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Vincent Bérézowski declares that he has no conflict of interest. All other authors were employed by HCS Pharma, and their research and authorship of this article was completed within the scope of their employment with HCS Pharma. Nathalie Maubon owns shares in HCS Pharma.

List of abbreviations: 6-OHDA: 6-hydroxydopamine; cAMP: cyclic Adenosine Monophosphate; FBS: Fetal Bovin Serum; iBMX: 3-isobutyl-1-methylxanthine; MEM/F12: Minimum Essential Medium/Ham F12 medium; RA: Retinoic Acid; SEM : Standard Error of the Mean; TH: Tyrosyne Hydroxylase; TPA: Tetradecanoylphorbol Acetate; vGLUT2: vesicular Glutamate Transporter 2.

Abstract

The SH-SY5Y cell line is commonly used for the assessment of neurotoxicity in drug discovery. These neuroblastoma-derived cells can be differentiated into neurons using many methods. The present study has compared 24 of these differentiation methods on SH-SY5Y cells. After morphologic selection of the three most differentiating media (retinoic acid in 10% fetal bovine serum (FBS), staurosporine in 1% FBS medium, and cyclic adenosine monophosphate (cAMP) in B21-supplemented neurobasal medium), cells were analyzed for pan-neuronal and specific neuronal protein expression by fluorescent automated imaging. The response of SH-SY5Y to a set of compounds of known toxicity was examined in these culture conditions performed in 2D, and also in a 3D hyaluronic acid-based hydroscaffold™ which mimics the extracellular matrix. The extent of neuronal markers expression and the sensitivity to neurotoxic compounds varied according to the differentiation medium. The cAMP B21-supplemented neurobasal medium led to the higher neuronal differentiation, and the higher sensitivity to neurotoxic compounds. The culture in 3D modified the neurotoxic response, through a lower sensitivity of cells compared to the 2D culture. The *in vitro* differentiation environment influences the neurotoxic response of SH-SY5Y cells and thus should be considered carefully in research as well as in drug discovery.

Keywords

SH-SY5Y cell line; *in vitro* differentiation; phenotypic analysis; neurotoxicity assessment.

1. Introduction

The pharmaceutical industry faces a low success rate in drug discovery. Indeed, 90% of clinical trials fail because of efficiency and safety issues. The Central Nervous System (CNS) is particularly involved in this outcome, since 34% of safety failures occur in CNS clinical trials, but only 7% in preclinical trials (Cook et al., 2014). This points to the relevance of preclinical models for the assessment of neurotoxicity as used upstream in the process, to select molecules of interest. Nevertheless, lots of *in vitro* models exist to evaluate drug candidates. Although considered more physiologic, primary cell cultures cannot be upscaled to an industrial level, in particular for high screening steps, because of limited access to biopsies, and reproducibility problems. Consequently, the pharmaceutical industry uses immortalized cell lines for the early assessment of neurotoxicity. Nevertheless, cell lines encounter differentiation problems possibly explaining their lack of relevance. Among them, the SH-SY5Y cell line is commonly used both in research and in the pharmaceutical industry for the assessment of neurotoxicity, with more than 1,500 publications including both "SH-SY5Y" and "neurotoxicity" terms listed on Pubmed in the last 20 years. Indeed, this cell line offers several advantages, including its human origin, its relatively homogenous population which promotes a good reproducibility, and its high proliferation rate, which makes it a suitable cell line for large-scale culture (Tieu et al., 1999). Since derived from a neuroblastoma, SH-SY5Y cells can be differentiated into neurons from several methods, ranging from the widely used Retinoic Acid (RA), to 12-O-tetradecanoyl-phorbol-13 acetate (TPA), N^6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (cAMP), staurosporine, and B27 (Kovalevich and Langford, 2013; Xicoy et al., 2017). This large panel of differentiation inducers might represent different extracellular environments leading to different cell responses to the same stimulus (Tieu 1999).

To provide a comparative insight into the influence of the differentiation environment on the neurotoxic response of SH-SY5Y cells, we cultured these cells in media containing commonly used differentiation factors at several concentrations: RA, staurosporine, TPA combined or not with RA, cAMP, 3-isobutyl-1-methylxanthine (iBMX). All of them were tested in MEM/F12 medium supplemented with 0%, 1% and 10% Fetal Bovine Serum (FBS), and in FBS-free neurobasal 2% B21 (a B27-derivated supplement) medium, for a total of 24 different culture conditions. After morphologic selection of the three most differentiating media, cells were analyzed for pan-neuronal and specific neuronal protein expression by fluorescent automated imaging, which allowed phenotypic analysis and marker intensity quantification.

Responses of SH-SY5Y to a set of compounds known as non-toxic, toxic but non-neurotoxic, or neurotoxic (Wilson et al., 2014) were examined in these culture conditions performed in 2D, but also in a 3D hyaluronic-based hydroscaffold™ which mimics the Extracellular Matrix (ECM).

2. Materials and methods

2.1.Cells and media

The SH-SY5Y cells came from European Collection of Authenticated Cell Cultures (ECACC). Two culture media were used: **1)** MEM/F12 medium: 50% Minimum Essential Medium (Eurobio cat. CM1MEM10 01) / 50% Ham F12 medium (Eurobio cat. L0136-500) (v/v), 100UI/mL penicillin and 100µg/mL streptomycin (Eurobio cat. CABPES01 0U), 2mM L-glutamine (Eurobio CSTGLU00 0U), 1% (v/v) Non-Essential Amino Acids (Eurobio, Cat. CSTAAN00 0U), supplemented with 1 or 10% (v/v) Fetal Bovine Serum (FBS) (Eurobio/Biowest n°S13439S1810, cat. S1810-500); **2)** Neurobasal 2% B21 medium: MACS Neuro (Miltenyi Biotec cat. 130-093-570), 2% MACS® NeuroBrew®-21 (B21) (Miltenyi Biotec cat. 130-093-566) 100UI/mL penicillin and 100µg/mL streptomycin (Eurobio cat. CABPES01 0U), 2mM L-glutamine (Eurobio cat. CSTGLU00 0U).

2.2.Screening of differentiation media

The SH-SY5Y cells were expanded in a T75 flask containing MEM/F12 10% FBS medium. Cells were then plated in 96-well-plates (Greiner cat. 655986) directly in the differentiation medium, at a density of 7 500 cells/well. Five differentiation factors were tested: 0.1, 1 and 10µM retinoic acid (TCI, cat. R0064-1G) prepared at 2mM in Dimethyl sulfoxide (DMSO, Sigma Aldrich, cat. 276855), 25nM staurosporine (Sigma Aldrich, cat. 37095), 16nM 12-O-tetradecanoyl-phorbol-13 acetate (TPA, Sigma Aldrich cat. P18139) with or without 0.1µM retinoic acid, 0.1 and 1mM N^6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (cAMP, Sigma Aldrich, cat. D0627), and 0.5 mM 3-isobutyl-1-methylxanthine (iBMX, Sigma Aldrich, cat. I5879). All of them were tested in MEM/F12 medium supplemented with 0%, 1% and 10% (v/v) FBS, and in neurobasal 2% B21 medium.

2.3.Cell culture and differentiation

Before differentiation in RA or staurosporine, SH-SY5Y were expanded in a T75 flask (Corning cat. 353136) in MEM/F12 10% FBS (Figure 1A and B). Before B21+cAMP differentiation, cells were expanded in neurobasal 2% B21 medium, to avoid a complete media modification between expansion and differentiation steps, and also because it is interesting to culture cells in a chemically defined media, and thus without FBS, to enhance the reproducibility of tests (Figure 1A and C). After two weeks, cells were plated directly in differentiation media: MEM/F12 10% FBS 10µM RA, MEM/F12 1% FBS 25nM staurosporine, or neurobasal 2% B21 1mM cAMP. It should be noted that even neurobasal 2% B21 medium is adapted for cell expansion in flask, it induced a slight neuronal pre-differentiation. Consequently, proliferation control was in MEM/F12 10% FBS medium. In the 2D cell culture, cells were plated in 96-well plates (Greiner ref 655986) coated with 10 µg/cm2 with Poly-D-Lysine (PDL) (Sigma Aldrich cat. P7280), at a density of 5,000 or 7 500 cells/well. In the 3D cell

culture, cells were plated in 96-wells BIOMIMESYS® *Brain* plates (HCS Pharma, BIO_BRA_96_96_black) at a density of 30,000 cells/well.

2.4.Immunostaining

To characterize the differentiated SH-SY5Y cells, pan- and specific neuronal marker proteins were immunostained. For that, the cells were fixed with 2% paraformaldehyde (Fisher Scientific cat. 10260219) for 15 min. and then permeabilized with 0.5% (v/v) Tween-20 (Sigma Aldrich cat. P2287) in Phosphate Buffered Saline (PBS) (Eurobio cat. CS1PBS01) with 1% (w/v) Bovine Serum Albumin (BSA) (Dominique Dutscher cat. 871004) during 30 min. Primary antibodies were added for 1h at room temperature or overnight at 4°C, in PBS with 1% (w/v) BSA. Then anti-Rabbit secondary antibodies were added during 1.5h at room temperature with Hoechst 33342 (Invitrogen™ H3570, Fisher Scientific cat. 11534886) for nuclei visualization, in PBS with 1% (w/v) BSA (Table 1).

2.5.Neurotoxicity assays

In 2D, neurotoxicity assessments were performed in 384-well plates (Greiner, cat. 781091) previously coated with 10 µg/cm^2 PDL. Cells were seeded at 2,500 cells/well in the indicated differentiation media. In 3D, neurotoxicity assessments were performed in 96-wells BIOMIMESYS® *Brain* plates, seeded at 30,000 cells/well. In the 2D neurotoxicity assessment, cells were incubated with compounds at the 30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003 and 0.001 µM final concentrations. In 3D, cells were incubated with compounds at 100, 30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01 and 0.003 µM final concentrations. The powdered compounds (Table 2) were solubilized in DMSO, at 200 times the final concentrations, to reach a final dilution of 0.005% DMSO. Control cells were incubated in a medium containing 0.005% DMSO as a vehicle condition. Three days after seeding, cells were incubated with compounds solubilized in MEM/F12 10% FBS (for RA-differentiated cells), MEM/F12 1% FBS (for staurosporine-differentiated cells), or neurobasal 2% B21 medium (for B21+cAMP-differentiated cells). After 2 days of incubation, dead cells were stained with 1µg/mL propidium iodide (Sigma Aldrich, cat. P4170) for 5 min in 2D, or with 1.2 µM DRAQ7 (Biolegend® cat. 424001) for 30 min in 3D, in co-incubation with 10µg/mL Hoechst 33342 in basic media (MEM/F12 or neurobasal).

2.6.Image acquisition and analysis

The images were acquired by the ImageXpress Micro Confocal system automated microscope (Molecular Devices), with 4 acquisition sites per well, using widefield imaging method for 2D analysis, and confocal 60µm pinhole imaging method for 3D analysis. Then images were post-treated withMetaXpress software (6.63.55 version) using the appropriate application module. Four filters were used, DAPI (excitation 377/50nm, emission 447/60nm), FITC (excitation 475/34 nm, emission 536/40nm,), Cy5 (excitation 631/28 nm, emission 692/40 nm and Texas Red (excitation 560/32 nm,

emission 624/40 nm), with a binning of 2. The number of cells per well was calculated from the counted nuclei per acquisition site after Hoechst staining. Neurites were detected from the results of the TUBB3 staining. Neurite length was measured as the sum of all neurite lengths found in the acquisition sites divided by the number of cells, using the MetaXpress application module "Neurite Outgrowth". Briefly, the application module first detected cell bodies (FITC fluorescence above local background with round shape), then considered it as a cell if it detected a nucleus inside (DAPI fluorescence above local background). Then it analyzed elongations from the detected cells (FITC fluorescence above local background with elongated shape). Neurites were considered as such when the length was above 5µm. For neuronal marker analysis, the stain integrated intensity of differentiated cells was normalized to the stain integrated intensity of proliferating cells for the same marker. In 3D, the stain average of the maximal 2D projection was determined by the integrated intensity divided by the surface of spheroids projection to avoid the bias induced by spheroid size variation according to the medium, and normalized to the average intensity of non-differentiated SH-SY5Y. For the 2D neurotoxicity assessment, the number of live cells incubated in the compounds was normalized to the number of live cells in vehicle controls. In 3D, the percentage of live cells in the well was normalized to the percentage of live cells in the vehicle controls. The concentration for which 50% of cells were dead has been calculated (Lethal Concentration 50% - LC50).

2.7.Statistical analysis

Values were presented in mean ± Standard Error of the Mean (SEM). Error bar in the graphs corresponded to SEM. The statistical analyses were achieved with the R software version 3.6.1 (R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.URL https://www.R-project.org/), and R Studio environment version 1.3.1093). The normality of the samples was verified by Shapiro test, and the heteroscedasticity by the Fisher test. The equality of the means was analyzed by Student test for parametric samples with equal variances, by Welch test for parametric samples with different variances, or by the Wilcoxon test for non-parametric samples. *p-value < 0.05; **p-value>0.005; ***p-value<0.0005.

3. Results

3.1.Selection of differentiation media

We selected the differentiation media based on 2 requirements: cell proliferation slowdown and neurite elongation, compared to proliferation media (MEM/F12 10% FBS without any factor; 30,000 ± 1,200 cell/well and neurite length 9.6 ± 0.3 µm/cell at day 5). Even the differentiation effects were already visible at day 3 (data not shown), results were more pronounced at day 5 (Figure 2). In MEM/F12 0% FBS media, cells did not survive. In MEM/F12 10% FBS medium, the number of cells

per well was significantly lower, only in the presence of RA (all concentrations) and iBMX, (Figure 2A). However, the neurite length was significantly increased only with 10 µM RA (Figure 2B). The decrease in FBS concentration (MEM/F12 1% FBS) led to a significant decline in cell proliferation for all tested factors (Figure 2A). The outgrowth of neurites was significantly higher with all factors in this medium, except in 0.1 and 1µM RA (Figure 2B). The neurobasal 2% B21 medium without additional factor led to a significant decrease in the number of cells, and a significant increase in the neurite length (Figure 2A and B). The use of staurosporine, RA and iBMX induced an additional decrease in the cell proliferation compared to neurobasal 2% B21 alone (Figure 2A, black asterisks), but no neurite elongation (Figure 2B). With TPA (combined or not with RA), and with cAMP, the number of cells per well was equal to the one in neurobasal 2% B21 alone, and thus lower than the one in proliferation media. Only cAMP was associated to a supplementary increase in the neurite length (Figure 2B, black asterisks). Following these results, we chose the most efficient conditions in each medium: RA 10µM in MEM/F12 10% FBS medium (14,000 +/- 940 cells/well and neurite length = 21.1 ± 0.4 µm/cell) (called "RA medium"), staurosporine in MEM/F12 1% FBS (18,000 ± 2,200 cells/well and neurite length = 22.3 ± 0.4 µm/cell) (called "staurosporine medium"), and cAMP 1mM in neurobasal 2% B21 medium (18,000 ± 2,900 cells/well, and neurite length = 36.6 ± 0.4 µm/cell) (called "B21+cAMP medium"). In addition, fluorescent microscopy observations showed a difference in cell shape and cell organization according to these media. Cells in proliferation medium were more clustered, with short neurites. Cell cultured in RA and staurosporine media displayed elongated cell bodies, whereas cells cultured in B21+cAMP medium displayed more rounded cell bodies. Neurite morphology varied too, with more branching in B21+cAMP (Figure 2C).

3.2. Kinetic analysis of cell proliferation and neurite elongation

Cells in proliferation medium had a high proliferation rate, with a 25-fold increase in the number of cells, from 5,000 cell/well at seeding to 123,000 ± 5,600 cell/well after 7 days. Proliferation of cells was significantly slowed down in differentiation media. Among them, the highest cell number was found in staurosporine medium, with a 6-fold increase between seeding and day 7 (from 5,000 to 29,600 ± 1,800 cells/well). An intermediate cell proliferation was found in RA medium, with a 5-fold increase (from 5,000 cells/well at the seeding time to 26,500 ± 1,500 cells/well after 7 days). The lowest cell number was seen in B21+cAMP medium, with a 4-fold increase (from 5,000 to 22,200 ± 2,000 cells/well) between seeding and day 7 (Figure 3A and C). The neurite length has been quantified because neurite elongation was a relevant morphological marker of neuronal differentiation in 2D, whereas the neurite number or branching nodes did not vary significantly among our experimental conditions (data not shown). The neurite length in proliferation medium decreased from 12.1 ± 0.5 µm/cell at day 3, to 4.9 ± 0.1 µm/cell at day 7 (Figure 3B). On the contrary, neurite length in the three selected differentiation media was significantly enhanced (Figure 3B and C). In RA and staurosporine

media, neurite length reached 22.4 ± 1.0 µm at day 3 in RA, and 23.1 ± 0.4 µm at day 5 in staurosporine, and then remained constant until day 7. Cells in B21+cAMP medium presented evident longer neurites for all times, with a maximum at day 5 (36.2 ± 2.7 µm).

3.3. Kinetic analysis of pan-neuronal marker expression

The proteins TUBB3 and synaptophysin were highly expressed by SH-SY5Y cells in all media, including proliferation medium. The TUBB3 protein was widely expressed, both in cell bodies and in neurites, whereas synaptophysin was mostly expressed in neurites. On the contrary, NeuN was poorly expressed in non-differentiated cells, and localized only in the nucleus and perinuclear zone of differentiated cells (Figure 4A). Overall, the stain intensities for the three marker proteins were significantly higher in differentiation media than in proliferation medium (ratio > 1) and rose over time. Moreover, intensities were higher in B21+cAMP medium, and followed by the ones in the staurosporine medium, and the lower ones in the RA medium (Figure 4B and C, and supplemental Figures 1, 2 and 3). We chose day 5 as the time point for assessments in the next experiments, because the cells became too numerous in the wells at day 7, impacting the analysis of neurite length, cell viability and marker proteins.

3.4.Neuronal specificity

Tyrosine hydroxylase (TH) was widely expressed both in cell bodies and in neurites, in all media, including proliferation medium. On the contrary, vGLUT2 was weakly expressed in proliferation medium but appeared in differentiated media as puncted areas of cell bodies (Figure 5A). Staining intensities were significantly higher in B21+cAMP medium compared to that in proliferation medium (ratio = 3.4 ± 0.1 for TH and 58.9 ± 8.2 for vGLUT2), followed by the staurosporine medium (ratio = 2.9 ± 0.1 for TH and 31.4 ± 8.0 for vGLUT2). In the RA medium, vGLUT2 expression was lower, but enhanced when compared to that in proliferation medium (ratio = 5.6 ± 2.6). There was no enhancement in TH expression (ratio < 1) (Figure 5B and C). Differentiated SH-SY5Y cells expressed simultaneously dopaminergic (TH) and glutamatergic (vGLUT2) markers (Figure 5D).

3.5.Neurotoxicity assessment

No toxicity was observed for non-neurotoxic compounds, in all the differentiation media, except for troglitazone in the staurosporine medium ($LC50_{Troglitazone}$ = 26µM). Troglitazone induced a slight mortality from 10µM in B21+cAMP, which stabilized at a lower bound value of 81% of viability (Figure 6). Toxic effects were observed in all differentiation media for neurotoxic compounds. Cisplatin, 6-OHDA and colchicine induced a difference in LC50 depending on the medium. Cells were more sensitive in the B21+cAMP medium ($LC50_{cisplatin}$=2 µM; $LC50_{6\text{-}OHDA}$=5 µM; $LC50_{colchicine}$=0.0008 µM), followed by the staurosporine medium ($LC50_{cisplatin}$=18 µM; $LC50_{6\text{-}OHDA}$=17

µM; $LC50_{colchicine}$=0.008 µM), and less sensitive in the RA medium ($LC50_{cisplatin}$=37 µM; $LC50_{6\text{-}OHDA}$=36 µM; $LC50_{colchicine}$=0.03 µM). Moreover, in the staurosporine and RA media the last no-toxic-effect concentration was higher (3µM of cisplatin in RA and 1µM in staurosporine, and 0.003µM of colchicine for both RA and staurosporine) than in B21+cAMP (toxic effect from the lowest tested concentration). Furthermore, in the presence of colchicine, the viability dropped down to 26% in B21 + cAMP medium, to 34 % in the staurosporine medium, and to 39% in the RA medium (Figure 6). Interestingly, tamoxifen exerted a neurotoxic effect more efficiently on cells differentiated in staurosporine medium, and not in B21+cAMP, with LC50=11µM in staurosporine medium, 21µM in B21 medium, and 24µM in RA medium (Figure 6).

3.6.Cell proliferation in 3D

In the context of the growing development of 3D cell culture technologies, we also tested the effects of the previously selected differentiation media (RA, staurosporine and B21+cAMP media) in 3D cell culture, by using a hyaluronic acid-based hydroscaffold™ (BIOMIMESYS® *Brain* technology).

In BIOMIMESYS® *Brain* hydroscaffold™, SH-SY5Y formed spheroids in all tested media, with no neurite outside the spheroids (Figure 7A). There was no correlation between the number of spheroids per well and the diameter of spheroids (Figure 7.B.). Therefore, variations in spheroid size were not linked to variations in cellular clustering. The number of spheroids was linked to the number of cells which survived and adhered to the matrix, and the diameter indicated the proliferation of cells (Figure 7C). In the three differentiation media, spheroid diameters were significantly lower than in the proliferation medium (35.4 ± 0.3 µm in RA medium, 36.1 ± 0.7 µm in staurosporine medium, 38.4 ± 0.5 in B21+cAMP medium, and 42.8 ± 0.7 µm in proliferation medium) (Figure 7C). Thus, the differentiation media previously selected in 2D led also to a slowdown in cell proliferation in 3D cell culture. Interestingly, the number of spheroids was lower in the B21+cAMP medium.

3.7.Impact of differentiation media on neuronal marker expression in 3D

NeuN and vGLUT2 were not detected in SH-SY5Y in 3D (data not shown). In B21+cAMP medium, the intensities of the three markers (TUBB3, Synaptophysin and TH) were significantly increased compared to proliferation medium (TUBB3: ratio=2.6 ± 0.3, p-value=0.0001055; Synaptophysin: ratio=2.1 ± 0.2, p-value= 0.0008879; TH: 1.7 ± 0.2, p-value= 0.004435). In staurosporine, TUBB3 and TH expression were also significantly enhanced (TUBB3: ratio=1.6 ± 0.2, p-value=0.00007174; TH: ratio=1.4 ± 0.1, p-value= 0.03128), but not synaptophysin (ratio= 1.4 ± 0.2, p-value=0.05966). In the RA medium, there was no neuronal marker increased (ratios < 1 for the three markers) (Figure 8, all panels).

3.8.Neurotoxicity assessment in 3D

No toxicity was observed for non-neurotoxic compounds, in any differentiation media, except for troglitazone in staurosporine medium, like in 2D, but with a shift in the LC50 ($LC50_{Troglitazone}$=90µM in 3D, and 26µM in 2D) (Figure 9). All neurotoxic molecules led a toxic effect in the three media, with difference in LC50 according to the medium. Cells were more sensitive to 6-OHDA in B21+cAMP ($LC50_{6OHDA}$=41µM), followed by staurosporine ($LC50_{6OHDA}$=94µM), and RA (not determined). Under cisplatin exposure, viability of staurosporine and RA-differentiated cells remained at 100% until 0.3µM, whereas a toxic effect was detected from the first concentration (0.003µM) in B21+cAMP. However, the curve slopes were very different (-0.1 in B21+cAMP, -0.5 in RA, and -10.2 in staurosporine medium): cells were more sensitive to cisplatin for low concentrations (<1µM) with B21+cAMP-induced differentiation, then more sensitive with staurosporine-induce differentiation for the higher concentrations. The LC50 was not measurable with the tested concentrations. Under colchicine exposure, the viability stabilized to 83% at 0.01µM in B21+cAMP, to 85% in RA and to 68% in staurosporine medium both at 0.03µM. Tamoxifen exposure caused also observed a toxic effect in the three media, with a higher sensitivity in staurosporine (Figure 9).

4. Discussion

This is the first study that compares the neuronal phenotypes and the neurotoxic responses of SH-SY5Y cells cultured in different differentiation environments. It tended to respond to the concerns raised by the pharmaceutical industry about the use of a relevant *in vitro* cell model for CNS high content screening.

Among our results, it should be noted that even non-differentiated SH-SY5Y cells displayed some neuronal features, including neurite outgrowth and some neuronal marker expression like TUBB3, synaptophysin and TH, as found in cells originating from neuroblastoma. However, the differentiation media clearly enhanced the neuronal phenotype in 2D and in 3D cultures. Overall, differentiated SH-SY5Y cells displayed a dopaminergic neuron phenotype in all the tested media, as showed by the enhanced TH expression. Interestingly, the glutamatergic marker vGLUT2, which also present in some dopaminergic neurons as a secondary neurotransmitter, was also expressed in 2D cultures (Kawano et al., 2006; Trudeau, 2004). Still, the variations in the marker protein profile obtained here might evoke the highly variable results in neurotoxicity assays observed here and in the literature when a single method of differentiation is used (Tieu et al., 1999). Therefore, the action of each differentiating factor on SH-SY5Y cells needs to be considered in neurotoxicity assays, as discussed in the following sections.

Retinoic acid is a well-known morphogen able to induce cellular differentiation as well as a potent inhibition of cell proliferation (Lotan, 1996; Melino et al., 1997). It is the most commonly used

differentiating factor for SH-SY5Y cells (Xicoy et al., 2017). However, in our culture conditions and time frame, RA was the least effective compound in inducing a neuronal phenotype, when compared to staurosporine or B21+cAMP. Indeed, although RA slew cell proliferation down and promoted neurite elongation, the expression of neuronal proteins was only slightly increased compared to non-differentiated SH-SY5Y cells in 2D, and even not increased at all in 3D cell cultures. Since the lowest sensitivity to neurotoxic compounds was found in our both 2D and 3D cultures under RA, one might consider this result as a direct consequence of the less differentiated phenotype of SH-SY5Y cells induced by this compound. This is still consistent with the well-known RA-induced activation of the phosphatidylinositol 3-kinase/Akt signaling pathway in SH-SY5Y cells, which promotes cell survival (López-Carballo et al., 2002). Also the opposite upregulation of anti-apoptotic Bcl-2 protein and downregulation of apoptotic p53 protein by RA are potential modifiers of the neurotoxic response observed here (Itano et al., 1996; Tieu et al., 1999).

Staurosporine is a well-known PKC inhibitor, which promotes cell cycle arrest and neurite outgrowth in SH-SY5Y cells (Leli et al., 1993; Tieu et al., 1999). In our study, the differentiating action of staurosporine was confirmed, to an intermediate extent between B21+cAMP and RA, in 2D and 3D cultures. Consistent with these results, an intermediate sensitivity to neurotoxic compounds was observed in 2D cultures, except for tamoxifen, to which our SH-SY5Y cells were interestingly the most sensitive. This higher sensitivity, compared to the one in RA-differentiated cells, had already been reported and explained by the decreased levels of Bcl-2 and increased levels of p53 after staurosporine exposure, contrary to after RA exposure (Itano et al., 1996; Tieu et al., 1999). Of notice, staurosporine itself is a neurotoxic compound, which has been used in this study at non-toxic concentrations for differentiation purposes (Tieu et al., 1999). This might elicit an exacerbated neurotoxic response to the set of compounds tested. Indeed, the observed mortality of staurosporine-differentiated cells after troglitazone exposure, although intended as a non-neurotoxic exposure, warns about the use of staurosporine as a differentiating factor, and needs further investigation. In line with this observation, the higher sensitivity of our cells to tamoxifen despite a less differentiated phenotype of SH-SY5Y cells compared to the ones under B21-cAMP, imply yet unknown molecular interactions that need to be clarified.

The cAMP + B27 supplement is also known to promote SH-SY5Y differentiation (Kovalevich and Langford, 2013). Our combination of B21 (a B27-derivated supplement) with cAMP was particularly interesting because it induced the most potent neuronal differentiation regarding all the tested criteria (slowdown of cell proliferation, promotion of neurite outgrowth and neuronal marker expression) in 2D and in 3D cultures. The highest sensitivity to neurotoxic compounds found in 2D cultures, except for tamoxifen as previously discussed, strongly suggests the link between the level of differentiation into neurons and the neurotoxic response. As cAMP + B21 was a chemically defined medium (FBS-free and more generally animal-free), it holds strong potential for the relevance and reproducibility

required by the high content screening procedures in the pharmaceutical industry. As for staurosporine, cAMP is known to decrease Bcl-2 expression, and also to enhance TH expression (Kume et al., 2008). This was confirmed in this study. Otherwise, the SH-SY5Y cells under B21+cAMP displayed the strongest dopaminergic phenotype, supported by the highest TH expression among our conditions. Consistently, these cells were the most sensitive to OHDA, both in 2D and 3D cultures. This relates to the ability of this molecule to provoke neurodegeneration of dopaminergic neurons (Ungerstedt, 1968). Cisplatin and colchicine are both anti-proliferative and neurotoxic compounds. Cisplatin kills both cancerous and neuronal cells by similar apoptosis mechanisms (Gill and Windebank, 1998), whereas the toxic effect of colchicine is linked to an interaction with tubulin, leading to the disruption of microtubules (Alkadi et al., 2018). As proliferation of our SH-SY5Y cells was strongly slowed down whatever the differentiation medium, we might observe here the sole neurotoxic effect of these two compounds. Consistently, the global cytotoxicity observed here was higher under B21+cAMP, which also represented the most potent differentiating environment, the weakest being RA in 2D cell cultures.

Interestingly, results were different in 3D cultures. Exactly, a higher sensitivity of SH-SY5Y cells to colchicine and to high cisplatin concentrations was found when cells were previously differentiated with staurosporine than with B21+cAMP, despite a higher neuronal differentiation in B21+cAMP. These results could be explained by the anti-proliferative effect of colchicine and cisplatin, when the neuronal differentiation was not sufficient. Moreover, the cell sensitivity to compounds was overall lower in 3D compared to the 2D conditions, as often described in the literature (Ko et al., 2020). A shift in the LC50 or a sub-population of resistant cells was observed in 3D. This lower sensitivity can be explained by several hypotheses. First, the cell organization in spheroids can modify the exposition of cells to the compounds, although the spheroids were quite small. In addition, the strengthened cell-cell interactions in spheroids could lead to resistances to the toxic compounds. Moreover, the ECM impacts on molecule bioavailability, that might change the diffusion of both toxic compounds and differentiation factors (Clause and Barker, 2013; Sainio and Järveläinen, 2020; Su et al., 2017). Consequently, the neuronal differentiation can be delayed or reduced, as showed here by a lower expression of studied neuronal markers, in particular in RA medium where the neuronal marker expression was not increased, but also in staurosporine and B21+cAMP where NeuN was not detected. Therefore, the differentiation conditions in 2D should not be applied in 3D in a strictly similar way. It seems essential to optimize the differentiation protocols in 3D by adjusting concentrations and times to get an optimal and homogeneous cell maturation, as described (Harris et al., 2017).

To conclude, the given differentiation environment to SH-SY5Y cells *in vitro*, has a great impact on the acquisition of the neuronal phenotype, with consequences on the response to neurotoxic compounds. The B21+cAMP medium was particularly interesting for acute neurotoxicity assessment by high content screening in pharmaceutical industries, because it is chemically-defined and induced

the strongest neuronal phenotype, associated with an overall higher sensitivity to compounds. The 3D cell device mimicking the ECM also impacted differentiation and neurotoxic response and require a more optimized culture conditions compared to the straight forward 2D culture. The assessment of neurotoxicity with a neuronal cell line requires the *in vitro* differentiation environment to be considered carefully in research as well as in drug discovery.

References

Alkadi, H., Khubeiz, M.J., Jbeily, R., 2018. Colchicine: A Review on Chemical Structure and Clinical Usage. Infect. Disord. Drug Targets 18, 105–121. https://doi.org/10.2174/1871526517666171017114901

Clause, K.C., Barker, T.H., 2013. Extracellular matrix signaling in morphogenesis and repair. Curr. Opin. Biotechnol. 24, 830–833. https://doi.org/10.1016/j.copbio.2013.04.011

Cook, D., Brown, D., Alexander, R., March, R., Morgan, P., Satterthwaite, G., Pangalos, M.N., 2014. Lessons learned from the fate of AstraZeneca's drug pipeline: a five-dimensional framework. Nat. Rev. Drug Discov. 13, 419–431. https://doi.org/10.1038/nrd4309

Gill, J.S., Windebank, A.J., 1998. Cisplatin-induced apoptosis in rat dorsal root ganglion neurons is associated with attempted entry into the cell cycle. J. Clin. Invest. 101, 2842–2850. https://doi.org/10.1172/JCI1130

Harris, G., Hogberg, H., Hartung, T., Smirnova, L., 2017. 3D differentiation of LUHMES cell line to study recovery and delayed neurotoxic effects. Curr. Protoc. Toxicol. 2017. https://doi.org/10.1002/cptx.29

Itano, Y., Ito, A., Uehara, T., Nomura, Y., 1996. Regulation of Bcl-2 Protein Expression in Human Neuroblastoma SH-SY5Y Cells: Positive and Negative Effects of Protein Kinases C and A, Respectively. J. Neurochem. 67, 131–137. https://doi.org/10.1046/j.1471-4159.1996.67010131.x

Kawano, M., Kawasaki, A., Sakata-Haga, H., Fukui, Y., Kawano, H., Nogami, H., Hisano, S., 2006. Particular subpopulations of midbrain and hypothalamic dopamine neurons express vesicular glutamate transporter 2 in the rat brain. J. Comp. Neurol. 498, 581–592. https://doi.org/10.1002/cne.21054

Ko, K.R., Tam, N.W., Teixeira, A.G., Frampton, J.P., 2020. SH-SY5Y and LUHMES cells display differential sensitivity to MPP+, tunicamycin, and epoxomicin in 2D and 3D cell culture. Biotechnol. Prog. 36, e2942. https://doi.org/10.1002/btpr.2942

Kovalevich, J., Langford, D., 2013. Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. Methods Mol. Biol. Clifton NJ 1078, 9–21. https://doi.org/10.1007/978-1-62703-640-5_2

Kume, T., Kawato, Y., Osakada, F., Izumi, Y., Katsuki, H., Nakagawa, T., Kaneko, S., Niidome, T., Takada-Takatori, Y., Akaike, A., 2008. Dibutyryl cyclic AMP induces differentiation of human neuroblastoma SH-SY5Y cells into a noradrenergic phenotype. Neurosci. Lett. 443, 199–203. https://doi.org/10.1016/j.neulet.2008.07.079

Leli, U., Shea, T.B., Cataldo, A., Hauser, G., Grynspan, F., Beermann, M.L., Liepkalns, V.A., Nixon, R.A., Parker, P.J., 1993. Differential expression and subcellular localization of protein kinase C alpha, beta, gamma, delta, and epsilon isoforms in SH-SY5Y neuroblastoma cells: modifications during differentiation. J. Neurochem. 60, 289–298. https://doi.org/10.1111/j.1471-4159.1993.tb05850.x

López-Carballo, G., Moreno, L., Masiá, S., Pérez, P., Barettino, D., 2002. Activation of the phosphatidylinositol 3-kinase/Akt signaling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells. J. Biol. Chem. 277, 25297–25304. https://doi.org/10.1074/jbc.M201869200

Lotan, R., 1996. Retinoids in cancer chemoprevention. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 10, 1031–1039. https://doi.org/10.1096/fasebj.10.9.8801164

Melino, G., Thiele, C.J., Knight, R.A., Piacentini, M., 1997. Retinoids and the control of growth/death decisions in human neuroblastoma cell lines. J. Neurooncol. 31, 65–83. https://doi.org/10.1023/a:1005733430435

Sainio, A., Järveläinen, H., 2020. Extracellular matrix-cell interactions: Focus on therapeutic applications. Cell. Signal. 66, 109487. https://doi.org/10.1016/j.cellsig.2019.109487

Su, W., Foster, S.C., Xing, R., Feistel, K., Olsen, R.H.J., Acevedo, S.F., Raber, J., Sherman, L.S., 2017. CD44 Transmembrane Receptor and Hyaluronan Regulate Adult Hippocampal Neural Stem Cell Quiescence and Differentiation. J. Biol. Chem. 292, 4434–4445. https://doi.org/10.1074/jbc.M116.774109

Tieu, K., Zuo, D.M., Yu, P.H., 1999. Differential effects of staurosporine and retinoic acid on the vulnerability of the SH-SY5Y neuroblastoma cells: involvement of bcl-2 and p53 proteins. J. Neurosci. Res. 58, 426–435.

Trudeau, L.-E., 2004. Glutamate co-transmission as an emerging concept in monoamine neuron function. J. Psychiatry Neurosci. JPN 29, 296–310.

Ungerstedt, U., 1968. 6-hydroxy-dopamine induced degeneration of central monoamine neurons. Eur. J. Pharmacol. 5, 107–110. https://doi.org/10.1016/0014-2999(68)90164-7

Wilson, M.S., Graham, J.R., Ball, A.J., 2014. Multiparametric High Content Analysis for assessment of neurotoxicity in differentiated neuronal cell lines and human embryonic stem cell-derived neurons. Neurotoxicology 42, 33–48. https://doi.org/10.1016/j.neuro.2014.03.013

Xicoy, H., Wieringa, B., Martens, G.J.M., 2017. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. Mol. Neurodegener. 12. https://doi.org/10.1186/s13024-017-0149-0

Captions

Fig. 1. Protocol of SH-SY5Y expansion and differentiation. A. Main steps from cell thawing to image analysis. **B.** Culture conditions for non-differentiated cells, and for cells differentiated with RA or staurosporine in FBS-supplemented MEM/F12. C. Culture conditions for cells differentiated with cAMP in FBS-free neurobasal 2% B21 medium. D: Day, followed by the number of days before (-) or after seeding. RA: Retinoic acid; Stauro: Staurosporine; FBS: Fetal Bovine Serum; MEM/F12: Minimum Essential Medium/Ham F12 medium; cAMP: cyclic Adenosine monophosphate.

Fig. 2. Screening of 24 conditions for SH-SY5Y differentiation after 5 days. A. Cell proliferation. Cell nuclei count after Hoechst staining (n=8). Grey asterisks: number of cells per well significantly lower in comparison to proliferation medium. **B.** Neurite length. Neurite length after TUBB3 immunostaining (n=8). Grey asterisk: neurite length significantly higher in comparison to proliferation medium. Black asterisks: neurite length significantly higher in comparison to neurobasal 2% B21 medium. Mean values were significantly different according to Student test for parametric samples with equal variances, Welch test for parametric samples with different variances, or Wilcoxon test for non-parametric samples. *p-value < 0.05; **p-value>0.005; ***p-value<0.0005. Error bar represents the SEM. **C.** Morphology of SH-SY5Y cells in proliferation medium and in the three selected differentiation media. Nuclei were stained by Hoechst (blue) and TUBB3 was immunostained (green). Scale bar = 200µm. The inset is a 4-fold magnified image of the outlined area. RA: Retinoic Acid; TPA: Tetradecanoylphorbol Acetate; cAMP: cyclic Adenosine Monophosphate; iBMX: 3-isobutyl-1-

methylxanthine; MEM/F12: Minimum Essential Medium/Ham F12 medium; FBS: Fetal Bovine Serum.

Fig. 3. Kinetic analysis of SH-SY5Y cell proliferation and neurite elongation. **A.** Cell proliferation. Cell nuclei count after Hoechst staining (n=12). **B.** Neurite elongation. Neurite length after TUBB3 immunostaining (n=12). Error bar represents the SEM**. C.** Statistical analysis. Mean values were significantly different according to Student test for parametric samples with equal variances, Welch test for parametric samples with different variances, or Wilcoxon test for non-parametric samples. *p-value < 0.05; **p-value > 0.005; ***p-value < 0.0005. RA: Retinoic Acid; cAMP: cyclic Adenosine Monophosphate; *ns*: not significant

Fig. 4. Kinetic analysis of pan-neuronal marker expression in SH-SY5Y cells. A. Cellular localization of TUBB3, synaptophysin and NeuN at day 5. Cell were stained for nuclear DNA by Hoechst (in blue), for TUBB3 (in green), synaptophysin (in red), and NeuN (in yellow). Scale bar = 200µm. **B.** Quantitative analysis of immunostainings. The stain integrated intensity of differentiated cells was normalized to the stain integrated intensity of proliferating cells (n=12). Error bar represents the SEM. **C.** Statistical analysis. Mean values were significantly different according to Student test for parametric samples with equal variances, Welch test for parametric samples with different variances, or Wilcoxon test for non-parametric samples. *p-value < 0.05; **p-value > 0.005; ***p-value < 0.0005. Prolif.: Proliferation medium; RA: Retinoic Acid; cAMP: cyclic Adenosine Monophosphate; Syn.: Synaptophysin; *ns*: not significant

Fig. 5. Analysis of neuronal specificity of SH-SY5Y cells at day 5. **A.** Cellular localization of TH and vGLUT2. Cells were stained for nuclear DNA by Hoechst (in blue), for TH (in green), and for vGLUT2 (in red). Scale bar = 200µm. **B.** Quantitative analysis of immunostainings. The stain integrated intensity of differentiated cells was normalized to the stain integrated intensity of proliferating cells (n=24). Error bar represents the SEM. **C.** Statistical analysis. Mean values were significantly different according to Student test for parametric samples with equal variances, Welch test for parametric samples with different variances, or Wilcoxon test for non-parametric samples. *p-value < 0.05; **p-value > 0.005; ***p-value < 0.0005. **D.** Co-expression of TH and vGLUT2 in SH-SY5Y cells differentiated in B21+cAMP medium. Scale bar = 200µm. Prolif.: Proliferation medium; RA: Retinoic Acid; cAMP: cyclic Adenosine Monophosphate; TH: Tyrosyne Hydroxylase; vGLUT2: vesicular Glutamate Transporter 2.

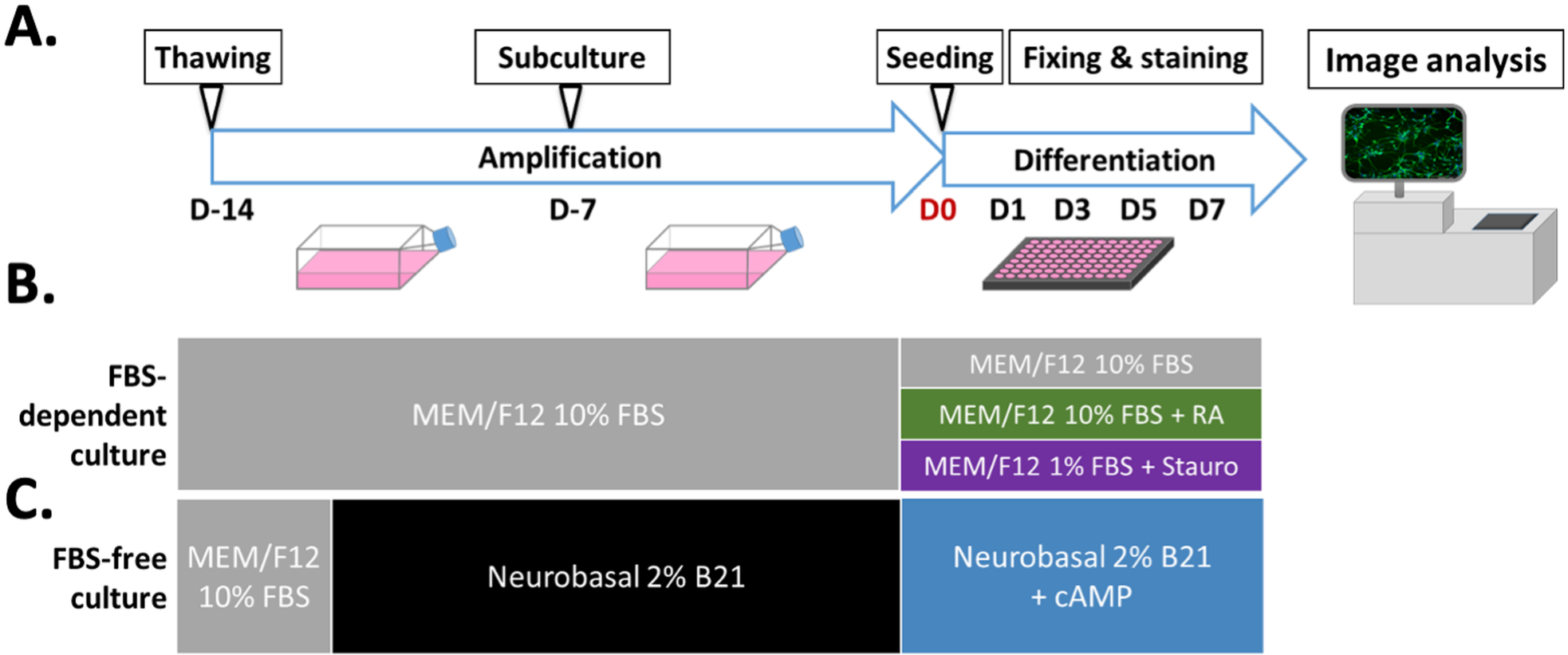
Fig. 6. SH-SY5Y cell viability after a 48h exposure to a set of compounds of known toxicity. Amoxicillin and L-ascorbic acid are known as non-toxic compounds. Acetaminophen and troglitazone

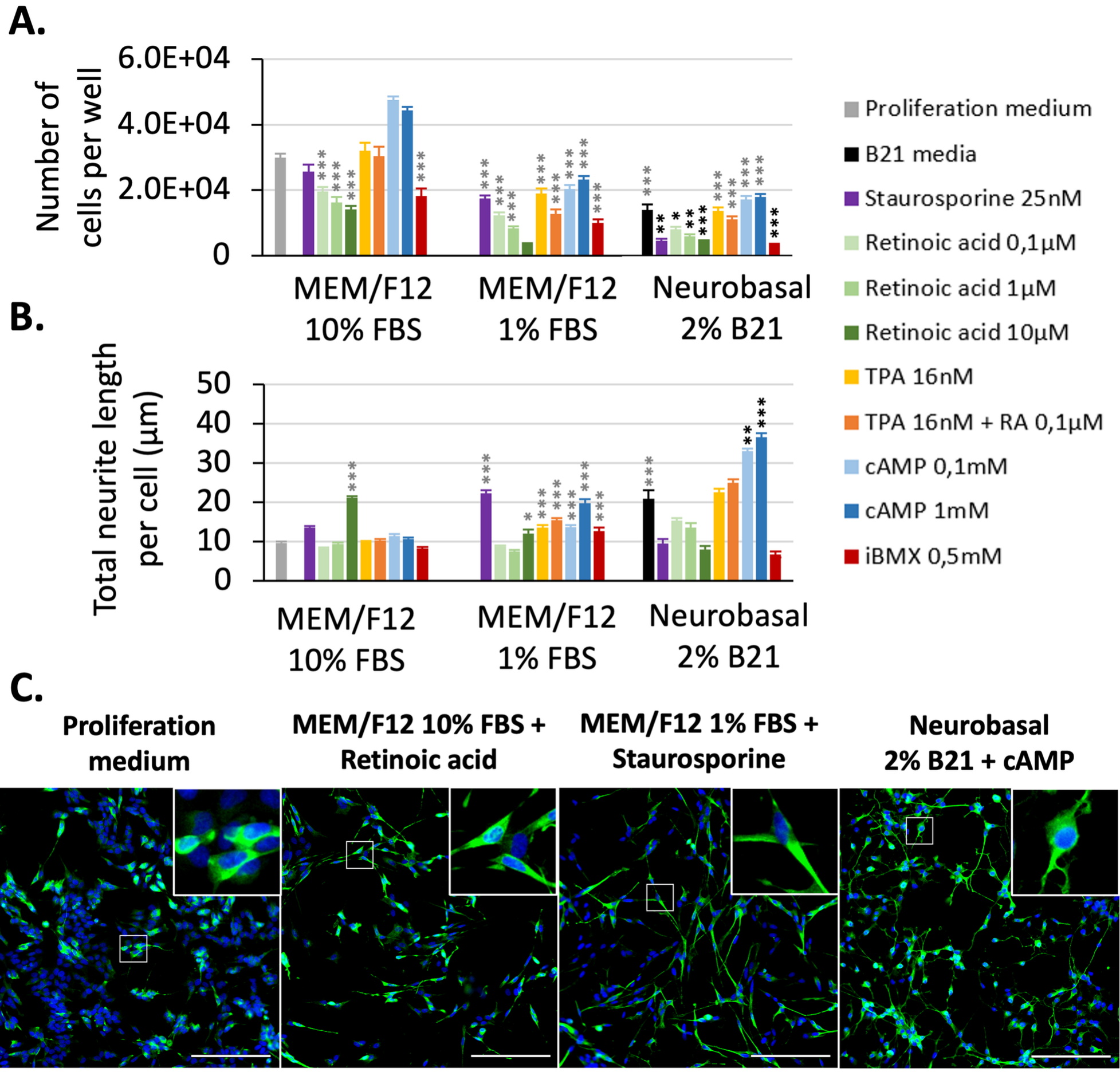
are known as toxic but non-neurotoxic compounds. 6-OHDA, colchicine, cisplatin and tamoxifen are known as neurotoxic compounds. The number of live cells exposed to the compounds was normalized to the number of live cells in vehicle control (n=12). Error bar represents the SEM. RA: Retinoic Acid; cAMP: cyclic Adenosine Monophosphate; 6-OHDA: 6-hydroxydopamine; *nd*: not determined; *nte*: no toxic effect.

Fig. 7. Culture of SH-SY5Y cells in an 3D hydroscaffold for 5 days. **A.** Spheroid organization. Nuclei were stained by Hoechst (blue) and TUBB3 was immunostained (green). Scale bar = 20µm. **B.** Correlation between the number of spheroids per well and their diameter. **C.** Cell proliferation (n=72). Mean values were significantly different according to Student test for parametric samples with equal variances, Welch test for parametric samples with different variances, or Wilcoxon test for non-parametric samples. *p-value < 0.05; **p-value > 0.005; ***p-value < 0.0005. Error bar represents the SEM. RA: Retinoic Acid; cAMP: cyclic Adenosine Monophosphate.

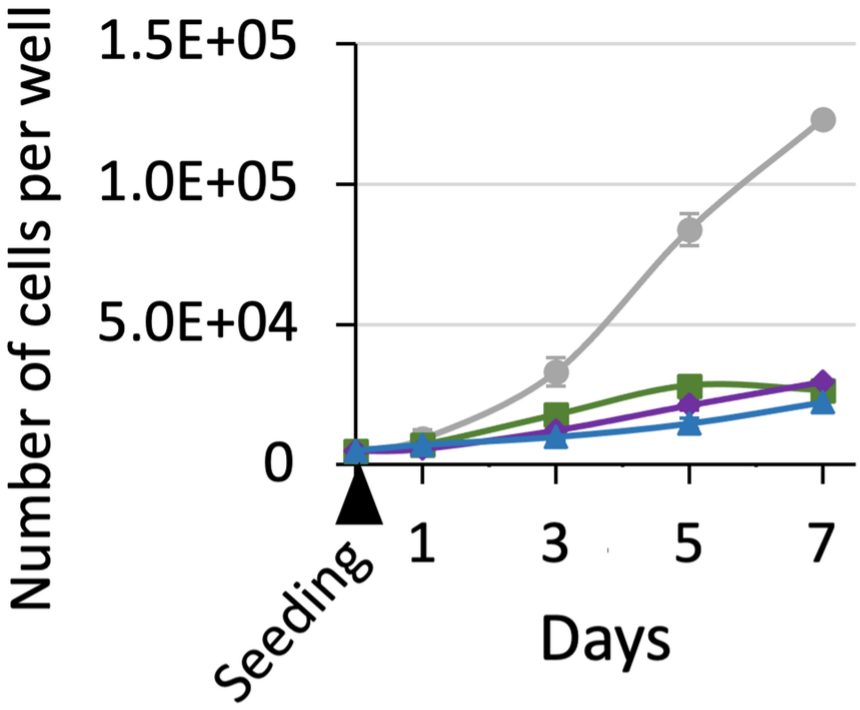
Fig. 8. Expression of neuronal markers in SH-SY5Y cells at day 5 in 3D culture. A. Neuronal marker expression. Cells were stained for nuclei by Hoechst (in blue), for TUBB3 (in green), Synaptophysin (in red), and TH (in green). Scale bar = 200µm. **B.** Quantitative analysis of immunostainings. The stain average intensity of differentiated cells was normalized to the stain average intensity of proliferating cells (n=12). Error bar represents the SEM. **C.** Statistical analysis. Mean values were significantly different according to Student test for parametric samples with equal variances, Welch test for parametric samples with different variances, or Wilcoxon test for non-parametric samples. *p-value < 0.05; **p-value > 0.005; ***p-value < 0.0005. Prolif.: Proliferation medium; RA: Retinoic Acid; cAMP: cyclic Adenosine Monophosphate; Syn.: Synaptophysin; TH: Tyrosine Hydroxylase; *ns*: not significant.

Fig. 9. SH-SY5Y cell viability after a 48h exposure to a set of compounds of known toxicity. Amoxicillin and L-ascorbic acid are known as non-toxic compounds. Acetaminophen and troglitazone are known as toxic but non-neurotoxic compounds. 6-OHDA, colchicine, cisplatin and tamoxifen are known as neurotoxic compounds. The percentage of live cells per well exposed to the compounds was normalized to the percentage of live cells per well in vehicle control. Error bar represents the SEM. RA: Retinoic Acid; cAMP: cyclic Adenosine Monophosphate; 6-OHDA: 6-hydroxydopamine; *nd*: not determined; *nte*: no toxic effect.

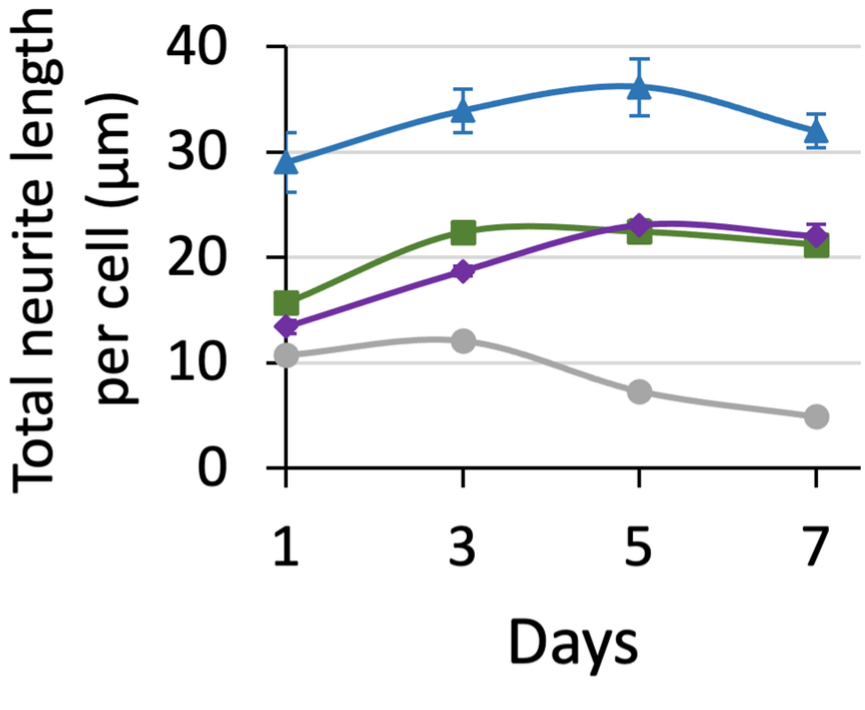

A.
Thawing
Subculture
Seeding
Fixing & staining
Image analysis
Amplification
Differentiation
D-14
D-7
D0
D1
D3
D5
D7
B.
FBS-dependent culture
MEM/F12 10% FBS
MEM/F12 10% FBS
MEM/F12 10% FBS + RA
MEM/F12 1% FBS + Stauro
C.
FBS-free culture
MEM/F12 10% FBS
Neurobasal 2% B21
Neurobasal 2% B21 + cAMP


A.
Number of cells per well
6.0E+04
4.0E+04
2.0E+04
0
MEM/F12 10% FBS
MEM/F12 1% FBS
Neurobasal 2% B21
Proliferation medium
B21 media
Staurosporine 25nM
Retinoic acid 0,1µM
Retinoic acid 1µM
Retinoic acid 10µM
TPA 16nM
TPA 16nM + RA 0,1µM
cAMP 0,1mM
cAMP 1mM
iBMX 0,5mM
B.
Total neurite length per cell (µm)
50
40
30
20
10
0
MEM/F12 10% FBS
MEM/F12 1% FBS
Neurobasal 2% B21
C.
Proliferation medium
MEM/F12 10% FBS + Retinoic acid
MEM/F12 1% FBS + Staurosporine
Neurobasal 2% B21 + cAMP

A.



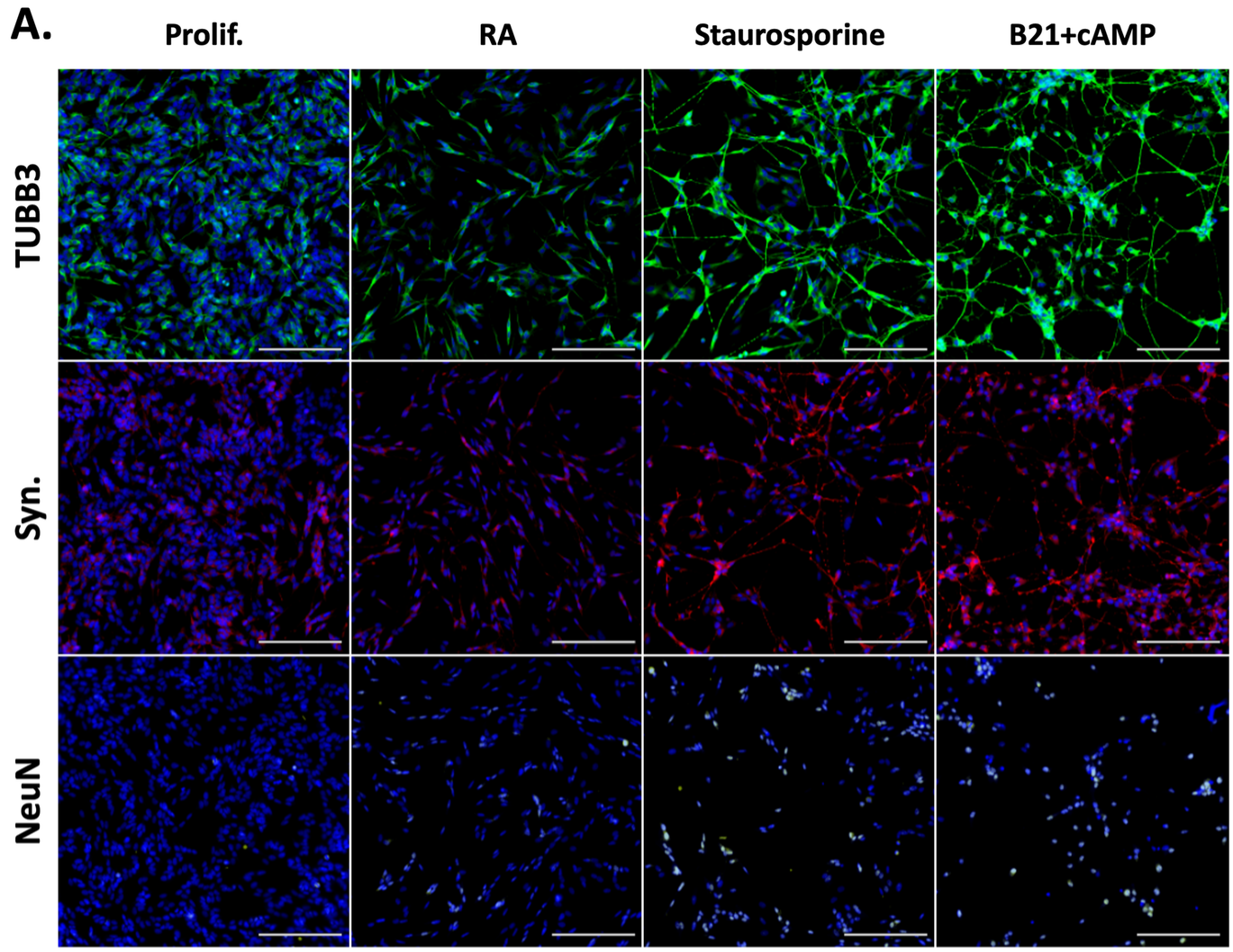
B.

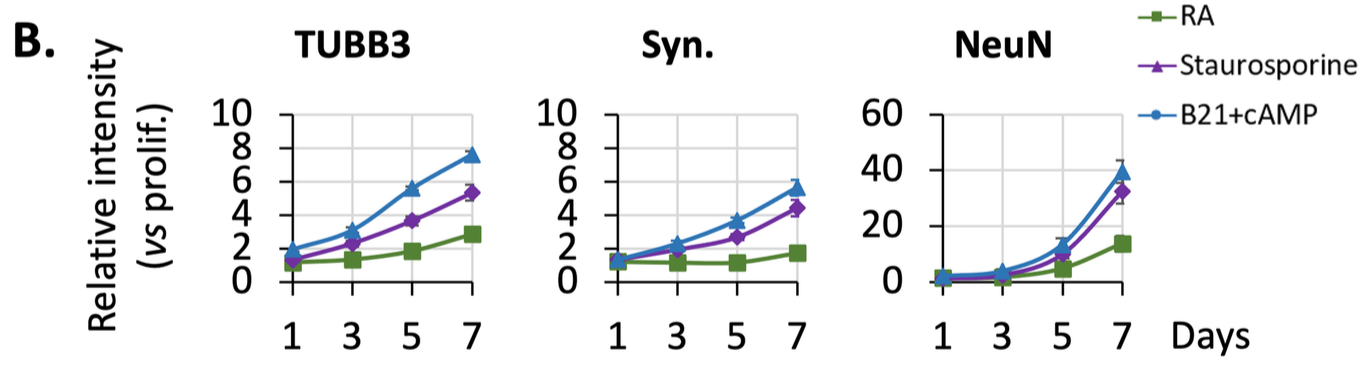


Proliferation RA Staurosporine B21+cAMP

C.

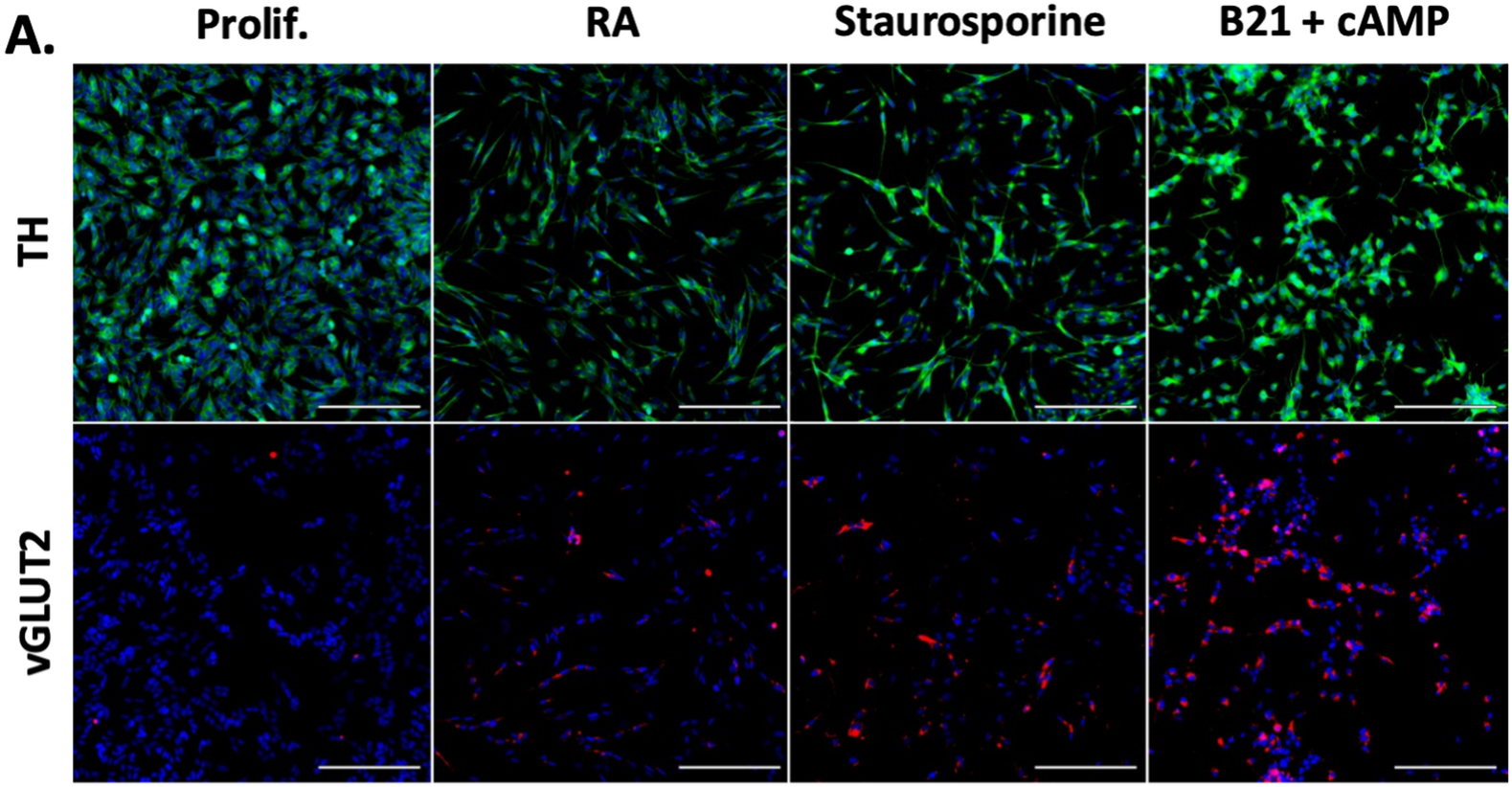
		D1			D3			D5			D7		
		RA	Stauro	B21 +cAMP	RA	Stauro	B21 +cAMP	RA	Stauro	B21 +cAMP	RA	Stauro	B21 +cAMP
Number	Prolif	*	***	*	**	***	***	***	***	***	***	***	***
	RA		*ns*	*ns*		*	**		*	***		*ns*	*ns*
	Stauro			*ns*			*ns*			**			*
Neurites	Prolif	***	**	***	***	***	***	***	***	***	***	***	***
	RA		*	***		**	***		*ns*	***		*ns*	***
	Stauro			***			***			***			***

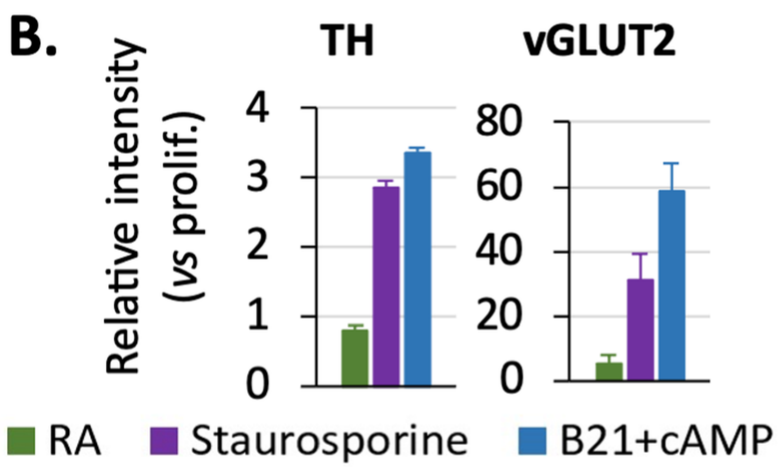




C.

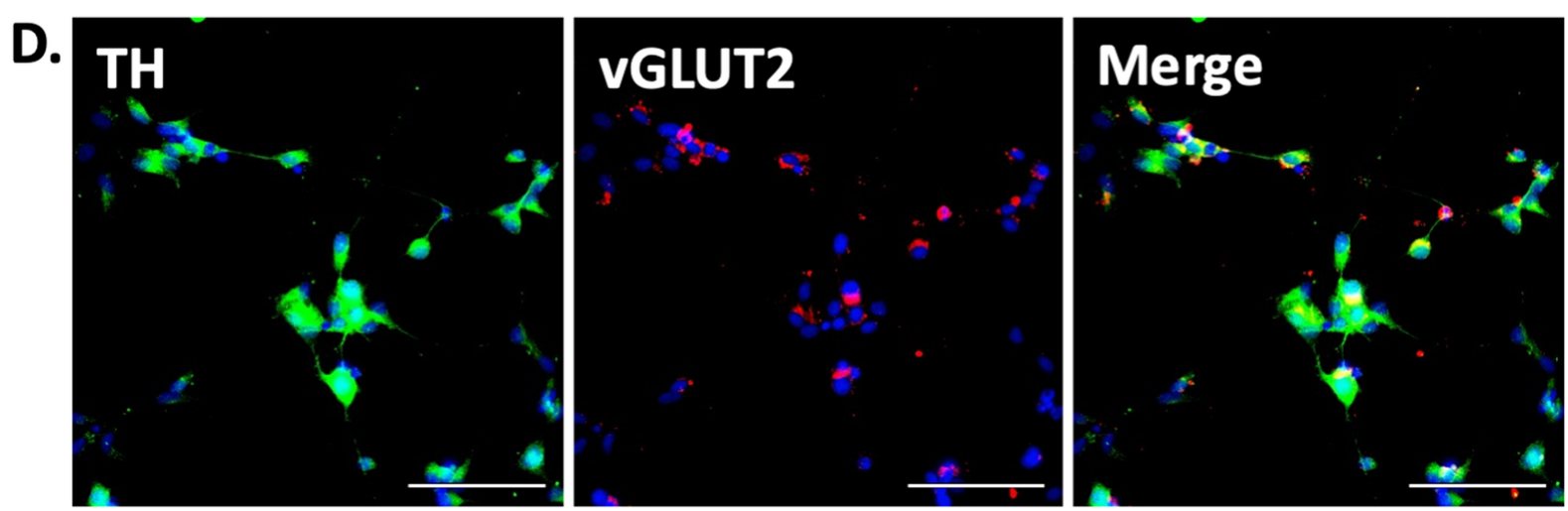
		D1			D3			D5			D7		
		RA	Stauro	B21 +cAMP	RA	Stauro	B21 +cAMP	RA	Stauro	B21 +cAMP	RA	Stauro	B21 +cAMP
B3T	Prolif	*	*	***	***	***	***	***	***	***	***	***	***
	RA		*ns*	***		***	***		***	***		***	***
	Stauro			**			*			***			***
Syn.	Prolif	*ns*	**	*	*ns*	***	***	*ns*	***	***	**	***	***
	RA		*ns*	*ns*		***	***		***	***		***	***
	Stauro			*ns*			*ns*			**			*ns*
NeuN	Prolif	*ns*	*ns*	*	*	***	**	***	***	***	***	***	***
	RA		*ns*	*ns*		*	**		*	***		**	***
	Stauro			*ns*			*			*ns*			*ns*



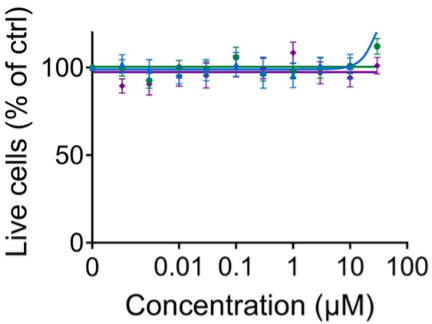


C.

		RA	Stauro	B21 +cAMP
TH	Prolif	*	***	***
	RA		***	***
	Stauro			**
vGLUT2	Prolif	***	***	***
	RA		***	***
	Stauro			**

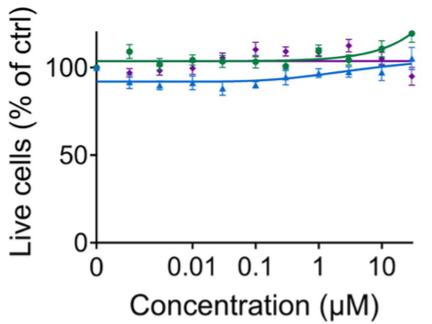


Amoxicillin



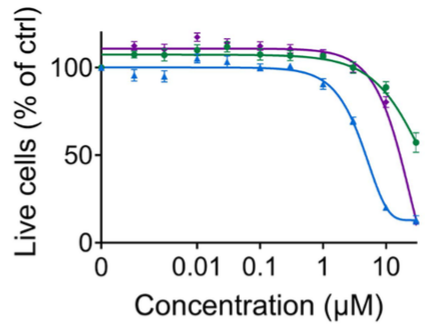
No toxic effect

L-Ascorbic acid



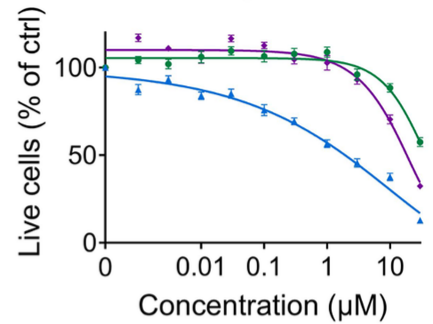
No toxic effect

6-OHDA



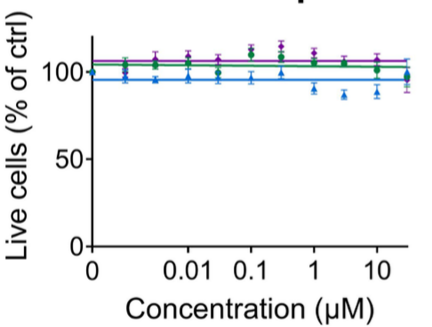
LC50 (μM)		
RA	Stauro	B21+cAMP
36	17	6

Cisplatin



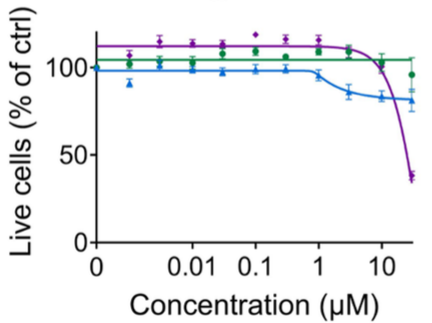
LC50 (μM)		
RA	Stauro	B21+cAMP
37	18	2

Acetaminophen



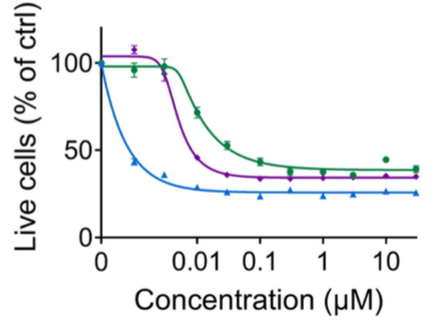
No toxic effect

Troglitazone



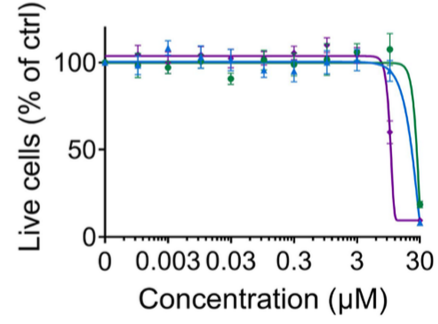
LC50 (μM)		
RA	Stauro	B21+cAMP
nte	25	*nd*

Colchicine



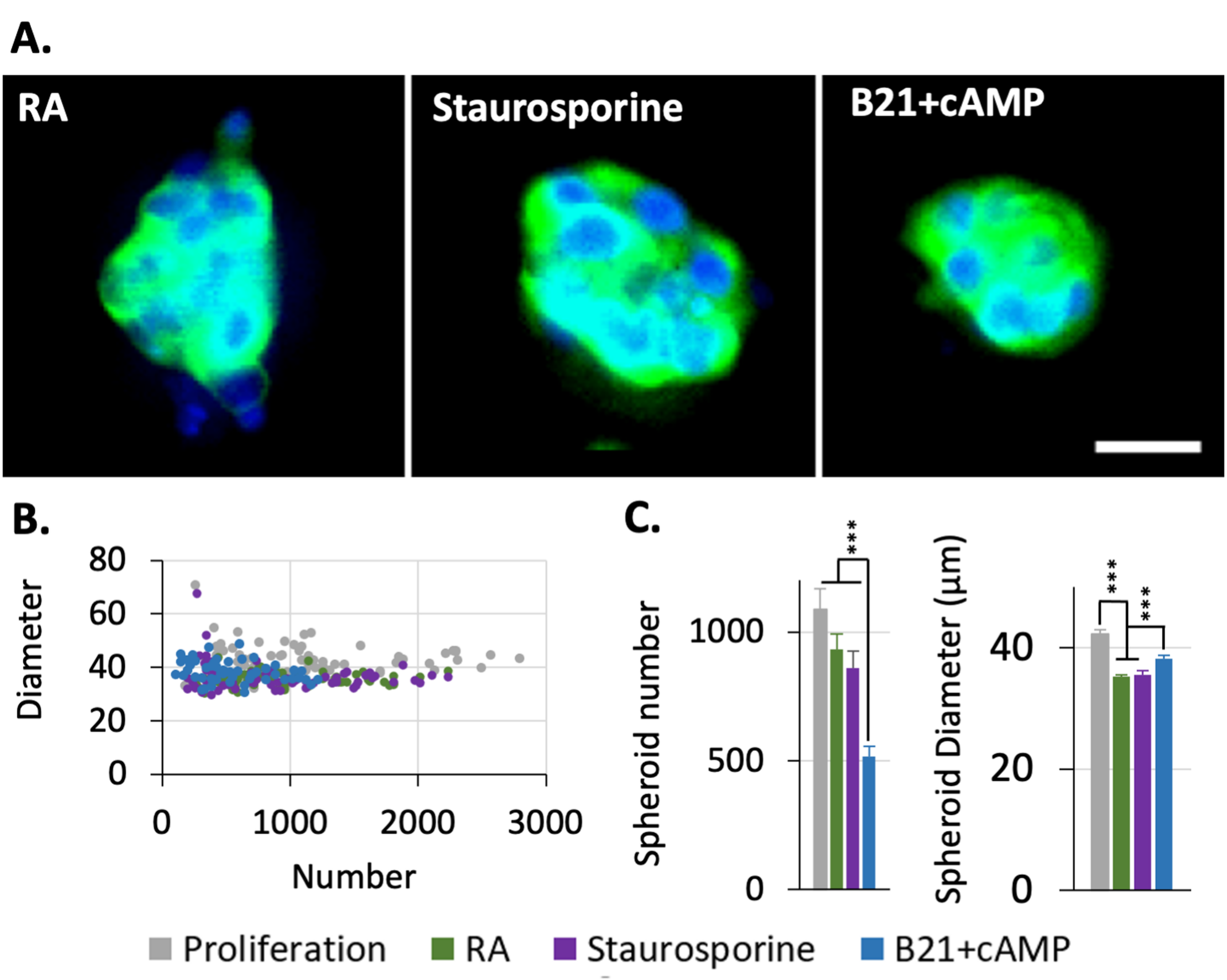
LC50 (μM)		
RA	Stauro	B21+cAMP
0.03	0.008	0.0008

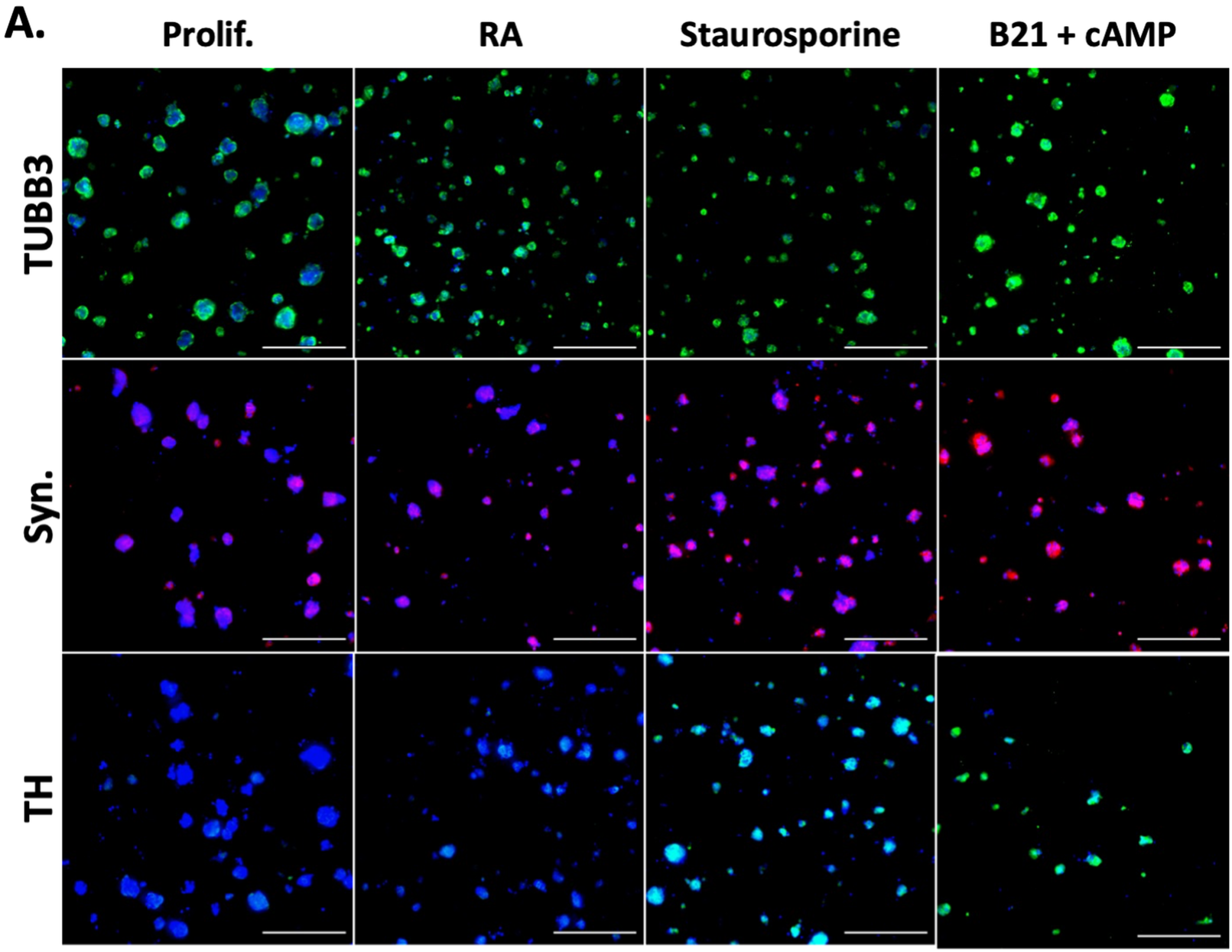
Tamoxifen



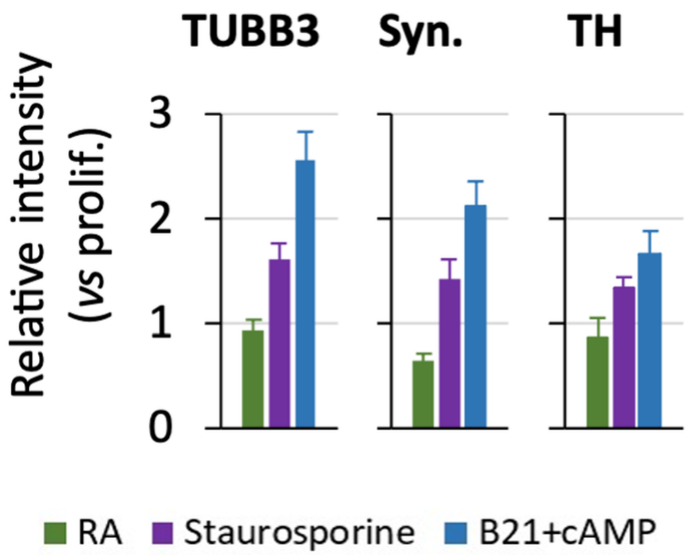
LC50 (μM)		
RA	Stauro	B21+cAMP
24	11	21

B21 + cAMP RA Staurosporine


A.
RA
Staurosporine
B21+cAMP
B.
Diameter
80
60
40
20
0
0
1000
2000
3000
Number
C.
Spheroid number
1000
500
0
***
Spheroid Diameter (µm)
40
20
0
***
***
Proliferation
RA
Staurosporine
B21+cAMP



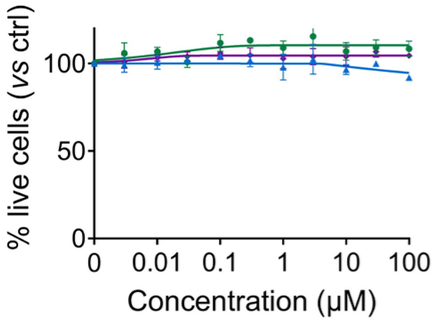
B.



C.

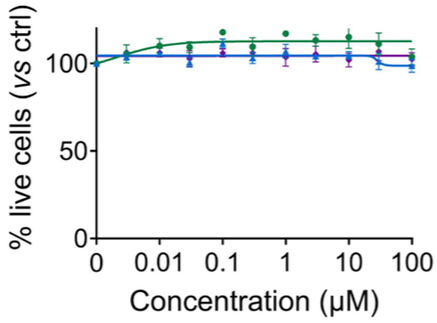
		RA	Stauro	B21 +cAMP
TUBB3	Prolif	*ns*	***	***
	RA		***	***
	Stauro			**
Syn.	Prolif	*	*ns*	**
	RA		***	***
	Stauro			*
TH	Prolif	*ns*	*	**
	RA		*	**
	Stauro			*ns*

Amoxicillin



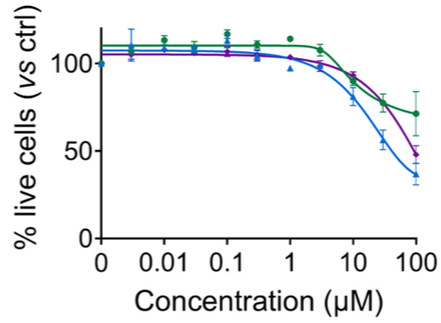
No toxic effect

L-Ascorbic acid



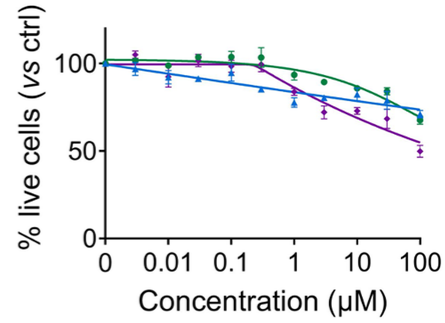
No toxic effect

6-OHDA



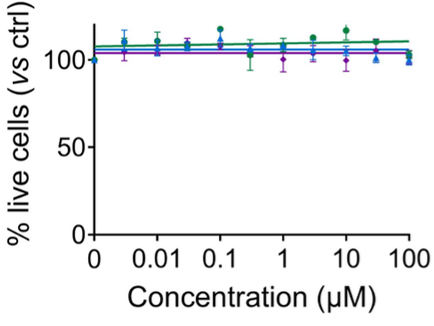
LC50 (µM)		
RA	Stauro	B21+cAMP
nd	25	41

Cisplatin



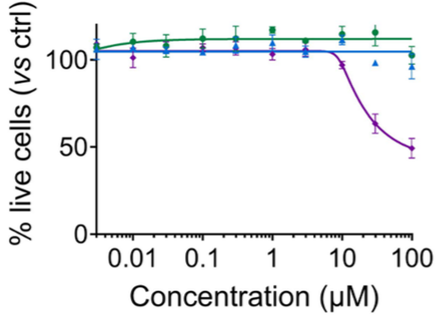
LC50 (µM)		
RA	Stauro	B21+cAMP
nd	262	*nd*

Acetaminophen



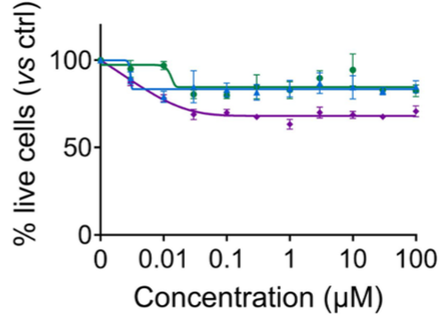
No toxic effect

Troglitazone



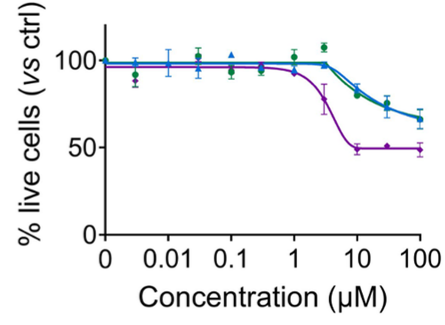
LC50 (µM)		
RA	Stauro	B21+cAMP
nte	90	*nd*

Colchicine



LC50 (µM)		
RA	Stauro	B21+cAMP
nd	*nd*	*nd*

Tamoxifen



LC50 (µM)		
RA	Stauro	B21+cAMP
nd	94	*nd*

B21 + cAMP RA Staurosporine

Table 1

List of primary antibodies used for immunocytochemistry procedures, respective dilution, provider and catalogue number.

	Antibody	Type	Host Species	Dilution	Provider	Cat.
Primary antibody	TUBB3	Polyclonal IgG	Rabbit	1:5,000 (2D) 1:1,000 (3D)	Abcam	Ab18207
	Synaptophysin	Monoclonal IgG	Rabbit	1:500 (2D) 1:250 (3D)	Abcam	ab32127
	NeuN D3S3I	Monoclonal IgG	Rabbit	1:500 (2D) 1:250 (3D)	Cell signaling	12943S
	TH	Polyclonal IgG	Rabbit	1:100	Sigma Aldrich	AB152
	vGLUT2	Monoclonal IgG	Mouse	1:100	Millipore	MAB5504
Secondary antibody	Alexa Fluor 488	IgG	Goat anti-rabbit	1:2,00	Cell signaling	4412S
	Alexa Fluor 488	IgG	Goat anti-mouse	1:2,000	Cell signaling	4408S

Table2
List of the compounds used for neurotoxicity assessment, CAS number, provider and catalogue number.

Compounds	CAS	Provider	Cat.
Acetaminophen	103-90-2	Sigma Aldrich	A7085-100G
Amoxicillin	61336-70-7	TCI	A2099-5G
L-Ascorbic acid	50-81-7	TCI	A0537
Cisplatin	15663-27-1	TCI	D3371-100MG
Colchicine	64-86-8	Sigma Aldrich	C9754-100MG
6-OHDA	28094-15-7	Sigma Aldrich	H4381-100MG
Tamoxifen	10540-29-1	TCI	T2510-1G
Troglitazone	97322-87-7	Sigma Aldrich	T2573-5MG

In vitro differentiation modifies the neurotoxic response of SH-SY5Y cells

Selection of the most differentiating conditions	Influence of the differentiation method	
	Neuronal phenotype	**Sensitivity**

Screening 24 conditions

Proliferation slowdown, neurite elongation

- **Retinoic acid**
  10% FBS medium
- **Staurosporine**
  1% FBS medium
- **cAMP**
  2% B21 medium

Neurite length, Pan and specific neuronal proteins

TUBB3 NeuN TH vGLUT2 Synaptophysin

Non-toxic, Toxic but non-neurotoxic And neurotoxic molecules

\+ Colchicine

++ 6-OHDA

+++ Cisplatin Tamoxifen

2D culture

3D culture in a hydroscaffold

Conclusion

The *in vitro* differentiation environment should be considered carefully in research as well as in drug discovery.