CAEN ISN 1B Research Report

April 2015 Round Notification
Principal Investigator: Cecilia Frecha

PROJECT TITLE

Development of a neurotropic vector for the study and treatment of Alzheimer's disease (AD)

OBJECTIVE

To develop a vector for specific and regulated NEP gene expression, as a potential tool to inoculate in a rat model of AD.

DESCRIPTION OF THE WORK

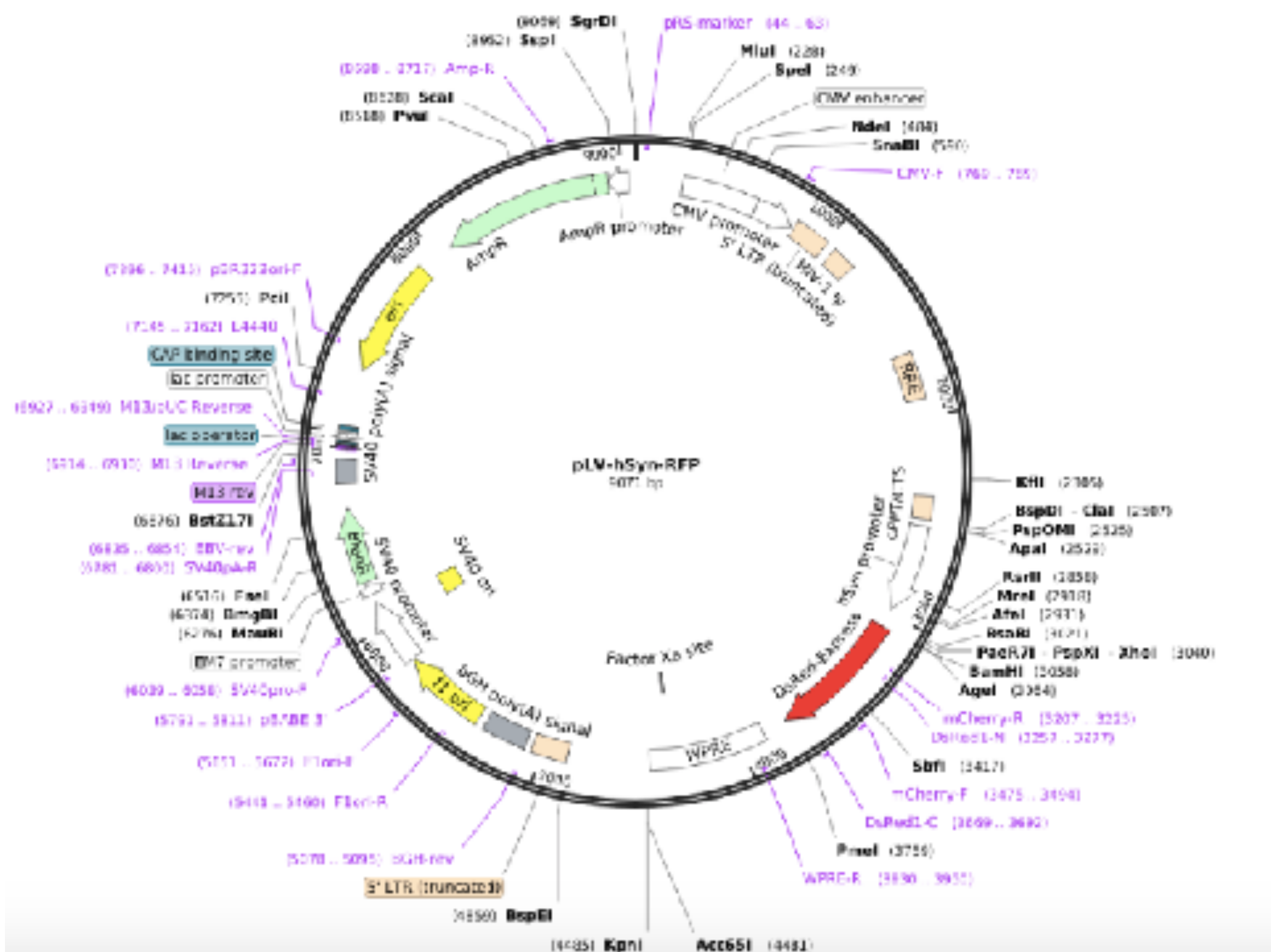
With the aim to generate an effective tool for the genetic modification neurons in culture, at the same time efficient for in vivo injection in a rat model of amyloidosis, we developed viral vectors containing neuron-specific promoter (synapsin; SYN) driving gene expression of neprilysin (NEP). In a first instance, it was intended to produce adenoassociated vectors, because of their natural neurotropism. However, due to technical complications for the implementation of this technology, we decided to choose lentiviral vectors instead, which are as well suited to deliver transgenes to the central nervous system, with high efficiency. Lentiviral vectors are derived from HIV-1 virus, they have tremendous plasticity to exchange promoters and envelope glycoproteins, which makes them extremely versatile for gene transfer to almost any type of eukaryotic cell. They consist of a plasmid vector containing a mutated version of the HIV-1 virus LTR with an exogenous promoter (in this case the SYN promoter) and the transgene of interest (in this case NEP cDNA). The production of lentiviral particles requires the cotransfection of the plasmid vector together with other plasmids required to form the capsid and the envelope, into 293T packaging cells. This results in the release of lentiviral vector particles to the culture supernatants within 48 hours.

We first constructed the lentiviral plasmid by restriction enzyme cutting and ligation of human NEP cDNA out of lentiviral plasmid p-BOB-NEP (containing NEP cDNA under a ubiquitous promoter), into the plasmid p-SYN-RFP in place of RFP reporter gene. We also cloned eGFP in place of RFP, to generate pSYN-GFP. As a result, the plasmid p-SYN-NEP was obtained, which express the gene under control of the neuron specific, SYN promoter (maps of both plasmids are depicted in figure 1).

Note: Due to regulatory restrictions by Argentinean Customs, every imported item (this applies to plasmids as well) requires the intervention of an official customs broker provided by the National Research Council. The purchase was done in July 2015, and the whole process took 3 moths to liberate the plasmids from Customs, meaning that we could start the project until beginning of October.

Figure 1.
Maps of p-BOB-NEP and p-SYN -RFP, with main features and single-cutter restriction enzymes. (dowloaded from www.addgene.org)

(11,088) SgrDI
pRS-marker (44 .. 63)
AmpR promoter
(10,717 .. 10,736) Amp-R
MluI (220)
SpeI (248)
CMV-F (769 .. 789)
(10,647) ScaI
(10,537) PvuI
(10,389) FspI
(10,157) AhdI
KasI (1015)
NarI (1016)
SfoI (1017)
PluTI (1019)
NotI (1519)
(9415 .. 9434) pBR322ori-F
(9164 .. 9181) L4440
CAP binding site
lac promoter
(8946 .. 8968) M13/pUC Reverse
lac operator
(8930 .. 8949) M13 Reverse
M13 rev
(8695) BstZ17I
(8854 .. 8873) EBV-rev
(8800 .. 8819) SV40pA-R
(8535) FseI
(8350) BmgBI
(8296) MauBI
EM7 promoter
(8058 .. 8077) SV40pro-F
(7810 .. 7830) pBABE 3'
(7670 .. 7691) F1ori-F
(7450 .. 7429) F1ori-R
(7097 .. 7114) BGH-rev
(6878) BspEI
(6504) KpnI
(6500) Acc65I
(6399) SacII
(5919 .. 5939) WPRE-R
(5778) PmeI
HpaI (5335)
AarI - BfuAI - BspMI (5114)
PspXI (4715)
XcmI (3890)
KflI (2305)
cPPT/CTS
CMV-F (3085 .. 3106)
LNCX (3032 .. 3056)
BamHI (3112)
AgeI (3118)
SfiI (3131)
PasI (3182)
AmpR
CMV enhancer
CMV promoter
HIV-1 Ψ
5' LTR (truncated)
RRE
CMV enhancer
CMV promoter
ori
SV40 poly(A) signal
SV40 promoter
SV40 ori
bGH poly(A) signal
f1 ori
5' LTR (truncated)
Factor Xa site
WPRE
pCSC-SP-PW-Nep (aka: pBOB-NEP)
11,098 bp



The amplification of two other plasmids, p8.91 and pVSVG essential for the production of vectors was performed:
1- p8.91 contains the gag-pol genes necessary for the formation of the capsid, polymerase and reverse transcriptase.
2- pVSVG contains the vesicular stomatitis virus surface glycoprotein gene. Since the receptor for VSVG is expressed ubiquitously, it gives the lentiviral particle the ability to transduce almost any type of eukaryotic cell.

Upon receipt of the plasmids from Addgene we immediately proceeded to plasmid amplification by transformation of Top-10 bacteria. We performed maxiculture and maxiprep of positive colonies, in order to clone NEP cDNA under the SYN promoter. Ligations were transformed into Top-10 bacteria, and ampicillin-agar colonies were picked, DNA was isolated, and checked by restriction with BamHI/KpnI. We obtained the expected profile in 16 of 20 analyzed colonies. Correct insertion and sequence was checked by sequencing.

Afterwards, the correct plasmid expression of NEP was evaluated. For this, the plasmid pSyn- NEP-1 transfected in HEK293 cells using Lipofectamine 2000 (Invitrogen). They were transfected in parallel with the pSYN-RFP (red fluorescent expressing a reporter gene), to assess the transfection efficiency by fluorescence microscopy.

RESULTS

HEK293 cells were efficiently transfected with pSYN-RFP (about 80%; Figure 2).

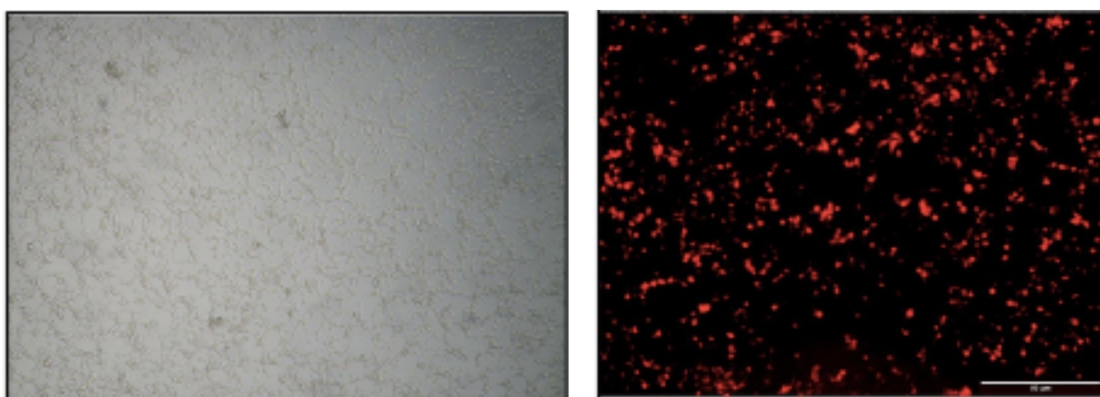


Figure 2
Photographs of bright field and fluorescent microscopy of HEK293T cells transfected with pSYN-RFP.

In order to evaluate the correct expression of NEP, HEK293T cells were transfected with pSYN-1-NEP. Western blot of cell lysates was performed, and a band of 110KD compatible with the expected molecular weight of NEP was seen (Figure 3).

These results indicate that the plasmid contains a correct form of NEP and thus is suitable to be incorporated into lentiviral vectors.

Figure 3

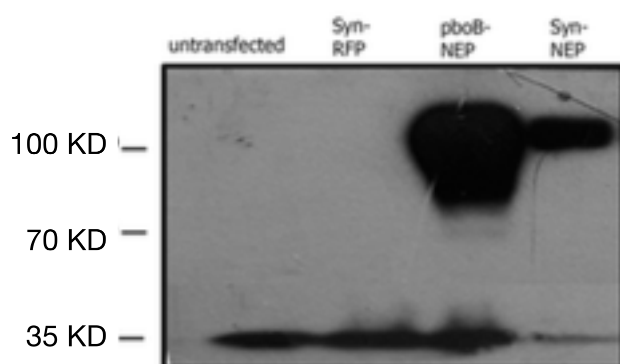


Figure 3: Western blotting. NEP detection of protein in cells transfected with pSYN-NEP and pBOB-NEP but not with pSYN-RFP. Lane 1: untransfected HEK-293T, second lane SYN-RFP, third lane pBOB-NEP and fourth lane pSYN-NEP. Detection of GAPDH protein, as loading control (35kb).

During the month of December, in collaboration with Dr Els Verhoeyen (Lyon, France) we received optimized versions of plasmid transfer and plasmid vector for the expression of reporter gene (GFP): Plasmid transfer PAX2, with 1 -2 log increased efficiency in lentiviral packaging, and pE1alfa-GFP plasmid, which contains a highly effective ubiquitous promoter in a variety of human cells and it is of human origin.

The lentiviral vectors are produced as follows:

1) lentiviral expression plasmids: GFP, RFP and NEP.
eIF1-GFP: GFP expressed under human promoter EIF1alfa, ubiquitously expressed.
SYN-RFP: RFP expressed under synapsin-1 promoter, ie, restricted to neuronal cells.
SYN-NEP: NEP Expressed under synapsin-1 promoter
CMV-NEP: NEP Expressed under a ubiquitous promoter fragment of the cytomegalovirus (CMV) promoter.

. 2) Packaging plasmid: p8.91, and pPAX2.

3) Envelope plasmid: pVSVG which allows transduction of almost all human cells.

Method: Vector production is done by co-transfection of producer cells, 293T, using calcium phosphate transfection reagent. Briefly, the day before transfection 2.6x106 cells are seeded in 10cm in DMEM 10% FCS, antibiotics. The day after, 8.6 ug of plasmid PAX2, 8.6 ug of expression plasmid (NEP, GFP, etc.), and 3ug of envelope plasmid (VSVG) are transfected and incubated for 48 hours. The supernatants are collected, filtered through 0,45um and stored. Vector titers are assessed by transduction of 293T cells (all vectors) or SHY5Y (SYN vectors with promoter), with 10- fold serial dilutions of supernatants of 293T producer cells

Lentiviral vectors can be directed to the desired cells either by exchange the surface glycoproteins (Pseudotyping) or by exchanging the internal promoter by a tissue specific one (as in this work). The specific promoter is important when designing vectors to restrict expression to the cell of interest to avoid potential side effects from ectopic transgene expression.

LV-SYN-RFP vector (neuron-specific promoter) and LV-EF1alpha-GFP vector (ubiquitous promoter) were transduced into 293T cells and human neural SHY5Y cell line to determine the specificity of the vectors. LV-EF1alpha-GFP vectors gave GFP expression in 293T (Figure 4) and SHY5Y cells, as expected, while LV- SYN-RFP do so only in cells of neuronal origin, and only when SHY5Y were put under neuron-differentiating conditions (Figure 5). A ratio between levels of GFP expression of each vector in neuronal cells versus non-neuronal is usually determined, and a "specificity index" is calculated. The qualitative analyses of images showed that SYN-RFP works only in neural cells, with no expression in 293T cells. Because differences in gene expression can arise from differences in the amount of integrated vectors, we compared the number of integration by qPCR. Primers covering the vector LTR region were synthesized. qPCR was performed in a LightCycler 2.0 (Roche) Using a SYBR Green master mix (Roche). A standard curve was done using know copy numbers of a lentiviral plasmid. We found similar number of integrations in 293T and SHY5Y cells, meaning that the observed differences in RFP intensity were due to differences in gene expresion, therefore, differences in promoter activity.

Figure 4.

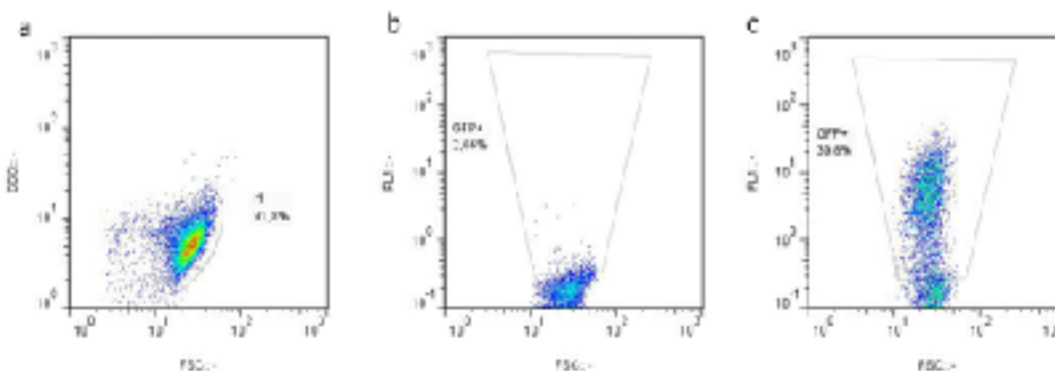


Figure 4: Transduction of HEK293T with G-EIF1alfa-GFP vector gives around 35% of GFP expression (Figure 4). FACS plots of a) 293T FSC vs SSC, b) non transduce 293T cells and c) 293T cells transduce 72 hours with G-EIF1alfa-GFP at MOI=1.

Figure 5.

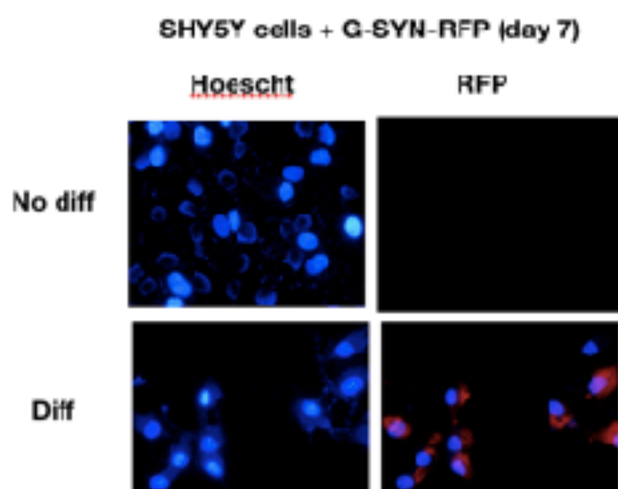


Figure 5: Transduction of SHY5Y cells with G-SYN-RFP. Cells were transduce with the vector for three days and further put (or not) under neuron-differentiating conditions. Cells were analyzed everyday following differentiation to investigate for RFP expression. RFP only appeared at day 7 of neuron differentiation.

We also intended to evaluate the effect of the lentiviral vector expressed NEP in the protection of Aß peptides treated cells. This test is used to determine whether the NEP expressed protein is able to protect cells from damage induced by Aß after 24 hours in vitro exposure to SH5Y5 transduce cells. Toxicity is measured by the number of viable cells (Trypan Blue or MTT assay). It is expected in to obtain a decreased exogenous Aß -mediated toxicity in those cells transduced with SYN-NEP. At the moment we re setting up the optimal conditions to obtain differentiated neurons in vitro to be transduce with the vectors and still viable to challenge with Aß peptides.

We will concentrate and purify the lentiviral vectors by a sucrose gradient in order inoculate them in Wistar rat transgenic model of amyloidosis. In the laboratory (LaNyN) the McGill-Thy1-R-APP transgenic rat model, which has a high production of Aß monomers that accumulate intracellularly is available, and it is considered ideal for the study of early processes of Alzheimer's Disease. The vectors will be injected stereotaxically in the hippocampus of the rats. Tg rats will be injected with the NEP expression vector and will be compared with the control vector GFP. We will study vector distribution in those rats injected with GFP vectors, while we expect a reduction in intracellular Aß species, mainly monomeric, in rats treated with the NEP vector, as a reflexion of the functional effect of recombinant NEP. This decrease would lead to a decrease in the formation of Aß oligomers and ultimately improve the behavioral signs of the model as well as the acquisition of new memories / learning.

In addition there has been progress in designing a strategy of generating an alternative rat model of amyloidosis by injection of a lentiviral vector for the expression of the mutated form of the precursor amyloid peptide-degrading enzyme (APP) containing the mutations

"Swedish" and "Indiana" in order to induce in the animals, the accumulation of amyloid Aß 40 and Aß 42 species in the brain. These rats would be producing a high number of amyloid peptides, as a result of a greater amount of mutated APP in the brain. This animal model will allow to study in a more direct way the function of the potentially therapeutic recombinant NEP in peptides derived from the cleavage of APP.

With all this, we made progress in designing an effective genetic tool for studying Alzheimer's disease with therapeutic potential.
We still need to define the degree of protection of the vector in vitro, as well as the distribution and function of the vectors in the transgenic rat model.