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Transcriptional targeting to brain cells: Engineering cell type-specific promoter containing cassettes for enhanced transgene expression ☆

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ABSTRACT

Transcriptional targeting using a mammalian cellular promoter to restrict transgene expression to target cells is often desirable for gene therapy. This strategy is, however, hindered by relatively weak activity of some cellular promoters, which may lead to low levels of gene expression, thus declining therapeutic efficacy. Here we outline the advances accomplished in the area of transcriptional targeting to brain cells, with a particular focus on engineering gene cassettes to augment cell type-specific expression. Among the effective approaches that improve gene expression while retaining promoter specificity are promoter engineering to change authentic sequences of a cellular promoter and the combined use of a native cellular promoter and other *cis*-acting elements. Success in achieving high level and sustained transgene expression only in the cell types of interest would be of importance in allowing gene therapy to have its impact on patient treatment.

Contents

1. Introduction

Disorders in the central nervous system (CNS) such as Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, epilepsy, and head and spinal cord trauma come with devastating effects on the individual and high social costs associated with chronic care and lost productivity. Many of the

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disorders are related to the absence, malfunction or ineffectiveness of one gene or more [1] and do not respond well to conventional therapeutic means. Gene transfer into the CNS has, therefore, been considered as a potential approach to treatment of these disorders [2–6]. This approach may alter expression levels of neurotrophic factors, anti-apoptotic proteins, antioxidant molecules and other therapeutic factors to restore, halt or prevent the degeneration of cells in the CNS, especially neurons. Gene therapy also offers much hope for the treatment of CNS malignancies.

To realize the goal of using gene transfer as a therapeutic approach for the treatment of CNS neurological disorders, several obstacles to successful gene transfer to target cells in the CNS must be overcome. These obstacles are related to unique attributes of the CNS. Anatomically, the CNS is sophisticatedly composed of many functional regions, each of which has its unique physiological role and is usually highly vulnerable to injury. Even limited damage to a small area of the CNS can harm a critical function, as evidenced in Parkinson's disease that results from the loss of dopamine neurons in a discrete anatomical region, the substantia nigra pars compacta. At cellular level, diverse types of cells constitute the brain parenchyma, in which neurons, astrocytes, oligodendroglia, microglia, and epithelial cells consist of complex, three-dimensional networks. Neurons, with their core function of processing and transmitting signals, can further be classified into a number of subtypes based on the type of neurotransmitter they are using. Other types of brain cells also play critical physiological functions, although they are not the core components in the nervous system. This cell type complexity underscores the importance of restricting expression of a therapeutic gene to a particular type of cells in CNS gene therapy, thus ensuring therapeutic effects in the desired cells while avoiding any unintended effects on non-target cells, either within or outside the CNS [7]. This is especially important when viral vectors, the most widely used delivery systems for CNS gene therapy, are employed for gene transfer. Natural infection spectrums of most viruses are not confined to just one type of cells. Leakage of locally injected viral vectors may lead to damage to nearby healthy cells and/or systemic toxicity, which might have severe consequences in sensitive organs like the brain and liver.

Given that the efficient targeted transduction of genes still represents a major barrier, the strategy to restrict transcription of transgenes to a specific cell population through the use of a mammalian cellular promoter is particularly attractive [8–10]. Transcriptional targeting is the term generally employed to address such a targeting strategy. In addition to offering cell-type specific gene expression, mammalian cellular promoters are less likely to activate host cell defense machinery because of their cellular authentic sequences, thus are usually less sensitive to cytokine-induced promoter inactivation than promoters derived from the genomes of viruses. The use of a cell type-specific promoter may avoid transgene expression in antigen-presenting cells, therefore abrogating unwanted immune responses against a desired transgene [11]. As such, the improved stability of gene expression can be expected. However, mammalian cellular promoters are in general relatively weak activators of transcription when compared to those derived from viruses, for example the commonly used cytomegalovirus immediate-early enhancer/promoter (CMV promoter). This inherent weakness in driving transgene expression could compromise the efficacy of certain targeted gene therapy applications that require high-level expression of therapeutic genes confined to a population of brain cells. To compensate for weak transgene expression, high doses of vectors will have to be used to produce the desired amounts of therapeutic molecules intracellularly. The approach is unfortunately problematic in the case of administration of viral vectors, due to the well-documented deleterious effects of a high dose of viral vectors, including virus-associated toxicity and strong host immune response to viral vectors. By using a potent expression cassette accommodating a cell type-specific promoter, it can be envisaged that titers of viral vectors used for *in vivo* transfer can be reduced and transgene expression will be limited to target cells, thus leading to attenuated viral toxicity and immune challenge. As such, therapeutic goals can be achieved with substantially fewer viral particles. With this aim in mind, several strategies have been developed to improve gene expression from a weak cellular promoter, yet retain its specificity. This review focuses specifically on the methods that have been successfully employed for enhanced transgene expression from a neural cell-specific promoter.

2. Neural cell-specific promoters for transcriptional targeting

Coordinated control of gene expression is central in almost all biological processes. In eukaryotes, it is governed by regulation of diverse elements into an integrated and harmonious process [12–14]. Gene expression is regulated in large part at the transcription level, with transcription factors (TFs) binding their specific DNA regulatory elements and activating or repressing transcription. Among those elements, the expression of a transgene requires *cis*-acting elements in both the 5' and 3' untranslated region (UTRs) of the expression cassette. 5'-UTR *cis*-regulatory elements include the core promoter, enhancer, silencers, insulators, matrix attachment regions, and locus control regions [15]. The core promoter is the simplest element to locate in the genome due to its upstream positioning of the transcription start site (TSS). The availability of the human genome sequence allows computer scientists to identify TSS, analyze promoter sequence data, and search for novel *cis*-regulatory elements. Experimental biologist can then clone proximal promoters to assess their activity and specificity. Often, because of the restriction of the size of an insert capacity in a given vectors, the core or minimal regions of a promoter is the best solution to drive activity of the transgene. This core promoter contains all the elements necessary and sufficient to generate basal levels of transcription *in vitro*. The activity of RNA polymerase II and the basal transcription machinery that is recruited to the core promoter elements found in every human gene are the main effectors in the control of eukaryotic gene expression. The RNA polymerase II together with its basal transcription factors are grouped around several core promoter elements spanning the region −35 to +35 surrounding TSS.

One of the major challenges in targeted gene transfer is the specificity of transgene expression only in the cell types of interest. As complex mechanisms regulate gene expression *in vivo* and most viral promoters do not have specific targeting capacities, a variety of tissue or cell specific promoters have been characterized. These promoters provide tissue or cell type-specific expression that is tightly regulated. Specific cellular promoters characteristic of different neural cell types, i.e. neurons, glial cells and oligodendrocytes, have been studied extensively [16,17]. Mammalian promoters are generally quite large owing to multiple, often complex regulatory identified elements governing the final activity and cell-type specificity of a promoter. To use a cellular promoter in gene transfer vectors, it is essential to identify a minimal promoter region that is important for cell-type specificity, and yet efficient in driving gene expression. To this end, several groups have been able to identify various cell type-specific promoters and test them for transcriptional targeting of transgenes. This section of the review elaborates on several of cell type-specific promoters used for transcriptional targeting in the nervous system (Table 1) and the current state of understanding with respect to the regulatory elements governing the specificity of these promoters (Fig. 1).

2.1. Neuron-specific promoters

Many neuron specific promoters have been used for transcriptional targeting [16–19], including those that control the expression of genes encoding neuron specific enolase (NSE), synapsin-1 (SYN), platelet-

Table 1
Strategies for engineering transcriptional targeting cassettes and examples of using the cassettes for gene transfer into the central nervous system.[a]

Promoter	Engineering native cellular promoters			Recombinant transcriptional activators	Cre activation	Viral regulatory elements		
	Multimerization	Chimeric promoter	Point mutation			CMV enhancer	AAV ITRs	WPRE
PDGF				[82] Baculovirus		[108] Plasmid [109] AAV [110] Baculovirus [111] Baculovirus [113] Lentivirus [129] Baculovirus	[111] Baculovirus [129] Baculovirus	[26] AAV [113] Lentivirus [146] AAV
Synapsin-1				[81] Lentivirus [82] Lentivirus [88] Lentivirus		[113] Lentivirus		[19] Adenovirus [113] Lentivirus [128] Adenovirus [146] AAV
NSE						[113] Lentivirus		[144] AAV [146] AAV
SCG10					[91] Adenovirus			
CaMKII						[113] Lentivirus		
DBH	[64] Adenovirus		[65] Adenovirus					
Tubulin alpha I						[113] Lentivirus		
NFH		[67] HSV-1 [70] HSV-1 [71] HSV-1						
GFAP	[64] Plasmid			[81] Lentivirus [88] Lentivirus	[92] Adenovirus	[112] Baculovirus	[112] Baculovirus	

[a] References and delivery vectors are listed. *Abbreviations*: CMV, Cytomegalovirus immediate early enhancer; AAV, Adeno-associated virus; ITR, Inverted terminal repeats; WPRE, Woodchuck hepatitis virus post-transcriptional regulatory element; PDGF, Platelet-derived growth factor; NSE, Neuron specific enolase; SCG10 Superior cervical Ganglion 10; CaMKII, Ca^{2+} /Calmodulin-Dependent Protein Kinase II; DBH Dopamine β-hydroxylase; NFH, Neurofilament heavy chain; GFAP, Glial fibrilliary acidic protein.

derived growth factor (PDGF), tyrosine hydroxylase (TH), and dopamine β-hydroxylase (DBH).

NSE is a glycolytic enzyme that is expressed in terminally differentiated neurons. A 1.8 kb promoter region at the 5' end of the gene has been shown to direct expression to all neurons [20]. Extensive characterization of the promoter region by Twyman et al. identified a 255 bp of 5' flanking sequence being sufficient to confer cell type-specificity on the reporter gene [21].

Most neuron specific promoters are either too large, and/or induce relatively weak gene expression [19]. Nonetheless, exclusive neuronal gene expression is conferred by a 495 bp SYN promoter *in vitro* and *in vivo* and, because of its relatively small size, is suitable for

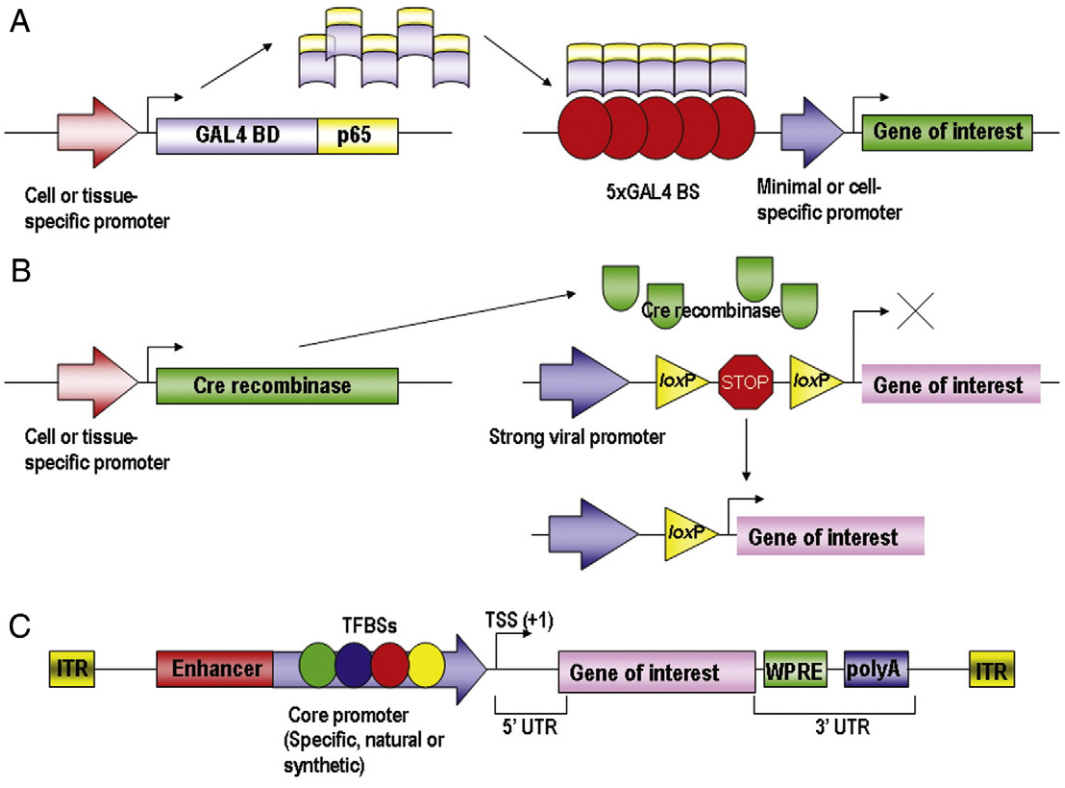

Fig. 1. Illustration of putative transcription factor binding sites, core promoter region containing of the TATA box and other elements in neural cell-specific promoters. Neuron specific restrictive element (NSRE) is an important restrictive element which restricts the expression in non-neuronal cell types and is generally present in neuron-specific promoters. Abbreviations: Zif, Zinc induced facilitator; CRE, cAMP response element; YY1, Ying Yang 1; PRS, PHOX2 response sites; SP1, Specificity protein1. The glial fibrilliary acidic protein (GFAP) gene promoter, an astrocyte specific promoter has a GATA binding site in the C region of the promoter that controls the specific expression in astrocytes. It also contains putative binding sites for CAAT/enhancer binding protein (C/EBP), acute myeloid leukemia 1a (AML1a) and nuclear factor-κB (NF-κB).

incorporation into small vector systems. SYN is a phosphoprotein that regulates synaptic vesicle formation [22], and has a high level of early transcription in primary hippocampal neurons [18] through a sequence containing a neuron restrictive silencer element (NRSE) region. Cells, like glia, fibroblasts, endothelial cells, contain a neuron restrictive silencer factor that binds to the NRSE present in many neuron-specific genes and blocks inappropriate gene expression in non-neuronal cells. Kugler et al. have demonstrated a higher activity for the SYN promoter when compared to the NSE promoter and its efficacy in driving a strong panneuronal expression in various rat brain regions [18].

PDGFs are growth-regulatory molecules that stimulate chemotaxis, proliferation, and increased metabolism of primarily connective tissue cells. PDGF β-chain is heavily expressed in neurons throughout the brain and the spinal cord, but not in glial cells [23]. Positive immunostaining of the polypeptide was observed in cytoplasmic, perinuclear regions and principal or secondary dendrites. The PDGF promoter has been used to direct the expression of transgenes to differentiated neurons in the cortex, cerebellum, brainstem, spinal cord and olfactory bulb in transgenic animals [23], including mouse models for Alzheimer's disease over-expressing a mutated beta-amyloid precursor protein [24] and apolipoprotein E [25]. The promoter has also been used to facilitate neuron-specific transgene expression in virus gene delivery systems [26,27]. The identified functional positive-regulatory transcription factor binding sites in the PDGF promoter include those for Sp1, Sp3, Egr-1, and NF-κB [28–31].

In the CNS, TH is expressed in catecholamine (CA) neurons, which include the midbrain dopamine (DA) neurons and noradrenaline (NA) neurons of the locus coeruleus (LC). In the periphery, TH is expressed in sympathetic ganglia and adrenal chromaffin cells [32]. In view of this wide distribution, distinct transcriptional control mechanisms are involved in regulating expression of the TH gene in different cell populations. A 2.5 kb fragment of the TH promoter, used in an adeno-associated virus, selectively drives the expression of transgene in NA neurons *in vivo* [33]. In another study, characterization of the TH promoter by Kim et al has shown that a NRSE represses the expression of the neuron specific genes in non-neuronal cell types [34]. They identify the presence of a silencing element(s) between −2164 and −1210 bp upstream in the 5' end of the TH promoter. Deletion mutant analysis of TH gene promoter/enhancer elements in TH-expressing cell lines and transgenic mice has indicated that basal TH expression is mediated by two highly conserved TH promoter sequences: a cyclic adenosine monophosphate (cAMP) response element (CRE) at −45 bp and an activator protein 1 (AP-1) site at −205 bp [35,36]. Transgenic studies using various reporter genes under the control of different regions of the TH promoter have demonstrated that, for both the rat and human TH genes, the 5.0 kb sequence immediately 5′ of the TH gene transcription initiation point can lead to a degree of tissue-specific expression in the embryo and adults *in vivo* [37,38]. However, the 5.0 kb rat TH promoter sequence did not lead to appropriate expression levels in peripheral and autonomic tissues, and, the 5.0 kb human TH promoter sequence led to marked ectopic expression in tissues which did not normally synthesize catecholamines. A sequence of 760 bp, immediately 3′ of the last exon 14 of the TH gene, has been shown to have transactivating properties, when used alone to drive reporter gene expression *in vitro* and *in vivo* [39,40].

DBH catalyzes the final step in noradrenaline synthesis and is expressed exclusively in noradrenergic and adrenergic cells. In order to identify elements within the DBH gene which contribute to the regulation of tissue-specific expression, the promoter was studied [41]. The activity of the promoter was localized to a region between −133 and −173 upstream of TSS. This element, however, also directed expression in non-DBH-expressing cell lines, but was inhibited when sequences between −212 and −388 were included. This inhibitory region contains sequences homologous to a silencer element recently identified in the human DBH gene, and shares homology with other previously identified silencer elements [41]. Ishiguro et al identified a 5'-flanking upstream domain, residing between −437 and −262 bp of the human DBH gene, has a cell type-specific silencer function that shares homology with NRSE [42].

2.2. Glial cell-specific promoters

Two commonly used glial cell specific promoters are the glial fibrilliary acidic protein (GFAP) promoter and the myelin basic protein (MBP) promoter. GFAP is an intermediate filament expressed in astrocytes. The specific cellular location of the GFAP protein in the CNS has encouraged the extensive use of a GFAP promoter to target transgene expression to cells of glial origin [43]. There have been several studies characterizing the regulatory elements governing the expression of GFAP in astrocytes [44–46]. A 2.2 kb region of the 5' UTR of human gene driving the LacZ expression has shown an astrocytes specific expression pattern [44,45]. A mouse GFAP promoter has also been shown to direct reporter genes in the astrocytoma derived cell lines. Lee et al characterized the promoter region of GFAP further and have found that a 448 bp GFAP promoter directs expression only in specific brain regions. It, however, drives expression in neurons as well. They elucidated a 45 bp sequence spanning bp −1443 to −1399 required for silencing expression in neurons. The same group has further characterized a novel 681 bp GFAP promoter, which has essentially the same expression pattern as the 2210 bp GFAP promoter and about two fold greater activity [46].

Mature oligodendrocytes play important roles in myelin synthesis during the development of the CNS. The oligodendrocyte lineage also encompasses the largest pool of postnatal proliferating progenitors whose behavior *in vivo* remains broadly elusive in health and disease. The promoters that drive gene expression specifically either in immature oligodendrocytes or in mature oligodendrocytes have been characterized [47]. Among them is the MBP promoter, which drives the synthesis of MBP exclusively in the oligodendrocytes. Wrabetz et al have extensively characterized the 5' end of the MBP gene and demonstrated that a 149 bp sequence 5' of the transcription start site of the gene is sufficient to drive the expression specifically in oligodendrocytes [47]. Interestingly, they also conclude that a region more distal at the 5' end is a negative regulator of the gene expression and may be a region restricting the expression of MBP in other cell types. Chen et al have shown that a 1.9 kb MBP promoter placed into an adenoviral vector could drive GFP expression exclusively in the white matter in a mouse model, with no expression in the astrocytes. The expression was shown to last for a period of 3 months [48,49]. The MBP promoter in the context of a lentiviral vector also directed transgene expression in oligodendrocytes [50].

2.3. Neural cell-specific promoters and CNS gene therapy

Transcriptional targeting using a neural cell-specific promoter has been tested for CNS gene therapy in pre-clinical animal models. Two commonly studied neurological diseases are Alzheimer's disease, which affects over 5% of population over 65 years old, and Parkinson's disease with a prevalence of 1% in the same age group. As the pathologic hallmark of Parkinson's disease is the loss of CA neurons in the substantia nigra (SN)-locus ceruleus (LC) tract and the TH promoter is mainly active in the CA system in the brain (cf 2.1), this promoter has been used in several studies to restrict the regional expression of a transgene in CA neurons. A previous study demonstrated that a 9 kb rat TH promoter could express lacZ for up to ten weeks in the SN and LC [51]. In a recent study, an 8 kb rat TH promoter has been used in the context of a non-viral plasmid vector to drive the expression of a human glial-derived neurotrophic factor (GDNF) cDNA [52]. The plasmid was delivered using Trojan horse liposomes modified with a monoclonal antibody to the rat transferring receptor

and rats with experimental PD were treated with intravenous injection of the complexes. Expression of the transgene was confined to CA cells and could be observed for up to 10 days. Alzheimer's disease is characterized by atrophy and death in the cholinergic neurons. A recent mouse study aimed to optimize transgene expression in sub-regions of the hippocampus, a brain region implicated in learning, memory, and also Alzheimer's disease and dementia. The authors found that a hybrid promoter consisting of the human translation elongation factor (EF1α) fused to the 5' UTR derived from the human T-cell leukemia virus (HTLV) was effective in restricting transgene expression to the CA2/3 region and dentate gyrus in the hippocampus, whereas the SYN promoter was particularly effective in the dentate gyrus [53].

Epilepsy is another neurological disease that may benefit from the use of targeted gene therapy. Epilepsy affects around 1% of the population and medical therapy for epilepsy is largely symptomatic, aiming primarily at controlling seizures. However, 30% of individuals do not respond to the anti-convulsant therapy. The only alternative is to surgically resect the epileptogenic tissue, a procedure that may cause functional impairment of the patient [54]. Therapeutic targets to suppress seizures in epilepsy include neuropeptides such as galanin, cholecystokinin and neuropeptide Y. Adeno-associated virus (AAV) vector with different promoters and post-transcriptional regulatory elements has been tested in the experimental models of epilepsy [55]. When expressed from AAV vectors, neuropeptide Y has inhibitory action on excitatory glutamate mediated neurotransmission and displays anti-convulsant properties in a large variety of acute models of seizures *in vitro* and *in vivo* [56,57]. NSE promoter has successfully been employed to drive the expression of both neuropeptide Y [58] and galanin [59]. A 3 kb promoter of the neuroactive peptide cholecystokinin (CCK), which is active in interneurons within cortical and subcortical regions, was also tested in epilepsy treatment [60]. CCK has been implicated in a variety of psychiatric disorders such as panic attacks and stress-related anxiety disorders. The peptide is also involved in satiety. Thus, the promoter can also be used to specifically drive expression of therapeutic genes with anxiolytic and anxiogenic effects.

The NSE promoter is also employed to drive expression of aspartylglucosaminidase (AGA) to correct the deficiency of the lysosomal enzyme in aspartylglucosaminuria (AGU), a lysosomal storage disease with severe neurodegenerative clinical features. In a mouse experimental model, the NSE-driven AGA achieved a 90% correction of storage up to 2 months after injection of the adenovirus vector [61]. The GFAP promoter is used to drive expression of transgenes mainly in glioma gene therapy. But it can also be used to drive expression of transgenes in astrocytes to assist survival of peripheral root ganglia cells or treat pain.

3. Augmenting the activity of neural cell-specific promoters

The level of transgene expression is critical for both gene therapies and biological studies to understand gene function. In many instances, sustained expression of a transgene is required to achieve therapeutic or biological effects. Therefore, enhancement of transgene expression may require increased level or duration of expression, or both. In general, transcriptional regulatory elements of a vector, including promoter, enhancer, intron, and poly(A) sequence, should be optimized first. To enhance transgene expression from a weak cellular promoter in cell or tissue type-specific manner, several specific strategies have been developed. These strategies involve either promoter engineering that changes authentic sequences of a cellular promoter or new design of an expression cassette that uses a native cellular promoter together with other *cis*-acting elements (Fig. 2). The strategies that have been used for targeted transgene expression in neural cells include (1) engineering native cellular promoters, including multimerizing positive regulatory promoters and/or enhancer elements and modifying native promoters through point mutation, (2) using recombinant transcriptional activators to achieve transcriptional amplification, (3) using Cre site-specific recombinase to activate transgene expression, and (4) constructing expression cassettes by combining viral regulatory elements and a native cellular promoter (Table 1).

3.1. Promoter engineering

3.1.1. Multimerization of cis-acting elements

A reasonable approach to augment promoter strength is using multiple copies of enhancer elements positioned upstream of a minimal promoter. This strategy has been used to improve transgene expression from the GFAP and DBH promoters. The human GFAP promoter contains three enhancer regions, A, B, and D. The A and B regions are located respectively from 1604 to 1757 and 1489 to 1612 bp upstream of the TSS, whereas the D region is immediately upstream of the basal promoter at −132 to −57 bp. The B region has the strongest individual activity [62]. Three copies of either the B region or the conjoined ABD region flanking the archetypical 2.2 kb human GFAP promoter drove expression of the LacZ transgene 75-fold higher than the parental GFAP promoter [63]. These results were obtained when transiently transfecting the corresponding plasmids in GFAP-expressing glioma cell line U251. When expressed in the context of a replication deficient adenoviral vector, the increase in activity was 10 times. However, there was no any increase in activity, when the adenovirus was used to transduce adult mice brain [63].

The strength of a human DBH promoter was also improved by using multimerization of *cis*-regulatory elements. Among all the TFs regulating DBH gene expression in NA neurons, the homeodomain (HD)-transcription factors PHOX2A/Arix and PHOX2B/NBPhox are central. There exist three PHOX2-binding sites (PRS1-3) in the promoter. When 8 or 12 copies of the PRS2 *cis*-regulatory element were multimerized and placed upstream of a minimal promoter containing a TATA box and a TSS, the generated artificial promoter was able to drive the reporter gene expression with an activity 50 times higher than that of the 1.15 kb human DBH promoter [64]. However, in the context of an adenoviral vector, the promoter failed to improve the activity *in vivo* when compared to the 1.15 kb human DBH promoter. In spite of the lack of improvement in increasing transgene expression *in vivo*, the synthetic promoters seemed to have cell-type specificity as expression in non-NA neurons was lower. Nevertheless, it has been noted that these synthetic promoters could perturb the normal physiology of NA cells by sequestering PHOX2 proteins that may needed to maintain NA neuronal phenotypes [65].

Edelman and co-workers devised a high-throughput selection procedure to allow synthesis of *cis* motifs that enhance the activity of a minimal promoter [66]. They took advantage of their synthetic promoter construction method (SPCM) to isolate more than a hundred DNA sequences that showed increased promoter activity in the neuroblastoma cell line Neuro2A [66]. Their random approach enabled them to unravel a predominance of eight motifs (AP2, CEBP, GRE, Ebox, ETS, CREB, AP1, and SP1/MAZ) after running the DNA sequences of selected synthetic promoters against database of known elements. The most active sequence among the selected synthetic promoters contained composites of a number of these motifs. Furthermore, they found that up to 10% of 133 selected active sequences had no match in currently available databases. This new combinatorial SPCM therefore may reveal new motifs and transcriptional regulatory proteins to which they bind.

3.1.2. Chimeric promoters with sequences from two different neural cell-specific promoters

Geller and co-workers have developed a strategy for promoter enhancement by generating chimeric promoters using promoter/enhancer elements from two different neural cell-specific promoters.

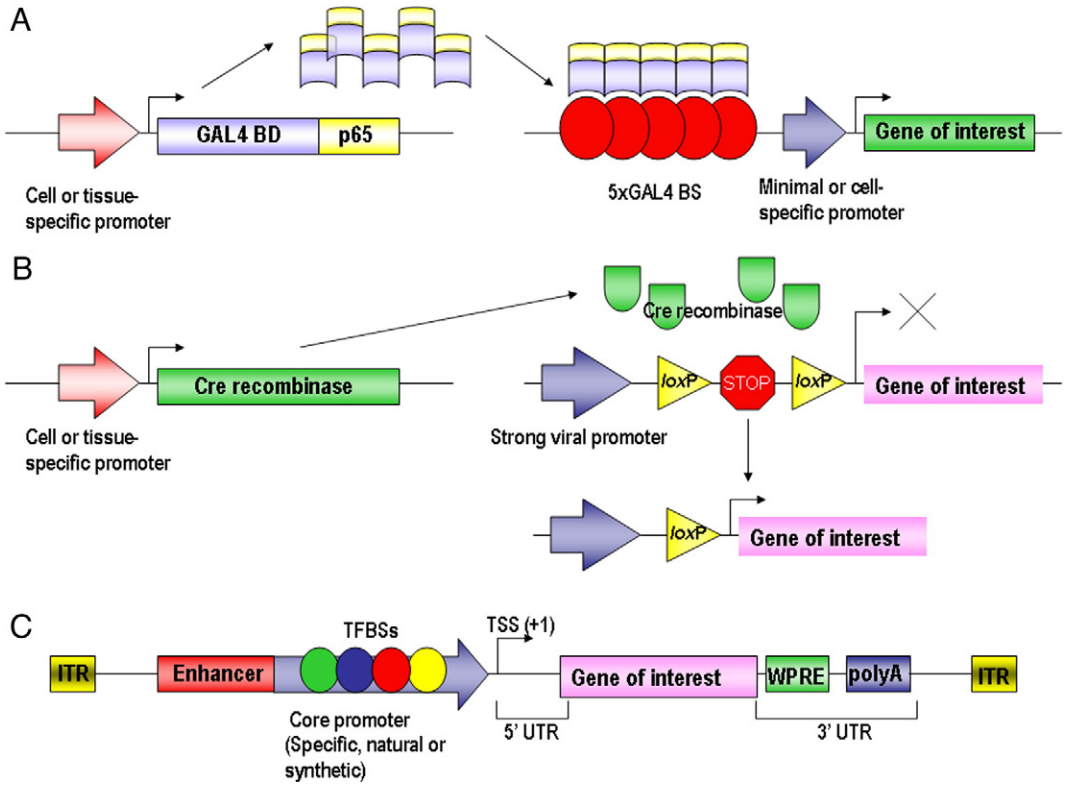

Fig. 2. Strategies for enhancing transgene expression from a weak cell type-specific promoter. (A). Two-step transcriptional amplification. In this example, the yeast GAL4 DNA binding domain (GAL4 BD) and the p65 subunit of the NF-κB transcription factor (p65) are fused to generate a recombinant transcriptional activator. A cell- or tissue specific promoter is used to drive the expression of the activator, which recognizes a unique GAL4 binding sequence. Five copies of the sequence (5xGAL4 BS) are incorporated upstream of either a minimal promoter or a cell-specific promoter. After binding to 5xGAL4 BS, the recombinant transcriptional activators work synergistically to enhance the activity of the nearby promoter. (B). Cre-mediated activation. The Cre recombinase expression is under control of a cell-type specific promoter. In those cells where the promoter is active, the Cre will recognize the specific loxP sequences that flank a STOP signal. After recombination, the translation inhibition sequence is removed, thus authorizing the expression of a transgene. (C). Viral regulatory element-mediated enhancement. Different elements can be incorporated in one expression cassette. These include inverted terminal repeats (ITR), the cytomegalovirus enhancer (CMVe), and the woodchuck virus post-transcriptional regulatory element (WPRE). Transcription factor binding sites (TFBSs), the transcription start site (TSS) and untranslated regions (UTR) are indicated.

In their initial study, a 6.3 kb fragment from the TH promoter was fused to the 5' end of a neurofilament heavy chain gene (NFH) promoter [67]. The generated TH-NFH chimeric promoter in a helper virus-free herpes simplex virus vector supported transgene expression for up to 6 months, much longer than 2 month expression supported by a preproenkephalin (ENK) promoter [68] and 2 to 2.5 month by a 6.8 or 9 kb fragment of the TH promoter [51,69]. They then identified two 100 bp fragments from the 6.3 kb TH promoter, each containing an enhancer and being able to support long-term expression from the NFH promoter [70]. Another chimeric promoter developed by the same lab contains a 5' upstream fragment of the ENK promoter fused to the NFH promoter [71]. This chimeric promoter appeared to support a higher level of long-term expression than the TH-NFH promoter, with more transduced neurons being observed at 1 month after gene transfer. It remains to be determined if the sequences from the TH and ENK promoters can improve long-term expression from other neuronal promoters.

3.1.3. Enhancement through point mutation

Modification of promoter architecture may improve the binding affinity of transcription factors or disrupt the binding of repressors, thus affecting promoter strength. Deletion mutants are often used to investigate the contribution of deleted elements to promoter strength. This could be problematic, as deletion may cause juxtaposition of different DNA elements, which might have a new regulatory effect. Point mutation through transitions or transversions provides more detailed insight into sequence requirement for promoter strength.

Hwang et al. managed to increase transgene expression from human DBH promoter by modification of transcription factor binding sites (TFBS) through point mutation [65]. They modified the native nucleotide sequence motifs of PRS2 and PRS3 in the human DBH promoter and observed significantly increased affinity of PHOX2A towards both sites. This resulted in greatly improved promoter strength (>20-fold) without loss of specificity, as non-NA cells did not express the EGFP transgene under control of the modified promoter *in vitro* as well as *in vivo*. Since transcriptional activity of a cell specific promoter is delicately coordinated by interactions among cell-specific and general transcriptional factors, an engineered promoter using a native promoter as the backbone would be a more physiologically relevant system than synthetic promoters that use repeated units of just one transcriptional binding site. However, to achieve successful activation by using this point mutation method, TFBS, as well as the structural basis of the binding of active TFs, need to be well characterized. Accumulation of data from experiments and computational results should allow the employ of this strategy more frequently in the future.

3.2. Two-step transcriptional activation

Two-step transcriptional activation approach, originally referred as recombinant transcriptional activation [10,72], is a relatively generalizable method [73–76]. This method utilizes artificial chimeric transcriptional activators to enhance transgene expression from a weak cell type specific promoter (Fig. 2). The basic principle of the method is to use a cell-specific promoter to drive the expression of a fusion protein containing a strong transcriptional activation domain and a DNA binding domain, which bind to a specific site in a modified promoter that drives transgene expression. The binding of the fusion

protein is expected to promote the assembly of RNA polymerase II complexes at the TATA box of the modified promoter and augment transgene expression. The most commonly used chimeric transcriptional activator is the herpes simplex virus transcriptional activator VP16 fused to the DNA binding domain of yeast transcriptional activator GAL4 [73–80]. This design takes advantage of the unique DNA binding sequence of GAL4 that exists in yeast but not in mammalian genomes. Thus, when included in a mammalian cellular promoter this sequence enables specific binding of artificial chimeric transcriptional activators. This results in augmented expression of the transgene while minimizing the chances of interference with the expression of other genes in mammalian cells.

To amplify the activity of a neural cell specific promoter, the transcriptional activation domain of the nuclear factor kappa B (NFκB) p65 has been used in the two-step transcriptional activation approach [81,82]. NFκB denotes a group of dimeric transcription factors, with the p50/p65 dimer as the most common complex regulating the expression of mammalian genes. In the central nervous system (CNS) neurons NFκB activity is constitutive and relatively high [83,84]. This transcription factor plays a crucial role in the survival of neurons in a variety of physiological and pathological settings [84–86]. For example, NFκB is activated in neurons in response to excitotoxic, metabolic, and oxidative stress. Activation of NFκB in neurons increases the levels of anti-apoptotic proteins and provides strong neuroprotection, whereas inhibition of NFκB activity leads to cell death after neurotoxic insults [84–87]. NFκB also plays a crucial role in both neuronal ontogeny and establishment of synaptic plasticity by regulating genes encoding neurotrophic factors, neurotransmitter receptors and calcium-regulating proteins [85].

In view of the potency of the transcription factor NFκB in regulating neuronal gene expression, we used neuron-specific promoters to drive expression of a chimeric transactivator consisted of a part of the transcriptional activation domain of NFκB p65 protein fused to the DNA-binding domain of GAL4 protein from yeast [82]. The second copy of the neuronal promoter is modified by introducing the unique GAL4 binding sequences at its 5' end and used to drive the expression of a transgene. Binding of the chimeric transcriptional activator upstream of the second promoter was expected to potentiate its transcriptional activity. The PDGF and SYN promoters were tested *in vitro* and *in vivo* using plasmid, lentiviral and baculoviral vectors. We observed up to a 100-fold improvement in reporter gene expression in cultured neurons and 20-fold improvement in the rat brain *in vivo*. The cell-type specificity of the two tested promoters was well preserved and restricted to neurons. The expression driven by the new lentiviral vectors with the p65-potentiated SYN promoter showed no signs of decline or cell damage 4 weeks after injection.

A modification of the above method was to use one synthetic bidirectional cell-specific promoter to drive the expression of both the artificial transcriptional activator and a transgene, with the promoter and the two transgenes being placed in the same vector [81]. This method overcomes the problem related to the use of two copies of a cell-specific promoter in the original design, which leads to generation of large expression cassettes that could be difficult to use in a viral vector with limited cloning capacity. When two cellular promoters, a 495 bp SYN promoter and a 681 bp GFAP promoter, were tested in the context of lentiviral and AAV vectors, enhanced transgene expression without loss of cell specificity was observed [81].

With an aim of incorporating an inducible mechanism, the powerful transcriptional amplification system has been modified to include the Tet-Off system that uses a tetracycline-controlled transactivator protein (tTA) and a tetracycline-responsive promoter element (TRE) to regulate expression of a target gene [88]. Two cellular promoters tested in that study were a 495 bp SYN promoter and a 681 bp GFAP promoter. Lentiviral vectors were constructed that contained two copies of one or the other of these 2 promoters. One copy was used to drive expression of a chimeric transactivator consisting of a part of the transcriptional activation domain of the NF-κB p65 protein fused to the DNA-binding domain of the yeast GAL4 protein. The second copy of the cell-specific promoter was modified by introduction of the GAL4 binding sequences at its 5' end. This copy was used to drive expression of tTA. A gene encoding a red fluorescent protein was cloned into another lentiviral vector under transcriptional control of TRE. Co-transduction with the two types of viral vectors provided doxycycline-regulated transgene expression in a neuron- or astrocyte-specific manner. Compared to control viruses without transcriptional amplification, these enhanced systems were approximately 8-fold more potent in cultured neurons and astroglial cells and at least 8- to 12-fold more potent in the rat brain *in vivo*. These results demonstrate the effectiveness of the transcriptional amplification strategy in developing viral gene delivery systems that combine the advantages of specific cell type targeting and Tet-inducible expression.

To make the two-step transcriptional activation approach more robust, several variables can be optimized, for example including (1) the number of GAL4-binding sites; (2) the space between the GAL4-binding sites and the downstream promoter; (3) the number of activator domains; (4) the orientation of the transgene and the transactivator-expressing cassettes and (5) the distance and insulator sequence between the two gene expression cassettes. Also, modifications of some regulatory sequence elements such as introns, translation initiation sites, the polyadenylation signal and posttranscriptional regulatory elements can be tested to further potentiate the system. Obviously, this approach is powerful and may be applied for generation of other viral vector gene delivery systems with high level of specificity for different types of neural cells.

3.3. Cre-mediated enhancement of gene expression

Yet another approach to amplify gene expression is the use of the Cre site-specific recombinase [89]. This method uses a relatively weak tissue-specific promoter to drive the expression of Cre, a bacteriophage P1-derived DNA recombinase that catalyzes recombination between two appropriately oriented 34-bp recognition sequences termed loxP sites. Once expressed, this DNA recombinase will function to induce the excision of a translational inhibition sequence that has been flanked with loxP sites and placed between a strong viral promoter and the transgene of interest. The removal of the inhibition sequence will then activate transgene expression from the viral promoter (Fig. 2). This process does not require any additional co-factor or accessory proteins. Commonly, Cre and the loxP component are placed into two separate vectors in order to maintain cell specificity. By co-transfection of the two vectors into target cells, the tissue-specific promoter could achieve high levels of gene expression (up to 300-fold) as a result of Cre-induced transcriptional activation [89].

The Cre/loxP system has been used to create a multicoloured mapping of the brain (Brainbow) in transgenic mice [90]. For that, the promoter for the Thy1 gene, a neuron-specific gene that expresses in projection neurons in many parts of the nervous system, was used to drive expression of Cre. Despite no reported applications of the system, the creation of a wiring diagram of the brain should be very useful to identify defective wiring in neurodegenerative disorders models. Namikawa and his colleagues employed the Cre/loxP-mediated gene amplification to achieve a highly specific neuronal expression in rat brains [91]. A modified promoter of the superior cervical ganglion10 (SCG10), with a very high neuronal specificity due to addition of two NSREs, was introduced into a Cre recombinase expressing adenovirus. This adenovirus in combination with another adenovirus vector expressing a Cre inducible enhanced green fluorescent proteins (EGFP), i.e. an EGFP gene flanked by loxP sites, was capable of mediating transgene expression at high levels both in neuronal cells of mixed cultures and in an animal model. Astrocyte-specific EGFP expression was achieved by using a Cre recombinase

expressing adenovirus containing the GFAP promoter. Maeda et al used a Cre/loxP-based adenovirus system in targeted gene therapy for astrocytoma [92]. In their system, the GFAP promoter was used to drive Cre expression in one viral vector and the CAG promoter to drive the expression of the herpes simplex virus thymidine kinase (HSV-TK) suicide gene in another vector. Co-injection of the two vectors induced HSV-TK expression, which in turn activated the prodrug ganciclovir to kill cancer cells. A notable finding from the study was low inflammatory response, probably due to the high level of specificity of the delivery system that restricted HSV-TK expression, thereby reducing side effects to the normal tissues around the tumor [92].

Several studies have reported that Cre-expression retroviruses or lentiviruses inhibit cell proliferation and induce chromosomal aberrations [93–95]. In the brain, constitutive expression of Cre causes the damage of brain structure and the presence of abnormal cavities in the lentiviral vector-injected striatum [94]. Transient expression of Cre from adenoviral vectors could also be deleterious to cell survival and growth [96]. This Cre cytotoxicity increased when Cre was tagged with a nuclear localization signal, indicating that Cre effects depend on the amount of Cre localized in the nucleus. Moreover, Cre toxicity was correlated with the strength of promoters used to drive Cre expression, with significant cytotoxicity in cells transduced with adenoviral vectors carrying the CAG promoter [96]. Thus, Cre cytotoxicity is dose dependent, suggesting the possibility that low levels of Cre expression mediate recombination without causing any adverse effects. Consistent with this notion, Kaspar et al. have reported that AAV vectors expressing Cre mediate extensive recombination in neural cells of defined brain regions without toxicity [97].

3.4. Viral regulatory elements to improve gene expression from a cellular promoter

Natural *cis*-acting elements from the genomes of viruses can interact with mammalian cell *trans*-acting factors. Once placed into a mammalian cell expression cassette, these viral elements can regulate transgene expression in mammalian cells. For example, viral enhancers are used to boost transcription and virus *cis*-acting sequences can act at the posttranscriptional level such as polyadenylation and RNA export to enhance transgene expression (Fig. 2).

3.4.1. Human cytomegalovirus immediate-early gene enhancer

Given that the immediate-early gene enhancer from the human cytomegalovirus (CMV) has the highest activity among other viral enhancers from herpes virus, Rous sarcoma virus and hepatitis B virus [98], several studies had added a CMV enhancer 5' to a cellular promoter to increase its transcriptional activity [99–106]. One of the most successful examples of this approach is the CMV enhancer/chicken beta actin (CAG) promoter that direct improved gene expression in several tissues [17,99,103,107]. In a study using plasmid vectors, enhancers and promoters from muscle-specific genes were substituted for or combined with the CMV enhancer/promoter and one of these chimeric vectors offered an expression level twice that of the parental plasmid [101]. An AAV-2 vector that uses the CMV enhancer/ promoter in combination with a 1.2 kb human skeletal actin promoter increased transgene expression in the muscle significantly, providing a therapeutic range of expression of coagulation factor IX with a 2- to 4-fold lower vector dose [102]. The CMV enhancer also stimulated the elongation factor 1α (EF1α) promoter and the ubiquitin promoter for increased levels of transgene expression [100]. For a human telomerase reverse transcriptase (hTERT) promoter, coupling the promoter with the CMV enhancer has much higher activity than wild-type hTERT promoter with retained hTERT specificity [105]. A recent study has reported that lentiviral vectors with the human CMV enhancer improves transgene expression levels in a cell type-specific manner from several cell type-specific promoters, including human atrial natriuretic factor and human ventricular myosin light chain promoters in cardiomyocytes and human surfactant protein C-specific promoter in type II alveolar epithelial cells [106]. The feasibility of this hybrid promoter approach in improving a neuronal specific promoter in the nervous system was first assessed in our lab.

We constructed a hybrid promoter by appending a 380 bp fragment of CMV enhancer upstream of the 1.5 kb PDGF promoter [108]. The plasmid containing the promoter was complexed with polyethylenimine for *in vitro* and *in vivo* gene transfer. In cultured cells, the plasmid with the hybrid promoter significantly augmented expression of a luciferase reporter gene, providing expression levels 8 to 90-fold and 7 to 178-fold higher than those from two baseline constructs containing the PDGF promoter alone and the CMV enhancer alone, respectively. In particular, the activities of the hybrid promoter in two neural cell lines were close to or higher than that of the CMV immediate-early gene enhancer/promoter, a transcriptional control element that has been considered to be the most robust one identified thus far. After stereotaxic injection into the hippocampus and striatum in rats, the hybrid promoter displayed a neuronal specificity, driving gene expression almost exclusively in neurons. Transgene expression in the brain driven by the hybrid promoter was detectable 24 h after injection, being 10-fold higher than that driven by the PDGF promoter alone. The expression peaked around 5 days at 1.5×10^5 RLU per brain and lasted for at least 4 weeks. This differed strikingly from the expression driven by the PDGF promoter, which was no longer detectable on day 3.

The hybrid CMV enhancer/PDGF promoter was then tested in an AAV vector [109]. In cultured cortical neurons, the AAV vector augmented transgene expression up to 20-fold over that mediated by titer-matched AAV vectors with the PDGF promoter alone and 4-fold over the CMV enhancer/promoter. Injection of the AAV vector with the hybrid promoter into the rat striatum resulted in neuron-specific transgene expression, the level of which was about 10-fold higher than that provided by the two control AAV expression cassettes at 4 weeks post-injection and maintained for at least 12 weeks. Gene expression in the substantia nigra through possible retrograde transport of the AAV vectors injected into the striatum was not obvious. After direct injection of AAV vectors into the substantia nigra, transgene expression driven by the hybrid promoter was observed specifically in dopaminergic neurons and its level was about 3 and 17 times higher than that provided by the PDGF promoter alone and the CMV enhancer/promoter, respectively. These results suggest possible application of the vector for gene therapy of Parkinson's disease. Moreover, when the hybrid promoter was tested in the context of baculoviral vector, several hundred-fold improvements *in vitro* and around 10-fold improvement *in vivo* were achieved over the control PDGF promoter without the CMV enhancer [110,111].

We also used this hybrid promoter approach to improve the activity of the GFAP promoter [112]. We constructed an expression cassette containing a hybrid CMV enhancer/GFAP promoter and placed it into baculovirus vectors, a type of viral vectors capable of transducing astrocytes. The recombinant baculoviruses with the hybrid promoter improved gene expression levels over two orders of magnitude in glial cell lines and by 10-fold in the rat brain when compared to the baculoviruses with the GFAP promoter alone. The astrocyte specificity of the GFAP promoter was preserved in the engineered expression cassette, as demonstrated by immunohistological analysis of brain samples and an axonal retrograde transport assay.

In a recent study [113], the CMV enhancer was fused to 5 neuron-specific promoters, including the SYN, calcium/calmodulin-dependent protein kinase II, tubulin alpha I, NSE and PDGF promoters, with the purpose of comparing and isolating the most efficient CMV enhancer/neuron-specific promoter combination in the context of VSV-G pseudotyped lentivirus for gene delivery into the rat brain. Among all the tested constructs, the hybrid CMV enhancer/SYN promoter was

the most effective construct in terms of *in vivo* specificity and activity, which provided a retained neuronal specificity, a gene expression level as high as that provided by the CMV promoter and sustained transgene expression for up to 8 weeks.

The possible mechanism underlying the increased transcriptional activity of these hybrid CMV enhancer/neural promoters has yet to be identified. It is well recognized that enhancer and promoter recognition by RNA polymerase, transcription factors, and auxiliary proteins involves both primary and secondary sequence characteristics of the regulatory DNA. The number, diversity, orientation, and placement of transcription factor-binding sites within promoters are critical parameters that define gene expression. In the case of the CMV-PDGF hybrid promoter, the PDGF promoter contains the transcription factor binding sites for Sp1, Sp3, Egr-1, NF-κB [28–31] and the CMV enhancer contains at least six NFκB regulatory elements and one Ap1 site that are recognized by NF-κB and Fos-Jun, respectively. These sites might act synergistically, resulting in a favorable interaction between the transcription factors and auxiliary promoters attracted by the PDGF promoter and CMV enhancer.

Maintenance of cell-type specificity after insertion of the CMV enhancer appears to depend on the promoter used. In addition, not all promoters benefited from the CMV enhancer, as some promoters actually displayed declined activity when flanked by the CMV enhancer. When trying to use this approach to improve the strength of the NSE promoter, we were unable to detect improved transcriptional activity (unpublished observation), probably because the NSE promoter is already highly active in neurons. We also noticed the specificity of the NSE promoter was somehow affected by the CMV enhancer. The decreased neuronal specificity in the NSE promoter after fusing with the CMV enhancer was confirmed by a recent report [113]. The authors further demonstrated that the neuronal specificity of the calcium/calmodulin-dependent protein kinase II, tubulin alpha I promoters, but not the SYN and PDGF promoters, decreased significantly after promoter/CMV enhancer fusion [113]. In a study using simian immunodeficiency virus (SIV)-derived lentiviral vectors, the cell-type specificity of GFP expression from a heart-specific human α-MHC promoter was severely compromised in combination with the CMV enhancer fragment [106]. In the brain, CMV promoter activity is remarkably different in different groups of cells, especially when it is incorporated in adenoviral and LV vectors [114,115]. While it is highly active in glial cells, only some neuronal phenotypes exhibit high level of expression when targeted with CMV-based constructs. Thus, it is likely that the incorporation of the control elements of CMV enhancer into another promoter may bias gene expression in favor of certain cellular phenotypes and distort the expression profile of the original promoter.

3.4.2. Inverted terminal repeats of adeno-associated virus

Among the viral vectors that are able to provide long-term stable transgene expression are recombinant adeno-associated virus (AAV), which are constructed by replacing the AAV *rep* and *cap* genes with a transgene cassette and flanking the cassette with two AAV *cis*-acting inverted terminal repeats (ITRs) of 145 nucleotides. Such recombinant AAVs do not integrate into chromosome site-specifically, as their wild-type counterparts do, and exist predominantly as episomal concatemers [116,117]. ITRs are considered as a crucial AAV element, serving as primers for host cell-mediated DNA synthesis to convert the single-stranded virion DNA into double-strand DNA templates for transcription and replication [117]. A number of reports also suggested their roles in mediating substrate DNA integration into the host DNA and in defining integration boundaries of viral genome [118–120]. The presence of ITRs appears to be the only requirement for the formation of the episomally stable concatamers of recombinant AAV genome [121,122]. Long-term transgene expression mediated by AAV vectors was found to be associated with the molecular conversion of single-stranded viral genomes to high-molecular-weight circular concatamers and the prolonged episomal persistence of the concatamers [121]. Inspired by the performance and structure of the AAV vectors, several groups have developed viral or plasmid vectors with expression cassettes of interest flanked by AAV ITRs and reported the improved efficiency of transgene expression in mammalian cells [123–126], Xenopus embryos [127] and fishes [128].

We tested whether using the AAV ITRs together with the hybrid CMV enhancer/neural promoters may lead to further improvement in transgene expression over the hybrid promoters alone [129]. We initially developed a baculovirus vector in which ITRs were used to flank a luciferase reporter gene cassette harboring the PDGF promoter. When tested in the rat brain, the baculoviral vector was able to provide transgene expression for at least 90 days [129]. Incorporating the AAV ITRs into our baculovirus vectors harboring the hybrid CMV enhancer/GFAP promoter also leads to further improvement in transgene expression [112]. Using this recombinant baculoviral vector, we observed extended *in vivo* transgene expression in the rat brain at 90 days postinjection, the longest time point examined, by which time the gene expression from baculovirus vectors with the GFAP or CMV promoter had already become undetectable. Immunohistological analysis demonstrated that ITR flanking and the incorporation of the CMV enhancer did not affect the cellular preference of the neural promoters in the context of baculovirus (Fig. 3).

Our findings are consistent with the previous observations obtained by using HSV-1 amplicon vectors and plasmid vectors containing the AAV ITRs [123–128,130–133]. The AAV ITRs used in our studies, however, appeared to be unable to change the dynamics of transgene expression. With or without the sequences, cultured cells and brain tissues transduced by the baculovirus vectors harboring the hybrid CMV enhancer/neural promoters displayed similar patterns of time-dependent decrease in gene expression. Several mechanisms might be responsible for the loss of expression. Baculovirus genomic DNAs remain episomal in transduced cells. They cannot replicate in mammalian cells and would be diluted by cell division over time, thus resulting in a decrease in gene expression. This is more likely for *in vitro* transduction, as cultured cells divide much faster than cells in the brain. Alternatively, baculovirus genomic DNA might activate host cell defense machinery, which would ultimately eliminate the viral vector together with the accommodated gene expression cassette. Although the PDGF and GFAP promoters are cellular transcription regulatory elements with an authentic DNA sequence and should be less sensitive to promoter silencing, the CMV enhancer part of our hybrid promoters would still be subjected to the regulation of transcriptional silencing mechanisms such as DNA methylation. It has been reported that transcriptional silencing can be caused by extensive methylation of the CMV promoter and enhancer following adenoviral gene transduction [134]. Nevertheless, the collaborative action of the AAV ITRs and the CMV enhancer significantly augmented initial expression levels, which might have made it possible to detect the prolonged expression of transgene up to 90 days postinjection. These findings establish an effective way to engineer baculovirus vectors in order to achieve relatively sustained expression of a functional gene for gene therapy for neurodegenerative disorders and physiological studies of neurons and astrocytes.

It is worthy of note that the effectiveness of using ITRs could be influenced by the choice of promoters, gene delivery vectors and/or targeted tissues. It has been reported that in the context of AAV carrying an astrocyte-specific promoter, most transduced brain cells appeared neuronal [27,107] probably because AAV ITRs, which can function directly as a promoter [135] had overridden the cellular promoter [16].

3.4.3. Woodchuck hepatitis virus post-transcriptional regulatory element

The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) is a *cis*-acting RNA element with powerful effects on

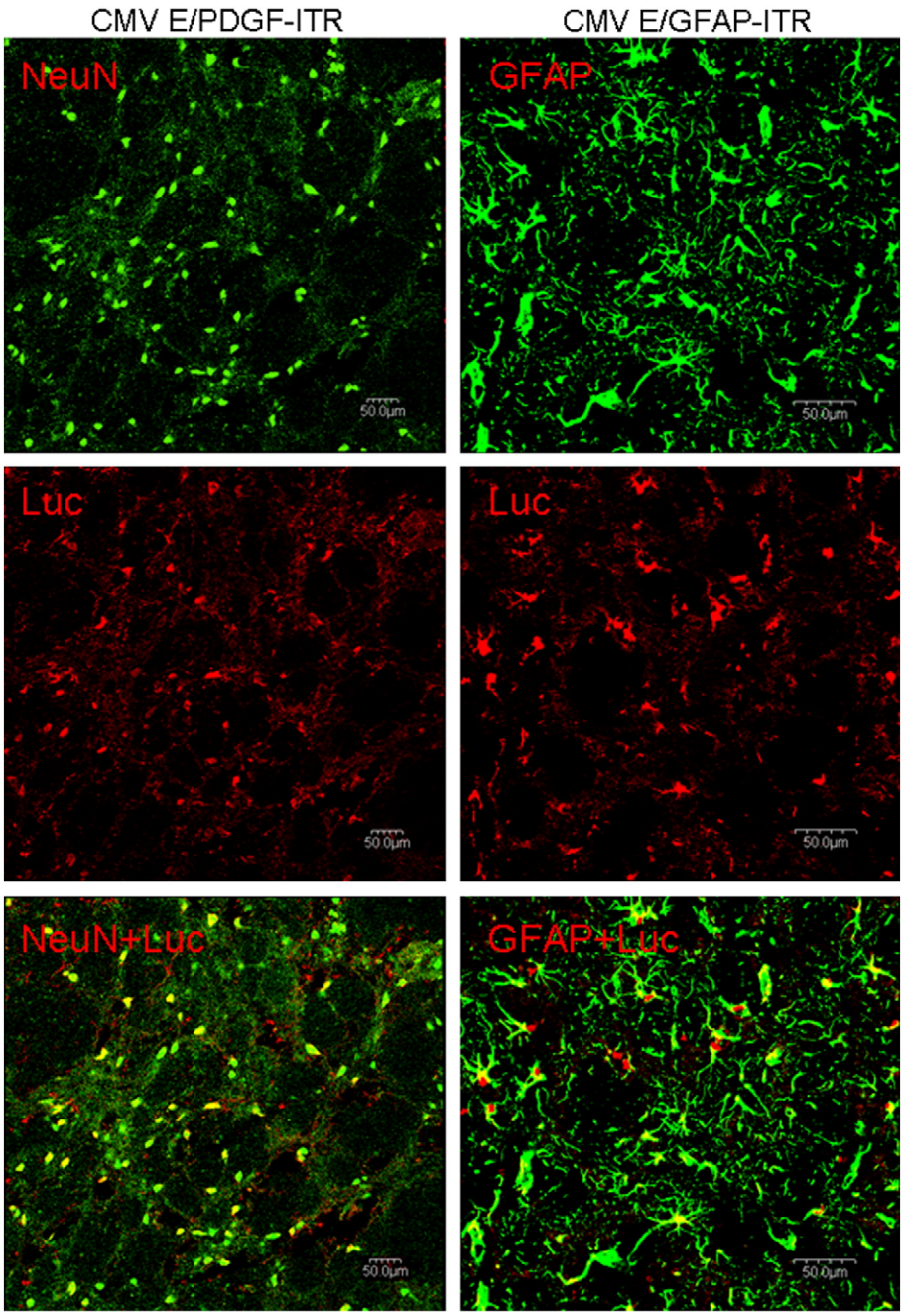

Fig. 3. Cell specificity of engineered expression cassettes. Immunohistological analysis was performed in the sections collected from rat brains injected with recombinant baculoviruses carrying an engineered expression cassette. In one vector, the inverted terminal repeats (ITRs) of AAV were used to flank a luciferase reporter gene cassette under the control of a hybrid CMV enhancer/PDGF promoter and in another vector the neuron-specific PDGF promoter was replaced by the GFAP promoter. Baculoviral vectors (1×10^8 pfu) were injected into the striatum of adult male Wistar rats. Two days after injection, brain samples were collected and frozen coronal sections of each brain, within 0.5 mm from the needle track, were cut at 30 µm thickness for free-floating double immunostaining. Anti-luciferase antibodies were used to visualize transducted cells, anti-neuronal nuclei protein (NeuN) and anti-glial fibrillary acidic protein (GFAP) antibodies to show neurons and astroglial cells, respectively. Note that luciferase-positive cells are co-stained either by antibodies against NeuN when baculoviral vectors with the PDGF promoter were used or by antibodies against GFAP in the case of using baculoviral vectors with the GFAP promoter, indicating that ITR flanking and incorporation of the CMV enhancer have no obvious effects on the cellular specificity of the two neural promoters.

nuclear and cytoplasmic accumulation of RNA [136]. In integrating RNA vectors, this post-transcriptional regulatory element improves transcript termination, likely from enhanced polyadenylation, thus augmenting viral titers and transgene expression [137]. WPRE can also increase transgene expression from DNA virus vectors based on AAV [26,138], adenovirus [19,139–141], and baculovirus [142,143]. WPRE is most effective when being inserted in 3' UTR of a transgene and upstream of the polyadenylation signal.

As the first example demonstrating the effect of WPRE on *in vivo* transgene expression from a viral vector in the brain, Bueler and his colleagues showed that an AAV vector containing the PDGF promoter and WPRE in the 3' UTR of the transgene outperformed vectors lacking WPRE and achieved significantly higher transduction in the rat brain, with the number of transduced dopaminergic neurons increasing twofold and stable transgene expression lasing for at least 41 week [26]. Their study further demonstrated that WPRE strongly increase transgene expression in individual neurons and GFP became detectable in axons emerging from the transduced neuronal bodies (while vectors lacking WPRE did not). Using an AAV vector containing a 1.8 kb NSE promoter to transduce the rat brain,

During's lab demonstrated a 26-fold increase in transgene expression level in the striatum by insertion of WPRE into the expression cassette [144]. The transgene expression was maintained for at least 15 months at a level similar to that observed at 1 month postinfection. In the same study, the preproenkephaline promoter driven luciferase also benefited from WPRE addition as expression in the striatum and hippocampus was amplified 13- and 35-fold respectively in the presence of WPRE. Klein et al. also used an AAV vector to deliver the EGFP gene to the rat brain [145]. The expression cassette contains a CAG promoter. The purpose of the study was dual: to evaluate the dose response (in terms of delivered virus particles) and potency of the promoter compared to the NSE promoter. The CAG promoter could increase the number of GFP expressing neurons when compared to the NSE promoter, with the expression of GFP for at least 18 months when the former vector was used. Incorporation of WPRE into the CAG driven cassette improved expression levels by 11-fold in the hippocampus. Kugler and colleagues tested WPRE together with the SYN, NSE and PDGF promoters in AAV vectors [146]. Comparison of the expression kinetics of the SYN promoter plus WPRE and the CAG promoter plus WPRE in their study revealed that the SYN-WPRE cassette expressed at least equal, or even higher, amounts of the transgene in primary hippocampal neurons. The above findings from AAV vectors suggest that the beneficial effect of WPRE on gene expression is probably promoter-independent in a DNA virus vector.

The WPRE capacity to enhance transgene expression was also tested in the context adenoviral vectors. Glover et al. developed an adenoviral expression cassette comprising a 495 bp human SYN promoter and WPRE [19]. Both *in vitro* and *in vivo* experiments showed the 3' addition of WPRE greatly enhanced transgene expression with no loss of neuronal specificity. This adenoviral vector also outperformed the vector containing the CMV promoter/WPRE combination in terms of specificity and activity *in vivo*. In a follow up study, the EGFP transgene was still expressed in neurons 9 months in the rat brain after injection of the SYN/WPRE vectors whereas in the brain injected with vectors using the CMV/WPRE combination there was a dramatic fall in expression levels and very little expression was detectable 9 months postinjection [140]. A similar approach showed the efficiency and specificity of an Ad5 (Adenovirus pseudotype 5 vector) containing a SYN-promoter-EFGP-WPRE cassette in driving long term expression of EGFP in neurons of mixed adult rat dorsal root ganglion (DRG) and retinal cell cultures [141]. After *in vivo* injection into the rat DRG, this adenoviral vector mediated restricted EGFP expression in neurons, while an adenoviral vector with the CMV promoter transduced both glial cells and neurons. However, Boulos et al. reported that the activities of the rat and human SYN promoters in adenoviral vectors were considerably lower than the CMV promoter in rat primary cortical neuronal cultures, despite the presence of WPRE in the expression cassettes [147]. Nevertheless, the authors demonstrated that WPRE enhanced transgene expression from the Rous Sarcoma Virus (RSV) promoter in adenovirus transduced rat primary cortical neurons.

Recently, WPRE has been tested in baculoviral expression cassettes. Mahonen et al. examined expression of three independent transgenes (EGFP, lacZ and lodavin) from the CAG promoter placed into a baculoviral vector in several cell lines, including a non-permissive cell line that is generally considered to be refractive to baculoviral transduction [142]. They showed that WPRE significantly boosted baculovirus-mediated transgene expression in all tested cell lines. We have generated a recombinant baculoviral vector equipped with a human elongation factor 1α promoter and WPRE. When using baculoviral vectors to transduce human embryonic stem (hES) cells, we observed an increase in efficiency from 40% in the cells transduced with the vector without WPRE to 80% in the cells transduced with the vectors with WPRE [143]. However, with or without the WPRE sequences, hES cells transduced by the baculoviral vectors displayed similar patterns of transient gene expression, diminishing toward background within one week irrespective of initial transgene expression levels. Baculovirus genomic DNA normally remains episomal in transduced cells. They cannot replicate in mammalian cells and would be diluted by cell division over time, resulting in the decrease of gene expression. Indeed, in non-proliferating hES-derived human neurons transduced with baculoviral vector harboring a human elongation factor 1α promoter transgene expression lasted for 1 month. With WPRE, the expression duration was prolonged to 3 months (unpublished observation). It remains to be determined whether WPRE can improve gene expression from a neural cell-specific promoter placed into baculoviral vectors.

WPRE is not the only post-transcriptional regulatory element. The flanking regions of eukaryotic mRNAs contain signal elements that also confer the function of increasing the stability of mRNA. In a study by Brun and colleagues [148], the efficiency of WPRE in increasing transgene expression in neuronal and glial cells was tested against three mammalian UTRs, the 3' UTRs of tau and tyrosine hydroxlase mRNA and the 5' UTR of human Alzheimer amyloid precursor (APP) mRNA. Among the four elements, WPRE is the most effective one in neural cells and glial cells in the context of plasmid and lentiviral vectors. Interestingly, WPRE, tau 3' UTR and APP 5' UTR had an additive effect on expression in neuronal cells and the combination of the three elements improved expression by 26-fold. This finding suggests a new approach to enhance gene expression through combined incorporation of appropriately selected viral and cellular post transcriptional regulatory elements into one expression cassette.

4. Outlook and future perspectives

High level and sustained transgene expression in target cells remains a great challenge for gene therapy. In this review, we have discussed published examples in the area of gene delivery into the nervous system in the context of engineering strategies to increase the strength of an expression cassette. While some of the strategies, such as the use of WPRE and the CMV enhancer, have been widely tested in both integrating and non-integrating vectors, many others that possibly contribute to effective expression of a transgene are less studied (Table 1). Apart from these, modification of UTR regions and polyadenylation signals and interruption of coding sequences with heterologous introns have not been exploited to their full capacity in transcriptional targeting of brain cells. In view of the critical role of dynamic chromatin alterations in gene expression, design of an expression cassette for integrating retroviral and lentiviral vectors requires special consideration to include other regulatory elements capable of counteracting silencing effects of heterochromatin. These elements may include locus control regions (LCR) that impose an active chromatin configuration in heterochromatic surroundings and matrix attachment region elements (MARs) and chromatin insulators that function as barrier elements to physically bock the passage of signals from the surrounding chromatin [149].

Apparently, the use of a cell type-specific promoter for transcriptional targeting will benefit from combination in a single construct of diverse regulatory elements that overcome different obstacles to transgene expression. It might be also desirable for certain applications to incorporate into such expression cassettes regulatable systems that control gene expression in an inducible manner in response to temporal or pathophysiological signals. To this end, development of delivery vectors with large packaging capacities would allow gene cassettes to be tailored to meet desired therapeutic needs, thus harnessing the full potential of transcriptional targeting.

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