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Review

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Abstract

Expression of a foreign gene in cerebellar Purkinje cells in vivo is a powerful method for exploring pathophysiology of the cerebellum. Although using developmental engineering many gene-modified mice have been generated, this approach is time-consuming and requires a lot of effort for crossing different lines of mice, genotyping and maintenance of animals. If a gene of interest can be transferred to and efficiently expressed in Purkinje cells of developing and mature animals, it saves much time, effort and money. Recent advance in viral vectors has markedly contributed to selective and efficient gene transfer to Purkinje cells in vivo. There are two approaches for selective gene expression in Purkinje cells: one is to take an advantage of viral tropism for Purkinje cells, which includes the tropism of adeno-associated virus and the vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped lentivirus. Another method, which might be used in combination with the first one, is utilization of a Purkinje-cell-specific promoter. Focusing mainly on these points, recent progress in viral-vector-mediated transduction of Purkinje cells in vivo is reviewed.

Key words:

Purkinje cell, viral vector, lentivirus, adeno-associated virus, gene therapy

Introduction

The cerebellum plays important roles in coordinated movement, motor learning and vestibular function, and cerebellar damage results in impaired body balance and disturbance in gait and posture. A major input to the cerebellar cortex is mossy fibers that originate from thalamus, brain stem and spinal cord, and make excitatory synapses with cerebellar granule cells. The activity of granule cells is transferred through their axons, called parallel fibers, to Purkinje cells. A Purkinje cell has ramified dendrites studded with hundred thousands of spines, on which parallel fibers make excitatory synapses. Parallel fiber-Purkinje cell spine synapses are tightly covered with processes of Bergmann glia so as to quickly take up glutamate released from parallel fiber terminals and thereby to prevent the glutamate from spilling over and activating the adjacent synapses. Another input to the cerebellar cortex is climbing fibers that originate from neurons in the inferior olivary nucleus and make excitatory synapses with Purkinje cells as well as neurons in the deep cerebellar nuclei. Purkinje cells, the sole source of output from the cerebellar cortex, exert inhibitory actions on the neurons of the deep cerebellar nuclei. The excitatory activity of granule cells and Purkinje cells is modulated by 3 types of inhibitory interneurons, Golgi cells, stellate cells and basket cells.

It is well known that long-term depression of synaptic transmission (LTD) at parallel fiber - Purkinje cell synapses can be induced upon conjunctive stimulation of parallel fibers

and climbing fibers, which is thought to provide a cellular basis for motor learning. Purkinje cells are thus key elements of higher brain function in the cerebellum as well as in maintaining the regulation of coordinated movement and body balance. These important cells are easily damaged by toxins, ischemia, infection or inherited disorders.[1] Accordingly, efficient and targeted expression of foreign genes in Purkinje cells is a powerful method for basic and translational research, such as studies of synaptic plasticity or gene therapy for diseases affecting Purkinje cells, including several major types of spinocerebellar ataxia. Gene transfer to Parkinje cells might not appear to be a big challenge, because the surface area of Purkinje cells is much larger than that of other cortical cells. However, it is indeed a challenge, partly because the well-developed dendrites occupying most of the surface area of Purkinje cells are tightly wrapped with the radial processes of Bergmann glia. Considerable effort has been devoted to transduce Purkinje cells using various types of viral vectors. In this mini review, I summarize the recent progress in viral-vector-mediated gene transfer into Purkinje cells in vivo.

Transduction of Purkinje cells using adenoviral vectors

Early adenoviral vectors were associated with toxicity that led to an inability to achieve stable transgene expression. Thereafter, the vectors were substantially altered to improve the stability of transgene expression by eliminating the

expression of viral proteins that cause the immune response. Consequently, many studies have succeeded in expressing foreign genes efficiently in neurons of various brain regions, including the striatum, hippocampus and cerebral cortex.[2-4] In line with this, the potential of adenoviral vectors to transduce Purkinje cells was tested. Adenoviral vectors expressing LacZ under the control of the Purkinje-cell-specific L7 promoter demonstrated Purkinje-cell-specific transduction.[5] However, the efficiency was very low: in that study, only two transduced Purkinje cells in one section (285 μm x 285 μm) were presented. The low tranduction efficiency of Purkinje cells in vivo using adenoviral vectors was confirmed by a different group,[6] in which virus-mediated LacZ expression was detected in very few Purkinje cells upon cortical injection, and the majority of cells transduced were Bergmann glia. Since we also observed essentially the same expression pattern: preferential transduction of Bergmann glia when adenoviral vectors were injected into the cerebellar cortex (unpublished data), we gave up using adenoviral vectors for gene transfer to Purkinje cells in vivo. Ozawa's group took advantage of the fact that adenoviral vectors preferentially infect Bergmann glia.[7] Native Bergmann glia express only GluR1 and GluR4 subunits of AMPA-type glutamate receptors (AMPA receptors), and lack GluR2, which confers Ca^{2+}-permeability on AMPA receptors of Bergmann glia. Using adenoviral vectors, Ozawa's group succeeded in expressing exogenous GluR2 subunit selectively in Bergmann glia and thereby replacing the Ca^{2+}-permeable AMPA receptors with

Ca^{2+}-impermeable ones. Thus, the adenoviral vectors currently used are effective for selective gene transfer to Bergmann glia, but have substantially limited potential for transducing Purkinje cells in vivo, although Purkinje cells in culture could be transduced efficiently using these vectors.[5,8]

Transduction of Purkinje cells using herpes simplex viral vectors

A unique feature of herpes simplex virus 1 (HSV-1) vector is long neuronal transport through axons using microtubule motors of the infected neurons.[9,10] Because HSV-1 is a neurotropic virus that causes encephalitis when introduced into the brain, much effort was made to modify HSV-1 so that the virus could be safely used as a gene transfer vector.[11] The defective helper-dependent amplicon vector is a toxicity-attenuated mutant of HSV-1 that lacks all portions of the genome except for an origin of DNA replication and DNA packaging signal sequences.[12] Agdo et al.[13] used HSV-1 amplicon vectors carrying the lacZ gene to transduce Purkinje cells, and showed that injection of HSV-1 vectors into the inferior olivary nucleus, rather than into the cerebellar cortex, led to efficient transduction of Purkinje cells. The vector transport from the inferior olivary complex to the cerebellar cortex is discussed in a later section. The HSV-1-vector-mediated expression of the lacZ gene within Purkinje cells was persistent and was maintained at the same level for at least 40 days with no significant signs of toxicity or inflammation. However, viral injection into the inferior

olivary nucleus located in the deep medulla has potential to cause significant, and in the worst case, life-threatening damage to the medulla, which could be a big problem in clinical application. As an alternative way, it is intriguing to test whether the injection of HSV-1 vectors into the deep cerebellar nuclei could cause efficient transduction of Purkinje cells.

Transduction of Purkinje cells using adeno-associated viral vectors

Recombinant adeno-associated virus (rAAV) is a promising gene therapy vector because wild-type adeno-associated virus is nonpathogenic, and expression of transgenes is sustained without integration of the viral genome into the host genome: integration of proviruses into a host chromosome could cause insertional mutagenesis associated with severe side effects. One major limitation of rAAV is the insert capacity, which primarily depends on the genome size of wild-type AAV.[14] Wild-type AAV genome with the size of 4.7 kb is replaced by a transgene cassette consisting of a promoter plus transgene, which should be theoretically less than 4.7 kb; otherwise, virus production is significantly affected due to impaired packaging. Thus, from the point of packaging capacity, the ideal size for rAAV-mediated-gene transfer is thought to be 4 kb or less, although it differs depending on the size of the promoter accommodated. Currently, at least 10 serotypes of AAV have been used to engineer recombinant viral vectors.[15,16] As different AAV serotypes enter cells via distinct cell surface receptors,

the tropism of different serotypes for neurons has been tested. rAAV2 is the serotype most widely used for gene transfer to neuronal cells because it transduces mostly neurons.[16-19] rAAV1 and rAAV5 have been shown to have higher transduction efficiency and higher levels of transgene expression than rAAV2 in various brain regions.[20-22] rAAV-mediated transduction of adult Purkinje cells in vivo has been shown by two groups.[23-25] Low's group showed transduction of thousands of Purkinje cells upon cortical injection of rAAV2 vectors,[24] while Davidson's group achieved comparable or even better results by injecting rAAV5[23] or rAAV1[25] vectors into the cerebellar cortex. We have also succeeded in transducing adult Purkinje cells in vivo by cortical injection of rAAV2 vectors expressing GFP (Fig. 1). On the other hand, Sena-Esteves's group showed efficient transduction of newborn mouse Purkinje cells by rAAV1, rAAV2 or rAAV8 after viral injection into cerebral lateral ventricle.[26] Among the serotypes tested, rAAV8 was found to cause most efficient transduction as well as the highest intensity of gene expression in Purkinje cells. Thus, in addition to developing neurons, rAAV8 may be promising for gene delivery to mature Purkinje cells.

Transduction of Purkinje cells using lentiviral vectors

Retroviruses have a single-stranded RNA genome. The genome RNA is replicated to a double-stranded DNA intermediate that is integrated in the nuclear chromosome of the infected cell. The retroviridae family is divided into three groups: the

oncoviridae, the lentiviridae and the spumaviridae. Because a double-stranded DNA intermediate of retroviruses of the oncoviridae cannot be transferred to the nucleus except during the period of cell division, the retroviruses cannot transduce post-mitotic cells, and therefore have limited utility in the brain.[14] On the other hand, lentiviridae retroviruses can pass through the pore of the nuclear membrane and transduce post-mitotic neurons, a feature that distinguishes them from oncoretroviridae. Lentiviridae include human immunodeficiency virus (HIV)-1, HIV-2, feline immunodeficiency virus (FIV) and simian immunodeficiency virus. Like rAAV vectors, lentiviral vectors have strict limitations of the insert capacity, but their capacity is almost twice (~ 8 kb) as large as that of AAV vectors (~ 4.7 kb).

Using FIV-derived vectors, Alisky et al. showed efficient transduction of Purkinje cells as well as stellate cells and Golgi cells in vivo.[23] We used HIV-1-derived lentiviral vectors for the transduction of Purkinje cells. The original HIV selectively infects CD4-positive cells such as T cells and macrophages. In order to gain broader tropism than that of the wild-type virus, the native envelope glycoprotein of HIV-derived lentiviral vectors is replaced with that of vesicular stomatitis virus (VSV-G), a method called pseudotyping. As VSV-G binds to phosphatidylserine of the plasma membrane, VSV-G-pseudotyped lentiviral vectors are expected to infect all types of cells: however, they did not transduce cerebellar granule cells when injected into the

cerebellar cortex.[27] Moreover, we experienced that lentiviral-tropism was significantly influenced by the period of cultivation of HEK293FT cells after transfection of the cells with viral plasmids.[28] Lentiviruses are harvested 2 days (40 h) after transfection of HEK293FT cells (1st harvest). As the HEK293FT cells look healthy and adhere tightly to the culture dish (Fig. 2A), additional virus can be obtained again the next day (64 h) by further incubating the cells after the addition of fresh medium (2nd harvest). The HEK293FT cells are substantially damaged at this time(Fig. 2C). In a previously published protocol for lentiviral production,[29] the authors proposed pooling the virus-containing supernatant from the 1st and 2nd harvests, presumably to double the viral vectors obtained. Initially, we used lentiviral vectors concentrated from the 1st harvest for the transduction of cerebellar cells, resulting in Purkinje-cell-preferential transduction (Fig. 2B). Thereafter, we began to use lentivirus solution prepared from the 2nd harvest, which unexpectedly resulted in Bergmann-glia-preferential transduction (Fig. 2D). We then attempted to identifying factor(s) that substantially decreased the lentiviral tropism for Purkinje cells, and found that the pH of the medium used to cultivate HEK293FT cells is critical for the viral tropism of Purkinje cells.[28] Although the mechanism underlying this phenomenon has not been fully clarified, our current hypothesis is that modification of VSV-G by a protease, which is probably released into the culture medium from lysed HEK293T cells, affects the viral tropism for

Purkinje cells.

Another factor that critically affects lentiviral tropism for Purkinje cells is variations of the lot of fetal bovine serum (FBS) preparations used to supplement the culture media.[30] We used 8 different serum lots (S1 – S8) to supplement the culture medium, generated lentiviruses and injected the resultant 8 viral solutions into mouse cerebellar cortices. Lentiviral vectors produced using FBS S1, S2 and S3 showed neuron-preferential transduction: those produced using S1 transduced stellate cells and basket cells in addition to Purkinje cells, while those produced using S2 or S3 selectively transduced Purkinje cells.[30] In contrast, viral vectors produced using other lots of FBS resulted in glia-preferential or non-selective transduction. Representative examples of the transduction patterns of cerebellar cells are shown in Fig. 3. It should be noted that the titers of the 8 lentiviral solutions that were determined using HeLa cells were almost identical. Further studies will be needed to elucidate the mechanisms underlying the pH- and serum-quality-dependent differences of the tropism of VSV-G-pseudotyped lentiviral vectors for Purkinje cells.

Relationship between injection sites and transduced regions in the brain

In addition to the transduction of cerebellar cortical cells, adenoviral vectors injected into the cerebellar cortex cause transduction of neurons in the pontine nuclei and inferior

olivary nuclei.[6] This is mediated by the retrograde axonal transport of the viral genome through mossy fibers and climbing fibers projecting to the cerebellar cortex, as shown in figure 4A. Unlike injection of adenoviral vectors, injection of HSV-1 vectors into the cerebellar cortex does not transduce cortical cells, including Purkinje cells, efficiently. However, injection of the HSV-1 vectors into the inferior olivary complex was shown to result in the transduction of thousands of Purkinje cells as well as transduction of deep cerebellar nuclei neurons as described in an earlier section (Fig. 4B).[13] The authors of that study speculated that HSV-1 in the inferior olivary complex was initially delivered to the cerebellar nuclei neurons by axonal transport. In the deep cerebellar nuclei, the virus went over the synaptic cleft to axon terminals of Purkinje cells and was transported retrogradely to the soma of Purkinje cells through their axons.

Both rAAV vectors[23] and lentiviral vectors[27] injected into the cerebellar cortex transduced cells in the cerebellar cortex, but not neurons in the pontine nuclei or inferior olivary nuclei (Fig. 4C, D). Interestingly, neurons in the deep cerebellar nuclei were transduced by rAAV vectors (Fig. 4C), but not by lentiviral vectors (Fig. 4D). This finding is puzzling because rAAV vectors are not transported transsynaptically. One possible and simple explanation to account for this observation is that the rAAV particles injected into the cerebellar cortex spread over the deep cerebellar nuclei. More careful studies will be required to clarify this issue.

Conclusion

It has been a big challenge to achieve efficient gene transfer into Purkinje cells in vivo. Recent progress in viral vector development, however, has allowed us to accomplish this to a certain extent, although the transduction efficiency and reproducibility in gene-modified mice generated by developmental engineering are still superior to those achieved using viral vectors. One promising application of viral vectors is rescue of a gene in a knock-out mouse, followed by evaluation of the functional recovery as shown in our recent report.[31] Most genes are expressed in variety of tissues or cell types, and therefore even if a simple knock-out of a gene leads to some abnormal phenotype, it is difficult to determine the region or cell type responsible for the abnormal phenotype. Thus, viral-vector-mediated rescue of a defective gene at a specific site or in a specific cell type in a knock-out mouse is a powerful means to dissect the roles of a gene of unknown function in various regions. Furthermore, viral vectors enable us to express various types of mutant genes such as a mutant lacking a phosphorylation site, a glycosylation site or a domain interacting with particular signaling molecules. Subsequent functional assays of the transduced cells could provide useful information about the signals mediated by the modified gene product. Viral-vector-mediated gene transfer to Purkinje cells in vivo will be a major approach in the near future for exploring cerebellar function and therapeutic targeting of disorders that affect Purkinje cells.

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Figure legends

Fig. 1

Transduction of Purkinje cells in vivo using rAAV vector. rAAV vector expressing GFP was injected into the cerebellar cortex. Cerebellar sections were made 7 days after the viral injection. Scale bar, 50 μm.

Fig. 2

Striking decrease in the lentiviral tropism for Purkinje cells upon prolonged cultivation of HEK293FT cells. HEK293 cells were transfected with lentiviral plasmid carrying the GFP gene. (A, C) GFP fluorescence images overlaid on the phase contrast images of HEK293FT cells 40 h (A) and 64 h (C) after transfection. Note that a substantial number of HEK293FT cells were detached from the dish surface 64 h after transfection. The viral solutions prepared from medium 40 h and 64 h after transfection were named as 40-h virus and 64-h virus, respectively. The biological titer of the 40-h virus stock was almost identical to that of the 64-h viral stock (~5 x10^8 transducing units/ml). (B, D) Confocal GFP fluorescence images of a sagittal section of cerebellar vermis 7 days after 40-h (B) or 64-h (D) virus injection into the cerebellar cortex. Note that the 40-h virus transduced mainly Purkinje cells (B), whereas the majority of cells transduced by the 64-h virus were Bergmann glia. Scale bar, 50 μm.

Fig. 3

Distinct patterns of transduction in cerebellar cells depending

on the batch of lentiviral vectors produced using different lots of FBS. Four microliters of lentiviral vectors produced using serum from Equitech-Bio (A) or from HyClone Laboratories (B) were injected into a mouse cerebellar cortex. Mice were sacrificed 7 days after injection. The panels show GFP fluorescence of a sagittal section of the cerebellar vermis. Lentiviral vectors produced using serum from Equitech-Bio showed preferential transduction of neurons (A): those produced using serum from HyClone Laboratories transduced mainly glial cells (B). Scale bar, 50 μm.

Fig. 4

Schematic drawing depicting the viral injection site and resultant transduced area in the brain. (A) LacZ expression in cerebellar cortex, potine nuclei and inferior olivary complex upon adenoviral injection into the cerebellar cortex.[6] (B) LacZ expression in Purkinje cells illustrated as blue dots, deep cerebellar nuclei and inferior olivary nuclei upon HSV-1 injection into the inferior olivary complex.[13] (C) LacZ expression in cerebellar cortex and deep cerebellar nuclei upon adeno-associated-viral injection into the cerebellar cortex.[23] (D) GFP expression confined to the cerebellar cortex upon lentiviral injection into the cerebellar cortex.[27]

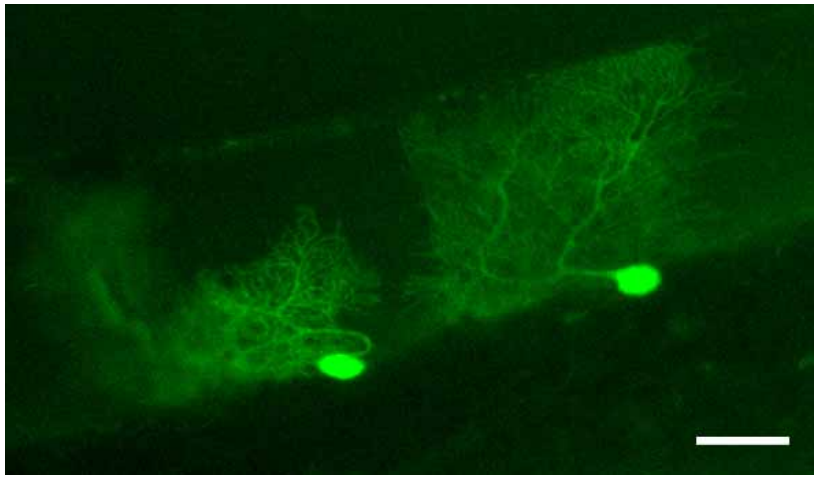

Fig. 1

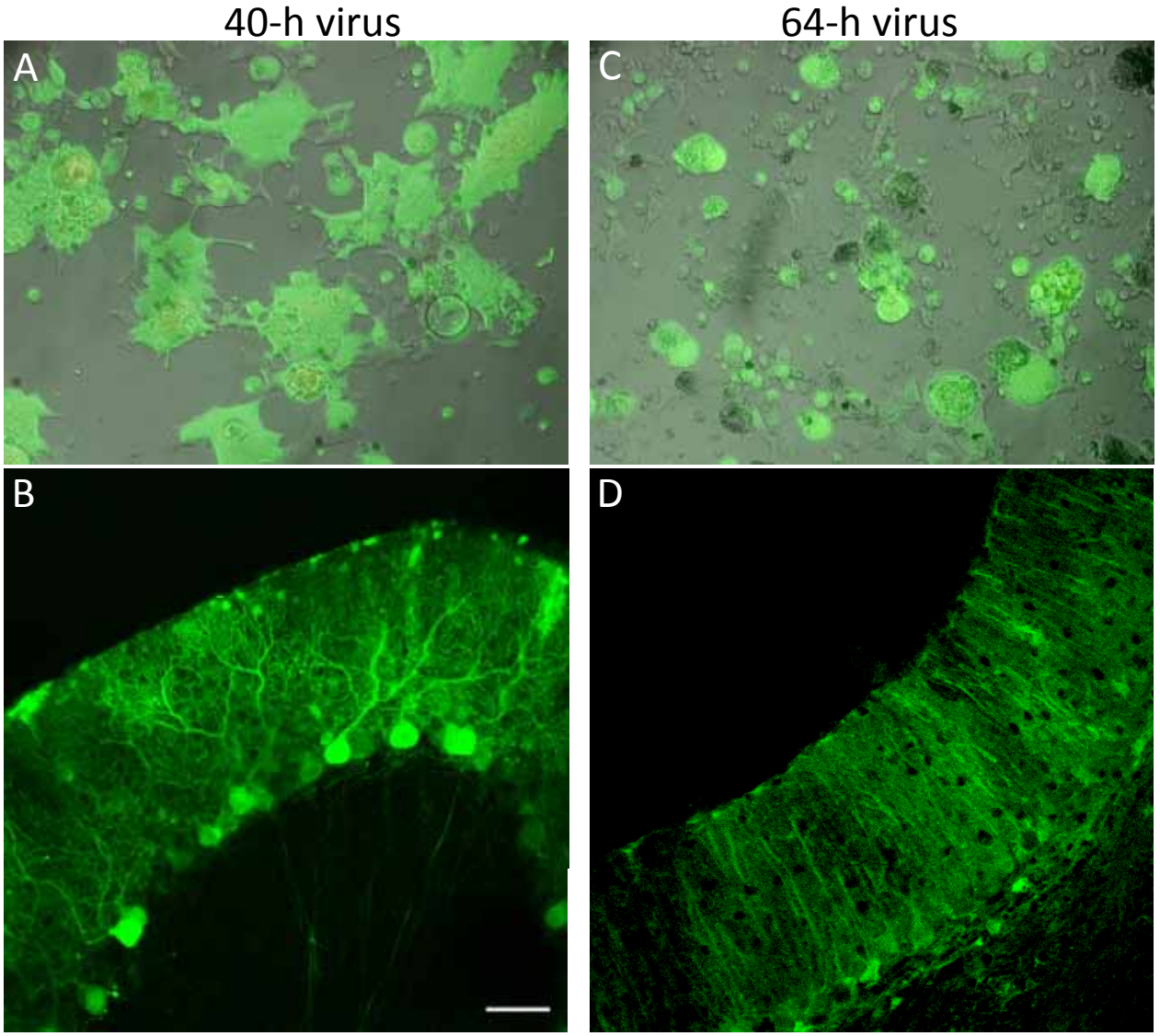

Fig. 2

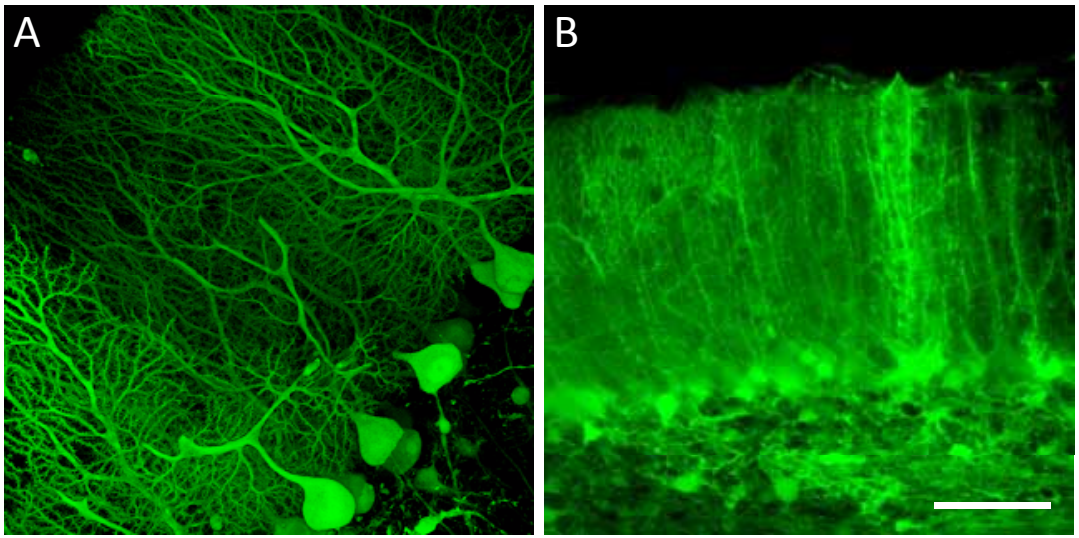

Fig. 3

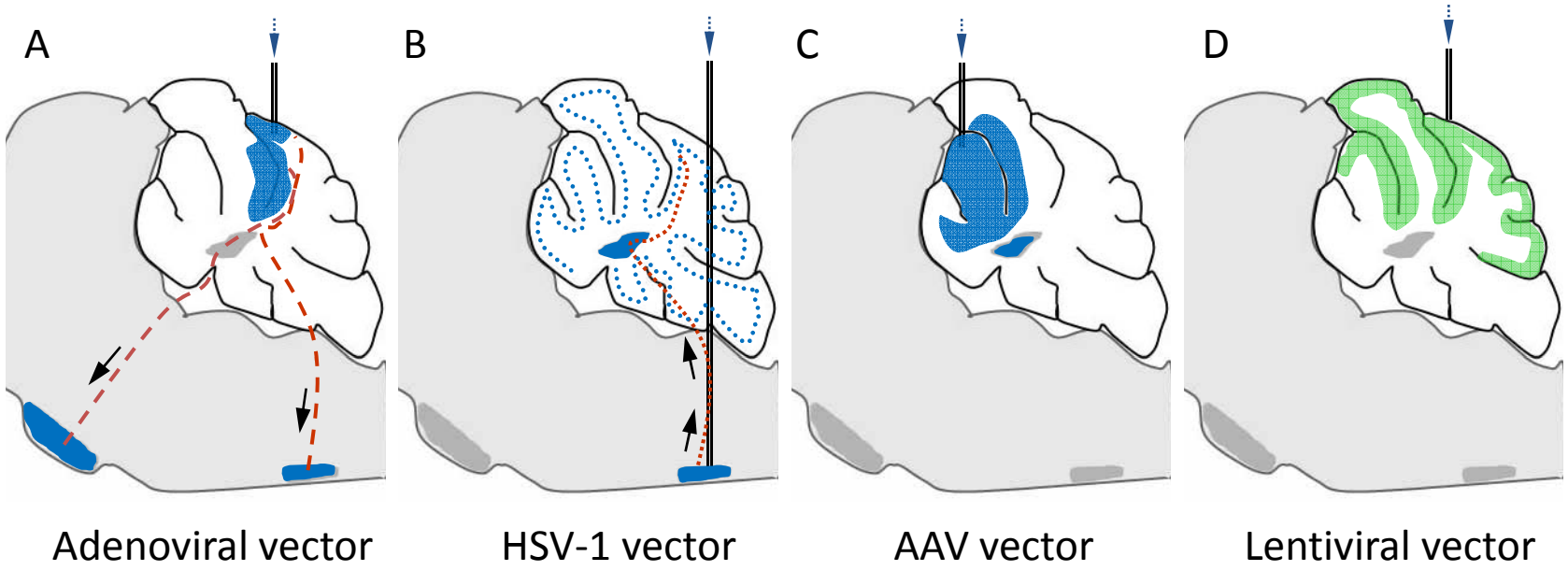
A
B
C
D
Adenoviral vector
HSV-1 vector
AAV vector
Lentiviral vector

Fig. 4