Motor and Visuospatial Abilities in a Model of Olivocerebellar and Retinal Degeneration – Lurcher Mutant Mice of C3H Strain

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Abstract: Lurcher mutant mice represent a natural model of olivocerebellar degeneration. They suffer from loss of Purkinje cells and decreased number of granule cells and inferior olive neurons. The degeneration leads to cerebellar ataxia and deterioration of cognitive functions. Some animals of the C3H strain have also the retinal degeneration. The aim of the study was to analyze the morphology of cerebellar and retinal degeneration and to evaluate the ability of motor coordination and visuospatial orientation in C3H Lurcher mutant mice. Cerebella of Lurcher mutant and wild type mice were examined with several histological, histochemical and immunohistochemical methods. Motor coordination was tested on a bar, ladder and rotarod. Spatial orientation and learning were tested in the Morris water maze with visible or hidden platform. Histological examinations showed decreased numbers of Purkinje cell in Lurchers. Various histological methods brought different information about the course or stage of the cerebellar degeneration. Retinal degeneration was identified with hematoxyline-eosine staining very well. Lurchers performed worse in motor coordination tests and in both the spatial orientation and learning test. Retinal degeneration influenced negatively both the spatial learning and orientation. Motor tests were influenced by retinal degeneration only in the wild type mice. Wild type mice showed some ability of idiothetic navigation, which was not found in Lurchers.

Introduction

Lurcher mutant mice represent a natural model of genetically determined olivocerebellar degeneration [1]. They carry a spontaneous mutation in the δ2-glutamate-receptor subunit (GluRδ2) gene [2]. GluRδ2 is expressed mainly in cerebellar Purkinje cells [3]. Heterozygous individuals (+/Lc) suffer from complete postnatal loss of Purkinje cells and a substantial decrease in cerebellar granular cells and inferior olive neurons. Purkinje cells die by apoptosis triggered by glutamate excitotoxicity resulting from the δ2-glutamate-receptor malfunction [2]. As a consequence of the loss of Purkinje cells the cerebellar ataxia develops in Lurcher mutants at the end of the second postnatal week. Their cognitive functions are deteriorated; some of their neurons are more sensitive to neurotoxic agents [4]. Unaffected homozygous littermates (+/+) of Lurcher mutants, so called wild type mice, are completely healthy and serve as ideal controls. Affected homozygots (Lc/Lc) are not viable. In mice of the C3H strain a hereditary retinal degeneration determined by homozygous combination of rd1 gene (rd1/rd1) occurs. Retinal degeneration leads to almost complete loss of photoreceptors before animals reach the age of 30 days [5]. Previous experiments with mice of the C3H strain in the Morris water maze showed that retinal degeneration influences negatively visual orientation [6]. The aim of the work was to assess the morphology of cerebellar and retinal degeneration in Lurcher mutant mice of C3H strain and evaluate ability of motor coordination and visuospatial orientation in these mice.

Materials and Methods

We tested the motor coordination, spatial orientation and spatial learning in adult (older than 60 days) wild type and Lurcher mutant mice of the C3H strain, both males and females. They were kept at 12/12 light/dark cycle. Food and water were available *ad libitum*. For histological examination mice of various ages were used.

Motor coordination tests

The motor coordination was tested with a set of three methods: horizontal bar, ladder and rotarod. We measured time that the animals were able to stay on the apparatus (fall latency) and assessed criterion meeting to reach latency 60 s or leave the apparatus actively. If the animal fell down within 60 s, the trial was considered as unsuccessful. All procedures were repeated four times in each session. Between the trials the mice spent 5 minutes resting in their breeding cages. Mean latencies of the fall and mean success rate of the experiment were evaluated. For statistical analysis of measured data Mann-Whitney test was used. 35 wild type mice without retinal degeneration (WT rd–), 38 wild type mice with retinal degeneration (WT rd+), 23 Lurcher mutants without retinal degeneration (Lc rd–) and 44 Lurchers with retinal degeneration (Lc rd+) were used for motor coordination test.

Spatial orientation test

Spatial orientation was examined with Morris water maze [7] with a visible platform. The platform (diameter: 7.5 cm) placed in the round plastic pool (diameter: 95 cm) was hidden 0.5 cm under the water surface but it was visualized by a cylindrical label (high: 5.0 cm, diameter: 3.5 cm) with black and white vertical stripes mounted 7 cm above the platform. The width of the stripes on the label was 0.5 cm. The water temperature was set at 29 ± 1 °C to prevent algidity of the mice. Four trials a day were performed from different starting points called as cardinal points (N, S, W and E). Each mouse was allowed to search the maze for 60 s. If it did not reach the platform within this time it was placed there. On the platform mice were left always for 30 s. The movements of the mice in the water maze were automatically registered by the EthoVision tracking system. Experiment was repeated for 5 days (D1–D5). The platform was located in the centre of the south-west quadrant during the first four days (D1–D4) and in the centre of the north-east quadrant on the fifth day (D5). Between individual trials mice spent 10 min resting in breeding cages. Mean latencies and swimming velocity on individual days of the experiment were evaluated (ANOVA). 23 wild type mice without retinal degeneration (WT rd–), 36 wild type mice with retinal degeneration (WT rd+), 27 Lurcher mutants without retinal degeneration (Lc rd–) and 14 Lurchers with retinal degeneration (Lc rd+) were used for spatial orientation test.

Spatial learning test
For spatial learning test Morris water maze method with hidden platform was used. The arrangement was the same as in the spatial orientation test except the visualization of the platform with the label. In this experiment the platform was invisible so that the mice had to localize its position according to distal landmarks (window, laboratory furniture). Four trials a day were performed from different starting points (N, S, W, E). Each mouse was allowed to search the maze for 60 s. If it did not reach the platform within this time it was placed there and on the platform mice were left always for 30 s. The experiment was repeated for 5 days (D1–D5). The platform was located in the centre of the south-west quadrant for all 5 days. Between trials mice spent 10 min resting in breeding cages. Mean latencies and swimming velocity on individual days of the experiment were evaluated (ANOVA). 12 wild type mice without retinal degeneration (WT rd–), 39 wild type mice with retinal degeneration (WT rd+), 11 Lurcher mutants without retinal degeneration (Lc rd–) and 36 Lurchers with retinal degeneration (Lc rd+) were used for spatial learning examination.

Histological examination
Finally mice were deeply anaesthetized with thiopental and transcardially perfused with phosphate buffer (pH 7.4) and 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde for at least 2 hours. The cerebellum and the brainstem were sectioned in the frontal plain with cryostat. Then the slices were processed with following histological, histochemical and immunohisto-chemical methods: Nissl staining with gallocyanin (14 days old mice), Fluoro-Jade B and staining with bisbenzimide simultaneously (14 days old mice), Anticalbindin (21 days old mice), fluorescent double staining with Lucifer Yellow and DiD oil (14 days old mice) [8]. The adult animals which brains were processed with Golgi impregnation in modification according to Ramón-Moliner [9] were sacrificed by overdosing with thiopental and the brains were removed without transcardial perfusion.

The eye bulbs of adult sacrificed experimental animals were dissected and stored in 4% paraformaldehyde in phosphate buffer for several days for fixation. Then the eyes were sectioned with cryostat (16 μm). The sections were stained with hematoxyline-eosine and retinas were examined microscopically.

All experiments were performed in full agreement with the EU Guidelines for scientific experimentation on animals and with permission of the Ethical Commission of the Faculty of Medicine in Pilsen.

Results

Histological examination
Nissl staining visualized the structure of the cerebellar cortex and showed reduced amount of Purkinje cells in young (14 days) Lurcher mutants and complete absence

of these cells in adult Lurchers. Identification of shrunken dying Purkinje cells was complicated. Nevertheless, reduced cell density in the granular layer in Lurchers was marked (Figure 1).

Anticalbindin staining marked specifically Purkinje cells in the cerebellum. Their number was reduced in 21 days old Lurchers as compared with wild type mice of the same age (Figure 2).

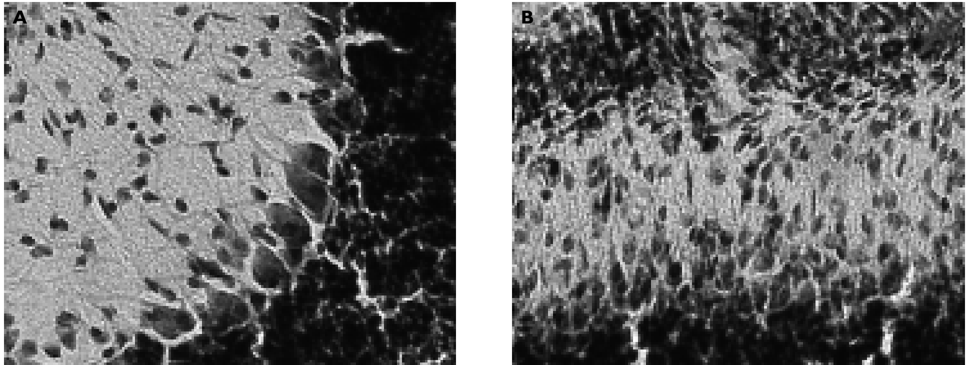

Figure 1 – Cerebellar cortex of 14 days old wild type (A) and Lurcher mutant (B) mouse. Nissl staining.

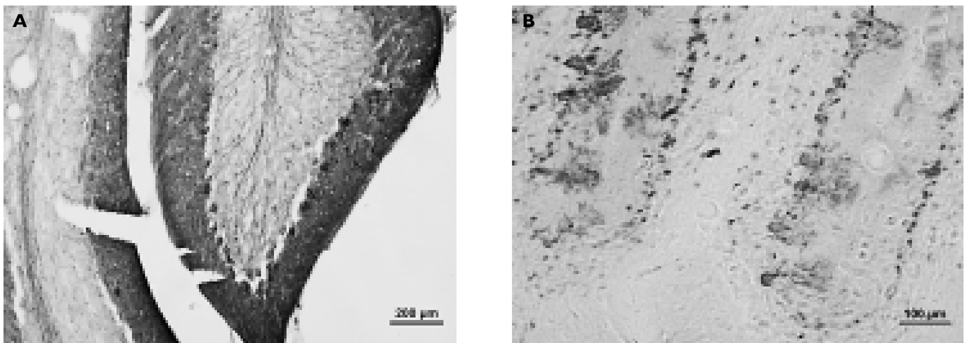

Figure 2 – Cerebellum of 21 days old wild type (A) and Lurcher mutant (B) mouse. Anticalbindin staining.

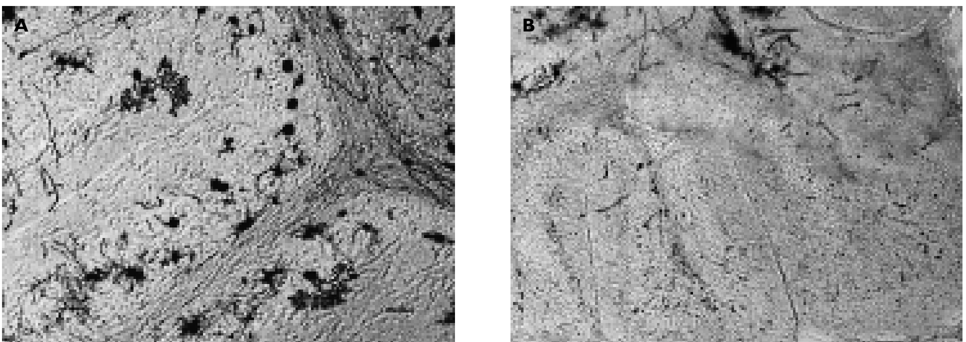

Figure 3 – Cerebellum of adult wild type (A) and Lurcher mutant (B) mouse. Golgi impregnation.

Golgi impregnation showed Purkinje cells with abundant dendritic trees in the cerebellar cortex of wild type mice. No Purkinje cells were found in adult Lurcher mutant mice (Figure 3).

In slices of the cerebellum of 14 or 21 days old Lurcher mutants processed with Fluoro-Jade B method only solitary positive Purkinje cells were found, though staining with bisbenzimide showed marks of apoptosis (fragmentation of nuclei) in much more cells (Figure 4).

Fluorescent double staining enabled to visualize detailed cell morphology with confocal fluorescent microscope. Purkinje cells of normal and typical appearance were displayed in wild type mouse (Figure 5A). In 14 days old Lurcher normal-shaped Purkinje cells were also found but most of the Purkinje cells showed signs of various stages of apoptosis. Both normal Purkinje cells and apoptotic cells (Figure 5B, C), and even empty spaces with size and shape of Purkinje cells (Figure 5D) were present together in one Lurcher mutant individual.

Retina examination showed completely missing outer nuclear layer in some of the wild type and Lurcher mutant mice (Figure 6B). These mice were considered as affected with retinal degeneration. Mice with all retinal layers spared were considered as unaffected (Figure 6A).

Motor coordination

In the motor coordination tests Lurcher mutant mice reached significantly worse results than wild type mice. In Lurchers, fall latencies were shorter and success rate was lower and the differences were evident in both with retinal degeneration affected (latencies: U value=24.5, $p<10^{-6}$, success rate: U value=58.0, $p<10^{-6}$) and unaffected mice (latencies: U value=4.5, $p<10^{-6}$, success rate: U value=0.5, $p<10^{-6}$) (Figure 7). Presence of retinal degeneration influenced the results of the motor test only in wild type mice, when mice affected with retinal degeneration showed shorter latencies (U value=420.0, $p<0.007$) and lower success rate (U value=424.5, $p<0.008$) than those with normal retina. In Lurchers there were no significant differences between animals with and without retinal degeneration (Figure 7).

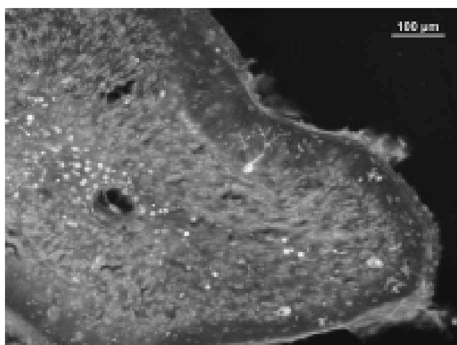

Figure 4 – Cerebellum of 14 days old Lurcher mutant mouse. Fluoro-Jade B.

Figure 5 – Normal Purkinje cells in cerebellum of 14 days old wild type mouse (A) and Purkinje cells in various stages of apoptosis in a Lurcher mutant mouse of the same age (B, C, D). Fluorescent double staining with hydrophilic Lucifer Yellow (green colour) and lipophilic DiD oil (red colour). Preparates were made by V. Štenglová. Photo: J. Reischig.

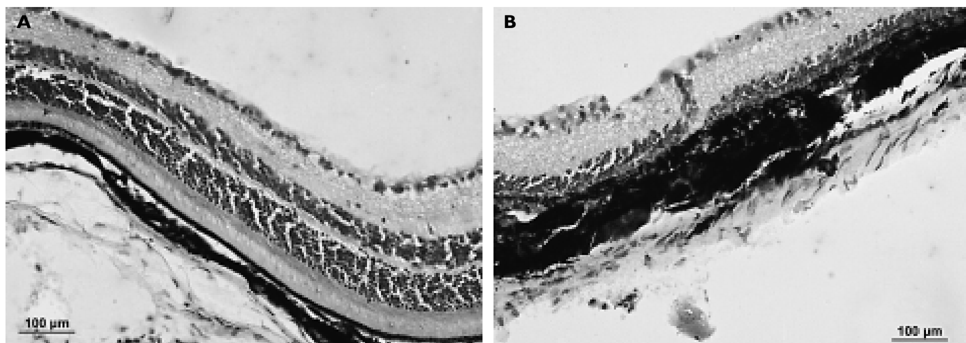

Figure 6 – Normal retina (A) and retina affected with the degeneration (B) in adult C3H mice. In degenerated retina outer nuclear layer is missing. Hematoxylin-eosin.

Spatial orientation

In the spatial orientation test in the water maze with the visualized platform, the influence of both retinal and cerebellar degeneration was observed. Latencies of mice with normal retina were significantly shorter then latencies of individuals with retinal degeneration in both wild type (F value=66.889, $p<10^{-6}$) and Lurcher mutant mice (F value=25.968, $p<9\times10^{-6}$) (Figure 8A). In mice with normal retina wild type mice reached significantly shorter latencies than Lurcher mutants (F value=26.674, $p<5\times10^{-6}$). In mice with retinal degeneration we found no significant differences in latencies between wild type and Lurcher mice (F value=2.437, $p>0.05$). In swimming velocity less marked differences were observed. Wild type mice with retinal degeneration swam more quickly than unaffected ones (F value=8.397, $p<0.006$) and Lurcher mutants with normal retina swam more quickly than wild type mice with normal retina (F value=5.858, $p<0.02$) (Figure 8B).

Spatial learning

The influence of both retinal and cerebellar degeneration was observed also in the spatial learning test using the water maze with hidden platform. Absolutely best results were observed in wild type mice with normal retina. Their latencies were significantly shorter than latencies of wild type individuals with retinal degeneration (F value=9.979, $p<0.003$). In Lurchers the difference between animals with normal and affected retina was less marked (F value=8.733, $p<0.005$). Lurcher mutants showed worse learning ability than wild type mice. The differences were significant in both animals with normal (F value=6.877, $p<0.02$) and affected retina (F value = 30.763, $p<10^{-6}$) (Figure 9A). Wild type mice with retinal degeneration were even better than Lurchers with normal sight; however, the difference was insignificant. Swimming velocity in wild type mice with retinal degeneration was higher than in Lurchers also with retinal degeneration (F value=10.192, $p<0.003$). Other differences with regard to retinal or cerebellar degeneration were insignificant (Figure 9B).

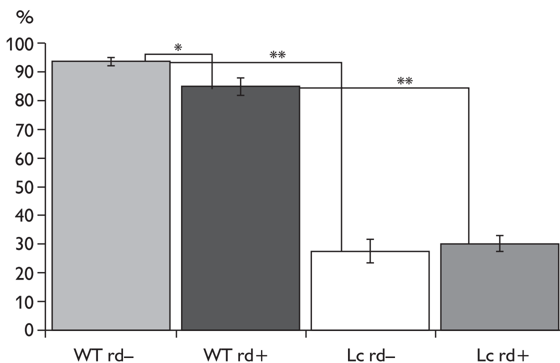

*Figure 7 – Mean success rate in all motor coordination tests (in %) ± S.E.M. in wild type mice without the retinal degeneration (WT rd–), wild type mice with the retinal degeneration (WT rd+), Lurcher mutants without the retinal degeneration (Lc rd–) and Lurchers with the retinal degeneration. (Lc rd+). * $p<0.008$, ** $p<10^{-6}$*

Discussion

As the Lurchers suffer from cerebellar ataxia [15, 16], worse performance in motor coordination tests was expected. The effect of retinal degeneration and subsequent visual problems (in fact blindness) played not so important role as cerebellar degeneration. Contrary to that, wild type mice are able to solve the task without visual control significantly worse than mice with normal sight. Due to the cerebellar malfunction Lurcher mutant mice are not able to use visual information for motor coordination as wild type mice and that is why retinal degeneration does not lead to more sever motor impairment than is that one caused by the cerebellar degeneration itself.

For both navigation to a visible goal and for navigation to a hidden goal which requires information about distal landmarks the visual orientation is essential [6]. The cerebellum plays role in cognitive functions including spatial learning [14, 17, 18, 19, 20, 21] and spatially oriented behaviour [22]. Analysis of swimming velocity in both types of experiments with visible and hidden platform showed that longer latencies in Lurcher mutants are not due to motor problems and they should be attributed to learning or spatial orientation ability deterioration in these mice.

In wild type mice with retinal degeneration we observed shortening of latencies during the experiments in the water maze – what is a sign of learning – and therefore they were more successful than Lurchers with retinal degeneration. As completely developed retinal degeneration in adult mice causes practical blindness

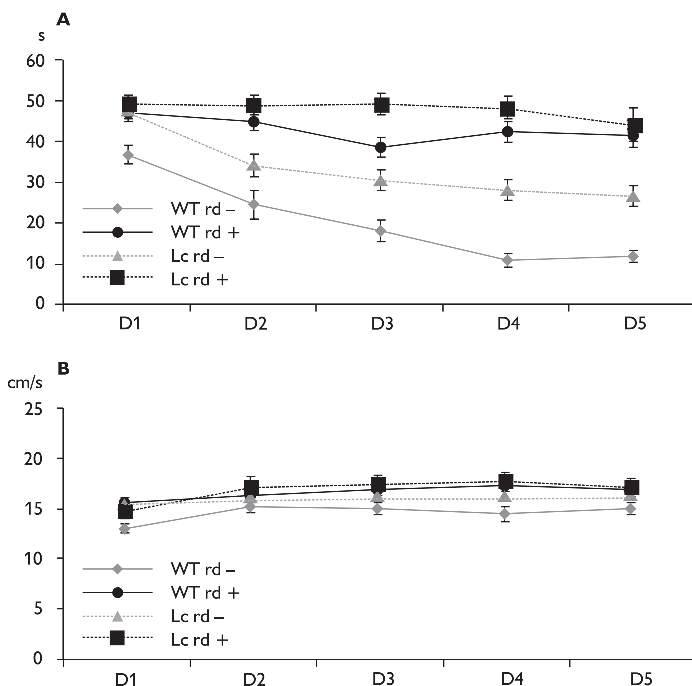

Figure 8 – Spatial orientation in the water maze with visualized platform in wild type mice without the retinal degeneration (WT rd–), wild type mice with the retinal degeneration (WT rd+), Lurcher mutants without the retinal degeneration (Lc rd–) and Lurchers with the retinal degeneration (Lc rd+). Mean latencies (in s) ± S.E.M. (A) and mean swimming velocity (in cm/s) ± S.E.M. (B).

this fact leads us to hypothesis that wild type mice are able to use idiothetic navigation (based on integrating selfmotion generated information) while Lurcher mutants have no ability to use such mechanisms of orientation. Because Lurchers with normal retina were able to learn to reach a visible platform, however not as easy as the wild type mice, their ataxia is not so critical for reaching the goal and they are able to solve the task when the goal is visible and the animals can use visual allothetic navigation (based on spatial relationships between visually perceived remote landmarks and the subject). When Lurchers loose the possibility of direct visual navigation due to the hidden goal or due to retinal degeneration they fail in the spatial orientation tasks. Although both above mentioned types of navigation systems (idiothetic, allothetic) require the hippocampal formation, it is probable that different circuits implement them. Among them probably the cerebellum and its functional connections play the role as the results showed [23].

Cerebellar degeneration in Lurcher mutants can be visualized with several histological, histochemical or immunohistochemical methods. All of them have some advantages and disadvantages and they are suitable for different purposes. Nissl staining shows the structure of the neural tissue. For Purkinje cell quantification anticalbindin staining is more applicable. Golgi impregnation displays only small part (about 1 %) of all cells. That is why it can inform only about relative representation of individual types of neurons. Because only some of the cells are stained there are no problems with overlaying of cells and the shape of

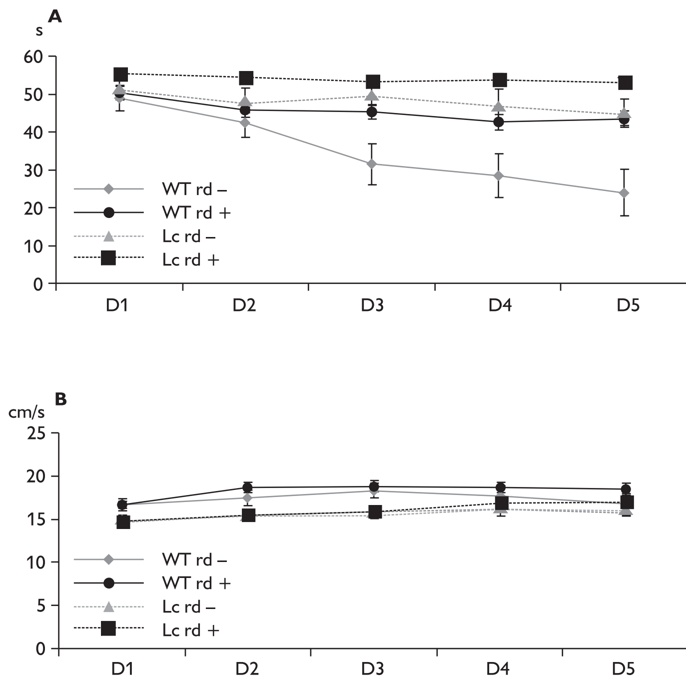

Figure 9 – Spatial learning in the water maze with hidden platform in wild type mice without the retinal degeneration (WT rd–), wild type mice with the retinal degeneration (WT rd+), Lurcher mutants without the retinal degeneration (Lc rd–) and Lurchers with the retinal degeneration (Lc rd+). Mean latencies (in s) ± S.E.M. (A) and mean swimming velocity (in cm/s) ± S.E.M. (B).

the cell and the arborisation of its dendrites is visible very well in Golgi impregnation.

Fluoro-Jade method labels injured and degenerating neurons and it is considered to be a specific indicator of lethal neuronal injury [10, 11, 12, 13]. We used this method to assess the intensity of apoptotic extinction of Purkinje cells in young Lurchers. The degeneration starts at the age of 8 days and in 26 days old mice only 10 % of Purkinje cells remain [4, 14]. That is why in 14 and 21 days old Lurchers high intensity of the apoptotic process can be expected. In spite of this assumption we found only small number of Fluoro-Jade positive cells in the cerebellum of these mice. On the other hand, bisbenzimide staining and fluorescent double staining confirmed, that most of the Purkinje cells showed signs of proceeding apoptosis. This discrepancy leads us to the opinion that Fluoro-Jade B staining marks only cells in short period of the apoptotic process in the case of Lurcher mutant cerebellum and that this method is not useful for assessment of number of dying Purkinje cells in Lurcher mutant mice.

Findings of Purkinje cells with normal appearance and those with signs of apoptosis in the cerebellum of one Lurcher mutant individual together with decreased number of Purkinje cells indicates that the affection by degenerative process is not uniform. It is in agreement with consecutive decrease of Purkinje cells since 8th to 90th postnatal day [14].

Conclusion

Motor skills of Lurcher mutant mice were markedly worse than in wild type mice. The impact of retinal degeneration was essentially inferior and it was observed only in wild type animals. Navigation to the visible goal depended on eyesight considerably and was also deteriorated expressively with cerebellar degeneration. Navigation to the hidden platform was also dependent on both eyesight and cerebellum. The cerebellum may play a role in both alothetic and idiothetic orientation processes. The cerebellar degeneration can be evaluated with many histological methods. Some methods are suitable to visualize of the tissue structure, some of them for Purkinje cells quantification and other for evaluation of individual cell morphology and detection of apoptotic process.

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