Genetic conversion of proliferative astroglia into neurons after cerebral ischemia. A new therapeutic tool for the aged brain.

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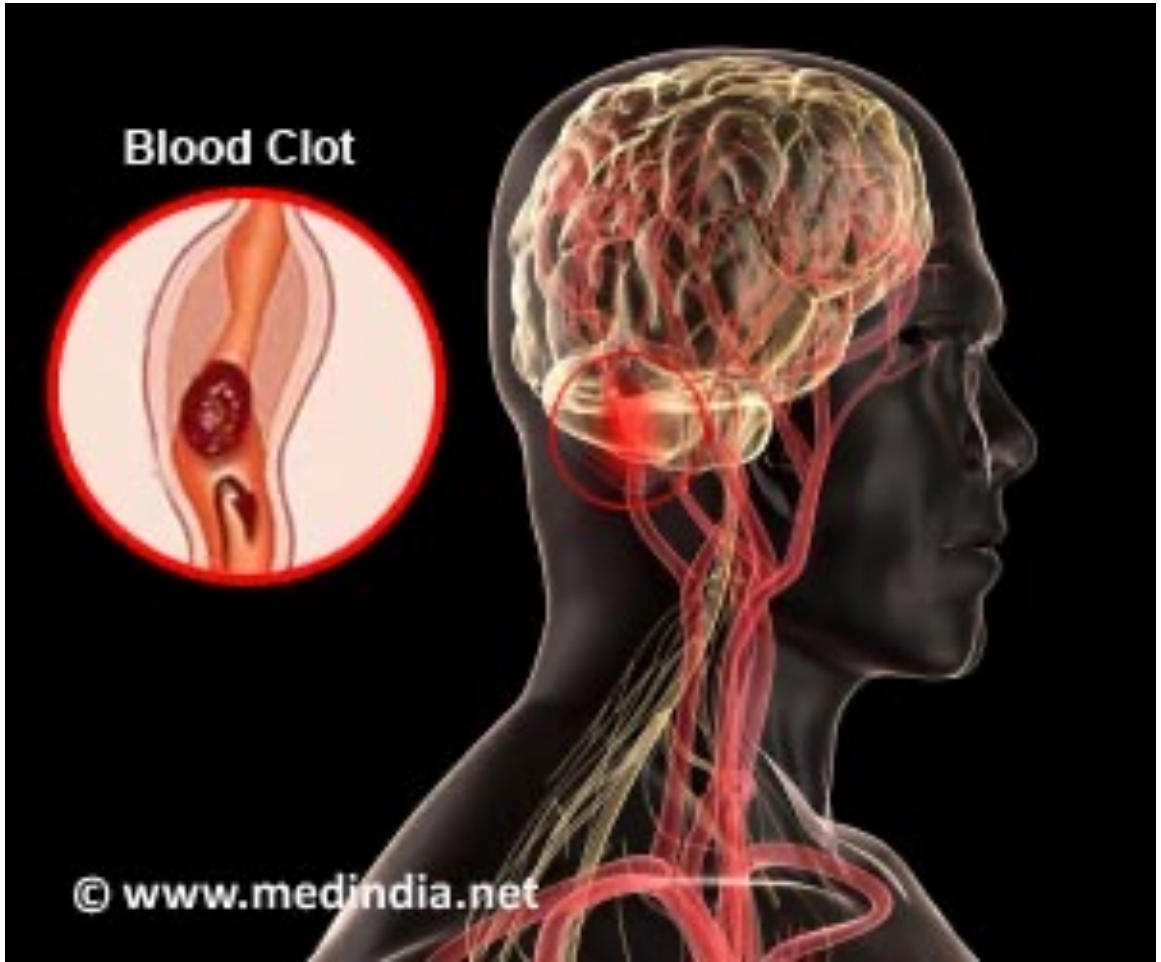
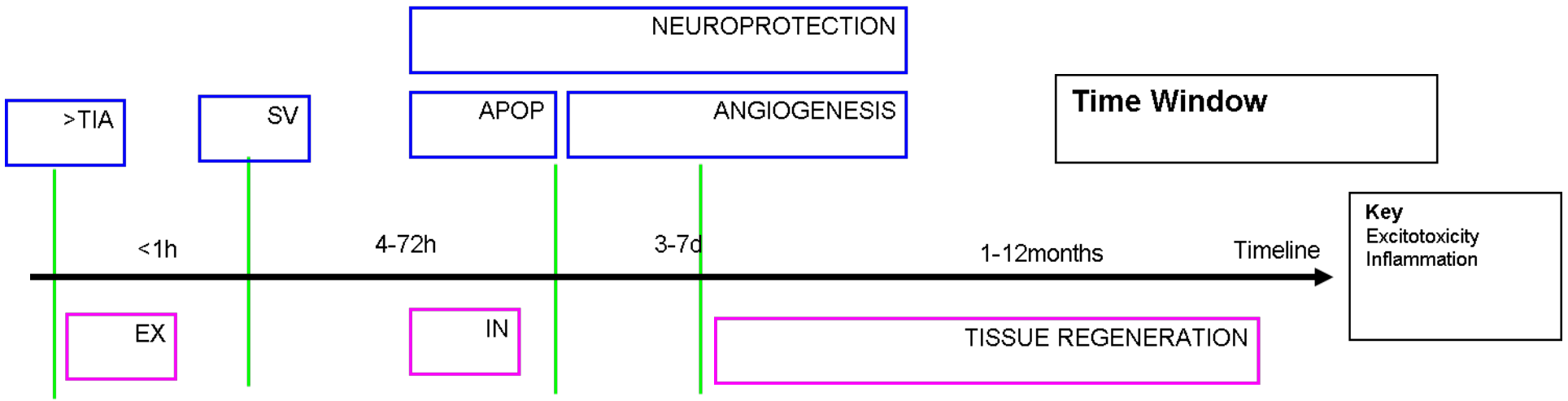
Current therapies

- Treatment options following acute ischaemic stroke have not improved significantly over the last two decades.

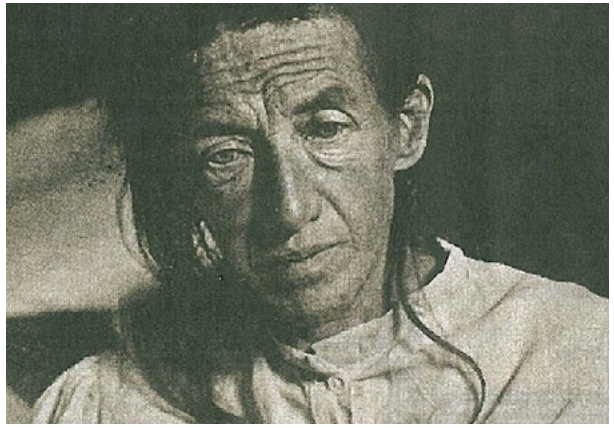


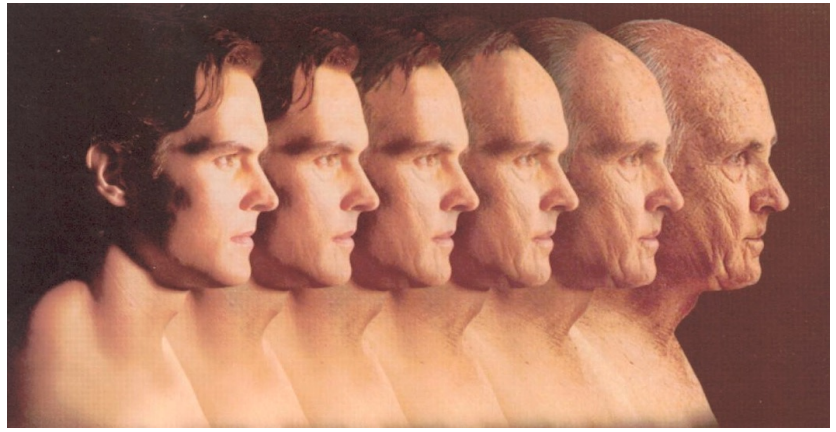
Combinational time-sensitive therapy

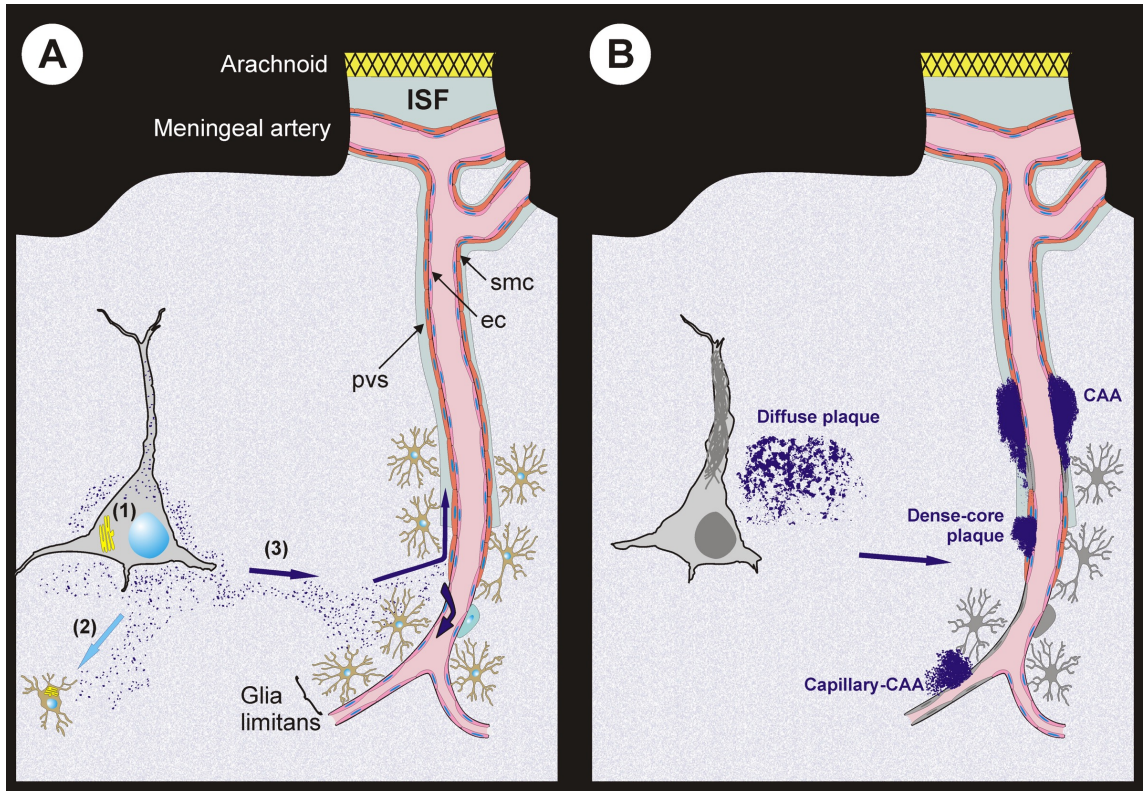


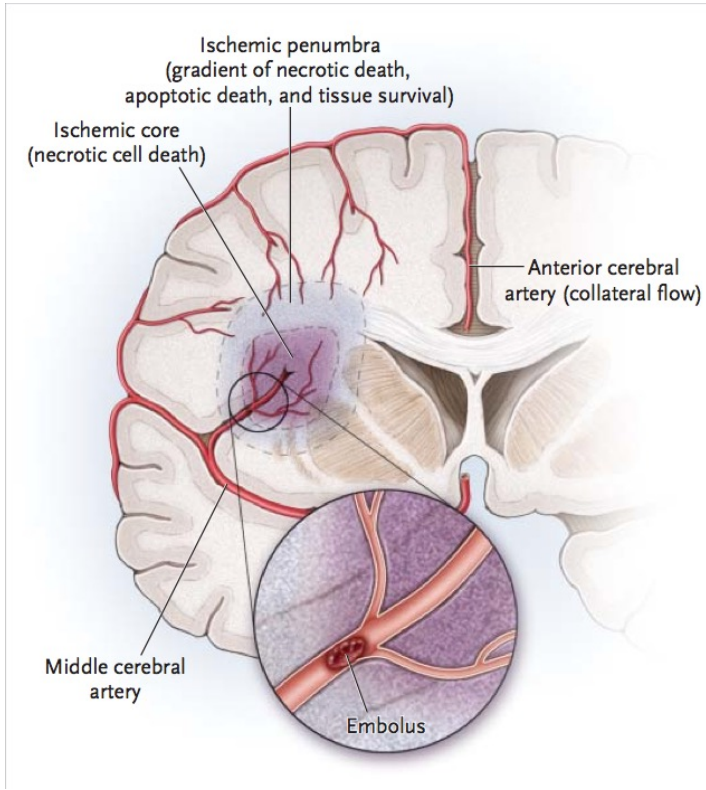
Key: Blue; Intervention points; Green; time limits; Purple; main pathobiological processes

Age is the principal nonmodifiable risk factor for cerebrovascular diseases









STAIR Criteria

(Stroke Academic and Industry Round Table)

- Reproducibility of results proven in many different laboratories wordlwide
- Efficiency in many different species
- Testing on aged animals
- Efficiency in both transient and permanent ischemia
- Establish the therapeutic time-window
- Establish the dose-efficiency relationship
- Monitoring of physiologic parameters during the experiments
- Does the infarct volume correlate with functional recovery ?
- Longterm studies of the above parameters (min. 4 Weeks)

STAIR 1999

Lifestyle risk factors

- *Being overweight or obese*
- *Physical inactivity*
- *Heavy or binge drinking*

Medical risk factors

- *Cigarette smoking or exposure to secondhand smoke*
- *High cholesterol*
- *Diabetes*
- *Personal or family history of stroke, heart attack or transient ischemic attack.*

Other factors associated with a higher risk of stroke include:

- *Age —People age 55 or older have a higher risk of stroke than younger people do.*
- *Sex — Men have a higher risk of stroke than women.*

A stroke can sometimes cause temporary or permanent disabilities:

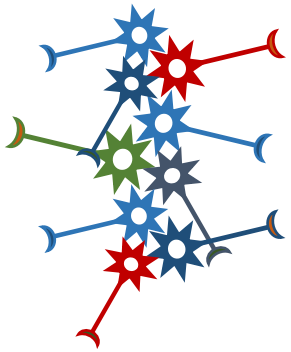
- *Paralysis or loss of muscle movement*
- *Difficulty talking or swallowing*
- *Memory loss or thinking difficulties*
- *Emotional problems*
- *Pain*
- *Changes in behavior and self-care ability*

Cellular balance in the post-stroke brain:

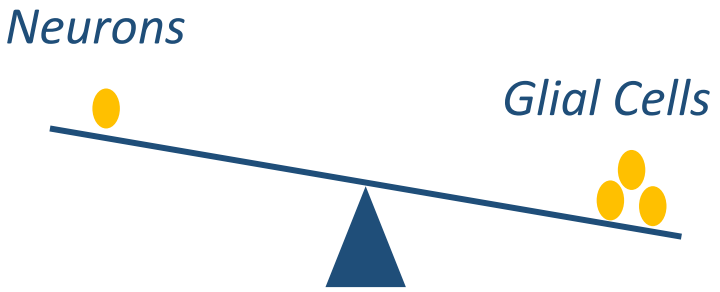
Following stroke, *neurons are lost in the infarct core. Neurons are post-mitotic cells therefore, once lost because of injury or degeneration, they do not regenerate in most regions of the central nervous system.*

Exceptions:

- ✓ ***the subventricular zone (SVZ) of the lateral ventricles***
- ✓ ***the sub granular zone (SGZ) of the dentate gyrus***



In response to neurodegeneration adult astrocytes become reactive. This sequence of events leads to a change in astrocyte morphology and proliferation status (Sofroniew and Vinters 2010) disrupting the neuronal vs non-neuronal cell balance in the injured area, especially in the aged brain.



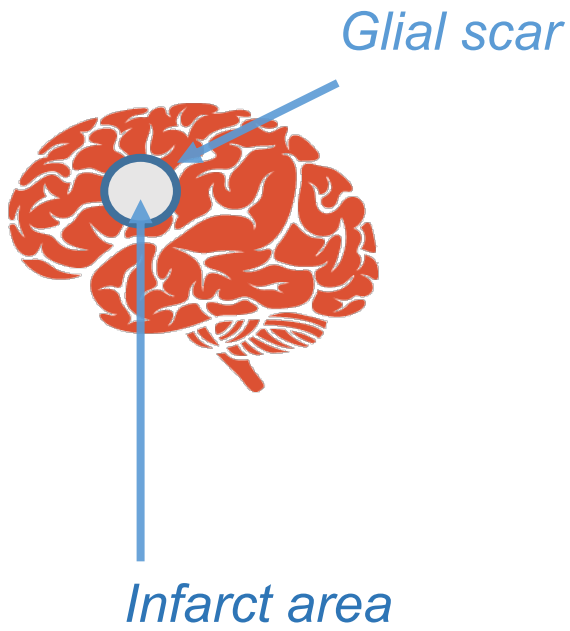
NG2-glia also respond to traumatic injury - including ***stab wound lesion*** (Buffo et al. 2005) and ***ischemia*** (Zhang et al. 2013) very fast (within one day; (Horky et al. 2006) and not only with morphological changes, but also with increased proliferation rate.

Restoring cell balance within the peri-lesional area is consequently crucial for post-stroke recovery of brain tissue.

Ischemic stroke also induces neurogenesis *but, although newly generated neurons can facilitate brain repair by integrating into local and distal neural networks,* the newly born neurons in these regions unfortunately have a very restricted distribution and function.

What is more proliferating glia cells build up gliotic scars that are initially protective by confining the damaged area. In the long-term, however, **the gliotic scar acts as a barrier to axonal and neural regeneration.**

“Melting” glial scars has been attempted with little success. Alternative strategies include transforming inhibitory gliotic tissue into an environment conducive to neuronal regeneration and axonal growth.



Neuroprotection studies in young animals have demonstrated the efficacy of a variety of pharmacological interventions.

Yet, all strategies that have clinically been tested in larger trials **failed to show benefits in aged humans**.

One possible explanation for this discrepancy between experimental and clinical studies may be the role that age plays in the recovery of the brain from stroke insults (Popa-Wagner et al., 2014; Hermann et al., 2019).

Direct lineage reprogramming

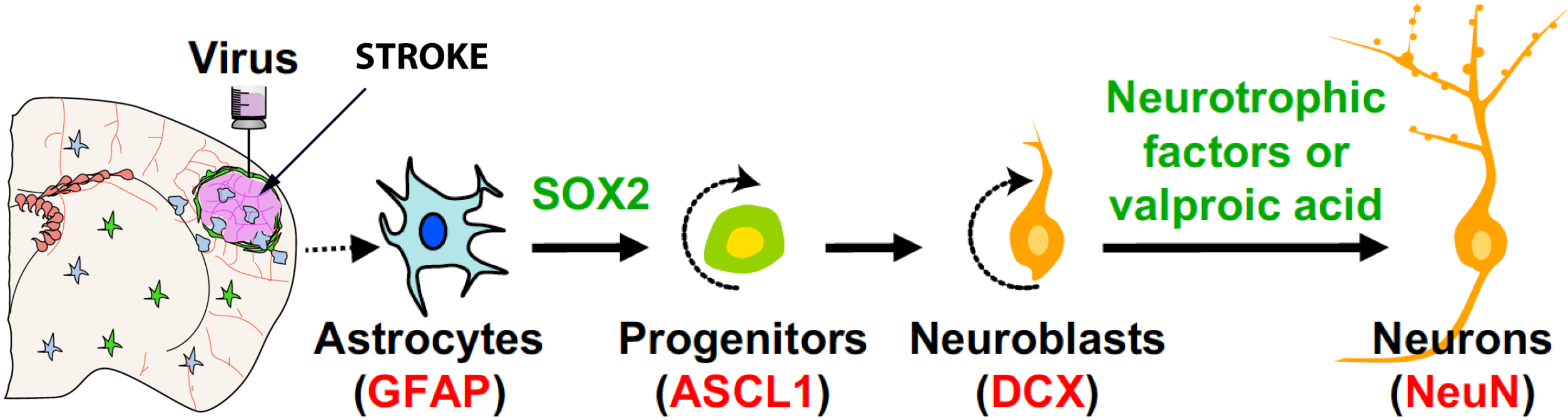
In order to restore the cell balance in vivo ***direct lineage reprogramming in the adult mammalian brain might be a feasible strategy*** *for* ***reprogramming non-neuronal cells into neurons.*** (Guo et al.2014; Kronenberg et al. 2010).

- *This technology has emerged as a new approach to circumvent cell transplantation.*

- ***Reactive astrocytes induced by ischemia exhibit properties of neural stem cells and can transdifferentiate into neurons.***

What is more, previous work in vitro showed that different types of **somatic cells, including astroglia and pericytes of brain origin**, can be **directly converted into functional neuronal** cells by forced expression of key neurogenic transcription factors

HOW it WORKS?


Virus
STROKE
Astrocytes
(GFAP)
SOX2
Progenitors
(ASCL1)
Neuroblasts
(DCX)
Neurotrophic
factors or
valproic acid
Neurons
(NeuN)

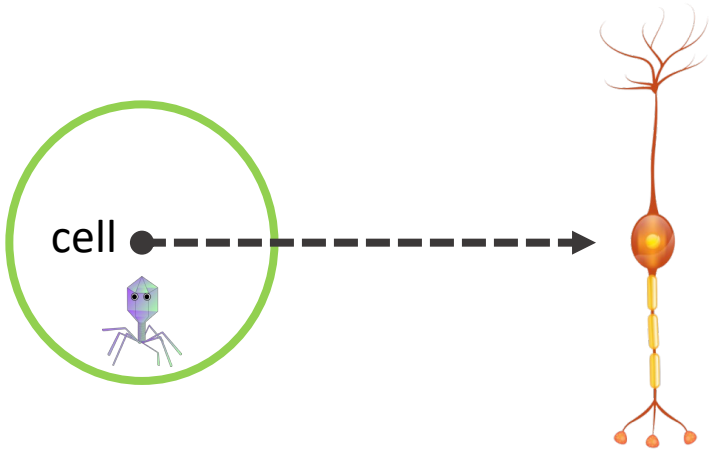
For instance, **astroglia and oligodendrocyte progenitor cells (OPCs)** isolated from the postnatal mouse cerebral cortex can be directed toward a neuronal identity by forced expression of the transcription factors Pax6, **Neurog2**, Ascl1, NeuroD1, or Pou3f4nand Sox11 and that astroglia-to-neuron conversion is facilitated by high levels of Sox2 expression.

Moreover, similar to newborn neurons from the subgranular and subventricular zones, astrocytes-derived neurons possess the characteristics of morphological and functional mature neurons.

The aged rodent brain develops a fulminant inflammatory response to cerebral ischemia and an accelerated delimitation of the infarct zone by capillary-derived nestin-positive cells, further complicating the efficacy of any stroke therapy (Popa-Wagner et al., 2007). Therefore, it is also hoped that converting reactive astrocytes into neurons may also reduce microglia-mediated neuroinflammation and restore the neurovascular unit along with the blood-brain-barrier

To this end, we used a retroviral delivery system encoding the **transcription factor *Neurog2-IRES-RFP*** alone or in combination with the **antiapoptotic factor *Bcl-2-IRES-GFP*** to target **proliferating cells.**

Of the previously tested genetic conversion factors, we chose Neurog2, as it instructs glutamatergic neurons from astrocytes in vitro and after stab injury in vivo (Gascón et al., 2016).



- The **Neurog2** *gene encodes the protein Neurogenin 2 which is a member of the neurogenin subfamily of basic helix-loop-helix (bHLH) transcription factor genes that play an important role in* ***neurogenesis.***

- The **BCL2** *gene encodes in humans the Bcl-2 which is the founding member of the Bcl-2 family of regulator proteins that regulate cell death (apoptosis).*

YOUNG C57BL6

Sham

Mix

Neurog2

Bcl-2

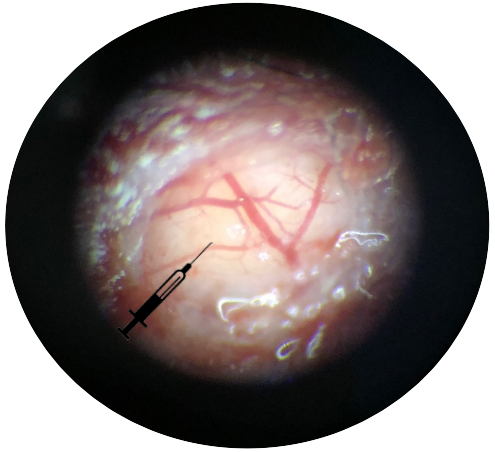
OLD Transgenic TYFF

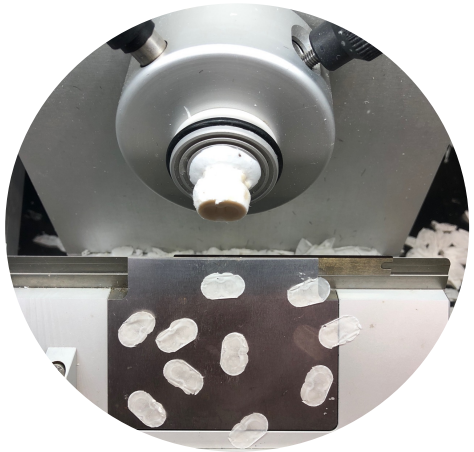
Mix

All experiments were performed in accordance with ARRIVE Guidelines for the Care and Use of Laboratory Animals", and animal procedures were approved by the University of Medicine and Pharmacy Craiova.

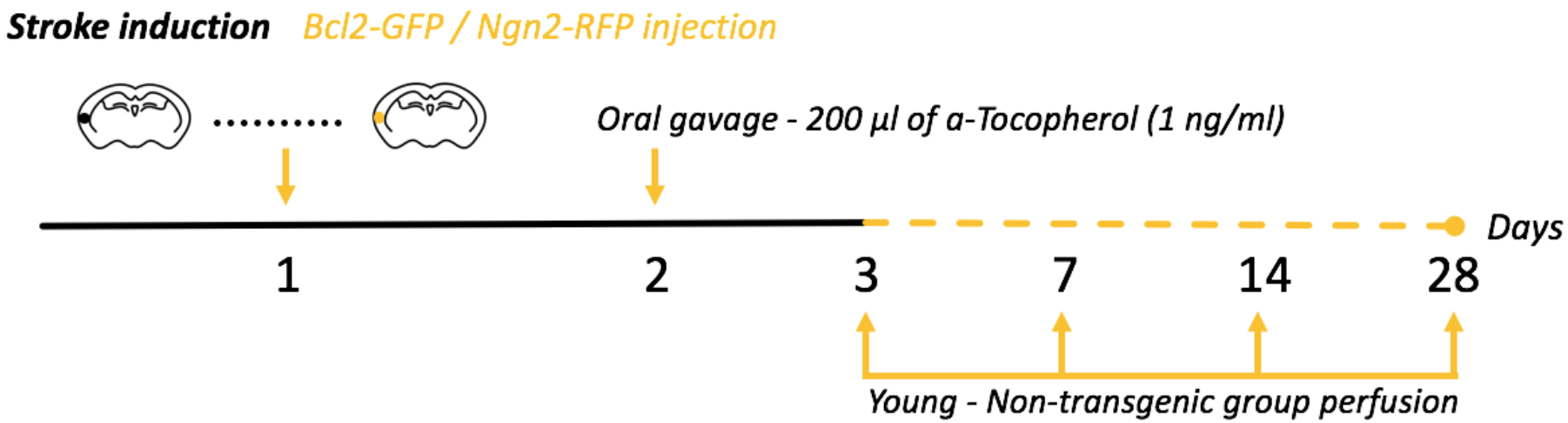
Induction of reactive glial cells by stroke injury in young and aged animals was performed using the craniotomy model and **permanent occlusion of the MCA** by electro-cauterization.

The injection of retroviruses into the peri-infarct area was made **by carefully avoiding the hippocampus or subventricular zone**.



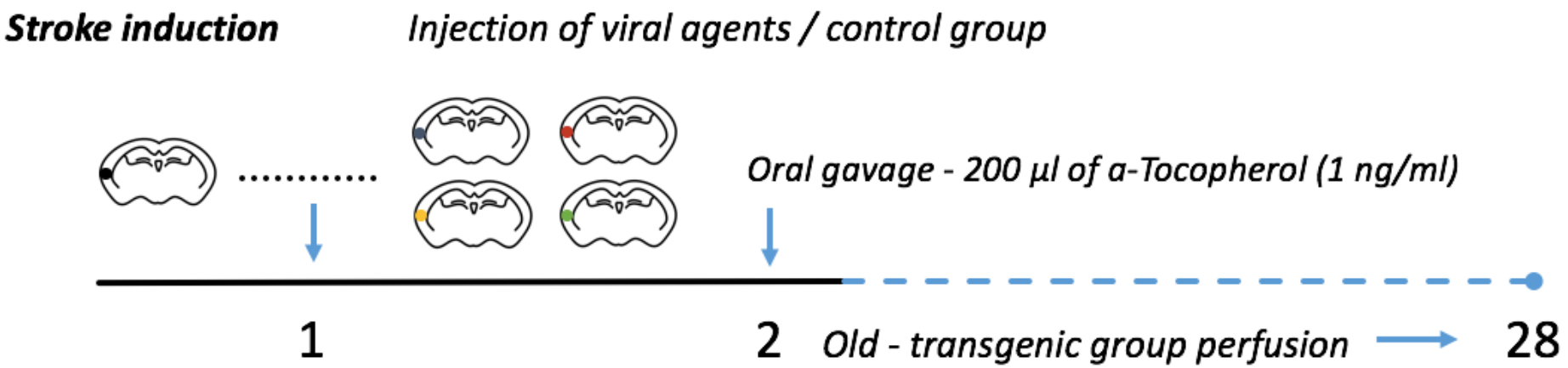


Perfusion young



We took into consideration that the efficiency of conversion could be low. ***To increase the number of conversion to NeuN-positive neurons, we used Tocoferol****, a vitamin that was reported to double proportion of NeuN+ iNs in Neurog2-transduced cells*

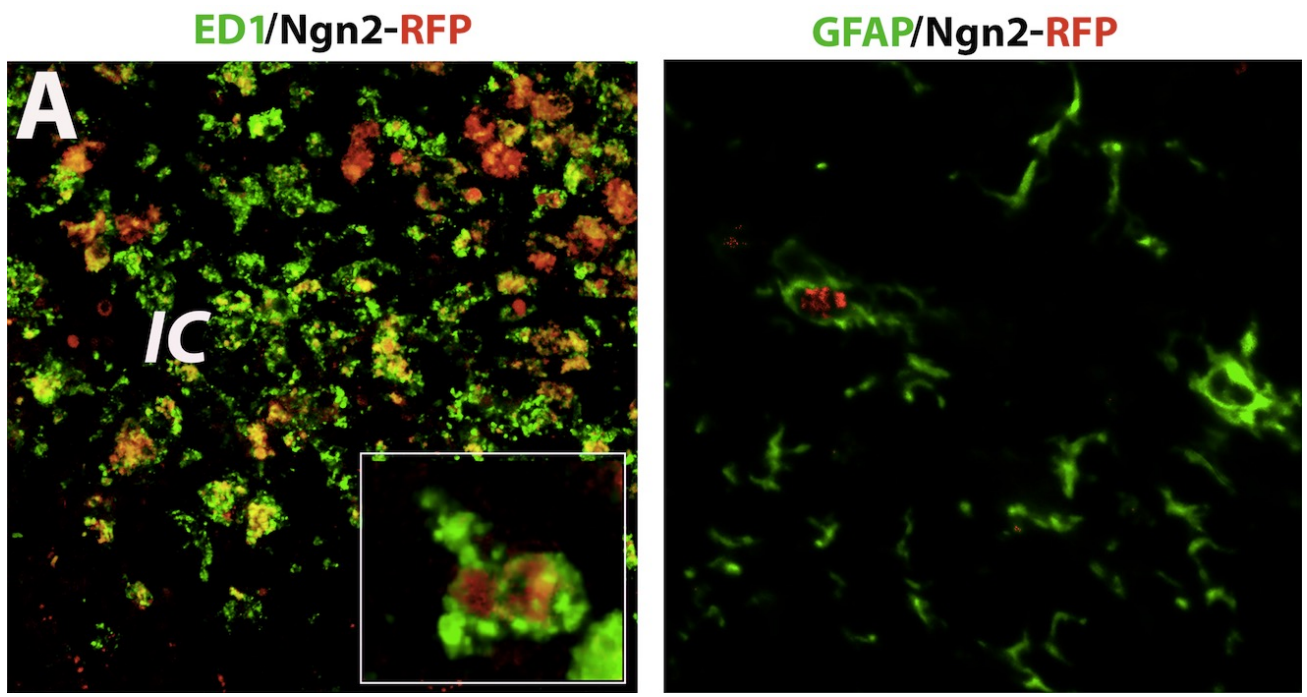
Perfusion old

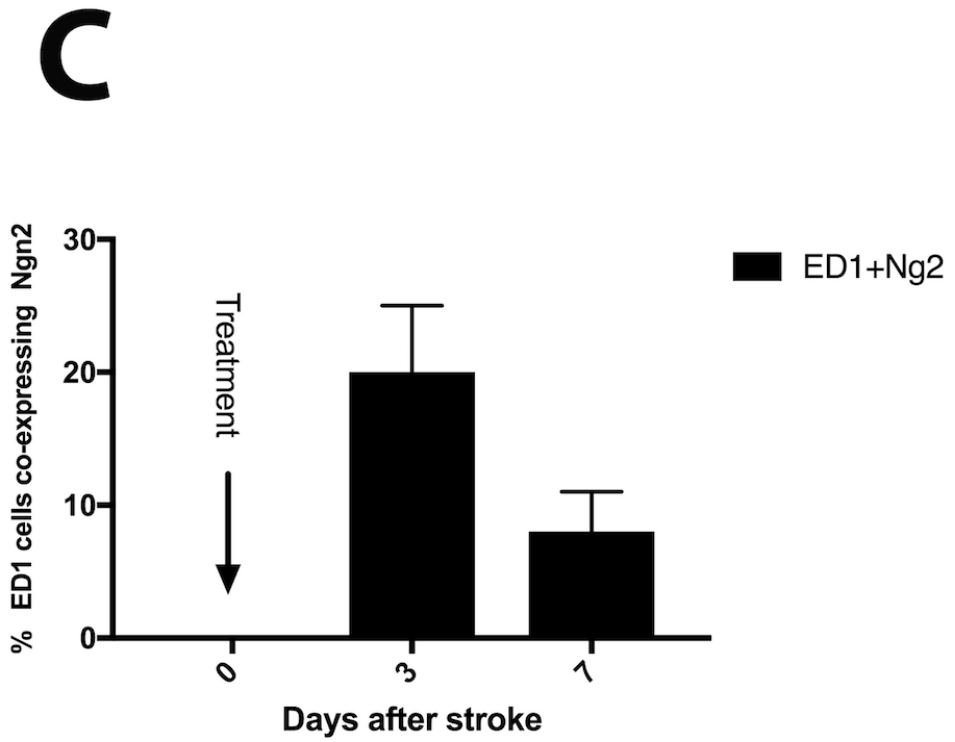


For ***phenotyping***, the tissue was incubated with:

1. *macrophages marker, rabbit anti-ED1* (1:000, abcam, UK),
2. *neuronal nuclei marker, rabbit anti-NeuN* (1:1000, Millipore, Germany)
3. *astrocytic marker, rabbit anti-GFAP* (1:1000, abcam, UK)

Results

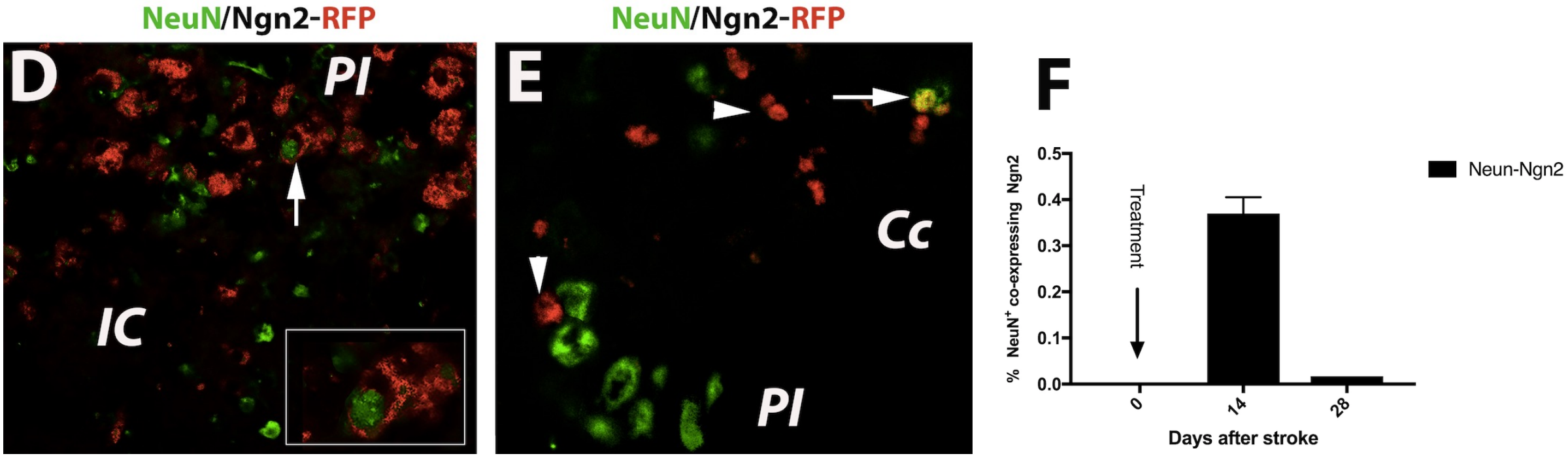




To our surprise the large majority (19.4%) of the RFP+ cells were ***microglia*** *cells having a* ***phagocytic phenotype*** *at day 3 after stroke. FIG A*

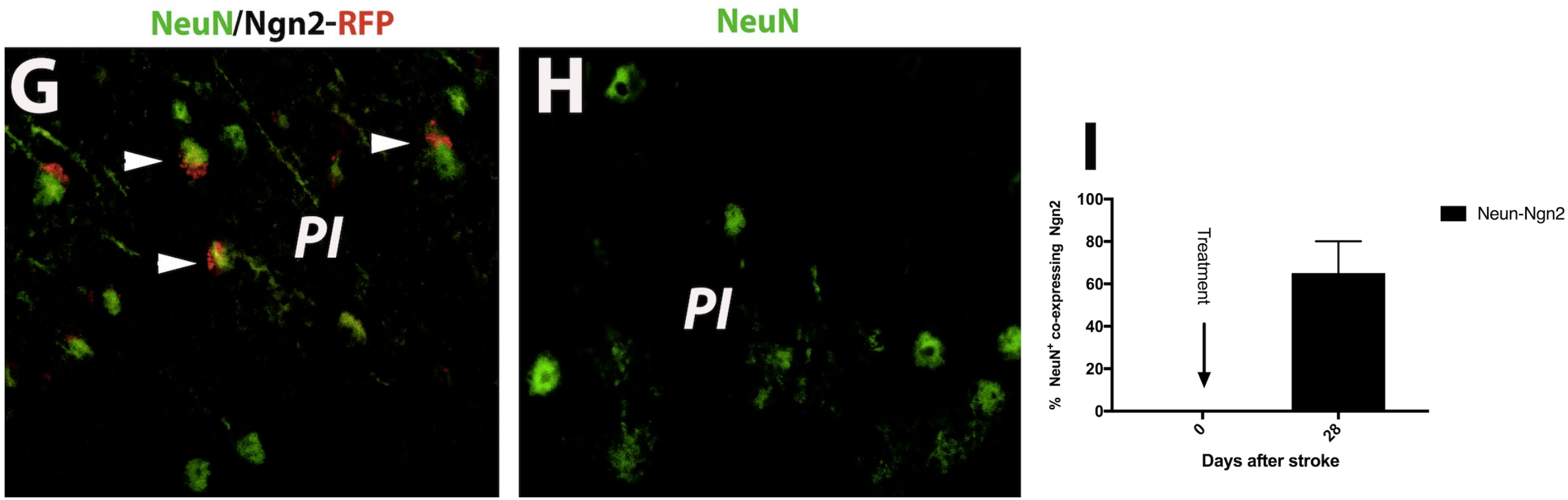
Thereafter, by day 7 post-stroke the number of cells co-expressing ED1 and RFP+ dropped to 8%.

Only a very small fraction of ***cells (less than 0.1%) co-expressed GFAP and RFP*** *in the peri-infarcted area (Fig. B).*

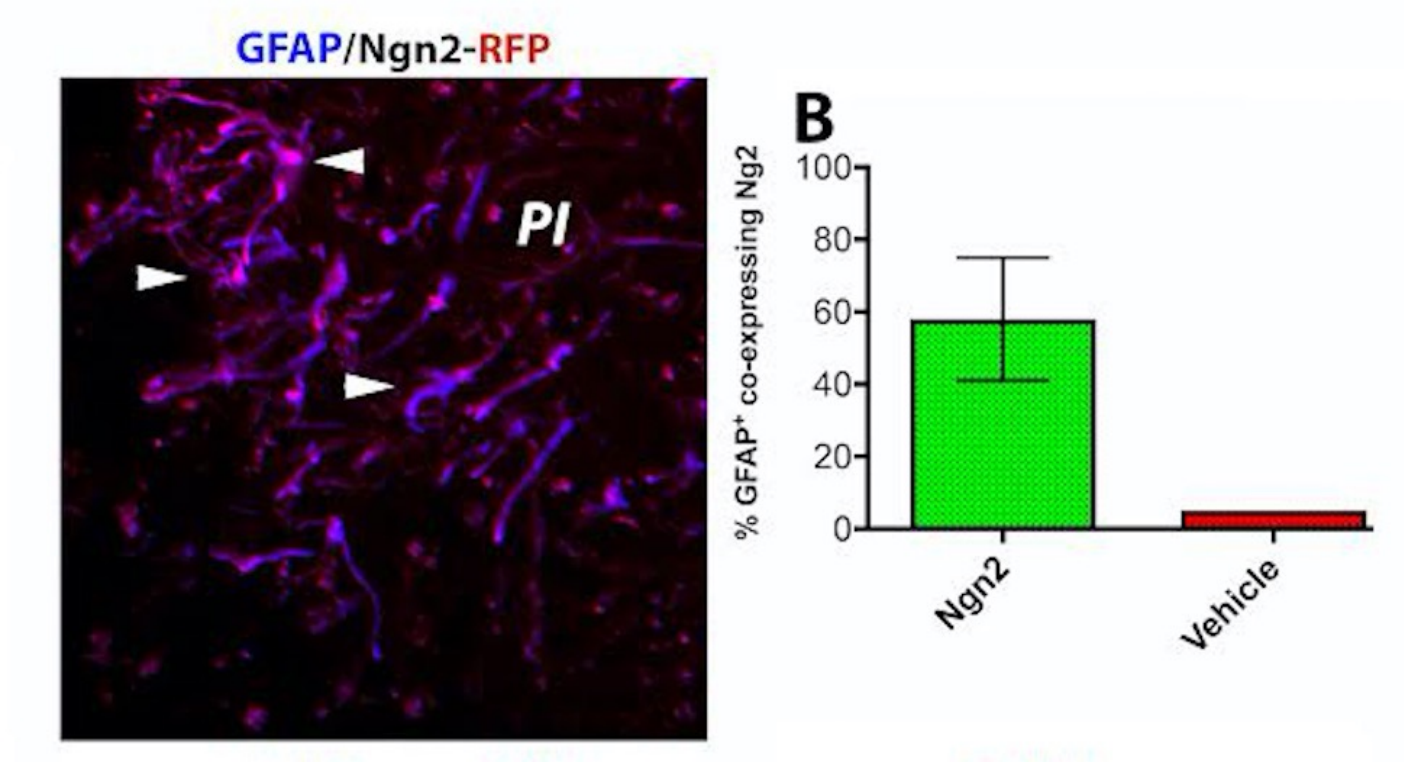


Recent studies have reported that ***about 75% of co-transduced cells had turned into NeuN+ neurons*** *with a clear neuronal morphology at day 10 after stab injury to the brain.*

In our experiments using the mouse model of focal ***ischemia which causes a severe injury, the conversion efficiency in the infarcted area was, about 0.35%.***

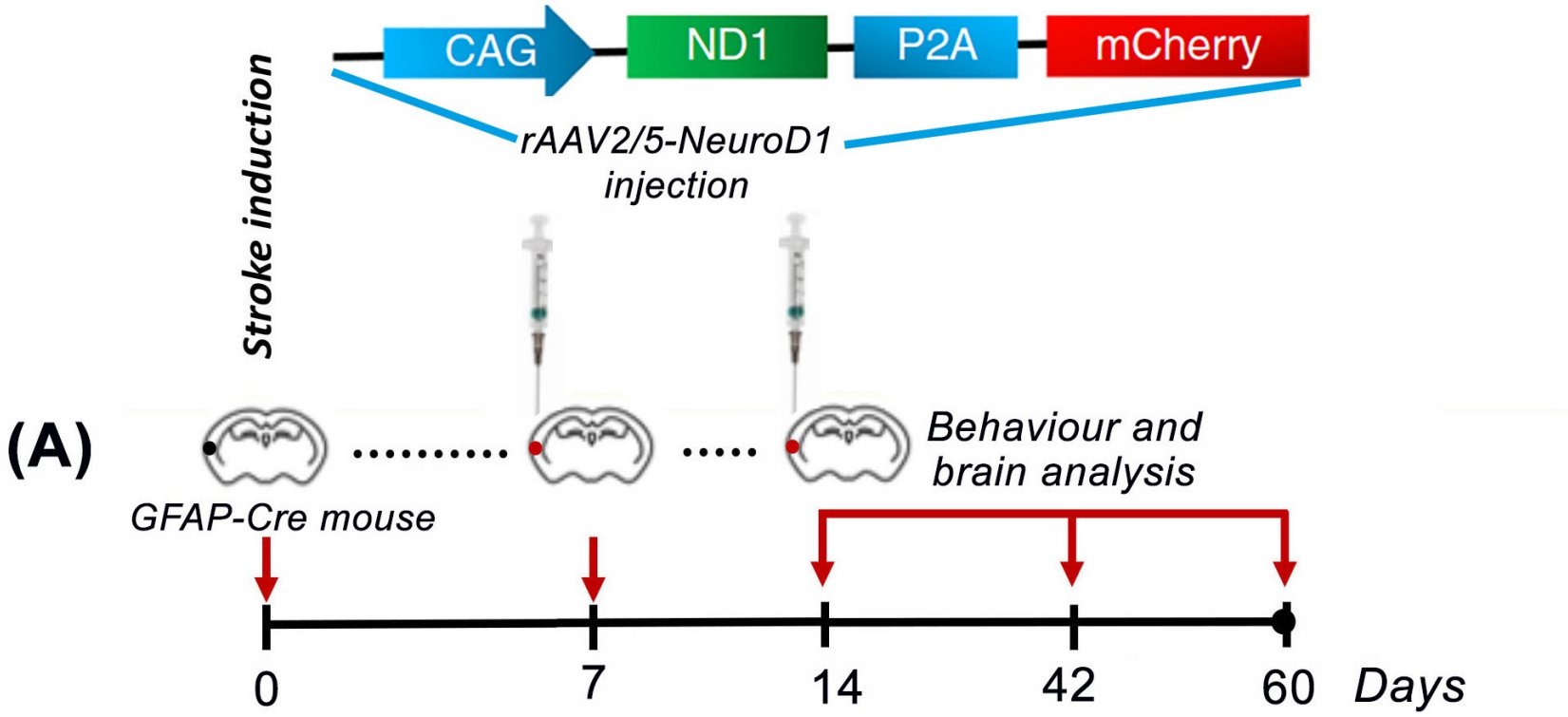


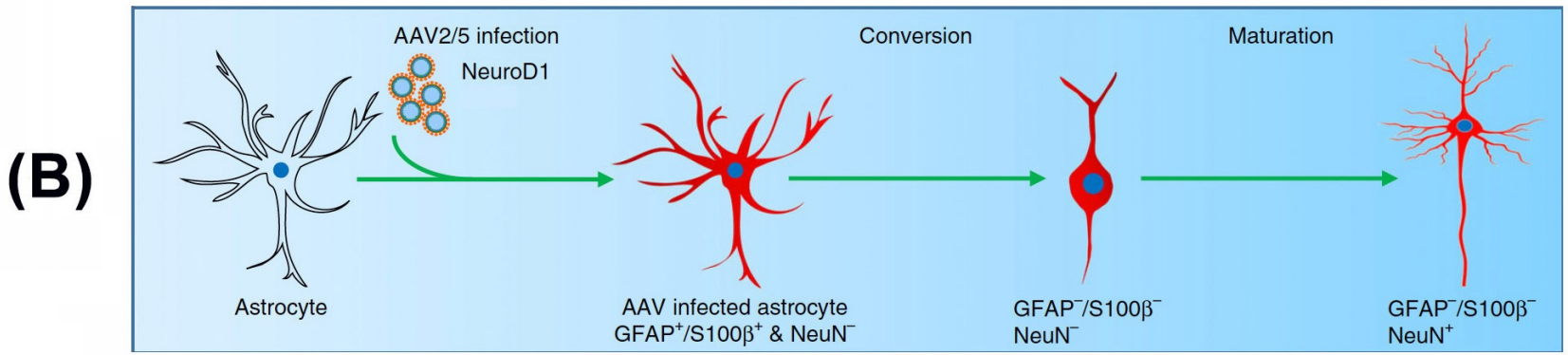
*In the old transgenic group at **28 days post stroke** we found a surprising number of neurons that **co-expressed** NeuN **and** Ngn2-RFP in the peri-infarcted area (Fig G) compared to the **Sham group** (Fig H). However, due to their morphology, we concluded that these were adult cells that only got infected by the viral agent without any actual conversion taking place.*

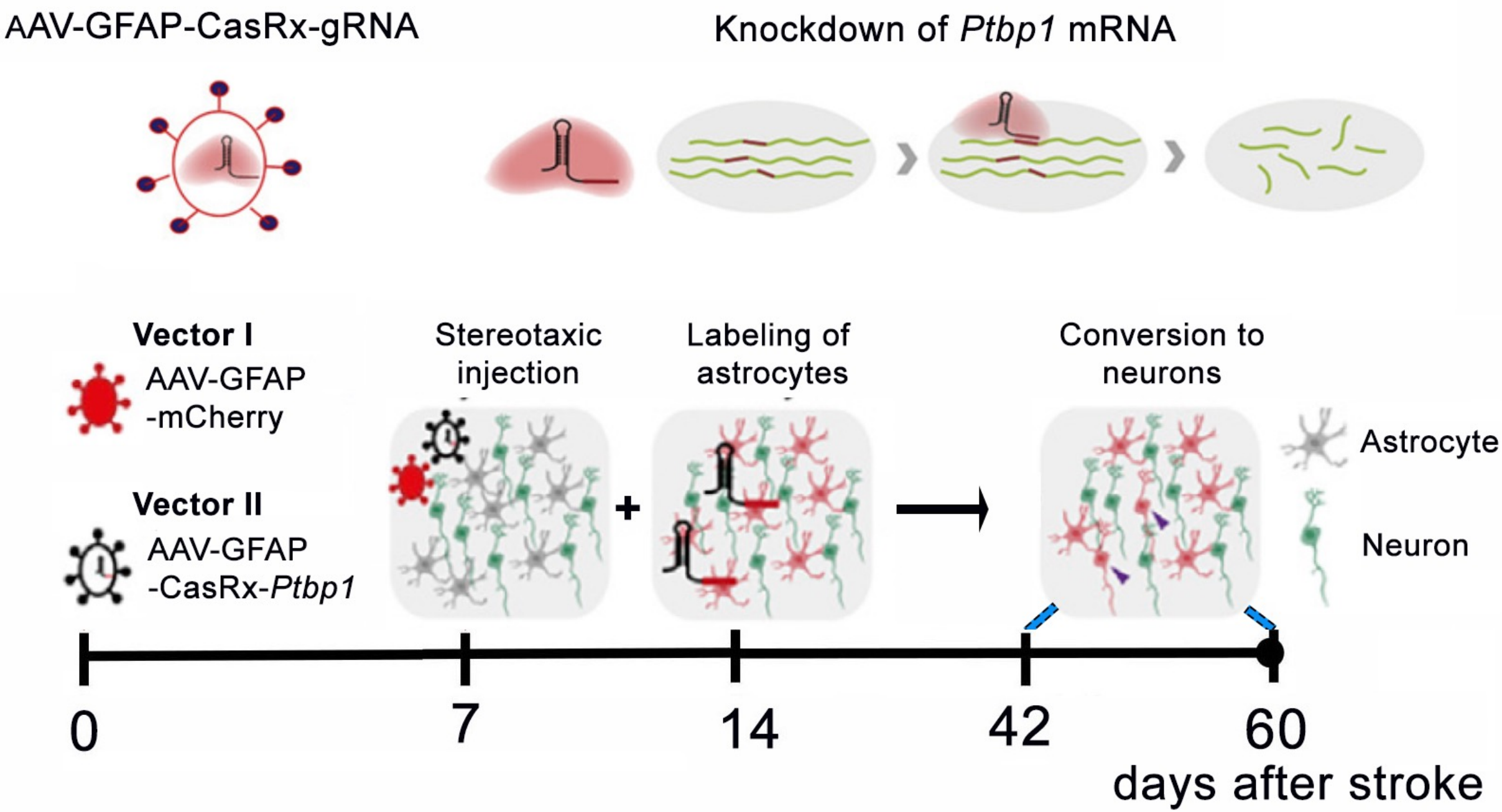


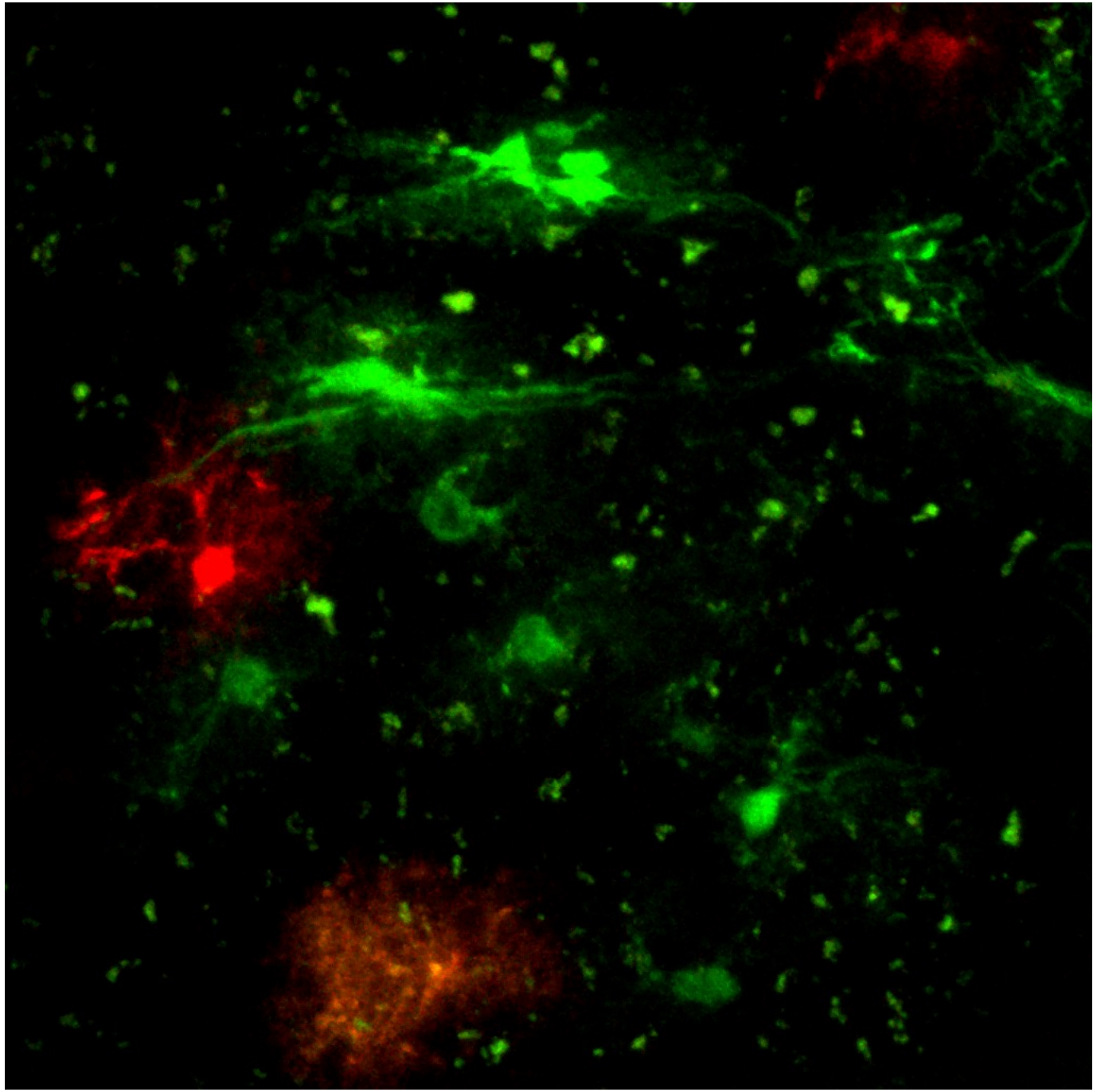
As expected, astroglia was highly proliferative after cerebral ischemia (Fig. 2A) and induce a highly inflammatory environment, especially in the brains of the aged rats (Sandu et al., 2016). At day 3 after stroke, in the aged brain the fraction of infected astrocytes with the retrovirus carrying the Ngn2-RFP sequence was high (57%, on the average, Fig. 2B) in the periinfarcted area as compared to young animals. At day28, in the lesioned brains of aged post-stroke transgenic mice displaying GFP-neurons we found indeed a large number of co-labelled Ngn2-RFP/NeuN-GFP cells. However, a closer examination of their nuclei revealed that Ngn2-RFP expression was confined to the neuronal NeuN-GFP neuronal nuclei (Fig. 2C, arrowheads). Sham-operated animals showed NeuN-GFP neuronal nuclei only (Fig. 2D).

Current Approach





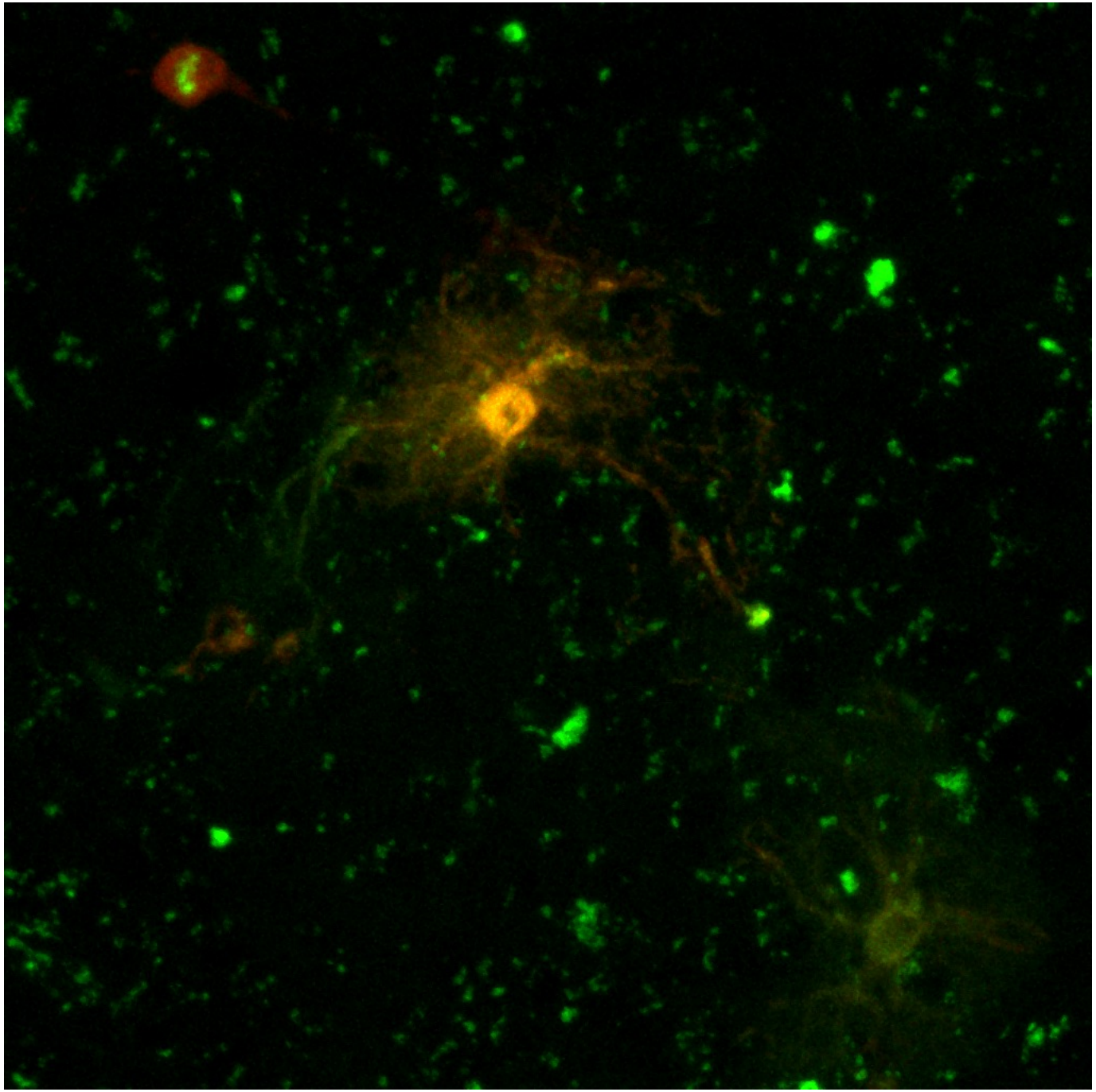

AAV-GFAP-CasRx-gRNA
Knockdown of *Ptbp1* mRNA
Vector I
AAV-GFAP
-mCherry
Vector II
AAV-GFAP
-CasRx-*Ptbp1*
Stereotaxic injection
Labeling of astrocytes
Conversion to neurons
Astrocyte
Neuron
0
7
14
42
60
days after stroke



Perilesional area.

RED = Reporter; converted cells

GREEN = infected cells

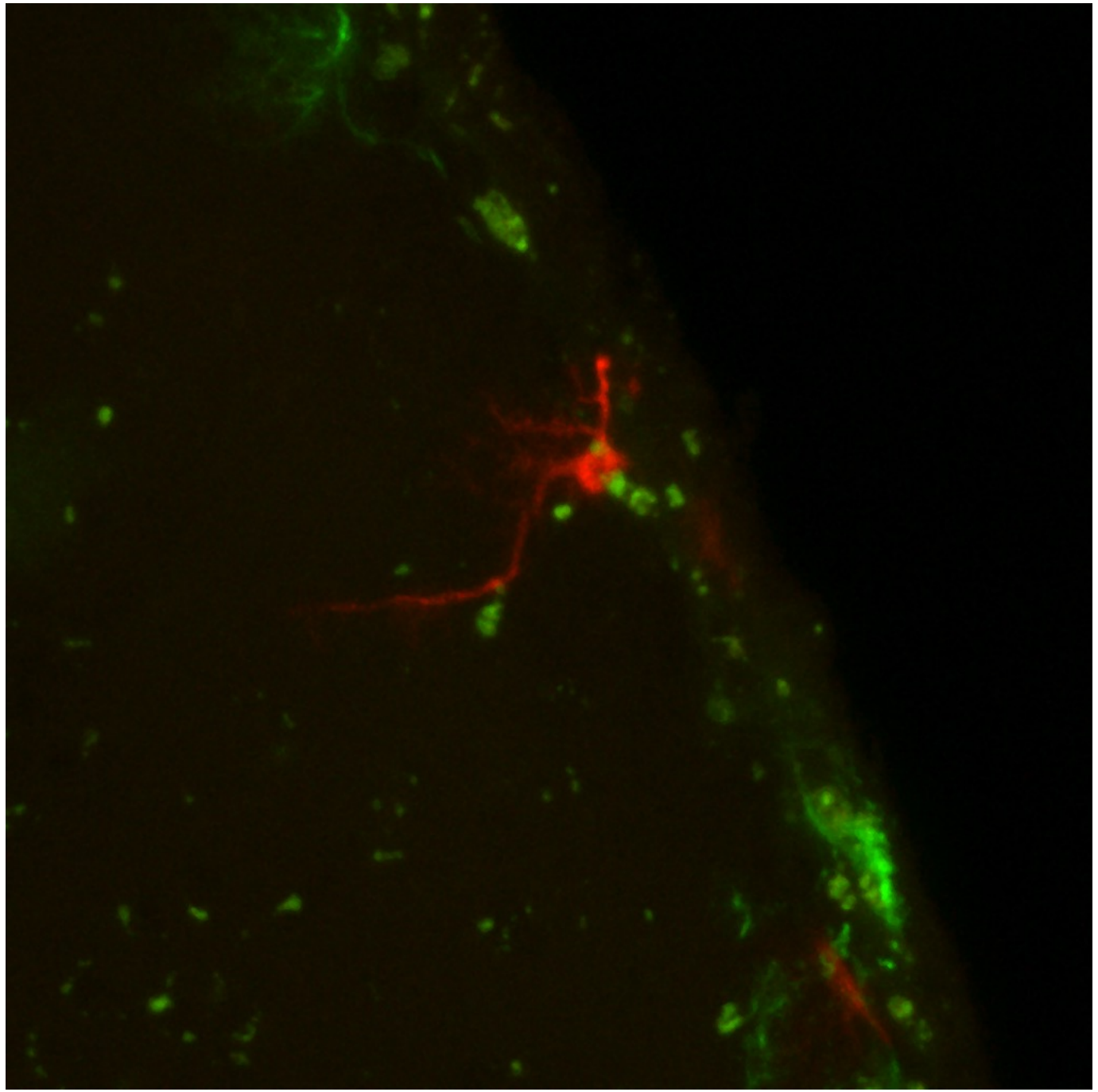


Perilesional area.

YELLOW = OVERLAY

Reporter; converted cells

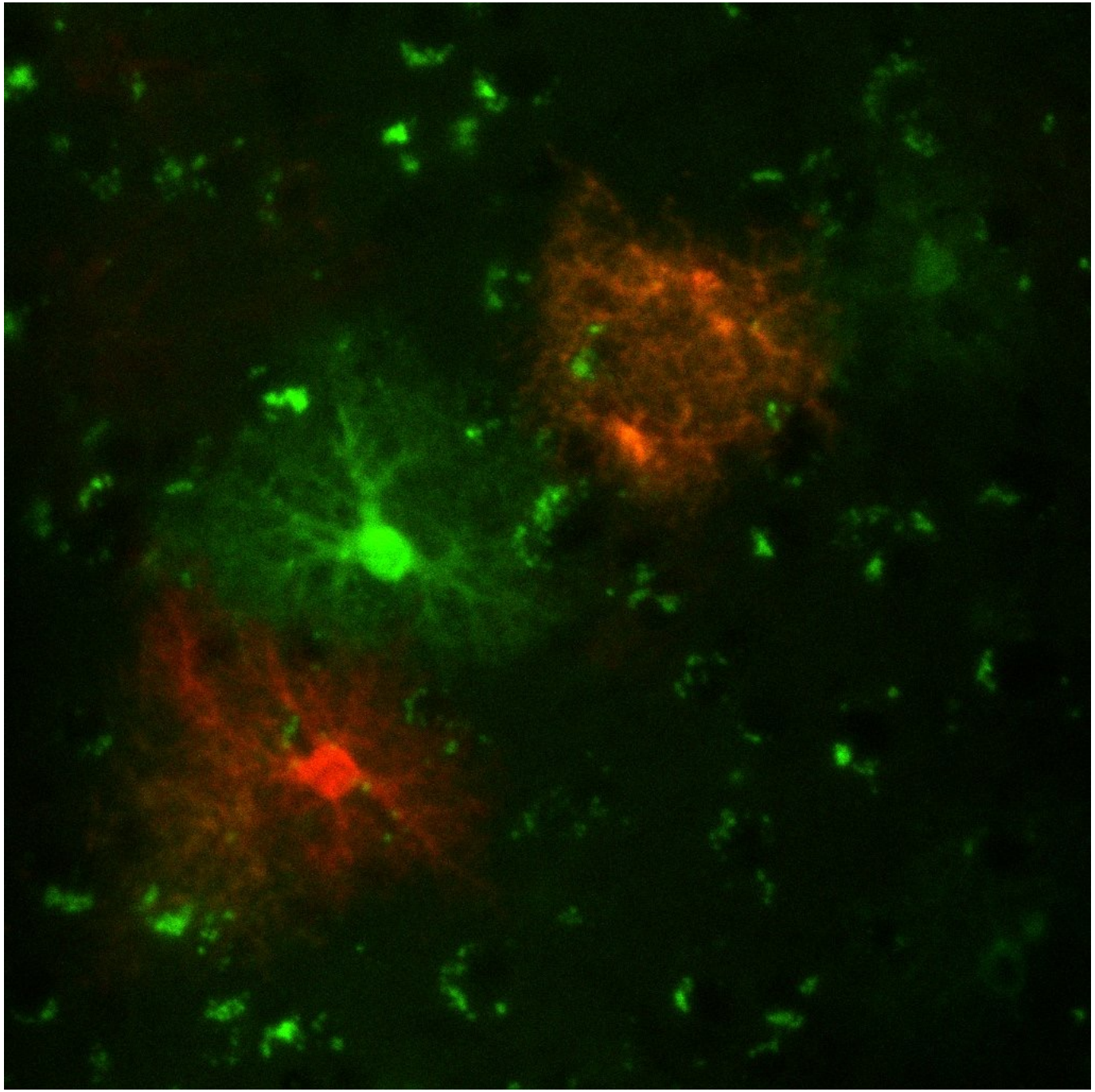
GREEN = infected cells



Perilesional area.

RED = Reporter; Neuron

GREEN = infected cells

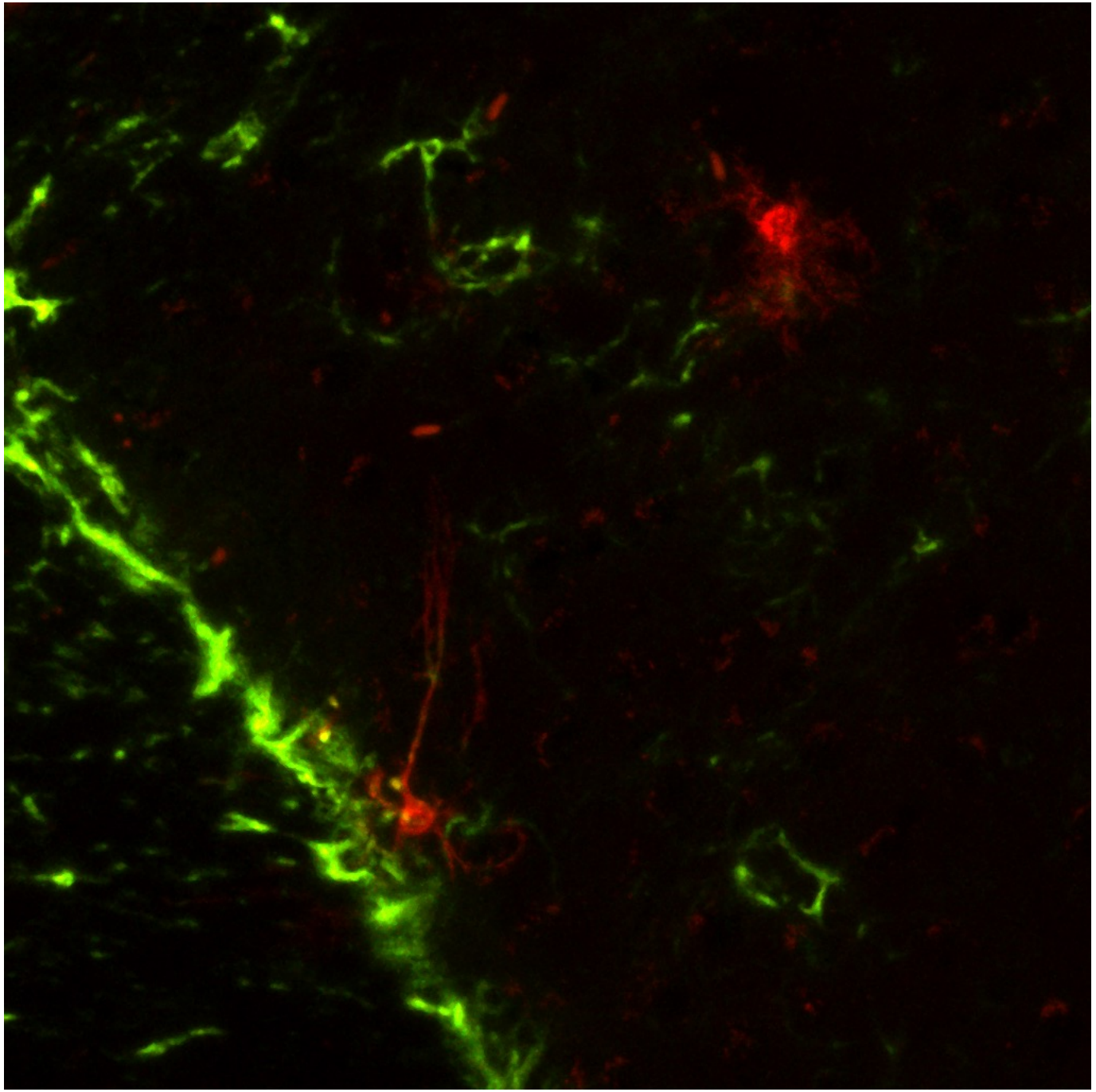


Perilesional area.

RED = Reporter; converted Glial

GREEN = infected cells

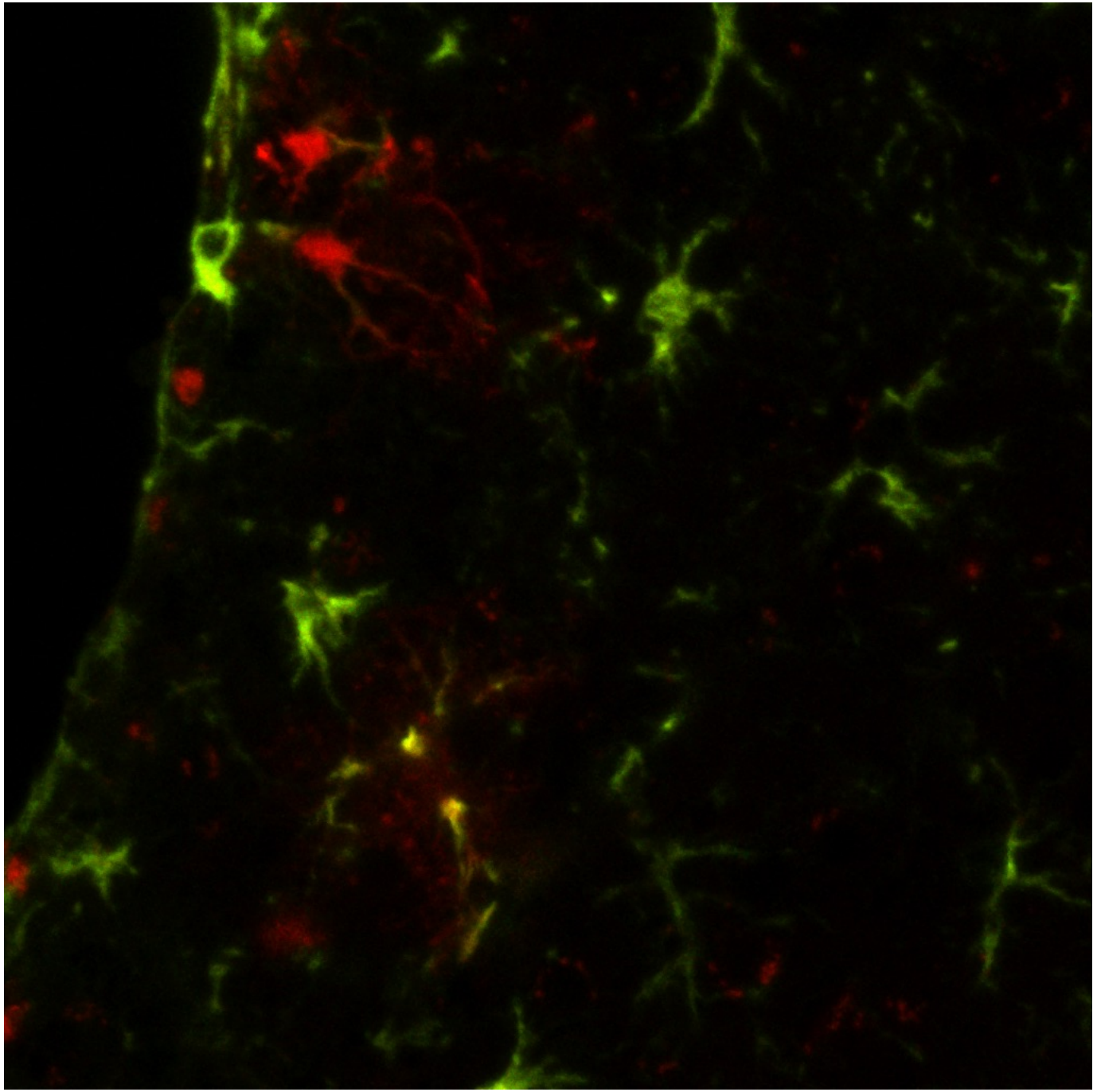
PROMOTER, GFAP, human



Corpus callosum.

RED = Reporter; converted cells

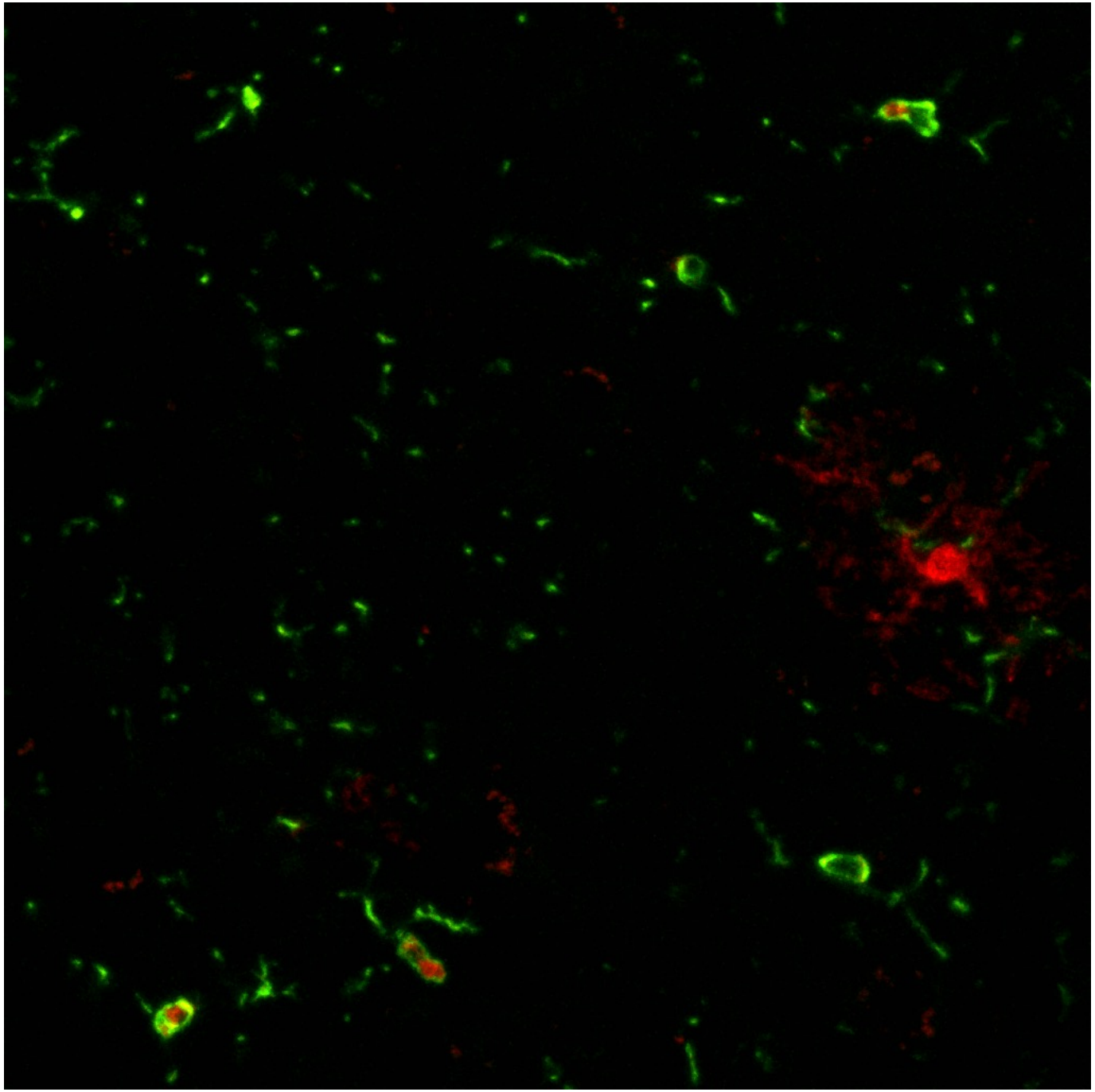
GREEN = infected cells



Subventricular area.

RED = Reporter; converted cells

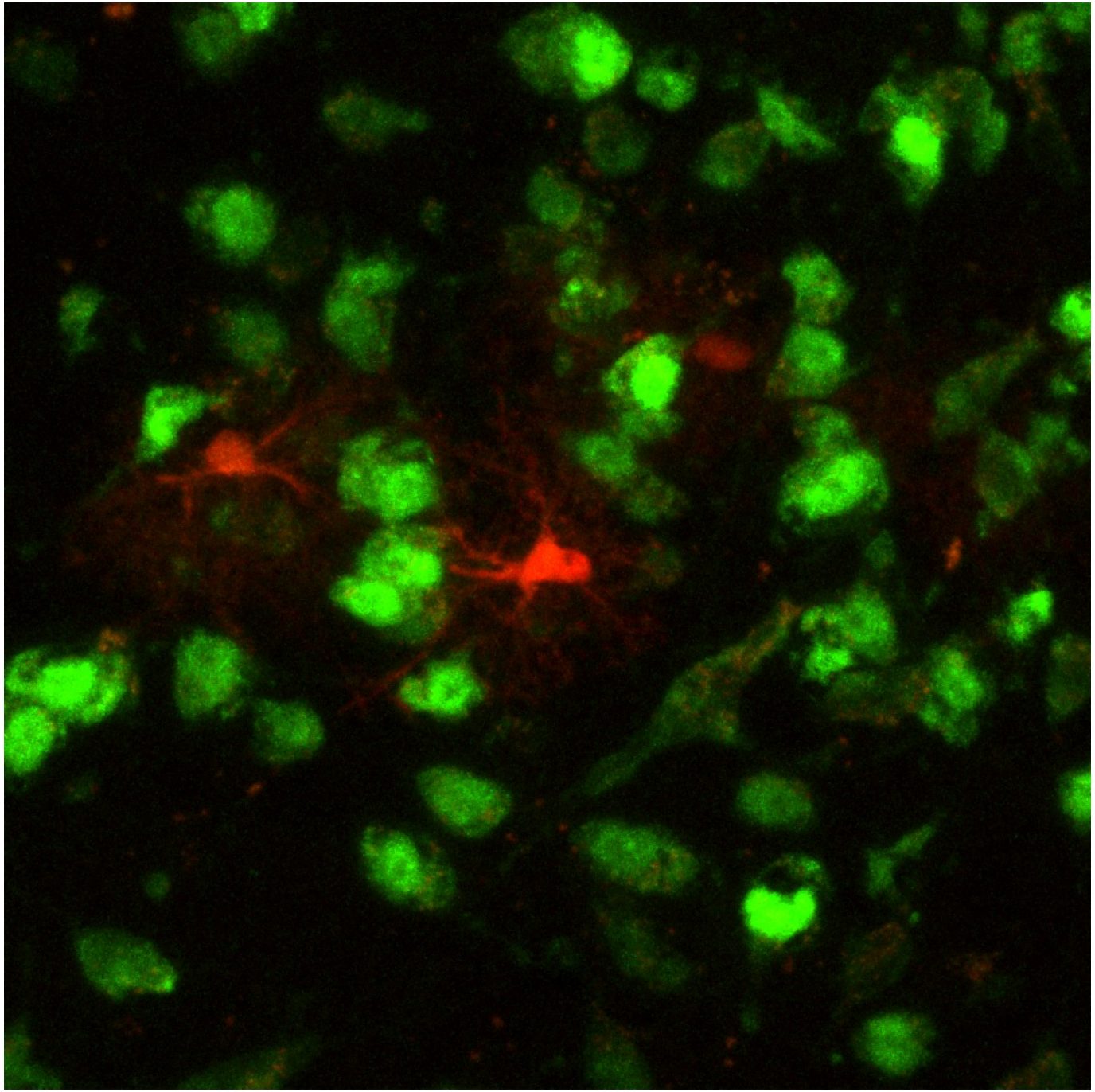
GREEN = infected cells



Cortical unlesioned

RED = Reporter; GFAP-like converted cells

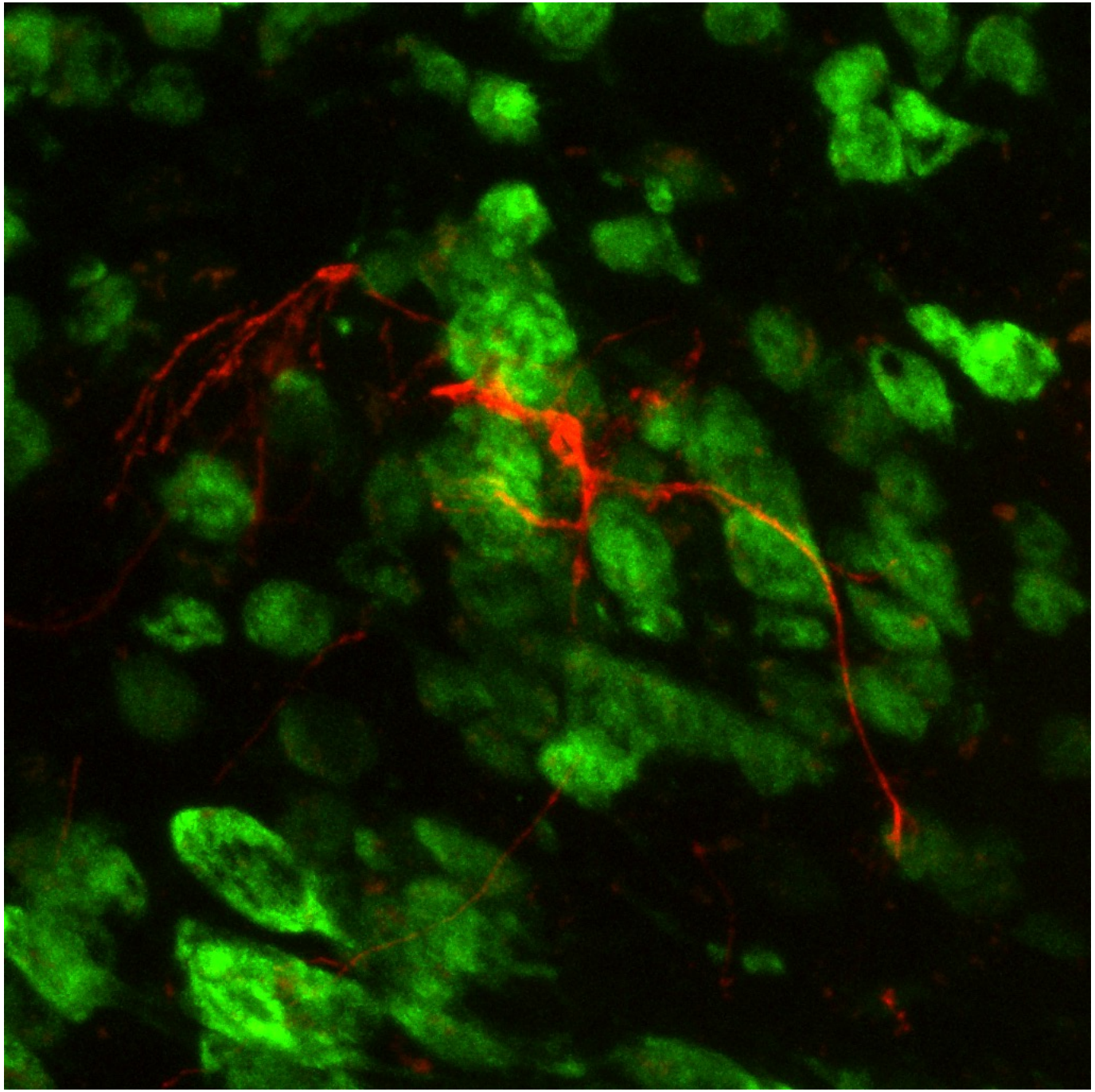
GREEN = Iba-positive
Co-localized cells



Perilesional area.

RED = Reporter; converted cells

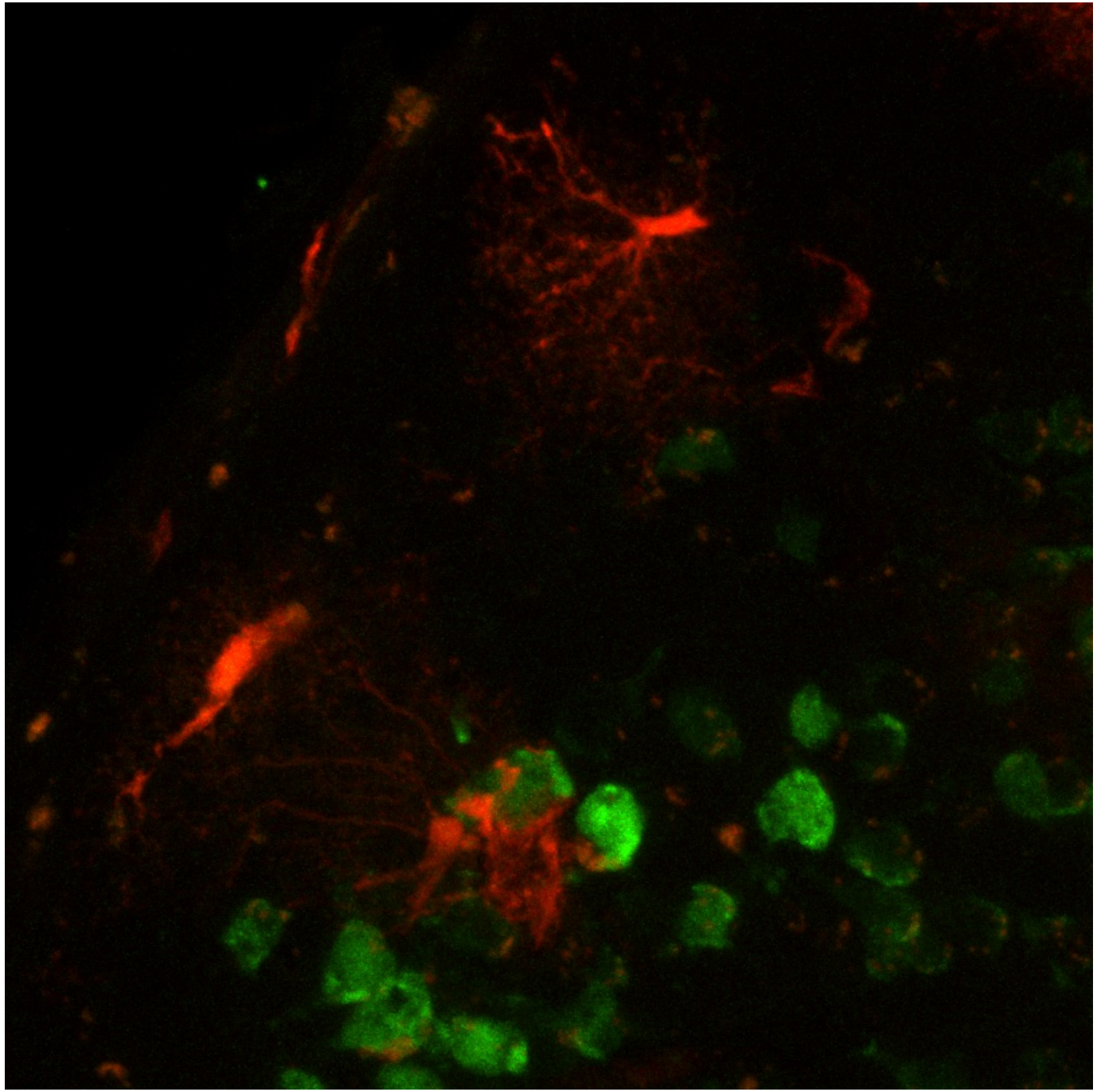
GREEN = Neurons: NeuN-positive



Subventricular zone

RED = Reporter; converted cells; neuron

GREEN = Neurons: NeuN-positive



Subventricular zone

RED = Reporter; converted cells; neuron

GREEN = Neurons
Co-localized cell

Conclusions

- **The conversion efficacy of proliferating astrocytes into neurons after cerebral ischemia in aged mice is disappointgly low**, most likely because the **therapeutical vectors carrying the conversion gene are engulfed by phagocyting macrophages shortly** after intracortical administration.

- Other viral vectors such as **adeno-associated viruses might be more efficient in promoting the conversion of reactive astrocytes.**

- **It is not clear to what extent the converted cells will be intergrated into existing network and to what extend could stimulate neurorestoration after ischemic stroke**

“Everything is possible except for sleeping on the ceiling”

Thank you all and thanks to my team!