Optimising the fibroblast-to-sensory-neuron differentiation protocol

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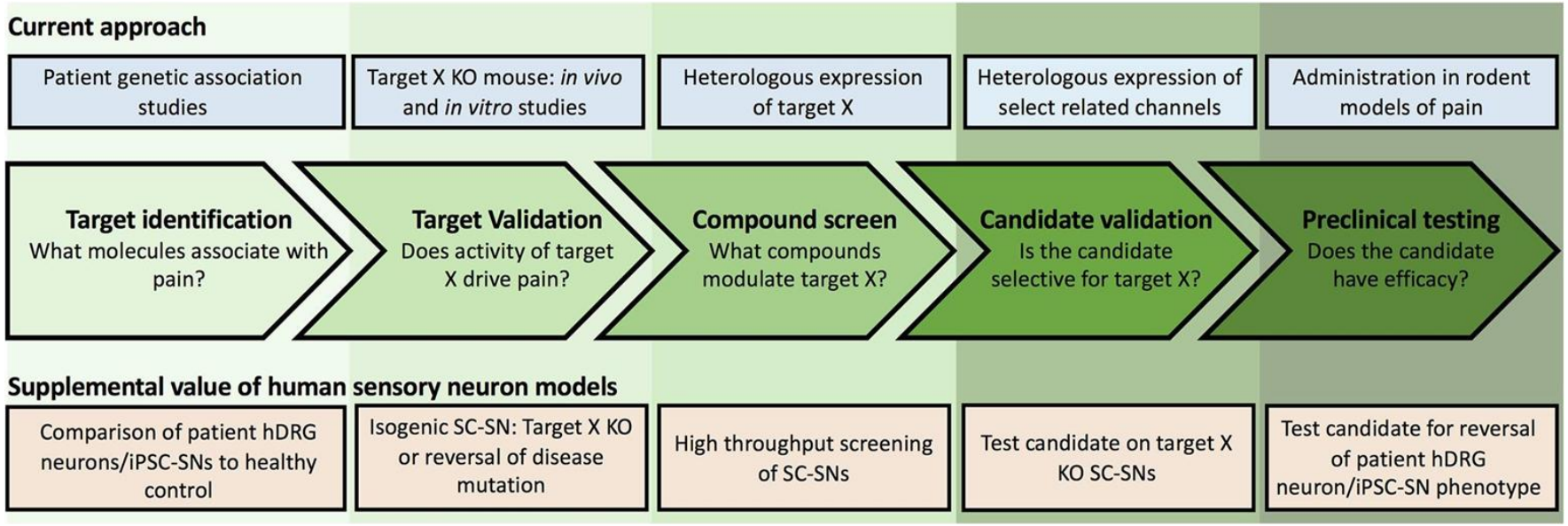
Associate scientist virtual work experience

LifeArc

Introduction

- *In vitro*-derived sensory neurons are gaining momentum in pain research and drug development



Chrysostomidou et al., *Neurobiol Pain* 2021

Introduction

- A fibroblast-to-sensory-neuron differentiation protocol (Blanchard et al., *Nat Neursci 2015,* with modifications)

1. **Day 0:** Infect human fibroblasts with lentiviruses expressing BRN3A and NGN1.
2. **Day 2:** Induce transcription factor expression with Doxycycline.
3. **Day 4:** Switch to N3 neural induction medium (+ Doxycycline).
4. **Day 7:** Withdraw Doxycycline.
5. **Day 10:** Switch to DMEM/F12:Neurobasal medium + supplements (B27, NGF, BDNF, GDNF). Optionally add NT3.
6. **Day 14:** Assess differentiation (e.g., MAP2 staining).

Aims

Optimise the differentiation protocol conditions with respect to:

- NGN2 virus concentration, measured by Multiplicity of Infection (MOI).
- Neurotrophin 3 (NT3) supplement concentration.

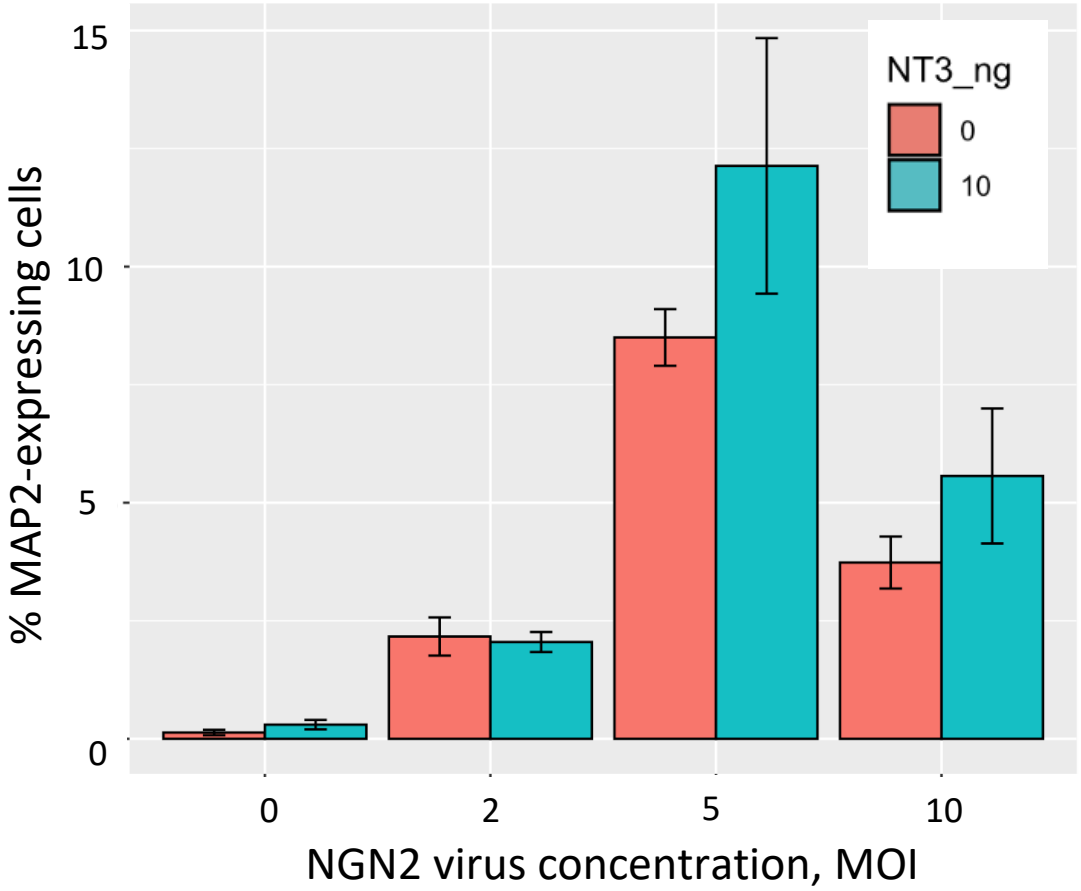
Experimental design

Key Features:

- Human fibroblasts were used as the starting cell type.
- Tested different NGN2 lentivirus Multiplicities of Infection (MOIs): 0, 2, 5, and 10.
- Tested two concentrations of NT3 supplement: 0 ng/mL and 10 ng/mL (Note: Protocol mentions 25 ng/mL optionally).
- Experiment performed in 96-well plates with ~20,000 cells/well.

- Assessing the expression of mature neuronal marker MAP2 (by immunostaining with anti-MAP2 Ab) after 14 days

Results



(Note how the labels & layout of the plot generated in R were modified slightly in PowerPoint for better readability on slides)

- The fraction of MAP2-positive cells increases with NGN2 MOI, peaking at an MOI of 5.
- An MOI of 10 resulted in lower MAP2 expression than an MOI of 5.
- Addition of NT3 (10 ng/mL) generally increased the fraction of MAP2+ cells, with the strongest effect observed at NGN2 MOI 5.
- One outlier replicate was removed from the 2 MOI + 10 ng/mL NT3 condition.

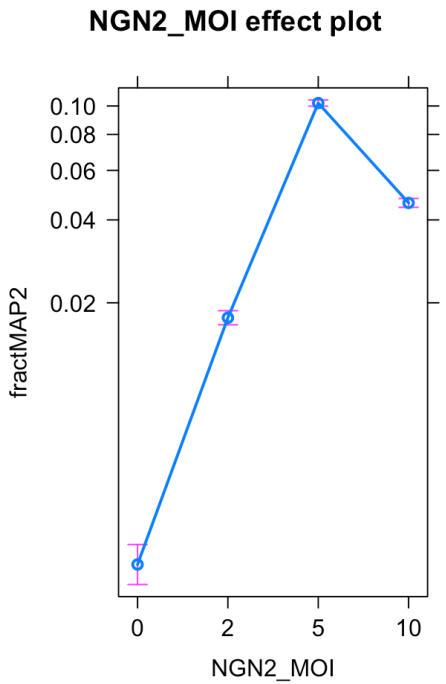
- One outlier replicate (~0% MAP2+ cells) was removed from 2 MOI+NT3, without much effect on the overall results

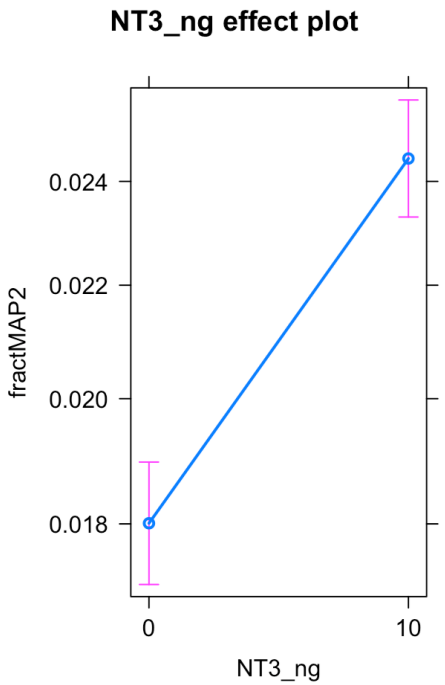
Results

IF YOU SKIPPED LOGISTIC REGRESSION ANALYSIS IN TASK 2, CONSIDER OMITTING THIS SLIDE

- Establish the statistical significance of differences between conditions using **logistic regression**

- Chose logistic regression, because:
  - The outcome is a proportion (over 10,000 scanned cells)
  - Have combinations of treatments and want to establish the effect of each one





Logit(Fraction MAP2+) = k1*NGN2_MOI + k2*NT3_ng

- Using treatment doses as factors to allow for discontinuous trends

Key Conclusions (using results after outlier removal):

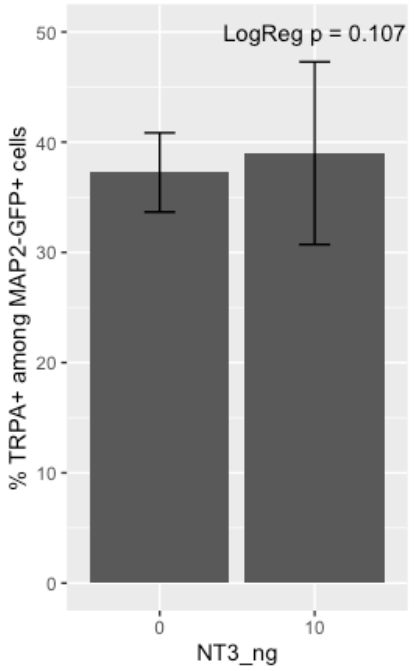
- Both NGN2 MOI and NT3 concentration significantly impact the likelihood of MAP2 expression (all p-values < 0.001).
- NGN2 MOI 5 yields the highest MAP2 expression, significantly greater than MOI 0, 2, and 10.
- Adding NT3 (10 ng/mL) significantly increases the fraction of MAP2-positive cells across NGN2 concentrations.

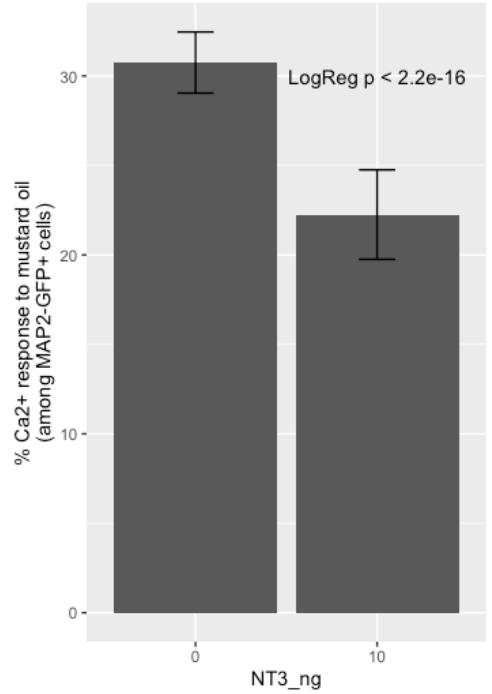
Follow-up analysis (performed by Ela)

- MAP2 is not a specific marker of sensory/nociceptor neurons
- Does NT3 help generate nociceptors?
- **Ela's analyses using MAP2-GFP fibroblasts**

Summary Points:

- TRPA1 Expression: Adding NT3 (10 ng/mL) did not significantly change the percentage of TRPA1-positive cells within the MAP2-positive population (p = 0.107).
- Functional Response (Mustard Oil): Adding NT3 (10 ng/mL) significantly decreased the percentage of MAP2-positive cells showing a Ca2+ response to mustard oil (a functional assay for some nociceptors) (p < 2.2e-16).





Conclusions

- What are the optimal NGN2 virus and NT3 supplement concentrations for maximising the yield of MAP2-positive neurons?
  - NGN2 MOI of 5 combined with 10 ng/mL NT3 provides the highest fraction of MAP2-positive cel (~12%).

- What is your recommendation for the optimal conditions to use to generate nociceptor neurons?
  - While NT3 increases overall MAP2+ cell number, it significantly reduces the proportion of functionally responsive nociceptors (mustard oil assay).
  - Therefore, NGN2 MOI 5 without NT3 addition appears optimal for generating functional nociceptors based on current data.

- What is the overall efficiency of the protocol with respect to generating nociceptor neurons? (Provide an approximate percentage and your calculations)
  - Efficiency = (% MAP2+ at MOI 5, 0 NT3) * (% Mustard Oil Responders at 0 NT3)
  - Efficiency ≈ 8.5% * 31% ≈ 2.6%

Next steps

- What is a potential disadvantage of using MAP2 as a marker to monitor the differentiation?
  - **Disadvantage of MAP2:** It is a general neuronal marker, not specific for sensory or nociceptor subtypes.
- What markers/assays would you propose as potential alternative(s)? For routine assessment of protocol performance? What are their advantages/disadvantages compared with MAP2?
  - **Markers**: Peripherin, Islet1, TRPA1, TRPV1 (specific sensory/nociceptor markers). Advantages: Higher specificity. Disadvantages: May require different antibodies, potentially lower expression levels.
  - **Assays**: Calcium imaging with specific agonists (capsaicin, menthol, mustard oil), Patch-clamp electrophysiology. Advantages: Assess function directly. Disadvantages: More complex, lower throughput.
- Is the overall protocol efficiency sufficiently high in your opinion?
  - 2.6% for functional nociceptors is likely too low for reliable high-throughput screening.
- Should the team consider further optimising the protocol?
  - Yes, consider exploring: other transcription factors, different timing for induction/withdrawal, additional media components or growth factors.
- What strategies could be considered for enriching the cultures for neurons post-hoc?
  - Fluorescence-Activated Cell Sorting (FACS) using a nociceptor-specific surface marker (if available), or selective survival protocols that eliminate non-neuronal cells.