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**An *in vitro* model for neuroscience - differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons**

**Running title:** A novel *in vitro* model for neuroscience

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**Abstract**

Neuroscience, including research on Alzheimer's disease, is hampered by the lack of suitable *in vitro* models to study the human nervous system. To counteract this, many attempts to differentiate cell lines into more neuron-like cells have been performed, resulting in partial expression of neuronal features. Furthermore, it has been reported that neuroblastoma cell lines lack mature isoforms of tau. Our aim was to develop an improved *in vitro* model, generating sustainable cells with morphology and biochemistry of human, mature neurons. To obtain cells with neuronal differentiation and function, we investigated the effect of combining three-dimensional culturing of SH-SY5Y cells in extra cellular matrix (ECM) gel with several factors reported to have neuro differentiating effects. This resulted in cells with apparent neuronal morphology with long, extensively branched neurites. Further investigation revealed expression of several neurospecific markers including synapse protein Sv2 and nuclear marker NeuN, as well as the presence of synapses and axonal vesicle transport. In addition, these cells expressed mature tau isoforms, and tau protein expression was significantly increased compared to undifferentiated cells, reaching levels found in adult human brain. In conclusion, we found that pre-treatment with retinoic acid followed by ECM gel culturing in combination with brain derived neurotrophic factor, neuregulin  $\beta$ 1, nerve growth factor, and vitamin D<sub>3</sub> treatment generated sustainable cells with unambiguous resemblance to adult neurons. These cells also express adult splicing forms of tau with neuronal localization, making this cellular *in vitro* model useful in many areas of neuroscience research, particularly the Alzheimer's field.

Keywords: neuroblastoma, *in vitro* model, differentiation, Alzheimer's disease, tau

## Introduction

Many areas in the field of neuroscience are hampered by the lack of relevant *in vitro* models resembling functionally mature neurons that express human proteins. This is especially true in Alzheimer's disease (AD) where commonly used cell models do not include axons, synapses (e.g. most cell-lines) and human proteins (e.g. primary neurons from rodents), all implicated in the pathology. Live, adult human neurons for functional and interventional studies are not readily available, so different immortalized cell lines are used as an alternative. However, these cell lines, including the frequently used neuroblastoma cell line SH-SY5Y, lack many of the features that define neurons, including neuronal morphology, inhibited cell division, and expression of neuron-specific markers. Furthermore, neuron-specific proteins are rarely expressed and distributed in SH-SY5Y cells at levels comparable to mature neurons. A diversity of proteins are used as markers for mature neurons, such as neuron specific  $\beta$ -III tubulin, which is almost exclusively expressed in neurons and it is a marker for differentiation and decreased proliferation [1]. A marker for synapses is Synaptic vesicle protein Sv2, a glycoprotein present in pre-synaptic vesicles of neurons and endocrine cells, involved in synaptic structure and transmitter release (reviewed in [2]). Furthermore, neurospecific nuclear marker NeuN is a marker for post-mitotic neurons [3], and a marker for maturation [4].

Tau is a microtubule binding protein, important for microtubule stability, axonal development and transportation [5]. Tau is involved in several neurodegenerative diseases, commonly named tauopathies, including AD. In the adult human brain, the microtubule binding protein tau has six isoforms, products of alternative splicing [6]. Only the shortest isoform is expressed in fetal brain [7], also reported to be the case with undifferentiated SH-SY5Y cells

[7, 8], these are thus unrepresentative of the adult brain. For example; fetal tau, although being heavily phosphorylated, does not form tangles as seen in AD [9]. Furthermore, the expression levels and cellular localization of tau are different in neurons of the human adult brain compared to cultured cells. Thus, in mature neurons, tau is mainly found in the axons whereas in cultured cells, tau is present throughout the cells including the cell soma [10].

SH-SY5Y cells can, depending on treatment, be differentiated into several different phenotypes. Commonly used differentiation agents include phorbol esters and retinoic acid (RA) [11], growth factors such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neuregulins [12-14]. Other less frequently used differentiation factors include the Vitamin D metabolite 1,25-dihydroxycholecalciferol (VitD<sub>3</sub>), and cholesterol [15-17]. Another way of improving neuronal differentiation is to culture SH-SY5Y cells in a three dimensional (3D) environment [18] such as an extracellular matrix (ECM) gel, which provides cell support and trace amounts of substances that promote differentiation [19, 20]. Each of these stimuli induces a certain tendency towards neuronal differentiation; however, none results in cells with extensive neuron-like morphology and markers of mature neurons. One untested approach is to use a combination of several stimuli to generate functionally mature neurons.

As there are increasing number of studies on AD focusing on microtubule function and axonal transport [21] as well as synaptic events and failure [22], there is a great need for human cell models suitable for studying these events here as well as in other areas of neuroscience. In the present study, we present a method to differentiate a readily available human neuroblastoma cell-line into cells with a high resemblance to adult human neurons. Hence offering a

reproducible, easily available, and affordable method with qualities that enable studies of cellular structures and events that are of great importance in neuroscience.

## **Material and methods**

### *Cell culture and differentiation*

SH-SY5Y cells (ECACC; Sigma Aldrich, St. Louis, MO, USA) were cultured in MEM-Glutamax medium supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Pasching, Austria), 50 U/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine (all from Gibco, Paisley, UK), in 37 °C, humidified air with 5% CO<sub>2</sub>. The medium was changed twice a week and cells were split at about 80% confluence. Two different batches of cells were used for experiments, and cells were never cultivated beyond passage 25. Cells to be treated with RA or BDNF were pre-differentiated with RA (10 µM; Sigma Aldrich) for 1 week. Cells were harvested by trypsination and the cell suspension was mixed 1:1 with pre-chilled ECM gel (Sigma Aldrich) and seeded in pre-cooled 24-well plates (BD Falcon, Franklin Lakes, NJ, USA) at the density of 200'000 cells/well. The gel was left to set at 37 °C before the culture medium (supplemented with RA when applicable) was added, and the cells were allowed to settle in the ECM gel for two days prior to stimulation. Subsequently, different combinations of RA (10 µM), BDNF (50 ng/ml; Peprtech, Rocky Hill, NJ, USA), neuregulin β1 (NRG; 10 ng/ml), NGF (10 ng/ml, R&D systems, Minneapolis, MN, USA), vitamin D<sub>3</sub> (VitD<sub>3</sub>; 24 nM), and cholesterol (10 µg/ml; Sigma Aldrich) were added to the culture medium (see table 1 for details). The culture medium was changed every 3-4 days, supplemented with fresh additives. Serum-free culture medium was used whenever BDNF was included [12]. Since differentiation of SH-SY5Y cells with RA is the most commonly used regime, this treatment was used as reference in all experiments apart from the tau mRNA

and protein analysis. These undifferentiated cells were used instead due to the earlier reported findings regarding tau expression in these cells.

### *Morphological classification*

The cells were differentiated for 10 or 21 days, and cellular morphology of living cells was visualized at day 0, 5, 10, 15, and 20 using a Nikon TMS-F phase contrast microscope (Nikon Instruments, Melville, NY, USA), equipped with an Olympus ALTRA 20 digital camera and the AnalySIS GetIT software (Olympus Soft Imaging Solutions, Münster, Germany). Two independent examiners performed the morphological classification blind based on pre-determined criteria described below. Three samples from at least two separate experiments were analyzed based on the following three features; growth inhibition, neurite length, and neurite branching. Growth inhibition was determined based on the number of cells on day 0 compared to the number of cells on day 10. Treatments resulting in unlimited growth, represented by cells covering almost all of the available space were given +. Treatments resulting in complete growth inhibition, with fewer cells than at day 0 were given +++, and treatments resulting in intermediate growth inhibition were given ++. The criteria for neurite length were as follows; treatments resulting in a majority of cells with short, blunt neurites were given +. Treatments resulting in a majority of cells having neurites stretching longer than half a picture frame were given +++, and treatments resulting in cells with intermediate neurite length were given ++. The criteria regarding neurite branching were ranked separately, where treatments resulting in a majority of cells having no branched neurites were given +. The treatments resulting in a majority of cells having neurites with branches, but short extensions after branching were given ++, and treatments resulting in a majority of the cells having neurites with multiple branches and extensive growth after the point of branching were given +++.

### *Live cell imaging*

Cells were seeded in glass bottom culture dishes (MatTek, Ashland, MA, USA) and treated as described above. For plasma membrane staining, cells were incubated with 2  $\mu$ M lipophilic dye FM 1-43 (Invitrogen, Paisley, UK) in medium for 5 min and visualized using a Zeiss Axiovert 200 inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany). For time-lapse imaging, neurites were visualized using a Zeiss axiovert 130 M microscope (Carl Zeiss) and images were captured once every minute.

### *Immunocytochemistry*

Cells were seeded in 4-well sonic seal slides (Nunc, Rochester, NY, USA) and treated as described above. The staining was performed directly in the gel as described previously [23], with slight modifications. Briefly, the gel was washed in ice cold PBS and fixated with 4% paraformaldehyde (PFA, Histolab, Gothenburg, Sweden) for 10 min. Subsequently, the gel was washed with PBS glycine (100 mM) and then incubated in blocking medium (10% goat serum (Gibco), 1% IgG Goat F(ab')<sub>2</sub> anti-mouse (Pierce Biotechnology, Rockford, IL, USA)) in immunofluorescence (IF) buffer (0.2% Triton X-100, 0.1% BSA, 0.05% Tween 20 (all from Sigma Aldrich) in PBS) for 1.5 h at room temperature. The samples were incubated with the primary antibody, diluted in IF buffer or PBS, overnight at 4 °C. The antibodies used were mouse anti NeuN 1:50 (Millipore, Billerica, MA, USA), mouse anti Sv2 1:500 (DSHB, University of Iowa, Iowa City, IA, USA), mouse anti tau clone T46 1:100 (Invitrogen), and rabbit anti neuron specific  $\beta$ -III tubulin 1:2000 (Abcam, Cambridge, UK). After washing with IF buffer, the gel was incubated with secondary Alexa Fluor-488 and -594 conjugated goat anti mouse and goat anti rabbit antibodies (Invitrogen), diluted 1:400 in IF buffer, for 1 h at room temperature. The nuclei were stained using 1.5  $\mu$ M ToPro3 (Invitrogen) for 15 min, and after washing in PBS, the samples were mounted using Vectashield mounting medium

(Vector laboratories, Burlingame, CA, USA). Cells were examined using a Nikon eclipse E600 laser scanning confocal microscope with three lasers at 488, 594, and 633 nm, together with the EZC1 3.7 software (Nikon instruments).

### *Quantification*

The intensity of NeuN immuno staining was quantified using ImageJ software (NIH). For each treatment, ten images (five each from two separate experiments) were analyzed. The nuclei area was selected (from images capturing ToPro3 stained nuclei), and the intensity within each nuclei area was analyzed. The mean value of the nuclei areas from each image was recorded, and thereafter the overall mean was calculated (n=10).

### *Reverse transcription PCR*

The gel was disrupted by the addition of dispase (2.2 U/ml in PBS; Gibco) for 2 h at 37 °C. Subsequently, the cells were washed twice in PBS, lysed with 350 µl buffer RLT (Qiagen, Germantown, MD, USA), vortexed for 1 min, and stored at -70 °C until mRNA extraction. The mRNA was extracted using the RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. The RNA concentration was determined using a nanodrop ND 1000 spectrophotometer. Approximately 200 ng of RNA were used for cDNA synthesis using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA from human adult brain (BioChain, Hayward, CA, USA) was used as a positive control. For PCR analysis, 2 µl of cDNA was added to the reaction mix containing 25 µl HotStarTaq mastermix (Qiagen), 19 µl DEPC treated water, and 2 µl of each primer (forward and reverse). One pair of primers for tau exons 2 and 3 (gaagatcacgctgggacgta (forward) and gtgtctcaatgcctgcttc (reverse)) and one pair of primers for tau exon 10 (gtccgtactccaccaagtc (forward) and atgagccacacttggaggtc

(reverse) were designed using Primer3 [24]. Amplification was carried out with the following protocol: 15 min at 95 °C for denaturation, followed by 30 s at 94 °C, 30 s at 58.5 °C, and 1 min at 72 °C for 35 cycles, finishing with 10 min at 72 °C. In addition, exon 10 was amplified using primer sequences and cycle protocols described by Uberti et al. [8]. The PCR products were visualized on 2% Agarose E-gel (Invitrogen) using the GelDoc XR software (BioRad Laboratories, Hercules, CA, USA).

#### *Real time quantitative PCR*

RNA was extracted, and cDNA was amplified as described above. Real time PCR assays were performed in MicroAmp Fast Optical 96-Well Reaction Plates using the 7500 fast real time PCR system (Applied Biosystems). PCR analyses were carried out using primers and FAM-labeled probes specific to exon 2 only (Hs00902978\_m1), exon 2 and 3 (Hs00902314\_m1), exon 10 (Hs00902312\_m1), total tau (Hs00213491\_m1), and cyclophilin (Hs99999904\_m1), all designed by Applied Biosystems. Amplification was carried out with the following protocol: 2 min at 50 °C, 10 min at 95 °C, and 15 s at 95 °C for 40 cycles, finishing with 1 min at 60 °C. The expression levels of the exons of interest were calculated using the  $\Delta\Delta C_t$ -method. Sample  $C_t$ -values were normalized to  $C_t$  values of the housekeeping gene cyclophilin A, chosen to be the most stable endogenous control under the present experimental conditions (Human endogenous control array, Applied Biosystems). The results were further normalized using untreated cells grown in culture flasks as a reference sample, and the relative amplification thus calculated for each exon.

#### *De-phosphorylation*

The gel was digested as described above, and cells were harvested and washed in PBS. Cells were lysed in 50 mM Tris-HCl supplemented with protease inhibitor cocktail (Sigma Aldrich)

by repeated freeze thaw cycles. This method of cell-lysis was found to yield the most consistent results on de-phosphorylated proteins when compared to other methods for cell-lysis. The protein concentration was determined using a DC protein assay (BioRad laboratories, Hercules, CA, USA), and the protein samples were thereafter de-phosphorylated using lambda phosphatase (10 U/  $\mu\text{g}$  total protein; New England Bioscience, Ipswich, MA, USA) at 30 °C for 30 min. The samples were thereafter prepared for western blotting as described below.

### *Western blotting*

Samples not subjected to de-phosphorylation prior western blot analysis were harvested as described above and lysed in Radio immune precipitation assay (RIPA) buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate (SDS)) supplemented with protease inhibitor cocktail, centrifuged to remove cell debris, and protein concentrations was determined. This method of cell-lysis was found to yield slightly more protein, than the freeze thaw method used for the de-phosphorylated proteins. Protein from human brain (Novus biologicals, Littleton, CO, USA) was used as positive control. Samples were corrected to 15  $\mu\text{g}$  total protein, 5x Western blot loading buffer (50 mM Tris-HCl pH 6.8, 32% Glycerol, 3.2% SDS, 0.16% bromphenol blue, 250  $\mu\text{M}$  DTT) was added, and the samples were denatured at 95 °C before application to 10 or 12% SDS gel (CBS scientific company, Solana Beach, CA, USA). When examining de-phosphorylated samples, 15  $\mu\text{g}$  total protein was used for negative control and human brain samples, and 25  $\mu\text{g}$  total protein was loaded for all other samples. Also, one  $\mu\text{l}$  of recombinant tau protein mix (rPeptide, Bogart, GA, USA), consisting of all six isoforms, was used as reference. Proteins were blotted onto a 0.45  $\mu\text{M}$  nitrocellulose membrane (BioRad laboratories), blocked in 5% BSA in TBS-Tween 20, and incubated with anti tau primary

antibody, clone Tau5 (1:2000; Covance research products, Princeton, NJ, USA) diluted in blocking buffer. After washing, membranes were incubated with HRP conjugated goat anti mouse antibody (1:3000; DAKO, Glostrup, Denmark) in 5% BSA, and after washing, protein bands were visualized using Western Blot Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Amersham Hyperfilm ECL (GE healthcare, Chalfont St. Giles, UK). Membranes analyzed for total tau were incubated in glycine stripping buffer (0.2 M glycine, 0.1% SDS, 1% Tween-20, pH 2.2) at 55 °C for 1 hour, and re-probed with anti GAPDH antibody (GeneTex, Irvine, CA, USA). Blots were quantified using the ImageJ software (NIH) and the ratio between tau and GAPDH was calculated. Mean values +/- SD from three experiments were analyzed.

### *Statistics*

The SPSS 16.0 package (SPSS Inc, Chicaco, IL, USA) was used for all statistical calculations. Immunohistochemical- and western blot data was analyzed by students t-test. GraphPad Prism 5 (GraphPad software Inc. La Jolla, CA, USA) was used for graphical presentation.

### **Results**

*Neuronal morphology is achieved by culturing cells in a 3D ECM gel with the addition of BDNF, NRG, NGF, and VitD<sub>3</sub>.*

Cells, cultured in an ECM gel for 10 days, displayed varying levels of growth inhibition, neurite length, and neurite branching, depending on the stimulatory factors added. The cellular morphology obtained with each treatment was determined based on those three features, and was given a grade between one and three, where three corresponds to features found in mature neurons. The levels of differentiation for all treatment combinations are

presented in table 1. Extended treatment in ECM gel for up to 21 days was possible, but did not further improve cellular morphology, thus 10 days of stimulation was chosen as the time point for further characterization, leaving a sufficient remaining time window for experimental procedures.

Prior to seeding cells in the ECM gel, all experiments (except where otherwise stated), were started with RA pre-treatment, which is known to result in some differentiation [25], and is required for a response to BDNF [26]. As described previously, BDNF had an additive effect to RA treatment when culturing in dishes [27] (Fig. 1A-B). This additive effect was also seen during gel culturing (Fig. 1C-D). Cells cultured in an ECM gel displayed improved neuron-like morphology compared to cells grown in traditional culture dishes, when otherwise given the same treatment (Fig. 1A-B compared to C- D). Furthermore, growth factor-treated cells cultured in the ECM gel displayed better survival for longer times than cells cultured on glass or plastic surfaces. This difference became apparent already after 5 days of culturing, when traditionally cultured, BDNF treated cells started to shrink and detach (Fig. 1B). Cells cultured in the ECM gel, treated with RA displayed intermediate growth inhibition and development of short neurites with few branches (Fig. 1C). A combination of RA with NRG, NGF, and VitD<sub>3</sub> did not generate more developed neurites (Fig. 1E). Cells that were pre-treated with RA, cultured in ECM gel, and stimulated with BDNF alone exhibited intermediate developed morphology in all terms as well, but the cells differed in morphology compared to RA treated cells by having thinner neurites and smaller cell bodies (Fig. 1D). However, contrary to RA treatment, an improvement in morphological appearance was seen when BDNF treatment was combined with NGR and NGF or VitD<sub>3</sub>, respectively, and an even more pronounced effect was seen when all four factors were added to the gel-cultured cells (Fig. 1E). Accompanying outgrowth of neurites and inhibition of cell division, the cell bodies

of differentiated cells also became smaller compared to undifferentiated cells. This was especially apparent in BDNF treated cells cultured within the ECM gel (Fig. 1D, F) This combination resulted in cells having a high resemblance to neurons as determined by morphological appearance, with elongated and well-branched neurites, as well as extensive growth inhibition. Treatment with BDNF and cholesterol resulted in cells with an equally developed neuronal morphology, but also caused unwanted deposits in the gel (not shown). This phenomenon was, for unknown reasons, apparent in treatments where cholesterol was combined with BDNF, and this combination was therefore discarded. Thus, treatment with BDNF in combination with NRG, NGF and VitD<sub>3</sub> was chosen as the best treatment to generate neuronally differentiated cells from a morphological standpoint.

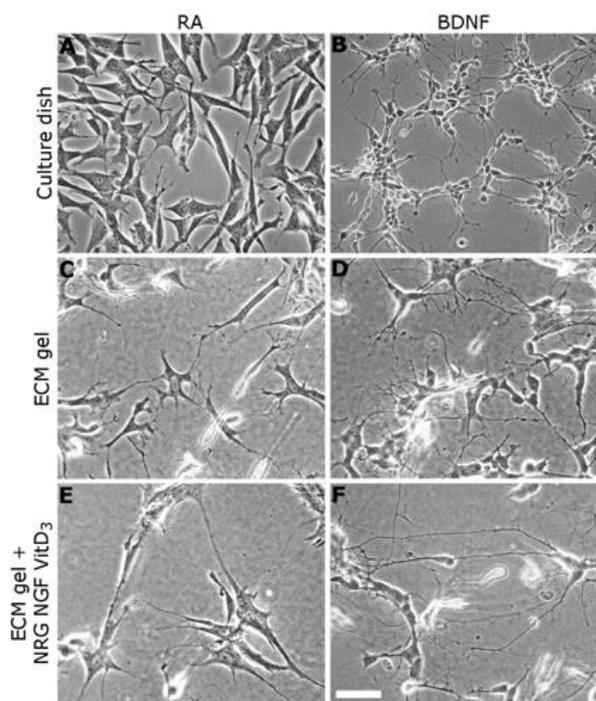


Figure 1  
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**Figure 1.** Cellular morphology of SH-SY5Y cells after one week pre-treatment with retinoic acid (RA; 10  $\mu$ M) followed by differentiation with RA or brain derived neurotrophic factor (BDNF; 50 ng/ml), in an extra cellular matrix (ECM) gel combined with neuregulin  $\beta$ 1 (NRG; 10 ng/ml), nerve growth factor (NGF 10 ng/ml), and vitamin D<sub>3</sub> (VitD<sub>3</sub> 24 nM) as specified. Cells cultured in dishes, treated with RA for 17 days (A) or treated with RA for 7 days followed by BDNF for 5 days (B). Cells cultured in an ECM gel in combination with RA treatment alone (C) or in combination with NRG, NGF and VitD<sub>3</sub> (E) for ten days. Cells cultured in an ECM gel in combination with BDNF treatment alone (D) or in combination with NRG, NGF and VitD<sub>3</sub> (F) for 10 days. Representative images out of at least three separate experiments are shown. Scale bar, 50  $\mu$ m.

*Cells with neuron-like morphology express synapse-specific proteins and display synaptic structures.*

To further investigate the appearance of neuronal features after differentiation, we immunolabeled ECM gel cultured cells with antibodies against neuron specific  $\beta$ -III tubulin and synapse protein Sv2. To be able to compare traditional RA treatment with our treatment regime, we seeded culture flask grown cells, treated with RA for 7 days, in the ECM gel and performed immunohistochemistry after 24 h in the gel. By doing so, all samples could be stained using the same protocol, but with minimal effect of the gel itself on the cells.

Traditional RA treatment resulted in an overall weak  $\beta$ -III tubulin staining, and very few  $\beta$ -III tubulin positive neurites. Furthermore, Sv2 staining was absent or weak, and localized around the nuclei (Fig. 2A, panels I-III). Treatment with RA within the ECM gel did improve  $\beta$ -III tubulin staining somewhat. Sv2 immunoreactivity was slightly stronger, but the localization remained primarily in the cell soma (Fig. 2A, panels IV-VI, arrow and Fig. 2B, panel I).

Addition of NRG, NGF and VitD<sub>3</sub> results in increased  $\beta$ -III tubulin staining, as well as more intense Sv2 immunoreactivity. The localization of Sv2 was still mainly somal (Fig. 2A, panels VII-IX, arrow), although some neuritic staining was detected (arrowhead, and Fig. 2B, panel II). BDNF treatment of ECM gel cultured cells resulted in strong  $\beta$ -III tubulin staining, and outgrowth of long neurites. Sv2 was still localized around nuclei, but was also enriched at sites of neurite contacts (Fig 2A, panel X-XII, arrowhead and Fig. 2B, panel III), indicating synaptic structures. Further addition of NRG, NGF and VitD<sub>3</sub> resulted in an elegant web of  $\beta$ -III tubulin positive neurites. This treatment greatly increased the immunoreactivity for Sv2 compared to BDNF alone, and strong staining was especially apparent in neurites and sites of contact between cells (Fig. 2A, panels XIII-XV, arrowheads and Fig. 2B, panel IV).

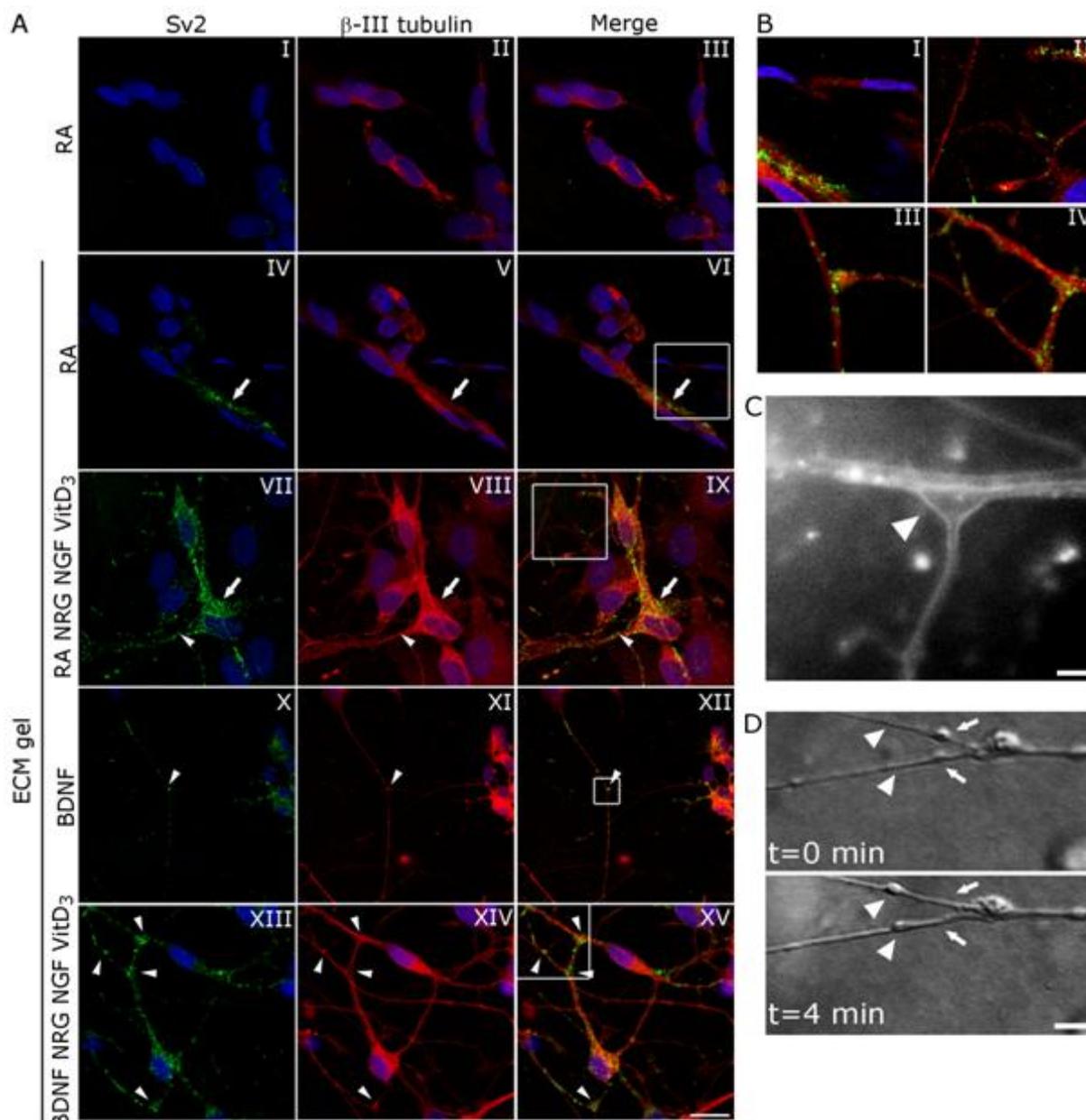


Figure 2  
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**Figure 2.** Expression of synapse specific protein Sv2, as well as presence of synaptic structures and functional vesicle transport in differentiated extra cellular matrix (ECM) gel cultured SH-SY5Y cells. **A**, SH-SY5Y cells were treated with retinoic acid (RA; 10  $\mu$ M) for 10 days and thereafter subjected or immunocytochemistry shortly after transferred to ECM gel, or cultured in an ECM gel, treated with RA (10  $\mu$ M) or brain derived neurotrophic factor (BDNF; 50 ng/ml) alone, or in combination with neuregulin  $\beta$ 1 (NRG; 10 ng/ml), nerve growth factor (NGF 10 ng/ml), and vitamin D<sub>3</sub> (VitD<sub>3</sub> 24 nM) for 10 days and thereafter subjected for immunocytochemistry, using antibodies against neuron specific  $\beta$ -III tubulin (red), and synapse protein Sv2 (green). Nuclei were stained with ToPro3 (blue). Diffuse  $\beta$ -III tubulin staining of cells with few neurites and very weak Sv2 staining, mainly localized around the nuclei in cells treated with RA alone (panels I-III, arrow). Sv2 immunoreactivity is somewhat increased, but remains somal in ECM gel cultured cells treated with RA for

additional 10 days (panels IV-VI, arrow). Increased  $\beta$ -III tubulin and Sv2 immunoreactivity is detected when NRG, NGF and VitD<sub>3</sub> are added to RA treatment (panels VII-IX). Staining remains mainly somal (arrow), but some neuritic Sv2 was detected (arrowhead). Long  $\beta$ -III tubulin positive neurites, with synapse-like structures is seen in ECM gel cultured cells treated with BDNF, (panels X-XII). Sv2 staining is enriched in neurites and synapse-like contacts (arrowhead and enlargement in B, panel III). Further increased Sv2 immunoreactivity and increased numbers of  $\beta$ -III tubulin positive neurites is detected with addition of NRG, NGF and VitD<sub>3</sub> to BDNF (panels XIII-XV) together with accumulation of Sv2 in contact areas between cells (arrowheads and enlargement in B panel IV). Bar = 20  $\mu$ m in panels I-IX and XIII-XV, 40  $\mu$ m in panels X-XII **B**, Magnified images from **A**, highlighting the difference in Sv2 localization in RA treated cells alone (panel I) or in combination with NRG, NGF and VitD<sub>3</sub> (panel II) compared to BDNF treated cells alone (panel III) or in combination with NRG, NGF and VitD<sub>3</sub> (panel IV). **C**, The presence of synapse-like structures were further investigated in differentiated ECM gel cultured cells, stained with the lipophilic dye FM 1-43, and visualized by fluorescence microscopy. Arrowhead indicates synaptic like contacts between cells. Scale bar, 2.5  $\mu$ m. **D**, Functional vesicle transportation along neurites from differentiated ECM cultured cells were analyzed by time-laps imaging of living cells. Pictures were taken once every minute and movement of vesicles is obvious when comparing images taken at time 0 (arrows) and time 4 min (arrowheads). Scale bar, 5  $\mu$ m.

To further characterize these synapse-like structures, well-differentiated ECM gel cultured cells, displaying neuritic contacts, were live-stained within the gel with the lipophilic dye FM1-43 and visualized with a high-magnification fluorescence microscope. This revealed distinctive synapse-like structures at the site of neurite junctions (Fig. 2C, arrowhead).

Furthermore, light-microscopy live cell imaging of differentiated cells clearly exposed the presence of vesicles within neurites. Time lapse imaging of living cells revealed that these vesicles were transported along neurites (Fig. 2D). This indicates that BDNF treatment in combination with NRG, NGF and VitD<sub>3</sub> not only results in cells with neuronal morphology, but also generates cells having a well developed neuritic network, with synaptic structures enriched in synaptic proteins, as well as having normal neuronal functions.

*Differentiation of SH-SY5Y cells is accompanied by increased expression of neuron-specific protein NeuN.*

To further characterize SH-SY5Y cells cultured under our conditions, we performed immunocytochemical staining for the neurospecific nuclear marker NeuN. As expected, traditionally RA treated cells (prepared as described above) were negative for NeuN (Fig. 3A, upper left panel). A weak positive NeuN staining was seen in some ECM gel cultured cells treated with RA, alone or in combination with NRG, NGF, and VitD<sub>3</sub>, (Fig. 3A upper middle

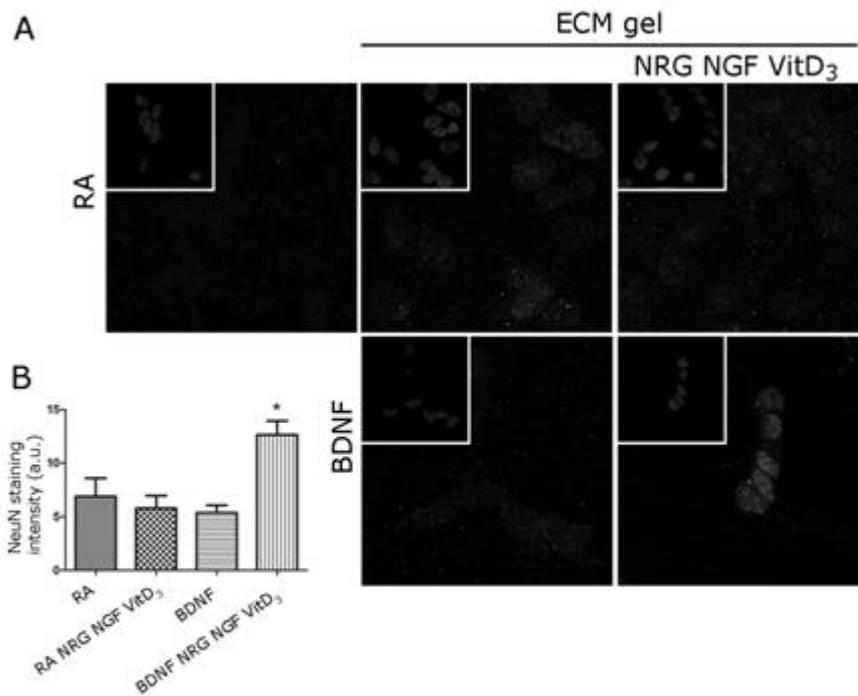


Figure 3  
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**Figure 3.** NeuN immunocytochemistry of SH-SY5Y cells treated with retinoic acid (RA; 10  $\mu$ M) for 10 days and thereafter shortly transferred to extra cellular matrix (ECM) gel, or ECM gel cultured cells differentiated with RA (10  $\mu$ M) or brain derived neurotrophic factor (BDNF; 50 ng/ml) alone or in combination with neuregulin  $\beta$ 1 (NRG 10 ng/ml), nerve growth factor (NGF 10 ng/ml) and vitamin D<sub>3</sub> (VitD<sub>3</sub>; 24 nM) for 10 days. Nuclei were stained with ToPro3 (blue). **A**, No NeuN immunoreactivity was detected in cells treated with RA, cultured outside the gel (upper left panel). ECM gel cultured cells treated with RA alone, or in combination with NRG, NGF and VitD<sub>3</sub> resulted in weak NeuN immunoreactivity (upper middle and right panels), and the same pattern was seen in ECM gel cultured cells treated with BDNF alone (lower left panel). More intense immunoreactivity was detected when BDNF treatment was combined with NRG, NGF and VitD<sub>3</sub> (lower right panel). Representative images from one out of at least two separate experiments are shown. **B**, Quantification of nuclear NeuN immunoreactivity. A significant increase in NeuN intensity was only observed in cells treated with BDNF, NRG, NGF and VitD<sub>3</sub> compared to all other treatments investigated (\* $p$ <0.05). For each treatment, mean intensity of five images each from two separate experiments were analyzed, and overall mean was calculated. Bars represent mean  $\pm$  SEM.

and right panels) as well as BDNF alone (Fig. 3A lower left panel), with other cells being negative. In contrast, BDNF treatment in combination with NRG, NGF, and VitD<sub>3</sub> resulted in

strong NeuN staining in many cells (Fig. 3A, lower right panel). To quantify these results, we measured the NeuN intensity in nuclear areas using the ImageJ software. The mean intensity from ten images (five each from two separate experiments) was calculated. NeuN immunostaining intensity was significantly higher in cells treated with BDNF combined with NRG, NGF and VitD<sub>3</sub> (mean 12.66 +/- 1.30 SEM) compared to cells treated with RA alone (mean 6.89 +/- 1.69 SEM, p=0.015) or in combination with NRG, NGF, and VitD<sub>3</sub> (mean 5.80 +/- 1.17 SEM p<0.001) as well as BDNF treated cells (mean 5.38 +/- 0.69 SEM, p<0.001). These results show that BDNF in combination with NRG, NGF and VitD<sub>3</sub> is the best treatment also in respect to the expression of markers specific for mature, post-mitotic neurons in addition to neuronal morphology and function.

*Differentiation of SH-SY5Y cells in ECM gel changes the expression of tau isoforms.*

Expression of all six mature tau isoforms is not only a marker for adult neurons, but is also desirable in a cellular model when studying microtubule function in general, and in AD related research. Human CNS splicing results in three N-terminal variations, 0N, 1N, and 2N, depending on the inclusion of exons 2 and 3, and two C-terminal variations, 3R and 4R, relating to exon 10. Since exon 3 is never present without exon 2 [28], inclusion of exons 2, 3, and 10 mRNA can be analyzed with only two sets of primers. With reverse transcriptase PCR analysis, we detected mRNA expression of exon 2 alone (1N) in cells from all culture conditions in the ECM gel (Fig. 4A, lanes 2-5), as well as untreated cells cultured in dishes (Fig. 4A lane 1). The expression of exons 2 and 3 (2N) was only detected in the ECM gel-cultured cells treated with RA alone, or in combination with other factors (Fig. 4A, lanes 2-3), and not with BDNF treatment. Furthermore, we were able to detect mRNA for exon 10 (4R) from cells exposed to all different culture conditions, including undifferentiated cells cultured in dishes (Fig. 4A, lanes 1-5). As a positive control, mRNA from human brain was used, and

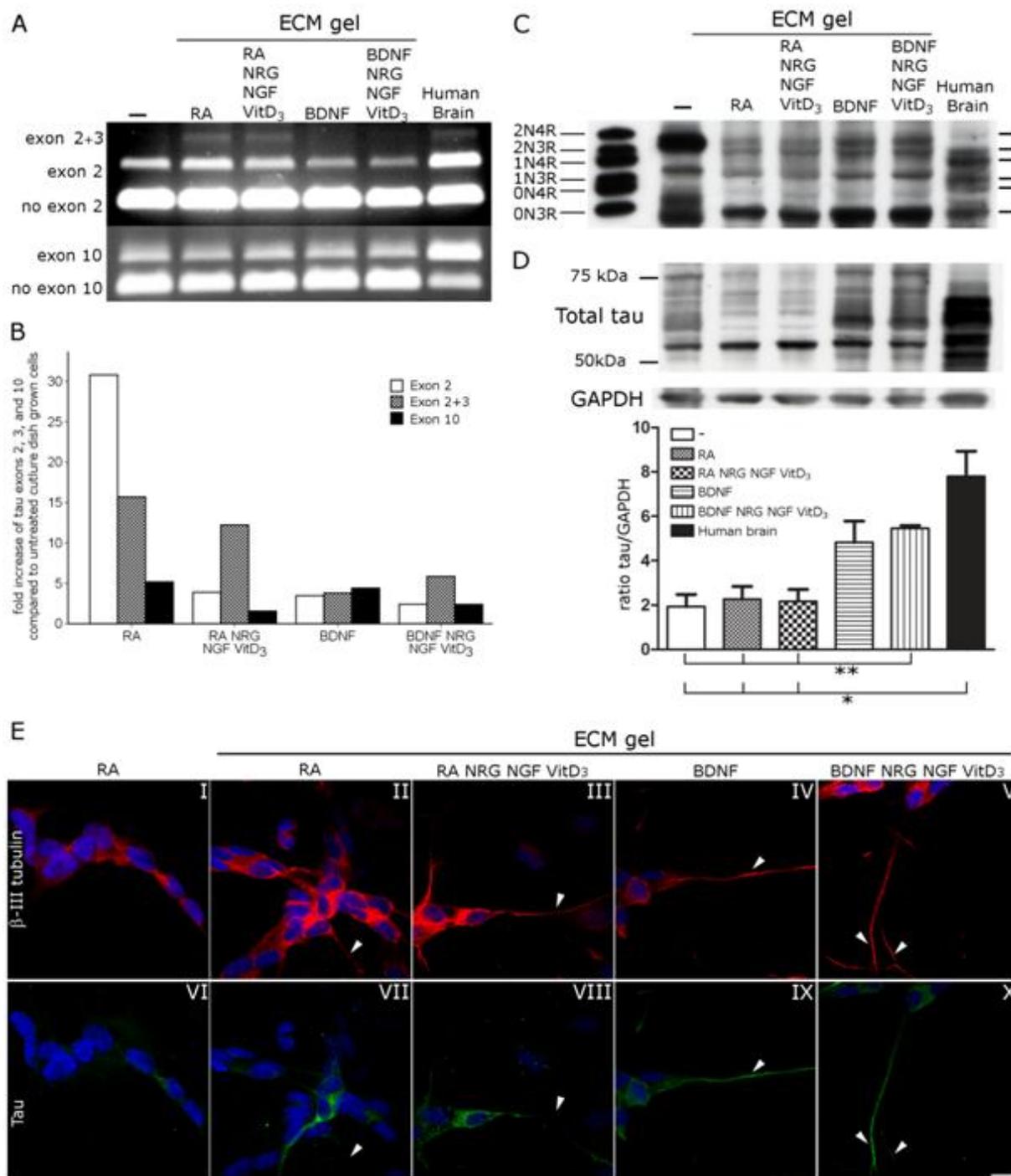


Figure 4  
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**Figure 4.** Differentiation of SH-SY5Y cells cultured in an ECM gel results in changes of tau isoform expression and localization. **A**, mRNA expression of tau exons 2, 2 and 3, and 10 in untreated cells grown in culture dishes (-) or ECM gel cultured cells differentiated with retinoic acid (RA; 10  $\mu$ M) or brain derived neurotrophic factor (BDNF; 50 ng/ml), alone or in combination with Neuregulin  $\beta$ 1(NRG; 10 ng/ml), nerve growth factor (NGF; 10 ng/ml), and vitamin D<sub>3</sub> (VitD<sub>3</sub>; 24 nM) was investigated using rPCR. As a positive control, mRNA from human brain was used. Representative rPCR results from at least two sets of experiments are shown. **B**, Quantitative rPCR analysis of tau exons 2, 3, and 10 of ECM gel cultured cells. Relative increase in mRNA was calculated using the  $\Delta\Delta C_t$  method, where untreated cells grown in culture dishes were used as reference sample. Means

from two separate experiments is shown. **C**, Protein expression of tau isoforms were investigated using western blot after de-phosphorylation. Recombinant human tau (left lane) was used as a reference, and protein from human brain as positive control. 15  $\mu\text{g}$  total protein from culture flask cells and human brain was loaded, 25  $\mu\text{g}$  total protein from all other samples. **D**, Expression of total tau in was investigated using western blot. ECM gel cultured cells treated with BDNF in combination with NRG, NGF, and VitD<sub>3</sub> resulted a significant increase in tau expression ( $p < 0.01$ ) compared to untreated SH-SY5Y cells (-), and ECM gel cultured cells treated with RA, alone or in combination with NRG, NGF and VitD<sub>3</sub>. Tau expression of human brain was significantly higher than untreated and the different RA treated cells ( $p < 0.05$ ), while no significant difference was detected compared to ECM gel treated cells differentiated with BDNF alone or in combination with NRG, NGF, and VitD<sub>3</sub>. One representative blot out of three is shown; bars represent mean values + SD. **E**, Cellular localization of tau was investigated by immunocytochemical staining of cells cultured as described before, using antibodies against neuron specific  $\beta$ -III tubulin (red) and tau (green). Nuclei were stained with ToPro3 (blue). Weak tau immunoreactivity was detected in traditionally cultured, RA treated SH-SY5Y cells (panels I, VI). Increased tau immunoreactivity was observed in ECM gel cultured cells, treated with RA alone, or in combination with NRG, NGF and VitD<sub>3</sub>, localized mainly around the nucleus. Furthermore, several  $\beta$ -III tubulin positive neurites were negative for tau (panels II-III, VII-VIII, arrowheads). Strong positive tau staining co-localized with  $\beta$ -III tubulin in BDNF treated cells (panels IV, IX, arrowhead), and when BDNF treatment was combined with NRG, NGF and VitD<sub>3</sub> (panels V, X arrowhead). Representative images from one out of at least two experiments are shown. Bar = 20  $\mu\text{m}$ .

as expected, inclusion of exon 2, exons 2 and 3, as well as exon 10 was detected (Fig. 4A, lane 6). Expression of exon 10 was also increased in the control sample compared to SH-SY5Y cells (Fig. 4A, lane 6). Exon 10 has previously been reported to be absent in cultured SH-SY5Y cells [8], and we repeated our experiments using their primers and cycling conditions. The outcome of this experiment was the same as described above (data not shown), and we conclude that undifferentiated SH-SY5Y cells express mRNA of tau exons 2 and 10. Detectable expression of exon 2 and 3 however, requires the ECM gel culture and differentiation with RA.

PCR products from each visualized band on the gel were extracted and sequenced (Cybergene AB Stockholm, Sweden). This confirmed that the cDNA sequence of the bands correlated to the mRNA products our primers were designed for, and that the weak bands corresponding to the inclusion of exon 3 indeed contained this product.

Since expression of exon 2 (1N) and exon 10 (4R) could be detected in all culture conditions, we decided to further quantify the mRNA expression of tau exons 2, 3, and 10 using

quantitative real-time PCR. The results showed a surprisingly high increase in the expression of all three exons in ECM gel cultured cells, with no additional differentiation factors (data not shown). ECM gel culturing combined with RA treatment alone gave the highest increase (over 30 times) of exon 2 expression (Fig. 4B), and addition of NRG, NFG, and VitD<sub>3</sub> as well as all BDNF treatments, yielded a much lower increase in exon 2 expression. In general, the expression of exon 2 and 3 (2N) was increased to a higher extent in cells treated with RA in combination with other factors, compared to the corresponding BDNF-treated cells (Fig. 4B), thus further corroborating the rtPCR results. Expression of exon 10 was increased about 5-fold in cells treated with RA or BDNF alone, and was not further increased when NRG, NGF, and VitD<sub>3</sub> was added (Fig. 4B). In conclusion, the culture of SH-SY5Y cells in an ECM gel increases the expression of all three exons included in mature tau, compared to traditionally cultured cells. However, we could not find an obvious correlation between the mRNA levels of the investigated exons and the morphological and biochemical maturity described above.

To confirm that the presence of the respective mRNA splicing variant also result in the expression of the protein isoforms, we analyzed tau protein expression using western blot. Since phosphorylation of tau changes the travelling pattern on SDS gel [29], we de-phosphorylated the protein samples before SDS-PAGE. Protein from human brain was used as positive control, and recombinant tau consisting of all six isoforms as reference. Since earlier studies have reported that undifferentiated SH-SY5Y cells only express fetal tau [7, 30], this treatment was also included. By western blotting, six bands are detected in de-phosphorylated protein samples from human brain (see bars, Fig. 4C, lane 7), corresponding to the six bands visualized from recombinant protein (Fig. 4C, lane 1). This indicates that tau is adequately de-phosphorylated and travels as expected within the gel. Cells cultured in traditional culture dishes exhibits several bands, but these do not correspond to recombinant

tau isoforms regarding molecular weight (Fig. 4C lane 2). All ECM gel cultured cells investigated, independent of treatment, displayed bands corresponding to all six tau isoforms, (Fig 4C lanes 3-6) although the expression pattern differed between treatments, and from human brain.

*Differentiation of SH-SY5Y cells increases tau expression and induce a neuritic distribution.*

Since the expression levels and cellular localization of tau are different in neurons of the human adult brain compared to cultured cells [10], the correct amount and cellular distribution is an important criteria for mature neurons. Thus, we further quantified tau protein using western blot without de-phosphorylation. This method was chosen since it resulted in a more complete extraction of tau. As before, protein from human brain was used as a positive control. Our results show that ECM gel culturing and differentiation of cells with BDNF, NRG, NGF and VitD<sub>3</sub> significantly increases the amount of tau expressed (5.46 +/- 0.23) compared to traditionally cultured cells (1.93 +/- 0.55) and ECM gel cultured cells treated with RA alone (2.27 +/- 0.98) or in combination with NRG, NGF and VitD<sub>3</sub> (2.17 +/- 0.92), p<0.01. Tau expression was somewhat higher in human brain (7.80 +/- 1.95) compared to ECM gel cultured cells treated with BDNF, NRG, NGF and VitD<sub>3</sub>, although no statistical significance was found (p=0.11) (Fig. 4D).

Furthermore, we investigated the intra-cellular localization of tau protein in different culturing conditions. Traditionally cultured cells treated with RA for 7 days displays poor tau staining, mainly localized around the nucleus (Fig. 4E, Panels I, VI). This pattern of staining mainly in the cell soma is also seen in ECM gel cultured cells treated with RA alone, or in combination with NRG, NGF and VitD<sub>3</sub> (Panels II-III, VII-VIII arrowheads), but his treatment seem to increases overall tau immunoreactivity compared to traditionally cultured cells. BDNF

treatment alone within the ECM gel is sufficient to increase tau immunoreactivity and increase the co-localization with  $\beta$ -III tubulin positive neurites (Fig. 4E panels IV, IX, arrowhead). Similar results is seen when NRG, NGF and VitD<sub>3</sub> is added, where tau immunoreactivity is localized to neurites (Fig. 4E panels V, X, arrowhead) in concert with tau's microtubule-binding function.

## Discussion

In this study we present a human neuronal *in vitro* model, differentiating SH-SY5Y neuroblastoma cells into sustainable neuronal morphology by combining 3D ECM gel culturing with several differentiating factors. These well-differentiated cells express features specific to mature neurons, such as synaptic structures and functional axonal vesicle transport. The most well-differentiated cells also express neurospecific proteins including nuclear protein NeuN, neuron specific  $\beta$ -III tubulin and synaptic protein Sv2. In addition, tau is expressed at levels comparable to adult human brain, and includes mature isoforms, altogether making this new concept for *in vitro* differentiation valuable for many neuroscientific research areas, including AD.

There is a great need for *in vitro* models having a high resemblance to mature human neurons. The most appealing solution is the use of human neuronal crest stem cells, but their use is however accompanied with difficulties. Apart from the apparent ethical issues, neuronal crest cells crave specific culturing conditions and displays high heterogeneity in culture [31]. Hence, immortalized cell-lines will continue to be an important scientific tool for the foreseeable future. There is increasing knowledge of the importance to use model systems with high resemblance to the real *in vivo* situation, especially when studying the nervous system. Accordingly, several attempts have been made to transform e.g. neuroblastoma cells,

into more neuron-like cells [12, 18, 19, 27, 32]. We show that 3D culture improved neuronal morphology compared to traditionally cultured cells with the same stimuli. The ECM gel culture alone however, was not enough to obtain extensive neuronal differentiation. Very limited data exists on the synergistic effects of combining multiple factors, however BDNF treatment is known to be dependent on the tropomyosin receptor kinase B, which is up regulated by RA [26]. Also, BDNF and NGF are both known to act on specific tropomyosin receptor kinases [33], whereas neuregulins are known to activate erbB3 and erbB4 of the epidermal growth factor receptor family [34]. All these receptors activate intracellular kinase signaling cascades making synergistic effects on downstream effectors a possible explanation of our results. This line of reasoning is corroborated by the induced expression of neuro-specific proteins after our differentiation, being of great importance in a relevant *in vitro* model. In an earlier report [32], SH-SY5Y cells were cultured on poly-D-lysine-coated coverslips treated with RA followed by mitotic inhibitors. This resulted in an up-regulated expression, and changes in cellular location, of several neurospecific proteins, a strong reduction in division rate, and somewhat better morphological features compared to other studies. Thus, although there is evidence for the possibility to differentiate neuroblastoma cells, none of these studies present as extensive neuronal morphology, with very long, functionally active neurites and synaptic structures, as shown in the present study.

From a morphological standpoint, the use of the ECM gel and BDNF seemed to be the most crucial factors. However, we show a clear additive effect for the different factors, and the best morphological results were obtained when NGF, NRG, and VitD<sub>3</sub> were added, supporting earlier reports of these factors' neuro differentiating properties. We show that neuron specific  $\beta$ -III tubulin was present in low amounts in undifferentiated cells, but with increased neuronal morphology, the expression became stronger and the location became more neuritic, in

concert with its reported expression in differentiating neuroblasts [1]. The nuclear marker NeuN is reported to only be expressed in mature, post mitotic neurons [3]. The presence of NeuN has only been shown during *in vitro* conditions once before [32]. We now show that culturing cells in an ECM gel is sufficient to result in some NeuN expression, but further differentiation with BDNF, NRG, NGF and VitD<sub>3</sub>, significantly increases the expression compared to other treatments.

One of the most characteristic features of neurons is their long processes with an extensive transport system and the interaction with other neurons through synapses. Lately, numerous studies in the Alzheimer's field focus on microtubule transport (reviewed in [35]) as well as synaptic targeting and deficits [36, 37]. Our model offers the possibility to study these events, as we show transportation of vesicles along the long neurites using real-time imaging of live, morphologically-differentiated cells within the ECM gel. In addition, these well differentiated cells showed obvious synapse-like structures, and intense staining of synaptic vesicle protein Sv2, important for both synaptic structure and neurotransmitter release [2], were present at sites of neuritic contacts. These data provide evidence for the appearance of synapses in differentiated cells although the definite proof of functional synapses would be electrophysiological experiments. Unfortunately, due to the nature of the ECM gel, patch-clamping experiments were technically difficult to perform. Further studies on the functionality of these would be interesting.

The microtubule binding protein tau is thought to be one of the key players in the progression of AD. The expression and splicing of tau is different in human adult brain neurons compared to fetal neurons [38]. This is believed to reflect the different needs; mobility and growth versus stability. The frequently used SH-SY5Y cells have been shown to only contain the

fetal isoform of tau [7, 8, 30] which has different characteristics compared to mature forms. For example, this fetal form is readily phosphorylated without forming tangles [9], being unrepresentative in AD. Contrary to this, we show that untreated cells, both cultured in dishes and in the ECM gel express both 3R and 4R, as well as 0N and 1N tau mRNA. Repeating these experiments with primer sequences from earlier studies we could explain these differences with improvements in PCR technique during the last decade. Nor by quantifying mRNA expression of different isoforms could a relation to morphology be found. It must thus be concluded that tau mRNA isoform analysis is not a relevant method for analyzing cellular maturation. When examining protein expression of tau isoforms, we found poor correlation with mRNA expression. Contrary to earlier results, protein expression of some mature tau isoforms was also found in undifferentiated cells. ECM gel differentiation of SH-SY5Y cells resulted in expression of all six tau isoforms, being more comparable to human brain than undifferentiated cells. Some variability in isoform expression was seen between treatments, however none of the conditions tested resulted in a pattern completely comparable to human brain.

However, we show that differentiation of SH-SY5Y cells, resulting in neuronal appearance and function, also increases the total expression of tau protein to levels slightly lower, but not differing statistically, from levels in human brain. One alternative to increase tau expression further would be transfection. However, achieving normal cellular levels of all six tau isoforms would be challenging but important for microtubule function [39]. The presented method of differentiation also results in neuronal distribution of tau protein, being co-localized with tubulin along neurites. This is best with BDNF differentiations in the ECM gel. In adult neurons, tau is richly expressed in the axonal compartment contrary to normal cultured cells [10] and the proper cellular distribution of tau is important for microtubule

function [39, 40]. This is highly important when investigating the role of tau and microtubule function in Alzheimer's disease. It is well known that SH-SY5Y cells express the AD relevant proteins A $\beta$ PP and A $\beta$  [41], and the expression of these proteins was also confirmed in the present model with no indication of differentiation related changes (data not shown).

In conclusion, we show that SH-SY5Y neuroblastoma cells can be differentiated into neuron-like cells displaying morphological and biochemical features of mature neurons. Furthermore, these cells display axonal expression of mature tau protein isoforms. Taking all aspects into consideration we found the best overall neuronal differentiation was achieved using RA pretreated SH-SY5Y cells followed by ECM gel culturing with addition of BDNF, NRG, NGF and VitD<sub>3</sub> to the medium. This cellular *in vitro* model has the potential to be useful in many, diverse areas of neuroscience research.

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**Table 1.** The morphological features graded based upon the levels of growth inhibition, neurite length and neurite branching

Stimulation <sup>1</sup>	Growth inhibition	Neurite length	Neurite branching
-	+	+	+
VitD <sub>3</sub>	+	+	+
RA	++	++	++
RA, VitD <sub>3</sub>	+++	++	++
RA, NGR	++	++	++
RA, NGF	++	++	++
RA, NGR, NGF	++	++	++
RA, NGR, NGF, VitD <sub>3</sub>	++	++	++
RA, cholesterol	++	++	++
RA, VitD <sub>3</sub> , cholesterol	++	++	++
BDNF	++	++	++
BDNF, VitD <sub>3</sub>	++	+++	+++
BDNF, NGR, NGF	++	+++	+++
BDNF, NGR, NGF, VitD <sub>3</sub>	+++	+++	+++
BDNF, cholesterol	+++	+++	+++
BDNF, VitD <sub>3</sub> , cholesterol	- <sup>2</sup>	++	++

**Table 1**  
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<sup>1</sup>Vitamin D<sub>3</sub> (VitD<sub>3</sub>; 24 μM), retinoic acid (RA; 10 μM) neuregulin β1 (NRG; 10 ng/ml) nerve growth factor (NGF; 10 ng/ml), brain derived neurotrophic factor (BDNF; 50 nM), cholesterol (10 mg/ml). <sup>2</sup>Inconclusive data due to diverging results.