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Original Article

From human dental pulp stem cells to functional cholinergic neurons: An optimized neurogenic differentiation protocol for new approach methodologies

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KEYWORDS
All-trans-retinoic acid;
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Cholinergic neuron;
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Neuronal differentiation;
New approach methodologies

Abstract *Background/purpose:* Drug discovery is now supported by new approach methodologies (NAMs), creating a growing need for in vitro non-animal models that mimic human physiology and, most importantly, function. Secretory function in exocrine organs like salivary glands relies mostly on cholinergic innervation. This study aims to establish an effective NAMs protocol to generate a robust percentage of functional cholinergic neurons by minimizing fetal bovine serum (FBS) and optimizing essential cues like all-trans-retinoic acid (ATRA) and brain-derived neurotrophic factor (BDNF).

Materials and methods: Human dental pulp stem cells (hDPSCs) were differentiated using a two-step media protocol: (1) ATRA (10 μM) in 10 % FBS-enriched medium for 5 days, and (2) BDNF (50 ng/ml) for 7 days. FBS-free and 1 % FBS were compared. Neuronal differentiation efficiency was determined via immunocytochemistry (β-III tubulin, Neurofilament M), Western blot and CellProfiler cell image analysis software (Broad Institute). Committed neurons were assessed for morphology (CellProfiler), viability (Calcein AM, Ethidium homodimer-1), subtype identity (cholinergic receptor muscarinic 1 (CHRM1), cholinergic receptor muscarinic 3 (CHRM3), and choline acetyltransferase (ChAT), and functional activity (glutamate assay).

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Results: Treating hDPSCs with ATRA, followed by BDNF under 1 % FBS, resulted in higher expression of β-III tubulin and Neurofilament M, and cells exhibited lower proliferation rate, developed neuron-like morphology, and maintained high viability at endpoint. CHRM1, CHRM3, and ChAT were expressed in the committed neurons, and the latter two displayed the highest levels. Approximately 97 % of the cells were functional cholinergic neurons.
Conclusion: A two-step protocol using ATRA with 10 % FBS for 5 days, followed by BDNF with 1 % FBS for 7 days, effectively generated a high number of cholinergic neurons.

Introduction

The growing demand for human-relevant and cost-efficient drug discovery and toxicology assessments has driven global efforts to develop and implement new approach methodologies (NAMs). NAMs are not limited to a single test or platform. They encompass in vitro, in silico, and in chemico techniques that do not rely on animal testing and can be applied individually or in combination with other methods.[1,2] In vitro models such as 2D culture, organoids and organ-on-a-chip are among these methods.[3–5]

The utilization of NAMs in dental research remains limited, particularly for both diseased and healthy salivary gland models. To generate models with increased complexity that better replicate human physiology, it is necessary to incorporate relevant cells or tissues. Cholinergic neurons, as a source of parasympathetic innervation essential for salivary gland development and function,[6,7] have been proposed.

Among mesenchymal stem cells, hDPSCs have strong self-renewal and differentiation potential. They are valued for easy surgical access, no reported tumorigenicity, and suitability for cryopreservation.[8,9] Since they originate from the neural crest, hDPSCs have the potential to differentiate into various neural cell types.[10–12]

Retinoic acid and BDNF are two widely utilized agents for neuronal differentiation.[13–17] ATRA is a biologically active derivative of vitamin A.[18] Pre-induction with ATRA promoted the neuronal differentiation of mesenchymal stem cells by activating the retinoic acid receptor beta (RARβ) signaling pathway.[19] BDNF is a member of the neurotrophin family known to exert neurotrophic effects as it regulates neuronal survival, development, and function.[16,20] A study with adult rat hippocampal stem cells showed that retinoic acid induced neuronal differentiation, and subsequent BDNF treatment promoted maturation.[21] In chick embryos, retinoic acid treatment increased the expression of TrkB, the receptor for BDNF. Sequential application of retinoic acid followed by BDNF increased neurite outgrowth two-to three-fold compared to retinoic acid alone.[22] hDPSCs and human neuroblastoma (SH-SY5Y) cells were found to commit to a differentiated phenotype of cholinergic sensory neuronal-like cells after sequential treatment with ATRA and BDNF.[15] Moreover, FBS has been reported to affect neuronal differentiation,[23] but its use is being subdued with the implementation of non-animal NAMs. Hence, the effects of ATRA, BDNF and FBS require further investigation in the establishment of preclinical NAMs for recapitulating functional cholinergic neurons.

Therefore, this study aimed to develop an effective protocol for generating functional cholinergic neurons by optimizing ATRA and BDNF and minimizing FBS use. The resulting neurons will be analyzed for their morphology, neuronal subtype, and functionality. This protocol will benefit the development of NAMs-compatible approaches for generating robust and functional tissue constructs containing cholinergic neurons.

Materials and methods

Culture and characterization of human dental pulp stem cells

This study protocol was approved by the Research Ethics Committee (HREC-DCU 2025-042) and the Institutional Biosafety Committee (DENT CU-IBC 009/2025) of Chulalongkorn University Faculty of Dentistry. All procedures adhered to the Declaration of Helsinki. hDPSCs were purchased from AllCells (Alameda, CA, USA). Cells were cultured in 10 % FBS-enriched medium [DMEM/F12 (Sigma—Aldrich, Merck KGaA, Darmstadt, Germany), 1 % antibiotic-antimycotic (Thermo Fisher Scientific, Waltham, MA, USA), and 10 % FBS (Cytiva, Logan, UT, USA)] and maintained at 37 °C with 5 % CO_2 in a humidified incubator. Cell morphology, population doubling time and surface markers were analyzed as described in Supplementary Data 1 and 2.

Optimization protocol for human dental pulp stem cell differentiation into neurons

In this study, hDPSCs at passages 6—8 were used. The protocol was optimized based on the approach outlined by Al-Maswary et al.[15] Cells were seeded at a density of 625 cells/cm^2, and 5 neuronal differentiation protocols were set up: M1, M2, M3, M2+ATRA + BDNF, and M3+ATRA + BDNF (Fig. 1A). Each protocol consisted of two stages. First stage (5 days), cells in the M1, M2, and M3 groups were cultured in 10 % FBS-medium with 0.1 % DMSO (PanReac AppliChem, Darmstadt, Germany), while cells in the M2+ATRA + BDNF and M3+ATRA + BDNF groups were cultured in 10 % FBS-

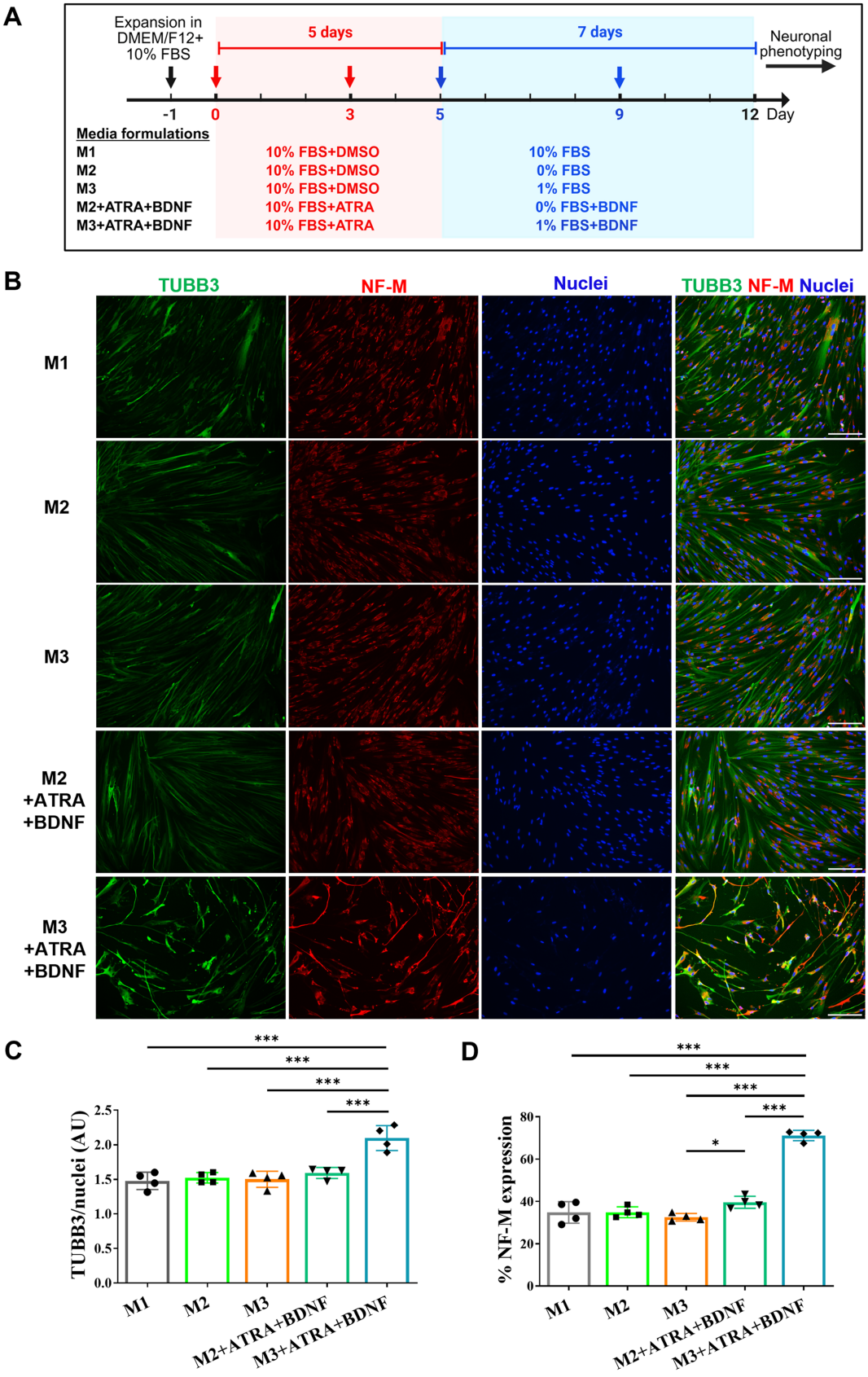

Figure 1 The combination of ATRA, BDNF and 1 % FBS promoted optimal conditions for neuronal differentiation of hDPSCs. (A) A schematic diagram illustrating the experimental conditions used to optimize neuronal differentiation. The differentiation process consisted of two stages, and five media formulations were tested. First stage, cells in the M1, M2, and M3 groups were

medium containing 10 μM ATRA (Sigma—Aldrich). Cells were washed twice with 1X PBS before the next step. Second stage (7 days), cells in the M1, M2, and M3 groups were cultured in 10 % FBS-medium, FBS-free medium (DMEM/F12 and 1 % antibiotic-antimycotic), and 1 % FBS-medium (DMEM/F12, 1 % antibiotic-antimycotic and 1 % FBS), respectively. Cells in the M2+ATRA + BDNF group were cultured in FBS-free medium containing 50 ng/ml BDNF (Sigma—Aldrich), while those in the M3+ATRA + BDNF group were cultured in 1 % FBS-medium containing 50 ng/ml BDNF.

Cell viability and morphological evaluation

To assess cell viability, cells were incubated with 2.5 μM Ethidium Homodimer I (EthD-I; Biotium, Fremont, CA, USA), 1 μg/ml Calcein AM (Thermo Fisher Scientific), and 1 μg/ml Hoechst 33342 (Thermo Fisher Scientific) in an incubator for 40 min. Cells pre-treated with 70 % ethanol for 15 min at 37 °C served as positive controls for dead cells. Micrographs were captured using a fluorescence microscope, EVOS FL Auto (EVOS, Thermo Fisher Scientific). Numbers of dead and live cells were counted by Image J version 1.54 g (NIH, Bethesda, MD, USA) and reported as a percentage of cell viability. Nuclear and cellular morphology were evaluated using CellProfiler version 4.2.1 (Broad Institute, Cambridge, MA, USA).

Immunocytochemistry

Cells were processed under standard immunocytochemistry protocol. Cells were fixed, blocked and stained with specific primary antibodies and fluorescent conjugated secondary antibodies, respectively. Next, nuclei were stained with Hoechst 33342. Cellular membranes were stained with PhenoVue Fluor 488-Concanavalin A (Revvity, Waltham, MA, USA). Full immunocytochemistry protocol and antibodies are detailed in Supplementary Data 3 and Supplementary Table 1. Samples were visualized using an EVOS FL fluorescence microscope and a Zeiss LSM 980 confocal microscopy with Airyscan (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Fluorescence intensity was quantified with ImageJ version 1.54 g and CellProfiler version 4.2.1.

Western blot analysis

For protein extraction, cells were lysed in cold RIPA buffer (Thermo Fisher Scientific) with protease inhibitor cocktail (HiMedia Laboratories, Mumbai, Maharashtra, India). Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific). Proteins were separated by 10 % SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific). Membranes were blocked with 3 % skim milk, then stained with primary and peroxidase-conjugated secondary antibodies as shown in Supplementary Table 1. Protein bands were visualized using Luminol reagent (Santa Cruz Biotechnology) and analyzed using ImageJ version 1.54 g.

Glutamate release assay

Committed cholinergic neurons were seeded at 6000 cells/well into 24-well plates. On day 12, media were completely removed and replaced with fresh media consisting of DMEM/F12,1 % FBS, 2.5 mM glutamine (Cytiva), and 1 % antibiotic-antimycotic. Cells were incubated 24 h, before culture media and cellular proteins were collected and stored at −80 °C until analysis. Glutamate levels in the media were measured using a glutamate assay kit (#AB1388883, Abcam). Glutamate release was reported as the amount of glutamate per μg of total protein.

Statistical analysis

All data were analyzed using GraphPad Prism 10.0 software (GraphPad Software, San Diego, CA, USA) and are presented as mean ± standard deviation (SD). A P-value of <0.05 was considered statistically significant. Statistical analyses included unpaired Student's t-test, one-way and two-way ANOVA, as indicated in each figure caption.

Results

Efficiency of neuronal commitment was promoted by all-trans-retinoic acid (ATRA) and brain-derived neurotrophic factor (BDNF)

The population doubling time and morphology of hDPSCs were consistent and stable between passages 6—8 (Supplementary Fig. 1). Consistent with mesenchymal stem cell phenotype, hDPSCs were positive for mesenchymal stem cell-specific markers including CD90, CD44, CD73, and CD105, and negative for the hematopoietic marker, CD34 (Supplementary Fig. 2).

cultured in 10 % FBS-medium with 0.1 % DMSO (vehicle control) while cells in the M2+ATRA + BDNF and M3+ATRA + BDNF groups were cultured in 10 % FBS-medium containing 10 μM ATRA. Second stage, cells in the M1, M2, and M3 groups were cultured in 10 % FBS-, FBS-free, and 1 % FBS-medium, respectively. Cells in the M2+ATRA + BDNF group were cultured in FBS-free medium containing 50 ng/ml BDNF, while those in the M3+ATRA + BDNF group were cultured in 1 % FBS-medium containing 50 ng/ml BDNF. During the process, the medium was changed twice in the first stage (red arrows) and twice in the second stage (blue arrows). (B) Immunofluorescence images showing the expression of neuronal proteins TUBB3 and NF-M on day 12 of differentiation. Scale bar: 200 μm. (C, D) Graphs presenting the quantitative analysis of TUBB3 (C) and NF-M (D) expression. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test (n = 4 per group). Bars represent mean ± SD. *$P < 0.05$, ***$P < 0.001$. ATRA: all-trans-retinoic acid, AU: arbitrary units, BDNF: brain-derived neurotrophic factor, DMEM/F12: Dulbecco's modified eagle's medium/nutrient mixture F-12 Ham, DMSO: dimethyl sulfoxide, FBS: fetal bovine serum, hDPSCs: human dental pulp stem cells, NF-M: neurofilament M, TUBB3: β-III tubulin. Fig. 1A was created in BioRender. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

β-III tubulin is widely used as a pan-neuronal marker since it's expressed throughout neuronal development.[24] Neurofilament M is a marker of mature neurons. Increased Neurofilament M expression typically correlates with neuronal maturation, including axonal growth and stability.[25] Hence, an increase in both the expression of β-III tubulin and Neurofilament M indicates an effective neuronal commitment. Cells cultured in media formulations without ATRA and BDNF (specifically M1, M2, and M3) exhibited low expression levels of β-III tubulin and NF-M, with no significant differences between them. This suggests that FBS alone does not promote neuronal differentiation. While the M2+ATRA + BDNF group slightly increased Neurofilament M expression, the M3+ATRA + BDNF group significantly increased both β-III tubulin and Neurofilament M expression (Fig. 1B–D). Therefore, treatment with ATRA in 10 % FBS followed by BDNF in 1 % FBS is the most effective media formulation protocol for the neuronal commitment of hDPSCs.

Differentiated cells exhibited neuronal characteristics

The cell viability was measured using Calcein AM and Ethidium homodimer-1 staining. The positive control for cell death in Ethidium homodimer-1 staining was cells treated with 70 % ethanol (Supplementary Fig. 3). Both the M3 and M3+ATRA + BDNF groups maintained a high proportion of viable cells (>97 %) and a low number of dead cells up to day 12 of differentiation (Fig. 2A and B). However, the M3+ATRA + BDNF group exhibited a lower overall cell number than the M3 group (Fig. 2A). In terms of morphology, cells in the M3+ATRA + BDNF group began to present neuronal characteristics, such as a phase-bright cell body, long, thin axons, and sprouting dendrites displaying a bipolar morphology, from day 1 to day 5 following ATRA supplementation. These features became more pronounced after BDNF treatment from day 6–12 (Figs. 2A and 3A). These results were supported by cellular and nuclear

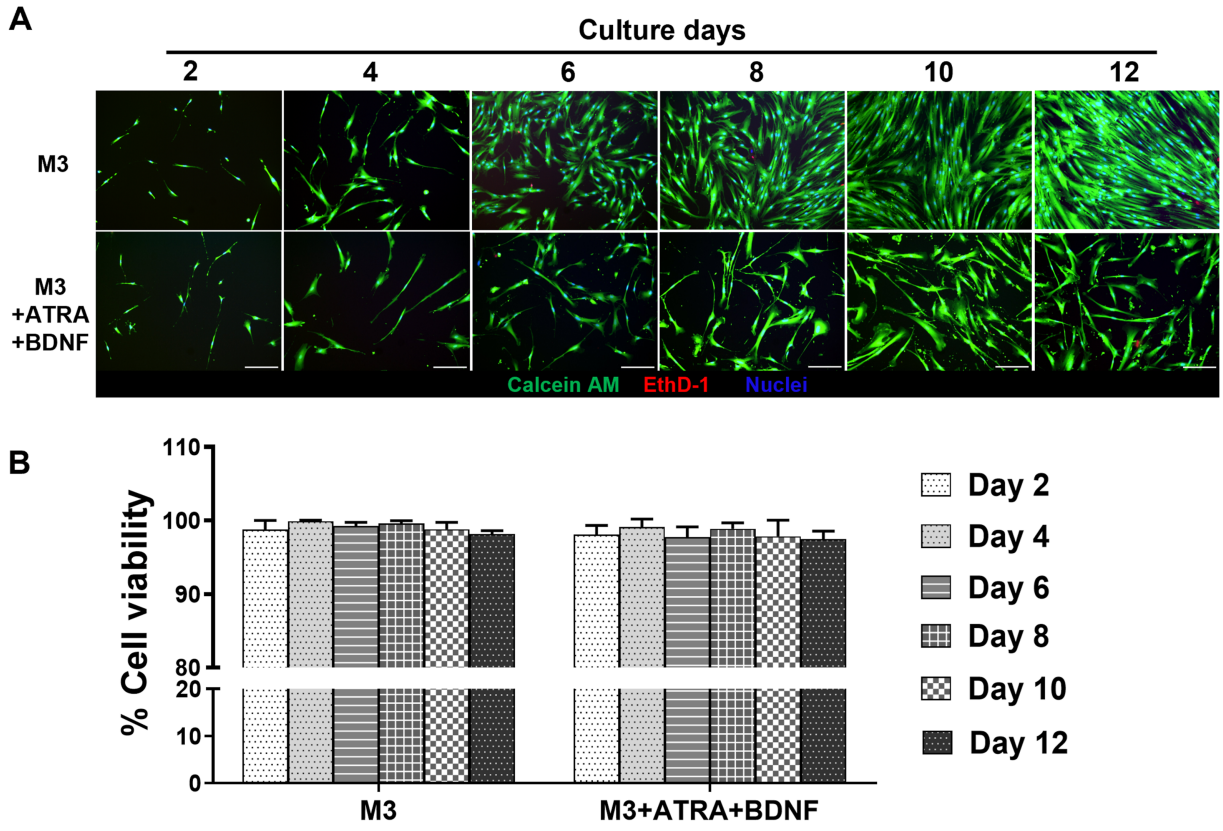

Figure 2 A lower proliferation rate and a shift toward cell differentiation, along with high cell viability, were observed in hDPSCs under the M3 condition supplemented with ATRA and BDNF. (A) Representative images showing cell viability. Calcein AM, Ethidium homodimer-1 (EthD-1), and Hoechst 33342 stained live cells (green), dead cells (red), and nuclei (blue), respectively. (B) Graph illustrating the percentage of viable cells. Data were analyzed using two-way ANOVA. Values at each time point were compared to day 2 within the same group (n = 5 per group). Bars represent mean ± SD. Scale bar: 200 μm. ATRA: all-trans-retinoic acid, BDNF: brain-derived neurotrophic factor, hDPSCs: human dental pulp stem cells, M3: The condition in which cells were first cultured in 10 % fetal bovine serum (FBS)-medium with 0.1 % dimethyl sulfoxide (DMSO; vehicle control) for 5 days, then switched to 1 % FBS-medium for 7 days, M3+ATRA + BDNF: The condition in which cells were first cultured in 10 % FBS-medium with 10 μM ATRA for 5 days, then switched to 1 % FBS-medium with 50 ng/ml BDNF for 7 days. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

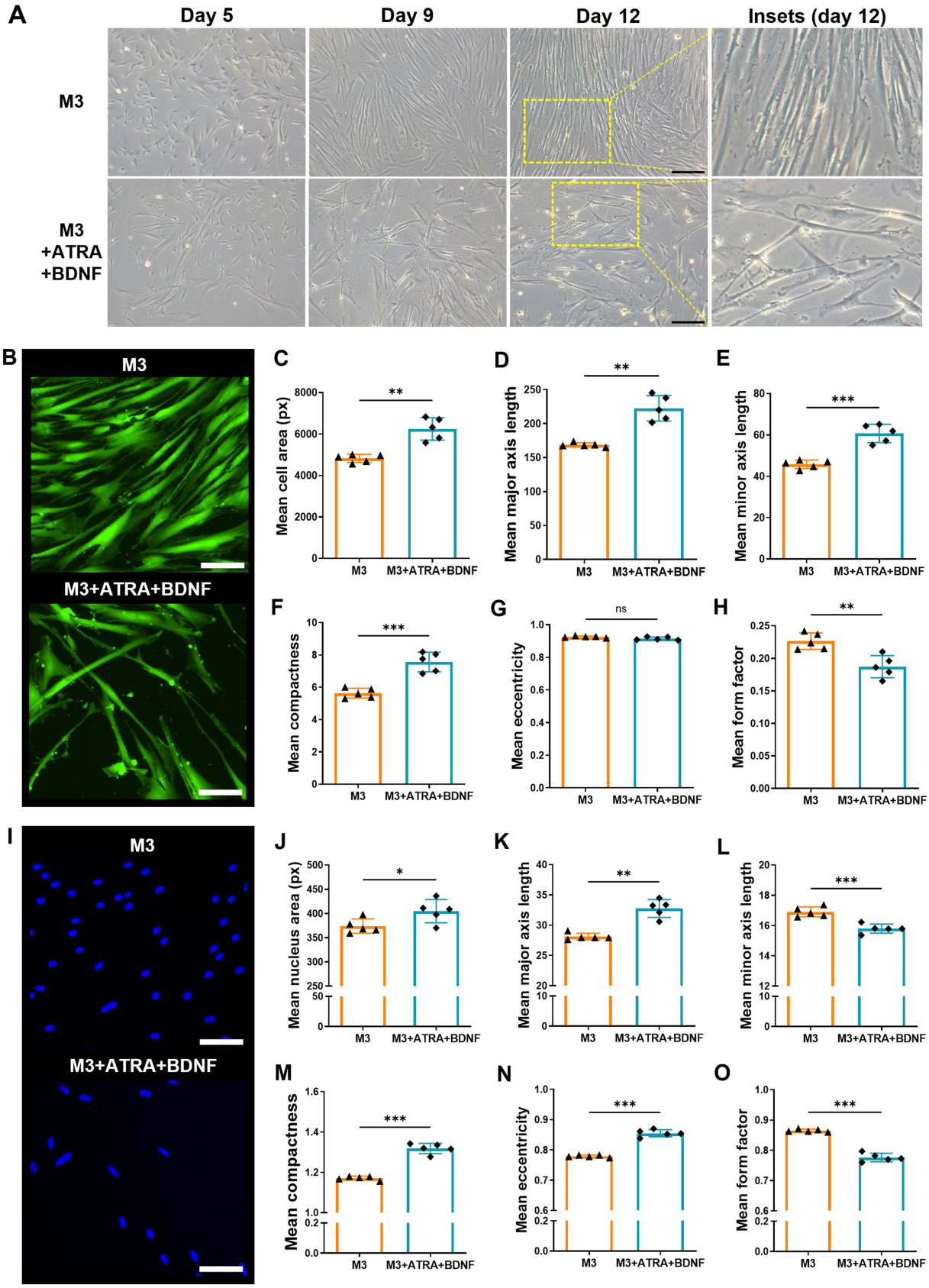

Figure 3 Sprouting neuron-like, especially bipolar, morphology was observed in hDPSCs cultured in M3 with ATRA and BDNF. (A) Morphological changes observed during the differentiation of hDPSCs into neurons in phase-contrast light microscopy. (B) Fluorescence micrographs of Calcein AM (green) and Hoechst 33342 (blue) staining of hDPSCs at day 12 of differentiation were

morphological imaging analysis using CellProfiler. In the M3+ATRA + BDNF group, cells showed larger area, major and minor axis lengths, and greater compactness, but a lower form factor than in M3, suggesting these cells were larger and more elongated (Fig. 3B–H). The nuclei in M3+ATRA + BDNF also had greater area, major axis length, compactness, and eccentricity, while lower minor axis length and form factor compared to M3, suggesting longer but slimmer nuclei (Fig. 3I–O). Definitions for all parameters are available in Supplementary Table 2. Based on the combination of cellular morphological changes and reduced proliferation, cells treated with the M3+ATRA + BDNF protocol were confirmed to have differentiated into neurons.

Neurons derived from human dental pulp stem cells displayed cholinergic features

To characterize hDPSC-derived neuron subtypes, the expression of cholinergic receptor muscarinic 1 (CHRM1), cholinergic receptor muscarinic 3 (CHRM3), and choline acetyltransferase (ChAT), which are key markers of cholinergic neurons, was examined. Immunocytochemistry and Western blot analyses showed higher CHRM3 and ChAT expression in the M3+ATRA + BDNF group than in the M3 group, while CHRM1 expression was similar in both (Fig. 4A–D, E and 5A–F). The proportion of cholinergic to non-cholinergic neurons was assessed based on ChAT expression by immunocytochemistry. In Fig. 4B and C, the M3+ATRA + BDNF group showed a higher percentage of cholinergic neurons (96.8 % ± 3.8 %) compared to the M3 group (44.1 % ± 21.0). These findings suggest that the combination of ATRA, BDNF, and a limited FBS (1 %) effectively drives the commitment of hDPSCs into cholinergic neurons.

Neurons derived from human dental pulp stem cells exhibited neuronal function

Glutamate serves as an excitatory neurotransmitter and measuring its release can validate the excitatory neuronal function of the neuron-like cells. Cells in the M3+ATRA + BDNF formulation robustly released higher levels of glutamate than those in the control M3 group (Fig. 6), indicating that these cells were functionally mature and capable of excitatory neurotransmitter release.

Discussion

Due to their simple isolation, clonogenic potential, rapid proliferation, lack of reported tumorigenicity, and absence of major ethical concerns, hDPSCs represent a promising postnatal source of mesenchymal stem cell-like cells for tissue biofabrication via NAMs to conduct preclinical studies.[8,9,26] The neural crest origin of hDPSCs makes them a promising candidate for neuronal differentiation.[10,11] Our study aimed to assess the effects of ATRA and BDNF while minimizing FBS on the neuronal commitment of hDPSCs. In addition to optimizing the differentiation protocol, we also characterized the resulting neuron subtypes.

To minimize the use of FBS, a second stage with either FBS-free and 1 % FBS were compared. We demonstrated that using ATRA in 10 % FBS (first stage), followed by BDNF in 1 % FBS (M3+ATRA + BDNF) resulted in optimal neuronal differentiation, as indicated by the higher expression of neuronal markers β-III tubulin and Neurofilament M as compared to FBS-free protocol (M2+ATRA + BDNF).

ATRA and BDNF are among the inducers that promote the differentiation of stem cells into neurons. ATRA regulates the cell cycle to halt proliferation and initiate differentiation.[14,17] BDNF plays a crucial role in differentiation, maturation, synaptic function, and the survival of neurons.[27,28] Our results were consistent with these functions. The morphological change from fibroblast-like cells to neuron-like cells (phase-bright cell body, long, thin axons, and sprouting dendrites), along with the lower cell number in the M3+ATRA + BDNF group compared to the M3 control group, indicated that hDPSCs shifted from proliferation to differentiation. Cells in M3+ATRA + BDNF group still retained high viability (97.4 % ± 1.1), comparable to M3 group (98.2 % ± 0.5), indicating that the survival rate of neurons remained high, like the control group. Moreover, Neurofilament M expression was markedly elevated in the M3+ATRA + BDNF group, further supporting the successful differentiation and maturation of hDPSC-derived neurons. Importantly, cells from the M3+ATRA + BDNF condition were predominantly cholinergic, with a high yield of ~97 %. The M3 control condition also produced cholinergic neurons (~44 %), likely due to the presence of FBS and the neural crest origin of hDPSCs, as both support neuronal differentiation.

In neurons, glutamate is the most abundant excitatory neurotransmitter, released by presynaptic vesicles through calcium-dependent vesicular exocytosis.[29] Therefore, glutamate release indirectly indicates neuronal function. Cholinergic neurons generated under the M3+ATRA + BDNF experimental setup exhibited greater glutamate release than those from the M3 formulation, demonstrating that they are not only neuron-like in terms of morphology but also functionally active. Hence, this result may imply that combining ATRA and BDNF under 1 % FBS conditions

analyzed for cellular and nuclear characteristics using CellProfiler software. Micrographs illustrate cells (B) and nuclear (I) morphologies. Plots represent in the Y-axis the mean area, major axis length, minor axis length, compactness, eccentricity, and form factor of cells (C–H) and nucleus (J–O), respectively. Data were analyzed using an unpaired Student's t-test. n = 5 per group, with 6–14 selected regions of interest (ROIs) of each sample. Bars represent mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: not significant. Scale bar: 200 μm. ATRA: all-trans-retinoic acid, BDNF: brain-derived neurotrophic factor, hDPSCs: human dental pulp stem cells, M3: The condition in which cells were first cultured in 10 % fetal bovine serum (FBS)-medium with 0.1 % dimethyl sulfoxide (DMSO; vehicle control) for 5 days, then switched to 1 % FBS-medium for 7 days, M3+ATRA + BDNF: The condition in which cells were first cultured in 10 % FBS-medium with 10 μM ATRA for 5 days, then switched to 1 % FBS-medium with 50 ng/ml BDNF for 7 days, px: pixels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

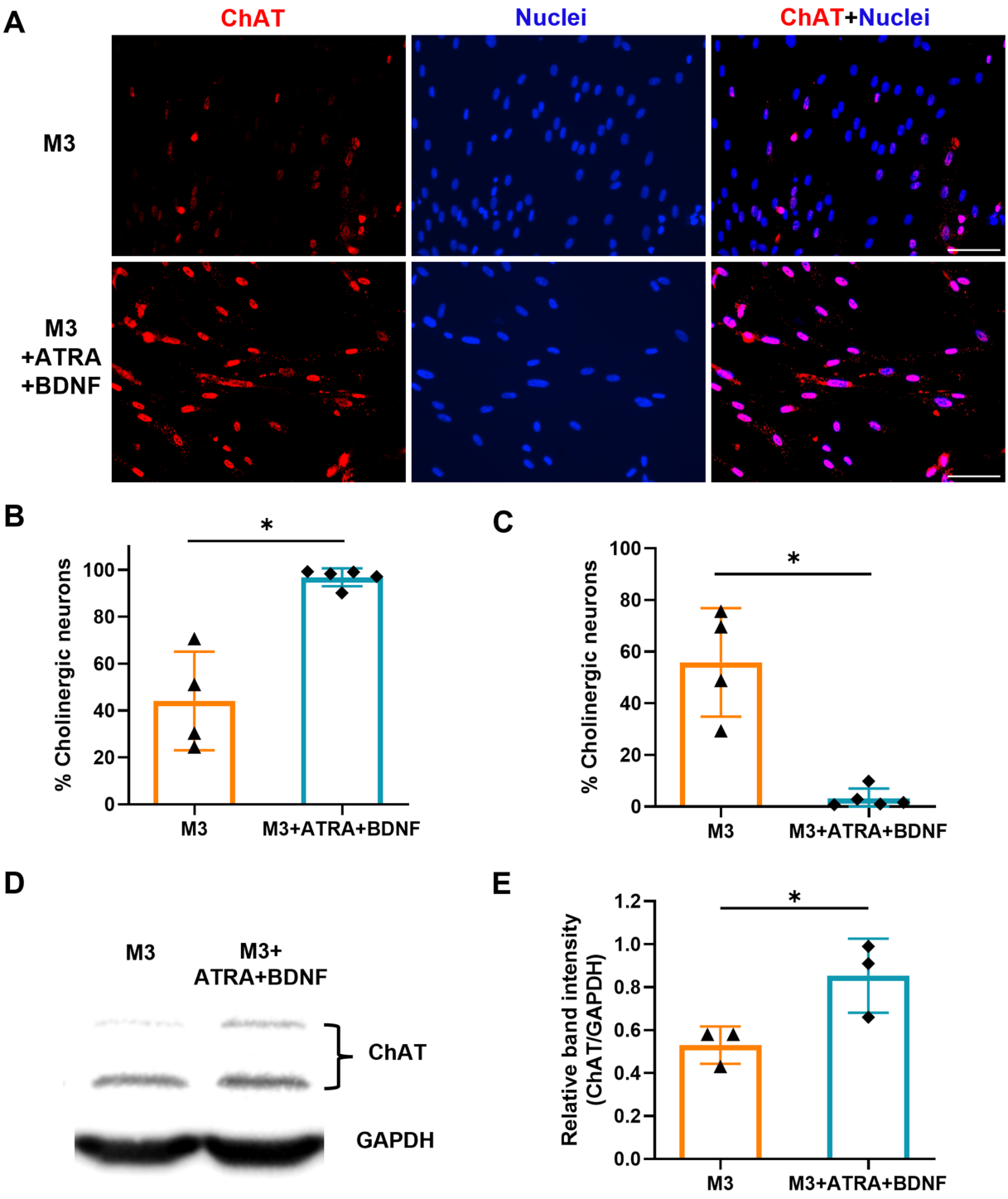

Figure 4 Majority of cells under the M3 condition supplemented with ATRA and BDNF were cholinergic neurons. (A) Immunocytochemistry showing choline acetyltransferase (ChAT) expression in differentiated cells. Based on ChAT expression, the percentages of cholinergic and non-cholinergic neurons are shown in (B) and (C), respectively. (D) Western blot showing ChAT protein bands, with corresponding quantification presented in (E). Data were analyzed using an unpaired Student's t-test (n = 4–5 per group). Bars represent mean ± SD. *$P < 0.05$. Scale bar: 200 μm. ATRA: all-trans-retinoic acid, BDNF: brain-derived neurotrophic factor, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, M3: The condition in which cells were first cultured in 10 % fetal bovine serum (FBS)-medium with 0.1 % dimethyl sulfoxide (DMSO; vehicle control) for 5 days, then switched to 1 % FBS-medium for 7 days, M3+ATRA + BDNF: The condition in which cells were first cultured in 10 % FBS-medium with 10 μM ATRA for 5 days, then switched to 1 % FBS-medium with 50 ng/ml BDNF for 7 days.

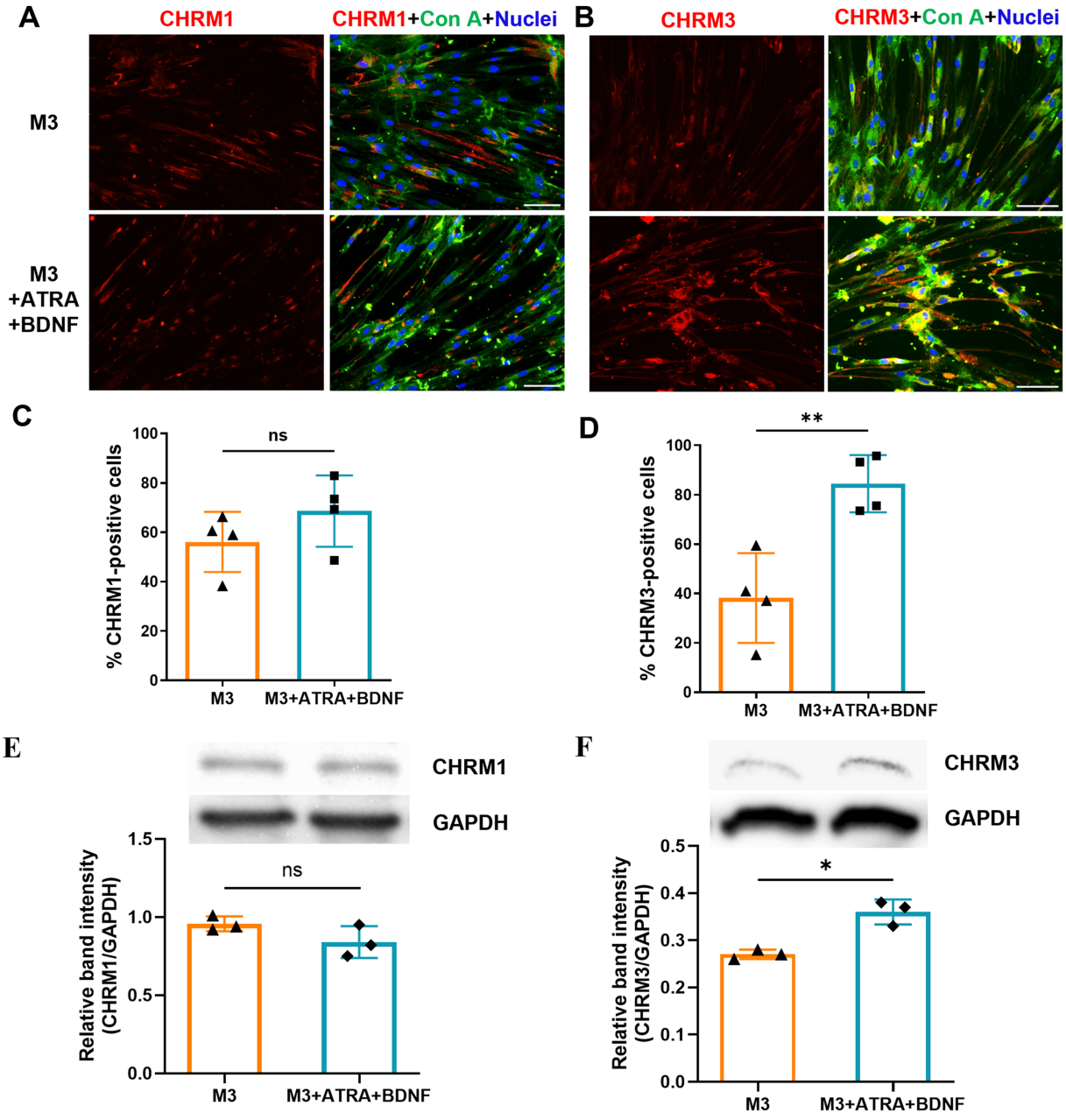

Figure 5 The M3 condition supplemented with ATRA and BDNF induced a high percentage of CHRM3-positive cells. Fluorescence micrographs display the expression of cholinergic receptor muscarinic 1 (CHRM1; red) (A) and cholinergic receptor muscarinic 3 (CHRM3; red) (B) on day 12 of culture. The cell membrane was stained with PhenoVue Fluor 488—Concanavalin A (Con A; green). Graphs display the expression of CHRM1 (C) and CHRM3 (D) based on immunocytochemistry (n = 4 per group). Representative Western blot bands and corresponding quantification plots exhibit CHRM1 (E) and CHRM3 (F) expression (n = 3 per group). Data were analyzed using an unpaired Student's t-test. Bars represent mean ± SD. *$P < 0.05$, **$P < 0.01$, ns: not significant. Scale bar: 100 μm. ATRA: all-trans-retinoic acid, BDNF: brain-derived neurotrophic factor, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, M3: The condition in which cells were first cultured in 10 % fetal bovine serum (FBS)-medium with 0.1 % dimethyl sulfoxide (DMSO; vehicle control) for 5 days, then switched to 1 % FBS-medium for 7 days, M3+ATRA + BDNF: The condition in which cells were first cultured in 10 % FBS-medium with 10 μM ATRA for 5 days, then switched to 1 % FBS-medium with 50 ng/ml BDNF for 7 days. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

promoted synaptic maturation and neurotransmitter processing.

Previously, Al-Maswary et al. reported using a similar differentiation protocol but without FBS during the BDNF induction stage and reported that hDPSCs have the potential to differentiate into cholinergic neuron-like cells; however, the efficiency of such commitment (the percentage of differentiated neuron-like cells) was not determined.[15] In our study, using FBS-free media during the BDNF induction stage resulted in reduced neuronal differentiation when

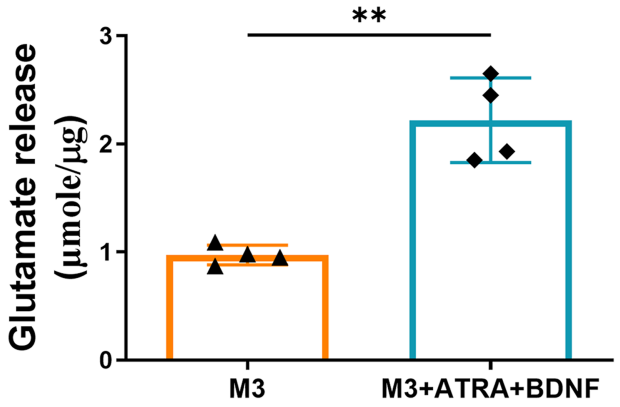

Figure 6 Cholinergic neurons under the M3 condition supplemented with ATRA and BDNF exhibited functional neuronal activity. Data were analyzed using an unpaired Student's t-test. n = 4 per group. Bars represent mean ± SD. $^{**}P < 0.01$. ATRA: all-trans-retinoic acid, BDNF: brain-derived neurotrophic factor, M3: The condition in which cells were first cultured in 10 % fetal bovine serum (FBS)-medium with 0.1 % dimethyl sulfoxide (DMSO; vehicle control) for 5 days, then switched to 1 % FBS-medium for 7 days, M3+ATRA + BDNF: The condition in which cells were first cultured in 10 % FBS-medium with 10 μM ATRA for 5 days, then switched to 1 % FBS-medium with 50 ng/ml BDNF for 7 days.

compared 1 % FBS. This suggests that FBS is still holding a few essential biological factors that drive cholinergic neuron commitment of hDPSCs, and such factors require further identification to establish a 100 % chemically defined media without non-human-derived components. Basic fibroblast growth factor (bFGF), one example of such factors present in FBS, has been reported to affect cholinergic neuron differentiation.[30] The limitation of FBS is that it is a natural medium with a complex, variable composition that may differ between brands and batches.[31] Therefore, its use should be controlled to ensure consistency.

In conclusion, our work confirmed the roles of ATRA and BDNF in effectively driving neuronal differentiation. Culturing hDPSCs with 10 μM ATRA in 10 % FBS, followed by treatment with 50 ng/ml BDNF in 1 % FBS, efficiently generated highly functional cholinergic neurons. These findings advance our understanding of cholinergic differentiation from hDPSCs, but more key biological cues need to be identified, if one needs to establish a serum-free media. While our current study does not include co-culture experiments with salivary gland tissue, the protocol may offer potential for future integration into salivary gland-on-chip systems. Further studies are needed to validate this application.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2025.10.025.

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