SAPIENZA
UNIVERSITÀ DI ROMA

Project of Gene Therapy for Alzheimer's Disease

"Life without memory is no life at all...Our memory is our coherence, our reason, our feeling, even our action. Without it, we are nothing…"
Luis Buñuel

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Course of Gene Therapy
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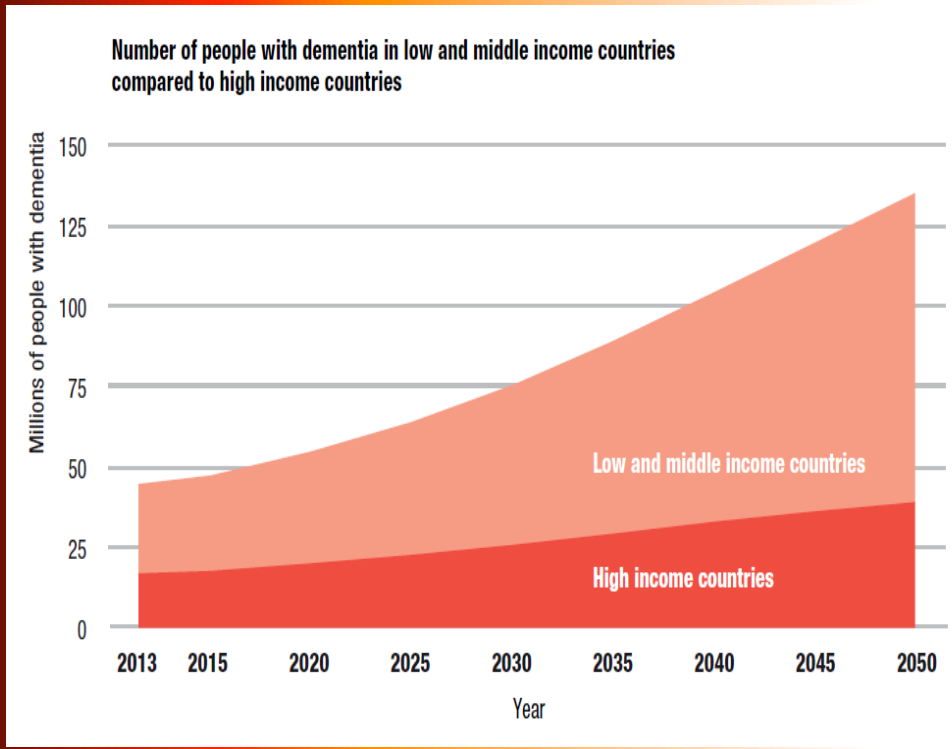
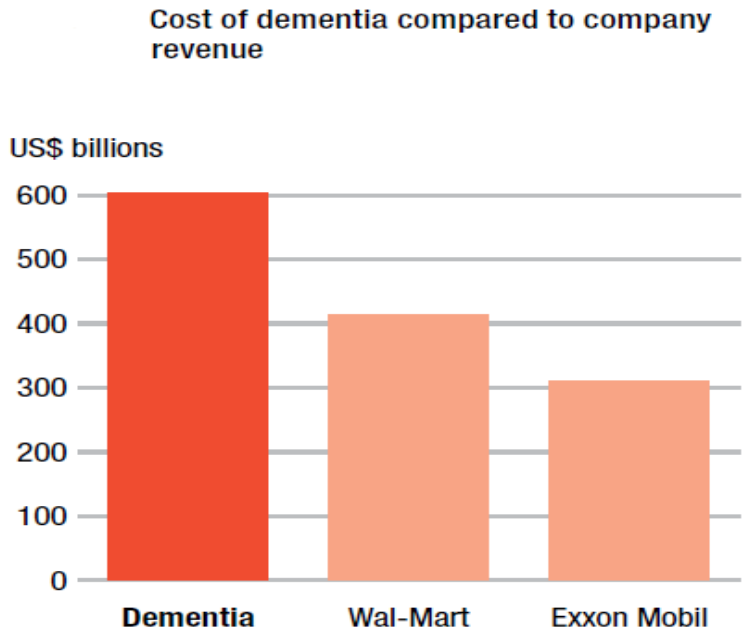
Academic Year: 2013/2014

Why Alzheimer's Disease?

- In last year's World Alzheimer Report, Alzheimer's Disease International estimated that there are 44 million people living with dementia worldwide in 2013
- If dementia care were a company, it would be the world's largest by annual revenue





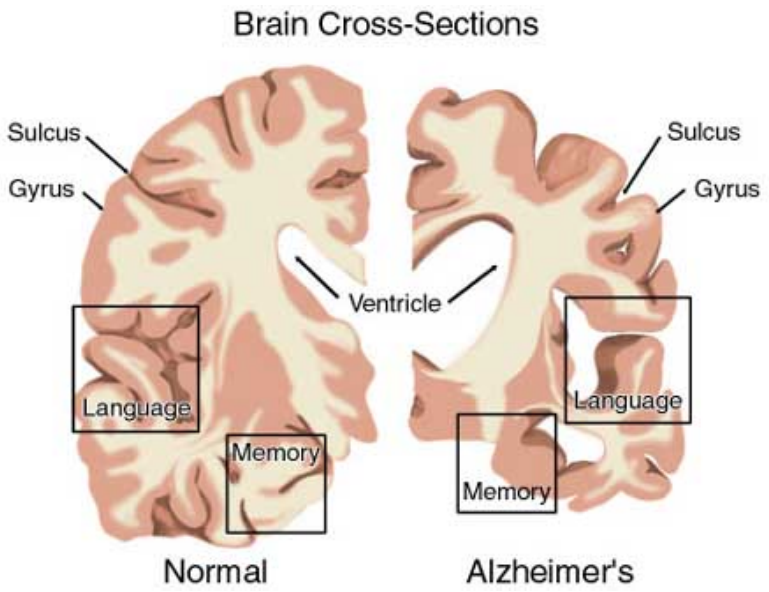
"Alzheimer's Disease International:World Alzheimer Report 2013." M. Price *et al.* (2013).

Alzheimer's Disease

- The Alzheimer Disease is the most common form of degenerative dementia disabling.
- It was first described in 1906 by German psychiatrist and neuropathologist Alois Alzheimer.
- The average age that people get Alzheimer's is after the age of 60.

Table 1. The Stages of Alzheimer's Disease

Not Alzheimer's	Early-stage
■ Forgetting things occasionally ■ Misplacing items, like keys, eye glasses, bills, paper work ■ Forgetting the names or titles of some things, like movies, books, people's names ■ Some reduction in ability to recall words when speaking ■ Being "absent-minded" or sometimes hazy on details ■ "Spacing out on things," such as appointments	■ Short-term memory loss, usually minor ■ Being unaware of the memory lapses ■ Some loss, usually minor, in ability to retain recently learned information ■ Forgetting things and unable to dredge them up, such as the name of a good friend or, even, family member ■ Function at home normally with minimal mental confusion, but may have problems at work or in social situations ■ Symptoms may not be noticeable to all but spouse or close relatives/friends
Middle-stage	**Late-stage**
■ Short-term memory loss deepens, may begin to forget conversations completely, or names of loved ones ■ Mental confusion deepens, trouble thinking logically ■ Some loss of self-awareness ■ Friends and family notice memory lapses ■ May become disoriented, not know where you are ■ Impaired ability to perform even simple arithmetic ■ May become more aggressive or passive ■ Difficulty sleeping ■ Depression	■ Severe cognitive impairment and short-term memory loss ■ Speech impairment ■ May repeat conversations over and over ■ May not know names of spouse, children, or caregivers, or what day or month it is ■ Very poor reasoning ability and judgment ■ Neglect of personal hygiene ■ Personality changes; may become abusive, highly anxious, agitated, delusional, or even paranoid ■ Needs extensive assistance with activities of daily living



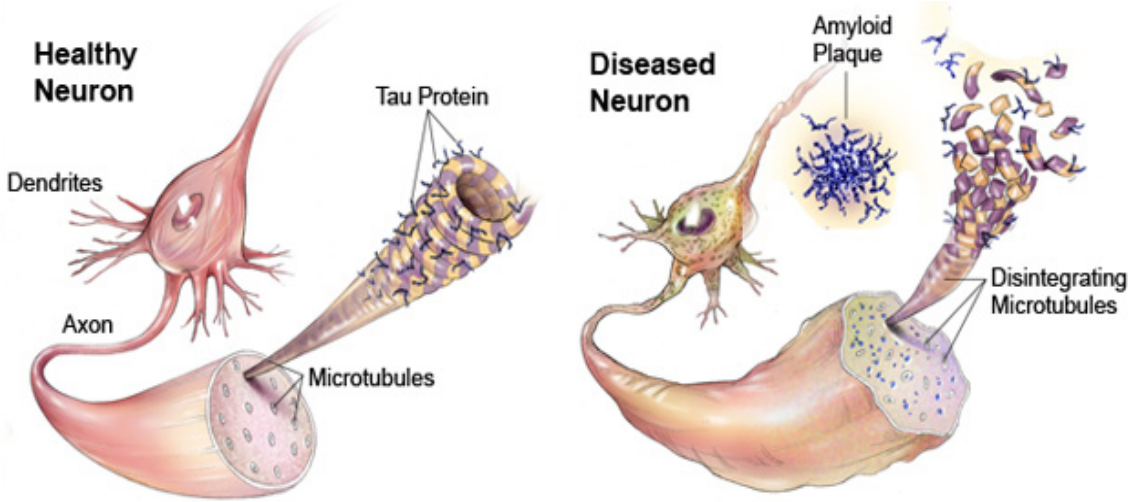
"Evaluating Prescription Drugs Used to Treat: Alzheimer's Disease." Consumer Reports Best Buy Drugs (2013)

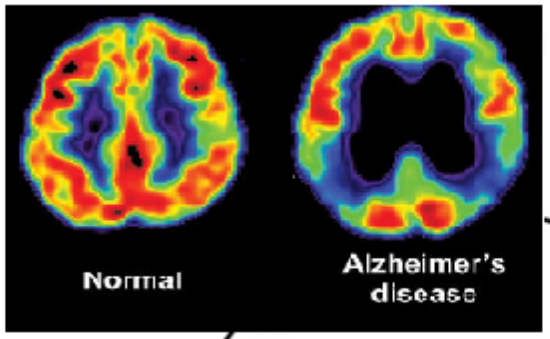
General Aspects

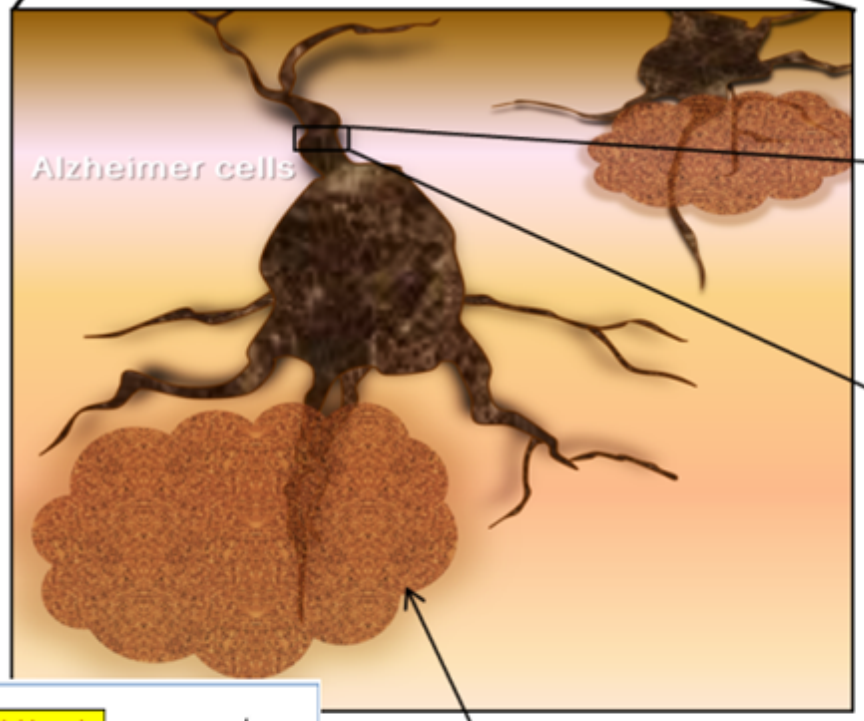
In more than 90% of cases the onset is sporadic (LOAD: Late-onset Alzheimer's). While in 5-10% of cases is observed familiarity (FAD: Familial Alzheimer's disease). Both are characterized by the accumulation of plaques and tangles. The cause for most Alzheimer's cases is still mostly unknown except for 1% to 5% of cases where genetic differences have been identified.

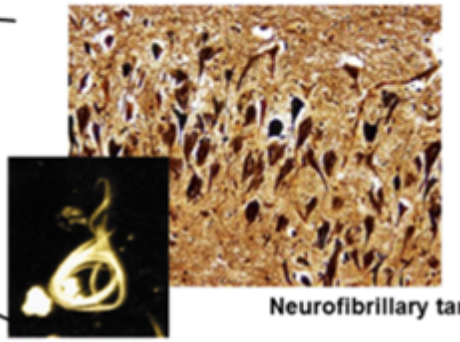
⇨ We decided to treat LOAD

Two major hypotesis:
1)Amyloid plaques
2)Neurofibrillary tangles









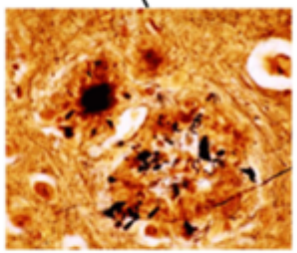
Neurofibrillary tangles

Paired helical filaments

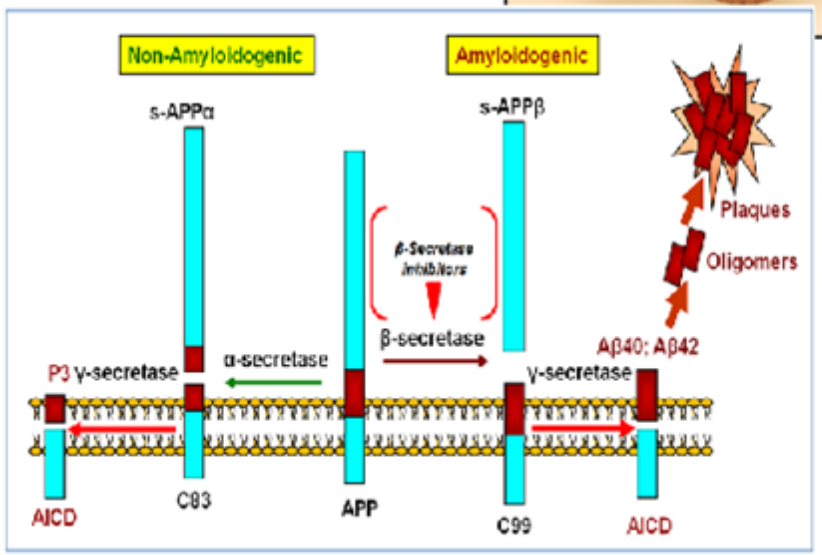
Hyperphosphorylated τ protein

Kinases (e.g. GSK3β) ⇅ Phosphatases (e.g. PP2A)

τ protein



Amyloid plaque



Which main molecules had been studied for a possible gene therapy?

Target protein	Function in AD	Vector	Site of expression	Effect
NGF	Neurotrophic, synaptic plasticity	MLV (*Ex vivo*)	B. F. (Fibroblasts)	No acceleration of Aβ deposition
		rAAV	Intraseptal/Medial septum	Protection of lesion-induced degeneration
		rAAV-2	Septum	Neurotrophic, increased synaptic activity
BDNF	Neurotrophic, synaptic plasticity	Lentivirus	Entorhinal cortex	Neurotrophic, cognitive improvements
Neprilysin (membrane-bound form)	Aβ degradation, neuroprotection	rAAV	Hippocampus, dentate gyrus	Reduced soluble Aβ and Aβ burden
ECE	Aβ degradation	rAAV-5	Hippocampus, Cortex	Reduced Aβ burden
Cathepsin B	Aβ degradation	Lentivirus	Hippocampus	Reduced Aβ burden
APOE2	Lipoprotein metabo-lism, Aβ burden	Lentivirus	Hippocampus	Reduced Aβ levels, and reduced Aβ burden
BACE1	Aβ generation	Lentivirus (siRNA)	Hippocampus	Reduced soluble Aβ, and reduced Aβ burden
APP	Aβ generation	HSV (siRNA)	Hippocampus	Reduced Aβ burden

"Gene therapy in Alzheimer's disease –potential for disease modification" Per Nilsson *et al.* (2010).

Aims and chosen molecules

In this gene therapy's project we decided to take action on two fronts:

- Degrade the beta amyloid plaques
- Protect neurons

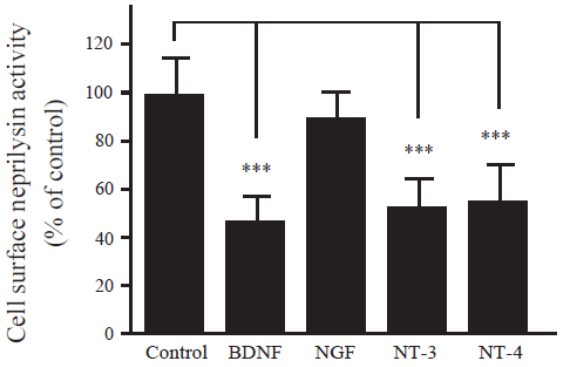
So, after careful research, we chose to treat the two molecules:

- Neprilysin (NEP)
- NGF

But why NGF and NEP?

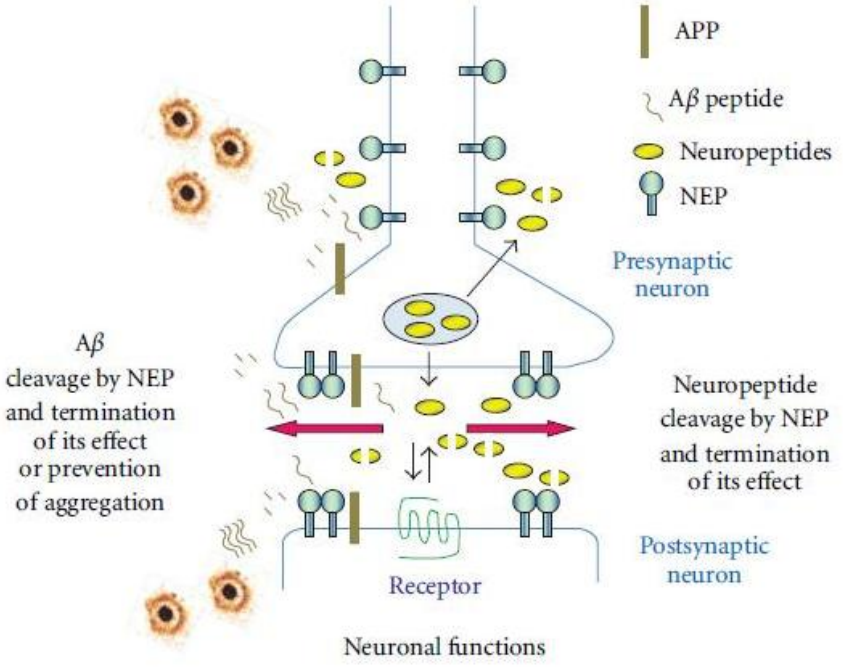
- NEP is the major Aβ degrading enzyme.
- NGF is the only neurotrophin that doesn't interfere with NEP

⇒ The novelty in our approach is the combined expression of both.



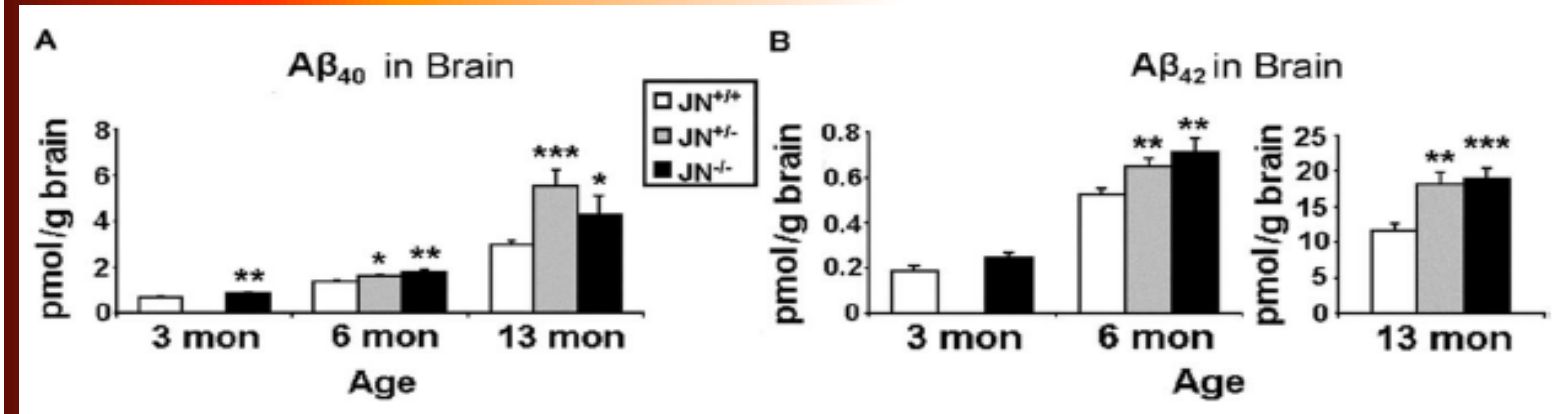
"Cell-surface expression of the major Aβ degrading enzyme, neprilysin, depends on phosphorylation by MEK and dephosphorylation by protein phosphatase 1a" N. Kakiya et al., (2012).

Neprilysin



"The Alzheimer's Amyloid-Degreding Peptidase, Neprilysin: Can we control it?" Nalivaeva N.N. *et al.* (2012).

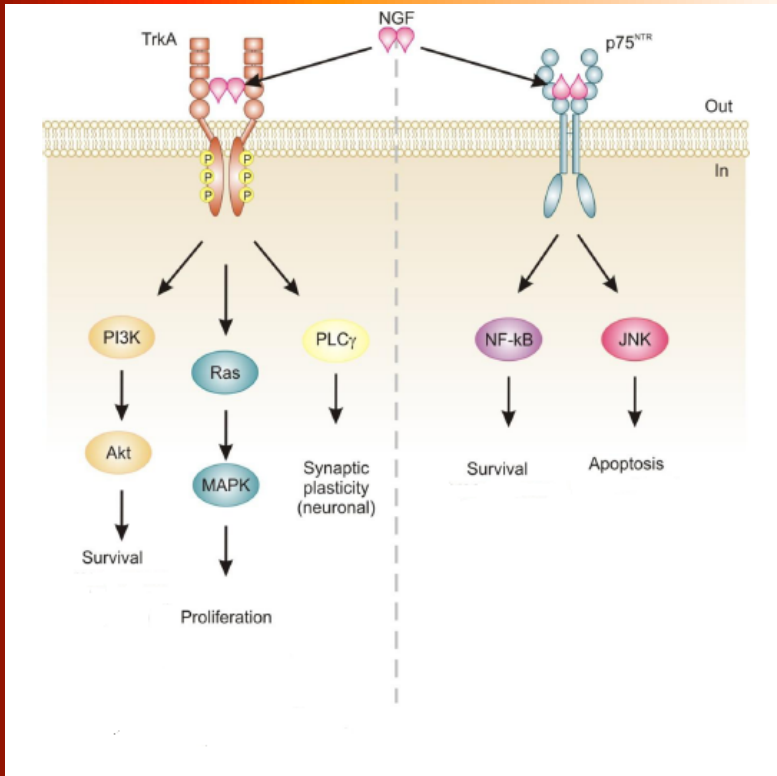
- Defective Aβ degradation is involved in late-onset AD
- NEP is a 90 ~ 110 kDa plasma membrane glycoprotein of the zinc metalloendopeptidase family that degrades Aβ peptides
- NEP levels decline in an age-dependent manner and inversely correlate with levels of insoluble Aβ

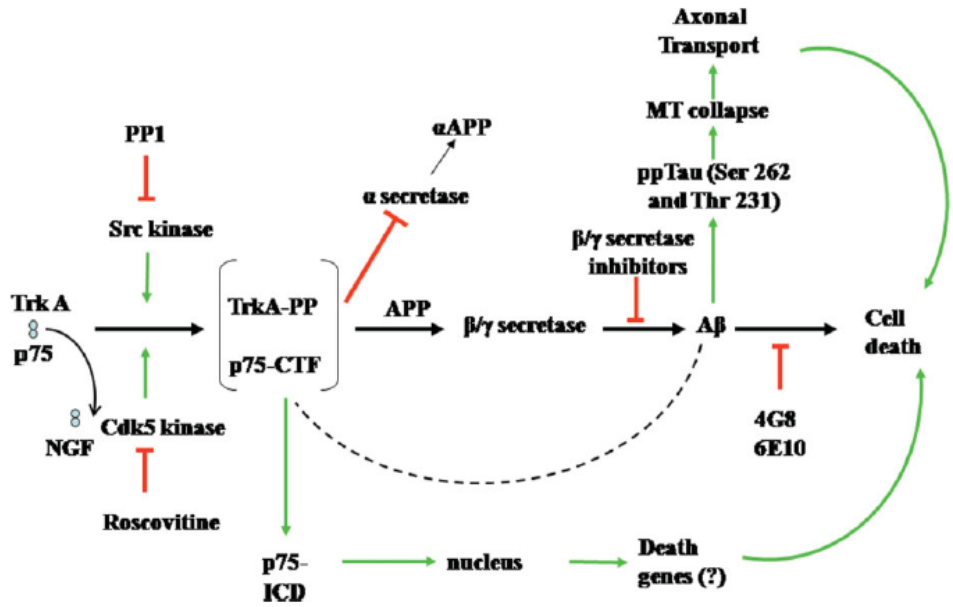


"Loss of Neprilysin Function Promotes Amyloid Plaque Formation and Causes Cerebral Amyloid Angiopathy", Farris et al. (2007).

NGF

- Nerve growth factor (NGF) is a small secreted protein that is important for the growth, maintenance, and survival of neurons.
- NGF binds two receptors, TrkA and p75. The binding and the interaction of these two receptors activates a cascade of downstream signals that allows cell survival.
- The lack of NGF causes the formation of Aβ, hyperphosphorylation of Tau protein and cell death.



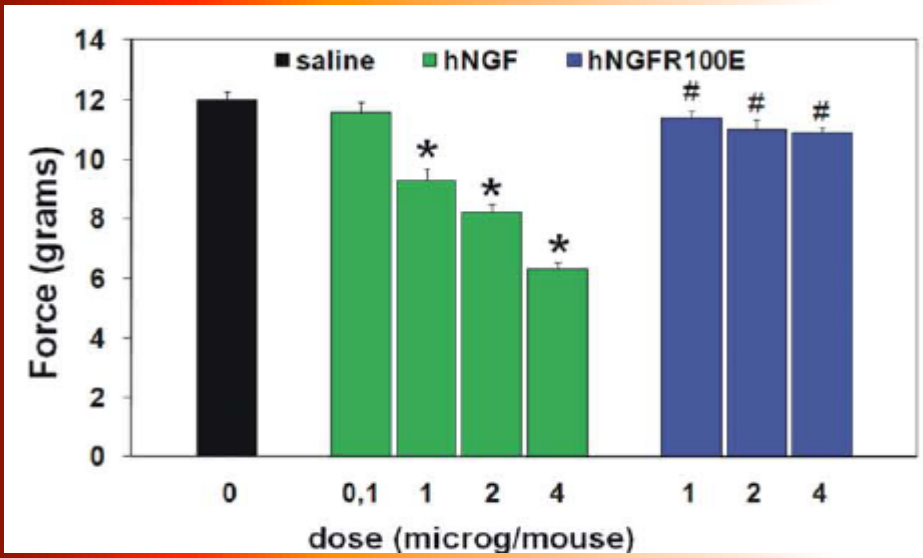


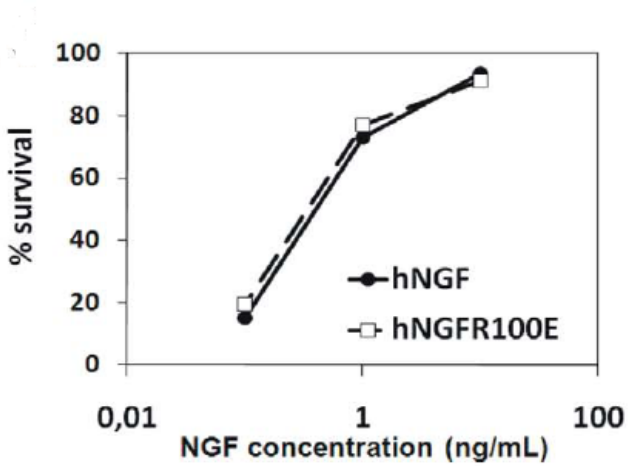
"Nerve Growth Factor As a Paradigm of Neurotrophins Related to Alzheimer's Disease", P. Calissano *et al.* (2009).

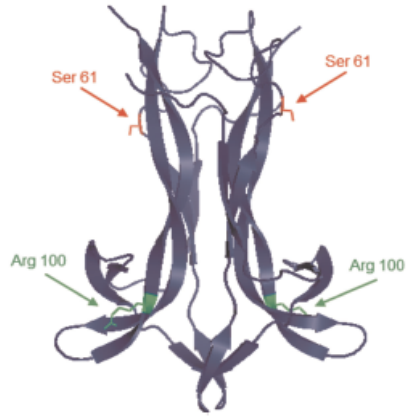
NGF “painless”

The previous studies have shown that the neurotrophin Nerve Growth Factor (NGF) sensitizes nociceptors, thereby increasing the response to noxious stimuli.

➡ NGF “painless”is a molecule engineered by a research group of EBRI (European Brain Research Institute) that maintains its ability to confer cell survival but not conducive to the pro-nociceptive stimulation.



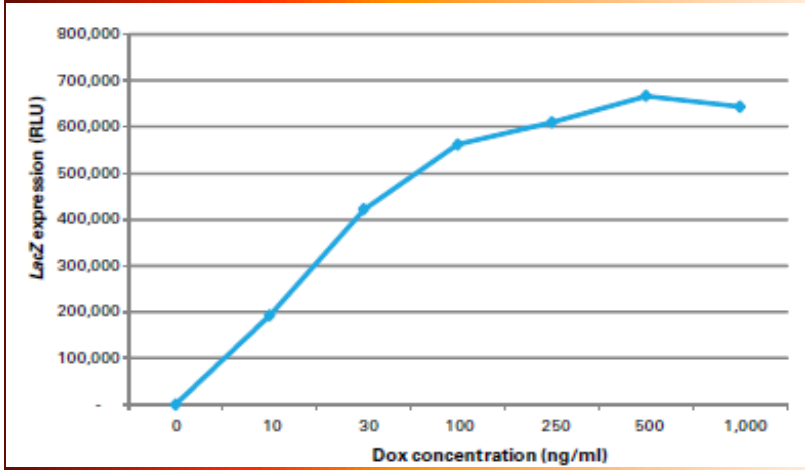




“Intranasal “painless” Human Nerve Growth Factors Slows Amyloid Neurodegeneration and Prevents Memory Deficits in App X PS1 Mice” Simona Capsoni *et al.* (2012).

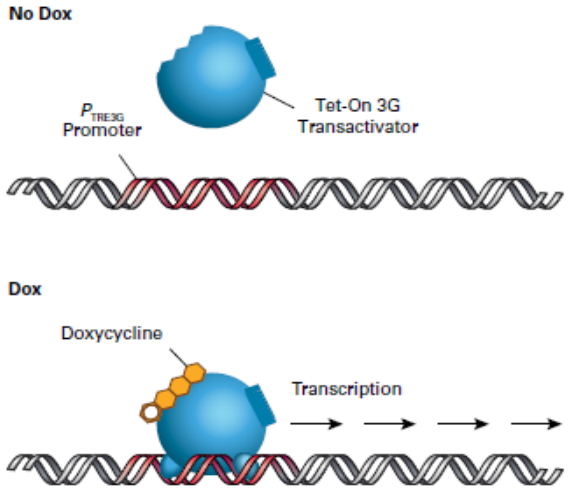
Choice of promoters

Tet-On 3G for Nep



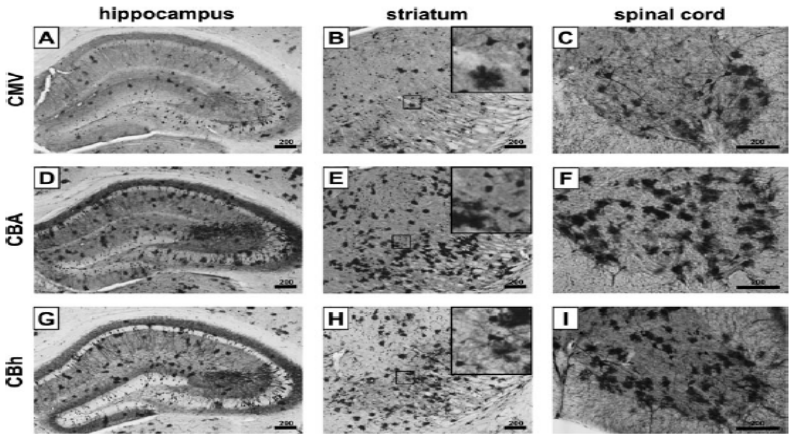
Tet-On 3G is a highly sensitive tetracycline inducible expression system.

Tet-On® 3G Inducible Expression Systems , Clontech® Laboratories, Inc.



CBh for NGF

Enh	Promoter	5' UTR/intron	Strength	Size	Specificity
CMV	CMV	SV40	High	800 bp	Ubiquitous
CMV	CBA	SV40	High	800 bp	Ubiquitous
CMV	CBA	CBA-MVM	High	800 bp	Ubiquitous
None	UBC	None	Weak	430 bp	Ubiquitous
None	GUSB	None	Weak	378 bp	Ubiquitous
None	NSE	None	Strong	2.2 kb	Neuron
None	Synapsin	None	Medium	470 bp	Neuron
None	MeCP2	None	Weak	229 bp	Neuron
None	GFAP	None	Medium	681 bp	Astrocyte



Compared with the CBA and CBh promoters, the CMV promoter showed reduced expression in hippocampal neurons 4 weeks postinjection

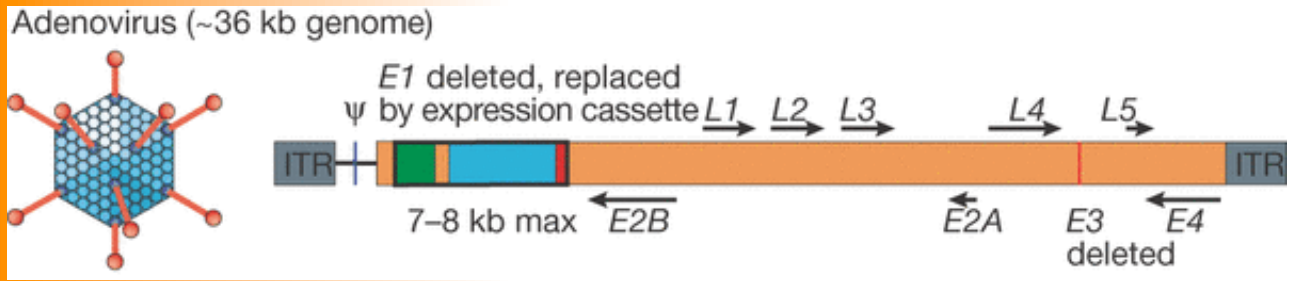
"Optimizing Promoters for Recombinant Adeno-Associated Virus-Mediated Gene Expression in the Peripheral and Central Nervous System Using Self-Complementary Vectors", Gray et al. (2011)

Viral vectors – Adenovirus

The viral system is the most widely used in gene therapy, allowing the delivery of a transgene in the target cells.

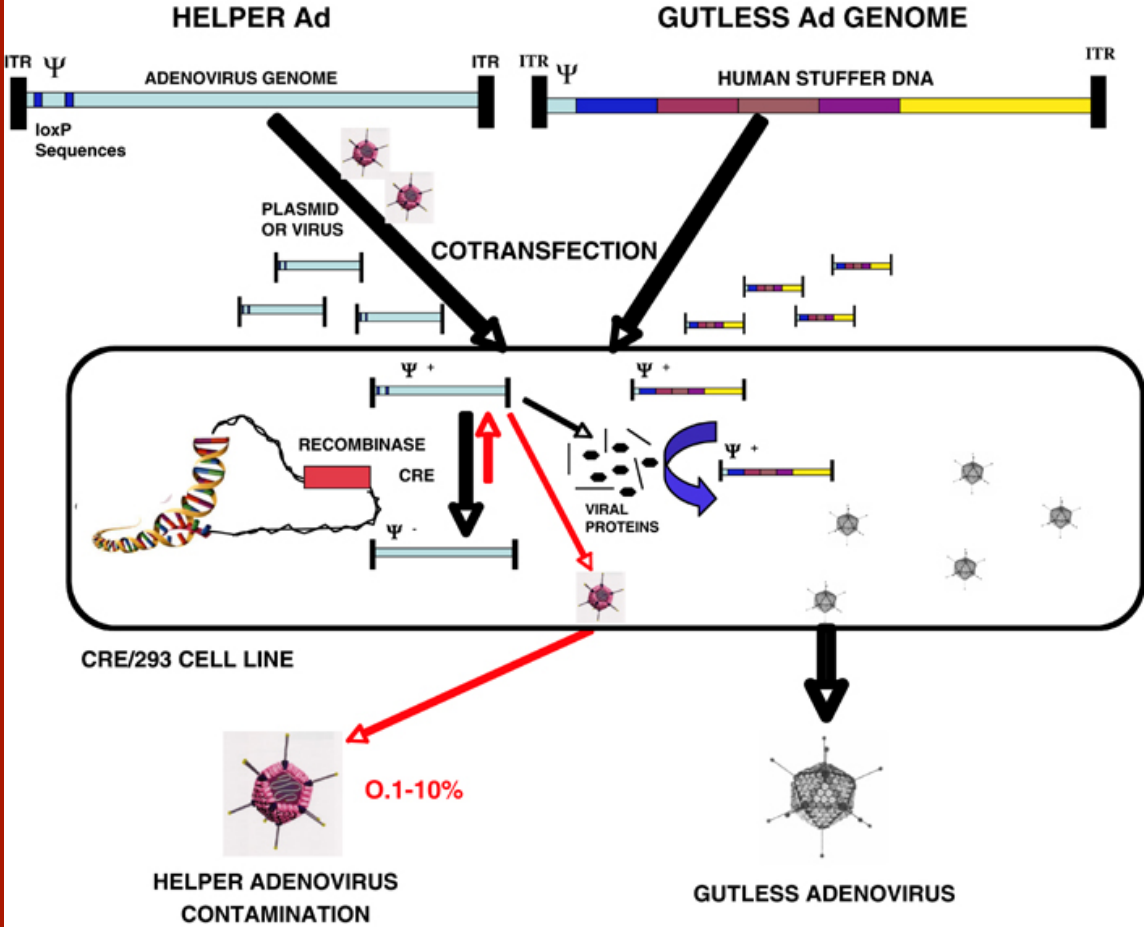
VIRUS CLASS	ADVANTAGE	DISADVANTAGE
Retrovirus	Easy to prepare	Can only infect dividing cells Low vector titers Limited capacity for foreign DNA
Adeno-associated virus (AAV)	High vector titers No viral gene expression Can infect nondividing cells Low immunogenicity	Low capacity for foreign DNA Tedious to prepare large quantities
Adenovirus	High vector titers High capacity for foreign DNA Can infect nondividing cells	High immunogenicity Limited duration of *in vivo* gene expression
Herpesvirus (HSV)	High vector titers High capacity for foreign DNA Can infect nondividing cells	High immunogenicity Limited duration of *in vivo* gene expression
Lentivirus	Can infect nondividing cells	Not very well studied Relatively low vector titers Limited capacity for foreign DNA

"Delivery of Neurotrophic Factors to Neuronal Targets: Toward Gene Therapy in the CNS", Blesch A. (2000).



"Gene therapy finds its niche", Sheridan C. (2011)

Viral vectors – Why HdAd?



To overcome the disadvantages of the 1st and 2nd adenovirus a helper-dependent system was developed.

HdAd:

- Capacity to integrate app 36kb of DNA;
- Long-term stability and expression in many tissues;
- Low immunogenicity;
- Low integration rate;
- Easily produced in high titers in the laboratory.

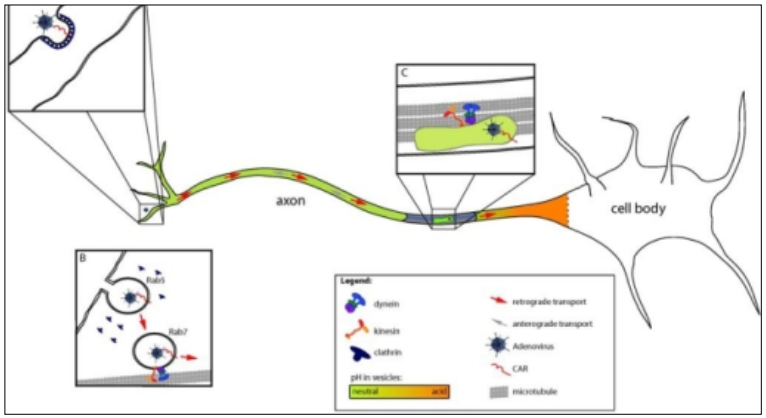
"Gutless adenovirus: last-generation adenovirus for gene therapy" Alba R., *et al. (*2005)

LoxP/Cre System – Provides 90-99% purity of the Hd vector

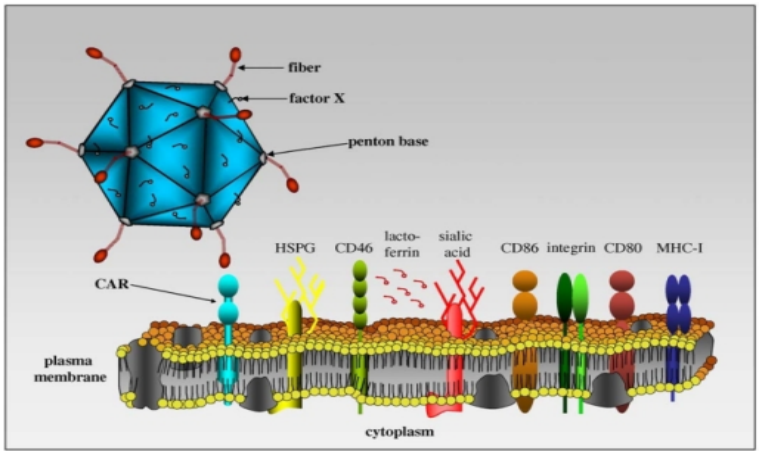
Vector choice: CAV-2

Specificity for cells in the CNS

- High tropism for neurons in the brains of rodents, dogs and primates and preferentially transduced human neurons ex vivo in organotypic cortical slices;
- Efficiently traffic to afferent structures via retrograde axonal transport (100-fold more efficient than HAdV5 or lentivirus);
- Use of the CAR (coxsackievirus adenovirus receptor) which is expressed by neurons in the brain parenchyma;
- Expression of a transgene for at least 1 year in vivo;
- Absence of immunogenicity in the CNS of immunologically naïve animals.



Scheme of axonal transportation

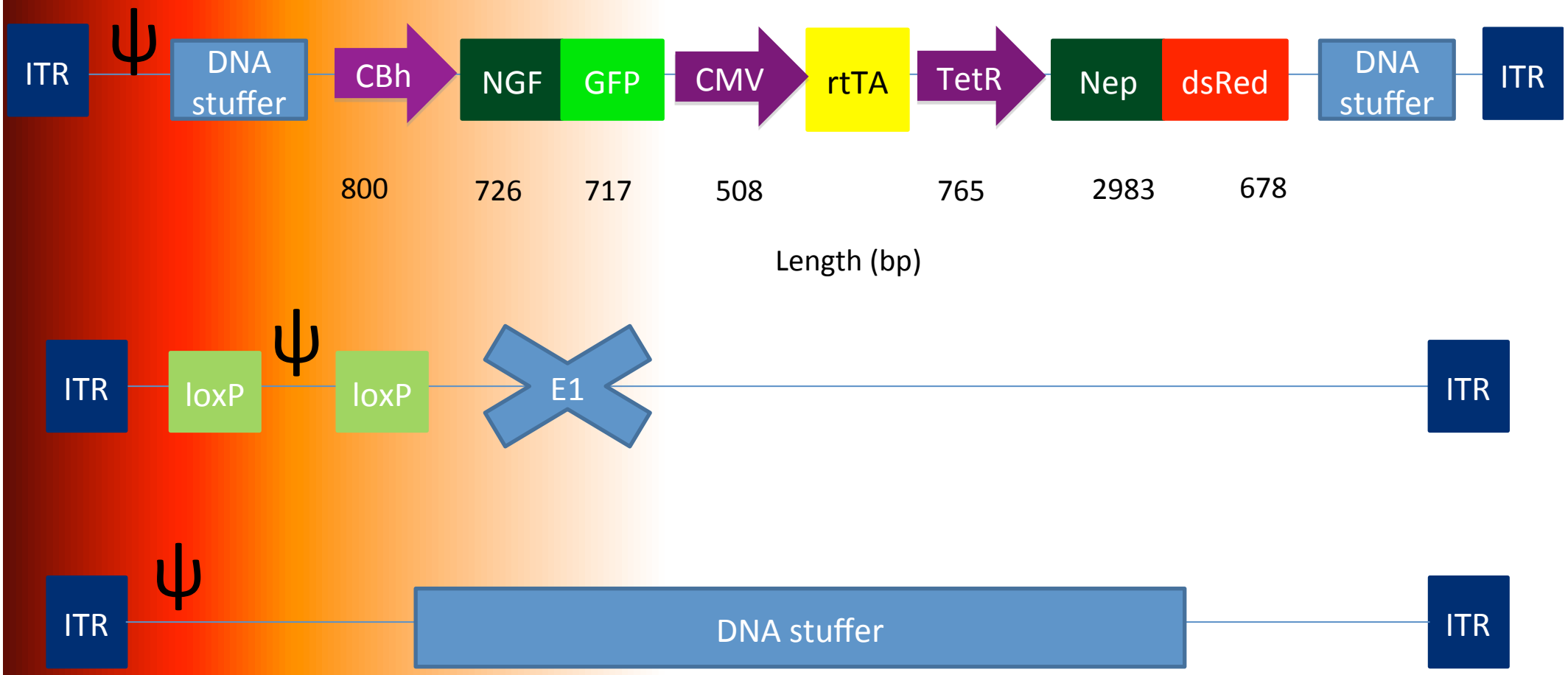


Scheme of ligation to CAR

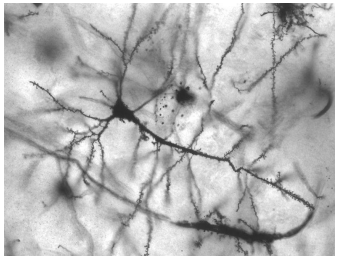
"An Update on Canine Adenovirus Type 2 and Its Vectors", Kremer E., et al (2010)

Vector's construction approach : A combine therapy

Bicistronic Gutless Adenovirus Vector:

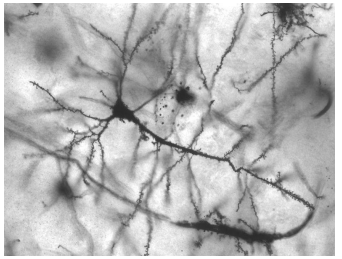


Experimental plan: *in vitro*



Preliminary tests

-**Fluorescence analysis** with GFP and dsRed ⟶ **Promoters efficiency**
-**Propidium iodide** ⟶ Dose-dependen**t Neurotoxicity**



Human and mice APP mutated hippocampal cell line transduced with:
- NEP wt/NGF wt
- NEP mut/NGF wt
- NEP wt/NGF mut
- NEP mut/ NGF mut
-Only DNA stuffer

Measurements and Expected Results

Test	Purpose	NEPwt/NGFwt	NEPmut/NGFwt	NEPwt/NGFmut	NEPmut/NGFmut (or DNA stuffer)
Northern blot	NEP and NGF trascription	↑	↓ ↑	↑ ↓	↓
Western blot/ ELISA-test	NEP and NGF translation	↑	↓ ↑	↑ ↓	↓
	Aβ40, Aβ42	↓	?	↓	↑
Enzymatic assay (DAGNPG)	NEP proteolitic activity	↑	↓	↑	↓
TrkA kinase assay	NGF bond to its receptor	↑	↑	↓	↓
Propidium iodide	Neuronal death	↓	↓	?	↑

Experimental plan : *in vivo*

Intracardiac injection in APPSwDI/NOS2 mice

Terapeutic vector

Six transgenic mice

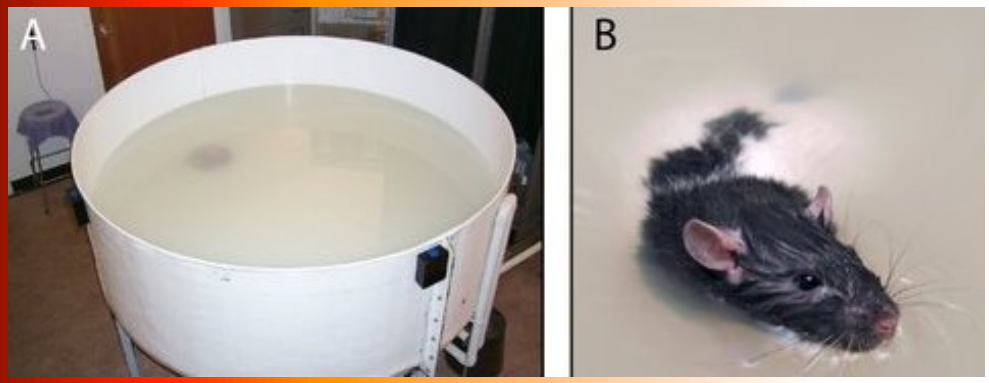
Six wild-type mice

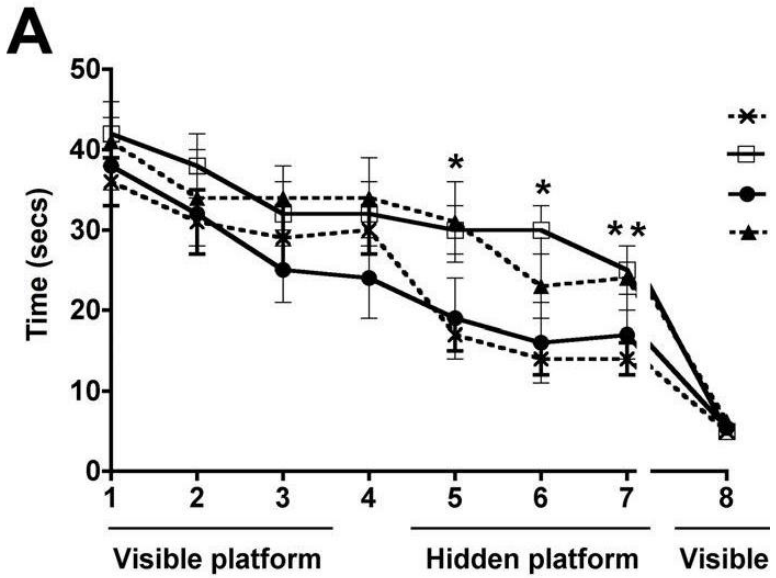
Empty vector

Six transgenic mice

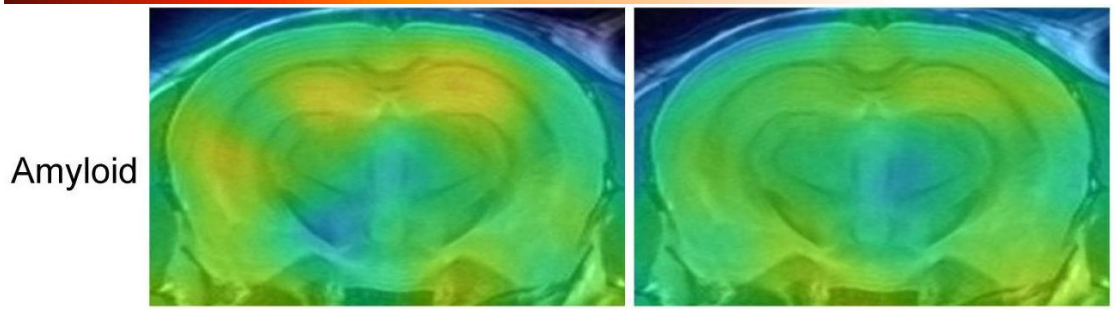
Six wild-type mice

Behavioral measurement: water maze test





"Long-term neprilysin gene transfer is associated with reduced levels of intracellular Abeta and behavioral improvement in APP transgenic mice", Spencerr, et al., 2008



"Global brain delivery of neprilysin gene by intravascular administration of AAV vector in mice", Iwata,et al., 2013

- Immunological tests in order to check the immunogenicity of our vector
- Brain morphological analysis by PET-scan

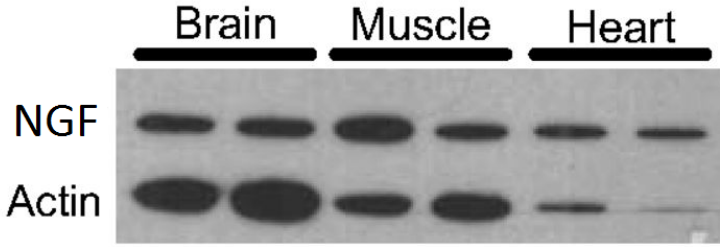
In order the study whether the expression of our transgene lasts in time, we'll sacrifice the mice in three different times: after 3, 6 and 12 months after the vector injection.

Measurements and Expeted results

Test	Purpose	NEPwt/NGFwt	NEPmut/NGFmut
Northern blot	NEP and NGF trascription	↑	↓
Western blot/ELISA-test	NEP and NGF translation	↑	↓
	Aβ40, Aβ42, tau levels	↓	↑
Enzymatic assay (DAGNPG)	NEP proteolitic activity	↑	↓
TrkA kinase assay	NGF bond to its receptor	↑	↓
Propidium iodide	Neuronal death	↓	↑

Pitfalls and solutions

- Constitutive promoter +
  - intravascular injection
  - synapsin 1 promoter
- Time-dependent decrease in expression of NGF or NEP
  - second injection with a different viral vector



Costs

Product	Company	Price
CAV-2 vector production kit	Microbix Biosystem Inc.	1400 €
DKCre cells	ATCC	640 €
Hyppocampal neuronal cells from human and mice (1 million)	Innoprot	1500 €
DAGNPG	Sigma	162 €
TrkA kinase assay	Promega	770 €
Propidium iodide (10 mL)	Life technologies	90 €
ELISA-kit for NEP and NGF	Sigma	1000 €
Antibodies for NEP, NGF, Aβ40, Aβ42, tau-protein, NeuN, GFAP, IBA1	Herk millipore / Sigma	4000 €
APPSwDI/NOS2 transgenic mice (one)	The Jackson Laboratories	1000 €

References

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