A novel neuroprotective therapy for Parkinson’s disease using a viral noncoding RNA that protects mitochondrial Complex I activity

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Parkinson’s disease (PD) is a neurodegenerative disorder that results in the loss of nigrostriatal dopamine neurons. The etiology of this cell loss is unknown, but it involves abnormalities in mitochondrial function. In this study, we have demonstrated that the administration of a novel noncoding p137 RNA, derived from the human cytomegaloviral β2.7 transcript, can prevent and rescue dopaminergic cell death in vitro and in animal models of PD by protecting mitochondrial Complex I activity. Furthermore, as this p137 RNA is fused to a rabies virus glycoprotein peptide that facilitates delivery of RNA across the blood–brain barrier, such protection can be achieved through a peripheral intravenous administration of this agent after the initiation of a dopaminergic lesion. This approach has major implications for the potential treatment of PD, especially given that this novel agent could have the same protective effect on all diseased neurons affected as part of this disease process, not just the dopaminergic nigrostriatal pathway.

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such delivery systems, such as the invasive nature of the intracerebral procedure to administer therapeutic agents and the nonspecific expression of these agents outside neural cells. Recently, Kumar et al. (2007) described a method to deliver short interfering RNA (siRNA) to the brain using a small peptide derived from the rabies virus glycoprotein (RVG). This peptide binds to the acetylcholine receptor (AChR) exclusively expressed in CNS cells (Hanham et al., 1993). Although the RVG peptide itself has no RNA binding affinity, a derivative containing nonamer arginine residues (RVG9R) binds RNA efficiently and delivers the RNA cargo across the blood–brain barrier after peripheral administration (Kumar et al., 2007). We therefore also sought to test whether the transvascular administration of the p137 RNA could be successfully delivered in this way to prevent dopaminergic cell loss in models of PD.

RESULTS AND DISCUSSION

The RVG9R–p137 system protects dopaminergic cells in both in vitro and in vivo models of PD

Our initial experiments clearly showed that the p137 RNA complexed with RVG9R peptide could protect SH-SY5Y cells from exposure to rotenone, a highly selective inhibitor to mitochondrial Complex I (Fig. 1 c; Betarbet et al., 2000). Conjugation with the RVG9R peptide enabled the delivery of p137 RNA into neurons of both dopaminergic and nondopaminergic systems (Fig. 1, d and e) and protected primary fetal dopaminergic cells from a 6-OHDA insult (Fig. 1 f). Such protection was not observed using a range of RNA and peptide controls of various sizes, which included RVG9R–antisense p137, RVMat9R–p137 (comprising a control peptide unable to bind to AChR), or control RVG9R–pXef, which encodes the Xenopus laevis elongation factor 1α (Fig. 1).

Figure 1. Delivery of RVG9R–p137 to cultured cells in vitro prevents neurotoxin–induced cell death. (a) Representative photomicrographs on the expression of α3/α5 nicotinic AChR (green) on HEK293, U373, and SH-SY5Y cells. Nuclei were counterstained with Hoechst (blue). (b) Representative photomicrographs showing the delivery of unlabeled p137 RNA in U373 cells treated with p137 alone, control RVMat9R peptide–conjugated p137, RVG9R–p137, or RVG9R–pXef, as detected using FISH to detect p137 RNA (red). (c) Treatment of RVG9R–p137 but not RVG9R complexed with the control antisense p137 RNA (a.s. p137), or the control RVMat9R peptide complexed with the p137 RNA significantly protected SH-SY5Y cells from rotenone (Rot)-mediated cell death analyzed by TUNEL staining. (d and e) FITC-tagged p137 RNA could be detected in both cholinergic (d) and dopaminergic (e) neurons after incubation with rat E13.5 primary cortical (CTx) and VM cells, respectively. FITC signals (yellow) were colocalized with Hoechst (blue), AChR (green), and ChAT/TH (red). (f) Numbers of TH+ cells in untreated VM cultures (con) or in VM cultures insulted with 6-OHDA, in the presence of increasing concentrations of RVG9R–p137 or RVG9R–pXef, were quantified. The proportion of TH+ cells was calculated against the total number of Hoechst+ cells. (a–f) Data were obtained from three (a–c), two (d and e), and six (f) independent experiments. Data represent mean ± SEM; **, P < 0.005; ***, P < 0.001, compared with the neurotoxin only group. Bars: (a and b) 30 µm; (d) 25 µm; (e) 40 µm.
Similarly, p137 RNA complexed with RVG9R peptide could also be delivered to the α3/α5 nicotinic AChR + U373 cell line (Fig. 1, a and b). In contrast, incubation of p137 RNA alone, with the RVMat9R–p137 complex, or with the RVG9R–pXef complex failed to result in p137 delivery to U373 cells (Fig. 1 b).

Based on the in vitro results, we tested the ability of RVG9R–p137 to protect dopaminergic cell loss against 6-OHDA neurotoxicity in vivo. Adult male rats were stereotactically injected with peptide–RNA complex containing 2.5 µg RNA into the dopaminergic cell bodies at the level of the substantia nigra pars compacta (SNc) 3 d before an acute, intranigral 6-OHDA insult. We observed the expected functional (Table 1) and phenotypical (not depicted) deficits in those rats that were given RVG9R–pXef pretreatment, whereas rats pretreated with equivalent doses of RVG9R–p137 showed significant attenuation of the lesioning effects.

As the RVG peptide was originally engineered to enable its RNA cargo to cross the blood–brain barrier and target its delivery to the brain, we examined the possibility of using a transvascular approach for RVG9R–p137 administration. Rats were treated with an intravenous injection of 300 µg of RVG9R–p137, via the tail vein, 3 d before an intranigral 6-OHDA lesion. Consistent with our observations using intranigral delivery, rats pretreated transvascularly with RVG9R–p137 performed significantly better than those receiving RVG9R–pXef on a battery of behavioral tests (Table 1). This correlated well with immunohistochemical analysis, showing that the RVG9R–p137 pretreatment significantly protected the nigrostriatal dopaminergic system from the lesion (not depicted).

**Table 1. Summary of scores in the drug-induced rotation tests, forelimb cylinder test, and forelimb stepping tests in the RVG9R–p137 and RVG9R–pXef receiving groups**

| Groups                          | Treatment (no. of rats) | Amphetamine-induced rotation | Apomorphine-induced rotation | Contralateral forelimb use | Contralateral forelimb stepping (no. of adjustment steps) |
|--------------------------------|-------------------------|------------------------------|------------------------------|---------------------------|----------------------------------------------------------|
|                                |                         | turns/min                    | turns/min                    | %                         |                                                          |
| Pre-lesion treatment           | pXef (10)               | 6.45 ± 1.04                  | 4.63 ± 1.16                  | 10.37 ± 3.57              | 2.00 ± 0.31                                               |
| (intranigral)                  | p137 (10)               | 0.32 ± 0.69c                 | −1.57 ± 1.08b                | 40.24 ± 7.95c             | 11.22 ± 0.81c                                             |
| Pre-lesion treatment           | pXef (10)               | 10.93 ± 6.80                 | 4.34 ± 0.71                  | 1.47 ± 2.31               | 2.33 ± 1.03                                               |
| (intranavenous)                | p137 (12)               | 1.61 ± 4.53c                 | 0.35 ± 1.32c                 | 41.61 ± 19.96c            | 10.33 ± 2.81c                                             |
| Post-lesion treatment          | pXef (9)                | 4.72 ± 1.65                  | 0.85 ± 0.72                  | 16.98 ± 16.74             | 6.22 ± 1.20                                               |
| (intranigral)                  | p137 (10)               | 0.44 ± 1.82c                 | −0.53 ± 0.40                 | 40.56 ± 12.74c            | 7.91 ± 1.38                                               |
| Post-lesion treatment          | pXef (6)                | 5.75 ± 3.41                  | 0.70 ± 0.31                  | 19.07 ± 11.89             | 4.17 ± 1.94                                               |
| (intranavenous)                | p137 (6)                | 0.82 ± 0.77c                 | −0.12 ± 0.28c                | 57.03 ± 8.19c             | 7.50 ± 1.05c                                               |

p137 encodes the neuroprotective domain of the human cytomegaloviral β2.7 transcript. Data shown for all treatment groups are derived from one observation, involving all lesioned animals, from four independent experiments over a time span of 2 yr, using multiple batches of RVG9R peptide and RNAs. Data represent mean ± SEM.

**:P < 0.05.
**:P < 0.01.
**:P < 0.001.

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We then examined whether RVG9R–p137 delivery offered protection at the level of enzyme activity of Complex I in the SNc in the presence of the 6-OHDA insult (Fig. 2 d).
Semipurified mitochondria were isolated from the nigra of rats treated with RVG9R–p137 or RVG9R–pXef before an acute 6-OHDA lesion. Univariate analysis of variance showed that there was a significant difference in Complex I activity between groups (P = 0.013), and post-hoc analysis revealed that there was a significant reduction in Complex I activity in rats receiving an acute 6-OHDA insult. A similar attenuation on Complex I activity was seen in the lesioned rats receiving RVG9R–pXef pretreatment. In contrast, mitochondria from rats pretreated with RVG9R–p137 demonstrated a significant protection of Complex I enzyme activity. Such protection in mitochondrial function was also confirmed using a resazurin reduction–based assay (Fig. 2 c). Respiration in purified mitochondria from rats treated with 6-OHDA was substantially inhibited compared with mitochondria from sham-treated rats, as expected. In contrast, rats pretreated with RVG9R–p137 demonstrated a protected mitochondrial function that was not observed in the RVG9R–pXef-pretreated rats.

Collectively, these results suggest that RVG9R–p137 pretreatment clearly mediates a robust protective effect to enhance dopaminergic cell survival against an acute 6-OHDA lesion. Such protection is associated with a specific interaction between p137 and Complex I, as well as the attenuation of 6-OHDA–mediated impairment of Complex I activity and mitochondrial respiratory function.

Repeated RVG9R–p137 treatment attenuates 6-OHDA toxicity in a progressive lesion model without eliciting a host immune response

Although our data showed that we could prevent cell death using RVG9R–p137 when delivered before 6-OHDA lesion, it is clinically more relevant to show protective effects after a lesion had been initiated. Therefore, we investigated whether the RVG9R–p137 treatment could mediate a neuroprotective function to attenuate cell loss after the onset of lesion by using a progressive lesion model of PD. It has been well reported that a partial and retrograde nigrostriatal dopaminergic degeneration can be achieved by 6-OHDA injection into the ventrolateral striatum (Kirik et al., 1998; Blandini et al., 2007), which causes a progressive cell death over a 2–6-wk period. Similar to the aforementioned pretreatment experiments, a significant correction of behavioral deficits (Table 1), as well as a rescue of nigral and striatal tyrosine hydroxylase (TH) expression (not depicted), was observed with repeated intranigral administrations of RVG9R–p137 starting 3 d after the intrastriatal 6-OHDA lesion. Such protection was not observed in rats receiving RVG9R–pXef under an equivalent treatment regimen. Clearly, RVG9R–p137 treatment was able to attenuate 6-OHDA toxicity in a progressive lesion model when delivered directly to the SNc after the initiation of lesion.

We then evaluated whether a comparable neuroprotective effect of RVG9R–p137 could be obtained by an intravenous route of administration. Rats were given four separate injections of 300 µg RVG9R–p137 via the tail vein 1–2 d after the lesion, with a further injection 8 d after the lesion, and with this last treatment, some animals were given FITC–tagged RVG9R–p137 for confocal microscopy analysis to evaluate the distribution of p137. Consistent with our observations using repeated intranigral delivery, a significant neuroprotection was detected only in animals receiving RVG9R–p137 treatment (Fig. 3, a–c; and Table 1). Densitometric analysis of striatal TH expression revealed significantly higher TH levels in rats receiving RVG9R–p137 (85.3 ± 3.9% of the contralateral side) compared with those given RVG9R–pXef (51.4 ± 4.1% of the contralateral side; P < 0.001). This correlated with the unbiased stereological counting showing that the number of dopaminergic cells in the SNc (6,182 ± 221 per contralateral side) was 54.2 ± 8.0% in the RVG9R–pXef, compared with 71.2 ± 3.8% in the RVG9R–p137-treated group (P < 0.01). Fluorescent detection of FITC, TH, and α3/α5 nicotinic AChR in the nigra revealed that many, but not all TH-expressing neurons were α3/α5 nicotinic AChR positive (Fig. 3 d, arrows) and that detection of p137 RNA was exclusively restricted to the AChR–expressing cells (Fig. 3, d and e). p137 could also be found associated with cells other than dopaminergic neurons (Fig. 3 e, arrows), supporting the possibility that RVG9R–mediated p137 delivery could also be used to target cell populations other than the dopaminergic system. No FITC signal could be found in rats given untagged RVG9R–p137 treatment (not depicted). There was a tendency for the p137 RNA to be recruited toward the site of a more severe lesion, i.e., the ipsilateral nigra, after intravenous RVG9R–p137 delivery. This preferential recruitment of p137 corresponds well with our immunoprecipitation data (Fig. 2 a). To determine whether repeated intravenous injections with a large RNA–protein complex stimulates a host immune reaction, we compared the level of CD3 and ED1 expression between sections from RVG9R–p137 (ipsilateral/contralateral sides), RVG9R–pXef (ipsilateral side), and naive animals. Immunostaining revealed no overt changes in the levels of T cell infiltration (CD3; Fig. 4 a) and microglial activation (ED1; Fig. 4 b) after repeated delivery of RVG9R–p137, other than those elicited by the lesioning procedure itself. This is consistent with a lack of immune responses observed by Kumar et al. (2007). We also ruled out the possibility that RVG9R–p137 treatment generated peptide–specific T cell or antibody responses. PBMCs from rats treated with RVG9R–p137 by repeated tail vein injections and subsequently stimulated with RVG9R peptide failed to elicit an IFN-γ response. In contrast, positive controls stimulated with ionomycin and PMA generated a strong response, as expected (Fig. 4 c). We also analyzed serum from the same animals for RVG9R–specific antibodies by ELISA (Fig. 4 d) and found that there was no difference in the antibody responses to RVG9R peptide between rats injected with RVG9R–p137 and saline. Overall, it is clear that the RVG9R–p137 complex delivered transvascularly does protect TH+ nigral neurons.
neurons in a progressive PD model and that this treatment is nonimmunogenic.

Since the first demonstration that mitochondrial Complex I activity was impaired in the nigra of patients with PD (Schapira et al., 1990), there has been interest in the etiological role of mitochondria in PD. Therapeutic agents that enhance mitochondrial functions, such as coenzyme Q10, have been reported to have a moderate effect in slowing disease progression in patients with early PD (Shults et al., 2002). Of late, several Mendelian genetic causes for PD have been identified, including abnormalities in the α-synuclein, LRRK2 (leucine-rich repeat kinase 2), DJ-1, PINK1 (PTEN-induced putative kinase 1), and parkin genes. Many of these genes are thought to have effects on mitochondrial function, reinforcing the view that mitochondrial dysfunction may lie at the heart of this disorder, even though the majority of patients with PD have an idiopathic disease origin. As such, it would have been useful to study the effects of RVG9R–p137 in transgenic models of PD, except that none of these models show consistent dopaminergic cell loss (Dawson et al., 2010), hence our use of neurotoxin-based models. These models, although a poor imitation of the clinical condition, have nevertheless been important in the development of a whole range of therapeutic agents such as dopaminergic cell transplants.

![Figure 3](image-url)
and, more controversially, glial cell line–derived neurotrophic factor (Gill et al., 2003; Lang et al., 2006). The defining pathological feature of PD is traditionally considered to be the loss of the nigrostriatal pathway, even though the disease is now recognized to have a range of nonmotor features and extranigral pathology. Indeed, this has even led some to consider PD as a multistage disease with nigral pathology only emerging halfway through that disease cascade (Braak et al., 2006). This, coupled with the heterogeneous nature of PD, may limit the potential benefits of current cell replacement (Freed et al., 2001; Olanow et al., 2003) and neuroprotective (Nutt et al., 2003; Lang et al., 2006) therapies that only target neurons of the dopaminergic system. The RVG9R–p137 treatment reported in this paper may therefore offer a more useful alternative by targeting mitochondrial stress in both dopaminergic and nondopaminergic cells affected by the disease process, all of which could be reached through the systemic delivery of the agent assuming that the relevant cell populations affected express the right AChR. This has also been reinforced by a recent study, showing that the RVG peptide can target siRNA delivery to different CNS cells such as neurons, astrocytes, and oligodendrocytes via transvascular administration (Alvarez-Erviti et al., 2011).

Although we did not examine the expression of p137 outside the brain after transvascular delivery, Kumar et al. (2007) used flow cytometry to analyze the RVG9R–mediated GFP expression in the peripheral tissues and concluded that the cargo delivery was restricted to the CNS. Such a CNS-targeted method of transvascular delivery suggests that our RVG9R–p137 complex may be helpful for clinical application in the future treatment of PD and related neurodegenerative conditions. However, more work is needed to determine the pharmacokinetic profile of the RVG9R–p137 conjugate, and work is ongoing to improve the half-life of siRNA in vivo, such as lipid encapsulation (Morrissey et al., 2005) and chemical modification for polyethylene glycol addition (Dassie et al., 2009).

In conclusion, we have developed a novel therapeutic agent that specifically targets Complex I and when given is neuroprotective in a range of model systems of dopaminergic cell loss mimicking PD, while not inducing any immune reactions. As such, this agent has great potential as a disease-modifying therapy for this common, incurable neurodegenerative condition.

Figure 4. Delivery of RVG9R–p137 via the intravenous route stimulates no apparent immune responses after repeated administration. (a) The striatum or substantia nigra of rats repeatedly treated with RVG9R–pXef or RVG9R–p137 via the tail vein after the initiation of intrastriatal 6-OHDA lesion. Immunostaining using anti-CD3 was performed on naive rats, the contralateral side of rats receiving RVG9R–p137 (p137 contra), the ipsilateral side of rats receiving RVG9R–p137 (p137 ipsi), and the ipsilateral side of rats receiving RVG9R–pXef (pXef ipsi). The minor up-regulation of CD3 signal was caused by the surgical procedure in generating the lesion. (b) Immunostaining for ED1 was performed as in panel a, and again the only apparent up-regulation of CD3 was caused by the lesioning procedure. Immunohistochemical analysis was carried out once on all animals receiving no lesion (n = 3), repeated intravenous treatments of RVG9R–p137 (n = 6), and RVG9R–pXef (n = 6). The data shown are from one representative animal of each group. Bars: (a and b, top) 1,000 µm; (a and b, bottom) 500 µm. (c) PBMCs from duplicate rats receiving RVG9R–p137 or saline by repeated tail vein injection were stimulated with media alone, RVG9R peptide (peptide), or ionomycin + PMA (positive control). Cells expressing IFN-γ were then assayed by ELISPOT, read on an AID ELISPOT reader, and enumerated using ImageJ. (d) Sera from the same duplicate rats receiving RVG9R–p137 or saline, used in c for the IFN-γ ELISPOT analysis, were also analyzed for RVG9R-specific antibodies using RVG9R-coated ELISA plates. Wells coated with ZNF785 peptide (anti-ZNF) were used as positive controls for peptide-specific detection. ELISA plates (OD) were read at 450 nm on a Multitek ELISA plate reader. (c and d) Assays were performed from three independent experiments. Data represent mean ± SEM; *, P < 0.05; and ***, P < 0.001 compared with immune responses stimulated in animals receiving repeated RVG9R–p137 treatments.
Animals were sacrificed by pentobarbital sodium and transcardially perfused 4 wk (2 wk for the rats receiving a transvascular, postlesion treatment) after lesion. The brains were removed, postfixed, and then transferred to 30% sucrose until they sank. 40-μm coronal brain sections were cut, and a 1:6 series of sections were immunostained for mouse anti-TH (1:500; Millipore), anti-AChR α3/α5 (1:1,000; Sigma–Aldrich), anti-ED1 (1:1,000; AbD Serotec), and anti-CD3 (1:1,500; Abcam) as described previously (Kuan et al., 2006, 2007). The cultures or sections were visualized either by immunofluorescence (Alexa Fluor 568 and Alexa Fluor 688; 1:1,000; both from Invitrogen) or diaminobenzidine. Staining omitting primary antibodies was performed in all immunohistochemical experiments to serve as a negative control. For FISH to detect p137 RNA, cells were washed, fixed with 4% paraformaldehyde, and permeabilized with 70% cold ethanol. Cells were then rehydrated in 2× SSC (saline sodium citrate), and incubated with blocking buffer (50% formamide, 2× SSC, 10% dextran sulfate, 1× Denhardt’s solution, 2 mM ribonucleoside vanadyl complexes, 50 μg/ml of yeast tRNA, and 50 μg/ml of salmon sperm DNA) for 30 min at 37°C in a humidified incubator followed by overnight hybridization with 0.4 ng/ml Cy3-labeled antisense probe for p137 in the same buffer. After hybridization, cells were counterstained with 1 mg/ml Hoechst and mounted with Citifluor (Dako) and analyzed by fluorescence microscopy (Eclipse TE300; Nikon).

**Behavioral tests.** The extent of striatal dopaminergic denervation was examined by 2.5 mg/kg amphetamine– and 1.6 mg/kg apomorphine–induced rotational behavior. The number of full ipsi- and contralateral rotations was measured for 90 min after drug administration. The paw-reaching test was developed to evaluate the sensorimotor function of the animal’s forelimb. In brief, the experimenter firmly held both hindlimbs and one forelimb of the animal and moved the rat sideways across a platform over 1 m in 5 s. The unrestrained forelimb was in contact with the platform and the number of adjustment steps performed by the rats counted in the forehand direction. The animals were habituated to the handling associated with this test for three consecutive days before lesion. The cylinder test evaluated the spontaneous forelimb use during vertical exploration. In brief, the animal was allowed to move freely in a Plexiglas cylinder during a 5-min session. The observation was videotaped, with mirrors placed behind the cylinder to ensure full visibility of paw placements during this time. Data were presented as the proportion of contralateral paw touches against the total. Behavioral tests were assessed between the third and fourth week after the 6-OHDA lesion. Further details can be found in Kuan et al. (2007).

**Immunohistochemistry and FISH.** Animals were sacrificed by pentobarbital sodium and transcardially perfused 4 wk (2 wk for the rats receiving a transvascular, postlesion treatment) after lesion. The brains were removed, postfixed, and then transferred to 30% sucrose until they sank. 40-μm coronal brain sections were cut, and a 1:6 series of sections were immunostained for mouse anti-TH (1:500; Millipore), anti-AChR α3/α5 (1:1,000; Sigma–Aldrich), anti-ED1 (1:1,000; AbD Serotec), and anti-CD3 (1:1,500; Abcam) as described previously (Kuan et al., 2006, 2007). The cultures or sections were visualized either by immunofluorescence (Alexa Fluor 568 and Alexa Fluor 688; 1:1,000; both from Invitrogen) or diaminobenzidine. Staining omitting primary antibodies was performed in all immunohistochemical experiments to serve as a negative control. For FISH to detect p137 RNA, cells were washed, fixed with 4% paraformaldehyde, and permeabilized with 70% cold ethanol. Cells were then rehydrated in 2× SSC (saline sodium citrate), and incubated with blocking buffer (50% formamide, 2× SSC, 10% dextran sulfate, 1× Denhardt’s solution, 2 mM ribonucleoside vanadyl complexes, 50 μg/ml of yeast tRNA, and 50 μg/ml of salmon sperm DNA) for 30 min at 37°C in a humidified incubator followed by overnight hybridization with 0.4 ng/ml Cy3-labeled antisense probe for p137 in the same buffer. After hybridization, cells were counterstained with 1 mg/ml Hoechst and mounted with Citifluor (Dako) and analyzed by fluorescence microscopy (Eclipse TE300; Nikon).

**Determination of Complex I activity.** To compare changes in mitochondrial Complex I activity, mitochondria were isolated from the SNc. To do this, rats were decapitated under general anesthesia. Upon removal of the brain, a 70–90-mg nigral tissue was acutely dissected and immediately submerged in ice-cold artificial cerebrospinal fluid (125 mM NaCl, 5 mM KCl, 1.25 mM NaH2PO4, 28 mM NaHCO3, 0.5 mM CaCl2, 4 mM MgCl2, 25 mM D-glucose, and 1 mM kynurenic acid). Mitochondrial preparations were obtained from brain sections using a proprietary mitochondria isolation kit (BioChain Institute) as described by the manufacturer. For analysis of Complex I activity in these mitochondria, a Complex I Enzyme Activity Microplate Assay kit (MitoSciences) was used exactly as described by the manufacturer. In all cases, samples were corrected for total mitochondrial protein assayed.

Mitochondrial respiration was also analyzed directly using a resazurin reduction assay of isolated mitochondria in a 96-well plate format, as
RNA/protein immunoprecipitation. Nigral tissue was prepared exactly as detailed in the previous section for preparation of mitochondria and enzymatically digested with trypsin (Worthington). The digestion was terminated with a mixture of trypsin inhibitor and DNase (both from Sigma-Aldrich). After centrifugation, the pellet was resuspended in DMEM and mechanically triturated until a single cell suspension had formed. The cells were then fixed in paraformaldehyde, quenched in glycine, and stored at ~80°C until used. To determine any interaction of p137 RNA with Complex I, cells derived from the SCNs of rats treated intrantragously with RVG9R–p137 or RVG9R–pXef were subject to RNA immunoprecipitation using Complex I and Complex IV immunocapture beads (MitoSciences) as described by the manufacturer. The anti–Complex I antibody used only recognizes fully formed native Complex I assembled in the mitochondria. IgG2a antibody–coated beads were also used as an additional control for the immunoprecipitation. After immunocapture of Complex I or Complex IV from the samples, the presence of interacting p137 RNA was analyzed by RT-PCR, as previously described (Reeves et al., 2007).

To assess the relative levels of localization of p137 RNA in mitochondria and the cytosol, mitochondria were isolated using the proprietary mitochondria isolation kit (BooCell), and RNA isolated from each cellular subfraction by TRIZOL extraction was analyzed by p137-specific RT-PCR (Reeves et al., 2007). Relative purity of the fractions was assessed using Western blot analysis with antibodies to HSP60 (specific for mitochondria) and GAPDH (specific for cytosol).

Assessment of peptide-specific T cell and antibody responses in treated rats. Blood was collected from rats by heart puncture (8 ml blood in 2 ml of 100 U/ml heparin sodium), and PBMCs were isolated by density barrier centrifugation using a 2:5.2 vol/vol ratio of Optiprep (Axis-Shield) and tricine buffer (10 mM tricine and 0.145 M NaCl, pH 7.0). Heparinized rat blood was diluted 1:1 with tricine buffer and 6 ml of diluted blood layered onto 3 ml of a density barrier and centrifuged at 800 g for 20 mm. The PBMCs were removed from the interface with a Pasteur pipette and diluted in PBS (Oxoid) and washed twice before resuspension and counting in RPMI 1640 10% FCS.

Rat IFN-γ ELISPOT assays were performed according to the manufacturers protocols (BD). In brief, PBMCs from two RVG- and two saline-treated rats were cultured in triplicate, in four wells, each well containing a 1:10 (100 µg/ml) ratio of RVG9R peptide and 50 µl of 2N sulfuric acid was added per well, incubated for 30 min at 37°C, and then washed five times. 50 µl of TMB substrate (eBioscience) was added per well, incubated overnight at 37°C in 5% CO2, and then developed according to the manufacturers recommendations. The plates were read at 450 nm on a Multititer ELISA plate reader.

Microscopy, densitometric, stereological, and statistical analysis. Imaging was performed using a DMi6000 microscope or a TCS SP2 confocal microscope (Leica). The mean OD was determined by subtracting the OD of the background from the region of interest defined by TH staining. Results were expressed as the ratio between the OD of the ipsi- and contralateral side. Total cell numbers in the SNc were estimated by unbiased stereology sampling using the Stereo Investigator 9.10.3 (MBF Bioscience). Densitometric, stereological, and behavioral data between all groups of animals was analyzed using univariate analysis of variance, and a post-hoc Bonferroni test was used as appropriate with significance set at P < 0.05. All the analysis was performed using ImageJ 1.42q and SPSS Release 16.0.0.

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