

ENCALS
18th-20th May 2017

Doris Lou DEMY

Implication of Nefl in the pathogenesis of ALS

Edor KABASHI lab

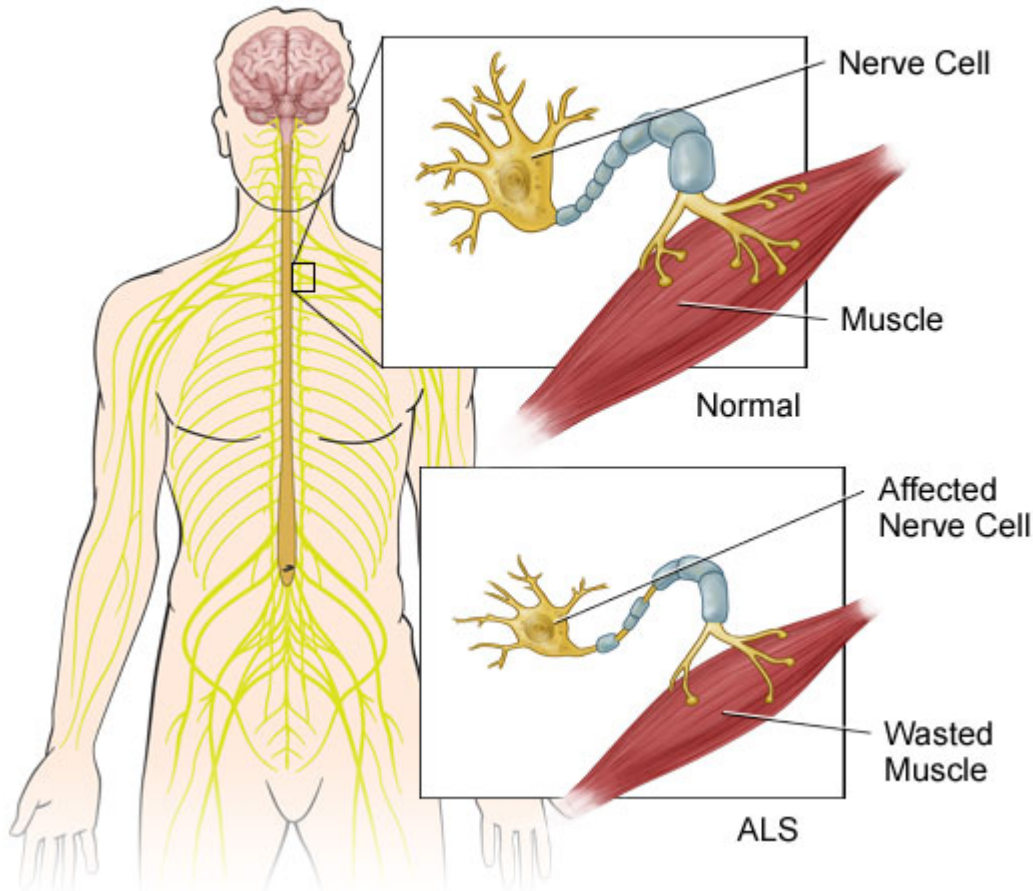
“Amyotrophic lateral sclerosis: from genetics to treatment”

Paris, France



ALS : state of the art

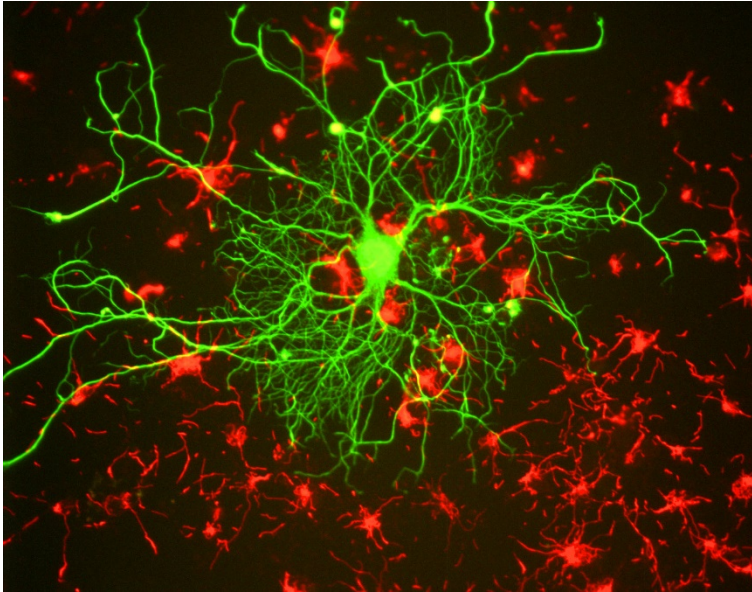
Amyotrophic Lateral Sclerosis (ALS)



- Neurodegenerative disorder
- Degeneration of motorneurons => muscle weakness/paralysis
- Changes in gene expression in both fALS and sALS
- Most genes identified : proteins with functions relevant to RNA and/or protein metabolism (transcription, splicing, transport, aggregation...)
- Aberrant expression of microRNAs in ALS

⇒ Evaluate the role of RNA dysregulation in ALS

NEFL : Neurofilament, light polypeptide



NF-L (EnCor Biothechnology Inc.)

- Most reliable biomarker for ALS diagnosis & disease severity (Gaiani et al., 2017)
- High neurofilament (NF) content in motorneurons (MN) for differentiation, perikaria, dendrites and axons bulk and physical strength ; ALS vulnerability
- Neurofilaments disrupted (organization, subunit expression, RNA regulation) in multiple forms of ALS ; neurofilamentous inclusions
- NF assembly requires NEFL
- TDP43 interacts with the 3'UTR of Nefl mRNA (Strong et al., 2007)

⇒ Nefl, a disease relevant target

Developing zebrafish models

⇒ Inhibition :

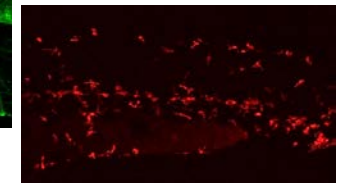
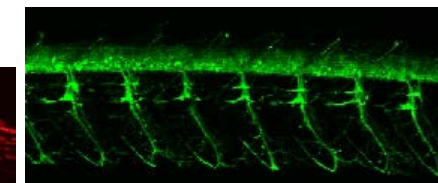
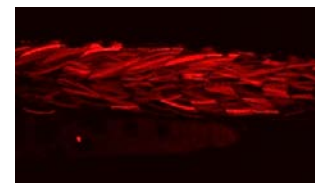
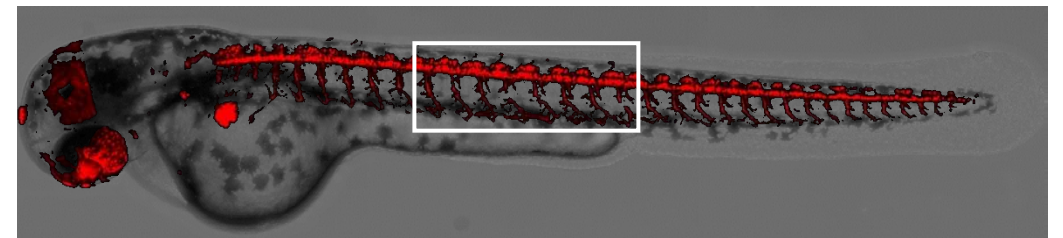
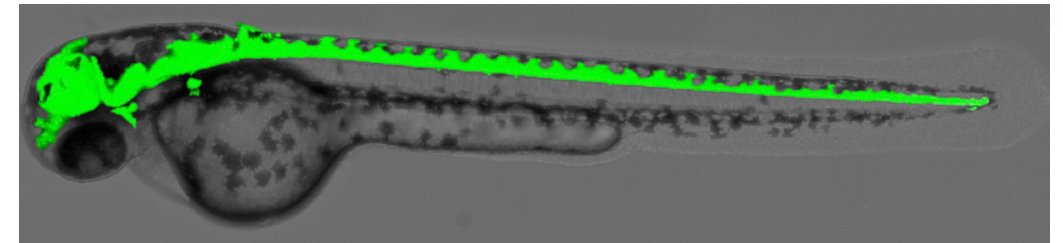
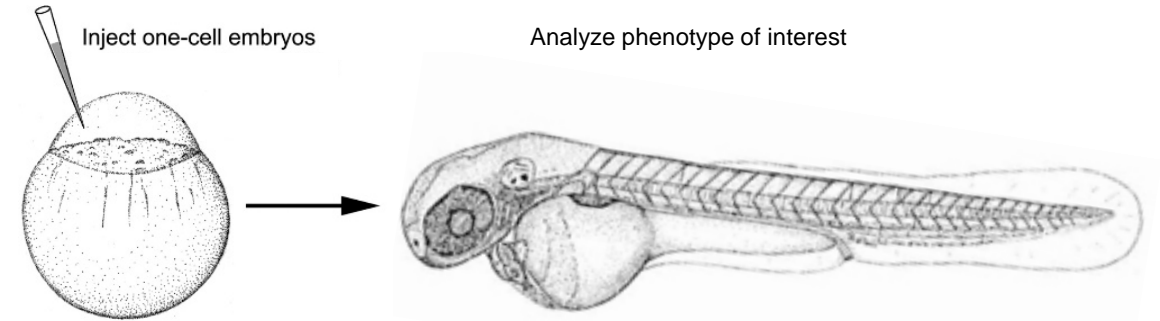
- ⇒ Antisense morpholinos
- ⇒ Specific inhibitors
- ⇒ Mutant lines (chemical mutagenesis, CRISPR/Cas9)

⇒ Overexpression :

- Injection at 1-cell stage - mRNA
- miR/pre-miR
- construct (promoter, reporter)

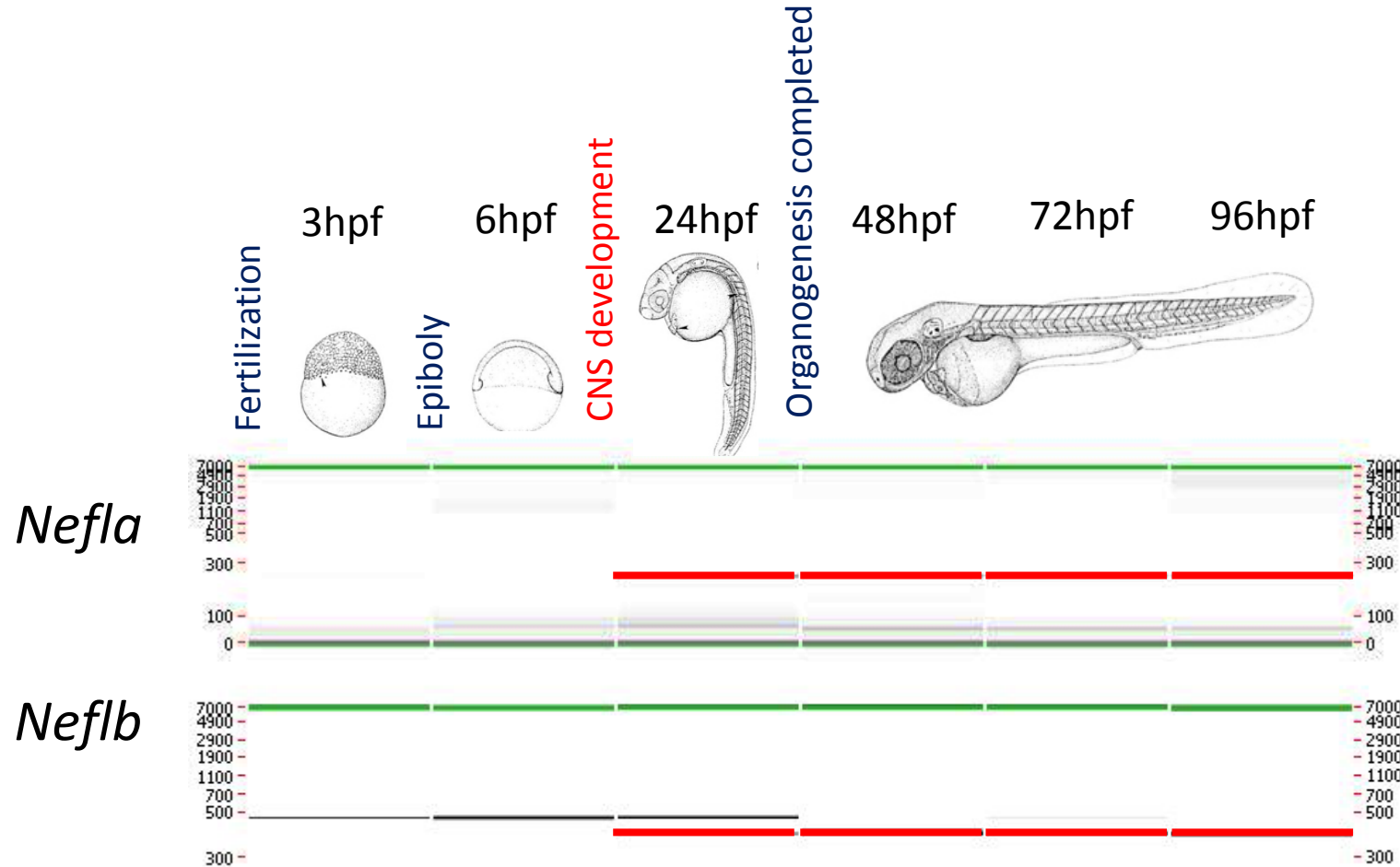
⇒ Assess phenotype :

- ⇒ Behavior
- ⇒ Fluorescent constructs ; genetic tools
(transient expression and/or stable transgenic lines)
- ⇒ *In situ* hybridization / immunostaining
- ⇒ qPCR, WB



Nefl in zebrafish : identification

⇒ Two previously uncharacterized *Nefl* isoforms identified in zebrafish



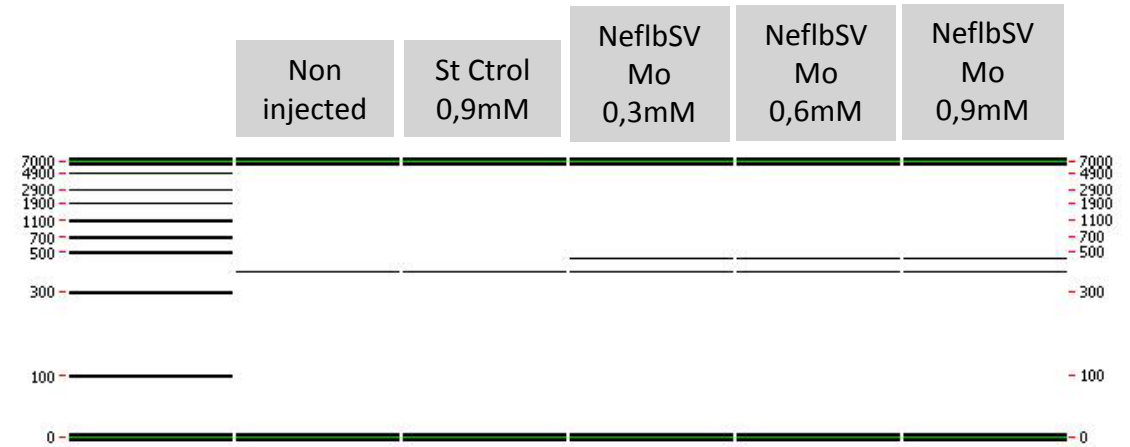
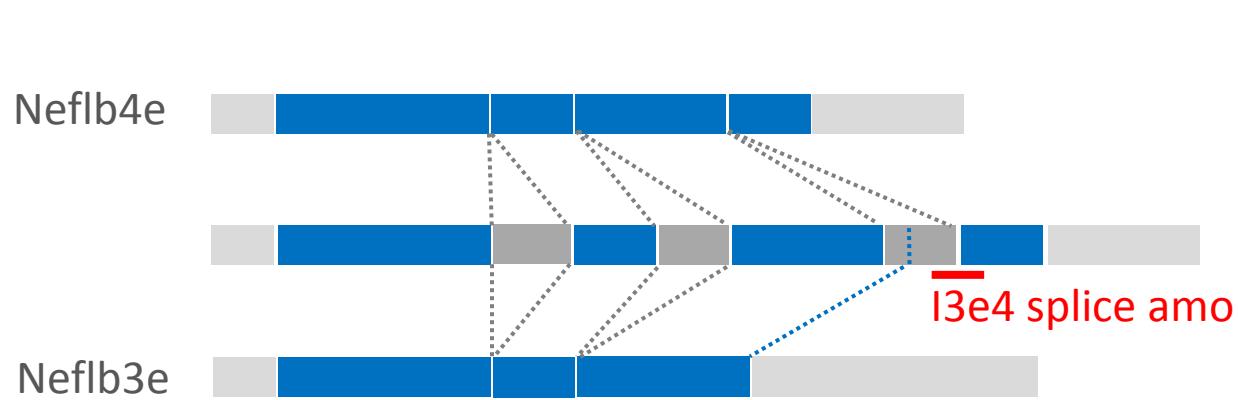
⇒ Stages of expression

⇒ Identification of splice variants :
Neflb3E and Neflb4E

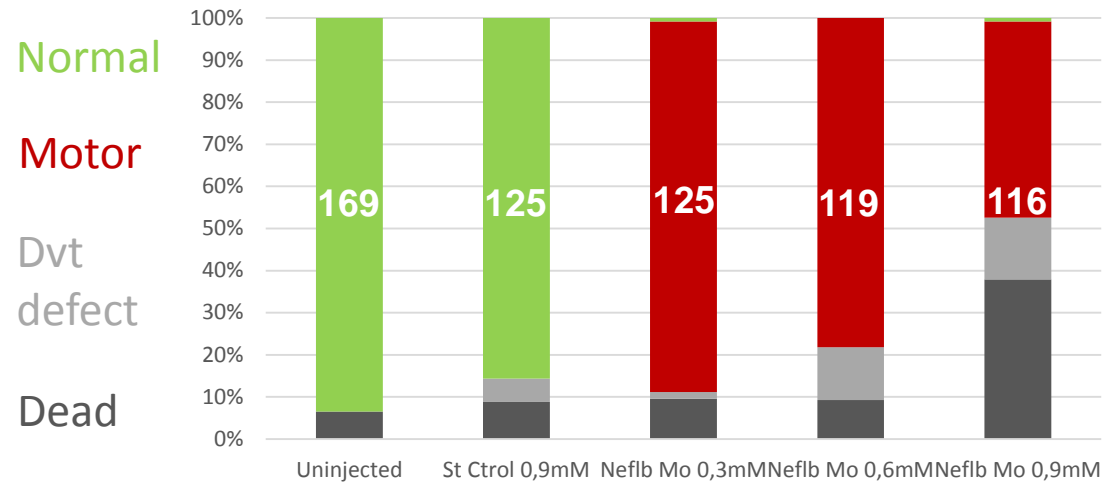
⇒ Consistent with an expression in the
nervous system

Nefl in zebrafish : role

⇒ Knock down : antisense oligo-morpholino



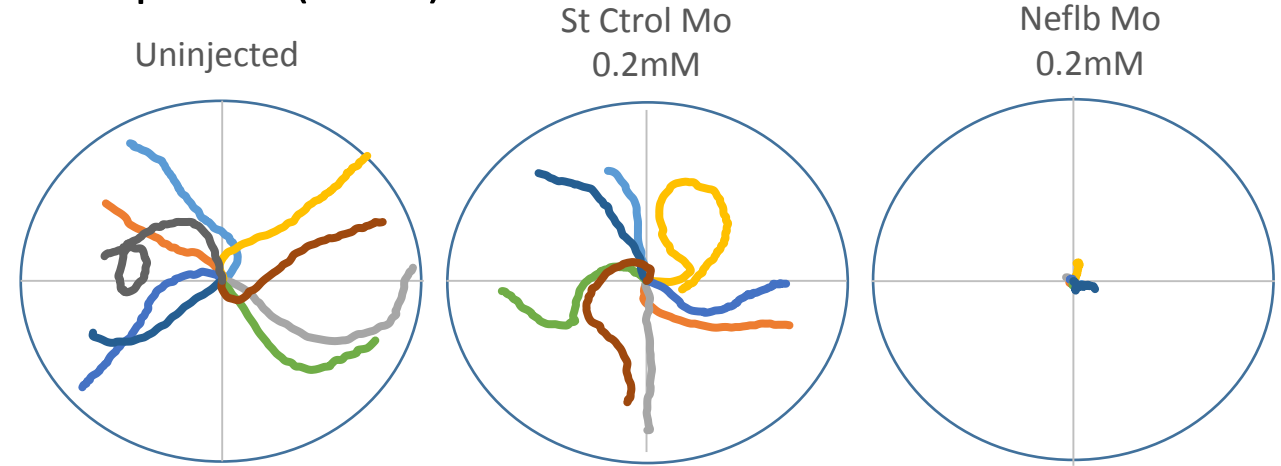
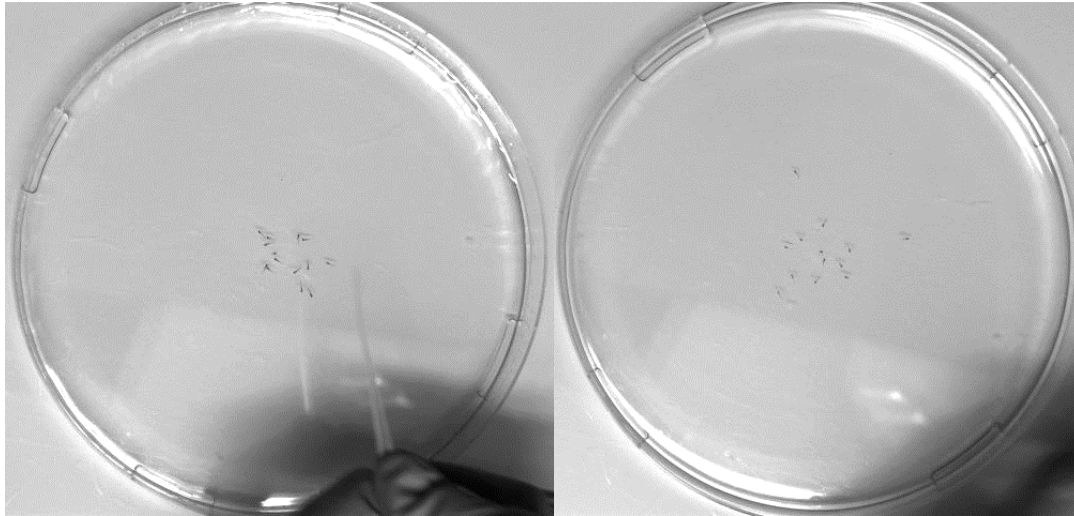
Global morphology 48hpf



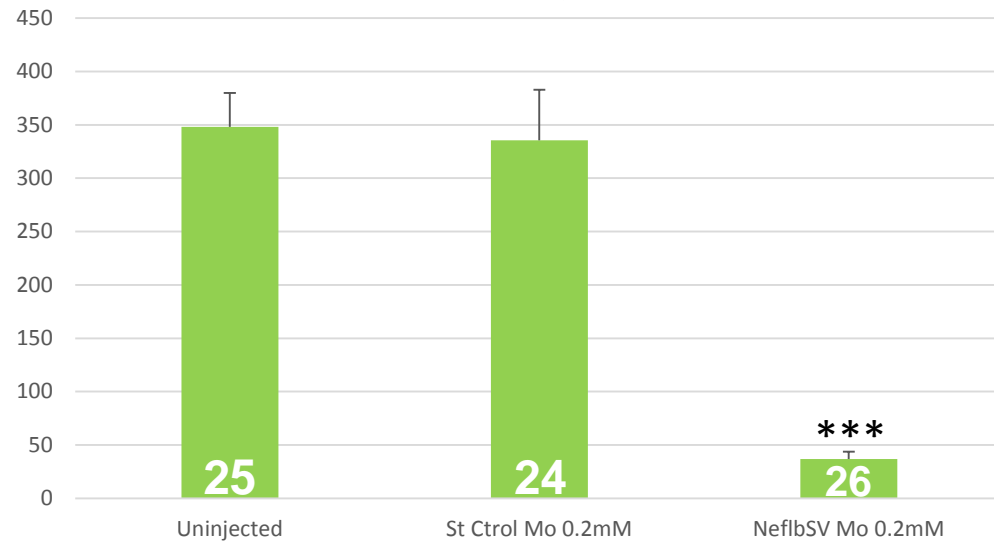
- ⇒ Splice antisense oligo-morpholino
- ⇒ Disruption of the Neflb3E/Neflb4E balance
- ⇒ Motor phenotype

Nefl in zebrafish : role

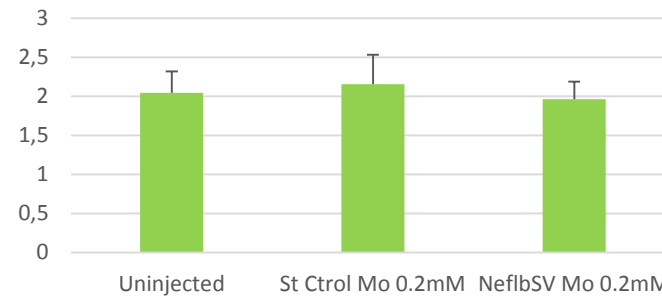
⇒ Assess motor phenotype : Touch evoked escape response (TEER)



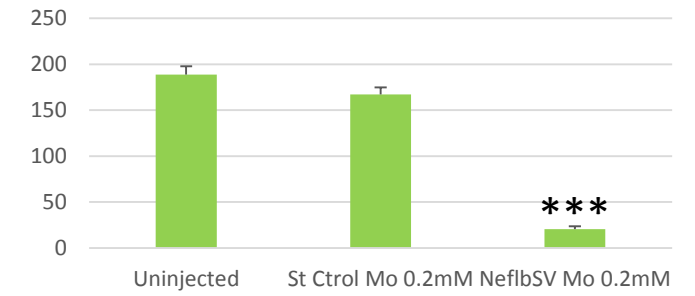
Track length



Duration



Speed

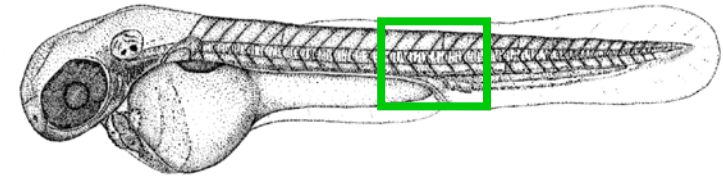


⇒ Response to the stimulus

⇒ Similar response duration

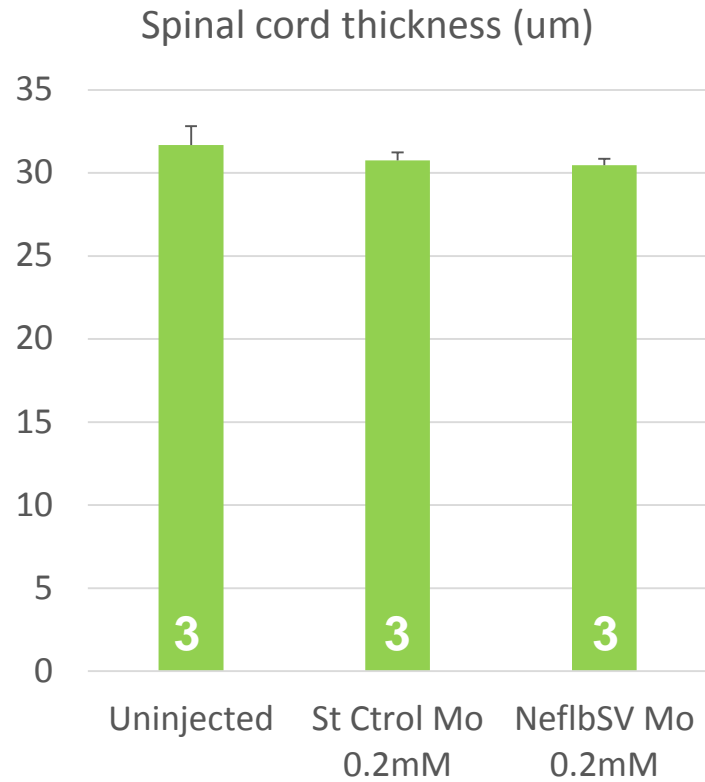
⇒ Specific motor phenotype

Nefl in zebrafish : role

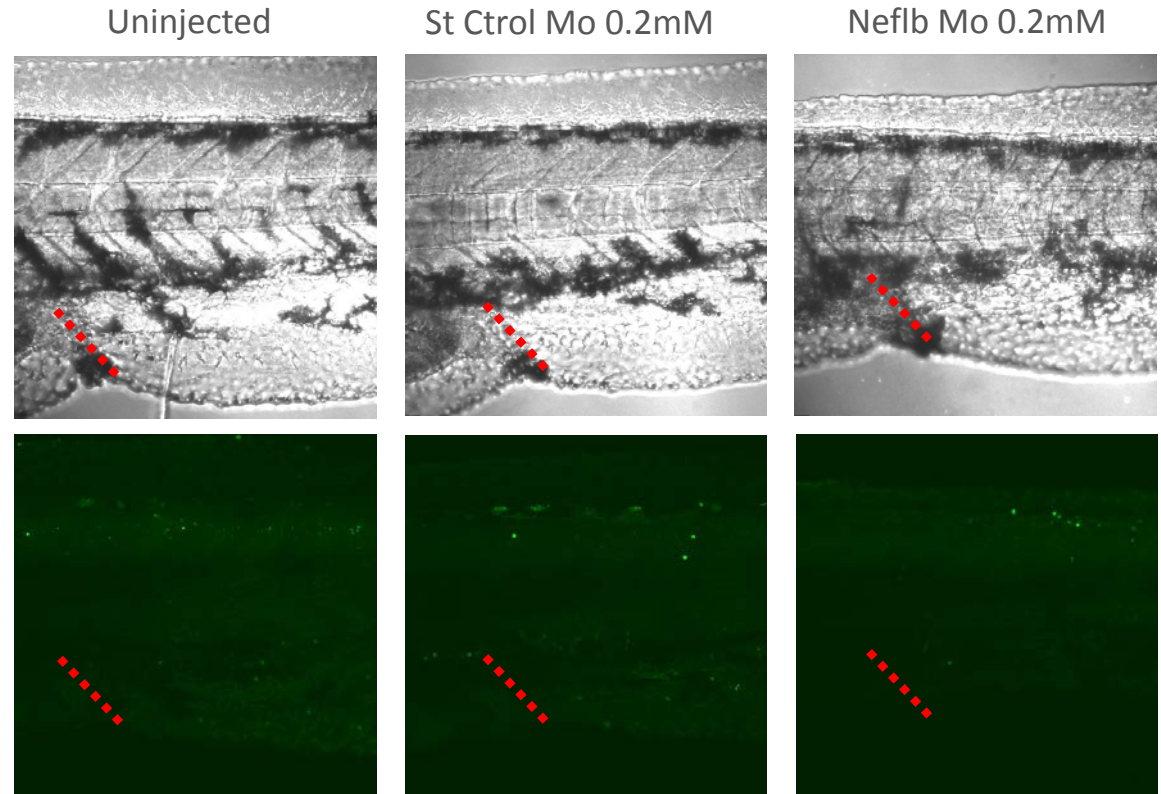


⇒ Assess neuronal phenotype : CNS development

In vivo **Hb9:GFP (motorneurons)**



In vivo **Acridine Orange (cell death)**

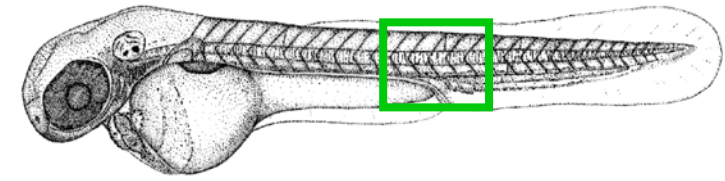


⇒ No toxicity

⇒ No developmental abnormalities

⇒ No neuronal death

Nefl in zebrafish : role



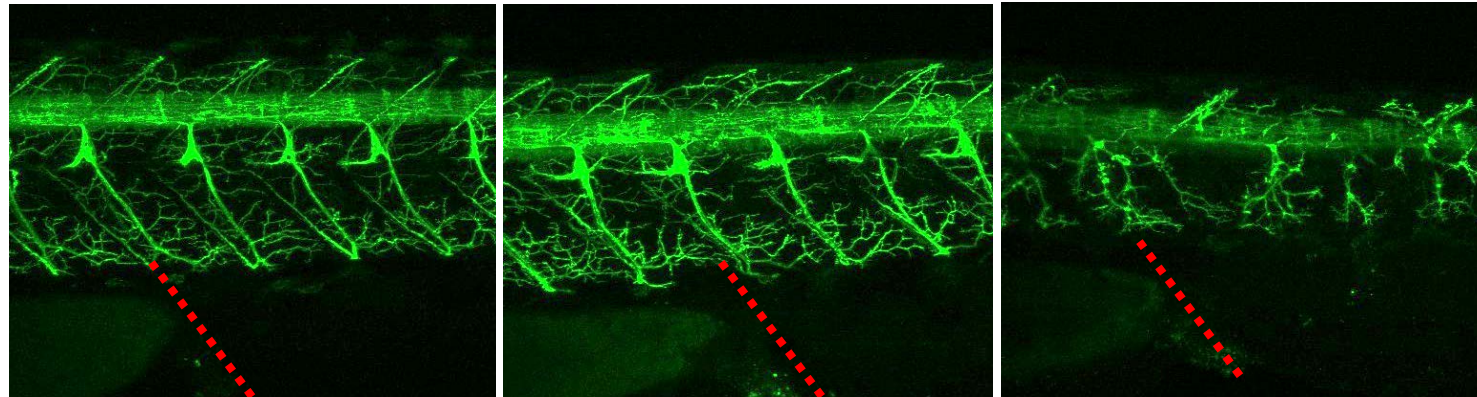
⇒ Assess neuronal phenotype : motor neurons development

In vivo Hb9:GFP (motorneurons)

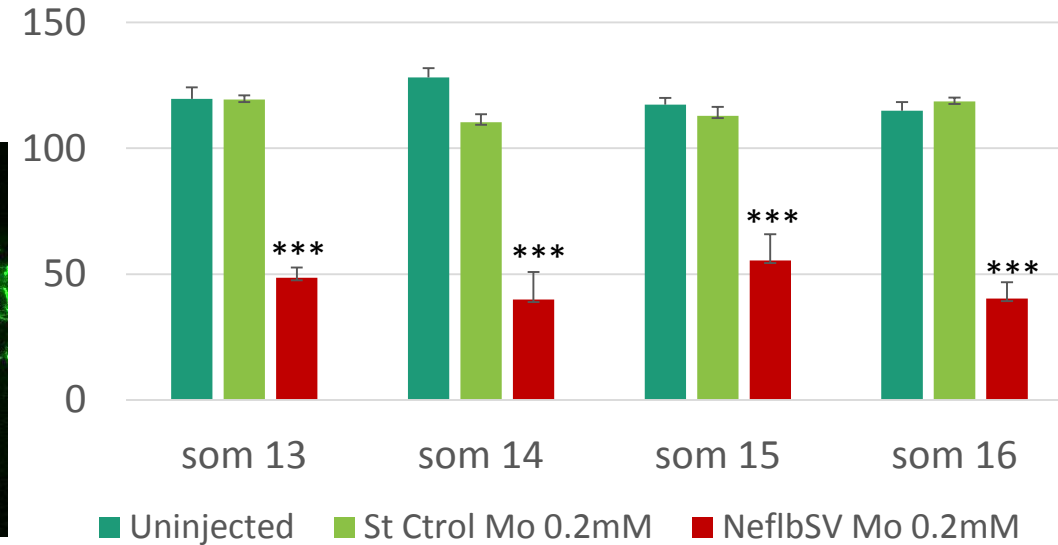
Uninjected

St Ctrl Mo
0.2mM

Neflb Mo
0.2mM



Axonal length (um)

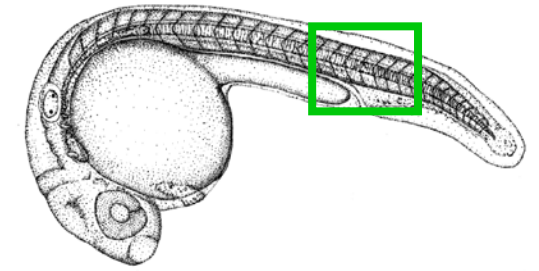


⇒ Irregular, shorter

⇒ Branching abnormalities

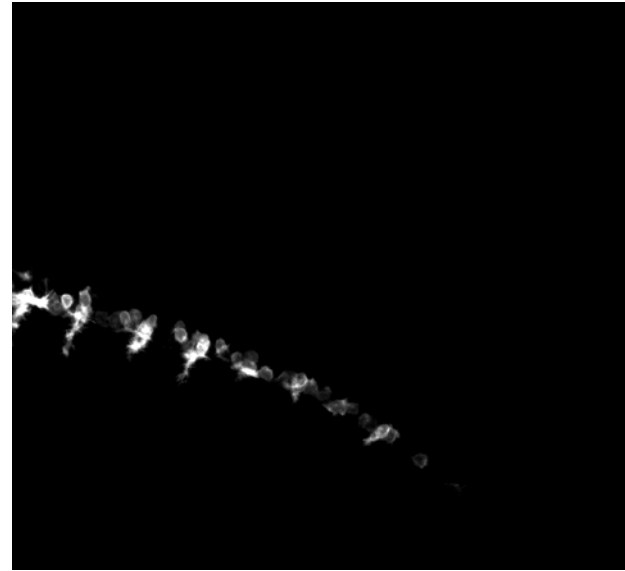
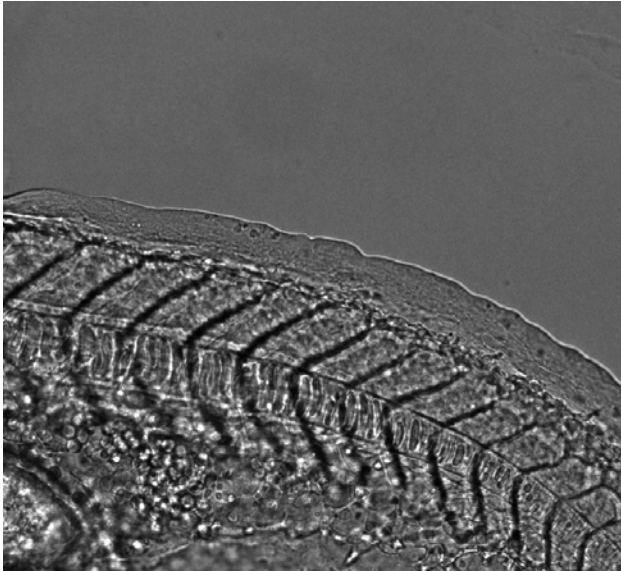
⇒ Motor axons are strongly affected in NeflbSV morphants

Nefl in zebrafish : role

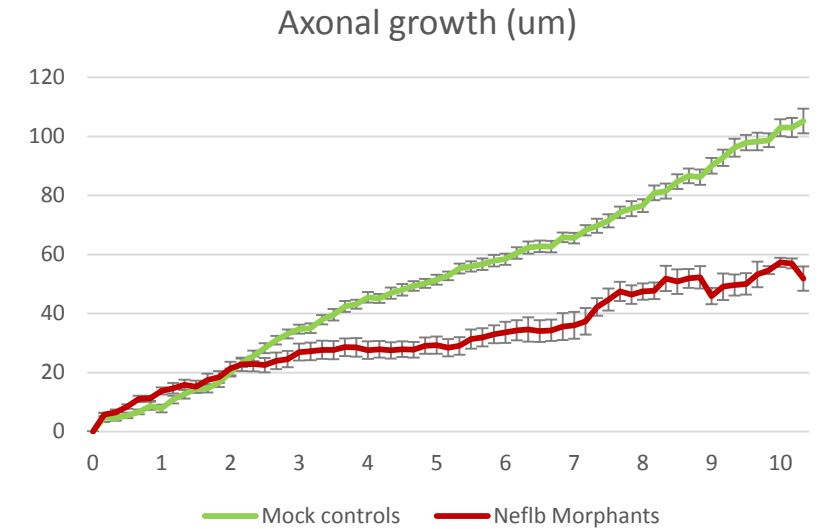
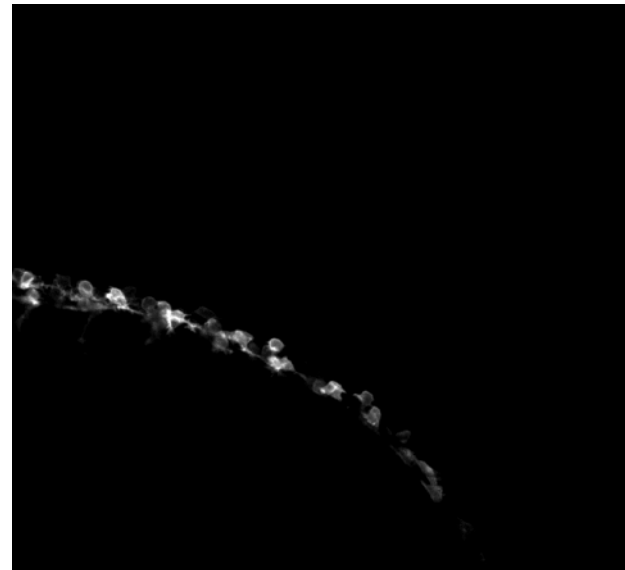
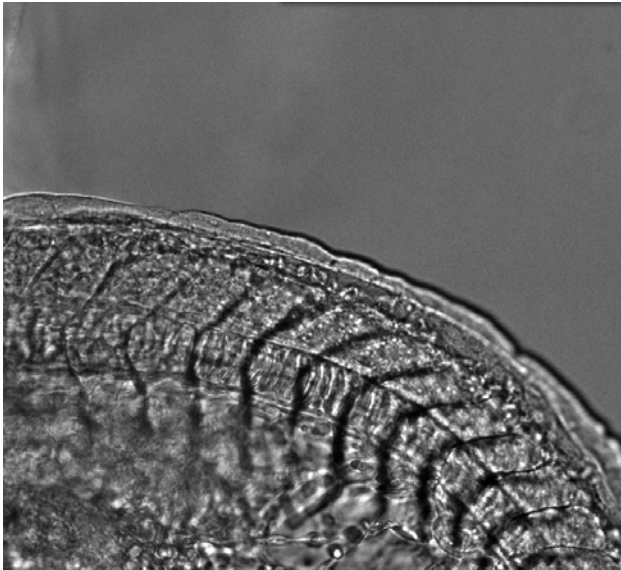


In vivo Hb9:GFP (motorneurons) 24 => 31hpf

St Ctrl Mo
0.2mM



Neflb Mo
0.2mM

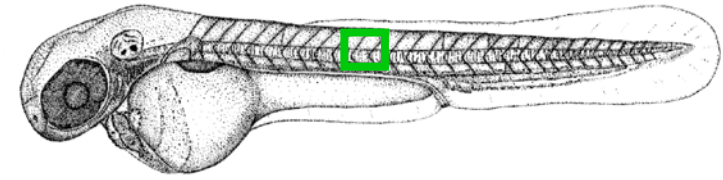


⇒ Signaling ok

⇒ Mechanical defect :
(de)polymerization? Stability?

⇒ Motor axons are strongly affected
in NeflbSV morphants

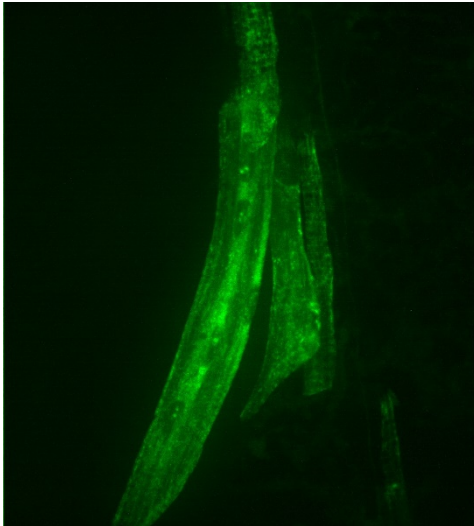
Nefl in zebrafish : role



⇒ Assess molecular phenotype :

tag Neflb4E (WT?) and Neflb3E (pathologic?) with eGFP

pCS2-SP6:EGFP-
Neflb4E (WT)

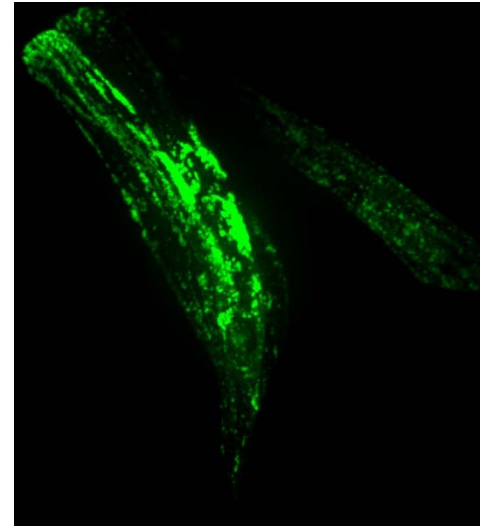


(muscle fibers)

⇒ Homogenous repartition

⇒ Normal assembly?
Polymerization?

pCS2-SP6:EGFP-
Neflb3E (pathologic?)



(muscle fibers)

⇒ Heterogenous repartition

⇒ Abnormal assembly?
Aggregates?

Nefl in zebrafish : role

⇒ Nefl exists in Zebrafish

⇒ Two paralogues Nefla and Neflb (2 splice variants)

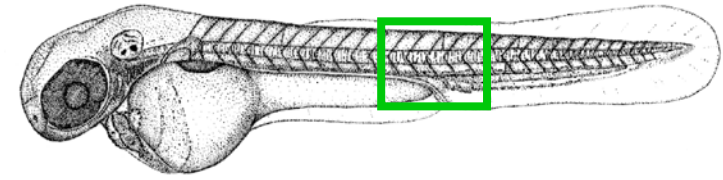
⇒ Expression correlates with the nervous system formation

⇒ Disrupting the Neflb3E/Neflb4E balance results in a specific motor phenotype without CNS damage

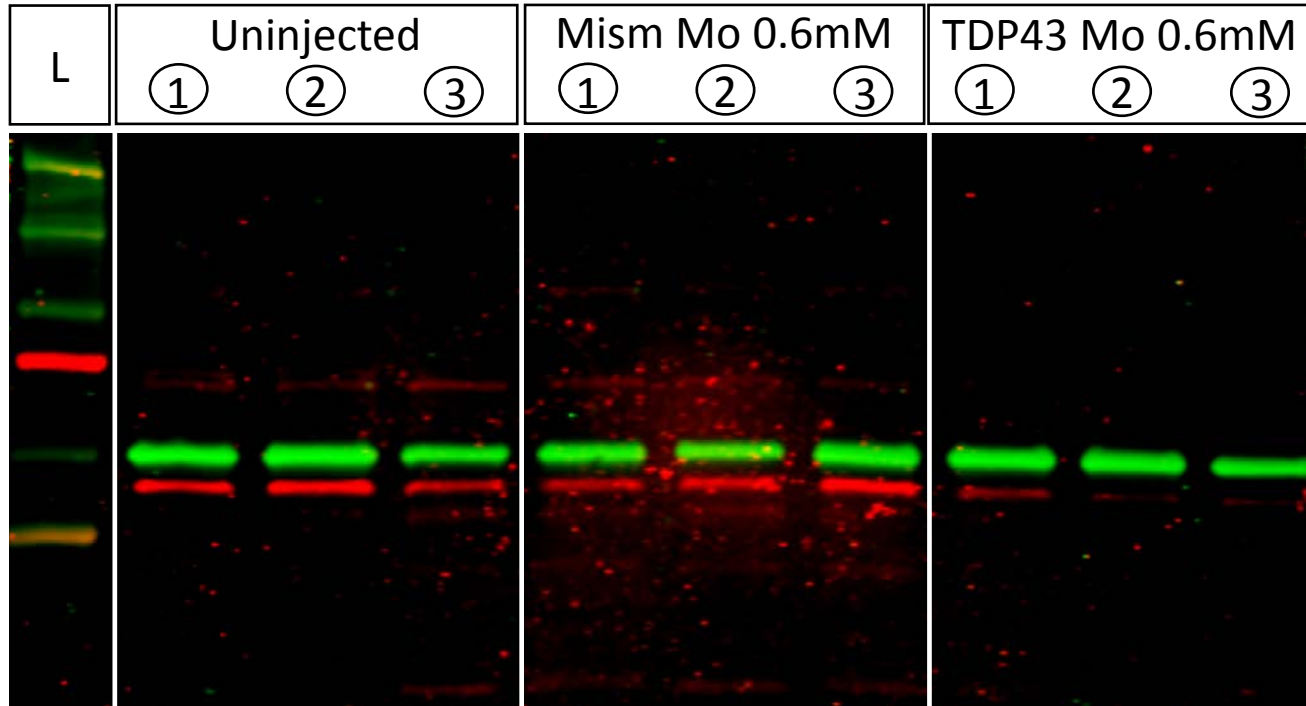
⇒ Motor neurons are strongly affected (shape, length, dynamics) in Neflb morphants

⇒ Neflb3E splice variants encodes for a protein that aggregates

Nefl in zebrafish : role



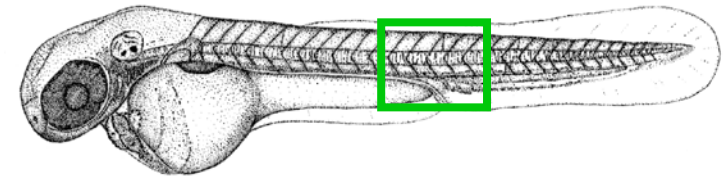
⇒ Characterize Nefl in zebrafish ALS models



Tub Ab
TDP43 Ab

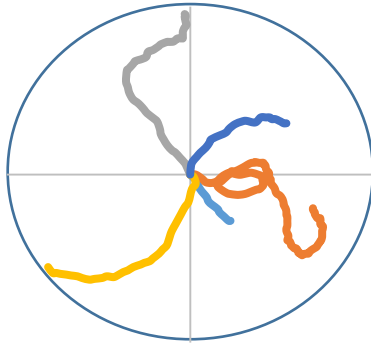
- ⇒ KD of known ALS- associated genes
- ⇒ Characterize phenotype
- ⇒ Implication of Nefl : quantification? Rescue?

Nefl in zebrafish : role

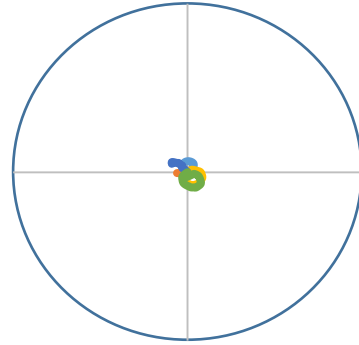


⇒ Characterize Nefl in zebrafish ALS models

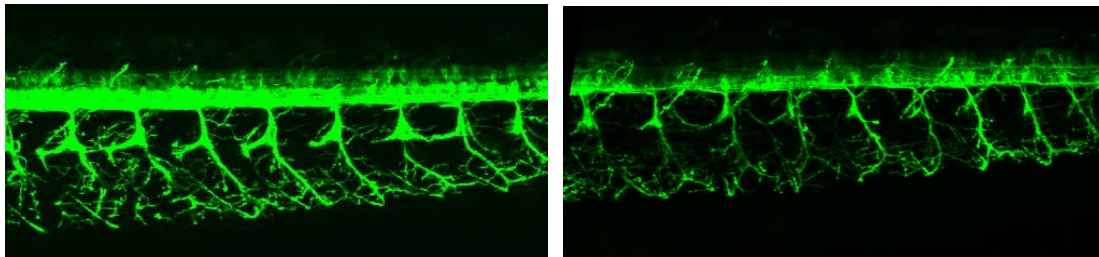
St Ctrol Mo
0.6mM



TDP43 Mo
0.6mM

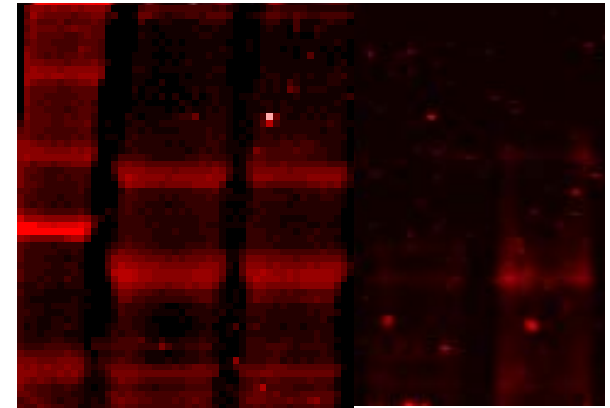


In vivo Hb9:GFP (motorneurons)

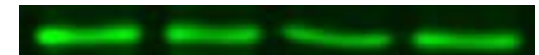


Uninjected
Mock
TDP43 Mo
Nef1b SV Mo

Nefl 68 Ab



GAPDH Ab



- ⇒ KD of known ALS- associated genes
- ⇒ Characterize phenotype
- ⇒ Implication of Nefl : quantification? Rescue?

Conclusion/Perspectives

⇒Nefl characterization

- ⇒ Stable mutant lines (Sanger, CRISPR/Cas9)
- ⇒ Visualize Nefl (RNA base-scope, dCas9)
- ⇒ Pursue NF assembly phenotype (SW13 cells)

⇒Nefl implication in ALS models

- ⇒ Characterize Nefl defects in ALS models
- ⇒ Rescue with Neflb3E? With Neflb4E?

⇒Nefl implication in ALS

- ⇒ Detect and quantify isoforms in patient tissues/CSF
- ⇒ Link to identified actors

Thank you and...

Kabashi lab

Letizia Campanari

Raphael Munoz-Ruiz (Session 7)

Hortense de Calbiac (Session 7)

Annis Bourefis (Poster n°104)

Nicolas Bayle

Corinne Besnard-Guerin

Sorana Ciura

Anca Marian

Durham lab

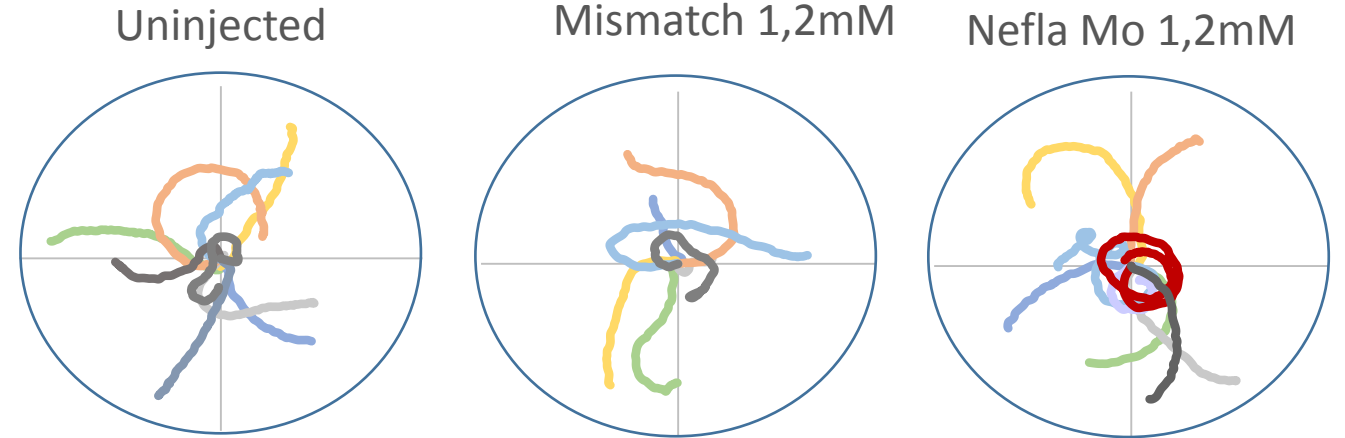
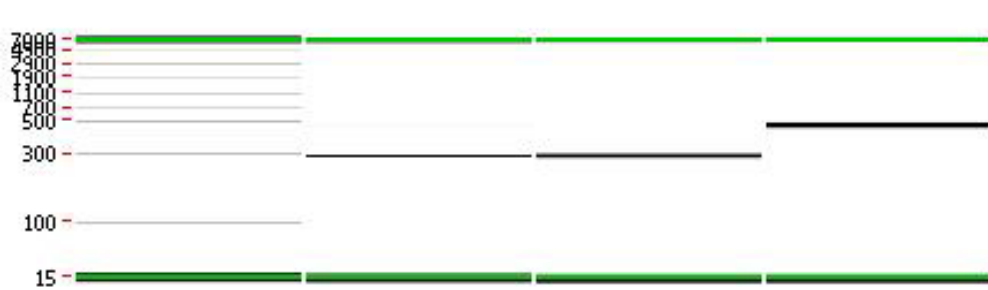
Horstein lab

Strong lab

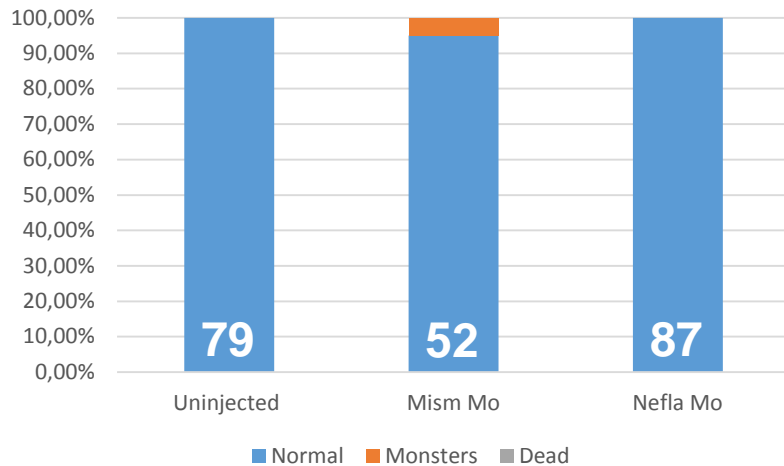


Nefla PCR Primers Splice AMO (3)

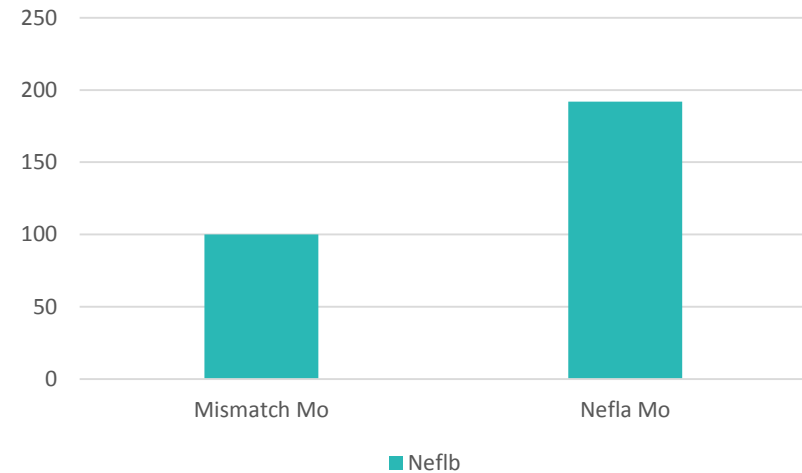
Ladder	Non injected	Nefla Mo 0,3mM 1nL	Nefla Mo 0,6mM 1nL
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Morphology Nefla KD



Neflb qPCR

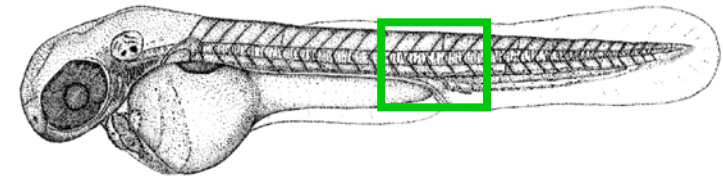


⇒ Compensation by Neflb?



Kabashi lab

Assess phenotype

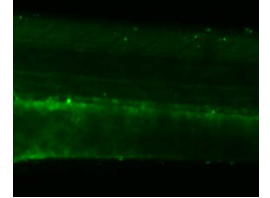
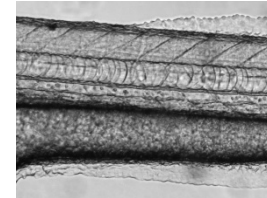
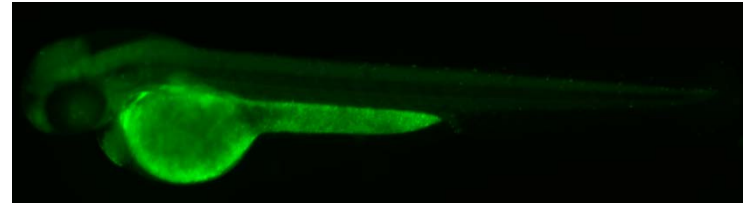


In vivo Acridine Orange staining @48hpf

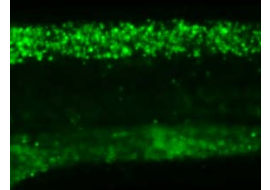
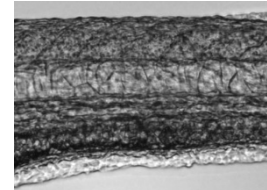
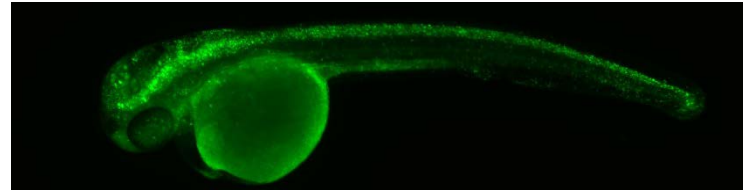
Non injected	Neflb Mo 1,2mM 3nL
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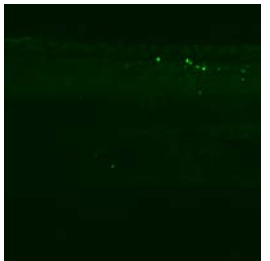
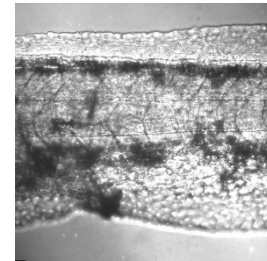
Control



Neflb AUG Mo

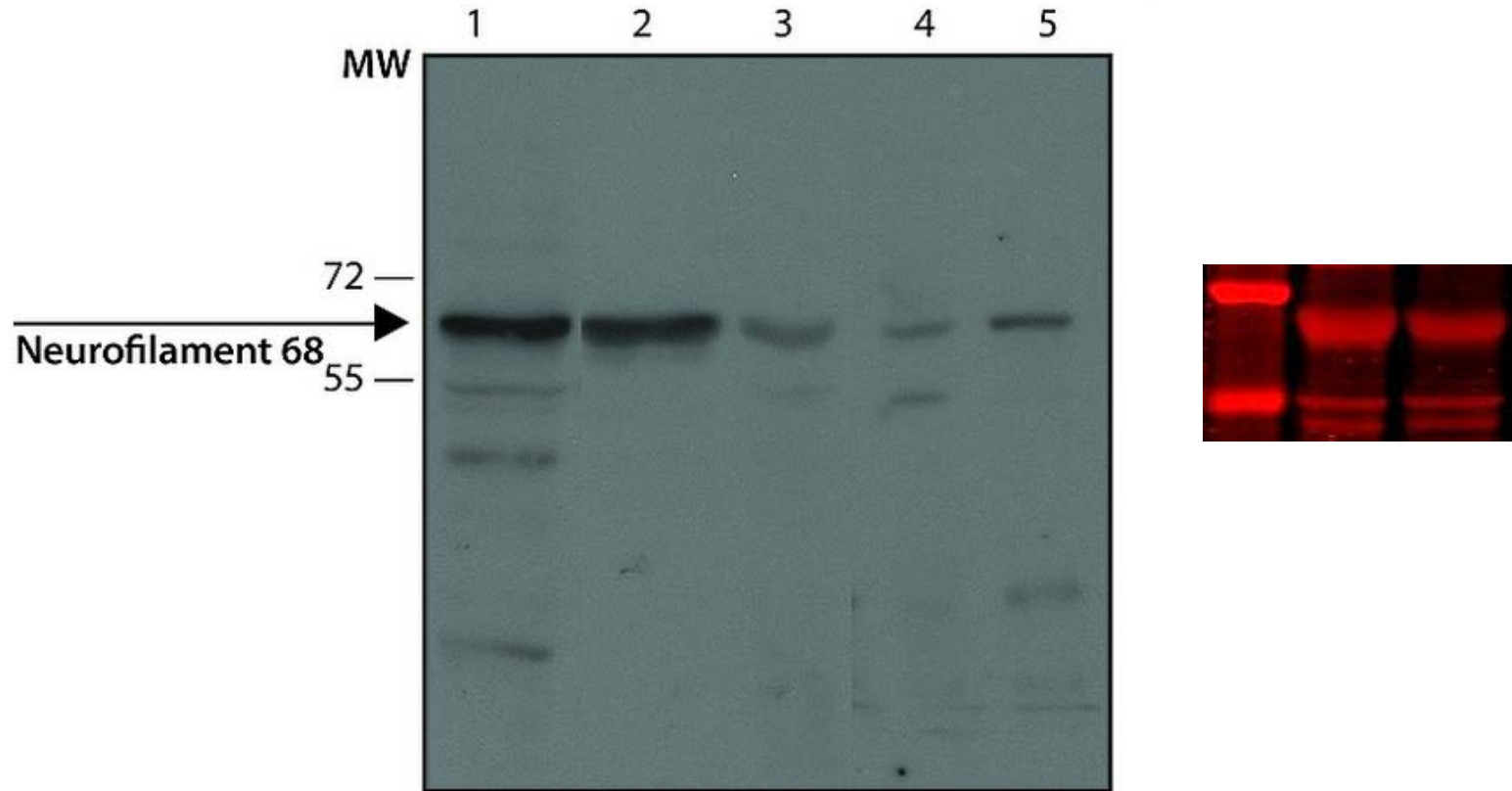


Neflb splice Mo



⇒ CNS damage, not MN specific

Anticorps



Immunoblotting

Cell line lysates were separated on SDS-PAGE and probed with 1:400 Monoclonal Anti-Neurofilament 68, Clone: NR4 (**Cat. No. N5139**). The antibody was developed using Goat Anti-Mouse IgG-Peroxidase (**Cat. No. A2304**) and a chemiluminescent substrate.

Lanes

1. PC3
2. SHSY-5Y
3. HeLa
4. MDBK
5. MDCK

Protein structure



Helix II

```

7 277 W Y R S K C E E I K A T V I R H G E T L R R T K E E I N E L N R V I Q R R T A E V E
8 W Y I R Q T E E L N K Q V V S S E Q L O C N Q E E I I E L R R T V N A L Q V E L O A Q H N L R D S L
E 242 W F F T K T E E L N R E V A T N S E L V Q S G K S E I S E L R R T M Q N L E I E L O S Q L S M K A S L
D 286 W Y K S K V S D L T Q A A N K N N D A L R Q A K Q E M L E Y R H O I Q S Y T C E I D A L K G T N D S L
V W Y K S K F A D L S E A A N R N N D A L R Q A K Q E S N E Y R R Q V Q S L T C E V D A L K G T N E S L
NF W F K S R F T V L T E S A A K N T D A V R A A K D E V S E S R R L L K A K T L E I E A C X G M N E A L

```

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7 (K6, R2, B9, T4, S9, Z15, P3, G11, A11, V9, L12, Y1) K A K Q N M A C L L K E Y Q E V M N S K L
8 E N T L T E T E A R Y S C O L N Q V Q S L I S N V E S Q L A E I R G D L E R Q N Q E Y Q V L L D V R A
E 293 E N S L E E T K G R Y C M Q L A Q I Q E M I G S V E E Q L A Q L R C E M E Q Q N Q E Y K I L L D V K T
D 337 M R Q M R E M E E R F A G E A G G Y Q D T I A R L E E E I R H L K D E M A R H L R E Y Q D L L N V K M
V E R Q M R E M E E N F A V E A A N Y Q D T I G R L O D E I Q N M K E E M A R H L R E Y Q D L L N V K M
NF E K Q L O E L E D K Q N A D I S A M Q D T I N K L E N E L R T T K S E M A R Y L K E Y Q D L L N V K M

```

Tail

```

7 G L D I E I A T Y R R L L E G E E Q R L C Q G V - G A V - N V (V, S, S, S) - R
8 R L E C E I N T Y R G L L D S E D C K L A C G K P L T P C I S S P - - - C A
E 344 R L E Q E I A T Y R R L L E G E D A H L S S S - - Q - F - S S G S Q S S R D V T S S S R Q I - - - R
D 388 A L D V E I A T Y R K L L E G E E E N R I S I P M H Q T F A S A L N F R E T - S P D Q R G S E V H T K K
V A L D I E I A T Y R K L L E G E E S R I S L P L - P N F - S S L N L R E T N L E S L P L V D T H S K R
NF A L D I E I A A Y R K L L E G E E T R L S F T S V G S L T T G Y S Q S S Q V F G R S A Y G G L Q T S S

```

← domain →

```

7 (R1, C9, B3, T4, S8, Z1, P3, G13, A3, V7, L5) R C
8 P A A P C T T - - - - C V V P S S C G (R, R, Y)
E 387 T K V M D V H - - - D G K V V S T H E Q V L R T K N 409
D 438 T V M I K T I E T R D G E V V S E A T Q Q Q H E V L 463
V T L L I K T V E T R D G Q V I N E T S Q H H N D L E
NF Y L M S T R S F P S Y Y T S H V Q E E Q I E V E E T I E A A K A E E A K D E P P S E G E A E E E G K E

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← domain →

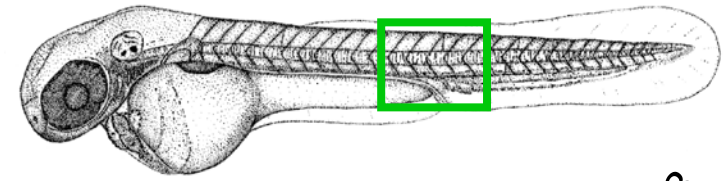
```

NF K E E A E A E A E E E G A Q E E E E A A E K E E S E E A K E E E G G E G E Q G E E T K E A E E E E
NF K K D E G A G E E Q A T K K K D

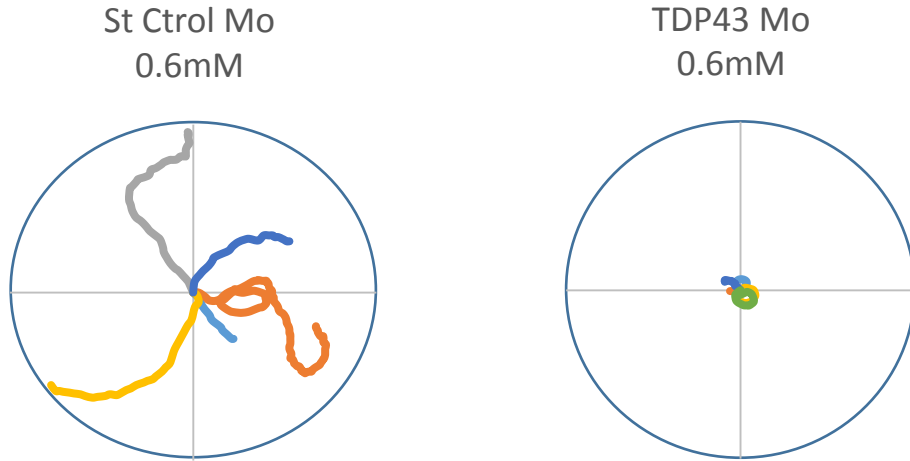
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- ⇒ Polymerization
- ⇒ Interaction with neuronal components
- ⇒ Axonal length, strenght?
- ⇒ Proteolytic resistance, stability
- ⇒ Protein degradation, autophagy?

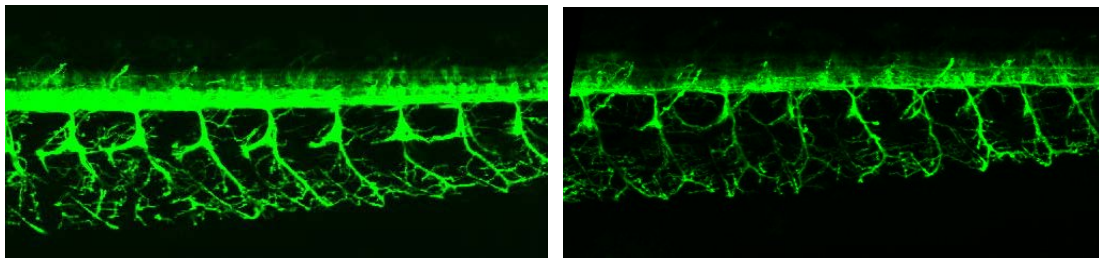
Nefl in zebrafish : role



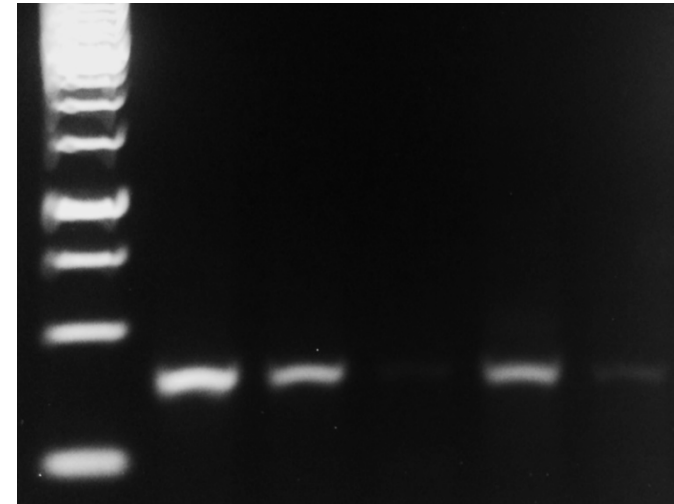
⇒ Characterize Nefl in zebrafish ALS models



In vivo Hb9:GFP (motorneurons)

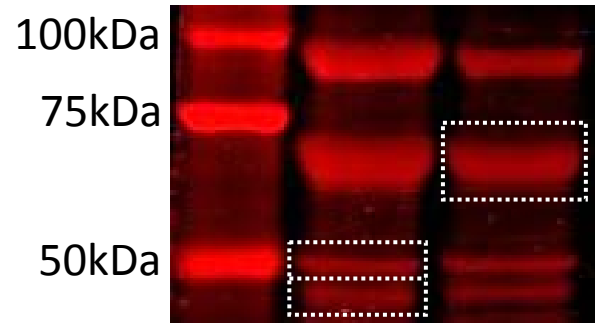


Mouse brain
Emb mouse brain
Human cells + eGFP
Human cells + wt TDP43
Human cells + mut TDP43

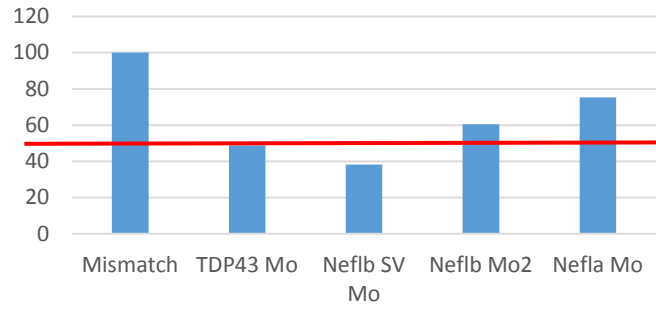


- ⇒ KD of known ALS- associated genes
- ⇒ Characterize phenotype
- ⇒ Implication of Nefl : quantification? Rescue?

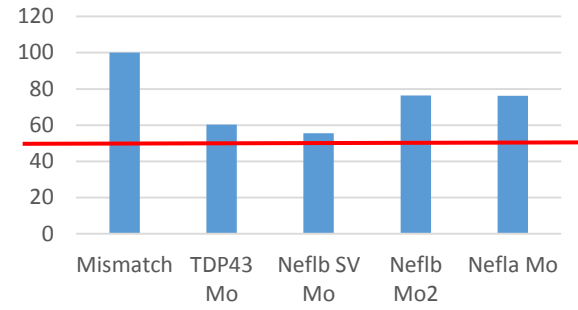
Western blot quantification of Nefl bands :



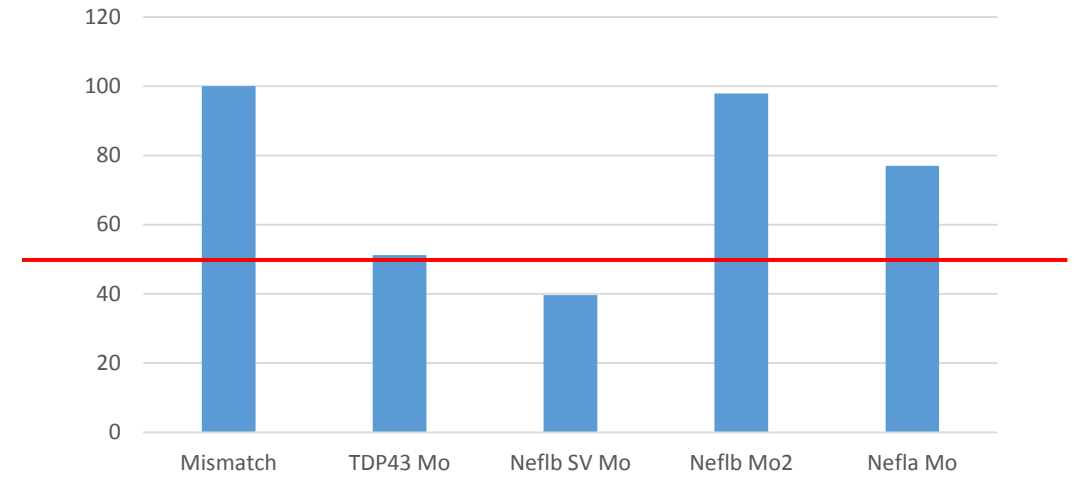
Nefl 50 kDa high



Nefl 50 kDa Low



Nefl 70 kDa



Ratio

