AN *IN-VITRO* MODEL TO STUDY THE ROLE OF SURVIVAL MOTOR NEURON (SMN) PROTEIN ISOFORMS IN NEURONAL DIFFERENTIATION

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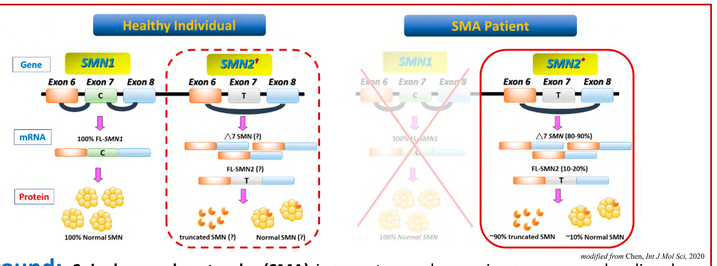
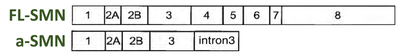


modified from Chen, Int J Mol Sci, 2020

Background: **Spinal muscular atrophy (SMA)** is an autosomal recessive neuromuscular disorder, caused by mutations in survival motor neuron (SMN) 1 gene. SMA has an incidence of 1 in 6,000 live births, and it is characterized by motor neuron degeneration, leading to progressive amyotrophic paralysis, respiratory deficiency and, in more severe cases, death. Recently approved SMN-targeted therapies have revolutionized the approach to SMA, but their efficacy is only partial, emphasizing the need for understanding the mechanisms of SMA pathogenesis, in order to find targets for additional therapies.

We have identified a new isoform of the SMN gene, preferentially expressed in the axon, called a-SMN (Setola et al, PNAS 2007).



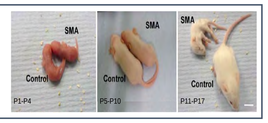
SiRNA silencing of either the full length FL- or the a-SMN isoform in rat hippocampal neurons led to loss of normal neuronal polarity with abnormal ankyrin G (AnkG) labelling, found in none or in multiple neurites, including those positive for the dendrite-specific MAP2 protein or in the terminal portion of the axon (Pletto et al., PlosONE 2018).

Ankyrins are modular proteins comprised of conserved and specialized domains, needed for organizing membrane microstructure and directing membrane traffic. AnkG is required for proper axon growth and axo-dendritic polarity, through the structure of the axonal initial segment (AIS). In normal neurons, AnkG is specifically localized in AIS.

Aim: We aim to study the molecular mechanisms that link the two SMN isoforms and AnkG functions in AIS formation. As siRNA specificity for the two SMN isoforms is only partial, we characterized a model of SMA hippocampal neurons prepared from a murine model (SMA Δ7 mice).

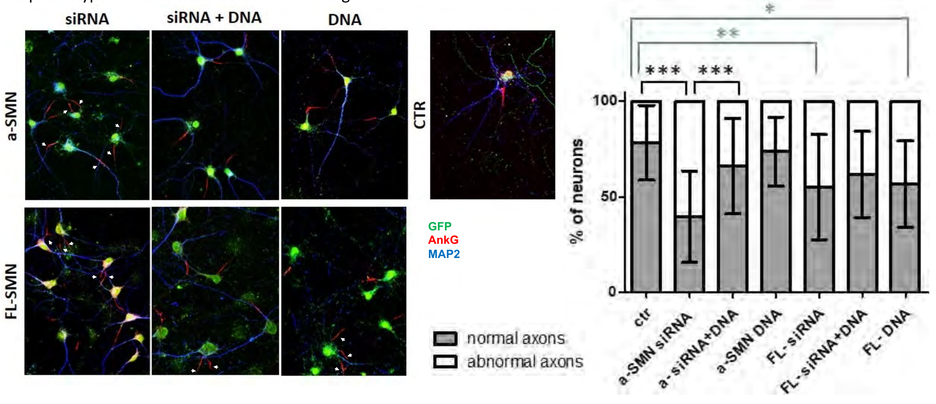
The Δ7 SMN mouse

Genetic background: ***SMN2$^{+/+}$; SMNΔ7$^{+/+}$; Smn$^{-/-}$***



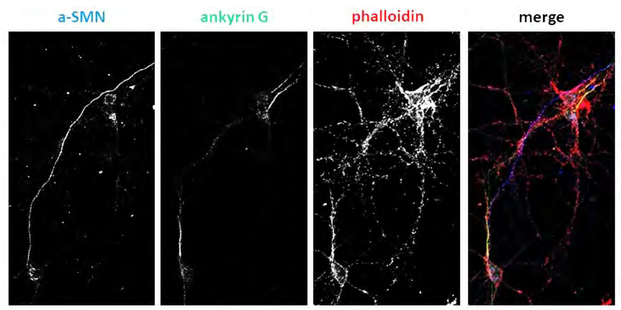
1 - Rescue experiments in a-SMN or FL-SMN silenced neurons

In rat hippocampal neurons where FL-SMN or a-SMN siRNA was co-transfected with a plasmid for the expression of FL-SMN or a-SMN, respectively, the ratio of cells displaying abnormal AnkG labelling was significantly lower than in silenced neurons transfected with empty cDNA vector, and similar to non-silenced control. a-SMN was more effective than FL-SMN in rescuing the phenotype. Arrows show abnormal labeling



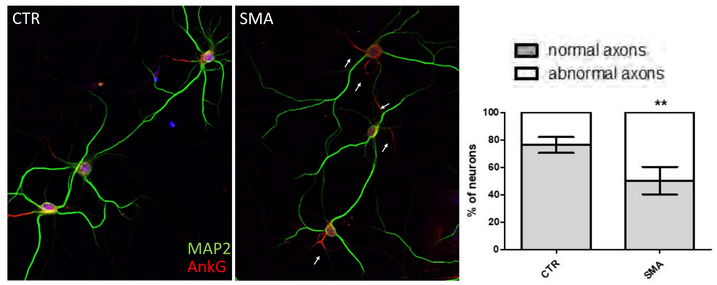
Representative images of rat hippocampal neurons silenced and/or co-transfected as indicated, fixed at 11div and immunostained for AnkG and dendritic marker MAP2. The number of neurons displaying abnormal AnkG labeling was counted in 30 images acquired from two independent experiments. Mean % of normal neurons ± StDev: CTR 78.28 ± 19.63; a-SMN siRNA 39.52 ± 23.88; a-SMN siRNA + DNA 66.09 ±24.95; a-SMN DNA 73.63 ± 18.01; FL-SMN siRNA 55.10 ± 27.57; FL-SMN siRNA + DNA 61.70 ± 22.50; FL-SMN DNA 56.54 ± 22.58. Kruskal-Wallis test and Dunn's multiple comparison test; * p<0.05; ** p<0.01; *** p<0,001

2- a-SMN and AnkG have different localizations in the axon



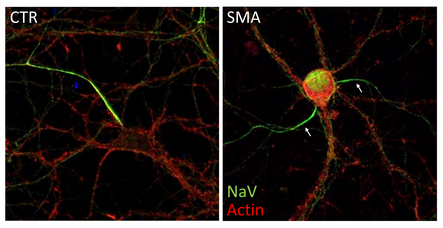
Immunofluorescence in hippocampal neurons from control mice (CTR) fixed at 8DIV and stained for a-SMN, AnkG and phalloidin. Direct interaction between the proteins is unlikely, given that immunofluorescence analysis of WT mouse neurons showed AnkG highly localized in the proximal AIS, while a-SMN is distributed along the axon but absent from AIS as well as growth cone and dendrites.

3- Mouse SMA neurons show abnormal AnkG labeling



Representative images of hippocampal neurons from CTR or SMA mice fixed at 8DIV and immunostained for AnkG and MAP2. The number of neurons displaying abnormal AnkG labeling (arrows) was counted in 134 images for CTR and 121 for SMA cultures acquired from six independent experiments. Mean % of normal neurons ± StDev: CTR 76.23 ± 5.81; SMA 50.32 ± 10.04. Mann Whitney test; ** p<0.01.

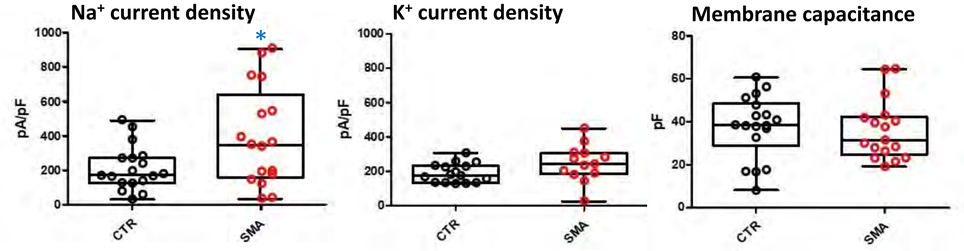
4- Voltage-gated Na$^+$ channels



Hippocampal neuron immunostaining with antibodies against voltage-gated sodium channels (NaV, green) and phalloidin (which binds actin, red). Similarly to what was observed with AnkG, many neurons in SMA cultures show an abnormal localization of Nav staining in 2 or more neurites, supporting the hypothesis of SMN playing a role in neuronal polarization and AIS formation.

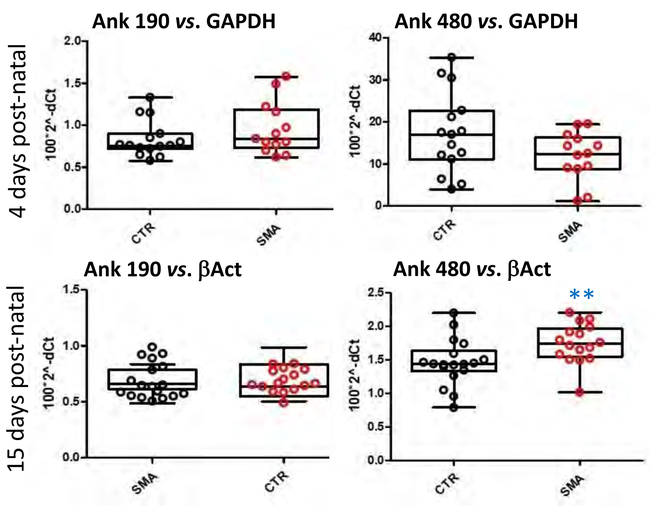
Methods: for detailed methods please take an handout

5- Na$^+$ current density is increased in hippocampal neurons from SMA mice



Whole-cell patch-clamp recordings on 11/12 DIV hippocampal neurons from control (CTR) and SMA mice. n= 18 (CTR) and 17 (SMA) cells from three different cultures. Na+ current density is increased in SMA neurons vs. CTR (mean ± StDev: CTR 213.1 ± 127.6, SMA 395.9 ± 286.2) while K+ current density did not change (mean ± StDev: CTR 190.0 ± 55.41, SMA 247.9 ± 106.5). Unpaired t-test, * p<0.05
Membrane capacitance was not statistically different between the two groups (mean ± StDev: CTR 37.66 ± 14.60, SMA 36.24 ± 13.97).

6- Hippocampal expression of AnkG isoforms is altered in SMA mice at P15



AnkG is expressed in different isoforms with distinct functions. AnkG isoform of 480 kDa (Ank480) is required for proper axon growth and axo-dendritic polarity, synapse maturation and fast and integrated neuronal signaling. In neurons, a shorter form of 190 kDa (Ank190) is also present, with different localization. When Ank480 is absent, Ank190 can compensate for its functions only partially, but is not able to restore AIS structure.

Age	Target	CTR		SMA	
		Mean	StDev	Mean	StDev
P4	Ank190	0.8353	0.2151	0.9608	0.3114
	Ank480	17.31	9.583	11.97	5.801
P15	Ank190	0.6869	0.1583	0.6883	0.1007
	Ank480	1.462	0.3433	1.746	0.2953

RT-PCR analysis for the two different AnkG isoforms in SMA or CTR mice hippocampi, at 4 and 15 post-natal days, expressed as 2^-dCT*100. Mann Whitney test; ** p<0.01. For mean ± StDev see the table above.

Conclusions and perspectives:

- Hippocampal neurons from SMA Δ7 mice are a **suitable *in-vitro* model** to study the role of SMN isoforms in neuronal differentiation. Evidence of a differential effect of the FL- and a-SMN isoforms on axon polarization would give new significance to neuronal susceptibility to SMN loss.
- Our model to study the molecular mechanisms underlying SMA pathogenesis is of particular significance as concerns are increasing among clinicians regarding **cognitive and neurobehavioral abnormalities** in SMA patients.
- Our data suggest **Ankyrin G isoforms involvement in SMA neuron alterations**. This could lead to the identification of so far unexplored mechanisms and the **individuation of new therapeutic targets**. Ankyrins are present in many specialized structures, such as neuromuscular junctions, skeletal muscles and cardiac membranes. Clarifying SMN/AnkG functional relation may enlighten **mechanisms that are important for other tissues involved in SMA pathology**.

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