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Recent Developments in Gene Therapy Research Targeted to Cerebellar Disorders

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1. Introduction

The cerebellum plays an important role in coordinated movement, motor learning and vestibular function. Cerebellar damage results in impaired body balance and disturbance in gait and posture. The cerebellum is impaired by various genetic diseases, such as spinocerebellar ataxia and mucopolysaccharidosis, and these diseases could be good candidates for gene therapy. There are at least two challenges that should be overcome before the clinical application of gene therapy is used to treat cerebellar disorders. The first challenge results from its large size, as the cerebellum is the second largest component of the central nervous system. The cerebellum can be subdivided into three main parts: the cerebrocerebellum, the vestibulocerebellum and the spinocerebellum (see Section 2 for details). Each subdivision in the cerebellum plays a distinct role and, to attain a satisfactory rescue of the cerebellar function by gene therapy, a therapeutic gene should be delivered efficiently and extensively into the large cerebellum.

The second challenge is to deliver a gene into specific target cell populations in the cerebellum. In Parkinson's disease, which is caused by degeneration of nigra-striatal dopaminergic neurons, cell type-specific delivery of a therapeutic gene is a secondary matter, as the supply of sufficient amounts of dopamine, irrespective of neurons or glia, in the striatum is most critical for the functional recovery of the basal ganglia. By contrast, specific cell populations within the cerebellum, such as cerebellar Purkinje cells, Bergman glia or neurons in the deep cerebellar nuclei, are selectively impaired in most cerebellar diseases, such as spinocerebellar ataxia. Thus, affected cell types differ depending on the disease type, and the selective delivery of a therapeutic gene to a subset of affected cell types could be a key advance for rescuing cells that are degenerating from progressive damage, restoring cerebellar function and, ultimately, helping patients to recover from cerebellar ataxia.

To this end, we have developed methods that allow for Purkinje cell-specific and Bergmann glia-specific gene expression in mice by modifying the culture conditions of lentiviral vector-producing human embryonic kidney (HEK) 293FT cells in combination with cell-type-specific promoters in lentiviral vectors. Moreover, a new injection technique for efficient and widespread gene delivery into the cerebellar cortex has been devised, which takes advantage of the anatomical location of the cerebellum. Using the newly developed gene transfer method for the cerebellum, we aimed to restore the abnormal phenotypes of two types of ataxic mice, both of which are affected in Purkinje cells by different

pathologies. The results showed that the efficient and widespread delivery of therapeutic genes into Purkinje cells significantly restored ataxia and morphological and functional abnormalities of these cells in mutant mice. In this chapter, we describe our method that efficiently permits the selective gene delivery to cerebellar Purkinje cells or Bergmann glia and the rescue of two examples of ataxic mice. We also describe existing problems that should be resolved for the future clinical application of lentiviral vectors to cerebellar disorders.

2. Cerebellar organization and neural circuits in the cerebellum

The cerebellum can be subdivided into three main parts based on differences in their sources of input. The largest subdivision in humans is the cerebrocerebellum, which occupies most of the lateral hemisphere and is involved in the regulation of highly skilled movements, including speech. The vestibulocerebellum is the phylogenetically oldest part of the cerebellum; it comprises the caudal lobes of the cerebellum, including the flocculus and the nodulus, and is primarily engaged in the regulation of movements underlying posture and equilibrium. The last of the major subdivisions is the spinocerebellum, which occupies the median and paramedian zone of the cerebellar hemispheres. The spinocerebellum is the only part that receives input directly from the spinal cord. The lateral part and central part (vermis) of the spinocerebellum are involved in the movements of distal and proximal muscles, respectively. The vermis also regulates eye movements in response to vestibular inputs.

The cerebellar cortex contains 5 neurons, the granule cell, the Purkinje cell and three inhibitory interneurons (stellate cell, basket cell and Golgi cell) and is divided into three morphologically distinct parts: the granule cell layer, the Purkinje cell layer, and the molecular layer (Fig. 1a and b). The granule cell layer contains numerous granule cells, in which Golgi cells are scattered. The Purkinje cell layer is a monolayer that consists of the soma of Purkinje cells and Bergmann glia; the Purkinje cells extend their well-differentiated dendrites into the molecular layer (Fig. 1c), where stellate cells and basket cells are located. The Bergmann glia also extend their processes into the molecular layer (Fig. 1b).

A major input to the cerebellar cortex are the mossy fibers, axon bundles projecting from neurons in the thalamus, the brain stem and the spinal cord (Fig. 1b). The excitation of granule cells triggered by mossy fibers is transferred through the axons called parallel fiber to Purkinje cells. One Purkinje cell has more than one hundred thousand dendritic spines (Fig. 1d, arrows), on which parallel fiber terminals make excitatory synapses. Parallel fiber - Purkinje cell synapses are tightly wrapped by processes of Bergmann glia, so as to quickly take up the glutamate released from parallel fiber terminals. In this context, the Bergmann glia prevent the prolonged excitation of Purkinje cells and the spillover of glutamate that would activate the adjacent synapses.

Another excitatory input to the cerebellar cortex is the climbing fiber that originates from neurons in the inferior olivary nucleus of the medulla oblongata. The climbing fiber makes excitatory synapses directly on proximal dendrites of Purkinje cells and neurons in the deep cerebellar nuclei. Excitatory activity of granule cells and Purkinje cells is modulated by 3 types of inhibitory interneurons: Golgi cells, stellate cells, and basket cells. The Purkinje cells eventually integrate the information entered into the cerebellar cortex and send an inhibitory signal as the sole source of output from the cerebellar cortex to neurons in the deep cerebellar nuclei.

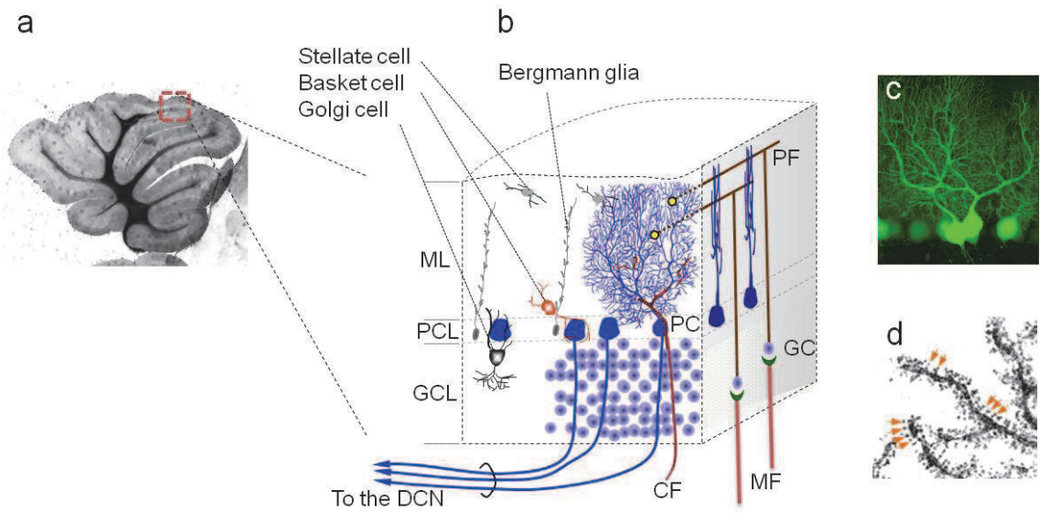

Fig. 1. Projections to and neural circuits in the cerebellar cortex. (a) A sagittal section of the vermis of a mouse cerebellum. (b) A schematic diagram of the cerebellar cortex that enlarges the square region in (a). ML, molecular layer; PCL, Purkinje cell layer; GCL, granule cell layer. A Purkinje cell (PC) receives excitatory inputs via parallel fibers (PF) and climbing fibers (CF) and sends inhibitory signals to neurons of the deep cerebellar nuclei (DCN). GC, granule cell, MF, Mossy fiber. (c) Well-differentiated dendrites of Purkinje cells are visualized by GFP expression. (d) Dendritic spines of Purkinje cells (arrows).

3. Disorders of the cerebellum

As cerebellar defects can be easily detected from overt ataxia, such as widespread gait and motor coordination deficits, numerous types of mutant mice with cerebellar degeneration have been isolated over the past 50 years. These mutant mice have contributed significantly to the elucidation of cerebellar physiology. At present, in parallel with advances in the development of viral vectors, we may be able to restore cerebellar defects of ataxic mice by a potential therapeutic gene delivery. Such rescue experiments have significant implications in terms of the future clinical application of gene therapy for patients suffering from cerebellar diseases. The following subsections summarize the cerebellar abnormalities and genetic defects identified in ataxic mice and humans.

3.1 Spontaneously occurring ataxic mice

Table 1 summarizes well-known murine mutants that cause cerebellar impairment. Recently, the genes responsible for those mutants have been identified and include missense mutations, nonsense mutations or frameshift mutations in the causative genes. The *hotfoot* mice are spontaneously occurring recessive mutants (Guastavino et al., 1990) that carry mutations in *Grid2*, which encodes the δ2 glutamate receptor (GluRδ2) (Wang et al., 2003). To date, more than 10 mutant alleles have been identified; among these, *hotfoot5J* mice have been shown to carry a point mutation in exon 12 of *Grid2*, which creates a stop codon in the region encoding transmembrane 3 of GluRδ2 protein (Wang et al., 2003). The aberrant GluRδ2 protein is easily degraded and is not detected in Purkinje cells of *hotfoot5J* mice; therefore, *hotfoot5J* mice are mutants lacking GluRδ2 function.

Mouse name	Chromosome	Gene	Major cell defect	Age of onset
Hotfoot (*Grid2*^{ho})	6	*Grid2* deletion (Loss of function)	Purkinje cell	3 weeks postnatal
Staggerer (*ROR*^{sg})	9	*Rora* deletion (Loss of function)	Purkinje cell	1 week postnatal
PCD (*Agtpbp1*^{pcd})	13	*Agtpbp* deletion (Loss of function)	Purkinje cell	3 weeks postnatal
Lurcher (*Grid2*^{Lc})	6	*Grid2* missense mutation (Gain of malfunction)	Purkinje cell	2 weeks potnatal

Table 1. Genetic abnormalities and affected cell types in spontaneously occurring ataxic mice.

Other loss-of-function mutants are *Staggerer* and Purkinje cell degeneration (pcd) mice. The *staggerer* mutation causes a functional impairment in a transcription factor, retinoid-related orphan receptor α (RORα), which belongs to the nuclear receptor superfamily (Boukhtouche et al., 2006; Gold et al., 2007). *Staggerer* mice have a 122-base pair deletion within the ligand binding domain (LBD) of the RORα gene (Hamilton et al., 1996). For PCD mice, 8 independent alleles have been identified, which carry genetic mutations in the *Nna1* gene, encoding a putative nuclear protein (Fernandez-Gonzalez et al., 2002; Wang & Morgan, 2007). Most of the *pcd* alleles represent complete loss-of-function mutations. Because it is reasonable to postulate that aberrant phenotypes in loss-of-function mutants may be restored by introducing the intact gene, and, as Purkinje cells are the main cell type impaired in the loss-of-function mutants discussed here (*hotfoot*, *staggerer* and *pcd*), the efficient delivery of the intact gene into the specific subset of cells (Purkinje) may be a promising therapy for the recovery of those mutant mice from cerebellar degeneration and, ultimately, ataxia.

There are two important points that should be addressed. First, gene delivery should be performed before Purkinje cells are substantially lost or irreversibly damaged. Purkinje cells of *pcd* mice die gradually from approximately 18 days of age and are virtually lost by 4 months of age. Similarly, the Purkinje cell number in *staggerer* mice begins to decrease in the first week after birth, and at least 75-90% of Purkinje cells are lost in adult mutants (Vogel et al., 2000). The degeneration of Purkinje cells causes secondary defects of granule cells and neurons in the inferior olivary nucleus. Therefore, later gene delivery results in less or no functional recovery in *staggerer* and *pcd* mutants.

Another important point is that gene delivery should be carried out before the Purkinje cell loses its plasticity. The compensation of a missing gene during an inappropriate time may result in the insufficient restoration of Purkinje cell abnormalities. By the second to third postnatal week, Purkinje cells extend dendrites and make a hundred thousand synapses with different counterparts. A series of developmental processes requires the strictly regulated expression of numerous genes, suggesting that the exogenous delivery of an intact gene after termination of this period may have little therapeutic impact. This period, in which the brain displays a heightened sensitivity to exogenous stimuli (and still maintains capacity to respond to gene therapy), is referred to as the "critical period". The critical period differs depending on the defects of the mutant animals; thus, a careful examination using animal models can provide clues to deduce the critical period for the human cerebellum.

The Lurcher (*Lc*) mouse is an autosomal semidominant mutant that displays the degeneration of virtually all of the cerebellar Purkinje cells (Vogel et al., 2007). In heterozygous mice (*Lc*/+), cerebellar Purkinje cells specifically start to degenerate in a cell-autonomous manner by postnatal day 8 (P8), and most die during the second postnatal week.; other cell types are eventually affected by secondary mechanisms (Wetts & Herrup, 1982). *Lc*/+ mice exhibit severe ataxia during the third postnatal week, when approximately 90% of the Purkinje cells have disappeared. *Lc* is caused by an alanine to threonine mutation in the highly conserved third hydrophobic segment of GluRδ2 (Zuo et al., 1997). As this region works as the gate of a cation channel (Kohda et al., 2000), the mutation converts the receptor into a constitutively leaky cation channel. Thus, lurcher is a gain-of-function mutation, suggesting that a simple delivery of the wild-type GluRα2 would not have a significant therapeutic impact on *Lc* Purkinje cells. A suppression of the mutant protein expression via, for example, RNA interference would be a promising gene therapy against disorders caused by a toxic-gain-of-function mutation.

3.2 Human cerebellar diseases potentially treatable with gene therapy

3.2.1 Spinocerebellar ataxia

The cerebellum is impaired by various diseases, including neurodegenerative and enzyme-deficient disorders. Spinocerebellar ataxia (SCA) is one of the representative diseases that affect the cerebellum. Approximately one third of the SCA in patients is hereditary. So far, at least 29 types of SCA result from chromosomal loci of the causal genes (Carlson et al., 2009), in which the major lesion of SCA type 1 (SCA1), SCA2, SCA6, SCA14, SCA17 and SCA31 affects Purkinje cells. Neurons in the deep cerebellar nuclei are impaired in SCA3, and cortical Bergmann glia are primarily degenerated in SCA7. The well-known cause of hereditary SCAs is the abnormal expansion of trinucleotide (CAG) repeats in the coding region of genes responsible for the diseases. This expansion produces mutant proteins having an abnormally expanded polyglutamine stretch, leading to the formation of nuclear aggregates with other proteins that are critical for cellular functions. This type of hereditary SCAs is called polyglutamine disease and includes SCA1, SCA2, SCA3, SCA6, SCA7 and SCA17. As these diseases are caused by the production of toxic polyglutamine proteins (polyQ), a potential therapeutic approach would be to reduce the mutant polyQ by, for example, RNA interference (Xia et al., 2004) or the facilitation of their degradation. The latter may include the enhancement of the ubiquitin-proteasome pathway (Al-Ramahi et al., 2006; Matsumoto et al., 2004; Torashima et al., 2008; Wang & Monteiro, 2007) and autophagy (Menzies et al., 2010; Menzies & Rubinsztein, 2010; Williams et al., 2006).

Autosomal dominant SCA14, characterized by severe cerebellar atrophy and ataxia, is caused by a missense mutation of the *PRKCG* gene, which encodes the protein kinase C (PKC) γ gene. γPKC-deficient mice show only mild ataxia and no gross morphological abnormalities in the cerebellum (Chen et al., 1995; Kano et al., 1995), suggesting that the gain-of-toxic function, rather than loss-of-function, of γPKC underlies the pathology of SCA14. Therefore, a decrease in the amount of mutant γPKC protein is thought to be an effective therapy for SCA14. Indeed, recent studies have shown the allele-specific inhibition of mutant gene expression (Alves et al., 2008; Hu et al., 2009), and such mutant allele-targeted RNA interference may result in better therapeutic efficacy.

3.2.2 Mucopolysaccharidosis

Lysosomal storage diseases (LSDs) are inherited metabolic disorders characterized by the accumulation of undigested macromolecules in the lysosomes due to a significant decrease

or a complete absence in the activity of soluble lysosomal enzymes (Neufeld, 1991). As most lysosomal enzymes are ubiquitously expressed, a deficiency in a single enzyme affects peripheral organs, as well as various regions of the brain, including the cerebellum. There are approximately 50 forms of inherited LSDs in humans with incidences of 1 in 7,000 live births (Haskins, 2009). LSDs are usually grouped biochemically by the accumulated metabolite into 3 subgroups: mucopolysaccharidoses (MPS), sphingolipidoses, and mucolipidoses.

The potential treatments for LSDs include bone marrow or cord blood transplantation, enzyme replacement and gene therapy. Among the LSDs, those due to soluble lysosomal enzyme deficiencies are generally considered good candidates for gene therapy. These include MPS I, MPS IIIB and Niemann-Pick AB diseases (Sands & Davidson, 2006). To overcome the blood-brain barrier (BBB), viral vectors should be applied intrathecally (Watson et al., 2006) or directly into the brain parenchyma (Dodge et al., 2005), except in neonates having an incomplete BBB (Hartung et al., 2004; Kobayashi et al., 2005). Recent studies using knockout mouse models of MPS I and Niemann-Pick type A disease have shown a significant rescue of the phenotypic manifestations of the diseases upon intravenous and intrathecal application of adeno-associated virus (AAV) or lentiviral vectors expressing a deficient enzyme (Dodge et al., 2005; Hartung et al., 2004; Kobayashi et al., 2005; Watson et al., 2006). In addition, the injection of AAV vectors expressing the deficient gene, acid sphingomyelinase, into the deep cerebellar nuclei alleviated storage accumulation and corrected behavior deficits in the mouse model of Niemann-Pick type A disease (Dodge et al., 2005). These results suggest that viral vector-based gene transfer is promising for clinical gene therapy of patients with LSDs.

4. Viral vector-mediated gene delivery to the cerebellum

Vectors derived from retrovirus, Sindbis virus, adenovirus, lentivirus and AAV are widely used for gene transfer to mammalian cells; the properties of these vectors are summarized in Table 2. Retroviral vectors can express a foreign gene only in proliferating cells because they cannot pass through the nuclear membrane of host cells: the viral genome that enters into the cytoplasm of an infected cell can access the host chromosome only when the nuclear membrane disappears during mitosis. Exploiting this unique feature of retroviral vectors, they have been used to label neural stem cells (Kageyama et al., 2003; Levison et al., 2003; Namba et al., 2007).

Vectors	Gene expression	Insert size	Exchange of a promoter	Toxicity	Tropism	Period for gene expression	Inflammatory potential
Lenti	Stable	8 kb	○	(-)	broad	~7 days	Low
Retro	Stable	8 kb	○	(-)	Dividing cells only	~7 days	Low
AAV	Stable	<4.7 kb	○	(-)	broad	5~7 days	Low
Adeno	Transient (2months)	30 kb	○	(+)	Glia tropic	3~5 days	High
Sindbis	Cell death in 1-2 days	6 kb	×	(+++)	Neuro tropic	< 1 day	Very high

Table 2. Properties of different viral vectors.

Vectors based on Sindbis virus, a member of the Togaviridae family in the alphavirus subfamily, can infect non-dividing cells, including neurons, and replication occurs entirely in the cytoplasm of the infected cell as an RNA molecule (Griffin, 1998). Therefore, a high level of gene expression starts quickly after infection. One drawback of the Sindbis virus vector is high toxicity, as infected cells deteriorate within a couple of days.

Adenoviral vectors are widely used as gene transfer vectors. They can accommodate a large gene of ~30 kb and infect both neurons and glia, with a higher tropism for glia. Therefore, adenoviral vectors have been used to modify the function of Bergmann glia (Iino et al., 2001). Adenoviral vectors can exert toxicity to infected cells by triggering immune responses, and gene expression generally lasts only for ~2 months (Terashima et al., 1997). The features of adenoviral vectors, including their high infectivity in glial cells and immunogenicity, in combination with the introduction of an apoptosis-triggering gene are often applied to the gene therapy of malignant glioma (Horowitz, 1999; Jiang et al., 2009; Parker et al., 2009; Shinoura & Hamada, 2003).

Vectors derived from lentiviruses, a genus of slow viruses of the Retroviridae family, can replicate in non-dividing neurons with little toxicity to the infected cells (Escors & Breckpot). Moreover, stable gene expression lasts for more than several years. In the cerebellum, lentiviral vectors pseudotyped with vesicular stomatitis virus glycoprotein (VSVG) can infect various types of cortical cells that include Purkinje cells, stellate cells, basket cells, Golgi cells, Bergmann glia and astrocytes (Croci et al., 2006; Torashima et al., 2006a). The properties of lentiviral vectors are described in more detail in the next section (Subsection 4.1).

AAV is a non-pathogenic, small (20 nm), icosahedral and non-enveloped virus that belongs to the genus Dependovirus of the Parvoviridae family. Similar to lentiviral vectors, it infects both dividing and non-dividing cells with very little toxicity and a minimal immune response in the infected cells. To date, a number of AAV serotypes and over one hundred AAV variants have been isolated from adenovirus stocks or from human and non-human primate tissues (Gao et al., 2005; Wu et al., 2006). For gene transfer to cerebellar cells, AAV serotype 1, serotype 2 and serotype 5 (AAV1, AAV2 and AAV5) vectors have been used for the successful transduction of Purkinje cells (Alisky et al., 2000; Hirai, 2008; Kaemmerer et al., 2000; Xia et al., 2004). Thus, lentiviral vectors and AAV vectors, which have the potential to transduce Purkinje cells with almost no substantial toxicity, are promising as gene therapy vectors for cerebellar disorders that affect Purkinje cells. Because of the lack of pathogenicity, AAV vectors are increasingly becoming the vectors of choice for a wide range of gene therapy approaches. One major limitation of AAV vectors is the insert capacity, which is less than 4.7 kb, whereas lentiviral vectors have a higher capacity, up to 8 kb, for transgene accommodation (Table 2) (Hirai, 2008).

4.1 Factors that control the tropism of lentiviral vectors for cerebellar neurons and glia

As the VSVG binds to the phospholipids that constitute cellular membranes, VSVG-pseudotyped lentiviral vectors are thought to infect mammalian cells without cell-type preference. In the cerebellar cortex, lentiviral vectors have successfully transduced Purkinje cells, three types of interneurons, Bergmann glia and astrocytes, but not granule cells. The efficient transduction of Golgi cells and astrocytes present in the granule cell layer suggest that the injected viral solution is accessible to granule cells: however, no or few transduced granule cells, if any, were detected upon injection of lentiviral vectors to the adult cerebellum *in vivo* (Croci et al., 2006; Torashima et al., 2006a).

The observed lentiviral tropism for Purkinje cells is affected by the serum-lot quality and cultivation period of the HEK293FT cells used for lentiviral production (Torashima et al., 2006b). A three-day culture that causes the degeneration and death of HEK293FT cells results in the production of glia-tropic lentiviral vectors. Although the mechanism underlying this phenomenon has not been clarified, increases in the protease activity in the culture medium seems to be involved because the addition of a protease inhibitor in medium reversed the shift of lentiviral tropism from Purkinje cells to Bergmann glia (our unpublished observation). These results suggest that VSVG is modulated by a protease released from dead HEK293FT cells, leading to the alteration of lentiviral tropism.
The L7/PCP2 promoter is a Purkinje cell-specific promoter, and the Gfa2 promoter works as an astrocyte-specific promoter. Therefore, the combination of the cultivation period of HEK293 FT cells for lentiviral production and the accommodation of the L7 or Gfa2 promoter into lentiviral vectors permits us to specifically transduce Purkinje cells or Bergmann glia, respectively. However, these cell-type specific promoters generally have weak promoter strength. Therefore, the modification of these promoters, by the addition of an enhancer sequence and/or a significant increase in the viral titer by ultracentrifugation, is needed to attain sufficient levels of transgene expression.

4.2 A method that enables efficient and widespread gene delivery to the cerebellum

The cerebellum is a second largest organ in the mammalian CNS. Neurodegenerative diseases and congenital enzyme deficiency usually affect the entire cerebellar cortex, ranging from the vermis to the hemisphere, lobule 1 to lobule 10.
For effective gene therapy, a wide range of therapeutic gene delivery methods is indispensable. Figure 2a is a photo of our viral injection system for the rodent brain.
The injection of viral solutions to the brain parenchyma mechanically damages the tissue around an injection site, and ~1 μl is usually the limit for mouse brain regions, such as the striatum and hippocampus. However, we found that when injected at a speed of 0.2 – 0.3 μl/minute, it was possible to apply 10 μl of viral solution to the subarachnoidal space over lobule 6 of the cerebellar cortex (Fig. 2b) without substantial damage to the cortical tissue. Injected viral particles spread through subarachnoidal spaces and infect Purkinje cells via their well-differentiated dendrites, leading to markedly efficient transduction of the Purkinje cells (Fig. 3). We have also verified that this injection method is applicable to neonatal pups and mature mice (Fig. 2c) (Sawada et al., 2010; Torashima et al., 2006a; Torashima et al., 2006b).

5. Lentiviral vector-based rescue of mice with cerebellar ataxia

5.1 Hotfoot5J mice

Hotfoot mice are spontaneously occurring recessive mutants (Guastavino et al., 1990), and mice homozygous for the mutation showed severe ataxia with jerky tapping of the hindlimbs, which can be noted by two weeks after birth. The *Hotfoot5J* allele possesses a point mutation in exon 12 of the GluRδ2 gene, which creates a stop codon in the region encoding transmembrane 3 (Wang et al., 2003). Aberrant GluRδ2 protein is easily degraded and not detected in the Purkinje cells of *hotfoot5J* mice. Therefore, *hotfoot5J* mice are thought to exhibit a similar phenotype to that of GluRδ2 knock-out mice (Kashiwabuchi et al., 1995). Accordingly, we determined whether the ataxia of *hotfoot5J* mice could be reliably rescued by lentiviral-vector-based expression of the recombinant wild-type GluRδ2 gene (Iizuka et al., 2009).

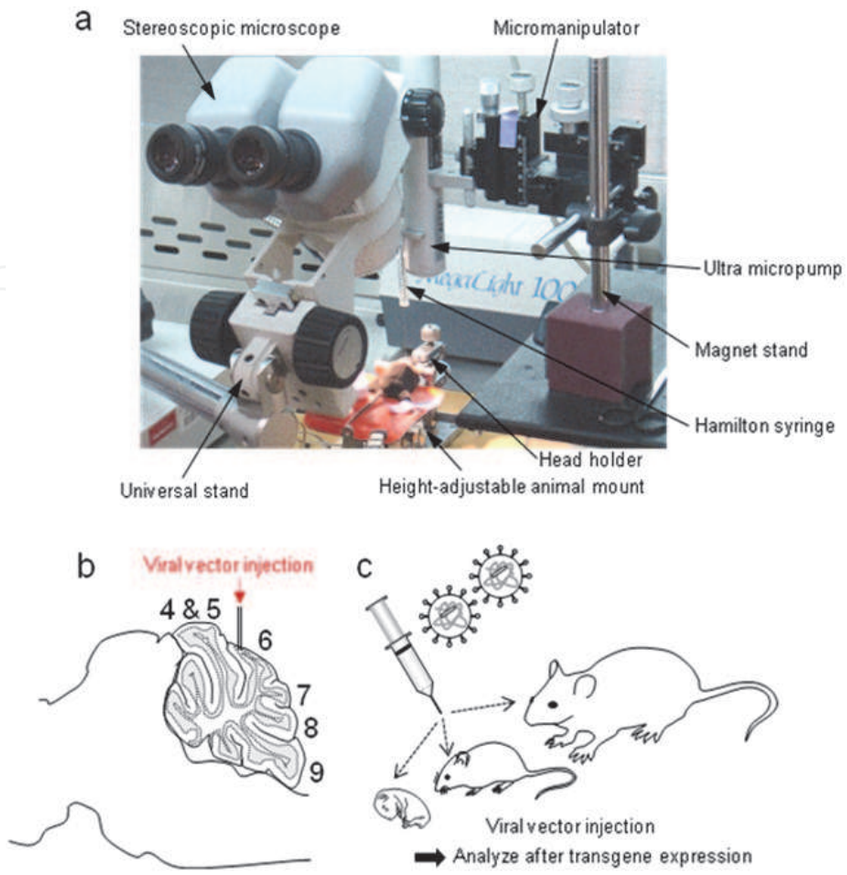

Fig. 2. Injection of viral vectors into the mouse cerebellum. (a) A viral vector injection setup. Viral vectors are injected very slowly with a speed of 0.2-0.3 µl/minute using an ultramicropump. (b) A schematic of the sagittal view of a mouse cerebellum depicting a viral injection site. (c) A schematic showing the availability of this injection method from a neonatal pup to a mature mouse.

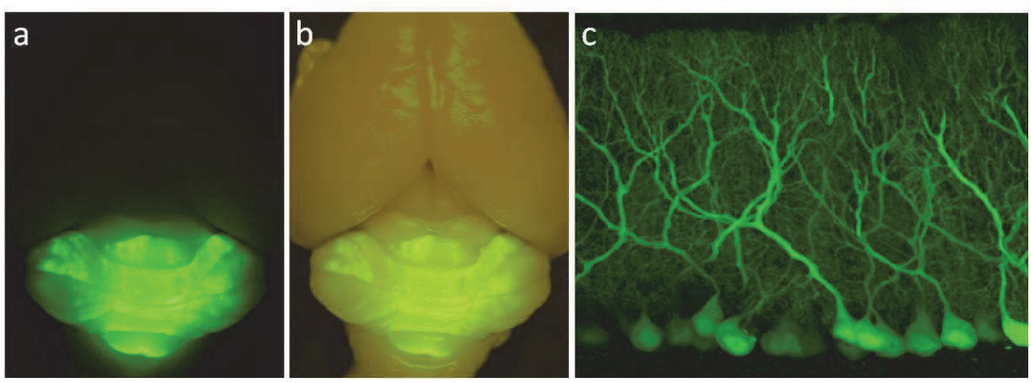

Fig. 3. Highly efficient transduction of Purkinje cells. Lentiviral vectors expressing GFP was injected as shown in Fig. 2. (a and b) Stereoscopic images of GFP fluorescence (a) and the superimposition with the whole brain (b) 7 days after the viral injection. (c) A GFP fluorescent image of Purkinje cells in the sagittal section.

Lentiviral vectors expressing GluRδ2 plus GFP or GFP alone were injected into lobule 6 of the *hotfoot5J* cerebella at P6, and the motor control ability was assessed at P30 by footprints and a rotarod test. The footprint pattern of mutant mice was markedly ameliorated by the expression of GluRδ2 plus GFP (Fig. 4).

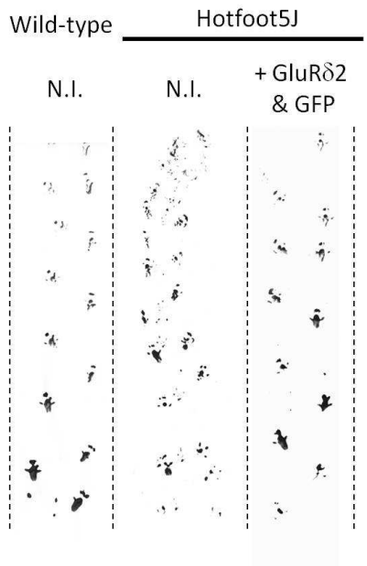

Fig. 4. Footprints of wild-type and *hotfoot5J* mice at P30. Ink was placed on the hindpaws of a non-injected wild-type mouse, a non-injected *hotfoot5J* mouse and *hotfoot5J* mice treated with GluRδ2 plus GFP (+ GluRδ2 & GFP); their footprints are shown. N.I., non-injected.

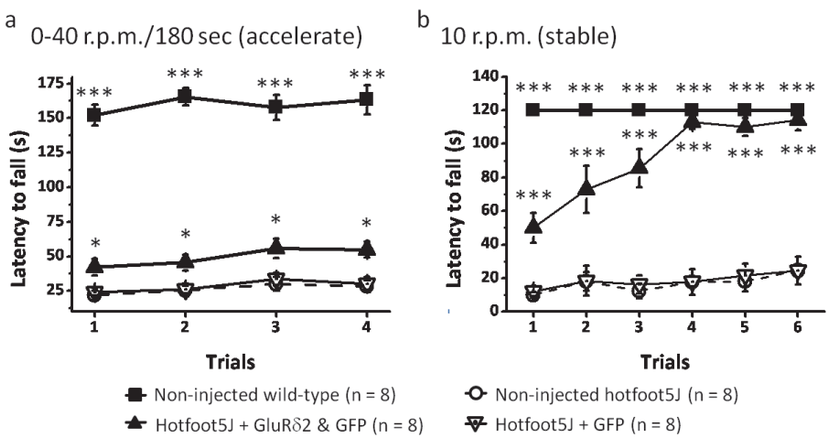

Fig. 5. Rescue of rotarod performance of *hotfoot5J* mice treated with GluRδ2 plus GFP. (a and b) Mice were assessed by two different tasks, an accelerating rod that reached 40 rpm from 0 rpm in 3 min (a) and a stably rotating rod with a speed of 10 rpm (b). The results of non-injected wild-type mice, non-injected *hotfoot5J* mice and *hotfoot5J* mice injected with lentiviral vectors expressing GluRδ2 plus GFP or GFP alone are presented. Asterisks indicate statistically significant differences compared with non-injected *hotfoot5J* mice: *$p < 0.05$, ***$p < 0.001$ (One-way ANOVA).

In the rotarod analysis, mice treated with GluRδ2 plus GFP showed significantly better performance at both acceleration and fixed-rod-speed tasks than non-injected mutant mice (Fig. 5), whereas neither the footprint pattern (not presented) nor the rotarod performance (Fig. 5) of *hotfoot5J* mice was altered by the injection of lentiviral vectors expressing only GFP. However, the rescue of ataxia by GluRδ2 expression was obviously incomplete; GluRδ2-treated *hotfoot5J* mice showed far poorer rotarod performance, particularly in the accelerating rod task (Fig. 5a), than wild-type mice. This was due partly to the expression of recombinant GluRδ2 in restricted lobules of *hotfoot5J* cerebellum.
Following the immunohistochemical examination, the GluRδ2 immunoreactivity was absent in the Purkinje cells from the non-injected *hotfoot5J* mice, whereas efficient GluRδ2 expression was detected in the dendritic spines of Purkinje cells from *hotfoot5J* mice treated with lentiviral vectors expressing GluRδ2 plus GFP.

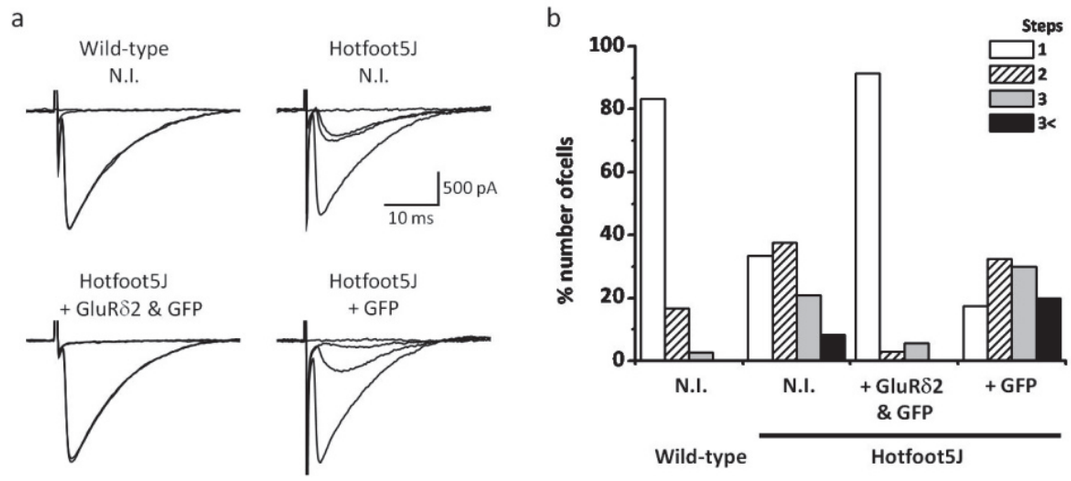

Fig. 6. Rescue from the persistent multiple CF innervation of *hotfoot5J* Purkinje cells by GluRδ2 expression. (a) Representative CF-EPSCs recorded from Purkinje cells clamped at −10 mV in non-injected wild-type, non-injected *hotfoot5J*, GluRδ2/GFP-treated *hotfoot5J*, and GFP-expressing *hotfoot5J* mice are shown. Scale bar, 500 pA, 10 ms. (B) Frequency histograms of Purkinje cells in terms of the number of discrete CF-EPSC steps in Purkinje cells from non-injected wild-type (42 cells, 3 animals), non-injected *hotfoot5J* (48 cells, 4 animals), GluRδ2/GFP-treated *hotfoot5J* (35 cells, 3 animals), and GFP-expressing *hotfoot5J* (40 cells, 3 animals) mice. N.I., non-injected.

Previous electrophysiological studies indicated that the multiple climbing fiber innervation of Purkinje cells continued even after maturation in the GluRδ2 knockout mice. GluRδ2$^{-/-}$ Purkinje cells were innervated persistently by multiple climbing fibers (Hashimoto et al., 2001; Ichikawa et al., 2002; Kashiwabuchi et al., 1995), and we examined whether the multiple innervations of *hotfoot5J* Purkinje cells were restored by the expression of recombinant GluRδ2 using a patch-clamp technique. Only 18% of the Purkinje cells in P31-P35 wild-type mice, and more than 60% of the Purkinje cells from age-matched GluRδ2-null mice, were innervated by multiple climbing fibers (Fig. 6). The failure of the developmental removal of surplus climbing fibers was completely rescued by the lentiviral vector-mediated expression of GluRδ2 plus GFP. However, no significant rescue was observed in *hotfoot5J*

cerebella expressing GFP alone. These results suggest a therapeutic potential of lentiviral vector-based gene therapy for cerebellar disorders that result from a loss-of-function gene mutation.

5.2 The SCA model mice

Polyglutamine diseases, including several autosomal dominant types of SCA, are inherited neurodegenerative diseases caused by expanded polyQ accumulation in neurons (Koshy & Zoghbi, 1997). Recent studies have identified proteins that facilitate the degradation of polyQ aggregates through a ubiquitin-proteasome pathway in cultured cells. Previously, Yanagi and colleagues have identified a novel guanosine triphosphatase (GTPase), CRAG, as one of those proteins. Furthermore, these authors have shown that CRAG triggers the nuclear translocation of a CRAG-polyQ complex, leading to the degradation of polyQ in HeLa cells (Qin et al., 2006). Because the expression of CRAG decreases in the adult brain, it is plausible that a reduced level of CRAG could underlie the onset of polyglutamine diseases. Therefore, we examined the potential of CRAG expression for treating polyglutamine disease and tested our hypothesis by lentivirally introducing CRAG into the cerebellar neurons of mice overexpressing polyQ in the cerebellum.

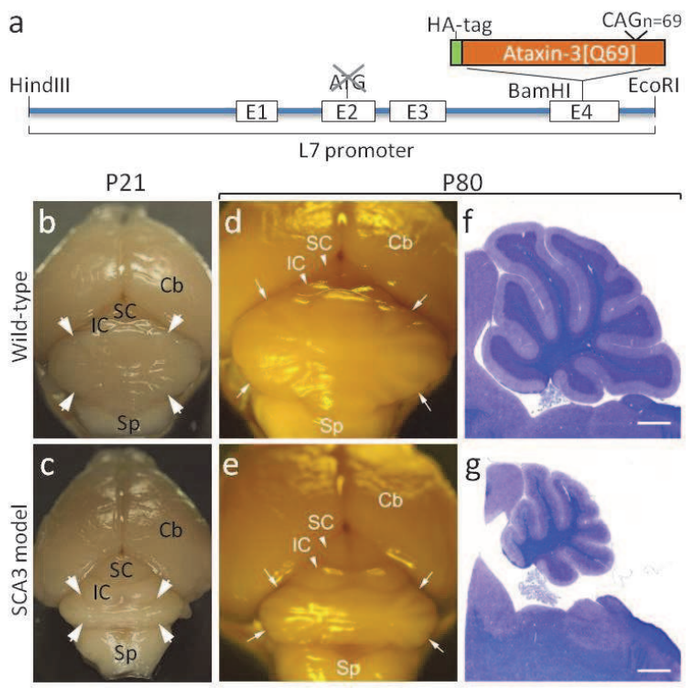

Fig. 7. Severe cerebellar atrophy in the PolyQ mouse at P21 and P80. (a) A schematic depicting the transgene that drives the mutant ataxin-3(Q69) under the control of the L7 promoter; an HA-tag was fused at the N-terminus of the truncated ataxin-3. Wild-type (b d, and f) and SCA3 model (c, e, and g) mice were fixed at P21 (b and c) or P80 (d-g). (b-e) A dorsal view of the whole brain; cerebella are indicated by arrows. Cb; cerebrum, IC; inferior colliculus, SC; superior colliculus, Sp; spinal cord. (f and g) Klüver–Barrera staining of sagittal sections of the cerebellum from a P80 SCA3 model mouse (g) and a wild-type littermate (f). Scale bars, 500 μm.

For this project, we generated transgenic mice (SCA model mice) expressing an expanded polyQ in cerebellar Purkinje cells using a truncated form of human ataxin-3, the gene responsible for Machado-Joseph disease (SCA3) with 69 CAG triplet repeats (ataxin-3[Q69]) (Kawaguchi et al., 1994; Yoshizawa et al., 2000) (Fig. 7a). The transgene expression was driven by a Purkinje cell-specific L7 promoter (Hirai et al., 2005). The SCA model mice started to show ataxic gait at approximately P10, which became more obvious as they developed further.

The cerebella of SCA model mice at P21 and P80 were substantially smaller than that of wild-type littermates (Fig. 7b-g). A low magnification of the sagittal sections of the SCA model mouse cerebellum showed that the overall structure of the cortex was not grossly affected (Fig. 7g). However, examination at a higher magnification revealed that the Purkinje cells were markedly disarranged, concomitant with a substantial impairment of dendritic differentiation (Fig. 8).

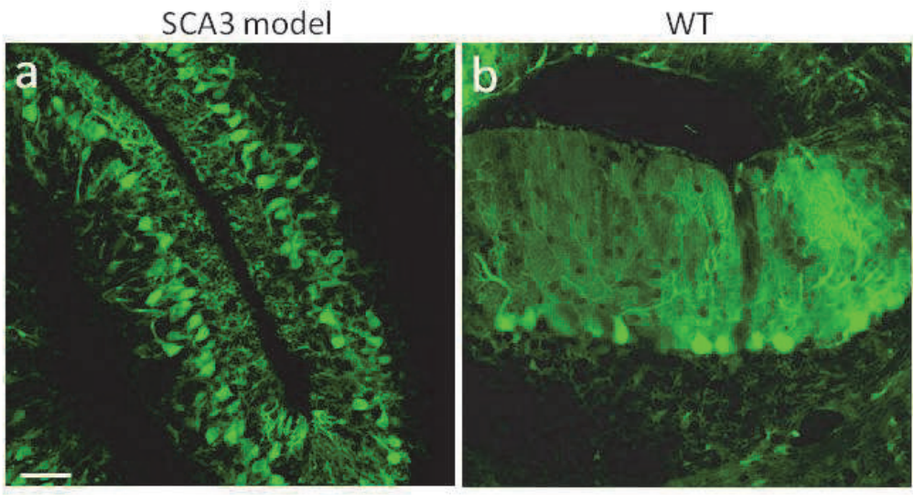

Fig. 8. Drastic morphological alteration in Purkinje cells of the SCA3 model mouse. Cerebellar sections from a P21 SCA3 model mouse (a) and a wild-type littermate (b) immunolabeled for calbindin. Note the markedly decreased thickness of the molecular layer and disarrangement of the Purkinje cell soma in the SCA3 model mouse cerebellum. Scale bar, 50 μm.

Immunostaining of the cerebellar sections for the hemagglutinin (HA)-tag at the N-terminus of polyQ revealed a weak and diffuse accumulation of polyQ mainly in the nucleus of Purkinje cells at P21. The polyQ was markedly increased and formed numerous inclusion bodies in or around the Purkinje cell bodies by P80. In addition to the immunoreactivity in the Purkinje cell layer, small inclusion bodies with strong immunoreactivity for polyglutamine and ubiquitin were detected in the axon terminals of the Purkinje cells in the deep cerebellar nuclei.

Lentiviral vectors expressing CRAG GTPase were injected into the midline cerebellar lobules of P21-P25 mice. The effect of CRAG or GFP expression was assessed by a rotarod test, in which mice were challenged with an accelerating rod paradigm just before or 4 or 8 weeks after the viral injection (Fig. 9a). The rotarod performance of the non-injected SCA3 model mice and the model mice expressing GFP alone deteriorated slightly at 8 weeks. In

contrast, the performance of mice treated with CRAG was significantly improved at both 4 and 8 weeks after the injection, as compared with the results of non-injected mice. To examine the effect of CRAG expression on motor learning, mice were evaluated again by a rotarod test with a different paradigm, in which the rod speed was fixed at 5 rpm, and the trial was repeated 6 times. Non-injected SCA3 model mice and those treated with GFP showed almost no improvement in the performance even at the 6th trial (Fig. 9b). In contrast, mice treated with CRAG learned quickly how to stay on the rod, indicating the rescue of motor learning ability.

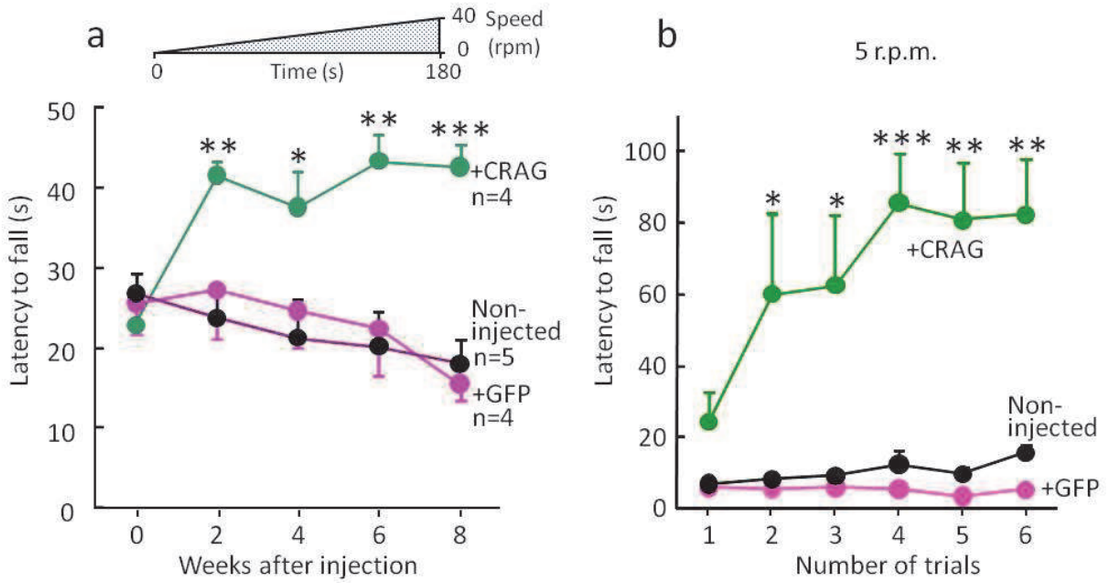

Fig. 9. Rescue of the ataxic phenotype in polyQ mice upon the lentivector-mediated expression of CRAG. (a and b) Results of the rotarod test. The rod was accelerated from 0 rpm and reached the maximum speed (40 rpm) in 3 min, as depicted above the graph (a). Mice treated with wild-type CRAG, but not those treated with GFP, exhibited significant improvement (a) (n = number of individual mice in each cohort). In the stable rod speed paradigm (5 rpm) administered 8 weeks after the injection, mice treated with CRAG learned quickly how to walk on the rod and showed a drastically better performance, compared with the non-injected and GFP-expressing mice (b). *p<0.05, **p<0.01, ***p<0.001, compared with results of non-injected mice.

We next examined the cerebellar sections from untreated mice or mice treated with the lentiviral vectors by immunohistochemistry. Whereas strong labeling with numerous polyQ inclusions was observed in the cerebellar slices from non-injected polyQ mice and those injected with virus expressing GFP, the overall labeling with an anti-HA antibody for polyQ was faint and diffusely distributed in the cytoplasm and nuclei of the Purkinje cells in CRAG-expressing slices (Fig. 10a-d). Notably, the arrangement and dendritic differentiation of the Purkinje cells was altered upon the expression of CRAG. Double immunolabeling for calbindin and Flag-tag fused with CRAG showed that only the CRAG-expressing Purkinje cells extended dendrites. Consistently, the molecular layer was significantly wider in the cerebella of polyQ mice treated with CRAG than in those of non-injected animals ($p<0.01$, Fig. 10e). These *in vivo* data substantiated previous cell-culture-based results and further

extended the usefulness of the targeted delivery of genes facilitating the ubiquitin-proteasome pathway as a gene therapy against polyglutamine diseases and other neurodegenerative disorders.

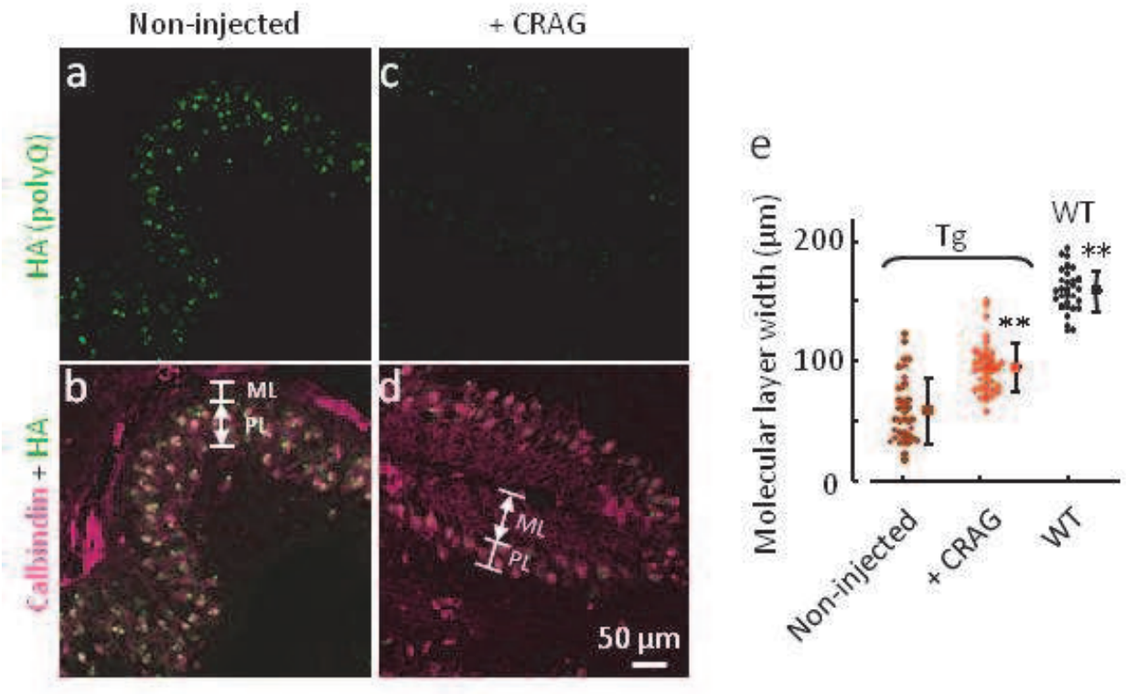

Fig. 10. Degradation of polyQ aggregates in Purkinje cells by lentiviral-vector-mediated expression of CRAG. (a-d) Cerebellar sections from mice receiving no injection (a and b) or treated with CRAG (c and d). Upper panels are fluorescent images of polyQ immunolabeled for HA (green), which were merged with those of Purkinje cells immunolabeled for calbindin (magenta, lower panels). ML; molecular layer, PL; Purkinje cell layer. (e) A graph of the thickness of the molecular layer. The thickness of the molecular layer in the cerebellum from SCA3 model mice (Tg) treated without (Non-injected) or with CRAG and their wild-type littermates (WT) was measured, and the data were plotted on the graph. The average ± SD is presented beside the each plot. Three animals in each experimental group and virus vectors obtained from at least two independent cultures were used for quantitative analysis. Asterisks indicate significant differences compared with results of non-injected mice, **$p<0.01$.

6. Underlying problems for the clinical application of lentiviral vectors to cerebellar diseases

6.1 The significantly larger size of human cerebellum compared with the mouse cerebellum

Figure 11 is a comparison of the mouse cerebellum with that of the cynomolgus monkey. Although our injection method allowed us to deliver a transgene very efficiently to mouse cerebellar cells, the human cerebellum is much larger than that of the cynomolgus monkey. Therefore, it is a tremendous challenge to attain the efficient transduction of Purkinje cells in

the human cerebellum. To overcome this volume problem, we are exploring ways to increase the amount of the viral solution from 10 µl to 1,000 µl and the number of injection sites from one to, for example, three points.

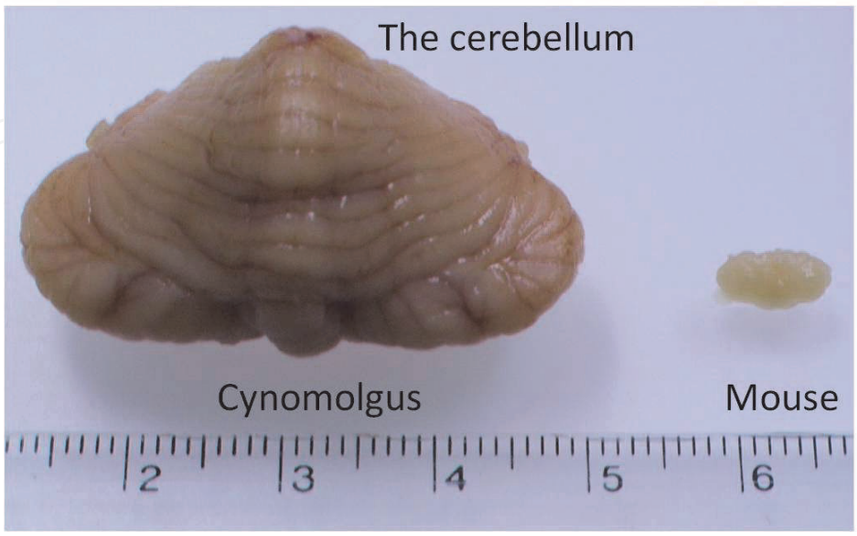

Fig. 11. Comparison of the mouse cerebellum (right) with that of the cynomolgus monkey (left).

6.2 Side effects that might be caused by the use of lentiviral vectors

6.2.1 Toxicity of lentiviral vector infection on Purkinje cells

Infection with Borna disease virus, an RNA virus tropic for cerebellar neurons, has been shown to cause developmental, neuroanatomical and behavioral abnormalities (Rubin et al., 1999). HIV infection has also been shown to cause a decreased expression of mRNA and protein of AMPA-type glutamate receptors in cerebellar Purkinje cells (Everall et al., 1995). Compared with adenoviral vectors that have immunogenicity, lentiviral vectors cause almost no immune responses to infected cells and are thought to exert little toxicity on host cells. However, it has not been fully clarified whether the infection of high-titer lentiviral vectors lacks an adverse influence on neurons *in vivo*. To clarify the influence of high-titer lentiviral vector infection and the subsequent expression of the transgene, we injected lentiviral vectors having a titer of 1.0×10^{10} transduction units (TUs) into the neonatal rat cerebellum. Neonates were used for examining lentiviral toxicity because the brain is extremely vulnerable to developmental damage following perinatal insult.

Lentiviral vectors expressing GFP under the control of the murine stem cell virus (MSCV) promoter were injected into the cerebellar cortex of neonatal rat pups. Three weeks after treatment, the GFP-expressing Purkinje cells were compared with control Purkinje cells from phosphate-buffered, saline-injected mice. An analysis of the dendritic tree showed that the total dendrite length in the GFP-expressing Purkinje cells was almost 80% of that in the control Purkinje cells. Furthermore, an electrophysiological examination showed that the short-term synaptic plasticity at the parallel fiber–Purkinje cell synapses and climbing fiber–Purkinje cell synapses was significantly altered in the GFP-expressing Purkinje cells. In contrast, the morphological and functional maldevelopment of infected Purkinje cells was attenuated substantially when lentiviral vectors with much weaker promoter activity were

used. These results suggest that the maldevelopment of the Purkinje cells was caused mainly by the subsequent expression of a high amount of GFP driven by the strong MSCV promoter and that the toxic influence of lentiviral vector infection itself was minimal.

6.2.2 Insertional mutagenesis

Upon infection of a retrovirus into a cell, the viral RNA is inserted into the cytoplasm, where the RNA is reverse-transcribed into DNA by reverse transcriptase, which is then inserted into the host genome by an integrase. The viral genome sequence integrated into the host chromosome is called a "provirus". The provirus insertion may disrupt regions of the host genome that are critical for cellular functions, such as the control of the cell cycle or apoptosis; this process is called "insertional mutagenesis". In fact, *ex vivo* gene therapy using a murine leukemia virus (MLV) vector caused leukemia in 3 of the 11 children that were being treated for X-linked SCID (Hacein-Bey-Abina et al., 2003). However, lentiviral vectors are considered to be less likely to disturb the regulation and expression of host genes because of a difference of integration sites between the MLV vectors and lentiviral vectors: MLV vectors integrate primarily in promoter regions and CpG islands, whereas lentiviral vectors integrate into transcriptionally active genes (Mitchell et al., 2004; Schroder et al., 2002). Although it is not clear whether insertional mutagenesis can lead to the transformation of postmitotic neurons, lentiviral vectors do infect and cause insertional mutagenesis in glial cells with proliferative properties. Therefore, the risk of insertional mutagenesis should be considered when lentiviral vectors are used clinically to treat neurological diseases (Jakobsson & Lundberg, 2006).

7. Conclusion

The cerebellum develops significantly after birth (Goldowitz & Hamre, 1998), and, therefore, the expression of various genes is strictly regulated. Cerebellar granule cell precursors that proliferate vigorously in the external granule cell layer migrate along the processes of Bergmann glia to form the internal granule cell layer during the first postnatal two weeks in rodents. The migrating granule cells supply trophic factors, such as brain-derived neurotrophic factor (BDNF), which stimulates the Purkinje cells to form differentiated dendrites (Schwartz et al., 1997). During the migration process, parallel fibers, granule cell axons, (Granule cell axons are called "parallel fibers") make synapses with extending dendrites of Purkinje cells. Synaptic clefts between the parallel fibers and dendritic spines of a Purkinje cell are wrapped by processes of Bergmann glia that reuptake released glutamate, thereby modulate the synaptic transmission. Thus, 5 neurons and Bergmann glia in the cerebellar cortex concertedly elaborate the functional cerebellar neuronal circuit. One attractive therapy against diseases that impair Purkinje cells is the transplantation of Purkinje cells or their precursors engineered from stem cells into the damaged cerebellum. However, unless other cells surrounding Purkinje cells, such as the granule cells and interneurons, have sufficient plasticity, the transplanted Purkinje cells are not properly integrated to form a functional network, resulting in little therapeutic impact.

In contrast, gene therapy aims to salvage degenerating Purkinje cells by delivering a therapeutic gene. Accordingly, when Purkinje cells are not lost, this approach is theoretically more effective than stem cell-based cell replacement therapy for diseases that impair Purkinje cells; this is despite the fact that the surrounding cells have only limited plasticity. To attain sufficient therapeutic efficacy in gene therapy, the broad and efficient gene

delivery to cerebellar neuronal or glial cells is indispensable, and this has been a significant challenge for decades. However, the problem is being solved by recent marked progress in both lentiviral and AAV vectors. The further accumulation of knowledge, including therapeutic genes and the critical period corresponding to distinct cerebellar defects, along with the development of animal models, would facilitate the clinical application of viral vector-based gene therapy for patients with various cerebellar disorders.

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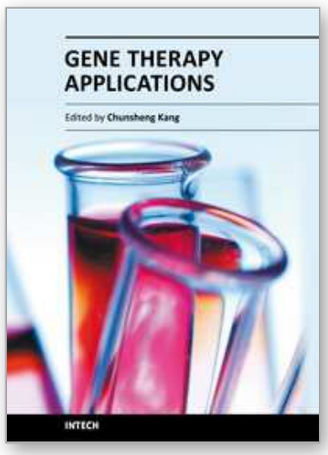

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The aim of our book is to provide a detailed discussion of gene therapy application in human diseases. The book brings together major approaches: (1) Gene therapy in blood and vascular system, (2) Gene therapy in orthopedics, (3) Gene therapy in genitourinary system, (4) Gene therapy in other diseases. This source will make clinicians and researchers comfortable with the potential and problems of gene therapy application.

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