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Brain-derived neurotrophic factor (BDNF) promotes molecular polarization and differentiation of immature neuroblastoma cells into definitive neurons

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ABSTRACT

Throughout development, neuronal progenitors undergo complex transformation into polarized nerve cells, warranting the directional flow of information in the neural grid. The majority of neuronal polarization studies have been carried out on rodent-derived precursor cells, programmed to develop into neurons. Unlike rodent neuronal cells, SH-SY5Y cells derived from human bone marrow present a sub-clone of neuroblastoma line, with their transformation into neuron-like cells showing a range of highly instructive neurobiological characteristics. We applied two-step retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) protocol to monitor the conversion of undifferentiated SH-SY5Y into neuron-like cells with distinctly polarized axon-dendritic morphology and formation of *bona fide* synaptic connections. We show that BDNF is a key driver and regulator of the expression of axonal marker tau and dendritic microtubule-associated protein-2 (MAP2), with their sorting to distinct cellular compartments. Using selective kinase inhibitors downregulating BDNF-TrkB signaling, we demonstrate that constitutive activation of TrkB receptor is essential for the maintenance of established polarization morphology. Importantly, the proximity ligation assay applied in our preparation demonstrates that differentiating neuron-like cells develop elaborate synaptic connections enriched with hallmark pre- and post-synaptic proteins. Described herein findings highlight several fundamental processes related to neuronal polarization and synaptogenesis in human-derived cells, which are of major relevance to neurobiology and translational neuroscience.

1. Introduction

Throughout development, neuronal precursors undergo a complex transformation, involving their migration, differentiation, and integration into neural circuits *via* synaptogenesis and pruning. In this course, cellular polarization and formation of functional connections present crucial steps. In neuronal precursors, the process starts with a breakdown of molecular and cellular symmetry, which leads to the development of distinct processes - dendrites and axons, with specialized synaptic contacts. The fundamental relevance of molecular and functional asymmetry of neurons to their functions has been recognized by S. Ramon y Cajal, and formulated as a principle of dynamic polarization [1], which ensures the unidirectional flow of information from the driver to follower neurons, enabling coordinated operation of neural networks.

During differentiation, the transformation of precursor cells to neurons involves remodeling and redistribution of cytoskeletal proteins, especially actin, tubulin, and intermediate filaments. Actin in particular, with an array of chaperon and auxiliary proteins, drives the growing cone whereas tubulin and filaments support the stability of intracellular matrix with molecular and organelle transport [2]. Microtubule-associated protein tau, on the other hand, is one of the important players in axonal stability and neuronal integration [3,4]. Under physiological settings, tau is localized primarily in axons, with

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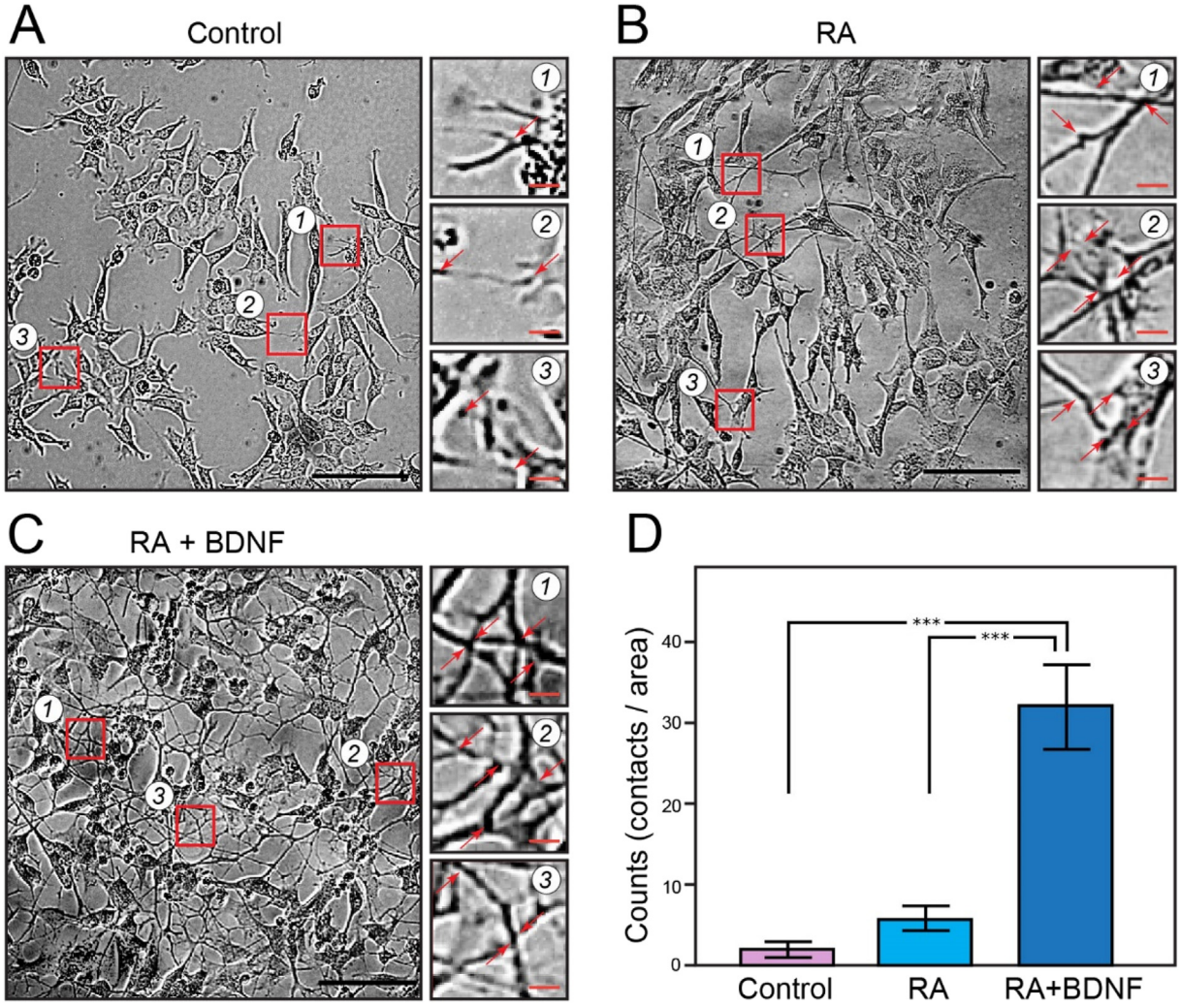

Fig. 1. Transformation of undifferentiated neuroblastoma cells into neurons by BDNF. **(A)** Undifferentiated neuroblastoma cells (SH-SY5Y) before exposure to differentiation media (Control) have amorphous cell bodies with short processes and large soma. **(B)** Undifferentiated neuroblastoma cells treated with RA (6 days) adopting spindle-like morphology with cytoplasmic extensions and usually one or two more developed neurite making distal contacts. **(C)** Fully differentiated neuroblastoma cells after sequential RA and BDNF (RA-BDNF) exposure resembling mature neurons with multipolar soma and multiple neurites branching and forming contacts. The red squared 0.05 cm^2 areas are expanded besides large panels (A-C) to show zoomed varicosities and contacts marked with arrowheads. Scale bars: 100 μm. **(D)** Summary histogram of the number of putative contacts (crossings) normalized over the area ($N = 3$). One-way ANOVA with Tukey *post hoc* test of the number of contacts calculated from multiple images. *** $p \leq .001$. Data are presented as mean values ± SD.

only trace amounts present in dendrites and soma [5,6]. Such specialized distribution is in agreement with its main function - maintenance of the microtubule stability and axonal integrity, controlled by phosphorylation at specific residues. The latter appears to be especially important for axonal functions and the long-range transmission of electrochemical signals, with dysregulations of tau phosphorylation implicated in the breakdown of axons, leading to collapse and degeneration of the connectivity in Alzheimer's disease and other tauopathies [7–9]. Due to the fundamental role in neuronal biology and pathobiology of several diseases, elucidating cellular and molecular correlates of neuronal polarization and synaptogenesis are of major research and translational interest.

Differentiation and integration of neurons into functional networks is a complex process, governed by a range of factors, amongst others by neurotrophins. The latter are known to bind to the tropomyosin receptor kinase (Trk) receptors and p75 neurotrophin receptor to activate various signaling cascades [10–12]. Amongst neurotrophins, brain-derived neurotrophic factor (BDNF) plays an important role in development and extension of neurites, plus formation and stabilization of synaptic connections [10,13] involving PI3-K/Akt downstream signaling, which takes central stage in governing the neurite outgrowth and axon formation [14–17]. Accordingly, low amount of BDNF in aging [18] and diseased brain appears to have the opposite effect, leading to axonal breakage and synaptic loss, implicated in the pathobiology of Alzheimer's disease ([19–21]). Although the specific mechanisms underlying these processes remain to be elucidated, they seem to be partly due to hyperphosphorylation of tau and its abnormal sorting and distribution. Indeed, aberrant phosphorylation of tau facilitates its accumulation in soma and dendrites, with BDNF capable of reversing this process [22]. Changes in tau distribution in neurons, therefore, warrant in-depth research of the mechanisms governing its sorting to various neuronal compartments.

SH-SY5Y cells, originating from neuroblastoma of a 4-year-old female, present an attractive model for neurobiological research. As a subline that originates from SK-N-SH cells, SH-SY5Y cells under physiological settings do not express generic markers of mature neurons but can be induced into neuron-like cells displaying numerous relevant characteristics [23]. Differentiation of SH-SY5Y cells into dopaminergic neurons, for instance, has been used for Parkinson's disease research, in which retinoic acid (RA) was applied as the main inducing agent [24].

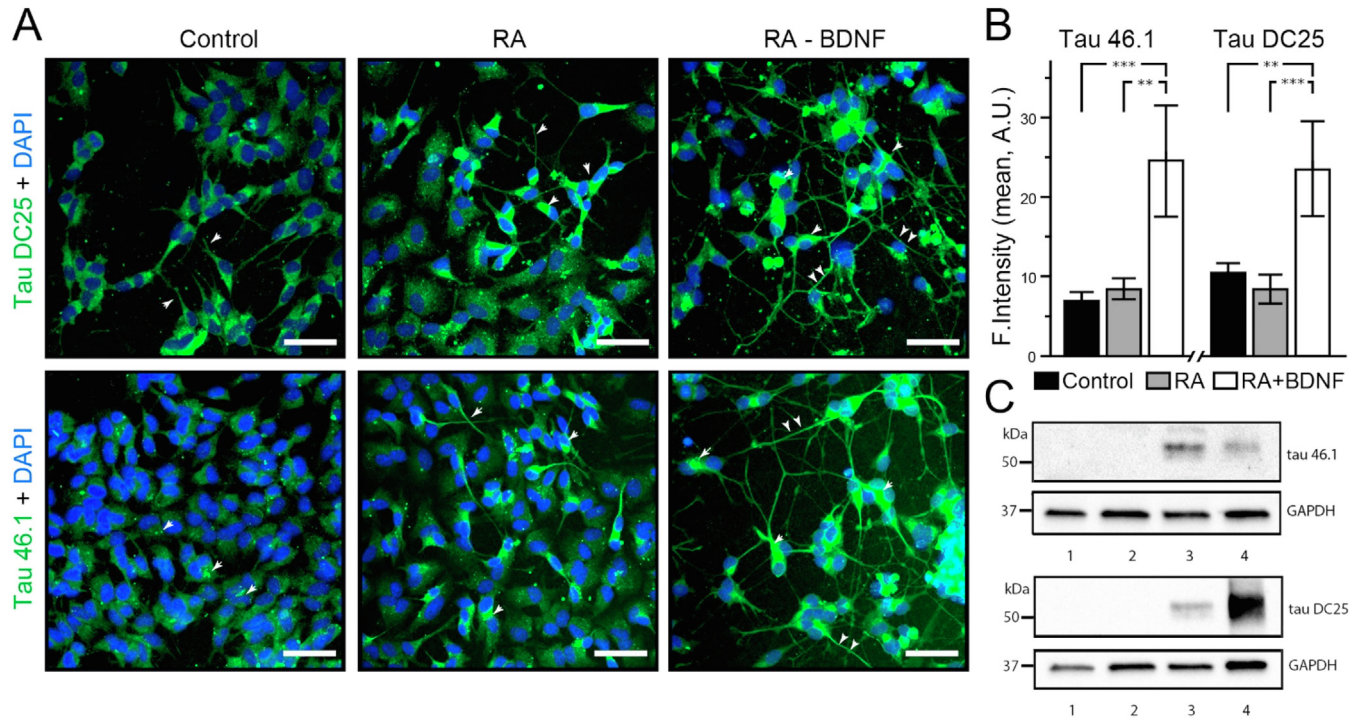

Fig. 2. Enrichment and distribution of tau protein in neuroblastoma cells differentiating into neurons. **(A)** Representative images (cold methanol fixation) of immunostaining for tau protein (green) by monoclonal anti-tau antibodies, DC25 and tau 46.1, of all three experimental groups: Control, RA and, RA-BDNF, respectively. Cellular nuclei in blue – DAPI. Note that in RA differentiated cells, a significant change in the expression and distribution of tau protein is evident, with tau sorting into the longer processes (RA panel). After RA-BDNF treatment, tau expression and enrichment in long, newly formed neurites (putative axons) are more visible (RA-BDNF panel). White arrows point to tau rich somata, while double arrowheads point onto putative axons. Scale bars: 50 μm. **(B)** Summary histogram showing an increase in tau signal (mean fluorescence intensity) in RA-BDNF cells. One-way ANOVA with Turkey *post hoc* test of the number of mean fluorescence intensity values calculated from multiple images. ** $p \leq .005$; $p \leq .001$. Data are presented as mean values ± SD. **(C)** Representative Western blots illustrating an increase in tau expression in RA-BDNF treated cells. (Top) Tau antibody clone 46.1 that binds to epitope 428–441 aa, the longest form of human tau, the band corresponding to tau was only detectable in RA-BDNF treated cells and brain homogenate. (Bottom) Tau antibody clone DC25 binds to all isoforms of tau, regardless of the phosphorylation status, detecting tau in RA-BDNF treated cells and brain homogenate. (lane 1: control, 2: RA, 3: RA-BDNF, 4: human brain homogenate). GAPDH used as a loading control.

RA treatment has been also found to induce the expression of NF-kB-related proteins necessary for the initiation of neurite outgrowth [25] leading to characteristic neuronal morphologies with activation of an array of neuron-specific genes [26]. The expression of hallmark proteins such as tau, NeuN, MAP2, and beta III tubulin, as well as synaptic proteins, however, remains sparse, implying only a partial transformation [27]. To replicate more closely neuronal differentiation, a two-step method has been introduced through a combination of RA with BDNF treatment [27–30]. The latter involves activation of TrkB and stimulation of downstream signaling pathways essential for the establishment and maintenance of neuronal polarization.

We took advantage of the two-step method of differentiation to investigate the expressional dynamics of tau, MAP2, and synaptic proteins, and to characterize their distribution in SH-SY5Y cells. We analyzed the process of polarization and development of distinct neurite types as well as the formation of *bona fide* connections, with the main focus on the distribution of tau, MAP2, and hallmark synaptic proteins. We also studied the effects of selective phosphoinositide 3-kinase (PI3-K) and glycogen synthase kinase 3 beta (GSK-3β) inhibitors on RA-BDNF differentiated SH-SY5Y cells. Unlike the majority of studies in neurons from murine brain, our results shed light on important neurobiological processes in human-derived neuron-like cells which are of potential relevance to neurodevelopmental research and mechanisms of neurodegenerative diseases.

2. Results

2.1. BDNF induces neuron-like morphology in neuroblastoma cells

Undifferentiated SH-SY5Y cells cultured in DMEM containing FBS maintain distinctive neuroblast-like morphology with non-polarized appearance and large soma, and a few short processes (Fig. 1A). Under these conditions, cells rapidly grow and proliferate. To prevent the disorderly overgrowth and formation of dense cellular clumps, we applied FBS starvation plus RA (Table S1) and monitored cell growth using phase-contrast imaging. In the presence of RA and sequential FBS starvation, we observed a transient reduction in cell density. The remaining cells acquire polarized appearance and spindle-like morphologies, with occasional shafts and projections extending from soma (Fig. 1B), typically one per cell. Subsequent exposure of these cultures to BDNF with the withdrawal of FBS prompted a rapid and dramatic extension of multiple cellular processes, which acquired distinct neurite-like features, with extensive branching and varicosities at points of contacts and crossings. Occasionally, these processes formed putative contacts reminiscent of synaptic connections in primary neuronal cultures, and developed dense networks (Fig. 1C). Analysis and comparison of numbers of putative synaptic contacts revealed their significantly higher number in cultures sequentially treated with RA and BDNF (Fig. 1D). To investigate if morphological changes prompted by BDNF are associated with molecular alterations characteristic to neurons, we carried out a series of immune-histochemical tests with neuron-specific markers using confocal microscopy and Western blotting, at various differentiation stages.

2.2. BDNF promotes molecular polarization in differentiating neuroblastoma cells

To investigate if differentiation and outgrowth of neurites in SH-SY5Y cells are associated with molecular alterations involved in neuronal polarization, we examined the expression and distribution of microtubule-associated proteins tau and MAP2, known as hallmarks for neuronal axons and dendrites, respectively [31]. Two tau antibodies recognizing different epitopes (DC25 epitope 347–353 aa, tau 46.1 epitopes 428–441 aa, counted for the longest human isoform htau40 with 1–441 aa) were applied. Both immunofluorescence and Western blotting data showed significantly higher expression of tau in cultures exposed to RA-BDNF, as compared to undifferentiated controls, or only RA treated cultures (Fig. 2A-C). The mean fluorescence intensity of tau signal calculated for tau 46.1 and tau DC25 in RA-BDNF cells was twofold higher compared to that of undifferentiated groups (Fig. 2B). Of note, in RA-BDNF differentiated cells, there was visibly stronger tau enrichment in neurites, suggesting active sorting of newly produced tau to these compartments. Importantly, RA-BDNF differentiated SH-SY5Y were immune-negative for glial marker GFAP (data are not shown), which is in agreement with previous studies [29], confirming the homogeneity of tested cultures, with a decisive predominance of neuronal phenotype. BDNF-dependent enrichment of SH-SY5Y cells with tau protein was also confirmed in Western blotting experiments, with the highest levels of tau detected in RA-BDNF treated cultures. In the RA-differentiated and undifferentiated control groups, the level of tau was below the detection threshold (Fig. 2C).

Neurons are characterized not only by the expression of microtubule-associated proteins tau and MAP2 but also by specific distribution of these proteins, reflecting their fundamentally polarized nature. To investigate if in RA-BDNF treated cultures these ubiquitous neuronal markers display asymmetric distribution, we performed double fluorescence labeling for tau and MAP2. Figs. 3 and S2 summarize the results of these studies. In both sets of data, cells show a polarized distribution of tau and MAP2. While MAP2 was enriched in the soma and short projections, the level of tau was much higher in longer projections, corresponding to putative axons (Fig. 3A). Importantly, similar to tau protein, MAP2 in undifferentiated SH-SY5Y showed the ubiquitous presence in the soma and short processes. This observation confirms distinct molecular polarization, in addition to characteristic neuronal morphology in RA-BDNF treated SH-SY5Y cells. The results of co-localization analysis are also in agreement with the general molecular asymmetry of differentiated SH-SY5Y cells, with tau protein selectively enriched in elongated axon-like processes, while MAP2 present in the soma and shorter and less elaborated projections (Fig. 3D). Of note, the mean fluorescence for MAP2 in RA treated cells was comparable with undifferentiated control cells, but rapidly increased in the presence of BDNF over the consecutive six days (Fig. 3B). This phenomenon, occasionally associated with the reduction of MAP2 levels in cells, was observed previously by Pezzini et al. [32]. To verify the role of TrkB-dependent downstream signaling in the maintenance of BDNF induced polarization of RA-pretreated SH-SY5Y cells, we used covalent PI3-K/Akt inhibitor wortmannin and canonical GSK-3β inhibitor lithium chloride (LiCl). Application of wortmannin in the presence or absence of LiCl in differentiated SH-SY5Y caused gradual retraction of neurites and reduction of their branching, as compared to control and LiCl-treated SH-SY5Y cells differentiated under standard two-step protocol (Fig. 4A, C). The depletion of tau-positive neurites and loss of elaborate branching by wortmannin and by co-application of wortmannin and LiCl were also confirmed by immunofluorescence imaging (Fig. 4B, D).

We also investigated and compared the distribution of MAP2 and tau under several different treatment conditions (Method S1, Table S3). It was found that while undifferentiated cultures survive without exposure to RA or BDNF (cultured in standard DMEM medium supplemented with FBS) for 12 days (the longest time point we tested), intensive proliferation and high density of cells forming dense clumps made their microscopic analysis problematic (data not shown). Although cultures exposed to RA alone with FBS starvation for 12 days maintained relatively stable cell density, no increase in the expression of microtubule-associated proteins tau and MAP2 levels was observed. Thus, under specified experimental settings, distinct morphological and molecular polarization of SH-SY5Y was observed only in RA-BDNF treated cultures (Figs. S1, S2).

2.3. BDNF promotes synaptogenesis and formation of specialized connections

Polarization of mature neurons *in vivo* warrants directional information flow *via* synaptic connections [33]. We tested if SH-SY5Y differentiation induced by RA-BDNF protocol is associated with the formation of genuine synaptic contacts enriched with pre- and post-synaptic proteins. The expression of hallmark proteins such as synaptophysin, bassoon, SHANK3, and PSD-95 was tested using immunofluorescence imaging, Western blotting, and proximity ligation assay (PLA) to verify the formation of synaptic connections. As illustrated in Figs. 5A and S3, cultures treated with RA-BDNF show high expression of synaptophysin and PSD-95 protein in the somatic compartment and neurites of SH-SY5Y cells, with multiple visible puncta, corresponding to putative sites of synaptic contacts. In stark contrast, in undifferentiated control cultures and cultures treated with RA only, the expression of these proteins was lower and showed predominantly somatic localization. The results of the comparison of the immunofluorescence signal across different experimental groups revealed a significant increase in synaptic markers under RA-BDNF treatment (Fig. 5B). While enrichment of synaptic proteins was evident in the soma, both synaptophysin and PSD95 signals becoming also clearly visible in neurites (Fig. 5A). Noteworthy, labeling of neurites positive for synaptophysin revealed punctate character, reminiscent of axonal varicosities and synaptic contacts (Fig. 5A). The results of Western blot analysis confirmed the highest levels of synaptophysin and PSD95 in RA-BDNF differentiated cells (Fig. 5C). Similar data were obtained also in studies of the expression of Basson and SHANK3 synapse-specific proteins (not shown).

To determine if enrichment of synaptic proteins in differentiated SH-SY5Y cells is associated with the formation of *bona fide* synaptic connections, we applied PLA for combinations of two sets of pre- and post-synaptic markers (Fig. 6A-C). PLA, also referred to as Duolink® PLA technology, enables visualization of two proteins in high proximity. Antibodies covalently linked with DNA oligomers are applied, which under sufficient closeness follow hybridization step and PCR amplification with fluorescent probes [34,35] This technique was recently used for detection of transsynaptic protein pairs reflecting synaptic density in primary neuronal cultures [36]. We took advantage of PLA to investigate if the enrichment of synaptic proteins in differentiated SH-SY5Y cells is associated with the formation of synaptic connections. The number of fluorescence PLA dots for different combinations of synapse-specific markers was counted and compared with those in undifferentiated cells and negative control experiments, and normalized for the number of nuclei per image frames (Fig. 6B, C). Negative control cells in PLA experiments were stained without primary antibodies, hence revealing only non-specific fluorescence spots. As can be seen, RA-BDNF differentiated cultures show a strong increase in PLA dots compared to negative controls for combinations of bassoon-PSD95, synaptophysin-SHANK3, and especially bassoon-SHANK3 pairs (Figs. 6B and S3). Overall, these results confirm the important role of BDNF in driving molecular polarization in differentiating SH-SY5Y, reminiscent of neuronal polarization and formation of synaptic connections.

3. Discussion

In this study, we combine biochemistry, immunofluorescence

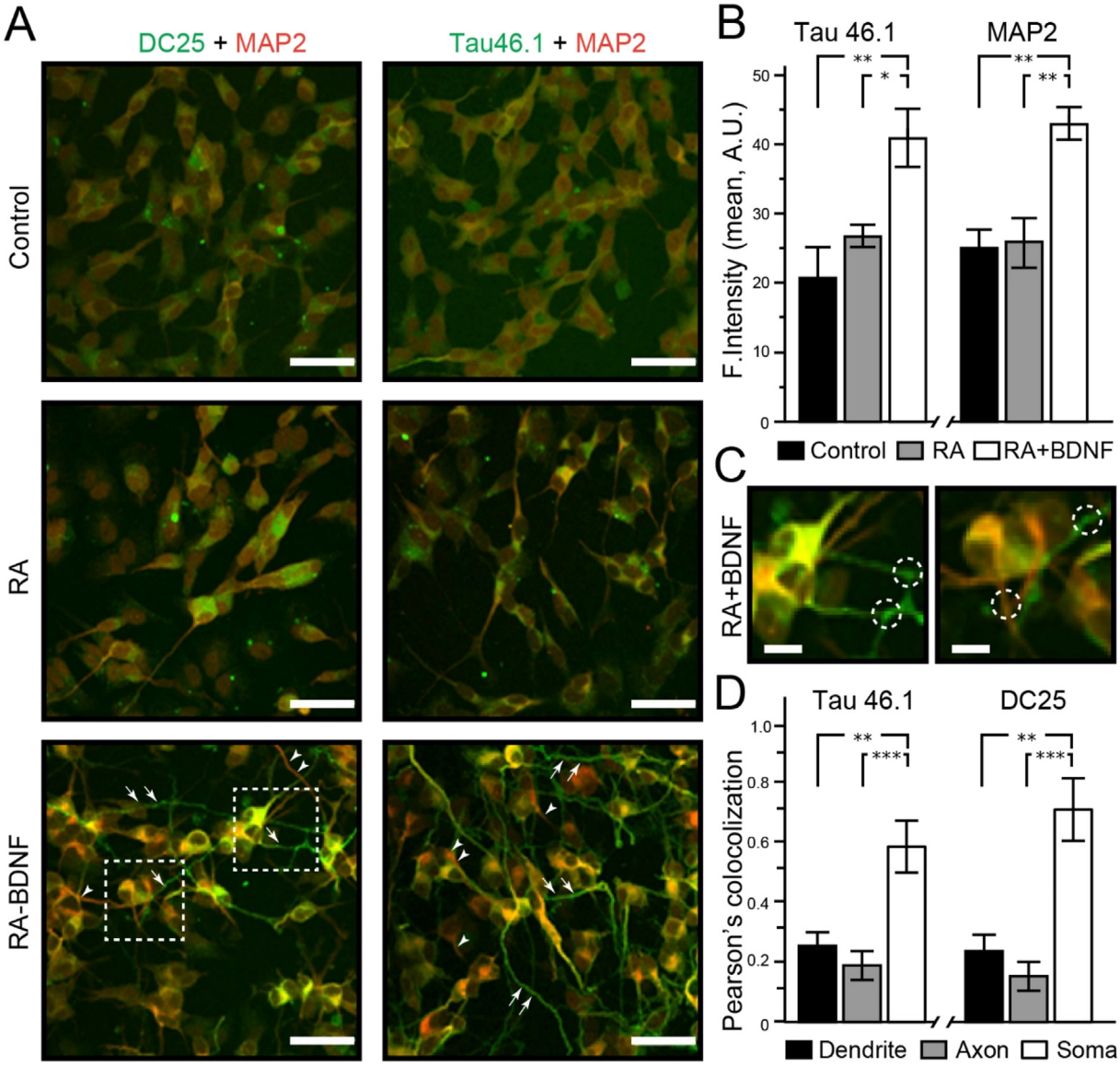

Fig. 3. Relative expression and localization of tau and MAP2 during development and differentiation of SH-SY5Y neuroblastoma cells. **(A)** Representative images (4% paraformaldehyde fixation) of double-stained cells for tau and MAP2 protein during differentiation of neuroblastoma cells show their enrichment with both, tau and MAP2 proteins. Note, that after RA-BDNF treatment, neuron-like neuroblastoma cells show two distinct types of neurites, based on the expressional levels of tau (green) and MAP2 (red) proteins. Scale bars: 50 μm. **(B)** Summary histogram of the intensity of the fluorescence signal under three different experimental conditions. The paired Student's test of mean fluorescence intensity for tau and MAP2 shows a strong increase in the expression of both proteins in differentiated neuroblastoma cells. ** $p < .005$; * $p < .05$. **(C, D)** Summary histograms of the results of the co-localization analysis of various compartments of RA-BDNF treated SH-SY5Y cells showing their differential enrichment with tau and MAP2 proteins, respectively. ROIs from various compartments of SH-SY5Y cells were analyzed **(C)**, with mean values of Pearson's correlation coefficient plotted (D). Scale bars: 15 μm. *** $p < .001$; ** $p < .005$.

imaging and proximity ligation assay to monitor the differentiation and polarization of SH-SY5Y into neuron-like cells with distinct sets of neurites, and synaptic connections. We demonstrate that while under RA treatment, SH-SY5Y cells develop rudimentary neurites, only when exposed to a combination of RA-BDNF, they differentiate and display molecular asymmetry and expression of high amounts of neuronal markers such as MAP2, tau, synaptophysin, SHANK3, PSD95, and others. We show that maintenance of polarized state of SH-SY5Y cells depends on constitutive activation of PI3-K/Akt, downstream to TrkB receptors. Finally, we demonstrate that BDNF signaling is essential for the formation of dense neurite network with synaptic contacts.

Neuroblastoma SH-SY5Y cells provide an instructive model for cellular and molecular biology studies and have been widely used as a tool for *in vitro* research, including neurodegenerative disorders such as Parkinson's disease [37–39] and Alzheimer's disease [28,40,41]. Non-differentiated SH-SY5Y cells are characterized as immature catecholaminergic neurons [42] enriched with nestin, a marker for neuro-glial progenitors [37,38] with the potential to differentiate into mature neurons. The principal advantage of this model is its human origin [42], especially relevant to exploring species-specificity of neurobiological processes. Moreover, their stable karyotype [43] with the possibility of use as post-mitotic cells makes this line advantageous for large scale neurochemical analysis with minimal genetic variability. Last but not least, the convenience of their manipulation to produce transiently or stably transfected cells makes SH-SY5Y cells useful as an expression system for drug screening studies [44–46]. Depending on differentiation protocol and culture conditions, relatively homogenous genetic populations of SH-SY5Y cell-derived adrenergic, dopaminergic, glutaminergic, and cholinergic phenotypes can be obtained [47]. Two-step differentiation used in the current study takes advantage of pre-

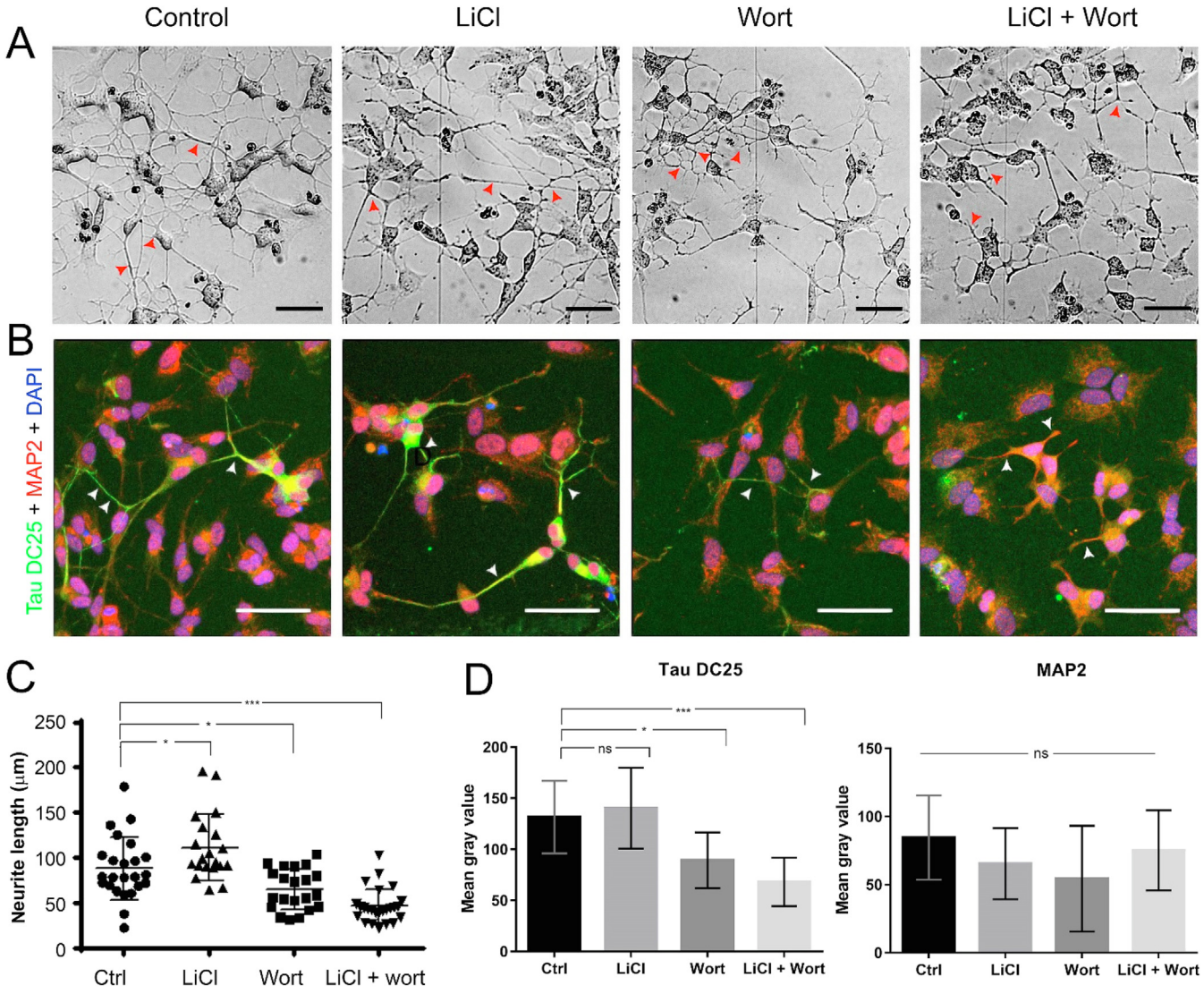

Fig. 4. Inhibition of PI 3-K/Akt signaling leads to degeneration of neurites of differentiated SH-SY5Y cells. **(A)** Representative phase-contrast images of SH-SY5Y cells differentiated by two-step protocol (Control), treated with LiCl (5 μM), wortmannin (Wort, 100 nM) and their combination (Wort + LiCl). Control group and group treated with LiCl possessed developed neurites compared to groups treated by wortmannin, where the retraction of neurites occurred. Scale bars: 50 μm. **(B)** Representative maximum intensity confocal imaging of SH-SY5Y cells under conditions specified in (A). Tau-positive projections in differentiated SH-SY5Y were reduced in the presence of wortmannin, causing a significant decline of tau expression in neurites- without notable changes in cell body appearance. Scale bars: 50 μm. Arrowheads pointed neurites and their branching. **(C, D)** Summary histograms of the analysis of the mean neurite lengths (C) and signal intensity corresponding to Tau DC25 and MAP2 (D) pulled from at least 3 independent images. One-way ANOVA with Tukey's multiple comparison test, * $p < .05$, *** $p < .001$, ns – not significant.

treatment of SH-SY5Y with RA with sequential FBS decrease, which supports the growth of neuroblastic phenotype (N-type) and suppress the substrate-adherent subpopulation (S-type) cells present in original SH-SY5Y population [29,30,48]. As Encinas et al. showed, the long-term exposure of SH-SY5Y cells to RA can shift the balance towards the undesirable S-type. Importantly, RA also withdraws neuroblastoma cells from the cell cycle [29,49] and induces the expression of functional TrkB receptor [50] with its maximum peak at days 5–6 [29,51]. RA-dependent TrkB expression is therefore crucial for cell response to BDNF, which plays an important role in neuronal signal transduction in this model. Accordingly, our data show that maintenance of polarization of SH-SY5Y cells depends on the constitutive activity of TrkB receptors and downstream PI3-K/Akt signaling. Indeed, irreversible inhibition of PI3-K with wortmannin caused a retraction of elaborate neurites of differentiated SH-SY5Y. Of note, the neuritic architecture and integrity can be altered by wortmannin even in presence of lithium, but not by lithium alone. Thus, the maintenance of established neurite-like network in differentiated SH-SY5Y is not dependent on inhibiting phosphorylation of GSK-3β by activated Akt, but is likely to involve other downstream targets of PI3-K-dependent signaling. While our findings are in general agreement with published literature, the precise mechanism of regulation of neuronal differentiation and polarization by BDNF signaling remains to be established. Indeed, inhibition of class I PI 3-K/Akt signaling, activated *via* BDNF-TrkB receptor [17] has been shown to impede the axonal growth but not axonal polarity, as reported recently by using more selective PI 3-K blockers [15]. Moreover, under certain conditions, GSK-3β is known to regulate axon-dendrite polarity independently from PI 3-K/Akt [52].

Taken as a whole, our results of the analysis of general morphology and molecular characterization of SH-SY5Y cells under RA treatment agree and advance the data reported by Nishida et al. [53]. We also confirm that the treatment of cells with BDNF enhances considerably their neuron-like appearance. Importantly, major morphological changes in our study are associated with fine molecular adjustments,

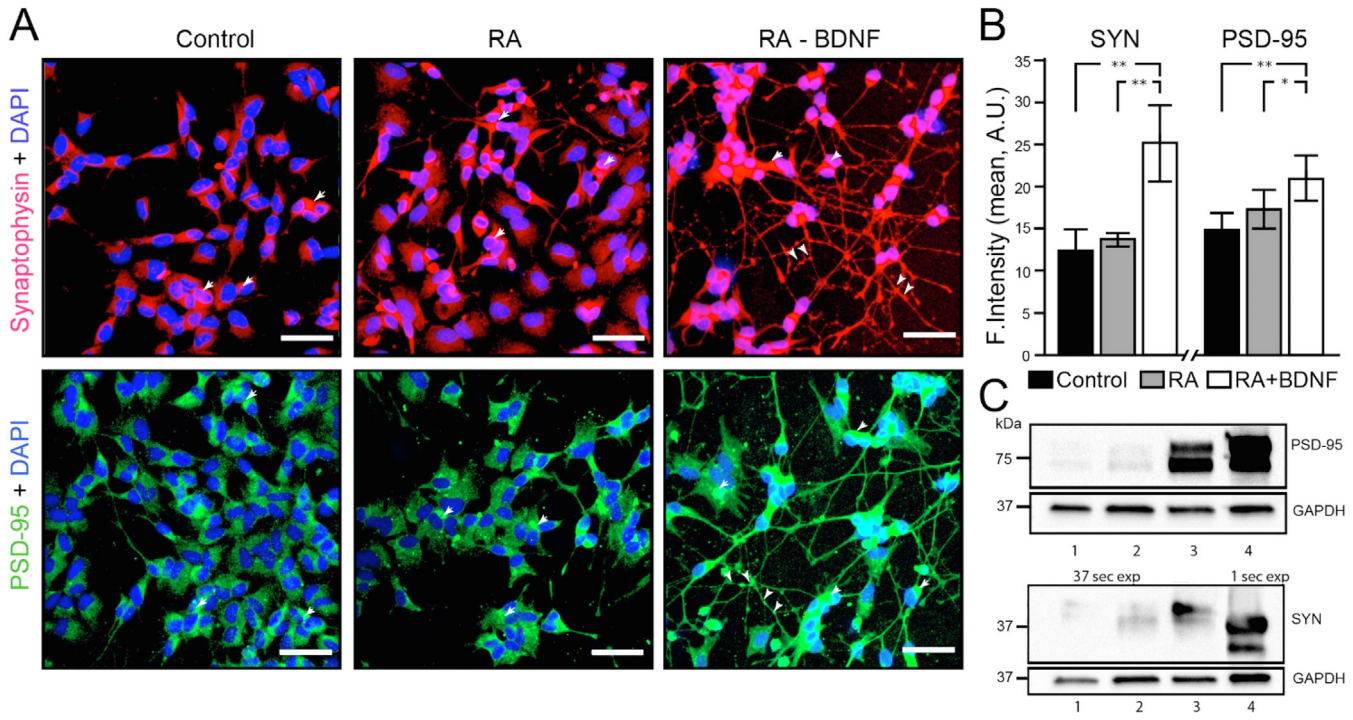

Fig. 5. Enrichment of differentiating neuroblastoma cells with hallmark synaptic proteins. **(A)** Representative confocal micrographs of SH-SY5Y cell cultures maintained under different experimental conditions: Control, RA, and RA-BDNF, respectively. White arrows indicate enrichment of synaptic markers in cell soma; double arrowheads point varicosities and putative terminals, also enriched with synaptic proteins. Scale bars: 50 μm. **(B)** Summary histogram of mean fluorescence intensity of synaptophysin (SYN) and PSD95 under different experimental conditions. Note the significantly higher expression of two synaptic proteins in RA-BDNF treated cells compared to control and RA-treated SH-SY5Y cells. Paired Student's *t*-test. ** $p < .005$, * $p < .05$. **(C)** Representative Western blots showing a strong increase in expression of both synaptic proteins in RA-BDNF treated cells (lane 1: control, 2: RA, 3: RA-BDNF, 4: human brain homogenate). GAPDH used as a loading control.

leading to polarization and formation of differentiated axons with sophisticated dendritic apparatus, as revealed by neuron-specific markers [31]. Such dramatic BDNF-dependent molecular rearrangements imply substantial modifications at multiple levels, from gene expression to translation, protein sorting and trafficking, and targeting to specific subcellular compartments. It is worth noting that these complex changes caused by BDNF have been implicated also in the development of specific molecular scaffolds at *bona fide* synaptic contact [54,55].

In the context of molecular polarization, there has been much interest in research of the biology of tau protein, with the focus of its abnormal sorting and propagation from axon to the somatodendritic compartment [6]. Such changes have been attributed to the disruption of the retrograde axonal barriers [56,57]. While tentatively assigned to aberrant phosphorylation of tau by kinase MAPK/Par1, which seem to be isoform-specific [6], the precise underlying mechanisms of these effects remain unknown. Selective sorting of tau and MAP2 to the various compartment of polarized SH-SY5Y cells under BDNF treatment implies not only the possible role of BDNF signaling in regulating tau phosphorylation but also its significance in promoting molecular polarization with enrichment of tau in developing axons. The utility of SH-SY5Y cells for research of tau pathology in Alzheimer's disease has been shown by multiple reports, although a wide range of methods and protocols used in these studies renders data comparison and interpretation complicated [22,28,41,58–60]. Human tau forms six isoforms, *via* alternative splicing of mRNA, differing by two N-terminal inserts of 29 amino acids, each generating 0 N, 1 N or 2 N tau isoforms, and of the second repeat in C-terminal part to form 3R or 4R isoforms [61]. In undifferentiated SH-SY5Y, like in the fetus, phosphorylated tau is detected as the exclusive tau isoform localized in soma region [41,59] and nucleus [60]. RA-treatment of SH-SY5Y cells, on the other hand, leads to the upregulation of tau levels [59,60] and promotes its movement from the soma into putative axons [22]. Interestingly, an earlier report showed that RA-differentiation did not cause any change in tau isoforms expression in SH-SY5Y cells [59], although another study demonstrated the presence of higher molecular weight tau [60]. Whether the arrival of higher molecular weight tau variants is responsible for its sorting to axons remains to be shown. The fact that short exposure of RA-pretreated SH-SY5Y to BDNF (48 h) significantly increased the tau content and phosphorylation rate but had no effects on a generation of additional tau isoforms [41] suggest that BDNF induced enrichment of this protein to axons could be due to changes in molecular apparatus of axons. The latter along with transient phosphorylation appears to be linked with the period of intense neurite outgrowth, as reported in developing neurons [62]. The work of Chen and co-workers showed that short treatment of RA-differentiated SH-SY5Y cells with BDNF (24 h) reduced phosphorylation at S262, which enhanced tau association with alpha-tubulin [22], that in turn could present an alternative mechanism for redistribution of tau into neurites during differentiation. The coincidence of an overall increase in tau levels and tau redistribution from the soma into developing processes with neurite outgrowth was also observed by Agholme et al. [28], using 3D extracellular matrix gel and sequential exposure of cells to RA and combination of BDNF, neuregulin beta1, NGF, and vitamin D3.

In conclusion, we presented several sets of data demonstrating that sequential RA-BDNF conditioning of SH-SY5Y cells causes rapid morphological and molecular polarization, mimicking differentiation of *bona fide* neurons, where BDNF-TrkB-dependent signaling pathways are essential. The approach presented herein shows that, in addition to addressing specific questions relevant to disruption of neuronal polarization and differentiation under diseased conditions, this model has much to offer for addressing fundamental questions of neuronal biology and neurodevelopment, with molecular and functional changes. Also, due to genetic homogeneity, described herein approach presents a useful stand for exploring the potential impact of epigenetic and

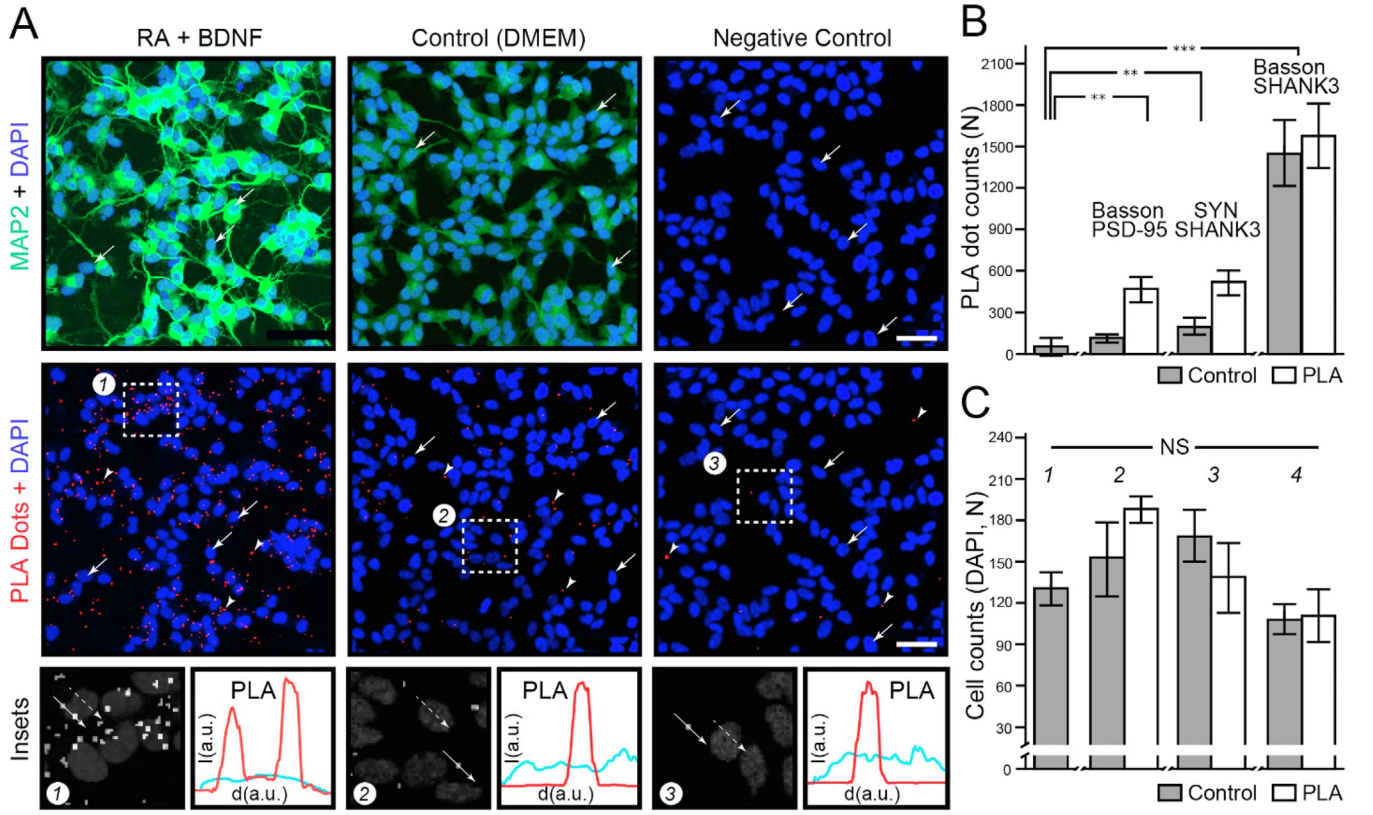

Fig. 6. Visualization of putative synaptic contacts in differentiating neuroblastoma cells using proximity ligation assay (PLA). Proximity ligation assay performed with combinations of synaptophysin and bassoon as pre-synaptic markers, and PDS95 and SHANK3 as post-synaptic markers, respectively. **(A)** Representative images of nuclear (DAPI) and MAP2 staining, and PLA puncta in RA-BNDF differentiated cells, as compared to non-differentiated control cells (Control DMEM). The negative staining controls were treated according to the same protocol without adding primary antibodies (Negative Controls). Insets below illustrate boxed areas expanded (left) with intensity profile graphs for DAPI and PLA dots (dashed arrow - blue and continuous arrow - red, respectively). **(B, C)** Summary histograms of counts and comparison of PLA dots between various experimental groups and cell numbers within the same groups. Paired Student's *t*-test was used for statistical comparison; *** $p < .001$, ** $p < .005$, ns – not significant. There was a statistically significant increase in PLA dots in bassoon - PSD95, and synaptophysin - SHANK3 pairs, but no changes in the number of pairs of bassoon - SHANK3 in controls and RA-BDNF treated cultures. Scale bars: 50 μm.

environmental factors on neuronal differentiation and polarization, as well as pharmacological and molecular interference, with relevance to a range of basic and translational questions.

4. Materials and methods

4.1. Materials and reagents

SH-SY5Y cell line (ECACC, 94030304), trypsin-EDTA solution, *all–trans*–retinoic acid (RA), human brain-derived neurotrophic factor (BDNF), RIPA lysis buffer, protease inhibitor cocktail, and Lithium Chloride (LiCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM, with high glucose, L-glutamine, and phenol red), Neurobasal™-A Medium, L-glutamine, N-2 supplement, Penicillin-Streptomycin (10,000 U/mL), normal goat serum (10%, NGS), Pierce™ ECL Western Blotting Substrate, and Pierce™ BCA Protein Assay Kit were ordered from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was from Biosera Europe (Nuaille, France). Tau protein ladder of all six isoforms was acquired from rPeptide (Bogart, GA, USA). Duolink® *In Situ* Red Starter Kit Mouse/Goat for proximity ligation assay (PLA) and Duolink® *In Situ* Mounting Medium with DAPI were ordered from Sigma-Aldrich (St. Louis, MO, USA). Western blot reagents, 0.2 μm nitrocellulose membrane, and 4–15% Criterion™ TGX Stain-Free™ Protein Gel (18 well, 30 μl) were acquired from Bio-Rad (Hercules, CA, USA). Corning® BioCoat™ Poly-D-Lysine 8-well Culture Slide was ordered from Corning (Corning, NY, USA). Centrifugal units Vivacon 500 (2000 MWCO) were purchased from Sartorius (Gottingen, Germany). Wortmannin was purchased from Acros organics (Geel, Belgium).

4.2. Culturing and differentiating of neuroblastoma cells

SH-SY5Y cells from the European collection ECACC were cultured in T75 flasks in standard medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin mixture) at 37 °C in 5% CO_2. After reaching 80–90% confluence, cells were trypsinized by trypsin-EDTA solution and split at density of 1×10^4 cells/cm^2. As the biochemical properties of SH-SY5Y may depend on number of passages [63], we kept the passage number below 20 in all performed experiments. The differentiation protocol to obtain neuron-like cells over 12 days was modified from Shipley et al. [30]. In brief, cells were seeded at a density of 1×10^4 cells/cm^2. For confocal microscopy, poly-D-lysine pre-coated 8-well slides were used. Cells cultured in T25 flasks with seeding density 3.6×10^5 were applied for lysis and Western blot. All cells were seeded at day 0 in DMEM with 5% FBS and 1% penicillin-streptomycin mixture, then the differentiation followed according to (Table S1). Three groups of cells were analyzed at certain time points during the differentiation process: non-differentiated controls cultured for 6 days without RA (Ctrls), 6 days RA-differentiated cells (RA), 6 days RA-differentiated cells followed by additional 6 days of treatment with BDNF (RA-BDNF).

In experiments involved the inhibitor treatment, SH-SY5Y cells were treated as described in Table S1 till day 9 of the differentiation, 6 days with RA under gradual FBS starvation and 3 days with BDNF. The

following 3 days of the differentiation protocol, fresh neurobasal media with inhibitor combinations were added: control group (no inhibitor), LiCl group (5 μM of LiCl), wort group (100 nM of wortmannin) and LiCl + wort group (5 μM of LiCl and 100 nM of wortmannin). After 3 days of incubation, the cells were fixed and photographed using Leica DMI1 microscope with phase contrast microscopy followed by immunocytochemistry staining.

4.3. Immunocytochemistry

Cultures in 8-well plates were rinsed with phosphate-buffered saline pH 7.4 (PBS) and fixed by the addition of 4% paraformaldehyde or 100% cold methanol for 10 min. Fixed cells were washed and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature (RT), followed by blocking in 10% NGS for 30 min at RT. Fixed cells were then incubated overnight with primary antibodies (Table S2) at 4 °C. After three 5-min PBS-T wash steps, they were exposed to fluorescent-conjugated secondary antibodies for 1 h at 37 °C, followed by three 5-min washes with PBS-T, mounted in medium containing DAPI and covered for microscopic analysis. Images were obtained with Leica Microsystems TCS SP8 X confocal laser scanning microscope, using a plan apo 40×/1.3 oil immersion objective. The images were acquired using appropriate excitation wavelengths and spectral detection ranges for the different applications.

4.4. Proximity ligation assay

PLA protocol was applied according to the manufacturer's instructions (Duolink®, Sigma-Aldrich) with slight modifications described in detail previously [64]. In brief, formalin-fixed and permeabilized cells were blocked with blocking solution for 30 min at 37 °C, followed by overnight incubation at 4 °C with primary antibodies against selected pre- and post-synaptic markers and MAP2. After washing, PLA probes and secondary antibody were added for 1 h at 37 °C. Cultures were then washed and incubated with ligation-ligase solution for 30 min at 37 °C. The amplification-polymerase reaction followed for 100 min at 37 °C, with a wash followed by mounting with DAPI-containing mounting medium and covered with coverslips. Images were obtained using 40 × 1.3 NA oil objective with Leica confocal laser scanning microscope (TCS SP8).

4.5. Western blot

Human brain samples used for Western blotting were taken at post-mortem intervals < 24 h from the Psychiatric Hospital Bohnice. All experiments involving human tissue were approved by Ethics Committee of the Third Faculty of Medicine, Charles University in Prague and were conducted in accordance with the Laws 129/2003 and 130/2003 of the Czech Republic. Cells cultured in T25 flasks were briefly washed twice with ice-cold PBS and lysed using RIPA buffer containing 1% (*v*/v) protease inhibitor cocktail and incubated on ice for 30 min. The lysate was spun down at 12000 ×*g* at 4 °C for 5 min. The samples were then concentrated using concentration tubes (2000 MWCO) for 1 h 15 min. Total protein concentration was determined by BCA assay. Cell lysates and human brain homogenate (20 μg for each sample) were loaded on 4–15% gradient Criterion stain-free gel. After electrophoresis, proteins were transferred onto 0.2 μm nitrocellulose membrane and the membrane was blocked in 10% (*w*/*v*) soy milk in PBS-T for 1 h at RT. Membranes were then cut in half, incubated with respective primary antibodies overnight at 4 °C, and washed three times in PBS-T (5 min each). The incubation with secondary antibodies followed for 1 h at RT, membranes were washed three times with PBS-T (5 min each). The chemiluminescence detection by Pierce™ ECL Western Blotting Substrate was performed according to the manufacturer's instructions. The bottom parts of the membranes were then stripped and re-probed with GAPDH antibody as a loading control.

4.6. Image processing and data analysis

The mean fluorescence intensity was measured from multiple regions of interest (ROIs) of fluorescence micrographs using LAS X software (Leica Microsystems) or ImageJ 1.47 software (NIH), with background subtracted from each image. In case of PLA assay, the images were presented as maximum intensity projections from 25 z-stacks. All images for PLA dots were processed exactly in the same manner by ImageJ 1.47 software (NIH), with fluorescence intensity threshold set at the same level, with the total amount of dots counted and compared. For cell counting, DAPI channel was transformed into a binary image. For PLA dot and nuclear counting, we used Analyze particles (size set as 20-infinity μm^2, exclude on edges) function. All data were analyzed with GraphPad Prism software Version 5.0 from GraphPad (San Diego, CA, USA). For comparison of mean intensity values, PLA dots and nuclei amount/frame paired Student's *t-test* was applied, with P values $\leq .05$ defining a significant difference. Colocalization analysis of tau and MAP2 fluorescence signals was done separately for anti-tau antibodies DC25 and Tau46.1 co-stained with MAP2 in cultures of RA-BDNF differentiated cells, with three sets of 15 ROIs (diameter 5 μm) marked in somatic, dendritic and axonal compartments. *Z*-stacks of 5 optical sections as a maximum projection (1.38 μm) for both Ch1 (green, tau fluorescence signal) and Ch2 (red, MAP2 fluorescence signal) channels were applied with threshold and background values set as 30% and 20% intensities, respectively. Pearson's correlation coefficient was applied for quantification of tau and MAP2 signal colocalization in soma, dendritic and, axonal regions. For comparison of neurite length and fluorescence intensity, One-way ANOVA with multiple comparisons was applied, with P values $\leq .05$ defining significant difference.

Credit authorship contribution statement

Lenka Hromadkova:Investigation, Methodology, Formal analysis, Writing - original draft.**Dagmar Bezdekova**:Investigation, Methodology, Formal analysis, Writing - original draft.**Jan Pala**:Formal analysis.**Sophia Schedin-Weiss**:Methodology, Writing - review & editing.**Lars O. Tjernberg**:Methodology, Writing - review & editing.**Cyril Hoschl**:Investigation, Writing - review & editing.**Saak V. Ovsepian**:Methodology, Formal analysis, Writing - original draft.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2020.118737.

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