REPORT: Edward H. Schuchman ASMD Research Fellowship

National Niemann-Pick Disease Foundation

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SUMMARY OF RESEARCH PERFORMED DURING THE PERIOD OF THE FELLOWSHIP

The goal of this project was to assess the suitability of intranasal delivery of chitosan-encapsulated hydrocortisone to treat brain pathology in mice lacking the acid sphingomyelinase (ASMko), which mimic the infantile neurovisceral ASMD. During the period of the fellowship, (from October 1 2022-October 1 2023 and in the months of extension we requested until April 2024) I have followed the experimental design proposed and performed some additional experiments as detailed below.

1. Generation of chitosan nanocapsules (NC) containing hydrocortisone (NC-HC).

To reach this objective we collaborated with the laboratory of Dr. Jesús Martínez de la Fuente (ICAM Institute, University of Zaragoza, Spain), who is an expert in NC synthesis. The synthesized NC consisted of a hydrophobic core capable of encapsulating hydrophobic molecules (such as hydrocortisone), surrounded by a polymeric shell of chitosan that provides mucoadhesive properties to the system. This type of nanocapsules were selected for this project since it has been described that, upon intranasal delivery, chitosan-based NC adhere to the nasal mucosa allowing the sustained release of the drug in the brain.

Although chitosan-based NC can reach the brain, we first determined whether neurons in culture were able to internalize them. To this aim, we loaded the NC with the fluorophore (DiD) and incubated primary cultures of neurons from wild type (WT) mice with different NC concentrations. The results showing fluorescence signal inside the cells confirmed that neurons uptake the chitosan NC in a concentration and time dependent manner (Figure 1).

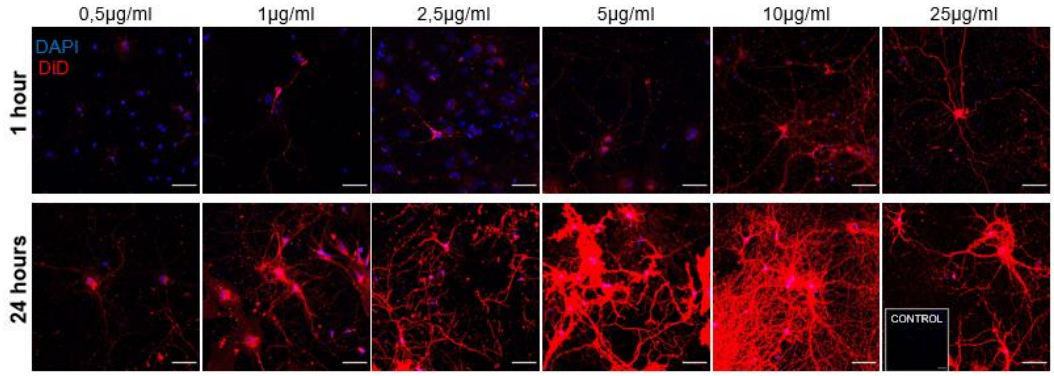

Figure 1. Neurons can uptake chitosan nanocapsules. *Representative images of neurons in culture treated with chitosan nanocapsules labelled with DiD fluorophore (red) at different concentrations and times of treatment. Cell nuclei were stained with DAPI (blue). Scale bar 50µm.*

To load hydrocortisone into the chitosan nanocapsules (NC-HC) we followed the protocol illustrated in Figure **2**. Briefly, a nanoemulsion between an organic phase (ethanol, oleic acid, and Span® 85), hydrocortisone and an aqueous phase (water and Tween® 20) was prepared under magnetic stirring. Chitosan was added to the nanoemulsion and combined with sodium sulphate to favor the ionotropic gelation by which chitosan is converted into a three-dimensional network structure conferring a solid shell to the nanoemulsion. Ultracentrifugation was performed to clear the NC-HC, which were freeze-dried with mannitol as cryoprotectant and stored at 4ºC until use.

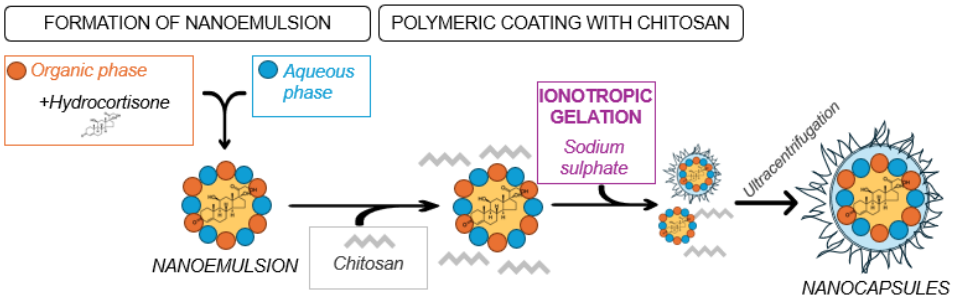

Figure 2. Schematic representation of NC-HC synthesis.

2. <u>Assessment of the safety and the efficacy of different doses of NC-HC to reduce SM levels in cultured neurons.</u>

To assess the safety and efficacy of NC-HC in ASMko neurons we treated primary neuronal cultures from ASMko mice with vehicle (CTL) or with free hydrocortisone (HC), empty chitosan nanocapsules (NC-empty) or NC-HC for five days at different concentrations: 0.1, 0.5 and 1μM.

Toxicity of nanocapsules was assessed with the DeadEnd™ Fluorometric TUNEL System–Promega assay, which measures DNA fragmentation. The result showed no toxicity of the treatment at any of the concentrations used (Figure 3).

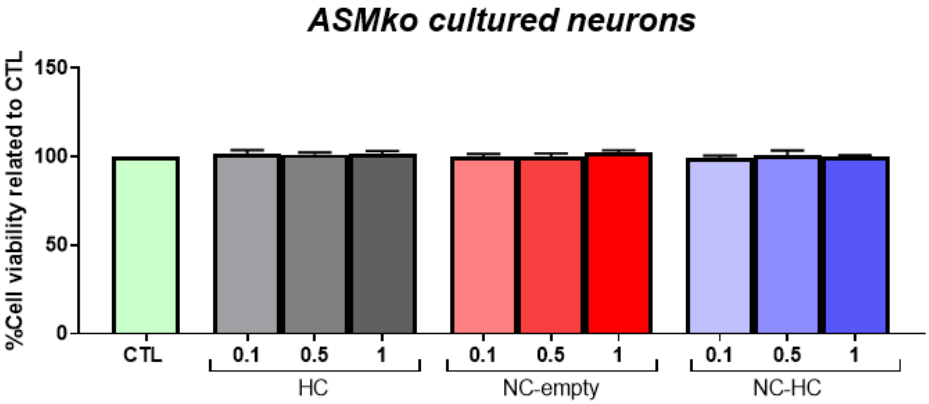

Figure 3. Chitosan nanocapsules are not toxic to ASMko neurons in the concentrations tested. *Graph shows mean±SEM cell viability as % of living cells related to the vehicle treated (CTL) ASMko neuronal cultures.*

Efficacy of the treatment was assessed by measuring sphingomyelin (SM) levels with a fluorometric assay. The results showed NC-HC encapsulating HC at the highest concentration (1μM) reduced SM levels by 24% in the ASMko neurons while the empty NC did not have any effect (Figure 4).

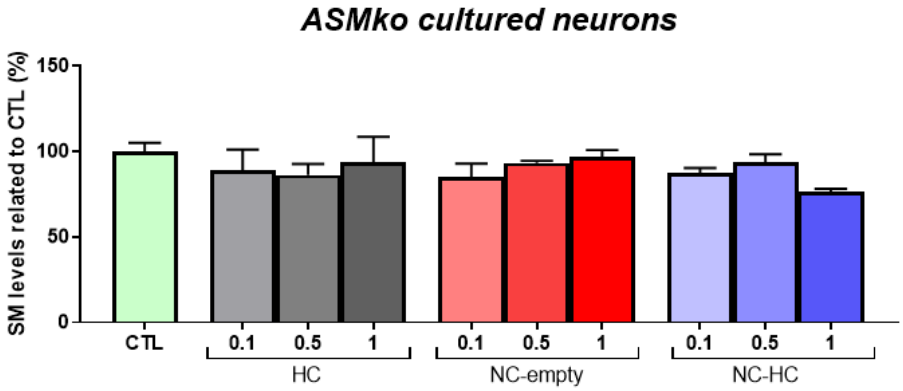

Figure 4. Effects of NC-HC incubation on SM levels in ASMko cultured neurons. *Graph shows mean±SEM SM levels as % related to the vehicle treated (CTL) ASMko neuronal cultures.*

3. <u>Assessment of the safety and the efficacy of intranasal delivery of NC-HC to reduce brain SM levels, neuroinflammation and neurodegeneration and to improve motor abilities and behavior in ASMko mice.</u>

To assess the efficacy of NC-HC *in vivo*, a first trial was performed following the experimental plan initially proposed, which is illustrated in Figure 5. Briefly, 1,5-month-old wt and ASMko mice, in groups of 6 animals each, were intranasally administered with empty NC (considered the vehicle) or with NC containing HC (NC-HC). NCs were administered to anesthetized mice twice a week during a total of nine weeks. NC-HC contained a final concentration of 30 μg per dose in a final volume of 20μl, which is the maximum allowed for intranasal delivery in mice. This volume limitation obliged us to use a high concentration of nanocapsules making the final solution quite dense and showing aggregates.

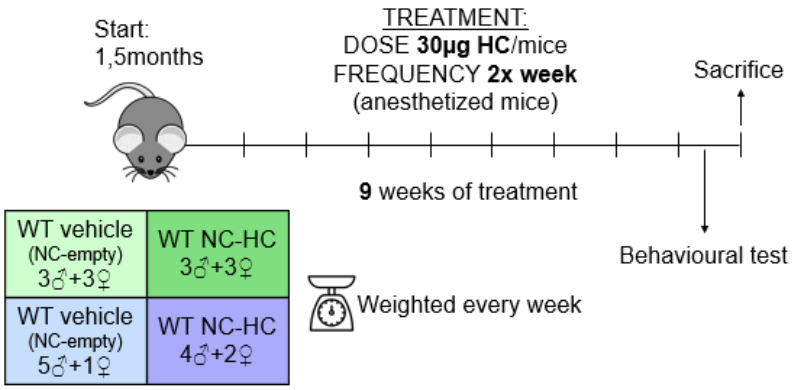

Figure 5. Scheme of the first in vivo trial using intranasal delivery of NC loaded with 30µg HC.

a) Weight gain analysis

All mice were weighted once a week during the whole trial. NC-HC treatment did not affect weight gain except for a slight reduction in ASMko mice at week 6 that was recovered in the following week (Figure 6).

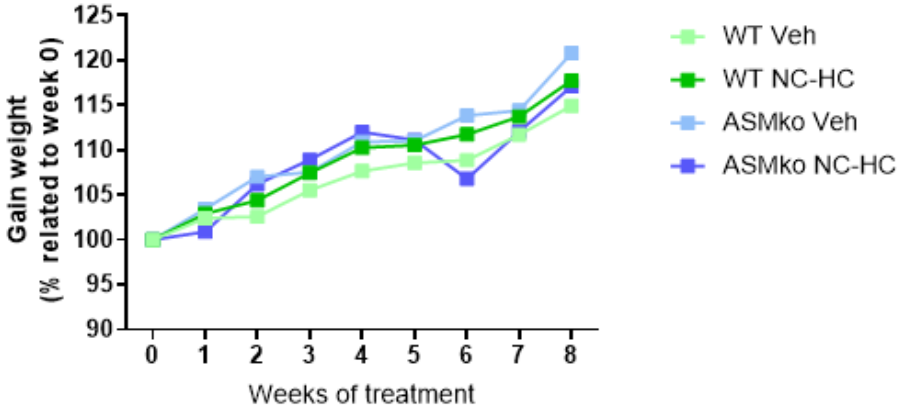

Figure 6. WT and ASMko gain weight during the trial. *Graph shows mean±SEM weight gain in wt and ASMko mice treated with vehicle or with NC-HC.*

b) Behavioral analysis

In the last week of treatment mice were subjected to different tests to assess the effects in motor abilities (Rota-rod test) and depressive-like behavior (Tails suspension test). The results confirmed the motor impairment (Figure 7A) and the altered behavior (Figure 7B) in the ASMko compared to wt mice. Intranasal delivery of NC containing 30 mg HC did not improve these alterations (Figure 7).

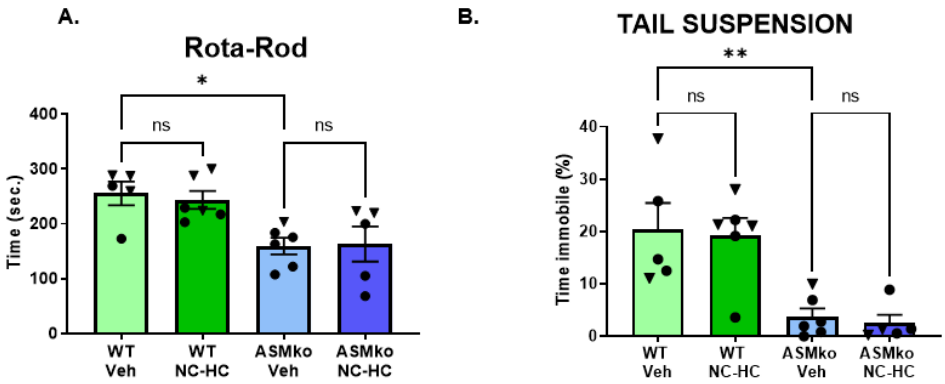

Figure 7. Nasal delivery of NC-HC twice a week does not improve motor and behavior anomalies in ASMko mice*. Graphs show mean±SEM of the time spent in the rod in the Rota-rod test (A) or of the immobility time in the Tail suspension test (B). Circles: males; triangles: females. *p-value<0.05, **p-value<0.01.*

c) Neurodegeneration, neuroinflammation and lysosomal size

Neuronal death was monitored by immunofluorescence against Calbindin in Purkinje cells of the cerebellum, which are the most vulnerable in ASMko mice. The results confirmed the drastic loss of these cells in ASMko mice compared to wt. Intranasal delivery of NC containing 30 µg HC did not increase neuronal survival (Figure 8).

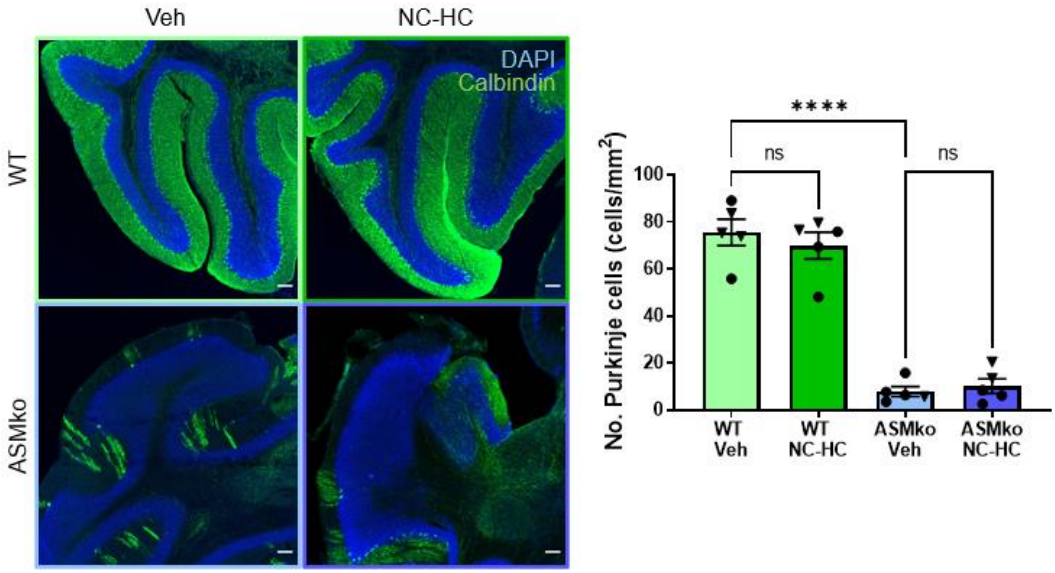

Figure 8. Nasal delivery of NC-HC twice a week does not prevent Purkinje cell loss in ASMko cerebellum. *Representative images from calbindin (green) immunostaining. Cell nuclei were stained with DAPI (blue). Scale bar 100µm. Graph shows mean±SEM number of Purkinje cells per area. Circles: males; triangles: females. ****p-value<0.0001.*

Neuroinflammation was determined in the cerebellum by immunostaining against the microglia marker iba-1. Increased number and size of microglia was confirmed in ASMko mice compared to wt (Figure 9). Intranasal delivery of NC containing 30 µg HC did not reduce these parameters (Figure 9).

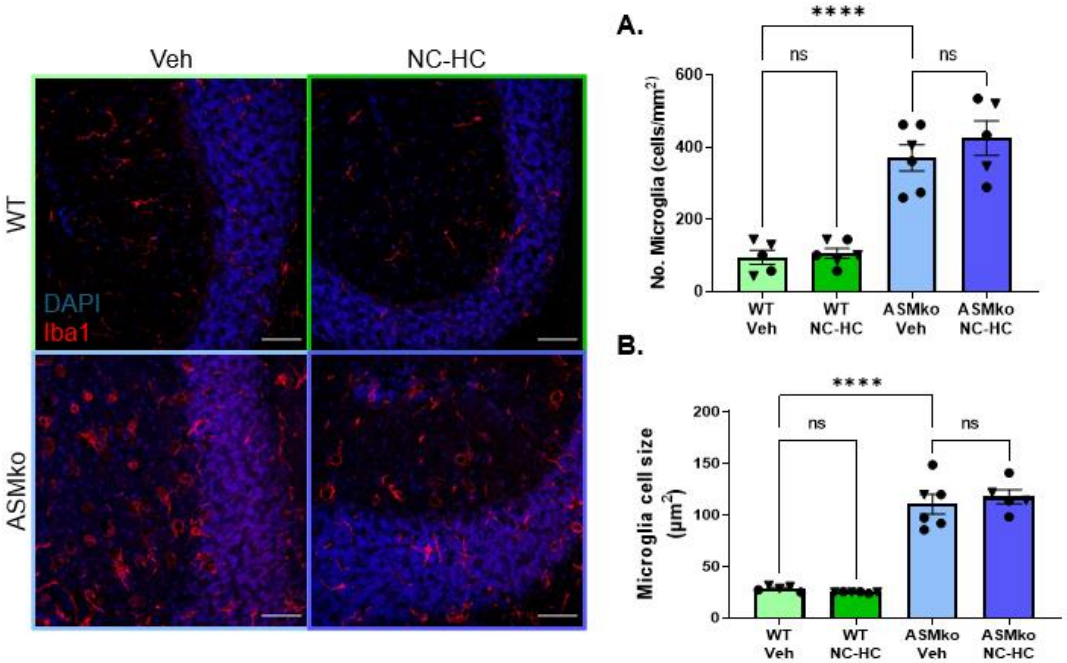

Figure 9. Nasal delivery of NC-HC twice a week does not ameliorate microgliosis in ASMko cerebellum. *Representative images from Iba1 (red) immunostaining. Cell nuclei were stained with DAPI (blue). Scale bar 50µm. Graphs show mean±SEM microglia number (A) and size (B). Circles: males; triangles: females. ****p-value<0.0001.*

Lysosomal size was determined in the Purkinje cells by immunostaining against the lysosomal protein Lamp1. Larger lysosomal size was confirmed in ASMko mice compared to wt (Figure 10). Intranasal delivery of NC containing 30 µg HC did not reduce lysosomal size in ASMko Purkinje cells (Figure 10).

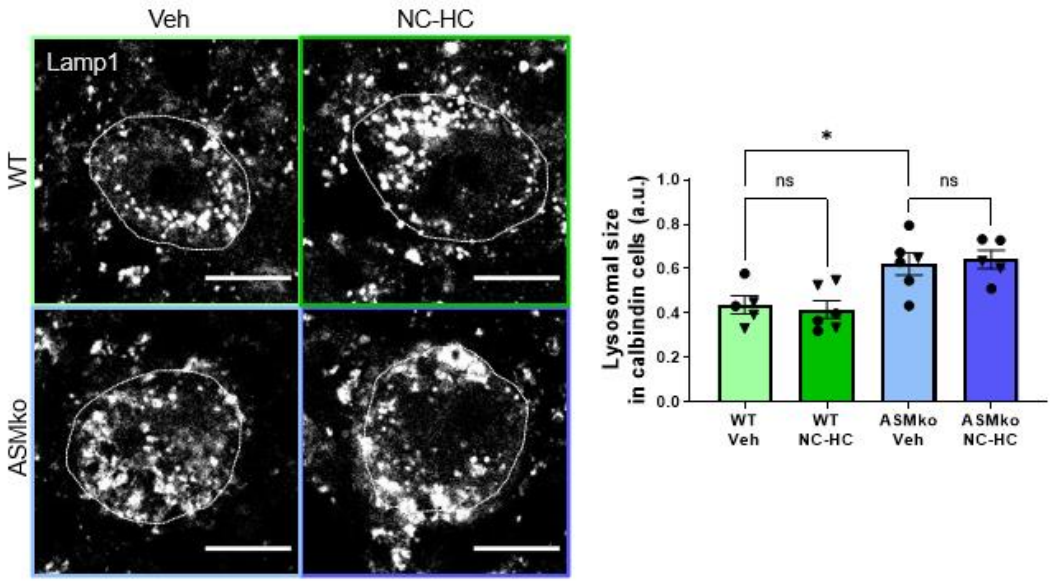

Figure 10. Nasal delivery of NC-HC twice a week does not reduce lysosomal size in ASMko cerebellum. *Representative images from Lamp1 (grey) immunostaining. Scale bar 10µm. Graph shows mean±SEM lysosomal size in calbindin+ Purkinje cells, which are outlined by a white line. Circles: males; triangles: females. *p-value<0.05.*

d) Sphingomyelin accumulation

SM levels were quantified in different brain areas (cortex, cerebellum, and hippocampus), the liver and the lungs by enzymatic assays. The results confirmed the accumulation of this lipid in ASMko compared to wt mice in all organs analyzed (Figure 11). Intranasal delivery of NC containing 30 µg HC did not reduce SM levels in any instance (Figure 11).

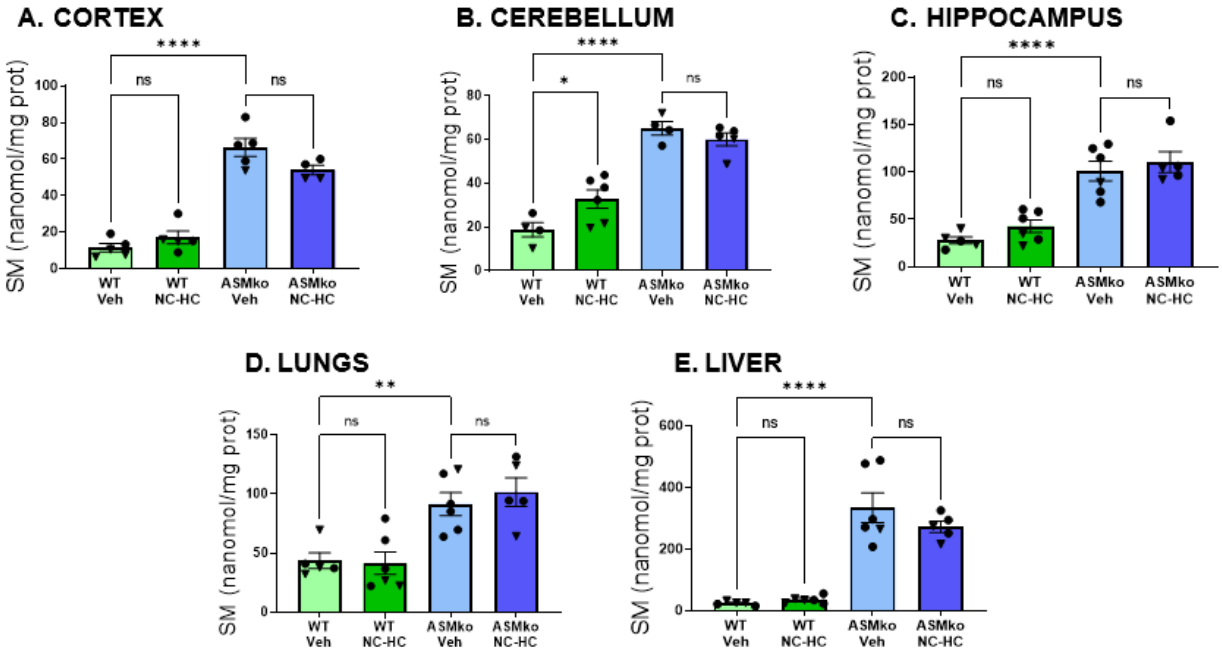

Figure 11. Nasal delivery of NC-HC twice a week does not reduce SM accumulation in ASMko mice. *Graphs show mean±SEM SM levels in A. Cortex, B. Cerebellum, C. Hippocampus, D. Lungs, E. Liver. Circles: males; triangles: females. *p-value<0.01, ****p-value<0.0001.*

This first *in vivo* trial showed no efficacy of the NC-HC tested in the ASMko mice. The negative results could be due to the problems of aggregation of the NC-HC solution. We thus decided to optimize the loading method to generate NCs able to encapsulate higher proportion of HC. The maximum loading capacity we could achieve was 23% compared to the initial 4%. We could thus use lower number of NCs for the maximum volume allowed in intranasal administration (20µl/dose) facilitating the solubilization of the nanocapsules and avoiding aggregation with respect to the first trial. Another change we implemented was to increase the frequency of administration from two to five times a week. To avoid problems with anesthesia the intranasal delivery was performed in awake mice. The rest of the trial protocol was similar to the first one as illustrated in Figure 12.

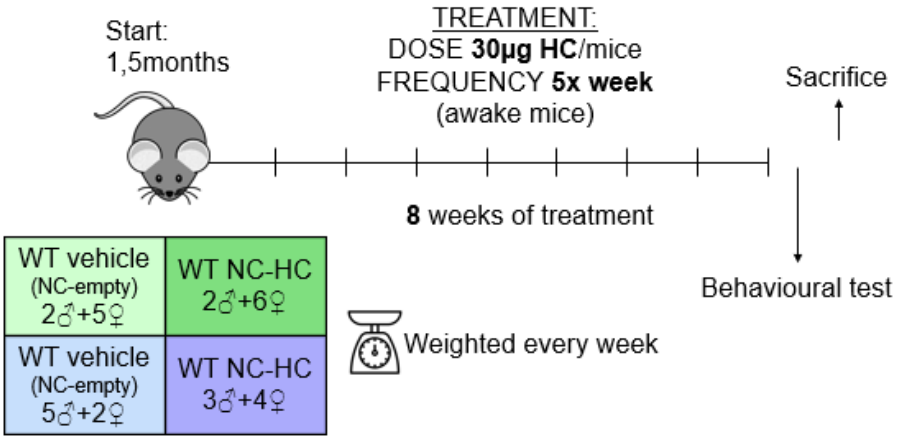

Figure 12. Scheme of the second in vivo trial using intranasal delivery of highly loaded NC-HC

a) Weight gain analysis

Weight did not increase significantly during the trial in all mouse groups (Figure 13). This unexpected finding could be due to the high frequency (5 times a week) of the intranasal administration in the awake mice, which could be very stressful to the animals.

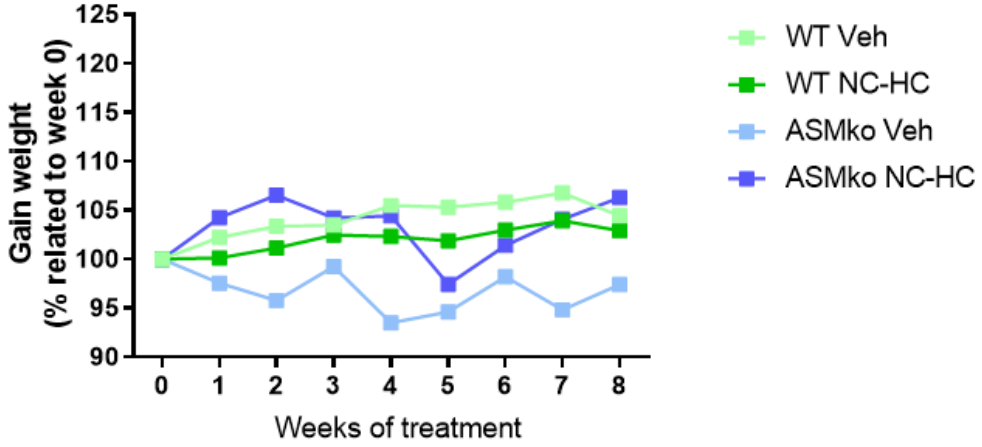

Figure 13. Nasal delivery five times a week prevents gain weight in wt and ASMko mice. *Graph shows mean±SEM weight gain in wt and ASMko mice treated with vehicle or with NC-HC.*

b) Behavioral analysis

Intranasal delivery of NC containing 30 µg HC five times a week did not improve the impaired motor abilities (Rota-rod test) nor normalized behavior (Tail suspension test) in the ASMko mice (Figure 14).

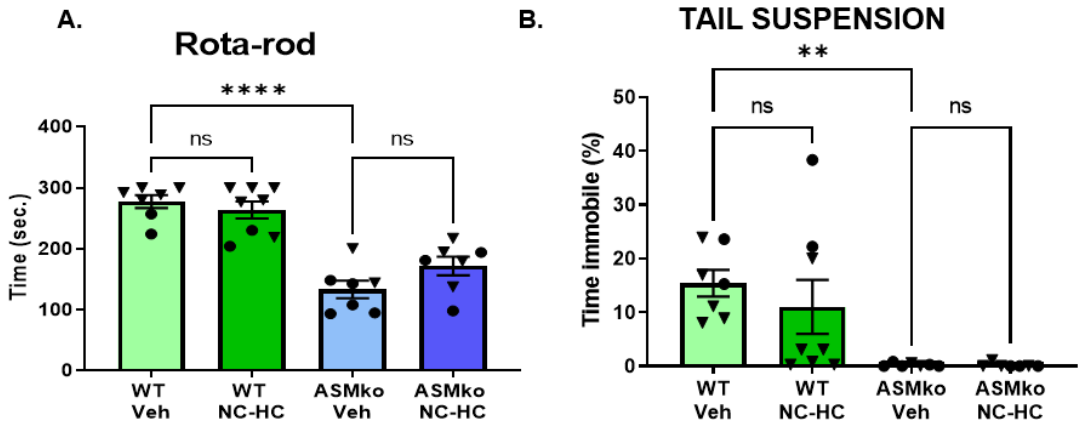

Figure 14. Nasal delivery of NC-HC five times a week does not improve motor and behavior anomalies in ASMko mice. *Graphs show mean±SEM of the time spent in the rod in the Rota-rod test (A) or of the immobility time in the Tail suspension test (B). Circles: males; triangles: females. **p-value<0.01, ****p-value<0.0001.*

c) <u>Neurodegeneration and neuroinflammation</u>

Intranasal delivery of NC containing 30 µg HC five times a week had no effects on neuronal survival measured by calbindin immunostaining (Figure 15) or in neuroinflammation measured by iba-1 immunostaining (Figure 16).

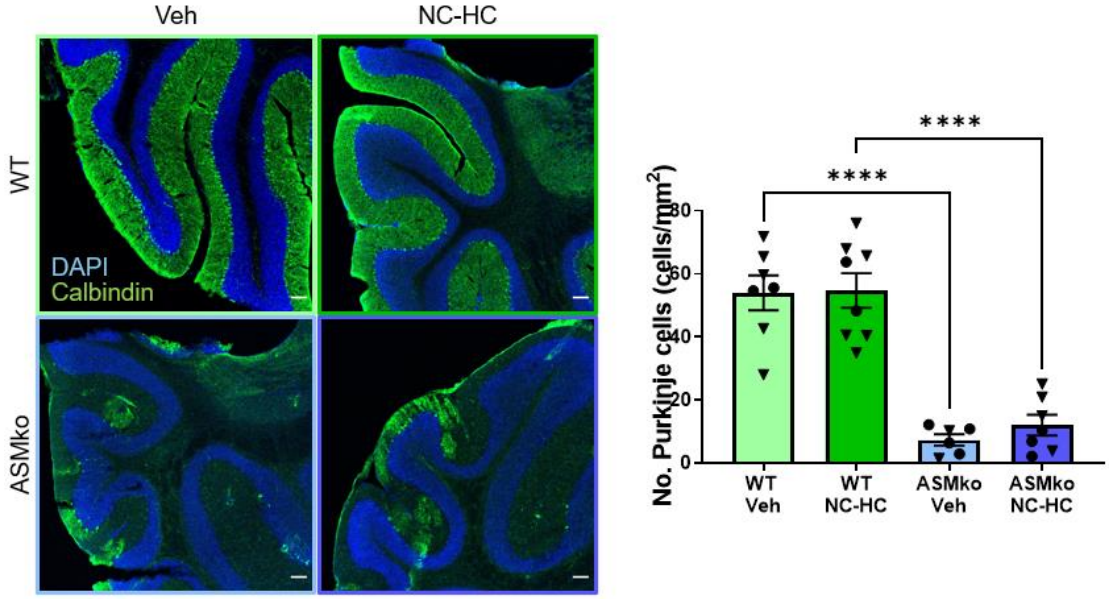

Figure 15. Nasal delivery of NC-HC five times a week does not prevent Purkinje cell loss in ASMko cerebellum
*Representative images from calbindin (green) immunostaining. Cell nuclei were stained with DAPI (blue). Scale bar 100µm. Graph shows mean±SEM number of Purkinje cells per area. Circles: males; triangles: females. ****p-value<0.0001.*

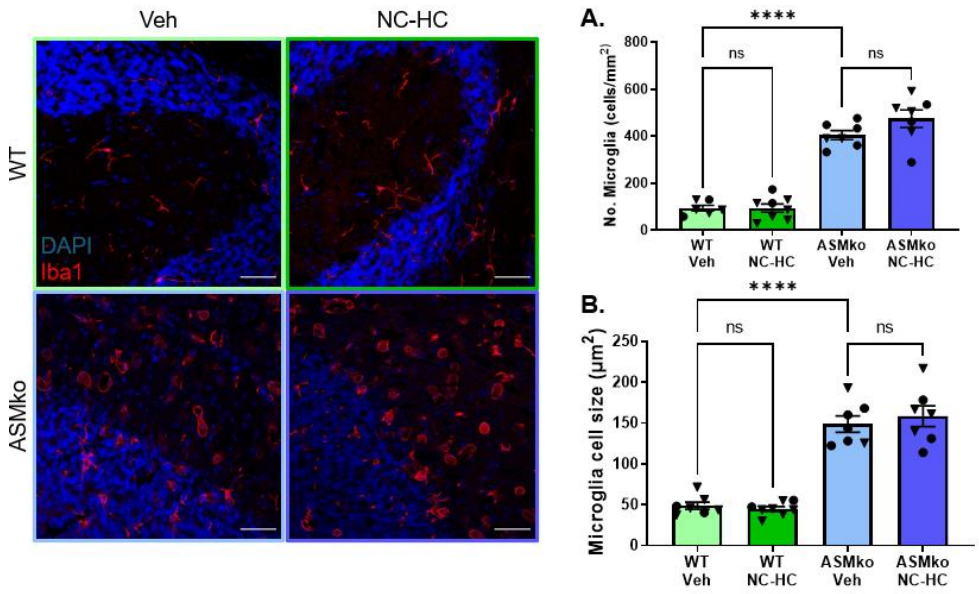

Figure16. Nasal delivery of NC-HC five times a week does not prevent microgliosis in ASMko cerebellum. *Representative images from Iba1 (red) immunostaining. Cell nuclei were stained with DAPI (blue). Scale bar 50µm. Graphs show mean±SEM microglia number (A) and size (B). Circles: males; triangles: females. ****p-value<0.0001.*

d) Sphingomyelin accumulation

SM levels were quantified in the cortex and in the lungs by enzymatic assays. Intranasal delivery of NC containing 30 µg HC five times a week did not reduce SM levels in the cortex but promoted a significant 31% reduction in the lungs (Figure 17).

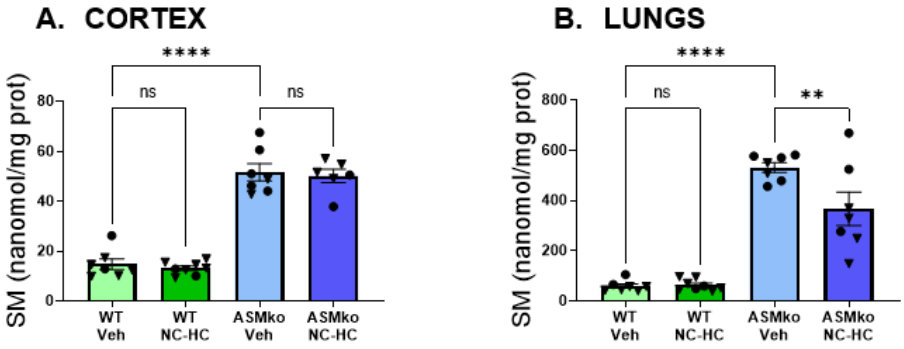

Figure 17. Nasal delivery of NC-HC five times a week does reduce SM levels in the lungs but not in the brain of ASMko mice. *Graphs show mean±SEM SM levels in A. Cortex, B. Liver. Circles: males; triangles: females. **p-value<0.001****p-value<0.0001.*

The results of this second *in vivo* trial showed that treatment with 30µg hydrocortisone in chitosan NC intranasal administered five times a week had a positive effect reducing SM levels but only in lungs from ASMko mice. This treatment had no overt effects on brain pathology in the ASMko mice. The lack of positive effects in this organ could be due to NC-HC not reaching the brain in enough amounts, while getting in to the lungs.

To analyze this possibility, we administered intranasally NCs loaded with the fluorophore DiD. No positive fluorescent signal was detected in the olfactory bulb with confocal microscopy or flow cytometry (Figure 18 A and C). In contrast, we could observe fluorescence in the liver of mice that were intraperitoneally injected with DID labelled NC, which were used as positive controls. DID-associated fluorescence could also not be detected by flow cytometry in the olfactory bulb of mice intranasally treated with labelled NCs. These results confirmed the inability of the NC-HC to reach the brain at the conditions used.

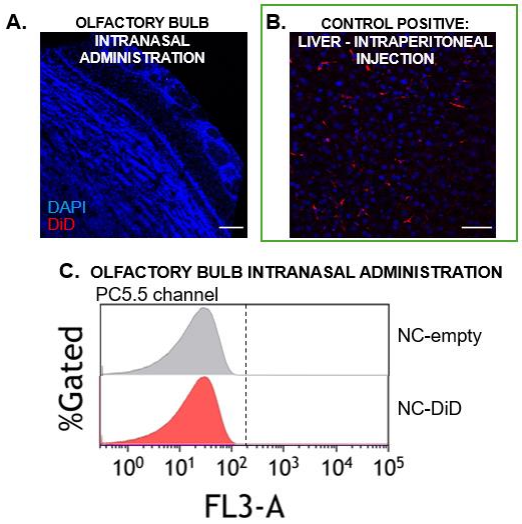

Figure 18. NC-DiD fluorescent signal was not detected in olfactory bulb of nasal administered mice by confocal microscopy or flow cytometry. *A-B Representative images of NC-DiD signal (red) A. olfactory bulb from mouse intranasal administered (scale bar 100µm), B. liver from mouse intraperitoneally injected (scale bar 50µm). Cell nuclei were stained with DAPI (blue) C. Flow cytometry histogram: olfactory bulb from mice intranasal administered with NC-empty (grey) or NC-DiD (red).*

In a final attempt to assess the suitability of HC to treat ASMD brain pathology we decided to use intranasal administration of non-encapsulated HC. This strategy is being used in the clinics for the treatment of allergy.

Figure 18 illustrates the protocol followed, which was similar to the other trials, except for the use of doses of 50µg free HC or vehicle (ethanol 10% in buffer saline) administered twice a week to awake mice.

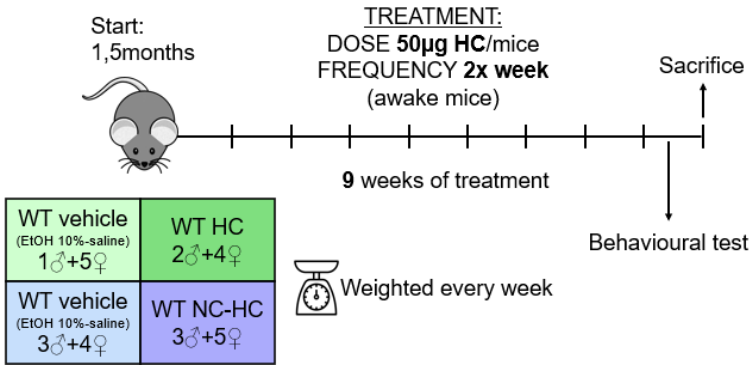

Figure 18. Scheme of the in vivo trial using intranasal delivery of 50µg free HC.

a) Weight gain analysis

All mice gained weight with the highest increase observed in the vehicle treated ASMko mice (Figure 19).

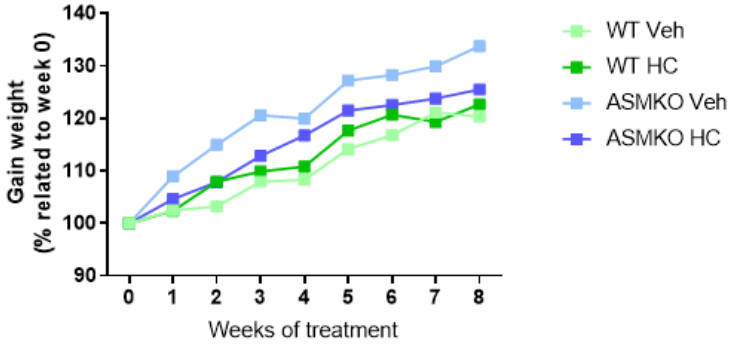

Figure 19. Nasal administration of free HC does not affect gain weight. *Graph shows mean±SEM weight gain in wt and ASMko mice treated with vehicle or with NC-HC.*

a) Behavioral analysis

Intranasal delivery of 50 µg free HC two times a week did not improve the impaired motor abilities (Rota-rod test) nor normalized behavior (Tail suspension test) in the ASMko mice (Figure 20).

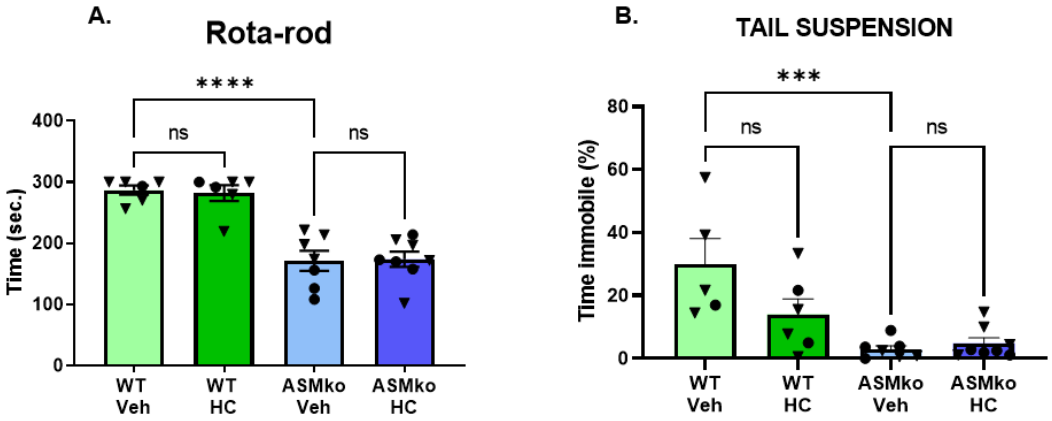

Figure 20. Nasal delivery of free HC does not improve motor and behavior anomalies in ASMko mice. *Graphs show mean±SEM of the time spent in the rod in the Rota-rod test (A) or of the immobility time in the Tail suspension test (B). Circles: males; triangles: females. ***p-value<0.001, ****p-value<0.0001.*

b) Neurodegeneration and neuroinflammation

Intranasal delivery of 50µg HC two times a week had no effects on neuronal survival measured by calbindin immunostaining (Figure 21). However, when neuroinflammation was measured by iba-1 immunostaining we observed no changes in the size but a 15% reduction in the number of microglia in the HC treated ASMko mice compared to the vehicle treated (Figure 22).

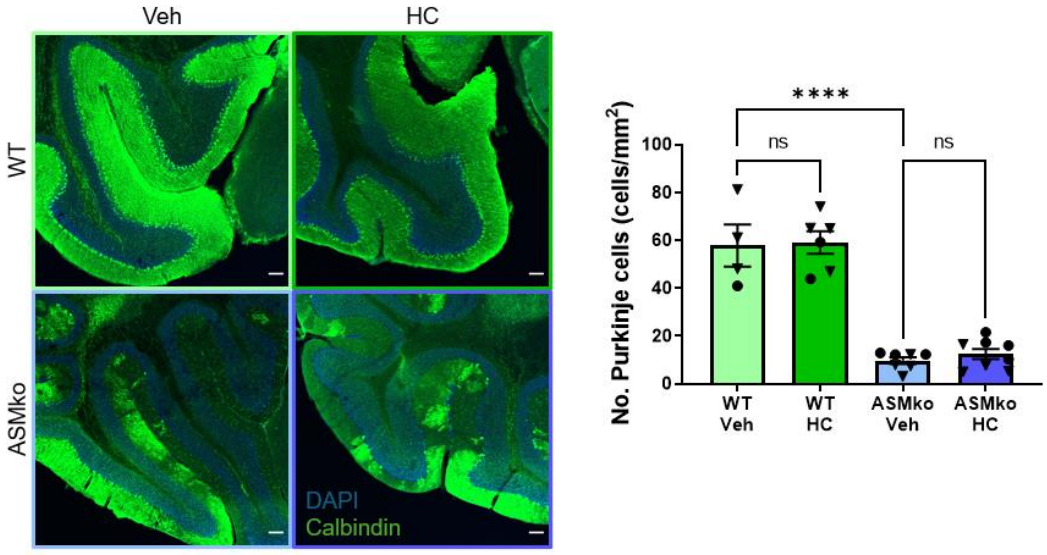

Figure 21. Nasal delivery of free HC does not prevent Purkinje cell loss in ASMko cerebellum. *Representative images from calbindin (green) immunostaining. Cell nuclei were stained with DAPI (blue). Scale bar 100µm. Graph shows mean±SEM number of Purkinje cells per area. Circles: males; triangles: females. ****p-value<0.0001.*

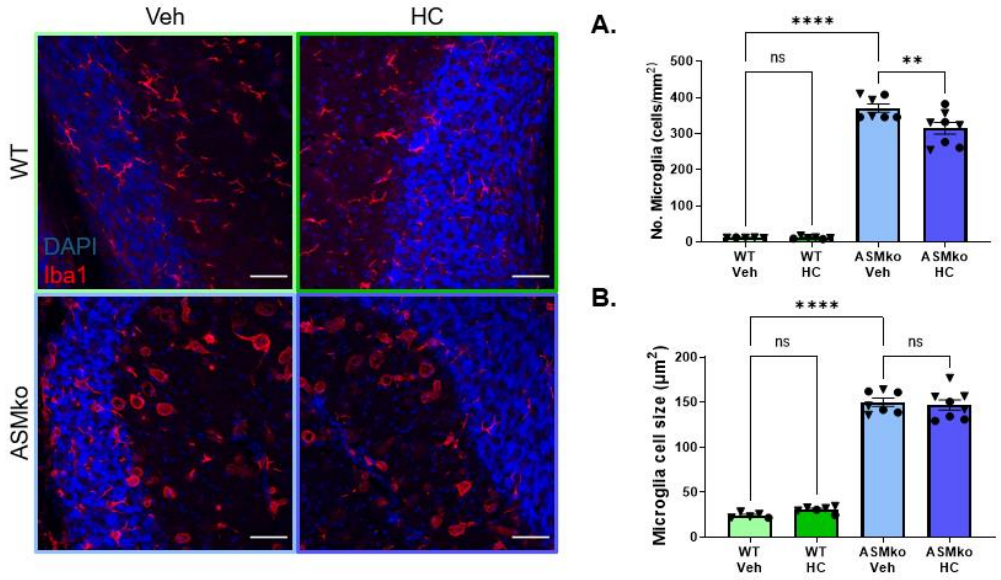

Figure 22. Nasal delivery of free HC reduces the number but not size of microglia in the cerebellum of ASMko mice. *Representative images from Iba1 (red) immunostaining. Cell nuclei were stained with DAPI (blue). Scale bar 50µm. Graphs show mean±SEM microglia number (A) and size (B). Circles: males; triangles: females. **p-value<0.01, ****p-value<0.0001*

c) <u>Sphingomyelin accumulation</u>

SM levels were quantified in the cerebellum and in the lungs by enzymatic assays. Intranasal delivery of 50µg HC two times a week did not reduce SM levels (Figure 23).

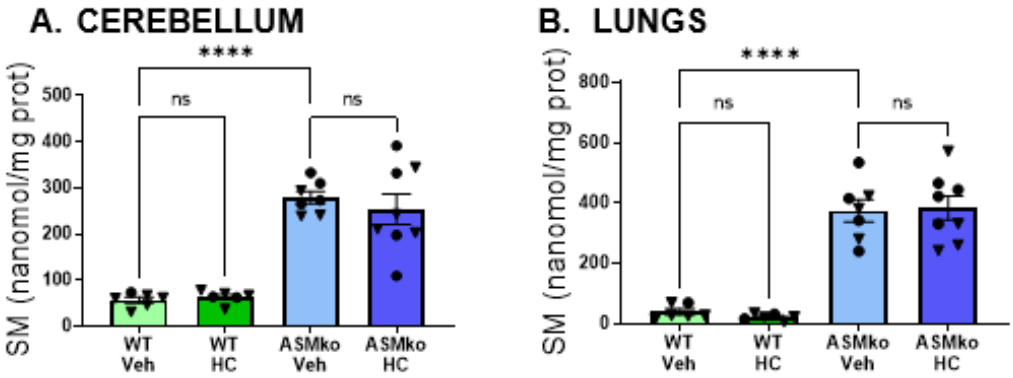

Figure23. Nasal delivery of free HC does not reduce SM accumulation in brain or lungs in ASMko mice. *A. Graphs show mean±SEM SM levels in A. Cerebellum, B. Lungs. Circles: males; triangles: females. ****p-value<0.0001*

CONCLUSIONS

- Chitosan nanocapsules (NC) can be uptaken by neurons in culture.
- NC loaded with HC (NC-HC) can be generated by ionotropic chelation with a maximum drug loading of 23%.
- High concentration of NC-HC reduces SM levels in ASMko cultured neurons.
- Intranasal delivery of NC containing 30µg HC, two times a week, has no effects in weight gain, motor abilities, depressive-like behavior, Purkinje cell survival and lysosomal size or neuroinflammation in the brain of ASMko mice. This strategy does not affect SM levels in brain, lungs, or liver of ASMko mice.
- Intranasal delivery of NC containing 30µg HC, five times a week, prevents weight gain and has no effect in motor abilities, depressive-like behavior, Purkinje cell survival or neuroinflammation in the brain of ASMko mice. This strategy does not affect SM levels in the brain but reduces SM in the lungs of ASMko mice.
- Intranasal delivery of 50µg free HC, two times a week, has no effects in weight gain, motor abilities and depressive behavior or Purkinje cell survival but reduces neuroinflammation in the brain of ASMko mice. This strategy does not affect SM levels in brain or lungs of ASMko mice.
- The results obtained do not differ between males and females.

Altogether, these results confirm the safety and ability to reduce SM levels of NC-HC in cultured ASMko neurons. However, intranasal delivery of free or chitosan-encapsulated HC to ASMko mice at the conditions used do not reduce SM levels or pathological signs in the brain. These evidences do not support intranasal delivery of encapsulated or free HC as a suitable therapy for neurological ASMD.

LAY SUMMARY

The acid sphingomyelinase deficiency (ASMD) is a lysosomal storage disorder caused by mutations in the gene encoding for the sphingomyelin (SM)-degrading enzyme acid sphingomyelinase (ASM). SM accumulation is a pathological hallmark in the disease. In the neurovisceral ASMD forms (types A and A/B) SM accumulates in brain cells leading to neuroinflammation and neurodegeneration that cause motor and behavioral alterations. Recently, intravenous infusion of recombinant ASM has proven successful to treat the visceral condition in type B ASMD patients. However, since the recombinant enzyme does not cross the brain blood barrier, the brain pathology remains unaddressed.

In this project we proposed to treat ASMD brain pathology using glucocorticoids. Previous work in mice lacking ASM (ASMko), which mimic ASMD type A, showed that oral treatment with the synthetic glucocorticoid dexamethasone reduced SM accumulation and ameliorated brain alterations. However, systemic long-term treatment with high doses of glucocorticoids is not an applicable option due to serious side effects including growth arrest. In this project we proposed to encapsulate the physiological glucocorticoid hydrocortisone (HC), which is more suitable than dexamethasone for treatment in children, in chitosan nanocapsules to facilitate direct delivery of low glucocorticoid doses to the brain upon nasal administration without affecting peripheral organs.

The research performed in this project has included: 1) the generation of chitosan nanocapsules loaded with hydrocortisone (NC-HC), 2) treatment with NC-HC cultured neurons from ASMko mice, and 3) Intranasal delivery of free HC and of different doses of NC-HC to control and ASMko mice.

The results obtained have confirmed the safety and ability to reduce SM levels of NC-HC in cultured ASMko neurons. However, intranasal delivery of free or chitosan-encapsulated HC to ASMko mice at the conditions used did not reduce SM levels or pathological signs in the brain. These evidences do not support intranasal delivery of encapsulated or free HC as a suitable therapy for neurological ASMD.

FINANCIAL SUMMARY

REF.: NATIONAL NIEMANN-PICK FOUNDATION

Principal investigator MARIA DOLORES LEDESMA MUÑOZ

Fundación Severo Ochoa

PROJECT

Intranasal delivery of chitosan-encapsulated hydrocortisone to treat brain pathology in ASMD

PERSONAL

SALARY+FRINGE BENEFITS	FECHA	AMOUNT
NAYA FORCANO, SARA	01/01/23 - 15/04/24	31.478,47 €
	TOTAL	**31.478,47 €**

REAGENTS

	PROVEEDOR	CONCEPTO	AMOUNT
1	QUIMIGEN S.L.	MATERIAL LABORATORY	377,52 €
2	QUIMIGEN S.L.	MATERIAL LABORATORY	365,76 €
3	FARMACIA SANTAOLALLA C.B.	MATERIAL LABORATORY	169,12 €
4	FISHER SCIENTIFIC S.L.	MATERIAL LABORATORY	829,33 €
5	FISHER SCIENTIFIC S.L.	MATERIAL LABORATORY	252,65 €
6	FISHER SCIENTIFIC S.L.	MATERIAL LABORATORY	1.199,35 €
7	INNOVACIONES GENETICAS, S.L.	MATERIAL LABORATORY	90,15 €
8	MERCK LIFE SCIENCE S.L.U.	MATERIAL LABORATORY	502,15 €
9	MERCK LIFE SCIENCE S.L.U.	MATERIAL LABORATORY	726,00 €
10	NIRCO, S.L.	MATERIAL LABORATORY	51,30 €
11	NIRCO, S.L.	MATERIAL LABORATORY	109,77 €
12	PROMEGA BIOTECH IBERIA, S.L	MATERIAL LABORATORY	896,61 €
13	PROMEGA BIOTECH IBERIA, S.L	MATERIAL LABORATORY	942,59 €
		TOTAL	**6.512,30 €**

OTROS

SERVICIO DE:		AMOUNT
ANIMAL FACILITY		1.642,52 €
	TOTAL	**1.642,52 €**

SUMA	**39.633,29 €**

Madrid 31 de Marzo de 2024

FDO:MARIA DOLORES LEDESMA MUÑOZ
PRINCIPAL INVESTIGATOR

Fdo.: PAOLA BOVOLENTA NICOLAO
FSO SECRETARY

LEDESMA MUÑOZ M.DOLORES - DNI 02622280G

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BOVOLENTA NICOLAO PAULA - DNI 33534698P

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