

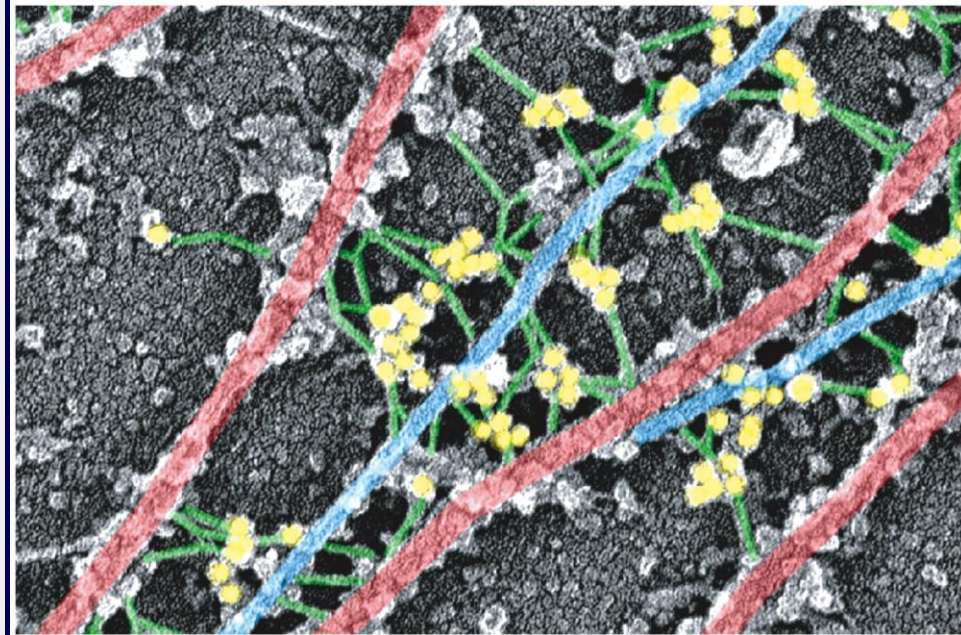
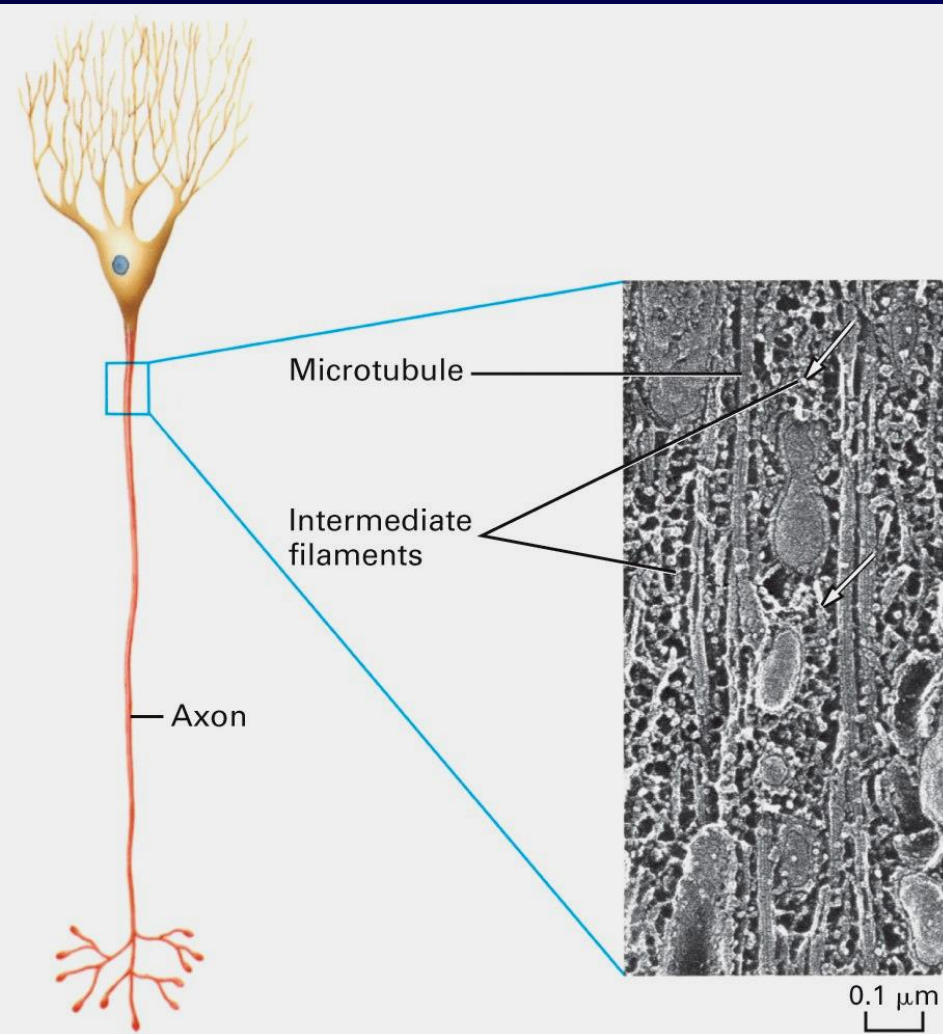
Animal and Cellular Models for the Neuronal Degeneration:

Misaccumulation of Neuronal Intermediate Filaments

台大醫學院
解剖學暨細胞生物學研究所
錢宗良

Neuronal Cytoskeletons

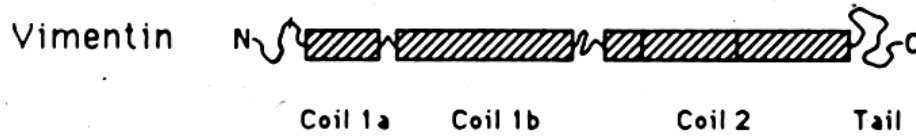
Microtubule



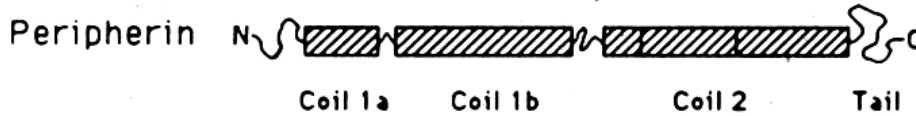
Intermediate filament:
Neurofilaments

Plakin family:
cytoskeleton linker proteins

Seven Intermediate Filament Proteins in Neural Differentiation



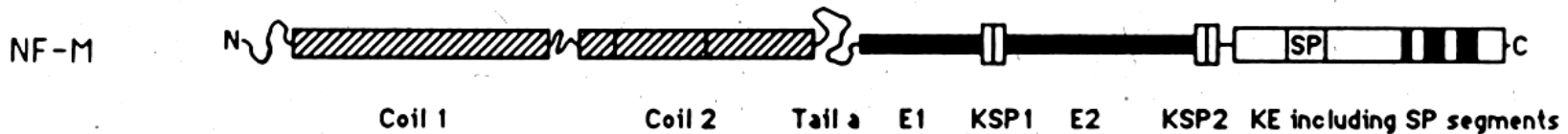
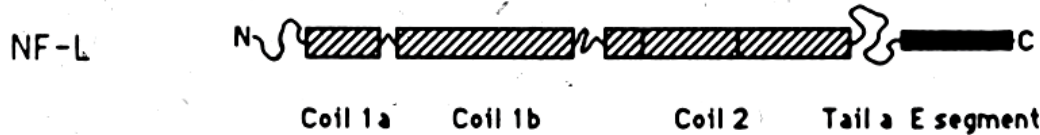
Neuroepithelial stem cells



First neuronal IF in PNS



First neuronal IF in CNS



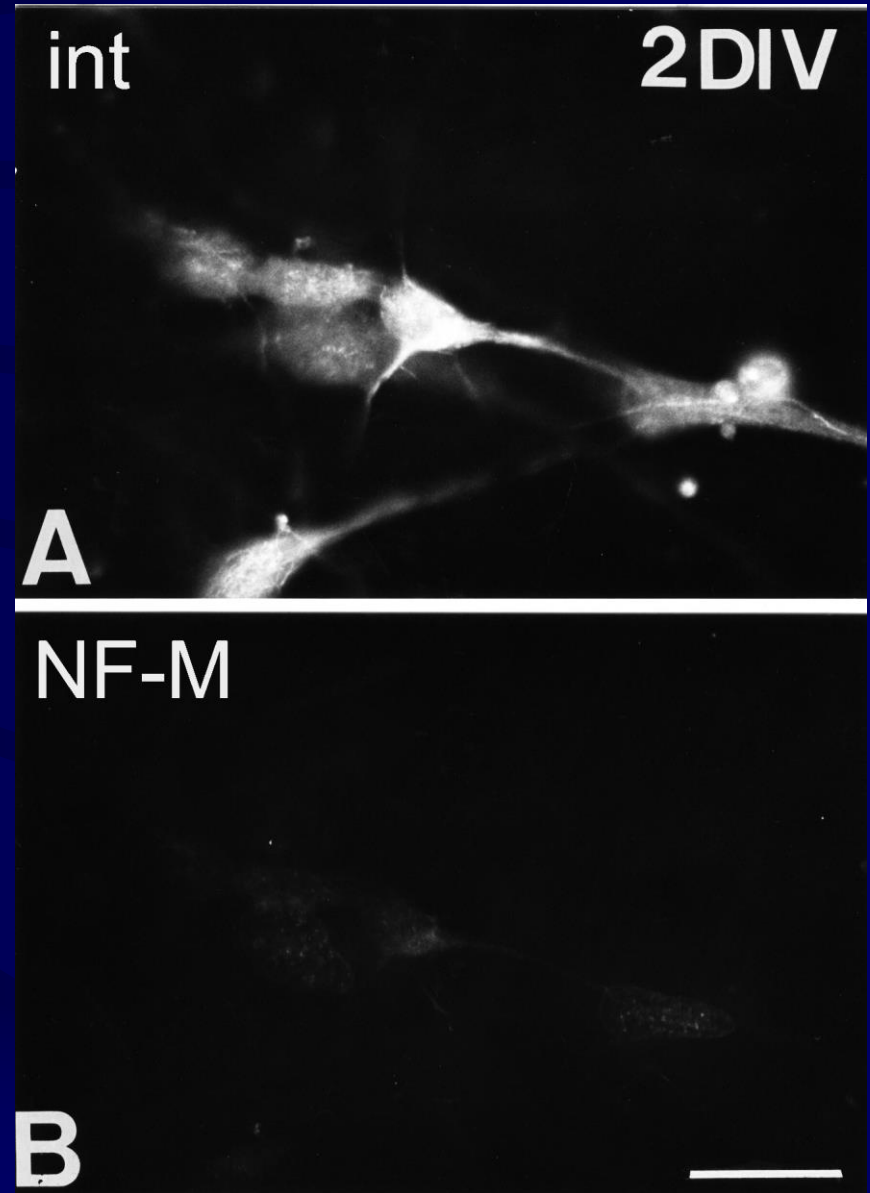
Latest



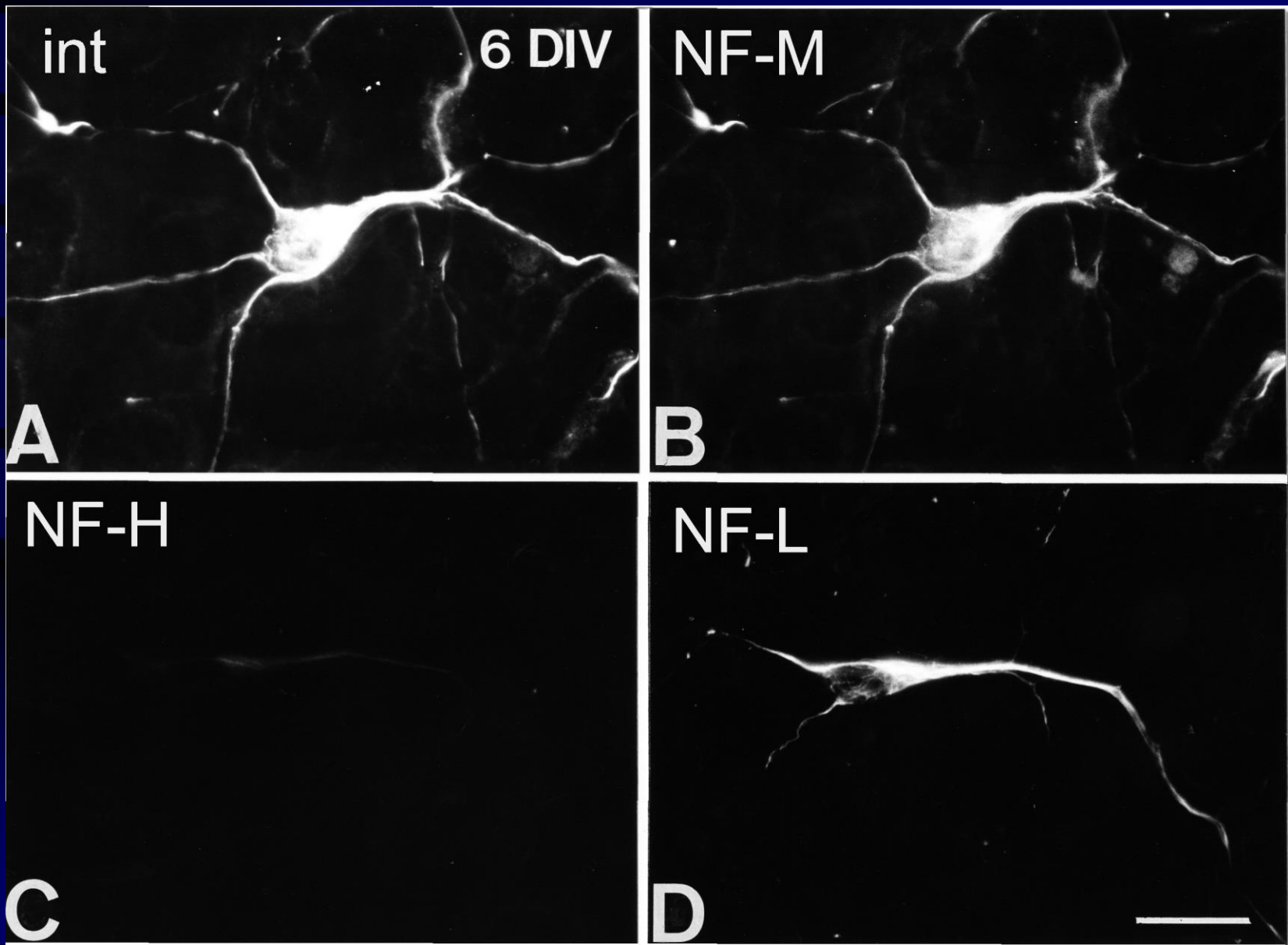
Neuroepithelial stem cells

Primary culture of embryonic (E15) hippocampal cells

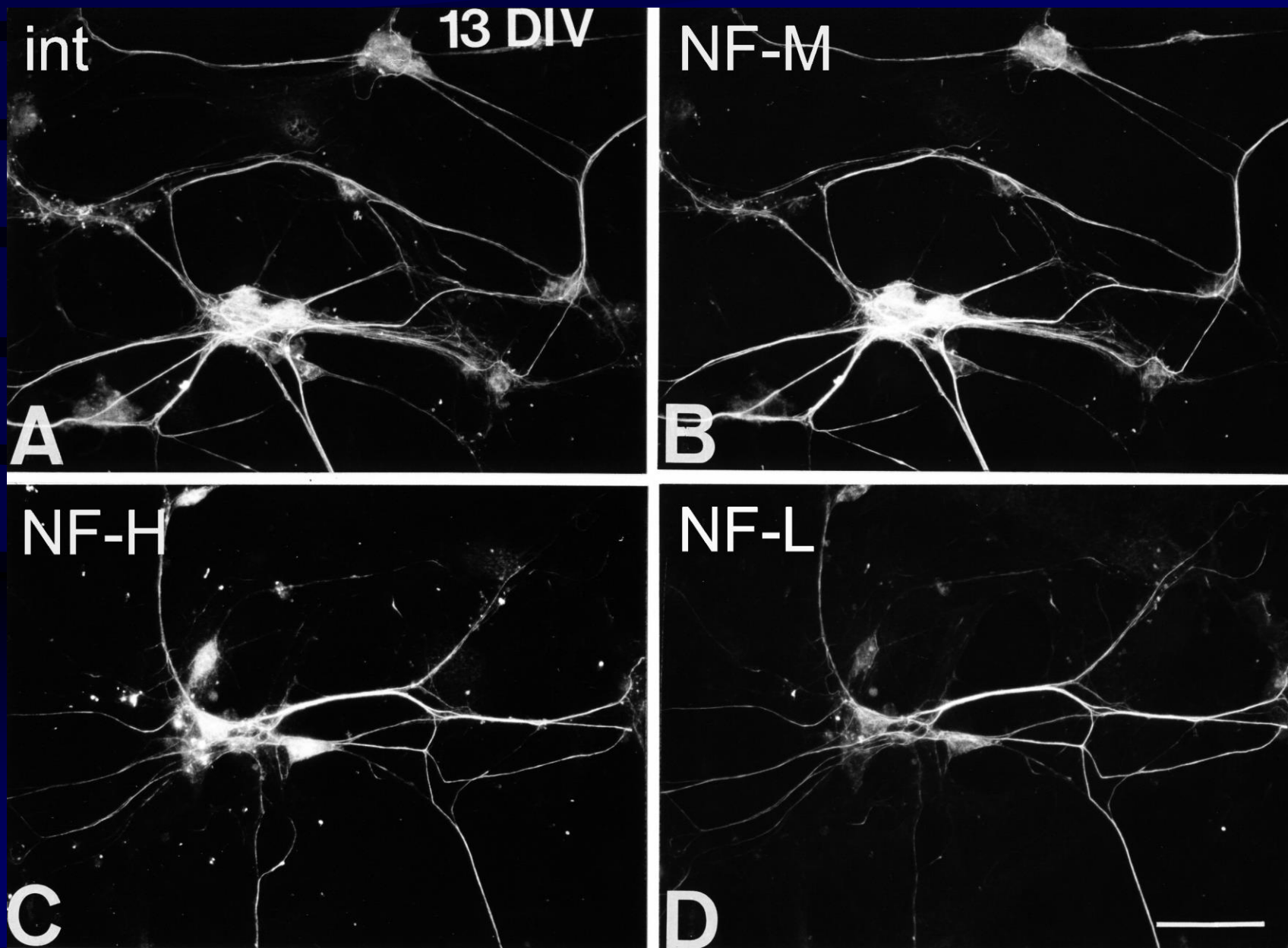
α -internexin: a 66 kD protein,
the first neuronal intermediate
filament protein expressed in the
post-mitotic neurons of
developing mammalian central
nervous system



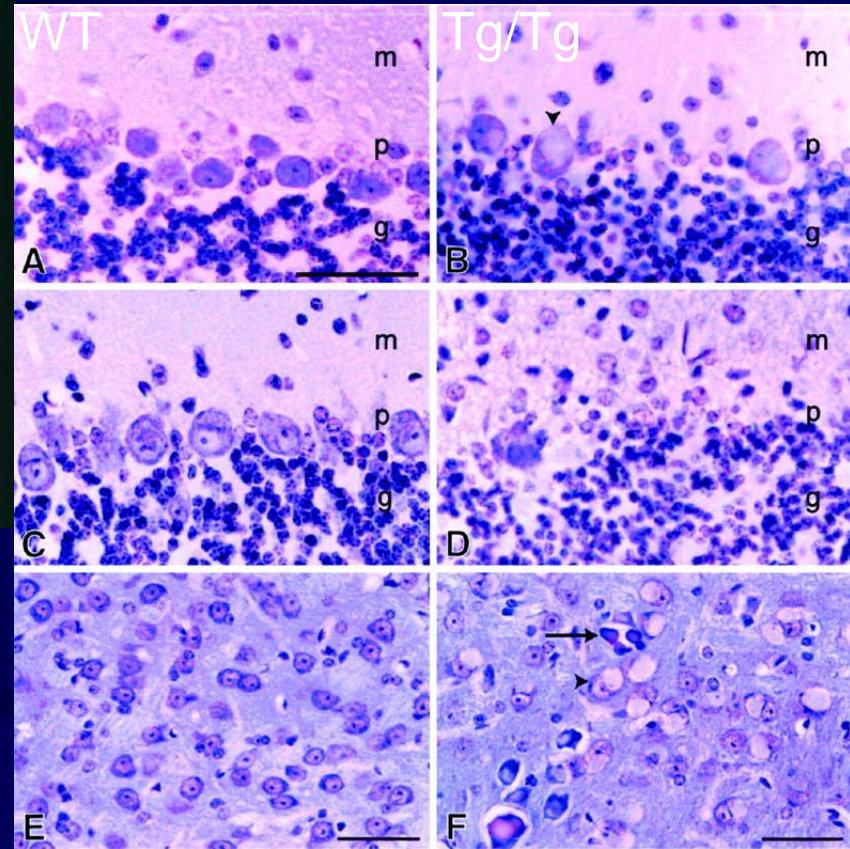
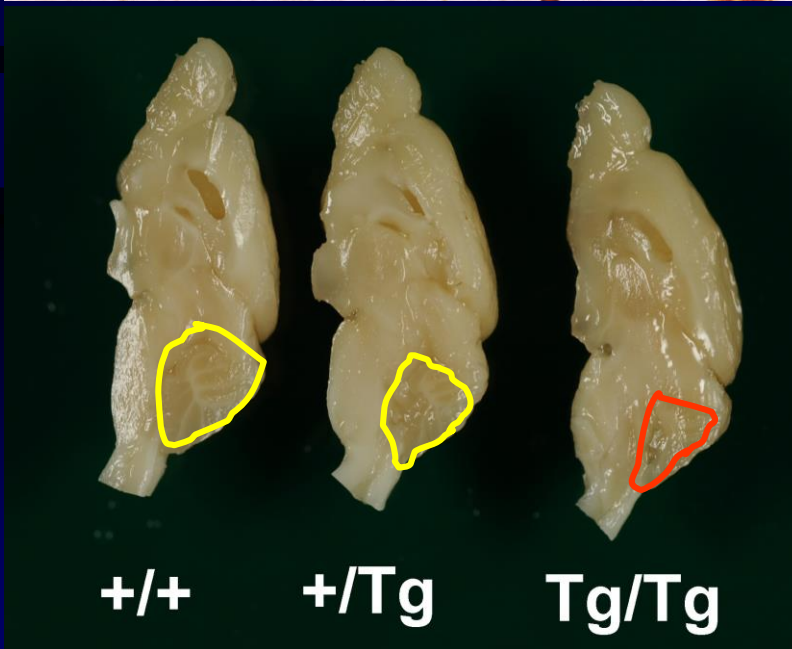
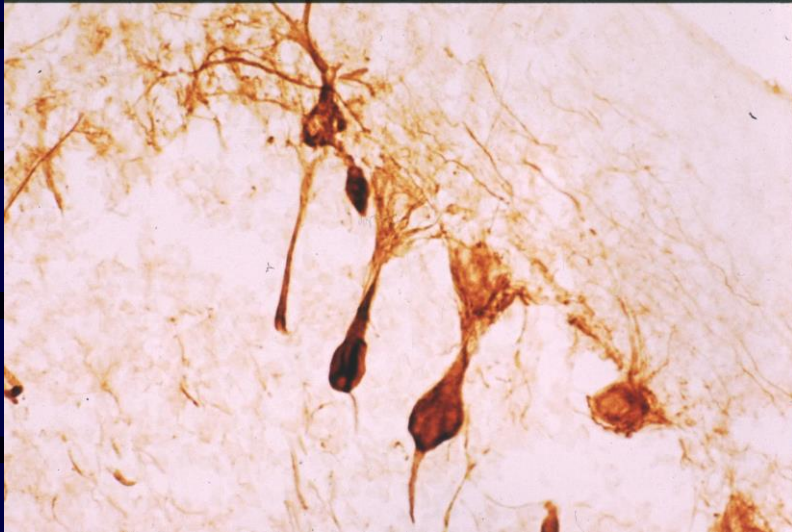
Internexin, NF-M, NF-L but not NF-H expressed in the 6 days *in vitro* (DIV) culture of hippocampal neurons



Internexin and Neurofilament Triplet Proteins (NF-L, NF-M and NF-H) all expressed in the 13 DIV hippocampal neurons



Animal model for cerebellar atrophy (J. Neurosci. 19:2974-2986, 1999)



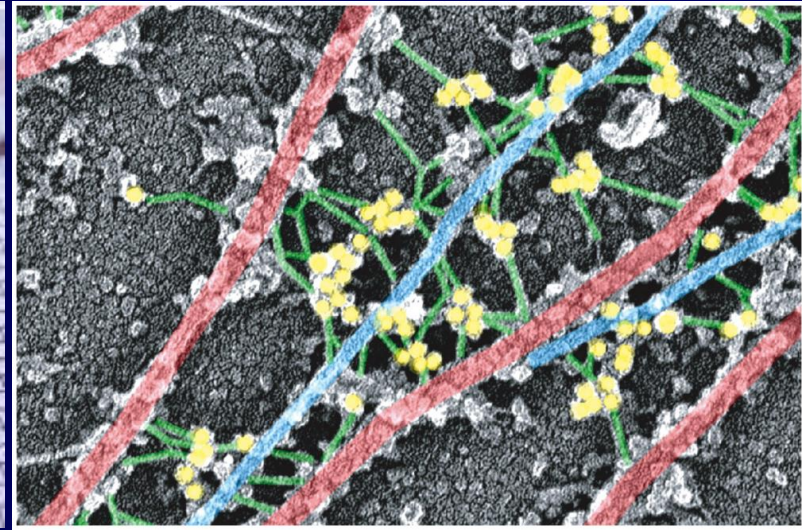
12 m
cerebella

18 m
cerebella

18 m
thalamus

Neuronal loss in the cerebella and thalamus of transgenic mice

Nature Mutant for Neuronal Degeneration

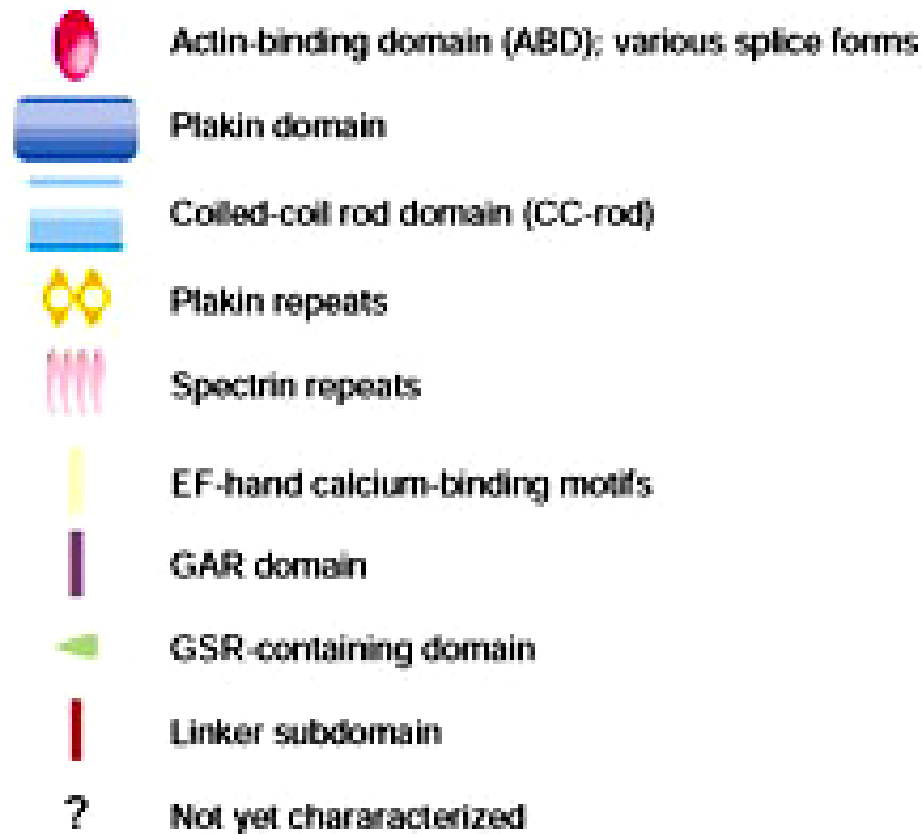
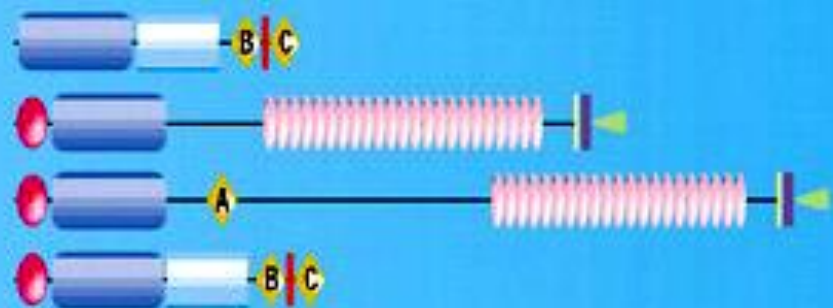


- *Dystonia musculorum (dt)* mouse is a recessive hereditary sensory neuropathy of the mutant mouse, which is defective in *BPAG1* gene.
- Mice affected with *dt* are seemingly normal at birth, but by 10–12 days they begin twitching, writhing, and exhibiting uncoordinated movements.
- **BPAG1** cross-links the intermediate filaments and other cytoskeletons.

BPAG1 is known as **dystonin**.

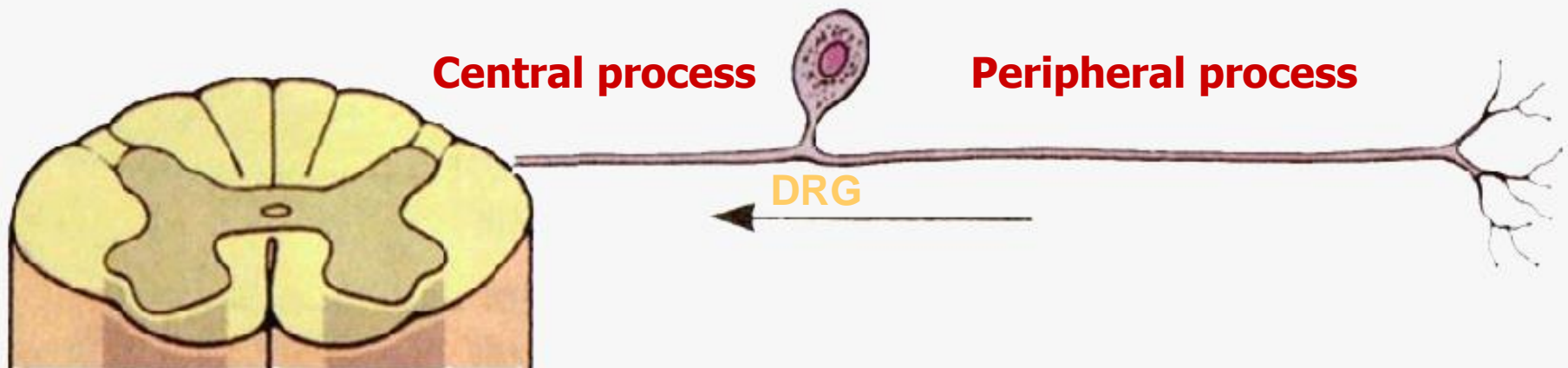
Dystonin, a neural isoform of BPAG1, contains actin-binding domain (ABD) at N-terminus, and is a cytoskeletal crosslinker protein.

BPAG1-e
BPAG1-a
BPAG1-b
BPAG1-n
(dystonin)



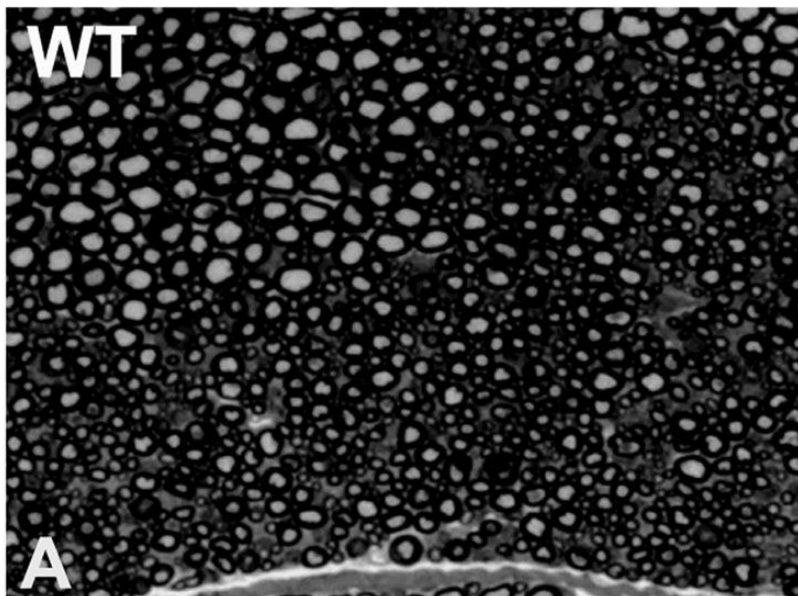
* The gene structures are not drawn to scale and do not represent the actual number of exons

To study the neural dysfunction and degeneration of **primary sensory neurons in dorsal root ganglia** in *dt* mice.

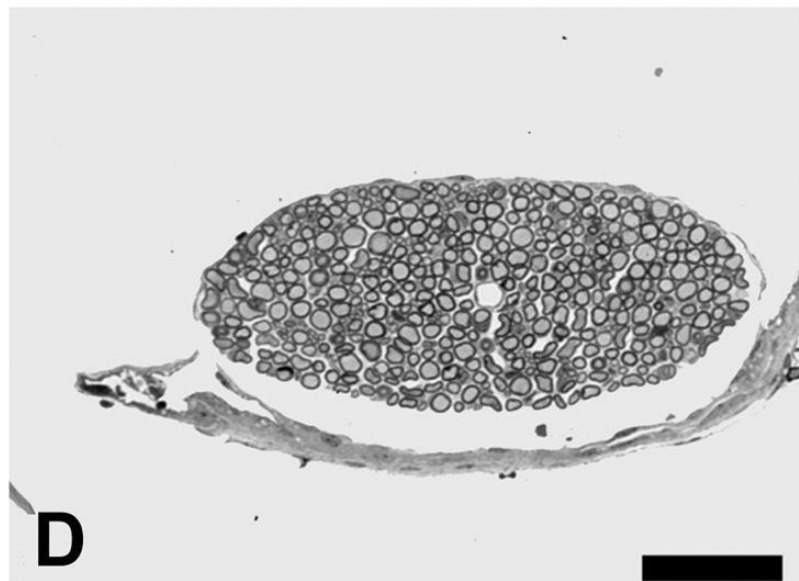
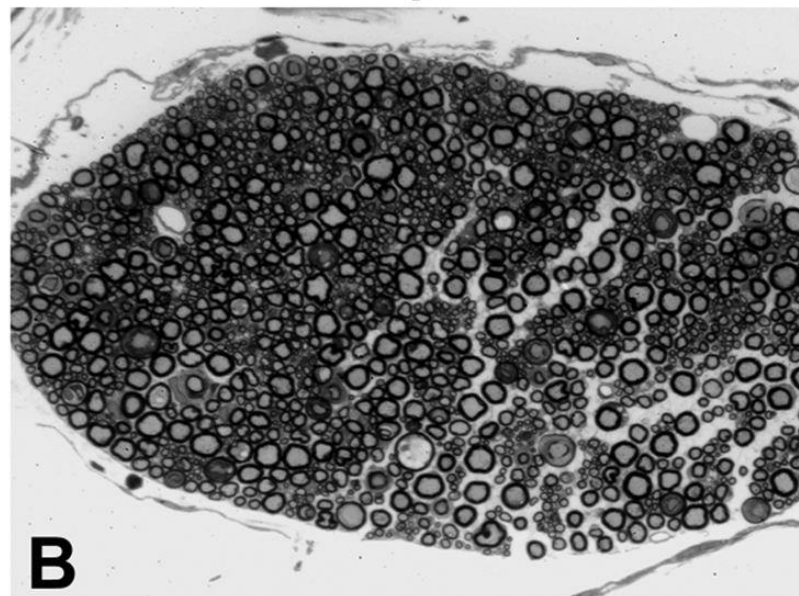


Peripheral and central processes from WT and *dt/dt* mice

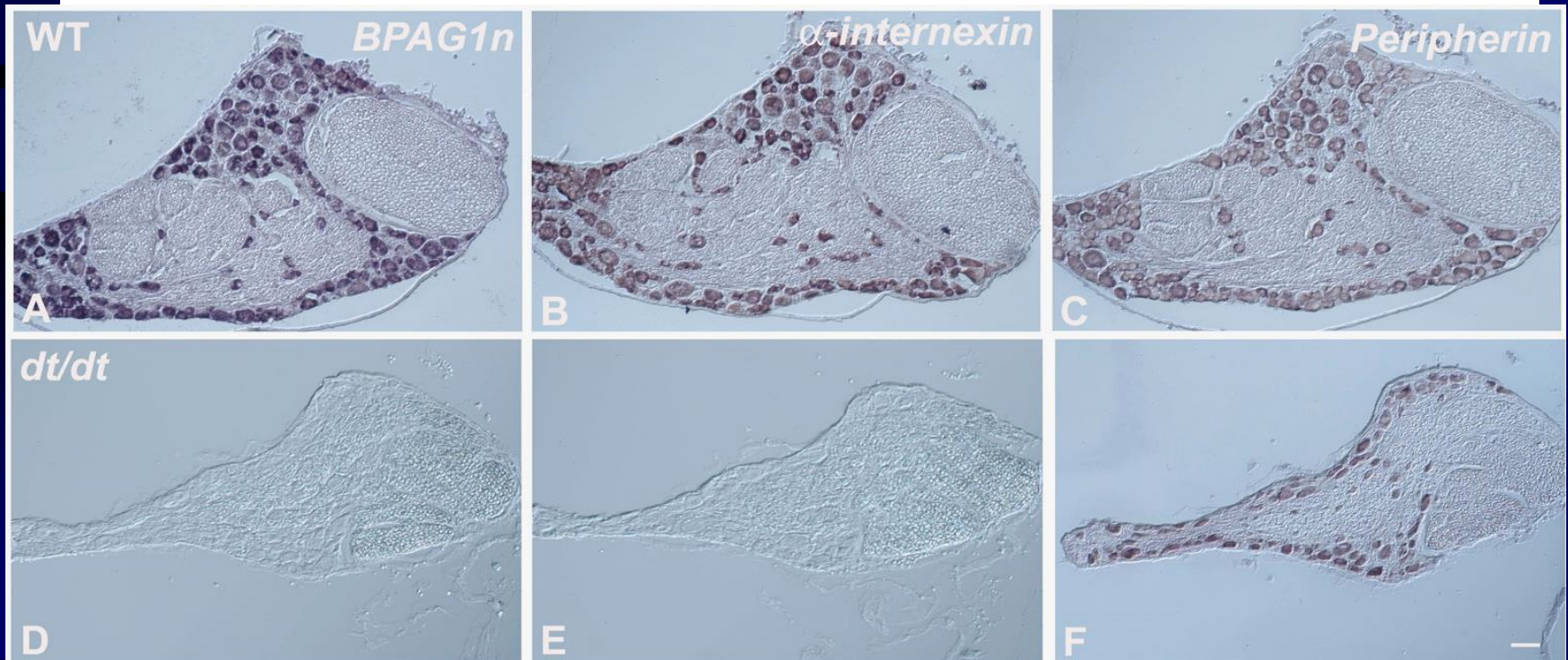
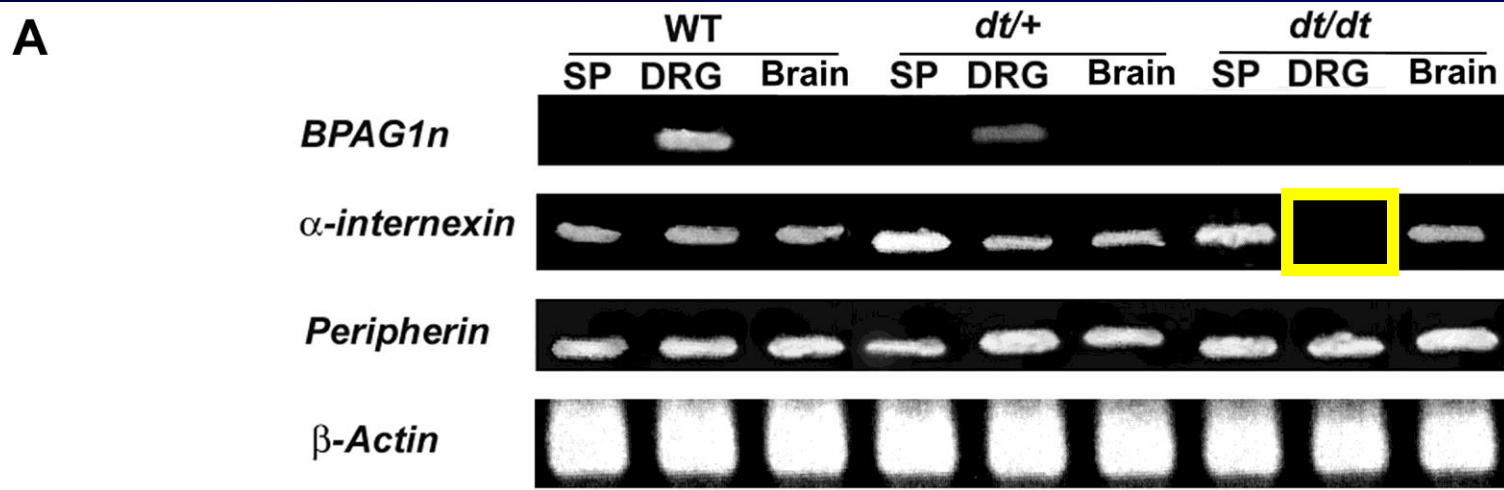
Peripheral process



Central process

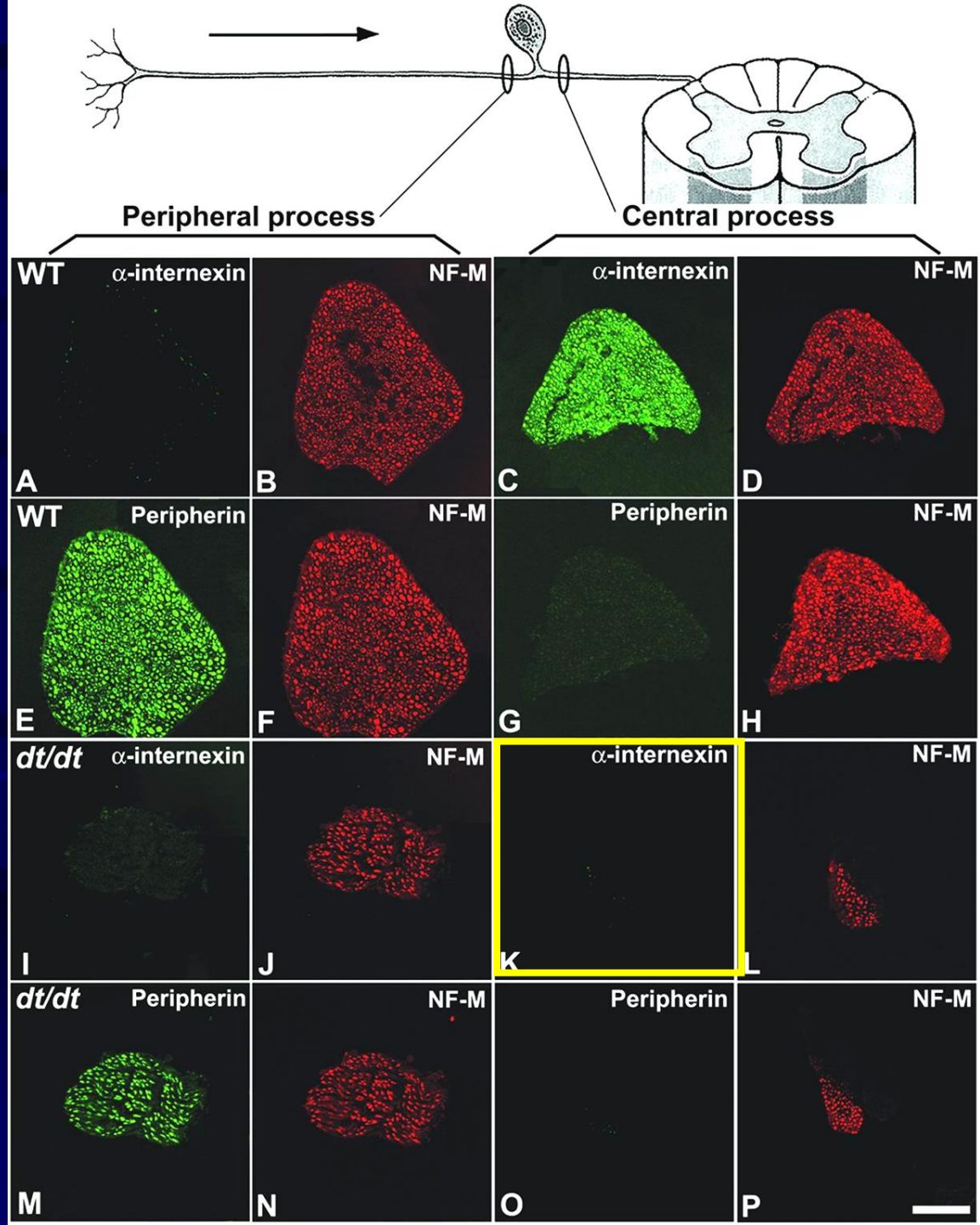


RT-PCR and in situ hybridization analysis



Expression of neuronal intermediate filaments in WT and *dt/dt* mice

α -interenxin is absent in the central process of adult *dt/dt* mice



Sensory and autonomic nerves degenerated in the skin of *dt* mutant

Fig. 6

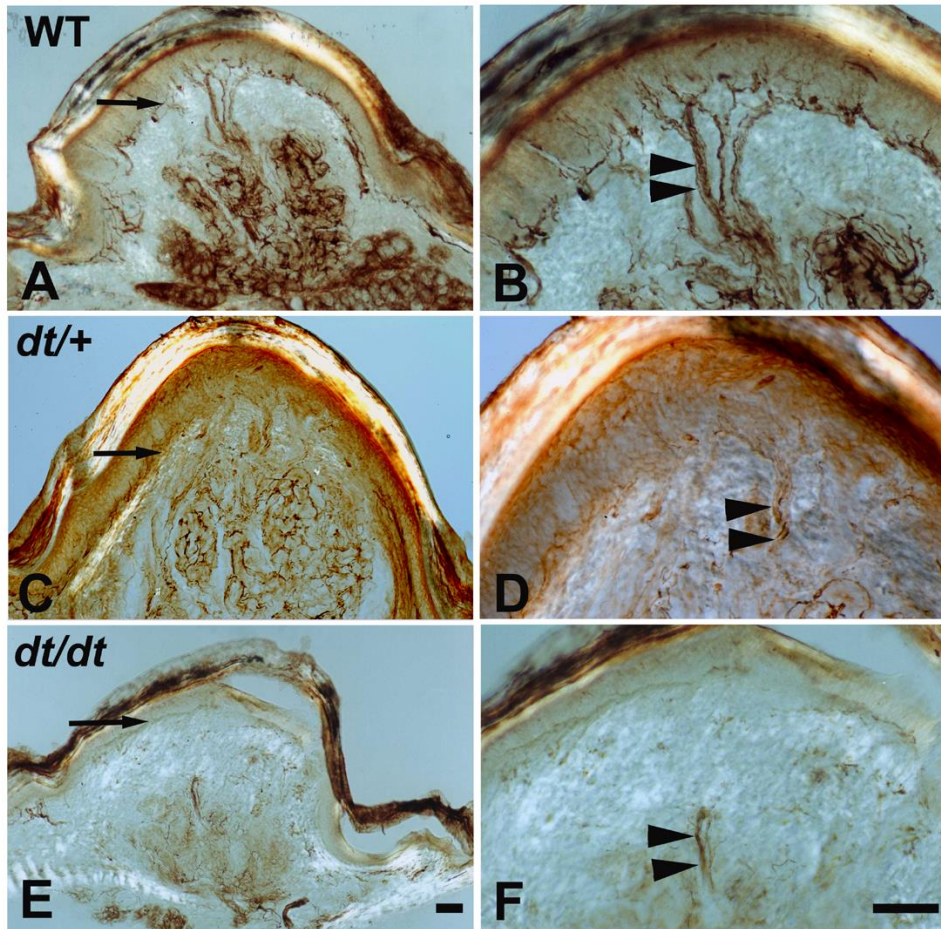
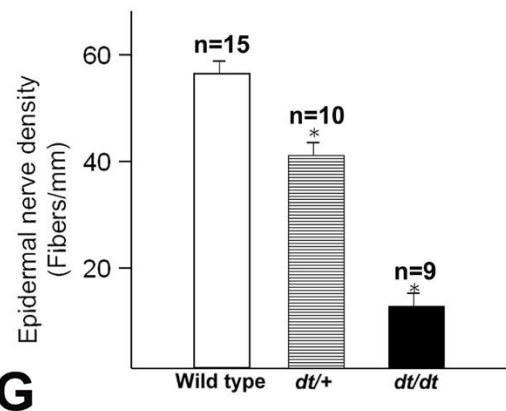
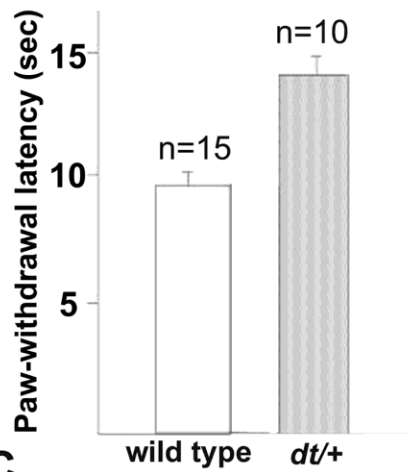
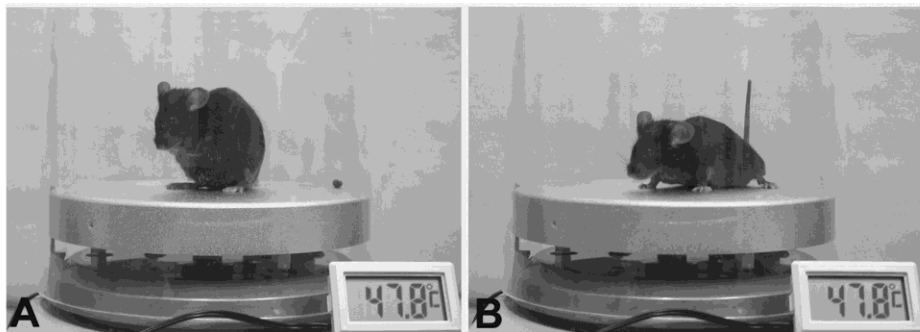


Fig. 7

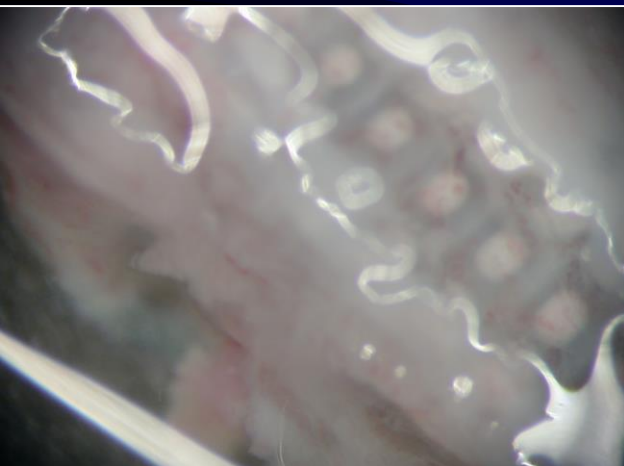


Primary culture DRG neurons

1. Take DRGs and transfer DRGs to a fresh ependroff tube with 0.5 ml HBSS (CMF) on ice.
2. Add 0.5 ml 0.25% Trypsin-EDTA and incubate in rotating incubator at 37°C for 15 min.
3. Resuspend with 40% FBS L15
4. Spin for 5 min at 1500 rpm, remove supernatant..
5. Resuspend with 1.5 ml 40% FBS L15 in incubator at 37°C for 15 min.
6. Spin for 5 min at 1500 rpm.
7. Resuspend in 2 ml NB1 with FBS, glucose, 100ng/ml NGF.
8. Transfer containing neurons medium to 30 mm poly-L-lysine coated Petri dish and then incubate 10-20 min (preplating).
9. Transfer the medium to 35 mm Petri dish containing poly-L-lysine coated slide.

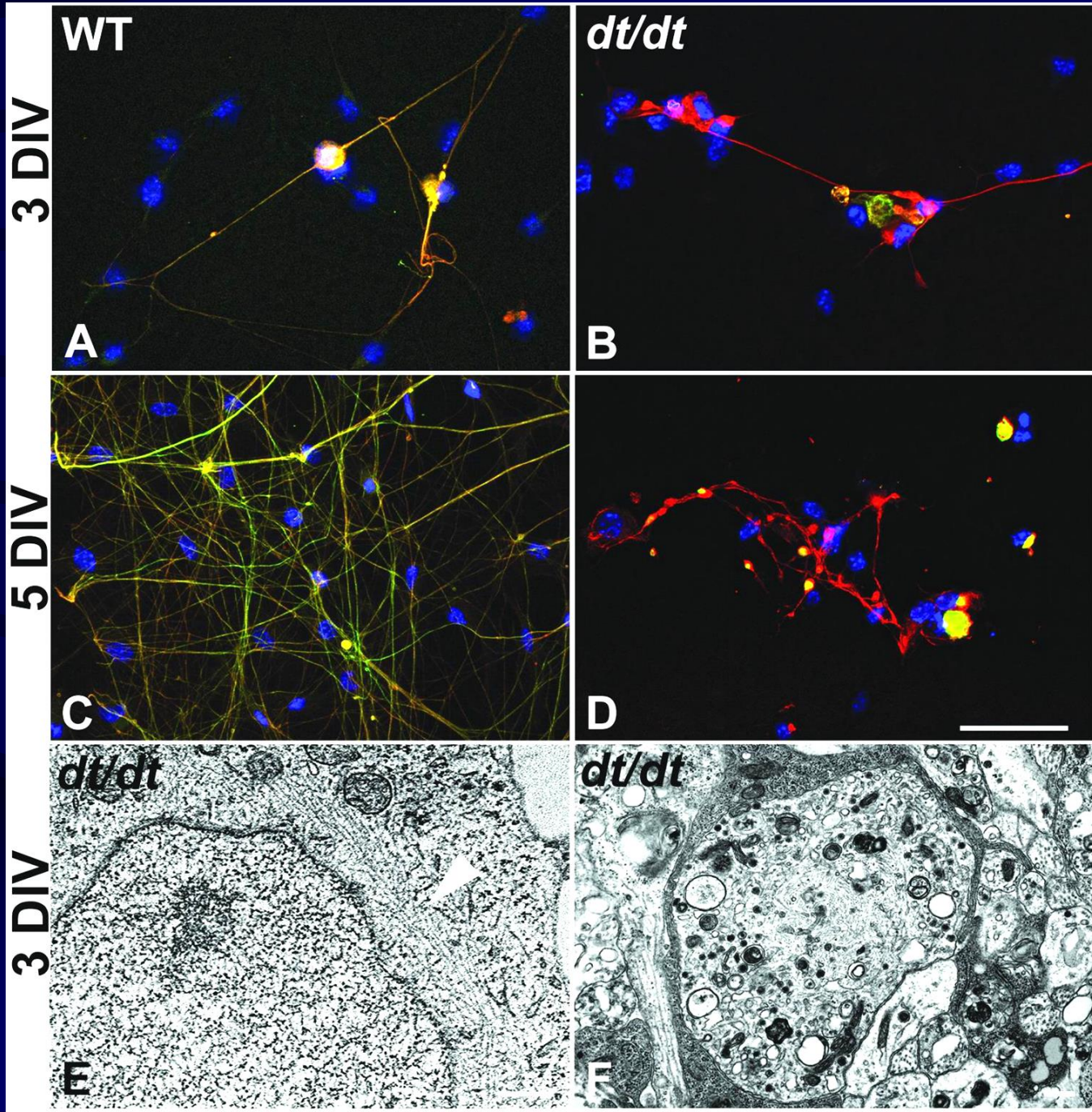
DRG neurons culture

E15.5

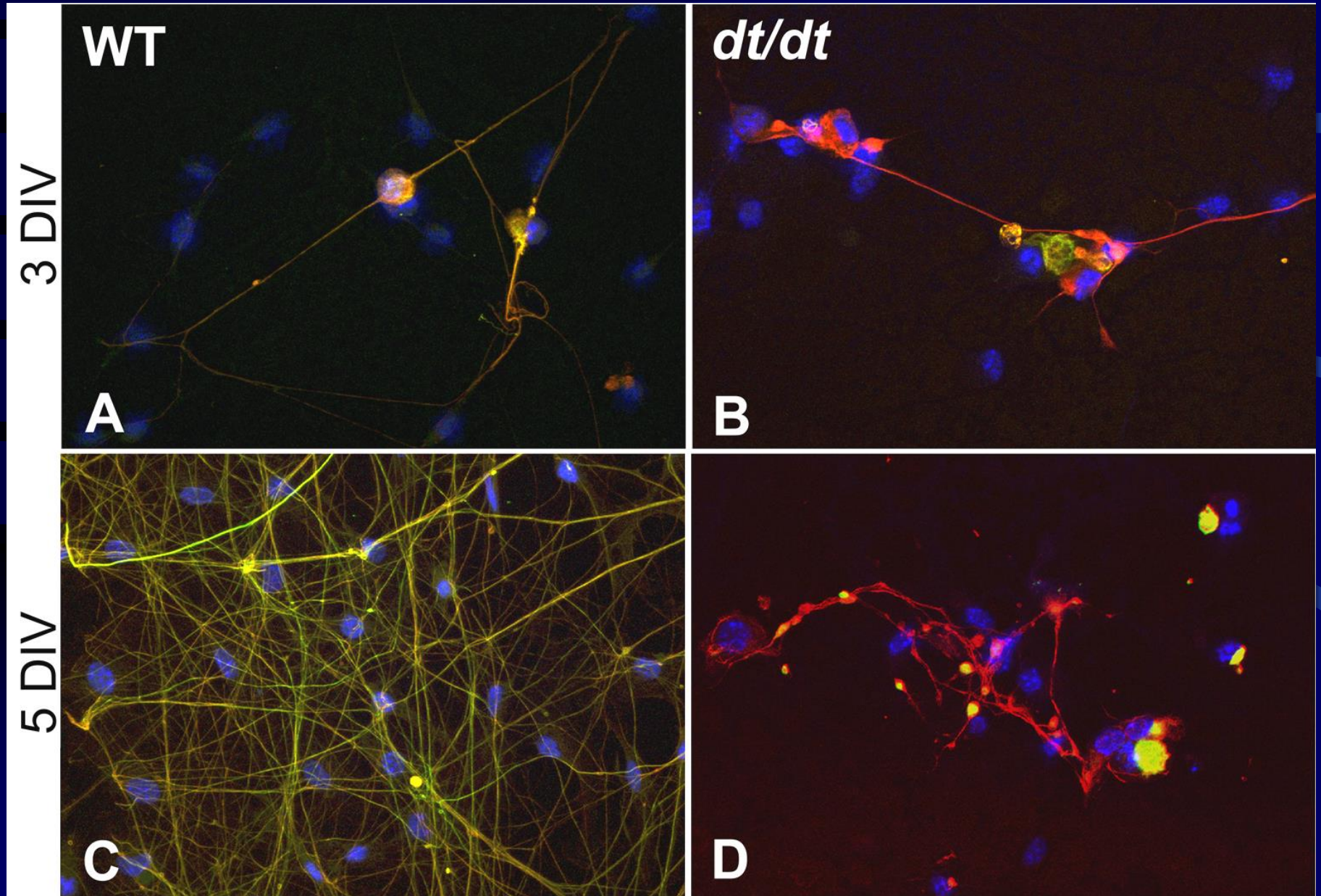


Cultured DRG
neurons
from E15.5
embryos

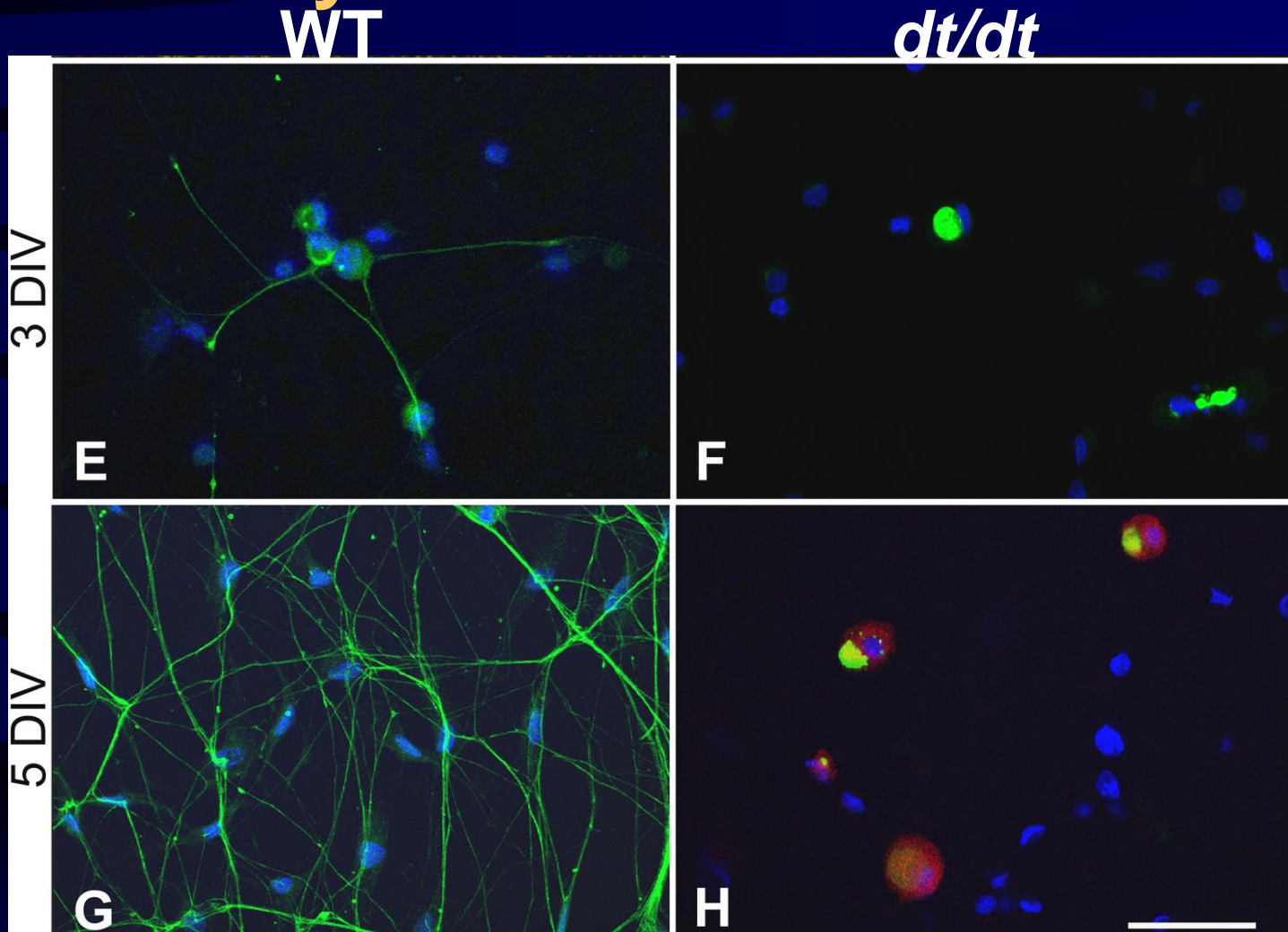
α -interenxin proteins
are accumulated in
the cell bodies as
well as in the
processes of *dt/dt*
neurons.



α -Internexin aggregates in cultured DRG neurons

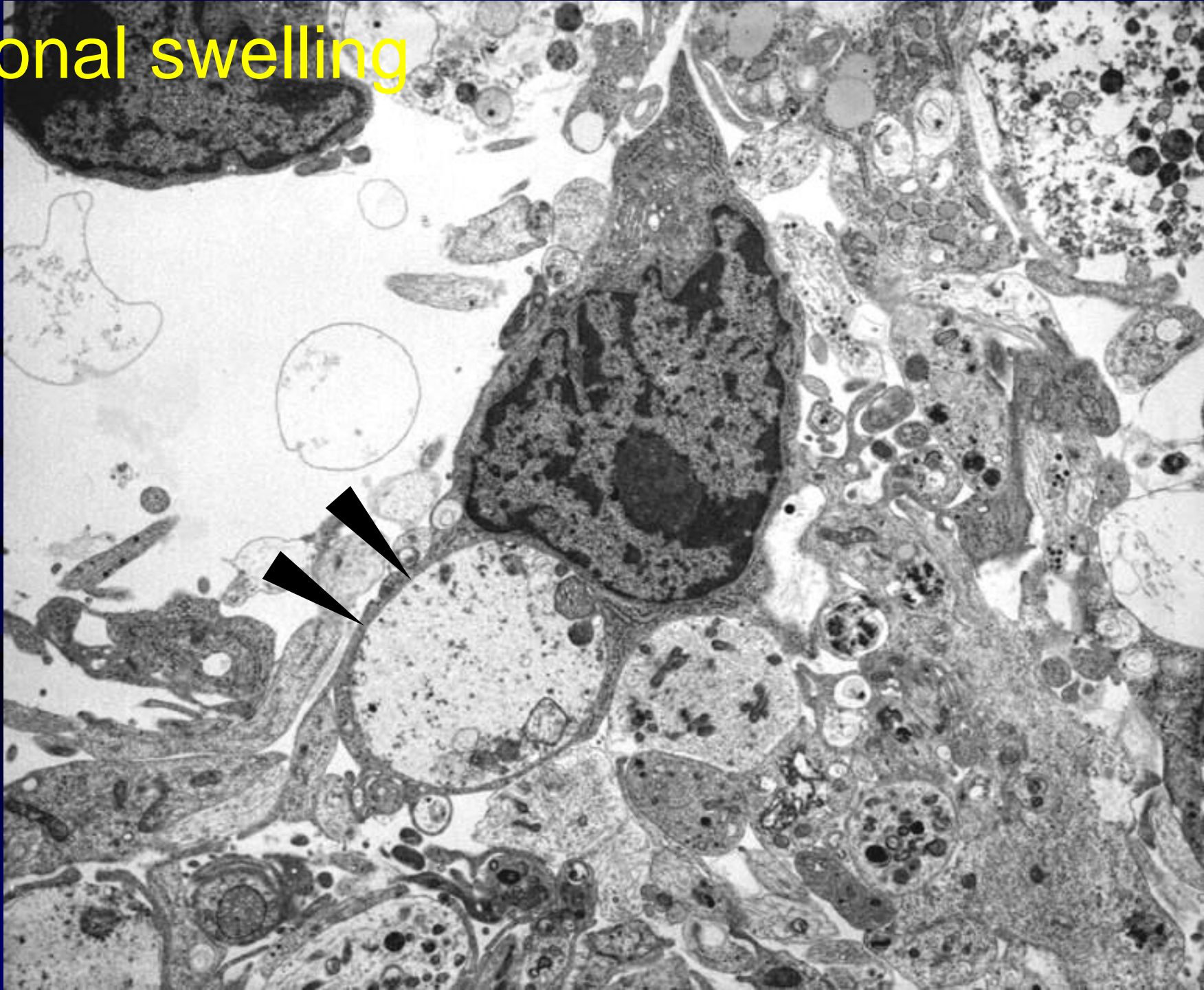


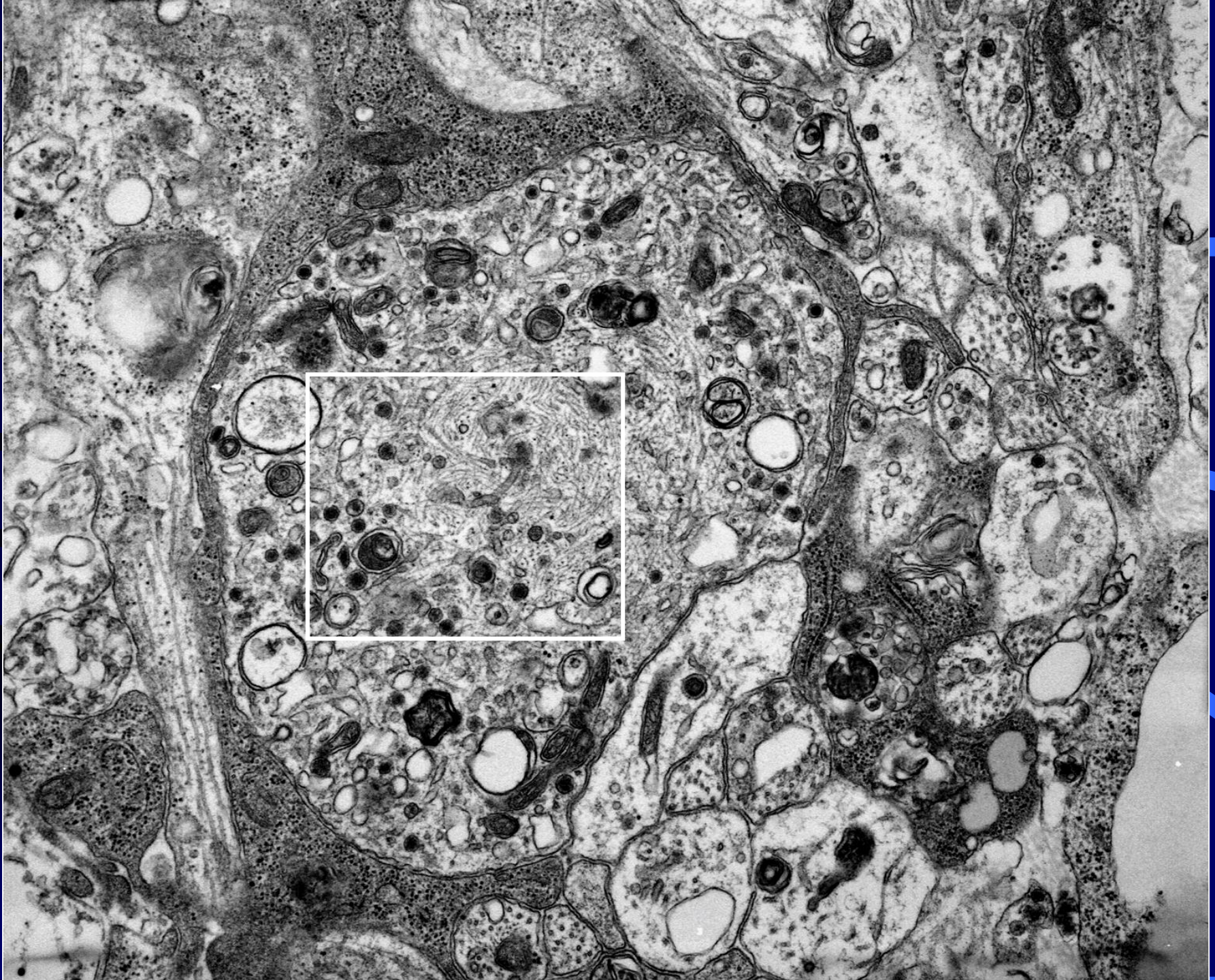
Primary culture of DRG neurons



	WT	<i>dt/dt</i>
Internexin	+	++ Aggregations
Activated Caspase-3	-	+

Axonal swelling



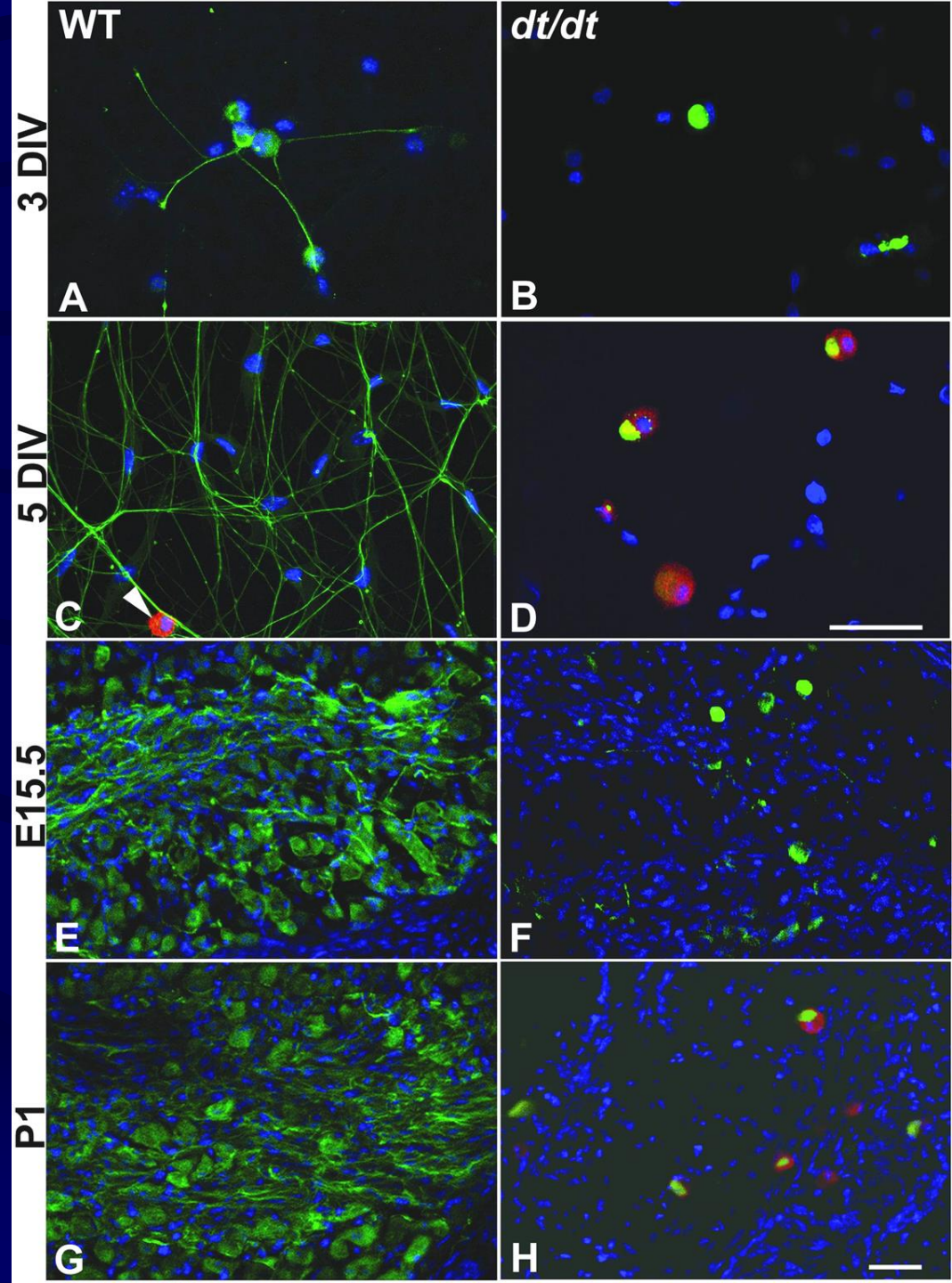


Primary culture of DRG neurons

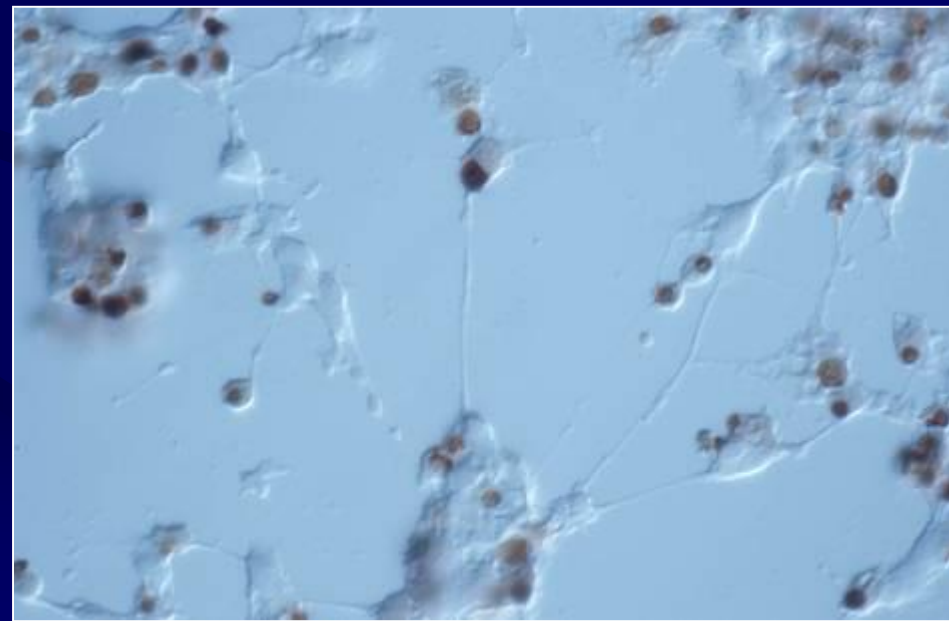
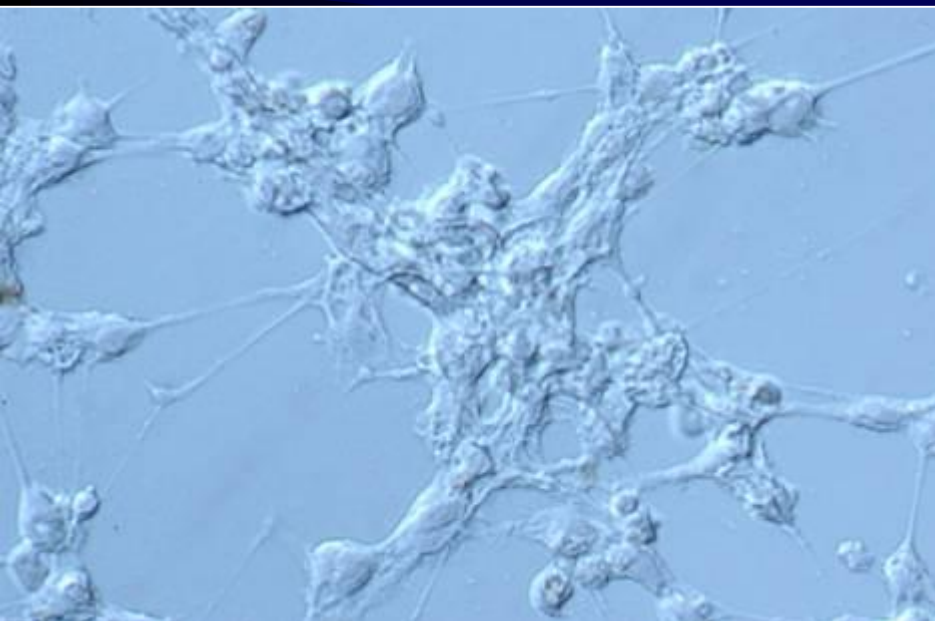
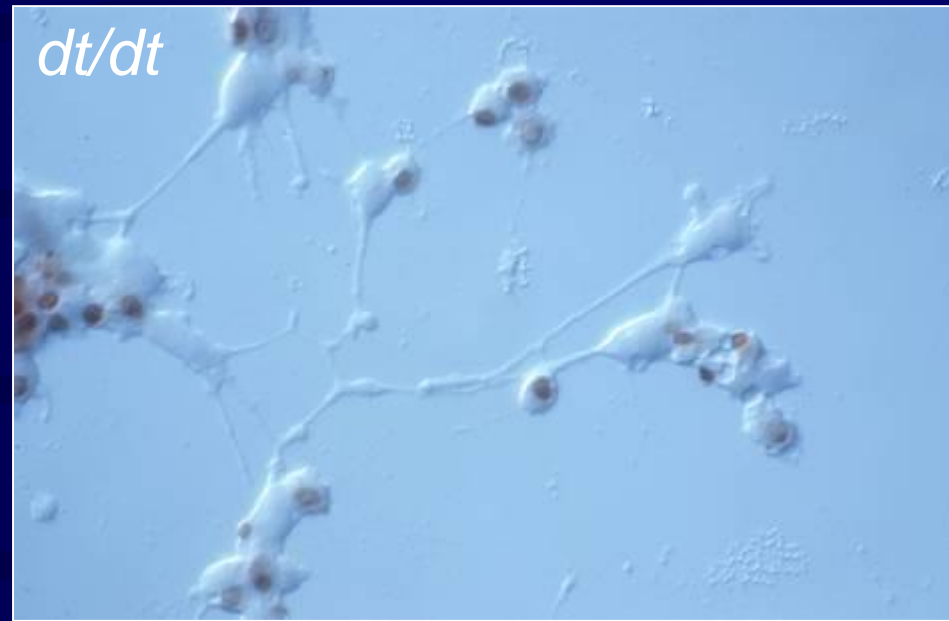
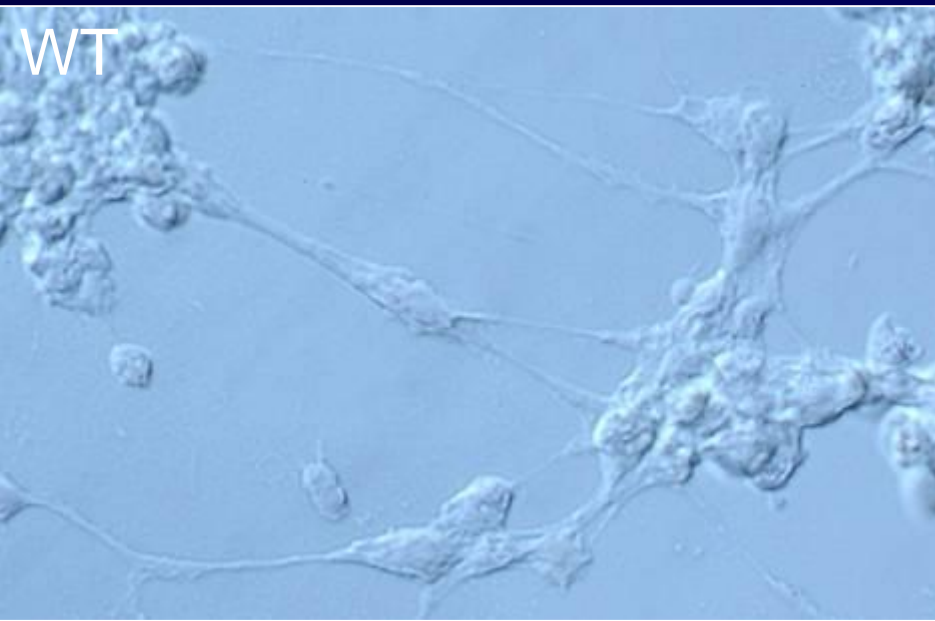
	WT	<i>dt/dt</i>
Internexin	+	++ Aggregations
Activated Caspase	-	+

Perinatal development

	WT	<i>dt/dt</i>
Internexin	+	++ Aggregations
Activated Caspase	-	+



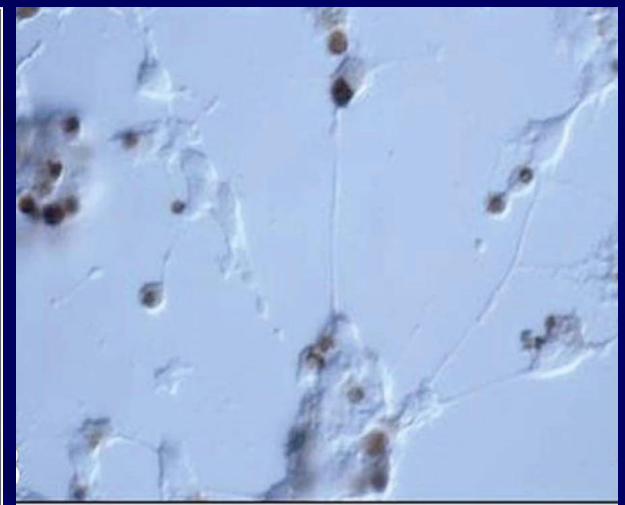
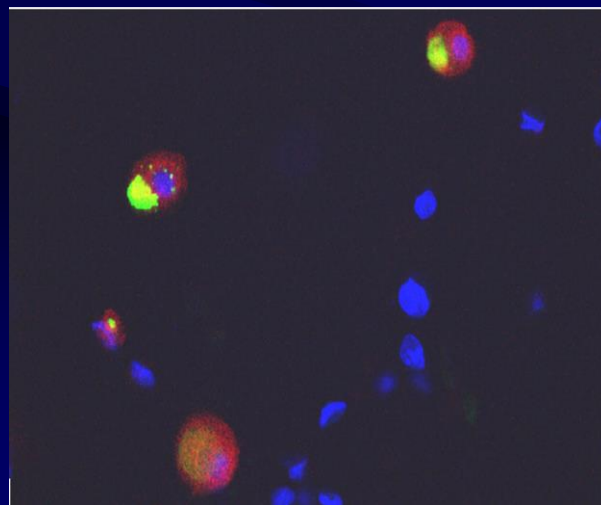
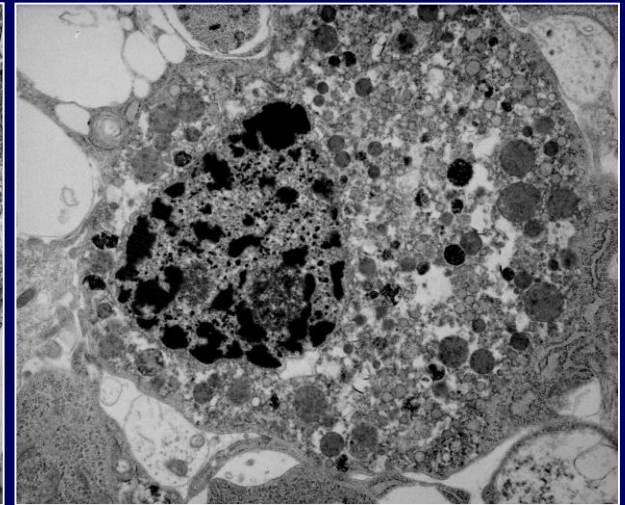
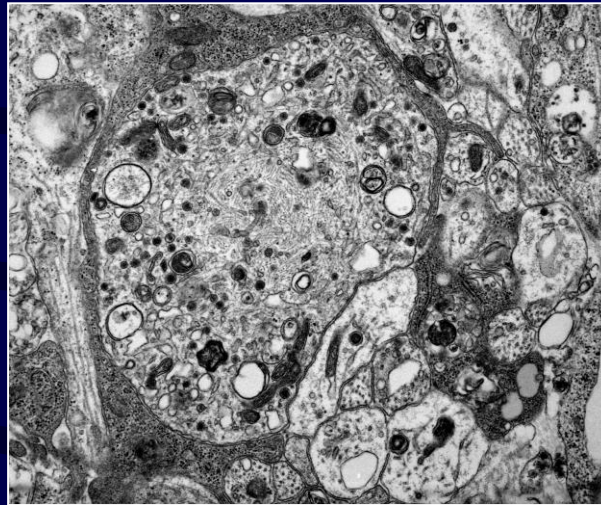
TUNEL Assays



Primary culture of DRG neurons

Cell death of cultured DRG neurons of *dt/dt*

- Axonal swelling
- IFs accumulation
- Expression of active caspase-3
- Chromatin condensation
- TUNEL-positive neurons
- DNA fragmentation
- Cell death in apoptosis pathway



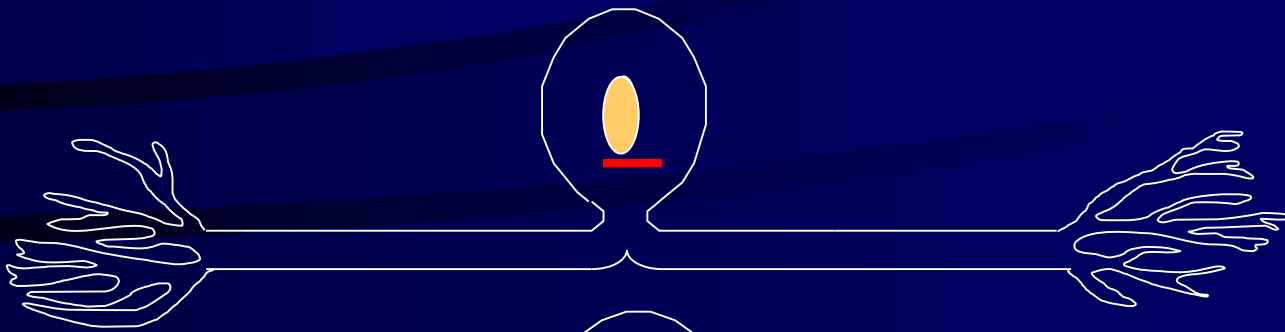
dt/dt WT

Summary I

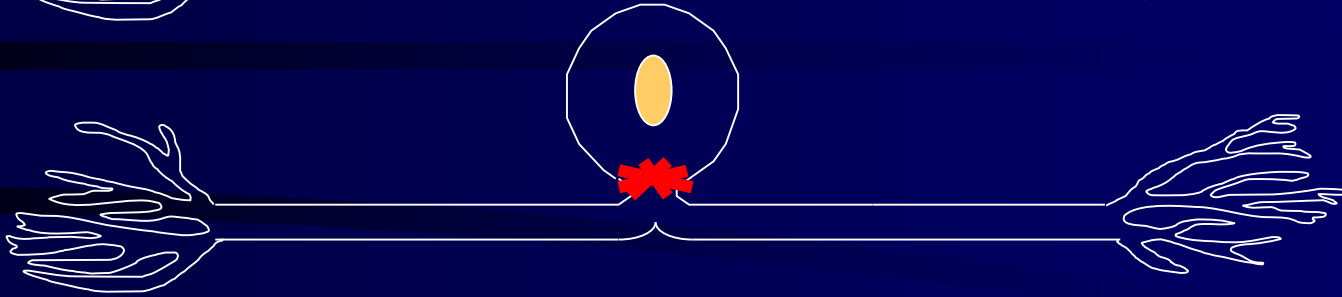
- The interaction between BPAG1 and α -internexin may be one of the key factors involved in the neuronal degeneration of DRG in the *dt* mutant.
- Abnormal accumulation of α -internexin and other cytoskeletal components may impair the axonal transport and subsequently turn on the cascade of neuronal apoptosis during development.

(J. Neuropathol. Exp. Neurol. 65:336-347 , 2006)

WT



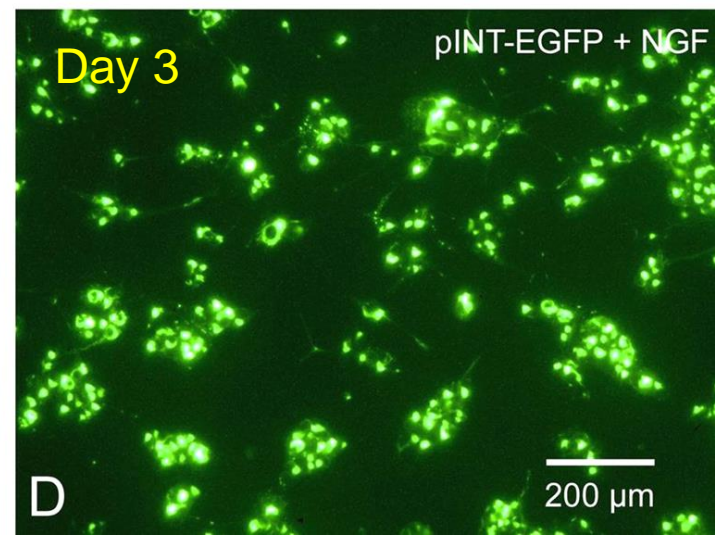
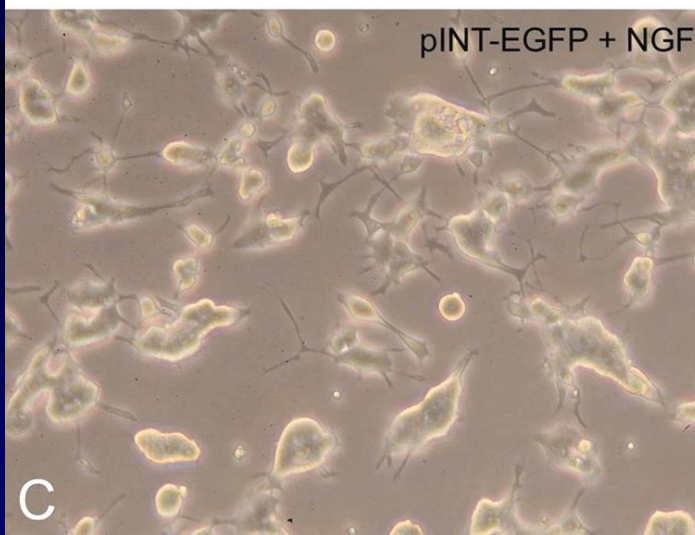
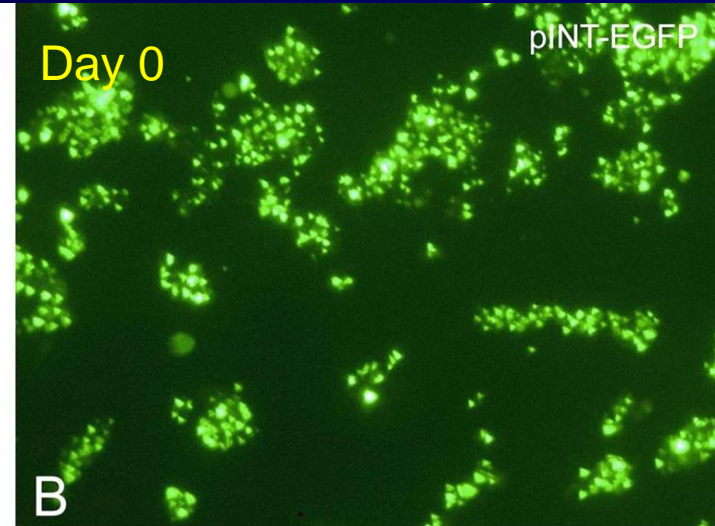
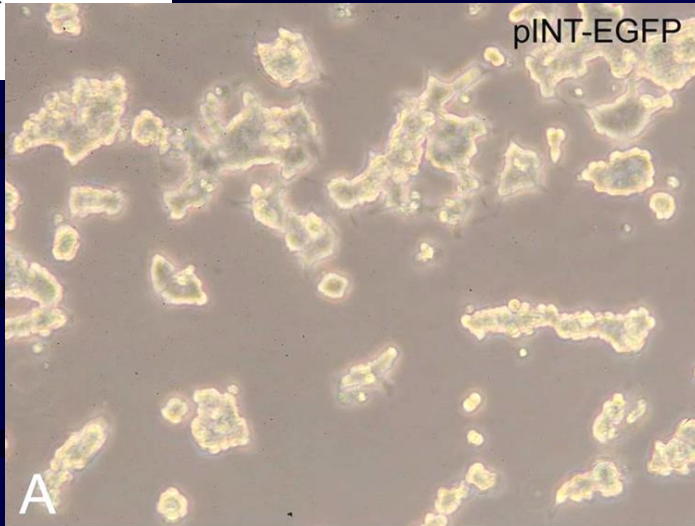
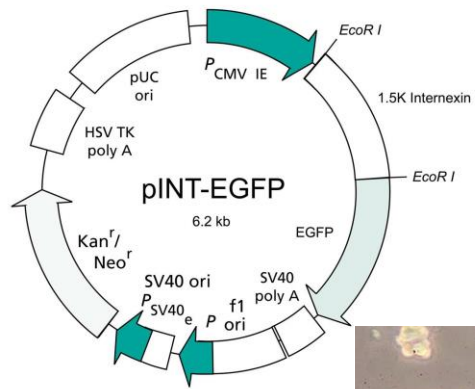
dt/dt



Abnormal accumulation of IFs impairs the axonal transport and subsequently turns on the cascade of neuronal apoptosis in *dt* mice

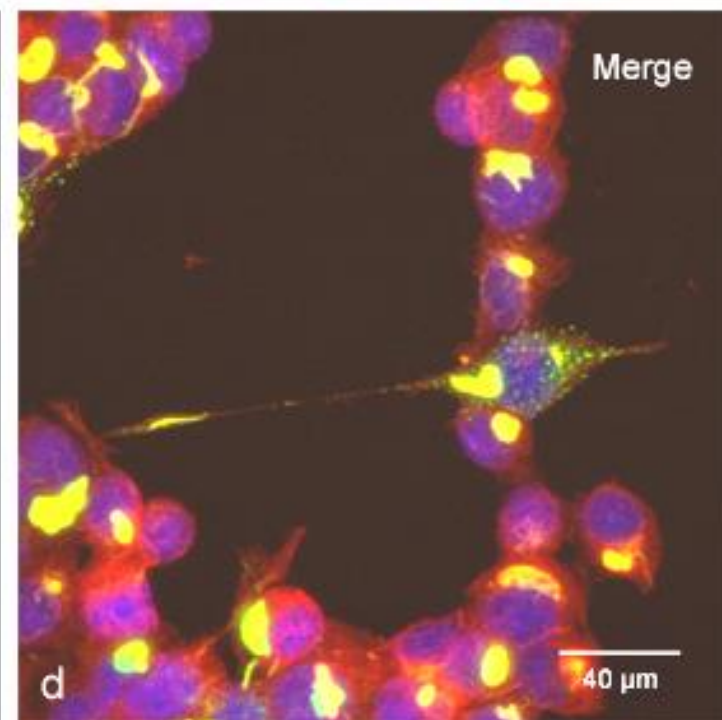
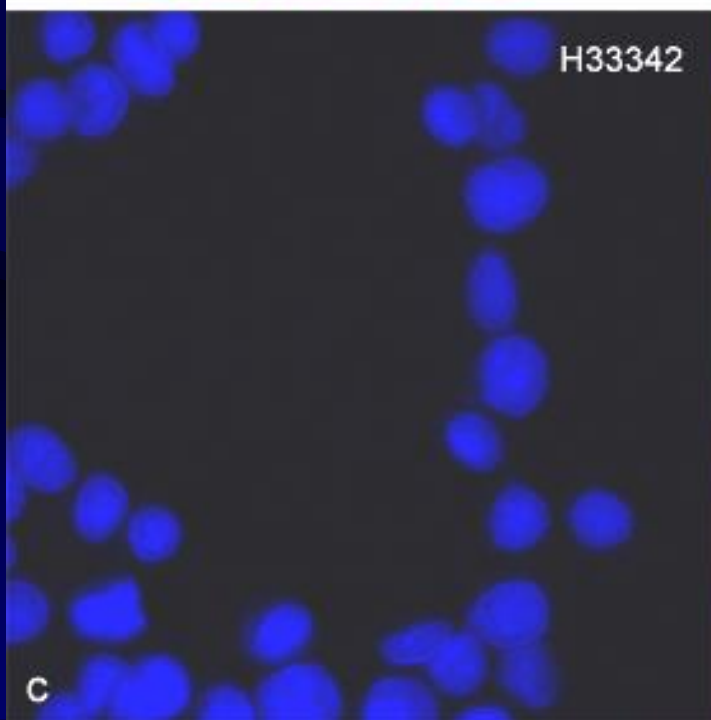
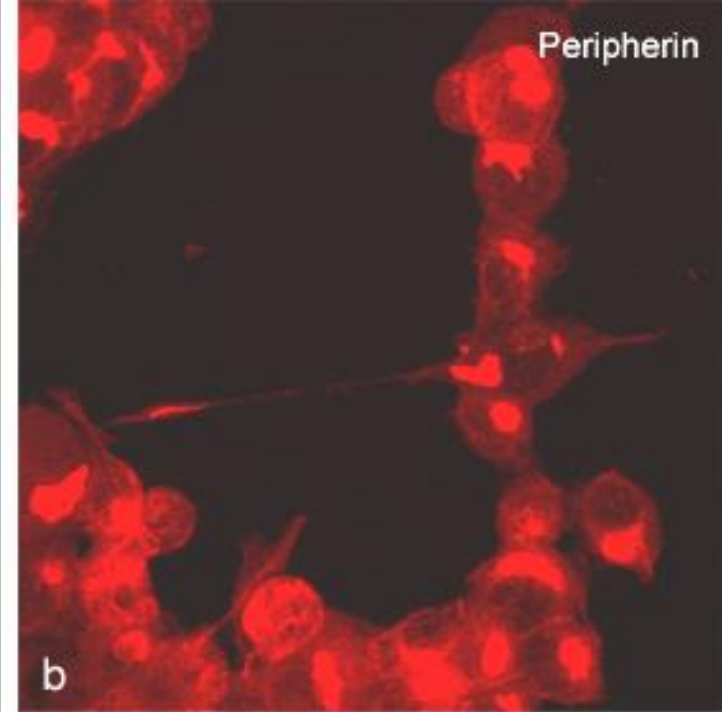
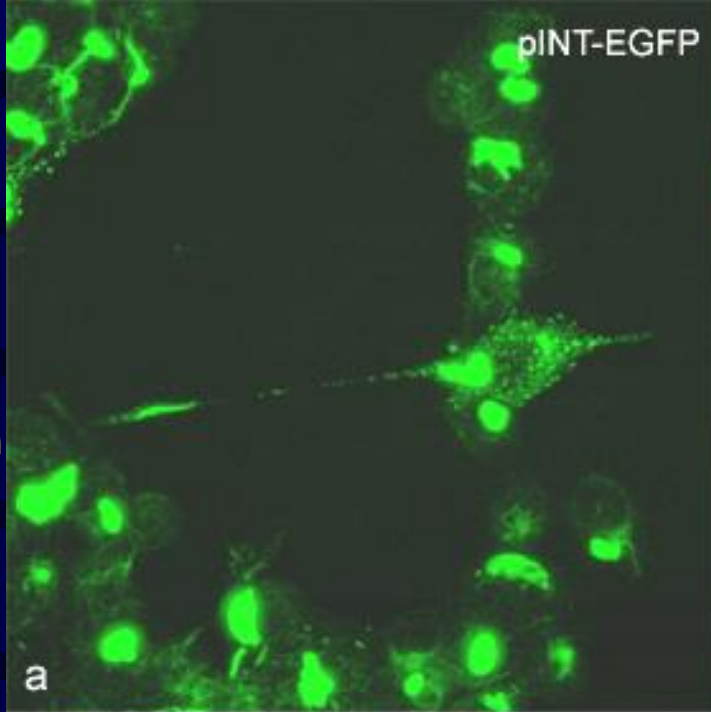
(*J. Neuropathol. Exp. Neurol.* 65:336-347 , 2006)

Overexpression of neuronal intermediate filament α -internexin in the PC-12 cell line (*J. Neurosci. Res.* 80:693-706, 2005)

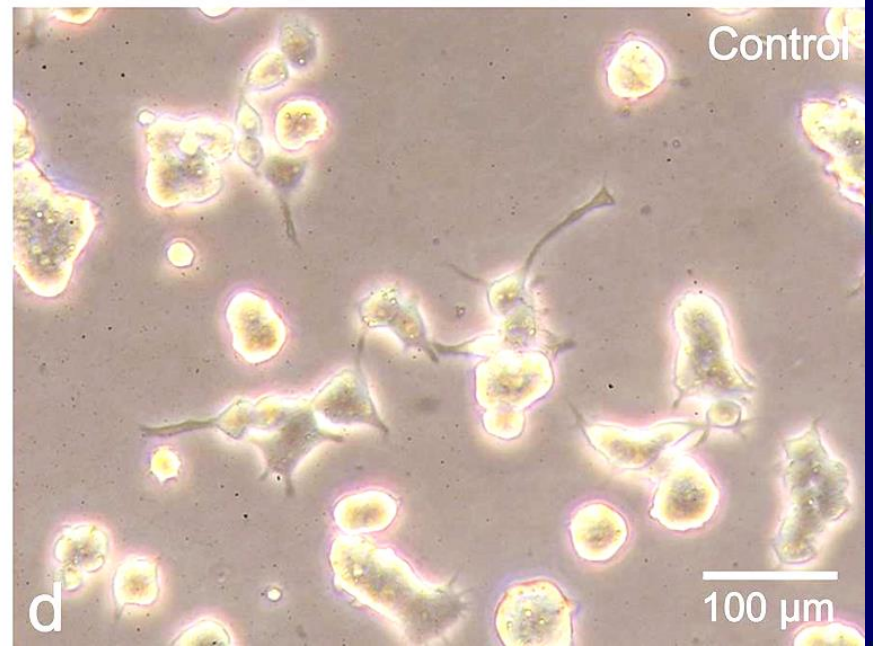
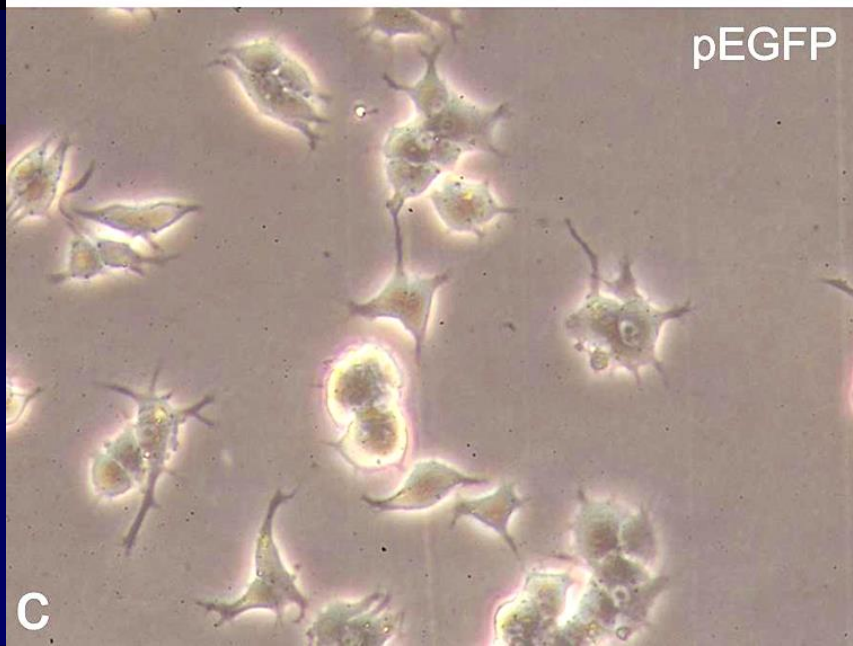


Confocal Patterns

3-day NGF induction



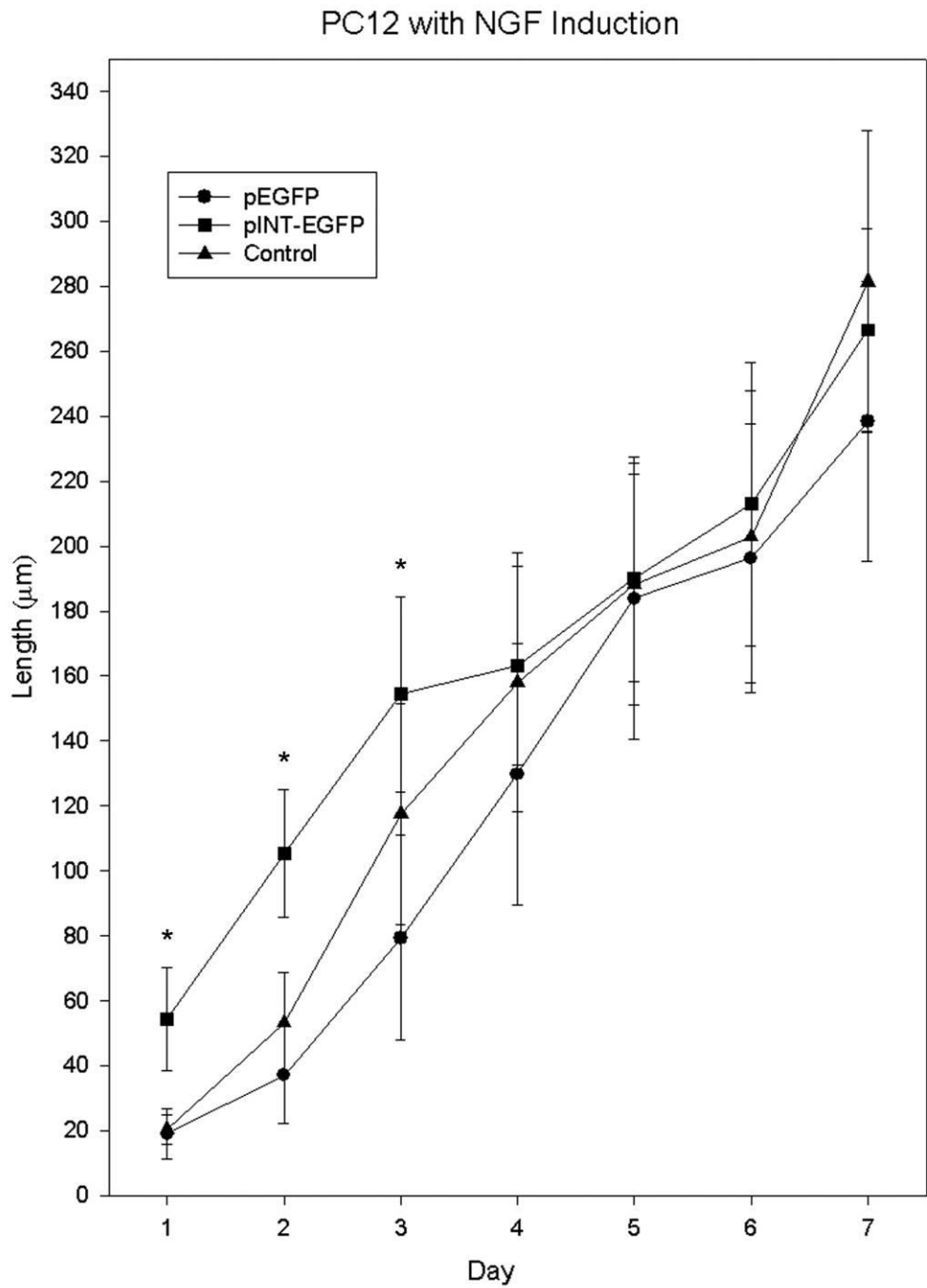
Cells after 2-day NGF induction



PC-12 Neurite outgrowth after NGF induction

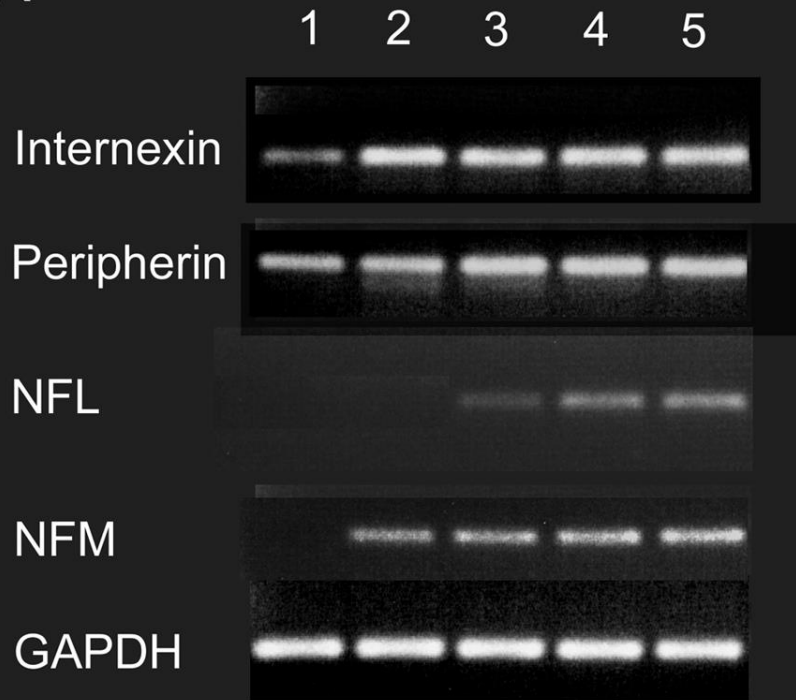
The longest neurite from each single cell was measured at different time points (n=25).

A



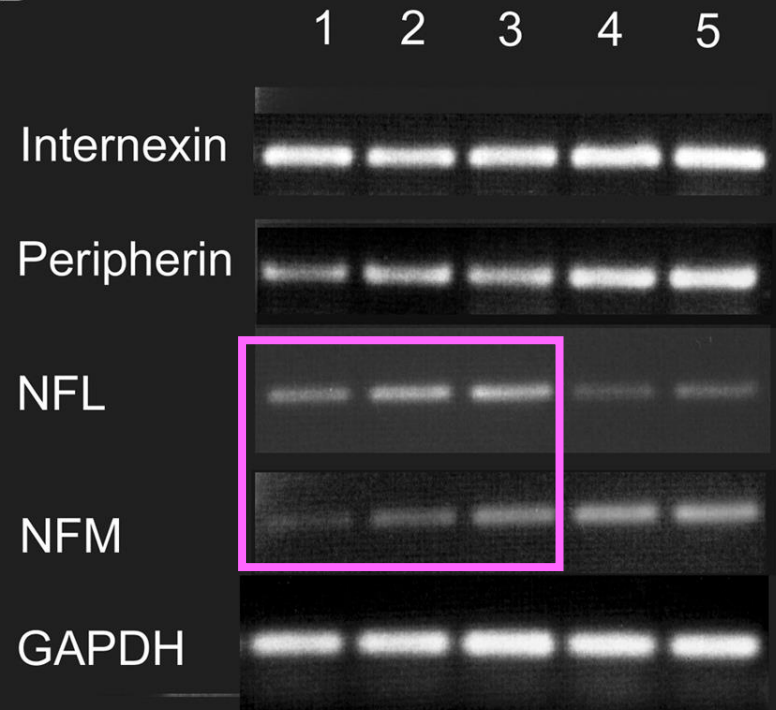
RT-PCR

A



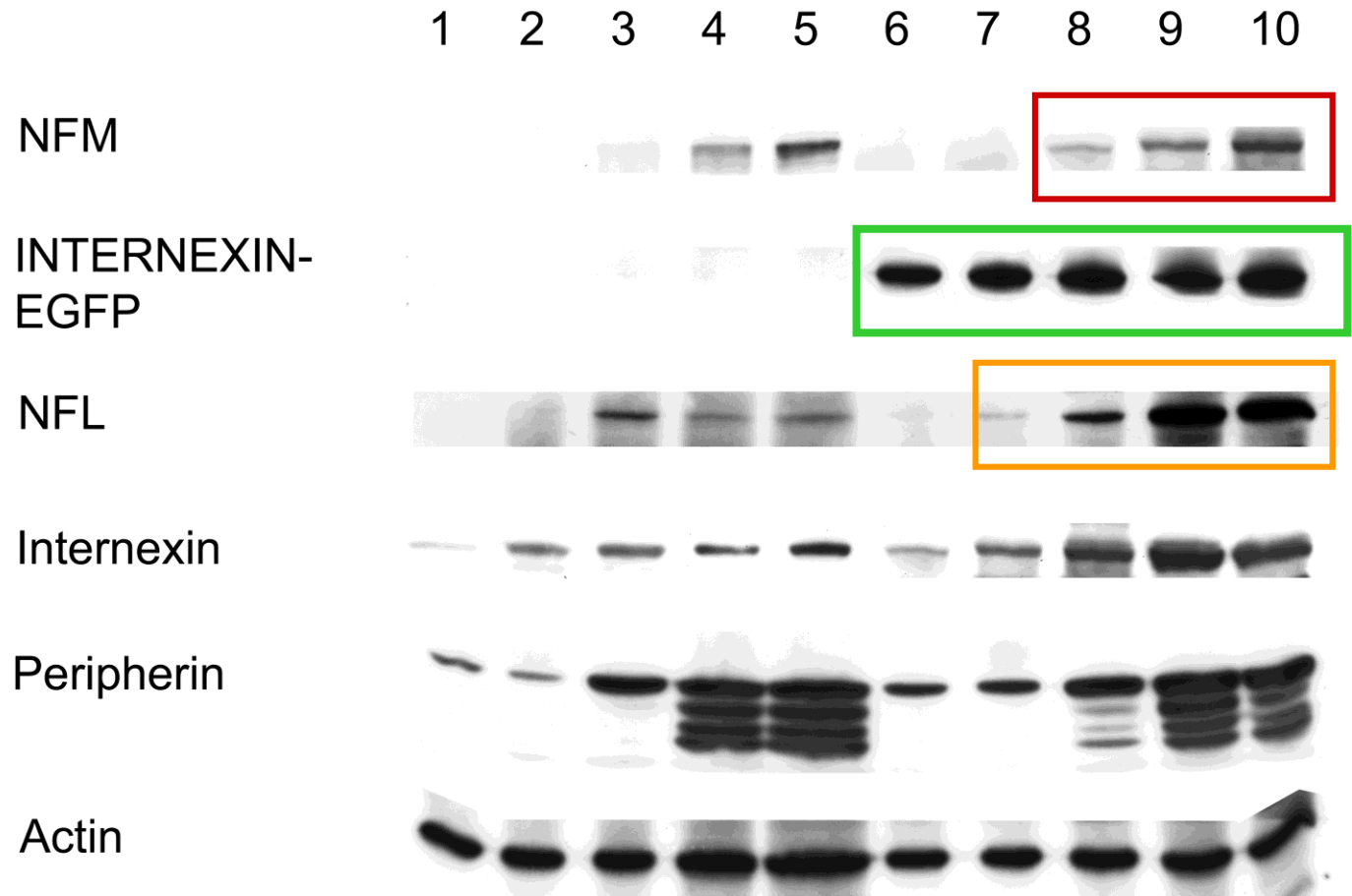
1. Control, Day 0
2. Control + NGF, Day 1
3. Control + NGF, Day 3
4. Control + NGF, Day 7
5. Control + NGF, Day 10

B



1. pINT-EGFP, Day 0
2. pINT-EGFP + NGF, Day 1
3. pINT-EGFP + NGF, Day 3
4. pINT-EGFP + NGF, Day 7
5. pINT-EGFP + NGF, Day 10

Western Blot



1. Control, Day 0
2. Control + NGF, Day 1
3. Control + NGF, Day 3
4. Control + NGF, Day 7
5. Control + NGF, Day 10

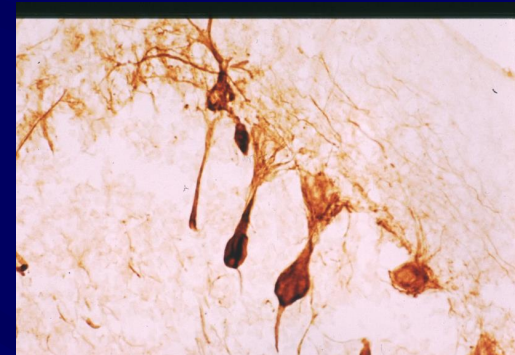
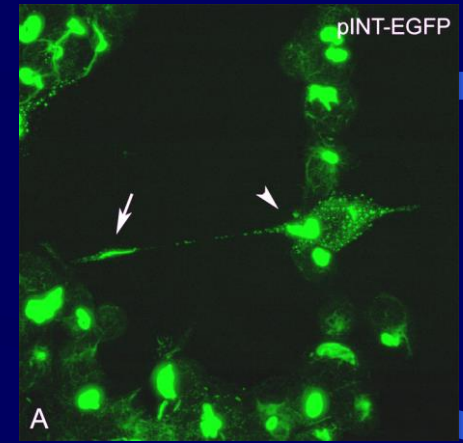
6. pINT-EGFP, Day 0
7. pINT-EGFP + NGF, Day 1
8. pINT-EGFP + NGF, Day 3
9. pINT-EGFP + NGF, Day 7
10. pINT-EGFP + NGF, Day 10

Summary II

1. Overexpression of pINT-EGFP enhances neurite outgrowth, it could be suggested that internexin may play an important role in early neuronal differentiation.
2. Internexin may regulate the expression of other neurofilaments during neuronal development, since overexpressed internexin-EGFP enhanced the expression of NF-L and NF-M.

Cell Death vs. α -internexin Overexpression

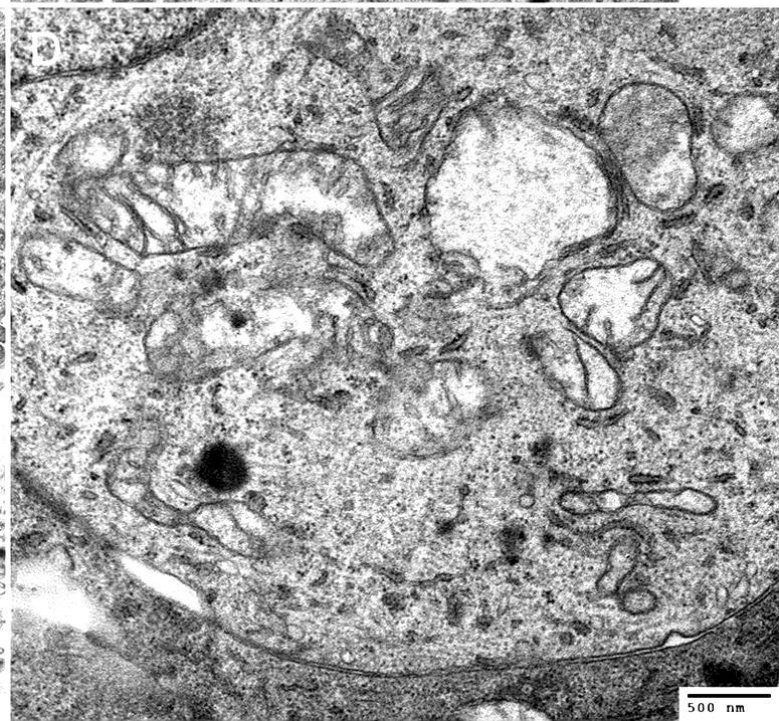
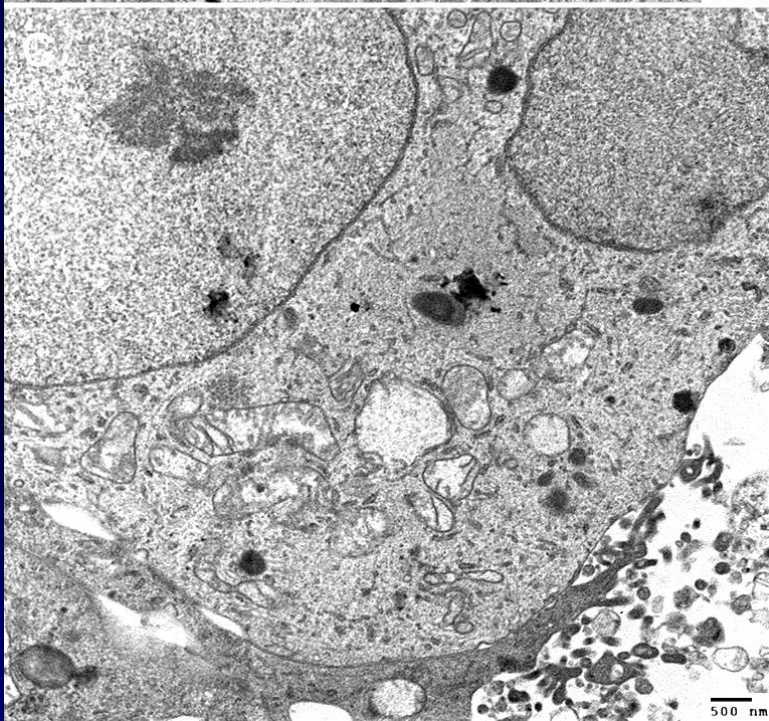
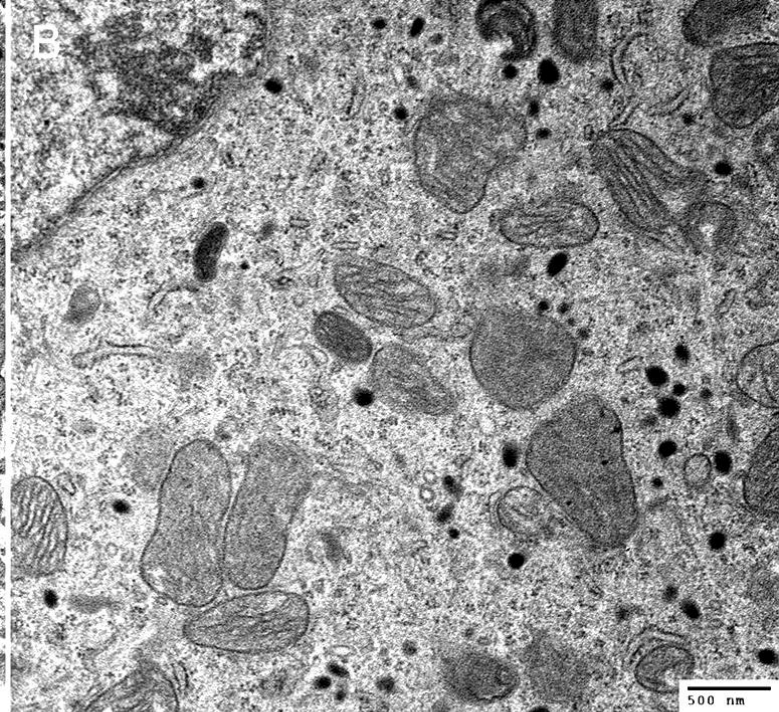
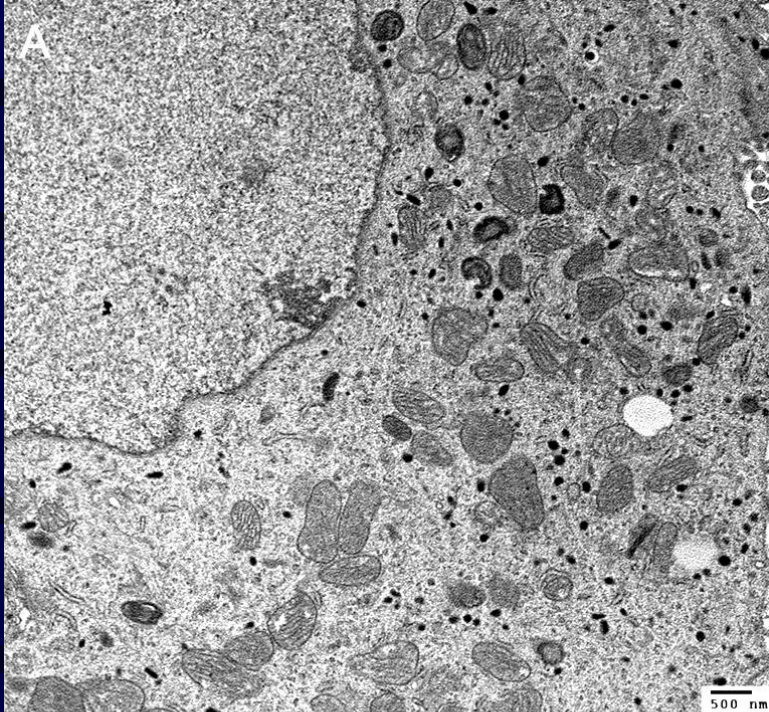
- From our observations, cells transfected with **pINT-EGFP** were found obviously detached from the culture plates after 5-day NGF induction.
- α -internexin-overexpressing transgenic mice show neuronal dysfunction, progressive neurodegeneration and **loss of neurons** in the neocortex, thalamus, and cerebellum of aged transgenic mice (Ching et al., 1999).



Ultrastructure
patterns
(5-day NGF
induction)

Control
cells

pINT-EGFP
tranfected
cells

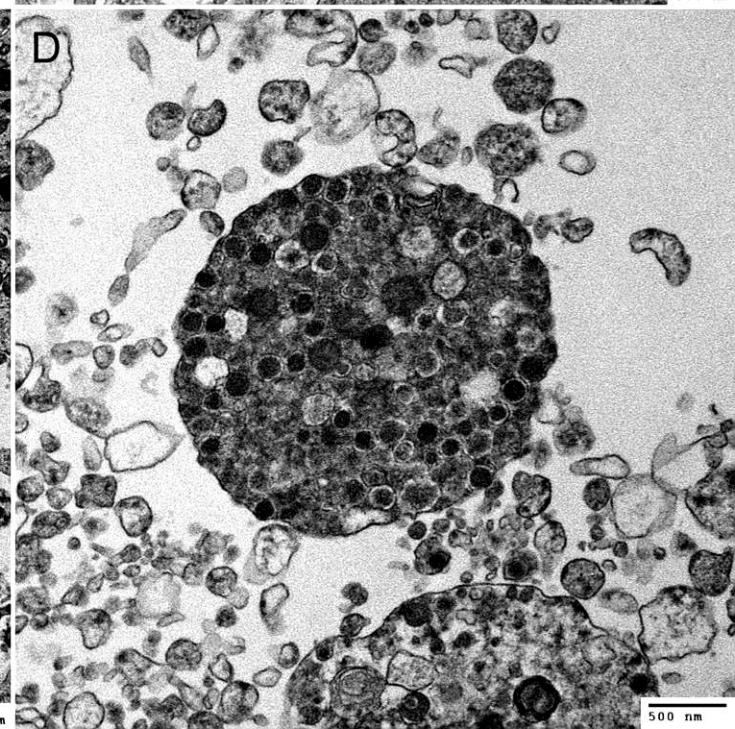
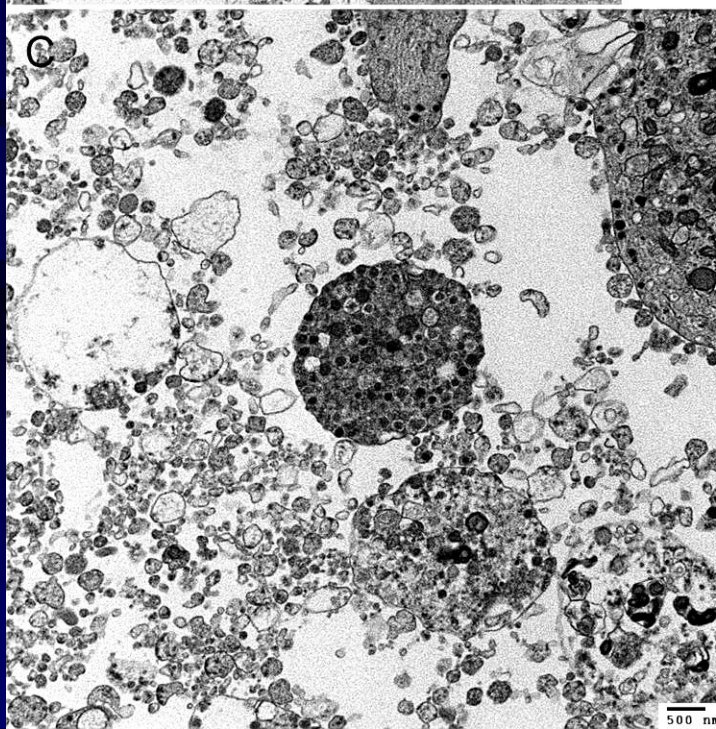
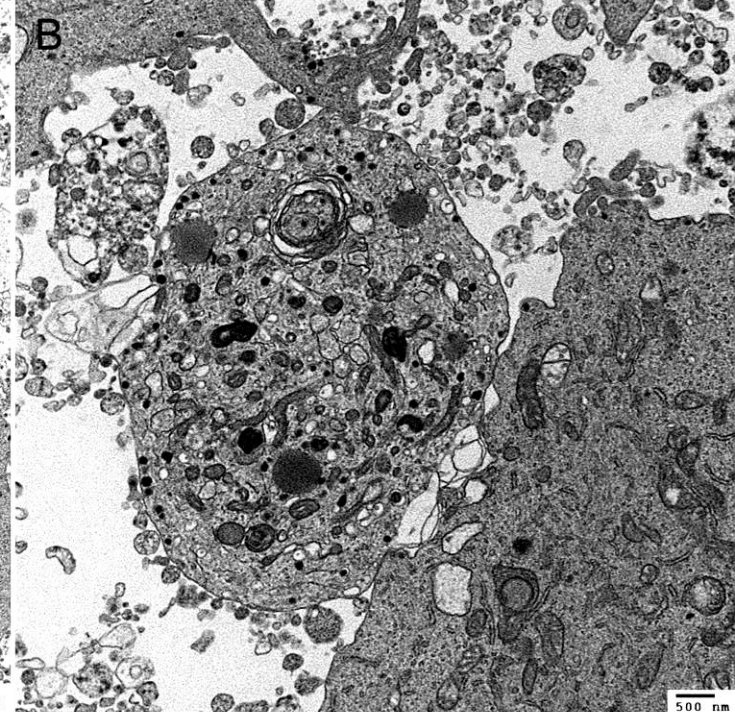
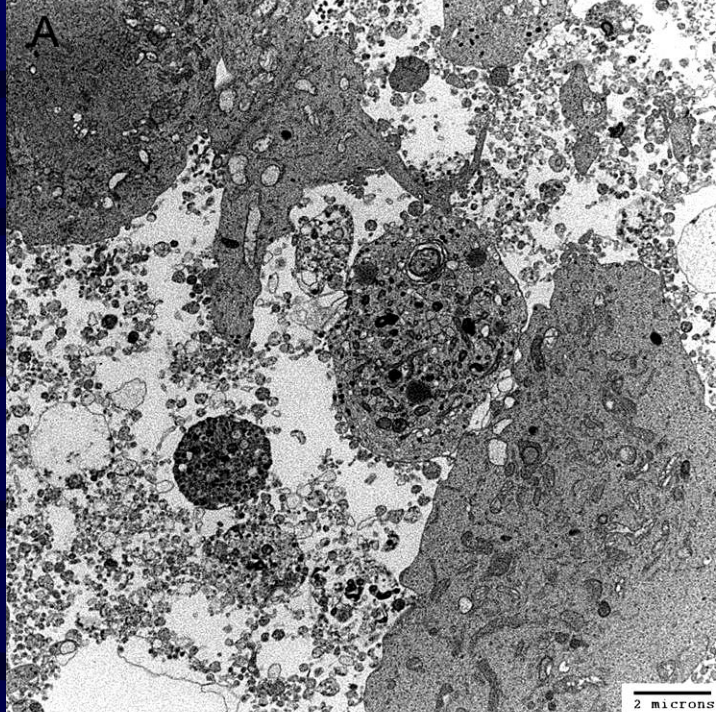


Ultrastructural
patterns
(5-day NGF)

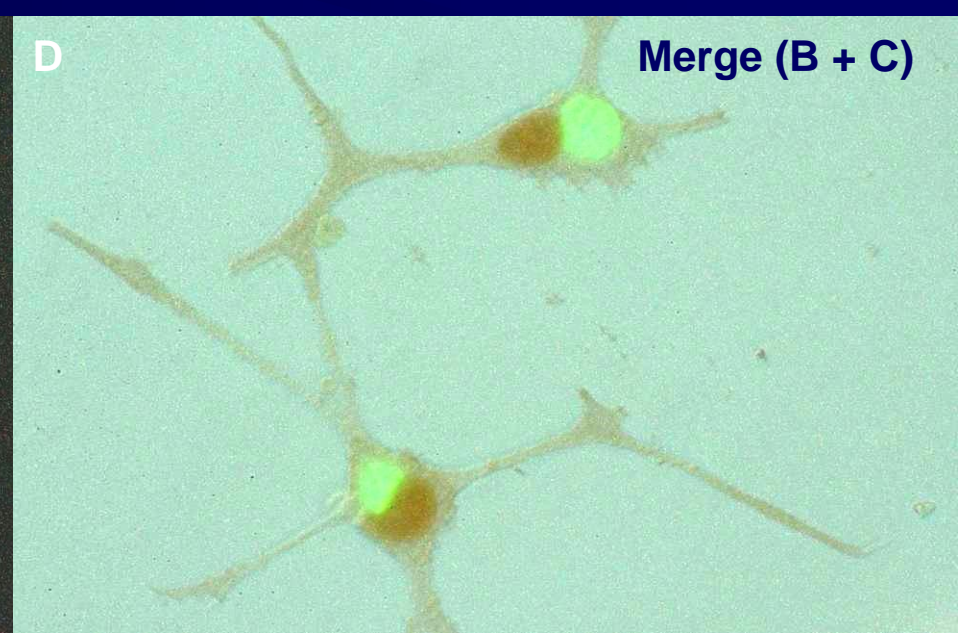
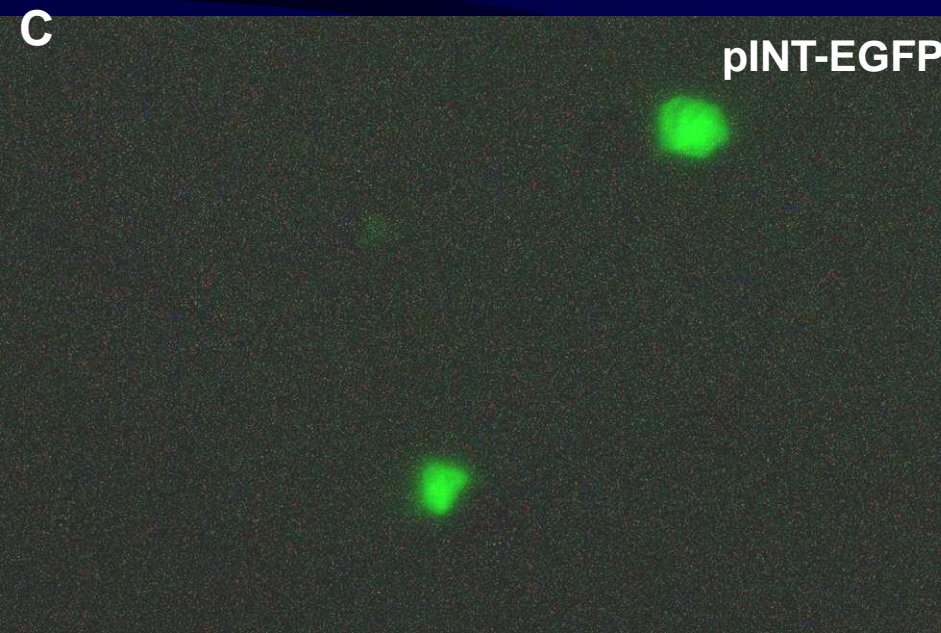
pINT-EGFP
transfected
cells

Degenerating
neurite

Degenerated
neurite



TUNEL assay at the 5th day of NGF induction



Summary III

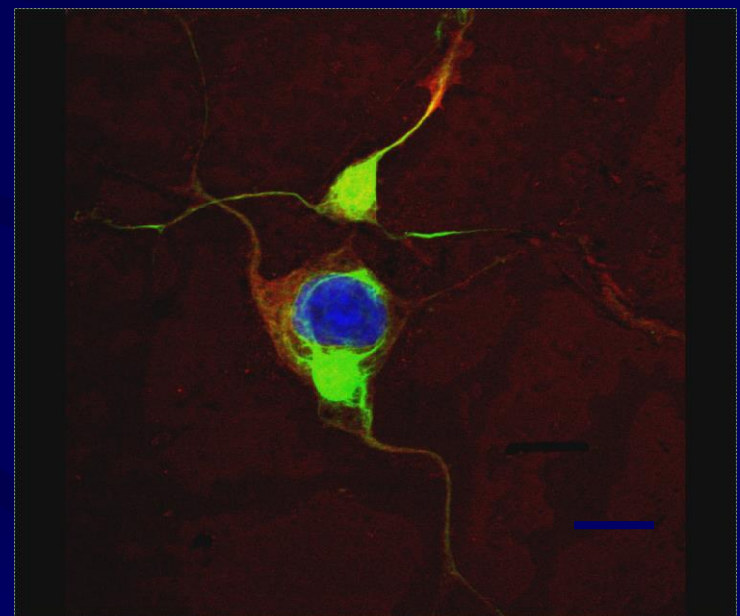
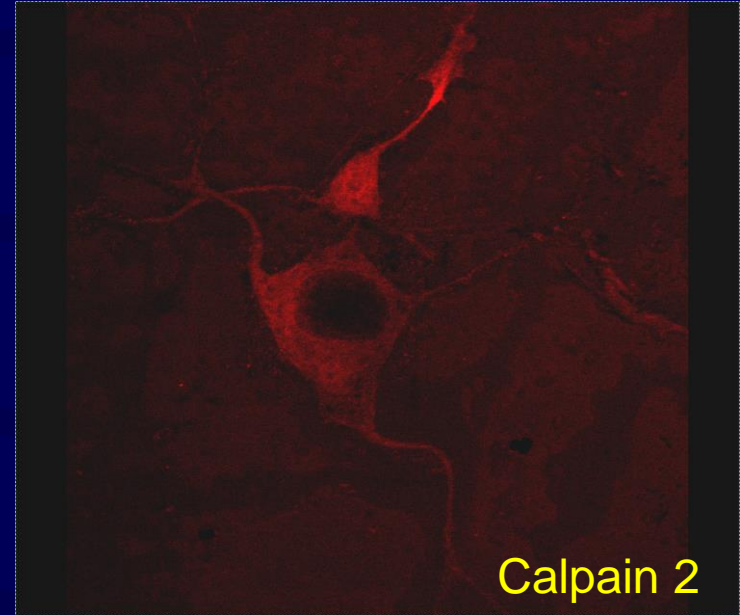
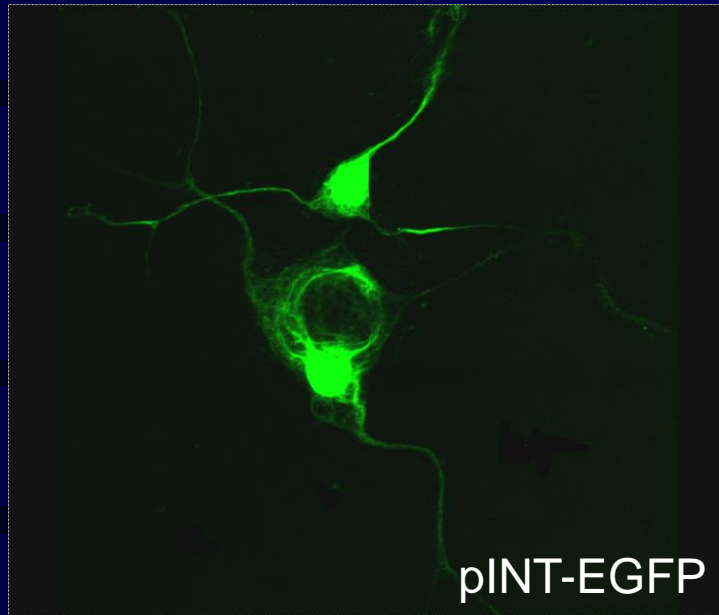
1. Overexpression of **pINT-EGFP** may induce swelling mitochondria and massive intermediate filament accumulations in cell bodies and processes.
2. Early events of apoptosis could be characterized in the **pINT-EGFP** transfected cells by the caspase activity and TUNEL positive patterns.

Ongoing Study: Microarray data (by Center of Genomic Medicine):

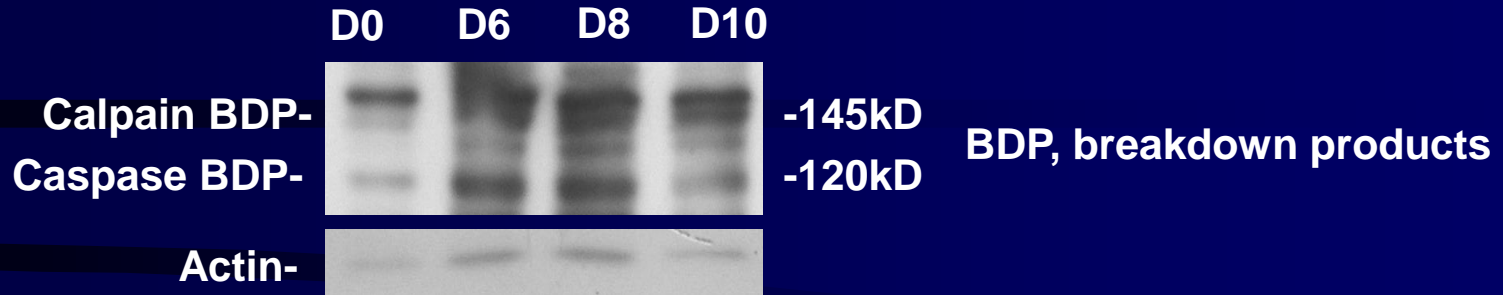
INT +NGF D6 vs. PC12 +NGF D6

Neuronal proteins		
internexin, alpha	UP	414.961
neurofilament 3, medium	UP	4.85568
neurofilament, light polypeptide	UP	6.28745
nestin	UP	3.34591
peripherin 1	UP	2.25822
microtubule-associated protein 1 light chain 3 alpha	UP	2.48439
Microtubule-associated proteins 1A/1B light chain 3	DOWN	3.38617
synapsin II	DOWN	3.02512
Regulation of cell cycle		
Cdk5 and Abl enzyme substrate 1 (predicted)	DOWN	3.12932
CDK5 regulatory subunit associated protein 3	DOWN	2.48071
mitogen activated protein kinase 3 (Erk1)	DOWN	2.68089
Calpain family of proteases		
calpain 1	UP	2.55472
calpain 2	UP	2.31951
Inhibitor of calpain		
calpastatin	UP	2.50921
Caspase family of proteases		
caspase 6	UP	2.19194
caspase 9	UP	3.8618

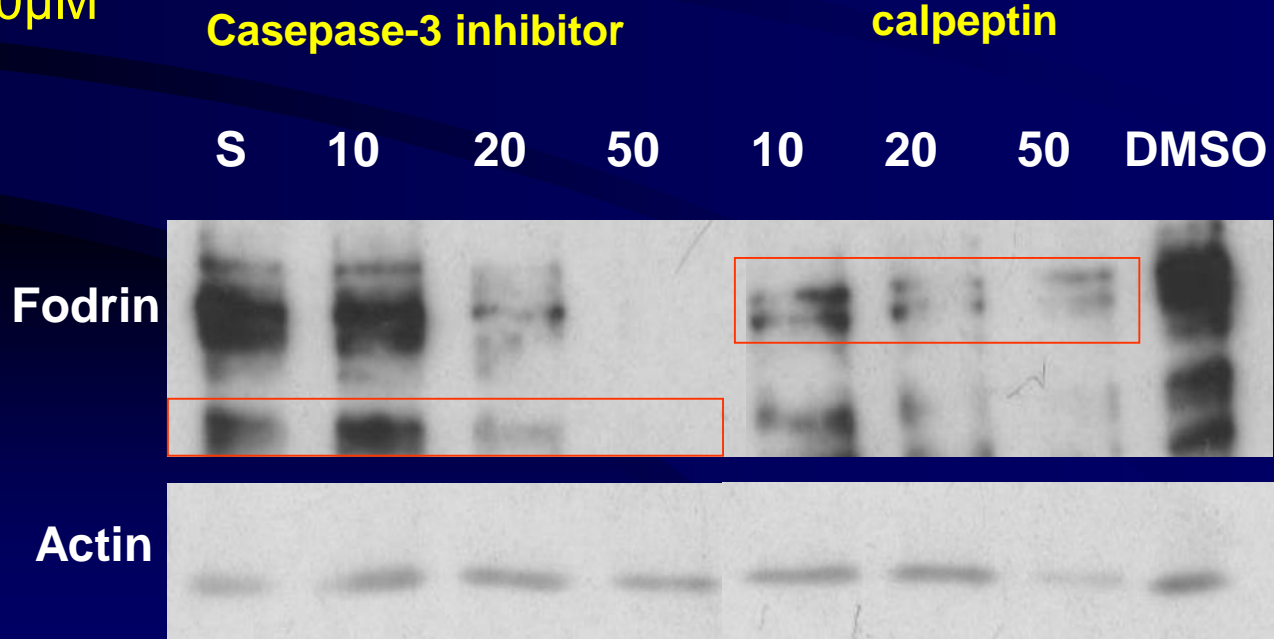
pINT-EGFP clone with NGF for 6 days



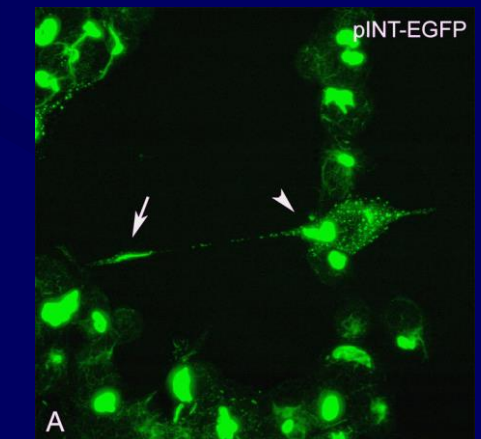
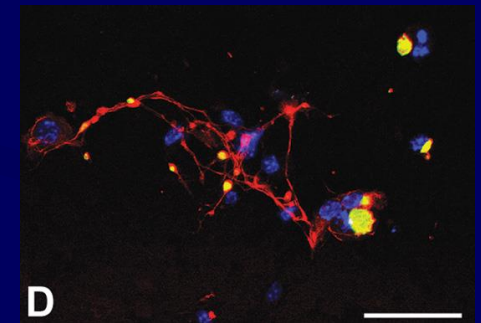
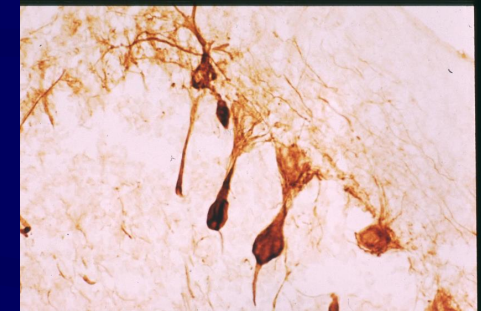
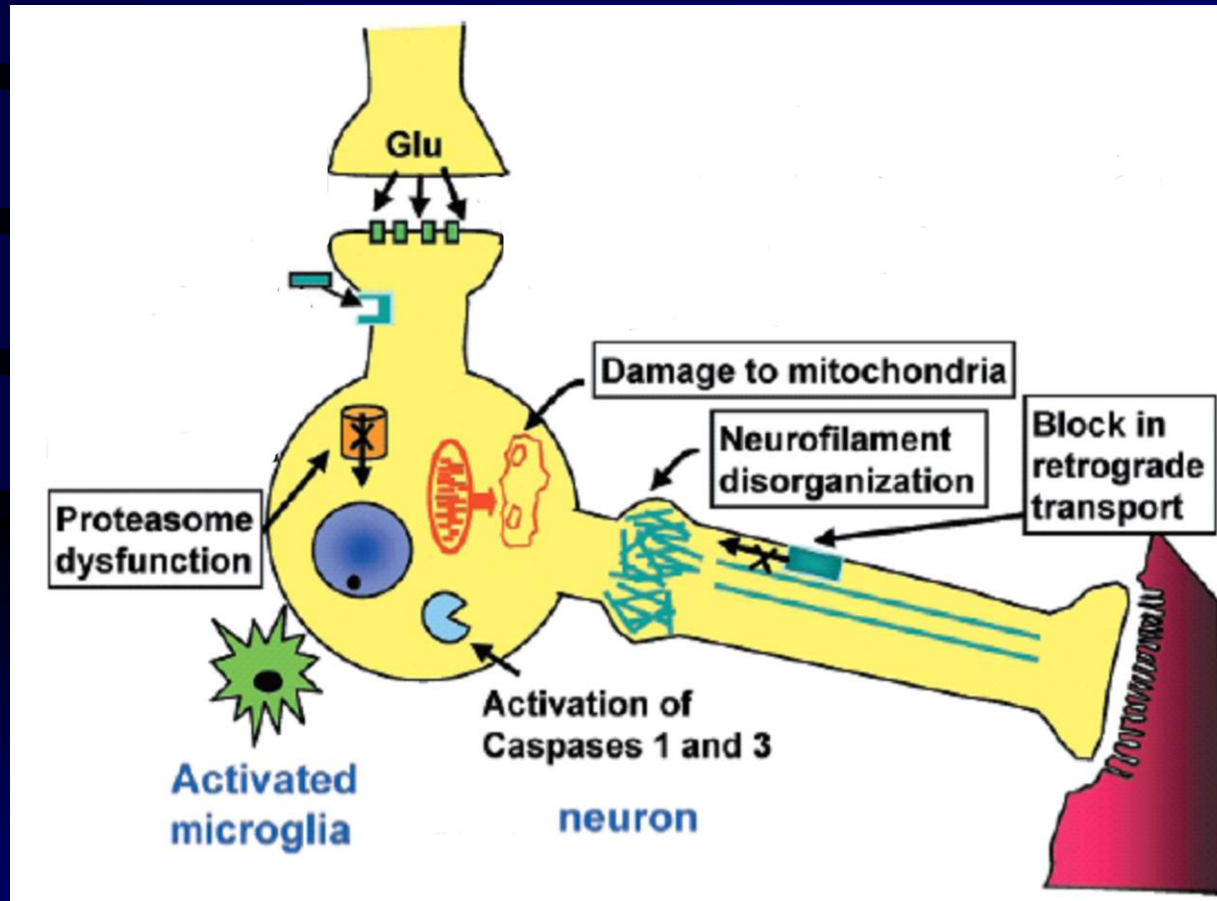
pINT-EGFP



Casepase-3 inhibitor: 20μM
Calpeptin: 20μM



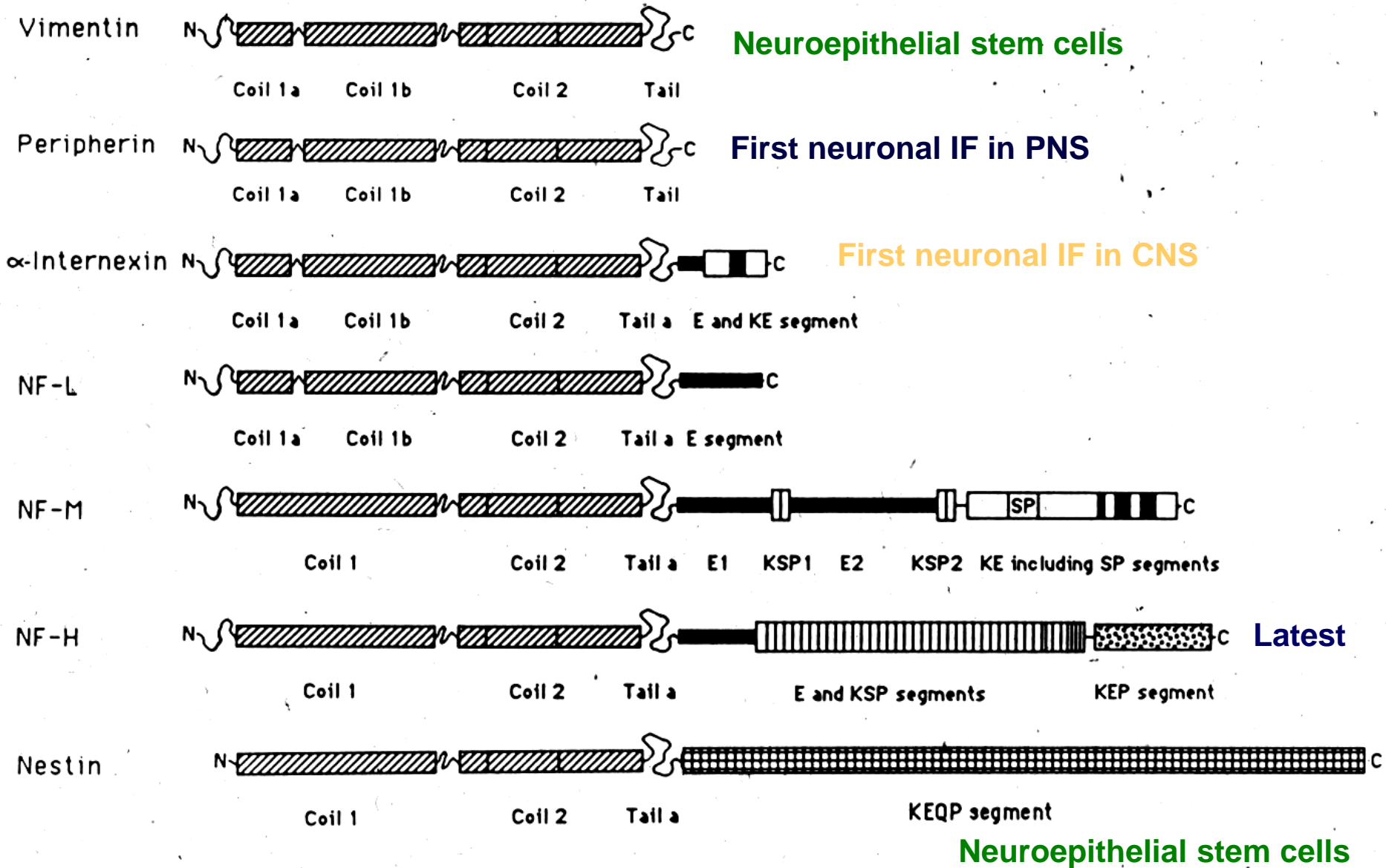
Abnormal accumulation of α -internexin and other cytoskeletal components may impair the axonal transport and subsequently turn on the cascade of neuronal apoptosis during development.



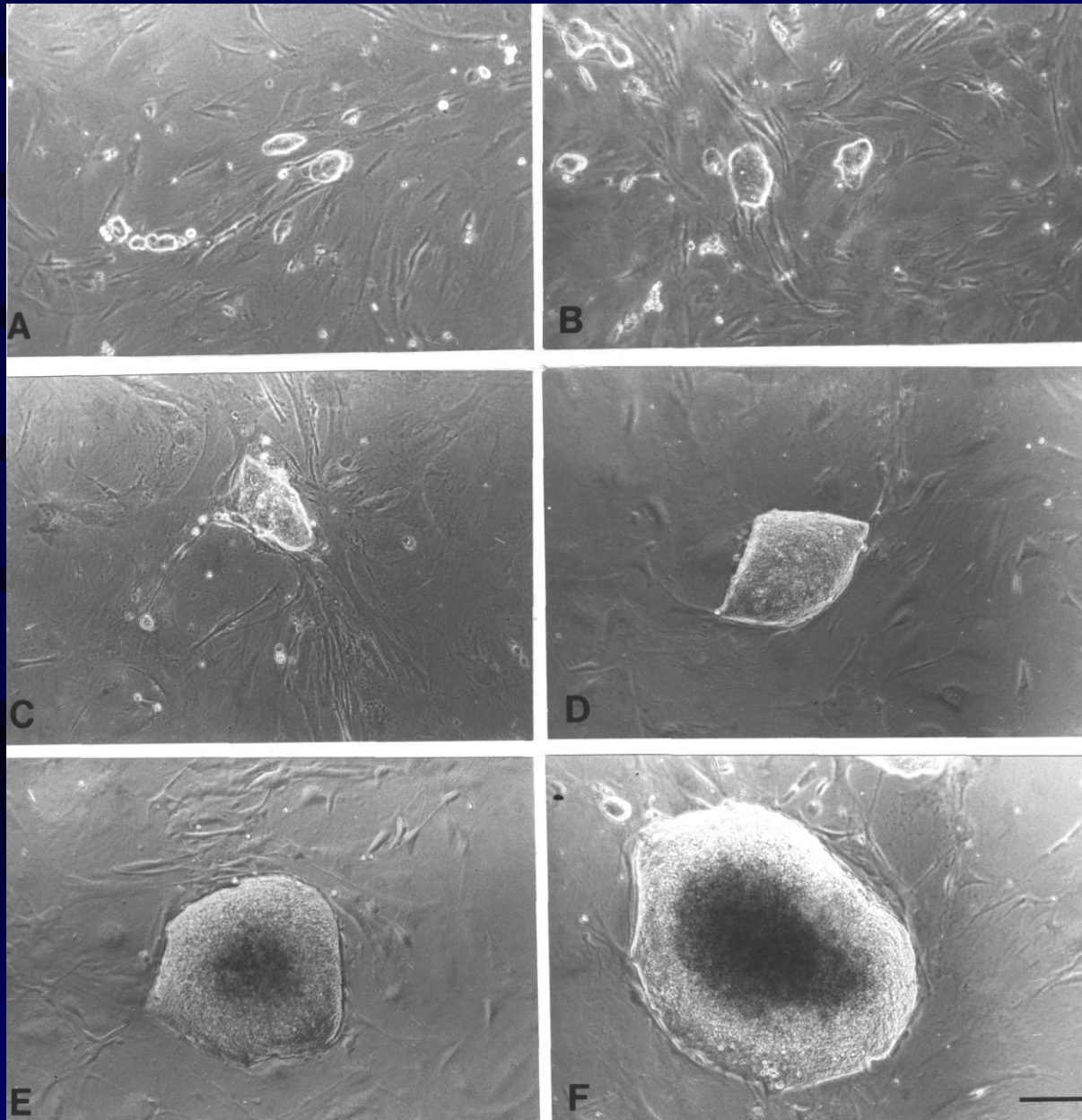
Thank you for your attention!



Seven Intermediate Filament Proteins in Neural Differentiation



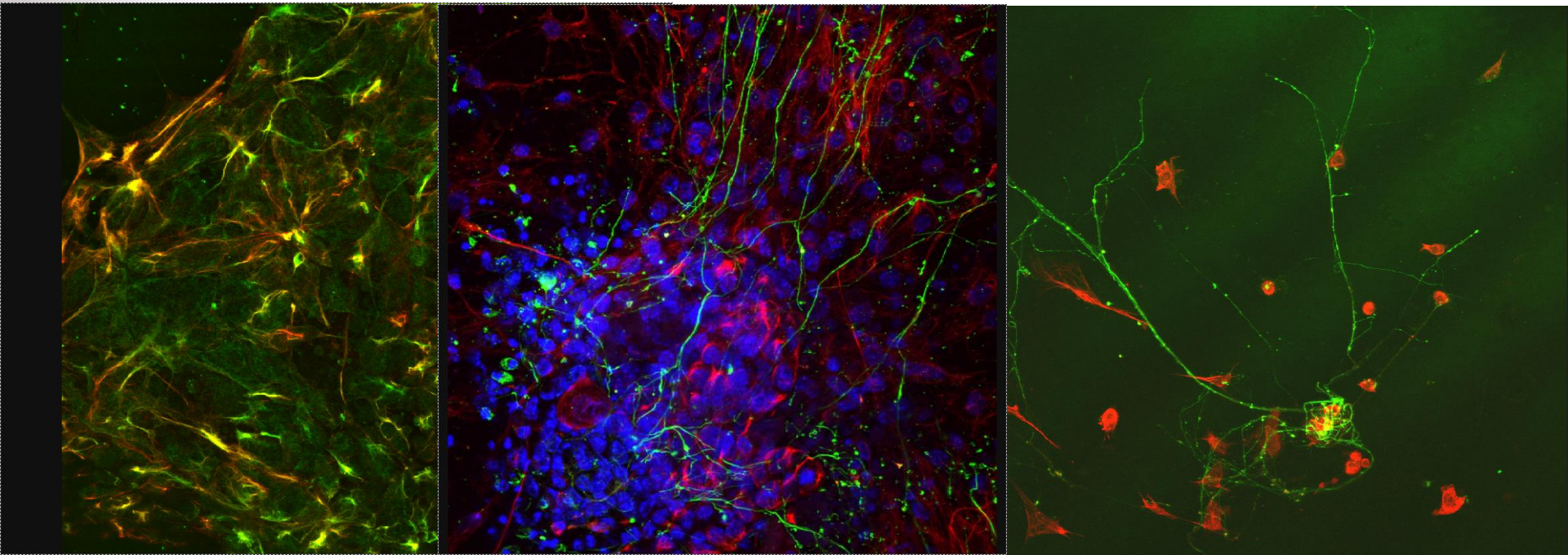
Neural Differentiation of Mouse Embryonic Stem Cells



Embryonic Stem Cells



Figure 1.2 Stem cells derived from the blastocyst of an activated monkey egg can differentiate into functioning neurons. (Modified from Cibelli, J. B., et al. [2002]. Parthenogenetic stem cells in nonhuman primates. *Science* 295: 819.)



Mouse R1 ES cells
plated on a feeder layer of primary mouse embryonic fibroblasts
(in DMEM with 20% FBS supplemented by 10 ng/ml recombinant human LIF)

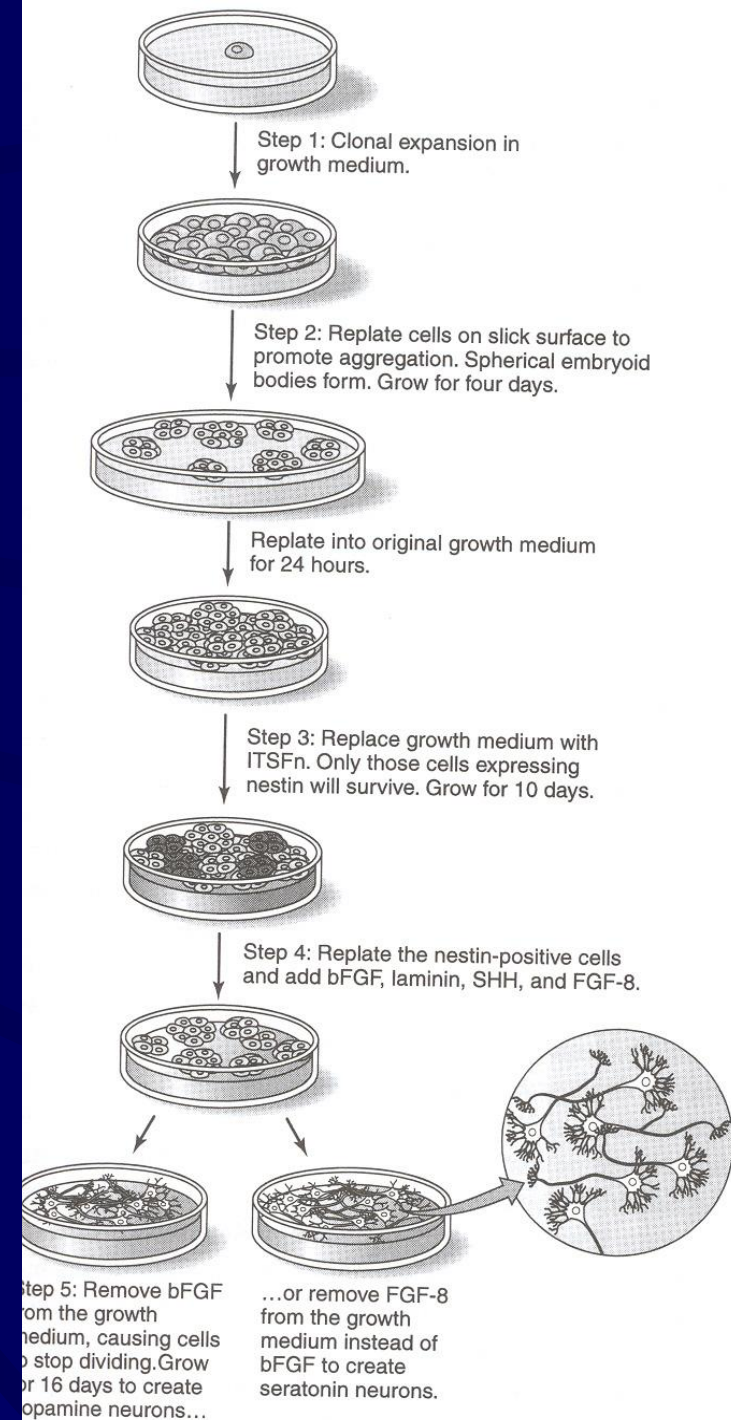
↓
Embryoid body formation:
Cultivation of 5×10^4 /ml ES cells in DMEM+20% FBS in petri dish for 4 days

↓
EBs plated and attached onto gelatin-coated cover-slide

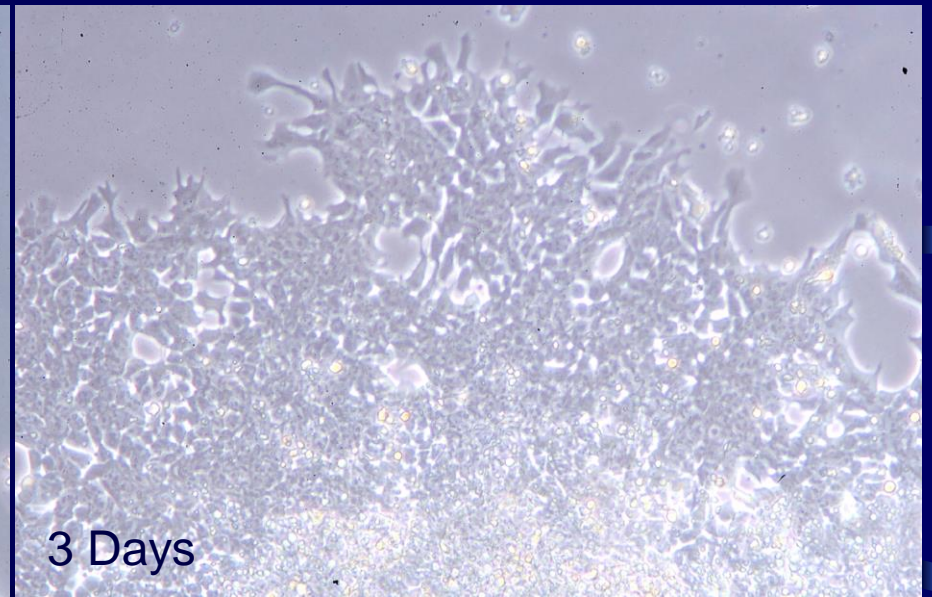
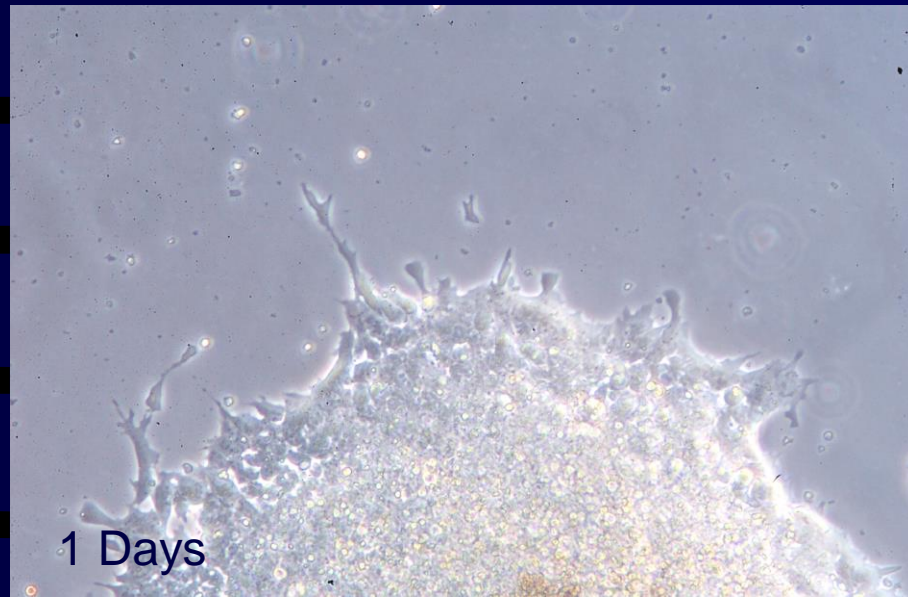
↓
DMEM/F12 media supplemented with ITSF (5 ug/ml Insulin, 50 ug/ml Transferrin, 30 nm sodium Selenite, 5 ug/ml Fibronectin)

↓
After 7 days, media changed into DMEM/F12 supplemented with N2 (bFGF)

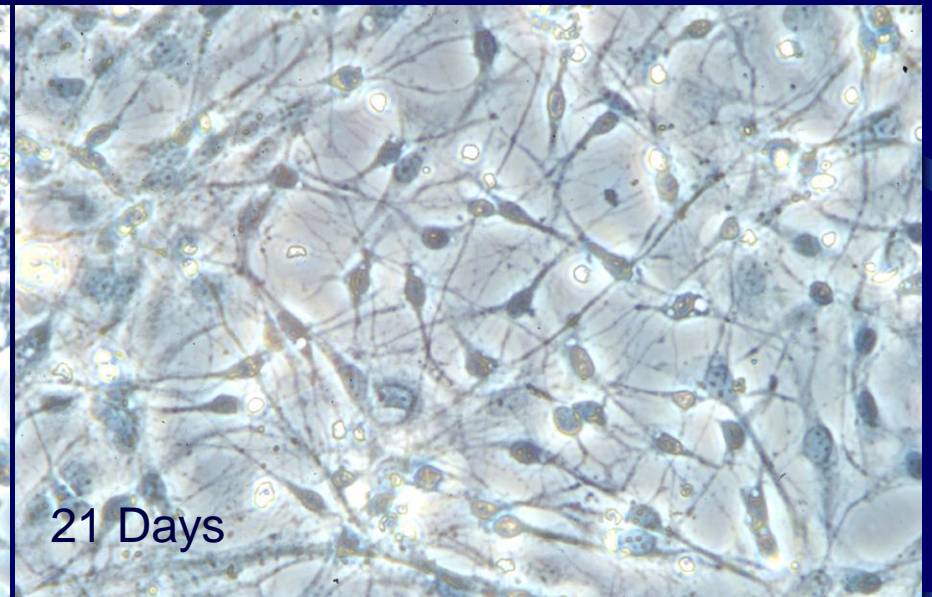
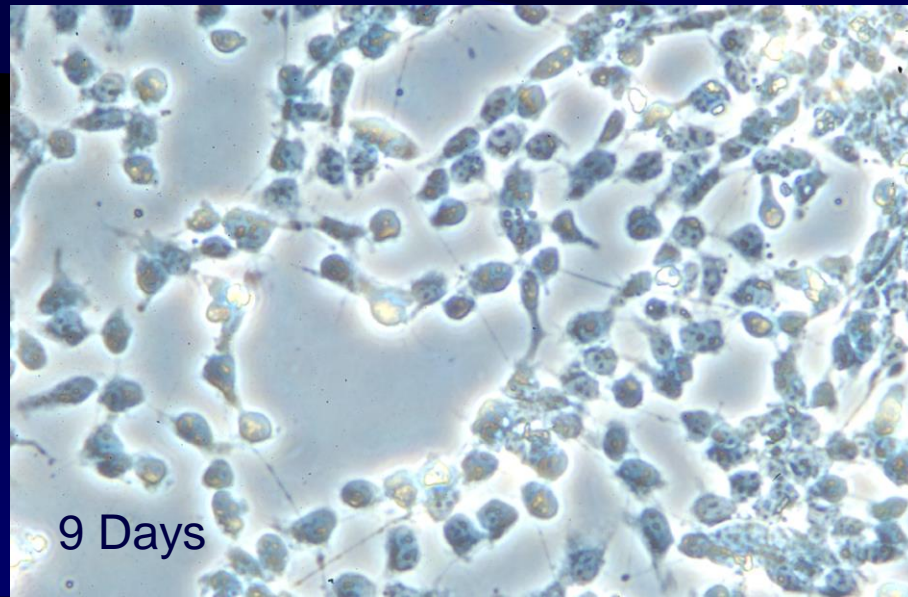
↓
After 7 days, media changed into DMEM/F12 supplemented with N2



Embryoid body in DMEM/F12 media supplemented with ITSF



Differentiating cells after neural induction with N2 supplement



Intermediate Filament Proteins are good markers for determining the differentiation status of neural stem cells

Neural Stem Cells: Nestin, Vimentin

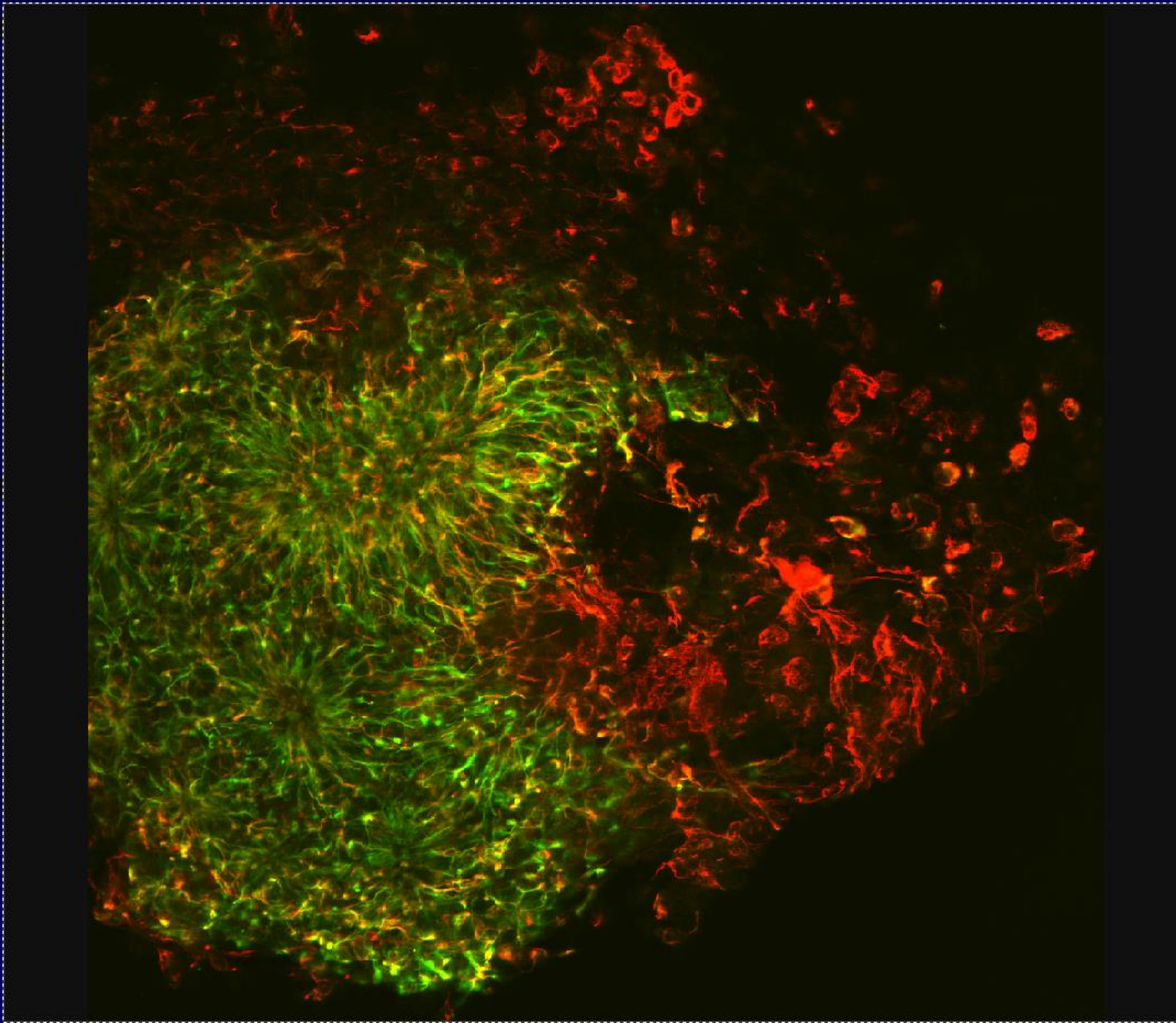
Glial cells: Vimentin, GFAP

Post-mitotic Young Neurons
Internexin, Peripherin

*Muscular cells :
Nestin, Vimentin, and Desmin

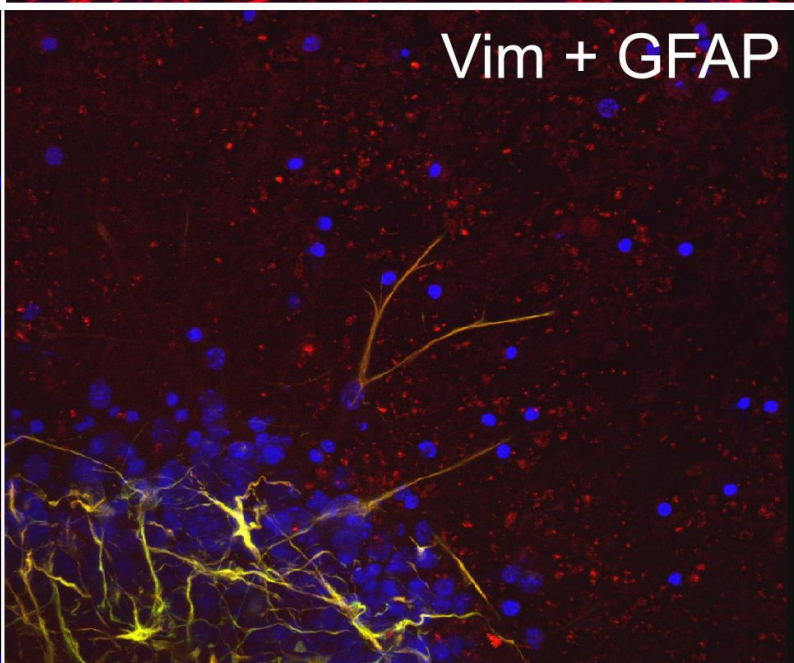
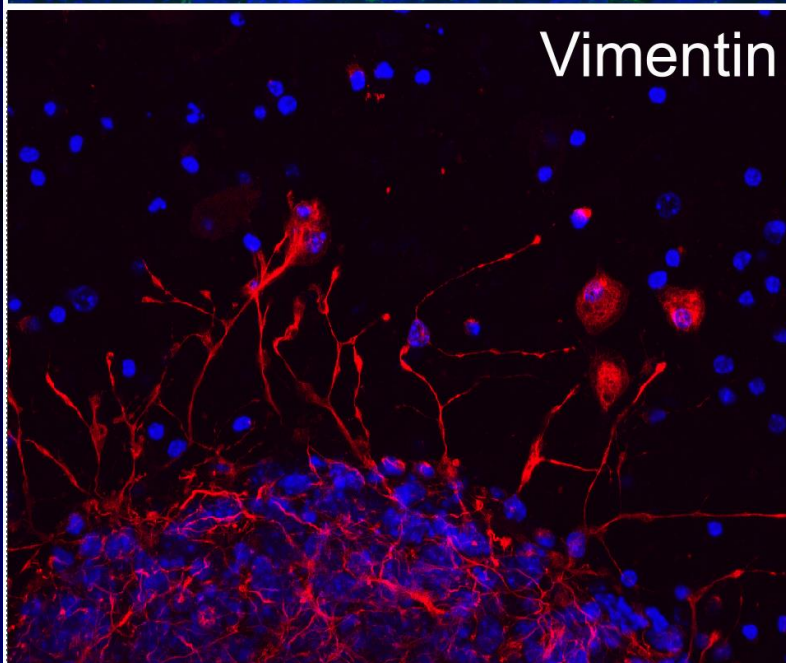
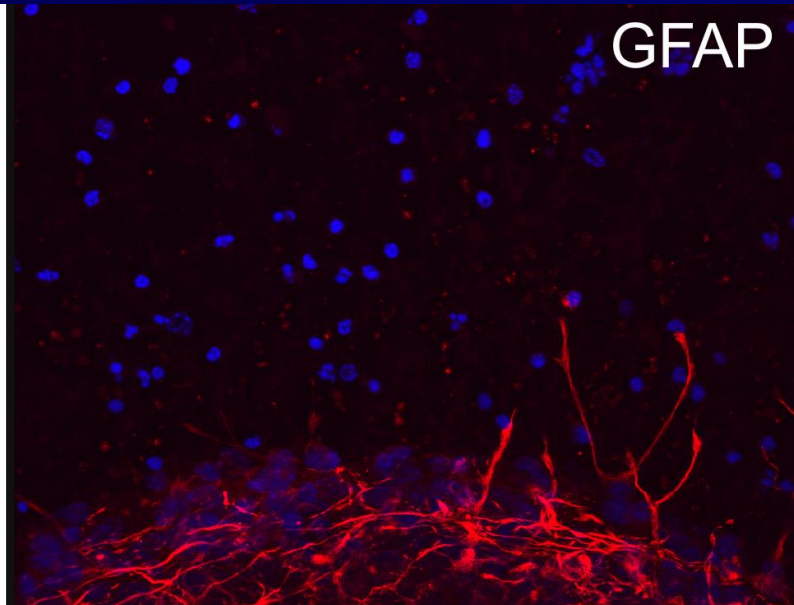
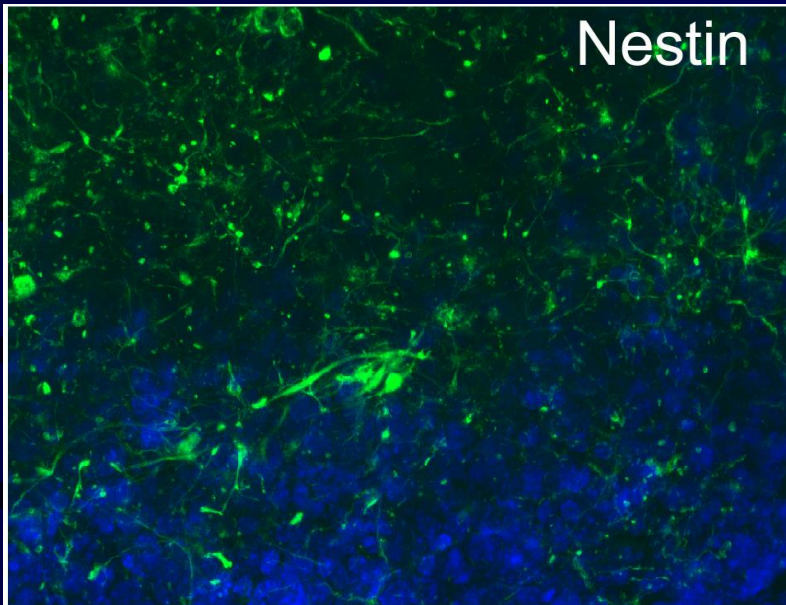
Differentiated Mature Neurons
Internexin, Peripherin
Neurofilament triplet Proteins
(NF-L, NF-M, and NF-H)

Embryoid body in DMEM/F12 media supplemented with ITSF for 7 days

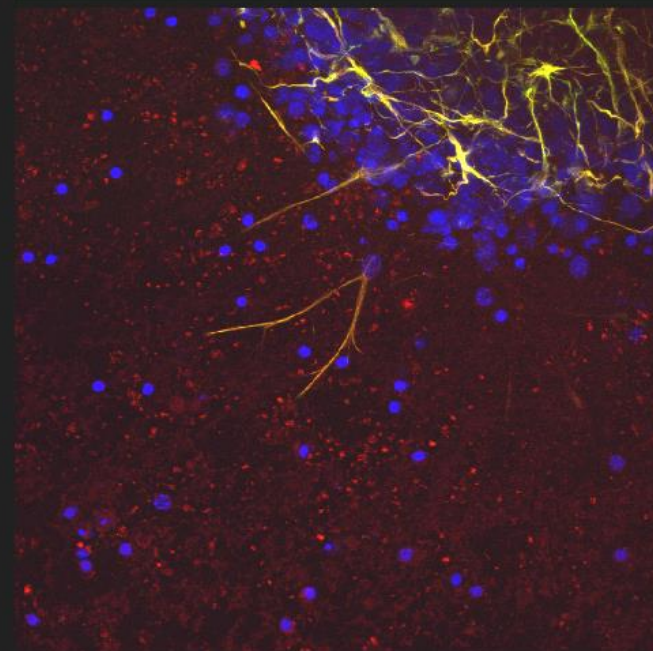
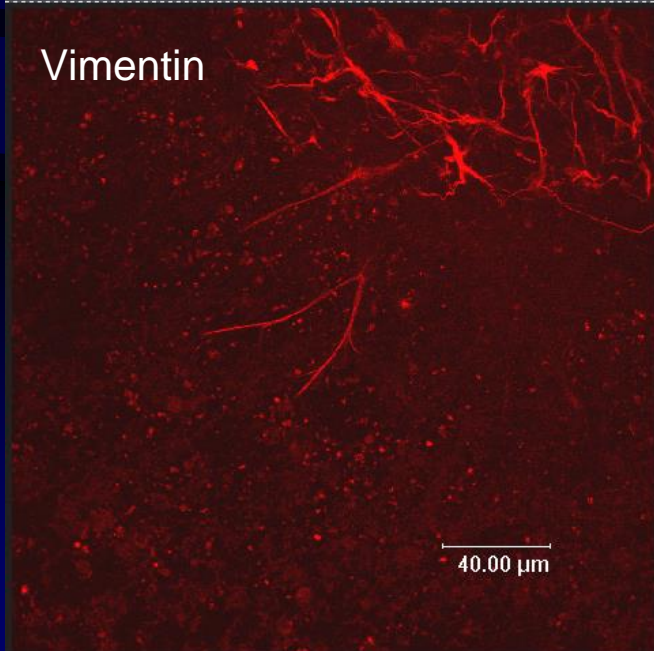
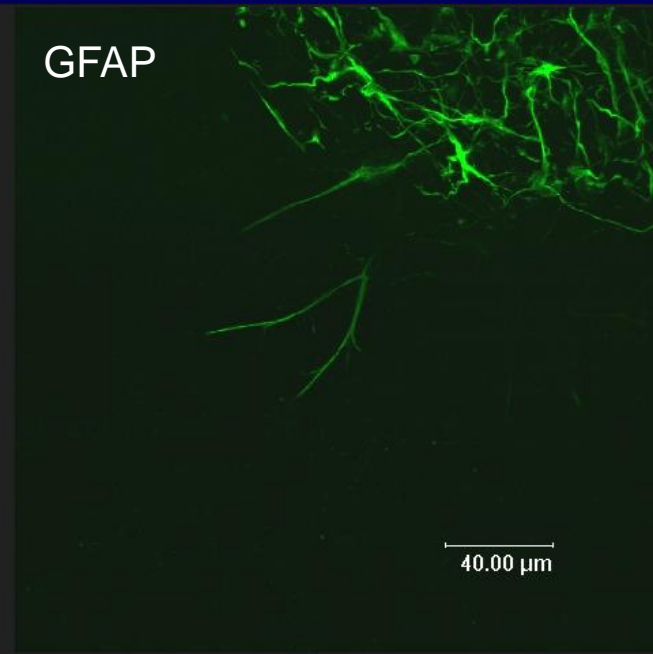
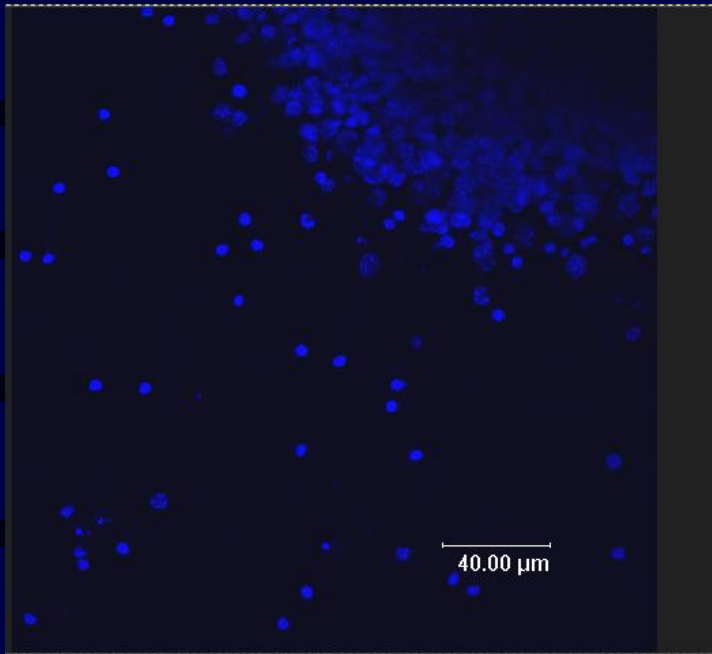


Green: Nestin
Red: Vimentin

Embryoid body in DMEM/F12 media supplemented with ITSF for 7 days



GFAP and Vimentin co-expressed in the differentiating glial cells (17days)



Intermediate Filament Proteins are good markers for determining the differentiation status of neural stem cells

Neural Stem Cells: Nestin, **Vimentin**

```
graph TD; A["Neural Stem Cells: Nestin, Vimentin"] -.-> B["Glial cells: Vimentin, GFAP"]; A --> C["Post-mitotic Young Neurons  
Internexin, Peripherin"]; C --> D["Differentiated Mature Neurons  
Internexin, Peripherin  
Neurofilament triplet Proteins  
(NF-L, NF-M, and NF-H)"]; A -.-> E["*Muscular cells:  
Nestin, Vimentin, and Desmin"];
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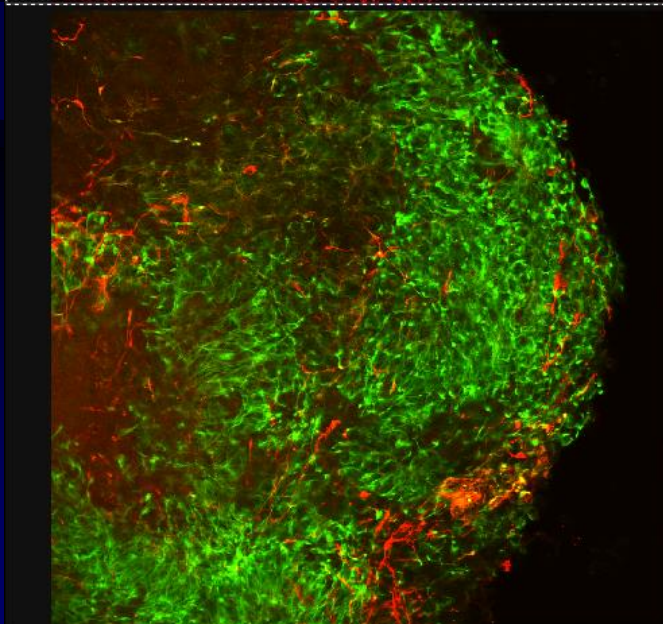
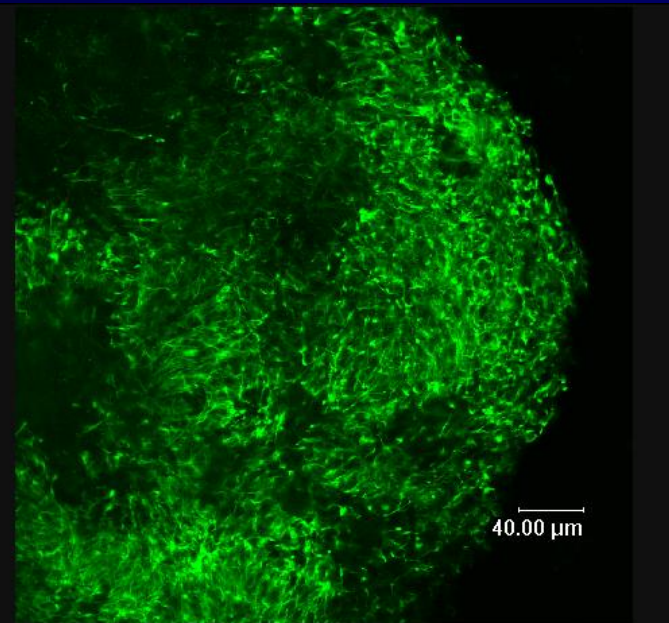
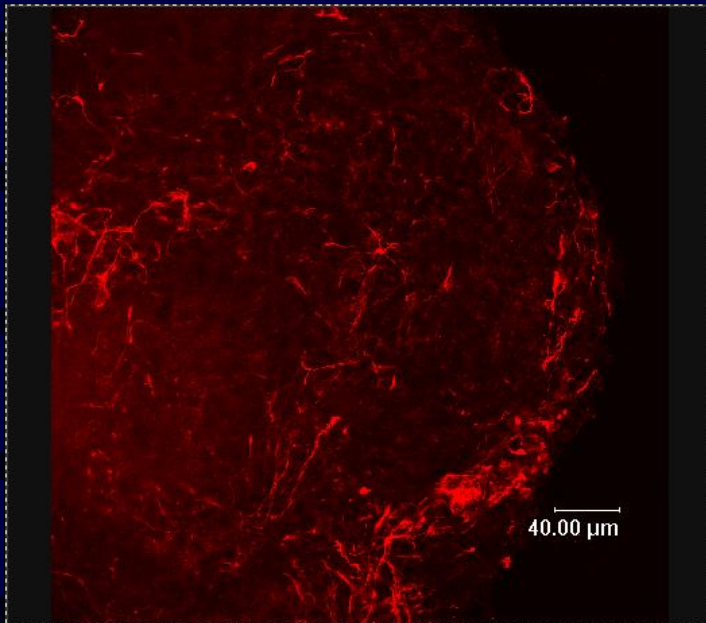
Glial cells: Vimentin, GFAP

Post-mitotic Young Neurons
Internexin, Peripherin

*Muscular cells:
Nestin, **Vimentin**, and **Desmin**

Differentiated Mature Neurons
Internexin, Peripherin
Neurofilament triplet Proteins
(NF-L, NF-M, and NF-H)

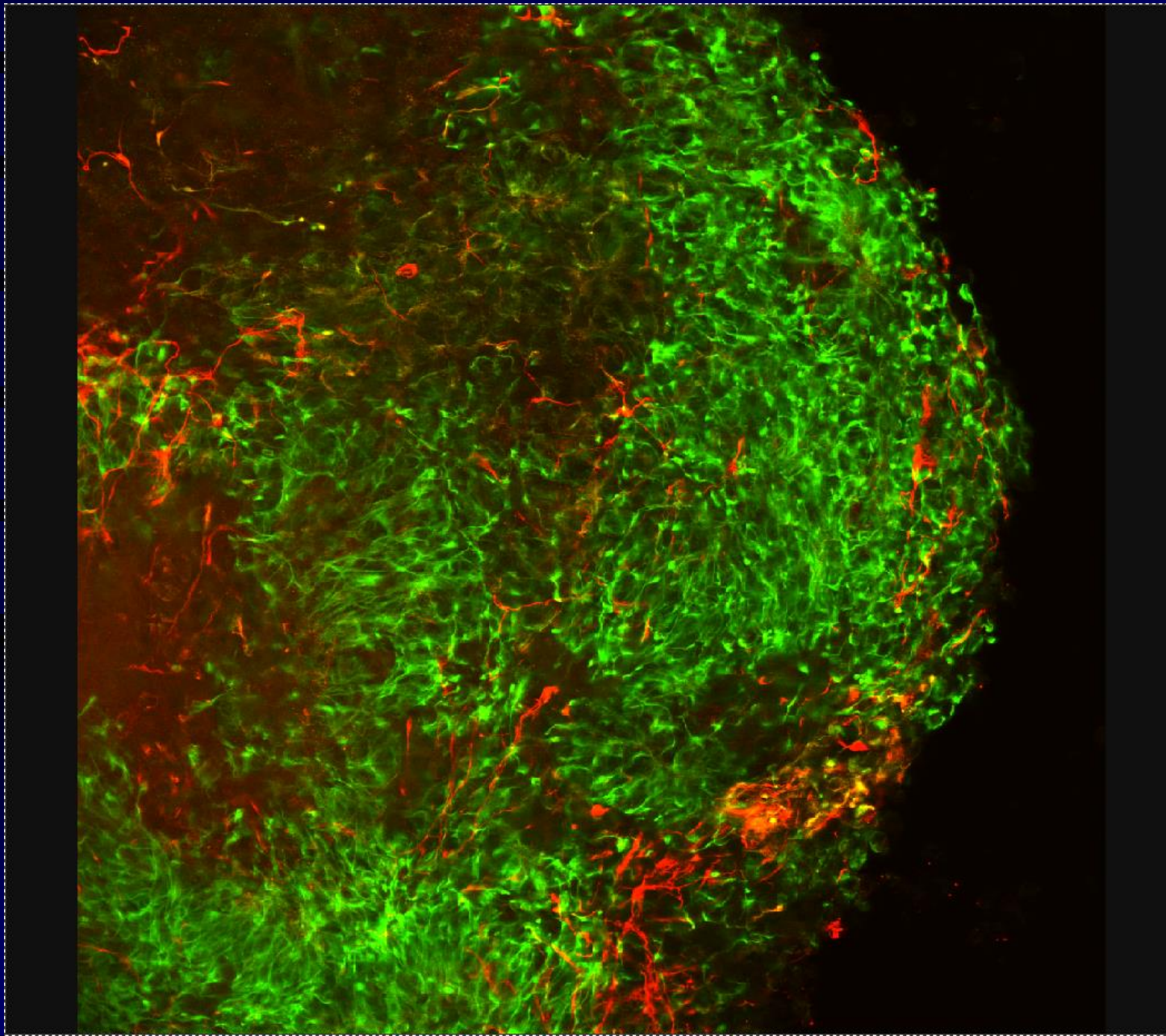
Neural induction of embryoid body for 7 days



Green: Nestin

Red: Internexin
Neuronal Marker

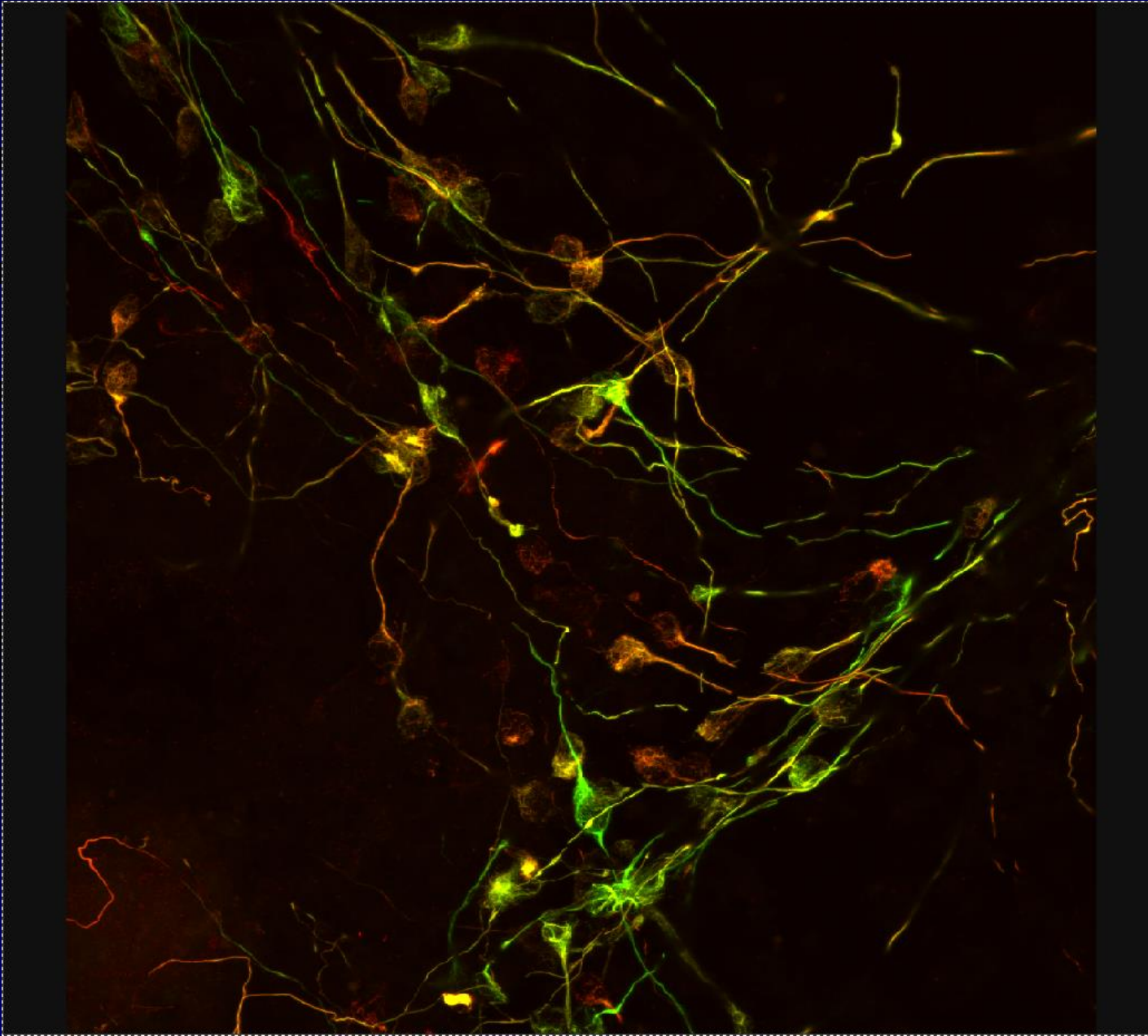
Neural induction of embryoid body for 7 days



Green: Nestin

Red: Internexin
Neuronal Marker

Neural induction of embryoid body for 7 days



Green: NF-L
Red: Internexin

Neuronal Markers

Intermediate Filament Proteins are good markers for determining the differentiation status of neural stem cells

Neural Stem Cells: Nestin, Vimentin

```
graph TD; A[Neural Stem Cells: Nestin, Vimentin] --> B[Glial cells: Vimentin, GFAP]; A --> C[Post-mitotic Young Neurons  
Internexin, Peripherin]; C --> D[Differentiated Mature Neurons  
Internexin, Peripherin  
Neurofilament triplet Proteins  
(NF-L, NF-M, and NF-H)]; E[*Muscular cells:  
Nestin, Vimentin, and Desmin];
```

Glial cells: Vimentin, GFAP

Post-mitotic Young Neurons
Internexin, Peripherin

*Muscular cells:
Nestin, Vimentin, and Desmin

Differentiated Mature Neurons
Internexin, Peripherin
Neurofilament triplet Proteins
(NF-L, NF-M, and NF-H)

MAP2A and Tau

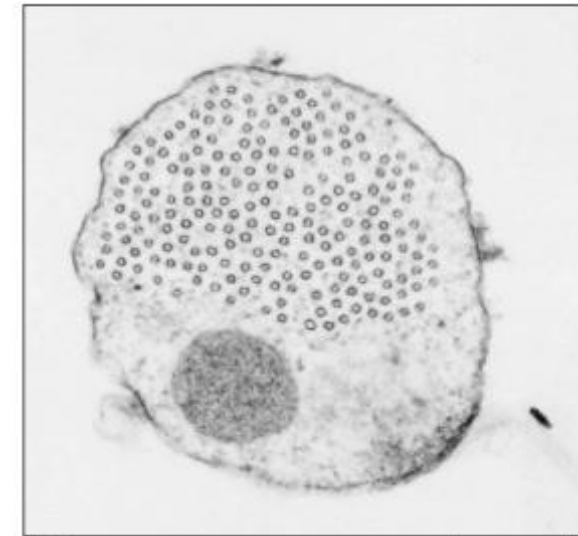
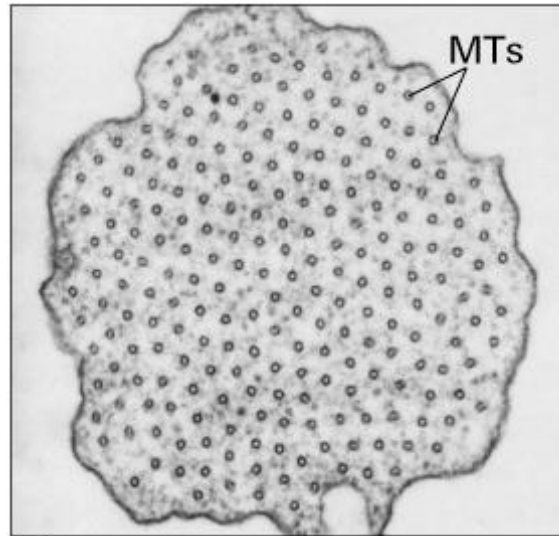
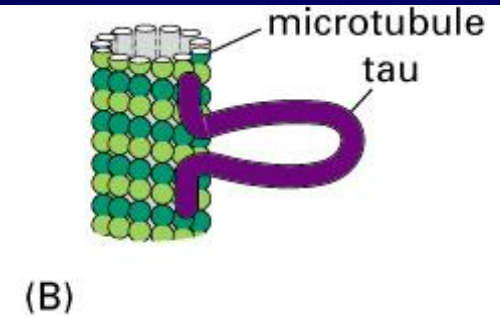
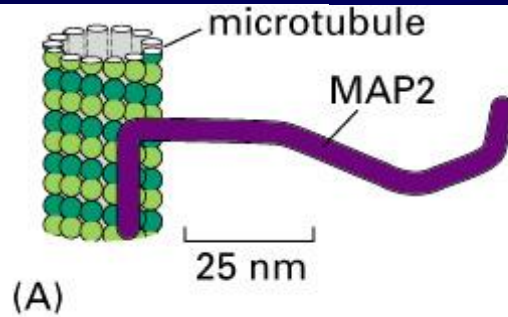
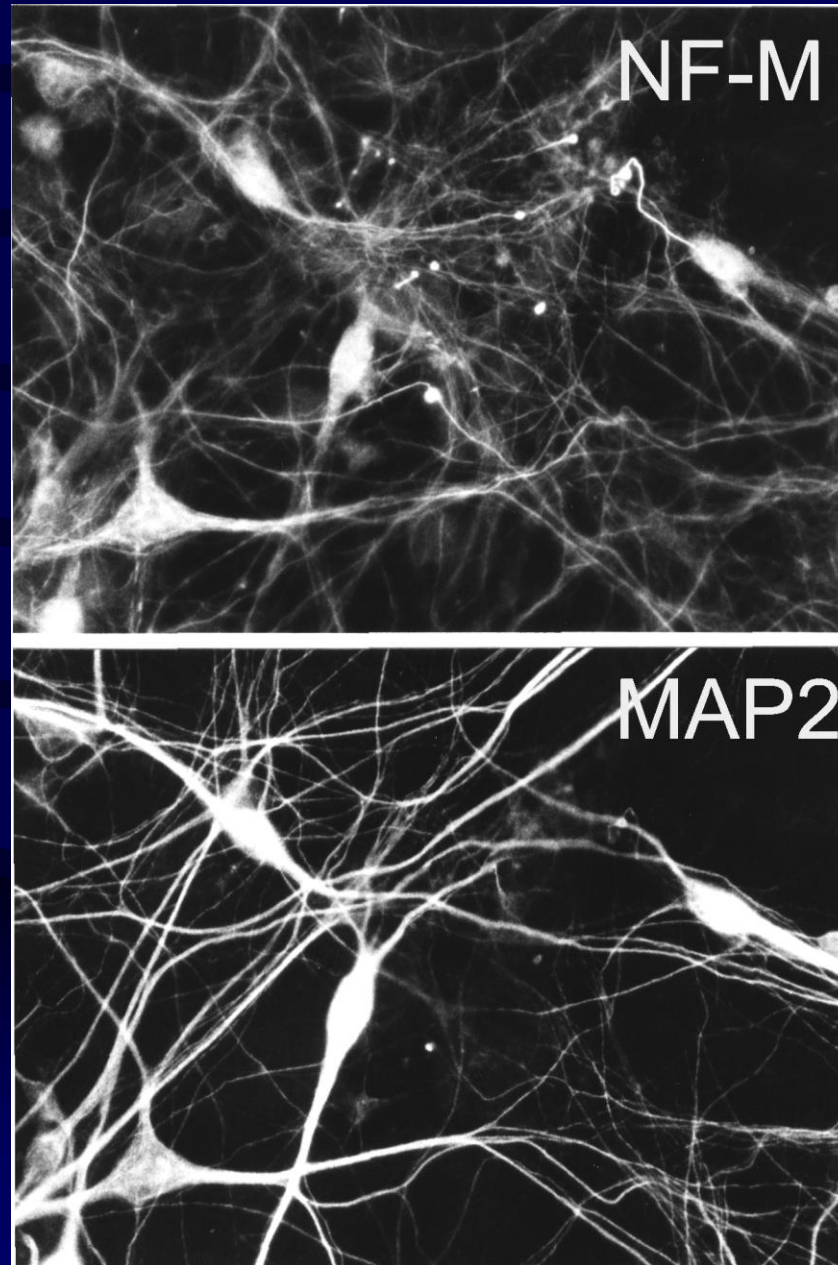
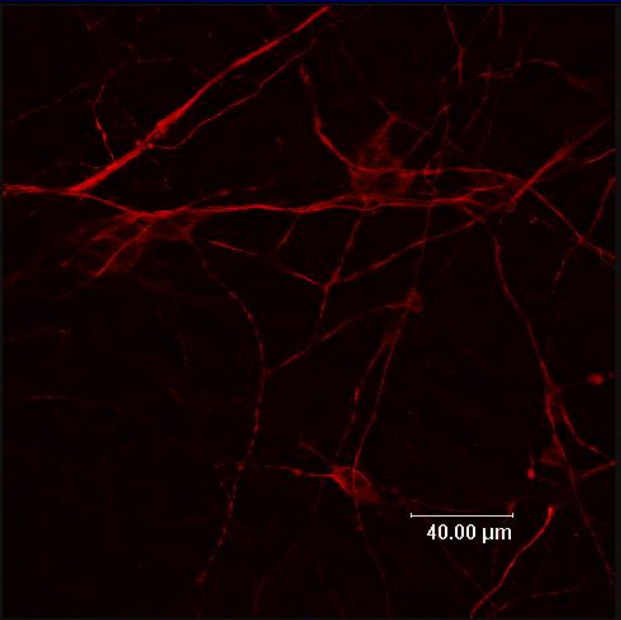
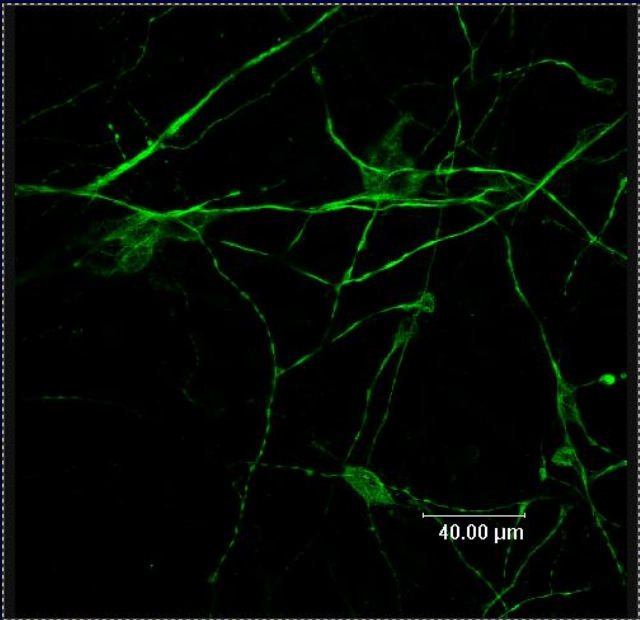


Figure 16–33. Molecular Biology of the Cell, 4th Edition.

Primary culture of embryonic hippocampal Neurons



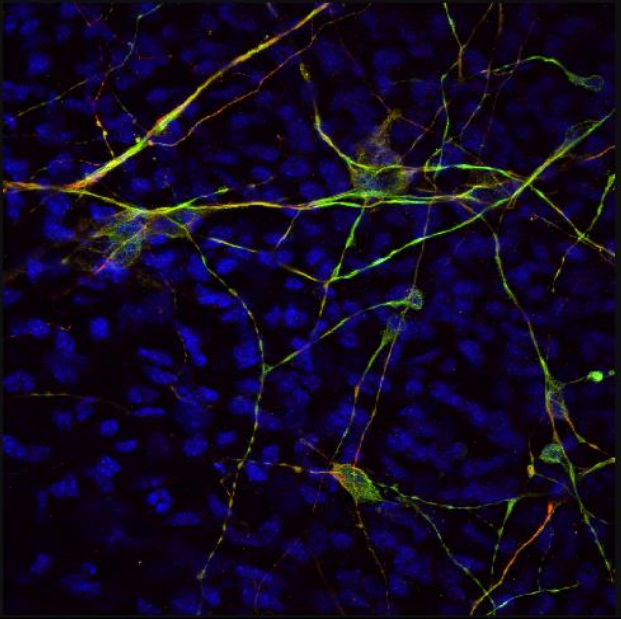
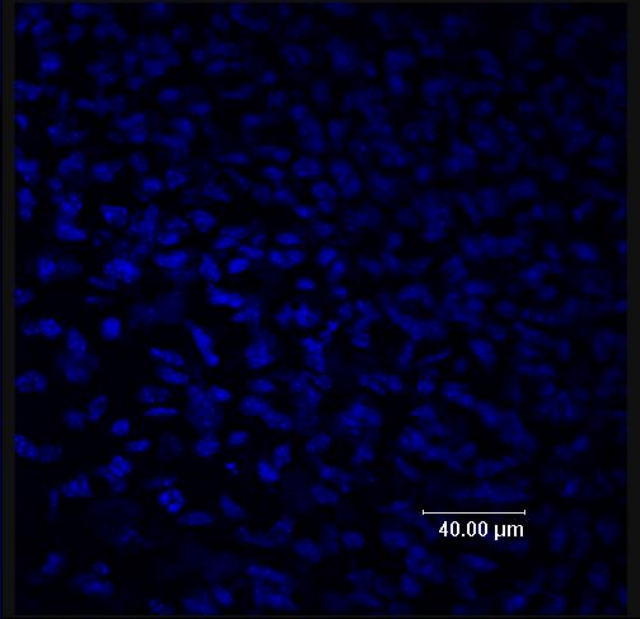
Neuronal differentiation from Embryoid Body in DMEM/F12 media supplemented with N2 for 9 days



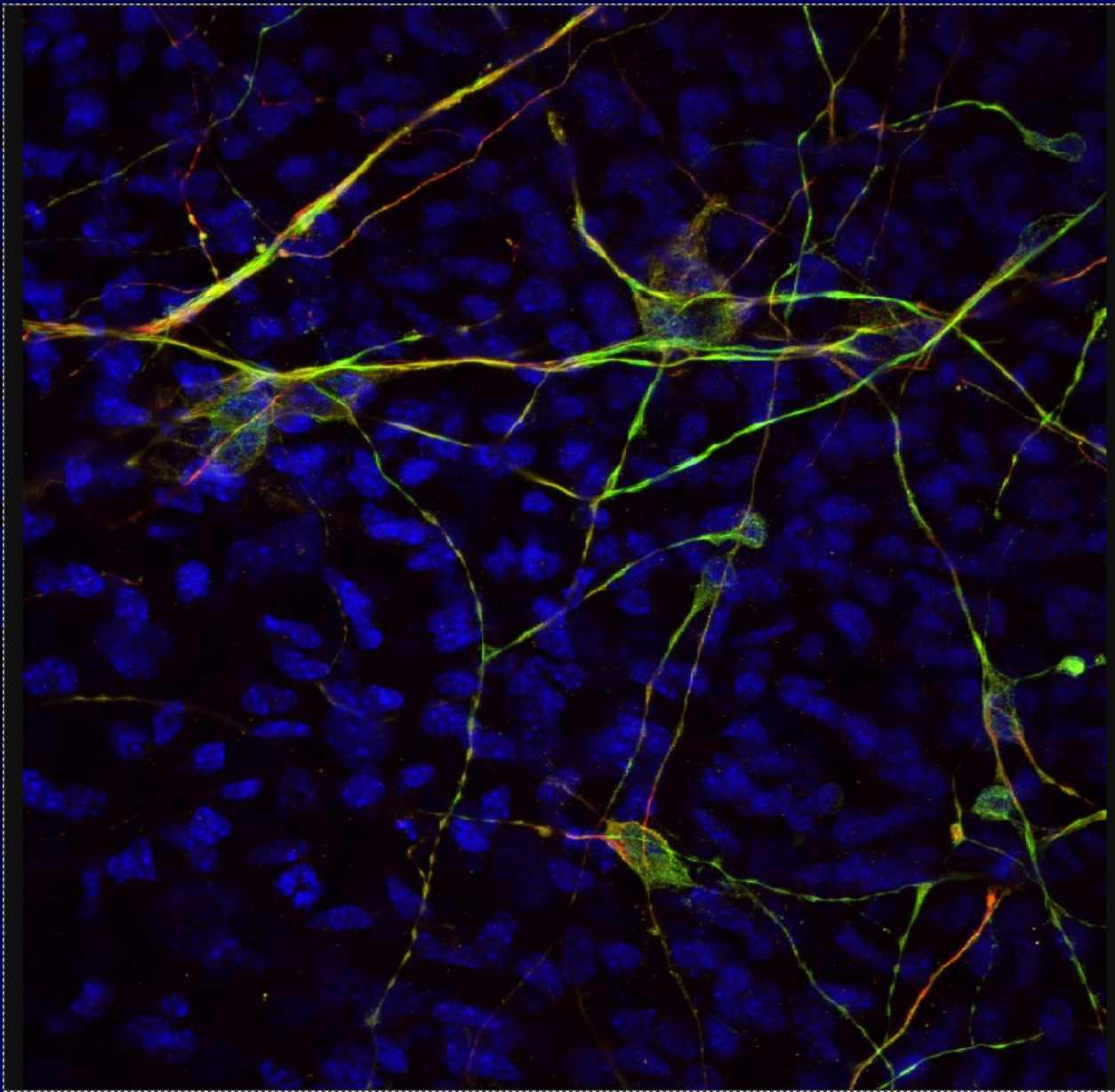
Neuronal Markers

Green:
Tubulin β III (Tu J)

Red: Internexin



Neuronal differentiation from Embryoid Body in DMEM/F12 media supplemented with N2 for 9 days



Neuronal Markers

Green:
Tubulin β III (Tu J)

Red: Internexin