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IMPORTANT POUR LE BIOREACTEUR: <https://www.sciencedirect.com/science/article/pii/0026286281900844>  
 Old paper that shows that in brain capillaries, the average velocity of blood is about 1.4ml/hr (or 0.5mm/s). In our model, impossible to reach (would consume too much media, or should find a way to get a closed system, which would be ideal!), so instead we go for a static culture conversion (change medium 3 times in 7 days with 500ul: flow of 8ul/hr).

*"The development of a tumour is guided by interactions in the microenvironment including the dynamics of interstitial fluid, and microcirculation of blood vessels. This complexity leads to tumour heterogeneity and heterogeneity between tumours of various origins and at differing stages of progression. Development of new therapeutics relies on screening in an accurate recapitulation of the tumour microenvironment, which is currently not reflected in the majority of current preclinical models. Therapeutics. After in vivo studies 60% of cancer therapeutics proven efficient in two-dimensional in vitro models are excluded due to ineffectiveness. Previously in our lab, a prototype bioreactor culture platform capable of supporting 3D micro-tissues formed either from established cancer cell lines or from small amounts of patient tissue has been developed. The small amount of tissue needed makes this system ideal for sustaining cancers isolated from the brain or pancreas as clinical samples are rare. The bioreactor design incorporates the perfusion of culture media to mimic the flow of the interstitial environment of tumours and resultant fluid dynamics. Effects of therapeutics with potential impact on the tumour microenvironment will be observed on the micro-tissue, which allows for screening and validation of cancer therapeutics in a physiologically relevant microenvironment prior to in vivo testing."*

## 1 Introduction

- parler de SVZ et de son utilite first off mais aussi de son lien a la formation de cancer (the closer the tumour is from the stem cell niche, the higher the recurrence - probably a link there, Sherlock).

- parler des therapies existantes pour brain tumours et dire les limites du field et les advances (WBRT, SRS).

- justifier que la neurogenese n'est pas un phenomene qui se reduit a l'enfance. Que nenni. Par exemple, mentionner le paper avec the adult rat. + mentionner les techniques qui ont permis d'en apprendre plus (BrdU)

INTRODUCTION routes: SY5Y: to see a differential effect compared to the healthy cells. All the more relevant since neuroblastomas occur mostly in young children (which have a bigger stem cell niche). Ren VM: damage to normal tissue (stem cell niche) evaluate the effects of radiation on ren VM (model for healthy stem cell niche) say for head and neck cancer patients. Evaluation of the combination of radiation and drug treatment on the morphology and differentiation state of neural (stem) cells Maybe try and get some data/stats on the characterisation of stem cells. Also interested to look into data from Retinoblastoma patients (on the long run), maybe if there are cohors/data available, could be cool to compare whole brain irradiation vs beam). This allows us to finally propose that by irradiating the SVZ region, one might exhaust the stem cell pool, leading to more cognitive issues while growing up.

In and of itself, having brain metastasis is going to affect your cognitive capacities (correlation between the size of the tumour/s and the extent of the disability), but still important to value this quality of life with treatment.

Ecrire sous la forme "l'hypothese c'etait bim et du coup on a teste boom, et ca a valide l'hypothese avec ces P-values ou rejet, mais il y a eu des limites parce que tant. On souhaiterait faire telle experience pour compenser".

TRO COOL: memantine, une drug qui a ete combinee a radiation pour faire un trial et qui a montre un protective effect of cognitive resources; ca a l'air d'augmenter le nombre de radial-like glial cells ce qui peut etre le meme effet que les DYRK1A inhibitors, peut-être que c'est pour ca qu'on a un effet de protection, ca stimule l'envie de se diviser? Mais pourquoi la differentiation....

STATISTICS: - Test for the normal distribution (check on google) -

### 1.1 DYRK1A, green tea and down-syndrome

The DYRK (dual-specificity tyrosine-regulated kinase) family covers a group of kinases found both in human and more distant organisms such as yeast or drosophila. They autophosphorylate a Tyrosine (Y) residue during translation leading to kinase activation, and become available for Serine and Threonine phosphorylation once fully translated. In 1997, a headline published in Nature read: 'Why drinking green tea could prevent cancer'. While this article focused on urokinase and its role in cancer, it also cast light on polyphenols, here epigallocatechin-3 gallate (ECGC). ECGC finds relevance in the DYRK family as it was later discovered that it is a strong inhibitor of DYRK1A.

These recent years have led to a growing interest for DYRK1A in particular as its gene sits in the Down Syndrome critical region (DSCR) of chromosome 21, leading to a 1.5-fold overexpression. Such increase has been associated to neuronal development aberrations although the intrinsic pathways involved remain unclear.

DYRK1A has many substrates and so far, evidence has shown its involvement in at least 3 distinct pathways: apoptosis, cell cycle and differentiation.

- 1.1.1 *DYRK1A and apoptosis*
- 1.1.2 *DYRK1A and the cell cycle*
- 1.1.3 *DYRK1A and differentiation*
- 1.1.4 *DYRK1A and cancer: an ambiguous bond*

It is both tumour suppressor and oncogene. Because of cell cycle block at the beginning (which means lower tendency to proliferate out of control) but becomes dangerous as the disease progresses since it protects it from being eradicated (pro-survival signals).

DYRK1A is constitutively active, but is extremely dose-sensitive. Its transcripts or protein levels can be altered very efficiently on various (?) levels: indeed, its mRNA transcript contains a destabilising sequence (AU-rich elements, or AREs) and the kinase has a PEST region which is a common tag for protein degradation through proteasome or calpain.

Where the intuition comes from, how DS individuals all have mental retardation to some extent, found of the genes in the overexpressed (on Chr21)

Extremely sensitive and dose-dependent gene: over or under expression leads to decreased neonatal viability.

DYRK1A is an inhibitor of proliferation so treatment with inhibitors should increase proliferation rates? Its loss of function leads to overproliferation and mass cell death ( [?]). DYRK1A overexpression leads to Hippo inhibition (which is a break on organ expansion)! Depending on the context, completely different effect.

**ATTENTION: "overexpression of DYRK1A is necessary to induce neural differentiation" which would mean that treatment with inhibitors should block any differentiation (we should have less markers for it if cells have been treated).**

what we know about it (neurons, etc), how DYRK1A inhibitors came about.

#### 1.1.5 *Radiation and DYRK1A*

DYRK1A is a negative regulator of intrinsic death (= mitochondria, check caspase 9). So inhibition should SENSITISE cells to radiation (would be nice to check for apoptosis).

apoptosis

### 1.2 Culture methods

- 1.2.1 *2D and 3D*
- 1.2.2 *Perfusion models*

DO MORE RESEARCH.

### 1.3 Aims and Objectives

## 2 Materials and methods

### 2.1 Cell lines and culture conditions

#### 2.1.1 *ReNcell VM cell line*

The neural stem cell line ReNcell VM cells (from now on referred to as VM cells) was kindly gifted by Delia Koennig, originally purchased from Millipore (Merck). They were thawed according to the manufacturer's protocol. Briefly, a T75 culture flask was coated with Laminin (Sigma-Aldrich) at 20ug/ml diluted in DMEM/F12 (without HEPES). After letting the laminin incubate for at least 4 hours at 37C and 5% CO<sub>2</sub>, the DMEM was gently aspirated and the coated flask was rinsed with PBS. After warming up ReNcell Maintenance Medium, both growth factors were added to [FGF] = [EGF] = 20ng/ml to obtain Complete Medium (CM is not stable and must be freshly prepared). 10 ml of this medium was added to the coated flask which was left to incubate at 37C until use. The cells were thawed quickly in a water bath and transferred to a 15 ml conical tube before adding 9 ml of ReNcell MM progressively (to protect the cells from osmotic shock). They were centrifuged at 300g for 5 minutes and the supernatant was aspirated. The cells were resuspended in 5 ml of CM, plated on the laminin-coated flask and incubated at 37C, 5%CO<sub>2</sub>. The medium was changed the following day.

**Table 1:** Data used for ReNcell VM culture

| Dishes        | Seeding density   | Accutase (ml) | DMEM-diluted laminin (ml) | Growth medium (ml) |
|---------------|-------------------|---------------|---------------------------|--------------------|
| 10cm dish     | $2.5 \times 10^6$ | 3             | 3                         | 10                 |
| 6-well plate  | $3 \times 10^5$   | 2             | 2                         | 3                  |
| 12-well plate | $1 \times 10^5$   | 1             | 1                         | 1                  |
| 24-well plate | $5 \times 10^4$   | 0.3           | 0.3                       | 0.5                |
| 96-well plate | $2 \times 10^4$   | 0.1           | 0.1                       | 0.2                |
| T25 flask     | $7 \times 10^5$   | 3             | 3                         | 4                  |
| T75 flask     | $2 \times 10^6$   | 5             | 5                         | 8                  |

The cells were passaged when they reached 80% confluence according to the manufacturer's protocol. Briefly, the cells were washed once with PBS and incubated in Accutase for 3 minutes at 37°C, 5%CO<sub>2</sub>. The Accutase was neutralised with MM and the cells were transferred to a 15 ml conical tube which was centrifuged at 300g for 5 minutes. The supernatant was aspirated and the cells were resuspended in 2 ml of CM. They were counted manually and seeded accordingly (see Table 2) on laminin-coated plates.

For cryopreservation, the cells were counted to obtain  $4.10^6$  cells/ml, centrifuged at 300g for 5 minutes and resuspended in ReNcell freezing medium (Millipore, Merck) to freeze 1 ml of cells per cryogenic vial. They were slowly frozen down with a Mr. Frosty (Sigma-Aldrich) and kept at -80C or in liquid nitrogen for longer storage (over 3 months).

Differentiation of ReNcell VM cells was induced by culturing the cells in growth factor-free ReNcell Maintenance Medium. Cells were allowed 24 hours to attach in complete medium before changing to maintenance medium.

### 2.1.2 SH-SY5Y cell line

The human bone marrow neuroblastoma SH-SY5Y cell line (from now on referred to as SY5Y cells) was purchased from Sigma-Aldrich. The growth medium recommended by the company is Ham's F12:EMEM (EBSS) (1:) + 2mM Glutamine + 1% Non Essential Amino Acids (NEAA) + 15% Foetal Bovine Serum (FBS). However, the literature reports that using DMEM-F12 (Cat. no. 21041025, Gibco) + 10% FBS allows normal cell growth [?], which is what was used here as the medium was readily available.

They frozen cells were resuscitated according to the manufacturer's protocol. Briefly, the cryogenic vial was wrapped in a 70% ethanol soaked tissue and opened in a ventilated hood to make sure that any residual liquid nitrogen was released before thawing. The cells were then transferred in a water bath until almost completely thawed (1-2 minutes). They were gently transferred to a 15 ml sterile tube and 5 ml of growth medium was added drop-wise (to avoid osmotic shock). The 15 ml tube was centrifuged at 300 g for 5 minutes and the cell pellet was resuspended in the appropriate complete medium. Finally, the cells were transferred to a T25 flask and the medium was changed the following day.

For cryopreservation, the cells were harvested and counted to obtain  $3 \times 10^6$ /cryovial, centrifuged at 300g for 5 minutes and resuspended in growth medium + 5% DMSO to obtain a final cell concentration of  $3 \times 10^6$ /ml. After transfer of 1ml/cryogenic vial, the cells were slowly frozen down with a Mr. Frosty and kept at -80C or in liquid nitrogen for longer storage.

Differentiation of SY5Y cells was induced by adding Retinoic Acid () to the growth medium. This differentiation medium is not stable and must be prepared freshly before every use, and RA must be kept in dark conditions as it is light-sensitive. Cells were allowed 24 hours in growth medium before differentiation induction.

## 2.2 DYRK1A Inhibitors

Leucettine 41 was purchased from Biovision (Cat. no. 2617), INDY was from Sigma-Aldrich (Cat. no. 1169755-45-6) and Harmine was from Cayman Cheminal (Cat. no. 442-51-3).

**Table 2:** Data used for SH-SY5Y culture

| Dishes        | Seeding density   | Trypsin (ml) | Growth medium (ml) |
|---------------|-------------------|--------------|--------------------|
| 10cm dish     | $7.5 \times 10^5$ | 3            | 10                 |
| 96-well plate | $2 \times 10^4$   | 0.1          | 0.2                |
| T75 flask     | $7.5 \times 10^5$ | 5            | 10                 |
| T175 flask    | $2 \times 10^6$   | 10           | 20                 |

All inhibitors were diluted in DMSO (Sigma-Aldrich) to obtain 10mM stock concentrations, aliquoted and kept in -20C.

During DYRK1A inhibitor treatment, DMSO was used as a negative control and each condition was topped up with DMSO to normalise and account for the effect of the toxic solvant.

### 2.3 Transient siRNA knockdown of DYRK1A

The ON-TARGETplus Human DYRK1A SMARTpool (cat no. L-004805-00-0005) siRNA was purchased from Dharmacon (Horizon Discovery Ltd).

#### 2.3.1 Electroporation

ReNcell VM cells were reverse transfected following AmaxaTM 4D-NucleofectorTM Protocol for P3 Primary Mammalian Neurons. Briefly,  $2.5 \times 10^5$  wells were centrifuged at 300g for 5 minutes and resuspended in 20 ul of P3 4D-NucleofectorTM solution before adding siRNA and pmaxGFP plasmid (provided by the kit), respectively 100-300 nM and 0.4 ug. This mix was carefully added to the 20 ul NucleocuvetteTM Strip (also from the kit) which was inserted in the Nucleofector 4D with the programme CM-162 (previously optimised by Delia Koennig). Following the nucleofection, the plate was left to incubate for 10 minutes at RT before adding 100 ul of complete medium. With a Pasteur pipette, the 120 ul were collected and added drop-wise in laminin-coated plates. The medium was changed the following day.

#### 2.3.2 Lipid transfection

SY5Y and ReNcell VM cells were transfected using Lipofectamine™ 2000 (Invitrogen) and Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's protocols. Briefly, cells were plated the day prior to transfection and on the day, rinsed with PBS and incubated in OptiMEM™ (Cat. no. 11058021, Gibco). Then, in a first 1.5ml eppendorf tube, 5 ul of Lipofectamine 2000 Reagent was diluted in 145 ul of OptiMEM™ and in another, 100, 200 or 300 nM SiRNA and 2 ug of GFP plasmid (TROUVER!!) were diluted in OptiMEM™ to a total volume of 150 ul. The Lipofectamine™ mix was combined to the SiRNA/plasmid tube and left to incubate for 15 minutes at room temperature. Finally, the mix was added drop-wise on previously plated cells. After 4 hours of incubation at 37C, 5%CO2, fluorescence was assessed with (NOTER LE MINI MICRSCOPE) and the cells were rinsed with PBS and transferred in growth medium to limit cell toxicity. The medium was changed again the following day.

### 2.4 Cell viability assay

Cell viability was assessed with alamarBlue(R) (CHECK!) following the manufacturer's protocol. Briefly, 10% of the total medium volume of the fluorescent solution was added to the cells. After incubation at 37C, 5%CO2 for 1-4 hours (depending on average cell number), 200 ul of media were extracted and split in a regular and a black 96-well plate (CHECK!). The first plate was used to determine the absorbance of the samples at  $\lambda_1 = 570$  nm and  $\lambda_2 = 600$  nm. The second plate allowed verification of the first data by obtaining the fluorescence of the samples at  $\lambda = 585$  (CHECK). Both measurements were obtained with POLARstar Omega plate reader (BMG LABTECH).

### 2.5 Immunofluorescence

Coverslips (Cat. no. 631-0149, VWR) were coated with 20ug/ml of laminin (L2020, Sigma-Aldrich) for ReNcell VM cells. The cells are washed twice with PBS and fixed with 4% PFA (Sigma-Aldrich) for 15 minutes at RT shielded from light. The coverslips are washed 3 x 5 minutes

**Table 3:** Antibodies used in IF Also I used TUB3 for SY5Y second staining, for the rest, the us'

| Protein | Species | Dilution | Cat. no. | Company    | Target                             | Secondary antibody |
|---------|---------|----------|----------|------------|------------------------------------|--------------------|
| SOX2    | Rabbit  | 1:400    | 3579P    | CST        | Marker for stem cells              | Alexa Fluor 594    |
| Nestin  | Goat    | 1:100    | sc-21248 | Santa Cruz | Marker for stem cells              | Alexa Fluor 488    |
| MAP2    | Rabbit  | 1:200    | 8707     | CST        | Marker for mature neurons          | Alexa Fluor 594    |
| Tuj1    | Mouse   | 1:100    | 801202   | Biolegend  | Marker for mature neurons (fibers) | Alexa Fluor 488    |
| Olig1   | Mouse   | 1:100    | MAB1327  | RD Systems | Marker for mature oligodendrocytes | Alexa Fluor 647    |
| GFAP    | Rat     | 1:100    | 13-0300  | Invitrogen | Marker for glial cells             | Alexa Fluor 647    |

with PBS and permeabilised with 0.2% Triton X-100 (Thermo Fisher Scientific) in PBS for 10 minutes and washed 3 x 5 minutes in PBS. They are blocked in 3% BSA (Invitrogen) for 1 hour at RT before primary antibody incubation o/n at 4C (dilutions in table 3). The cells are washed 3 x 5 minutes in PBS and incubated in the secondary antibodies away from light for 1 hour at RT. The coverslips were washed 3 final times with PBS and were mounted with DAPI (ABCAM MAIS CHECK MIEUX) and sealed with nail varnish. Image acquisition was done with a Zeiss LSM710 microscope and processed with ZEN software.

## 2.6 Western blot

Cells were put on ice and rinsed with ice-cold PBS twice. After removing any trace of PBS, lysis buffer (composition of LB in Table 5 and ??) was added and the plates were scraped, transferred in chill 1.5ml tubes. We vortexed the samples thoroughly, let them sit 30 minutes on ice and sonicated 5 for 5 pulses at power = 20. Cells were centrifuged at 14 000 rpm for 10 minutes at 4C. Transfer the supernatant in clean chilled tubes and determine protein concentration with a BCA assay.

### 2.6.1 BCA assay

Protein concentration was established following the Pierce BCA Protein Assay Kit (Thermo Scientific) with Pierce bovine serum albumin standards. In a 96-well plate, samples and standards were duplicated. After adding the Working Reagent (Thermo Scientific), the plate incubated at 37C for 30 minutes before measuring the absorbance ( $\lambda = 620$  nm) with a POLARstar Omega plate reader (BMG LABTECH).

### 2.6.2 Bradford assay

Protein concentration for limited volume samples was established using Coommassie Blue (.). In a 96-well plate, samples and standards were pipetted in triplicates of 1ul and 200ul of Coommassie Blue were added. After mixing thoroughly and removing any bubble, the absorbance at  $\lambda = 595$ nm was measured with a POLARstar Omega plate reader.

### 2.6.3 Running the gel

The samples were denatured at 90C for 10 minutes and centrifuged briefly before loading on pre-cast NuPAGE™ 4-12% Bis-Tris Protein gels. They were run at 120V for approximately one hour in SDS MOPS running buffer (NuPage) and transferred on a methanol-activated nitrocellulose membrane at 100V for one hour in ice-cold transfer buffer. The membrane was then washed and blocked for an hour at room temperature in blocking buffer (PBS + 0.1% Tween + 5% milk) before incubating overnight at 4C in the primary antibodies. (?) Checker Tween The membrane was then

**Table 4:** Primary antibodies used in WB. They were diluted in blocking buffer with 1:500 sodium azide.

| Protein           | Species | Dilution | Cat. no.  | Company    |
|-------------------|---------|----------|-----------|------------|
| Caspase 9         | Mouse   | 1:1000   | 9508S     | CST        |
| Cleaved Caspase 3 | Rabbit  | 1:1000   | 9661S     | CST        |
| DYRK1A            | Rabbit  | 1:1000   | sc-130741 | Santa Cruz |
| GAPDH             | Rabbit  | 1:1000   | AB128915  | Abcam      |
| p-Thr-Pro         | Mouse   | 1:1000   | 9391S     | CST        |
| RASSF1A           | Mouse   | 1:1000   | AB23950   | Abcam      |
| SIRT1             | Mouse   | 1:1000   | 8469S     | CST        |
| YAP               | Rabbit  | 1:1000   | 4912S     | CST        |

washed three times in PBS-T and incubated 1 hour at room temperature in secondary antibodies. Finally, the membrane was washed again and covered in ECL for one minute to then be developed.

**Table 5:** Composition of lysis buffer used for the western blots (scaled for 1.5ml)

| Component                       | Stock | Volume     |
|---------------------------------|-------|------------|
| Na <sub>3</sub> vO <sub>4</sub> | 1x    | 1.5 uL     |
| β-glycerophosphate              | 1X    | 15 uL      |
| Protease inhibitors             | 50X   | 30 uL      |
| NaF                             | 1X    | 150 uL     |
| RIPA buffer                     | 1X    | 1 303.5 uL |

**Table 6:** Composition of radioimmuno-precipitation assay (RIPA) buffer.

| Component           | Stock |
|---------------------|-------|
| Sodium deoxycholate | 0.5%  |
| EDTA                | 5mM   |
| Tris-HC             | 50mM  |
| NaCl                | 1X    |
| NP-40               | 1%    |

## 2.7 Bioreactor simulation

Using Autodesk Inventor Professional 2019 and Autodesk CFD 2018 with a student licence - maybe COMSOL Multiphysics??

The prototypes were built on Autodesk Inventor Professional 2019 using the same technique catered to various designs: drawing the 2D base, extruding it to obtain a 3D model then exporting this raw file to the CFD 2018 Module. One must then set the material (here, we approximated the medium to plain water) and the boundary conditions. For the inlet, we set the volume flow to 10uL/hr. This value stems from static culture comparison:

$$\text{inlet flow} = \frac{\text{volume per well}}{\text{frequency of medium change}} = \frac{500 \text{ uL}}{48 \text{ hours}}$$

The outlet condition is pressure since the our set up does not use a pump on the outlet side. To visualise the mixing capacity of a prototype, the initial volume is filled with water and the scalar "0" (colour red), and the inlet lets scalar "1" in, mimicking the influx of new medium. This is a way to show two liquids mixing, with assumed mass diffusivity constant of  $2.10\exp{-5}$ . This is the diffusion coefficient of oxygen in water. Note: our bioreactor simulation has a capacity of about 785uL (and we add about 10uL/hr).

## 2.8 Casting bioreactor

The bioreactor is made of polydimethylsiloxane, or PDMS, which is a form of silicone. It is achieved by mixing the polymers (Sylgard 184 Silicone Elastomer, Dow Corning) and the cross-

linking reagent (Sylgard 184 Silicone Elastomer Curing Agent, Dow Corning) with a 10:1 ratio, which is then poured in the mould. It is then put into a vacuum chamber for one hour to allow encased bubbles to surface up and be removed. Finally, the bioreactor must bake in a 60C oven for 24 hours. It is then removed from the mould and autoclaved before use.

## 2.9 Radiation

Either with concentrating beam Or with the shielding thing. DIFFERENT. - changed the medium of all cells before radiation every time. (IF ADDING DRUGS, should add right before the radiation rather than after, that means no need to change the milieu after)

- briefly explain how x-ray generators work, to factor in the heel effect (check dans les favoris, le site est super clair nde-ed). - explain why we chose x-rays and not alpha-particles for instance: x-rays are the most relevant form of radiation in the in vitro context since the purpose would be to transfer such findings in the clinic, where x-rays are still the standard treatment. - use of 225kv and 17mA on the EXP 1 (96 well plate, for 1.25min - more or less 2Gy). - use of 250kV and 12mA on the EXP 2 (24 well plate, exactly 2Gy).

"PROTOCOL": - new 'machine' to automate the movement of the plate depending on how it must be radiated (so that one needn't enter the room every time to change the placement of the lead - ultimately renders the experiments a lot more reproducible). Still in development so we chose to sacrifice the 8th column in half.

### 2.9.1 Dosimetry

Initial step was to put the motors and set them to the plate+field size. Then add a film (just to get a feel of the dose distribution) -; got a good distribution (cannot see a heel effect just by looking at the film transcript).

Heel effect: The end result is that the field intensity towards the cathode is more than that towards the anode (due to electrons traversing the target and having to escape it - many are resorbed). Described in my book. This is compensated (partially) by a 'heel effect compensation' filter (which also hardens the beam to get rid of 'useless' soft x-rays) which is made of concentric copper rings.

Use of collimator to get a fairly high dose-rate while limiting scatter and achieving a homogeneous dose distribution.

### 2.9.2 Protocol?

## 2.10 Oxidative stress assay

The extent of oxidative stress was determined with a dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. The DCFH-DA (Sigma-Aldrich) was diluted in DMSO to a stock concentration of 10mg/ml (1000X).

### 2.10.1 Microscopy

The cells were rinsed gently twice with PBS, before adding DCFH-DA further diluted to 1X in PBS. They were incubated at 37C, 5%CO2 shielded from light for 30 minutes. Image acquisition was performed with a (CHECK WHICH MICROSCOPE, LITTLE OR RHOD?).

### 2.10.2 Flow Cytometry

The cells were washed gently twice with PBS and detached with Trypsin (SY5Y cell line) or Accutase (ReNcell VM). They were then spinned down at 300g x 5 minutes at 4C and washed twice with FACS buffer (2% FBS + 2mM EDTA in PBS) in round-bottom 96-well plates. They were resuspended in DCFH-DA further diluted to 1X in PBS and incubated at 37C, 5%CO2 for 30 minutes before being exposed to 2 Gy of x-rays in suspension. They were kept on ice and analysed straight-away with an Attune Autosampler NxT Flow Cytometer (Thermo Fisher).

## 2.11 Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 (version 7.1, GraphPad Software, Inc.). Shapiro-Wilk normality test was performed on every data set to assess the distribution. The standard variation of comparable sets was checked to justify the use of Welch's correction on the unpaired T-test. A parametric or non-parametric (Mann-Whitney for non-Gaussian distributions) t-test was then performed to investigate any significant difference.

### 3 Results

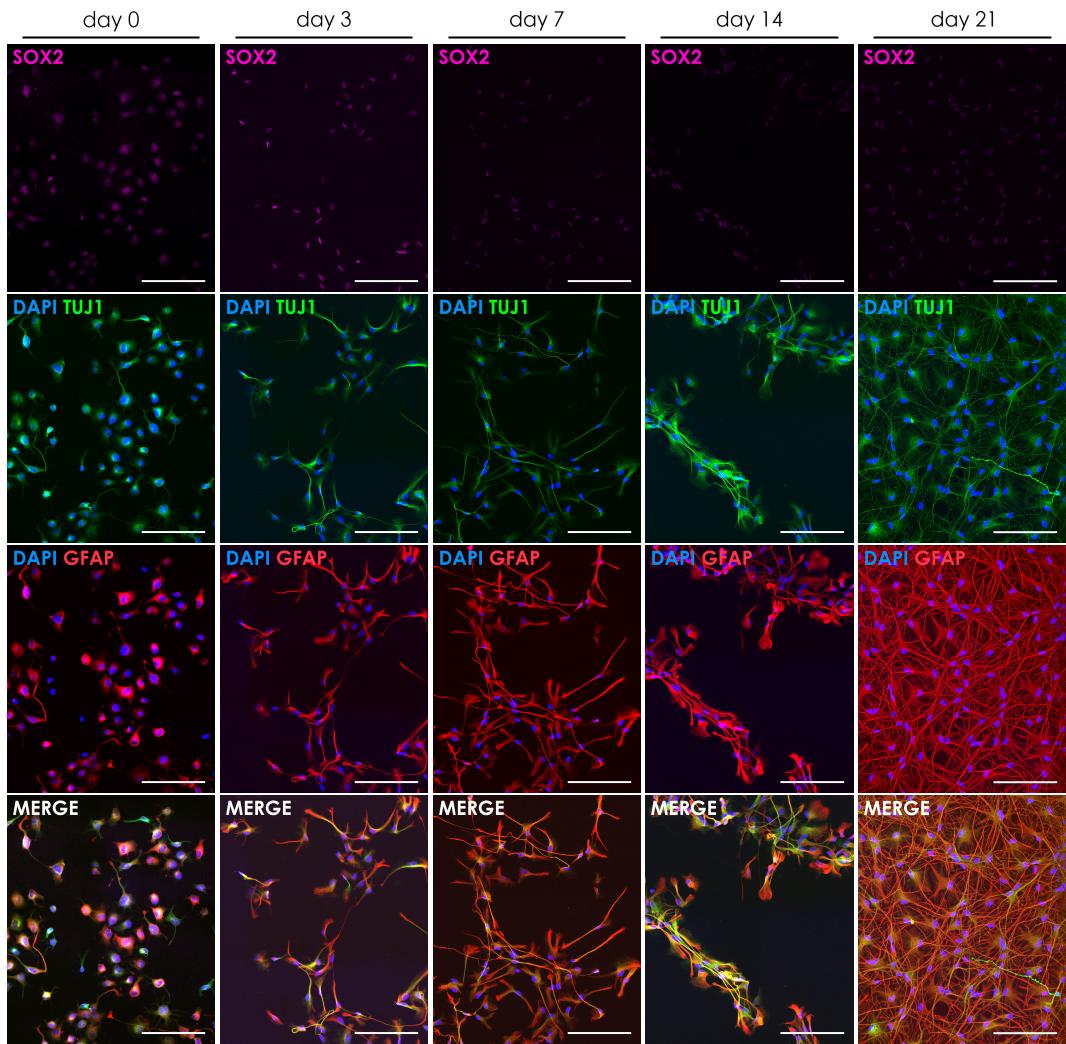
#### 3.1 Characterisation of the SY5Y and ReNcell VM cell lines

##### 3.1.1 The ReNcell VM cell line

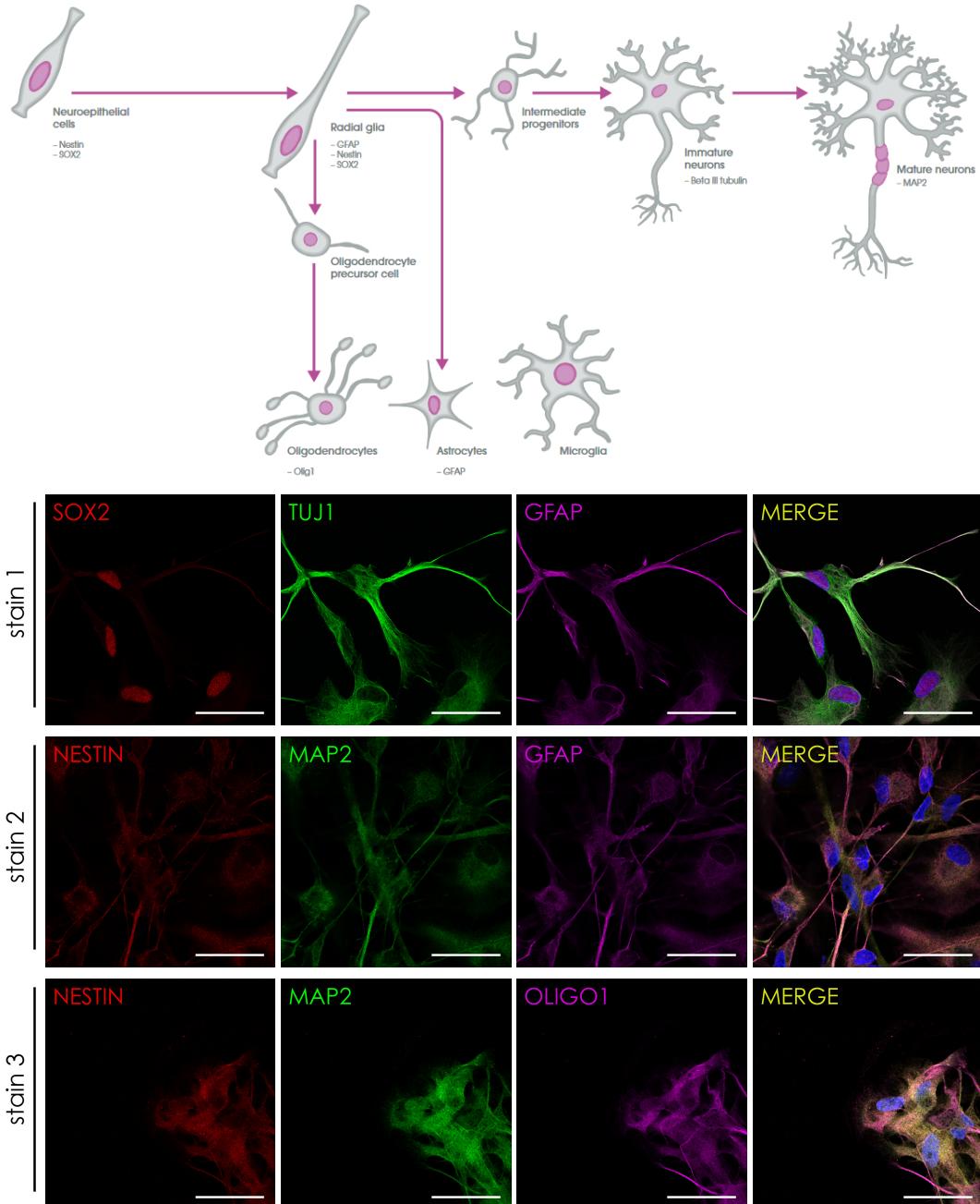
According to the workflow depicted in Figure 3.8, the cells were plated on Day 0 in complete medium which was changed to differentiation medium on Day 1 (with or without drug inhibitors). This was maintained for 4 days to allow significant differentiation. Finally, the cells were radiated with 2 Gy x-rays and kept to grow for 2 more days before being harvested.

In this context, their differentiation pattern was assessed with immunofluorescence over 21 days of culture in maintenance medium. The results of the 5 time points can be found in Figure 3.1. This characterisation assay revealed an important feature of ReN VM cells which is their ability to express a wide array of markers. The Figure 3.2.A sums up the markers used in neural stem cell culture to determine differentiation. Instead of the binary dogma of positive or negative cells, the VM cells were positive for each marker. This was further verified by comparing different antibody stains on Day 14 and Day 21 (not shown) differentiated cells on 3.2.B. The antibodies were chosen according to the Abcam's neural marker guide and availability in the laboratory's stock. Once again, the cells were positive for both stem cell markers (Nestin and SOX2) and an array of differentiated neural cells (immature neurons with  $\beta$ -III tubulin, now referred to as Tuj1, oligodendrocytes with Oligo1 and radial glia with GFAP).

It was thus tricky to rely exclusively on the immunofluorescence signal of each marker. Instead, the differentiation stage was determined using the morphology. Indeed, the cells increasingly elongate as they differentiate and form neurites, which is reflected in both Tuj1 and GFAP stains. The whole cell body size decreases as well as they thin out and extend to form connexions with nearby cells.



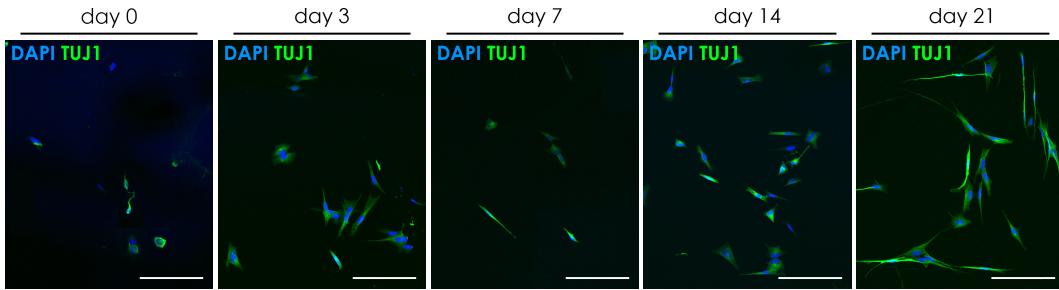
**Figure 3.1: Ren VM cells express a wide array of neural differentiation markers.** The differentiation capacity of ReNcell VM cells was assessed with immunofluorescence over 21 days with the indicated antibodies for n = 3 biological. Scale bars represent 500 um.



**Figure 3.2: Optimisation of IF stain on D-14 differentiated cells.** (A) Cells were cultured in growth-factor free medium for 7 days. (B) In parallel, cells were culture for 4 days in differentiation medium, radiated with 2 Gy and stained 3 days later. Scale bars represent 40um. These cells are basically positive for all the markers (at least at day 14) which shows they go from progenitor cells to more differentiated progenitor cells but do not reach full maturation (at least not at day 14). (A) adapted from Abcam's neural marker website. TUJ1: marker for mature neurons. SOX2: marker for stem cells. In blue, counterstained with DAPI.

### 3.1.2 The SY5Y cell line

The SY5Y cells are equally capable of differentiation which was assessed in the same manner as before. Cells were harvested on days 0, 3, 7, 14 and 21 and stained with the same markers as Figure 3.1. However, this neuroblastoma cell line only generates (?) neurons, justifying why GFAP and SOX2 were used as negative controls. Indeed, the cells did not express either of these markers (not shown here), but were positive for Tuj1. Once again, no dramatic increase or decrease of Tuj1 expression was witnessed over 21 days in retinoic-acid supplemented medium. Similarly to the VM cells, the morphology of the SY5Y cells provided a reliable measure of the differentiation stage, where the cells elongated drastically to form neurites.



**Figure 3.3: SY5Y cells express neuronal differentiation markers.** The differentiation capacity of ReNcell VM cells was assessed with immunofluorescence over 21 days with the indicated antibodies for n = 3 biological repeats. Scale bars represent 500 um.

## 3.2 Drug inhibition

### 3.2.1 DYRK1A inhibition and transient knockout

To apprehend the functional implications of DYRK1A in the differentiation pathway of neural cells, the VM cells were transfected with an siRNA on the kinase. However, the neural stem cells are especially fragile and difficult to transfect so three protocols were tested: 4d Nucleofector, Lipofectamine 2000 and Lipofectamine RNAiMAX. In both lipofections, a GFP plasmid was inserted to allow assessment of transfected cells by fluoroscope microscopy. As shown in Figure 3.4, the electroporation yeilded significantly more GFP positive cells (p value < X, 4d Nucleofector vs Lipofectamine 2000, p < 4d Nucleofector vs Lipofectamine RNAiMAX)(?).

The SY5Y cells were transfected solely with lipofections without GFP (according to Thermo Fisher's protocols) which is a cheaper alternative to electroporation which is quite efficient on robust cell lines such as cancer cells.

To verify the specificity of the siRNA and the efficiency of the transfection, the cells were harvested 48 hours after transfection and DYRK1A expression was evaluated by Western Blot. As Figure 3.4 shows, the transfection on the VM cells did not yield a significant decrease of the kinase. Due to limited time, the focus of the project shifted away from transient knockout of DYRK1A.

Instead, three well-known DYRK1A inhibitors were investigated. The initial step to pinpoint the effect of these drugs on both VM and SY5Y cells was to run a cytotoxicity assay. This was carried out with a colorimetric AlamarBlue assay on both cell lines. The VM responded a lot quicker than the SY5Y, and a lot more heterogeneously. From preliminary data (not shown here), the third drug of choice, INDY, pointed to interesting results, which justifies why the following three repeats of this experiment used a range of three concentrations on this drug solely.

Drug inhibition was maintained for 4 days in maintenance medium before half of cells were exposed to radiation (2 Gy x-rays) and the other half were not. The latter serves as a cytotoxic assay on each drug: Harmine and INDY at 1 uM and 0.1 uM did not lead to significant cell death while L41 (at 3uM) was significantly toxic (ADD SOME STATS (?)). This assay, supported by the literature [?], led us to decrease the concentration of L41 at 1 uM for any subsequent experiment. The populations exposed to radiation gave insight as to the effect of each drug which were revealed as radioprotectors. Indeed, each population over the three days following radiation showed an increase in survival compared to the control.

### 3.3 DYRK1A knockdown in traditional cell culture

To investigate the effect of radiation on neural differentiation, a comparison between 2 Gy radiated cells and non radiated cells was drawn up from immunofluorescence staining. As mentioned previously, the VM cells are positive for TUJ1 from day 0 to day 21 of differentiation, so the measure of differentiation was defined as morphology. Here specifically, a significant decrease in the average cell body area ( $p < ??$ , test) betrays the elongation of the VM cells following x-ray exposure. This differentiated phenotype was supported by the formation of neurites; any cell projection longer than two thirds of the cell body width was counted as a positive cell for this trait. A significant increase was witnessed from control to radiated cells ( $p < X$ ). The SOX2 stain decreased in exposed cells, which is coherent feature of differentiated phenotype although brightness was rejected as a reliable quantitative measure due to its ambiguity. Another qualitative trait which differs in these two populations is the appearance of the signal: it is distinctly more diluted in the control group whereas radiated VM cells express a sharper stain.

### 3.4 Engineering and biology

## 4 Discussion

### 4.1 Cell characterisation

The cells were positive for everything because they are progenitor cells moving towards a more differentiated progenitor phenotype. Limit: should have differentiated for longer (maybe one to two months) to see if these cells can be used as a model of fully differentiated cells. One could have used the brightness as a criteria but it's not very reliable, was used by previous student and did not yield interesting results.

### 4.2 DYRK1A inhibitors in traditional cell culture

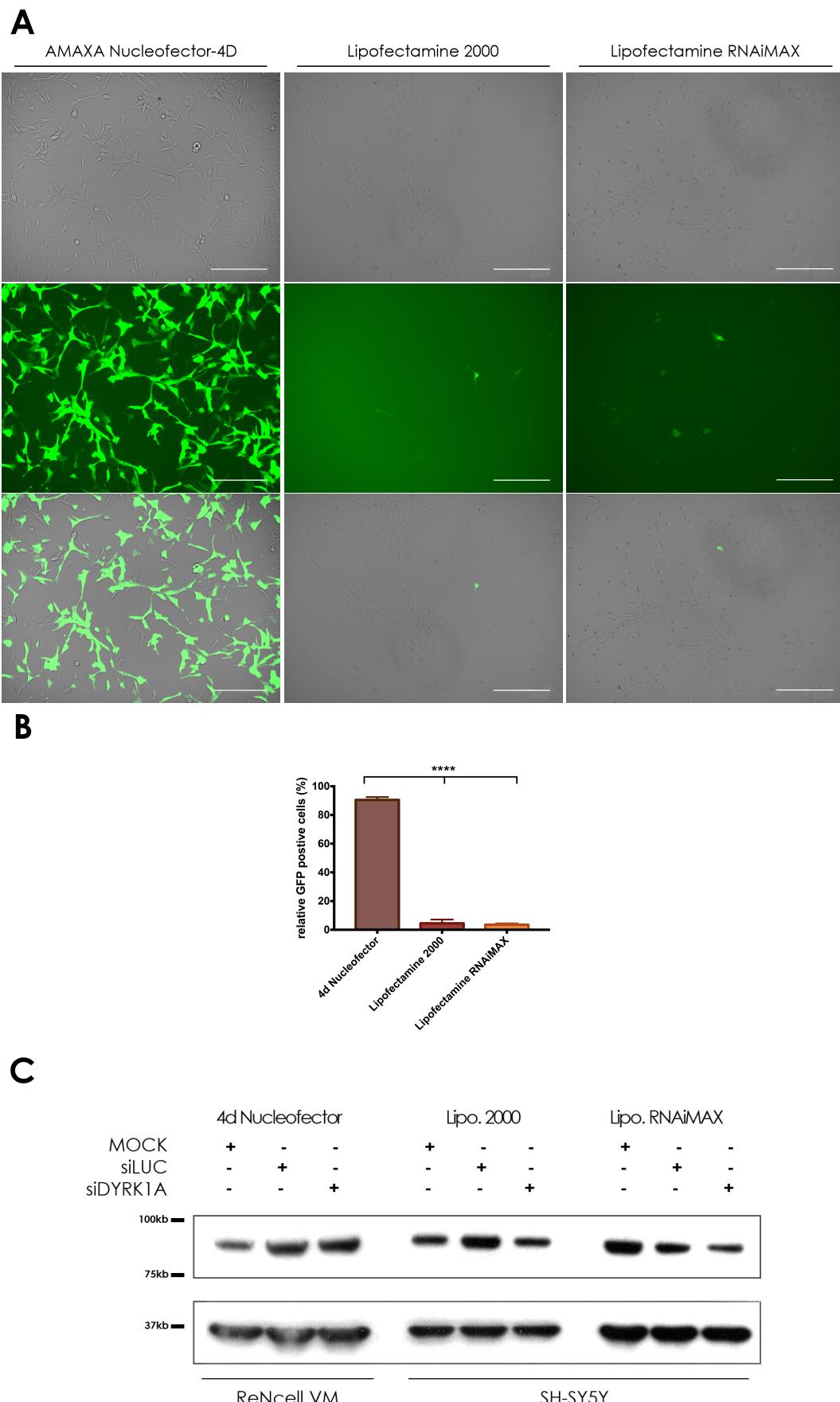
### 4.3 Engineering and biology

### 4.4 Conclusion and outlook

## 5 Conclusion

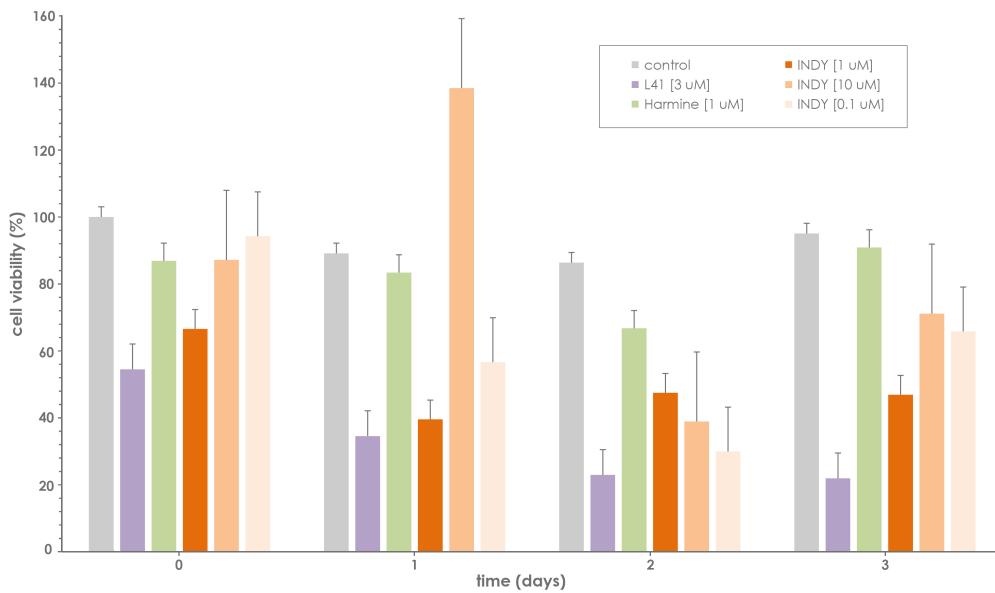
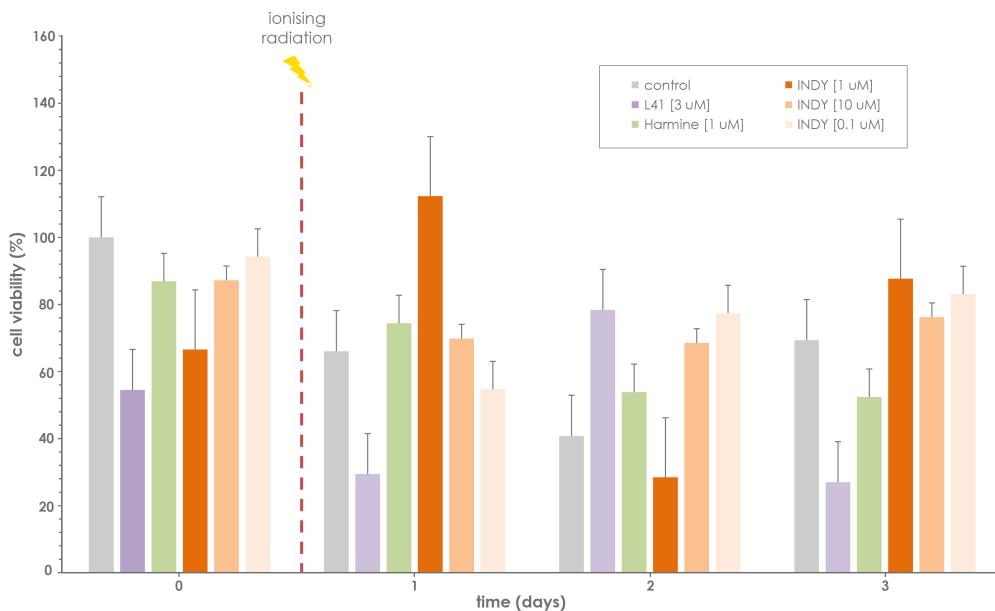
Comme quoi quand tu irradies, tu recrutes aussi des microglias qui vont engendrer un inflammed environment, ce qui n'est pas reproduit ici, une limite de notre study.

La conclusion c'est qu'on a trouve une piste qui justifie pourquoi apres radiation le SVZ il galere a catch up, c'est parce que les cells perdent tout leur potentiel d'aider.

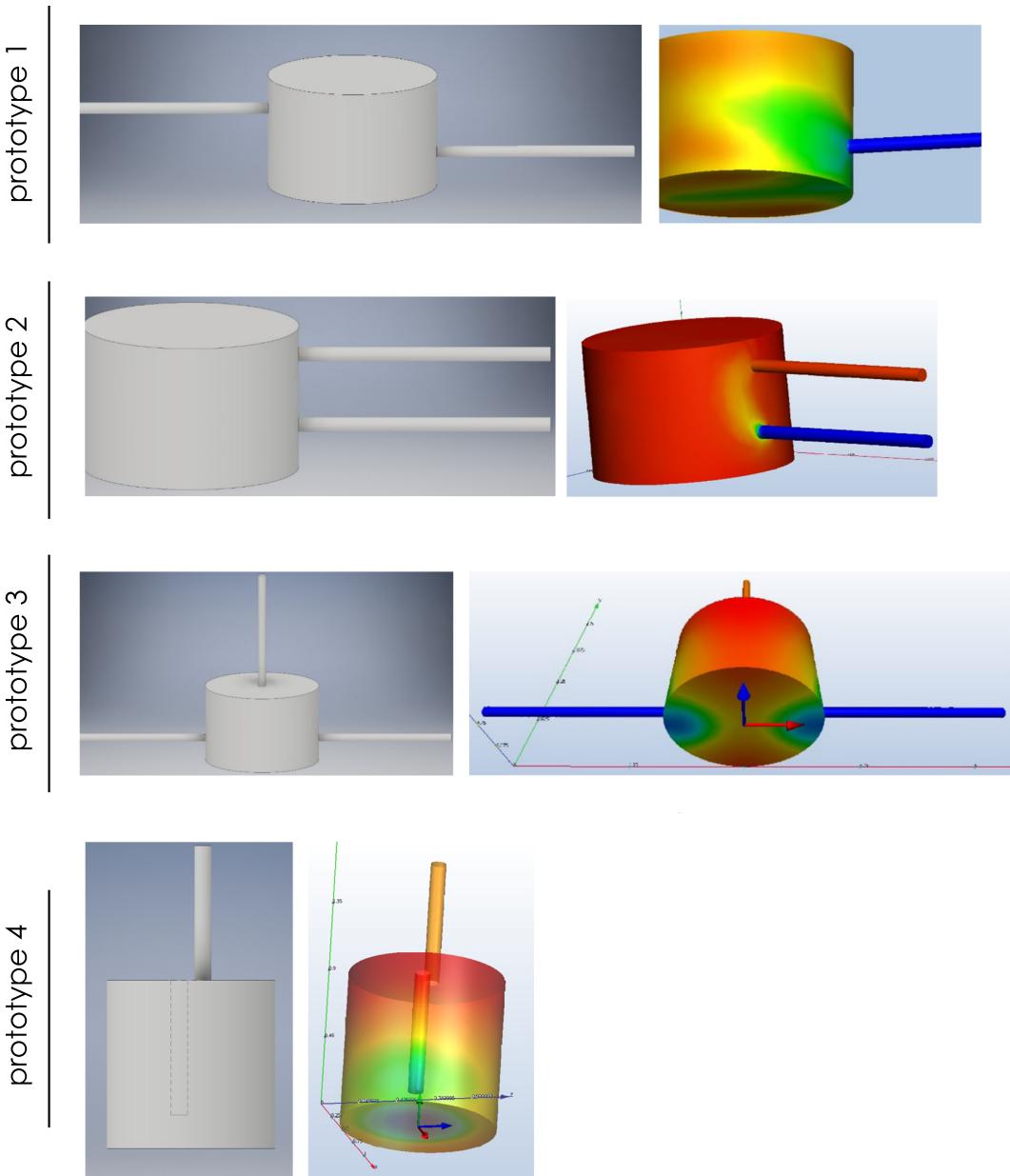


**Figure 3.4: Transfection of Ren VM cells with DYRK1A siRNA.** Cells were transfected with DYRK1A siRNA by using the LONZA 4d Nucleofector protocol on primary cell lines. This was done with three different siRNA concentrations (100, 200 and 300 nM), with a mock (no DNA was added) and a negative control (luciferase). For  $n = 4$ , transfection efficiency is 33.75% Unfortunately, the cells died before any quantifiable data was acquired - to be repeated. Might want to try with Lipofectamine considering electroporation is pricey (the cuvettes). Maybe put the WB in the annexe?

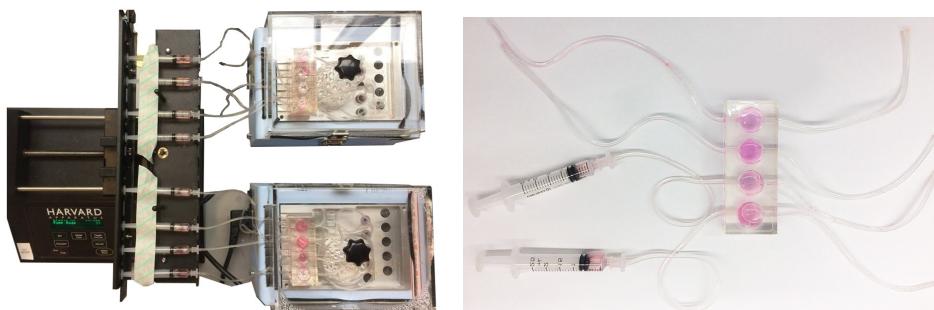
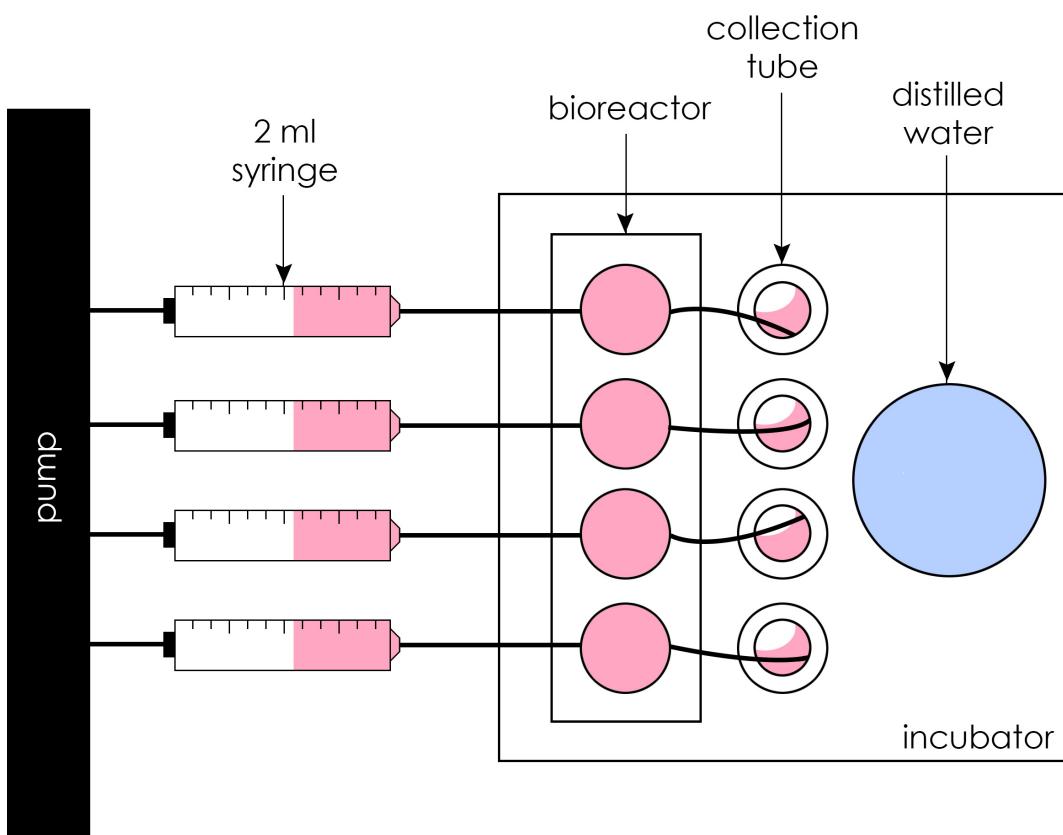
Scale bar = 200 um.

**A****B**

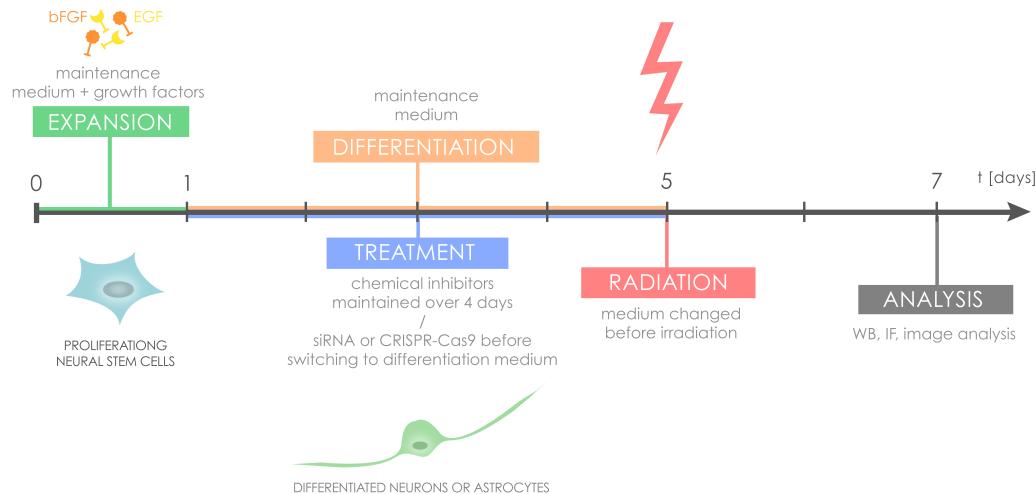
**Figure 3.5: measures from absorbance only. AlamarBlue assay to measure cell viability following DYRK1A treatments (n=8 technical repeats).** Side note: there were issues with this very first experiment which might affect these results (must be repeated): 1) the controls (IR or not) stayed in the complete medium for a day longer than treated cells (differentiation difference). 2) on day 2 and day 3, 20ul and 30ul were added instead of the usual 50ul, which might modify the signal altogether (might justify the drop in the 48hrs signal). **(A)** DYRK1A inhibition followed by radiation: INDY seems to have a protection effect (quick recovery by 72hrs, almost as good as the control) whilst Harmine and L41 show an opposite effect (more damage). One should confirm which one inhibits most (TBD soon, running an experiment with AlamarBlue, WB and IF on the same samples - more comprehensive). **(B)** Radiation followed by DYRK1A inhibition: INDY appears to have a protective effect on radiated cells, leading to a quicker recovery post-IR. On the other hand, **(B)** DYRK1A inhibition only: this graph illustrates the toxicity of each inhibitor. Harmine is the most toxic drug, shown by the drastic drop in viability compared to the control (respectively 7 210 and 12 860).



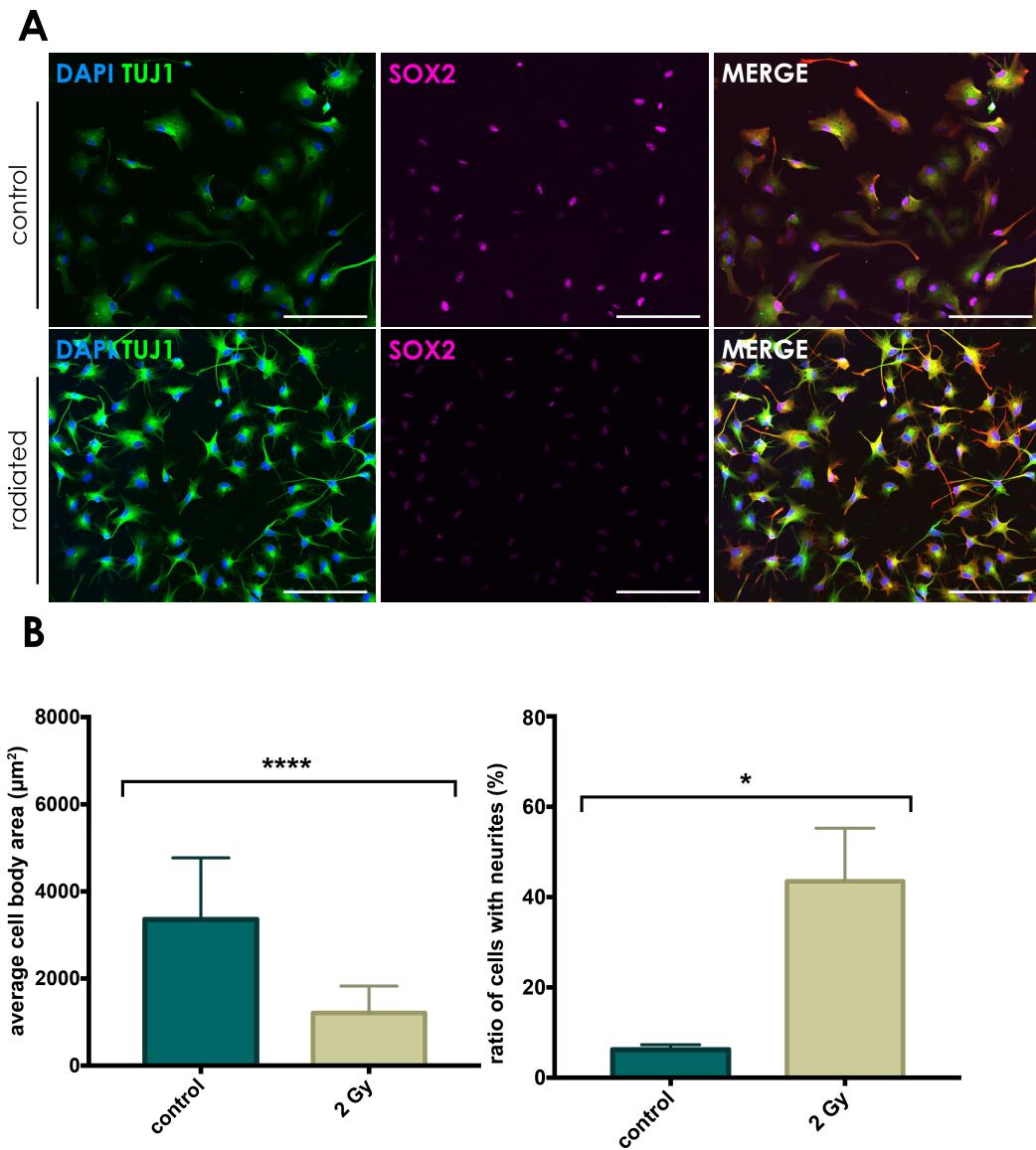
**Figure 3.6: Prototypes for bioreactor designs.** The first column represents the moulds created with Autodesk Inventor 2019, the second are shots of simulations run on the Autodesk CFD 2018 module. The colour red represents the initial medium while the blue is the new medium flowing in through the outlet(s). These simulations were run before building the moulds to determine the most efficient way to mix new medium in the well without disrupting the cells. So far, model 4 is the most likely to be built (it is the most practical design which would allow one to use regular 24-well plates and simply add modified lids to start a perfused culture).



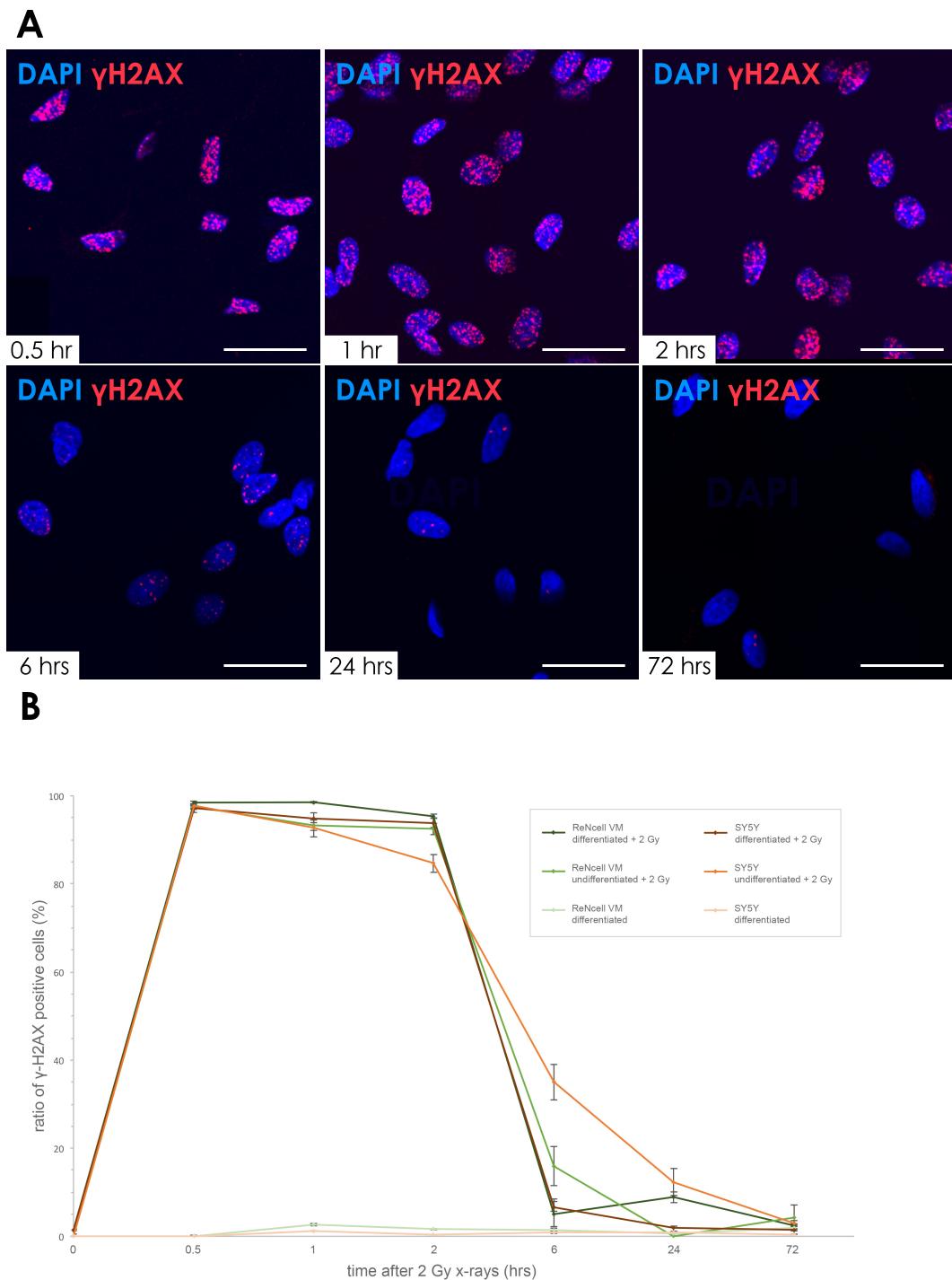
**Figure 3.7: Prototypes for bioreactor designs.** The first column represents the moulds created with Autodesk Inventor 2019, the second are shots of simulations run on the Autodesk CFD 2018 module. The colour red represents the initial medium while the blue is the new medium flowing in through the outlet(s). These simulations were run before building the moulds to determine the most efficient way to mix new medium in the well without disrupting the cells. So far, model 4 is the most likely to be built (it is the most practical design which would allow one to use regular 24-well plates and simply add modified lids to start a perfused culture).



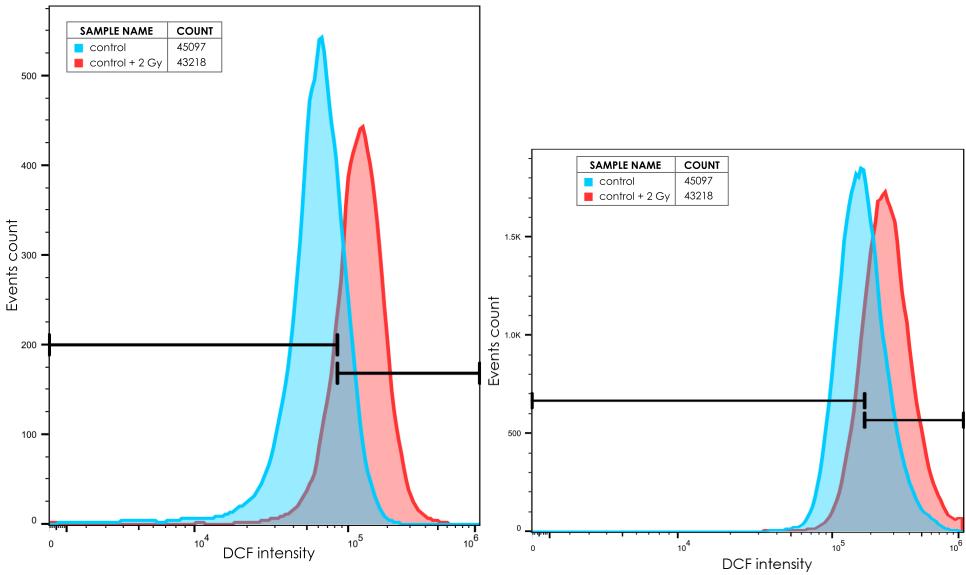
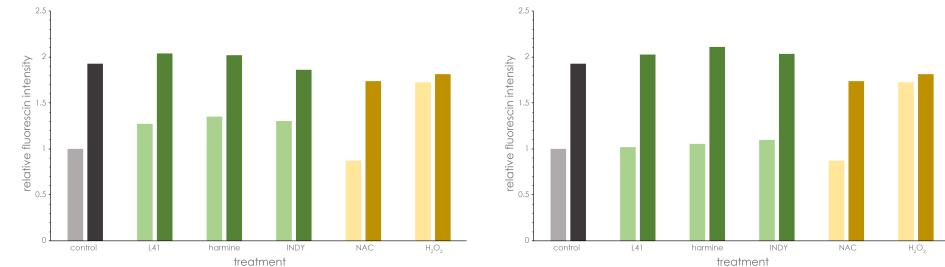
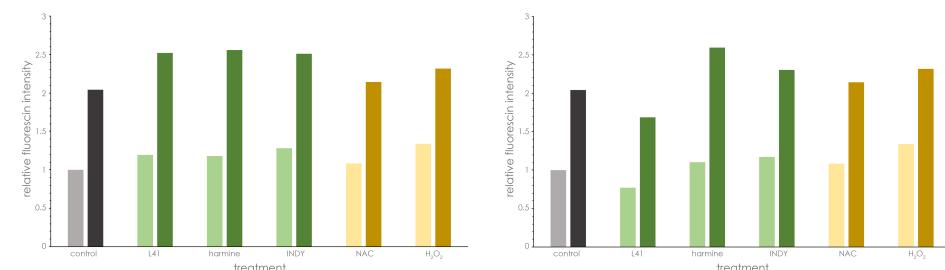
**Figure 3.8: Workflow for Ren VM cells.** After seeding the cells, they are left to attach (on pure laminin) overnight in complete medium (MM + GFs). The next day, the medium is removed, the cells are washed with PBS once covered in maintenance medium (with or without DYRK1A inhibitors). This is repeated every two days for 4 days. The cells are then radiated, and 2-3 days later, they are ready for analysis. We change the medium of cells right before radiation but do not change it anytime between IR and analysis to benefit from the full effect of radiation (mostly free radicals), as many studies have shown the importance of the bystander effect (radiation effect in medium!).



**Figure 3.9: Immunofluorescence output of Ren VM cells following 7 days of differentiation medium.** (A) Cells were cultured in growth-factor free medium for 7 days. (B) In parallel, cells were culture for 4 days in differentiation medium, radiated with 2 Gy and stained 3 days later. TUJ1: marker for mature neurons. SOX2: marker for stem cells. In blue, counterstained with DAPI.



**Figure 3.10: DNA damage on ReNcell VM and SY5Y cells following 2 Gy of x-ray radiation.** (A) Cells were cultured in growth-factor free medium for 7 days. (B) In parallel, cells were culture for 4 days in differentiation medium, radiated with 2 Gy and stained 3 days later. These images are from differentiated Ren VM cells.  $\gamma$ -H2AX positive cells are cells with more than 10  $\gamma$ -H2AX foci. Scale bars represent 30um. !! AJOUTER LES PHOTOS 63X. Limites dans la discussion: le fait qu'on aurait pu compter le nombre de foci pour avoir un resultat plus précis mais ici ce n'est pas ce qui nous intéressait.

**A****B****C**

**Figure 3.11: The DCF-DA assay does not prove a strong antioxidant capacity in any drug inhibitor.** Due to time constraints, the protocol for this experiment varied from the typical workflow depicted in Figure 3.8. Instead, cells were seeded on day 0 and treated on day 1 with the 3 drug inhibitors for 3 hours. NAC and H<sub>2</sub>O<sub>2</sub> were added respectively one hour and 20 minutes before detaching the cells, which were then assayed and analysed by flow cytometry.