Published in final edited form as:

J Neurosci Methods. 2012 October 15; 211(1): 77–83. doi:10.1016/j.jneumeth.2012.08.012.

Construction and Validation of Lentiviral Vector Carrying Rat Neuronal Nitric Oxide Synthase *In Vitro* and *In Vivo*

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Abstract

In the present study, we developed a lentiviral vector with human cytomegalovirus promoter permitting high-level of nNOS expression. Neuronal cell line NG108 was used as an *in vitro* model to check the validity of gene transfer. The cells were infected with lenti-EGFP or lenti-nNOS particles for 24h. Lenti-nNOS infection in the NG108 cells induced dose dependent increase in mRNA and protein for nNOS; with a dose of 2.5×10^4 pfu/ml, nNOS mRNA expression increased by 40-fold while protein expression was increased by 2.5-fold compared to lenti-EGFP. Moreover, lenti-nNOS infection caused a greater increase in nNOS immunoreactivity in NG108 cells compared to lenti-EGFP as shown by immunocytochemistry. nNOS expression showed time dependent increases with lenti-nNOS infection with maximum up-regulation observed after two weeks of infection. Moreover, *in vivo*, unilateral injection of lenti-nNOS into the paraventricular nucleus (PVN) of rats induced a 27-fold increase of nNOS protein level in the injected side compared to non-injected side and this escalation was sustained up to three weeks. Overall, lenti-EGFP injection in the PVN did not show any significant change in nNOS expression. Furthermore, NADPH-diaphorase staining of nNOS in the PVN infected with lenti-nNOS induced a visible increase in nNOS expression compared with contralateral non-injected side up to three weeks. These results indicate that this approach of lentiviral mediated gene transfer of nNOS may provide a new means to up-regulate the nNOS expression for longer periods of time compared to Adeno-viral transfection and can be used as a research tool and potentially a therapy for chronic diseases involving impaired nNOS expression.

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Conflict of interest

None

Disclosures

None

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Keywords

lentivirus; nNOS; PVN; heart failure

Introduction

Nitric oxide (NO) synthesized by nitric oxide synthase (NOS) enzymes has a variety of biological actions both positive and negative under physiological and pathological conditions. Neuronal NOS (nNOS) is constitutively expressed in variety of neuronal cells, produces NO, which can serve as a retrograde second messenger for neuron-to-neuron communication. Malfunctioning of NOS enzymes is associated with various pathological conditions, including hypertension (DiCarlo et al., 2002; Node et al., 1997), heart failure (Li and Patel, 2003), diabetes (Kurioka et al., 2000; Zheng et al., 2006), coronary arteriosclerosis (Tanaka et al., 1997) and metabolic diseases in humans (Sun et al., 2006). In rats with heart failure, we have observed downregulation of nNOS as well as reduced NO mediated sympatho-inhibition mediated by the paraventricular nucleus (PVN) of the hypothalamus (Li and Patel, 2003; Sharma et al., 2011).

PVN is connected to areas of brain involved in the control of cardiovascular functions (Kannan and Yamashita, 1985) and is known to be a site of integration for autonomic and endocrine mediated cardiovascular responses (Swanson and Sawchenko, 1983, 1980). In our previous studies, we evaluated the effect of gene transfer of nNOS using adenoviral vector into the PVN (Li et al., 2002). Adeno.nNOS mediated up-regulation of nNOS expression in the PVN inhibits increase in renal sympathetic nerve activity in rats with heart failure. Although the adenoviral vectors provide a tool for localized overexpression of nNOS protein in the PVN, the efficiency in transferring the nNOS gene into the cells within the PVN is relatively short term, limiting this method of gene transfer to acute studies (Li et al., 2002).

Lentiviral vectors efficiently transduce both dividing and post-mitotic cells, and stably integrate into the host genome allowing long-term expression of the transgene, and therefore documented as good vehicles for long term gene delivery (Dittgen et al., 2004; Naldini et al., 1996; Van den Haute et al., 2003). In the present study, we describe a novel method of long-term up-regulation of nNOS using lentiviral system in the PVN of rat. To date, there is no report for the use of lentivirus to over-express nNOS in the PVN of rats. This method for overexpressing nNOS in the brains of rats will be a very useful tool to investigate the consequence/s of nNOS up-regulation in chronic diseases such as hypertension, heart failure, diabetes, neurodegenerative diseases etc. where the NO has been suggested to play a pathophysiological role.

Methods

Animals

The University of Nebraska Medical Center Institutional Animal Care and Use Committee approved all procedures utilized in this study, and the experiments were conducted according to the APS "Guiding Principles for Research Involving Animals and Human Beings". Male Sprague-Dawley rats weighing 280–300g were obtained from Sasco Breeding Laboratories (Omaha, NE). Each rat was caged individually in an environment with ambient temperature maintained at 22°C and humidity at 30–40%. Laboratory chow (Purina) and tap water were available *ad libitum*.

Cell Culture

The NG108-15 (ATCC HB-12317), a neuronal cell line, was grown in DMEM (high glucose Dulbecco's Modified Eagle's medium) complemented with 10% fetal bovine serum (FBS), penicillin G and streptomycin in a humidified atmosphere of 5% CO₂ at 37°C as described earlier (Sharma et al., 2011).

nNOS-lentiviral Vector Production and Titration

Total RNA was isolated from the PVN of rat using an RNeasy kit (Qiagen, Valencia, CA). RNA was reverse-transcribed to cDNA using a Verso cDNA kit (Thermo Scientific, Foster City, CA) with verso enzyme mix and random primers. The product of this reaction was amplified using nNOS forward primer (5'-CACCGATACCATGGAAGAGAAC-3') and a reverse primer (5'-GGAGCTGAAAACCTCATCTGCG-3'). Amplified nNOS cDNA was ligated into the pLenti6/V5-D-TOPO vector using invitrogen pLenti6/V5-D-TOPO cloning kit as per the manufacturer guidelines. The forward primer contains CACC sequence at 5' end for directional cloning. The four nucleotides CACC base pair with the overhang sequence GTGG in pLenti6/V5-D-TOPO vector. Briefly the vector has human cytomegalovirus (CMV) immediate/early promoter permitting high-level expression of nNOS, rous sarcoma virus (RSV) promoter for tat independent production of viral mRNA in the producer cell line and modified HIV-1 5' and 3' long terminal repeats (LTR) for viral packaging and reverse transcription of the viral mRNA. The new vector, designated as pLenti-nNOS, was sequenced using CMV forward (5'-CGCAAATGGGCGGTAGGCGTG-3') and V5 (C-term) reverse (5'-ACCGAGGAGAGGGTTAGGGAT-3') primers to confirm the validity of the polymerase chain reaction (PCR), amplification and ligation. Lentiviral particles containing plenti-nNOS were generated using Vira Power Lentiviral Expression System (Invitrogen, Carlsbad, Calif., USA) in Gene Transfer Vector Core facility, University of Iowa. Briefly, pLenti-nNOS was co-transfected with ViraPower Packaging Mix constituting pLP1 plasmid (containing the gag/pol), pLP2 plasmid (encoding the Rev protein) and pLP/VSVG (encoding the envelope protein) into 293FT packaging cell line (Invitrogen). Culture medium was collected at 48 h after transfection for 3 consecutive days at 24-hour intervals. The supernatant was centrifuged at 25,000 g for 90 min. The viral vector pellet was re-suspended in Hanks' balanced salt solution, aliquoted, titered and kept frozen at -80°C. Lentiviral vector expressing the enhanced green fluorescence protein (Lenti-EGFP) via human CMV promoter was kindly supplied by Gene Transfer Vector Core facility, University of Iowa.

Overexpression of nNOS in NG108 Cells via Lentiviral Infection

NG108 cells were infected with increasing 2.5×10^4 to 1×10^5 plaque-forming units (pfu) per cell of lentiviral vectors carrying either nNOS (lenti-nNOS) or EGFP (lenti-EGFP; as a control vector) tagged to human CMV promoter in serum-free medium for 24 h. Cultures were also continued for 1 to 3 weeks with the dose of 2.5×10^4 pfu/ml of lenti-nNOS particles.

In vivo Lentivirus Injection

Lenti-nNOS or lenti-EGFP suspended in CSF was delivered into the PVN of rats by microinjection unilaterally. Rats were assigned to two groups, lenti-nNOS and lenti-EGFP. For the transduction of lentivirus in the PVN, rats were anesthetized with cocktail (47.6mg ketamine, 11.9mg xylazine, 1.2mg acepromazine, 0.006mg atropine/kg body weight) and then placed in a stereotaxic apparatus (Davis Kopf Instruments). The coordinates for the PVN (1.5 mm posterior to bregma, 0.4 mm lateral to the midline, and 7.8 mm ventral to the dura) were determined from the atlas of Paxinos & Watson (Paxinos and Watson, 1986) and

used previously (Leite et al., 2012; Zheng et al., 2011b). A cannula (outer diameter, 0.2mm) connected to a micro-syringe (Hamilton) was advanced into the right side of the PVN. A 100nl solution (final concentration 2.5×10^4 pfu/ml) of lenti-nNOS or lenti-EGFP was injected into the PVN over a 10-minute period. After the injection, the needle was left in place for additional 5–10 minutes. Following the surgical procedure, analgesic (Buprenex 0.01–0.05 mg/kg body weight, sc) was administered to the rats on each of the next 2 days. No damage to the neurons within the PVN was observed as evaluated by light microscopy (Li et al., 2002).

Micropunch of the PVN

Lentiviral infected rats were euthanized by overdose of pentobarbital sodium (150 mg/kg, ip) and the brains were removed and quickly frozen on dry ice. Six coronal sections (100 μ m each) of the brain were cut using a cryostat following the Palkovits technique (Palkovits and Brownstein, 1983). The PVN was punched on injected as well as on non-injected side using a diethylpyrocarbonate-treated blunt 18-gauge needle attached to a syringe (Li et al., 2006).

Real-time PCR

Tri-Reagent (MRC) was used to isolate total RNA from NG108 cells as per the manufacturer's instructions (Kleiber et al., 2010). Following extraction of RNA, cDNA was produced using 100 U MMLV-RTase in the presence of 1.5 M random hexamers at 37°C for 40 min. Real-time PCR measurements were made using the iCycler iQ Multicolor Real-Time Detection System with output to a computer-based acquisition system (Bio-Rad) as described earlier (Sharma et al., 2011). The sequence of primers used for amplification of nNOS and ribosomal protein L19 (rpl19) were 5'-ATCACAAGCCTATGCCAAGACC-3' (F), 5'-TCTCCATTGCCAAAGGTGCTG-3' (R) 5'-CCCCAATGAAACCAACGAAA-3' (F), 5'-ATGGACAGTCACAGGCTTC-3' (R), respectively. Relative expression nNOS was calculated using the $2^{-\Delta\Delta C_T}$ method, which relates expression of the target gene to expression of a reference gene (Livak and Schmittgen, 2001).

Western blot

Protein was extracted from NG108 cells or PVN punches in 100 μ l of extraction buffer (10 mM Tris, 1 mM EDTA, 1% SDS, 0.1% Triton X-100 containing complete protease inhibitor cocktail). Bicinchoninic acid method with bovine serum albumin as standard (Pierce, Rockford, IL) was used to estimate the protein content of lysate. SDS loading buffer (63mM Tris-HCL, 10% glycerol, 2% SDS, 0.0025% bromophenol blue pH 6.8) was added after normalization the concentration of protein in each sample with 1% SDS. Protein lysates (20–30 μ g) were fractionated on 7.5% SDS-polyacrylamide gel and electrophoretically transferred onto PVDF membranes (Sharma et al., 2011). Membrane was incubated overnight with mouse monoclonal nNOS (0.4 μ g/ml) from Santa Cruz at 4°C after blocking with 5% nonfat dry milk (w/v) in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20) for 1h. Blots were washed three times with TBS-T and then incubated with horseradish peroxidase conjugated anti-rabbit IgG (1:3333, Pierce) at room temperature for 1h. Enhanced chemiluminescence substrate (Pierce), followed by an exposure within an Epi Chemi II darkroom (UVP Bio imaging, Upland, CA) was used for visualization of protein bands. NIH Image J program was used to highlight the bands and quantify the signal.

Immunofluorescent Staining of nNOS

NG108 cells were grown on laminin coated 6mm Transwel-ClearTM inserts (Corning, Costar) and then treated with 2.5×10^4 pfu/ml of lenti-nNOS particles. Cells were permeabilized with 0.2% Triton X-100 for 20–30 min after fixation in 4% paraformaldehyde in PBS for 10 min.

Following blocking with 10% goat serum for 1h, the cells were incubated with anti mouse monoclonal nNOS (sc-5302) (1.0 μ g/ml) from Santa Cruz Biotechnology overnight at 4°C. After washing with PBS, the cells were incubated with fluorescence-conjugated secondary antibodies for 1h at room temperature. Fluoromount G (Southern Biotechnology) was used to mount the coverslips onto frosted glass microscope slides. Leica-DMR microscope with corresponding filters was used to observe the slides.

NADPH-diaphorase Histochemistry

Sections of the PVN were stained for NADPH-diaphorase activity to demonstrate the presence and distribution of functional NOS protein after one, two and three weeks of lenti-nNOS infection by previously described method (Li et al., 2005). Briefly, the rats were deeply anesthetized with pentobarbital sodium (65 mg/kg, ip) and perfused transcardially with 150 ml of heparinized saline as a vascular rinse, followed by 250 ml of freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brain was removed from each rat and placed in 20% sucrose for 24 h after post-fixing at 4°C for 4 h in 4% paraformaldehyde solution. The brain was blocked in the coronal plane and sectioned at 30 μ m thickness in a cryostat. The sections were collected in 0.1 M phosphate buffer (pH 7.4) containing 0.3% Triton X-100, 0.1 mg/ml nitroblue tetrazolium, and 1.0 mg/ml β -NADPH. The sections in nitroblue tetrazolium solution were then incubated at 37°C for 60 min. After the reaction, the sections were rinsed in phosphate buffer (pH 7.4) and mounted onto chrome-alum-coated slides. The slides were air dried overnight, rinsed in distilled water, and then dried again. Coverslips were then mounted directly with Permount. The intensity of staining was assessed using Kodak 1D Image Analysis software.

Statistics

The data was expressed as mean $\pm$ SEM and statistical significance was set at P 0.05. Differences between groups were determined by a one-way or two-way ANOVA followed by the Newman-Keuls test for *post hoc* analysis of significant differences between individual groups.

Results

Expression of nNOS in NG108 cell line infected with lenti-nNOS

NG108 cells were treated with three different doses (2.5×10^4 , 5×10^4 , 1×10^5 pfu/ml) of lenti-EGFP or lenti-nNOS and changes in nNOS mRNA expression were analyzed by real time PCR after 24h (Figure 1). Relative nNOS mRNA expression normalized to rpl19 and calculated as ratio to control without any treatment was not significantly different in lenti-EGFP treated cells. On the contrary, in lenti-nNOS treated cells, nNOS mRNA expression is about 40 fold increased without any significant difference between the three doses (2.5×10^4 : 42.56 ± 13.55 ; 5×10^4 : 44.53 ± 11.09 ; 10^5 : 41.38 ± 12) in comparison to both control and lenti-EGFP (2.5×10^4 : 0.69 ± 0.19 ; 5×10^4 : 0.41 ± 0.13 ; 10^5 : 0.72 ± 0.50) (Figure 1). Parallel to the mRNA expression, infection of NG108 cells with increasing doses of lenti-nNOS also revealed an increase in nNOS protein expression (Figure 2). nNOS protein expression was upregulated significantly even at the lower concentrations of lenti-nNOS compared with the control (without treatment) as well as with lenti-EGFP infected. At the doses of 2.5×10^4 and 5×10^4 , 2-fold increase in nNOS protein expression was observed compared with the control without any treatment and 2.5 fold as compared to lenti-EGFP treatment. To further confirm the upregulation of nNOS after lentiviral infection, nNOS immunofluorescent staining was performed in lenti-EGFP-treated (Figure 3A) and lenti-nNOS-treated (Figure 3B) cells after 24h of infection. Consistent with the Western blotting, nNOS staining was pronounced in lenti-nNOS treated cells compared to lenti-EGFP cells suggesting that lentiviral nNOS infection up-regulate the expression of nNOS in NG108 cell line.

Expression of nNOS in NG108 cell line infected with lenti-nNOS for 1–3 weeks

We evaluated the nNOS expression pattern in NG108 cells treated with lenti-EGFP or lenti-nNOS for one, two or three weeks (Figure 4). Western blot analysis showed progressive increase in expression of nNOS in lenti-EGFP as well as lenti-nNOS infected cells up to two weeks and then it starts decreasing.

Overexpression of nNOS using lenti-nNOS in the PVN

We next examined whether lenti-nNOS can up-regulate nNOS expression in the PVN of rats. NADPH diaphorase histochemistry was performed in the PVN of rats microinjected with lenti-nNOS or lenti-EGFP (2.5×10^4 pfu/ml) unilaterally to check the efficacy and distribution of gene transfer within the PVN. Representative pictures showing the difference in staining of the uninfected versus lenti-nNOS infected PVN are shown in the Figure 5. The injected side of the PVN infected with lenti-nNOS induced an increase in NADPH-diaphorase staining compared with contralateral non-injected side (Figure 5A). Cumulative data showed that the ratio of intensity of injected to non-injected site peaked after one week of lenti-nNOS injection (4.1-fold) and was sustained even after three weeks of infection (2.1-fold) (Figure 5B). To quantify this up-regulation more vigorously, nNOS expression was monitored via Western blotting in the PVN punches of lenti-nNOS or lenti-EGFP injected rats (Figure 6) after one, two or three weeks of infection. Lenti-EGFP transduction after one week of infection caused an increase in nNOS gene expression compared to contralateral non-injected side (1.28 ± 0.57 vs. 0.25 ± 0.07). On the other hand, the PVN lysates of non-injected side and contralateral injected side of lenti-EGFP had similar increase in nNOS protein levels after 2 and 3 weeks of infection. Lenti-nNOS transduction after 1 week of infection caused approximately 27-fold increase in nNOS gene expression compared to contralateral noninjected side (4.19 ± 1.47 vs. 0.16 ± 0.07). Such an increase remained significantly high even at three weeks of infection (1.94 ± 0.73 vs. 0.32 ± 0.12). The non-injected side of the PVN from rats infected with lenti-EGFP on the contralateral side expressed similar nNOS protein to the non-injected side of the PVN from rats infected with lenti-nNOS on the contralateral side. The injected side of the PVN infected with lenti-nNOS induced a significant increase in nNOS protein expression in comparison with lenti-EGFP injected side of the PVN after one and two weeks (3.70 ± 0.70 vs. 1.49 ± 0.55) of infection.

Discussion

In this study we developed and validated a lentiviral-based method of efficient delivery of nNOS in the PVN of rats. This study demonstrates that lentiviral delivery of nNOS can efficiently be used for nNOS up-regulation in the PVN for a longer period of time, i.e. 3 weeks. This system will be very valuable to study the regulatory role played by nNOS in chronic diseased conditions such as heart failure (Kleiber et al., 2008), hypertension (DiCarlo et al., 2002; Node et al., 1997; Wang et al., 2004) and diabetes (Kurioka et al., 2000; Zheng et al., 2006).

The NG108-15 (neuroblastoma X glioma hybrid) cell line used in the present study has neuronal properties and has endogenous expression of nNOS (Grant et al., 2001; Sharma et al., 2011; Zheng et al., 2011a) and, therefore, serves as good *in vitro* model system for studying the mechanism of activation of nNOS in diseased conditions involving compromised levels of nNOS. Lentivirus vector for nNOS administered to NG108 cells line significantly increase the nNOS protein expression as soon as after 24h following the infection, being this effect not related with the dose. In fact, when the concentration reached 1×10^5 pfu/ml there was no longer a significant increase in nNOS in the cell line. It may well be that, as described with neural stem cells (Abdellatif et al., 2006; Englund et al., 2000), as

viral loads increase, nuclear entry and integration of lentivirus may be limited, leading to decreased transgene production. Therefore, 2.5×10^4 pfu/cell was found to be the optimum concentration for NG108-15 cells, and thus this dosage was also used for *in vivo* microinjections. The nNOS immunofluorescent staining of cells treated with such concentration also detected an abundance of nNOS in lenti-nNOS treated cells compared to lenti-EGFP cells, suggesting an up-regulation in the expression of nNOS. Such up-regulation most likely occurs at the stage of transcription as indicated by equivalent changes in nNOS mRNA level that are consistent with changes in protein expression level. Taking all these results together, our results support the concept that lenti-nNOS integrated in the genome of target cells (Dittgen et al., 2004; Naldini et al., 1996) allowing for high-level expression of nNOS.

We have also observed an increase in nNOS expression with lenti-EGFP infection after 2 weeks of infection in NG108 cells. As mentioned above, the NG108 cells have endogenous expression of nNOS (Sharma et al., 2012), therefore, it is reasonable to suggest that increased differentiation and aging of neurons may be the cause of increase in nNOS expression after lenti-EGFP treatment. It should be noted that following three weeks of treatment with either lenti-nNOS or lenti-EGFP, the expression of nNOS decreased significantly probably as a consequence of cell death associated with extended time of culture. As cells develop, nutrients are depleted, waste products accumulate and pH decreases to intolerable levels. Even the implementation of periodic media exchanges may be ineffective in preventing the oscillation in pH and nutrient concentrations as well as accumulation of waste products in long-standing cultures, resulting in media instability and cell death (Dhurjati et al., 2006).

Our *in vitro* cell data from NG108 cells correlate well with results from *in vivo* studies in the PVN of whole animal. NADPH-diaphorase staining of sections of PVN reveals significant enhancement of positively stained neurons in the lenti-nNOS injected side of the PVN compared with contralateral non-injected side after one week of infection and this was sustained even after three weeks. This histochemical technique is based on the ability of NOS enzymes to catalyze the reduction of pale-yellow nitroblue tetrazolium to dark colored insoluble formazon compound in NADPH manner. The presence of functional NOS coincides with that of NADPH diaphorase activity, therefore, NADPH diaphorase staining is widely used to detect NOS-containing cells in neural and non-neural tissues (Dellacorte et al., 1995). Our Western blotting data are consistent the diaphorase staining data demonstrating that the increase in nNOS with lenti-nNOS injection in the PVN peak after one week of infection, being also high up to three weeks. Consequently, our study has demonstrated good efficacy and distribution of lentiviral vector harboring nNOS for long-term nNOS up-regulation in the PVN of rats.

Similar to the present findings, previous work from our laboratory has demonstrated increased NADPH-diaphorase stained cells in culture of fibroblasts for 72 hours after infection with adeno.nNOS (Li et al., 2002). More recent findings demonstrate that the intensity of nNOS staining was increased in NG108 cells also infected with adeno.nNOS (10^4 to 10^8) for 24 hours (Sharma et al., 2012; Zheng et al., 2011a). Nevertheless, time course results *in vivo* demonstrated that there was slight change in NADPH-diaphorase in the injected PVN of rats treated with adeno.nNOS for 1 day, a robust increase after 3 and 5 days, but a decrease 6 days after the infection when comparing the contralateral uninfected PVN (Li et al., 2002; Zheng et al., 2011a). Taken together, these data indicate that adeno.nNOS is efficient in transferring the nNOS gene in the PVN, however, for a shorter period of time, perhaps a week. Other studies suggest that extended inflammatory response triggered after infection with high concentrations of adenovirus is responsible for ceasing

cells transgene expression over 2 weeks after administration or inducing cell degeneration (Abdellatif et al., 2006; Hermens and Verhaagen, 1997).

Based on the current data and on our previous studies with adenovirus (Li et al., 2002; Sharma et al., 2012; Zheng et al., 2011a) no difference in the ability of lentivirus and adenovirus to infect the PVN was verified. Lentiviral delivery of nNOS to mammalian cells in culture or *in vivo* is advantageous over traditional adenoviral-based systems because it provides stable expression of a target gene with minimal inflammatory response and tissue damage, making this approach ideal for situations that require long term delivery of therapeutic molecules to the CNS (Abdellatif et al., 2006). Most retroviral vectors are limited in their usefulness as gene delivery vehicles by their restricted tropism (host cell specificity) and generally low titers (lowest concentration of virus that still infects cells). We used Vira Power Lentiviral Expression System to generate lentiviral particles containing plenti-nNOS package that utilize G glycoprotein gene from Vesicular Stomatitis Virus as a pseudotyping envelope. pLP/VSVG, thus, allows the production of a high titer lentiviral vector with a significantly broadened host cell range (Burns et al., 1993). The limitation of this technique is that such a lentiviral transfection was not specific for neurons but infected all cells types including glia and endothelial cells. The use of specific promoters in the future would increase the specificity of the targeted cells within the PVN.

There is a fair amount of evidence to indicate that decreased levels of nNOS in the PVN during disease states, such as heart failure, hypertension etc, leads to increased sympatho-excitation (Li et al., 2006). NO synthesized mainly by nNOS in the PVN, a major site that directly controls sympathetic outflow, has also been suggested to play an important role in the regulation of cardiovascular functions (Swanson and Sawchenko, 1983, 1980; Zheng et al., 2011b). Increased nNOS concentration in the PVN via adeno.nNOS was shown to reduce sympathetic outflow (Li et al., 2002). In addition, in our recently published study, increased expression of nNOS using adeno.nNOS transduction in neuronal cell line significantly decrease intracellular calcium concentration in responses to angiotensin II, suggesting an important modulating effect of NO on AT₁R expression at a cellular level (Sharma et al., 2012). nNOS/AT₁R interaction indicate a possible mechanism for the increase in AT₁R and subsequent increase in sympathetic outflow in disease states such as CHF and hypertension and may provide a potential therapeutic target. Considering that the effect of lenti-nNOS infection on nNOS expression in the PVN is similar to adenovirus infection, its influence on sympathetic activity seems predictable, but is yet to be determined. Still, the manipulation of NO in the CNS with lenti-nNOS, which induces long-term expression of nNOS, provide a potentially important candidate gene for cardiovascular gene therapy in chronic diseases states lacking central nNOS, such as heart failure and hypertension.

Here we have demonstrated that lentivirus mediated nNOS gene transfer in the PVN produces high level of nNOS and this expression is sustained for 3 weeks at least. Therefore, lenti-nNOS injection into the PVN may provide a novel approach to inhibit increased sympathoexcitation in the PVN of pathological conditions involving compromised nNOS levels.

Acknowledgments

The technical assistance of Dr. Xuefei Liu and Dr. Lirong Xu is greatly appreciated.

Sources of Funding

Funding from NIH grant HL62222, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) supported this work.

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Highlights

We developed a lentiviral vector with human cytomegalovirus promoter to express nNOS.

Lenti-nNOS infection up-regulates the expression of nNOS *in vitro* and *in vivo*.

Lenti-nNOS infection showed time dependent increase in expression of nNOS.

Lenti-nNOS can be used as a therapy for diseases known to have impaired nNOS expression.

Male Sprague-Dawley rats (Sasco Breeding Laboratories, Omaha, NE) were fed and housed according to approved guidelines of the University of Nebraska Medical Center Institutional Animal Care and Use Committee, which confirms to the guidelines for the care and use of laboratory animals of the National Institutes of Health and the American Physiological Society. Anesthesia, surgery, postoperative care, maintenance, and euthanasia of animals were carried out according to Guiding Principles for the Care and Use of Animals approved by the Council of the American Physiological Society, and by policies and procedures approved by the American Association for the Accreditation of Laboratory Animal Care.

Figure 1.

Real time PCR analysis of nNOS in NG108 cells treated with 3 concentrations of lenti-EGFP and lenti-nNOS for 24h. Values are mean $\pm$ SE of three different experiments. * Significantly different from control without any treatment; # Significantly different from corresponding dose between different groups, $P < 0.05$.

Figure 2. nNOS expression in NG108 cells treated with 3 concentrations of lenti-EGFP and lenti-nNOS for 24h. Top: a representative Western blot, bottom: densitometric analyses of nNOS level normalized to GAPDH. Values are mean±SE of four different experiments. * Significantly different from control without any treatment; # Significantly different from corresponding dose between different groups, P<0.05.

Figure 3. Representative immunofluorescent photomicrographs of Lenti-EGFP (A) and Lenti-nNOS (B) (2.5×10^4 pfu/ml) treated NG108 cells for 24h and stained for nNOS (green). Blue spots show the nucleus stained by 4', 6'-diamidino-2-phenylindole (DAPI)

Figure 4.

Time course of nNOS in lenti-EGFP-treated (A) and lenti-nNOS-treated (B) NG108 cells after 1, 2 and 3 weeks. Top: a representative Western blot, bottom: densitometric analyses of nNOS level normalized to GAPDH. Values are mean $\pm$ SE of four different experiments. * Significantly different from control without any treatment, $P < 0.05$.

Figure 5.

(A) Representative histochemical photomicrographs of NADPH-diaphorase labeled neurons (blue staining) in the PVN at similar coronal level. Left side: lenti-nNOS injected side. Scale bar=200µm. 3V: third ventricle. (B) Ratio of nNOS intensity in the PVN of two independent experiments. Values expressed as mean $\pm$ SEM, n=2 rats. NI: non-injected side; I: injected side.

Figure 6.

Time course of nNOS in the PVN of rats microinjected unilaterally with lenti-EGFP and lenti-nNOS after 1, 2 and 3 weeks. Values expressed as mean $\pm$ SEM, n=4 rats. NI: non-injected side; I: injected side. * Significant difference from non-injected side within the same week and group; # Significant difference from corresponding PVN side and week between different groups, P<0.05.