Morphological changes and selective loss of motoneurons in the lumbar part of the spinal cord in a rat model of familial amyotrophic lateral sclerosis (fALS)

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Abstract
Morphological alterations and the course of changes in motoneuron counts were studied by light microscopy (cresyl violet staining) in the L2/L3 region of the spinal cord of hemizygotic transgenic rats carrying the amyotrophic lateral sclerosis-associated mutant human gene for Cu,Zn superoxide dismutase (hSOD1G93A) and of their non-transgenic littermates. In 60-day old transgenic rats, a few ischaemic-looking α-motoneurons and occasional vacuolization and accumulation of tigroid in some of the cells were apparent. On day 93 of life more distinct cellular pathology was found in transgenic rats, including moderate gliosis, neuronophagy of α-motoneurons, and occasional neuronophagy of γ-motoneurons. In 120-day-old transgenic rats, abundant gliosis and profound neuronophagy of α-motoneurons were observed combined with occasional neuronophagy of other cells. Some loss of α-motoneurons was also apparent in 120-day-old non-transgenic littermates of the transgenic rats. No difference in α-motoneuron and γ-motoneuron counts was found between the rats on day 60 of life (early presymptomatic stage of the model disease in the transgenic rats). At 93 days of age (late presymptomatic stage), α-motoneuron count, but not γ-motoneuron count, tended to be lower (p=0.06) in the transgenic rats. On day 120 of life (symptomatic stage), α-motoneuron count in the transgenic rats was about half that in their nontransgenic littermates (p<0.001); at this time point the relative decline in α-motoneuron number in the former was 57% (day 120 versus day 60; p<0.001). A smaller decline in α-motoneuron count was also found in nontransgenic rats (day 120 vs day 60: 24%, p<0.05); this was not associated with the emergence of neurological symptoms or distinct changes in the cell morphology of the spinal cord region studied.

Key words: motoneuron disease, α-motoneuron, γ-motoneuron, gliosis, reactive astrocyte.

Introduction
Transgenic rats carrying the amyotrophic lateral sclerosis (ALS) -associated mutant human gene for Cu,Zn superoxide dismutase (hSOD1G93A), which were created by Howland et al. [5], develop, beginning usually at the end of the 4th month of life, symptoms of an incurable illness that imitates ALS in most respects, and are an established model of familial ALS with early onset and a short symptomatic phase. A colony of these transgenic rats has been maintained in the animal facility of the...
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Medical Research Centre of the Polish Academy of Sciences since 2003 [4]. This study was aimed at establishing the course of the model disease-associated morphological changes in the lumbar part (intumescentia lumbaris) of the spinal cord of these transgenic rats.

Materials and methods

Details of transgenic rat propagation, maintenance and genotyping, and animal use procedures, are given in the preceding paper [4].

Twelve transgenic rats (hSOD1+) and 12 of their non-transgenic (hSOD1-) littermates of either sex were used for the study. Four rats each of these groups were decapitated under chloral hydrate anaesthesia at 60, 93 and 120 days of age (early presymptomatic, late presymptomatic and late symptomatic stage of the model disease, respectively). Most rats in this model, including all symptomatic rats used for this study, developed first neurological signs of the model disease in a hindlimb. Therefore, the lumbar parts of the spinal cords corresponding to the L2-L3 region were used for this study. The samples were fixed in 4% formaldehyde solution, pH 7.4, dehydrated in a series of ethanol dilutions, embedded in paraffin and cut transversally into 8 μm thick serial sections. Each 8th section was mounted on a gelatin-coated coverslip, stained with cresyl violet and examined under a light microscope. Different sections from the same segment of the spinal cord can contain varying numbers of motoneurons. Therefore, four such sections from each rat, each section covering the whole of both ventral horns of the spinal cord, were used for quantification of changes in the numbers of "large" motoneurons (cells of >25 μm approximate diameter, classified as α-motoneurons) and "small" motoneurons (18 μm<cells<25 μm approximate diameter, classified as γ-motoneurons) using x40 magnification. Only motoneurons with both the nucleus and nucleolus visible in the cross-section were counted. To facilitate the counting and avoid double counting the same cells, especially the relatively small γ-motoneurons, a square 10x10 mm reticle with 20 divisions along each side was placed in the microscope eyepiece to overlap with the cross-section view of the spinal cord.

Statistics

Motoneuron count data from four sections of the spinal cord of the same rat were averaged prior to further statistical analyses. The averaged results were analyzed by two-way ANOVA with hSOD1 transgene status (hSOD1+ or hSOD1-) and rat age (60, 93, or 120 days) as independent variables, followed by Fisher’s protected least significant difference test when appropriate. In all cases, p<0.05 was considered significant. All statistical tests were performed using Statistica for Windows v. 6.1 statistical software package (Statsoft, Tulsa, OK, USA).

Results

Clinical findings

No obvious neurological symptom of the model disease (an abnormality in gait/posture or evidence of limb weakness) was seen in transgenic rats sacrificed on day 60 or day 93 of life, or in their hSOD1-littermates of any age. The hSOD1+ rats sacrificed when 120 days old showed substantial weight loss (data not shown) and clear neurological deficits that included partial or complete paralysis of one or two hindlimbs.

Morphological/morphometric findings

γ-Motoneurons were found dispersed over the entire cross-sections of the ventral horns and at the base of the dorsal horns, whereas most α-motoneurons were situated at the ventral, ventrolateral and ventromedial parts of the spinal cord ventral horns (Fig. 1A-B). No clear morphological changes were apparent in 60-day-old hSOD1+ rats except for few ischaemic-looking, dark cells and occasional minor vacuolar rarefaction of the cytoplasm in some α-motoneurons (Fig. 1C; see also ref. 13). Among intensely stained cells a few pale α-motoneurons were seen that showed cytoplasmatic accumulation of fine-grain tigroid.

No marked difference in the number of α-motoneurons was apparent between hSOD1+ rats sacrificed at 60 and 93 days of age (cf. Figs. 1A and 2A; see also Fig. 4A). Some distinct qualitative morphological changes, including moderate gliosis only along the ventral border of the ventral horns, neuronophagy of some α-motoneurons (Fig. 2B) and occasional neuronophagy of γ-motoneurons, were
Fig. 1. Ventral horn of the spinal cord from a 60-day-old transgenic hSOD1<sup>G93A</sup> rat. Panel A – Note symmetrical distribution of α-motoneurons in the transverse section of the cord; original magnification: x100. Panel B shows the distribution of large motoneurons (α-motoneurons) and small motoneurons (γ-motoneurons) in the ventral horn of the spinal cord; orig. magn.: x40. Panel C – Single pale neurons (arrowheads) are dispersed among intensely stained cells. Most of the neurons show no distinct damage except for occasional small vacuole-like cytoplasm rarefactions (arrows); orig. magn.: x400; cresyl violet staining.
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Fig. 2. Ventral horn of the spinal cord from a 93-day-old transgenic hSOD1<sup>G93A</sup> rat. Panel A shows a minor decrease in α-motoneuron number; orig. magn.: x40. Panel B – The presence of glial cell clusters and neuronophagic foci (arrows) among normal-looking cells is apparent; orig. magn.: x200. Panel C – A rarely seen neuronophagy of a γ-motoneuron is shown (arrow); orig. magn.: x400; cresyl violet staining.
apparent in these rats (Fig. 2C), but not in their hSOD1-littermates.

Profound morphological aberrations were seen in hSOD1+ rats sacrificed on day 120 of life. The changes included abundant gliosis that was visible throughout the entire ventral horns’ cross-section, and particularly at the location of large motoneurons (Fig. 3A). There was an obvious decrease in the number of surviving α-motoneurons, but not of surviving γ-motoneurons, as compared to those at earlier time points. A decrease in the number of α-motoneurons, but not γ-motoneurons, was also apparent in the 4-month-old hSOD1+ rats. α-Motoneurons of normal appearance were only occasionally seen in the spinal cord of 4-month-old hSOD1+ rats, whereas there were abundant neuronophagic glia clusters (Fig. 3B) and glia-encircled empty beds that marked the supposed location of extinct α-motoneurons (Fig. 3C). Whereas most of the gliosis involved astrocytes, infrequently some microglia activation was apparent as well (Fig. 3D). Occasional neuronophagy of non-motor cells was also seen in the dorsal horns of the spinal cord of 120-day old hSOD1+ rats (not shown).

ANOVA revealed significant effects of rats’ age and of the presence of the hSOD1G93A transgene on α-motoneuron count (F2,18=18.9, p=0.0004, and F2,18=20.7, p=0.00002, respectively), but not on γ-motoneuron count (p>0.36), and no significant age x transgene interaction effect on the motoneuron counts (p=0.12 and 0.81, respectively). However,
there was a significant age x transgene interaction effect ($F_{2,38}=6.18$, $p=0.009$) on the relative size of the $\alpha$-motoneuron subset: $R_{\alpha MN} = \alpha$-motoneuron count / ($\alpha$-motoneuron count + $\gamma$-motoneuron count). $\alpha$-Motoneuron count was significantly lower both in 120-day-old $hSOD1^+$ rats and their $hSOD1^-$ littermates than in their 60-day-old counterparts. $R_{\alpha MN}$ tended to be lower in the transgenic rats than in their nontransgenic littermates already at day 93 of life, and was significantly lower in 120-day-old $hSOD1^+$ rats than in their non-transgenic littermates of the same age or in 60-day-old $hSOD1^+$ rats. A tendency for age-related decline in $R_{\alpha MN}$ was evident also in non-transgenic rats (Fig. 4A-C).

**Discussion**

The ratio of $\alpha$-motoneuron number to $\gamma$-motoneuron number in the ventral horns of the spinal cord of 60-day-old presymptomatic $hSOD1^{G93A}$ transgenic rats was approximately 1 to 2 and was

**Fig. 4.** Time-dependent changes in the numbers of $\alpha$-motoneurons (panel A) and $\gamma$-motoneurons (panel B) and in the relative size of the $\alpha$-motoneuron subset (panel C) in untreated transgenic $hSOD1^{G93A}$ rats and their nontransgenic littermates. Data shown represent (or are derived from) means±S.D. of the respective averaged neuronal counts acquired in four transverse cross-sections (at the L2/L3 level) from each rat, each section covering the whole of both ventral horns of the spinal cord. Four rats were used for each experimental group; for more details see Methods and Materials. * - $p<0.05$, *** - $p<0.001$ vs the respective 60-day-old rats; ### - $p<0.001$ vs the non-transgenic littermates.
closely similar to that in their nontransgenic littermates of the same age. Despite some differences in the cell soma sizes used for classification of spinal cord motoneurons in rats and mice, a similar α-motoneuron to γ-motoneuron number ratio was found also in the spinal cord of age-matched transgenic mice overexpressing the same mutant human gene and their nontransgenic littermates [9]. Taking into account differences in motoneuron labelling and counting, and in examining the animals for signs of motor impairment, the time course of the α-motoneuron count changes in our study considerably resembled that reported in the aforementioned murine ALS model [9]. In a “twin” transgenic rat ALS model employing the same mutant human gene, a similar relative decline (by ≈50%) in spinal cord motoneuron count was reported at the time of subjective onset of the model disease denoted by general muscle weakness and limb paralysis. These symptoms followed shortly (by somewhat over 1 day on average) the objective onset of the disease as defined by muscle weakness in inclined plane test. However, in that study no stratification into “large” motoneuron and “small” motoneuron subsets was used and the numbers reported were summed total motoneuron (choline acetyltransferase-positive cells >20 μm approximate diameter) counts in C6, T5, and L3 segments [8].

Notably, the number of α-motoneurons showed a tendency for decrease at the end of the 3rd month of life in transgenic rats, i.e. prior to the emergence of a visible neurological deficit, and declined at an accelerated rate thereafter. This rapid decline is in line with the fulminant ALS characteristics of human carriers of the mutant transgene expressed in these rats [3]. The reduction in α-motoneuron number reached ≥50% at or shortly after the onset of a neurological deficit (muscle weakness or abnormalities in gait/posture). Interestingly, muscle weakness in poliomyelitis anterior acuta (Heine-Medin’s disease) victims was also observed when the number of α-motoneurons in their spinal cords fell below 49% of that in healthy persons of the same age [16].

A fall in α-motoneuron count was also apparent in hSOD1- rats in this study. This may suggest an aging-related phenomenon, but we believe it was due to individual variability and its related statistical fluctuation in the motoneuron count in that particular rat group. This is because the apparent decline rate in α-motoneuron count (-24% over two months, as calculated from the data for 60-day-old and 120-day-old rats, and -21% during the last pre-sacrifice month, as calculated from the data for 93-day-old and 120-day-old rats) seemed much too rapid for the apparently healthy nontransgenic rats. Moreover, no similar phenomenon was observed in the “twin” rat line created by Nagai and his colleagues [8]. Those authors have also stressed the possibility of some confounding factors causing phenotype and clinical course variability in that rat line. These factors may include the heterogeneous genetic background of the Sprague-Dawley rat employed to generate the transgenic rat lines, epigenetic regulation of the transgene expression, and others [8]. Actually, with the passing of time, some unexpected deviations from the original characteristics were observed in the hSOD1G93A transgenic rat line propagated at our animal facility [4].

In agreement with the findings in ALS victims [7], transgenic ALS mice [9] and other transgenic ALS rats [1], morphological analysis revealed that the decline in motoneuron number was mostly limited to α-motoneurons in this study. As stressed by many, whereas other cell types are also affected in both the human disease and rodent ALS models [1,7,12, also this study], the early death of large motoneurons in the ventral horns of the spinal cord is the dominant histopathological feature of the disease. This is also in accordance with previous reports on this and similar rodent models of fALS [8,9] and appears to be due to some peculiarity in the biochemical characteristics of the cells. Notably, the mutant hSOD1 transgene can be expressed at high levels in some brain regions (e.g. cerebellum and cortex) and peripheral organs of transgenic rodents [5,10] without causing an apparent local pathology or the respective “clinical” symptoms. These observations are also in accordance with those made in the human disease [6]. However, one should remember that the apparent stability of spinal cord γ-motoneuron counts may be related in part to some shrunken α-motoneurons being included in the “small” motoneuron counts [7,9].

The other pathomorphological changes found in the transgenic rats in this study included neuron vacuolization and gliosis which affected both the astrocytic and microglial subsets. A recent study in transgenic mice carrying the same mutant
transgene has shown that microgliosis present in the spinal cord of diseased mice is primarily due to an expansion of resident microglia and not to the recruitment of bone marrow-derived microglial precursors from the circulation [17]. Interestingly, the gliosis effects also preceded the worsening of locomotor performance in the transgenic rats. This observation is in agreement with those reported by others in this and similar ALS models [1,2,5,10,14]. The vacuolization is supposed to result from degeneration of mitochondria in rodent fALS models [18], whereas the microglia and reactive astrocytes most likely contribute to motoneuron injury and ALS progression [2,11,15]. All the pathomorphological changes found in this study were typical of neurodegeneration; no signs of an inflammatory process were visible.

In summary, this study has corroborated the morphological findings reported by others in the same and akin transgenic rodent models of ALS, showing that the pathomorphological changes precede the emergence of neurological deficits and that the latter occur due to exhaustion of the “spare” motoneurons’ capacity of the spinal cord.

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