



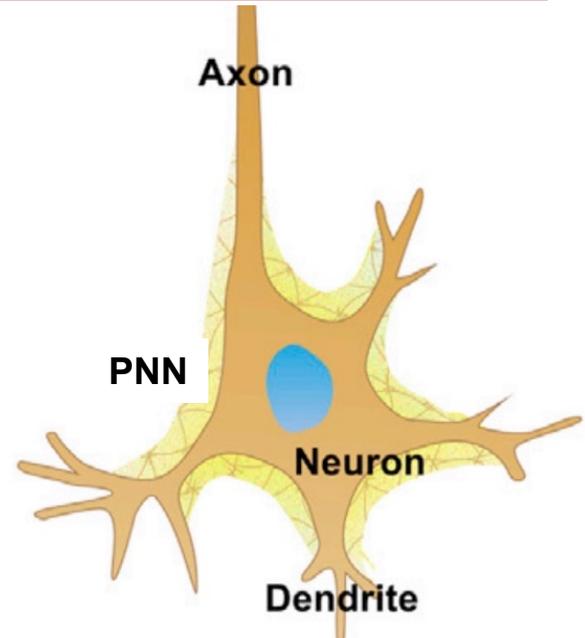
The Effect of Substrate Stiffness on Perineuronal Net Development *in vitro*

**Riley Flores, supervised by George Touloumes, Kevin Kit Parker
Disease Biophysics Group**

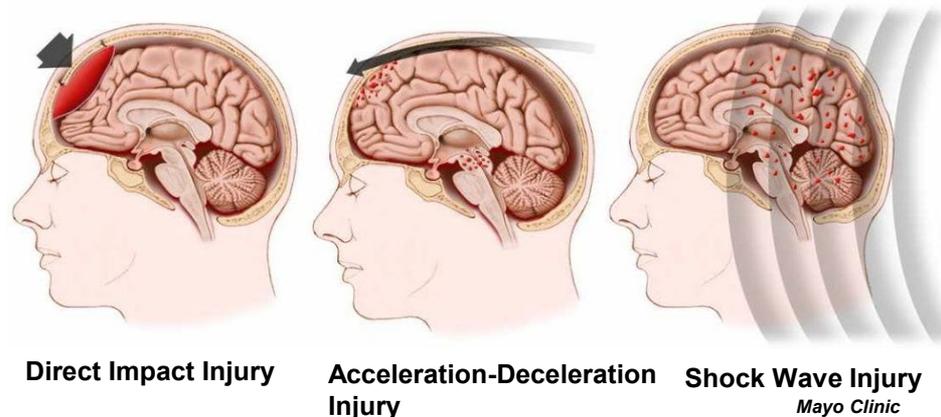


Motivation

- Perineuronal nets (PNNs) are specialized structural components of the ECM surrounding inhibitory neurons that play a role in maintaining synaptic stabilization and inhibitory/excitatory balance in the adult brain.
- Traumatic Brain Injury (TBI) alters the structure of the ECM, particularly through PNN degradation.
- TBI has been modeled *in vitro* to study its effects on neuronal network behavior, but there is limited literature on its effects on PNNs.
- PNN development has been demonstrated *in vitro*, but there are no studies reporting the effect of substrate stiffness.



Perineuronal net structure.
M Hemphill., 2015, *Neuron*



We aim to promote the natural growth and development of PNNs *in vitro* by mimicking the physiological stiffness of the brain *in vivo*.

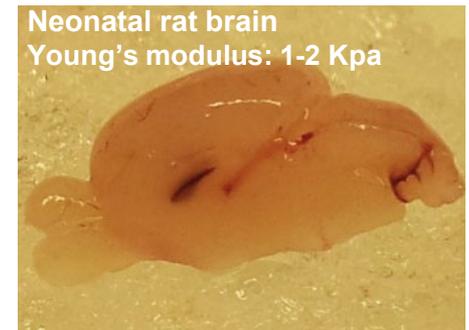
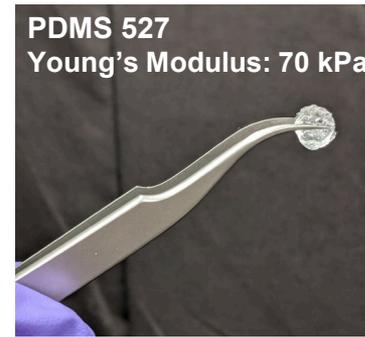
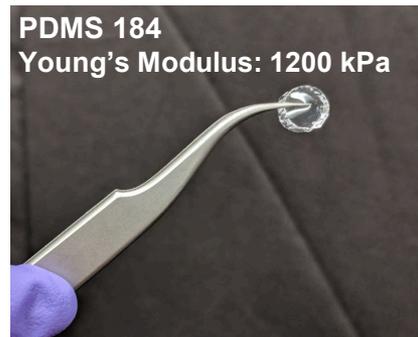
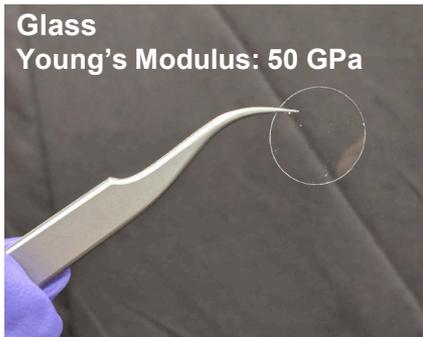


Objectives and Aims

Objective: Compare the development of PNNs on substrates of varying stiffness

Hypothesis: A physiologically soft substrate will be more conducive to the growth and development of PNNs *in vitro*.

- ✓ Aim 1: Culture rat primary neurons that develop PNNs
- ✓ Aim 2: Compare PNN development *in vitro* to PNNs *in vivo*
- Aim 3: Quantify the effects of substrate stiffness on PNN development *in vitro*



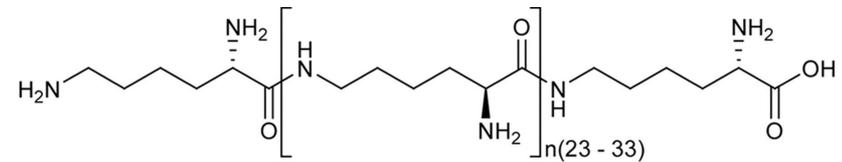
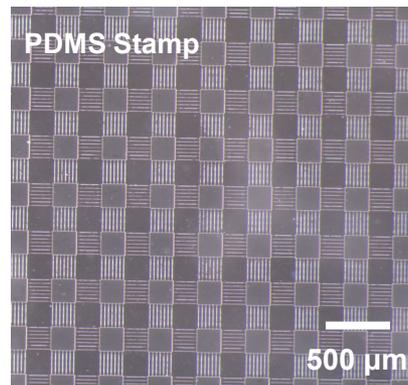
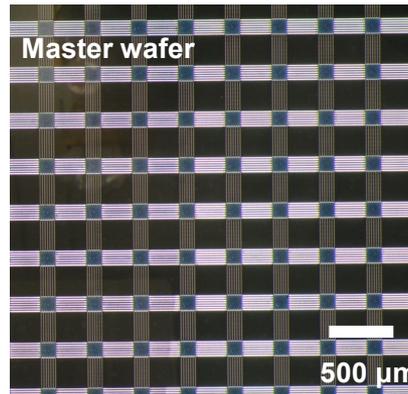
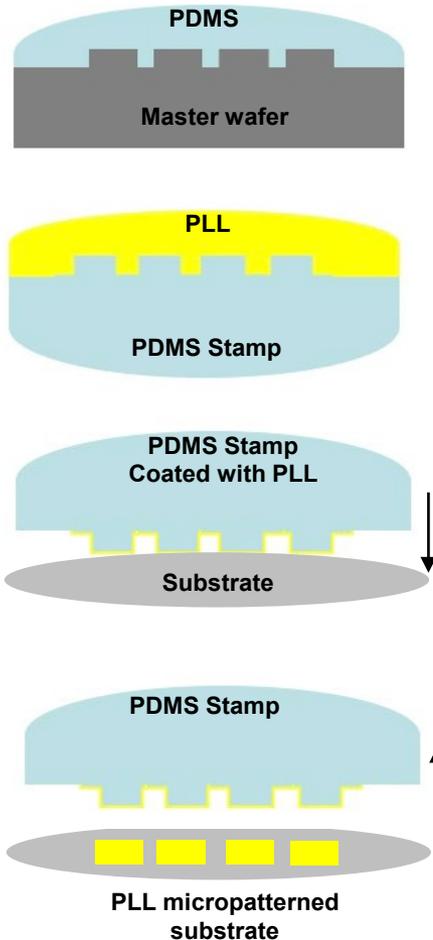
Experimental Design Considerations:

- PDMS is less stiff than glass, but still more stiff than brain tissue.
- PDMS 184, PDMS 527, and glass all have different surface chemistry.
- Neurons *in vitro* are extremely sensitive to: infection, temperature change, and mechanical trauma.
- Cell culture lifespan on PDMS is no more than 3 weeks.

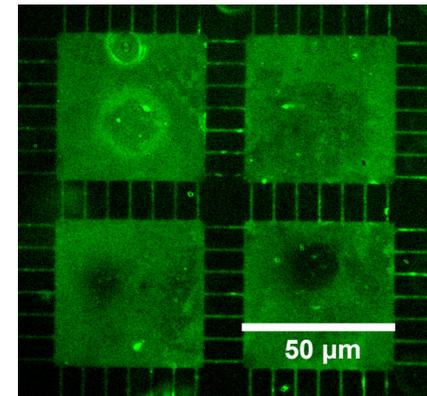


Methods

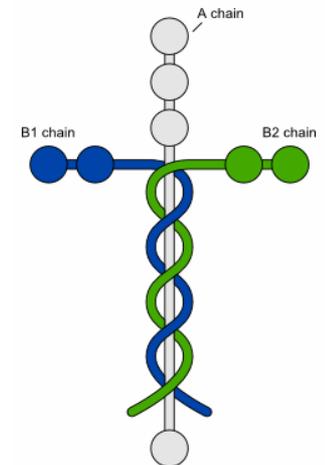
- **Microcontact print substrates with PLL (Poly-L-lysine) and laminin coated stamps**
 - PLL: positively charged polymer to which negatively charged cells and proteins adhere
 - Laminin: ECM protein, influences cell adhesion



Chemical structure of Poly-L-lysine
K. Yamanaka, 2010, *ASM*



Microcontact printed PDMS cover slip to be used for cell culture.
Green: Laminin



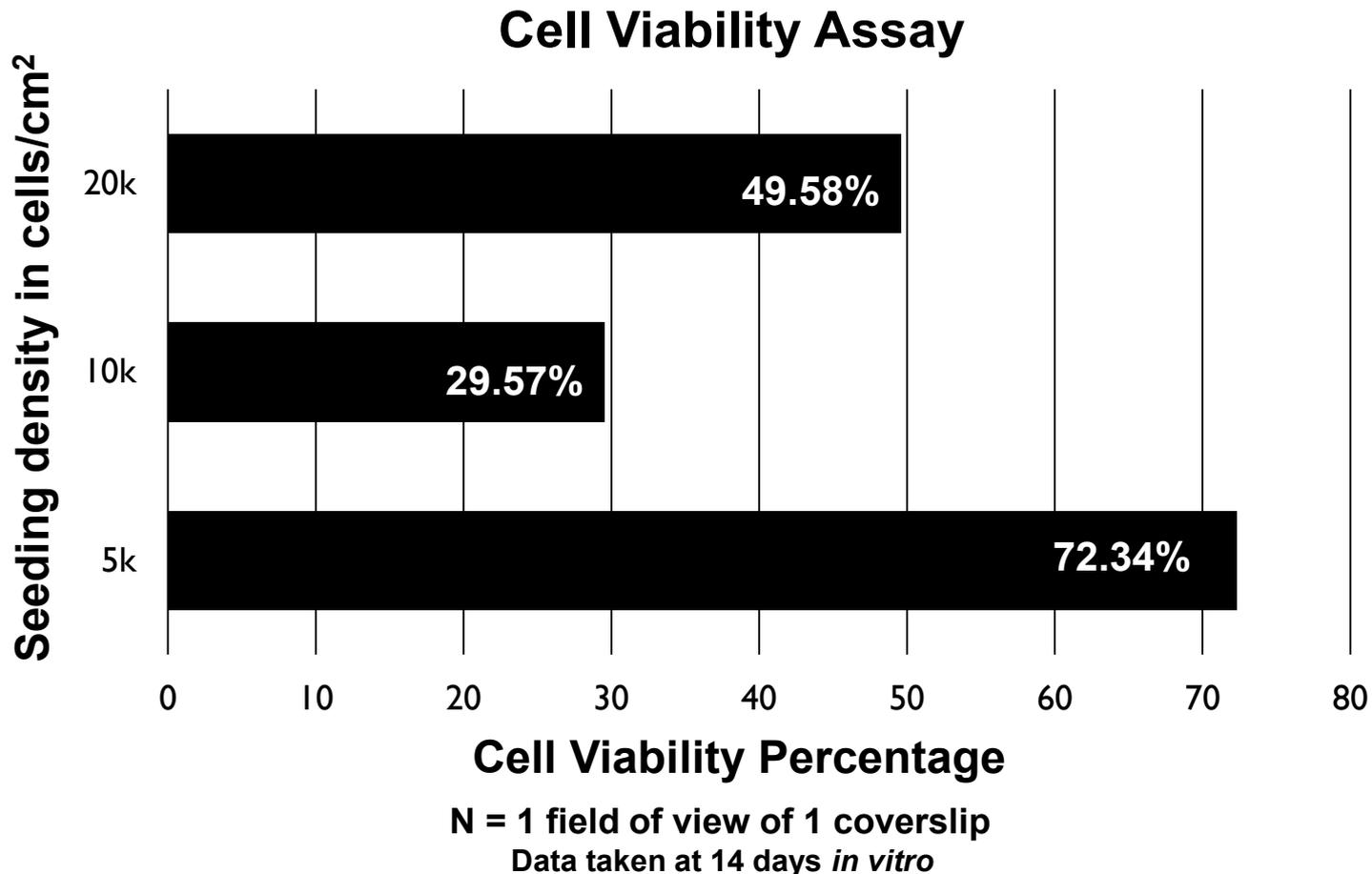
Laminin protein structure
G. Purdom, 2007, *Science*

Microcontact printing creates a defined growth area. This helps with visualization and quantification of cellular growth and network development.



Cell viability data according to seeding density

- Cells seeded at 5k cells/cm² resulted in the highest viability but sparse, underdeveloped networks. Cells seeded at 20k cells/cm² resulted in denser networks.
- This assay did not take into account cell type, which could have an impact on the data.

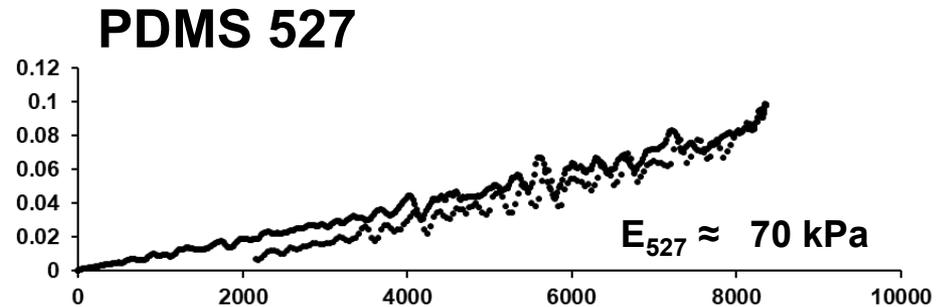
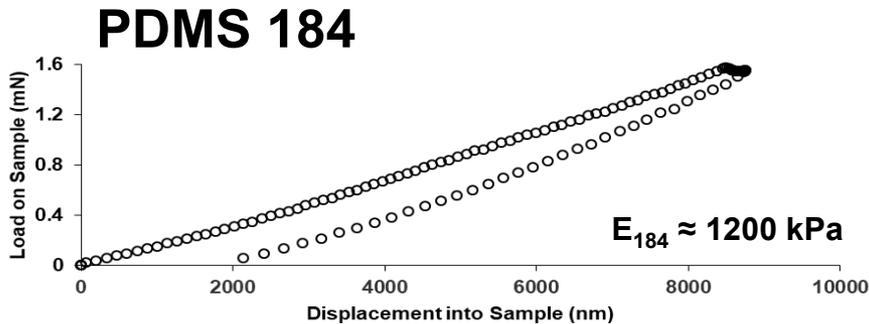


Low density resulted in good viability in the short term, but neurons need network formation to survive. Seeding at a higher density resulted in denser networks.



PDMS Mechanical Testing

- Stiffness testing shows differences in displacement based on load between PDMS 184 and PDMS 527.
- Results show that PDMS 527 is much less stiff than PDMS 184. A substrate of low stiffness, comparable to brain tissue, would be more conducive to neuron growth and development.



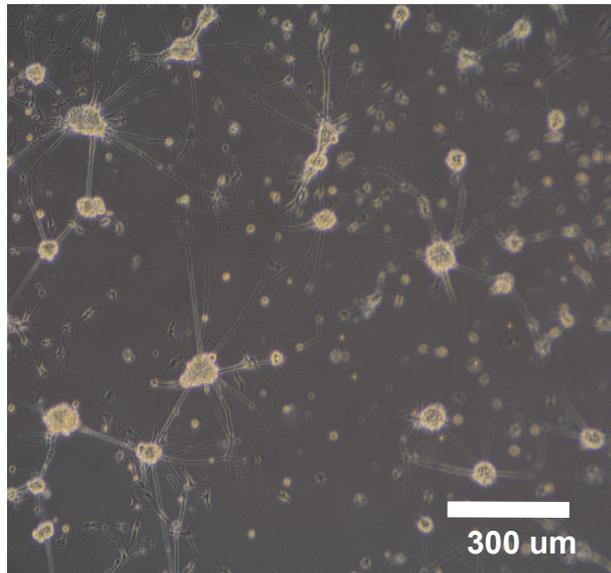
Data plotted is mean of $n = 4$ test points from $N = 1$ sample

Tissue stiffness increases upon injury. Immature brain is physiologically stiffer; with higher plasticity.

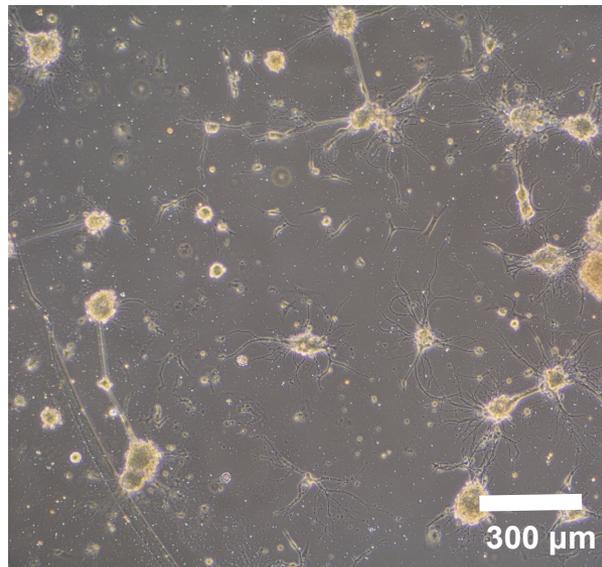


Comparing neuronal network development on different substrate stiffnesses

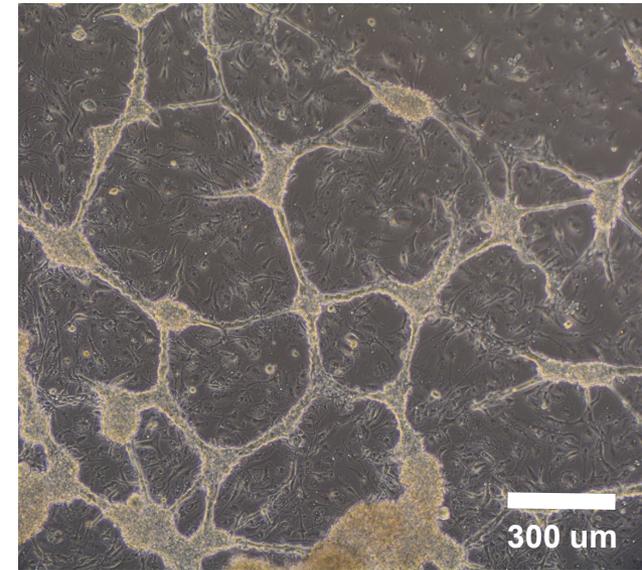
Glass
Young's Modulus: 50 MPa



PDMS 184
Young's Modulus: 1200 kPa



PDMS 527
Young's Modulus: 70 kPa



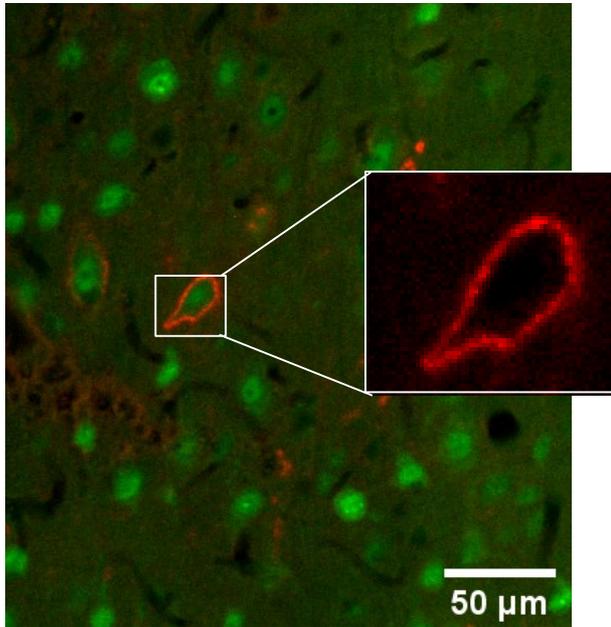
Rat primary neurons day 7 in vitro
Seeded at 25k cells/cm² on PLL coated microcontact printed substrates of various stiffness.

Low stiffness leads cells to adhere to each other, forming dense clusters, as opposed to adhering to the substrate.



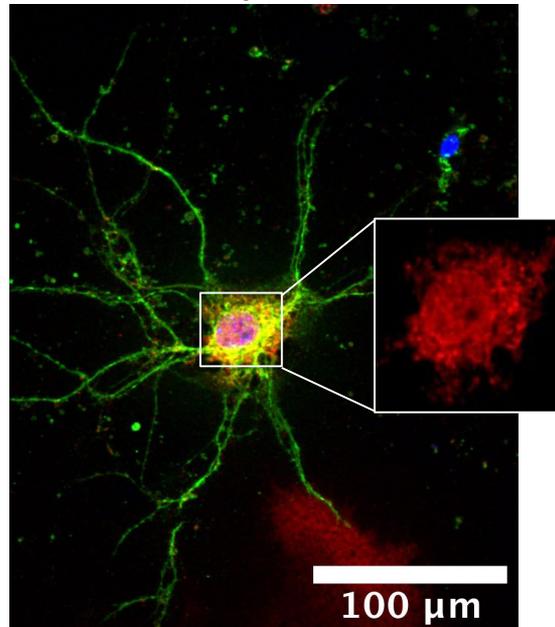
PNN development in vitro vs in vivo

PNN Growth *in vivo*



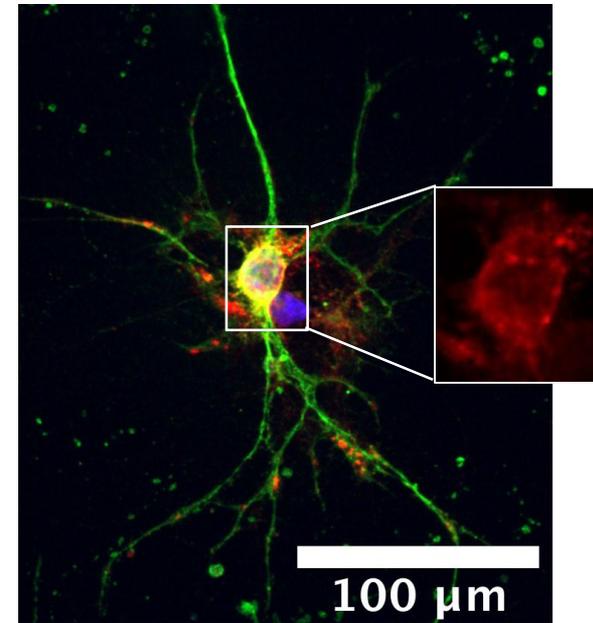
Red: WFA (PNN), Green: PV (Inhibitory Neurons),
Adult rat

PNN Growth day 21 *in vitro*



Red: WFA (PNNs), Green: MAP2 (All Neurons)
Blue: DAPI (Nuclei, all cells)
Rat primary neurons seeded on glass

PNN Growth day 21 *in vitro*



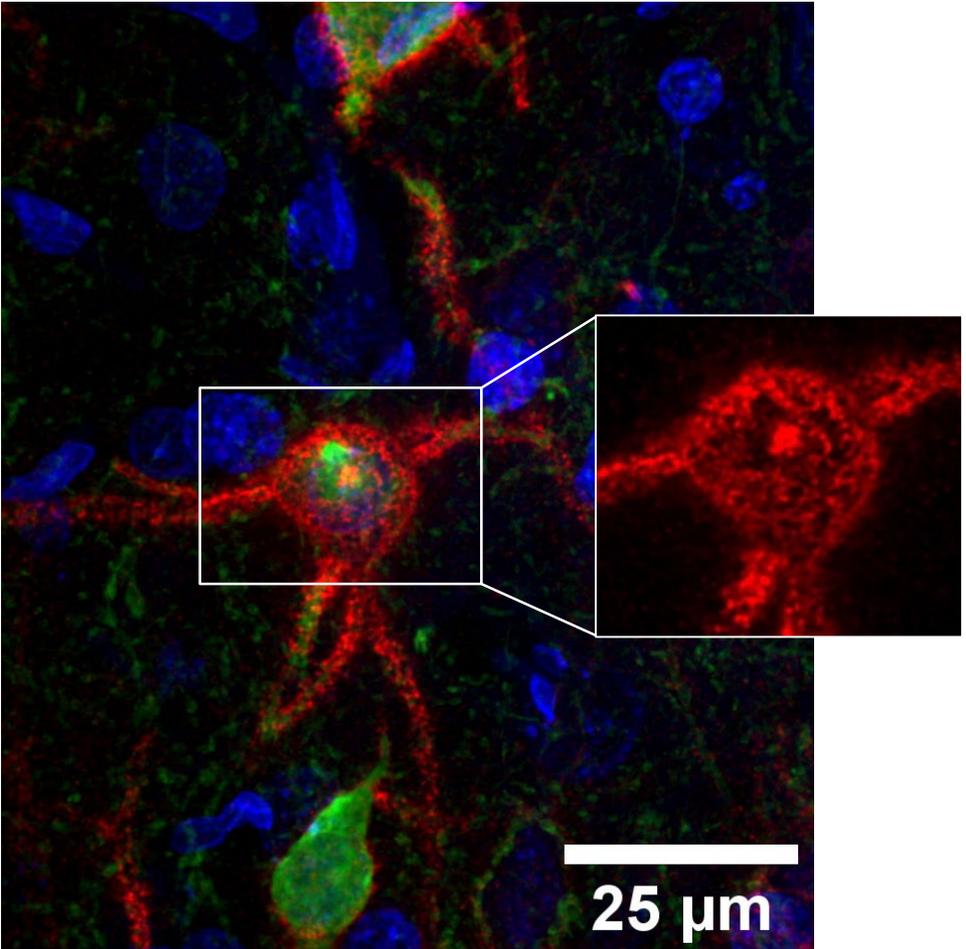
Red: WFA (PNNs), Green: MAP2 (All Neurons)
Blue: DAPI (Nuclei, all cells)
Rat primary neurons seeded on glass

We have successfully cultured and identified neurons with PNNs *in vitro*.



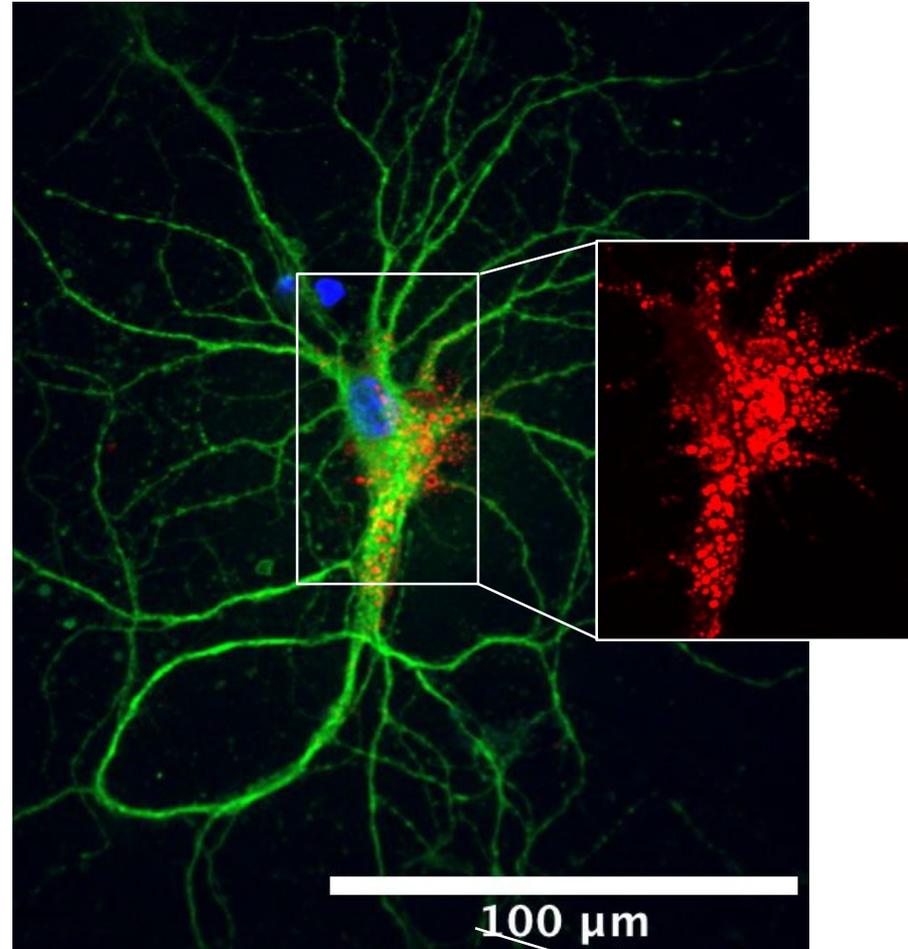
PNN development in vitro vs in vivo

PNNs in adult rat neurons *in vivo*



Red: WFA (PNNs), Green: Parvalbumin (Inhibitory Neurons) Blue: DAPI (Nuclei, all cells)

PNNs in rat primary neurons day 21 in vitro.



Red: WFA (PNNs), Green: MAP2 (All Neurons) Blue: DAPI (Nuclei, all cells)

Key observations: PNN morphological variance *in vivo*



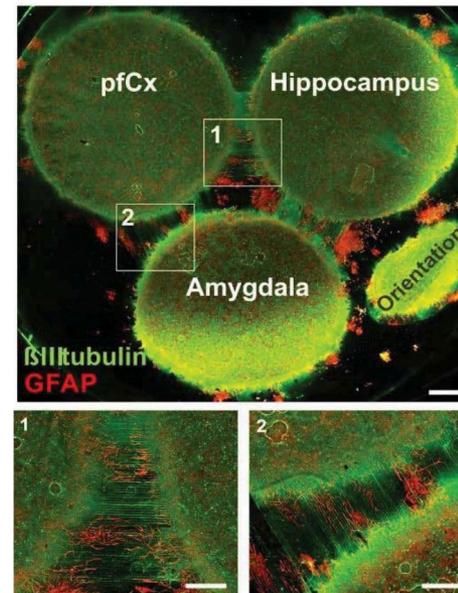
Observations

Key Observations: PNN morphological variance *in vivo*

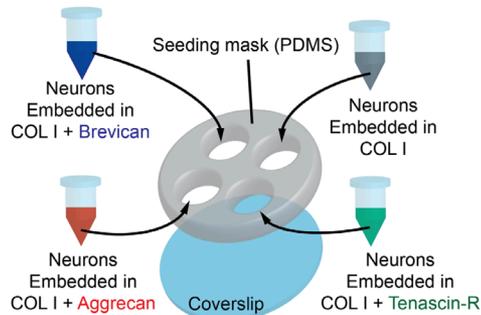
- There are several structural differences between PNNs *in vivo* and PNNs *in vitro*.
- The optimal time frame for the development of PNNs is at least 21 days. 21 days is also the upper limit for culturing cells on PDMS.

Limitations

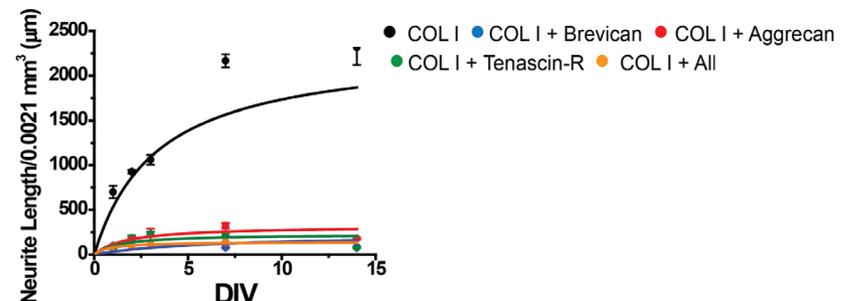
- *In vivo*, any one neuron has about a thousand times the connections any one neuron *in vitro* does. Differences in network connectivity could effect PNN structure.
- The fact that we are culturing 2D networks may also have an effect on the physical structure of the PNNs we have identified.
- Further study is required to effectively quantify the effects of substrate stiffness on PNN development *in vitro*.



S. Dauth, *J Comp Neurol*, 2016



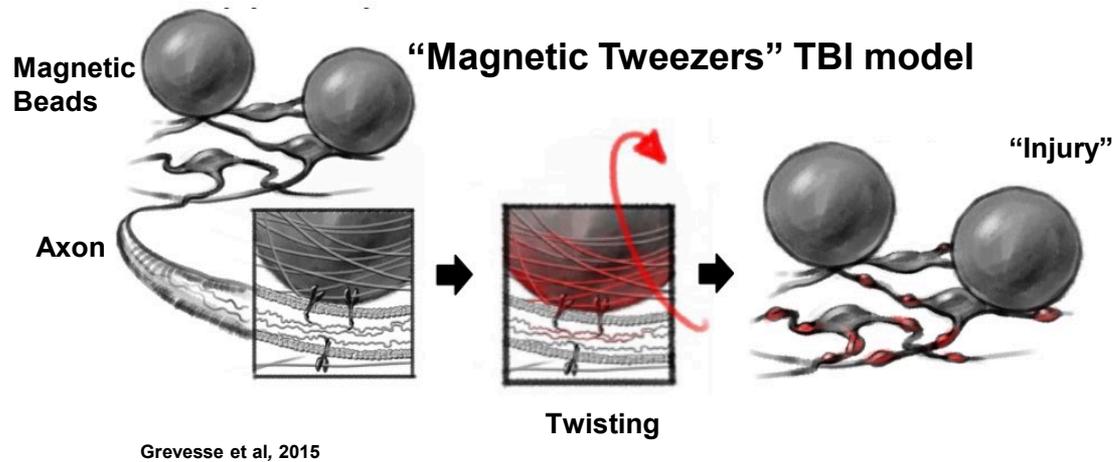
S. Dauth, *J Neurophysiol*, 2017





Conclusions

- Changes in the microenvironment due to damage to the ECM could affect PNN recovery and contribute to secondary TBI symptoms such as epilepsy.
- A successful model of the ECM will allow us to extend the current model of TBI *in vitro* to study how PNNs affect the sensitivity of neurons to mechanical trauma.



A successful model of the ECM will allow us to eventually apply our current model of TBI *in vitro* to study how PNNs affect the sensitivity of neurons to mechanical trauma.