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CNS Gene Therapy Applications of the Semliki Forest Virus 1 Vector Are Limited by Neurotoxicity

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The Semliki Forest virus (SFV) 1 vector system is highly efficient at gene transduction in a broad range of host cells, including neurons. To determine the potential of SFV1-based vectors to mediate gene expression in substantia nigra neurons, we inoculated d1EGFP-expressing SFV virus-like particles stereotaxically into the mouse brain. This system selectively and extensively mediated gene expression in dopaminergic neurons of the substantia nigra. Continual reporter gene expression was evident in neuronal cell bodies for up to 3 weeks postinoculation and d1EGFP-positive neuronal processes were apparent for 12 weeks. There was no evidence of an apoptotic response to infection, but with time cell degeneration and an axonopathy, indicative of neuronal loss, were increasingly apparent. This system has potential for experimental studies requiring efficient transient gene transduction of mouse CNS neurons. The current SFV1 vector system is, however, limited in its potential for CNS gene therapy by neurotoxicity.

Key Words: gene therapy, CNS, substantia nigra, alphavirus, Semliki Forest virus, axonopathy

Virus vector-mediated CNS gene therapy has important therapeutic potential in Parkinson disease [1]. Semliki Forest virus (SFV) vectors have been used successfully in gene therapy, vaccination, and recombinant protein production [2–4] and can target CNS cells [5]. The SFV vector system has a broad host cell range and can express heterologous genes of up to 7 kb in length [3]. The current generation of SFV vectors, designated SFV1, is derived from the prototype strain of SFV. Infectious virus derived from the full-length prototype cDNA is known as SFV4. Replication-deficient virus-like particles (VLPs) can be prepared by cotransfecting cells with the pSFV1 plasmid and a plasmid containing the virus structural genes or, for increased biosafety, with two helper plasmids separately encoding the virus capsid and spike genes [6]. VLPs are identical to SFV4 in structure, ability to infect cells, and replication of RNA; however, VLP-infected cells produce no new virus particles. Transcription of the "foreign" gene is under the control of the virus subgenomic promoter and requires transcription and translation of the vector replicase gene.

In continuously cultured eukaryotic cell lines SFV1 vectors are cytopathic; as with infectious virus they induce apoptotic cell death [7,8]. However, the propensity of cells to undergo apoptosis is highly dependent upon their cellular differentiation state, particularly in highly specialized tissues such as the CNS [9]. Whereas the A7(74) strain of SFV induces rapid apoptosis of cells in culture and immature neurons of the mouse brain, it is able to persist in the highly differentiated cells of the adult mouse brain [9,10]. This raises the possibility that the SFV vector system may be able to mediate transient heterologous gene expression within differentiated postmitotic cells in areas of the CNS that are potential targets for gene therapy. Prominent among these areas is the substantia nigra (SN), which is progressively damaged in Parkinson disease. To investigate the ability of the SFV vector system to target neurons of the SN and mediate gene expression in this discrete nucleus, we inoculated SFV1-VLPs encoding the short-half-life (~1 h) green fluorescent protein reporter gene (d1EGFP) stereotaxically into the SN of the mouse brain and determined the course of events over time.

We prepared VLPs using the split-helper system [6]. The replicon-encoding plasmid pSFV1-d1EGFP, along with pSFV-helper-spike and pSFV-helper-capsid, was a kind gift from Professor Peter Liljeström (Microbiology and Tumor Biology Centre, Karolinska Institute, Stockholm, Sweden). We transcribed linearized plasmids and capped them *in*

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vitro and electroporated them into BHK-21 cells using a 140-V square-wave pulse with a pulse length of 25 ms on a Bio-Rad Gene Pulser X cell electroporator. We cultured electroporated cells at 33°C for 48 h prior to clarifying the supernatant by ultracentrifugation through 20% sucrose.

All animal experiments were carried out under the authority of a UK Home Office License and were approved by the University of Edinburgh Ethical Review Committee. We kept all mice under SPF conditions, housed in environmentally enriched boxes, and monitored them regularly. We inoculated groups of four Balb/c mice (10 to 20 weeks of age) stereotaxically into the right SN with 0.3 μl of saline and into the left with 0.3 μl of saline containing 2×10^7 pSFV1-d1EGFP-VLPs. Prior to surgery, we deeply anesthetized the mice with Avertin and placed them on a body temperature regulator. Using a stereotaxic rig, we targeted the SN using the coordinates vertical −4.4 mm, horizontal −3.1 mm, lateral ±1.0 mm, all relative to bregma [11]. We confirmed these coordinates targeted the SN in a series of pilot studies using pontamine sky blue. We delivered all inoculates slowly over a 2-min period; the needle was then left in place for 4 min before being slowly withdrawn. Postoperatively, we administered to the mice physiological saline and an analgesic and allowed them to recover in a thermostatically controlled box. After surgery, all mice remained healthy with no clinical signs of disease. We sampled the mice at 4 or 10 days or at 3, 6, or 12 weeks postinoculation. We removed the brains and immersion-fixed one in 10% neutral buffered formalin and sectioned it transversely at 50 μm using a Vibratome, cryopreserved two in sucrose and cut them into 10-μm frozen sections, and embedded one in paraffin wax and cut it into 5-μm sections.

At day 4, tissue trauma, hemorrhage, vacuolation, and d1EGFP-positive cells were apparent along the needle tract and at its termination in the SN (Fig. 2). Outside the SN, d1EGFP-positive cells were particularly evident where the needle tract crossed the corpus callosum; these cells had a morphology characteristic of oligodendrocytes. SFV is known to infect both oligodendrocytes and neurons

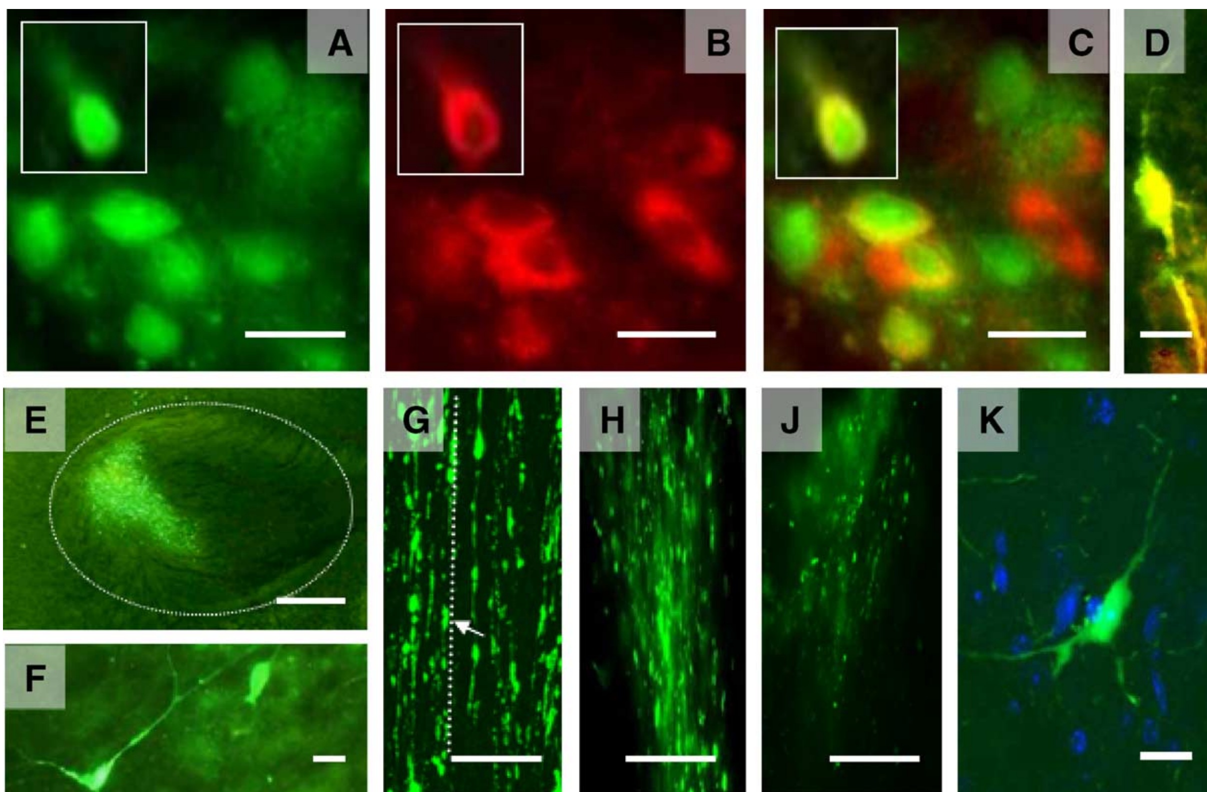

FIG. 1. (A to D) Vibratome sections demonstrating d1EGFP-positive cell bodies (A, green) and staining for tyrosine hydroxylase (B, red). Tyrosine hydroxylase was detected by staining free-floating 50-μm Vibratome sections with a rabbit anti-tyrosine hydroxylase followed by an Alexa Fluor-594-conjugated goat anti-rabbit antibody. (C and D) Double-labeled (yellow) cell bodies and processes, confirming infection of dopaminergic neurons in the SN. (E to J) Vibratome sections demonstrating time course of d1EGFP expression in the SN. (E) Focal area of d1EGFP expression (green) in the SN (dotted ellipse in E) at day 4 (a similar distribution was observed at day 10). (F) d1EGFP-positive cell body and axons at 4 days. (G, H, and J) d1EGFP expression in axons at 3, 6, and 12 weeks, respectively, demonstrating the reduction in d1EGFP signal over time. Note axons in these images run in bundles as depicted by the dotted line (G). In each case the axons are interrupted by d1EGFP-positive swellings (arrow in G). (K) Confocal microscope image showing a d1EGFP-positive cell body at 3 weeks. The cell has d1EGFP-positive axons with a normal morphology. Nuclei are stained with TO-PRO 3 (blue). Bar in E, 500 μm; all other bars, 20 μm.

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[12,13]. At days 4 and 10, we observed clear, large, and discrete areas of d1EGFP-positive cells in the SN (Fig. 1). We immunostained Vibratome and cryostat sections for tyrosine hydroxylase (TH), a marker of dopaminergic neurons in the SN. This demonstrated many double-labeled cell bodies and confirmed the correct targeting of the inoculation (Fig. 1). As expected, some TH-negative cells, presumably other types of neuron or oligodendrocytes, were also d1EGFP-positive. In all mice at these time points, the percentage of cells positive for both d1EGFP and TH varied with distance from the inoculation site and reached a maximum of approximately 50%. Localization of d1EGFP was evident in the nucleus, as well as in the cytoplasm and at day 10 in the processes, whereas tyrosine hydroxylase was exclusively located in the cytoplasm (Fig. 1). These results demonstrate the capacity of SFV1 VLPs to infect and mediate foreign gene expression in dopaminergic neurons of the mouse brain, allowing the possibility of gene therapy of these neurons by this vector. SFV vectors have previously been shown to direct gene expression to neurons in rat hippocampal slice cultures [14–16] and stereotaxic inoculation of rats with a different (SFV-PD) EGFP-expressing vector also resulted in rapid EGFP expression in neurons of the SN [17]. In mice, expression of β-galactosidase by Sindbis virus vectors has been observed to be neuron specific [18].

At 4 and 10 days, an inflammatory response was present in the VLP-inoculated SN. This was characterized by perivascular cuffing, infiltrating mononuclear cells, and many cells with the morphology of activated microglia/

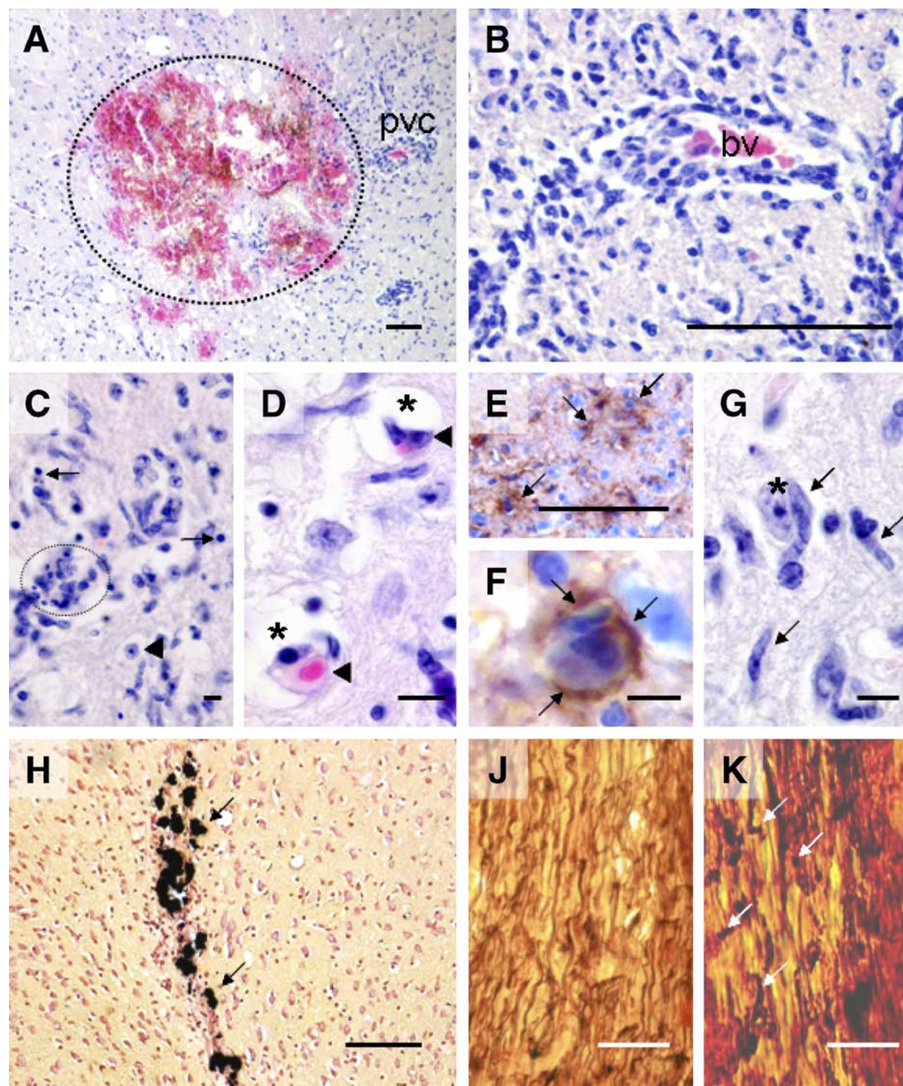

FIG. 2. (A to D) 5-μm paraffin-processed sections stained with hematoxylin and eosin. (A) Tissue trauma, hemorrhage (red), vacuolation, and inflammation at the site of inoculation in the VLP-inoculated SN (dotted ellipse). A perivascular cuff (pvc) indicates an active inflammatory response, day 4. (B) A higher power perivascular cuff showing many infiltrating mononuclear cells (arrows) spreading out from the blood vessel (bv) into the surrounding tissue, day 4. (C) Areas of vacuolation (e.g., arrowhead) with several abnormal cellular profiles, condensed nuclear material (circled area), and pyknotic nuclei (arrows), all consistent with neuronal degeneration; SN, day 10. (D) Areas of vacuolation (*) containing abnormal cell profiles of putative degenerating neurons (arrowheads); a pyknotic nucleus can also be observed (arrow); SN at 3 weeks. (E and F) Focal F4/80-positive (brown) microglia/macrophages (arrows) surrounding degenerating cells in the VLP-inoculated SN; 3 weeks postinoculation; 10-μm cryostat sections counterstained with hematoxylin. (G) Cells with elongated nuclei characteristic of activated microglia/macrophages adjacent to a cell with a large nucleus (asterisk); 3 weeks, 5-μm paraffin-processed section stained with hematoxylin and eosin. (H) Lesion in SN at 12 weeks postinoculation showing black calcification (arrows) by Von Kossa silver nitrate stain; counterstained with neutral red, 10-μm cryostat section. (J and K) Silver staining of axonal tracts in deep mesencephalic nuclei, 6 weeks postinfection. (J) Contiguous, unbroken, silver-stained (dark brown/black) axonal processes extend down the entire field of view on the control, saline-inoculated side of the brain. (K) A bundle of axons from the VLP-inoculated side of the same brain. These processes are not contiguous but are disrupted by bulbous swellings (arrows) indicative of an axonopathy. These axons were d1EGFP-positive (cf., Fig. 1H) prior to silver staining; the EGFP signal was obscured by the silver stain. Bars in A, B, E, and H, 100 μm; all other bars, 10 μm.

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macrophages that stained positive for the F4/80 marker characteristic of these cells. Serum antiviral antibodies were detectable by ELISA (data not shown) in all mice at day 4 and at subsequent time points. Following intraperitoneal inoculation, SFV gains access to the brain by 2 days and CNS inflammatory responses and serum antibodies are apparent from 4 days [19]. The parallel temporal course of events indicates that stereotaxic brain inoculation, as with peripheral inoculation, results in rapid priming of immune responses. We also observed an inflammatory response following stereotaxic inoculation of the SFV(PD) vector into the SN of the rat brain [17] and this is observed with other virus vectors such as adenoviruses [20]. Priming of peripheral immune responses following stereotaxic inoculation into the brain appears to be dose related and in some cases occurs only weeks after inoculation [21].

By day 10, as characterized by cells with abnormal, swollen, or condensed cytoplasmic and nuclear profiles (Fig. 2), we observed degenerating cells in the SFV1 VLP-inoculated SN (Table 1). At 3 weeks, d1EGFP-positive cell bodies remained detectable in the SN. As at 4 and 10 days, the majority of these were TH-positive; however, numbers were reduced relative to the earlier time points (Table 1). This relatively prolonged (>3 weeks) d1EGFP expression is in contrast to expression in rat SN cells mediated by the SFV-PD vector, in which expression in TH-positive cells diminished sometime between 1 and 11 days [17]. At 3 weeks, as at 10 days, throughout the VLP-inoculated SN, we observed scattered vacuoles and degenerating cells; in some cases putative degenerating neurons were surrounded by F4/80-positive activated microglia/macrophages (neuronophagia, Fig. 2). We observed no comparable lesions on the control saline-inoculated side of these brains. TUNEL staining and activated caspase-3 staining [12] of six 50-μm Vibratome sections spanning the inoculation site from brains at 4 days, 10 days, and 3 weeks, and serial cryostat sections cut throughout the entire rostrocaudal length of the SN of two mouse brains with remaining EGFP-positive cells at 3 weeks postinfection, did not demonstrate any apoptotic neurons. No d1EGFP-positive cell bodies remained at 6 or 12 weeks (Table 1), but we did observe degenerating cells, cell debris surrounded by F4/80-positive cells, small accumulations of F4/80-positive cells, glial scars, and areas of calcification in the VLP-inoculated SN (Fig. 2).

At 3 weeks, many d1EGFP-positive cells had fluorescent axons (Fig. 1, Table 1). Bundles of fluorescent axons with focal fluorescent swellings were readily observed both in the SN and in other brain regions (Fig. 1). Fewer bundles of d1EGFP-positive axons were present at 6 weeks and these were rare by 12 weeks (Table 1). The d1EGFP-positive neurite swellings suggested an axonopathy and resembled the segmentation of axons into linear rows of granules observed in Wallerian degeneration [22]. To investigate this further, we silver stained cryostat brain sections to visualize axons using a modification of the technique of Bielschowsky [23]. An axonopathy was clearly present in selected tracts on the VLP-inoculated side of the brain starting at 3 weeks and was increased at 6 and 12 weeks (Table 1, Fig. 2). We observed no axonal degeneration in corresponding tracts on the contralateral side. Unfortunately, the sliver deposits obscured the d1EGFP signal; however, examination of adjacent sections indicated that tracts with axonal degeneration did have d1EGFP-positive axons with swellings. We observed degenerating axons up to 12 weeks postinoculation, after the presence of d1EGFP positive cell bodies. A similar slow Wallerian degeneration is observed in the *Wld^s* mouse model, in which distal portions of transected *Wld^s* axons remain viable for up to 3 weeks [24]. At 6 and 12 weeks, the degree of axonopathy was inversely proportional to the number of d1EGFP axons remaining.

Whereas it is difficult to quantify neuronal loss, taken together, the degenerating SN cell bodies, the neuronophagia, and the axonal degeneration are indicative of death of infected (d1EGFP-positive) neurons. This is likely to be a necrotic death since no TUNEL-positive nuclei or

TABLE 1: Time course of d1EGFP expression and pathological changes following stereotaxic inoculation of SFV1-d1EGFP-VLPs into the substantia nigra

	d1EGFP		Pathology	
PID	Cell bodies	Axons	Degenerating SN cells	Axonopathy
4 days	++++	−	−	−
10 days	+++	+	+	−
3 weeks	+	+++	++	++
6 weeks	−	++	++	+++
12 weeks	−	+	−	++++

Four mice were stereotaxically inoculated into the right substantia nigra (SN) with 0.3 μl of physiological saline and into the left with physiological saline containing 2×10^7 pSFV1-d1EGFP-VLPs. Mice were sampled at 4 or 10 days or at 3, 6, or 12 weeks postinoculation. For three mice, a series of transverse sections (50 μm Vibratome or 10 μm cryostat) through the SN were screened for the presence of d1EGFP; for each section with d1EGFP-positive signal a score from − to ++++ was given for the extent of positive signal in cell bodies and axons. Sections throughout the area of d1EGFP positivity were stained with hematoxylin and eosin to assess cell morphological changes or with silver nitrate to assess axonopathy. Scoring was again on a scale from − to ++++. In the SN, cells with an abnormal profile that were swollen or condensed or that were highly eosinophilic were scored as degenerating. Broken, interrupted, or swollen axonal processes were considered indicative of an axonopathy. The mean scores for all d1EGFP-positive sections for each of three mice at each time point are shown. The brain from the fourth mouse sampled at each time point was processed for paraffin histology. This destroys the EGFP signal but allows greater resolution of the neuropathology. Sections from these brains also demonstrated degenerating cells in the SN (Fig. 2).

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activated caspase-3-positive cell bodies were observed. EGFP has been much used as a reporter gene and has only rarely been implicated as being cytotoxic [25]. Studies in the mouse brain, at least in the context of other vectors, indicate that EGFP expression is not toxic to neurons [26,27]. It is most likely therefore that the neuronal toxicity observed is attributable to the SFV vector itself. The SFV4 virus and the derived SFV1 vector system both induce rapid apoptotic cell death in a number of cell types *in vitro* [7,8]. The present study demonstrates that postmitotic neurons of the CNS also undergo cell death when replicating the SFV1 vector; however, this death is necrotic and considerably delayed relative to that observed in cultured neurons [9]. That this death is necrotic is consistent with our earlier proposal that postmitotic neurons do not readily undergo apoptosis upon virus infection [9]. d1EGFP has a short half-life and fluorescence is therefore indicative of active synthesis of this reporter protein and an indication of continual basal cell functions; however, this does not preclude the possibility that these CNS neurons are compromised in their differentiated functions at some point before cell death. In summary, SFV1 VLPs can target, replicate in, and mediate heterologous gene expression for as long as 3 weeks in SN neurons but infection also initiates neuronal damage, as evidenced by cell degeneration, neuronophagia, and axonopathy, which progress to neuronal loss.

The ability of SFV1 vectors to target neurons of the SN, the absence of an apoptotic response, and the relatively sustained neuronal transgene expression do show some promise for gene therapy and may have applicability in experimental studies. Studies in the rodent CNS do not always accurately predict events in humans; however, the cytotoxicity of the current SFV1 vector, as with the SFV-PD vector [17], mandates caution in consideration of the current generation of SFV vectors for replacement gene therapy in humans. In contrast, exploiting the cytotoxicity of the current SFV vector systems for rapidly dividing tumor cells has been advocated for human tumor therapy, including CNS tumors, and studies in experimental model systems are encouraging [28,29]. If SFV vectors are to be useful for long-term replacement gene therapy, as with adenoviruses [30], development of less cytopathic systems, perhaps based on the A7(74) strain of SFV [16], is necessary.

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