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Enriched Environmental Conditions Ameliorate Age-Dependent Alterations in the Somatosensory System, but Do Not Affect the Motor System of the Rat Spinal Cord

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Key Words
COX-2 · Degraded myelin basic protein · Lipofuscin · Nitrotyrosine

Abstract
At the behavioral level, old rats are known to show a number of age-related changes such as characteristic impairments in the sensorimotor system, which is most strikingly expressed in a walking impairment involving the hind limbs. We have previously shown that sensorimotor impairments involving the hind limbs are in part reversible following housing in an enriched environment. The question arises as to whether these effects are accompanied by cytological alterations in the spinal cord. During aging, we found an increase in cyclooxygenase with nitrotyrosine in the fasciculus gracilis and lateral funiculus. The somatosensory tracts and dorsal horn were more affected by myelin-degrading processes than the other parts of the spinal cord. The accumulation of lipofuscin seemed to depend on age, but independent of the environment. In contrast, the age-related calretinin immunoreactivity loss in the substantia gelatinosa from both the cervical and the lumbar enlargements was significantly reduced in stimulating environmental conditions. Morphologically, the lectin binding sites of the perineuronal nets seemed to be decreased. The results provide evidence that the somatosensory system of the spinal cord is affected by age-dependent alterations independently of the motor system.

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Introduction

We have previously shown that sensorimotor impairment in the hindlimb can be ameliorated by housing in enriched environmental conditions [1–4]. In the present study, we tried to find neural substrates in terms of age-dependent cytological alterations at the level of the spinal cord.

Non-pathological age-related impairments in the motor system have rarely been reported [5–7], whereas impaired sensory perception, including nociceptive perception, is a well-established stigma of aging [1, 8–11]. Age-related changes in nerve fibers from the human fasciculus gracilis have been reported recently [12]. A functional and structural decline in the neuromuscular system with aging has been recognized as a cause of impairment in physical performance in the elderly [13]. Muscle strength

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is a marker of physical performance. Tests on handgrip and knee extension in men have shown an evident decline in strength by the age of 65. A comparable age-related decline in peak force development had also been shown in hindlimb muscles of aged rats. Motor neurons and consequently motor units decrease with age [see ref. 14 for review]. Delbono [13] reviewed neural control of aging skeletal muscle, establishing that the reciprocal influences of neurons on gene expression in muscles as well as those of muscles on neurons were poorly understood. This begs the question as to whether sensorimotor impairment is accompanied or even caused by alterations in the spinal cord. Here, we studied cervical and lumbar enlargements in the spinal cord from 3-year-old rats comparing those housed under standard living conditions with those housed for the last 3 months of life under enriched environmental conditions. In these animals, expression of glial fibrillary acidic protein (GFAP), degraded myelin basic protein (dMBP), cyclooxygenase-2 (COX-2), nitrotyrosine (3-NT), calretinin, binding sites of both *Wisteria floribunda* agglutinin (WFA) and *Lycopersicon esculentum* agglutinin (LEA) as well as the accumulation of lipofuscin were studied semiquantitatively. Where semiquantitative analysis indicated significant differences, gray values were measured.

GFAP has been shown to be expressed by reactive astrocytes [15]. Recently, we reported a correlation between GFAP expression and lipofuscin accumulation in the cerebral cortex of aged rats [16]. Lipofuscin accumulation is regarded as a sign of normal aging [17].

Since gliosis could interfere with the glia-neuron interfaces in aged brain, we attempted to visualize these interfaces using WFA. WFA is known as a marker of glia-associated perineuronal nets that are identical to the glia-neuron interface or extracellular matrix [18]. Plastic changes in the extracellular space affect glia-neuron communication, the spatial relation of glial processes towards synapses and synaptic crosstalk [19]. Previous studies have shown that the neuronal microenvironment is not homogeneously distributed throughout the central nervous system, especially in the brain [20, 21]. High densities of WFA-labeled structures in the motor and primary sensory areas correspond to the relative resistance to neurofibrillary changes [22]. On the other hand, proteoglycans undergo structural alterations after focal cortical ischemia [23]. Blood vessels constitute another component of the neuronal microenvironment. Vascular changes are evident in the senescent mammalian brain. Loss of capillary endothelial cells has been reported [see ref. 24 for review]. We used LEA for endothelial cell visualization. Normal aging is affected by decreased blood flow in the whole cortex, associated with diminished metabolic activity [25, 26]. Age-related changes in cerebrovascular regulation are discussed as one of the more important factors, and COX-2 and 3-NT have been identified as mediators of functional hyperemia. COX-2 activity is a key player in the regulation of functional and pathology-related hyperemia [27, 28]. COX-2 is the inducible isoform of prostaglandin H synthetase. COX-1 is constitutive [29]. COX-2 activity is associated with the formation of radical oxygen species such as peroxynitrite and following 3-NT. Especially COX-2 metabolites have been identified as major neurotoxic mediators for ischemic damage of neurons and axons [30]. The structural protein, myelin basic protein, is degraded during axon demyelinization. The antibody against dMBP recognizes the degraded, but not normal myelin from Schwann cells and oligodendrocytes in the rat spinal cord [31].

Calcium-binding calretinin protein is present in the spinal cord, including sensory pathways, where it may play an important role in the regulation of cellular activity [32]. Calcium-binding protein content in interneurons could explain the relative resistance of interneurons to acute ischemia [33]. The authors reported selective neuronal vulnerability following mild focal brain ischemia, which is a thoroughly normal age-dependent event.

The present study combined these approaches in order to establish whether enriched environmental conditions may specifically prevent or reverse age-related degenerative processes in the spinal cord.

Materials and Methods

Experimental Animals

We used 32 male hybrid Fischer 344 × Brown Norway (FBNF1) rats. The care and use of the animals were approved by the University Animal Care Committee. The principles of laboratory animal care (NIH publication No. 86-23, revised in 1985) and the German law concerning the protection of animals were observed. Ten 1-year-old animals were used as adult controls. Ten animals served as aged controls and were kept their entire life under standard housing conditions until 36 months of age. Twelve animals were raised and kept under standard housing conditions until 33 months of age. Subsequently, the animals were kept under enriched conditions for 3 months. Groups of 5 rats were kept under standard housing conditions in standard type IV cages (54 × 33 × 18.5 cm, length × width × height). The dimensions of the cages containing the 'enriched environment' were 100 × 60 × 80 cm (length × width × height). The interior was changed weekly and consisted of large irregular-shaped extended polystyrene

blocks of variable size, cardboard boxes and several wooden ladders. Food pellets were supplied at variable locations to reinforce extensive explorative behavior. At 3 years of age (or 1 year, as appropriate), the rats were transcardially perfused with saline under ether anesthesia, followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The spinal cords were removed and postfixed.

Preparation of Spinal Cord Sections

From the spinal cords, 5 mm of the cervical and lumbar enlargements each were dissected and cryoprotected. Thirty-micrometer coronal cryosections were used for histochemistry, immunohistochemistry and lipofuscin visualization. For lipofuscin visualization by autofluorescence, the sections were mounted on slides, dried, embedded in Entellan (Merck, Darmstadt, Germany) and sealed with coverslips. The patterns of lipofuscin could be directly detected under UV light. For light-microscopic staining, neighboring sections were rinsed several times with PBS. Endogenous peroxidase was blocked by incubation in PBS containing 0.3% H_2O_2 for 30 min. Following three rinses with PBS (15 min each) and an overnight treatment with normal goat serum (10% in PBS), sections were incubated overnight at 4°C with the following monoclonal, primary antibodies: against GFAP (code Z334, DAKO, Hamburg, Germany) at a final dilution of 1:1,000, against COX-2 (1: 200, NatuTec, Frankfurt, Germany), against 3-NT (1:100, Alexis, Grünberg, Germany), against dMBP (1:1,000, Chemicon, Temecula, Calif., USA) or with the polyclonal, primary antibody against calretinin (1:1,000, Swant, Bellinzona, Switzerland) or, for lectin histochemistry, with biotinylated WFA (b-WFA, Sigma L-1766, Munich, Germany) at a concentration of 10 μg b-WFA/ml PBS containing 2% bovine serum albumin, and with biotinylated LEA (b-LEA, Sigma, Munich, Germany) at the same concentration. After several rinses with PBS, biotinylated goat anti-rabbit or goat anti-mouse (diluted 1:50, Linaris, Wertheim-Bettingen, Germany) served as secondary antibodies. Sections labeled with WFA and LEA were incubated in extravidin peroxidase (Sigma-Immunochemicals, Munich, Germany) for 1 h. Visualization of the reaction products was performed with DAB/H_2O_2. Sections were rinsed with Tris buffer, mounted on glass slides, air-dried and embedded in Entellan (Merck) and sealed with coverslips. Well-stained sections were examined semiquantitatively. Gray values of the calretinin immunoreactivity in the spinal cord were assessed as described previously [16]. The Mann-Whitney U test was used to analyze the level of significance.

Some sections were fluorescence-immunohistochemically stained to study colocalization of the autofluorescent lipofuscin. In these cases, Cy3-conjugated secondary antibody (Amersham, diluted 1:50) and the Cy3-conjugated primary antibody against GFAP (Sigma) were used.

Results

Comparing the spinal cords from 3-year-old rats with those of 1-year-old rats disclosed the following main observations. Firstly, we did not find any alterations regarding vascularization since the amount of LEA binding sites revealing blood vessel walls seemed to be stable during life (fig. 1). We noted strong vascularization in the substantia gelatinosa (layer 2 of the spinal cord). Secondly, we found both age- and environment-dependent alterations in the expression of calretinin, GFAP and WFA (fig. 1, 2), and thirdly, our study revealed age-dependent and environment-independent accumulation of lipofuscin (fig. 2). In some regions of the spinal cord, the differences were more prevalent in the lumbar than in the cervical segments (fig. 3). All changes occurred both uni- and bilaterally, as demonstrated in figures 1, 2, 4 and 5.

Age- and Environment-Dependent Alterations

In all animals, the expression of calretinin and the number of perineuronal nets revealed by WFA binding sites had decreased, though to different extents, in the spinal cord compared to younger rats (fig. 1, 2). Significant differences in calretinin immunoreactivity were found between the two age groups. The age-dependent loss of calretinin immunoreactivity in both the cervical and the lumbar enlargements could be reduced by enriched environmental conditions. The averaged gray values measured were 188.54 ± 1.81 in the substantia gelatinosa of the cervical enlargement and 186.66 ± 3.02 in the lumbar enlargement in the aged rats living under standard environmental conditions, and 196.88 ± 2.11 (cervical) and 195.67 ± 4.01 (lumbar) in the rats kept under enriched environmental conditions. The Mann-Whitney U test revealed significant differences ($p < 0.05$) between 'standard housing' and 'enriched housing' rats and between the cervical and lumbar enlargements.

One-year-old rats did not undergo any remarkable oxidative stress in the spinal cord. In the 3-year-old rats, the spinal cord showed a strongly correlated increase in COX-2 with 3-NT in the fasciculus gracilis and lateral funiculus (indicating oxidative stress). Representative examples (fig. 4) display greater, more widespread damage in the rats kept under standard environmental conditions (circles in fig. 4) compared to those from enriched housing. In some regions of the spinal cord, the expression of COX-2 and 3-NT was stronger in lumbar than in cervical segments (fig. 3).

Age-Dependent but Environment-Independent Alterations

In the spinal cord, the first dusty lipofuscin granules occurred at the age of about 1 year in large motor neurons of the ventral horn. With progressing age, enlarged lipofuscin granules accumulated in the somata of many neurons, in both the ventral and dorsal horn of the spinal cord. Additionally, the walls of blood vessels were labeled

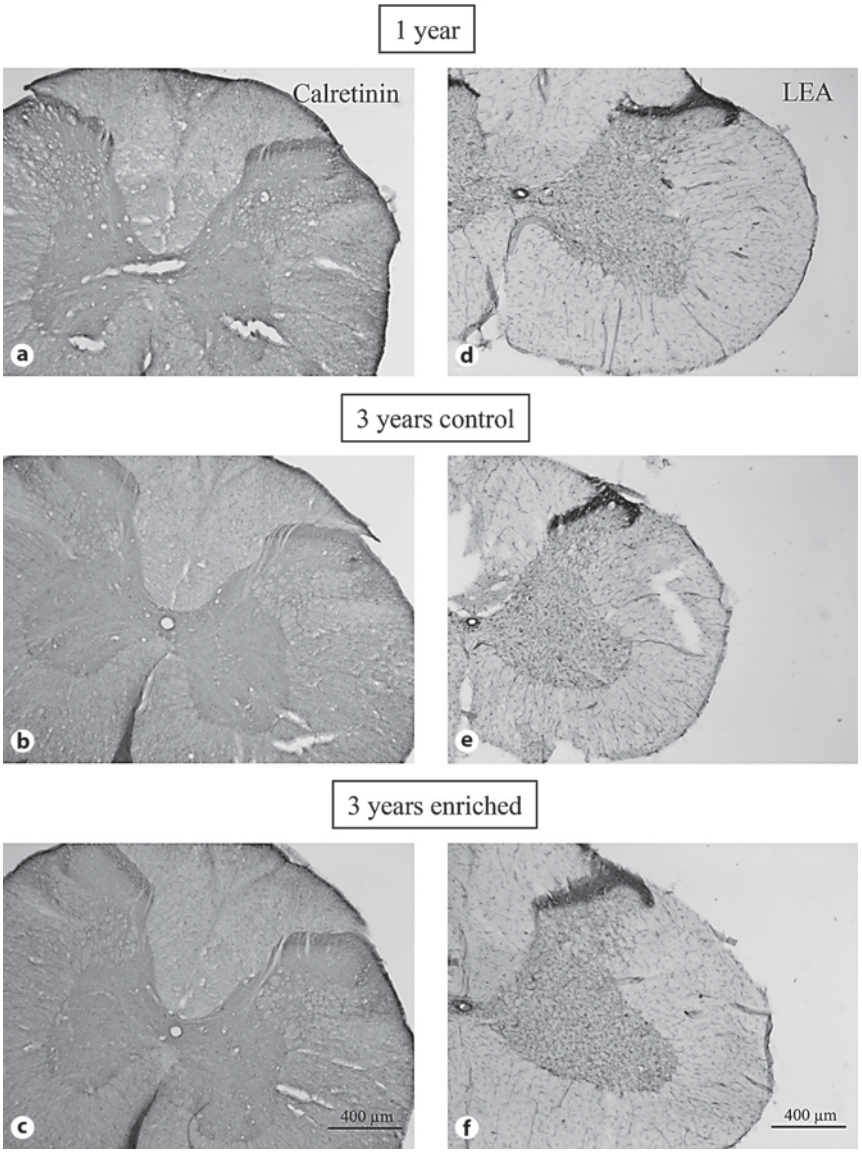

Fig. 1. Overview of calretinin- and LEA-labeled spinal cords during aging. The distribution pattern of blood vessels revealed by LEA (**d–f**)evidences strong vascularization of the calretinin-immunoreactive structures (**a–c**) in the substantia gelatinosa. Calretinin expression decreased with aging but to smaller amounts in the animals kept in enriched environment. Vascularization patterns did not change.

by the accumulation of lipofuscin granules. The accumulation of very large lipofuscin granules in the somata of the motor neurons (fig. 2) was accompanied by a loss of perineuronal nets surrounding the somata of those neurons, which correlates to decreased calretinin immunoreactivity (fig. 1) described in the normal rat [34].

During aging, GFAP expression slightly increased in the white matter (including the membrana limitans gliae superficialis) and outer parts of the gray matter (fig. 2), but the increase was not found in regions of COX-2 and 3-NT expression (fig. 4).

The most impressive was the fact that the expression of dMBP increased with age but did not change with the enriched environment (fig. 5). Examples of 3 animals per group are given in figure 5. Obviously, the somatosensory tracts and the dorsal horn were more affected by myelin-degrading processes than the other parts of the spinal cord.

To summarize, our data indicate that, in contrast to the cerebral cortex of aged rats, neuronal and glial alterations are moderate at spinal cord level. The structure most severely affected is the substantia gelatinosa, as it contains the highest amount of the calcium-binding protein calretinin.

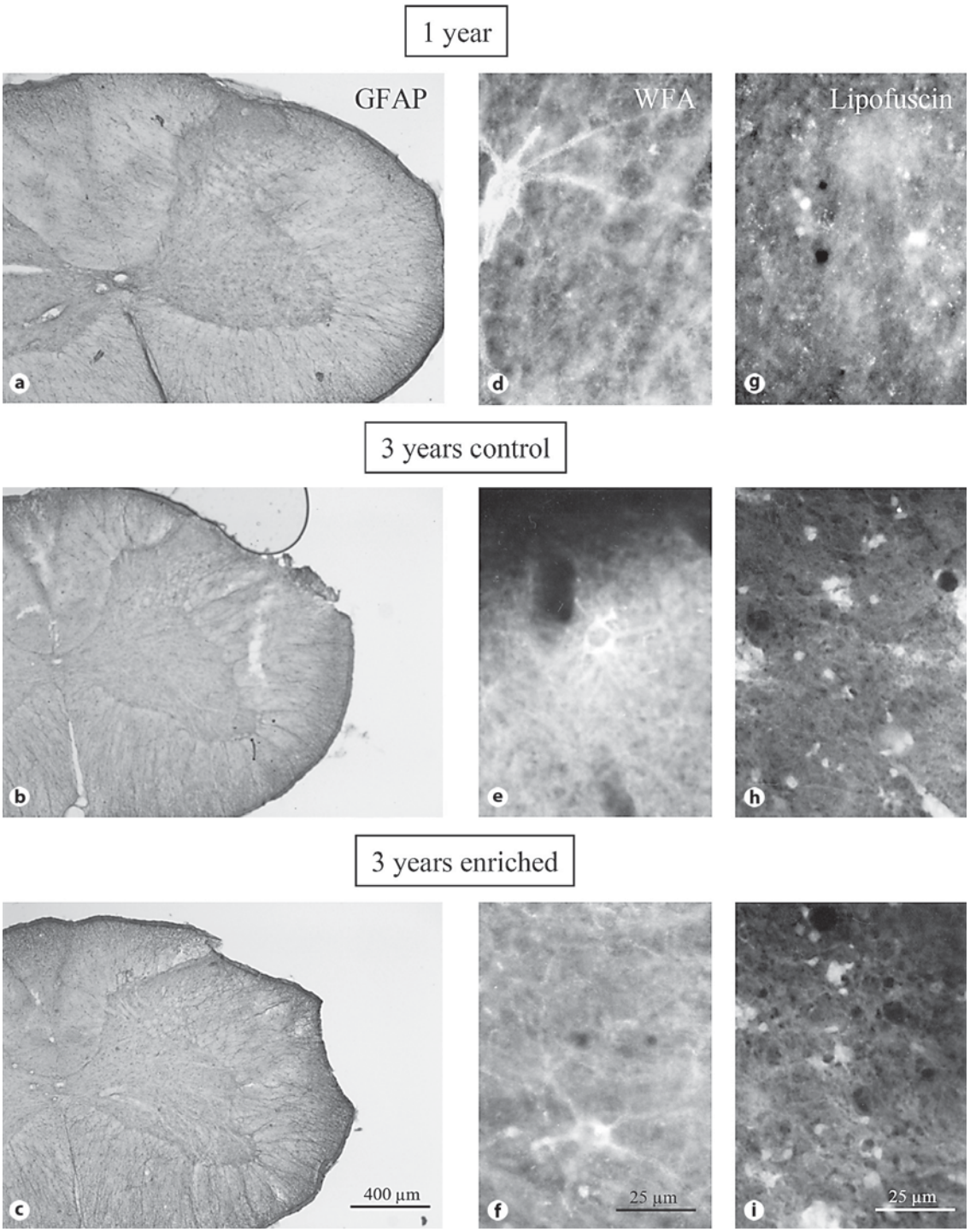

Fig. 2. a–c Overview of GFAP expression during aging. GFAP expression is slightly increased in the control animals in the white matter. **d–f** Age- and environment-dependent alterations in WFA binding sites revealed by the length of dendritic branches of neurons surrounded by perineuronal nets. **g–i** Higher magnifications display the age-dependent change in dusty lipofuscin granules at the age of 1 year to large granule accumulation of lipofuscin in the motor neurons of the lumbar ventral horn at the age of 3 years (for both control rats and rats kept the last 3 months of their life in an enriched environment). White dots reveal lipofuscin by autofluorescence.

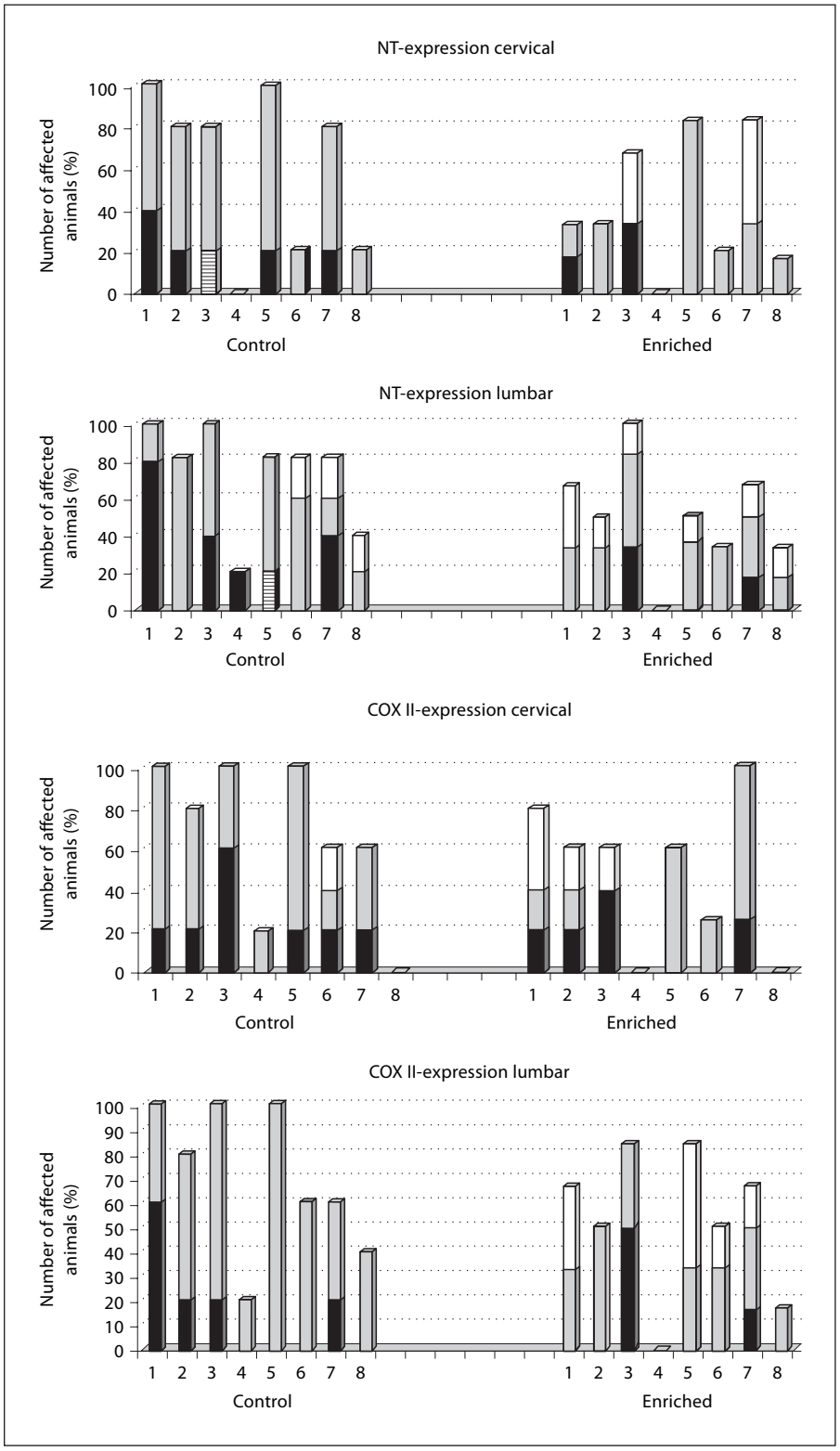

Fig. 3. Semiquantitative analysis of alterations in the spinal cord of aged rats. White column = just labeled; gray column = weakly labeled; striped gray column = moderately labeled; black column = strongly labeled; 1 = ventral horn; 2 = lateral horn; 3 = dorsal horn; 4 = ventral funiculus; 5 = lateral funiculus; 6 = fasciculus cuneatus; 7 = fasciculus gracilis; 8 = corticospinal tract.

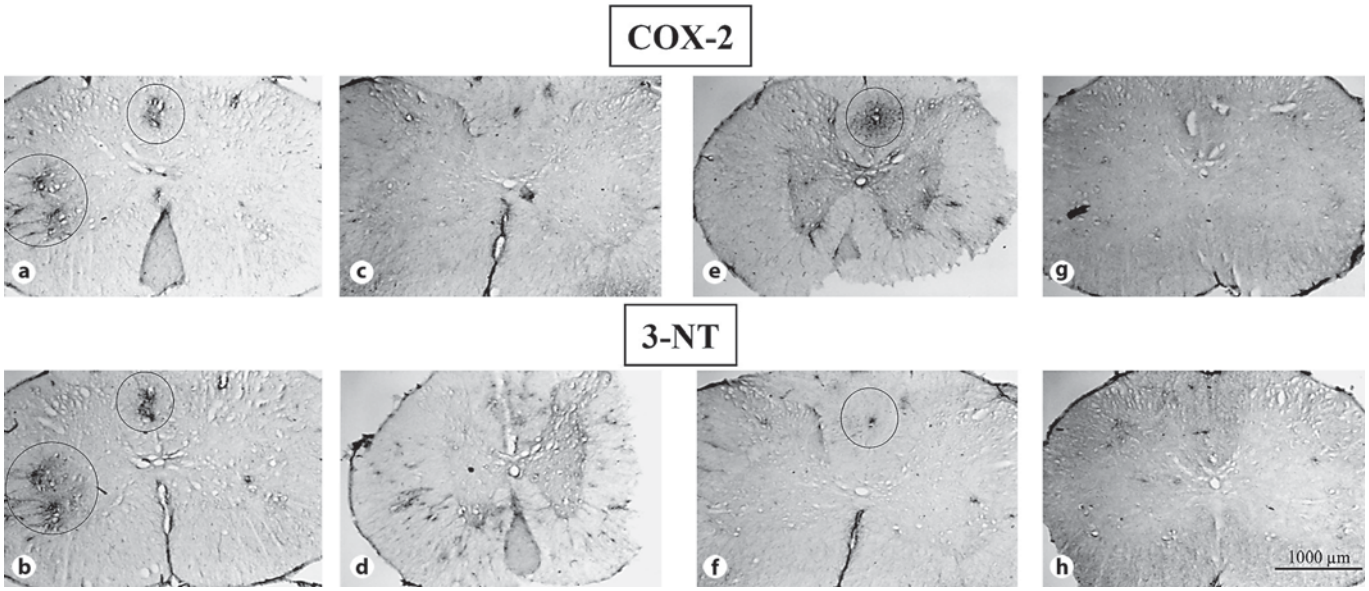

Fig. 4. Characteristic examples of the distribution patterns of COX-2 (**a**, **c**, **e**, **g**) and 3-NT (**b**, **d**, **f**, **h**) in cervical (**a–d**) and lumbar enlargements (**e–h**) of the spinal cord of 3-year-old rats demonstrating the correlation of alterations (indicated by circles). **a**, **b**, **e**, **f** Control rats. **c**, **d**, **g**, **h** Rats kept the last 3 months of their life in an enriched environment.

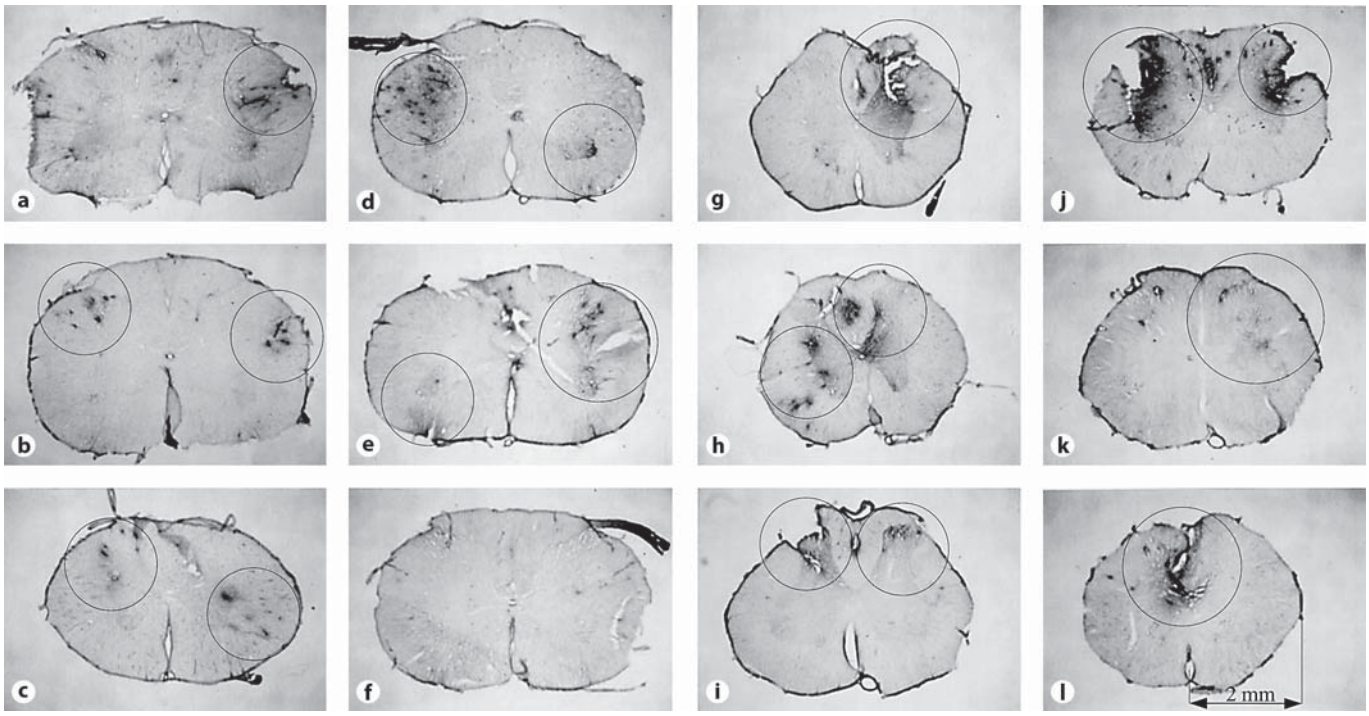

Fig. 5. Examples of demyelinization (indicated by circles) in cervical (**a–f**) and lumbar enlargements (**g–l**) of the spinal cord of 3-year-old rats revealed by dMBP immunoreactivity showing a high degree of individuality independently from the housing conditions. **a–c**, **g–i** Control rats. **d–f**, **j–l** Rats kept the last 3 months of their life in an enriched environment.

Discussion

Our results indicate that age-related changes in the spinal cord are more pronounced in lumbar segments, being compatible with age-related alterations in the cortical hindlimb region [16]. Furthermore, our present results provide evidence that the somatosensory system is earlier affected by age-dependent alterations than the motor system, because we found solely affected somatosensory tracts and both affected somatosensory and weakly affected motor tracts (less than 8%), but we never found solely affected corticospinal tracts. Most importantly, housing the animals under enriched environmental conditions for only a few months may ameliorate some of the age-related changes described.

Neurons in the forelimb area of the somatosensory and motor cortices project to the cervical enlargement, whereas those in the hindlimb area project to the lumbar enlargement [35]. Use of sensitive retrograde tracers has shown that corticospinal neurons are not restricted to the primary motor or somatosensory cortices, but are also found in a number of other cortical regions (such as Fr2, see [36]). Corticospinal axons terminate in all spinal laminae contralateral to the cells of origin, with dense terminations in laminae 3–7 of the dorsal horn and less dense terminations in the ventral horn of the spinal cord [37–39]. The corticospinal tract of the rat plays a role in the control of movement through its terminations in the intermediate gray matter and the ventral horn. This control is exerted primarily through interneurons. However, corticospinal axons were shown to make synaptic contacts with motor neurons in the rat spinal cord, as they do in primates [40]. We suggest that the sensory impairments in the periphery first affect the sensory ascending pathways, explaining the correlation between sensory impairments in the hind limbs, enlargement of their representational fields in the somatosensory cortex [2, 41] and – in our view – the resulting motor impairments. The most significant age-related impairments are restricted to the hind limbs (findings about the effects of enriched housing reported by Coq and Xerri [42] refer to young animals kept under enriched conditions). We assume that three processes are involved: firstly, age-dependent accumulation of lipofuscin which only has a marginal effect on neuron function, if any; secondly, age-dependent alterations such as oxidative stress influencing the function of the neuron (such as inducible NOS [43]), which were modified by environmental conditions, and thirdly, degenerative alterations in the neurons, resulting in the destruction of the neuron (e.g. loss of neuronal NOS [43, 44]). Interestingly, enriched environmental conditions were not able to reverse the degenerative alterations in the neurons. Accordingly, to explain our contradictory results regarding COX-2 and 3-NT on the one hand and dMBP on the other, we suggest that COX-2 and 3-NT may indicate oxidative stress [45]. dMBP expression is assumed to be a result of degeneration processes that are not reversible in enriched environmental conditions. We found marginal changes in various markers in the spinal cord, whereas calretinin immunoreactivity indicated significant differences in the amount of specialized terminals in the substantia gelatinosa, part of the nociceptive system. Impaired nociception is one of the most evident signs of aging [44]. Obviously, the loss of somatosensory abilities in the rats was both age and use dependent, and was indeed modified by enriched environmental conditions. Taken together, our results support previously proposed findings and suggestions [46] that target failure is a critical event in the aging process of both sensory and motor neurons. This is a critical, but not a fateful event.

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