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Direct MRI-guided stereotaxic viral mediated gene transfer of alpha-synuclein in the Göttingen minipig CNS

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The aim was to establish a non-primate large animal PD model by lentiviral vector mediated mutant alpha-synuclein overexpression in the substantia nigra. Lentivirus encoding A53T alpha-synuclein (6 × 2.5 µl) was stereotaxically injected into the substantia nigra of six adult female Göttingen minipigs. Contralateral control injections encoding enhanced green fluorescent protein (EGFP) were performed. Gait-analysis was performed pre- and postoperatively. PCR of the transgenes and immunohistochemical staining against alpha-synuclein, EGFP, GFAP and TH was performed after 20 weeks. Gait-analysis revealed a significant increase in step length and height, and a decrease in the double stand phase. PCR verified the mesencephalic presence of transgenes. IHC analysis showed alpha-synuclein expression in nigral neurons, around the injection tract and in related nigrostriatal projections. The alpha-synuclein positive neurons appeared swollen and vacuolated, in contrast to the EGFP-injected control side. To transduct all nigrostriatal cells with few microinjections, wider dissemination of the transgene must be achieved.

Key words: animal model, gene therapy, substantia nigra, *Sus scrofa*, transgenesis, Parkinson's disease

INTRODUCTION

Basic research in Parkinson's disease (PD) has profited greatly from rat and mice models. These models allow for experimentation on homogeneous populations with low costs relative to larger animal models (Meredith et al. 2008, Terzioglu and Galter 2008, Dawson et al. 2010). Larger animal models are, however, an important translational step toward clinical applications (Capitanio and Emborg 2008, Jenner et al. 2009). Several primate models of PD have been successfully developed (Langston et al 1984, Capitanio and Emborg 2008, Jenner et al. 2009) and have paved the way for current PD treatment paradigms such as continuous L-dopa treatment and subthalamic deep brain stimulation (DBS) (Capitanio and Emborg 2008, Jenner et al. 2009). The use of primate models has, however, been increasingly difficult and expensive, necessitating the search for alternative non-primate large animal models (Goodman and Check 2002). During the last fifteen years we have used pigs and especially the Göttingen minipig to examine neuromodulatory treatment modalities such as stem cell transplantation and DBS directed towards PD (Danielsen et al. 2000, Cumming et al. 2001, 2003, Bjarkam et al. 2005, 2008). This has been accomplished by the development of a MPTP-based Göttingen minipig model of PD having a substantial decrease in striatal dopamine and a stable parkinsonian syndrome characterized by rigidity, bradykinesia, and discoordination of the hind limbs (Mikkelsen et al. 1999, Danielsen et al. 2000, Bjarkam et al. 2005). The Göttingen minipig has a large gyrencephalic brain (6 × 5 × 4 cm) that both can be examined at sufficient resolution using conventional clinical scanning modalities and preclinical testing of DBS and other neuro-

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modulatory devices (Danielsen et al. 1998, 2000, 2001, Watanabe et al. 2001, Cumming et al. 2001, 2003, Røhl et al. 2002, Andersen et al. 2005, Bjarkam et al. 2004, 2005, 2008, 2009, 2010, Jensen et al. 2009, Rosendal et al. 2009, Fjord-Larsen et al. 2010). The instituted CNS changes can be evaluated through cystometry, gait analysis, neurological evaluation, and *post mortem* histological and stereological analysis (Danielsen et al. 2000, Sørensen et al 2000, Cumming et al. 2001, 2003, Røhl et al. 2002, Larsen et al. 2004, Dalmose et al. 2004, 2005, Andersen et al. 2005, Bjarkam et al. 2005, Rosendal et al. 2005, 2009, Nielsen et al. 2009, Ettrup et al. 2010). The MPTP model is, however, a toxic model that mimics PD pathogenesis (Terzioglu and Galter 2008), rather than the current genetic based models, which increases the accumulation of alpha-synuclein in the nigral neurons (Polymeropoulos et al. 1998, Bayer et al. 1999, Terzioglu et al. 2008).

The aim of the current study was to establish an alternative PD model in the Göttingen minipig based on lentiviral (LV) vector mediated A53T alpha-synuclein overexpression in the substantia nigra.

METHODS

The viral system

The LV vector system was developed in the laboratory of Professor Didier Trono, Lausanne, and has previously been described (Glud et al. 2010). The vectors utilized for direct viral mediated gene transfer harbour either a mutated human alpha-synuclein transgene (*SNCA-A53T*) or a transfer vector, pWPXL, encoding EGFP. The transgene is flanked by long terminal repeats (LTR) and the expression is driven by the human elongation factor 1α promoter (EF1-α). The cPPT (central polypurine tract signal) and WPRE (Woodchuck hepatitis virus posttranscriptional element) sequences incorporated in the vector enhance the transgene expression.

Stereotaxic implantation

Six female Göttingen minipigs aged 8–12 months, weighing 20–34 kg, were used in this study as approved by the Danish National Council of Animal Research Ethics. Animals were anesthetized with ketamine and midazolam according to weight (Olsen et al. 2010). Artificial ventilation and isoflurane (1–2%) anesthesia was used during the remainder of the procedure. After initiating isoflurane anesthesia the head of the animal was fixed in a stereotaxic localizer box (Mark 2.5 with TSE parallel rail and micromanipulator assembly, Neurologic, Denmark) (Bjarkam et al. 2005) (Fig. 1A). A MRI-visible fiducial marker was placed in bregma (Fig. 1B) and each animal was MR-scanned in order to calculate substantia nigra (SN) coordinates at six different positions, in each SN, relative to the fiducial marker (Fig. 1C). 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 (Fig. 1D). A 10 µl Hamilton syringe with attached glass needle was filled with a 6.0×10^7 transducting units per µl LV preparation and placed on the micromanipulator. Through a skull burr hole and dural incision, six 2.5 µl injections were made in the SN at the pre-determined coordinates, three injections encoding for alpha-synuclein and three encoding for EGFP. After suturing the skin, the animals were placed in a quarantine stable for 72 hours. Postoperative antibiotics and analgesics were administrated for 3 and 2 days, respectively.

Euthanasia and collection of brain tissue

After 20 weeks the animals were euthanized by an overdose of pentobarbital. Two brains were freshly removed for PCR analysis. The remaining four pigs were transcardially perfused with 5 l of phosphate buffered 4% paraformaldehyde (pH 7.4) fixative. The brains were removed and immersed in the fixative for 24 hours. The brains were then sectioned into 1-cm-thick coronal brain slabs and paraffin embedded. Embedded slabs were subsequently microtome sectioned into 40 µm thick coronal sections.

Nissl staining and immunohistochemistry

Tissue staining was performed according to previously established principles (Larsen et al. 2004). Sections for immunohistochemistry were incubated for 72 h with a primary polyclonal rabbit anti-GFP (ab290, Abcam Ltd., Cambridge, UK) diluted 1:1000 or a primary monoclonal mouse anti-GFAP (ab4648, Abcam Ltd., Cambridge, UK) diluted 1:500 or a primary polyclonal Sheep anti-alpha-synuclein (ab6162,

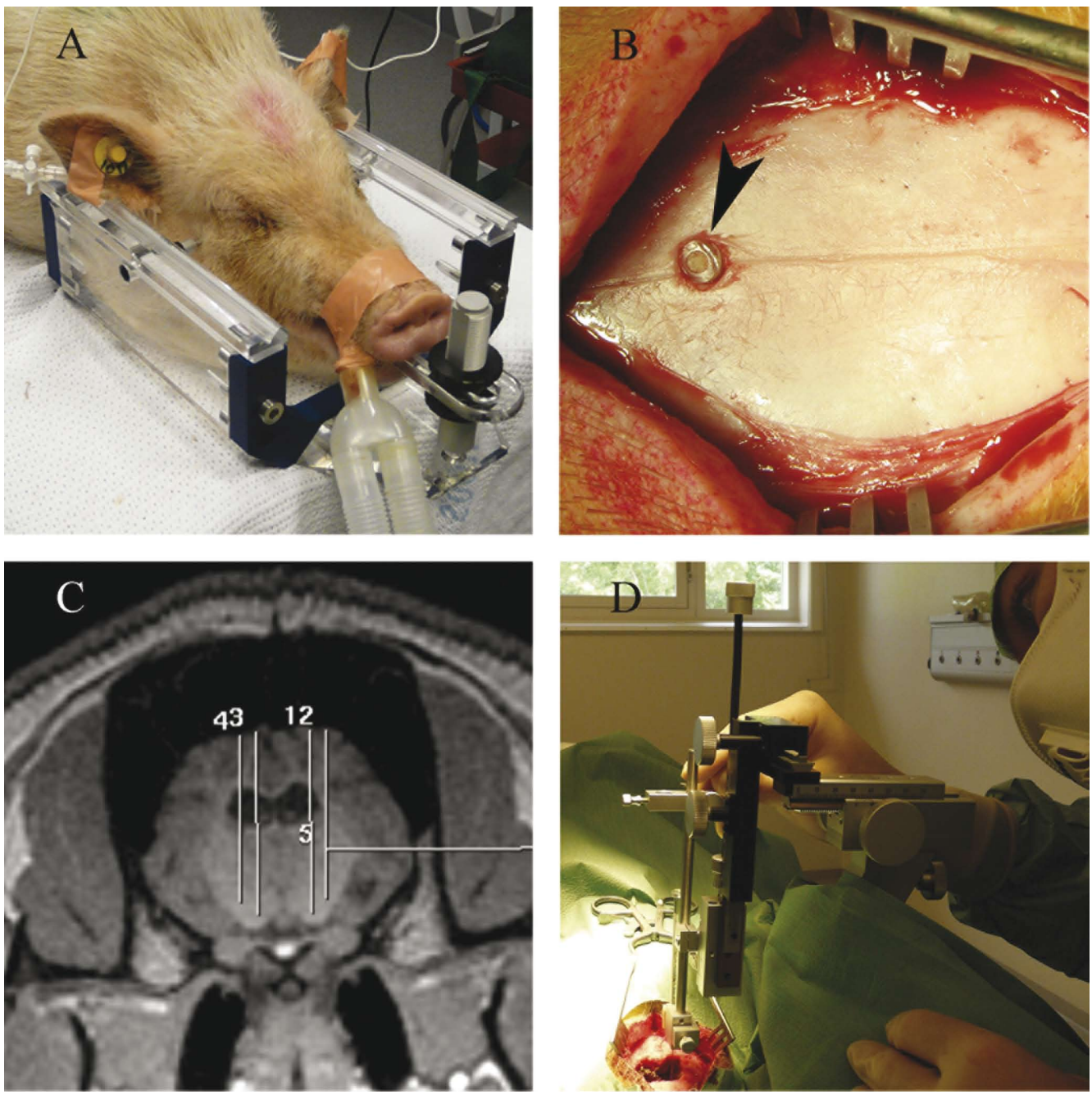

Fig. 1. Photographs of the surgical procedure. (A) Fixation of the head in the stereotaxic frame. (B) A copper sulphate filled fiducial is placed in bregma for MRI localization. (C) MR-image depicting the planned trajectories. The numbers 1–4 refer to the lines. The lines mark the injection tract. Number 5 refers to a measure-bar used to keep all injections in three planes parallel. On this picture four injection-tracts are seen. There are two more MR-slides (data not showed) with injections for the two other coronal planes, making the total of injections twelve, e.g. six in each SN. (D) The localizer box is converted into a stereotaxic device by addition of a stereotaxic frame with attached TSE-micromanipulator. The system is sterilely wrapped. It is possible to angle the system without derailing the manipulator, enabling a larger surgery area.

Table I

List of oligonucleotide primers used for PCR amplification of cDNA sequences

Primer	Sequence (5' - 3')	Product size (bp)
hSNCA5UTR-S	TGG AGA AGC AGA GGG ACT C	600
hSNCA3UTR-AS	ACT GGG CAC ATT GGA ACT GA	
GAPDH-F	TGGTGAAGGTCGGAGTGA	250
GAPDH-R	TTGATTTTGGCGGGATCT	

Abcam Ltd., Cambridge, UK. Made against human synuclein (aa116-131) coupled to diphtheria toxoid with a cysteine residue attached to the N-terminal of the peptide) diluted 1:2000. Primary antibody staining was followed by 1 hour incubation with a secondary anti-rabbit IgG biotinylated antibody (RPN1004, Amersham, Buckinghamshire, UK) diluted 1:400, a secondary anti mouse IgG biotinylated (RPN1177 GE Healthcare Europe GmbH) diluted 1:400 or a biotinylated anti-Sheep IgG (RPN1025 GE Healthcare Europe GmbH) diluted 1:400, respectively. Primary antibodies were omitted for negative control staining. Alpha-synuclein-positive striatum was counterstained with toluidine blue as previously described (Larsen et al. 2004).

Purification of nucleic acids from tissue samples

Total RNA was isolated from the minipig mesencephalon by the RNeasy method (Qiagen). The integrity of the RNA samples was verified by ethidium bromide staining of the ribosomal RNA on 1% agarose gels.

Synthesis of cDNA

Synthesis of cDNA used for detection of the transgene transcript by RT-PCR cloning was conducted with 5 mg of total RNA isolated from mesencephalon using SuperScript II RNase$^-$ reverse transcriptase (Invitrogen). The cDNA synthesis was initiated by the heating of total RNA, oligo(dT)$^{12\text{-}18}$ primer (Invitrogen), random hexamer primers (Invitrogen) and dNTP at 65°C for 5 min, followed by the addition of 200 U reverse transcriptase and incubation at 42°C for 50 min. cDNA synthesis reaction was terminated by heating at 70°C for 15 min.

RT-PCR amplification of cDNA sequences

Oligonucleotide primers were derived from the untranslated regions of the human *SNCA* mRNA sequence to ensure transgene-specific PCR amplification. The amplification product therefore covers a part of the 5'UTR of the entire coding sequence and a part of the 5'UTR of the human *SNCA* gene. The PCR reaction mix contained 1 ml cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 mM of each primer hSNCA5UTRs and hSNCA3UTRas (Table I) and 1 U Phusion DNA polymerase (Finnzymes), in a total volume of 10 ml. The PCR profile was as follows: 98°C for 1 min, 10 touchdown cycles of 98°C for 5 s, 65°C for 20 s (−0.5°C/cycle), 72°C for 1 min, followed by 20 cycles of 98°C for 5 s, 60°C for 20 s, 72°C for 1 min and finally an elongation at 72°C for 5 min.

The cDNA amplicons were visualized and isolated from an ethidum bromide stained 2% agarose gel. The PCR products were cloned directly into the pCR TOPO 2.1 vector (Invitrogen) and sequenced in both direc-

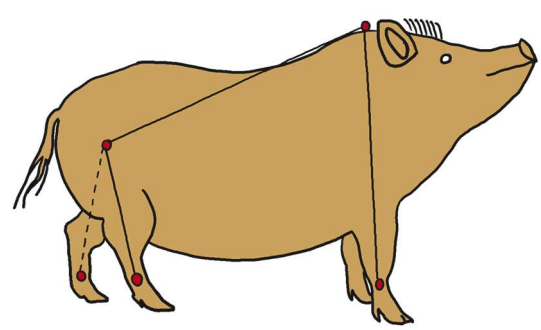

Fig. 2. Schematic drawing of the minipig with attached reflectors (red) on the hind limbs, the thigh, the neck and the right front limb.

tions. DNA sequencing was performed as previously described (Bjerre et al. 2006).

Gait analysis

All animals underwent open field pre-operative and post-operative [after 110 days (3 animals), and after 140 days (3 animals)] digital gait analysis, utilizing an infrared 3-D computerized Vicon system with 6 cameras to measure the temporospatial parameters of gait. Chocolate chips or pieces of apple were offered at the ends of the pen enclosure to promote animal movement. Reflectors were placed on hind limbs, the right front limb, the thigh and the highest point on the pig (the back of the neck) (Fig. 2). We obtained post OP double stand phase data from 5 of the 6 animals. The rest of the gait analysis data is for all 6 animals.

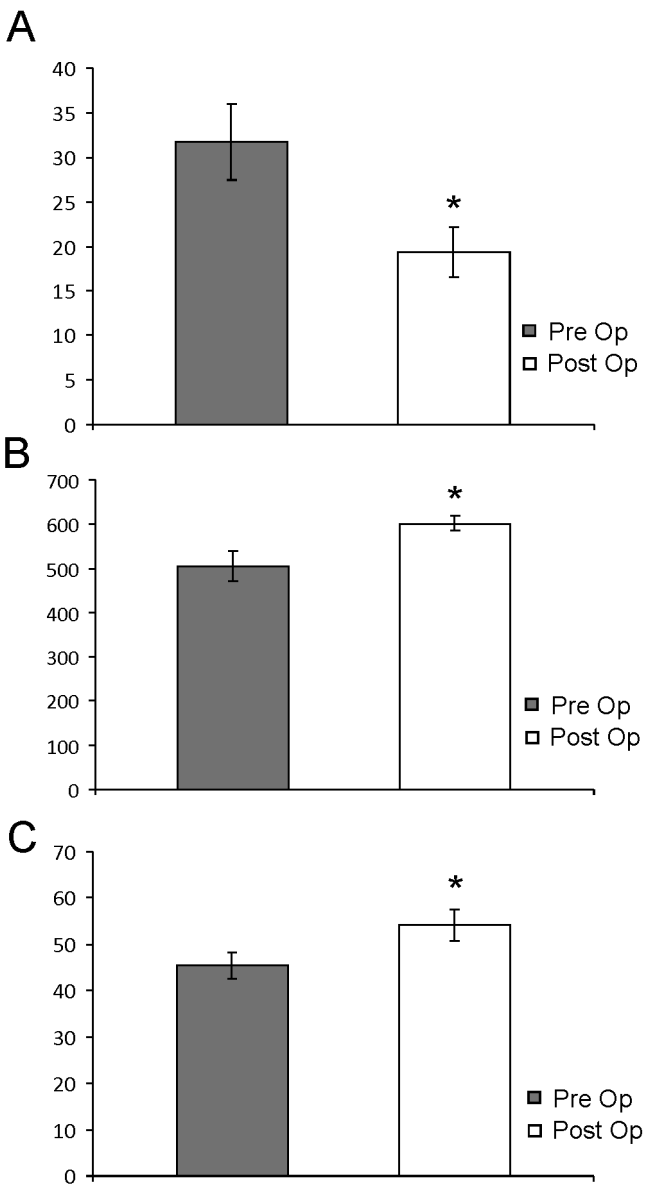

Fig. 3. Gait-analysis: Significant findings after pre- vs. post-operatively comparison. A *t*-test for paired data was used. (A) Double stand phase of the hind limb (P=0.04). (B) Step length (P=0.04). (C) Step height (P=0.02).

RESULTS

Postoperative behavior

The animals did not exhibit rotational behavior, increase in aggressiveness, decrease in weight or changes in vocalization.

Gait changes after injection

A change in gait pattern was noted after performing stereotaxic injections. Despite normal gait velocity, a significant decrease in the double limb stand phase of the hind limbs was quantified (Fig. 3A). Additionally, the animals took significantly longer steps (Fig. 3B), and raised their legs significantly higher from the floor (Fig. 3C) (Table II). A *t*-test for dependent samples was used.

IHC findings in nigrostriatal pathways

Needle trajectories targeting the substantia nigra without accompanying infection or hemorrhage were clearly visible in all animals (Fig. 4A). GFAP staining revealed bilateral normal representation of glial cells around the injection site indicating limited signs of astrogliosis or infection caused tissue damage (Fig. 4B). Alpha-synuclein-positive cells were noted immediately around the injection tract in the substantia nigra (Fig. 5A). These cells appeared to have swollen soma and nuclei (Fig. 5B), compared to surrounding non-transfected cells. Few alpha-synuclein-positive fibers were noted in the striatum (Fig. 5C). EGFP-positive staining was observed in neurons and glial cells of the contralateral substantia nigra (Fig. 5DE). EGFP was observed in the soma, axons and dendrites of the substantia nigra neurons. Numerous EGFP-positive boutons and fibers were found in the striatum (Fig. 5F). Tyrosine hydroxylase(TH)-staining showed that the

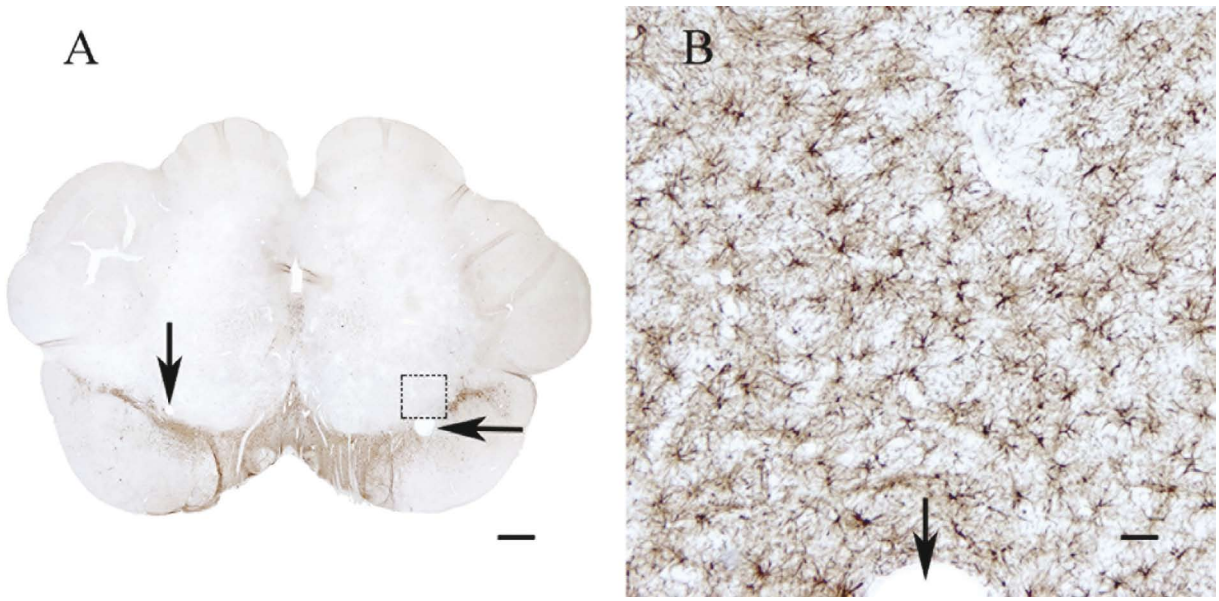

Fig. 4. (A) TH-stained mesencephalon showing the substantia nigra. Vertical arrow: Injection site of vectors encoding for EGFP. Horizontal arrow: Injection site of vectors coding for alpha-synuclein. (B) GFAP-stained tissue corresponding to the stippled area marked on (A). Scale bar is 1.4 mm (A), 35 µm (B).

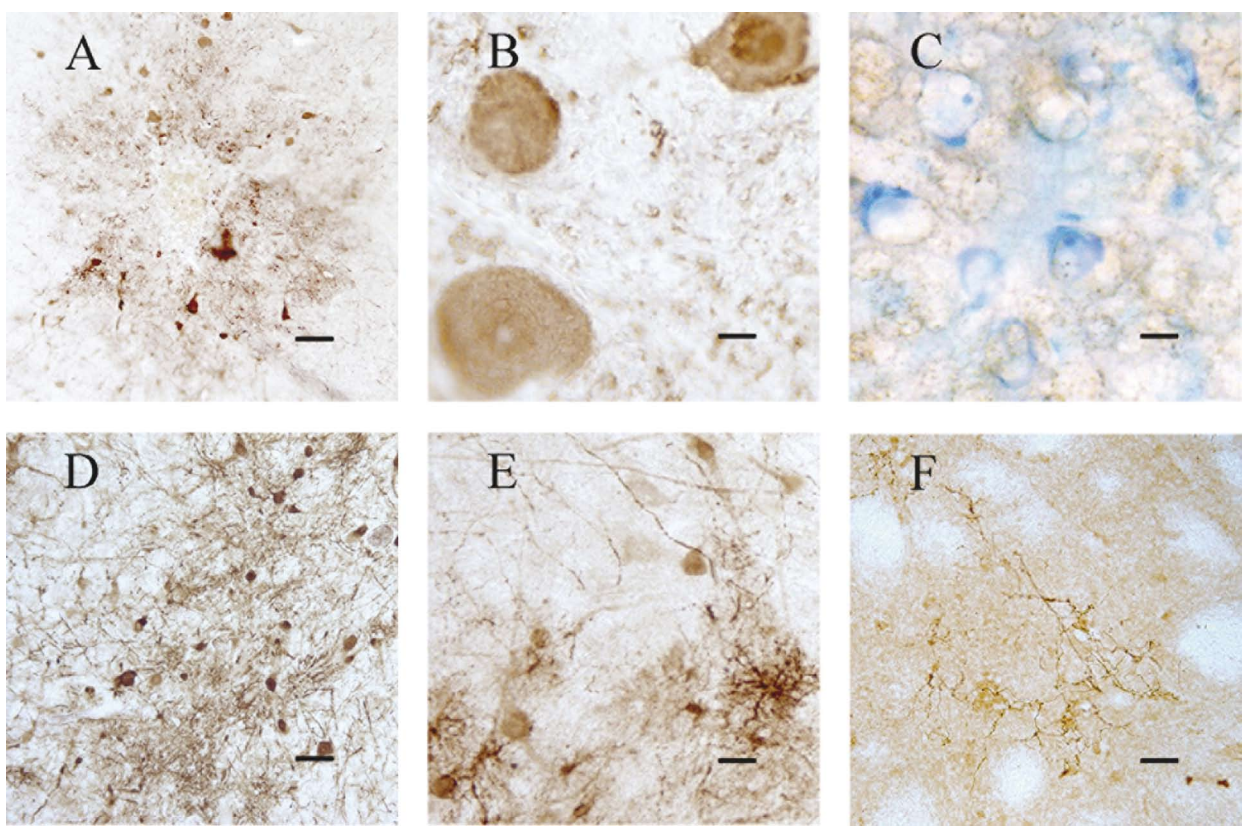

Fig. 5. (A) Alpha-synuclein-stained injection site in substantia nigra. (B) Alpha-synuclein-stained swollen neurons in substantia nigra. (C) Alpha-synuclein positive fibers surrounding toluidine-counterstained striatal neurons. (D) EGFP-stained injection site in substantia nigra. (E) EGFP-stained section from substantia nigra. (F) EGFP-stained varicose fibers in striatum. Scale bar is 55 µm (A, D and F), 10 µm (B), 5 µm and 35 µm (C and E).

Table II

Gait analysis						
Gait	Double stand phase [%]		Step length [mm]		Step height [mm]	
	Pre OP	Post OP	Pre OP	Post OP	Pre OP	Post OP
Mean	32.1	19.3	504.8	601.7	45.5	54.3
SEM	4.3	2.8	33.3	17.6	2.7	3.5
Student *t*-test, *P*-value	0.04		0.04		0.02	

trajectories targeted the SN on both the alpha-synuclein injection side and the contralateral control side (Fig. 4A). The expression was found in neuronal somas up to 1.5 mm. from the injection site and several cm away in axons and boutons of the nigrostriatal pathway.

Expression of transgene in the brain

The presence of the introduced alpha-synuclein transgene hSNCA A53T (SNCA) was examined using semi-quantitative RT-PCR with specific primers ensuring transgene amplification (Table I). Total RNA was isolated from the mesencephalon of 2 transduced minipigs and from 2 control animals. The tranduced mesencephalon was dissected into three equally sized parts. The oligonucleotide primers were derived from the human SNCA sequences and does not PCR amplify the endogenous porcine SNCA sequence. Expression of SNCA transcript was observed only in samples from the two transduced Göttingen minipigs (Fig. 6). A significantly higher expression of SNCA transgene was detected in mesencephalon samples from one transduced mini-pig (lanes 4–6). Sequencing of the amplified 600-bp DNA fragment revealed correctly the identity of the transgene introduced by the A53T mutation. No SNCA transcript was detected in the mesencephalon samples from the two non-transfected control pigs (lanes 7 and 8). Amplification of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript served as a control for the quality and concentration of the synthesized cDNA (Fig. 6).

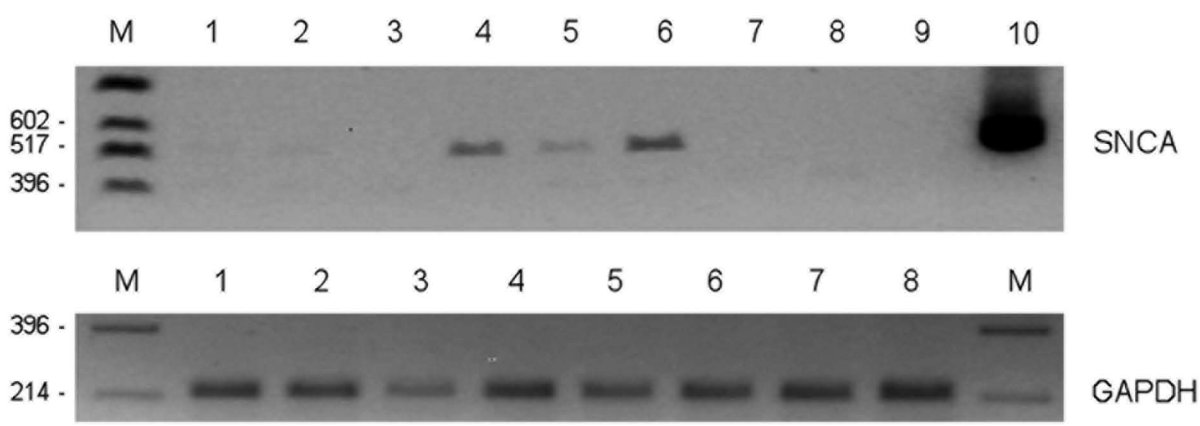

Fig. 6. Reverse transcriptase PCR analysis of transgene expression for SNCA (alpha-synuclein), (M) DNA molecular weight marker, (Lane 1) Mesencephalon sample 1 – pig 1, (Lane 2) Mesencephalon sample 2 – pig 1, (Lane 3) Mesencephalon sample 3 – pig 1, (Lane 4) Mesencephalon sample 1 – pig 2, (Lane 5) Mesencephalon sample 2 – pig 2, (Lane 6) Mesencephalon sample 3 – pig 2, (Lane 7) Mesencephalon non-transducted pig A, (Lane 8) Mesencephalon non-transducted pig B, (Lane 9) Negative (reagent) control, (Lane 10) Positive control (SNCA_A53T - plasmid used for transduction). GAPDH transcript with lanes equivalent to SNCA is used to serve as a control for the quality and concentration of the synthesized cDNA.

DISCUSSION

Our study has shown that cells in the substantia nigra of the Göttingen minipig can be transducted with an alpha-synuclein transgene using direct MRI-guided stereotaxic injection of viral vectors encoding for alpha-synuclein (Figs 4–5). Alpha-synuclein-positive neurons appeared swollen and vacuolated and had less intensely stained nigrostriatal projections (Fig. 5A–C), compared to the EGFP-positive control side (Fig. 5D–E). This indicate that the viral vector caused intracellular alpha-synuclein accumulation had a pathogenetic influence on the nigral neurons. Gait-analysis displayed that post-operative transducted animals had reduced double stand phase and increased step height and length.

Several caveats should be taken into consideration when interpreting our results. Firstly, the changes demonstrated by gait-analysis were not side dependent. We would expect that unilateral nigral degeneration would cause unilateral movement deficits and rotational behavior, as we have previously noted when STN stimulation was applied unilaterally to bilaterally MPTP intoxicated animals (Bjarkam et al. 2005). We would also expect primary symptoms of rigidity, imbalance and bradykinesia, as these symptoms are normally seen in MPTP intoxicated animals (Mikkelsen et al. 1999, Danielsen et al. 2000, Bjarkam et al 2005). We are therefore inclined to believe that the gait-analysis findings are mainly due to tissue damage caused by the injection procedure and are not transgene-induced. A wider spread of the vector and subsequently wider loss of dopaminergic cells may be required to cause unilateral symptoms. Accordingly, in humans it is known that symptoms of PD are seen after loss of around 80% of the dopaminergic neurons. Our TH staining revealed minimal loss of dopamanergic neurons bilaterally (Fig. 4). According, to the human findings we would expect the resulting symptoms to be non existent or mild in comparison with previously described minipig MPTP-induced parkinsonism. In future studies based on a widely disseminated viral vector and a larger cohort, stereology will be preferable for quantification of cell loss and pathology.

Secondly, although the alpha-synuclein-positive cells appeared swollen and vacuolated (Fig. 5A–C), we cannot definitively conclude that their pathology is due to the expression of alpha-synuclein. The cell pathology findings could have been caused by the injection procedure or the viral vector system. Against the latter, we would argue that a similar injection technique, viral vector system and injection volume was used on both sides. Alpha-synuclein-induced neurotoxicity in rodents has been reported to activate microglia and may induce robust neurotoxicity. In future studies staining of microglia activation markers and double staining with alpha-synuclein and TH/GFAP will be incorporated in the protocol.

Thirdly, the proven transduction and alpha-synuclein expression in nigral neurons was limited to neurons situated immediately around the injection tract. This indicates limited viral vector spread, and the necessity of numerous injections in order to obtain substantial infection of nigral neurons using the current vector system. Performing numerous injections would result in substantial mechanical damage, masking potential effects of alpha-synuclein. Limited dissemination of injected viral vectors is a general problem in the field of gene therapy as the diffusion of injected substances (e.g. viral vectors) diminishes exponentially with increasing distance from the injection site (Salvatore et al. 2006). Aside from performing more injections and therefore inducing mechanical tissue damage, alternative strategies could utilize viral vectors that are able to replicate more widely and engage in transsynaptic transduction (Cearley and Wolfe 2007). We have accordingly previously used the minipig model to develop an intracerebral microinjection instrument, which allows multiple angled injections from one central injection tract passing through the brain tissue (Bjarkam et al. 2010).

The selection of the viral vector is critical as viral vectors may be based on RNA-viruses (retrovirus, LV) or DNA-viruses [adenovirus, adeno-associated virus (AAV) or herpesvirus]. Viral vectors based on LV and AAV have proved to be particularly useful to generate transgenic animal models of neurological diseases. One disadvantage of the AAV vector is the limited insert size of usually less than 5 kb. This restricts the use of long promoters used to drive cell-specific expression. In addition, AAV also have a low packaging capacity (Verma and Somia 1997, Davidson and Breakefield 2003, Thomas et al. 2003). For the reasons mentioned above we preferred to initiate our study with vectors based on LV which have been claimed to be particularly well suited for transduction of neuronal cells. LV can transduct to both dividing cells and post mitotic cells like neurons (Lewis et al. 1992, Verma and Somia 1997, Naldini 1998, Trono 2000). LVs pos-

sess a large cloning capacity – between 7 and 9 kb (Verma and Somia 1997, Aebischer and Ridet 2001) and the ability to integrate the genes into the chromosomes of target cells. Generally, a long term expression from the transferred gene is observed (Blomer et al. 1997, Naldini 1998, Verma and Somia 1997). Furthermore, LVs have a low cytotoxicity and immunogenity, and do not compromise normal cellular functions *in vitro* and *in vivo* (Naldini et al. 1996, Blomer et al. 1997). LVs have accordingly been used to study long-term treatment by expression of therapeutic genes in animal models of neurological disorders, such as Parkinson's disease, Alzheimer's disease, Huntington's disease (Ralph et al. 2006). Our study have clearly confirmed that the selected LV based vector transduct nigral cells causing an over expression of alpha-synuclein. The dissemination of the vector was however to restricted and we will therefore need to identify a new vector that disperses the tissue more readily for future experimentation.

CONCLUSIONS

We are thus assured that the obstacles we have meet during this study with viral vectors in the Göttingen minipig reflect some of the problems that gene therapy directed towards brain disorders currently encounters, e.g. problems with delivery systems, viral dissemination and safety.

Further development of the described animal model will, accordingly, not only lead to a useful animal model of PD, but also provide valuable experiences and technical development for future clinical use of gene therapy directed against brain disorders.

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