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Direct gene transfer in the Göttingen minipig CNS using stereotaxic lentiviral microinjections

Andreas Nørgaard Glud[1*,#], Claus Hedegaard[2,#], Mette Slot Nielsen[1], Jens Christian Sørensen[3], Christian Bendixen[2], Poul Henning Jensen[4], Knud Larsen[2], and Carsten Reidies Bjarkam[1,3]

[1]Institute of Anatomy, Faculty of Health Sciences, University of Aarhus, DK-8000 Aarhus C, Denmark, *Email: angl@ana.au.dk; [2]Department of Genetics and Biotechnology, Faculty of Agricultural Sciences, University of Aarhus, DK-8830 Tjele, Denmark; [3]Department of Neurosurgery, Aarhus University Hospital, DK-8000 Aarhus C, Denmark; [4]Institute of Medical Biochemistry, Faculty of Health Sciences, University of Aarhus, DK-8000 Aarhus C, Denmark, [#] Both authors contribute equally.

We aim to induce direct viral mediated gene transfer in the substantia nigra (SN) of the Göttingen minipig using MRI guided stereotaxic injections of lentiviral vectors encoding enhanced green fluorescent protein (EGFP). Nine female Göttingen minipigs were injected unilaterally into the SN with 6×2.5 µl lentivirus capable of transducing cells and mediating expression of recombinant EGFP. The animals were euthanized after four (n=3) or twenty weeks (n=6). Fresh brain tissue from three animals was used for PCR. The remaining six brains were cryo- or paraffin-sectioned for fluorescence, Nissl-, and immunohistochemical EGFP visualization. EGFP was seen in nigral neurons, axons, glial cells, endothelial cells, and in nigral fibers targeting the striatum. PCR-based detection confirmed the presence of the transgene in SN, whereas all other examined brain areas were negative, indicating that the immunohistochemically detected EGFP in the striatum derived from transfected nigral cells.

Key words: animal model, gene therapy, substantia nigra, *Sus scrofa*, transgenesis

INTRODUCTION

During the last few years, the use of viral vectors to model central nervous system (CNS) diseases has been a rapidly emerging field (Kirik et al. 2003, Carlsson et al. 2005, Maingay et al. 2005, Popovic et al. 2005). Viral vectors such as adenovirus, adeno-associated virus, herpesvirus, and lentivirus have been modified to offer safe, specific gene transfer and expression in the target region of interest. This has led to the development of several new animal models, aimed at studying and potentially treating human CNS diseases. Thus, expression of a certain gene product in neurons has been performed by use of viral vectors in different species such as *Strongyloides stercoralis* (Junio et al. 2008), quail (Scott et al. 2005), mouse (Palella et al. 1989), rat (Huang et al. 1992), rhesus monkey (Doran et al. 1994), baboon (Goodman et al. 1996) and St. Kitts green monkey (Bohn et al. 1999). Recombinant lentivirus is known to function as a transgene delivery system in domestic pigs (Hofmann et al. 2003) and pseudotyped with vesicular stomatitis virus G-protein (VSV-G) lentiviral vectors have been demonstrated to efficiently transduce both glial and neuronal cells (Jakobsson et al. 2003). Furthermore adeno-associated viral vectors have been injected into CNS of the newborn piglet (Kornum et al. 2010). In this study, we performed injections of lentiviral vectors encoding enhanced green fluorescent protein (EGFP) in the substantia nigra of the adult Göttingen minipig. The large gyrated brain of the pig enables identification of cortical and subcortical structures by conventional magnetic resonance imaging (MRI) and direct use and testing of clinical neurosurgical equipment, including MRI guided stereotaxic procedures (Sørensen et al. 2000, Bjarkam et al. 2004, 2008, Dalmose et al. 2004, 2005, Larsen et al. 2004, Lind et al. 2007).

Correspondence should be addressed to A. N. Glud
Email: angl@ana.au.dk

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Additionally, the pig genome resembles that of primates more than mice (Wernersson et al. 2005). Furthermore the SN of the Göttingen minipig is well described (Nielsen et al. 2009) and the pig is easily trained to accept neurological testing and behavioral analysis (Mikkelsen et al. 1999). The use of minipigs as opposed to larger domestic pig breeds enables easy handling even in chronic studies, as the Göttingen minipig retains its small size (30-40 kg) (Lind et al. 2007), and some of the ethical and economical considerations concerned with the use of primates in research may potentially be avoided by the use of pigs (Goodman and Check 2002). Establishment of a consistent and reproducible model for direct viral mediated gene transfer in the Göttingen minipig would hence potentially play a central role in further brain research and benefit CNS disciplines addressing Parkinson's and Alzheimer's disease.

METHODS

The viral system

The lentivirus system was developed in the laboratory of Professor Didier Trono, Lausanne. The recombinant viral system consisted of three dsDNA plasmids: a packaging vector, pBR8.91, harbouring genes responsible for virus matrix protein and enzyme expression; an envelope vector, pMDG, holding the VSV-G gene encoding the major viral envelope protein; and a transfer vector, pWPXL, carrying the transgene encoding EGFP. The transgene was flanked by Long Terminal Repeats (LTRs) of HIV-1 origin that mediated genomic integration facilitating stable, long-term transgene expression driven by the human elongation factor Iα promoter (Fig. 1). Only genetic segments within the LTRs were transferred to the virus particles, hence packaging and envelope genes were excluded resulting in replication deficient particles crucial for the biosafety of the system.

Figure 1: Schematic drawing of the transfer vector harboring the Enhanced Green Fluorescent Protein (EGFP) transgene used for direct viral mediated gene transfer. The transgene is flanked by Long Terminal Repeats (LTRs) and its expression driven by the human Elongation Factor 1-α promoter (EF1-a). The cPPT (central poly-purine tract signal) and WPRE (Woodchuck hepatitis virus post-transcriptional regulatory element) sequences enhance transgene expression.

Belonging to the group of retro viruses, lentivirus is a ssRNA type that is reversely transcribed upon infection of the host cell and before genomic incorporation.

Viral vector production and titer determination

Lentivirus particles were produced by triple transfection of human embryonic kidney cells (HEK 293T) with pBR8.91, pMDG, and pWPXL. Cells were grown in 10 ml Dulbeccos Modified Eagles Medium glutamax (Invitrogen, Carlsbad, CA) with 100 U/ml penicillin, 100 µg/ml streptomycin, 5 mM Hepes, and 10% foetal calf serum. Cells were seeded at a number of 2.2×10^6 per culture dish the day before transfection with 150 µg pBR8.91, 50 µg pMDG, and 200 µg pWPXL. Sterile water containing 2.5 mM Hepes was added to a volume of 2.5 ml followed by addition of 2.5 ml 0.5 M $CaCl_2$. This mixture was subsequently added dropwise into 5 ml of HeBS (Hepes Buffered Saline Solution) during thorough agitation. After a resting period of 25 min at room temperature, 1 ml of the final calcium phosphate-plasmid transfection precipitate was distributed into each of the HEK 293T containing dishes. The next day, the culture medium was discarded and substituted by fresh medium. On the following two days, the medium was collected and centrifuged for 10 min at 2 500 rpm and 4ºC for removal of cell remnants, and stored at 4ºC. The harvested medium was pooled, filtered at 0.45 µm, and ultra centrifuged for 90 min at 80 000 g. The pellet was re-suspended in medium before repetition of centrifugation. Finally, after decantation of the supernatant, the virus particle-containing pellet was re-suspended in 200 µl culture medium, aliquoted, and stored at -80ºC. For titer determination of the virus preparation, 10^5 HEK 293T cells per well were seeded in a six well plate. After 18 hours of cultivation, a serial dilution of the virus stock was added to transduce the cells. After 48 hours, GFP positive cells were counted and the ratio between positive and negative cells calculated. The dilution resulting in approximately 10% positive cells was used for calculation of the titer to 3.3×10^8 transducing units/ml.

Stereotaxic implantation

Nine female Göttingen minipigs aged 8-12 months, weighing 20-30 kg, were used in this study approved by the Danish National Council of Animal Research Ethics. The animals were deeply anesthetized before the head

was fixed in a stereotaxic localizer box (Mark 2.5 with TSE parallel rail and micromanipulator assembly, Neurologic, Denmark) (Bjarkam et al. 2005). A MRI-visible fiducial marker was placed in the bregma and each animal MRI-scanned enabling coordinate calculation of the SN at six different positions in relation to the fiducial marker. The anaesthetized animals were then transported to a class II virus facility, where the localizer box was converted to a stereotaxic device by addition of a stereotaxic frame with attached TSE-micromanipulator. A 10 µl Hamilton syringe with attached borosilicate-glass needle was filled with a dilute lentiviral preparation of 6.6×10^{7} TU/µl and placed on the micromanipulator. Through a burr hole in the skull and dural incision, six 2.5 µl unilateral injections in the SN, yielding a total lentiviral dose of 9.9×10^{8} TU, were carefully made at the pre-determined coordinates, e.g., two injections separated by 2 mm in the coronal plane at three different rostro-caudal levels. After suturing the skin, the animals were placed in a quarantine stable for 72 hours. Postoperative antibiotics and analgetics were administrated for 3 and 2 days, respectively.

Euthanasia and collection of brain tissue

Four weeks (3 animals) or four months (6 animals) postoperatively, the animals were euthanized with pentobarbital. A total of 3 brains (1 animal from the four week group and 2 animals from the 4 month group) were freshly removed for PCR analyses, with bilateral sampling from the SN, striatum, frontal lobe and the hypothalamus. The remaining 6 pigs (2 animals from the four week group, and 4 animals from the 4 month group) were transcardially perfused with 5 l phosphate buffered 4% paraformaldehyde (pH 7.4). The brains were removed and immersed in the fixative for 24 hours. The brains were then sectioned into five 1-cm-thick coronal brain slabs and immersed in 30% sucrose for 3-4 days, before freezing with dry ice and cryostat sectioning into 40-µm-thick coronal sections, or paraffin embedded and sectioned into 30 µm coronal sections.

Nissl staining and immunohistochemistry

Tissue staining was performed after previously established principles (Larsen et al. 2004). Sections for immunohistochemistry were, accordingly, incubated for 72 hr with a primary polyclonal rabbit anti-GFP (ab290, Abcam Ltd., Cambridge, UK) diluted 1:1 000 followed by incubation for 1 hour with the secondary anti-rabbit IgG biotinylated antibody (RPN1004, Amersham, Buckinghamshire, UK) dilut-

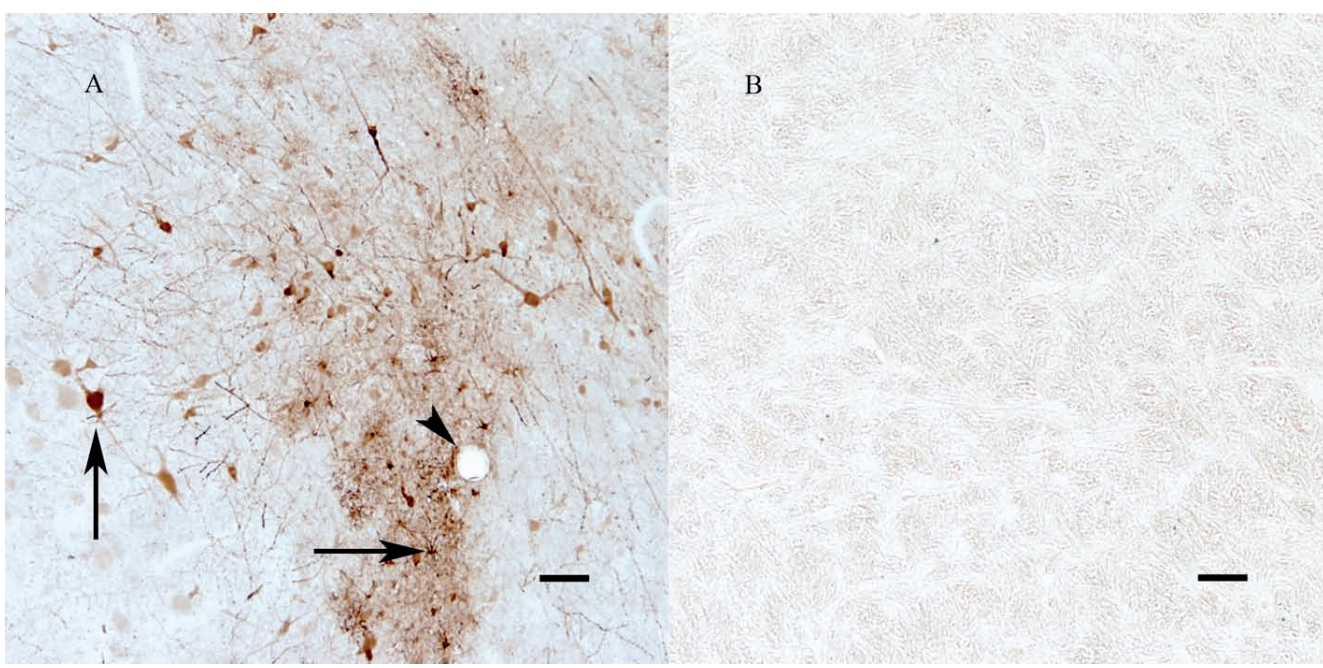

Figure 2: (A) Photograph of immunohistochemical anti-EGFP stained section demonstrating EGFP in both neurons and glial cells. Arrowhead: Injection trajectory from the tip of the glass needle. Horizontal arrow: microglia. Vertical arrow: neuron. (B) Negative control made by omitting the primary antibody. Scale bar 10 µm.

ed 1:400. Sections for flourescence immunohistochemistry against GFP, were incubated for 24 hour with primary polygonal rabbit anti-GFP (ab290, Abcam Ltd., Cambridge, UK) diluted 1:1000 and with monoclonal mouse anti NeuN (Millipore Billerica, MA, USA MAB377) diluted 1:500 or monoclonal mouse anti-GFAP (ab4648, Abcam Ltd., Cambridge, UK) diluted 1:500. The secondary antibodies were respectively Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 goat anti-mouse IgG, both diluted 1:1000 (Invitrogene, Life technologies, USA).

Negative control was performed by omitting the primary antibody from the staining protocol, resulting in no staining.

Purification of DNA from tissue samples

DNA was purified from fresh, snap-frozen brain tissue. Approximately 20 mg of sample was suspended in 1200 µl lysis-buffer and incubated overnight at 55ºC. Subsequently, 180 µl saturated NaCl was added, the sample mixed and centrifuged at room temperature for 15 min at 11,000 rpm. From the supernatant, 400 µl was withdrawn, 1 ml 96% ethanol added, and the sample mixed until a DNA precipitate was visually observed. After centrifugation for 10 min at 11 000 rpm, the DNA precipitate was washed with 200 µl 70% ethanol, isolated by centrifugation, and dried for 15 min at 37ºC. The recovered DNA was re-dissolved

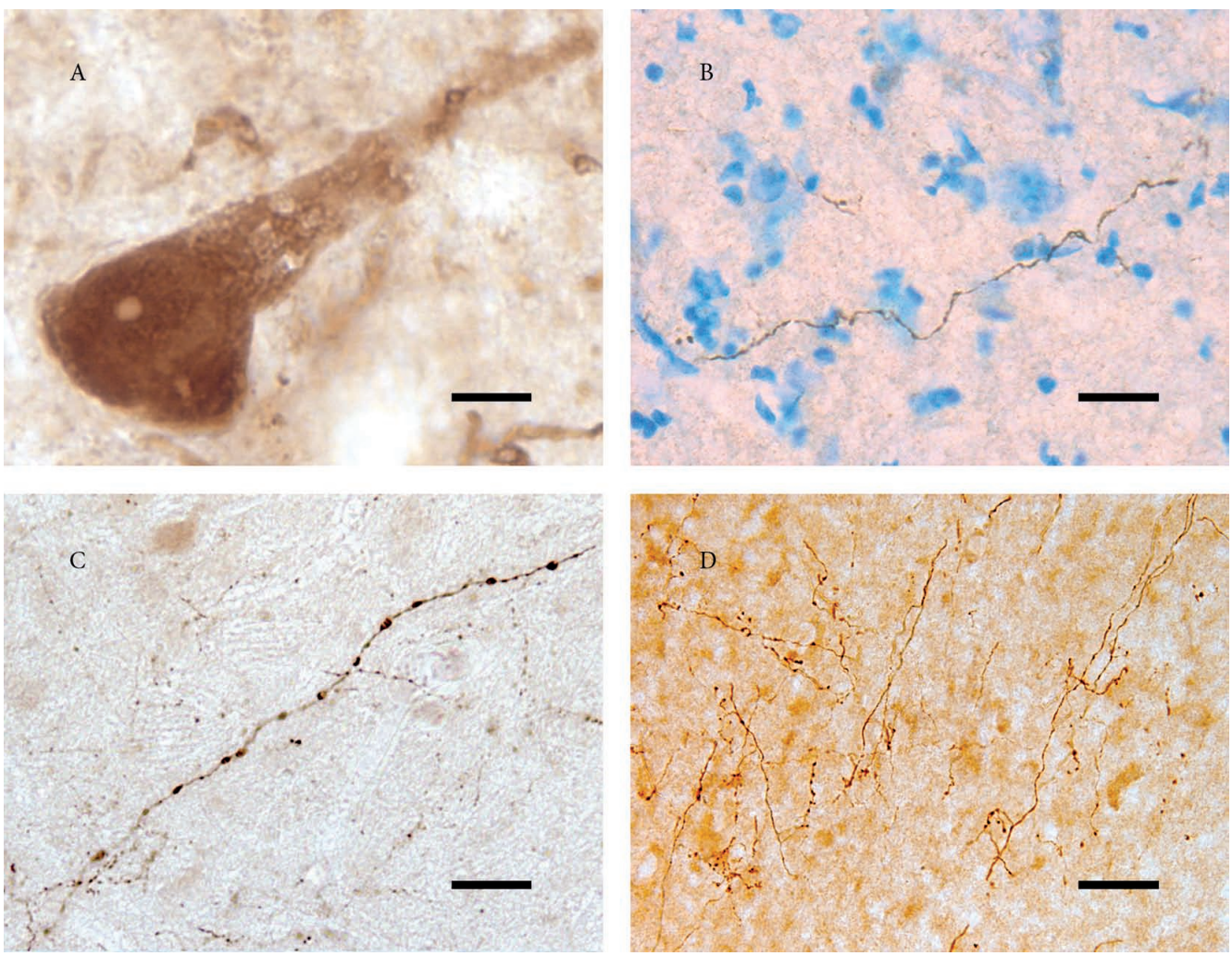

Figure 3: Photographs of immunohistochemical anti-EGFP (brown) and Nissl (blue) stained sections displaying a EGFP-positive SN neuron (A), EGFP-positive fibers loosely distributed in the medial forebrain bundle (B) and more compactly in the internal capsule (C), on their way to the striatum which bilaterally displays numerous EGFP-positive varicose fibers terminating around unstained striatal cell bodies (D). Scale bar 10 µm (A), 25 µm (BC) and 50 µm (D).

in 10 mM Tris buffer pH 8.0 containing 1 mM EDTA at 55ºC for 30 min. The DNA concentration was spectrofotometrically determined and its integrity investigated by 1% agarose gel electrophoresis.

PCR-based analyses

The purified DNA was tested as template (~50 ng) for PCRs by amplification of a 621 bp porcine elongation factor Iα promoter fragment using the oligo nucleotide primers porEF-FW, 5'-TAGCCAAGAGGTCAGCGCA-3' and porEF-RV 5'-GTTTACCCCGCGCCACCT-3' (DNA Technology, Aarhus, Denmark). The AmpliTaq Gold DNA polymerase with standard buffer (Applied Biosystems, Denmark) was applied under the thermal conditions: 94ºC for 12 min; 35 cycles of 94ºC for 15 sec, 61ºC for 15 sec, and 72ºC for 15 sec; 72ºC for 7 min; 4ºC. The samples were subsequently tested for the presence of the EGFP gene using the primers EGFP-FW 5'-GCAAGGGCGAGGAGCTGT-3' and EGFP-RV 5'-CGAGATCTGAGTCCGGACT-3' (DNA Technology, Aarhus, Denmark) amplifying 727 of the 738 bp constituting the EGFP coding sequence in pWPXL. The DynaZyme DNA polymerase with standard buffer (Finnzymes, Espoo, Finland) was applied under the thermal conditions: 94ºC for 4 min; 50 cycles of 94ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 40 sec; 72ºC for 5 min; 4ºC. Amplicons were identified by ethidium bromide staining and 1 or 2% agarose gel electrophoresis.

RESULTS

Behavior

The animals showed no behavioral changes during the four-week to four months follow-up period, except for mild transient rotational behavior of one animal the first day after surgery.

Histology

In the six transcardially perfused animals, parallel needle trajectories targeting the SN without accompanying infection or hemorrhage were clearly visible (Fig. 2A). EGFP-fluorescence (Fig. 4) and a positive immunohistochemical reaction were very dominant in the distal part of the needle trajectory, and faded and narrowed along the proximal part of the trajectory (Fig. 2A). In the SN, EGFP-positive staining was observed in both neurons (Figs. 2A, 3A, 4E-G), glial (Figs. 2A, 4A-C) and endothelial cells immediately

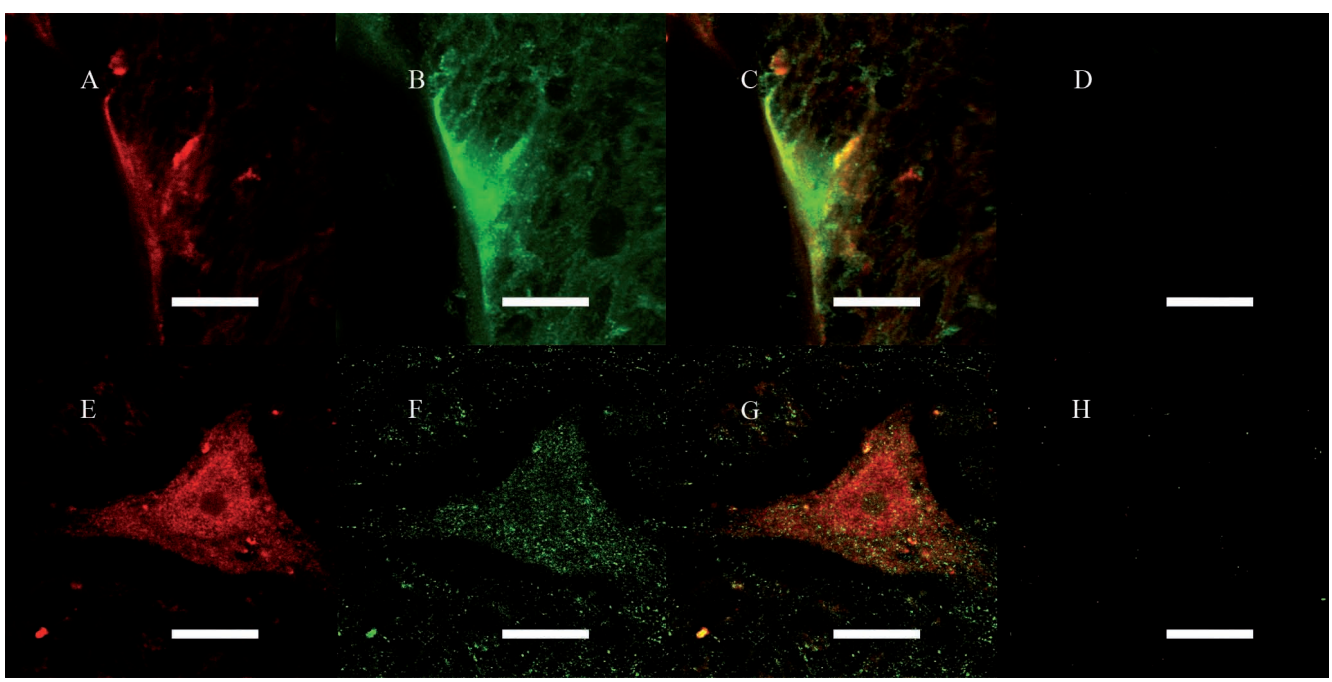

Figure 4: Confocal microscopy photographs of tranfected cells. (A) GFAP positive glial cell. (B) EGFP positive glial cell. (C) Merged BC. (D) Negative control. (E) NeuN positive neuron. (F) EGFP positive neuron. (G) Merged EF. (H) Negative control. Scale bar A,B,C,E,F and G 15 µm. Scale bar D and H 50 µm.

surrounding the distal part of the trajectory. In neurons EGFP especially localized to the nucleus, the soma and their branches (Fig. 3A). Outside the boundaries of the SN it was possible to follow EGFP-positive fibers running loosely through the medial forebrain bundle (Fig. 3B) and in more compact bundles through the internal capsule (Fig. 3C) towards the striatum, which bilaterally contained numerous EGFP-positive varicose fibers (Fig. 3D). No staining of neuronal or glial cell bodies was noted outside the right SN or the distal part of the injection trajectory (Fig. 3B-D).

PCR

The endogenous elongation factor 1α promoter fragment serving as control of the quality of the DNA preparations could be amplified from all samples obtained from the three animals reserved for PCR analysis (Fig. 5). Samples from the striatum, pons, and medulla oblongata on the injected side along with bilateral samples from rostral and caudal levels of the SN were tested for the presence of the EGFP gene. The rostral SN on the injected side was positive, whereas the rest of the samples were negative (Fig. 6).

DISCUSSION

We injected lentiviral vectors encoding EGFP in the SN of Göttingen minipigs (Figs. 2A, 3A, 4) and achieved cell transduction at the injection site with subsequent expression of EGFP not only in nigral neurons but also in their striatal projections (Figs. 2-4). We also demonstrated that injection of a rather large volume (6×2.5 µl) unilaterally in the SN is well tolerated without causing severe adverse behavioral or histopathological effects (Figs. 2-4).

Various methods for transferring a gene into the CNS and thereby obtaining expression of a specific gene product have been described; such as inserting vector-infected grafting cells (Kim et al. 2006, Yasuhara et al. 2006, O'Keeffe et al. 2008), inserting capsules containing genetically modified cells (Aebischer et al. 1994, Kordower et al. 1994) or as described here by directly injecting viral vectors *in vivo* into the brain (Maingay et al. 2005, Eslamboli et al. 2007). The direct stereotaxic injection technique with a small-gauge needle used in this study enables precise delivery of a certain amount of vector to a well-defined anatomical location with a minimum risk of hemorrhage. Avoiding this complication is very important, especially when using the technique for studying movement disorders, as a hemorrhage in the SN could in itself cause movement difficulties and thus seriously blur behavioral observations.

Cell transduction was demonstrated using both immunohistochemistry (Figs. 2-4) and PCR (Fig. 6). Combining these two techniques enabled detection of the inserted gene and related gene product in nigral cell bodies. In addition, the fact that only cell bodies

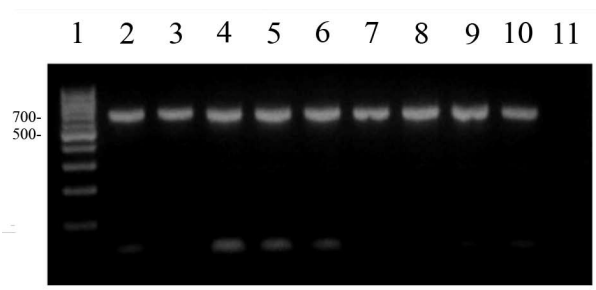

Figure 5: Control of suitability of the purified brain DNA from one animal for PCR based analysis by amplification of a 621 bp endogenous promoter fragment. Lane 1: size marker. Lane 2: nucleus caudatus. Lane 3: putamen. Lane 4: pons. Lane 5: medulla oblongata. Lane 6: caudal SN, injected side. Lane 7: caudal SN, contralateral to injection. Lane 8: rostral SN, injected side. Lane 9: rostral SN, contralateral to injection. Lane 10: positive control (template material from landrace pig). Lane 11: negative control (no template material added).

Figure 6: PCR-based test in one animal for the presence of the EGFP gene amplifying almost the entire coding region (727 bp). Lane 1: 1 kb marker. Lane 2: positive control (transgene harboring plasmid). Lane 3: negative control (non-infected pig). Lane 4: nucleus caudatus. Lane 5: putamen. Lane 6: Pons. Lane 7: Medulla oblongata. Lane 8: caudal SN, injected side. Lane 9: caudal SN contralateral to the injection. Lane 10: rostral SN , injected side. Lane 11: rostral SN contralateral to the injection. Lane 12: negative control (no template material added). In lane 10 (rostral SN on the injected side), a distinct band is observed indicating the presence of the EGFP gene.

immediately surrounding the injection tracts (up to 1,5 mm) were EGFP-positive seems to confirm that the recombinant lentivirus used here displays a limited degree of viral spread/diffusion in the tissue and only has the ability to integrate, but not to replicate (Pfeifer 2006). The PCR finding of the EGFP gene in the SN only and not in the striatum despite immunohistochemical observation of numerous EGFP-positive varicose axons, reflects the known fiber projections of the nigral cells (DeLong 1990). Our data accordingly support that the injected lentivirus has been localized in the nucleus of the nigral cells while the gene product not only is expressed locally but also transported axonally to the projection areas of these cells.

CONCLUSION

The findings of specific expression of a gene product in a discrete brain region and its efferent connections as demonstrated in this study may be used in future attempts to develop new animal models for CNS disorders with relatively localized primary pathology such as Parkinson disease. In disorders with more widespread CNS pathology, the method will, however, require multiple injections or the use of a different viral vector with the ability to transfect more than once or to transfect trans-synaptically as recently described (Cearley et al. 2007). Nevertheless, our study has introduced the Göttingen minipig as a candidate for direct viral mediated gene transfer to model CNS disorders, offering a non-primate large animal model that will span the gap between results obtained in basic science and clinical medicine.

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