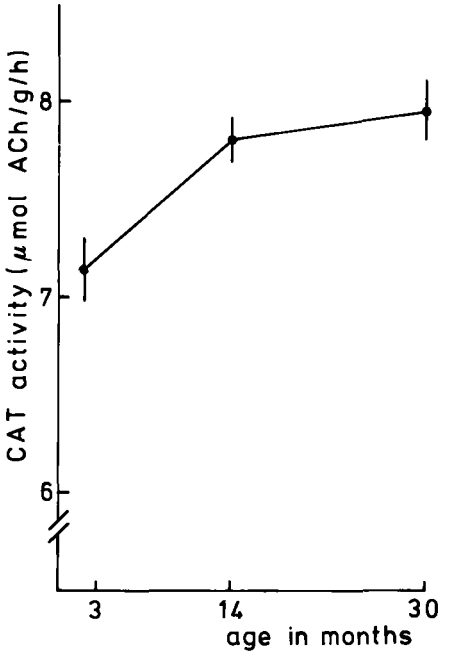



Fig. 29. The CAT activity (expressed as µmol ACh/g wet weight/hour) in the hippocampus of rats of 3 (n=6), 14 (n=6) and 30 (n=6) months old. The results are expressed as means ± S.E.M.

## 7.4. DISCUSSION

The results of the present measurements show that in our rats the hippocampal CAT activity slightly but significantly ($p < 0.05$, two tailed) increases with age. Since the oldest rats clearly exhibited age-related acquisition deficits, these results exclude that these deficits are due

to a decrease in hippocampal CAT activity. These results are in line with earlier findings of McGeer et al. (1971) who found no significant age-related decrease in CAT activity in brains minus caudates and cerebella of male Wistar rats; in the caudates a significant decrease with age was found. On the other hand the results are in contrast with those of Vyayan (1977), who found a decrease in CAT-activity in the hippocampi of mice with age. In normal old people also decreased activity of hippocampal CAT has been found (Perry et al., $1977^{b}$). This decrease in CAT activity is mostly pronounced in humans suffering from Alzheimer's disease (Davies, 1978; Davies and Maloney, 1976; Reisine et al., 1978). Bowen et al. (1979) showed in their study that the reduction in CAT activity in the neocortex is parallelled by a selective loss of neocortical cholinergic neurons. They suggested that since acetylcholine has been implicated in memory processes, the selective loss of cholinergic neurons might be relevant for the clinical features of senile dementia.

Since the decreased hippocampal CAT activity found by Perry et al. ($1977^{b}$) was not found in rats in the present study, this might mean that in this respect the rat may not be an optimal model for studying the relationship between CAT activity in the brain and acquisition deficits.

# CHAPTER 8

## GENERAL DISCUSSION AND CONCLUSIONS

The objective of this study was to gain more insight into the nature of the decline in acquisition of old rats as found by Wolthuis et al. (1976). From their results and from an experiment with rats of various ages (12, 18, 24 and 30 months) described in the present study (chapter 2), it appeared that the deficits in acquisition develop gradually with increasing age. In fact, these deficits became visible when the rats had reached the age of 18 months. The present results also demonstrate that the acquisition deficits found are not limited to the strain of rats used by Wolthuis et al. (1976), i.e. the albino small Wistar WAG/Rij rats. Pigmented BN/BiRij rats of 30 months old also showed a deficit in acquisition when challenged in the drinktest in comparison with 3 months old rats of the same strain. When compared with the WAG/Rij rats, the BN/BiRij rats clearly acquired the task much more slowly but ultimately reached the same level of performance. To examine whether age-related differences could also be demonstrated in more basic, adaptive functions of the central nervous system (CNS), the adaptation of drinking activity in reaction to reversed light-dark cycles was chosen as a paradigm. No appreciable differences in diurnal drinking rhythms with age appeared after light-dark reversal and after return to normal lighting conditions.

It is possible that the performance deficits seen in old rats are attributable to secondary factors such as changes in motor skill, susceptibility to interference, or alterations in motor activity level or footshock sensitivity. It will be clear that any task requiring athletic performance, such as active avoidance in a jump box (Gordon et al., 1968), passive avoidance in a runway (Ordy et al., 1978) or transfer of handedness (Rapaport et Bourlière, 1963) may measure the animals' motor skill rather than its capability to learn a task. Therefore, tasks that depend to a great extent on motor skill are not suitable to test acquisition in aged rats. Such tasks as used by Goodrick (1972) - maze learning without time limit - are much better suited but are extremely time consuming. Since Dye (1969) has shown that aged animals are more susceptible to interference than young ones, one has to aim at minimal interference levels

in the task the aged animals have to perform, a requirement that is fulfilled to a large degree by the drinktest used. The simplicity of the drinktest, based on conditioned suppression of drinking, makes it unlikely that the acquisition deficits found could be attributed to decreased motor skill or to a higher susceptibility to interference of the aged animals. Other factors which could contribute to the age-related differences in performance might be changes in activity levels and/or differences in sensitivity to footshock with age. Aged animals, in general, show reduced motor activity (Goodrick, 1966; Hofecker et al., 1978; Verzàr, 1965) and since the findings of Wolthuis et al. (1976) could not definitely answer the question whether decreased activity with age contributed to the observed acquisition deficits, the activity levels of rats of different ages were measured again, but this time in an open field situation. From the results of these measurements - described in chapter 2 - it can be concluded that acquisition of the different age groups and their activity levels are not related. In the drinktest used in the present experiments the training of the animals was based on punishment by electric footshocks. A decreased sensitivity to footshock would lead to a reduction in punishment level resulting in decreased motivation for avoidance. It is not very likely, however, that a difference in footshock sensitivity can be held responsible for the acquisition decrements observed, because similar decrements are found when no footshock is used (Wolthuis et al., 1976) and because the performance during the first week was comparable for all age groups and differences began to appear no sooner than several days after day 4, when maximum footshock levels had been reached. Furthermore, when the shock level was increased on day 14, this had no noticeable effect on performance. Because in the literature the results of the measurements of footshock sensitivity in rats of different ages were not uniform (Gordon et al., 1978; Parê, 1969) footshock sensitivity in rats of different ages was measured by determining the threshold current for a "flinch" reaction (Gispen et al., 1970). As described in chapter 2 no age-related differences in footshock sensitivity were found in the rats used; the observed acquisition deficits of old rats, therefore, cannot be explained by a decreased footshock sensitivity.

The acquisition deficits might be the consequence of gross over-

all metabolic changes in the brain during ageing. Therefore, three biochemical parameters have been measured in the brains of rats of different ages, i.e. glutamate metabolism, accumulation of lipofuscin and choline-acetyltransferase (CAT) activity. An attempt was made to gain an impression of the metabolic state of the brain, for which glutamate metabolism was taken as an index. Findings in the literature suggested age-related changes in the glutamate metabolism of the brain (Davies and Himwich, 1975; Fonda et al., 1973; Kanungo and Kaur, 1969; Kirzinger and Fonda, 1978). The results of the present study, however, do not confirm these suggestions. The turnover study of glutamate (described in chapter 4) show that age-related overall gross changes in glutamate metabolism do not occur, albeit that the turnover of glutamate in glutamine is somewhat delayed in the small compartment. The deficits in acquisition of old rats, therefore, cannot be simply explained by gross changes in cerebral metabolism, with glutamate turnover as an index.

Although no gross age-related metabolic changes were found in glutamate metabolism, the possibility remains that neuronal function in aged rats is impaired by the intracellular accumulation of the age-pigment lipofuscin. Lipofuscin is generally considered as metabolic "garbage". The results of the experiments described in chapter 5 do not point to a relationship between the lipofuscin accumulation and the deficits in acquisition found in aged rats. In the first place the age-related acquisition deficits were not accompanied by a concomitant lipofuscin increase in the brain. In the second place meclofenoxate caused a significant decrease in the amount of lipofuscin, which had no significant effect on acquisition of the old animals. Surprisingly, the present results do not show an age-related increase in biochemically determined lipofuscin in the CNS. This is in contrast with the results of almost all other authors, who find with microscopical methods that lipofuscin does increase with age. The present results suggest that this discrepancy might be due to a difference in the level of dispersion of the lipofuscin between young and old rats. It seems that in young rats the lipofuscin is present in a highly dispersed form which makes it very difficult to quantify by fluorescent microscopy. Theoretically it is also possible that with the method of biochemical analysis used in the present study lipofuscin precursors are measured together with "real" lipofuscin.

In hippocampi of healthy old human subjects a decreased activity was found of choline-acetyltransferase (CAT), an enzyme essential for acetylcholine synthesis (Perry et al., 1977[a]). In addition, such a decrease in CAT activity is most pronounced in humans suffering from Alzheimers' disease (senile dementia) (Davies, 1978; Davies and Maloney, 1976; Reisine et al., 1978) which is characterized by early intellectual deterioration. It was thought possible that a similar decrease in CAT activity might exist in the old rats in the present experiments. The results of measurements of hippocampal CAT activity, quite unexpectedly, showed that this activity did not decrease with age (chapter 7). In contrast the lowest activity was found in the youngest animals. An explanation for these findings cannot be given at present.

Taken together, these findings do not furnish a biochemical correlate for the acquisition deficits found in the old rats. In fact, the three parameters measured indicate that brain metabolism in these healthy old animals is not much different from that in the other age groups. Since the results suggest that metabolism and in particular protein metabolism was normal and since protein metabolism plays an essential role in the consolidation process it seems unlikely that disturbances in the consolidation mechanisms could account for the acquisition deficits found in the aged rats.

The lack of biochemical correlates, however, does not exclude that the acquisition deficits are caused by age-related defects in the first or registration phase of the learning process. During the registration phase the electrical activity in the neurons allegedly plays a dominant role whereas metabolic processes seem to be less important. An approach to investigate the registration phase is the study of electrophysiologically detectable responses of retina and visual cortex to visual stimulation. The present experiments showed no significant age-related changes in the shapes, amplitudes and latencies of the electroretinograms and visual evoked responses in the cortex. Therefore, it is unlikely that the registration of visual information is seriously hampered in old rats and that the acquisition deficits found can be explained by deficits in visual registration.

To gain insight in the short-term memory function of young and old rats, two age groups were subjected to alternation training and

testing in a T-maze with a chocolate reward. The results of this experiment (chapter 3) make it unlikely that the short-term memory of the rats was affected by age. With a passive avoidance learning task it was tried to test whether retention or retrieval of learned information exhibited age-related changes. Since the different levels of motor activity of the rats of different ages confound the results of retention testing, it appeared impossible to measure retention in relation to age in this way. The validity of results with passive avoidance tasks in the literature (McNamara et al., 1977; Ordy et al., 1978) are therefore difficult to interprete. A good retention test in which the results are not affected by the activity level of the animal has not yet been found.

The fact that all data acquired do not point at a single factor or a group of factors which can be held resonsible for the acquisition deficits found in the aged rats, is perhaps not surprising. The combination of considerable changes in behaviour with relatively minor biological changes in the brain becomes more acceptable when it is realized that the young and old rats do not only differ in age, but also differ vastly in behavioural experience. Moreover, biologically speaking, a selected population of the fittest survivors was tested with most of their adaptive and compensatory mechanisms still intact.

It is not often realised that the cost of research with senescent rats such as described in this study is rather high. The price of one female WAG/Rij rat of say 30 months is about Dfl. 700.--. As a consequence, in the literature far reaching conclusions are sometimes drawn on the basis of very few animals. Furthermore, the health status of the senescent rats is almost never mentioned. Personal communications suggest that in several instances experimental results have been obtained with a small number of animals of questionable health and the validity of the data, therefore, is doubtful. In contrast, the health status of the animals used in the present experiments is known in detail (Burek, 1979) and was carefully and repeatedly checked. Moreover, the group sizes almost always were 6 or more and almost each experiment was repeated. Therefore, it seems reasonable to assume that the present experiments provided results which are really representative and offer a true reflection of CNS function in the healthy senescent rat. On the whole, research in which it is

attempted to correlate altered behaviour with biological changes is still in an exploratory stage. The present work should be regarded as such a study.

Regardless of the nature of the behaviourally detectable deficits it is possible to investigate whether these deficits can be influenced in a positive way by pharmacological means. Piracetam (Nootropil®), etiracetam, Hydergine® and desglycinamide-arginine-vasopressin (DG-AVP, Org 5667) were tested. These drugs, except etiracetam, are already employed in geriatric practice. Of etiracetam nothing has been reported yet; of the other drugs results reported in the literature suggest that they cause an improvement in many types of performances of patients with memory deficits. Therefore, it was studied if they might have an effect on acquisition of old rats in the drinktest. None of these drugs were found to have a significant effect on drinktest learning of aged rats.

The following possibilities for the discrepancies observed between rat and man must be considered: the effects of these geriatric drugs in man are grossly overestimated, or, the observed acquisition deficit in old rats is not comparable with the memory deficits seen in aged man. However, with regard to the last mentioned possibility, recent studies in rats (reviewed in NAS, 1981) have indicated that in the hippocampus of aged rats morphological alterations are observed which are similar to those seen in the hippocampus of aged man. Therefore, even in the light of the outcome of the experiments described in this study, it seems worthwhile to explore further whether the ageing rat might serve as a model for studying age-related behavioural changes. As has been stated before, the studies described in this thesis have been conducted in healthy, aged rats. The age of these animals was around the observed 50% survival. It can be envisaged that further studies should employ even older animals. Also, it should be realised that, contrary to the situation in man, there are no atherosclerotic changes of vessels observed in even very old rats. In man it is believed that hypoxia is often the cause of diminished brain function in the aged (Schulze, 1980).

In conclusion, it can be said that further studies are necessary to explore the feasibility of using the ageing rat as a model for memory deficits in man. Both from this study and from the literature (NAS, 1981) it appears that ageing rat offers certain aspects which allow the deve-

lopment of models for studying aspects of brain ageing in man. Further studies are necessary to prove or disprove this.

## SUMMARY

The objective of this study was to gain more insight into the nature of the decline in acquisition of old female WAG/Rij rats as found by Wolthuis et al. (1976). From their results and from an experiment with rats of various ages (12, 18, 24 and 30 months) described in the present study, it appeared that the deficits in acquisition develop gradually with increasing age. Between pigmented female BN/BiRij rats of 3 and 30 months old - which clearly acquired the task much more slowly when compared with the WAG/Rij rats - the same kind of acquisition deficits were found. This finding demonstrates that this phenomenon is not limited to the WAG/Rij strain. To examine whether age-related differences could also be demonstrated in more basic, adaptive functions of the central nervous system (CNS), the adaptation of drinking activity in reaction to reversed light-dark cycles was chosen as a paradigm. No appreciable differences in adaptation speed of diurnal drinking rhythms with age appeared after light-dark reversal and after return to normal lighting conditions.

The acquisition deficit could not be attributed to differences in spontaneous motor and "open-field" behaviour between the different age groups. It was also not very likely that the observed acquisition deficits could be explained by differences in footshock sensitivity, since no significant age-related differences in footshock sensitivity were found.

It was attempted to obtain an answer to the question whether the decrease in acquisition might be the consequence of gross, overall metabolic changes in the brain during ageing. Therefore, three biochemical parameters have been measured in the brains of rats of different ages, i.e. glutamate metabolism, accumulation of lipofuscin and choline-acetyltransferase (CAT) activity. An attempt was made to gain an impression of the metabolic state of the brain, for which glutamate metabolism was taken as an index. The turnover study of glutamate and some additional findings show that age-related overall gross changes in glutamate metabolism do not occur, albeit that the turnover of glutamate in glutamine is somewhat delayed in the small compartment. The deficit in acquisition of old rats, therefore, cannot be simply explained by gross changes in cerebral metabolism, with glutamate turnover as an index.

Although no gross age-related metabolic changes were found in

glutamate metabolism, the possibility remains that neuronal function in aged rats is impaired by the intracellular accumulation of the age pigment lipofuscin. Lipofuscin is generally considered as metabolic "garbage". The results of the experiments do not point to a relationship between the lipofuscin accumulation and the deficit in acquisition found in aged rats. In the first place the age-related acquisition deficits were not accompanied by a concomitant lipofuscin increase in the brain. In the second place meclofenoxate caused a significant decrease in the amount of lipofuscin, which had no significant effect on acquisition of the old animals. Surprisingly, the present results did not show an age-related increase in biochemically determined lipofuscin in the CNS. This is in contrast with the results of almost all other authors, who found with microscopical methods that lipofuscin does increase with age. The present results suggest that this discrepancy might be due to a difference in the level of dispersion of the lipofuscin between young and old rats. It seems that in young rats lipofuscin is present in a highly dispersed form, which makes it very difficult to quantify by fluorescent microscopy. Theoretically it is also possible that with the method of biochemical analysis used in the present study lipofuscin precursors are measured together with "real" lipofuscin.

In hippocampi of healthy old human subjects a decreased activity was found of CAT, an enzyme essential for acetylcholine synthesis; such a decrease is most pronounced in humans suffering from Alzheimer's disease (senile dementia) which is characterized by early intellectual deterioration. It was thought possible that a similar decrease in CAT activity might exist in the old rats used in the present experiments. The results of measurements of hippocampal CAT activity, quite unexpectedly, showed that this activity does not decrease with age. In contrast, the lowest activity was found in the youngest animals. An explanation for these findings cannot be given at present.

Taken together, these findings do not furnish a biochemical correlate for the acquisition deficits found in the old rats. In fact, the three parameters measured indicate that brain metabolism in these healthy old animals is not much different from that in the other age groups. Since the results suggest that metabolism and in particular protein metabolism was normal and since protein metabolism plays an essential role in

the consolidation process it seems unlikely that disturbances in the consolidation mehanisms could account for the acquisition deficit found in the aged rats.

The lack of biochemical correlates, however, does not exclude that the acquisition deficit is caused by age-related defects in the first or registration phase of the learning process. During the registration phase the electrical activity in the neurons allegedly plays a dominant role whereas metabolic processes seem to be less important. An approach to investigate the registration phase is the study of electrophysiologically detectable responses of retina and visual cortex to visual stimulation. The present experiments showed no significant age-related changes in the shapes, amplitudes and latencies of the electroretinograms and visual evoked responses in the cortex. Therefore, it is unlikely that the registration of visual information is seriously hampered in old rats and that the acquisition deficit found can be explained by deficits in visual registration. The significant decrease of lipofuscin after treatment with meclofenoxate also has no effect on these parameters.

To gain insight in the short-term memory function of young and old rats, two age groups were subjected to alternation training and testing in a T-maze with a chocolate reward. The results of this experiment made it unlikely that the short-term memory of the rats was affected by age. With a passive avoidance learning task it was attempted to test whether retention or retrieval of learned information exhibited age-related changes. Since the different levels of motor activity of the rats of different ages confound the results of retention testing, it appeared impossible to measure retention in relation to age in this way.

Regardless of the nature of the behaviourally detectable deficit it was possible to investigate whether this deficit can be influenced in a positive way by pharmacological means. Piracetam (Nootropil®), etiracetam, Hydergine® and desglycinamide-arginine-vasopressin (DG-AVP, Org 5667) were tested. None of these drugs were found to have a significant effect on drinktest learning of aged rats.

## SAMENVATTING

Het doel van dit onderzoek was meer inzicht te krijgen in de leerdefecten die gevonden zijn bij oude vrouwelijke WAG/Rij ratten (Wolthuis e.a., 1976). Uit de resultaten van Wolthuis e.a. (1976) en van een experiment met ratten van 12, 18, 24 en 30 maanden oud bleek dat de leerstoornissen geleidelijk optreden na de leeftijd van 12 maanden. Met gepigmenteerde vrouwelijke ratten van de BN/BiRij stam van 3 en 30 maanden oud - die overigens langzamer leerden - werden soortgelijke verschillen gevonden, hetgeen erop wijst dat dit fenomeen niet specifiek is voor de WAG/Rij stam. De vraag rees of ook leeftijdsafhankelijke verschillen optreden in een meer basale vorm van adaptief gedrag zoals de aanpassing van de drinkactiviteit aan omkering van de licht-donker periodiciteit. De resultaten toonden aan dat er geen significante leeftijdsverschillen optreden in de snelheid waarmee de dieren zich aanpassen aan gewijzigde licht-donker omstandigheden.

De gevonden leerstoornissen bleken niet samen te hangen met verschillen in de spontane motorische en "open-field" aktiviteit tussen de verschillende leeftijdsgroepen. Bovendien leek het erg onwaarschijnlijk dat de gevonden leerdefecten te wijten zijn aan verschillen in pijngevoeligheid, aangezien de stroomdrempelgevoeligheid tussen de leeftijdsgroepen niet significant verschilde.

Getracht werd een antwoord te krijgen op de vraag of de achteruitgang van het vermogen tot aanleren bij oude ratten het gevolg zou kunnen zijn van afwijkingen in het metabolisme van de hersenen bij het ouder worden. Hiervoor werden drie biochemische parameters gemeten in de hersenen van ratten van verschillende leeftijd, n.l. het glutaminezuur metabolisme, de accumulatie van lipofuscine en de choline-acetyltransferase (CAT) activiteit. Als een index voor het hersen metabolisme werd het metabolisme van glutaminezuur bestudeerd in hersenen van ratten van verschillende leeftijden. De turn-over studie van glutaminezuur toonde geen grove overall veranderingen aan in het glutaminezuur metabolisme; slechts de omzetting van glutaminezuur in glutamine bleek enigszins vertraagd te zijn in het kleine glutaminezuur compartiment in de hersenen van de 30 maanden oude ratten. De resultaten van deze experimenten duiden er op dat de gevonden leerstoornissen niet op simpele wijze verklaard kunnen worden

door grove veranderingen in het hersenmetabolisme.

Hoewel grove leeftijdsafhankelijke veranderingen in het hersenmetabolisme niet werden gevonden bleef de mogelijkheid bestaan dat de functie van de zenuwcellen in oude ratten verslechterd is door opstapeling van het ouderdomspigment lipofuscine, dat in het algemeen als een metabool afvalprodukt wordt beschouwd. De resultaten van de experimenten wijzen niet op een relatie tussen de hoeveelheid lipofuscine en de gevonden leerstoornissen bij oude ratten. In de eerste plaats gingen de leeftijdsafhankelijke leerstoornissen niet gepaard met een toename van lipofuscine in de hersenen. Op de tweede plaats veroorzaakte behandeling met meclofenoxaat een significante daling van het lipofuscine in de hersenen, evenwel zonder dat dit gevolgen had voor het leergedrag van de dieren. De bevinding dat er geen leeftijdsafhankelijke veranderingen in het lipofuscine gehalte van de hersenen aan te tonen was met een kwantitatieve chemische methode, is geheel in strijd met die van vele andere onderzoekers. Deze discrepantie zou verklaard kunnen worden door aan te nemen dat het lipofuscine bij jongere dieren meer dispers in de hersencellen aanwezig is, waardoor het met de tot nu toe meestal gebruikte en op z'n best semikwantitatieve microscopische technieken niet valt aan te tonen. Theoretisch zou het ook mogelijk zijn dat met de chemische bepaling precursors van lipofuscine met dezelfde chemische eigenschappen worden meebepaald.

In de hippocampi van gezonde oude mensen is een verminderde activiteit aangetoond van CAT, een enzym dat betrokken is bij de acetylcholine synthese; deze verminderde activiteit is het meest uitgesproken bij mensen die lijden aan seniele dementie (type Alzheimer), een ziekte die o. a. gekarakteriseerd is door verminderde intellectuele functies. Het werd mogelijk geacht dat zo'n verminderde CAT activiteit ook in de gebruikte ratten aan te tonen zou zijn. De resultaten van de metingen in de hippocampi van jonge en oude ratten tonen, geheel onverwachts, aan dat de CAT activiteit niet daalde met de leeftijd; de laagste activiteit werd zelfs gevonden in de jonge ratten. Een verklaring voor deze bevindingen kan echter nog niet gegeven worden.

Samenvattend leken er in deze fase van het onderzoek dus geen duidelijke biochemische aanknopingspunten te bestaan die een mogelijke verklaring zouden kunnen opleveren voor de stoornissen in het leergedrag van de ouder wordende rat. Aangezien biochemische processen een heel be-

langrijke rol spelen in de consolidatie fase van het leerproces, leek het onwaarschijnlijk dat stoornissen in het consolidatie mechanisme het slechter leren van oude ratten zou veroorzaken. Zijn er wel deficienties in het leergedrag en geen aanknopingspunten voor biochemische stoornissen, zo was de gedachte, dan heeft het zin om het onderzoek te richten op de eerste fase van het leergedrag, waarin de electrische activiteit van de neuronen, naar men algemeen aanneemt een belanrijke rol speelt. Voor het onderzoek van de eerste fase - de zogenaamde registratie fase - van het leerproces werd het visuele systeem gekozen. Electrophysiologische responses werden bestudeerd van de retina en de visuele cortex na visuele stimulatie. Geen significante leeftijdsafhankelijke verschillen waren aantoonbaar in de gemeten parameters van de electroretinogrammen en de visuele "evoked responses". Ook in deze proeven had een door meclofenoxaat veroorzaakte daling van lipofuscine geen effect. Het leek dus onwaarschijnlijk dat de gevonden leerstoornissen veroorzaakt worden door ernstige stoornissen in de registratie van visuele informatie.

Een mogelijk defect in de "short-term memory" bij oude dieren als verklaring voor de leerdefecten lijkt eveneens onwaarschijnlijk. Jonge en oude ratten werden getraind op alterneer gedrag in een T-vormig doolhof; leeftijdsafhankelijke verschillen in dit experiment werden niet gevonden. Met een "passive-avoidance" procedure werd voorts getracht een indruk te krijgen over de retentie of "retrieval" van de aangeleerde informatie. Aangezien leeftijdsafhankelijke verschillen in motorische activiteit tussen de leeftijdsgroepen een interpretatie van de gevonden verschillen onmogelijk maakte, kon met deze gebruikelijke technieken het retentie proces in relatie met het ouder worden niet onderzocht worden in de rat.

Tot slot werd nagegaan of een aantal (potentiële) geriatrische therapeutica een invloed zou hebben op de gevonden leerstoornissen in oude ratten. Getest werden de effecten van piracetam (Nootropil®), etiracetam, Hydergine® en desglycinamide-arginine-vasopressine (DG-AVP, Org 5667) op het leergedrag van oude ratten in de drinktst. Geen van deze stoffen bleek een significant effect te hebben.

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## CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren te Breda op 24 januari 1950. Na het behalen van het eindexamen HBS-b aan de Newman-HBS te Breda in 1969 begon zij aan haar studie farmacie aan de Rijksuniversiteit te Leiden. Het kandidaatsexamen werd afgelegd in mei 1972. In april 1975 werd het doctoraalexamen behaald met bijvak farmacologie, waarvoor 8 maanden onderzoek werd verricht in het Medisch Biologisch Laboratorium TNO onder leiding van Dr. O.L. Wolthuis. In juli 1976 werd het apothekersexamen afgelegd. Van augustus 1976 tot mei 1980 is zij met steun van de stichting FUNGO werkzaam geweest in het Medisch Biologisch Laboratorium TNO te Rijswijk waar het in dit proefschrift beschreven onderzoek uitgevoerd werd in samenwerkingsverband met het Instituut voor Experimentele Gerontologie TNO te Rijswijk. Sinds december 1980 is zij werkzaam als tweede apotheker in een officine apotheek in Tilburg.