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Herpes simplex virus type 1-based amplicon vectors for fundamental research in neurosciences and gene therapy of neurological diseases

Diana Jerusalinsky [a,b,*,1], María Verónica Baez [a], Alberto Luis Epstein [c,*,1]

[a] Instituto de Biología Celular y Neurociencia (IBCN), CONICET-UBA. Buenos Aires, Argentina
[b] CBC, Universidad de Buenos Aires (UBA), Buenos Aires, Argentina
[c] Centre de Génétique et Physiologie Moléculaire et Cellulaire, Villeurbanne, France

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ABSTRACT

Somatic manipulation of the nervous system without the involvement of the germinal line appears as a powerful counterpart of the transgenic strategy. The use of viral vectors to produce specific, transient and localized knockout, knockdown, ectopic expression or overexpression of a gene, leads to the possibility of analyzing both *in vitro* and *in vivo* molecular basis of neural function. In this approach, viral particles engineered to carry transgenic sequences are delivered into discrete brain regions, to transduce cells that will express the transgenic products. Amplicons are replication-incompetent helper-dependent vectors derived from herpes simplex virus type 1 (HSV-1), with several advantages that potentiate their use in neurosciences: (1) minimal toxicity: amplicons do not encode any virus proteins, are neither toxic for the infected cells nor pathogenic for the inoculated animals and elicit low levels of adaptive immune responses; (2) extensive transgene capacity to carry up to 150-kb of foreign DNA; i.e., entire genes with regulatory sequences could be delivered; (3) widespread cellular tropism: amplicons can experimentally infect several cell types including glial cells, though naturally the virus infects mainly neurons and epithelial cells; (4) since the viral genome does not integrate into cellular chromosomes there is low probability to induce insertional mutagenesis. Recent investigations on gene transfer into the brain using these vectors, have focused on gene therapy of inherited genetic diseases affecting the nervous system, such as ataxias, or on neurodegenerative disorders using experimental models of Parkinson's or Alzheimer's disease. Another group of studies used amplicons to investigate complex neural functions such as neuroplasticity, anxiety, learning and memory.

In this short review, we summarize recent data supporting the potential of HSV-1 based amplicon vector model for gene delivery and modulation of gene expression in primary cultures of neuronal cells and into the brain of living animals.

1. Introduction

Controlled expression of proteins is a key experimental approach to a deeper understanding of molecular basis of neuronal function and the outcoming behavior. Strategies necessarily involve silencing or enhancement of expression or function of neuronal proteins. Methods currently used for these studies have included pharmacological and transgenic approaches, both of which have serious limitations.

The most frequently used approach has been the pharmacological one, based on the application of agonists/antagonists of different neurotransmitters, enzymes and second messenger inhibitors, channel blockers, neurotrophins and inhibitors of transcription factors. More recently, some authors have included immunological strategies involving the use of antibodies. However, selective and specific pharmacological agents are not always available. Besides, this approach has two disadvantages which are difficult to control: access to the brain and diffusion into the brain tissue.

There is no doubt that knockout and transgenic animals constitute a potentially powerful strategy for studying the physiopathological relevance of some genes and their products (Picciotto and Wickman, 1998). However, besides being expensive and time-consuming, transgenic approaches are often neither regionally nor temporally restricted enough for appropriate gene manipulation. Moreover, functional compensation can take place during development, masking the phenotypes that result from the chronic

* Corresponding authors. Addresses: Instituto de Biología Celular y Neurociencia (IBCN), Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155 3°Piso (1121), Buenos Aires, Argentina (D. Jerusalinsky), Université de Lyon, Lyon, F-69003, France. (A.L. Epstein).

E-mail addresses: djerusal@gmail.com (D. Jerusalinsky), alberto.epstein@univ-lyon1.fr (A.L. Epstein).

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absence or overexpression of particular genes. For this and many other reasons, it is extremely difficult to restrict temporally and/or spatially a genetic modification; furthermore, some of these changes can be lethal.

The use of viral vectors to produce specific, transient and localized knockout, knockdown, ectopic expression or overexpression of a gene, can help to overcome those difficulties leading to the possibility of analyzing, for example, molecular aspects of behavior and cognitive functions that have resisted precise characterization (Simonato et al., 2000). Furthermore, the possibility of somatic manipulation of the central nervous system (CNS) without the involvement of the germinal line appears as a powerful counterpart of the transgenic strategy. In this approach, viral particles engineered to carry transgenic sequences are delivered into discrete brain regions, to transduce a group of cells that will therefore express the transgenic products.

In this short review, we summarize recent data supporting the potential of HSV-1 based amplicon vector model for gene delivery and modulation of gene expression in primary cultures of neuronal cells and into the nervous system of living animals.

2. Applications of amplicon vectors

Amplicon vectors (Spaete and Frenkel, 1982) are advantageous tools in neuroscience research (reviewed in Jerusalinsky and Epstein, 2006; Cuchet et al., 2007; Marconi et al., 2010). Amplicons are replication-incompetent helper-dependent vectors derived from HSV-1. These vectors have several advantages that potentiate their use in neurosciences: (1) minimal toxicity: since amplicons do not encode any virus proteins, they are not toxic for the infected cells nor pathogenic for the inoculated animals and, in addition, amplicon infection elicits relatively low levels of adaptive immune responses; (2) extensive transgene capacity: amplicons are capable to carry up to and deliver almost 150-kb of foreign DNA to the nuclear environment of mammalian cells, which means that entire genes with regulatory sequences or combination of several genes could be delivered using these vectors; (3) widespread cellular tropism: HSV-1 (and amplicons) can experimentally infect a wide range of cell types including glial cells, though naturally this virus infects mainly neurons and epithelial cells; (4) since the viral genome does not integrate into cellular chromosomes, there is very low probability to induce insertional mutagenesis.

Several recent technological breakthroughs addressing both the possibility to produce large amounts of helper-free amplicon vectors (Saeki et al., 2001; Zaupa et al., 2003) and the ability to deliver very large pieces of foreign DNA (Wade-Martins et al., 2001, 2003) have, amongst other improvements, significantly favoured the application of these vectors in different settings of experimental gene therapy models, and for the study of complex neural functions. As shown both in the diagram and Table 1, and further developed below, recent applications of gene transfer into the brain using amplicon vectors have focused on (i) experimental gene therapy of inherited genetic diseases affecting the nervous system, such as ataxias, (ii) on neurodegenerative disorders, using experimental models of Parkinson's disease or Alzheimer's disease, (iii) on neuroprotection and synapse restoration, (iv) on brain cancer, and (v) on a group of complex functions of the nervous system related to anxiety, sexual behavior, and to learning and memory in animal models, using different tasks such as fear conditioning and inhibitory avoidance paradigms (Table 1).

2.1. Ataxias

The capacity of amplicons to deliver very large DNA fragments was used to treat an experimental model of Friedreich's ataxia (FA), the most common recessive form of ataxia in humans, which originates from a deficiency in frataxin, a protein encoded by the FRDA gene. In a first study, Lim et al. (2007) demonstrated that the synthesis of frataxin could be eliminated in neurons from transgenic mice carrying floxed FRDA (*loxP-frda*) genes by infection with amplicon vectors expressing CRE recombinase (CRE-amplicons). *In vivo* delivery was achieved by stereotaxic injection of the CRE-amplicons into the brainstem of loxP-frda mice, to generate a localized gene-knockout model. These mice developed a behavioral deficit detectable after 4 weeks, and when re-injected with amplicons expressing the frataxin cDNA, they exhibit behavioral recovery as early as 4 weeks after the second injection. In a second study, amplicons were used to deliver a 135-kb insert containing the entire 80-kb FRDA human genomic locus, including long upstream and downstream regulatory sequences (the FXN genomic DNA locus) into FA patient deficient primary fibroblasts (Gomez-Sebastian et al., 2007). Synthesis of frataxin in the FRDA-transduced FA-deficient cells was confirmed by immunefluorescence. Moreover, functional complementation studies demonstrated restoration of the wild-type cellular phenotype in the FRDA-transduced cells in response to oxidative stress. More recently, and to investigate the persistence of transgene expression in the brain provided by the amplicon-delivered 135-kb FXN genomic DNA locus, the same group constructed a second vector carrying the 135-kb FXN locus but with the E. coli lacZ gene inserted at the ATG start codon (Gimenez-Cassina et al., 2011). Direct intracranial injection of this vector into the adult mouse cerebellum resulted in a large number of cells expressing lacZ driven by the FXN locus, which persisted for at least 75 days. In contrast, synthesis of GFP expressed from the same vector but driven by the HSV-1 IE4/5 promoter, was strong but transient. This study demonstrated for the first time a sustained transgene expression *in vivo*, by amplicon-borne delivery of a very long genomic DNA locus.

Ataxia-Telangiectasia (AT) is an autosomal recessive disease with a pleiotropic phenotype, characterized by cerebellar degeneration, immunodeficiency, cancer predisposition, radiation sensitivity and premature aging. This disease is caused by a defect in the ATM (Ataxia Telangiectasia Mutated) gene, which is responsible for recognizing and correcting errors in duplicating DNA when cells divide. Currently, no treatment can stop progression of AT. Expression of the ATM cDNA from amplicons allows functional recovery of human AT fibroblasts (Cortes et al., 2003). In a further study from this team, an amplicon encoding both the enhanced green fluorescent protein (EGFP) and a human FLAG-tagged-ATM protein was inoculated in the cerebellum of $Atm^{-/-}$ mice. This amplicon was delivered to thousands of cerebellum cells, including Purkinje cells, as assessed by EGFP fluorescence. FLAG-tagged-ATM expression was demonstrated at transcriptional (qRT-PCR, in situ-hybridization) and translational (immune-precipitation of the full-length human protein) levels 3 days post-inoculation (Cortes et al., 2006). In order to achieve stable gene replacement, this group then generated an HSV/adeno-associated virus (AAV) hybrid amplicon, carrying the expression cassette for the ATM and EGFP cDNA, flanked by AAV inverted terminal repeats (ITRs). This hybrid vector, in the presence of AAV Rep proteins, mediated site-specific integration of the transgenic sequences into the AAV1 site of chromosome 19 in human cells and in $Atm^{-/-}$ mice carrying that human locus. The functional activity of the vector-derived ATM was confirmed *in vivo* by ATM autophosphorylation. Hence, HSV/AAV hybrid amplicon vectors are able to mediate functional targeted integration of the ATM cDNA into cultured AT cells and in $Atm^{-/-}$ mice *in vivo* (Cortes et al., 2008).

2.2. Neurodegenerative diseases

One of the most studied neurodegenerative disease is Alzheimer's disease (AD). In this pathology, it is believed that a peptide

Table 1
Applications in neuroscience.

Pathological disturbances	Application	Genes or proteins involved	Mechanism	Ref.
Neurodegenerative disorders	Alzheimer's disease	NMDAR-NR1	Relationship between NMDAR and Aβ oligomers. Silencing of NR1-NMDAR subunit expression through delivery of NR1 antisens sequences	Decker (2010)
		Aβ peptide Tau, Alpha-synuclein,	Vaccination against Aβ peptides to prevent or remove peptide deposition.	Frazer (2008), Peruzzi (2009)
	Parkinson's Disease	TH, GTP-CH-I, AADC, VMAT-2 Hexokinase II	Gene replacement in 6-hydrodopamine-lesioned or rotenone–treated rats	Sun (2004), Corona (2010)
	Narcolepsy	Pre-pro-orexin	Gene replacement in a KO model for orexin	Liu (2008)
Ataxias	Friedreich's ataxia	FRDA locus Frataxin	Gene replacement	Gimenez-Cassina (2011), Cortes (2006, 2008)
	Ataxia telangectasia	ATM cDNA		
Neuroprotection	Different types of neuroprotection	BDNF, NT-3 GDNF, Bcl-2, HSP72 Catalase, Peroxidase	Gene overexpression or replacement in lesioned or drug-treated model animals	Garrido (1998), Bowers (2002), Sun (2005), Arvanian (2006), Hoehn (2001), Zemliak (2006)
Cancer	Glioblastomas	Prodrugs HSV-1 ICP0	Cell toxicity	Rainov (1998), Cuchet (2005), Hoshi (2000), Ho (2010)
		Inhibitors of metalloproteinases	Inhibition of invasive activity	Saydam (2005), Shah (2003, 2005), Ho (2006, 2010)
		EGFR	Gene silencing (RNAi)	
		FasL, FADD, TRAIL	Induction of apoptosis in cancer cells	
	Shwannomas	Caspase-1	Selective apoptosis in cells infected with amplicons expressing the apoptosis-inducing enzyme, caspase-1 (ICE) driven by the Schwann cell-specific promoter P0	Prabhakar (2010)
Behavioral traits. Learning and memory	Inhibitory avoidance.	NMDA-NR1	Inhibition of NR1 subunit expression	Adrover (2003), Cheli (2006)
	Auditory reversal.	PKC beta II	Activation of PKC pathways	Neill (2001)
	Fear conditioning.	GluR1	AMPAR mobilization	
	Social transmission of food preference; Anxiety.	CREB	Manipulation of cAMP function in different regions of the brain	Rumpel (2005)
	Alcoholism.	GABA	Inhibition (iRNA) of GABA expression in the amygdala	Han (2007, 2008), Brightwell (2005, 2008), Barrot (2005), Liu (2011)

known as Aß (amyloid beta), acts as a neurotoxin that produces neurodegeneration. More precisely, a recently enunciated hypothesis states that soluble oligomers of Aß peptide (named ADDLs: Aß-derived diffusible ligands) bind to post-synapses, and that this binding would be responsible for triggering toxic effects that ultimately lead to neuronal death (De Felice et al., 2007; Shankar et al., 2007). Aß peptide is generated by degradation of Amyloid Precursor Protein (APP). Under physiological conditions, APP is first cleaved by an α-secretase, resulting in a non-amyloidogenic soluble peptide. However, under abnormal conditions or by blocking the normal degradation pathway, APP is cleaved by the β-secretase BACE-1, generating an amyloidogenic peptide of 40–42 amino acids (Thinakaran and Koo, 2008). Aß initially aggregates in soluble oligomers of 2–14 monomers (ADDLs), which can bind to the post-synaptic densities from very early stages, and then form the typical amyloid plaques (Haass and Selkoe, 2007; Klein, 2006; Lesne et al., 2006; Roselli et al., 2005; Shankar et al., 2007, 2008).

Two studies describe the use of amplicons for Aß vaccination in mice, as a possible therapeutic strategy for AD, aimed at preventing Aß fibrillogenesis and/or to enhance removal of parenchymal amyloid deposits. In the first study, the amplicons expressed either Aß 1-42 (HSVAß) or Aß 1-42 fused to the molecular adjuvant tetanus toxin Fragment C (HSVAß/TtxFC). Peripheral administration of both vaccines augmented humoral responses to Aß and reduced CNS Aß deposition in transgenic Tg2576 mice. However, HSVAß vaccination was found to be toxic, inducing expression of pro-inflammatory transcripts within the mouse hippocampus (Bowers et al., 2005).

A second amplicon vector was then constructed {HSV(IE)Aß(CMV)IL-4} that co-delivers Aß 1-42 and interleukin 4 (IL-4), a cytokine that promotes the generation of Th2 like T-cell responses. Triple transgenic AD (3XTg-AD) mice, which progressively develop both amyloid and neurofibrillary tangle pathology, were vaccinated with these amplicons. Increased Th2-related Aß-specific antibodies improved learning and memory, while prevention of AD-related amyloid and tau pathological progression were significantly more important in {HSV(IE)Aß(CMV)IL-4}-vaccinated mice than in control experimental groups, underlining the potential of amplicons for Aß immune-therapy of AD (Frazer et al., 2008).

The microtubule-associated protein tau (MAPT) and alpha-synuclein (SNCA) genes play central roles in neurodegenerative disorders. Peruzzi and colleagues recently generated amplicon vectors carrying either the 143-kb MAPT or the 135-kb SNCA locus. They have used these vectors to study regulation of gene expression of both, MAPT and SNCA transgenes, and have demonstrated functional complementation in cultured neurons and organotypic brain slices. They showed that cultured neurons transduced with either amplicon vector expressed the human loci similar to the endogenous gene. In particular, multiple MAPT transcripts were expressed under strict developmental and cell type-specific control. Primary cultures from Mapt−/− embryos had been shown to be resistant to Aβ peptide-induced toxicity suggesting that the tau protein may mediate the neurotoxicity of Aβ fibrils. To test the functionality of the *MAPT* transgene the authors examined whether it could restore the responsiveness to Aβ peptide in the

Mapt−/− neurons and organotypic brain slices. In both preparations from Mapt(−/−) mice, the MAPT vector expressed the tau protein, as detected by ELISA and immune-cytochemistry, and restored sensitivity of Mapt(−/−) neurons to Aß peptide treatment (Peruzzi et al., 2009). As stated by the authors, the faithful retention of gene expression and phenotypic complementation by this system provides a novel and powerful approach to analyze neurological disease genes (see Fig. 1).

Some neurodegenerative pathologies, such as AD or Parkinson's disease (PD), as well as some forms of depression, have been associated to dysfunction of receptor-neurotransmitter systems. L-glutamate is the major excitatory neurotransmitter in the CNS. For this reason, glutamate receptors represent an attractive molecular target in the treatment of these neurodegenerative diseases and also in epilepsy, schizophrenia and ischemia.

There is recent evidence that the transmembrane protein APP appears capable of interacting with N-methyl-D-aspartate receptors (NMDAR) (Cousins et al., 2009; Hoey et al., 2009). These ionotropic glutamate receptors are tetramers made of two NR1 subunits and different NR2 (A-D), and/or NR3 (A-B) subunits, with NR1 being essential for receptor assembly (Paoletti, 2011). Nowadays, association of NMDAR with several neuropathologies has been continuously growing up. Thus, the generation of novel tools that modify expression and structure of NMDARs should help us to understand both the normal functioning and the physiopathology of these receptors. It was proposed that ADDLs binds to NMDAR or to post-synaptic complexes containing it, acting as gain of function ligands (De Felice et al., 2007; Decker et al., 2010; Shankar et al., 2007). By targeting such post-synaptic complexes, ADDLs would activate a cascade of signals that lead to an increase in intracellular reactive oxygen species molecules (ROS) (De Felice et al., 2007). Recently, Decker and colleagues have demonstrated that blockade of NR1 expression through the infection of primary cultures of neurons with amplicon vectors encoding an anti-NR1 antisense RNA (Adrover et al., 2003), inhibited ADDLs binding to synapses (Decker et al., 2010). In the same study, they showed that there was a great reduction in ADDL-instigated ROS formation in neurons in which the expression of NR1 had been knocked down (Decker et al., 2010). Moreover, it has recently been reported that different NR2 subunits would also be involved in the binding of ADDLs to synaptic sites (Liu et al., 2010; Balducci et al., 2010). Liu and colleagues have suggested that increasing activity of NR2A and/or reducing that of NR2B, may alter or reduce the expression of cytotoxic effects mediated by ADDLs in neuronal cultures (Liu et al., 2010). On the other hand, Balducci and colleagues showed that there is an alteration in the trafficking of NR2A and NR2B subunits in mutant mice expressing an amyloidogenic human form of APP (Balducci et al., 2010). However, in the absence of more precise studies supporting a specific interaction between the different subunits of the NMDAR and the Aß peptide, neither in normal nor in pathological conditions, we cannot conclude which could be the specific site for ADDLs binding. It should be taken into account that the decrease in NR1, which is essential for assembly and for the membrane allocation of the receptor, produces a decrease of all the NMDAR subunits at the post-synaptic site (Paoletti, 2011).

Several studies have used amplicons in experimental settings of PD. A typical feature of PD is the progressive loss of dopaminergic neurons in the substantia nigra (SN). During et al. (1994) were the first to report the use of amplicons to deliver human tyrosine hydroxylase (TH) into the partially denervated striatum of 6-hydrodopamine-lesioned rats, used as model of PD. Efficient behavioral and biochemical recovery was maintained for 1 year after gene transfer. Further studies then achieved striatal dopamine level restoration by using complex amplicons expressing TH in combination with aromatic amino acid decarboxylase (AADC) (Sun et al., 2003) or TH in combination with AADC, GTP cyclohydroxyase I (GTP CHI) and vesicular monoamine transporter 2 (VMAT-2) (Sun et al., 2004). In a series of elegant studies, this group further compared the activities of tissue-specific promoters to drive gene expression, particularly the TH, the neurofilament and the vesicular glutamate transport 1 (VGLUT1) promoters (Zhang et al., 2000, 2011; Gao et al., 2007; Cao et al., 2008; Zhang and Geller, 2010).

The effect of amplicon-mediated transduction of the dominant-negative fibroblast growth factor (FGF) receptor 1 mutant protein (FGFR1(TK-)) into the rat SN, was evaluated *in vivo* as a possible strategy to model the reduced FGF signaling already documented to occur in PD. Following intra-nigral delivery of the FGFR1(TK-) expressing amplicon, the number of SN neurons expressing TH was significantly reduced, leading to the conclusion that reduced FGF signaling in the SN of Parkinsonian patients could play a role

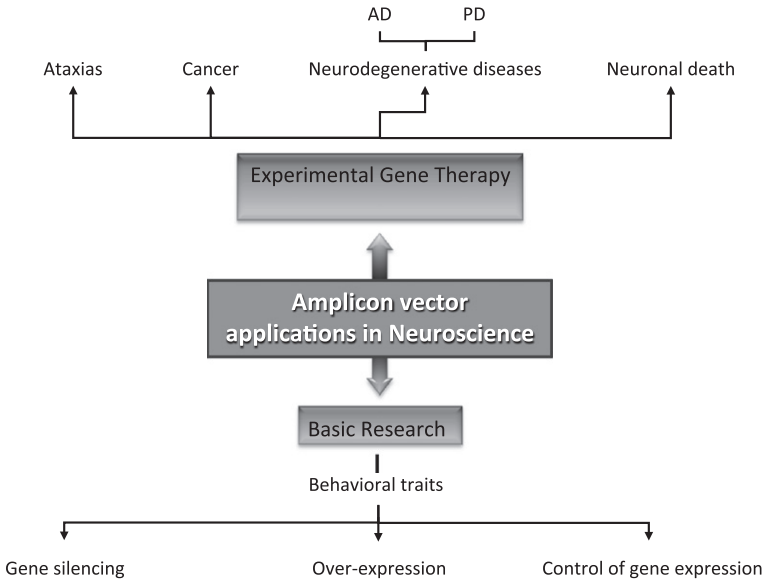

Fig. 1.

in the impaired dopaminergic transmission associated with PD (Corso et al., 2005). A further study from the same group analyzed the effects of *ex vivo* transduction of mesencephalic reaggregates with the anti-apoptotic protein bcl-2 on grafted dopamine neuron survival. Using an amplicon expressing bcl-2 under the control of the TH promoter (HSV-TH9bcl-2) to transduce mesencephalic reaggregates, it was shown that, in spite of the efficiency of the infection, since many cells were effectively transduced, amplicon-mediated overexpression of bcl-2 did not lead to an increase in grafted TH-immune-reactive neuron number (Sortwell et al., 2007).

Mitochondrial alterations are detected in most neurodegenerative disorders and may contribute to the dysfunction and demise of neurons. Rotenone or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridina (MPTP) inhibit the mitochondrial complex I, causing the death of SN dopaminergic neurons, and provide acute models of PD. It has been recently demonstrated that mitochondrial hexokinase II promotes neuronal survival in rotenone treated cells and that this enzyme acts downstream of glycogen synthase kinase-3 (GSK-3), which is considered to be a critical factor in regulating neuronal cell survival and death (Gimenez-Cassina et al., 2009). More recently, the same group generated amplicons expressing hexokinase II and showed that overexpression of this protein in SN of mice, subsequently administered with rotenone or MPTP, prevented neuronal cell death induced by both drugs and reduced the associated motor defects. These results provide the first proof that hexokinase II could protect against dopaminergic neurodegeneration *in vivo* and suggest that increase of hexokinase II expression could represent a promising approach to treat PD (Corona et al., 2010).

Narcolepsy is a neurodegenerative sleep disorder that is linked to the loss of neurons containing the neuropeptide orexin (also known as hypocretin). Liu and collaborators inoculated an amplicon vector expressing pre-pro-orexin into the lateral hypothalamus of orexin KO mice and showed that exogenous expression of orexin significantly improved sleeping in these animals (Liu et al., 2008).

2.3. Neuroprotection and synapse restoration

In several neuropathologies, traumas, or interventions in the brain, neuronal death is a common outcome. Therefore, delivery of transgenes that could prevent cell loss and progression of symptoms, using amplicons expressing neurotrophic and anti-apoptotic factors, or other approaches reducing neurotoxicity, has been widely explored.

Neurotrophins are a family of growth factors that play important roles in the development and maintenance of the nervous system. Amplicons expressing the human brain-derived neurotrophic factor (BDNF) cDNA were used in different studies. BDNF participates in the maturation and function of mammalian auditory neurons, and amplicons expressing this molecule were used to evaluate the feasibility of gene therapy of deafness. These vectors efficiently express BDNF in many cell types, including auditory neurons (Geschwind et al., 1996) and were used in mice to infect damaged spiral ganglion. Four weeks post-infection, stable production of BDNF was observed and supported the survival of auditory neurons by preventing their loss due to trophic factor deprivation-induced apoptosis (Staecker et al., 1998). In a model of dissociated cultures of avian cochlear neurons, the use of amplicons expressing BDNF promoted neuronal survival similar to the maximal level seen by adding exogenous BDNF (Garrido et al., 1998).

The capability of BDNF and of glial cell line-derived neurotrophic factor (GDNF) to protect nigrostriatal neurons was compared in a rat model of PD. According to this study, GDNF was significantly more effective than BDNF for both correcting behavioral deficits and protecting nigrostriatal dopaminergic neurons, and the expression of both neurotrophic factors was no more effective than expressing only GDNF (Sun et al., 2005). In a further study addressing the effect of this trophic factor, it was shown that intracerebral administration of amplicons expressing GDNF, prior occlusion of the middle cerebral artery, displayed neuroprotection of ischemic injury. Treated animals showed reduced motor deficits and, after 1 month, there was a reduction in tissue loss and in Glial Fibrillary Acidic Protein (GFAP) and caspase-3 immune-staining (Harvey et al., 2003).

Amplicons expressing neurotrophin-3 (NT-3) were used in murine cochlear explant models. After infection, the cochlear explants were exposed to cisplatin to induce destruction of hair cells and neurons in the auditory system. This toxicity, defined as ototoxicity, is a major dose-limiting side effect of cisplatin chemotherapy for cancer patients. Amplicon-mediated NT-3 transduction was shown to attenuate the ototoxic action of cisplatin, demonstrating the potency of NT-3 in protecting spiral ganglion neurons from degeneration (Chen et al., 2001). Moreover, amplicon-mediated NT-3 delivery showed similar therapeutic properties *in vivo* in the peripheral auditory system of the aged mouse (Bowers et al., 2002). Therefore, this approach seems to be a promising treatment for prevention of chemical-induced hearing disorders and potentially for hearing degeneration due to normal aging. Also related to NT-3, amplicon vectors expressing NR2D subunit of the NMDAR (HSVnr2d), were used to demonstrate that the combined delivery of NT-3 and NR2D strengthen monosynaptic connections in contused cords and induced the appearance of weak but functional multi-synaptic connections in double hemi-sected cords, while treatment with either NT3 or HSVnr2d alone failed to induce appearance of synaptic responses through the hemi-sected region (Arvanian et al., 2006).

Apoptosis also plays a critical role in many neurological diseases, including stroke, and many studies have shown that expression of bcl-2 using amplicons can protect neurons *in vivo* from adriamycin treatment (Lawrence et al., 1996) or from different ischemic injuries (Antonawich et al., 1999; Lawrence et al., 1997; Linnik et al., 1995; Zhao et al., 2004). Amplicon vectors expressing the inducible heat shock protein HSP72 also can attenuate cerebral ischemic injury, even in post-ischemia situations, when introduced in rat striatum (Hoehn et al., 2001). Moreover, amplicons expressing Hsp72 also protected neurons in CA1 hippocampal region from ischemia; this protection would be mediated, at least in part, by increased expression of bcl-2 (Kelly et al., 2002). Another study used amplicons to overexpress HSP70 in order to protect cultured hippocampal neurons from HIV gp120 induced neurotoxicity (Lim et al., 2003).

Amplicons expressing the rat brain glucose transporter were used to demonstrate that: (i) they can enhance glucose uptake in adult rat hippocampus and in hippocampal cultures (Ho et al., 1993), (ii) such vectors can maintain neuronal metabolism and reduce the extent of neuron loss in cultures after a period of hypoglycemia (Lawrence et al., 1995), and (iii) these vectors protected cultured hippocampal, spinal cord and septal neurons against various necrotic insults, including hypoglycemia, glutamate, and 3-nitropropionic acid (Ho et al., 1995).

Increases in cytoplasmic Ca^{2+} concentration can lead to neurotoxicity and neuronal death. The increase of Ca^{2+} can be induced by neurological trauma associated with aging and some neurological diseases. It was shown, both *in vitro* and *in vivo*, that amplicons expressing the calcium-binding protein calbindin D28 K decreased the neurotoxic impact of Ca^{2+} (Meier et al., 1998; Phillips et al., 1999).

Lastly, generation of reactive oxygen species (ROS) and oxidative damage plays an important role in neuron death, and vectors expressing different antioxidant enzymes were used to counteract

oxidative damages. Amplicons expressing catalase or glutathione peroxidase, two enzymes involved in degradation of hydrogen peroxide, were shown to decrease neurotoxicity induced by different agents in primary cultures of hippocampus or cerebral cortex cells (Wang et al., 2003). A further study using amplicons to express the antioxidant enzyme Cu–Zn–SOD, showed that these vectors were able to protect hippocampal neurons through the induction of glutathione peroxidase, though only in the case of neurons treated with sodium cyanide. The authors pointed out that when neurons were treated with kainic acid, another classical ROS inducer, the effect of the amplicon actually worsen the toxic effects, raising a cautionary note concerning gene therapy against oxidative damages (Zemlyak et al., 2006). Amplicons expressing glutamic acid decarboxylase (GAD67) were able to protect non-differentiated cortical neurons from glutamate toxicity mediated by oxidative stress (Lamigeon et al., 2003).

2.4. Cancer

Amplicons have been widely used to study or to treat experimental cancers, both in brain and in other tissues, using several anticancer strategies. Since these vectors can efficiently deliver genes to cancer cells but are diluted during successive cell divisions, most studies have used acute approaches, like direct cell killing using prodrugs or toxic proteins or induction of apoptosis. In rodent and human glioma cell lines, the fusion protein 4B1:EGFP was expressed from amplicons, in an attempt to combine advantages of expression of the cytochrome P450 4B1, a potent bioactivating "suicide" gene, with the EGFP marker gene. Amplicon-mediated delivery of the fusion protein, which converts cyclophosphamide (CPA) into toxic metabolites, to tumor cells was successfully demonstrated and, in addition, a strong bystander effect, mediated by cell-to-cell contact, was observed (Rainov et al., 1998). Amplicons were also used to transduce both TK and cytosine deaminase, followed by treatment with ganciclovir and 5-fluorocytosine (5-FC), in rat 9L gliosarcoma and in human Gli36 glioma cells (Jacobs et al., 2003).

In order to improve efficiency and safety of cancer gene therapies, efforts at specifically targeting proliferating cells were made in glioma models. The HSV-1 immediate-early protein ICP0 possesses E3-ubiquitin ligase activity (Boutell et al., 2005) and can induce the degradation of centromeric proteins (Lomonte et al., 2001). Amplicons expressing the HSV-1 ICP0 were used to infect human glioblastoma Gli36 cells and well-established models of non-dividing cells, such as primary cultures of either rat cardiomyocytes or brain cells. Results showed that ICP0 induced a strong cytostatic effect and significant cell death in Gli36 cells. In contrast, neither cell death nor any evidence of ICP0-induced toxicity was observed in both primary cultures of non-cycling cells. These observations suggest that ICP0 has gene therapy potential and could be the first member of a new family of cytostatic proteins that could be used to treat cancers (Cuchet et al., 2005).

In order to target the invasive activity of malignant glioma cells, an amplicon vector expressing the tissue inhibitor of metalloproteinase-2 was used. Results suggested that this strategy is potentially useful to treat malignant brain tumors (Hoshi et al., 2000). A different approach used amplicons expressing siRNA in order to mediate post-transcriptional silencing of the epidermal growth factor receptor (EGFR). Infected human glioblastoma cells with knockdown for EGFR expression displayed growth inhibition both in culture and in athymic mice (Saydam et al., 2005).

Another used strategy is to target tumor cells *via* transcriptional control of therapeutic genes. Ho et al. constructed a glioma-specific and cell cycle-regulated amplicon carrying the glial fibrillary acidic protein (GFAP) enhancer/promoter element, plus a cell cycle-specific regulatory element from the cyclin A promoter. Transgenic activity was mediated in a cell type-specific and cell cycle-dependent manner, both *in vitro* and *in vivo* in glioma-bearing animals (Ho et al., 2004). Anti-tumor efficacy of this vector system was assessed using the pro-apoptotic proteins (FasL- and Fas-Associated protein with a Death Domain, FADD), both *in vitro* and *in vivo* (Ho et al., 2006, 2010).

Efficiency of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in Gli36 cells and in subcutaneous glioma was evaluated upon delivery of this molecule using amplicons (Shah et al., 2003). In cultured cells, TRAIL induced apoptosis by 24 h post-infection. In addition, TRAIL-treated gliomas reduced in size over a period of 4 weeks, demonstrating the efficiency of TRAIL delivery by amplicons in tumors *in vivo*. In a similar experiment, expression of a secreted version of TRAIL (S-TRAIL) induced apoptosis in surrounding cells *in vivo*, resulting in a dramatic reduction of glioma size in mouse tumor models *via* a bystander effect (Shah et al., 2005). During these experiments, gene delivery was monitored *in vivo* in real time by dual enzyme substrate (Renilla-luciferase/Firefly-luciferase) imaging. More recently, using an amplicon vector codifying caspase 1 driven by the Schwann cell-specific promoter P0, the same team was able to induce selective apoptosis only affecting the schwannoma cells (Prabhakar et al., 2010). For an exhaustive review of previous work on *in vivo* imaging of amplicon vectors delivery and gene expression in tumor models, please refer to the review by Shah and Breakefield (2006).

2.5. Behavioral traits

Amplicon vectors designed to express or to block expression of neuroreceptor subunits or proteins involved in neuron signaling, have been delivered into distinct brain regions to investigate complex aspects of the normal functioning of the CNS. In this short review we will summarize some examples to illustrate the powerfulness of amplicon vectors to address these questions. For a more comprehensive review of previous works on the use of amplicons to study behavior, see Jerusalinsky and Epstein (2006).

Different challenges to find causal relationships between neuronal molecular mechanisms and learning and memory processes, have been solved by the use of amplicon vectors. These vectors were used, for example, to study the role of NMDAR in learning and memory. In these studies, amplicons were used to investigate the role of hippocampal NMDAR by modifying the expression of the essential NR1 subunit in the rat CNS. The vectors expressed sequences in either sense or antisense orientations of the NR1 subunit gene, in addition to EGFP. The ability to modify endogenous levels of NR1 was first tested in primary cultures of rat embryo neocortical neurons (Adrover et al., 2003; Cheli et al., 2002). Adult rats inoculated into the dorsal hippocampus with vectors expressing NR1 antisense performed significantly worse than control rats in an inhibitory avoidance task, and did not show habituation by repeated exposure to an open field. Immune-histochemistry performed in brain slices from the same animals, showed that the transduced cells represented approximately 6–7% of hippocampal pyramidal neurons in CA1 region (Cheli et al., 2006), indicating that a single gene knockdown of NR1 in a small number of those neurons could significantly impair memory formation.

Amplicons expressing a constitutively active catalytic domain of the rat protein kinase C (PKC) β II were used to transduce hippocampal dentate granule neurons. Activation of PKC pathways in a small percentage of these neurons was sufficient to enhance rat auditory discrimination reversal learning and suggests an hippocampal auditory mediated learning in the rat (Neill et al., 2001).

In order to elucidate the role of the AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid) receptor (AMPAR) in fear conditioning and, more generally, to study molecular, cellular and circuit changes that occur in the brain during learning, Rumpel

et al. (2005) used amplicons expressing the AMPA glutamate receptor subtype 1 (GluR1). This study showed that fear conditioning drives AMPAR into the synapse of a fraction of post-synaptic neurons in the basolateral amygdala. In treated animals, 10–20% reduction in AMPAR synaptic incorporation in the basolateral amygdala provoked an impairment of memories that depend on this structure (Rumpel et al., 2005). Also to investigate the molecular basis of fear conditioning and other behavioral paradigms, several groups have manipulated the function of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), using amplicons encoding the wild type or a dominant-negative form of this protein ($CREB^{S133A}$). In this way, it was shown that changes in CREB function could influence the probability of individual lateral amygdala neurons to be recruited into a fear memory trace, suggesting a competitive model underlying memory formation, in which eligible neurons are selected to participate in a memory trace as a function of their relative CREB activity at learning. Furthermore, Han et al. (2007, 2008) have shown that increasing CREB in the auditory thalamus enhances memory and generalization of auditory conditioned fear, implicating that CREB-mediated plasticity in the thalamus plays a role in this cognitive process. Other study used the same vectors to demonstrate that hippocampal overexpression of a dominant-negative form of CREB can block long-term – though not short-term – memory for a socially transmitted food preference, therefore involving hippocampal CREB function in this type of memory (Brightwell et al., 2005). This team has later shown that, in a task where rats were trained to make a consistent turning response in a water version of the cross maze, long-term memory of a response strategy requires CREB function in the dorsolateral striatum and is independent of CREB function in the dorsal hippocampus (Brightwell et al., 2008). Using a model of protracted social isolation in adult rats, Barrot et al. (2005) observed an increase in anxiety-like behavior and deficits in both the latency of the onset of sexual behavior and the latency to ejaculate. Using transgenic cAMP response element (CRE)-LacZ reporter mice, the authors showed that protracted social isolation also reduced CRE-dependent transcription within the nucleus accumbens (NAc). This decrease in CRE-dependent transcription was mimicked in non-isolated animals by local amplicon-based gene transfer of the dominant negative mutant of CREB. This study suggests a role for the NAc in anxiety responses and in specific aspects of sexual behavior, and provides novel insight into the molecular mechanisms by which social interactions affect brain plasticity and behavior (Barrot et al., 2005).

Finally, Liu and co-workers (2011) recently reported that an amplicon expressing small interfering RNA (siRNA) for the gamma-Aminobutyric acid A ($GABA_A$) receptor α2 subunit, infused into the central nucleus of the amygdala (CeA) of alcohol-preferring rats, (i) caused profound and selective reduction of binge drinking associated with inhibition of α2 subunit expression, (ii) decreased $GABA_A$ receptor density and (iii) inhibited Toll-like receptor 4 (TLR4) expression (Liu et al., 2011). Moreover, infusion of an amplicon expressing TLR4 siRNA into CeA also inhibited binge drinking, but neither vector caused such changes when infused into the ventral pallidum nucleus. On the other hand, binge drinking was effectively inhibited by a $GABA_A$ receptor α1 subunit siRNA expressing amplicon, when infused into the ventral pallidum nucleus, unrelated to TLR4. Those data indicate that $GABA_A$ α2-regulated TLR4 expression in the CeA contributes to binge drinking and may be a key for early neuroadaptation in excessive drinking (Liu et al., 2011).

3. Concluding remarks

Due to their very large transgenic capacity, amplicons are one of the most interesting, versatile, powerful, and promising gene transfer platforms. These vectors are able to deliver many copies of a small transgenic cassette, or a group of genes encoding the full set of proteins required to assemble complex structures, or to deliver one copy of a 150-kb genomic locus, including all exons, introns, and large upstream and downstream regulatory sequences. Due to several outstanding adaptations of HSV-1 to the nervous system environment, amplicons are particularly well suited to deliver genes, both to the CNS and the peripheral nervous system (PNS). In this context, and as described in this short review article, amplicon vectors have been used in several experimental gene therapy settings of neurologic disorders, as well as in basic research in neuroscience, as a new and powerful tool for modifying gene expression.

Several recent technological developments have significantly improved and are extending the use of amplicons to several aspects of neurosciences. We would like to stress in particular the development of different systems to produce vectors devoid of contaminating helper particles (Saeki et al., 2001) or carrying only a very low amount of completely defective and non-pathogenic helper particles (Zaupa et al., 2003). The demonstration that amplicons can be safely used to deliver very large DNA fragments to the nuclear environment of mammalian cells, which appeared an unattainable dream some years ago, is now a reality and many groups today are exploiting this unique property of amplicons in many different experimental settings (Wade-Martins et al., 2001, 2003; Gomez-Sebastian et al., 2007; Gimenez-Cassina et al., 2011). The introduction of the Epstein–Barr virus replicon elements (Wang and Vos, 1996), MARs (Lufino et al., 2007), or HAC (Moralli et al., 2006) sequences into the amplicon genome, to allow autonomous replication and segregation of the vector genome during S-phase, as well as the development of vectors that induce targeted (Wang et al., 2002; Heister et al., 2002; Bakowska et al., 2003) or not-targeted (Bowers et al., 2006) integration into the host chromosomes, have shown that it is possible to avoid dilution of the transgenic cassette delivered by the amplicons to proliferating cells.

We should also stress, however, the limitations of the amplicon vector system that should be resolved before these vectors could be safely and efficiently applied to human beings in gene therapy protocols. The production and purification procedures of amplicon vectors need to be, and actually can be, be further improved. We still do not completely understand the factors that affect control of gene expression, which can result in the silencing of the transgenic cassette delivered by amplicons, although the demonstration that the use of long native DNA regulatory sequences can confer long-term physiological control of expression (Wade-Martins et al., 2003; Gomez-Sebastian et al., 2007) opens a door for the possible resolution of this problem. The systems above described, which have been designed to avoid dilution of the transgenic cassette, with or without integration into host chromosomes, are still imperfect and can certainly be optimized. Several aspects of the biology of amplicons, related in particular to the cellular and host responses against infection or expression of transgenic proteins, are only now beginning to be explored (Olschowka et al., 2003; Suzuki et al., 2006, 2007, 2008; Tsitoura et al., 2009). Research and development on other domains of the amplicon biology or technology are just beginning, including the possibility of engineering the tropism of amplicons or the development of hybrid or combined vector systems that could eventually achieve transport and delivery of the transgenic cassettes to regions of the brain that are difficult to access without surgical intervention.

Amplicon research is quite dynamic and the very large transgenic capacity of these vectors offers unique possibilities for the resolution of many problems that cannot be done with smaller vector systems. Probably the strongest future challenge that will boost amplicon research and development will be the successful

application of these vectors to human beings. At the light of the outstanding progress achieved in the last 10 years, we have few doubts in that such an eventuality should arrive quite soon.

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