Agència de Gestió d'Ajuts Universitaris i de Recerca

AGAUR

# Memòria justificativa de recerca de les convocatòries BCC, BE, BP, CTP-AIRE, INEFC i PIV

## 1.- Dades bàsiques i resums

**Nom de la convocatòria**

**BP**

**Llegenda per a les convocatòries:**

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|---|---|
| BCC | Convocatòria de beques per a joves membres de comunitats catalanes a l'exterior |
| BE | Beques per a estades per a la recerca fora de Catalunya |
| BP | Convocatòria d'ajuts postdoctorals dins del programa Beatriu de Pinós |
| CTP-AIRE | Ajuts per accions de cooperació en el marc de la comunitat de treball dels Pirineus.<br>Ajuts de mobilitat de personal investigador. |
| INEFC | Beques predoctorals i de col·laboració, dins de l'àmbit de l'educació física i l'esport i les ciències aplicades a l'esport |
| PIV | Beques de recerca per a professors i investigadors visitants a Catalunya |

**Títol del projecte:** ha de sintetitzar la temàtica científica del vostre document.
CAV2 vector gene transfer into central nervious system

**Dades de l'investigador o beneficiari**

| Nom | Cognoms |
|---|---|
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**Número d'expedient**

BP 2009

**Paraules clau:**

Helper-Dependent Canine Adenoviral vectors, non-human primates, gene therapy, central nervious System and Sly Syndrome.

**Data de presentació de la justificació**

**08/04/2013**

Nom i cognoms i signatura Neus Bayó Puxan del/de la investigador/a

Vist i plau del/de la responsable de la sol·licitud

**Resum en la llengua del projecte**

En aquest projecte s'ha avaluat les avantatges dels vectors adenovirals canins tipus 2 (CAV2) com a vectors de transferència gènica al sistema nerviós central (SNC) en un model primat no-humà i en un model caní del síndrome de Sly (mucopolisacaridosis tipus 7, MPS VII), malaltia monogènica que cursa amb neurodegeneració.

En una primera part del projecte s'ha avaluat la biodistribució, l'eficàcia i la durada de l'expressió del transgen en un model primat no humà, (*Microcebus murinus*). Com ha vector s'ha utilitzat un CAV2 de primera generació que expressa la proteïna verda fluorescent (CAVGFP). Els resultats aportats en aquesta memòria demostren que en primats no humans, com en d'altres espècies testades anteriorment per l'equip de l'EJ Kremer, la injecció intracerebral de CAV2 resulta en una extensa transducció del SNC, siguent les neurones i els precursors neuronals les cèl·lules preferencialment transduïdes. Els vectors canins, servint-se de vesícules intracel·lulars són transportats, majoritàriament, des de les sinapsis cap al soma neuronal, aquest transport intracel·lular permet una extensa transducció del SNC a partir d'una única injecció intracerebral dels vectors virals.

En una segona part d'aquest projecte s'ha avaluat l'ús terapèutic dels CAV2. S'ha injectat un vector helper-dependent que expressa el gen la b-glucuronidasa i el gen de la proteïna verda fluorescent (HD-RIGIE), en el SNC del model caní del síndrome de Sly (MPS VII). La biodistribució i la eficàcia terapèutica han estat avaluades. Els nivells d'activitat enzimàtica en animals malalts injectats amb el vector terapèutic va arribar a valors similars als dels animals no afectes. A més a més s'ha observat una reducció en la quantitat dels GAGs acumulats en les cèl·lules dels animals malalts tractats amb el vector terapèutic, demostrant la potencialitat terapèutica dels CAV2 per a malalties que afecten al SNC.

Els resultats aportats en aquest treball ens permeten dir que els CAV2 són unes bones eines terapèutiques per al tractament de malalties que afecten al SNC.

Durada del projecte: Desembre 2010-Desembre 2012

**Resum en anglès**

Advantages of canine adenoviral vector type 2 (CAV2) as a CNS gene transfer vector have been assessed in a non-human primate model and in a canine model for Sly Syndrome; monogenic neurodegenerative disease.

Biodistribution, efficacy and transgene expression has been assessed in a non-human primate model. CAV2 first generation vector expressing GFP has been used. Results show a widespread CNS transduction of CAV2 in non-human primates, as in other many species previously tested. As in other species, CAV2 preferentially transduces neurons and neural precursor cells. CAV2 are naturally retrogradelly transported allowing widespread distribution along the brain.

Therapeutical use of CAV2 vectors has been also assessed. A helper-dependent vector expressing b-glucuronidase and GFP (HD-RIGIE) has been intracerebraly injected into Sly dogs. Biodistribution and therapeutical benefits have been analyzed. Enzymatic activity of b-glucuronidase has been restored on Sly-dogs treated with HD-RIGIE reaching to normal enzymatic levels. Moreover a reduction on GAG accumulation has also been observed on HD-RIGIE-treated dogs.

CAV2-based vectors are promising therapeutical tools to treat diseases affecting CNS.

**2.- Memòria del treball**

## I. TESTING GENE TRANSFER EFFICACY OF HD-CAV2 VECTORS IN PRIMATE NON-HUMAN CENTRAL NERVIOUS SYSTEM.

Efficacy and biodistribution of CAV-2 vectors into the CNS and skeletal muscle of *Microcebus murinus* has been assayed. *Microcebus murinus* has been used as a primate model: it is readly bred in captivity, self-sustained breeding colonies existing and are able to provide sufficient number of animals for each trial to reach significance in statistical test. Moreover *Microcebus murinus* can naturally show some signs of neurodegenerative diseases found in human. *Microcebus murinus* were housed at the primate facility at the University of Montpellier II. The protocol was in accordance with EU legislation and approved by regional ethic committee.

Helper-dependent CAV2 vectors expressing GFP were used. CAV2 based vectors have numerous advantages that make it suitable vector for brain-directed gene transfer: (i) CAV-2 preferentially transduces neurons and undergo efficient axonal transport in the CNS of rodents, dogs and primateds CAV-2, (ii) following intramuscular injection. CAV-2 preferentially transduces motoneurons and (iii) CAV-2 vectors lead to more than 1 year in vivo transgene expression in rodent CNS without accompaning immunosupression. HD vectors led to long-term expression in many tissues in immunologically naïve animals and is feasible to obtain nontoxic, concentrated and homogeneous HD preparation.

**Widespread neuronal expression of HD-CAVGFP vectors in CNS-primates.**
Male microcebes were injected with HD-CAVGFP into the caudate nucelus of the right hemisphere ($5x10^8$ viral particles, $5x10^7$ infectious units). Animals were sacrificied and brains were processed for immunostology, scanned for GFP expression and/or used to extract total DNA.

Transduced cells in diferent structures throughout the CNS were identified and quantified on 50-micron-thick sections screened by LSCM. GFP+ cells were found at the injection site (Figure 1A), at the frontal and the occipital cortex (Figure 1B-D) in both hemispheres. GFP+ cells were also found throughout the substantia nigra pars compacta (Figure 1E), the basal nuclei of Meynert (Figure 1F) as well as several other regions (Figure 1G). A 3D image of microcebe brain highliting areas that contained transduced cells was created to better represent transgene distribution (Figure 1H).

To identify GFP+ cells, brain sections were stained with antibodies that mark neurons (NeuN) and astrocytes (GFAP). HD-CAVGFP preferentially transduced neurons at the injection site (Figure 2A) as well as in the còrtex (Figure 2B). In the substantia nigra, co-labeling with tyrosin hydrolase (TH) demonstrated that GFP+ cells were dopaminergic neurons (Figure 2C).

These data demonstrated that HD CAV-2 vectors preferentially transduced neurons in the primate brain and were carried by axonal retrograde transport to afferent structures in both hemispheres.

**Transduction of multipotent neural stem cells.**
Ependymocytes lining the ventricles were transduced in some males (Figure 1G). In addition, in some microcebes transgene expression was consistent with the vector being deposited in the lateral ventricle (not shown). Among the cells near the surface of the ventricles are multipotent neural stem cells (NSCs). Adult periventricular NSCs give rise to glia and migrating neuroblasts that differentiate into interneurons in the olfactory bulb. To determine if CAV-2 vectors that reached the cerebral spinal fluid transduced primate NSCs we stained for GFAP and Sox2, putative markers for NSCs. We found GFP/Sox2/GFAP-positive cells in the ventricular zone in contact with the ventricular lumen (Figure 3), suggesting that CAV-2 vectors transduced NSCs.

**Efficient transduction of motoneurons following injection into the hind leg muscles.**
CAV-2 is retrograded transported in rodent motor neurons (MNs). To determine if primate motor neurons were also capable of being transduced *in vivo* via skeletal muscles, CAVGFP was injected into the gastrocnemius of three microcebes. Spinal chords were removed, fixed, sectioned and screened by LSCM for transduced cells. In the animals sacrificed at 2 weeks and 1 month post-injection, an average of ~130 transduced cells/animal in each ipsilateral anterior horn was found (Figure 4). The region in the chord, the location within the horn and the morphology of the GFP+ cells clearly identified the transduced cells as alpha-MNs.

**Transduction of MNs is due to CAR expression at the neuromuscular junction.**
CAR expression at the neuromuscular junction (NMJ) in rodents has been reported previously. Although these studies did not formally exclude the neuronal expression, they concluded that CAR was expressed and located on the muscle cells. To address the preferential transduction of MN following intramuscular injections in the microcebe we stained CAR on cryosectioned microcebe skeletal muscles. NMJ were stained using Tuj1, which is neuron specific; and $\alpha$-bungarotoxin which labels the acetylcholine receptor (muscle specific). We found that CAR colocalized with Tuj1 (Figure 5A). We detected no signal overlapping with $\alpha$-bungarotoxin or projecting into the muscle cell (Figure 5A-C). Although we cannot exclude a low level of muscle-derived CAR at the NMJ, the location of CAR at the primate NMJ was consistent with the essentially exclusive transduction of MNs following intramuscular injection.

## II. PRECLINICAL GENE THERAPY ASSAYS ON MPSVII-DOGS TREATED WITH HD-GUSB.

Potentiality of CAV2 vectors as a gene therapy vectors for CNS has been assessed on MPS VII-dogs. MPS VII is a monogenic neurodegenerative disease. Helper-dependent vector expressing b-glucuronisase and GFP has been used as a therapeutical vector.

**Widespread neuronal expression of HD-CAVGFP vectors in CNS-dogs.**
To determine the efficacy and level of retrograde transport in the healthy and MPS VII dog brain an initial cohort was injected with HD-GFP. At the site of striatal injections, GFP+ cells with quintessential neuron morphology were predominant (Figure 6A). We detected GFP+ cells throughout both hemisphere in particular on the ipsilateral frontal, the temporal, the occipital cortex; the thalamus, the hippocampus and the nuclei of Meynert. These data demonstrated the efficient in vivo retrograde transport of CAV-2, which mimics its transport in vitro and in vivo in rodents as previously described. To identify GFP+ cells, brain sections were stained with antibodies that mark neurons (NeuN) and astrocytes (GFAP) (Figure 6B). The data demonstrated that CAV-2 vectors preferentially transduced neurons in the dog CNS.

**Contruction of HD-CAVGFP expressing β-glucuronidase and GFP (HD-RIGIE).**
HD CAV-2 vector harboring a GUSB expression cassette followed by an IRES-GFP sequence (Figure 7A) was created, HD-RIGIE. The expression cassette allowed us to identify transduced cells via GFP expression, and compare that to the biodistribution of β-glu activity. It is important to note that in contrast to GFP, β-glu is released from cells, recaptured by neighbouring cells and transported to afferent and efferent brain regions.

**Gene therapy on MPS VII-dogs injected with HD-RIGIE.**
HD-RIGIE was injected into the brains of six MPS VII dogs that were sacrificed at 1 or 4 months postinjection. MPS VII brains were semi-fixed in vivo and were processed for vector biodistribution, β-glucurunodiase activity and immunohistology studies.
At 1-month postinjection of HD-RIGIE in MPS VII dogs, β-glucuronidase activity was detected in sections near the predicted injection sites in the hippocampus and in the caudate nucleus in most dog brains (Figure 7B). At 4-months postinjection staining was notably more intense in several areas of the MPS VII brain parenchyma (Figure 7B). A striking difference between 1 and 4 months was in ependymal cells, which were more intensely stained cells.

β-glucuronidase activity was quantified in tissue homogenates by a biochemical approach. β-glucuronidase activity was compared in healthy dogs, MPS VII dogs injected with HD-GFP and MPS VII dogs injected with HD-RIGIE (Figure 7C). In semi-fixed healthy brains, β-glu activity averaged ~3,500 4-MU nmol/mg protein/hr. In MPS VII dog brains injected with HD-GFP, we found an average of 200 4-MU nmol/mg protein/hr. β-glu activity was found in caudate nucleus, hippocampus and thalamus consistent with the in situ β-glu activity (Figure 7C). Consistent with the biodistribution of the HD-GFP vector (not shown) increased β-glu activity throughout the frontal, parietal, temporal and occipital cortexes was found. These data demonstrate the widespread distribution of β-glu activity, likely due to a combination of vector transport and β-glu transport.

Several MPSs, including Sly Syndrome, leads to upregulation of other lysosomal enzyme. β-hexosamindase (β-hex) activity was measured in healthy, HD-GFP and HD-RIGIE injected MPS VII-dogs (Figure 7D - E). In healthy dogs, β-hex activities were ~0.2 to 1 (4-MU nmol/mg protein/hr) in the cortex, caudate nucleus and hippocampus. In brain structures from HD-GFP-injected MPS VII dogs, β-hex activities were significantly higher than healthy dogs. β-hex activity on HD-RIGIE injected dogs was significantly reduced when compared to age-match HD-GFP injected dogs and was near normal level. β-hex activity in the hippocampus was returned to levels of healthy dogs in 3 of the 5 dogs injected with HD RIGIE.

Neuropathology on injected dog brains was indirectly assayed by toluidine blue staining, which allows to visualize GAG storage. In healthy neurons, toluidine blue staining appears homogeneous thought the cytoplasm, whereas in MPS VII cells cytoplasmic nonstaining extended vacuoles reflect GAG storage (Figure 8). As in MPS VII mice, cats, dogs, and human cells, MPS VII dogs injected with HD-GFP and sacrificed at 1 or 4 months, we detected nonstaining extended vacuoles throughout the CNS. In HD-RIGIE injected MPS VII dogs sacrificed at 1 month we detected a significant reduction of nonstaining extended vacuoles in cells throughout the caudate nucleus, hippocampus and cortex (Figure 8). Cells with glia and neuron-like morphology had no detectable distended storage vesicles and were not different from the cells in comparable region in healthy dogs. At 4-months postinjection of HD-RIGIE, we found a minority of cells with nonstaining extended vacuoles in the caudate nucleus, hippocampus and cortex.

All those data suggested CAV2-based vectors are promising therapeutical tools to treat diseases affecting CNS.