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# A microRNA embedded AAV alpha-synuclein gene silencing vector for dopaminergic neurons

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## Abstract

Alpha-synuclein (SNCA), an abundantly expressed presynaptic protein, is implicated in Parkinson disease (PD). Since over-expression of human SNCA (hSNCA) leads to death of dopaminergic (DA) neurons in human, rodent and fly brain, hSNCA gene silencing may reduce levels of toxic forms of SNCA and ameliorate degeneration of DA neurons in PD. To begin to develop a gene therapy for PD based on hSNCA gene silencing, two AAV gene silencing vectors were designed, and tested for efficiency and specificity of silencing, as well as toxicity *in vitro*. The same hSNCA silencing sequence (shRNA) was used in both vectors, but in one vector, the shRNA was embedded in a microRNA backbone and driven by a pol II promoter, and in the other the shRNA was not embedded in a microRNA and was driven by a pol III promoter. Both vectors silenced hSNCA to the same extent in 293T cells transfected with hSNCA. In DA PC12 cells, neither vector decreased expression of rat SNCA, tyrosine hydroxylase (TH), dopamine transporter (DAT) or the vesicular monoamine transporter (VMAT). However, the mir30 embedded vector was significantly less toxic to both PC12 and SH-SY5Y cells. Our *in vitro* data suggest that this miRNA-embedded silencing vector may be ideal for chronic *in vivo* SNCA gene silencing in DA neurons.

### Keywords

Parkinson's disease; tyrosine hydroxylase; vesicular monoamine transporter; gene therapy; cell death; neurodegeneration

## Introduction

Parkinson's disease (PD), the second most prevalent neurodegenerative disease, is a movement disorder characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta. There is no effective therapy that slows or reverses the pathogenesis of PD. Recent studies suggest that α-synuclein (SNCA), abundantly expressed in presynaptic terminals, plays a role in both sporadic and familial forms of PD. SNCA, a highly conserved 140 amino acid phosphoprotein, naturally associates with biomembranes



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and is involved in vesicle trafficking (Auluck et al.; Kahle et al., 2000; Ueda et al., 1993; Volles et al., 2001). SNCA protein is prone to aggregation and forms oligomers, fibrils and high molecular weight beta-sheets, however, it is not clear which form underlies the toxicity of SNCA to DA neurons although most evidence favors SNCA oligomers as the toxic forms (Cookson and van der Brug, 2008; Wood et al., 1999). Aggregated SNCA protein is the major component of Lewy bodies (LBs), the characteristic histopathological hallmark of PD brain tissue observed in over 90% of sporadic PD cases (Spillantini et al., 1998; Trojanowski and Lee, 1998). The association of SNCA to PD has been further revealed by the discovery of three point mutations (A53T, A30P and E46K), duplications and triplications, as well as promoter variations in the *SNCA* gene in families with a history of PD (Kruger et al., 1998; Maraganore et al., 2006; Polymeropoulos et al., 1997; Zarranz et al., 2004).

In addition to the clear implication of SNCA to PD in humans, the importance of increased levels of SNCA expression to PD pathology is borne out by several experimental models of PD. In mice treated with the PD neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the level of endogenous SNCA expression in substantia nigra is elevated and is associated with death of DA neurons (Kuhn et al., 2003). Neuronal inclusions of aggregated SNCA associated with DA neuronal cell death and motor deficits have also been reported in some, but not all, lines of SNCA transgenic mice (Fernagut and Chesselet, 2004; Giasson et al., 2002; Richfield et al., 2002; van der Putten et al., 2000), as well as in *Drosophil*a *Melanogaster* expressing human SNCA (hSNCA) (Feany and Bender, 2000). Experimental expression of hSNCA following injection of viral vectors into rat or non-human primate substantia nigra also leads to degeneration of DA neurons and motor deficits (Kirik et al., 2002; Kirik et al., 2003). In addition, an age-dependent increase in SNCA expression in nigral DA neurons that correlates with loss of tyrosine-hydroxylase (TH), the rate-limiting enzyme in DA synthesis has been reported to occur in both human and monkey (Chu and Kordower, 2007).

Therapeutic approaches designed to interfere with the toxic action of SNCA is an active area of research. Down-regulation of SNCA expression using a ribozyme has been shown to protect DA neurons against apoptosis(Hayashita-Kinoh et al., 2006). Another approach is to interfere with expression of wild type or mutant SNCA using RNA interference (RNAi), the highly conserved gene silencing process mediated by endogenous microRNAs (miRNAs) which has been exploited recently to study gene function using exogenously administered synthetic small interfering RNAs (siRNAs), short-hairpin RNAs (shRNAs) or artificial miRNAs (Chang et al., 2006; Davidson and Boudreau, 2007; Rodriguez-Lebron and Paulson, 2006). The RNAi gene silencing technique has been developed further as a potential therapeutic or experimental tool for diseases of the CNS, such as PD (Fishman-Jacob et al., 2009; Fountaine and Wade-Martins, 2007; McCormack et al., 2010; Sapru et al., 2006; Ulusoy et al., 2009), Huntington's disease (HD) (Harper et al., 2005), and others (Boudreau and Davidson, 2010). On the other hand, recent studies have revealed that some shRNA silencing vectors induce cell death *in vivo* (Boudreau et al., 2009; McBride et al., 2008) due to saturation of the endogenous RNAi system, which is mitigated by using artificial miRNA silencing vectors (Boudreau et al., 2009; McBride et al., 2008). In order to study the potential of SNCA gene silencing as a therapy for PD, it is therefore critical to identify silencing vectors that are not toxic to DA neurons. We report here the characterization of a silencing vector that specifically silences human, but not rat SNCA or markers of the DA phenotype, and that has negligible toxicity to DA cells *in vitro*.

## Results

### Efficiency of exogenous SNCA silencing in 293T cells

SNCA silencing cassettes were designed and cloned into adeno-associated virus (AAV) shuttle plasmids (Fig. 1a). The same hSNCA-specific short-hairpin silencing and loop sequence (shRNA) targeting (Sapru et al., 2006) was used in both vectors. This silencing sequence is homologous to hSNCA, but has three mismatches with rat and mouse nucleotides 288-309 of hSNCA as described previously SNCA. In one vector, the shRNA was embedded in a microRNA backbone and driven by a pol II promoter, CMV (pAAV-CMV-mir30-shRNA-SNCA). In the other vector, the shRNA was not embedded in a microRNA and was driven by the pol III promoter, H1. Non-silencing mir-30 embedded and non-embedded firefly luciferase (Luc) shRNA AAV shuttle plasmids and mock transfected cells were used as controls.

We first tested efficiency of mir30-shRNA and shRNA constructs for silencing of human SNCA. HEK293T cells were co-transfected with pAAV-CBA-hSNCA and pAAV-CMV-mir30-shRNA-SNCA or pAAV-H1-shRNA-SNCA. As controls, cells were transfected with pAAV-CBA-hSNCA alone or with pAAV-CMV-mir30-shRNA-NS or pAAV-H1-shRNA-Luc. After 72 hours, cells were harvested and the level of human SNCA expression was analyzed by western blotting using a human specific SNCA antibody and normalized to ß-actin. As shown in Figure 1b, both SNCA silencing vectors significantly silenced exogenous human SNCA in 293T cells (~75% reduction) to a similar extent while control shRNA vectors had no effect. These results are in agreement with our previous report that this same shRNA-SNCA effectively silences hSNCA *in vitro* and *in vivo* in rat striatum in the context of a lentivirus (Sapru et al., 2006).

### Silencing specificity of silencing vectors in DA neuronal cell lines

To examine the specificity of the AAV silencing vectors, western blotting for protein levels (Fig. 2a) and quantitative real-time RT-PCR for mRNA levels (Fig. 2b) of rat SNCA and phenotypic markers of DA neurons, including TH, phosphorylated TH, dopamine transporter (DAT) and/or the vesicular monoamine transporter expressed by PC12 phenochromocytoma, VMAT1, were determined. Rat SNCA expression was not significantly silenced in rat PC12 cells at either the protein or mRNA level by either vector design, indicating that silencing was specific to hSNCA. Moreover, no significant off-target silencing on expression of TH, phosphorylated TH, DAT or VMAT1 was observed.

The silencing effectiveness of mir30shRNA and shRNA vectors against endogenous hSNCA was tested in the human dopaminergic neuroblastoma cell line, SH-SY5Y. As shown in Fig.3, both the mir30-embedded and non-embedded shRNA-SNCA silenced endogenous human SNCA at 72 hours post transfection by approximately 60%, $p \leq 0.001$. In addition, as in PC12 cells, the expression of TH and phosphorylated TH were not affected by experimental or control silencing vectors.

These data suggest that either design of AAV shuttle plasmid displays appropriate specificity toward hSNCA gene silencing. Importantly, off target silencing of genes critical to DA neuronal function was not observed, although it is possible that off target silencing of other genes not studied here might have occurred.

### The mir30-embedded shRNA construct is less toxic than the un-embedded shRNA vector in DA neuronal cells

We also tested the effects of the silencing vectors on PC12 cell viability using an Alamar Blue assay. Findings were replicated in a duplicate experiment. Compared to mock

transfected cells, viability of cells transfected with the pAAV-H1-shRNA-SNCA was significantly decreased by 31% at 48 hrs and by 36%, at 72 hrs ($p \leq 0.001$, experiment 1; Fig. 4). Moreover, cell viability was also decreased, albeit to a lesser extent, in cells transfected with the H1-shRNA-Luc control vector by 15% at 48hrs and 17% at 72 hrs ($p \leq 0.01$; experiment 1; $p < 0.001$, experiment 2). In contrast, cell viability in cells transfected with the mir30 embedded shRNA NS or shSNCA vectors was not significantly different from that of mock transfected cells (Fig.4). The mir30-embedded SCNA silencing vector was significantly less toxic than the non-embedded vector at 48 and 72 hrs, $p \leq 0.001$.

Cell viability was further studied in duplicate experiments in SH-SY5Y cells transfected with pAAV-CMV-mir30-shRNA-NS, pAAV-CMV-mir30-shRNA-SNCA, pAAV-H1-shRNA-Luc or pAAV-H1-shRNA-SNCA. In contrast to the results in PC12 cells, silencing endogenous hSNCA in SH-SY5Y cells with either vector design resulted in significantly decreased cell viability (Fig 5). Nevertheless, the mir30 embedded silencing vector was significantly less toxic than the non-embedded vector in this cell type also ($p \leq 0.001$).

## Discussion

In this study, we have characterized an artificial AAV-SNCA miRNA-embedded hSNCA silencing vector that lacks toxicity in rat PC12 cells in which rat SNCA is not silenced and has reduced toxicity in human SHSY-5Y cells in which hSNCA is silenced. In addition, this vector does not display off-target effects on genes commonly expressed at high levels in DA neurons. Our data show that an shRNA silencing vector with the same silencing sequence, albeit driven by a different promoter, is not ideal for DA neurons due to displaying higher levels of cellular toxicity.

SNCA has been postulated to play important roles in a variety of cellular processes, including synaptic plasticity, neurotransmission, axonal transport, regulation of lipid uptake, chaperone-like activity and membrane associated processes (Abeliovich et al., 2000; Alerte et al., 2008; Chung et al., 2009; Kaplan et al., 2003; Murphy et al., 2000; Perez and Hastings, 2004; Saha et al., 2004; Scott et al.; Volles et al., 2001; Wersinger et al., 2003; Wood-Kaczmar et al., 2006). There are many reports demonstrating that over-expression of SNCA leads to death of DA neurons (Cookson, 2009), review. Conversely, a physiological level of SNCA expression appears to be critical to DA cell viability and function as reviewed recently (Perez and Hastings, 2004). In support of this, a recent report showed that specifically silencing endogenous rat SNCA *in vivo* in substantia nigra with an shRNA approach resulted in loss of DA neurons (Gorbatyuk et al., 2010). Another study showed that down-regulation of SNCA in MN9D cells decreases cell viability (Liu et al., 2008). On the other hand, it was recently reported that down regulation of endogenous SNCA in primate substantia nigra by pump delivery of siRNAs does not reduce the number and phenotype of nigral DA neurons (McCormack et al., 2010). In this monkey study, the level of SNCA was decreased by ~45%, whereas in our study, the silencing vectors decreased endogenous hSNCA expression by ~60% and also decreased cell viability. This suggests there may be a critical window of SNCA expression required to maintain DA cells with both lower and higher levels being toxic. Consistent with the concept that a large decrease in endogenous expression of SNCA promotes cell death, we observed our silencing vectors decreased cell viability in SH-SY5Y cells in which the expression of endogenous human SNCA was greatly reduced, but not PC12 cells in which expression of endogenous rat SNCA was not decreased. However, the non-miRNA embedded shRNA Luc control vector also displayed toxicity in both SH-SY5Y and PC12 cells. Thus, our data suggest that decreased cell viability of SH-SY5Y cells resulted from a combination of hSNCA gene silencing and specific vector design, whereas in PC12 cells, decreased cell viability was due

primarily to the non-miR embedded vector design. In both cell lines, the miRNA-embedded silencing vector displayed significantly lower toxicity than the other design.

In addition to the benefit of embedding the shRNA sequence in a miRNA backbone, these vectors can be driven by pol II promoters. The advantages of being able to use a pol II promoter for cell type specific or regulated gene silencing have been discussed previously (Giering et al., 2008) (Kesireddy et al., 2009; Nielsen et al., 2009; Stegmeier et al., 2005). Consistent with our data, others have also reported that shRNA silencing vectors are more toxic than artificial miRNA embedded silencing vectors in mouse striatal neurons and Purkinje neurons (Boudreau et al., 2009; McBride et al., 2008). Several differences in cellular action between shRNA and artificial miRNA vectors have been revealed by the studies of Davidson and co-workers. Compared to miRNA vectors, shRNA vectors are expressed at higher levels, produce a higher level of unprocessed precursors, and interfere with biogenesis of endogenous miRNAs (Boudreau et al., 2009; McBride et al., 2008). Furthermore, an shRNA vector targeting ataxin-1 was shown to compete with miRNAs in a reporter assay whereas an artificial miRNA with the same sequence had little effect (Boudreau et al., 2009). This interference with the miRNA intracellular machinery by shRNAs was further demonstrated in C2C12 muscle cells where elongation of differentiating myotubes was inhibited by an shRNA, but not by an artificial miRNA(Boudreau et al., 2009). In another study, acute liver toxicity resulted in mice when shRNAs were systematically delivered resulting in saturation of the endogenous RNAi system (Grimm et al., 2006). Recent studies in spinal cord also have revealed toxicity stemming from the use of AAV shRNA vectors (Ehlert et al., 2010). Alternative solutions to decrease the toxicity other than embedding the shRNA in a miRNA would be to deliver a lower dose of virus, use a weaker promoter, or regulate the expression of the shRNA. It has been reported that high titers of AAV virus, including both scrambled control and silencing vectors targeting TH showed toxicity, which was attenuated by using a lower dose of virus in DA cells in rats (Ulusoy et al., 2009). However, another study suggests that using a lower dose of AAV1 shRNA vector results in insufficient cell transduction and inefficient silencing (McBride et al., 2008). When considered together with our results, these findings illustrate the complexities and challenges inherent in gene silencing in the CNS in specific cell types.

AAV vectors are excellent for transduction of DA neurons and are currently being used in several clinical trials for PD patients for delivering growth factor genes or genes required for DA or glutamic acid decarboxylase synthesis. For this reason, we characterized hSNCA gene silencing constructs in the context of AAV. Our results *in vitro* suggest that future studies designed to target hSNCA in rat DA neurons should be performed with a mir30-embedded shRNA AAV vector.

## Methods and materials

### Shuttle plasmids: pAAV-H1-shRNA-SNCA, pAAV-H1-shRNA-Luc, pAAV-CMV-mir30-non silencing (NS) and pAAV-CMV-mir30-shRNA-SNCA

The AAV shuttle plasmids as shown in Figure 1 were cloned by standard techniques. In brief, the dual expression shuttle plasmids pAAV-H1-shRNA-SNCA and pAAV-H1-shRNA-Luc which harbor both an shRNA and the reporter gene, humanized green fluorescent protein (hrGFP), were generated by inserting an expression cassette designed to silence human SNCA or firefly GL2 luciferase (Luc), as described previously (Sapru et al., 2006), into the MLu1 and Ase1 sites of the AAV-hrGFP backbone (Stratagene, La Jolla, CA). The silencing portion of these vectors contains a 21-nucleotide RNA duplex shRNA corresponding to nucleotides 288-309 of human SNCA (GenBank Accession No. L08850) or nucleotides 153-173 of firefly GL2 luciferase (Luc; GenBank Accession No X65323) and

an 11-base pair duplex loop under control of the human H1 promoter. The hrGFP reporter gene is under control of the cytomegaloviral promoter (CMV).

The expression cassette, including CMV promoter, turboGFP, internal ribosome entry site (IRES), puromycin resistence gene (PuroR), mir30 embedded non silencing sequence, was extracted by *XbaI* and *Bsu36I* from pGIPz-CMV-NS plasmid (Open Biosystems, Thermo Scientific,Waltham, MA). The expression cassette was blunted by Klenow polymerase and ligated with *HindIII* and *RsrII* digested and Klenow treated pAAV-H1-shRNA-SNCA vector to create pAAV-CMV-NS.

SNCA shRNA silencing sequence embedded in mir30 backbone located in pUC57 vector was ordered from Genescript (Piscataway, NJ). This vector was digested by *XhoI* and *EcoRI* followed by ligation into pAAV-CMV-mir-NS to generate pAAV-CMV-mir30-shRNA-SNCA. Human SNCA was cloned into an AAV shuttle plasmid under control of the chicken ß-actin (CBA) to generate pAAV-CBA-hSNCA.

## Cell culture

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) in an incubator containing 5 % $CO_2$ at 37 °C. PC12 cells, a kind gift from Dr. Simon Halegoua, were grown on collagen coated polystyrene tissue culture dishes (NUNC, Roskilde, Denmark) and in DMEM/low glucose supplemented with 10 % heat inactivated horse serum (HS; Invitrogen, Carlsbad, CA), and 5% heat-inactivated FBS in a humidified atmosphere containing 5 % $CO_2$ at 37 °C. The human neuroblastoma SH-SY5Y cells were grown in 1:1 mixture of Eagle's Minimum Essential Medium (EMEM, ATCC) and F12 medium (Invitrogen) supplemented with 10% FBS. All cell lines were split every 4 days for maintenance.

## Transfection/Nucleofection

One day before transfection, $0.6 \times 10^6$ HEK293 cells were plated on 6-well plates. Cells were transfected with pAAV-CBA-hSNCA and/or pAAV-CMV-mir30-shRNA-SNCA, pAAV-CMV-mir30-NS, pAAV-H1-shRNA-SNCA or pAAV-H1-shRNA-Luc using lipofectamine 2000. One day before electrophoresis, PC12 cells were treated with 100 ng/ml nerve growth factor (NGF; Sigma-Aldrich) and maintained in NGF containing medium. PC12 and early passage SH-SY5Y cells were nucleofected with pAAV-CMV-mir30-shRNA-SNCA, pAAV-CMV-mir30-NS, pAAV-H1-shRNA-SNCA or pAAV-H1-shRNA-Luc using a Nucleofector device (Lonza, Cologne, Germany).

## Western Blot

Samples separated on SDS-PAGE gels were transferred to a nitrocellulose membrane at a constant voltage of 12v for 1hr. The membrane was blocked with 5% nonfat milk in TBST (Tris-buffered saline/Tween 20, 20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.05% Tween 20) for 2hr at room temperature and then incubated with primary antibody for 2h, including antibody against SNCA (BD Biosciences, Franklin Lakes, NJ; 1:250), panTH (Chemicon, Temecula, CA; 1:500), Ser 40 panTH (Millipore, Billerica, MA; 1:500), VMAT1 (Santa Cruz Biotechnlogy, Santa Cruz, CA; 1:200), actin (Sigma-Aldrich, St. Louis, MO; 1:20,000), turboGFP (Evrogen, Moscow; 1:40,000) or hrGFP (Stratagene; 1:5,000). The membrane was washed with TBST three times followed by incubation with horseradish peroxidase (HRP) conjugated goat anti-rabbit/mouse secondary antibody (Santa-Cruz, CA; 1:5000) for 1hr at room temperature. Following five washes, the membrane was developed by Supersignal West Pico Luminol/enhanced solution and West Pico stable peroxide solution (Pierce, Appleton, WI) and exposed to Kodak BioMax Light Film.

### Cell viability assay

Nucleofected PC12 or SH-SY5Y cells of 25,000 were plated in each well of collagen (Sigma-Aldrich) coated or uncoated 96-well plates, respectively. At 48 and 72 hrs post transfection, 11 μl of Alamar blue (Biosource, Camarillo, CA) was added to each well and plates were incubated for 1hr at 37°C followed by fluorometry.

### Quantitative, real-time RT-PCR

RNA was extracted 72 hours after PC12 cell transfection using TRI Reagent™ (Ambion, Austin TX). DNase-treated RNA was measured using quantitative TaqMan™ real time PCR on an Applied Biosystems (Foster City, CA) 7500 fast real-time PCR system. Each reaction contained 25ng of RNA, 12.5 μl of 2× TaqMan Universal PCR buffer, 7.8125 U of MuLV reverse transcriptase, 3.125 U of RNAse inhibitor, 0.25 μl of each primer (10 μM forward and 20 μM reverse except 10 μM for TH), and 0.5 μl of probe (5 μM) in a 25ul volume. Samples underwent incubation at 48°C for 30 min, 95°C for 10 min, then 40 cycles of 95°C for 15s and 59°C for 1min. Specific primers and probes were designed using Primer Express 3.0 (Applied Biosystems) and BLAST (blast.ncbi.nlm.nih.gov). Probes contained a FAM reporter dye and a TAMRA quencher dye. Data are expressed as delta Ct compared to β-actin Ct.

Rat primer and probes were: TH (fwd primer: CGG AAG AGA TTG CTA CCT GGA A, rev primer: GTA GCC ACA GTA CCG TTC CAG AA, probe: TCA CGC TGA AGG GCC TCT ATG CTA CC), DAT (fwd primer: TCC AGC AAT TCA GTG ATG ACA TC, rev primer: GCA TAG CCG CCA GTA CAG GT, probe: AGC AAA TGA CAG GGC AGC GAC CC), SNCA (fwd primer: ATG TCG TTG TAC CCA CTG TCC TAA G, rev primer: ATG ACT GGG CAC GTT GGA A, probe: CCC AGG TGT TCT TCC ATG GCG TAC AAG), β-actin (fwd primer: TCA CCC CTG TGC CCA TCT ATG, rev primer: CAT CGG AAC CGC TCA TTG CCG ATA G, probe: ACG CGC TCC CTC ATG CCA TCC TGC GT).

### Statistical analysis

Statistical analyses were performed by ANOVA using GraphPad Prism software, and the significance of intergroup differences was determined using Tukey's multiple comparison test or Bonferroni post-hoc test. Differences at the level of $p \leq 0.05$ were considered statistically significant.

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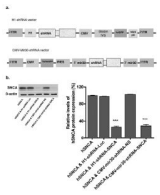

**Figure 1. The silencing efficiency of mir30-shRNA vectors for human SNCA**

a) Design of pAAV-H1-shRNA-SNCA or Luc (top) and pAAV-CMV-mir30-shRNA-SNCA or NS (bottom) shuttle plasmids. The SNCA silencing sequence in both constructs is designed to silence only human SNCA with three nucleotide mismatches to rat and mouse *SNCA*. Control plasmids contain either shRNA-Luc or shRNA-NS. b) Western blot using a human specific SNCA antibody and densitometry results of human SNCA in 293T cells transfected with pAAV-CBA-hSNCA (2 μg) alone or with 1 μg of one shRNA plasmid. hSNCA bands were normalized to ß-actin bands and expressed as percent of mock transfected. Both silencing vectors significantly decreased levels of human SNCA protein in 293T cells by approximately 75% (1-way ANOVA; Tukey's posthoc test, n=3; ***$p<0.001$ for silencing vectors compared to control groups). ITR, internal terminal repeat.

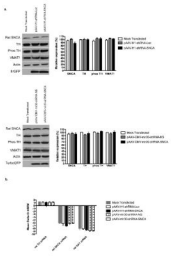

**Figure 2. Expression levels of rat SNCA and DA phenotypic markers in PC12 cells**

NGF treated PC12 cells were mock transfected or transfected with human SNCA silencing or control vectors as indicated. **a)** Representative western blot and densitometry of protein levels of rat SNCA, TH using a pan-TH Ab, Ser40P-TH and VMAT1 at 72 hrs post transfection as analyzed by Openlab software, normalized to ß-actin and expressed as percent of mock transfected (mean ±SEM, n≥3, 2-way ANOVA, nsd.) **b)** Rat TH, rat SNCA and rat DAT mRNA levels were determined by qRT-PCR and expressed as delta Ct values. The mean delta Ct values ± SEM relative to the Ct of β-actin are shown. (mean ±SEM, 1-way ANOVA, n≥3, nsd).

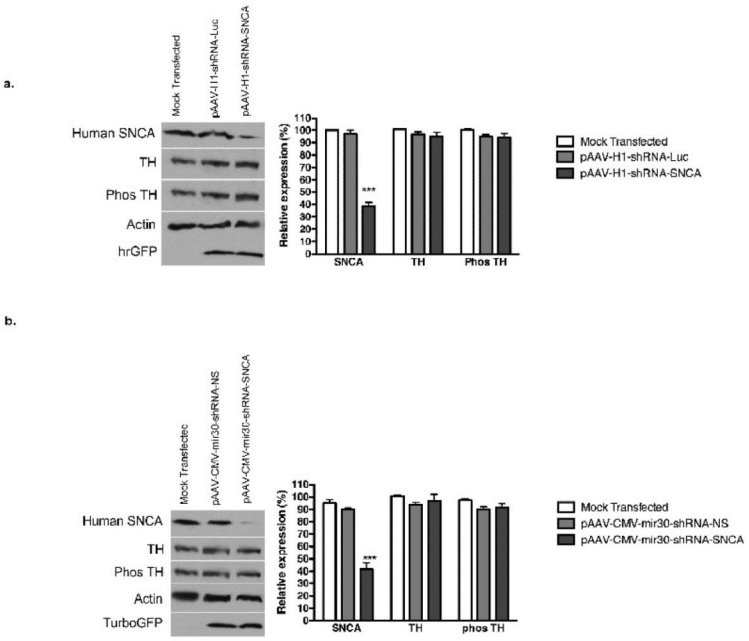


**Figure 3. Both shRNA SNCA vectors efficiently silence endogenous hSNCA in human neuroblastoma SH-SY5Y cells without changing expression of DA phenotypic markers**

Western blots and densitometry results for expression of hSNCA, pan TH and Ser40P-TH in SH-SY5Y cells of $2\times10^6$ transfected with 3 μg of pAAV-H1-shRNA-SNCA or pAAV-H1-Luc shRNA **(a)**, or, pAAV-CMV-mir30-shRNA-SNCA or pAAV-CMV-shRNA-NS **(b).** 2-way ANOVA $p<0.0001$ for vector and protein; ***$p<0.001$ compared to other bars.

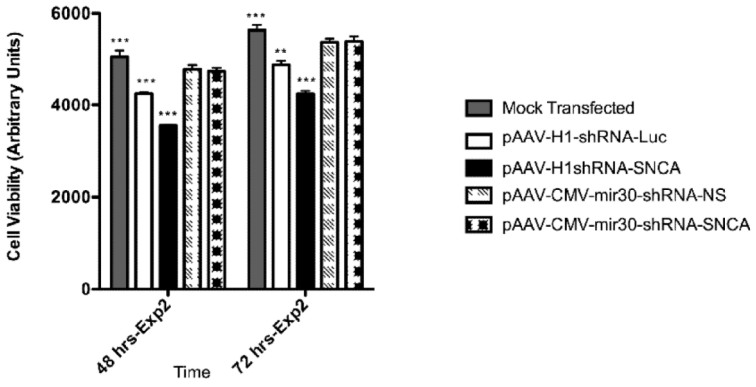


**Figure 4. Effects of silencing vectors on cell viability in PC12 cells**

NGF-induced PC12 cells were mock transfected or transfected with pAAV-H1-shRNA-Luc, pAAV-H1-shRNA-SNCA, pAAV-mir30-shRNA-NS or pAAV-mir30-shRNA-SNCA and harvested for Alamar Blue cell viability assays at 48 or 72 hrs post transfection. Graph shown is for one of two replicate experiments (n=3, mean ± SEM). Cells transfected with the H1-shRNA vector design showed the greatest reduction in cell survival compared to other treatment groups. 2-way ANOVA, $p<0.0001$ for vector; Bonferonni posthoc tests: ***$p\leq0.001$ for Mock vs H1shLuc and H1shSNCA; H1shSNCA vs mir30shNS and mir30shSNCA; ** $p\leq0.01$ for Mock vs mir30shNS and mir30shSNCA; *$p\leq0.05$ for Mock vs mir30shSNCA only 48hrs in 1 experiment; NSD, Mock vs mir30shNS and mir30shSNCA (72hrs); mir30shNS vs mir30shSNCA.

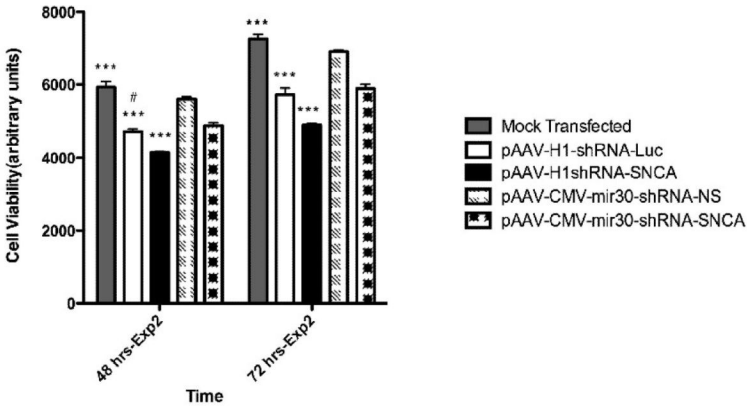


**Figure 5. Effects of silencing vectors on cell viability cells human SH-SY5Y cells**

Cells were mock transfected or transfected with pAAV-H1-shRNA-Luc, pAAV-H1-shRNA-SNCA, pAAV-mir30-shRNA-NS or pAAV-mir30-shRNA-SNCA and harvested for Alamar Blue cell viability assays at 48 or 72 hrs post transfection. Graph shown is for one of two replicate experiments (n=3; mean ± SEM). Non-embedded shRNA vectors had the greatest effect on reducing cell viability. Cells transfected with pAAV-mir30-shRNA-NS did not show increased cell death. Note significantly reduced cell viability in cells transfected with pAAV-CMV-mir30shRNA compared to mock-transfected and the mir30-shRNA-NS control, but this was significantly less than in cells transfected with the non-embedded SNCA silencing vector; 2-way ANOVA ($p<0.0001$ for vector and time); Bonferonni posthoc tests: ***$p\leq0.001$ for Mock vs H1shLuc, H1shSNCA and mir30shSNCA; H1shLuc vs H1shSNCA (72hrs), mir30shNS and mir30shSNCA; H1shSNCA vs mir30shSNCA; #$p\leq0.01$ H1shLuc vs H1shSNCA (48hrs); NSD, Mock vs mir30shNS; H1shLuc vs mir30shSNCA.