non coding RNAs (ncRNAs) in neurons

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IBPM CNR

LncRNAs in the nervous system

- LncRNAs show prominent expression in the nervous system and have been implicated in neural development, function and disease.

- Remarkably large number of annotated lncRNAs (approximately 40%) is expressed specifically in the brain (Derrien et al., 2012)

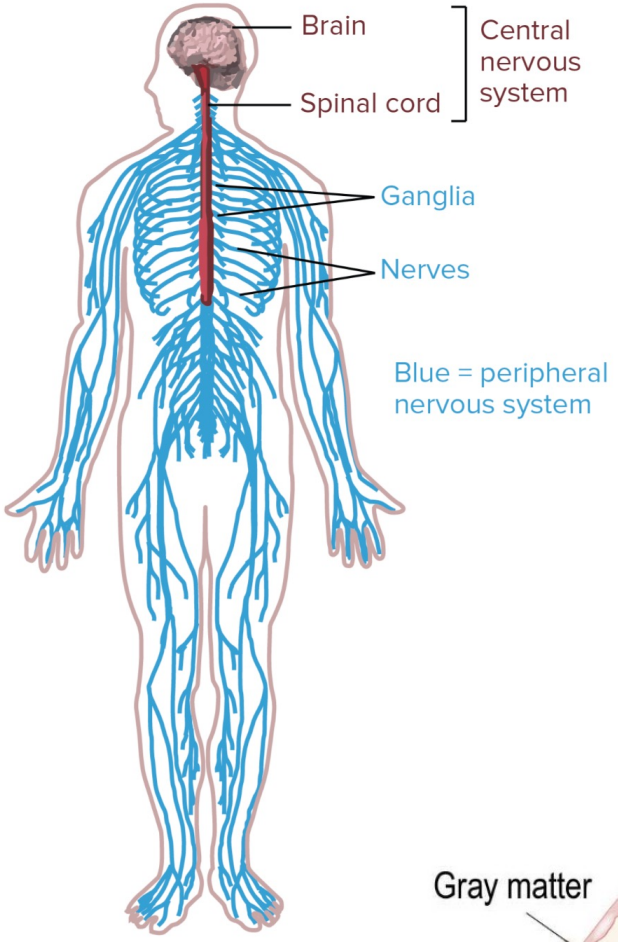
- Ubiquitously expressed lncRNAs are generally expressed at high levels, while cell type- or tissue-specific lncRNAs, such as those in MNs, are often expressed at lower levels (Jiang, Li,, et al., 2016)

- lncRNAs have been linked to processes such as neuron development, neurite growth, synaptic transmission, memory consolidation and ageing (Mehler & Mattick, 2007; Mercer et al., 2008; Pereira Fernandes et al., 2018; Shi et al., 2017)

Central nervous system

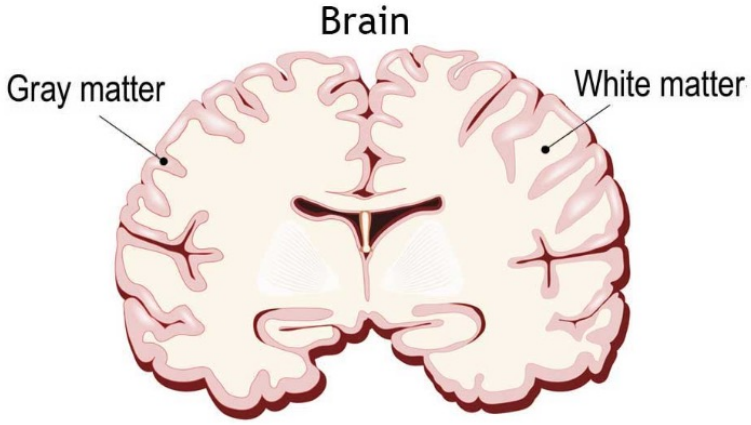
The brain is a complex organ that controls thought, memory, emotion, touch, motor skills, vision, breathing, temperature, hunger and every process that regulates our body. Together, the brain and spinal cord that extends from it make up the central nervous system, or CNS.

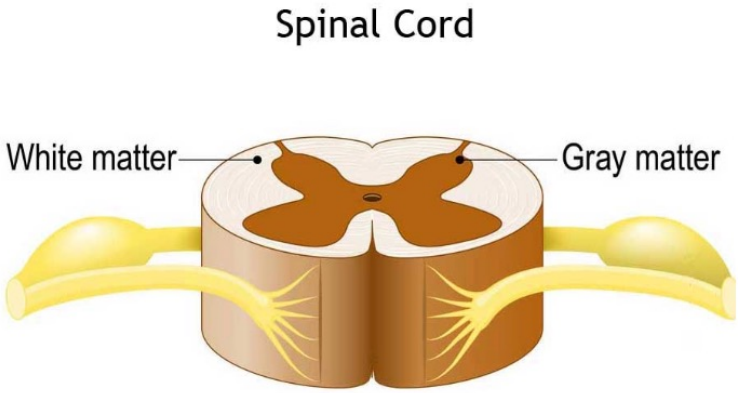
Central nervous system



Gray and white matter are two different regions of the central nervous system.

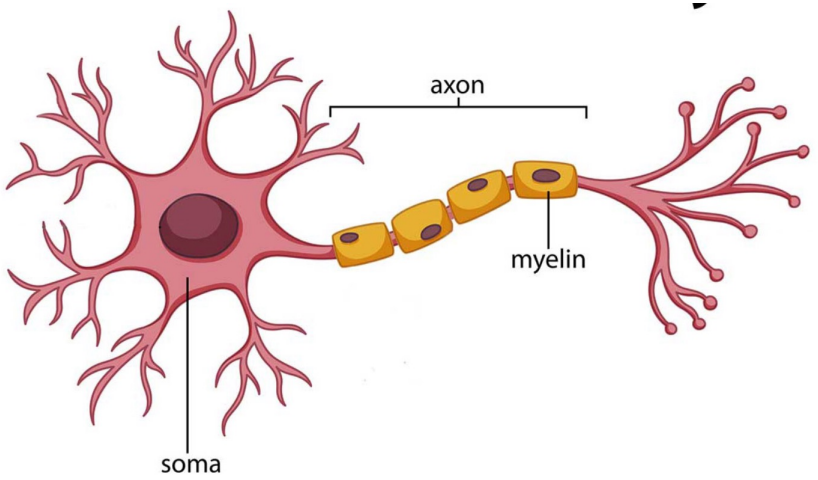
- In the **brain**, gray matter refers to the darker, outer portion, while white matter describes the lighter, inner section underneath.
- In the **spinal cord**, this order is reversed: The white matter is on the outside, and the gray matter sits within.





Gray and white matter

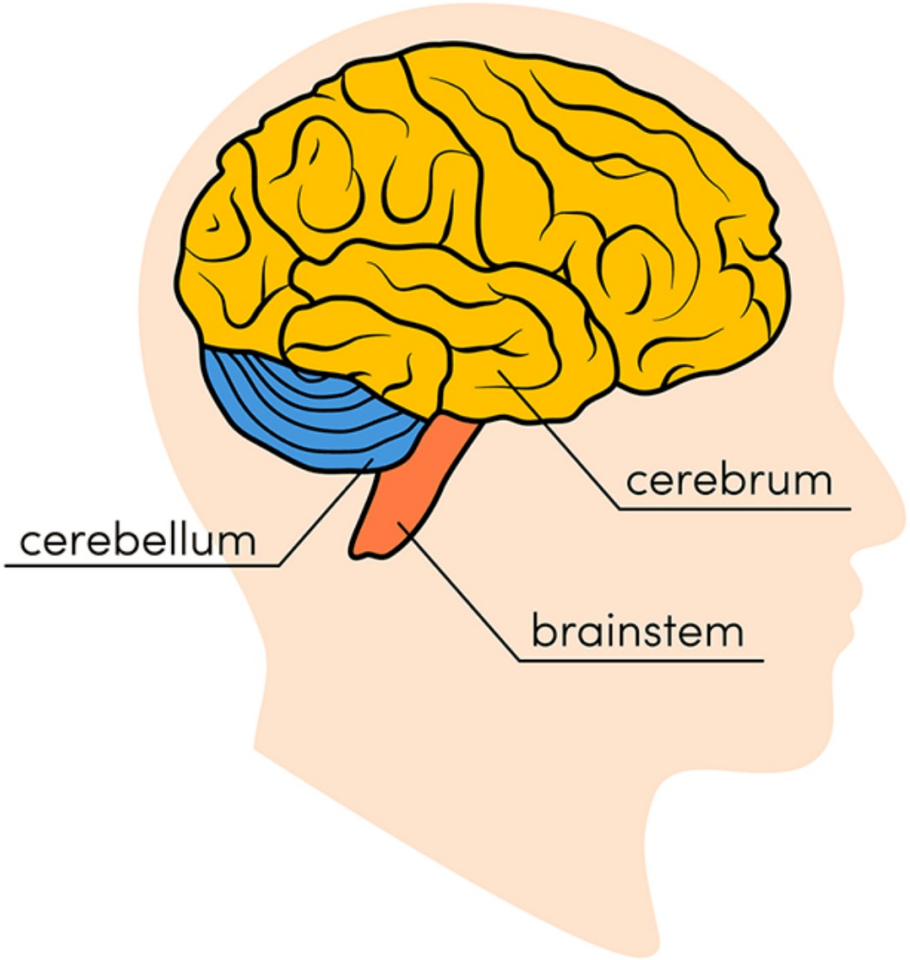
Gray matter is primarily composed of neuron somas (the round central cell bodies), and white matter is mostly made of axons (the long stems that connects neurons together) wrapped in myelin (a protective coating). The different composition of neuron parts is why the two appear as separate shades on certain scans.



Each region serves a different role:

- **Gray** matter is primarily responsible for **processing** and interpreting **information**,
- **White** matter **transmits** that **information** to other parts of the nervous system.

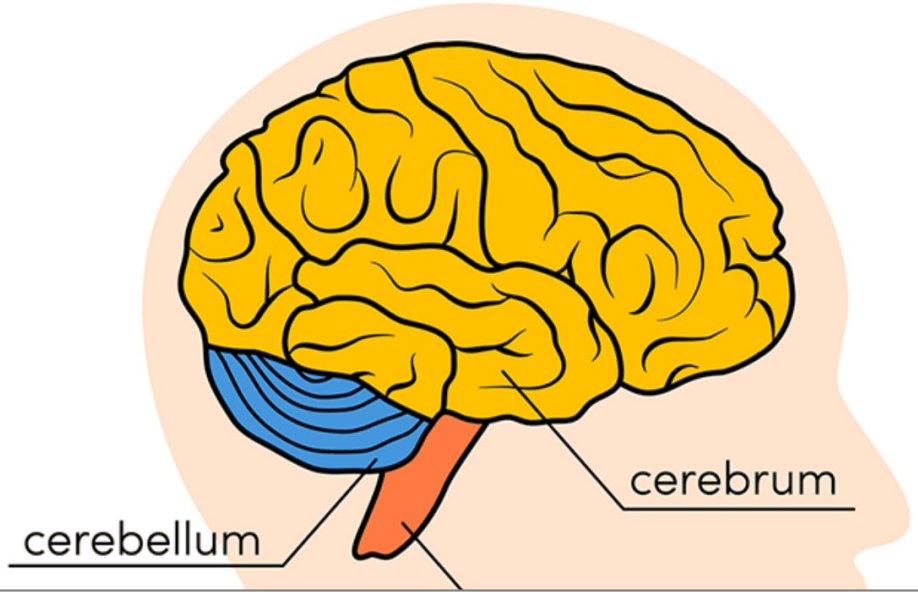
Brain



At a high level, the brain can be divided into the cerebrum, brainstem and cerebellum.

Adapted from: https://www.hopkinsmedicine.org/

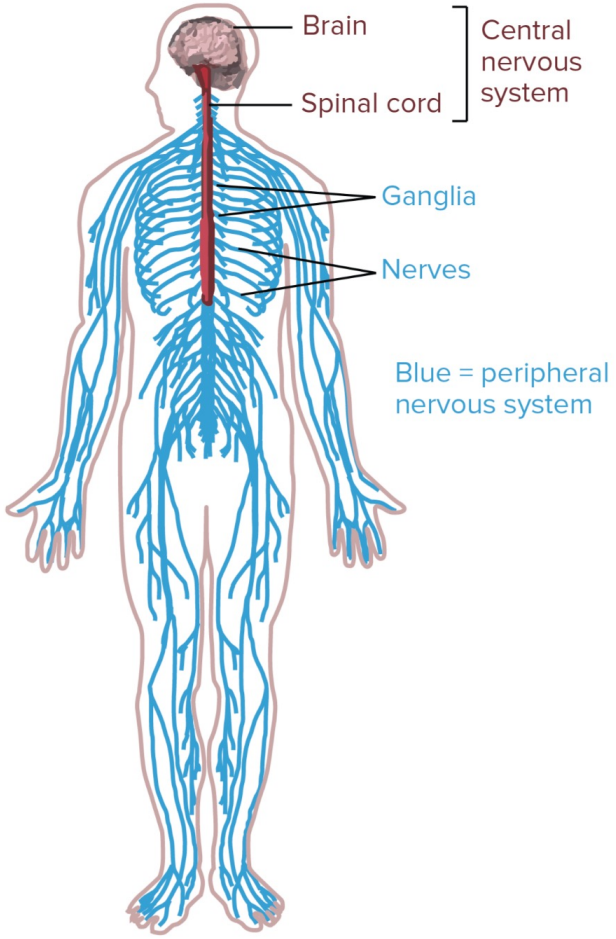
Brain

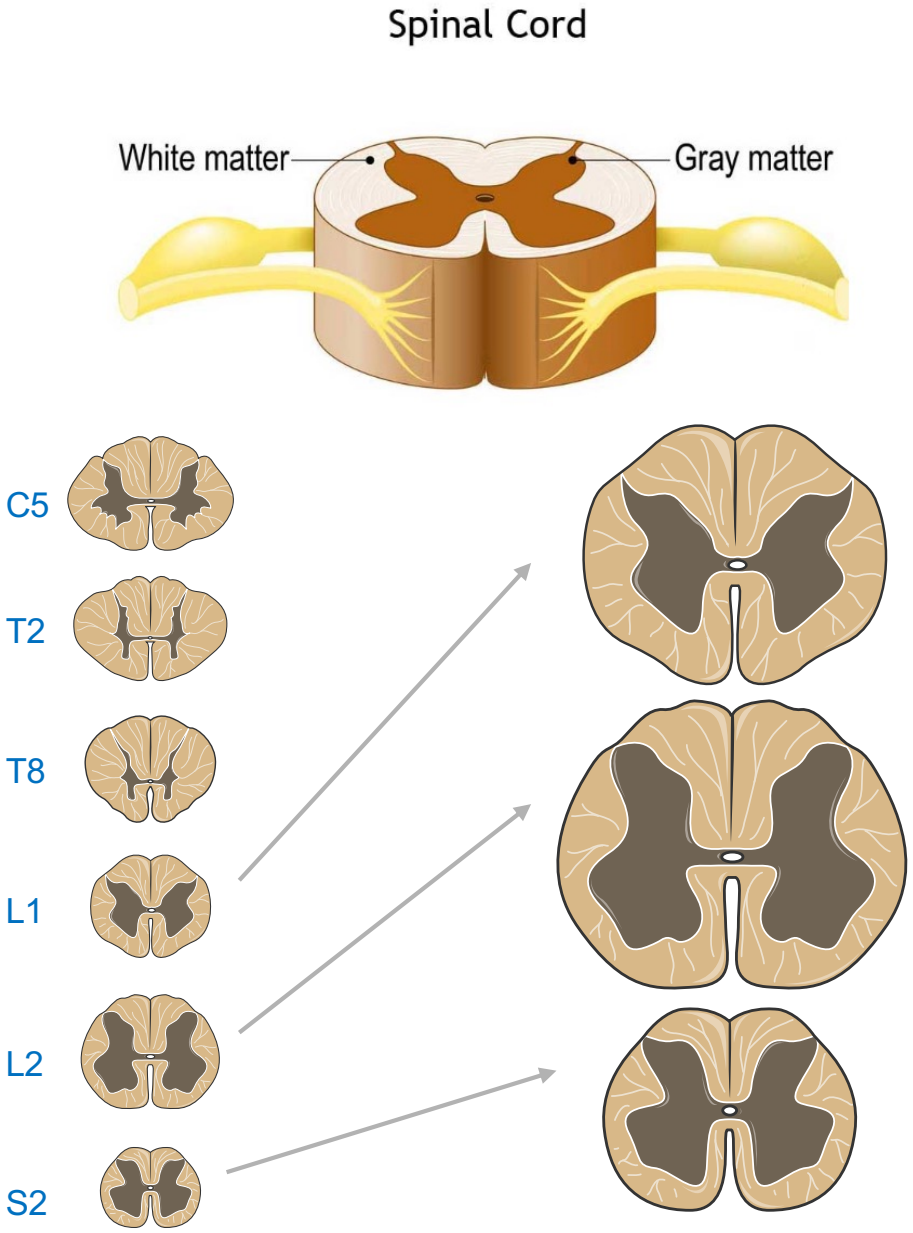


The **CEREBELLUM** ("little brain") is a fist-sized portion of the brain located at the back of the head, below the temporal and occipital lobes and above the brainstem. Like the cerebral cortex, it has two hemispheres. The outer portion contains neurons, and the inner area communicates with the cerebral cortex. Its function is to coordinate voluntary muscle movements and to maintain posture, balance and equilibrium.

New studies are exploring the cerebellum's roles in thought, emotions and social behavior, as well as its possible involvement in addiction, autism and schizophrenia.

Central nervous system





Synage

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Cell Death & Differentiation

ARTICLE

The long noncoding RNA *Synage* regulates synapse stability and neuronal function in the cerebellum

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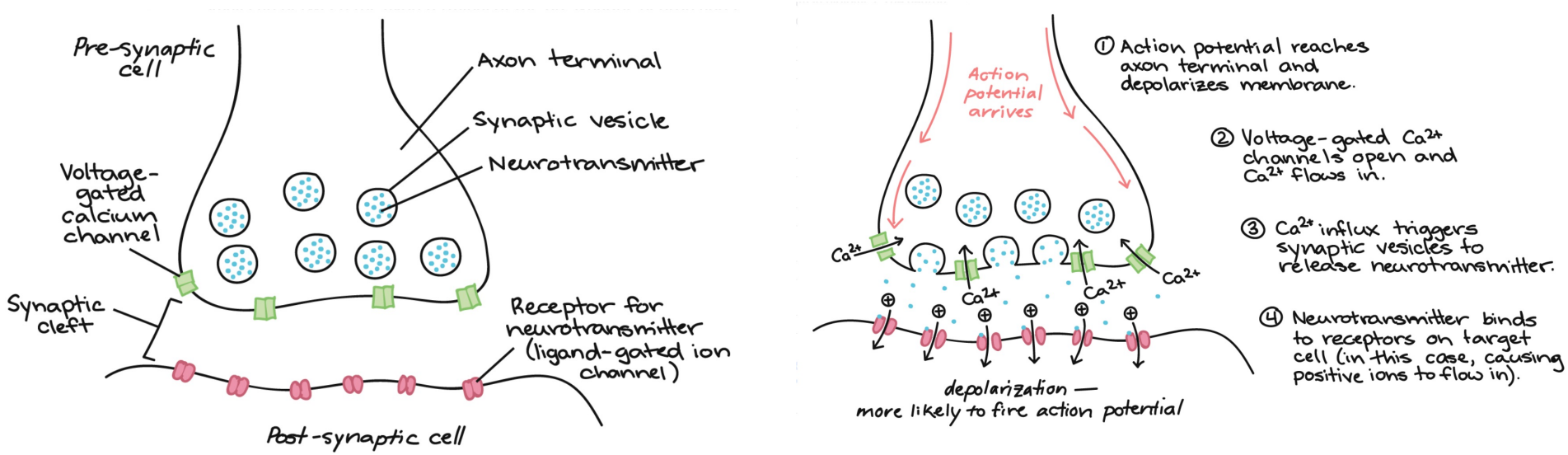
The long noncoding RNA Synage regulates:
- synapse stability
- neuronal function in the cerebellum

A synapse is a junction between two nerve cells



Neurons (or nerve cells) are specialized cells that transmit and receive electrical signals in the body.

Neurons are composed of three main parts:

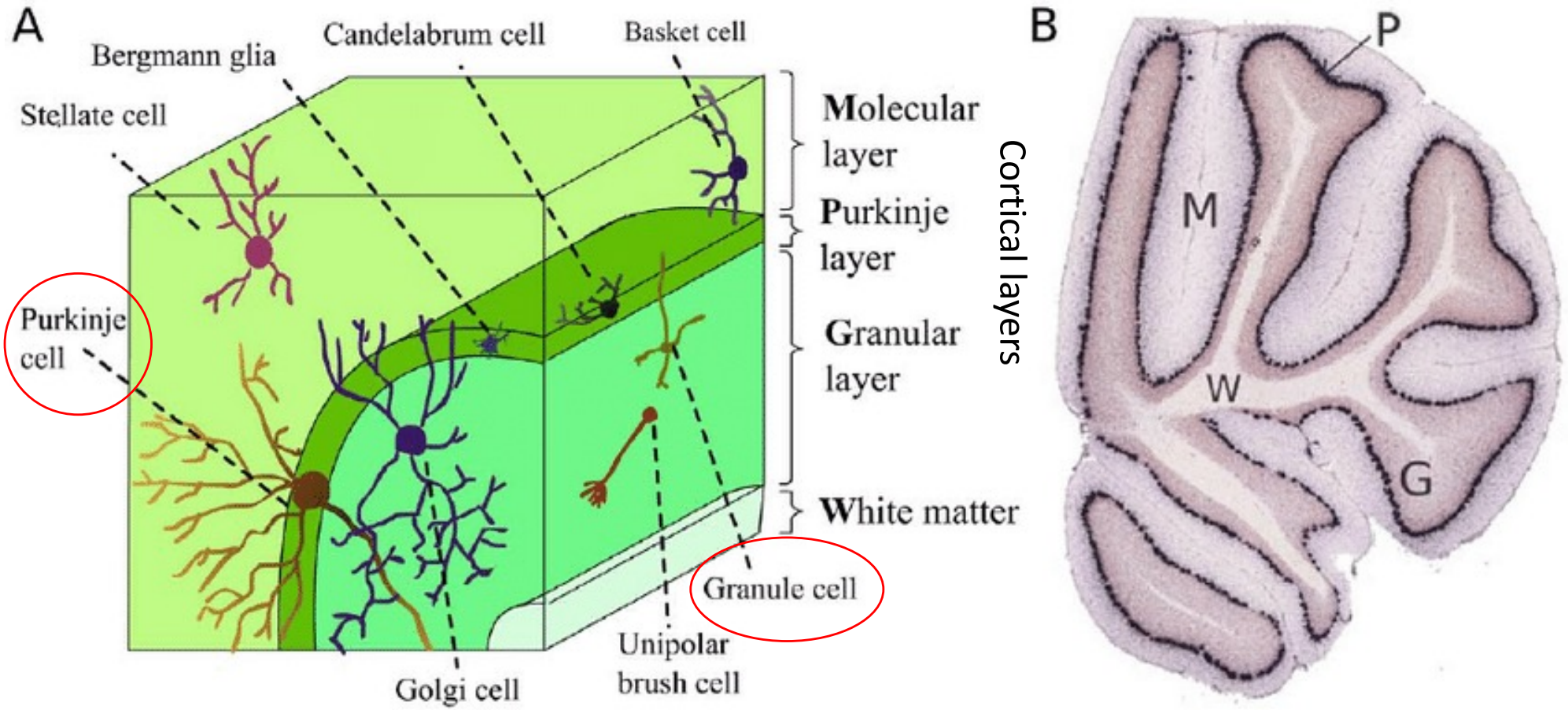
-dendrites,

-a cell body

-an axon.

Signals are received through the dendrites, travel to the cell body, and continue down the axon until they reach the synapse (the communication point between two neurons)

Cerebellum layers and cell types

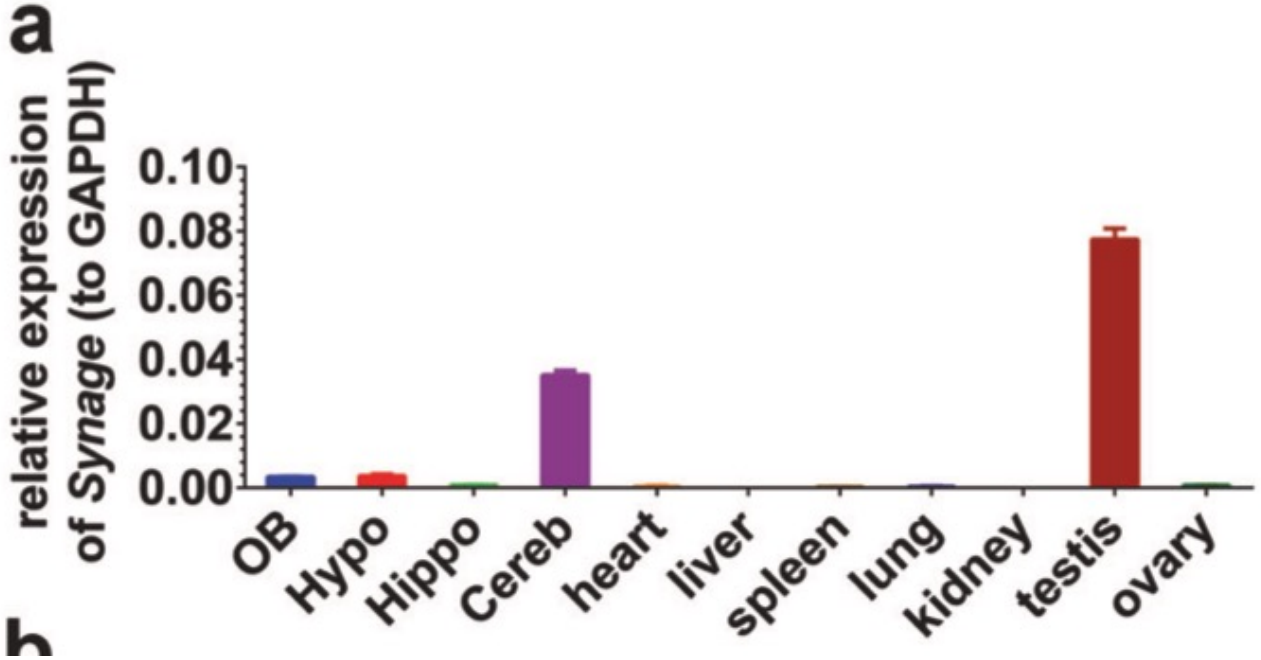


(A) Cell types and their location across the cerebellar cortical layers.
(B) Cerebellum in-situ hybridization image of Calbl. The different layers can be easily discriminated. P - the Purkinje layer; G - the granular layer; M - the molecular layer; W - the white matter.

Kirsch et al., 2012.. PLOS Computational Biology 8(12): e1002790. https://doi.org/10.1371/journal.pcbi.1002790

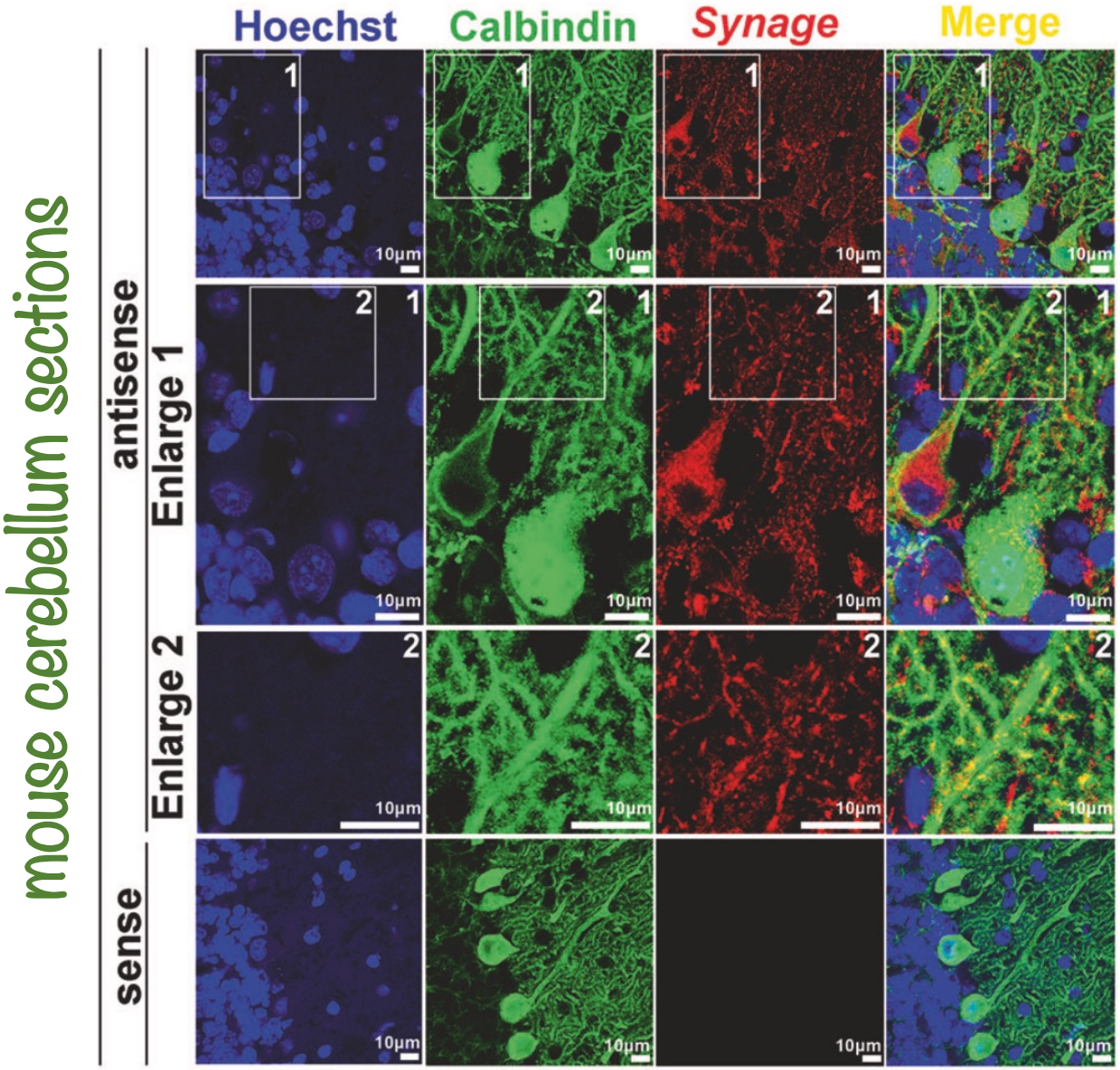
Synage is mainly expressed in cerebellum and testis

lncRNAs enriched in Mouse brain (RNA-seq)



Wang et al., 2021

Synage lncRNA is mainly distributed in the cytoplasm and dendrites of cerebellar neurons.



Green: Calbindin (a Purkinje cell marker)

Red: RNA-FISH using a biotin-labeled Synage antisense RNA probe

Blue: nuclei

Synage was localized primarily in cytoplasm and dendrites of cerebellar cells, including PCs

Wang et al., 2021

Synage conservation

Synage-homologous genes (LOC106995009 in rhesus macaque, and RP11-491F9.1 in human) were conserved in terms of their **LOCATIONS** in the genomes of mouse, rhesus macaque, and human (adjacent to the Cbln1 gene)

Synage lncRNA is conserved in its genomic location (adjacent to the Cbln1 gene) and in its **DISTRIBUTION** specificity in the cerebellum among mouse, rhesus maca- que, and human.

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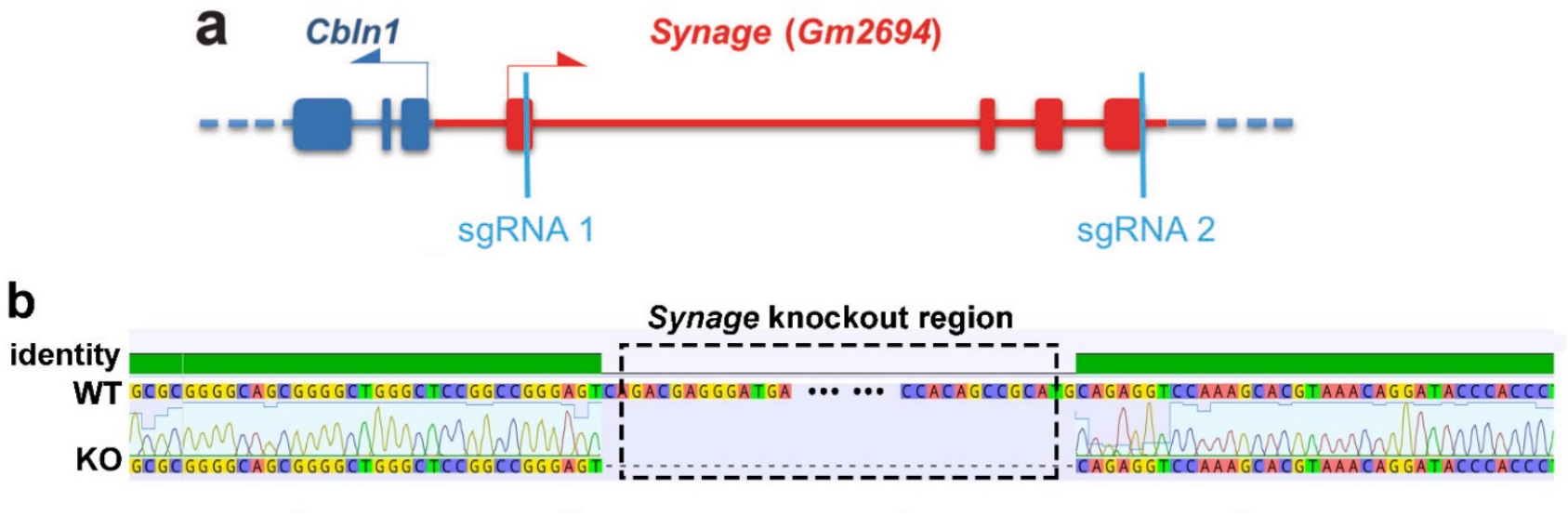
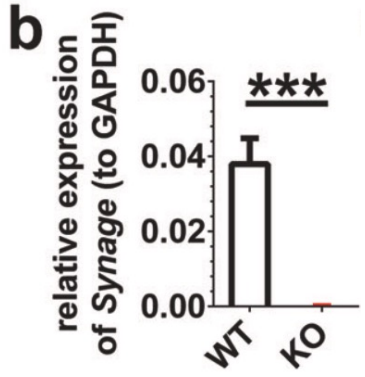
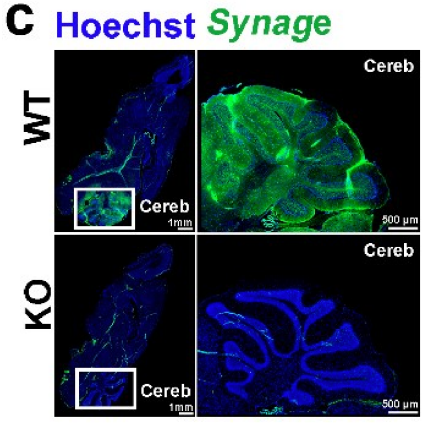
Synage KO mice show significant cerebellar atrophy and neuronal loss during cerebellar development







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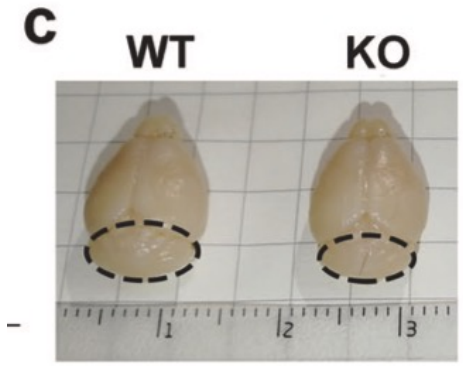
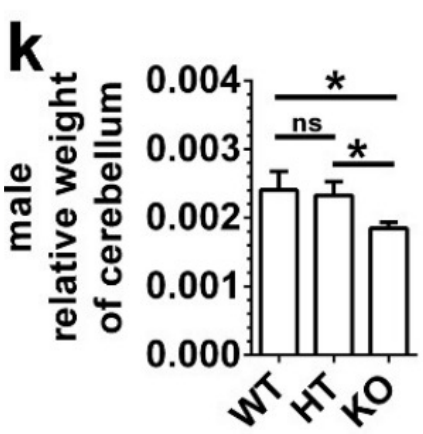
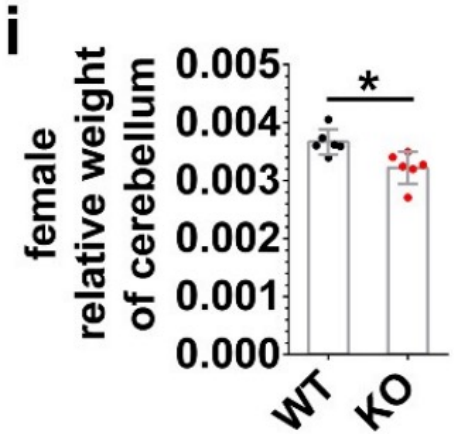
Synage KO mice phenotype

Between WT and Synage KO mice, there were no significant changes in the body appearance, body weight, or brain weight. However, the weight of cerebella relative to body weight was significantly decreased in both female and male KO mice





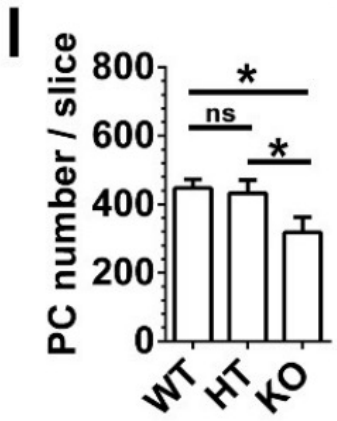


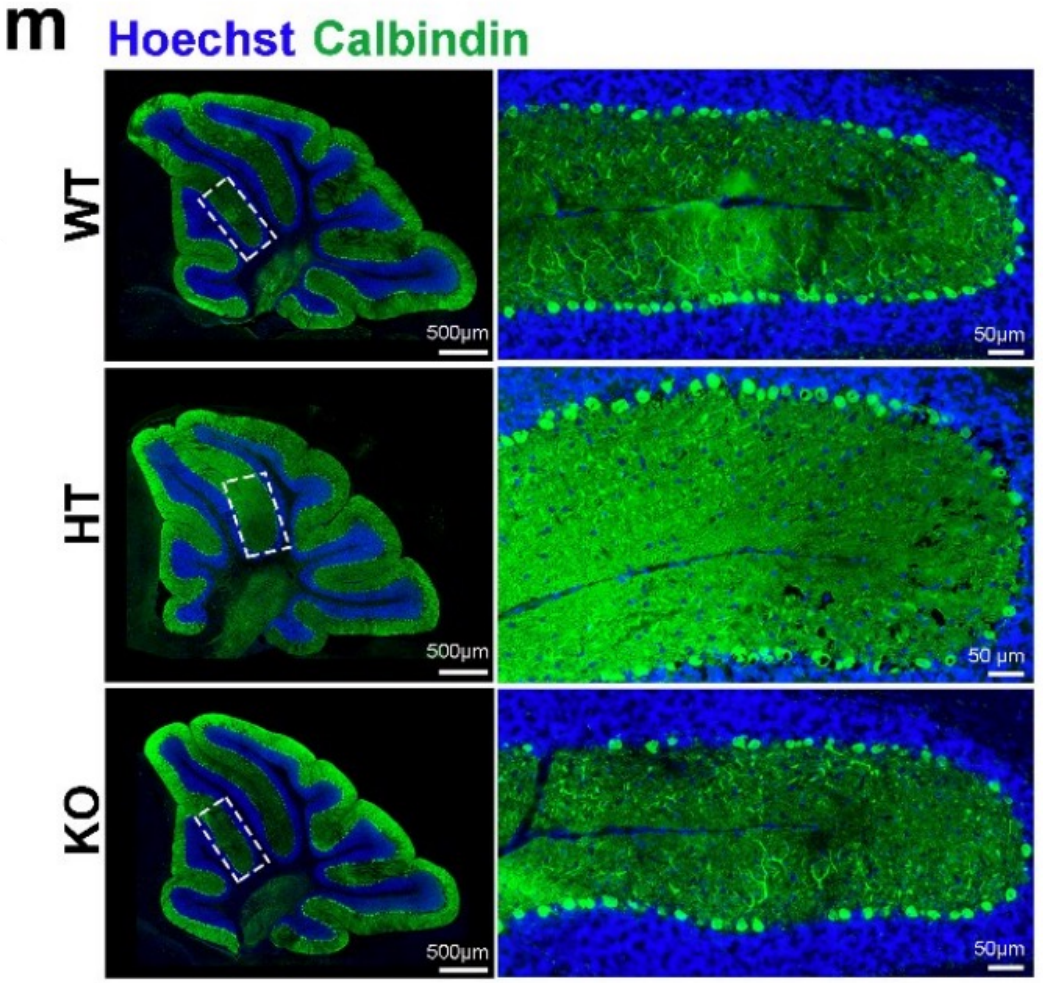
Wang et al., 2021

Synage KO mice phenotype

IF staining for PCs (using Calbindin, a specific marker for PCs) on cerebellar sections of 2-month-old mice.

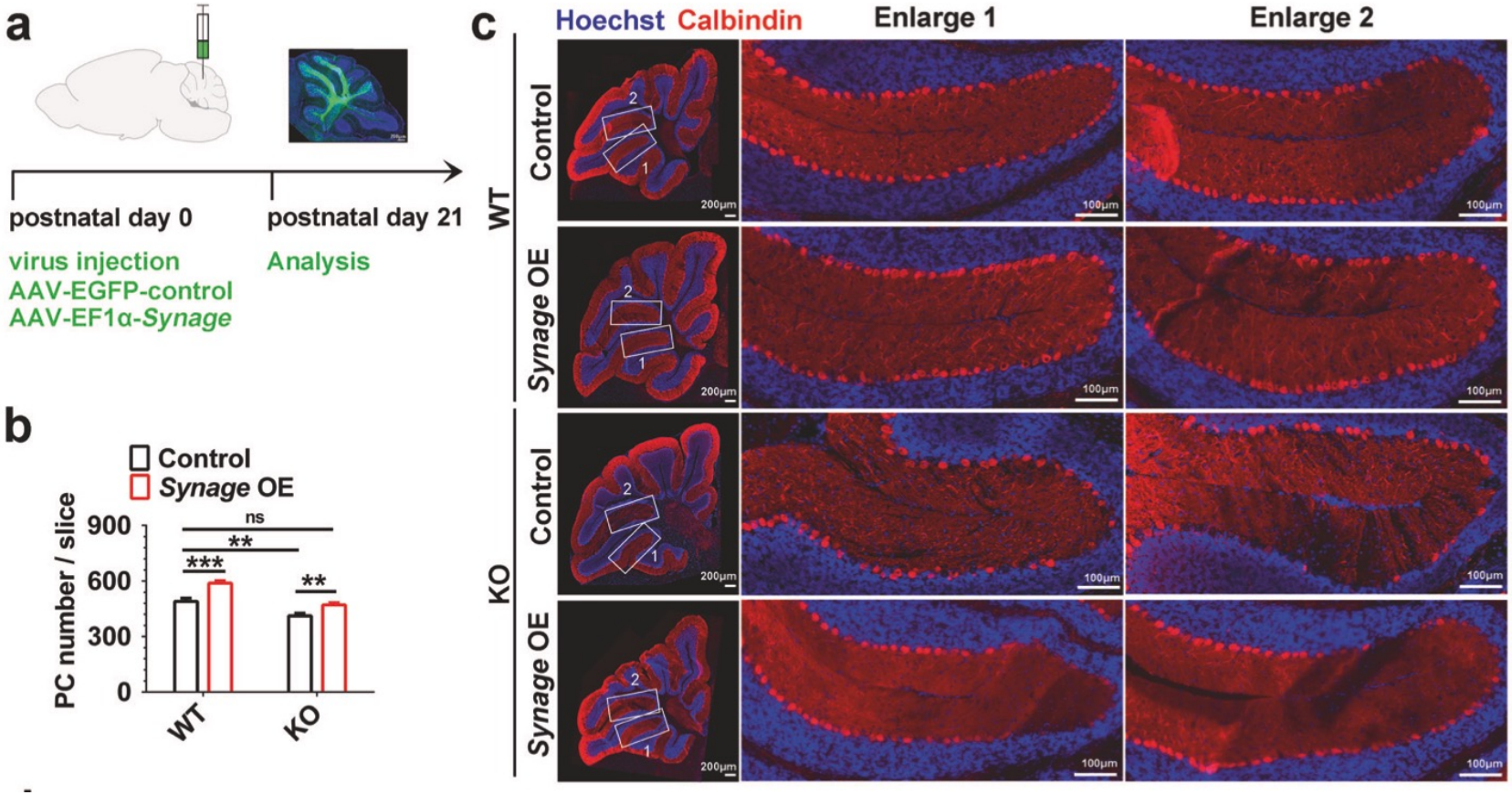
The number of PCs in adult KO mice was significantly decreased compared to those of both WT mice and HT mice, while it did not differ between WT and HT mice.





Wang et al., 2021

Rescue experiment in Synage KO mice



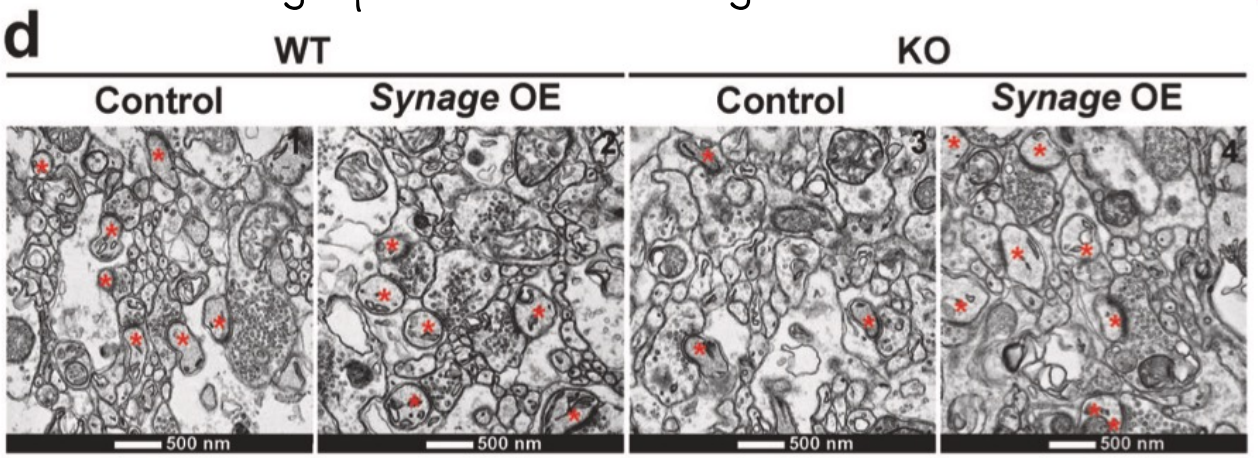
Overexpression of Synage in the cerebellum rescued the number of PCs to the level of WT mice at 3 weeks after injection by injecting AAV-EF1α- Synage into the cerebella of newborn Synage KO mice (Fig. a-c).
These results supported that Synage is necessary for cerebellar development and maturation.

Wang et al., 2021

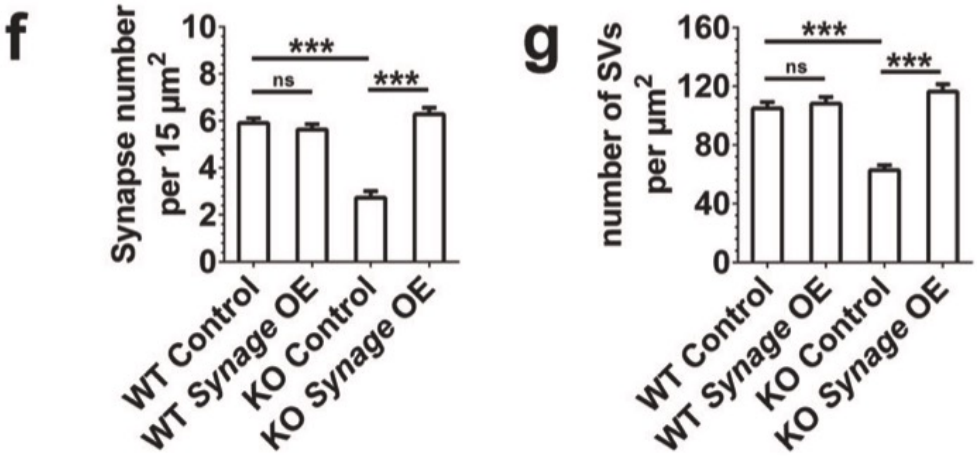
Synage deletion leads to morphological and functional defects in synapses

Transmission electron microscopy (TEM) analyses on cerebellar cortex slides in adult WT and Synage KO mic

Synapses are indicated by red asterisks



They found: significantly reduced numbers of both synapses and synaptic vesicles (SVs) in **PRESYNAPTIC TERMINALS** in the cerebellar cortex of KO compared to WT mice.



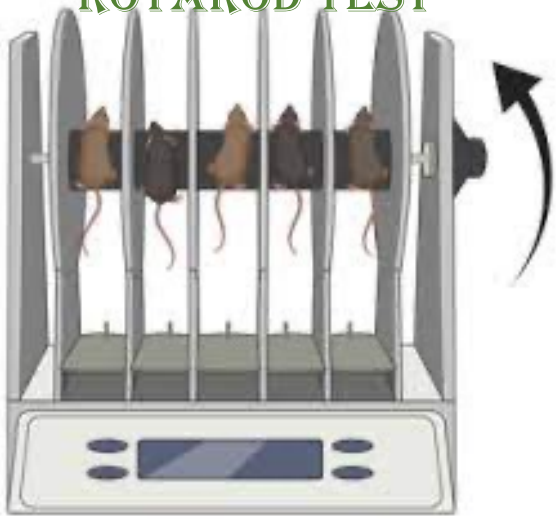
Wang et al., 2021

Motor behavior defects in Synage KO mice

Aberrant cerebellar morphology often leads to motor behavior defects.

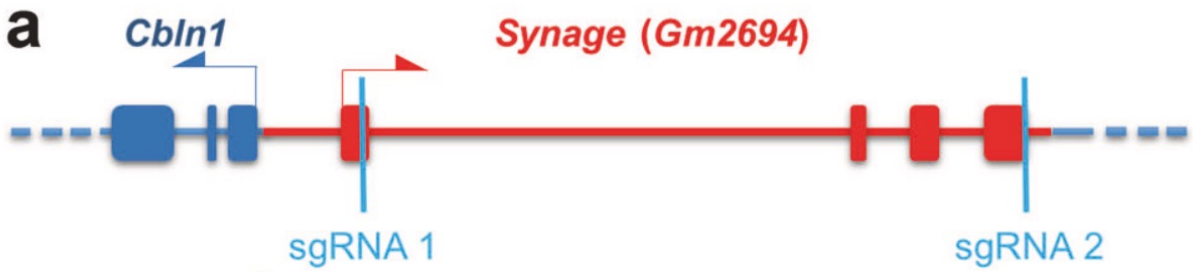
The **ROTAROD TEST** and the **BALANCE BEAM TEST**, well-established methods to evaluate motor coordination in rodents, showed that motor abilities and motor-dependent learning and memory were severely impaired in Synage KO mice. Taken together, their findings of the decrease in cerebellar neurons and synapses and the defects in neuronal synaptic function in Synage KO mice all strongly suggest that the severe morphological and functional defects in neurons and synapses are responsible for the observed motor dysfunction of Synage KO mice.

ROTAROD TEST



Wang et al., 2021

Synage lncRNA molecular function maintains stability and fun ction of cerebellar synapses partially by regulating Cbln1 mRNA



CBLN1, highly expressed in cerebellar granule cells, is a synaptic protein crucial for organization of parallel fibers, that are the axonal extensions of granule cells, with each fiber making single synapses on hundreds of thousands of Purkinje cells.

CBLN1 protein is secreted from cerebellar GCs to act as a critical synapse organizer between PFs and PCs

Since many lncRNAs regulate their neighboring protein-coding genes, we asked whether Synage lncRNA also modulates Cbln1 expression.

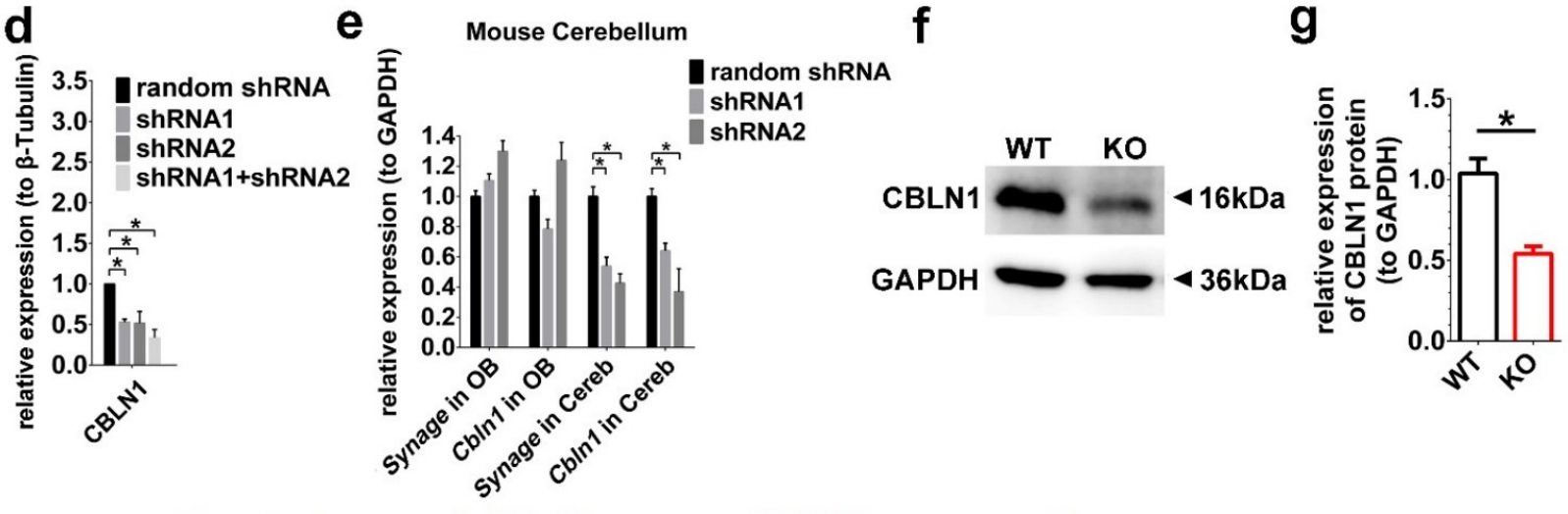
Wang et al., 2021

The formation of mature neurons and stabilized synapses during development is a prerequisite for proper nervous system functionality, which require synaptic proteins. For instance, CBLN1, highly expressed in cerebellar granule cell layer (GCs), is a synaptic protein crucial for organization of parallel fibers (PFs, axons of the GCs) and Purkinje layer (PCs)

Wang et al., 2021

Mice

- Injection of the Synage shRNAs into the cerebella of adult WT mice…..
- Two weeks later….



OB: olfactory bulb

Wang et al., 2021

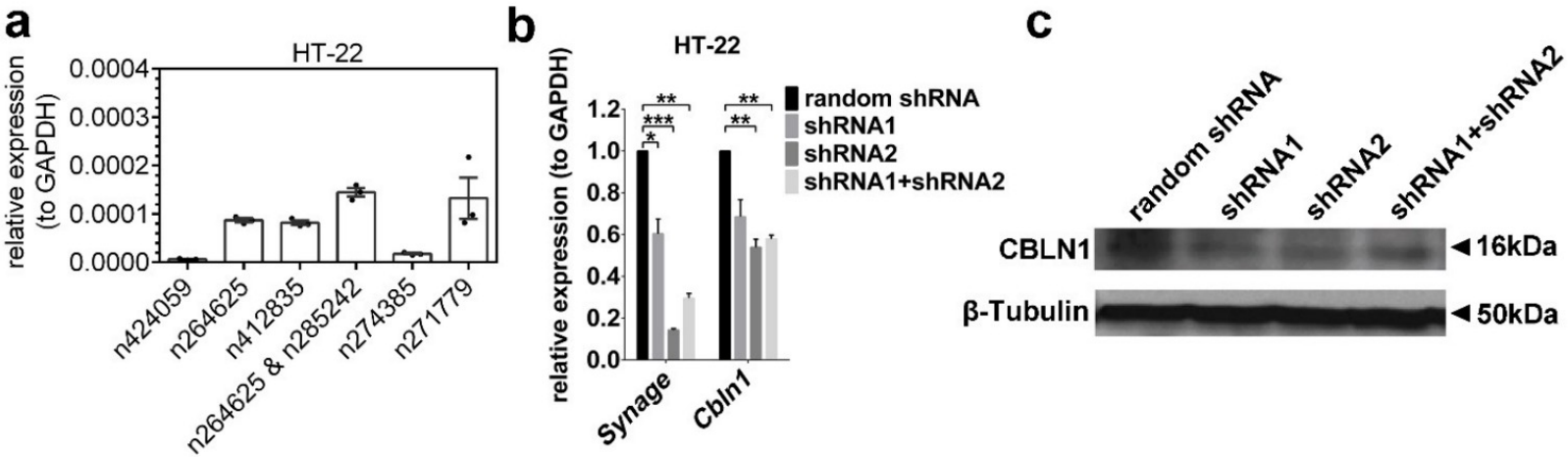
Controls

Given their spatial proximity in the genome, we asked whether Synage KO inhibiting Cbln1 expression is due to **INADVERTENT EXCISION** of some potential regulatory elements upstream of the Cbln1 gene. They verified that Synage deletion did not affect the number of nascent Cbln1 transcripts, although the total Cbln1 mRNA and protein levels were significantly decreased in the cerebella of 2-month-old Synage KO mice. These data suggest that Synage regulates Cbln1 expression at the mRNA and/or protein levels.

Wang et al., 2021

Cell line

- C8-DIA cell line (astrocyte type I cloned cell line from 8-day postnatal mouse cerebella) difficult to transfect…..
- Looking for other cells expressing the Synage lncRNA…
- Synage was robustly expressed in the HT-22 cell line, which is a mouse hippocampal neuronal cell line, easy to transfect



The results indicated that the expression levels of both Cbln1 mRNA and protein were significantly reduced upon Synage knockdown in the HT-22 cell line

Wang et al., 2021

Are Cbln1 protein and synage able to interact?

Synage deletion exerted a strong influence on both the mRNA and protein levels of Cbln1; however, the CBLN1 protein was not detected in in vivo RNA pull- down–MS experiment as a potential Synage-associating protein.

Considering the finding that Synage is localized in the cytoplasm of cerebellar cells, we explored the possibility that Synage may function as a **COMPETING ENDOGENOUS RNA** by competing with miRNAs was explored

the shared miRNA targets for Cbln1 and Synage were predicted using the miRNA- target (mRNA/lncRNA) interaction modules of both Star- Base v3 and DIANA-LncBase v2, which identified a perfectly conserved seed match, **MMU-MIR-325- 3P**, in the 3′UTR region of Cbln1 and the last exon of two isoforms of Synage

Wang et al., 2021

Luciferase assay

AGO2 CLIP-Seq data

We found that AGO2 had multiple binding sites located in the Cbln1 and Synage

AGO2-RIP-qPCR experiment

Synage acts as a sponge for mmu-miR-325-3p to regulate Cbln1 mRNA expression, which leads to the change of the CBLN1 protein levels.

Wang et al., 2021

CBLN1 KO MICE showed:
cerebellar ataxia and impaired performance accompanied by a significant reduction in the number of PF-PC synapses, as well as severe impairment to synaptic function.

SYNAGE KO MICE exhibited phenotypes consistent with these reports including synapse reduction and dysfunction, as well as motor defects, **BUT** otherwise showed more severe impairment than the phenotypes of Cbln1−/− mice, including **DECREASED SYNAPTIC VESCICLES, OBVIOUS NEURONAL LOSS, DECREASED CEREBELLAR WEIGHT, AND REDUCED FERTILITY**

The authors speculated that both Synage and Cbln1 likely have additional functions that are independent of one another. Synage probably modulates cere- bellar development and function through other mechanisms in addition to regulation of Cbln1 expression.

Wang et al., 2021

Looking for other molecular pathways….

RNA pull-down–MS experiments

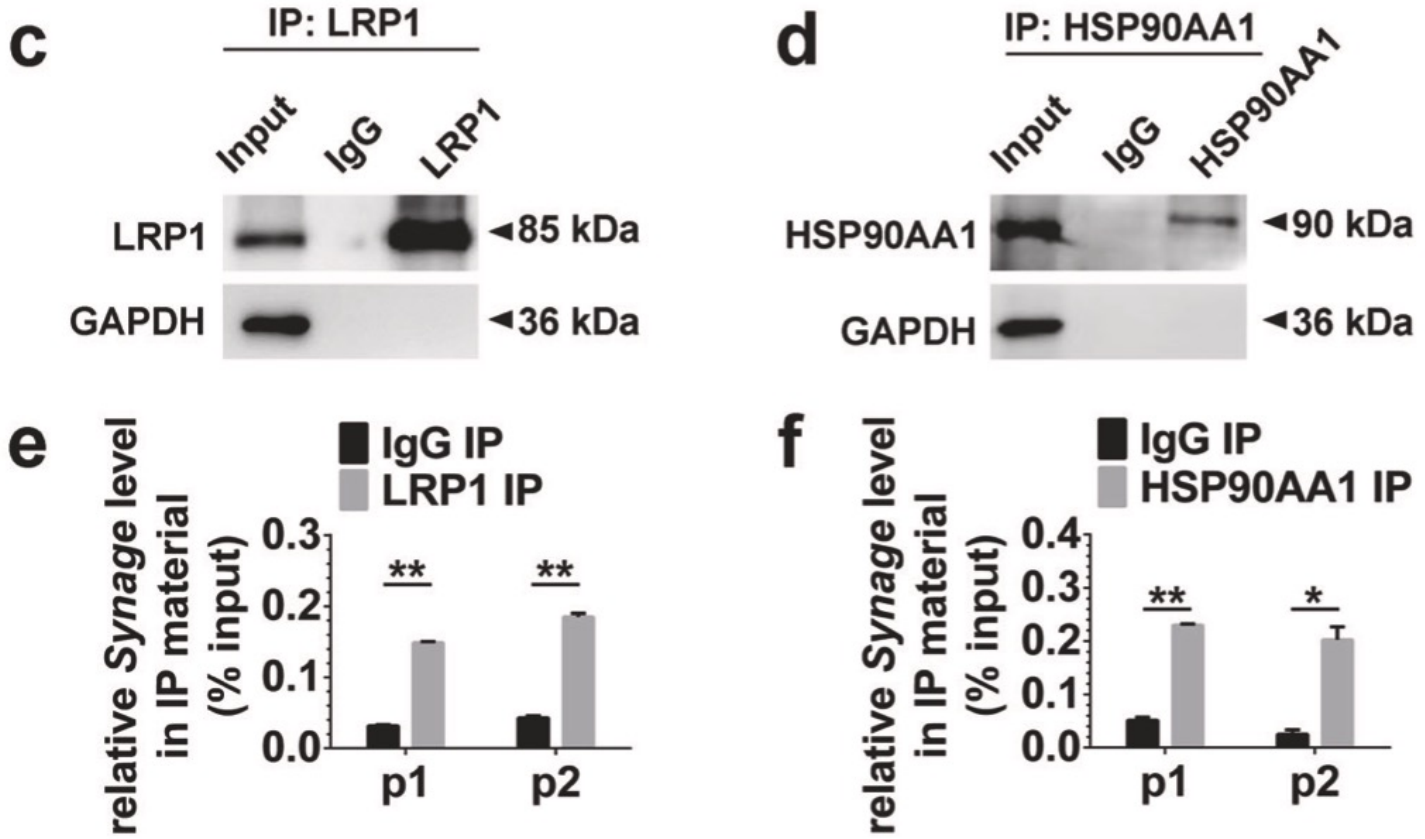
LRP1 and HSP90AA1 were the two strongest candidates identified by MS in the cerebellum

LRP1 is known to interact with PSD-95, through which it modulates synaptic function

Synage lncRNA modulates cerebellar synapses by orchestrating assembly of synaptic LRP1-HSP90AA1-PSD-95 complex

Wang et al., 2021

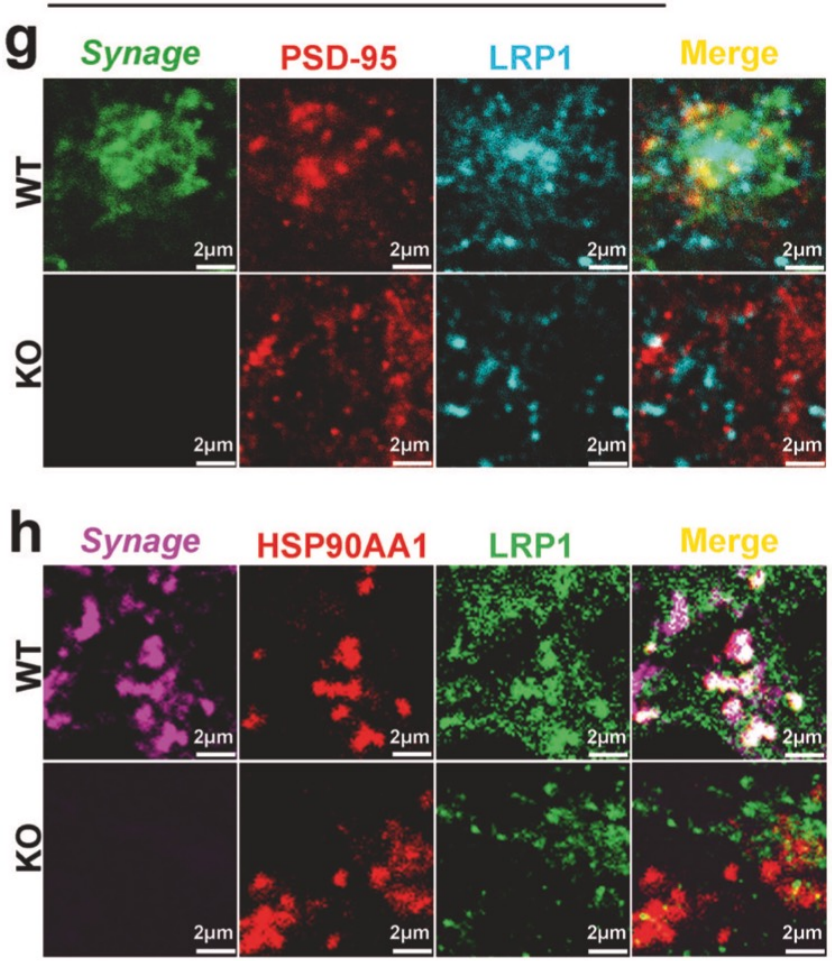
Interaction validation by RIP



We thus speculated that PSD-95 may also be in the complex of LRP1-HSP90AA1-Synage.

Wang et al., 2021

Interaction validation by FISH



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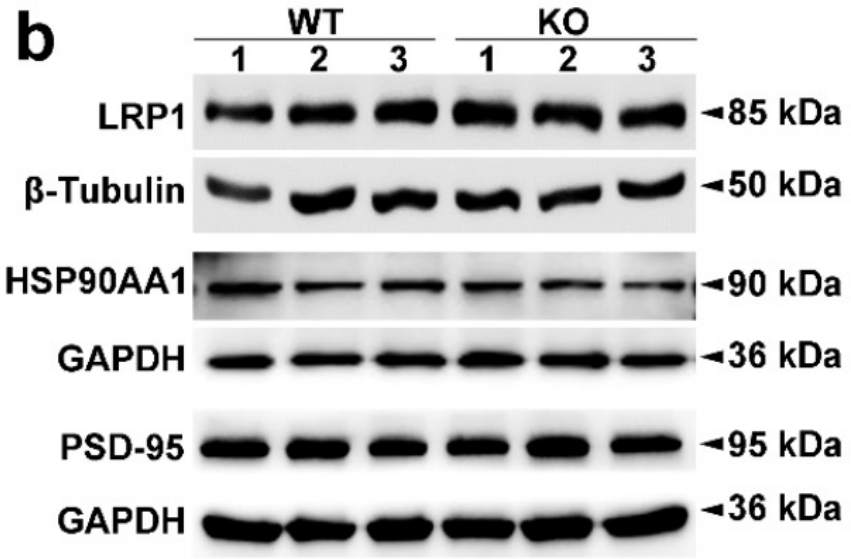
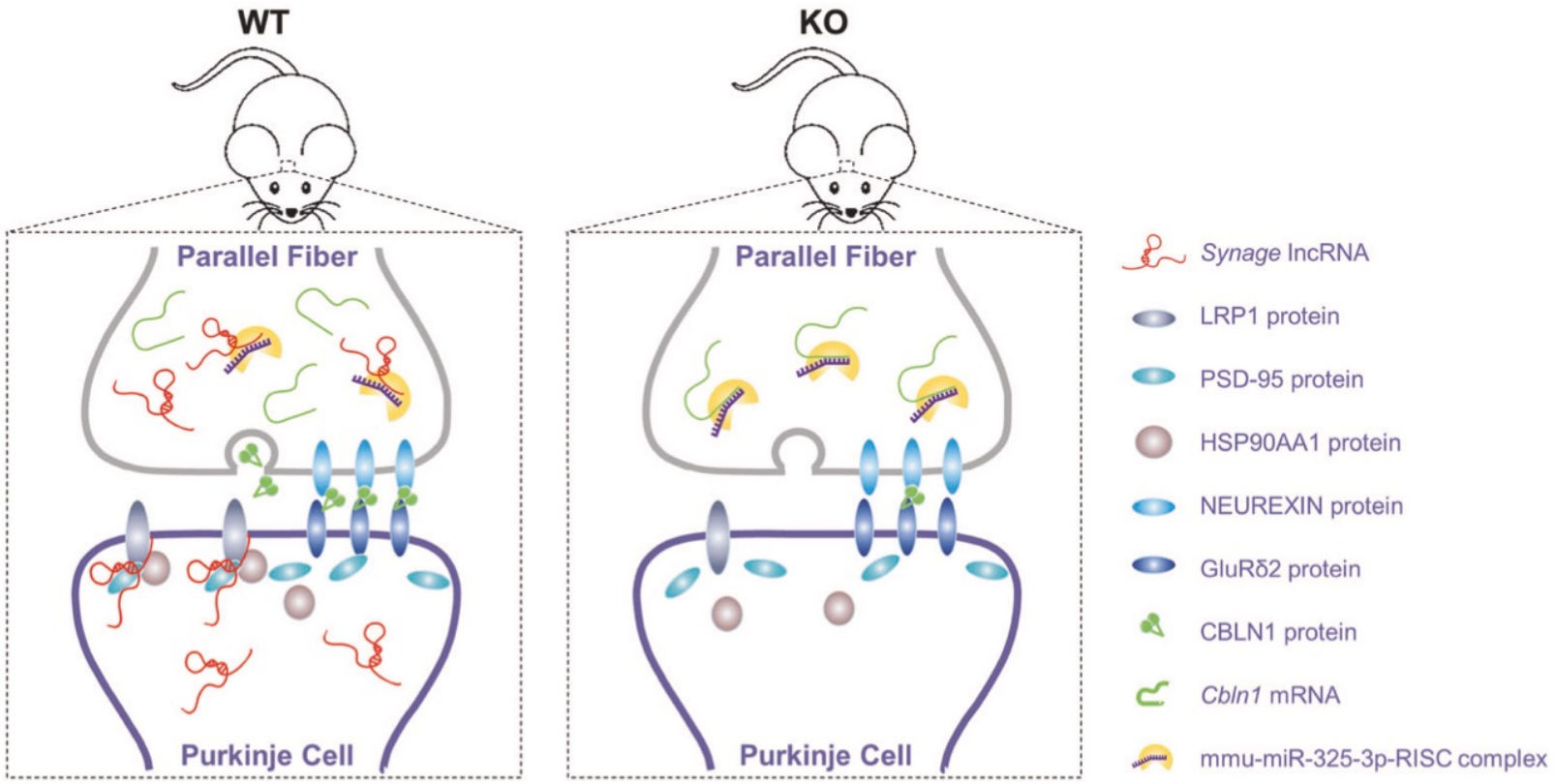


These data suggest that Synage functions in synapse stability not by reducing protein levels per se, but rather by somehow regulating Synage-dependent assembly of the LRP1- HSP90AA1-PSD-95 complex in the cerebellar cortex.

Wang et al., 2021

Model



Synage lncRNA regulates stability and function of cerebellar synapses via at least two mechanisms.

One is through the function of Synage as a sponge for mmu-miR- 325-3p to regulate Cbln1 mRNA expression, which leads to the change of the CBLN1 protein levels.

The other function is to serve as a scaffold for orchestrating the assembly of synaptic LRP1-HSP90AA1- PSD-95 complex.

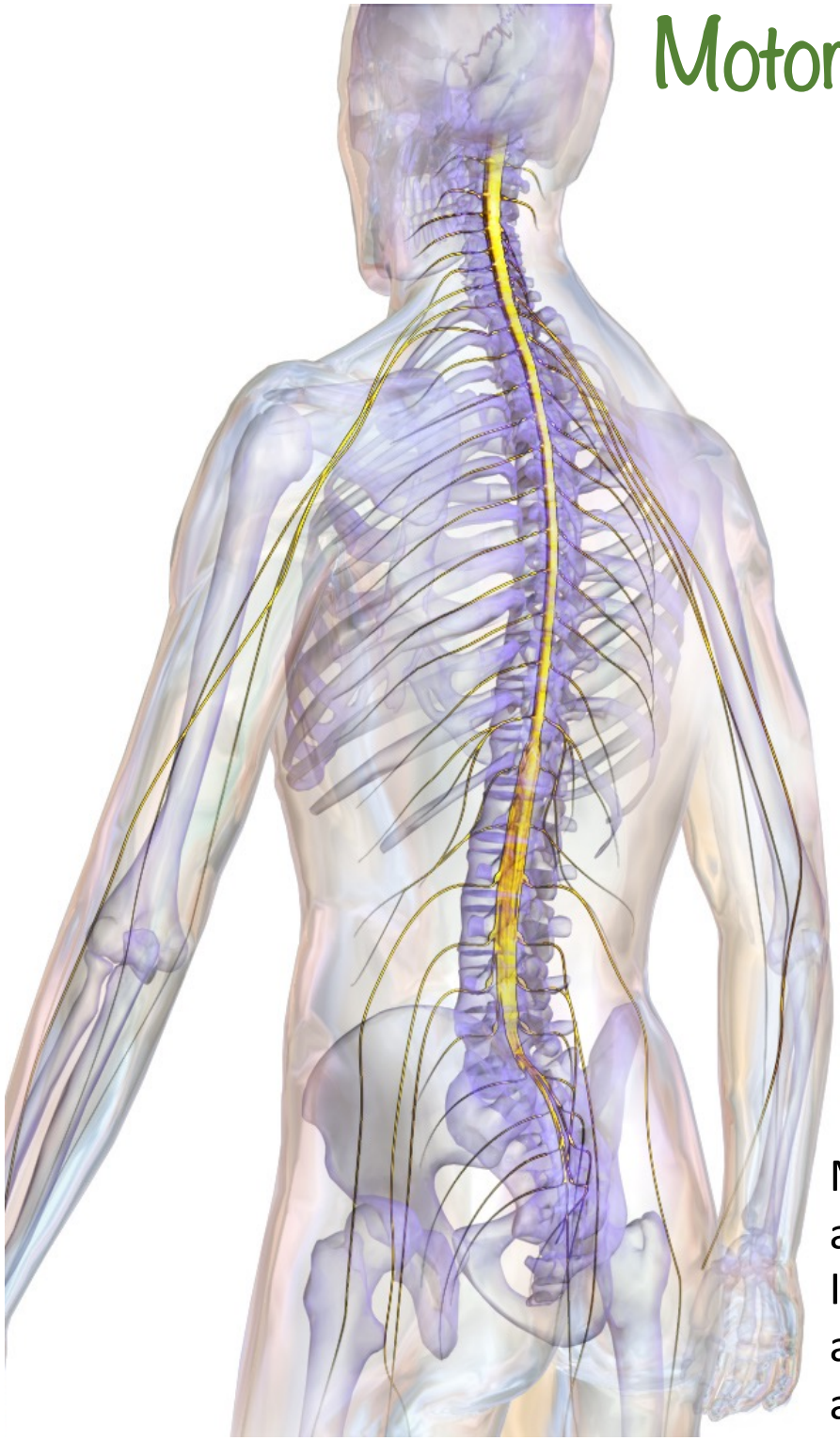
Wang et al., 2021

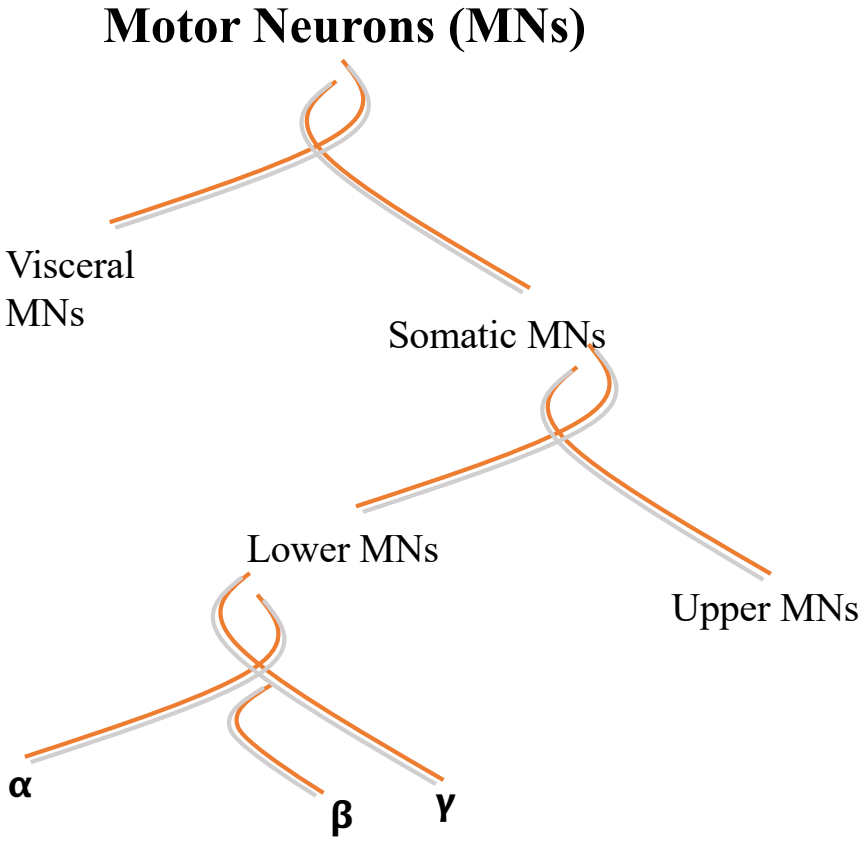
Motorneurons (MNs)

MNs are a group of neurons that have their cell bodies:

- in the cortex (upper MNs)
- in the brainstem and spinal cord (lower MNs) and project axons into the brainstem, spinal cord or towards peripheral muscles. These projections control essential functions such as movement, breathing and swallowing.

Motor neurons (MNs) features



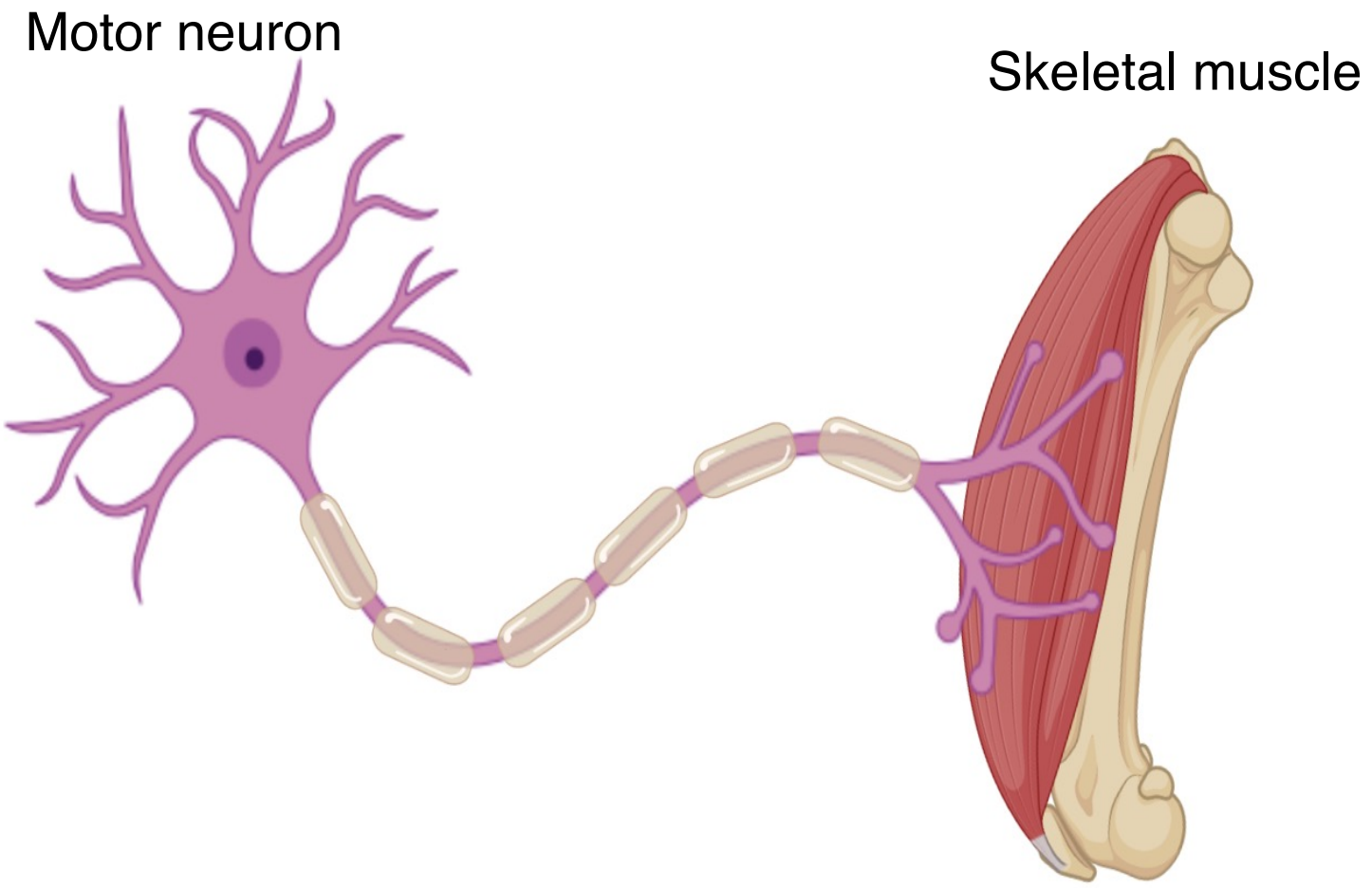


MNs are classified in visceral and somatic MNs that are in turn divided in upper and lower MNs.
In the end lower MNs are classified in alpha that are able to interact with extrafusal fibers and beta and gamma with the intrafusal fibers

Motor neurons (MNs) features

- Motor neurons are located in the central nervous system (CNS), specifically in the motor cortex, brainstem and spinal cord.
- Motor neurons are also known as efferent neurons, meaning they carry information from the CNS to muscles, and other peripheral systems such as organs and glands.
- This contrasts with afferent neurons, or sensory neurons, which carry information from sensory organs and tissues back to the CNS.

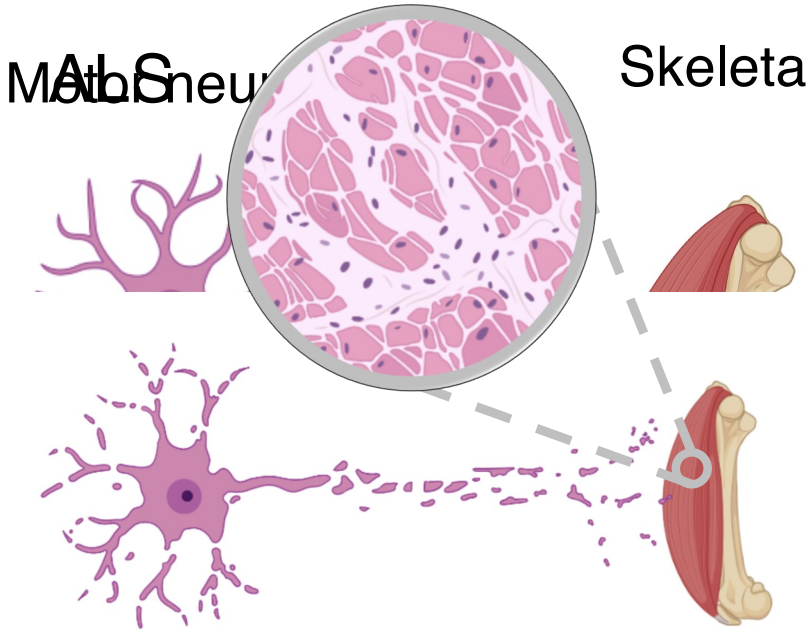
Lower MNs



Not surprising given their important functions, selective degeneration of MNs is a hallmark of motor neuron diseases (MNDs) such as:

amyotrophic lateral sclerosis (ALS)
spinal muscular atrophy (SMA)

MN and ALS



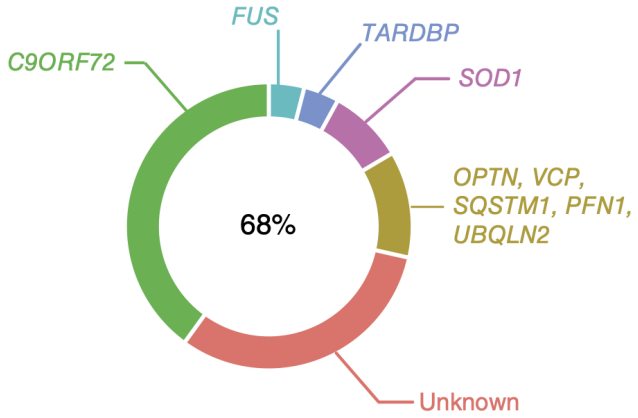
Neurodegenerative disease

Affects MNs

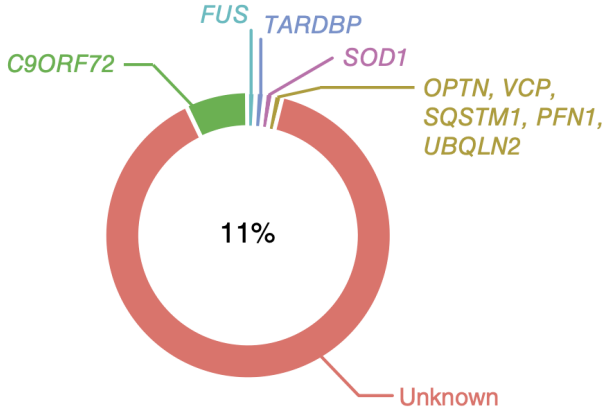
No cure

Da 1 a 2 per 100.000 persone l'anno

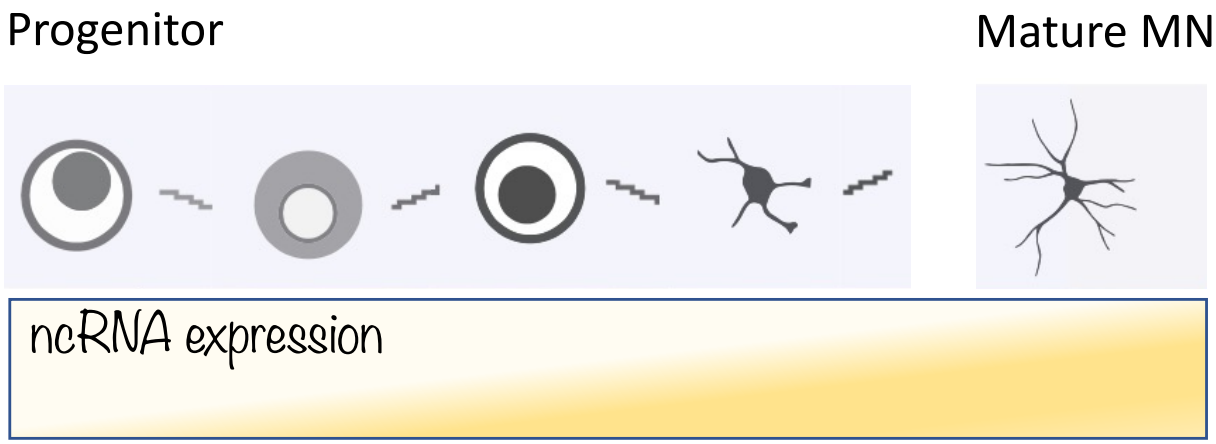
Familial



Sporadic



Motor neuron development



A role for lncRNAs in the specification of neuron subtypes has been proposed.

lncRNAs and motor neuron development

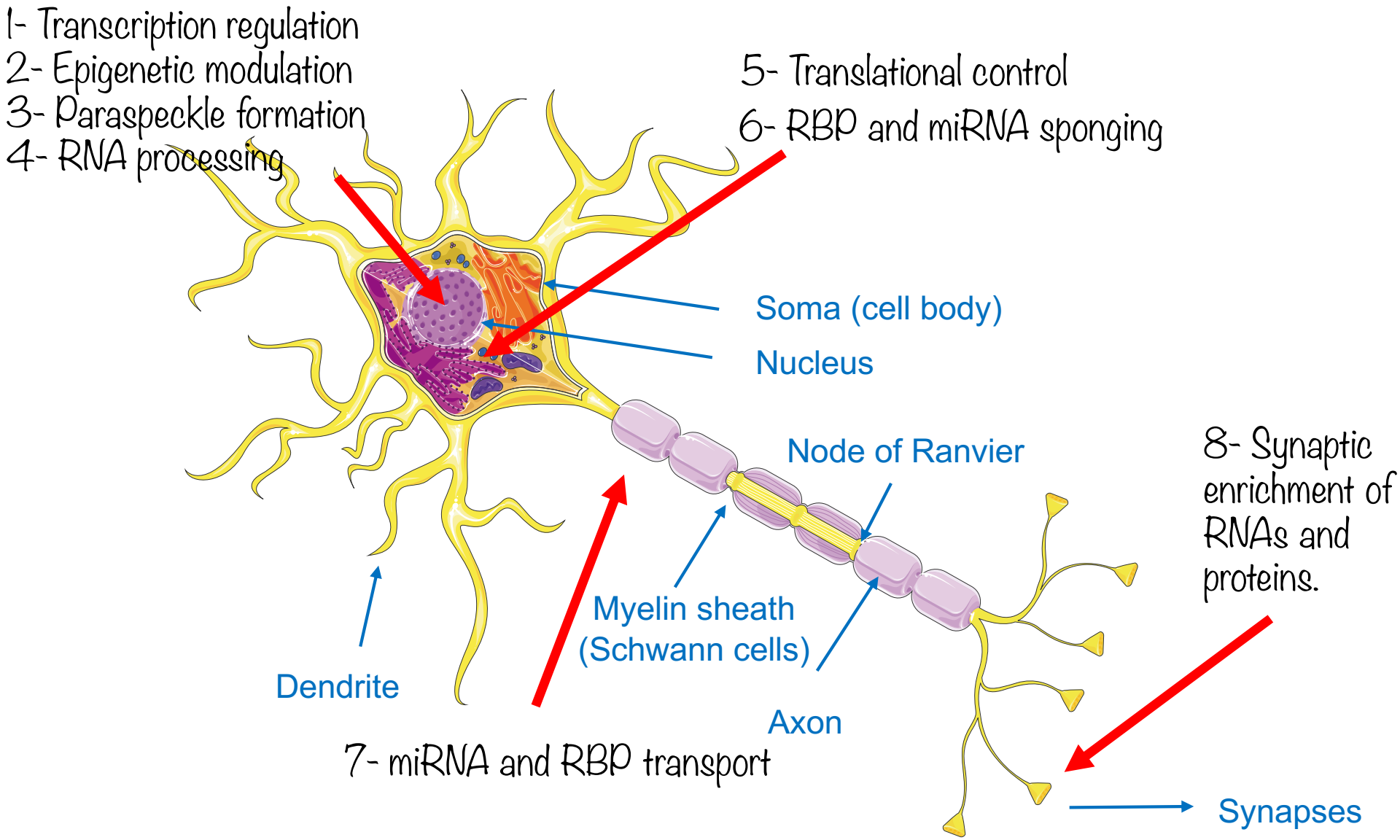
TABLE 2 Overview of the expression and proposed function of lncRNAs in motor neuron development

Name	ncRNA	Regulation	Observation	Mechanism	References
Meg3	lncRNA	Up-regulated; spatial regulation	Regulated throughout embryonic stem cells–motor neuron (ESC-MN) differentiation; enriched in the nucleus	Epigenetic regulation of *Hoxa4:Hoxc5* expression	Yen et al., (2018)
CAT7	lncRNA	Up-regulated	Regulated during early stages of human ESC-MN differentiation	Regulation of polycomb repressive complex 1 (PRC1) associated genes	Ray et al., (2016)
Hoxb5os	lncRNA	Up-regulated	Regulated throughout ESC-MN differentiation	Tbd	Rizvi et al., (2017)
Gm12688/Gm14204	lncRNA	Cell type-specific expression	Uniquely expressed in V1/V1 and V2b GABAergic interneurons	Tbd	Rizvi et al., (2017)
LncMN−1,−2,−3 and *Lhx1os*	lncRNA	Cell type-specific expression; up-regulated	Specifically enriched in MNs; regulated during differentiation of mouse ESC (mESC)/ human-induced pluripotent stem cells (hiPSC)-derived MNs	Tbd	Biscarini et al., (2018)
Lncrps25	lncRNA	Down-regulated	Knockdown reduces swimming activity because of defects in primary MNs	Via *olig2* (Tbd)	Gao et al., (2020)
Malat1, *Meg3*, *Rmst*, *Xist* and *Miat*	lncRNA	Spatial distribution	Specifically enriched in somatodendritic/axonal fractions	Tbd	Briese et al., (2016)
c−1, *c−2*, *c−13*, *c−16*, *c−48*, *c−80*, *c−82*, *c−84*, *c−88*	circRNA	Up-regulated	Regulated during mESC/hiPSC-derived MN differentiation	Tbd	Errichelli et al., (2017)
Human circSMN	circRNA	Multiple isoforms produced	Primate specificity of SMN-derived circRNAs	Tbd	Ottesen et al., (2019)

Abbreviations: hiPSC, human-induced pluripotent stem cells; Meg3, maternally expressed gene 3; mESC, mouse embroynic stem cells; Tbd, to be determined.

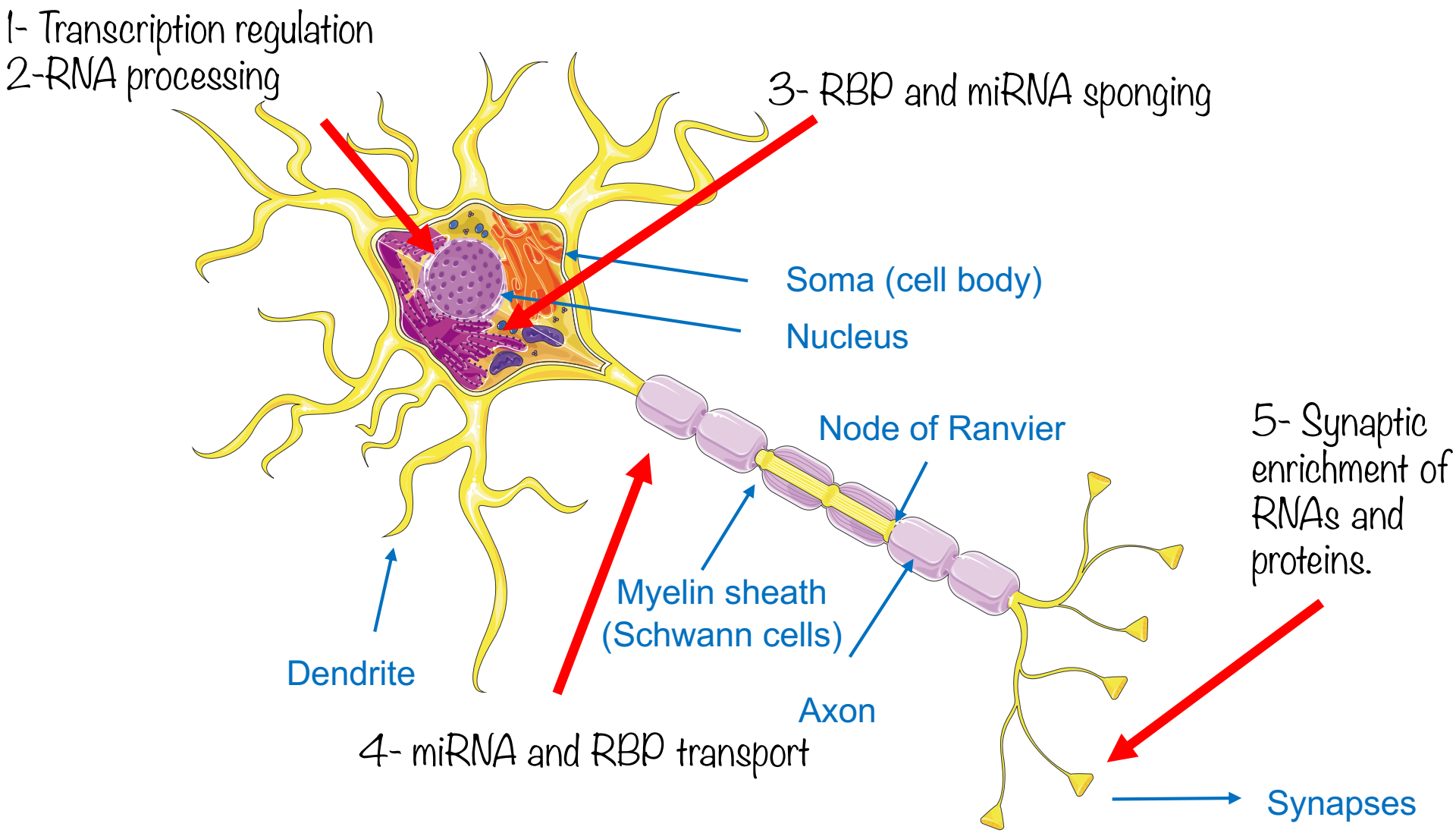
Vangoor et al. 2020

LncRNAs have been implicated in a wide range of functions in developing MNs



Adapted from Vangoor et al. 2020; Service medical art

circRNAs have been implicated in a wide range of functions in developing MNs



Adapted from Vangoor et al. 2020; Service medical art

lncRNAs have been linked to MN disease

TABLE 4 Overview of the expression and proposed functions of lncRNAs in motor neuron disease

Name	ncRNA	Disease	Regulation	Function	References
NEAT1	lncRNA	ALS	Up-regulated at early stage	Regulates paraspeckle formation, increased *NEAT1* expression leads to neurotoxicity	Clemson et al., (2009); Nishimoto et al., (2013) and Suzuki et al., (2019)
C9ORF72-AS	antisense RNA	ALS	Up-regulated	Forms RNA foci that recruit RBPs, DPR protein formation via repeat-associated non-ATG-initiated (RAN) translation leading to neurotoxicity	Cheng et al., (2019); Mizielinska et al., (2014); Mori, et al. (2013); Sareen et al., (2013); Swinnen et al., (2018) and Wen et al., (2014)
ATXN2-AS	antisense RNA	ALS	Up-regulated	Repeat expansion RNA induces neurotoxicity	Li, Sun, et al. (2016)
SMN-AS	antisense RNA	SMA	Up-regulated	Recruits polycomb repressive complex 2 (PRC2) complex to the *SMN* gene to suppress SMN expression	d'Ydewalle et al., (2017) and Woo et al., (2017)
ZEB1-AS, *ZBTB11-AS*	antisense RNA	ALS	Up-regulated in blood samples (peripheral blood mononuclear cells [PBMCs])	Tbd	Gagliardi, et al. (2018)
UBXN7-AS, *ATG10-AS*, *ADORA2A-AS*	antisense RNA	ALS	Up-regulated in blood samples (PBMCs)	Tbd	Gagliardi, et al. (2018)
hsa_circ_0001173, *hsa_circ_0043138*, *hsa_circ_0088036*	circRNA	ALS	Up-regulated in blood samples (PBMCs)	Biomarker potential	Dolinar et al., (2019)

Abbreviations: ATXN2, Ataxin-2; NEAT1, nuclear-enriched abundant transcript 1; SMN, survival motor neuron; Tbd, to be determined.

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Characterization of the lncRNA transcriptome in mESC-derived motor neurons: Implications for FUS-ALS

Silvia Biscarini [a], Davide Capauto [a], Giovanna Peruzzi [a], Lei Lu [b], Alessio Colantoni [c], Tiziana Santini [a], Neil A. Shneider [b], Elisa Caffarelli [d], Pietro Laneve [a,*,1], Irene Bozzoni [a,c,d,e,**]

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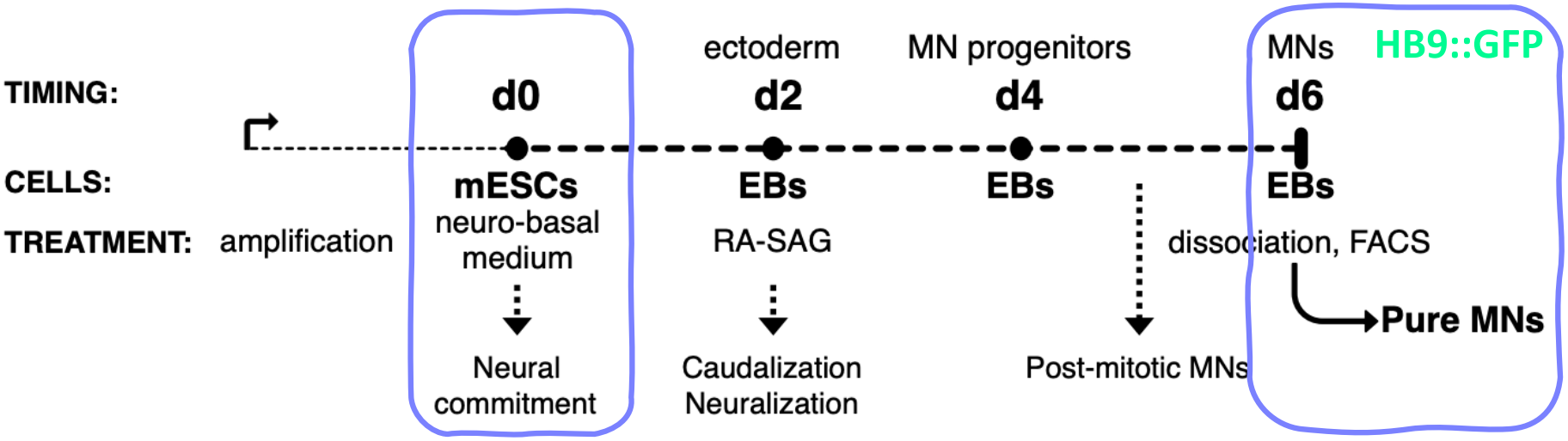
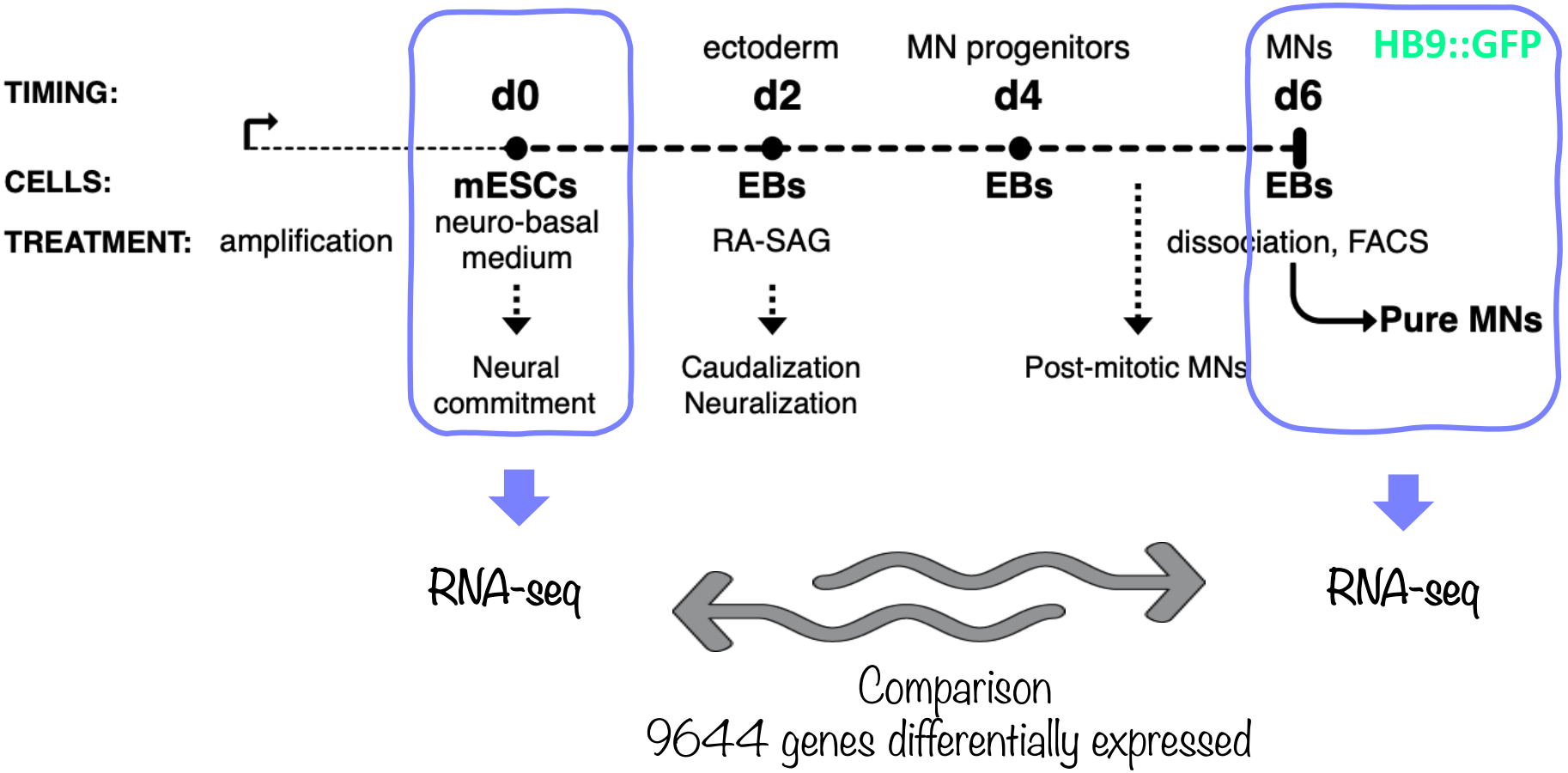
Starting from the the beginning...



Wichterle H. et al., 2002; Errichelli et al., 2017; Biscarini et al., 2018

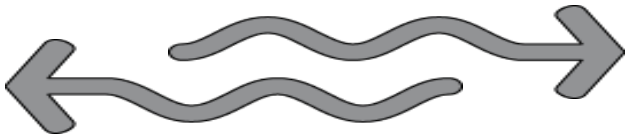
Starting from the the beginning...



Errichelli et al., 2017; Biscarini et al., 2018

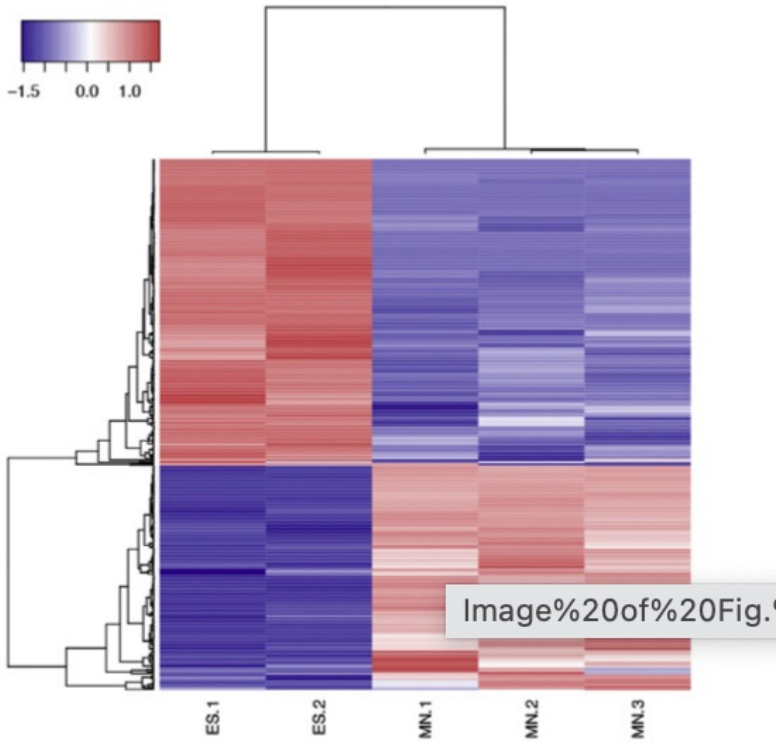
Starting from the the beginning...

RNA-seq



RNA-seq

Comparison
9644 genes differentially expressed

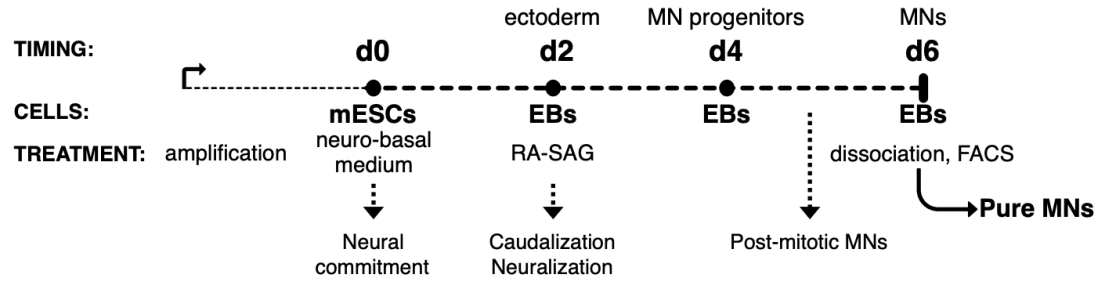


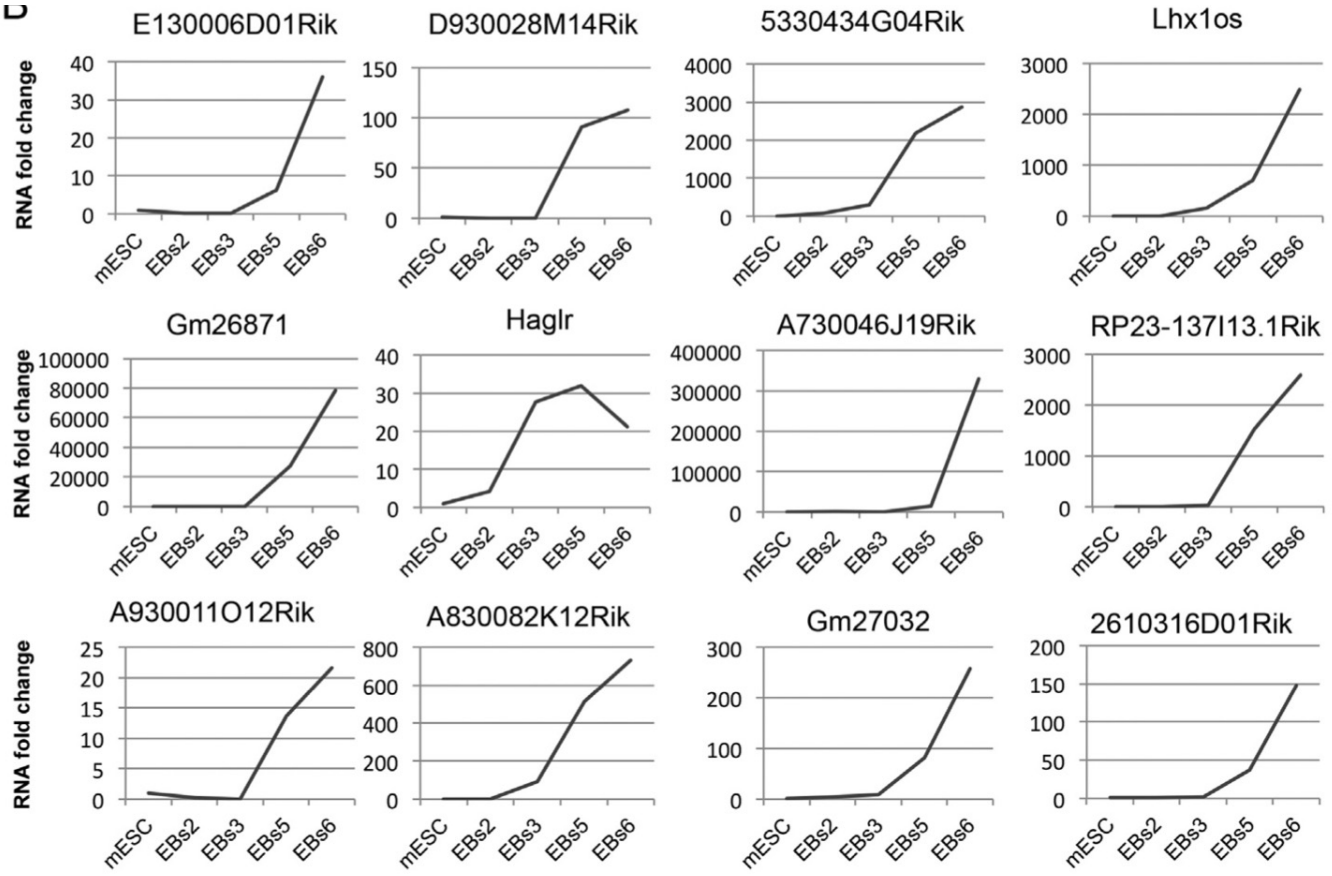
469 encoded for bona fide lncRNAs
The family of lncRNAs up-regulated in MNs derived from 270 loci and includes some species already known to play key roles in neurogenesis; among them, Miat, Rmst, Hotairm1, Meg3, Rian and Mirg.

lncRNAs involved in motor neural differentiation process (12).

Errichelli et al., 2017; Biscarini et al., 2018

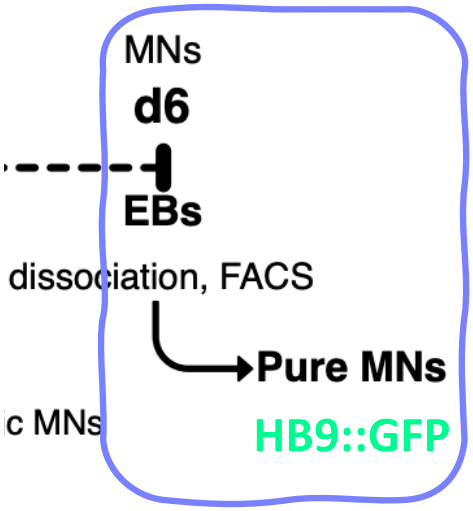
qRT-PCR analysis of the 12 lncRNAs up-regulated during MN differentiation

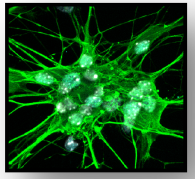




Biscarini et al., 2018

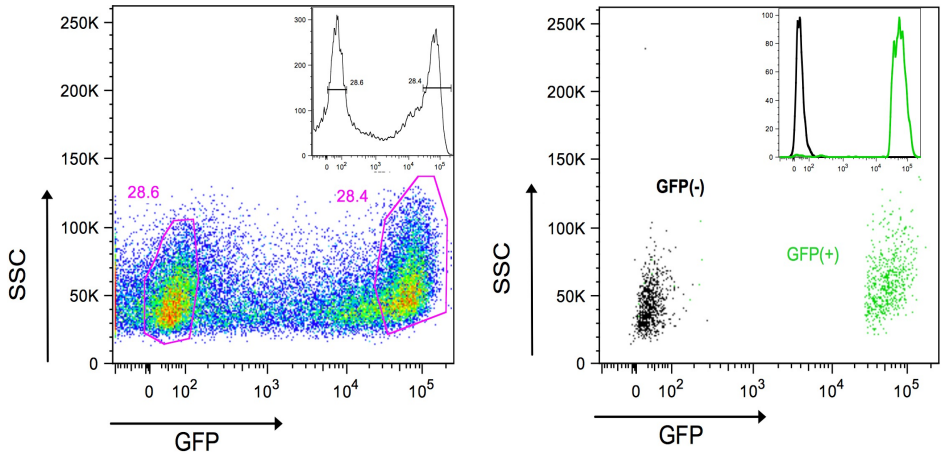
lncRNAs enriched in motor neurons

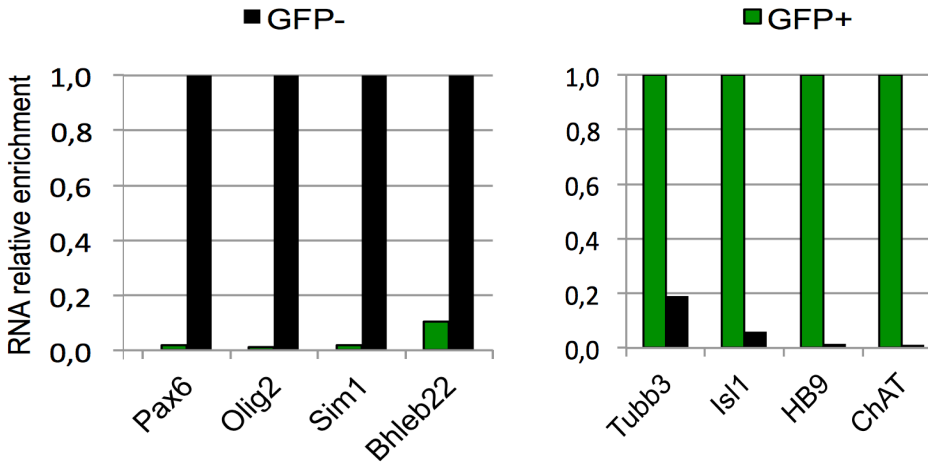




Pax6 and Olig2 transcription factors, responsible for establishing MN progenitors

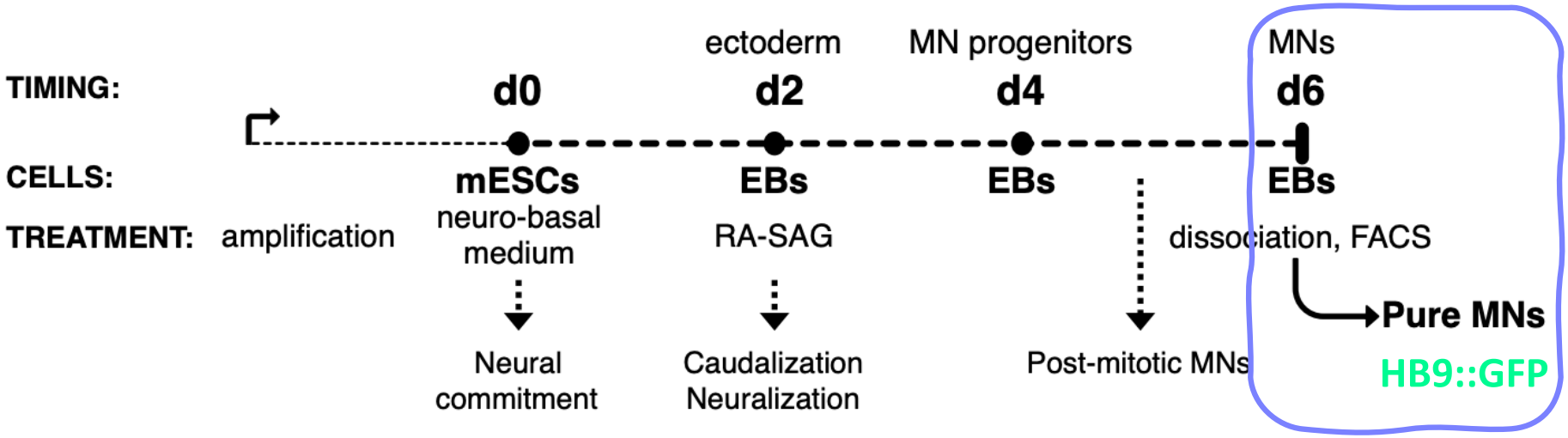
Genes required for consolidation of MN identity (Hb9) and for development (Islet-1) and function (ChAT) of spinal MNs were highly enriched in Hb9::GFP+ cells

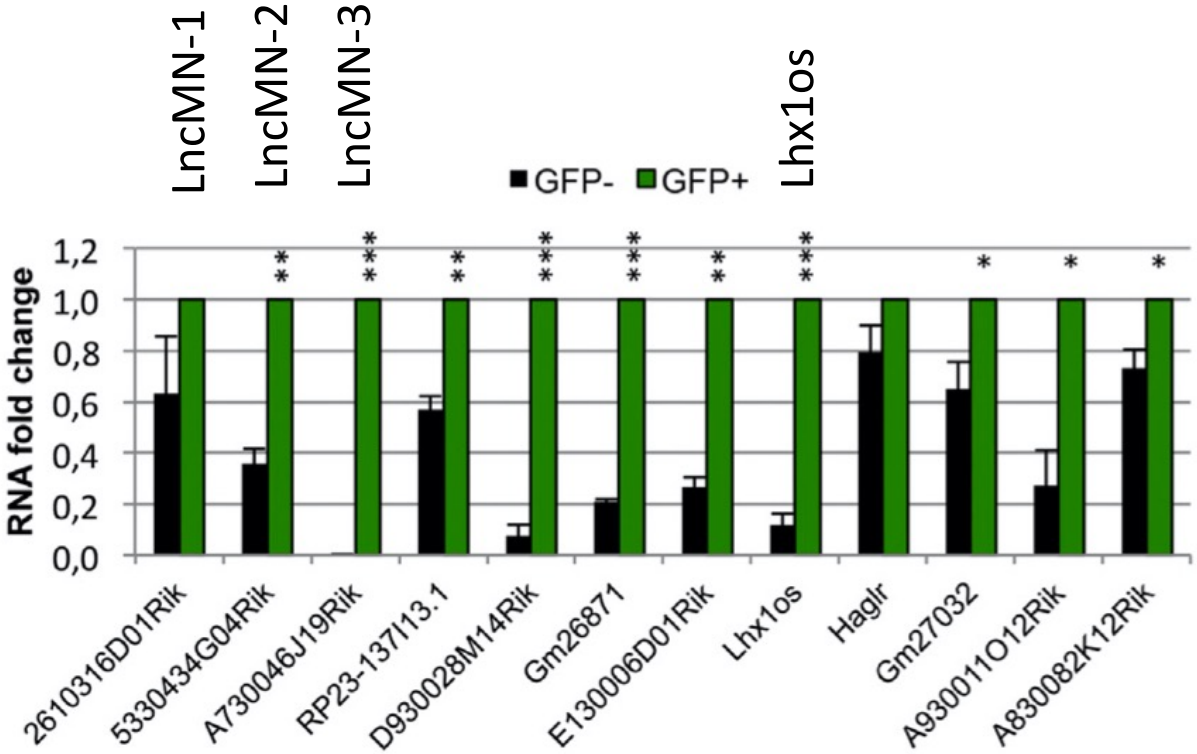




Biscarini S. et al., 2018

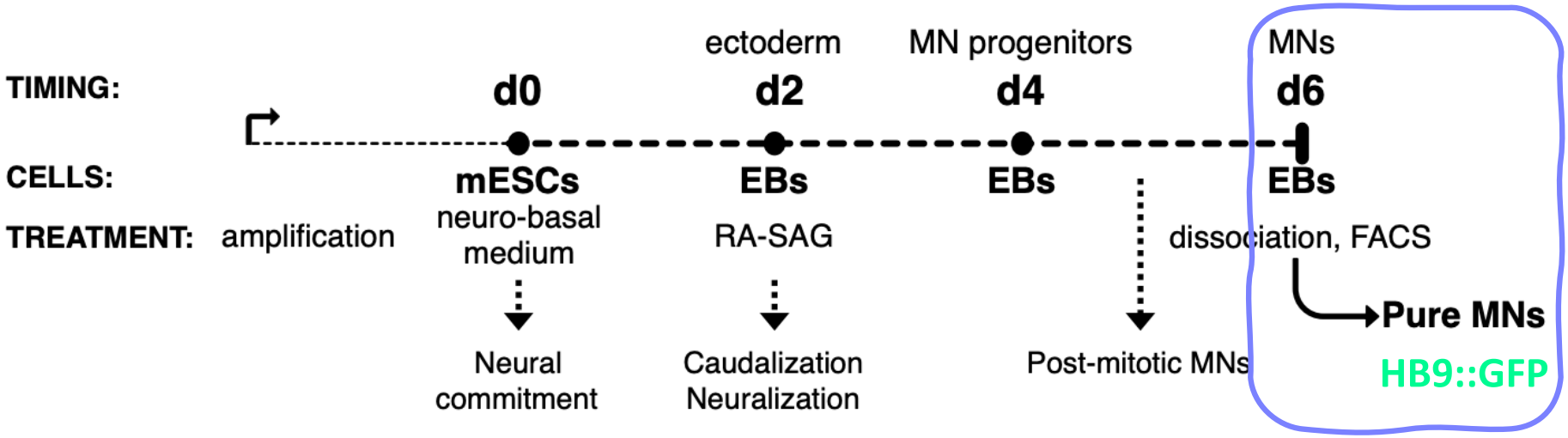
lncRNAs enriched in motor neurons

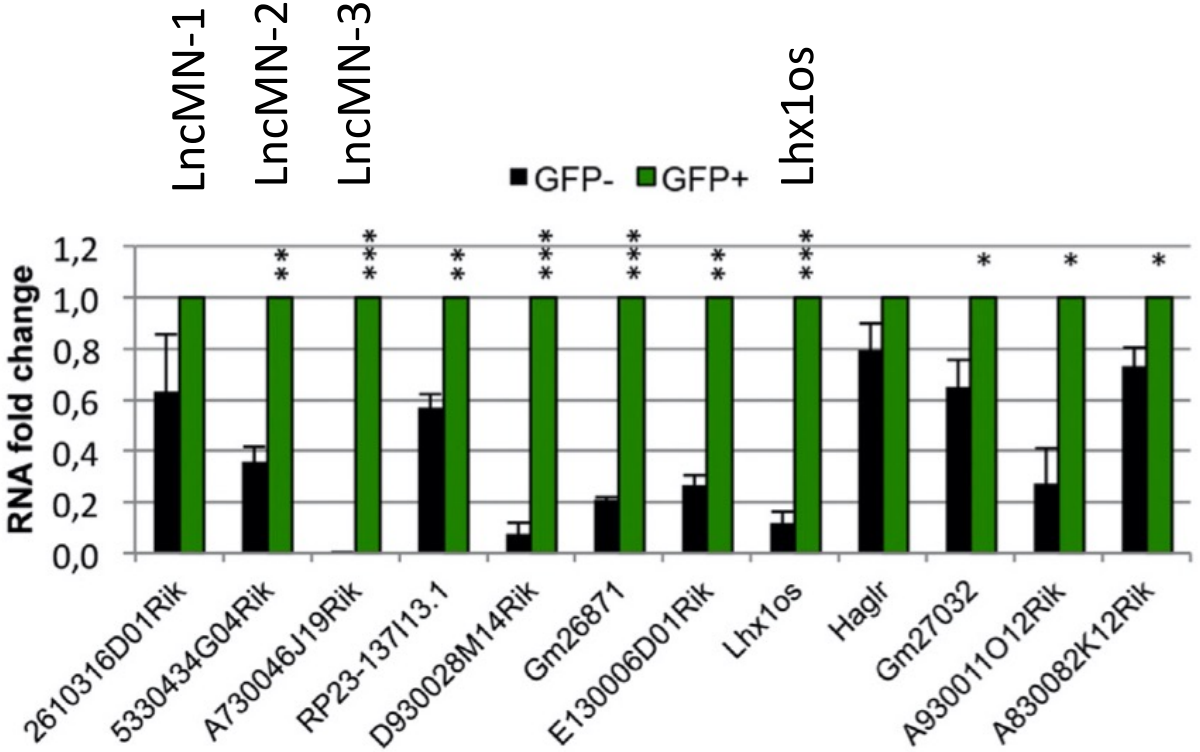


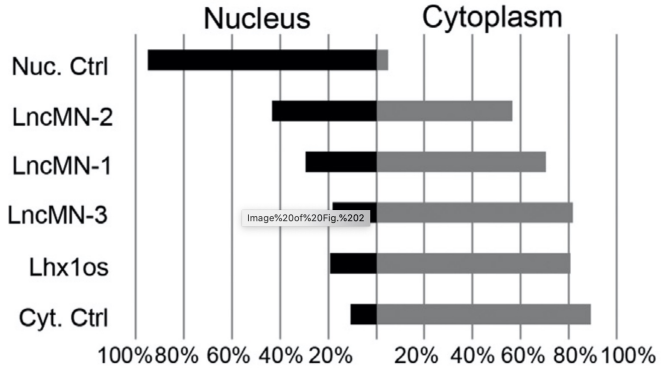


Biscarini et al., 2018

lncRNAs enriched in motor neurons

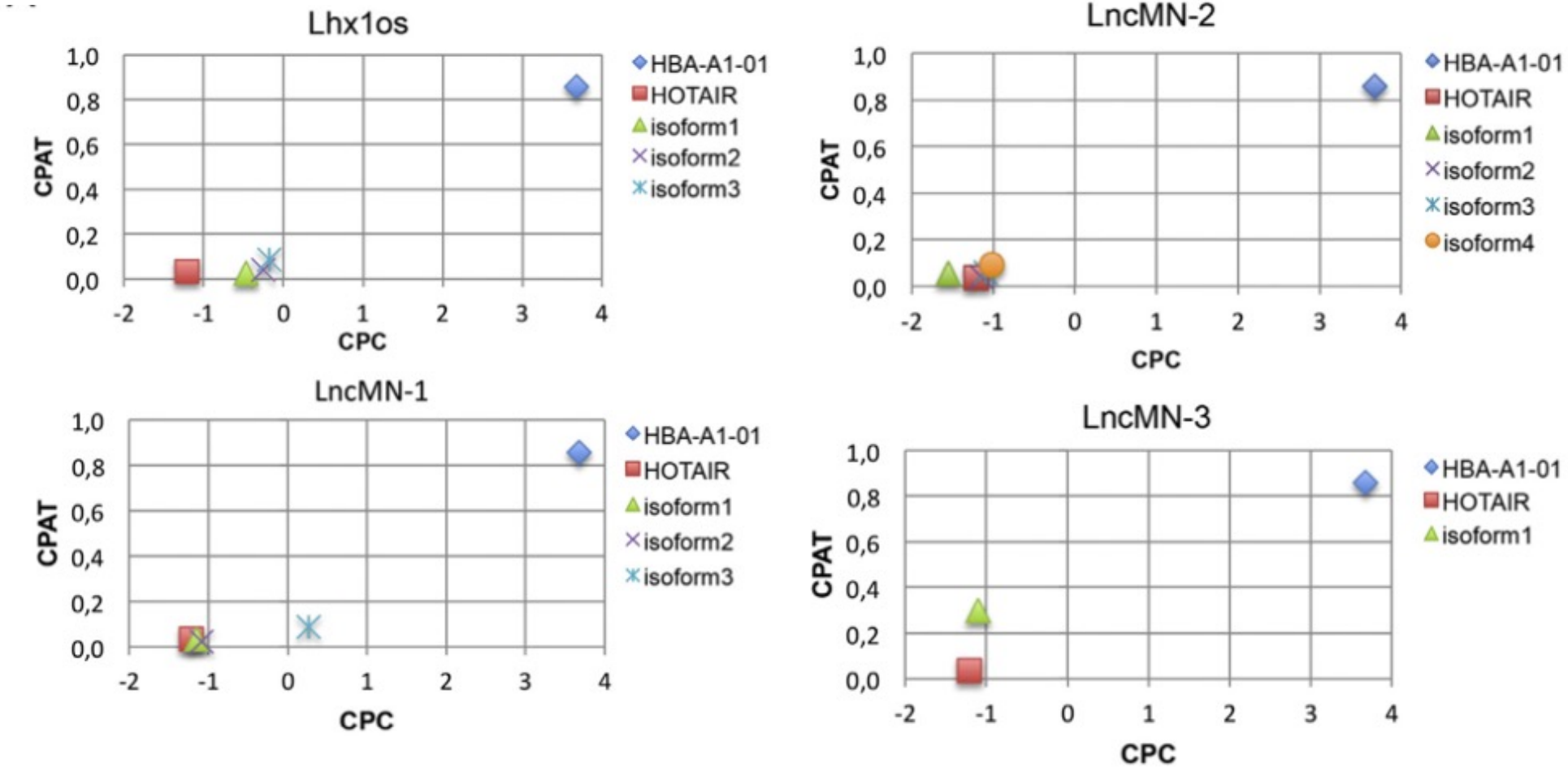






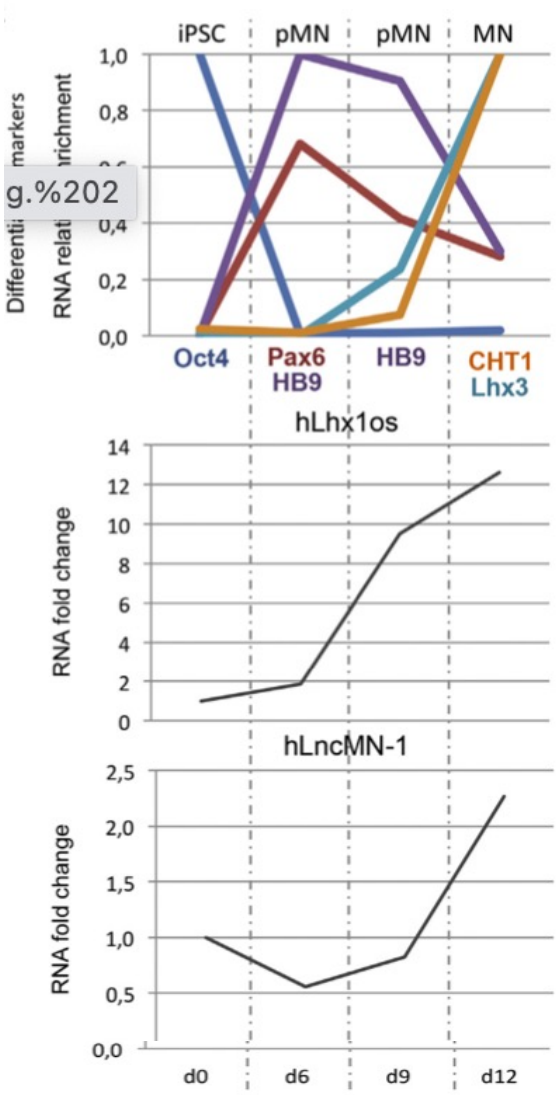
Biscarini et al., 2018

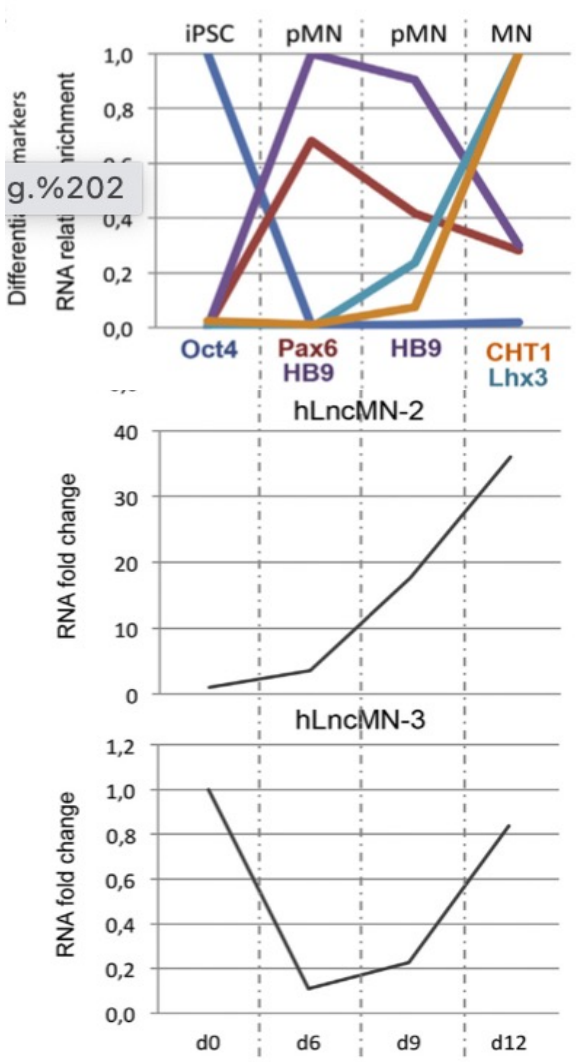
Codogeneity graph



Biscarini et al., 2018

Expression profile of selected lncRNAs during human MN differentiation from iPSCs.



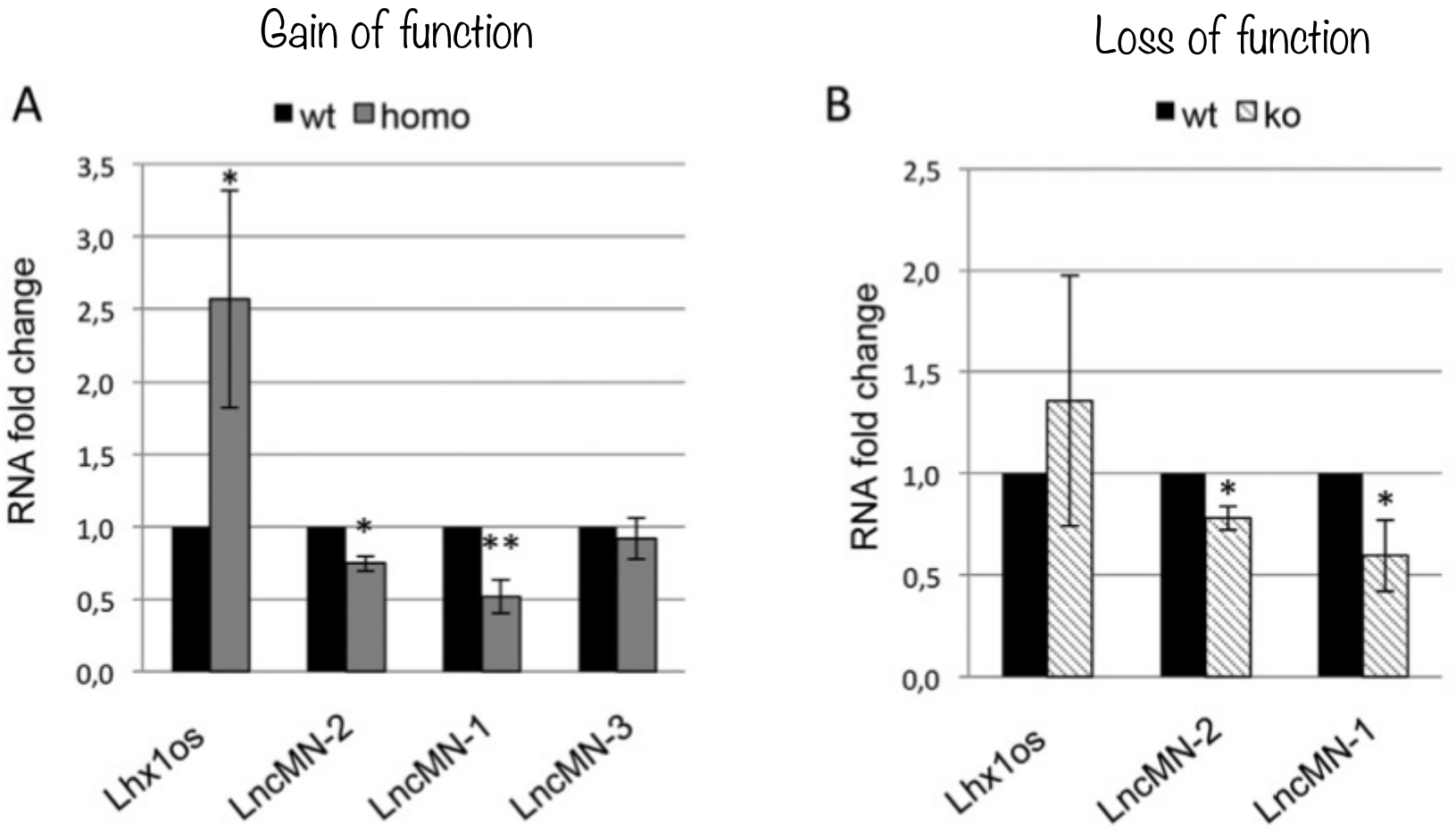


Biscarini et al., 2018

Als...

ALS is an incurable adult-onset neurodegenerative disease, which affects upper and lower motor neurons (MNs), and leads to paralysis and death in 3–5 years from diagnosis. Several genetic alterations are associated with ALS, including causative mutations in FUS, TDP-43 and expansions in C9ORF72 point to the essential role of aberrant RNA metabolism in ALS pathogenesis

LncRNA expression in FUS-ALS MNs



Fus mutant mouse MNs carrying the equivalent of one of the most severe human ALS-associated FUS alleles (P517L) MNs (homo, gray bars), relative to Fus+/+ MNs (wt, black bars). qRT-PCR analysis of specific lncRNAs in Fus−/− MNs (ko, striped bars), relative to Fus+/+ MNs (wt, black bars).

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