and genetic engineering of experimental mice [5–7]. Simultaneously we have tried to develop a new molecular therapy for lysosomal storage diseases, starting from Fabry disease [8], and then G_{M1}-gangliosidosis [7] and Gaucher disease [9], by using low molecular compounds acting as chemical chaperones that stabilize mutant enzyme proteins; 1-deoxygalactonojirimycin, *N*-octyl-4-epi-β-valienamine, and *N*-octyl-β-valienamine, respectively.

The therapeutic effects of these compounds have been well established at the cellular level for each disease [7]. However, during the course of mouse experiments, we faced some difficulty assessing the neurological status of individual experimental animals with progressive neurological deterioration. We therefore started a trial to establish a neurological assessment system by modifying various motor and reflex testing methods currently in use for clinical child neurology.

2. Materials and methods

2.1. Knockout (KO) and transgenic (Tg) mice

We prepared a C57BL/6-based congenic KO mouse strain with β-galactosidase deficiency (−/−) [6]. It is a mouse strain with complete deficiency of β-galactosidase, corresponding to infantile G_{M1}-gangliosidosis in humans (severe form) [6,7]. Female mice are fertile. However, feeding and breeding of the offspring are difficult as they have already developed neurological symptoms and signs. In this study we examined them at 5–9 months of age (body weight 20–40 g).

Then, a DNA fragment, containing β-actin CAG promoter and human mutant β-galactosidase cDNA (R201C), was injected into C57BL/6 fertilized eggs for preparation of a Tg mouse line, overexpressing mutant human β-galactosidase with an amino acid substitution R201C causing juvenile G_{M1}-gangliosidosis in humans (mild form) [7]. The Tg mouse (R201C mouse) for this study was obtained by cross-breeding of the KO mouse and original Tg mouse. We used the hemizygous Tg mouse (Tg/−) with the KO background, expressing detectable residual β-galactosidase activity (4% of the control mean). In this study we examined them at 4–11 months (body weight 20–40 g).

Wild-type (WT) mice (C57BL/6Cr) were purchased from Japan SLC (Shizuoka). They have the life span of 2 years in average, and reproduction is possible at 2–8 months of age. Their age and body weight were the same as the two types of disease model mice in this study.

The mice were kept in a temperature-controlled room (23 ± 1 °C) that was illuminated between 08:00 and 20:00 h. Commercial rodent chow and tap water were provided *ad libitum*.

2.2. Neurological assessment of mice

We chose 11 tests mainly by modification of reflex testing methods currently in use for clinical child neurology; spontaneous movement and posture observations, and testing of primitive, postural and equilibrium reflexes in infancy and young children (Table 1). We evaluated the neurological status by both individual and total scores for each mouse.

The tests depend on the physical and environmental conditions of individual mice. Testing was performed at night (20:00–22:00 h), and, if necessary, repeated on the same mouse for a few successive days.

The care of experimental animals was carried out in accordance with the Guidelines on Animal Experimentation of International University of Health and Welfare (Otawara).

2.3. Scoring of the test results

Individual test items were graded in 4 scores: 0 (normal), 1 (slightly abnormal), 2 (moderately abnormal), and 3 (highly abnormal) (Table 1). We designated each score based on gross qualitative observation and/or quantitative temporal–spatial parameters, such as staying time, walking distance, or staggering angle. We used Microsoft Excel (Microsoft, Seattle) and STATISTICA Ease (StatSoft Japan, Tokyo) for statistic analysis of the score data.

3. Results

3.1. Life span of KO and Tg mice

For confirmation of the severity and clinical course of the KO and Tg mice, we collected the natural death cases in both groups (Fig. 1). Death occurred at 7–11 months and 11–19 months of age, respectively, in the KO and Tg mouse groups. In general the clinical course of Tg mice were 1.5- to 2-fold longer than that of KO mice.

3.2. Reproducibility of individual test scores

Repeated testing revealed reproducible score results for each test (data not shown). Experimental conditions were kept identical in the test laboratory as far as possible with regard to temperature, light, sound, and other environmental factors. We performed neurological examinations at night (20:00–22:00 h).

3.3. Sex difference in test results

The animals were fed with normal nutritional food, avoiding overfeeding with high calorie diet. There was

Table 1
Neurological examination of genetically engineered G_{M1}-gangliosidosis model mice

1. Gait
   Score 0: normal
   Score 1: slight gait disturbance with hip abduction, knee extension, and lumbar elevation (0.5–1 cm); mild staggering and shivering (2–3 s; intermittent; localized to limbs)
   Score 2: marked gait disturbance with hip abduction, knee extension, and lumbar elevation (1–1.5 cm); moderate staggering and shaking (2–3 s; intermittent; generalized)
   Score 3: marked staggering and shaking (continuous and vertical); gait impossible
2. Posture: forelimb
   Score 0: normal
   Score 1: starting gait difficult and clumsy
   Score 2: dragging limbs; inversion of dorsum pedis
   Score 3: complete paralysis; no spontaneous movement
3. Posture: hind limb
   Score 0: normal; smooth joint flexion and extension
   Score 1: slight hip abduction (up to 10°) and external rotation; knee extension; wide-based (2–3 cm)
   Score 2: severe hip abduction (10°–20°) and external rotation; knee extension; wide-based (>3 cm)
   Score 3: no spontaneous movement
4. Trunk
   Score 0: normal
   Score 1: slight back hump
   Score 2: moderate back hump
   Score 3: severe back hump
5. Tail
   Score 0: normal
   Score 1: slight stiffness and elevation (up to 20°)
   Score 2: severe stiffness and elevation (up to 45°)
   Score 3: severe stiffness and elevation with persistent deformity
6. Avoiding response: pinching tail root with forceps for 1 s
   Score 0: strong rejection, avoidance, and squeaking
   Score 1: slight decrease of response
   Score 2: trunk torsion; hind limb extension
   Score 3: no response
7. Rolling over: turning the tail root three times to left and right
   Score 0: extending four limbs, resisting passive rolling
   Score 1: slow passive rolling; prompt recovery (within 1 s)
   Score 2: markedly slow passive rolling; delayed recovery (several seconds)
   Score 3: posture change impossible; slow body movement
8. Body righting acting on head: response to vertical hanging (head down by holding tail tip) and quick upward movements (three times) within 30 s
   Score 0: strong upward righting reaction of the head up to 180°
   Score 1: slight decrease in response up to 45°
   Score 2: marked decrease in response up to 20°
   Score 3: no response; trunk rotation only
9. Parachute reflex: response to vertical hanging (head down by holding tail tip) and quick downward movement (three times) within 30 s
   Score 0: extension and abduction of hind limbs (>45°); continuous knee extension
   Score 1: slight decrease in response (<45°); intermittent knee extension
   Score 2: marked decrease in response; flexion and adduction of hind limbs; slow movements
   Score 3: no response; continuous flexion and adduction of hind limbs
10. Horizontal wire netting: stepping through interstice during walking on horizontal wire netting for 30 s (size 23.5 × 23.5 cm; mesh 2 × 2 cm; wire diameter 1 mm, undulating)
    Score 0: no stepping into interstice
    Score 1: 21–30 s before stepping into interstice
    Score 2: 11–20 s before stepping into interstice
    Score 3: 0–10 s before stepping into interstice
11. Vertical wire netting: clinging and holding body on vertical wire netting for 30 s (size 23.5 × 23.5 cm; mesh 1 × 1 cm; wire diameter 1 mm, undulating)
    Score 0: stay for 30 s
    Score 1: stay for 21–30 s before falling
    Score 2: stay for 11–20 s before falling
    Score 3: stay for 0–10 s before falling

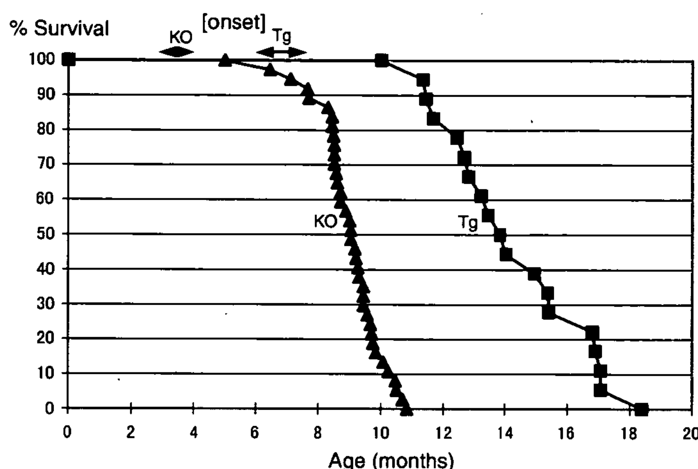

Fig. 1. Life span of genetically engineered G_{M1}-gangliosidosis model mice. ▲-▲: KO mouse, severe form of the disease, corresponding to human infantile G_{M1}-gangliosidosis ($n = 37$). ■-■: Tg mouse based on KO background, less severe form of the disease, corresponding to human juvenile G_{M1}-gangliosidosis ($n = 18$). Onset: clinical impression by gross observation; 3–4 months for KO, and 6–8 months for Tg.

no significant difference in score values between males and females (data not shown). Accordingly all test results in both sexes were collected together for further analysis.

3.4. Individual tests

All numeric data of individual tests are summarized in Fig. 2 (mean ± SEM). In the WT mice any of the mean test scores was never elevated more than 0.5 during the age period of this study (2–10 months).

The KO mice showed abnormally high scores even at the early stage of the disease in almost all tests. Gait and tail abnormalities were particularly remarkable already at 5 months of age (>1.5), and the high level persisted till the terminal stage of the disease. The other tests showed increasing abnormalities up to 2.0–2.5 with the progression of the disease.

The Tg mice showed less high scores for all tests as compared to the KO mice, but again the tail abnormality was evident (>1.0) at the early stage of the disease, and slowly increased till the end of the disease. Some other tests, such as trunk posture, parachute reflex, horizontal and vertical wire netting tests, became increasingly abnormal (>1.0–1.5) as compared to those for WT mice.

3.5. Total scores

The total scores are shown in Fig. 3 (mean ± SEM). The score never reached more than 0.5 in the WT mice during the course of this study till 10 months of age. It increased slowly with age in both KO and Tg mice, with always significantly higher scores in the KO mice. The scores of the Tg mice even before the onset of clinically detectable neurological signs were significantly higher than those of WT, mainly by the contribution of abnormal postures (gait, hind limb, and trunk) and abnormal parachute reflex.

4. Discussion

After the original studies on experimental dogs by Sherrington [10], the results of human studies were first reported by Magnus and de Kleijn [11], followed by many other physiologists, pediatricians, neurologists, and physiotherapists [12–18]. At present these techniques of neurological examination are used for routine motor assessment of early development in infants and young children in humans.

However, in spite or recent rapid progress of genetic and metabolic approaches to experimental animals, clinical assessment of their neurological status has not been well described till present. Thousands of genetically engineered disease model mice are left without clear and systematic description of phenotypic expression, although in some cases genotype–phenotype correlation has been elaborately analyzed using some test apparatuses. In fact the new fields of mouse behavioral genetics [19] and behavioral phenotyping [20] have been proposed.

A new assessment protocol SHIRPA was reported [21] for comprehensive phenotypic evaluation. This starts with the primary screen by behavioral observation, followed by the secondary screen involving a comprehensive behavioral screening battery for locomotor activity, together with pathologic and biochemical analyses, and then the tertiary screen utilizing test apparatuses for anxiety, learning and memory, electrophysiology and neuroimaging. Further a monograph was published for more detailed behavioral phenotyping of Tg and KO mice

[22]. This system consists of comprehensive testing, including motor and sensory functions, learning and memory, feeding and drinking, and various other behaviors (reproductive, social, and emotional). Both are useful for clinical examination of general and behavioral status of disease model mice.

Another study reported differences in behavioral performance among the seven mouse 129 substrains [23], particularly anxiety-related behaviors in the zero-maze, habituation to the open field, and cued fear conditioning. The authors concluded that behavioral differences may have implications for interpretation of data for KO mice that may retain a small portion of the original genome even after backcross to B6. We backcrossed the JCI/IcR KO mice to establish congenic B6. Clear and definite judgment was possible in our present study

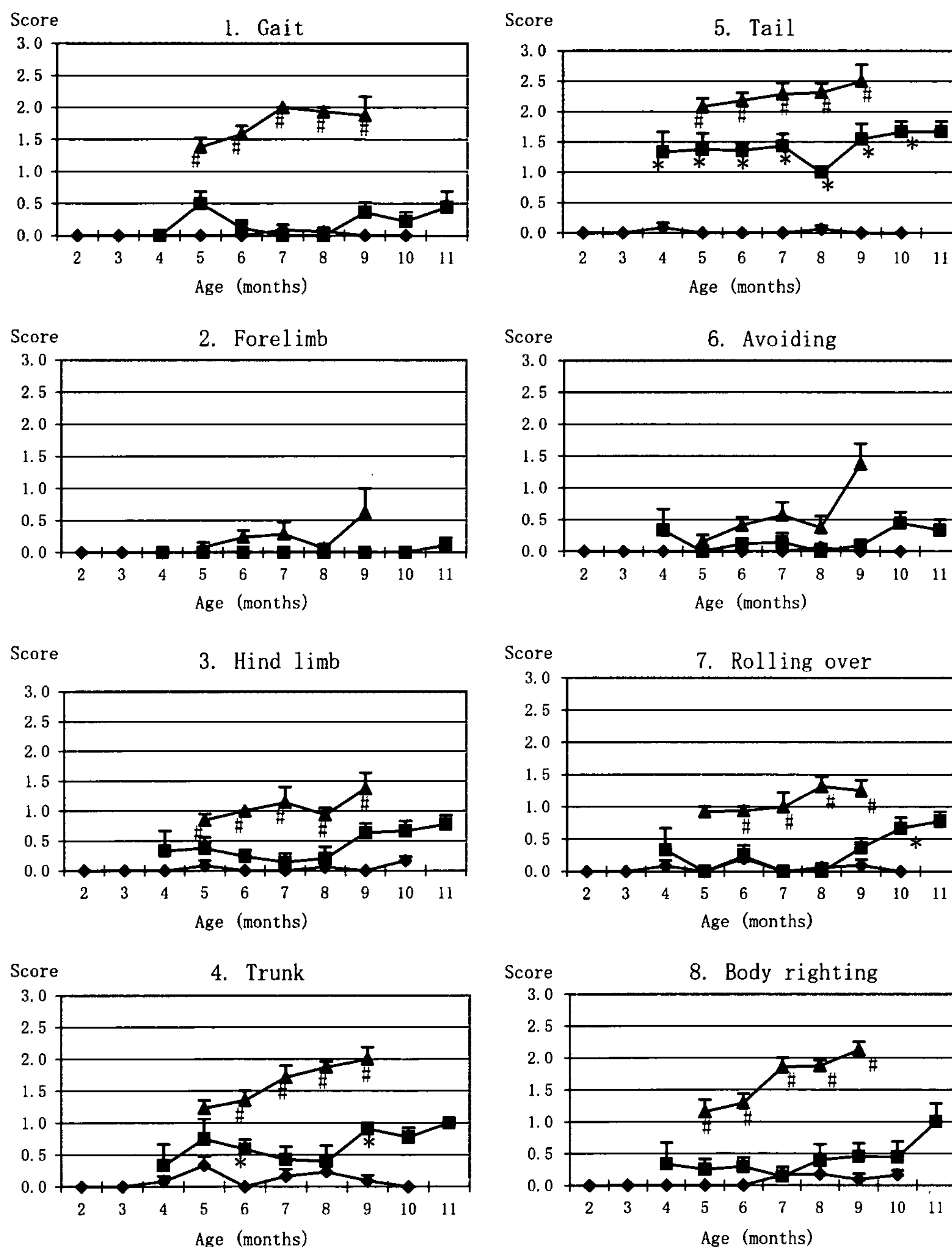

Fig. 2. Individual test scores in G_{M1}-gangliosidosis and WT mice. ▲-▲: KO mouse with severe clinical manifestations; $n = 13$ (5 m), 17 (6 m), 7 (7 m), 16 (8 m), and 8 (9 m). ■-■: Tg mouse with less severe clinical manifestations; $n = 3$ (4 m), 8 (5 m), 17 (6 m), 7 (7 m), 5 (8 m), 11 (9 m), 9 (10 m), and 9 (11 m). ◆-◆: commercially purchased WT mouse; $n = 12$ (4 m), 12 (5 m), 5 (6 m), 12 (7 m), 17 (8 m), 11 (9 m), and 6 (10 m). Each value represents the mean of the individual score values with SEM (vertical bar). $^*p < 0.05$ (Tg vs WT); $^\#p < 0.05$ (KO vs Tg); otherwise $p > 0.05$.

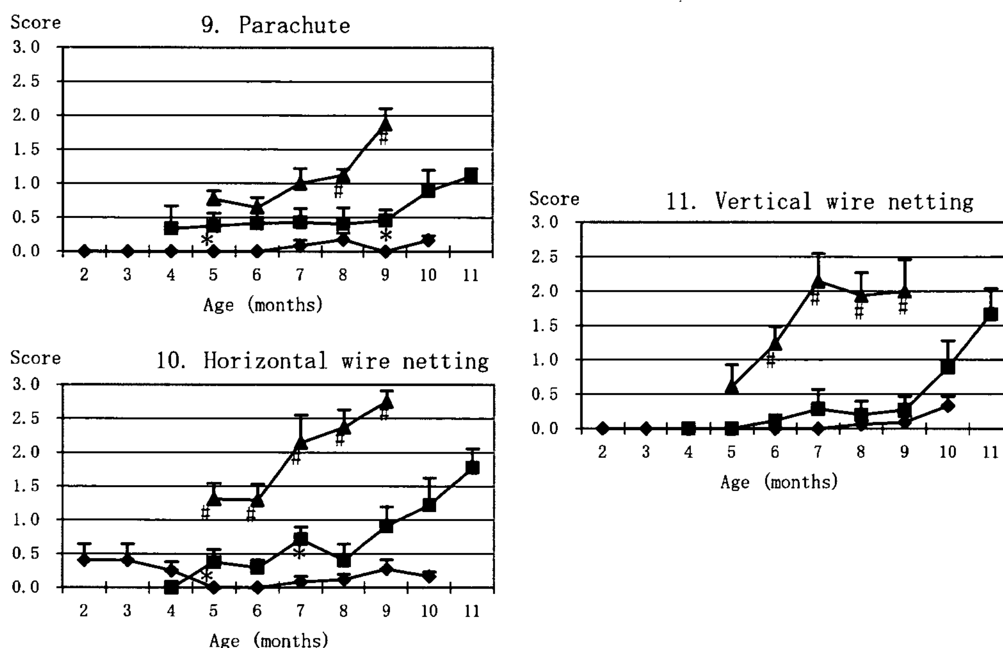

Fig. 2 (*continued*)

about chronological changes of neurological deterioration in both KO and Tg congenic strains originated from the same genetic background as compared to the C57BL/6Cr WT mice. We are aware that some sophisticated test apparatuses are commercially available mainly for learning, memory, and behavior analysis by repeated testing for a few days or more.

In spite of these previous reports, we needed a simple and quick assessment system for clinical experiments

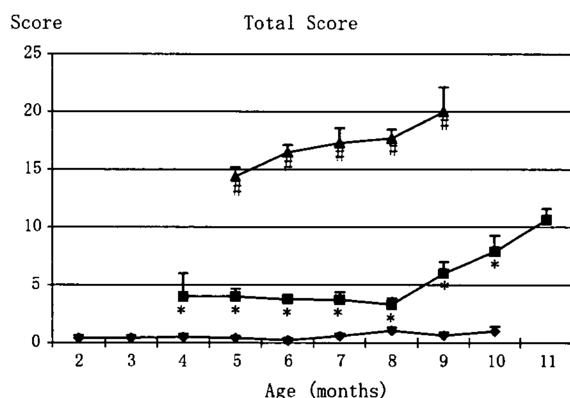

Fig. 3. Total test scores in G_{M1}-gangliosidosis and WT mice. ▲-▲: KO mouse with severe clinical manifestations; $n = 13$ (5 m), 17 (6 m), 7 (7 m), 16 (8 m), and 8 (9 m). ■-■: Tg mouse with less severe clinical manifestations; $n = 3$ (4 m), 8 (5 m), 17 (6 m), 7 (7 m), 5 (8 m), 11 (9 m), 9 (10 m), and 9 (11 m). ◆-◆: commercially purchased WT mouse; $n = 12$ (4 m), 12 (5 m), 5 (6 m), 12 (7 m), 17 (8 m), 11 (9 m), and 6 (10 m). Each value represents the mean of the total score values with SEM (vertical bar). $^{*}p < 0.05$ (Tg vs WT); $^{\#}p < 0.05$ (KO vs Tg); otherwise $p > 0.05$.

using model mice, presenting particularly with rapid deterioration of the nervous system like lysosomal storage diseases. G_{M1}-gangliosidosis is a classic neurogenetic disease in humans, occurring mainly in infancy, deteriorating rapidly to severe neurosomatic dysfunction within a few months after the onset of the disease. The neurological status of the animal counterpart in question may change in a short period, even within a week. We therefore excluded intentionally the test methods involving learning and memory, as they are not appropriate for assessment of such a rapidly progressive disease. Similar assessments were made for model mice and rats with amyotrophic lateral sclerosis, a less rapidly progressive neurological disease in humans, using several different non-invasive and objective methods [24–26].

We initially started this study with 16 test methods, including the tests utilizing commercially available simple apparatuses, such as open field test, Rotarod, and water maze tests, but finally reduced to 11 tests, discarding the others because of unstable and unreliable test results, questionable reproducibility, or insufficient test conditions in our preliminary study for G_{M1}-gangliosidosis. We will re-evaluate these tests, and hopefully add also other test items in order to establish more reliable assessment system of the brain function in genetic disease model mice with progressive neurological deterioration.

We anticipated that a quantitative analysis will give a more clear idea about the neurological status of a disease mouse strain at different clinical stages. We therefore tried scoring of the neurological tests. The clinical impression was found to be highly correlated with this

quantitative score data. Unfortunately, for technical reasons, the number of animals in this study was not systematically arranged, and not always sufficient for data analysis. The data presented in this report was based on random collection of the age groups available for clinical evaluation, although some animals were followed monthly for sequential changes of neurological abnormalities. In addition, the mice were not available at the very early stage of the disease (pre-symptomatic and early symptomatic), when this testing method became ready for use. At 4–5 months of age, both severe (KO) and mild (Tg) model mice already showed abnormal scores in some tests (tail and hind limb postures). We conclude that this assessment is necessary for accurate early diagnosis of G_{M1}-gangliosidosis model mice. The testing should be started as early as 2–3 months after birth for detection of clinical symptoms.

We have confirmed reliability of this new assessment method in G_{M1}-gangliosidosis model mice The main purpose of this study was to develop a clinical method for monitoring efficacy of a new molecular therapeutic approach, chemical chaperone therapy [1,7,27], for brain pathology in experimental model mice with G_{M1}-gangliosidosis and other lysosomal storage diseases. We expect that this method will reveal the effectiveness of chemical chaperone therapy for these diseases in the near future. This approach will be useful also for many other neurogenetic model mice.

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References

[1] Suzuki Y, Oshima A, Nanba E. β-Galactosidase deficiency (β-galactosidosis): G_{M1}-Gangliosidosis and Morquio B disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Vogelstein B, editors. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill; 2001. p. 3775–809.

[2] Oshima A, Tsuji A, Nagao Y, Sakuraba H, Suzuki Y. Cloning, sequencing, and expression of cDNA for human β-galactosidase. Biochem Biophys Res Commun 1988;157:238–44.

[3] Yoshida K, Oshima A, Shimmoto M, Fukuhara Y, Sakuraba H, Yanagisawa N, et al. Human β-galactosidase gene mutations in G_{M1}-gangliosidosis: a common mutation among Japanese adult/chronic cases. Am J Hum Genet 1991;49:435–42.

[4] Oshima A, Yoshida K, Shimmoto M, Fukuhara Y, Sakuraba H, Suzuki Y. Human β-galactosidase gene mutations in Morquio B disease. Am J Hum Genet 1991;49:1091–3.

[5] Matsuda J, Suzuki O, Oshima A, Ogura A, Naiki M, Suzuki Y. Neurological manifestations of knockout mice with β-galactosidase deficiency. Brain Dev 1997;19:19–20.

[6] Matsuda J, Suzuki O, Oshima A, Ogura A, Noguchi Y, Yamamoto Y, et al. β-Galactosidase-deficient mouse as an animal model for G_{M1}-gangliosidosis. Glycoconjugate J 1997;14:729–36.

[7] Matsuda J, Suzuki O, Oshima A, Yamamoto Y, Noguchi A, Takimoto K, et al. Chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis. Proc Natl Acad Sci USA 2003;100:15912–7.

[8] Fan JQ, Ishii S, Asano N, Suzuki Y. Accelerated transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. Nat Med 1999;5:112–5.

[9] Lin H, Sugimoto Y, Ohsaki Y, Ninomiya H, Oka A, Taniguchi M, et al. *N*-octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease. Biochim Biophys Acta 2004;1689:219–28.

[10] Sherrington CS. Decerebrate rigidity, and reflex coordination of movements. J Physiol 1898;22:319–32.

[11] Magnus R, de Kleijn A. Die Abhängigkeit des Tonus der Extremitätenmuskeln von der Kopfstellung. Pflüg Arch ges Physiol 1912;193:455–548.

[12] Landau A. Über motorische Besonderheitendes zweiten Lebnshalbjahrs. Mschr Kinderheilk 1925;29:555–7.

[13] Schaltenbrand G. Normale Bewegungs-und Lagereaktion be Kindern. Dtsch Ztschr Nervenheilk 1925;87:23–59.

[14] Gesell A, Amatruda CS. Developmental diagnosis. 2nd ed. New York: Hoeber; 1947.

[15] Thomas A, Dargassis S-A. Études neurologique sur le nouveau-né et jeune nourisson. Paris: Masson et Cie; 1952.

[16] Paine RS. Neurologic examination of infants and children. Pediat Clin North Amer 1960;7:471–510.

[17] Peiper A. Die Eigenart der Kindlichen Hirntatigkeit, 3 Auflage, Leipzig: G. Thieme; 1961.

[18] Fiorentino R. Reflex testing methods for evaluating CNS development. Springfield: Charles C. Thomas; 1963.

[19] Bucan M, Abel T. The mouse: genetics meets behaviour. Nat Rev Genet 2002;3:114–23.

[20] Van der Staay FJ, Steckler T. Behavioural phenotyping of mouse mutants. Behav Brain Res 2001;125:3–12.

[21] Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ, Martin JE. Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. Mamm Genome 1997;8:711–3.

[22] Crawley JN. What's wrong with my mouse? Behavioral phenotyping of transgenic and knockout mice. New York: Wiley-Liss; 2000.

[23] Cook MN, Bolivar VJ, McDadyen MP, Flaherty L. Behavioral differences among 129 substraints: implications for knockout and transgenic mice. Behav Neurosci 2002;116:600–11.

[24] Matsumoto A, Okada Y, Nakamichi M, Nakamura M, Toyama Y, Sobue G, et al. Disease progression of human SOD1 (G93A) transgenic ALS model rats. J Neurosci Res 2006;82:119–33.

[25] Barneoud P, Lolivier J, Sanger DJ, Scatton B, Moser P. Quantitative motor assessment in FALS mice: a longitudinal study. NeuroReport 1997;8:2861–5.

[26] Weydt P, Hong SY, Kliot M, Moller T. Assessing disease onset and progression in the SOD1 mouse model of ALS. NeuroReport 2003;14:1051–4.

[27] Iwasaki H, Watanabe H, Iida M, Ogawa S, Tabe M, Higaki K, et al. Fibroblast screening for chaperone therapy in β-galactosidosis. Brain Dev 2006;28:482–6.