From the Department of Neuroscience
Karolinska Institutet, Stockholm, Sweden

ON AGE RELATED CHANGES IN AXONS AND GLIA OF THE CENTRAL NERVOUS SYSTEM

Susanna Kullberg

Stockholm 2002

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7349-271-X

ABSTRACT

A growing body of evidence shows that phenotypic changes including axon aberrations, rather than loss of neurons, account for behavioral impairments during aging. The present thesis was undertaken to investigate the occurrence of axon aberrations in relation to transmitter identity, glial reaction and sensorimotor disturbances. To shed light on possible underlying mechanisms, signs of oxidative stress and inflammation were also examined. The studies were performed on behaviorally defined aged (30 months old) and young adult (2-3 months old) Sprague Dawley rats, by using electron microscopy, immunohistochemistry, *in situ* hybridizsation and reverse transcriptase-polymerase chain reaction.

The results show that many aged motoneurons lose a significant portion of their bouton covering, due to a decreased number of apposing boutons. Consistent with the more pronounced sensorimotor disturbances observed in the hind- in comparision with the forelimbs, lumbar motoneurons appeared more severely affected than cervical motoneurons. In the neuropil of the motor nucleus, aberrant axons were encountered. Ultrastructutal analysis of aberrant axons in relation to content of amino acid neurotransmitters and the free radical scavenger glutathione (GSH) revealed that many of the aberrant axons contained high levels of glutamate-immunoreactivity (–IR) and were often enriched with GSH-IR. Increased levels of GSH-IR were also encountered in glutamate-IR terminals with a preserved ultrastructure, suggesting that a changed redox status may be mechanistic in the development of axon aberrations. GABA- and glycine-IR terminals were more rarely affected, suggesting that excitatory and inhibitory pathways are differentially affected.

In the aged rats, immunohistochemistry showed a reduced fiber density and axon aberrations of cholinergic and monoaminergic axons in both the spinal cord and the hippocampus. In contrast, the innervation of α-motoneurons by C boutons was preserved in senescence. However, the C boutons showed a decreased labeling for cholinergic markers. Regions disclosing axon terminal loss and aberrations showed increased expression of glial fibrillary acidic protein (GFAP, the main intermediate filament of astrocytes).

Using Marchi staining on spinal cord sections, the outer parts of the white matter showed signs of a changed myelin metabolism and/or dysmyelination in aged rats. In the same regions, astro- and microglial cells showed conspicuous signs of activation, most pronounced in rats disclosing the most severe sensorimotor disturbances. The glial reaction appeared less pronounced in brain white matter compared to the spinal cord white matter.

The spinal cord white matter of aged rats also disclosed a changed expression of several cytokines, while the majority of investigated cytokines were unaltered in the hippocampus. One of the most prominent changes was an upregulation of the proinflammatory cytokine IFN- γ, encountered in both the hippocampus and the spinal cord. There was a robust upregulation of TGFβ-1 and IL1-β in astroglia of spinal cord white matter, while no change was evident in the hippocampus. CNTF levels were unaltered in aged rats, however, IR appeared reduced in oligodendroglia-like cells, while it seemed increased in astroglia of the spinal cord white matter. IGF-1, a molecule with similar effects as CNTF, was upregulated in hippocampus but not in the spinal cord.

Keywords: aging, synaptic input, spinal cord, hippocampus, amino acid neurotransmitters, astrocyte, microglia, oligodendroglia, C bouton, cholinergic, monoaminergic, cytokine

PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Kullberg S., Ramírez-León V., Johnson H., Ulfhake B. Decreased axosomatic input to motoneurons and astrogliosis in the spinal cord of aged rats. Journal of Gerontology. 1998; 53A(5):B369-379.

II. Kullberg S., Edström E., Hökfelt T., Villnow E., Ulfhake B. (2002) Do C boutons switch transmitter phenotype in senescence? Manuscript. 2002

III. Ramírez-León V., **Kullberg S.**, Hjelle O-P., Ottersen O-P., Ulfhake B. Increased glutathione levels in neurochemically identified fibre systems in the aged rat lumbar motor nucleus. European Journal of Neuroscience. 1999; 11:1-14.

IV. Kullberg S., Aldskogius H., Ulfhake B. Microglial activation, emergence of ED1-expressing cells and clusterin upregulation in the aging rat CNS, with special reference to the spinal cord. Brain Research. 2001; 899:169-186.

V. Kullberg S., Ming Y., Edström E., Ulfhake B. Changes in expression of cytokines in the spinal cord and hippocampus of aged rats. Manuscript. 2002

CONTENTS

INTRODUCTION

Aging

Aging can be defined as a process of gradual decrease in functional ability and is associated with growing old. During aging there is an accumulation of senescent cells and in most organisms, cell (tissue) functions are impaired.

With advancing age, the risk of acquiring diseases increases. However, aging is not a disease itself and needs to be delineated from effects of diseases. Since this is not easily managed in long-lived species like humans, animal models with a shorter life span may serve better for capturing mechanisms of aging. Distinct from many neurodegenerative diseases (which often have a late onset and are therefore confused with aging), deficits in nervous functions during normal aging do not correlate with loss of neurons. For decades, neuron loss was considered a hallmark of the aging mammalian nervous system, however, recent quantitative studies suggest that neuron loss is limited and cannot explain the functional impairments (Bergman and Ulfhake, 1998; Johnson et al., 1995; Morrison and Hof, 1997; Rapp and Gallagher, 1996; Šimić et al., 1997; Wickelgren, 1996). Instead, an increasing body of evidence implies that phenotypic changes including axon aberrations are mechanistic in the emergence of disabilities in senescence (see below). Aging affects different organisms as well as tissues within an organism differently, depending on whether they are postmitotic/have a retarded renewal pace (like the nervous system and skeletal muscles) or are rapidly replaced (e.g. damaged epithelial cells in the gut of mammals). Certain neurological symptoms and signs, such as weakness, alterations in gait cycle, unsteadiness and increased proprioceptive thresholds, are so common in a healthy elderly human population that they are often regarded as “normal”. A similar range of behavioral deficits is encountered in aged rodents (see Appendix), suggesting that rodents may serve as a useful model to dissect the underlying mechanisms. Both humans and rodents exhibit a considerable variability in the effects of aging on behavioral and cellular parameters, such that some individuals exhibit extensive impairments with age, whereas others show little or no symptoms (Appendix, Burek and Hollander, 1980; Koller at al., 1985; Lamberts et al., 1997). The fact that in several species it seems possible to distinguish “unsuccessful” and “successful” patterns of aging, may facilitate the identification of factors instigating disabilities during aging.

Over the years several theories on the mechanisms of aging have been put forward, some emphasizing intrinsic biological clocks or ”programs”, whereas others suggest environmental factors that damage cells and organs as mechanistic (Martin and Baker, 1993; Warner et al., 1987). The latter include cell impairment caused by reactive oxygen species (ROS) generated in metabolism (Finkel and Holbrook, 2000; Harman, 1981; Kirkwood and Austad 2000; Martin and Oshima, 2000; Sohal and Weindruch, 1996). Program theories hold that a biological timetable will regulate gene expressions as well as the function of e.g. endocrine systems and the immune system and in this way control the aging process (Kuro-o et al., 1997; Lamberts et al., 1997; Pennisi, 1998). These hypotheses are not mutually exclusive, and it seems likely to assume that a combination of genetic and epigenetic factors will affect the pace of aging (Hayflick, 2000). Given that life span has a continuous distribution in populations, aging is probably not governed by a single factor. It is more likely attributable to genetic variation at multiple quantitative trait loci (QTL) and sensitivity of QTL alleles to environmental factors (McClearn et al., 1997; Tower, 1996; see also Klebanov and Harrison, 2002). In *C. Elegance* and *D. melanogaster* several genes affecting aging have been identified

(e.g. Ewbank et al., 1997; Kimura et al., 1997; Lin et al., 1998). Many of these encode proteins regulating growth and energy metabolism (Guarente and Kenyon, 2000; Wolkow et al., 2000). In mammals, Pelicci and coworkers (Migliaccio et al., 1999) provided evidence indicating that the p66 isoform of the ShcA adaptor protein can regulate life span by its sensitivity to oxidative stress. The *Shc* gene family encodes adaptor proteins associated with signal transduction pathways found to regulate life span and phenotype in *C. Elegance* (for references see above). In common for all species examined so far is that caloric restriction will retard aging, implying that metabolic processes and generation of ROS may play a key role (Masoro, 2000; Roth et al., 1999, and references above).

"The free radical theory of aging"

According to the "free radical theory of aging" (Harman, 1981), cellular damage caused by reactive oxygen species (ROS) will accumulate during life time. Several cellular processes, such as mitochondrial respiration, generate ROS. Through reactions with DNA, RNA, lipids and proteins they can damage vital cell functions. The high oxygen consumption of the brain in relation to its weight indicates the potential generation of a high quantity of ROS during brain metabolism (Dringen, 2000 and references therein). If formation of ROS exceeds the scavenging capacity, a cell/tissue is under oxidative stress (Dringen, 2000; Sastre et al., 2000). Glutathione (GSH) is regarded as a major protectant against ROS in the brain (Cooper and Kristal, 1997). GSH is directly involved in redox cycling reactions, in which the reduced form is converted to its oxidized counterpart, glutathione disulfide (GSSG). Many studies have found an age related shift towards the oxidized form (GSSG) and an accumulation of oxidative damage to DNA in the aging brain (Barja and Herrero, 2000; de la Asuncion et al., 1996; Sohal et al., 1994; Soong et al., 1992 and references above). The level of oxidative damage seems to correlate with life span as well as age related impairments such as decline in cognitive function and motor skills (Forster et al., 1996; and references above). Thus, a great deal of experimental evidence supports the "free radical theory of aging". The mitochondria appear to be particularly vulnerable to the harmful effects of ROS. They disclose considerably more DNA damage than nuclear DNA in senescence (de la Asuncion et al., 1996 and references above). GSH plays a key role in protecting against oxidative damage in the mitochondria, and depletion of GSH leads to mitochondrial damage (Jain et al., 1991 and references above). Whether aging is associated with changes in the capacity of ROS scavenging has not been resolved (Ashok and Ali, 1999; Benzi and Moretti, 1995). Available data indicate that the CNS cells differ in their capacity to withstand attack of ROS. Presumably due to a high content of lipids and low concentrations of GSH, oligodendroglia appear to be especially vulnerable to ROS. Moreover, oligodendroglia contain high levels of iron, which catalyses generation of hydroxyl radicals ($OH^{\bullet}$), which are the most reactive species within the ROS family. Astroglia contain high levels of GSH compared to other cell populations of the central nervous system (CNS), and they seem to be able to protect both neurons and oligodendroglia from the detrimental effects of oxidative stress (Dringen, 2000 and references therein).

Neuroaxonal dystrophy

As mentioned above, a common stigmata in the mammalian nervous system is axonal aberrations. Neuroaxonal dystrophy (NAD) is a process that, at least initially, is restricted to the terminal part of the axon. At the electron microscopic level it is characterized by swelling,

increased density of organels and neurofilaments, accumulation of normal and enlarged mitochondria, patches of electrondense material, multivesicular bodies and dysmyelination (Jellinger and Jirásek, 1971; Suzuki and Suu, 1978). NAD was first described by Seitelberger and is encountered in the aging CNS of a number of species, including humans and rodents (Fujisawa and Shiraki, 1980; Seitelberger, 1971 and references therein). NAD has also been referred to as axonal spheroids (Jellinger and Jirásek, 1971; Suzuki and Suu, 1978), aberrant axons and axon dystrophy/dystrophic axons (Fujisawa and Shiraki, 1980; Johnson et al., 1993; van Luijtelaar et al., 1988). In the following text these terms will be used interchangeably.

Although NAD is occurring with increasing frequency and severity as age advances (Brannon et al., 1967; Fujisawa and Shiraki, 1978), there are considerable differences among individuals regarding the extent of changes (Brannon et al., 1967). Moreover, it appears to be a selective process involving certain axons but not others. The dorsal column nuclei, especially the gracile nucleus, appear to be severely affected. But NAD is also encountered in e.g. other sensory relay nuclei, grey matter of the spinal cord, substantia nigra, basal ganglia, hippocampus and cortex (Fujisawa and Shiraki, 1978; Jellinger and Jirásek, 1971; Johnson et al., 1993; van Luijtelaar et al., 1988; 1991; 1992). Despite the apparent selectivity of NAD, reports on its relation to neurochemical phenotype of the affected axons are few. The serotoninergic (5-HT) system was the only identified transmitter system when this project was initiated. In the 5-HT system both individual as well as regional differences in the extent of NAD have been described, e.g. cortex is more affected than hippocampus and in the spinal cord the lumbar region contains more NAD than the cervical regions (for references, see above). Mechanisms suggested to induce the age related NAD include metabolic disturbances, reduced axonal flow, vitamin E deficiency and the deleterious effects of free radicals (for references, see above). More recently, changes in interactions between axon terminal and target, retrograde signaling and trophic mechanisms have been added to the list of mechanisms that may underpin NAD (reviewed by Cowen and Gavazzi, 1998; Ulfhake et al., 2000, 2002).

Axon loss, astrogliosis and dysmyelination

In association with NAD there is a loss of axon fibers and terminals and it is suggested that NAD represents a “dying-back” process (Fujisawa and Shiraki, 1978; Fujisawa, 1988; Kikuchi et al., 1990). An age related loss of axon terminals has been established in several studies of hippocampus and various cortical regions (Glick and Bondareff, 1979; McWilliams and Lynch, 1984; Saito et al., 1994; Uemura, 1980; van Luijtelaar et al., 1988). The functional significance of the axon terminal loss is underscored by work correlating behavioral deficits to loss of axon terminals (Chen et al., 1995). Some regions affected by NAD and/or axon loss have been reported to disclose an increase in astroglia processes and elevated levels of glial fibrillary acidic protein (GFAP, the main intermediate filament of astrocytes), i.e. astrogliosis (Fujisawa and Shiraki, 1978; Goss et al., 1991; Lindsey et al., 1979; Nichols et al., 1993; Nichols, 1999). Dysmyelination was originally described in relation to NAD (for references see above), however more thorough studies on dysmyelination in the central nervous system (CNS) are scarce. There are reports on distended myelin sheaths in spinal cord white matter and some brain regions of aged rats and monkeys (Burek et al., 1976; Feldman and Peters, 1998; Peters et al., 1991, 1994; van Steenis and Kroes, 1971). Most studies on age related dysmyelination have been done on peripheral nerves and spinal cord roots. From these studies

it is clear that dysmyelination mainly affects large myelinated fibers and is associated with axon atrophy, dystrophy and loss of axons (Bergman and Ulfhake, 2002; Burek et al., 1976; Gilmore, 1972; Karlsson and Hildebrand, 1996; Kazui and Fujisawa, 1988; Knox et al., 1989; Krinke, 1983).

AIMS

With support of the background given above, we decided to examine NAD in greater detail using a rodent model described in the Appendix. Most of the work in this thesis focused on the spinal cord in order to enable correlation to behavioral sensorimotor deficits in the aged animals. More specifically the aims were as follows:

1. To study changes in axon terminals contacting spinal motoneurons and to examine the occurrence of NAD in neurochemically identified axons (papers I, II , III, unpublished observations).

2. To study signs of changed redox status and possible relationship with NAD and neurochemical phenotype (paper III).

3. To study signs of increased breakdown/changed turnover of myelin (paper I).

4. To study micro- and astroglial reaction (papers I, IV).

5. To study cytokine expression in relation to glial cells (paper V).

MATERIALS AND METHODS

Experimental animals (papers I-V)

Young adult (2-3 months old) and aged (30 months old) Sprague-Dawley rats of both sexes were used, see Table 1. For details on cohort characteristics and housing conditions, see Appendix. Rats show a progressive deterioration of sensorimotor behaviour, with symptoms usually emerging at an age of 24-26 months and mainly being confined to the hindlimbs. The symptoms are described according to a previously described staging protocol (Johnson et al., 1995) and compromise moderate muscle atrophy and an adduction insufficiency in the least affected cases (stage I). More severely affected animals disclose symptoms ranging from a prominent muscle atrophy and an ataxic gate (stage II), to a more or less complete hindlimb paralysis with severe muscle wasting (stage III). Furthermore, the rats are subjected to a panorama of behavioral tests in order to more thoroughly determine the degree of age related impairments, see Appendix. All experimental procedures were performed under deep chloralhydrate (300-420mg/kg i.p.) or pentobarbital (40mg/kg) anaesthesia. The experiments were approved by the local ethical committee (Stockholms Norra Djurförsöksetiska nämnd; project numbers N59/91, N75/93, N263/95, N90/97, N54/00).

Immunohistochemistry (papers I, II, IV, V)

Deeply anaesthetized animals were perfused through the ascending aorta with Ca^{2+}-free Tyrode´s solution and then with a fixative containing 4% w/v paraformaldehyde and 0.2% picric acid in 0.1M phosphate-buffered saline (PBS; pH 7.4) (Zamboni and De Martino, 1967) for 6-8 minutes. In addition, six animals included in paper IV were perfused as described above but for 12 minutes and without the addition of picric acid to the fixative. No difference in tissue morphology or labeling pattern was observed between the two methods of fixation. Tissue samples were then dissected out, put in the same fixative for 90 minutes, and then stored in 10% sucrose in PBS at 4ºC overnight. Sections were cut at 14µm in cryostat and proceeded according to the indirect immunofluorescence technique (Coons, 1958), or with modifications for double-labeling (Staines et al., 1988). Briefly, the sections were rehydrated in PBS and incubated at 4ºC overnight (polyclonal antibodies) or for 72 hours (monoclonal antibodies) in a humid chamber with the primary antibodies. See Table 2 for specification of used antibodies. Following thorough rinsing in PBS, the sections were incubated with fluorescent conjugated secondary antibodies for 30 minutes at 37ºC. Thereafter, the sections were rinsed and mounted in glycerol/PBS (3:1) containing 0.1% *p*-phenylendiamine in order to retard fading (Johnson and Nogueira Araujo, 1981; Platt and Michael, 1983).

For 11 and 8 animals included in paper IV and V respectively, a modified procedure for fixation was used. Fresh, i.e. unfixed tissue was quickly dissected out and immediately frozen on dry-ice. Following sectioning, the tissue specimens were fixed by immersion in 4% paraformaldehyde and then treated as the tissue fixed by perfusion. Although this method has the disadvantage of tissue morphology being less well preserved, it was used for a few antibodies that yielded a more distinct labeling with this method.

Immunohistochemistry is a powerful method for determining localization of substances in a tissue. However, the validity of findings obtained is directly related to the degree of specificity that can be achieved. Specificity can be controlled by preabsorbing the antibody with the appropriate antigen, which should abolish staining. Western blot demonstrating a single band is also an indicator of specificity. The different antibodies used here have been

tested with regard to specificity by preabsorption and/or Western blot (see papers for references). The specificity of the GFAP antibody used in paper I was not discussed. However, the labeling pattern obtained with that antibody have been confirmed in paper IV with two other GFAP antibodies (see Table 2), for which specificity have been tested. Additional controls, i.e. omission of primary or secondary antibodies resulted in no labeling. Despite these controls, it must be acknowledged that an element of uncertainty still remains as to the specificity of the staining. Consequently, antibody labeling is referred to as like immunoreactivity (LI) or immunoreactivity/immunoreactive (IR).

Section analysis
Tissue sections were examined in a Nikon Microphot-FX epifluorescence microscope equipped with the proper filters for FITC- and LRSC fluorescence. Photomicrographs were captured either using an Ultrapix 1600 CCD camera with a 1536x1024 element matrix connected to the microscope or a black-and white Kodak Technical Pan camera. Quantitative analysis of IR (paper IV) was performed by using the Optimas software (Optimas Co., Bothell, WA, USA). A representative slide for the marker to be analysed was used to set exposure time. Images from all sections subjected to analysis were then captured without changing the settings. The IR was recorded in a square-shaped area with the filters for FITC fluorescence. After subtraction of background labeling, sampled in the vicinity, and emitted fluorescence by lipofuscin granulae a sum of all pixel values above background within the square-shaped area was recorded.
In paper I, sections were also examined in a confocal laser scanning microscope, Sarasto 1000.

Marchi staining (paper I)
Deeply anaesthetized animals were perfused through the descending aorta. Following a brief rinse with Tyrode´s solution, fixation was performed with a mixture of 4% glutaraldehyde and 0.5% paraformaldehyde in 0.1M PBS. Spinal cord segments were dissected out, placed in a fresh fixative, then stored in PBS overnight and thereafter sectioned at 50µm on a Vibratome. They were then incubated with Marchi solution for 18 hours, dehydrated in alcohol with a final step in aceton and finally embedded in Epon (Agar 100 resin, Agar Aids, Essex, UK).

Section analysis
Sections were examined under a light microscope using an eye-piece grid and a ×60/1.4 Nikon oil-immersion planapochromate objective. Marchi-positive bodies were counted through the depth of the section within a pre-determined area placed in selected regions of the spinal cord sections.

In situ hybridization (papers II, IV)
Following decapitation of deeply anaesthetized animals, the unfixed tissue was dissected out and immediately frozen on dry ice. Sections were cut at 14µm in a cryostat and thaw mounted onto Probe-on or aminoalkylsilane-coated slides. In situ hybridization was then performed according to previously published protocols (Dagerlind et al., 1992; Young III, 1990). Oligonucleotide probes (see papers for details) were labeled at the 3´-end with α-[^{35}S]dATP using terminal deoxynucleotidetransferase. Without any pre-treatment the sections were hybridized for 16-18 hours at 42ºC in a humid chamber. The hybridization solution contained

10^6 cpm of the radiolabeled probe per 100µl; 50% formamide; 4xSSC; 1x Denhardt´s solution; 1% Sarcosyl; 0.02M sodium phosphate; 10% dextran sulphate; 500µg/ml sheared and heat denatured salmon sperm DNA and 200mM dithiotreitol. Following hybridization, sections were washed 4x15 min in 1xSSC at 56ºC, brought to room temperature in the final rinse and dehydrated in ascending concentrations of ethanol. Thereafter NTB2 nuclear track emulsion was applied by dipping and after 2-4 weeks exposure, the sections were developed in Kodak D-19 developer. Propidium iodide was used for counterstaining and, finally, the sections were cover-slipped in glycerol.
The specificity of the oligonucleotide probes are of crucial importance for the validity of the results. To control specificity, all oligonucleotide probes were synthesized against sequences published in GenBank and were not found to have any significant sequence similarities to other deposited sequences. It should be kept in mind, though, that far from all sequences have been cloned. However, with the hybridization stringency conditions used, unspecific binding may only occur if the probes have >90% homology with a certain mRNA. Thus, the probability of unspecific binding to an unrelated mRNA species is extremely small. Moreover, specificity of the oligonucleotide probes used have also been controlled by addition of excess unlabeled probe to the hybridization solution and resulted in an abolished signal.

Section analysis
Sections were examined in a Nikon Microphot-FX microscope equipped with an Ultrapix 1600 CCD camera with a 1536x1024 element matrix. In the quantitative analysis (paper II), it was assumed that the number of silver grains overlying a cell profile was proportional to the number of mRNA copies contained within the sectioned cell. Dark field illumination was used to visualize the silver grains, while epifluorescence was used to visualize the propidium iodide counterstain. Thus, every examined field yielded two separate images. Image analysis was then performed using the Optimas software. Briefly, the image pair generated from each field was presented as an RGB image, with the silver grain image presented in the blue and the counterstained image in the red channel. Cell profiles subjected to analysis had their cross sectional area recorded and thereafter a binary threshold was set for the silver grain image. Data was then extracted from the traced cell profiles, providing information about their cross sectional area and the percentage of that area occupied by silver grains (labeling density). Furthermore, in each recorded field the background labeling density was sampled in a representative portion of the neuropil.

Reverse transcriptase-polymerase chain reaction (RT-PCR) (paper V)
Total RNA was isolated from unfixed tissue with an RNA extraction kit according to the manufacturer (TRIZOL®-protocol; GibcoBRL, Life Technologies, Täby, Sweden). Reverse transcription was then conducted in a reaction volume containing 10µl containing 25ng of total RNA, 25 units MuLV reverse transcriptase, 2.5µM Oligo $d(T)_{16}$, 10 units Rnase inhibitor, 1mM of each dNTP (dATP, dCTP, dGTP, dTTP), 5mM $MgCl_2$ and 1xPCR Buffer II. The RT-reaction mixture was incubated for 10 minutes at room temperature, brought to 42˚C in a Perkin Elmer GeneAmp PCR system 2400 for 15 minutes and finally terminated by 5 minutes incubation at 99˚C. Polymerase chain reaction was carried out by addition of a PCR master mix to the RT-reaction mixture, yielding a reaction volume of 50µl containing the following components: 0.2 µM of each oligonucleotide primer, 5 units AmpliTaq Gold DNA

polymerase, 2mM $MgCl_2$, 200µM of each dNTP and 1xPCR Buffer II. For each experimental sample, three PCR reaction mixtures were prepared and subjected to three different numbers of PCR cycles (e.g. 31, 33 and 35). The three cycle points were chosen according to preceding experiments, so that the PCR amplification was found to be within the range of experimental progression. Despite a high number of cycles some mRNAs were too rare to be detected. Accordingly, the total RNA was increased up to 100ng. Hot start PCR was performed, and included an initial template denaturation and DNA polymerase activation step at 95˚C for 12 minutes. Each following cycle consisted of denaturation at 95˚C (15 sec) and primer annealing/ extension at 60˚C (30 sec, with an automatic increment of 3 seconds per cycle). At the end of the last cycle, the reaction was kept at 72˚C for 7 minutes and then brought to 4˚C. In all experiments RT-PCR was performed simultaneously on young adult and aged rat samples. The oligonucleotide primer pairs were synthesized against sequences deposited in GenBank and they were checked to avoid homologies with other sequences. All experiments included negative controls, where template RNA or reverse transcriptase was omitted, resulting in no detectable PCR product. Furthermore, a positive control was run to analyze failures during the PCR process. Intron spanning primers, producing different size products from DNA and cDNA, respectively, were used to check for possible DNA contamination of the samples. Ten µl of the PCR product was electrophoretically run on a 1.5% agarose gel containing ethidium bromide. The gels were visualized in an u.v. transilluminator and images were captured using an 8-bit CCD camera. Subsequent analysis, using the Optimas software, included measurement of the mean grey levels of each band, correction for local background and normalization against GAPDH levels. In the evaluation of the results, an arbitrary limit was set such that a difference between young adult and aged rats of a least 100% was considered significant.

Electron microscopy (paper I- III)

Rats under deep anaesthesia, were perfused through the descending aorta. Following a brief rinse with Tyrode´s solution, the tissue was fixed by 4% glutaraldehyde and 0.5% paraformaldehyde in 0.1M phosphate-buffered saline (PBS). Tissue was dissected out and placed in fresh fixative, then stored overnight in PBS. Sections were cut at 50µm on a Vibratome, treated with 1% OsO_4 in PBS for 1 h, dehydrated in a graded series of alcohol with a final step in aceton, then embedded in Durcupan ACM (Fluka, Buchs, Switzerland). Under inspection in a light microscope, appropriate areas were trimmed out and subjected to ultrathin sectioning on a LKB Ultratome. They were then mounted on formvar-coated nickel slot grids and counterstained with uranyl acetate and lead citrate.

Freeze-substitution and postembedding immunocytochemistry

Freeze-substitution is a method that in addition to providing good ultrastructure also allows good antigen preservation and is therefore suitable for immunocytochemistry (van Lookeren Campagne et al., 1991). Rats were perfused as described above with a fixative containing either 4% glutaraldehyde and 0.5% paraformadehyde (paper III) or 4% paraformaldehyde and 0.25% glutaraldehyde (paper II) in PBS. Since the antibodies used (glutamate, GABA, glycine, GSH) recognize the glutaraldehyde conjugated epitope, rats perfused with the fixative containing higher glutaraldehyde concentration was used for postembedding immunocytochemisry. Spinal cord segments were dissected out, then stored in a fixative with a concentration of 10% of that used for perfusion. Thereafter they were subjected to

cryopotection through immersion into a graded series of glycerol (van Lookeren Campagne et al., 1991). The specimens were then plunged into liquid propane cooled by liquid nitrogen, using a rapid-freeze apparatus (KF80; Reichert-Jung), and subsequently transferred to a pre-cooled chamber (-90ºC) of the rapid-freeze apparatus. Freeze-substitution was then performed according to the protocols by Müller et al. (1980). Ultrathin sections were cut on a LKB Ultratome, then mounted on formvar-coated nickel slot grids.
For sections subjected to immunocytochemistry, a postembedding procedure based on that of van Lookeren Campagne et al. (1991) with small modifications (see Hjelle et al., 1994) was used. Briefly, it concluded the following steps. (i) Tris buffered saline (0.05M Tris, with different NaCl concentrations depending on which antibody used) containing 0.1% (w/v) sodium borohydride and 50mM glycine, or Tris buffered saline with 1% Triton X-100 but no sodium borohydride if sections were incubated with antibody against glycine; (ii) Tris-buffered saline containing 2% human serum albumin; (iii) incubation with antibody; (iv) incubation with secondary antibodies coupled to colloidal gold particles; (v) counterstaining with uranyl acetate and lead citrate. The used antibodies have been previously characterized (see paper III for references). Preabsorption with the respective antigen abolished all labeling. As an additional specificity control, the tissue sections in each experiment were incubated along with the test systems of plastic-embedded amino acids/ peptide conjugates (see Hjelle et al., 1994 for details). Enrichment of gold particles was only encountered over corresponding conjugate.

Section analysis
The sections were examined in Philips EM 301 and CM 12 electron microscopes. Photomicrographs were captured and quantitative analysis was performed either by recording data directly on photomicrographs using a digitising tablet (Summagraphics^{TM}) and a commercially available software (Bioquant^{TM}) or, after scanning of negatives, with the Optimas software. A method previously described (Hackney et al., 1996), was used for analysing the subcellular distribution of immunoreactivity for GSH and the examined amino acid neurotransmitters (paper III). By the use of an overlay screen, gold particles were counted over non-mitochondrial vesicle-containing and vesicle-free axoplasmic regions located entirely within each bouton profile. Thus, the mean gold particle density for non-mitochondrial regions containing synaptic vesicles and those that did not was obtained. Similarly, the number of gold particles present in the corresponding adjacent postsynaptic area was recorded (Örnung et al., 1996). For the amino acid neurotransmitters, a quotient between the gold particle density in boutons and their adjacent postsynaptic region was calculated. Thus, an estimate on the enrichment of amino acid neurotransmitter immunoreactivity in the vesicle-containing region of each bouton was obtained. By applying a criterion for pre- to postsynaptic labeling ratio, most boutons could be classified as being enriched with one of the amino acids. In addition, GSH-IR was also quantified in pre- and postsynaptic mitochondria. In the analysis of aberrant and normal appearing axon profiles in the neuropil, all gold particles in the axolemma were recorded. This was due to the fact that mitochondria could not be distinguished in the aberrant axons and subsequently had to be included also in the analysis of normal appearing axon profiles.

Statistics (papers I-V)

Analysis of variance (ANOVA) with Fisher´s LSD was used to test differences between groups. Two-sample analysis was done with Student´s *t*-test or Mann-Whitney U test. Data clustering was performed using K-mean cluster analysis. The levels of significance were indicated as follows: $p>0.05$=not significant (ns), $p<0.05$=*, $p<0.01$=**, $p<0.005$=***. Correlations were done using linear regression analysis of the data sets.

Table 1. Animals used in the studies.

Gender	Age	No of rats	Tissue processing	Method	Used in study
male	2-3	2	perf., 4% GA + 0.5% PF	EM morph.	I, II
male	30	2	perf., 4% GA + 0.5% PF	EM morph.	I, II
male	2-3	2	perf., 4% GA + 0.5% PF	Marchi	I
male	30	2	perf., 4% GA + 0.5% PF	Marchi	I
male	2-3	4	perf., 4% PF + 0.2 PA	IHC	I
male	30	4	perf., 4% PF + 0.2 PA	IHC	I
male	2-3	4	4% GA + 0.5% PF	EM ICH	III
male	30	4	4% GA + 0.5% PF	EM ICH	III
female	2-3	4	perf., 4% PF + 0.2 PA	IHC	II
female	30	4	perf., 4% PF + 0.2 PA	IHC	II
female	2-3	3	perf., 4% PF+ 0.25% GA	EM morph.	II
female	30	3	perf., 4% PF+ 0.25% GA	EM morph.	II
female	2-3	3	fresh frozen	ISH	II
female	30	6	fresh frozen	ISH	II
male	2-3	3	perf., 4% PF	IHC	IV
male	30	3	perf., 4% PF	IHC	IV
male	2-3	4	perf., 4% PF + 0.2 PA	IHC	IV
male	30	3	perf., 4% PF + 0.2 PA	IHC	IV
female	2-3	4	imm., 4% PF	IHC	IV
female	30	7	imm., 4% PF	IHC	IV
male	2-3	3	fresh frozen	ISH	IV
male	30	4	fresh frozen	ISH	IV
female	2-3	3	perf., 4% PF + 0.2 PA	IHC	V
female	30	3	perf., 4% PF + 0.2 PA	IHC	V
female	2-3	4	imm., 4% PF	IHC	V
female	30	4	imm., 4% PF	IHC	V
female	2-3	4	frozen in liquid nitrogen	RT-PCR	V
female	30	8	frozen in liquid nitrogen	RT-PCR	V
male	30	5	frozen in liquid nitrogen	RT-PCR	V
female	2-3	3	perf., 4% PF	IHC	unpublished
female	30	4	perf., 4% PF	IHC	unpublished

The figures in the **Age** column represents months. The following abbreviations are used in the table: perf.= fixed by perfusion; GA= glutaraldehyde; PF= paraformaldehyde; PA= picric acid; imm.= fixed by immersion (i.e. after mounting on slides); EM morph.= electron microscopy, morphological analysis; IHC= immunohistochemistry; EM ICH= high resolution postembedding immunocytochemistry; ISH= in situ hybridization; RT-PCR= reverse transcriptase-polymerase chain reaction

Table 2. Primary antibodies used in the studies.

Antibody	Raised in	Dilution	Study	Source
ChAT	Mo	1:800	II	Mr. Hartman
Clusterin	Mo	1:500	IV	Upstate Biotechnology, USA
Clusterin	Rb	1:1000	IV	Mr. Griswold
CNTF	Rb	1:500	V	Mr. Sendtner
ED1	Mo	1:500	IV, V	Serotec, UK
ED2	Mo	1:500	IV, V	Serotec, UK
FGF-2	Mo	1:400	V	Mr. Reilly
GABA	Rb	1:1000	III	Mr. Ottersen
GFAP	Mo	1:50	I	Boehringer Mannheim, Sweden
GFAP	Mo	1:1000	IV, V	Sigma, USA
GFAP	Rb	1:1000	IV, V	Dako, Denmark
Glutamate	Rb	1:6000	III	Mr. Ottersen
Glutathione	Rb	1:300	III	Mr. Ottersen
Glycine	Rb	1:300	III	Mr. Ottersen
IL1-β	Go	1:40	V	R&D systems, UK
IL6	Go	1:40	V	Santa Cruz, USA
OX42	Mo	1:1600	IV, V	Seralab, UK
PGP 9.5	Rb	1:1000	IV	Ultraclone, Ltd, UK
Serotonin	Rb	1:400	unpublished	Mr. Verhofstad
TGFβ-1	Rb	1:80	V	Santa Cruz, USA
TNF-α	Go	1:80	V	R&D systems, UK
Transferrin	Rb	1:1000	IV, V	Nordic, The Netherlands
Tyrosin hydroxylase	Rb	1:1000	unpublished	Mr. Markey
VaChT	Go	1:2000	II	Chemicon Internatinal Inc., USA

RESULTS AND DISCUSSION

1. Changes in synaptic connectivity of spinal motoneurons and prevalence of NAD in neurochemically identified pathways (papers I, II, III)

Loss of synaptic input (paper I)

Using electron microscopy, spinal cord motoneuron somata were found to have a reduced bouton coverage. Since bouton size did not differ between young adult and aged animals and motoneuron size remains unchanged in senescence (Johnson et al.1995), it was concluded that the reduced coverage was due to a decreased number of axon terminals contacting the motoneurons (c.f. Conradi and Ronnevi, 1975). This age related deafferentation was not uniform across the motoneuron population. About 50% of the cervical and 40% of the lumbar motoneurons had values for the examined parameters similar to those in young adult rats. Furthermore, affected motoneurons were found intermingled with motoneurons that had a normal bouton coverage, indicating that age related loss of synaptic input may depend in part on the postsynaptic neuron and was not likely a process intrinsic to the presynaptic axons. Since the deafferentation appeared more extensive of lumbar than cervical motoneurons, it correlated with the pattern of behavioral motor impairment of the aged rats. Loss of synaptic input during aging has been described in other regions of the CNS (e.g. Glick and Bondareff, 1979; McWilliams and Lynch, 1984) and there is evidence for a correlation between cortical axon terminal loss and cognitive deficits (Chen et al., 1995).

The mechanism(s) by which spinal motoneurons become deprived of synaptic input during aging is not clear, however, several mechanisms may be in operation. In the neuropil axon profiles and terminals showed signs of dystrophy indicating that NAD may at least be one of the "deafferentation mechanisms". So far, only a few, of the different inputs to spinal motoneurons such as the bulbospinal serotoninergic system (Johnson et al., 1993) and primary afferents from the muscle spindles (Bergman and Ulfhake, 2002), have been described to be affected by NAD and/or axon loss. Thus, fairly little was known about the prevalence of NAD among different inputs to motoneurons and then also to what extent the loss was a selective process among afferent inputs.

C boutons are not lost during aging (paper II)

The C boutons constitute an enigmatic synaptic input to spinal cord motoneurons. They are large cholinergic boutons that form highly specialized somatic/juxta-somatic contacts with motoneurons (Conradi, 1969; Li et al., 1995; Nagy et al., 1993). Their origin and exact function remain unknown, but from lesion studies it has been suggested that they exert a trophic function on motoneurons (Pullen and Sears, 1983) and that they derive from local interneurons (Hellström et al., personal comm.; Hellström et al.,1999; Pullen and Sears, 1983). By using immunohistochemistry with antibodies against the vesicular acetylcholine transporter (VAChT) and choline acetyltransferase (ChAT), qualitative light microscopic analysis revealed a reduction of large cholinergic boutons in close apposition with motoneuron somata in aged rat spinal cord. However, analysis at the electron microscopic level failed to confirm a loss of C boutons and, in addition, showed that the characteristic morphological appearance of the C boutons was well preserved. This was also true for aged motoneurons that showed a substantial overall loss in bouton covering. Thus, C bouton input to motoneurons seems to be resistant to the processes of stripping motoneurons from synaptic input, which is interesting against the possibility that they may exert a trophic function. Our

data, however, indicates a loss/downregulation of their cholinergic phenotype in senescence, which contrasts to the preserved cholinergic phenotype of α-motoneurons of the same individuals. It can not be excluded that phenotypic changes in aged motoneurons (Bergman et al., 1999; Johnson et al., 1995, 1999) are of relevance here, since synaptic communication is bi-lateral (see also below) and ample evidence suggest that the target influence the phenotype of innervating fibers (Cooper and Sofroniew, 1996; Ernsberger et al., 1997; Guidry and Landis, 1998). An example of this is the changes in neuropeptide expression taking place in the serotoninergic neurons (which innervate motoneurons) following motor axon manipulation and, possibly, also during aging (Johnson et al. 1993, 1995; Van den Bergh, 1988, 1991; see also Piehl et al., 1991). Moreover, several recent studies have reported on the importance of retrograde synaptic signaling with trophic molecules for transmission properties and plasticity of synaptic axon terminals. It is believed that the target neuron release neurotrophins in an activity-dependent manner, which then can influence the function of the presynaptic terminal (Schinder and Poo, 2000; Schuman, 1999).
Since death of motoneurons in neurodegenerative diseases has been linked to the loss of C boutons (Nagao et al., 1998) it is plausible that the preservation of C boutons contributes to the survival of motoneurons in senescence (Johnson et al., 1995; 1999 and references therein). The hypothesized trophic function of C boutons does not necessarily have to be associated to the cholinergic transmission. The presence of dense core vesicles in C boutons and the close association to post synaptic endoplasmatic reticulum, suggest that C boutons can release other neuroactive molecules and influence protein synthesis.

Non C bouton cholinergic axons (paper II, unpublished observations)
In addition to the large C boutons, the spinal cord neuropil contains small cholinergic terminals. In the ventral horn many of these are recurrent axon collaterals innervating Renshaw cells but also motoneurons (Cullheim et al., 1977).

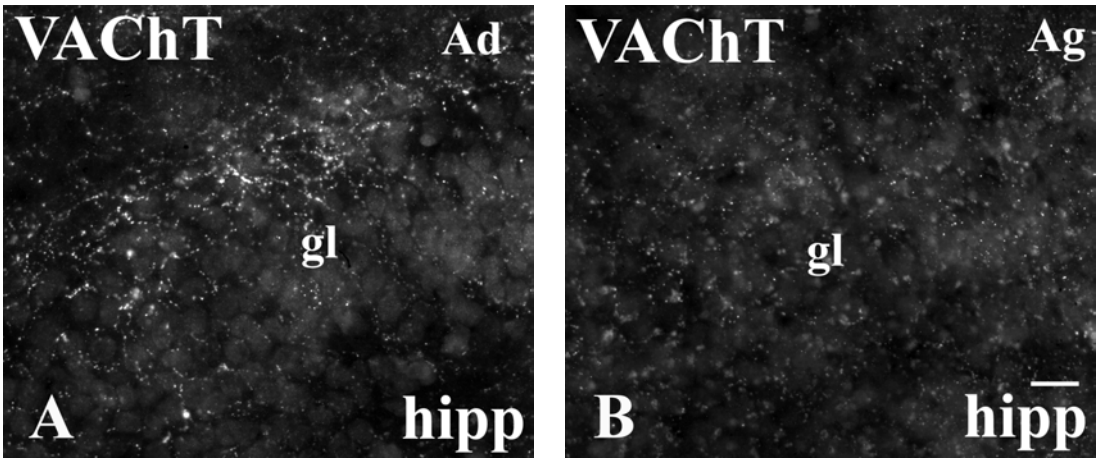

Figure 1. *Sections from the granule cell layer (gl) of the hippocampal dentate gyrus (hipp) from a young adult (Ad) and aged (Ag) rat. Cholinergic terminals are shown by using an antibody against the vesicular acetylcholine transporter (VAChT). Note decreased density of terminal-like profiles in (B) compared to (A). Scalebar corresponds to 20μm.*

There was no conspicuous difference between aged and young adult rats with respect to density of small cholinergic terminals, however, in the aged some of them had a dystrophic appearance, suggesting that NAD affects also cholinergic pathways.
In the hippocampus of aged rats, the density of cholinergic fibers was clearly reduced (Figure 1). This is in line with earlier reports (Lukoyanov et al., 1999), however, signs of NAD were more infrequent than in the spinal cord. Considering the findings on C boutons, the paucity of cholinergic fibers in the aged hippocampus may not necessarily reflect loss of fibers but could also be caused by neurotransmitter phenotype changes in senescence.

Monoaminergic axons (unpublished observations)
Using immunohistochemistry against tyrosine hydroxylase (TH; Markey et al., 1980, see Table 2 in Materials and methods), the rate-limiting enzyme in the synthesis of noradrenaline and dopamine, a decreased density of TH immunoreactive fibers was apparent in both the hippocampus and in the spinal cord of aged rats (Figure 2).

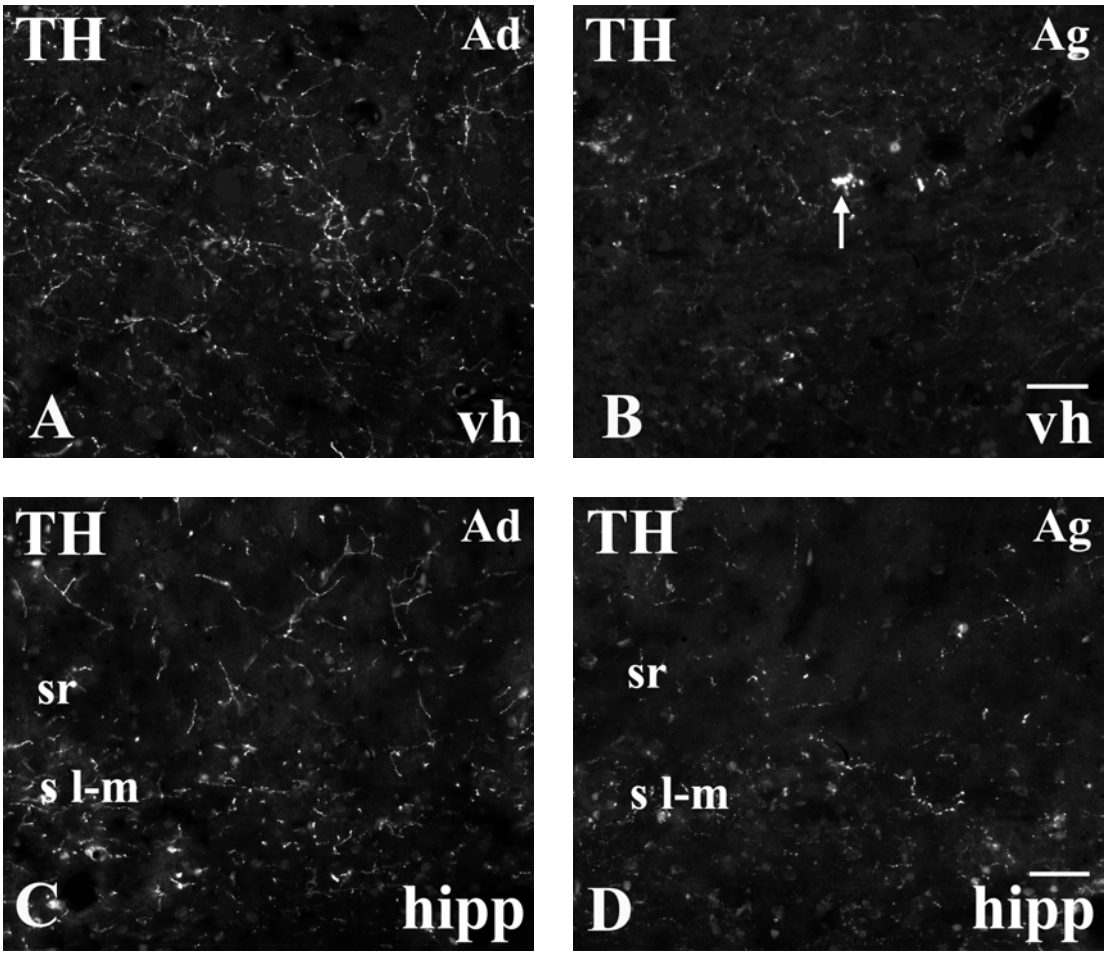

Figure 2. *Sections from young adult (Ad) and aged rat (Ag) spinal cord ventral horn (vh, shown in A and B) and the hippocampus (hipp, shown in C and D), labeled with the TH antibody. Note decreased fiber density in B and D. Arrow in B point at an aberrant profile. The hippocampal layers stratum radiatum (sr) and stratum lacunosum moleculare (s l-m) are indicated. Scalebars correspond to 50μm.*

Some of the TH positive fibers showed signs of NAD in the aged rats. This is in line with previous reports (Masuoka et al., 1979; van Luijtelaar et al., 1992; see also Olson et al., 1973). The NAD-affected TH positive axons were, however, more frequent in the spinal cord than in the hippocampus, whereas loss of immunoreactive fibers was severe in both regions. Since dopaminergic and noradrenergic fibers are present in both spinal cord and hippocampus (Hökfelt et al., 1979; Loughlin et al., 1986; Swanson, 1982), it cannot be resolved if both pathways are equally affected by NAD.
Using immunohistochemistry with an antibody against serotonin (Steinbusch et al., 1978; Verhofstad and Johnson, 1983, see Table 2 in Materials and methods), we could confirm earlier results of axon aberrations and loss of immunoreactive fibers in the aged serotoninergic system (Johnson et al., 1993; van Luijtelaar et al., 1992: see also Olson et al., 1973). However, in line with the findings on TH fibers, it appeared as if NAD of serotoninergic axons was more frequent in the spinal cord than the hippocampus, while fiber loss was extensive in both regions. One tempting explanation for the differences in the occurrence of NAD between the spinal cord and the hippocampus in senescence is a difference of influences from the target, e.g. expression of trophins or other molecules affecting innervation (Gavazzi et al., 1992 and references cited above).

Amino acid neurotransmitters (paper III)
About 90% of boutons apposing spinal motoneurons appear to use one or a combination of glutamate, GABA and glycine as fast neurotransmitters (Fonnum, 1984; Örnung et al., 1998). Quantitative analysis in the electron microscope, indicated that aberrant axons (only encountered in the aged rats) often were glutamatergic, while GABAergic and glycinergic fibers were only rarely affected. One mechanism that might contribute to the susceptibility for NAD among glutamatergic axons may be age related phenotypic changes in the target neurons. Evidence from studies on cortical and hippocampal cell cultures and brain slices, suggest that excitatory and inhibitory transmissions are differentially affected by retrograde neurotrophin signaling at synapses. Glutamatergic signaling is increased whereas GABAergic is depressed in response to neurotrophins (Leßman, 1998; Schinder and Poo, 2000; Schuman, 1999). If similar mechanisms operate in the spinal cord, phenotype changes in the target neurons may affect excitatory and inhibitory boutons differentially.

2. NAD, transmitter phenotype and oxidative stress (paper III, unpublished observations)
In the material used for analysis of NAD in axons using amino acid neurotransmitters, adjacent sections were labeled with antiserum against the free radical scavenger glutathione (GSH) to examine if GSH synthesis is upregulated pre- and/or postsynaptically in the lumbar motor nuclei of aged rats. GSH is a major protector against ROS in the brain and synthesis is regulated by feed-back inhibition of GSH (Cooper and Kristal, 1997, Dringen, 2000). Thus, changes in GSH expression are likely to reflect alterations in redox status. The quantitative analysis revealed an increase in GSH immunoreactivity in both pre- and postsynaptic compartments in the lumbar motor nuclei of aged rats. Presynaptically, the enrichment of GSH immunoreactivity was seen in axonal boutons of normal appearance as well as in dystrophic boutons, and was furthermore restricted to the extra-mitochondrial compartment. In particular glutamatergic axon terminals contained high levels of GSH, in fact, close to 50% of all glutamate immunoreactive boutons in the aged rats contained very high levels of GSH

immunoreactivity. Increase of GSH in both normal and dystrophic glutamatergic axon profiles in the aged rats, may indicate that oxidative challenge precede the occurrence of NAD. Despite the fact that mitochondria are reported to be the primary target of age associated oxidative damage (Ashok and Ali, 2000; Barja and Herrero, 2000; de la Asuncion et al., 1996; Ferrándiz et al., 1994; Sastre et al., 2000), mitochondria in the axon terminals did not show an increase in GSH immunoreactivity. Low levels of mitochondrial GSH have been linked to degeneration and impaired function (de la Asuncion et al., 1996; Dringen et al., 2000; Jain et al., 1991; Meister, 1995) and there is a similarity in the ultrastructure of mitochondria in aberrant axons and mitochondria subjected to experimentally induced oxidative damage (Jain et al., 1991; Meister, 1995). Mitochondrial deterioration may result in decreased formation of ATP, impairing energy dependent reactions such as transmitter release and degradation processes. Subsequently, terminal content and undegraded material may accumulate in the terminal and cause the development of NAD. Postsynaptically, GSH was increased both in the cytosolic and the mitocondrial compartments of aged rats, indicating a change in redox status also in motoneurons. All in all, the results support the notion that aging is associated with an increased oxidative stress (Harman, 1981) and indicate that different transmitter systems are differentially affected.

3. Dysmyelination (paper I)

Whereas only a few studies have addressed the issue of dysmyelination in the white matter of CNS during aging (Dickson et al., 1990; Feldman and Peters, 1998; Fuisawa, 1988; Peters et al., 1994), numerous studies have described and characterized age related dysmyelination in spinal roots and/or peripheral nerves (Berg et al., 1962; Bergman and Ulfhake, 2002, Gilmore, 1972; Johansson et al., 1996; Kazui and Fujiawa, 1988; Karlsson and Hildebrand, 1996; Knox et al., 1989; Krinke, 1983; Sharma et al., 1980; van Steenis and Kroes, 1971). We used staining with Marchi solution to examine myelination of the spinal cord at the light microscopic level. There was a marked increase of Marchi-positive bodies (MPBs), suggested to represent myelin degradation products (Corneliuson et al., 1988; Fransson and Ronnevi, 1984; Hildebrand 1977; Persson and Berthold, 1991), in the outer portions of the spinal cord funiculi of aged rats (Figure 3). MPBs in adult animals are found mainly along large myelinated fibers and probably reflect a normal turn-over in thick myelin sheaths (Hildebrand 1977). However, during Wallerian degeneration there is a drastic increase in number of MPBs (Franson and Ronnevi, 1984). Thus, our data suggest an increased breakdown of myelin but a defect in clearance of myelin debris can not be ruled out. However, the regions containing most MPBs had also vacuoles and a more pale appearance, indicating a low content of myelin (Figure 3) (Corneliuson, 1988). Thus, an increased breakdown seems to be a more likely explanation to the increased number of MPBs during aging.

From the figure below (Figure 3), it is evident that there is an overlap between regions affected more severely by dysmyelination and the regions harbouring ascending sensory pathways and some of the descending bulbospinal pathways. Some common features of these pathways are that they are far-projecting, they are myelinated and that they have a monoaminergic or a glutamatergic neurochemical phenotype.

In paper I, it was also noted that there appeared to be close coincidence of regions affected by dysmyelination (white matter; see above) or NAD/loss of axon terminals (motor nucleus, paper I; superficial part of the dorsal horn, Bergman and Ulfhake, 2002), on the one hand, and signs of astrogliosis, on the other.

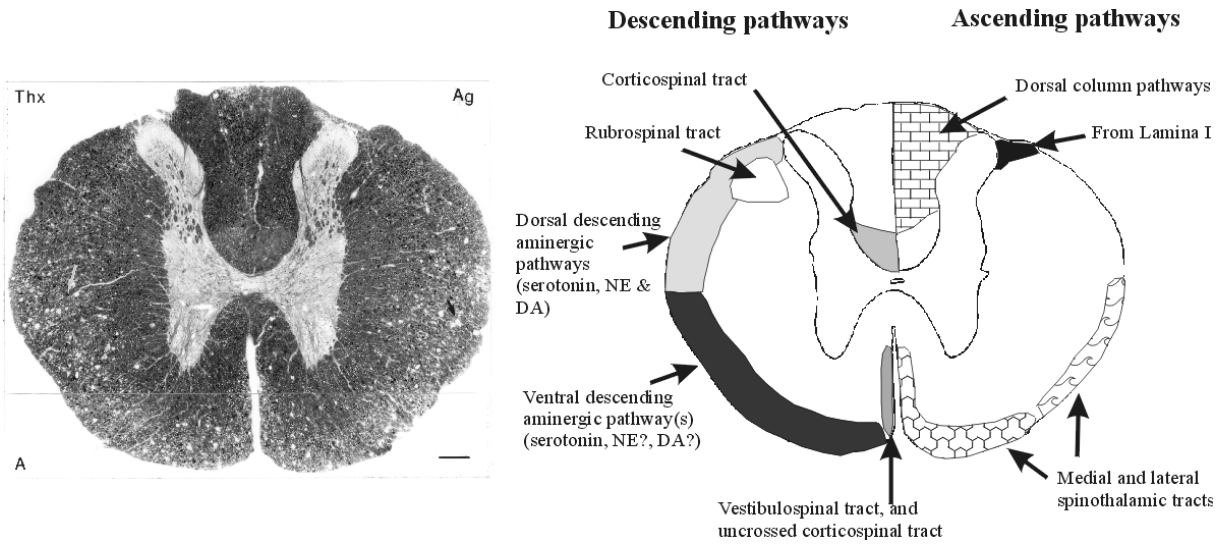

Figure 3. *A crossection through the thoracic spinal cord (reproduced from paper I) and a schematic representation of some major ascending and descending axon tracts (data replotted from "The rat nervous system", edited by Paxinos, 1995). Scalebar corresponds to 220µm.*

4. Astro- and microglial reaction (papers I, IV)

From the results discussed above it is evident that NAD and fiber/terminal loss are hallmarks of the aged rat grey matter, while signs of myelin changes are evident in the spinal cord white matter. Since astroglia and/or microglia are activated in a number of conditions with axon and/or myelin damage (Kreutzberg, 1996; Liedtke et al., 1996; Wilson and Molliver, 1994; Zielasek and Hartung, 1996), we decided to look for signs of astroglial and microglial activation in the spinal cord and, for comparison, also in more rostral brain regions.

Grey matter (papers I, IV)

In the aged rat spinal cord, GFAP expression and GFAP positive profiles were increased around motoneurons and in the superficial part of the dorsal horn. Signs of astrogliois were also evident in several brain regions, e.g. the hippocampus. Electron microscopic analysis of spinal motoneurons with a decreased bouton covering, showed that they were surrounded by pale processes, most likely of astroglial origin. Signs of microglial activation and phagocytic activity were not conspicuous in the spinal cord of aged rats, despite occurrence of axon dystrophy and loss of synaptic terminals. In the literature, there are some controversies concerning the role for microglia in bouton elimination. Following axotomy of motoneurons there is an elimination of bouton from the receptive domain of the lesioned motoneuron (Lindå et al., 1992). In some animal models, post axotomy shedding of synapses occur without microglial reaction (Svensson and Aldskogius, 1993). However, there is also evidence favouring that microglia are responsible for terminal degeneration (Blinzinger and Kreutzberg, 1968; Wilson and Molliver, 1994) or at least influence the displacement process (Aldskogius et al., 1999). Since microglial activation has been claimed to be a rapid and transient event in grey matter (Raivich et al., 1999 and references therein), microglial activation may not be easy to capture. Nevertheless, a low level activation of microglia was evident in our material, thus a role for them in bouton removal during aging cannot be ruled out. There is morphological evidence that immature astrocytes are responsible for the

postnatal removal of boutons from motoneurons (Ronnevi, 1978) and data indicates that also mature astrocytes are involved in the detachment of presynaptic terminals following axotomy (Aldskogius et al., 1999 and references therein). However, astroglia are also necessary for maintaining a number of other functions, e.g. synapse formation, synaptic plasticity and integrity, ion homeostasis, transmitter metabolism, regulation of extracellular matrix (Araque et al., 1999; Haydon, 2000; Pfrieger and Barres, 1996; Ridet et al., 1997; Ullian et al., 2001) and in the defence against oxidative stress (Dringen, 2000 and references therein). Thus, the observed astrogliosis of the grey matter may perhaps mainly serve other purposes than bouton removal during aging.

White matter (papers I, IV)

GFAP expression was increased in the outer portions of spinal cord funiculi of the aged rats, a pattern coinciding with that encountered for Marchi-positive bodies (see above). In the same regions, OX42 labeled profiles disclosed the characteristics of activated microglia (Kreutzberg, 1996) and ED1 positive profiles were numerous. Since ED2 labeling was similar in both age groups and restricted to perivascular locations, and the intact blood brain barrier does not allow entry of immune cells to any great extent (Becher et al., 2000), ED1 labeled profiles were considered as phagocytes derived from resident microglia. Thus, it appears as if signs of myelin changes (see above), activation of microglia (OX42), presence of phagocytic (ED1) microglia, and astrogliosis are related events during aging. This is not surprising, since astroglia and microglia together with MPBs have been suggested to be involved in normal myelin catabolism (Hildebrand et al., 1993 and references therein). In addition, expression of the multifunctional protein clusterin was increased in astroglia in the outer parts of spinal cord funiculi. Since one of its attributed functions is lipid scavenging in myelin clearing processes (Rosenberg and Silkensen, 1995), it is likely that the upregulation reflects an increased breakdown of myelin.

By measuring the photometric values of GFAP, OX42 and ED1, a positive correlation was recorded between these markers for gliosis. In this analysis it also became apparent that aged animals with severe sensorimotor impairment showed more extensive signs of gliosis than aged animals with mild behavioral deficits or young adult animals. This is consistent with results obtained in aged monkeys, where memory impairment correlated with the degree of degenerative changes in myelinated axons and microglial activation (Peters et al., 1994). In aged rats, a positive correlation between GFAP expression in hippocampus and corpus callosum and the extent of behavioral deficits have been observed (Soffié et al., 1999; Suguaya et al., 1996).

The findings made here that microglial activation is much more overt in the white than the grey matter of the spinal cord, is in accordance with results from other brain regions (Ogura et al., 1994; Perry et al., 1993; Peters et al., 1994; Sheffield and Berman, 1998; Sloane et al., 1999). Since myelin breakdown products are potent activators of microglia and microglia phagocytosis appears to be instrumental in the removal of myelin/myelin debris in de-/dysmyelinating conditions (Lawson et al., 1994; Zielasek and Hartung, 1996), it is likely that the microglial activation in the white matter during aging, is secondary to dysmyelination. Since microglial activation in a lesion situation is an inflammatory reaction, it may also cause harm. “Uncontrolled” inflammatory processes have been suggested to worsen the course of neurodegenerative diseases such as Alzheimer´s disease (Campbell et al., 1998). Judging from experimental studies, however, it seems as if a myelin debris clearance by macrophages is a

prerequisite for proper remyelination and functional restoration (Franklin and Hinks, 1999; Zhang et al., 2001). Similarly, astroglia seem to be important for successful remyelination as well as structural organization of the white matter (Hinks and Franklin, 1999; Liedtke, 1996; Woodruff and Franklin, 1999; Yao et al., 1995a). Thus, the activation of astroglia may reflect an attempt towards remyelination during aging.
From analysis of co-processed tissue, it appeared as if microglial activation and astrogliosis were less pronounced in brain white matter regions compared to the spinal cord. This may reflect a lesser extent of dysmyelination in rostral CNS regions, or a relatively lower density of large myelinated fibers in the white matter of the brain.

5. Cytokine expression and glial cells in the aged spinal cord and hippocampus (paper V)

Glial cell activation and inflammatory responses are tightly regulated by molecules referred to as cytokines and chemokines (Asensio and Campbell, 1999; Turrin and Plata-Salamán, 2000). Cytokines are divided into families, like pro-inflammatory or trophic. However, many cytokines as IL6 and TGFβ–1 are involved in both inflammatory and trophic signaling (Flanders et al., 1998; Van Wagoner and Benveniste, 1999). Since both inflammatory signaling and trophic support are highly relevant in a state of age related axon aberrations and gliosis, the expression and cellular distribution of several cytokines were examined in the spinal cord and, for comparison, the hippocampus of young adult and aged rats. The changes were more pronounced in the spinal cord than in the hippocampus and, in particular, the white matter of the spinal cord contained glial cells with increased content of cytokines. Among the examined cytokines, only IFN-γ was upregulated in both the hippocampus and the spinal cord. IFN-γ is a potent proinflammatory cytokine that can induce cytotoxic functions in microglia, and it is likely that an increased expression could be connected to the phagocytic activity of microglia observed in aged rats. However, the cellular source of the increased level of IFN-γ could not be determined here. Although T-lymphocytes and natural killer cells are regarded to be the source for IFN-γ (Aloisi, 2001; Popko et al., 1997), evidence indicates that both astro- and microglia can synthesize this cytokine (De Simone et al., 1998). Considering that at least T-lymphocytes are only rarely encountered in brains of young adult as well as aged rats (Perry et al., 1993), it appears plausible that IFN-γ is produced in the activated glial cells in the aged rats. Since dysmyelination/disruption of myelin sheath appears to be able to directly activate microglia (Lawson et al., 1994; Zhang et al., 2001), the contribution of peripheral immune cells for eliciting glial activation may not be needed. TNF-α, another molecule implicated in microglial cytotoxicity, mainly recognized for its destructive effects on oligodendroglia (Stoll and Jander, 1999 and references therein) did not show a consistent change in expression in the aged rats. However, a prominent feature in the spinal cord white matter of all aged rats studied, was TNF-α positive profiles tightly enclosed by large activated microglia, suggesting this molecule involvement in their phagocytic activity.
CNTF was encountered in astroglia and oligodendroglia of the white matter in aged and young adult rats. In the aged rats, the expression of CNTF appeared decreased in oligodendroglia of the white matter, whereas it was increased in astroglia processes. Since CNTF can protect oligodendroglia from cytotoxicity and also influence myelination (Dell´Albani et al., 1998; Linker et al., 2002), a decreased expression may render aged oligodendroglia more vulnerable to insults and less competent to handle the demand for remyelination. The increased expression of CNTF in astroglia might reflect its effects in

inducing astrogliosis (Kahn et al., 1997; Winter et al., 1995), or an effort to support oligodendroglia. IGF-1 can exert similar effects on oligodendroglia and myelination as CNTF, and has also been shown to reduce demyelination and up-regulate gene expression of myelin proteins (Yao et al., 1995b; Ye and D´Ercole, 1999). However, in contrast to CNTF, the expression of IGF-1 was upregulated in the hippocampus only. At least in part, an upregulation of IGF-1 in the hippocampus may explain the differences between this region and the spinal cord concerning degree of gliosis and differences in occurrence of dystrophic axons.

In addition to an increased expression of CNTF, astroglia in the spinal cord white matter also disclosed increased content of IL1-β, IL6, FGF-2 and TGFβ-1. FGF-2 was also increased in astroglia of the spinal cord grey matter and the hippocampus. Since all these molecules can induce astrogliosis (Baghdassarian et al., 1993; Chiang et al., 1994; Laping et al., 1994; Reilly et al., 1998), it is likely that their upregulation reflects a role in the age related astrogliosis. Interestingly, only FGF-2 was upregulated also in astroglia of the grey matter (spinal cord and hippocampus), which suggests that the mechanisms underlying the white matter and grey matter gliosis are different. In addition to their effects on astroglia, FGF-2 and TGFβ-1 can promote myelination (Franklin and Hinks, 1999). Thus, their increased expression may reflect mechanisms to promote repair processes. Following experimental induced demyelination, remyelination takes place in the aged rat CNS as extensively as in young adult rats, but at a slower pace (Shields et al., 1999), indicating that the capacity for remyelination is retained.

Aspects on gender

Although not specifically addressed, it was revealed that the senescence related changes in cytokine expression (and glia activation; paper IV) were similar in male and female rats, except for TNF-α, which was upregulated in spinal cord of male but not female aged rats. Whether this represents a "true" gender difference or rather reflects individual differences was not resolved. Although we have been unable to detect conspicuous differences in behavior between female and male rats (Appendix), it seems likely that the changes occurring in several hormonal systems during aging (Lamberts et al., 1997) may contribute to the phenotypic changes observed in senescence, an issue that deserves further attention.

GENERAL DISCUSSION

Results of this thesis work are compatible with the "free radical theory of aging" (see Introduction). However, the differences in the loss of axon terminals, NAD and dysmyelination between regions and neurochemically identified systems, can hardly be explained by a simple interpretation of this theory. Rather, it suggests that several and possibly diverse mechanisms contribute to the age related changes. Although direct evidence is missing, impairment of oligodendrocyte integrity is probably a key element in NAD of large (projecting) myelinated axons. Data presented here also indicates that NAD is associated with the neurochemical phenotype of the axon and, possibly, phenotypic changes of the target neurons/cells.

Neurochemical phenotype and prevalence for NAD.

The selective vulnerability for NAD in glutamatergic and monoaminergic axononal pathways may relate to the handling and the metabolism of the transmitters.

Glutamate signaling can initiate an oxidative challenge through stimulation of NMDA receptors, referred to as excitotoxicity (Leist and Nicotera, 1998; Michaelis, 1998). This mechanism has mainly been implicated in damage of the postsynaptic profile. Perhaps surprising, age related axon damage affects the glutamatergic axon itself. However, NMDA receptors are also expressed presynaptically (Liu et al., 1994; Michaelis, 1998 and references therein), and it has been suggested that glutamate can be detrimental to the glutamatergic terminals themselves (Mattson et al., 1998), which would be consistent with an increased susceptibility for NAD. The function of astroglia is critical for a normal function of glutamate synapses. For example, the glial glutamate transporter is responsible for the re-uptake of the transmitter from the synaptic cleft and this transporter has been shown to be especially vulnerable to the actions of ROS (Keller et al., 1997). Astroglia are also responsible for the further handling of glutamate and re-loading of the transmitter precursor to the presynaptic axon terminal (Hertz et al., 1999). In addition, evidence (see Introduction) indicate that astroglia support neurons in the protection against oxidative stress. If one or several of these functions become impaired during aging, the probability for glutamate toxicity will increase and may explain the higher prevalence for NAD in glutamatergic axons compared to axons using other amino acid transmitters.

During monoamine metabolism, ROS (hydrogen peroxide) are formed through the actions of the enzyme monoamine oxidase (MAO), situated at the outer mitochondrial membrane. This will add to the ROS generated in the electron transport chain during cell respiration, and may thus increase the probability for oxidative damage in monoaminergic axon terminals.

The major contribution to the ROS in neurons derives from cell respiration, thus, the metabolic demand in neurons that give rise to large and/or far-projecting axon arbors may be at greater risk for oxidative damage than locally projecting neurons with a less extensive axon arbor. This is consistent with the findings made here and earlier studies showing a high incidence for NAD in the dorsal column system and the serotoninergic system (for references see above).

Possible effects by target neurons and dysmyelination on NAD and axon terminal loss

As evident from the results of paper I, loss of synaptic input is selective among spinal motoneurons. Thus, some but not other motoneurons are affected indicating that the target

neuron may be instrumental in synaptic removal during aging as is the case when motoneuron axons are severed in young individuals. As discussed above, the target neuron at synapses influences the function of the presynaptic axon terminal through retrograde signaling mechanism(s). In fact, an intact retrograde signaling may be a prerequisite to maintain phenotype and integrity of the presynaptic terminal. Such a mechanism has also been put forward as an explanation for innervation deficits in PNS in senescence (e.g. Johansson et al., 1996; reviewed by Cowen and Gavazzi, 1998; Ulfhake at al., 2000, 2002).

Although according to its definition, NAD is a process in the axon, myelinated axons are dependent on oligodendroglia for integrity and NAD is associated with dysmyelination (see Introduction and references below). Studies on multiple sclerosis and experimental-induced encephalomyelitis have shown that NAD appears in the wake of demyelination, suggesting that dysmyelination can induce NAD (Giordana et al., 2002; Raine and Cross, 1989; Woodruff and Franklin, 1999). A possible explanation for this is that compact myelin/myelin components are necessary for neurofilament organization in the axonal cytoskeleton (Brady et al., 1999; de Waegh et al., 1992; Yin et al., 1998), subsequently dysmyelination may interfere with axonal transport. Others have discussed changes in axonal transport as a cause for NAD, although an exact mechanism by which this would occur has not been established (Jellinger and Jírasek, 1971; Schmidt et al., 1997). Despite controversy regarding changes that take place in the different components of axonal transport with age, there is evidence suggesting that the rate of slow anterograde transport, responsible for the movement of cytoskeleton and enzymes of intermediary metabolism may be decreased (Jacob, 1995; Komiya, 1980; McQuarrie et al., 1989). This can have implications for synaptic plasticity and possibly results in reduced activity at the terminal. Since neurofilament accumulation has been described in inactive nerve terminals of the goldfish (Bondar and Roots, 1977), it might be hypothesized that during aging reduced activity in axon terminals, through presynaptic and/or postsynaptic (see above) mechanisms, results in neurofilament accumulation and subsequent distortion of the axon architecture. Moreover, interactions between compact myelin and axon are suggested to influence neurofilament organization through activation of kinases and phosphatases (Brady et al., 1999). Hence, it is plausible that dysmyelination itself, disrupting the compact myelin, may result in abnormally phosphorylated epitopes that are more resistant to degradation and subsequently can accumulate in the distal axon, as seen in NAD. Indeed, there is evidence for an increased phosphorylation of neurofilaments in senescence (e.g. Gou et al., 1995; Uchida et al., 1999).

Factors that may contribute to dysmyelination

Dysmyelination (and thereby possibly also NAD) may be elicited by a number of factors, e.g. changes in trophic signaling (e.g. PDGF, IGF-1, FGF-2 and CNTF), cytotoxic molecules (e.g. TNF-α and IFN-γ), intrinsic changes including oxidative stress in the oligodendroglia and/or myelin sheath (Casaccia-Bonnefil, 2000 and references below).

Administration of exogenous IGF-1 and PDGF has been found to decrease demyelination in acute/semi-chronic experimental animal models (Allamargot et al., 2001; Jean et al., 2002; Yao et al., 1995b). The endogenous expression of TGFβ–1, FGF-2, PDGF and IGF-1 shows a distinct regulatory pattern in experimental demyelination–remyelination processes (Hinks and Franklin, 1999, 2000), suggesting that they play different supportive roles in the repair process. Of these molecules, only TGFβ–1 showed a consistent upregulation in the spinal cord during aging (paper V) and for CNTF our data indicates the possibility of a decreased

content of CNTF protein in oligodendrocyte-like profiles. Thus, it cannot be excluded that the progressive dysmyelination occurring during aging, in part, is due to a deficient/exhausted remyelination/repair process. Interestingly, IGF-1 was upregulated in the hippocampus of the aged animals and may be one factor contributing to the lower incidence of NAD and glial activation in this region compared to the spinal cord (paper IV and V). It should be mentioned here, that since the relative number of large myelinated axon probably is lower in the hippocampus than in the spinal cord less signs of glial changes were expected.

One, not mutually exclusive, explanation to the dysmyelination is the detrimental actions of ROS (see Introduction). This notion is supported by a study with monkeys in which brain white matter disclosed signs of oxidative damage (Sloane et al., 1999). By the reaction between ROS and membrane components, its properties are altered (Beckman and Ames, 1998) and products formed in these reactions can be directly harmful to oligodendroglia (Gard et al., 2001) and recognized by macrophages (Vlassara et al., 1988).

There are also evidence that changes in the fatty acid composition can activate microglia (Poulos, 1995 and references therein; Dubois-Dalcq et al., 1999). The brain is rich in very long chain fatty acids (VLCFA) and there is evidence favouring an age related increase in their length as well as an increased proportion of unsaturated compared to saturated VLCFA (Giusto et al., 1992). This pattern resembles the situation in adrenoleukodystrophy (ALD) an X-liked inherited demyelinating disease, where VLCFA accumulate due to disturbed transport into the peroxisomes, resulting in an impaired β-oxidation of VLCFAs. The accumulation of VLCFA is suggested to destabilize the myelin and cause an activation of microglia (Dubois-Dalcq et al., 1999). It is therefore tempting to speculate that the age related increase in the length of VLCFA can cause destabilization and subsequent dysmyelination. Also, results from mice deficient in CGT, the enzyme required for the synthesis of galactocerebroside (GalC, one of the most abundant myelin lipids) indicate that an intact lipid composition is necessary for myelin stability. Interestingly, the same mice exhibited differences between regions in myelin stability (Coetzee et al., 1996) indicating that phenotypic subpopulations based on morphological and/or biochemical differences (Anderson et al., 1999; Bjartmar et al., 1994; Norton and Cammer, 1984) may vary with respect to vulnerability to genetic and/or environmental changes.

Increased break down of myelin in de- and dysmyelination triggers a reactive inflammatory astro- and microgliosis. Several of the proinflammory cytokines involved in inflammation are potentially harmful to oligodendroglia and the inflammation process itself can be damaging to oligodendroglia function if it is not strictly controlled.

Results from knock-out and mutant mice, have also shown that the myelin proteins influence myelin sheath integrity (Klugmann, 1997; Nadon and Duncan, 1995; Weiss et al., 2000). In the aged rat CNS, expression of several of the myelin proteins is decreased (Sim et al., 2000; Virgili et al., 2001), which may reflect a decreased myelin content secondary to dysmyelination but possibly also a deficiency in remyelination processes.

As well as the changes in lipid composition, also a decreased synthesis/turn-over of proteins and/or posttranslational modifications of protein (Sato et al., 1999) may be primary events in the impairment of oligodendroglia function.

Natural history of NAD and dysmyelination during aging

In contrast to acute lesions where remyelination and/or re-innervation take place (Woodruff and Franklin, 1999), the reparative efforts during aging are not sufficient to ameliorate

impairment of function(s). However, this is probably not so initially. It is worthwhile to remember that signs of NAD are evident in human post mortem material, from subject less than 10 years of age and become progressively more abundant with advancing age. Thus for a considerable period of time (relative to the expected life span of the species), the NAD and dysmyelination process do not induce overt behavioral impairment in the individuals (see Appendix). At later time points, impairments emerge and become progressively worse but with considerable variations among individuals in a population. There are several explanations for this pattern; firstly it is likely that the instigating mechanism(s) can act in a chronic manner that over time may exhaust repair mechanisms. Secondly, with advancing age cells that are not replaced may become more vulnerable to insults. Thirdly, both environmental factors as well as genetic differences may affect both the susceptibility for NAD and dysmyelination.

ACKNOWLEDGEMENTS

The present work has been carried out at the Department of Neuroscience, Karolinska Institutet. I would like to express my sincere gratitude to all those who made this thesis possible. In particular I would like to thank:

Professor Brun Ulfhake, my supervisor, for sharing your vast knowledge in neuroscience, for excellent guiding through my thesis project and for always being ready to discuss scientific matters whenever something was brought up.
Professor Lars Olson, head of Department of Neuroscience, for providing excellent research facilities, and always being ready to put your signature under applications etc more or less immediately after asking.
Professor Tomas Hökfelt, head of Division of Chemical neurotransmission and co-author of paper II, for inspiration through your positive attitude and for making this thesis possible by letting me cut at your cryostat for endless hours.
Professor emeritus Gunnar Grant, for valuable comments on paper I and for always being so nice, and not the least…holding up doors when I carried the incubationbox through the corridors of the Anatomy building to the cool room.
Late Professor emeritus Lars-Gösta Elfvin, for taking sincere interest in my work and sharing a wide knowledge in neuroscience.
Vania and **Yu Ming**, my room mates in the girl´s room in the Anatomy building, for good laughs and discussions on both scientific and non-scientific matters. Especially thanks to Vania for introducing me to the mysteries of electron microscopy.
Elisabeth, technician and room mate, for sharing some of your positivism and technical skilfulness. Double thank you for always immediately piling down to me when I was in desperate need for help in the dark by the electron microscope.
Erik and **Mikael**, for making the corridor more vivid and for discussing both scientific and "less scientific" matters. **Therese** and **Veronique** for bringing new fresh spirit and thoughts. Former members of the group, **Göran** and **Birger** for creating a joyful atmosphere.
Siv Nilsson and **Katarina Åman**, for sharing your knowledge in technical matters and all help with localizing obscure antibodies in the big freezer. Especially thanks for nice small talks making my sessions in the cryostat a little easier to withstand. **Elzbieta** for technical assistance and for fun discussions on fashion.
Margareta Almström and the **staff of the Animal Department**, for taking excellent care of the animals.
All other people at the Department of Neuroscience, contributing to a nice and friendly atmosphere, making this such a pleasant place to work at!
Annika and **Ulrika,** thanks to both of you for a tremendous lot of fun during the past 11 years and also for always being ready to give support, good advice and not the least, sharing secrets. **Carolina**, for true and honest friendship, and for great fun during our girl´s trips. **Helena**, for taking my aggressions during ju-jutsu fighting. **Johanna**, for nice friendship, and arrangement of social events. **Bengt H** for sincere good advice when everything concerning research was black, for a lot of fun and for absolutely crazy moments during nightly events by copying machines…**AJAX** for being such an open-minded friend and for bringing me to nightly baths at Långholmen. **Bengt P with family**, **Hasse**, **Tomas C**, **Magnus W** and **Lasse** for wine

drinking (I mean testing…). **Peter Å** for fruitful discussions on life as a PhD student and nice lunch company.
Anna and **Michi**, two of my oldest friends, for lots of fun (as well as fighting) during childhood and nowadays for joining in more "adult interests"…
And a **lot of other good friends** outside the lab for a lot of fun**.**
My younger brother, **Mikael**, for a lot of playing and joyful moments during childhood and nowadays, being a true adult friend.
Mother and **father,** for always believing in me, endless love, constant support as well as good advice during the period when research wasn´t fun. Without your help this thesis would have been impossible.
Late **grandmother** and **grandfather**, for always being proud of me and for contributing to a lovely childhood.

REFERENCE LIST

Aldskogius, H.; Liu, L., and Svensson, M. Glial responses to synaptic damage and plasticity. J Neurosci Res. 1999 Oct 1; 58(1):33-41.

Allamargot, C.; Pouplard-Barthelaix, A., and Fressinaud, C. A single intracerebral microinjection of platelet-derived growth factor (PDGF) accelerates the rate of remyelination in vivo. Brain Res. 2001 Nov 9; 918(1-2):28-39.

Aloisi, F. Immune function of microglia. Glia. 2001 Nov; 36(2):165-79.

Anderson, E. S.; Bjartmar, C.; Westermark, G., and Hildebrand, C. Molecular heterogeneity of oligodendrocytes in chicken white matter. Glia. 1999 Jul; 27(1):15-21.

Araque, A.; Parpura, V.; Sanzgiri, R. P., and Haydon, P. G. Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci. 1999 May; 22(5):208-15.

Asensio, V. C. and Campbell, I. L. Chemokines in the CNS: plurifunctional mediators in diverse states. Trends Neurosci. 1999 Nov; 22(11):504-12.

Ashok, B. T. and Ali, R. The aging paradox: free radical theory of aging. Exp Gerontol. 1999 Jun; 34(3):293-303.

Baghdassarian, D.; Toru-Delbauffe, D.; Gavaret, J. M., and Pierre, M. Effects of transforming growth factor-beta 1 on the extracellular matrix and cytoskeleton of cultured astrocytes. Glia. 1993 Mar; 7(3):193-202.

Barja, G. and Herrero, A. Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. FASEB J. 2000 Feb; 14(2):312-8.

Becher, B.; Prat, A., and Antel, J. P. Brain-immune connection: immuno-regulatory properties of CNS-resident cells. Glia. 2000 Feb 15; 29(4):293-304.

Beckman, K. B. and Ames, B. N. The free radical theory of aging matures. Physiol Rev. 1998 Apr; 78(2):547-81.

Benzi, G. and Moretti, A. Age- and peroxidative stress-related modifications of the cerebral enzymatic activities linked to mitochondria and the glutathione system. Free Radic Biol Med. 1995 Jul; 19(1):77-101.

Berg, B. N.; Wolf, A., and Simms, H. S. Degenerative lesions of spinal roots and peripheral nerves in aging rats. Gerontologia. 1962; 6:72-80.

Bergman, E.; Kullberg, S.; Ming, Y., and Ulfhake, B. Upregulation of GFRalpha-1 and c-ret in primary sensory neurons and spinal motoneurons of aged rats. J Neurosci Res. 1999 Jul 15; 57(2):153-65.

Bergman, E. and Ulfhake, B. Evidence for loss of myelinated input to the spinal cord in senescent rats. Neurobiol Aging. 2002 Mar-Apr 30; 23(2):271-86.

Bergman, E. and Ulfhake, B. Loss of primary sensory neurons in the very old rat: neuron number estimates using the disector method and confocal optical sectioning. J Comp Neurol. 1998 Jun 29; 396(2):211-22.

Bjartmar, C.; Hildebrand, C., and Loinder, K. Morphological heterogeneity of rat oligodendrocytes: electron microscopic studies on serial sections. Glia. 1994 Jul; 11(3):235-44.

Blinzinger, K. and Kreutzberg, G. Displacement of synaptic terminals from regenerating motoneurons by microglial cells. Z Zellforsch Mikrosk Anat. 1968; 85(2):145-57.

Bondar, R. L. and Roots, B. I. Neurofibrillar changes in goldfish (Carassius auratus L.) brain in relation to environmental temperature. Exp Brain Res. 1977 Dec 19; 30(4):577-85.

Brady, S. T.; Witt, A. S.; Kirkpatrick, L. L.; de Waegh, S. M.; Readhead, C.; Tu, P. H., and Lee, V. M. Formation of compact myelin is required for maturation of the axonal cytoskeleton. J Neurosci. 1999 Sep 1; 19(17):7278-88.

Brannon, W.; McCormick, W., and Lampert, P. Axonal dystrophy in the gracile nucleus of man. Acta Neuropathol (Berl). 1967 Aug 2; 9(1):1-6.

Burek, J. D. and Hollander C.F. Experimental gerontology. The laboratory rat, vol II. Academic Press, Inc.; 1980; pp. 149-159.

Burek, J. D.; van der Kogel, A. J., and Hollander, C. F. Degenerative myelopathy in three strains of aging rats. Vet Pathol. 1976; 13(5):321-31.

Campbell, I. L.; Stalder, A. K.; Akwa, Y.; Pagenstecher, A., and Asensio, V. C. Transgenic models to study the actions of cytokines in the central nervous system. Neuroimmunomodulation. 1998 May-Aug 31; 5(3-4):126-35.

Casaccia-Bonnefil, P. Cell death in the oligodendrocyte lineage: a molecular perspective of life/death decisions in development and disease. Glia. 2000 Jan 15; 29(2):124-35.

Chen, K. S.; Masliah, E.; Mallory, M., and Gage, F. H. Synaptic loss in cognitively impaired aged rats is ameliorated by chronic human nerve growth factor infusion. Neuroscience. 1995 Sep; 68(1):19-27.

Chiang, C. S.; Stalder, A.; Samimi, A., and Campbell, I. L. Reactive gliosis as a consequence of interleukin-6 expression in the brain: studies in transgenic mice. Dev Neurosci. 1994; 16(3-4):212-21.

Coetzee, T.; Fujita, N.; Dupree, J.; Shi, R.; Blight, A.; Suzuki, K.; Suzuki, K., and Popko, B. Myelination in the absence of galactocerebroside and sulfatide: normal structure with abnormal function and regional instability. Cell. 1996 Jul 26; 86(2):209-19.

Conradi, S. Ultrastructure and distribution of neuronal and glial elements on the motoneuron surface in the lumbosacral spinal cord of the adult cat. Acta Physiol Scand Suppl. 1969; 332:5-48.

Conradi, S. and Ronnevi, L. O. Spontaneous elimination of synapses on cat spinal motoneurons after birth: do half of the synapses on the cell bodies disappear? Brain Res. 1975 Jul 18; 92(3):505-10.

Coons AH. Fluorescent antibody methods. General cytochemical methods. Academic press; 1958; pp. 399-422.

Cooper, A. J. and Kristal, B. S. Multiple roles of glutathione in the central nervous system. Biol Chem. 1997 Aug; 378(8):793-802.

Cooper, J. D. and Sofroniew, M. V. Increased vulnerability of septal cholinergic neurons to partial loss of target neurons in aged rats. Neuroscience. 1996 Nov; 75(1):29-35.

Corneliuson, O.; Berthold, C. H.; Persson, H., and Fredman, P. Aspects of the protein and the lipid composition of myelinoid Marchi- positive bodies from mammalian spinal cord. Neurochem Res. 1988 Dec; 13(12):1149-56.

Cowen, T. and Gavazzi, I. Plasticity in adult and ageing sympathetic neurons. Prog Neurobiol. 1998 Feb; 54(3):249-88.

Cullheim, S.; Kellerth, J. O., and Conradi, S. Evidence for direct synaptic interconnections between cat spinal alpha- motoneurons via the recurrent axon collaterals: a morphological study using intracellular injection of horseradish peroxidase. Brain Res. 1977 Aug 19; 132(1):1-10.

Dagerlind, Å.; Friberg, K.; Bean, A. J., and Hökfelt, T. Sensitive mRNA detection using unfixed tissue: combined radioactive and non-radioactive in situ hybridization histochemistry. Histochemistry. 1992 Aug; 98(1):39-49.

de la Asuncion, J. G.; Millan, A.; Pla, R.; Bruseghini, L.; Esteras, A.; Pallardo, F. V.; Sastre, J., and Vina, J. Mitochondrial glutathione oxidation correlates with age-associated oxidative damage to mitochondrial DNA. FASEB J. 1996 Feb; 10(2):333-8.

De Simone, R.; Levi, G., and Aloisi, F. Interferon gamma gene expression in rat central nervous system glial cells. Cytokine. 1998 Jun; 10(6):418-22.

de Waegh, S. M.; Lee, V. M., and Brady, S. T. Local modulation of neurofilament phosphorylation, axonal caliber, and slow axonal transport by myelinating Schwann cells. Cell. 1992 Feb 7; 68(3):451-63.

Dell'Albani, P.; Kahn, M. A.; Cole, R.; Condorelli, D. F.; Giuffrida-Stella, A. M., and de Vellis, J. Oligodendroglial survival factors, PDGF-AA and CNTF, activate similar JAK/STAT signaling pathways. J Neurosci Res. 1998 Oct 15; 54(2):191-205.

Dickson, D. W.; Wertkin, A.; Kress, Y.; Ksiezak-Reding, H., and Yen, S. H. Ubiquitin immunoreactive structures in normal human brains. Distribution and developmental aspects. Lab Invest. 1990 Jul; 63(1):87-99.

Dringen, R. Metabolism and functions of glutathione in brain. Prog Neurobiol. 2000 Dec; 62(6):649-71.

Dubois-Dalcq, M. Regeneration of oligodendrocytes and myelin. Trends Neurosci. 1995 Jul; 18(7):289-91.

Ernsberger, U.; Patzke, H., and Rohrer, H. The developmental expression of choline acetyltransferase (ChAT) and the neuropeptide VIP in chick sympathetic neurons: evidence for different regulatory events in cholinergic differentiation. Mech Dev. 1997 Nov; 68(1-2):115-26.

Ewbank, J. J.; Barnes, T. M.; Lakowski, B.; Lussier, M.; Bussey, H., and Hekimi, S. Structural and functional conservation of the Caenorhabditis elegans timing gene clk-1. Science. 1997 Feb 14; 275(5302):980-3.

Feldman, M. L. and Peters, A. Ballooning of myelin sheaths in normally aged macaques. J Neurocytol. 1998 Aug; 27(8):605-14.

Ferrándiz, M. L.; Martinez, M.; De Juan, E.; Diez, A.; Bustos, G., and Miquel, J. Impairment of mitochondrial oxidative phosphorylation in the brain of aged mice. Brain Res. 1994 May 2; 644(2):335-8.

Finkel, T. and Holbrook, N. J. Oxidants, oxidative stress and the biology of ageing. Nature. 2000 Nov 9; 408(6809):239-47.

Flanders, K. C.; Ren, R. F., and Lippa, C. F. Transforming growth factor-betas in neurodegenerative disease. Prog Neurobiol. 1998 Jan; 54(1):71-85.

Fonnum, F. Glutamate: a neurotransmitter in mammalian brain. J Neurochem. 1984 Jan; 42(1):1-11.

Forster, M. J.; Dubey, A.; Dawson, K. M.; Stutts, W. A.; Lal, H., and Sohal, R. S. Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. Proc Natl Acad Sci U S A. 1996 May 14; 93(10):4765-9.

Franklin, R. J. and Hinks, G. L. Understanding CNS remyelination: clues from developmental and regeneration biology. J Neurosci Res. 1999 Oct 15; 58(2):207-13.

Franson, P. and Ronnevi, L. O. Myelin breakdown and elimination in the posterior funiculus of the adult cat after dorsal rhizotomy: a light and electron microscopic qualitative and quantitative study. J Comp Neurol. 1984 Feb 10; 223(1):138-51.

Fujisawa, K. Study of axonal dystrophy. III. Posterior funiculus and posterior column of ageing and old rats. Acta Neuropathol (Berl). 1988; 76(2):115-27.

Fujisawa, K. and Shiraki, H. Study of axonal dystrophy. I. Pathology of the neuropil of the gracile and the cuneate nuclei in ageing and old rats: a stereological study. Neuropathol Appl Neurobiol. 1978 Jan-Feb 28; 4(1):1-20.

Fujisawa, K. and Shiraki, H. Study of axonal dystrophy. II Dystrophy and atrophy of the presynaptic boutons: a dual pathology. Neuropathol Appl Neurobiol. 1980 Sep-Oct 31; 6(5):387-98.

Gard, A. L.; Solodushko, V. G.; Waeg, G., and Majic, T. 4-Hydroxynonenal, a lipid peroxidation byproduct of spinal cord injury, is cytotoxic for oligodendrocyte progenitors and inhibits their responsiveness to PDGF. Microsc Res Tech. 2001 Mar 15; 52(6):709-18.

Gavazzi, I.; Andrews, T. J.; Thrasivoulou, C., and Cowen, T. Influence of target tissues on their innervation in old age: a transplantation study. Neuroreport. 1992 Aug; 3(8):717-20.

Gilmore, S. A. Spinal nerve root degeneration in aging laboratory rats: a light microscopic study. Anat Rec. 1972 Oct; 174(2):251-7.

Giordana, M. T.; Richiardi, P.; Trevisan, E.; Boghi, A., and Palmucci, L. Abnormal ubiquitination of axons in normally myelinated white matter in multiple sclerosis brain. Neuropathol Appl Neurobiol. 2002 Feb; 28(1):35-41.

Giusto, N. M.; Roque, M. E., and Ilincheta de Boschero, M. G. Effects of aging on the content, composition and synthesis of sphingomyelin in the central nervous system. Lipids. 1992 Nov; 27(11):835-9.

Glick, R. and Bondareff, W. Loss of synapses in the cerebellar cortex of the senescent rat. J Gerontol. 1979 Nov; 34(6):818-22.

Goss, J. R.; Finch, C. E., and Morgan, D. G. Age-related changes in glial fibrillary acidic protein mRNA in the mouse brain. Neurobiol Aging. 1991 Mar-Apr 30; 12(2):165-70.

Gou, J. P.; Eyer, J., and Leterrier, J. F. Progressive hyperphosphorylation of neurofilament heavy subunits with aging: possible involvement in the mechanism of neurofilament accumulation. Biochem Biophys Res Commun. 1995 Oct 4; 215(1):368-76.

Guarente, L. and Kenyon, C. Genetic pathways that regulate ageing in model organisms. Nature. 2000 Nov 9; 408(6809):255-62.

Guidry, G. and Landis, S. C. Target-dependent development of the vesicular acetylcholine transporter in rodent sweat gland innervation. Dev Biol. 1998 Jul 15; 199(2):175-84.

Hackney, C. M.; Osen, K. K.; Ottersen, O. P.; Storm-Mathisen, J., and Manjaly, G. Immunocytochemical evidence that glutamate is a neurotransmitter in the cochlear nerve: a quantitative study in the guinea-pig anteroventral cochlear nucleus. Eur J Neurosci. 1996 Jan; 8(1):79-91.

Harman, D. The aging process. Proc Natl Acad Sci U S A. 1981 Nov; 78(11):7124-8.

Haydon, P. G. Neuroglial networks: neurons and glia talk to each other. Curr Biol. 2000 Oct 5; 10(19):R712-4.

Hayflick, L. The future of ageing. Nature. 2000 Nov 9; 408(6809):267-9.

Hellström, J.; Arvidsson, U.; Elde, R.; Cullheim, S., and Meister, B. Differential expression of nerve terminal protein isoforms in VAChT- containing varicosities of the spinal cord ventral horn. J Comp Neurol. 1999 Sep 6; 411(4):578-90.

Hertz, L.; Dringen, R.; Schousboe, A., and Robinson, S. R. Astrocytes: glutamate producers for neurons. J Neurosci Res. 1999 Aug 15; 57(4):417-28.

Hildebrand, C. Presence of Marchi-positive myelinoid bodies in the spinal cord white matter of some vertebrate species. J Morphol. 1977 Jul; 153(1):1-21.

Hildebrand, C.; Remahl, S.; Persson, H., and Bjartmar, C. Myelinated nerve fibres in the CNS. Prog Neurobiol. 1993 Mar; 40(3):319-84.

Hinks, G. L. and Franklin, R. J. Delayed changes in growth factor gene expression during slow remyelination in the CNS of aged rats. Mol Cell Neurosci. 2000 Nov; 16(5):542-56.

Hinks, G. L. and Franklin, R. J. Distinctive patterns of PDGF-A, FGF-2, IGF-I, and TGF-beta1 gene expression during remyelination of experimentally-induced spinal cord demyelination. Mol Cell Neurosci. 1999 Aug; 14(2):153-68.

Hjelle, O. P.; Chaudhry, F. A., and Ottersen, O. P. Antisera to glutathione: characterization and immunocytochemical application to the rat cerebellum. Eur J Neurosci. 1994 May 1; 6(5):793-804.

Hökfelt, T.; Phillipson, O., and Goldstein, M. Evidence for a dopaminergic pathway in the rat descending from the A11 cell group to the spinal cord. Acta Physiol Scand. 1979 Dec; 107(4):393-5.

Jacob, J. M. Fast axonal transport rates are unchanged in 6- and 24-month F344 rats. Brain Res. 1995 Nov 13; 699(1):154-6.

Jain, A.; Mårtensson, J.; Stole, E.; Auld, P. A., and Meister, A. Glutathione deficiency leads to mitochondrial damage in brain. Proc Natl Acad Sci U S A. 1991 Mar 1; 88(5):1913-7.

Jean, I.; Allamargot, C.; Barthelaix-Pouplard, A., and Fressinaud, C. Axonal lesions and PDGF-enhanced remyelination in the rat corpus callosum after lysolecithin demyelination. Neuroreport. 2002 Apr 16; 13(5):627-31.

Jellinger, K. and Jirásek, A. Neuroaxonal dystrophy in man: character and natural history. Acta Neuropathol (Berl). 1971; 5:Suppl 5:3-16.

Johansson, C. S.; Stenström, M., and Hildebrand, C. Target influence on aging of myelinated sensory nerve fibres. Neurobiol Aging. 1996 Jan-Feb 28; 17(1):61-6.

Johnson, G. D. and Nogueira Araujo, G. M. A simple method of reducing the fading of immunofluorescence during microscopy. J Immunol Methods. 1981; 43(3):349-50.

Johnson, H.; Hökfelt, T., and Ulfhake, B. Expression of p75(NTR), trkB and trkC in nonmanipulated and axotomized motoneurons of aged rats. Brain Res Mol Brain Res. 1999 May 21; 69(1):21-34.

Johnson, H.; Mossberg, K.; Arvidsson, U.; Piehl, F.; Hökfelt, T., and Ulfhake, B. Increase in alpha-CGRP and GAP-43 in aged motoneurons: a study of peptides, growth factors, and ChAT mRNA in the lumbar spinal cord of senescent rats with symptoms of hindlimb incapacities. J Comp Neurol. 1995 Aug 14; 359(1):69-89.

Johnson, H.; Ulfhake, B.; Dagerlind, Å.; Bennett, G. W.; Fone, K. C., and Hökfelt, T. The serotoninergic bulbospinal system and brainstem-spinal cord content of serotonin-, TRH-, and substance P-like immunoreactivity in the aged rat with special reference to the spinal cord motor nucleus. Synapse. 1993 Sep; 15(1):63-89.

Kahn, M. A.; Huang, C. J.; Caruso, A.; Barresi, V.; Nazarian, R.; Condorelli, D. F., and de Vellis, J. Ciliary neurotrophic factor activates JAK/Stat signal transduction cascade and induces transcriptional expression of glial fibrillary acidic protein in glial cells. J Neurochem. 1997 Apr; 68(4):1413-23.

Karlsson, M. and Hildebrand, C. Postnatal development and aging of the rat ventral root L5: electron microscopic and immunohistochemical studies. J Comp Neurol. 1996 Jan 8; 364(2):211-8.

Kazui, H. and Fujisawa, K. Radiculoneuropathy of ageing rats: a quantitative study. Neuropathol Appl Neurobiol. 1988 MarApr 30; 14(2):137-56.

Keller, J. N.; Mark, R. J.; Bruce, A. J.; Blanc, E.; Rothstein, J. D.; Uchida, K.; Waeg, G., and Mattson, M. P. 4-Hydroxynonenal, an aldehydic product of membrane lipid peroxidation, impairs glutamate transport and mitochondrial function in synaptosomes. Neuroscience. 1997 Oct; 80(3):685-96.

Kikuchi, T.; Mukoyama, M.; Yamazaki, K., and Moriya, H. Axonal degeneration of ascending sensory neurons in gracile axonal dystrophy mutant mouse. Acta Neuropathol (Berl). 1990; 80(2):145-51.

Kimura, K. D.; Tissenbaum, H. A.; Liu, Y., and Ruvkun, G. daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science. 1997 Aug 15; 277(5328):942-6.

Kirkwood, T. B. and Austad, S. N. Why do we age? Nature. 2000 Nov 9; 408(6809):233-8.

Klebanov, S. and Harrison, D. E. Optimizing detection of QTLs retarding aging: choice of statistical model and animal requirements. Mech Ageing Dev. 2002 Jan; 123(2-3):131-44.

Klugmann, M.; Schwab, M. H.; Puhlhofer, A.; Schneider, A.; Zimmermann, F.; Griffiths, I. R., and Nave, K. A. Assembly of CNS myelin in the absence of proteolipid protein. Neuron. 1997 Jan; 18(1):59-70.

Knox, C. A.; Kokmen, E., and Dyck, P. J. Morphometric alteration of rat myelinated fibers with aging. J Neuropathol Exp Neurol. 1989 Mar; 48(2):119-39.

Koller, W. C.; Glatt, S. L., and Fox, J. H. Senile gait. A distinct neurologic entity. Clin Geriatr Med. 1985 Aug; 1(3):661-9.

Komiya, Y. Slowing with age of the rate of slow axonal flow in bifurcating axons of rat dorsal root ganglion cells. Brain Res. 1980 Feb 10; 183(2):477-80.

Kreutzberg, G. W. Microglia: a sensor for pathological events in the CNS. Trends Neurosci. 1996 Aug; 19(8):312-8.

Krinke, G. Spinal radiculoneuropathy in aging rats: demyelination secondary to neuronal dwindling? Acta Neuropathol (Berl). 1983; 59(1):63-9.

Kuro-o, M.; Matsumura, Y.; Aizawa, H.; Kawaguchi, H.; Suga, T.; Utsugi, T.; Ohyama, Y.; Kurabayashi, M.; Kaname, T.; Kume, E.; Iwasaki, H.; Iida, A.; Shiraki-Iida, T.; Nishikawa, S.; Nagai, R., and Nabeshima, Y. I. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature. 1997 Nov 6; 390(6655):45-51.

Lamberts, S. W.; van den Beld, A. W., and van der Lely, A. J. The endocrinology of aging. Science. 1997 Oct 17; 278(5337):419-24.

Laping, N. J.; Teter, B.; Nichols, N. R.; Rozovsky, I., and Finch, C. E. Glial fibrillary acidic protein: regulation by hormones, cytokines, and growth factors. Brain Pathol. 1994 Jul; 4(3):259-75.

Lawson, L. J.; Frost, L.; Risbridger, J.; Fearn, S., and Perry, V. H. Quantification of the mononuclear phagocyte response to Wallerian degeneration of the optic nerve. J Neurocytol. 1994 Dec; 23(12):729-44.

Leist, M. and Nicotera, P. Apoptosis, excitotoxicity, and neuropathology. Exp Cell Res. 1998 Mar 15; 239(2):183-201.

Leßman, V. Neurotrophin-dependent modulation of glutamatergic synaptic transmission in the mammalian CNS. Gen Pharmacol. 1998 Nov; 31(5):667-74.

Li, W.; Ochalski, P. A.; Brimijoin, S.; Jordan, L. M., and Nagy, J. I. C-terminals on motoneurons: electron microscope localization of cholinergic markers in adult rats and antibody-induced depletion in neonates. Neuroscience. 1995 Apr; 65(3):879-91.

Liedtke, W.; Edelmann, W.; Bieri, P. L.; Chiu, F. C.; Cowan, N. J.; Kucherlapati, R., and Raine, C. S. GFAP is necessary for the integrity of CNS white matter architecture and long-term maintenance of myelination. Neuron. 1996 Oct; 17(4):607-15.

Lin, Y. J.; Seroude, L., and Benzer, S. Extended life-span and stress resistance in the Drosophila mutant methuselah. Science. 1998 Oct 30; 282(5390):943-6.

Lindå, H.; Cullheim, S., and Risling, M. A light and electron microscopic study of intracellularly HRP-labeled lumbar motoneurons after intramedullary axotomy in the adult cat. J Comp Neurol. 1992 Apr 8; 318(2):188-208.

Lindsey, J. D.; Landfield, P. W., and Lynch, G. Early onset and topographical distribution of hypertrophied astrocytes in hippocampus of aging rats: a quantitative study. J Gerontol. 1979 Sep; 34(5):661-71.

Linker, R. A.; Maurer, M.; Gaupp, S.; Martini, R.; Holtmann, B.; Giess, R.; Rieckmann, P.; Lassmann, H.; Toyka, K. V.; Sendtner, M., and Gold, R. CNTF is a major protective factor in demyelinating CNS disease: a neurotrophic cytokine as modulator in neuroinflammation. Nat Med. 2002 Jun; 8(6):620-4.

Liu, H.; Wang, H.; Sheng, M.; Jan, L. Y.; Jan, Y. N., and Basbaum, A. I. Evidence for presynaptic N-methyl-D-aspartate autoreceptors in the spinal cord dorsal horn. Proc Natl Acad Sci U S A. 1994 Aug 30; 91(18):8383-7.

Loughlin, S. E.; Foote, S. L., and Bloom, F. E. Efferent projections of nucleus locus coeruleus: topographic organization of cells of origin demonstrated by three-dimensional reconstruction. Neuroscience. 1986 Jun; 18(2):291-306.

Lukoyanov, N. V.; Andrade, J. P.; Dulce Madeira, M., and Paula-Barbosa, M. M. Effects of age and sex on the water maze performance and hippocampal cholinergic fibers in rats. Neurosci Lett. 1999 Jul 16; 269(3):141-4.

Markey, K. A.; Kondo, H.; Shenkman, L., and Goldstein, M. Purification and characterization of tyrosine hydroxylase from a clonal pheochromocytoma cell line. Mol Pharmacol. 1980 Jan; 17(1):79-85.

Martin, G. M. and Oshima, J. Lessons from human progeroid syndromes. Nature. 2000 Nov 9; 408(6809):263-6.

Martin, G. R. and Baker, G. T. Aging and the aged: Theories of aging and life extension. Encyclopedia of bioethics. New York: MacMillan; 1993.

Masoro, E. J. Caloric restriction and aging: an update. Exp Gerontol. 2000 May; 35(3):299-305.

Masuoka, D.T.; Jonsson, G. and Finch, C.E. Aging and unusual catecholamine-containing structures in the mouse brain. Brain Res. 1979; 169: 335-341.

Mattson, M. P.; Keller, J. N., and Begley, J. G. Evidence for synaptic apoptosis. Exp Neurol. 1998 Sep; 153(1):35-48.

McClearn, G. E.; Johansson, B.; Berg, S.; Pedersen, N. L.; Ahern, F.; Petrill, S. A., and Plomin, R. Substantial genetic influence on cognitive abilities in twins 80 or more years old. Science. 1997 Jun 6; 276(5318):1560-3.

McQuarrie, I. G.; Brady, S. T., and Lasek, R. J. Retardation in the slow axonal transport of cytoskeletal elements during maturation and aging. Neurobiol Aging. 1989 Jul-1989 Aug 31; 10(4):359-65.

McWilliams, J. R. and Lynch, G. Synaptic density and axonal sprouting in rat hippocampus: stability in adulthood and decline in late adulthood. Brain Res. 1984 Feb 27; 294(1):152-6.

Meister, A. Mitochondrial changes associated with glutathione deficiency. Biochim Biophys Acta. 1995 May 24; 1271(1):35-42.

Michaelis, E. K. Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. Prog Neurobiol. 1998 Mar; 54(4):369-415.

Migliaccio, E.; Giorgio, M.; Mele, S.; Pelicci, G.; Reboldi, P.; Pandolfi, P. P.; Lanfrancone, L., and Pelicci, P. G. The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature. 1999 Nov 18; 402(6759):309-13.

Morrison, J. H. and Hof, P. R. Life and death of neurons in the aging brain. Science. 1997 Oct 17; 278(5337):412-9.

Müller, M.; Marti, T., and Kritz, S. Improved structural preservation by freeze substitution. Electron Microscopy. Proc. 7th Eur. Congr. Electron Microscopy 2. 1980; p. 720.

Nadon, N. L. and Duncan, I. D. Gene expression and oligodendrocyte development in the myelin deficient rat. J Neurosci Res. 1995 May 1; 41(1):96-104.

Nagao, M.; Misawa, H.; Kato, S., and Hirai, S. Loss of cholinergic synapses on the spinal motor neurons of amyotrophic lateral sclerosis. J Neuropathol Exp Neurol. 1998 Apr; 57(4):329-33.

Nagy, J. I.; Yamamoto, T., and Jordan, L. M. Evidence for the cholinergic nature of C-terminals associated with subsurface cisterns in alpha-motoneurons of rat. Synapse. 1993 Sep; 15(1):17-32.

Nichols, N. R. Glial responses to steroids as markers of brain aging. J Neurobiol. 1999 Sep 15; 40(4):585-601.

Nichols, N. R.; Day, J. R.; Laping, N. J.; Johnson, S. A., and Finch, C. E. GFAP mRNA increases with age in rat and human brain. Neurobiol Aging. 1993 Sep-1993 Oct 31; 14(5):421-9.

Norton, W. E. and Cammer, W. Isolation and characterization of myelin. In: Myelin. Ed. P. Morell. New York: Plenum Press; 1984; pp. 147-80.

Ogura, K.; Ogawa, M., and Yoshida, M. Effects of ageing on microglia in the normal rat brain: immunohistochemical observations. Neuroreport. 1994 Jun 2; 5(10):1224-6.

Olson, L.; Nyström. B. and Seiger, Å. Monoamine fluorescence histochemistry of human post mortem brain. Brain Res. 1973 63: 231-247.

Paxinos G. The rat nervous system. 2nd ed. Academic Press; 1995.

Pennisi, E. Do fateful circles of DNA cause cells to grow old? Science. 1998 Jan 2; 279(5347):34.

Perry, V. H.; Matyszak, M. K., and Fearn, S. Altered antigen expression of microglia in the aged rodent CNS. Glia. 1993 Jan; 7(1):60-7.

Persson, H. and Berthold, C. H. Cellular relationships of paranodal Marchi-positive bodies studied with monoclonal antibodies against partially degraded CNS myelin fragments. J Neurocytol. 1991 Apr; 20(4):277-89.

Peters, A.; Josephson, K., and Vincent, S. L. Effects of aging on the neuroglial cells and pericytes within area 17 of the rhesus monkey cerebral cortex. Anat Rec. 1991 Mar; 229(3):384-98.

Peters, A.; Leahu, D.; Moss, M. B., and McNally, K. J. The effects of aging on area 46 of the frontal cortex of the rhesus monkey. Cereb Cortex. 1994 Nov-1994 Dec 31; 4(6):621-35.

Pfrieger, F. W. and Barres, B. A. New views on synapse-glia interactions. Curr Opin Neurobiol. 1996 Oct; 6(5):615-21.

Piehl, F.; Arvidsson, U.; Johnson, H.; Cullheim, S.; Villar, M.; Dagerlind, Å.; Terenius, L.; Hökfelt, T., and Ulfhake, B. Calcitonin Gene-related Peptide (CGRP)-like Immunoreactivity and CGRP mRNA in Rat Spinal Cord Motoneurons after Different Types of Lesions. Eur J Neurosci. 1991; 3(8):737-757.

Platt, J. L. and Michael, A. F. Retardation of fading and enhancement of intensity of immunofluorescence by p-phenylenediamine. J Histochem Cytochem. 1983 Jun; 31(6):840-2.

Popko, B.; Corbin, J. G.; Baerwald, K. D.; Dupree, J., and Garcia, A. M. The effects of interferon-gamma on the central nervous system. Mol Neurobiol. 1997 Feb-Apr 30; 14(1-2):19-35.

Poulos, A. Very long chain fatty acids in higher animals--a review. Lipids. 1995 Jan; 30(1):1-14.

Pullen, A. H. and Sears, T. A. Trophism between C-type axon terminals and thoracic motoneurones in the cat. J Physiol. 1983 Apr; 337:373-88.

Raine, C. S. and Cross, A. H. Axonal dystrophy as a consequence of long-term demyelination. Lab Invest. 1989 May; 60(5):714-25.

Raivich, G.; Bohatschek, M.; Kloss, C. U.; Werner, A.; Jones, L. L., and Kreutzberg, G. W. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. Brain Res Brain Res Rev. 1999 Jul; 30(1):77-105.

Rapp, P. R. and Gallagher, M. Preserved neuron number in the hippocampus of aged rats with spatial learning deficits. Proc Natl Acad Sci U S A. 1996 Sep 3; 93(18):9926-30.

Reilly, J. F.; Maher, P. A., and Kumari, V. G. Regulation of astrocyte GFAP expression by TGF-beta1 and FGF-2. Glia. 1998 Feb; 22(2):202-10.

Ridet, J. L.; Malhotra, S. K.; Privat, A., and Gage, F. H. Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci. 1997 Dec; 20(12):570-7.

Ronnevi, L. O. Origin of the glial processes responsible for the spontaneous postnatal phagocytosis of boutons on cat spinal motoneurons. Cell Tissue Res. 1978 May 29; 189(2):203-17.

Rosenberg. M.E. and Silkensen, J. Clusterin: physiologic and patophysiologic considerations. Int. J. Biochem. Cell Biol. 1995; 27: 633-45.

Roth, G. S.; Ingram, D. K., and Lane, M. A. Calorie restriction in primates: will it work and how will we know? J Am Geriatr Soc. 1999 Jul; 47(7):896-903.

Saito, S.; Kobayashi, S.; Ohashi, Y.; Igarashi, M.; Komiya, Y., and Ando, S. Decreased synaptic density in aged brains and its prevention by rearing under enriched environment as revealed by synaptophysin contents. J Neurosci Res. 1994 Sep 1; 39(1):57-62.

Sastre, J.; Pallardo, F. V., and Vina, J. Mitochondrial oxidative stress plays a key role in aging and apoptosis. IUBMB Life. 2000 May; 49(5):427-35.

Sato, Y.; Kimura, M.; Yasuda, C.; Nakano, Y.; Tomita, M.; Kobata, A., and Endo, T. Evidence for the presence of major peripheral myelin glycoprotein P0 in mammalian spinal cord and a change of its glycosylation state during aging. Glycobiology. 1999 Jul; 9(7):655-60.

Schinder, A. F. and Poo, M. The neurotrophin hypothesis for synaptic plasticity. Trends Neurosci. 2000 Dec; 23(12):639-45.

Schmidt, R. E.; Dorsey, D.; Parvin, C. A.; Beaudet, L. N.; Plurad, S. B., and Roth, K. A. Dystrophic axonal swellings develop as a function of age and diabetes in human dorsal root ganglia. J Neuropathol Exp Neurol. 1997 Sep; 56(9):1028-43.

Schuman, E. M. Neurotrophin regulation of synaptic transmission. Curr Opin Neurobiol. 1999 Feb; 9(1):105-9.

Seitelberger, F. Neuropathological conditions related to neuroaxonal dystrophy. Acta Neuropathol (Berl). 1971; 5:Suppl 5:17-29.

Sharma, A. K.; Bajada, S., and Thomas, P. K. Age changes in the tibial and plantar nerves of the rat. J Anat. 1980 Mar; 130(2):417-28.

Sheffield, L. G. and Berman, N. E. Microglial expression of MHC class II increases in normal aging of nonhuman primates. Neurobiol Aging. 1998 Jan-1998 Feb 28; 19(1):47-55.

Shields, S. A.; Gilson, J. M.; Blakemore, W. F., and Franklin, R. J. Remyelination occurs as extensively but more slowly in old rats compared to young rats following gliotoxin-induced CNS demyelination. Glia. 1999 Oct; 28(1):77-83.

Sim, F. J.; Hinks, G. L., and Franklin, R. J. The re-expression of the homeodomain transcription factor Gtx during remyelination of experimentally induced demyelinating lesions in young and old rat brain. Neuroscience. 2000; 100(1):131-9.

Šimić, G.; Kostovic, I.; Winblad, B., and Bogdanovic, N. Volume and number of neurons of the human hippocampal formation in normal aging and Alzheimer's disease. J Comp Neurol. 1997 Mar 24; 379(4):482-94.

Sloane, J. A.; Hollander, W.; Moss, M. B.; Rosene, D. L., and Abraham, C. R. Increased microglial activation and protein nitration in white matter of the aging monkey. Neurobiol Aging. 1999 Jul-1999 Aug 31; 20(4):395-405.

Soffié, M.; Hahn, K.; Terao, E., and Eclancher, F. Behavioural and glial changes in old rats following environmental enrichment. Behav Brain Res. 1999 May; 101(1):37-49.

Sohal, R. S.; Agarwal, S.; Candas, M.; Forster, M. J., and Lal, H. Effect of age and caloric restriction on DNA oxidative damage in different tissues of C57BL/6 mice. Mech Ageing Dev. 1994 Oct 20; 76(2-3):215-24.

Sohal, R. S. and Weindruch, R. Oxidative stress, caloric restriction, and aging. Science. 1996 Jul 5; 273(5271):59-63.

Soong, N. W.; Hinton, D. R.; Cortopassi, G., and Arnheim, N. Mosaicism for a specific somatic mitochondrial DNA mutation in adult human brain. Nat Genet. 1992 Dec; 2(4):318-23.

Staines, W. A.; Meister, B.; Melander, T.; Nagy, J. I., and Hokfelt, T. Three-color immunofluorescence histochemistry allowing triple labeling within a single section. J Histochem Cytochem. 1988 Feb; 36(2):145-51.

Steinbusch, H. W.; Verhofstad, A. A., and Joosten, H. W. Localization of serotonin in the central nervous system by immunohistochemistry: description of a specific and sensitive technique and some applications. Neuroscience. 1978; 3(9):811-9.

Stoll, G. and Jander, S. The role of microglia and macrophages in the pathophysiology of the CNS. Prog Neurobiol. 1999 Jun; 58(3):233-47.

Sugaya, K.; Chouinard, M.; Greene, R.; Robbins, M.; Personett, D.; Kent, C.; Gallagher, M., and McKinney, M. Molecular indices of neuronal and glial plasticity in the hippocampal formation in a rodent model of age-induced spatial learning impairment. J Neurosci. 1996 May 15; 16(10):3427-43.

Suzuki, Y. and Suu, S. Spheroids (axonal dystrophy) in the central nervous system of the dog. II. Electron microscopic observations. Nippon Juigaku Zasshi. 1978 Oct; 40(5):549-58.

Svensson, M. and Aldskogius, H. Regeneration of hypoglossal nerve axons following blockade of the axotomy-induced microglial cell reaction in the rat. Eur J Neurosci. 1993 Jan 1; 5(1):85-94.

Swanson, L. W. The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. Brain Res Bull. 1982 Jul-1982 Dec 31; 9(1-6):321-53.

Tower, J. Aging mechanisms in fruit files. Bioessays. 1996 Oct; 18(10):799-807.

Turrin, N. P. and Plata-Salamán, C. R. Cytokine-cytokine interactions and the brain. Brain Res Bull. 2000 Jan 1; 51(1):3-9.

Uchida, A.; Yorifuji, H.; Lee, V. M.; Kishimoto, T., and Hisanaga, S. Neurofilaments of aged rats: the strengthened interneurofilament interaction and the reduced amount of NF-M. J Neurosci Res. 1999 Oct 15; 58(2):337-48.

Uemura, E. Age-related changes in prefrontal cortex of Macaca mulatta: synaptic density. Exp Neurol. 1980 Jul; 69(1):164-72.

Ulfhake, B.; Bergman, E.; Edström, E.; Fundin, B. T.; Johnson, H.; Kullberg, S., and Ming, Y. Regulation of neurotrophin signaling in aging sensory and motoneurons: dissipation of target support? Mol Neurobiol. 2000 Jun; 21(3):109-35.

Ulfhake, B.; Bergman, E., and Fundin, B. T. Impairment of peripheral sensory innervation in senescence. Autonomic Neuroscience Basic and Clinical. 2002; 96:43-49.

Ullian, E. M.; Sapperstein, S. K.; Christopherson, K. S., and Barres, B. A. Control of synapse number by glia. Science. 2001 Jan 26; 291(5504):657-61.

Van den Bergh, P.; Kelly, J. J. Jr; Soule, N.; Munsat, T. L.; Jackson, I. M., and Lechan, R. M. Spinal cord TRH deficiency is associated with incomplete recovery of denervated muscle in the rat. Neurology. 1988 Mar; 38(3):452-8.

Van den Bergh, P.; Octave, J. N., and Lechan, R. M. Muscle denervation increases thyrotropin-releasing hormone (TRH) biosynthesis in the rat medullary raphe. Brain Res. 1991 Dec 6; 566(1-2):219-24.

van Lookeren Campagne, M.; Oestreicher, A. B.; van der Krift, T. P.; Gispen, W. H., and Verkleij, A. J. Freeze-substitution and Lowicryl HM20 embedding of fixed rat brain: suitability for immunogold ultrastructural localization of neural antigens. J Histochem Cytochem. 1991 Sep; 39(9):1267-79.

van Luijtelaar, M. G.; Steinbusch, H. W., and Tonnaer, J. A. Aberrant morphology of serotonergic fibers in the forebrain of the aged rat. Neurosci Lett. 1988 Dec 19; 95(1-3):93-6.
van Luijtelaar, M. G.; Tonnaer, J. A., and Steinbusch, H. W. Aging of the serotonergic system in the rat forebrain: an immunocytochemical and neurochemical study. Neurobiol Aging. 1992 Mar-1992 Apr 30; 13(2):201-15.
van Luijtelaar, M. G.; Wouterlood, F. G.; Tonnaer, J. A., and Steinbusch, H. W. Ultrastructure of aberrant serotonin-immunoreactive fibers in the caudate putamen complex of the aged rat. Synapse. 1991 Jul; 8(3):162-8.
van Steenis, G. and Kroes, R. Changes in the nervous system and musculature of old rats. Vet Pathol. 1971; 8(4):320-32.
Van Wagoner, N. J. and Benveniste, E. N. Interleukin-6 expression and regulation in astrocytes. J Neuroimmunol. 1999 Dec; 100(1-2):124-39.
Verhofstad, A. A. and Jonsson, G. Immunohistochemical and neurochemical evidence for the presence of serotonin in the adrenal medulla of the rat. Neuroscience. 1983 Dec; 10(4):1443-53.
Virgili, M.; Monti, B.; Polazzi, E.; Angiolini, G., and Contestabile, A. Topography of neurochemical alterations in the CNS of aged rats. Int J Dev Neurosci. 2001 Feb; 19(1):109-16.
Vlassara, H.; Brownlee, M., and Cerami, A. Specific macrophage receptor activity for advanced glycosylation end products inversely correlates with insulin levels in vivo. Diabetes. 1988 Apr; 37(4):456-61.
Warner, H; Butler, RN; Sprott, RL, and Schneider, EL. Modern biological theories of aging. New York: Raven; 1987.
Weiss, M. D.; Hammer, J., and Quarles, R. H. Oligodendrocytes in aging mice lacking myelin-associated glycoprotein are dystrophic but not apoptotic. J Neurosci Res. 2000 Dec 15; 62(6):772-80.
Wickelgren, I. For the cortex, neuron loss may be less than thought. Science. 1996 Jul 5; 273(5271):48-50.
Wilson, M. A. and Molliver, M. E. Microglial response to degeneration of serotonergic axon terminals. Glia. 1994 May; 11(1):18-34.
Winter, C. G.; Saotome, Y.; Levison, S. W., and Hirsh, D. A role for ciliary neurotrophic factor as an inducer of reactive gliosis, the glial response to central nervous system injury. Proc Natl Acad Sci U S A. 1995 Jun 20; 92(13):5865-9.
Wolkow, C. A.; Kimura, K. D.; Lee, M. S., and Ruvkun, G. Regulation of C. elegans life-span by insulinlike signaling in the nervous system. Science. 2000 Oct 6; 290(5489):147-50.
Woodruff, R. H. and Franklin, R. J. Demyelination and remyelination of the caudal cerebellar peduncle of adult rats following stereotaxic injections of lysolecithin, ethidium bromide, and complement/anti-galactocerebroside: a comparative study. Glia. 1999 Feb 1; 25(3):216-28.
Yao, D. L.; West, N. R.; Bondy, C. A.; Brenner, M.; Hudson, L. D.; Zhou, J.; Collins, G. H., and Webster, H. D. Cryogenic spinal cord injury induces astrocytic gene expression of insulin-like growth factor I and insulin-like growth factor binding protein 2 during myelin regeneration. J Neurosci Res. 1995a Apr 1; 40(5):647-59.

Yao, D. L.; Liu, X.; Hudson, L. D., and Webster, H. D. Insulin-like growth factor I treatment reduces demyelination and up- regulates gene expression of myelin-related proteins in experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 1995b Jun 20; 92(13):6190-4.

Ye, P. and D'Ercole, A. J. Insulin-like growth factor I protects oligodendrocytes from tumor necrosis factor-alpha-induced injury. Endocrinology. 1999 Jul; 140(7):3063-72.

Yin, X.; Crawford, T. O.; Griffin, J. W.; Tu, P.; Lee, V. M.; Li, C.; Roder, J., and Trapp, B. D. Myelin-associated glycoprotein is a myelin signal that modulates the caliber of myelinated axons. J Neurosci. 1998 Mar 15; 18(6):1953-62.

Young III WS. In situ hybridization histochemistry. Handbook of chemical neuroanatomy, Volume 8. Amsterdam: Elsevier Science publishers BV; 1990; pp. 481-512.

Zamboni, I. and De Martino, C. Buffered picric acid-formaldehyde: A new, rapid fixative for electron microscopy. Journal of Cell Biology . 1967; 35(148A).

Zhang, S. C.; Goetz, B. D.; Carre, J. L., and Duncan, I. D. Reactive microglia in dysmyelination and demyelination. Glia. 2001 Apr 15; 34(2):101-9.

Zielasek, J. and Hartung, H. P. Molecular mechanisms of microglial activation. Adv Neuroimmunol. 1996; 6(2):191-22.

Örnung, G.; Ottersen, O. P.; Cullheim, S., and Ulfhake, B. Distribution of glutamate-, glycine- and GABA-immunoreactive nerve terminals on dendrites in the cat spinal motor nucleus. Exp Brain Res. 1998 Feb; 118(4):517-32.

Örnung, G.; Shupliakov, O.; Lindå, H.; Ottersen, O. P.; Storm-Mathisen, J.; Ulfhake, B., and Cullheim, S. Qualitative and quantitative analysis of glycine- and GABA-immunoreactive nerve terminals on motoneuron cell bodies in the cat spinal cord: a postembedding electron microscopic study. J Comp Neurol. 1996 Feb 12; 365(3):413-26.