

Original Article

The natural product 4,10-aromadendranediol induces neuritogenesis in neuronal cells *in vitro* through activation of the ERK pathway

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Abstract

Recent studies focus on promoting neurite outgrowth to remodel the central nervous network after brain injury. Currently, however, there are few drugs treating brain diseases in the clinic by enhancing neurite outgrowth. In this study, we established an NGF-induced PC12 differentiation model to screen novel compounds that have the potential to induce neuronal differentiation, and further characterized 4,10-Aromadendranediol (ARDD) isolated from the dried twigs of the *Baccharis gaudichaudiana* plant, which exhibited the capability of promoting neurite outgrowth in neuronal cells *in vitro*. ARDD (1, 10 $\mu\text{mol/L}$) significantly enhanced neurite outgrowth in NGF-treated PC12 cells and N1E115 cells in a time-dependent manner. In cultured primary cortical neurons, ARDD (5, 10 $\mu\text{mol/L}$) not only significantly increased neurite outgrowth but also increased the number of neurites on the soma and the number of bifurcations. Further analyses showed that ARDD (10 $\mu\text{mol/L}$) significantly increased the phosphorylation of ERK1/2 and the downstream GSK-3 β , subsequently induced β -catenin expression and up-regulated the gene expression of the Wnt ligands Fzd1 and Wnt3a in neuronal cells. The neurite outgrowth-promoting effect of ARDD in neuronal cells was abolished by pretreatment with the specific ERK1/2 inhibitor PD98059, but was partially reversed by XAV939, an inhibitor of the Wnt/ β -catenin pathway. ARDD also increased the expression of BDNF, CREB and GAP-43 in N1E115 cells, which was reversed by pretreatment with PD98059. In N1E115 cells subjected to oxygen and glucose deprivation (OGD), pretreatment with ARDD (1–10 $\mu\text{mol/L}$) significantly enhanced the phosphorylation of ERK1/2 and induced neurite outgrowth. These results demonstrated that the natural product ARDD exhibits neurite outgrowth-inducing activity in neurons via activation of the ERK signaling pathway, which may be beneficial to the treatment of brain diseases.

Keywords: 4,10-aromadendranediol; neurite growth; PC12 cells; N1E115 cells; cortical neurons; ERK1/2; OGD; brain diseases; NGF; PD98059; XAV939

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Introduction

During the process of forming a central network, progenitor cells extend neurites that eventually become axons and dendrites to establish connections with other neurons. In network development, axon elongation, directed by the growth cone at the tip of the neurite, stops and shapes the correct neural architecture through multiple molecules^[1]. In the beginning of neurite sprouting, several ECM-associated molecules, such as

laminin, fibronectin and neurotrophic factors, trigger neurite outgrowth and promote neuritogenesis^[2]. After the mature network construction has been achieved, inhibitory molecules are required to stop neuritogenesis to sustain the precise network^[3]. Thus, even though axonal regeneration is a key repair process leading to a reduction in neurological deficits after central nervous system (CNS) injuries, such as ischemic stroke and Alzheimer's disease, it is difficult to carry out. The growth suppressors Nogo (neurite outgrowth inhibitor) and MAG (myelin associated glycoprotein) are released by injured cells and limit the intrinsic capacity of mature cells, and are two main factors that prevent axonal regeneration^[4,5].

Toxicity factors induced by brain diseases suppress neuro-

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nal differentiation and survival by disturbing the functions of neurotrophins including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF)^[6, 7]. It is reported that BDNF reduction may exacerbate brain disease deterioration. Maintaining BDNF levels is important in rebuilding the CNS network. Although the exact pathway involved in the BDNF-induced regulation of neurite growth is not fully established, most research reveals that BDNF mediates several intracellular signaling pathways such as Akt/PI3K, phospholipase C, and MAPK/ERK, which regulate the transcription factor CREB and neurite growth-related gene expression^[8].

MAPK/ERK associates with many other signaling pathways, such as the Wnt/ β -catenin pathway involved in the inhibition of GSK-3 β activity, to regulate cell growth. In the regular canonical Wnt pathway, when the Wnt ligand does not combine with the Fzd1 receptor, GSK-3 β interacting with the APC complex accelerates β -catenin ubiquitination and degradation, but when the Wnt ligand combines with the Fz1 receptor, the activity of GSK-3 β is reduced and it dissociates from β -catenin, accumulates and translocates into the nucleus to induce target gene expression^[9]. Therefore, ERK pathway activation leads to inactive GSK-3 β phosphorylation, which is involved in neuronal outgrowth to regulate target gene expression.

Recent studies focus on promoting neurite outgrowth by regulating extracellular molecules to remodel the central nervous network after brain injury. BDNF and Ganglioside endogenous factors derived from the brain have prominent abilities in enhancing neurogenesis, but both of them show weak effects as exogenous treatments^[10]. There are few drugs treating brain diseases by enhancing neurite outgrowth in the clinic^[11]. In this study, based on an established *in vitro* neurite outgrowth model, we first discovered one natural product, 4,10-Aromadendranediol (ARDD), with the capability of promoting neurite outgrowth in neuronal cells. ARDD is isolated from the dried twigs of the *Baccharis gaudichaudiana* plant, which is used for the treatment of diabetes and gastrointestinal diseases, but the function of ARDD in brain diseases is unknown^[12]. Our group, for the first time, found its pharmacological function and related underlying mechanisms, which may provide insight into treating brain diseases that are associated with neuronal injury.

Materials and methods

Materials

The natural compound 4,10-Aromadendranediol (ARDD, powder purity over 98%) was obtained from Yunnan Xili Company (China). PD98059 (inhibitor of ERK), edaravone and mNGF (mouse nerve growth factor) were purchased from Sigma-Aldrich. XAV939 (inhibitor of β -catenin) was purchased from Selleck (Houston, TX, USA). For all the experiments, these compounds were prepared by diluting the stock with culture medium (the final concentration of dimethyl sulfoxide was less than 0.1%). Cell culture medium and supplements were purchased from Invitrogen (Carlsbad, CA, USA). All other regular reagents were obtained from Sigma-Aldrich.

Cell culture and drug treatment

PC12 cells, from a rat pheochromocytoma cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Undifferentiated PC12 cells were cultured in DMEM complete medium with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin and maintained at 37°C in a humidified incubator supplemented with 5% CO₂. To induce differentiation^[13], PC12 cells were seeded at a density of 2 \times 10⁴ cells/mL on a poly-L-lysine-coated 96-well plate and cultured in complete medium containing 2.5 ng/mL NGF or different concentrations of ARDD (0.1, 1, 10 μ mol/L) concomitant with 2.5 ng/mL NGF administration. Four days after incubation, morphometric analysis was performed on digitized images of live cells taken under phase contrast illumination with an inverted microscope linked to a camera. Images of five fields per well were taken with an average of 20 cells per field. The number of differentiated cells was determined by counting cells that had at least one neurite with a length equal to the cell body diameter and was expressed as a percentage of the total cells in the field. The counting was performed in a blinded manner. All experiments were performed at least three times^[14].

N1E115 cells, from a mouse neuroblastoma cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Undifferentiated N1E115 cells were cultured in DMEM complete medium with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin and maintained at 37°C in a humidified incubator supplemented with 5% CO₂. To induce differentiation, N1E115 cells were seeded at a density of 5 \times 10³ cells/mL on poly-L-lysine-coated 96-well plates, 12-well plates and 6-well plates and grown in serum-free medium^[15] or different concentrations (1, 5, 10 μ mol/L) of ARDD for four days. To test the inhibitor effects, N1E115 cells were pretreated with PD98059 (10 μ mol/L) or XAV939 (10 μ mol/L) for an hour and then cultured as before. Four days after incubation, immunofluorescence was performed on digitized images of cells taken under confocal microscopy (Olympus, Japan). Images of five fields per well were taken with an average of 100 cells per field. Cells that had at least one neurite with a length that was twice as long as the body diameter were expressed as a percentage of the total cells in the field. The counting was performed in a blinded manner. All experiments were performed at least three times.

Cortical neurons were isolated as previously described^[16]. Primary neuronal cells were separated from the cerebral cortices of Sprague Dawley rats at embryonic 16–18 d. Tissue was digested with trypsin (0.05% trypsin in EDTA maintained at 37°C) for 10 min. The trypsin solution was decanted, and the tissue was washed three times with DMEM supplemented with L-glutamine and glucose. Cultures were maintained in Neurobasal medium supplemented with 2% B27 and 1% Glutamax. The cells were dissociated mechanically and plated on 12-well plates pre-coated with poly-L-lysine. After 4 h of culture, neurons were treated with 100 ng/mL NGF or different

concentrations of ARDD (1, 5, 10 $\mu\text{mol/L}$) for 24 h. Immunofluorescence was performed on digitized images of cells taken under confocal microscopy (Olympus, Japan). Images of five fields per well were taken with an average of 50 cells per field. The counting was performed in a blinded manner. All experiments were performed at least three times.

Western blotting

To determine the levels of protein expression, N1E115 cells obtained from different treatment groups were washed with cold PBS and collected with Tris-Glycine SDS lysis buffer^[17]. The mixtures were collected as the whole cell extracts. Cell extracts were separated by 10% SDS-PAGE, and then transferred onto PVDF membranes. The membranes were blocked for 1 h with 3% bovine serum albumin (BSA) in Tris-buffered saline plus Tween 20 (TBST, pH 7.4) and then probed with the specific primary antibodies of rabbit anti-phospho-ERK1/2 (1:1000), rabbit anti-phospho-GSK-3 β -Ser9 (1:1000), mouse anti- β -actin (1:2000), rabbit anti- β -catenin (1:1000), rabbit anti-phospho-CREB (1:1000), and rabbit anti-GAP-43 (1:1000). After several washes with TBST, the membranes were incubated with the secondary antibodies of anti-rabbit IgG (1:10000; SunShineBio, China) or anti-mouse IgG (1:10000; SunShineBio, China) for 1 h at room temperature, then blots were visualized using chemiluminescence with a Bio-Rad ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). The resulting bands were quantified using densitometric analysis and were normalized to the levels of β -actin protein.

Reverse transcription-polymerase chain reaction (RT-PCR)

To determine the levels of gene expression, N1E115 cells obtained from different treatment groups were washed with cold phosphate buffer saline (PBS, pH 7.2) and total RNA was extracted using Trizol reagent (Invitrogen, USA). Reverse transcription was performed using a cDNA synthesis kit (Vazyme, Nanjing, China) following standard techniques.

Table 1. Primers sequence.

Gene	Sequence
Fzd1	
Forward	5'-CAGCAGTACAACGGCGAAC-3'
Reverse	5'-GTCCTCCTGATTCGTGTGGC-3'
BDNF	
Forward	5'-TCATACTCGGTTGCATGAAGG-3'
Reverse	5'-ACACCTGGGTAGGCCAAGTT-3'
Wnt3a	
Forward	5'-CTCCTCTCGGATACCTCTTAGTG-3'
Reverse	5'-CCAAGGACCACCAGATCGG-3'
GAPDH	
Forward	5'-GGCCTCCGTGTTCTAC-3'
Reverse	5'-TGTCATCATATCTGGCAGGT-3'

For PCR analysis, 5 μL of cDNA was used as a template and amplified using specific primers (Table 1). Each PCR mixture contained 5 μL of cDNA, 1 μL of each primer (10 $\mu\text{mol/L}$), 3.5 μL of ddH₂O, 10.5 μL of SYBR Green Premix (Vazyme, Nanjing, China). The relative quantitative expression was measured in triplicate on a Real-time PCR Applied Biosystems Step One Plus Detection System (USA) where the GAPDH levels served as a control. The amplification conditions were 95°C for 5 min, 40 cycles of 95°C for 10 s, 60°C for 30 s. The specificity of amplification was confirmed by a melting curve.

Immunofluorescence staining

N1E115 cells or primary cortical neurons exposed to drug treatment were seeded on coverslips pre-coated with poly-L-lysine. For immunofluorescence staining, the cells were fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized in PBS containing 0.1% Triton X-100 for 10 min and blocked with 5% bovine serum albumin. Then, the coverslips were incubated overnight at 4°C with anti-MAP-2 antibody (1:500; Millipore). Nuclei were labeled with Hoechst 33342 (Beyotime, Haimen, China) at room temperature. After washing three times with PBS, cells were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Life, USA) for 60 min. The fluorescence was then examined with confocal microscopy (Olympus FV1000, Japan).

Oxygen and glucose deprivation (OGD)

N1E115 cells were pretreated with ARDD at a density of 5×10^3 cells/mL for two days, and then the complete medium was replaced with glucose-free Earle's balanced salt solution (EBSS, pH 7.4) and transferred to a hypoxia chamber containing a mixture of 95% N₂ and 5% CO₂ for 30 min. After OGD, the complete medium was replaced for an additional 2 d under normal conditions with 95% air and 5% CO₂ at 37°C. Control cells without OGD were maintained under normal conditions^[18].

MTT assay

N1E115 cells were pretreated with 10 $\mu\text{mol/L}$ of ARDD or 50 $\mu\text{mol/L}$ of edaravone (Eda) at a density of 5×10^3 cells/mL for two days and then underwent the OGD procedure as described above. After OGD exposure, cells were incubated with 0.5 mg/mL MTT for 4 h in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. After 4 h, the medium was replaced with DMSO, and the absorbance was measured at 570 nm in a Bio-Rad plate-reader^[19].

Statistical analysis

Statistical analysis was performed using GraphPad 6.0 software (GraphPad Software, Inc, La Jolla, CA, USA), and data were obtained by applying a one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. The results were quantified and expressed as the mean \pm SEM. The data were considered to be statistically significant if the *P* value was less than or equal to 0.05.

Results

4,10-Aromadendranediol (ARDD) induces neurite outgrowth in PC12 cells

An NGF-induced PC12 differentiation model was established to screen our in-house natural product library for a novel natural product that would have the potential to induce neuronal differentiation as indicated by the appearance of neurite outgrowth. One natural product, 4,10-Aromadendranediol (ARDD, Figure 1A), was discovered to show excellent function in promoting neurite outgrowth in this model. ARDD

(0.1, 1, or 10 $\mu\text{mol/L}$) significantly increased the average neurite length in NGF-induced PC12 cells (Figure 1B, 1C), but it did not increase the number of cells with new neurites (Figure 1D). In particular, 10 $\mu\text{mol/L}$ of ARDD exhibited this effect in a time-dependent manner (Figure 1E, 1F). We also investigated compounds with similar structures to ARDD, such as 8 α -hydroxy- α -gurjunene and Palustrol, but they did not show neurite outgrowth-inducing effects in this model (data not shown).

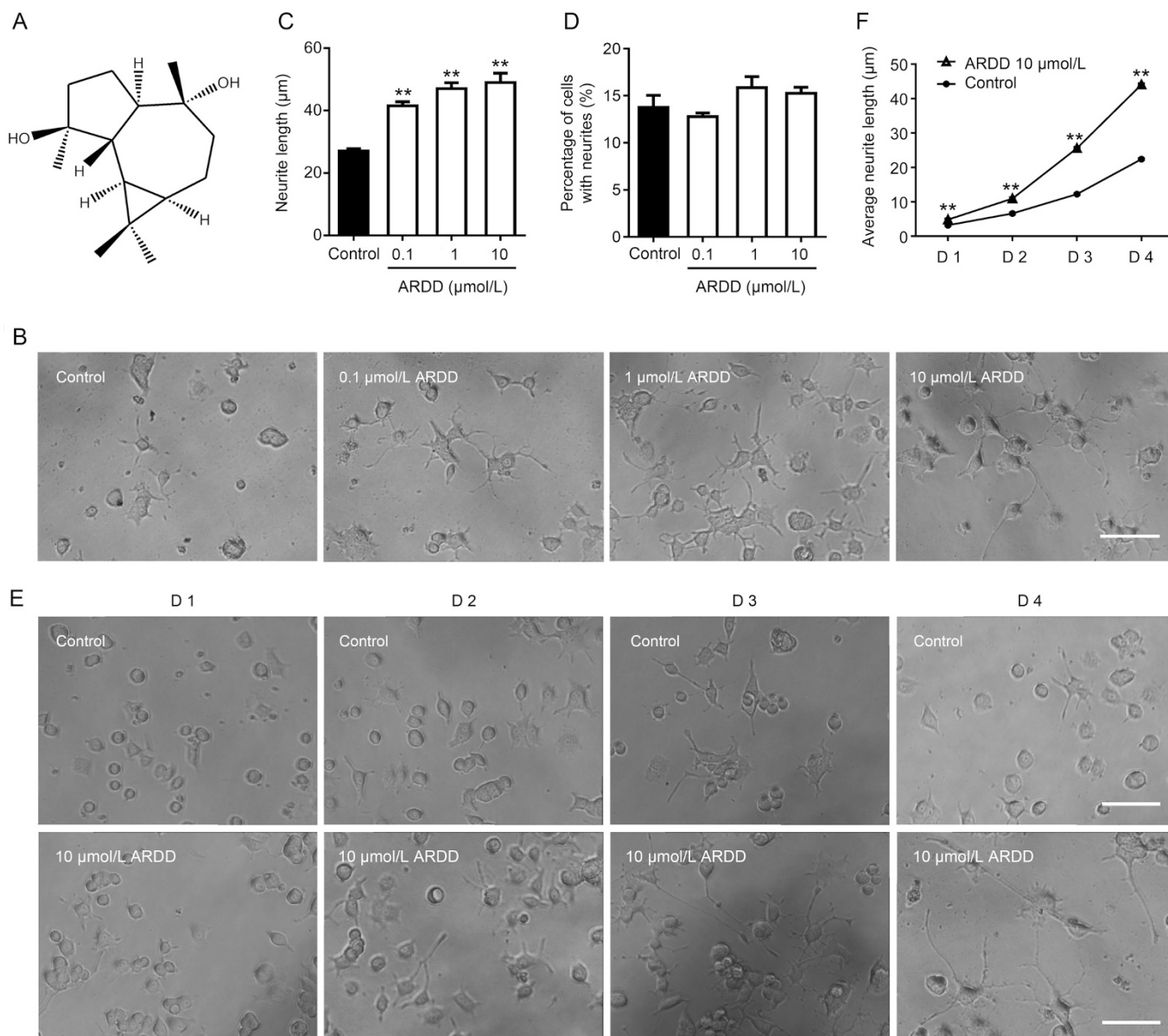


Figure 1. ARDD promotes NGF-induced PC12 neurite growth. (A) Structure of 4,10-Aromadendranediol (ARDD). (B) Effects of different doses of ARDD on neurite outgrowth of PC12 cells at d 4. PC12 cells were cultured in complete medium containing 2.5 ng/mL NGF with or without different concentrations of ARDD (0.1, 1, and 10 $\mu\text{mol/L}$) to induce neurite outgrowth. The scale bar represents 50 μm . (C) Statistics of average neurite length in the above groups at d 4. The data are expressed as the mean \pm SEM ($n=60-80$). ** $P<0.01$ vs control group. (D) Percentage of cells with neurites in the above groups at d 4. The data are expressed as the mean \pm SEM ($n=7$). (E) Time-course showing 10 $\mu\text{mol/L}$ ARDD promoting neurite outgrowth of PC12 cells. The scale bar represents 50 μm . (F) Statistics of average neurite length at different days. The data are expressed as the mean \pm SEM ($n=60-80$). ** $P<0.01$ vs control group.

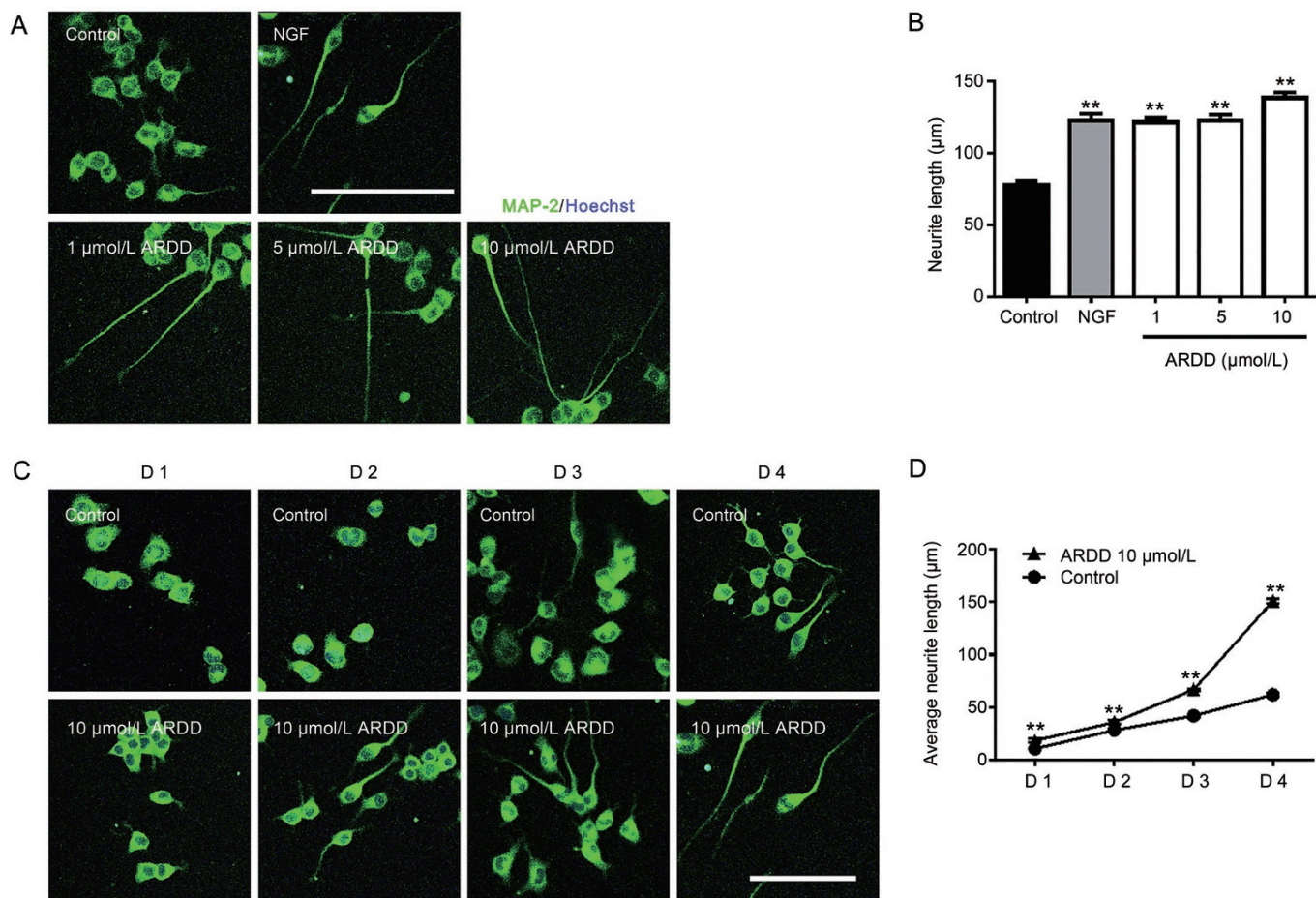


Figure 2. ARDD induces neurite outgrowth of N1E115 cells. (A) Effects of different doses of ARDD on neurite outgrowth of N1E115 cells at d 4. The scale bar represents 100 μm. (B) Statistics of average neurite length in the above groups at d 4. The data are expressed as the mean±SEM ($n=80-100$). ** $P<0.01$ vs control group. (C) Time-course showing 10 μmol/L ARDD promoting neurite outgrowth of N1E115 cells. The scale bar represents 100 μm. (D) Statistics of average neurite length at different days. The data are expressed as the mean±SEM ($n=80-100$). ** $P<0.01$ vs control group.

ARDD promotes neurite growth of N1E115 cells

ARDD (1, 5 or 10 μmol/L) significantly increased the average neurite length of N1E115 cells (Figure 2A, 2B), and 10 μmol/L of ARDD showed this effect in a time-dependent manner (Figure 2C, 2D). Moreover, 1 μmol/L of ARDD had a similar effect as the positive control (100 ng/mL NGF group).

ARDD enhances neurite growth of primary cortical neurons in a dose-dependent manner

To confirm the effects of ARDD in primary neurons, primary rat cortical neurons were treated with different concentrations of ARDD and cultured for 24 h. The immunofluorescence staining data show that ARDD significantly increased the average neurite length (Figure 3A, 3B) and also induced more branching of axons and dendrites than the control (Figure 3C, 3D).

Neurite outgrowth-inducing effect of ARDD is mediated by the ERK pathway in N1E115 cells and primary cortical neurons

Activation of the ERK pathway has been reported to play an

important role in neuronal differentiation. Thus, the possible involvement of this signaling pathway in ARDD-induced neurite outgrowth was investigated. As shown in Figures 4A and 4B, ARDD (10 μmol/L) stimulated ERK phosphorylation. A total of 10 μmol/L of PD98059, an inhibitor of the ERK pathway, completely reversed the neurite outgrowth-inducing effects of ARDD in both N1E115 neuronal cells (Figure 4C, 4E) and primary cortical neurons (Figure 4D, 4F), indicating that the ERK signaling pathway is involved in regulating neurite outgrowth induced by ARDD.

ARDD up-regulates the BDNF/CREB pathway in N1E115 cells

Previous studies have shown that the ERK signaling pathway could regulate CREB through direct CREB phosphorylation^[20]. ERK regulates the BDNF/CREB pathway, which is important for neurite outgrowth and neuronal differentiation. Since ERK is activated by ARDD, we wanted to know whether it regulates the BDNF/CREB pathway. As shown in Figure 5A, BDNF gene expression was remarkably increased when cells were treated with ARDD for 6 h or 12 h. CREB phos-

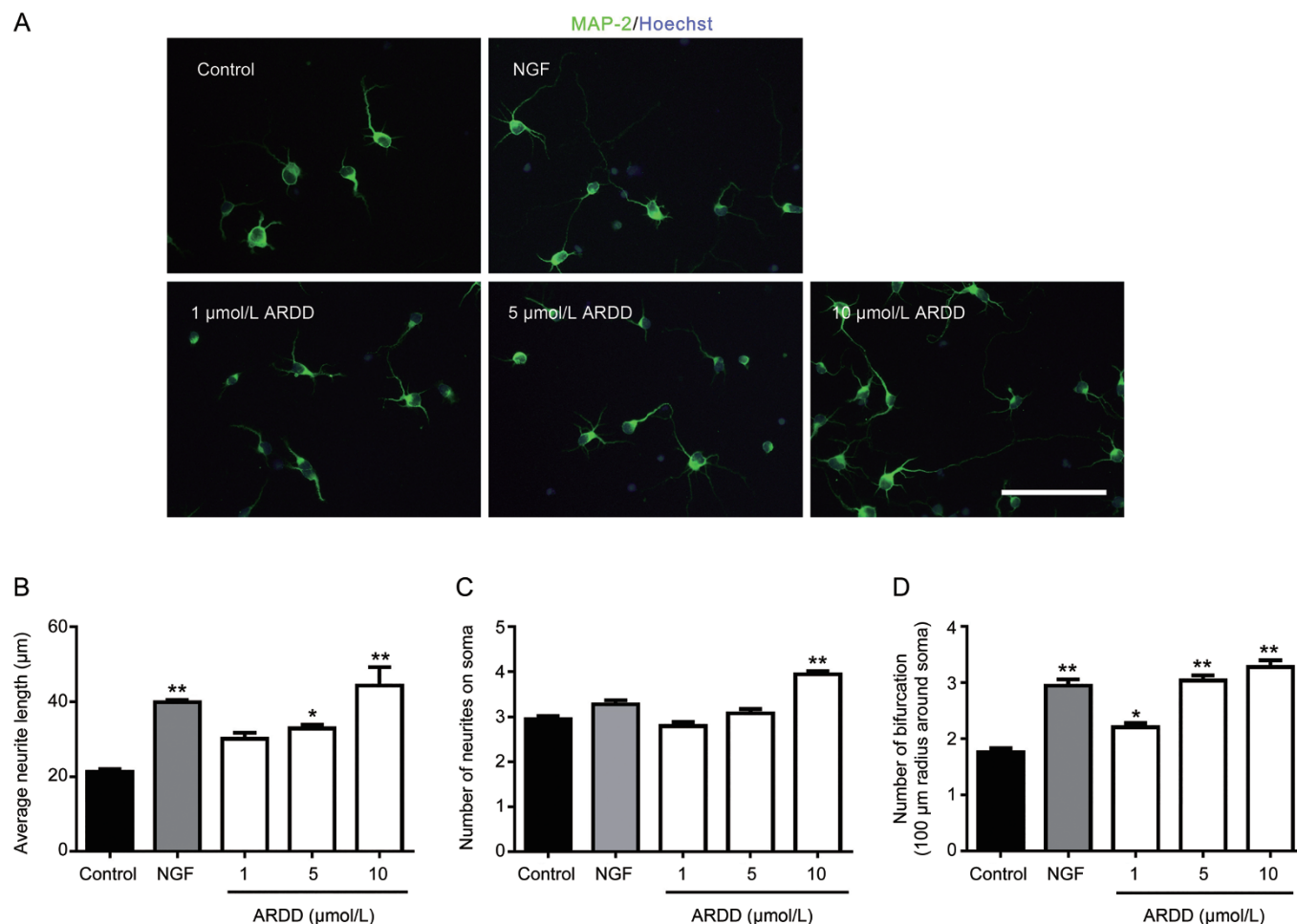


Figure 3. ARDD facilitates neuritogenesis in primary rat cortical neurons. (A) Dose-dependent manner of ARDD-induced neurite outgrowth of cortical neurons for 24 h. The scale bar represents 50 μm. (B) Statistics of average neurite length in the above groups. The data are expressed as the mean±SEM ($n=80-100$). * $P<0.05$, ** $P<0.01$ vs control group. (C, D) Number of neurites on the soma and bifurcations in cortical neurons incubated with 10 μmol/L ARDD. The data are expressed as the mean±SEM ($n=90-100$). * $P<0.05$, ** $P<0.01$ vs control group.

phorylation was also enhanced by ARDD treatment (Figure 5B). GAP-43 is highly expressed in neurons during neuronal outgrowth^[21]. Here, we found that ARDD also up-regulated GAP-43 protein expression in N1E115 cells (Figure 5C). However, PD98059 pretreatment reversed these effects (Figure 5B, 5C).

The GSK-3β/β-catenin pathway is involved in ARDD effects in N1E115 cells

GSK-3β phosphorylation and β-catenin accumulation levels were tested in ARDD-incubated N1E115 cells. As shown in Figure 6A, ARDD increased inactive GSK-3β phosphorylation, but PD98059 partly reversed this effect. We also found that ARDD treatment significantly increased β-catenin protein expression (Figure 6B) and gene expression of Fzd1 and Wnt3a compared with the corresponding time point group (Figure 6C, 6D). However, 10 μmol/L of XAV939, an inhibitor of the Wnt/β-catenin pathway, reversed the neurite outgrowth-inducing effects of ARDD in both N1E115 neuronal cells

(Figure 6E, 6F) and primary cortical neurons (Figure 6G, 6H), suggesting that the Wnt/β-catenin pathway may be involved in ARDD-induced neurite outgrowth.

ARDD prevents OGD-induced neurite disruption in N1E-115 cells

The OGD model was established to study whether ARDD prevents neuronal injury by promoting neurite outgrowth in N1E115 cells. After 30 min of OGD, the neurites were destroyed (Figure 7A). As shown in Figure 7B, the number of cells with neurites in the ARDD-treated group was significantly increased compared to the OGD group, while PD98059 inhibited the ARDD effect. We also investigated whether ARDD has a neuroprotective effect in N1E115 neuronal cells under OGD conditions. As shown in Figure 7C, ARDD pretreatment did not prevent OGD-induced neuronal death, but the positive control edaravone (Eda) showed a neuroprotective effect. OGD reduced ERK phosphorylation levels but ARDD enhanced ERK phosphorylation (Figure 7D). The above data suggest that ARDD could promote neurite outgrowth in neu-

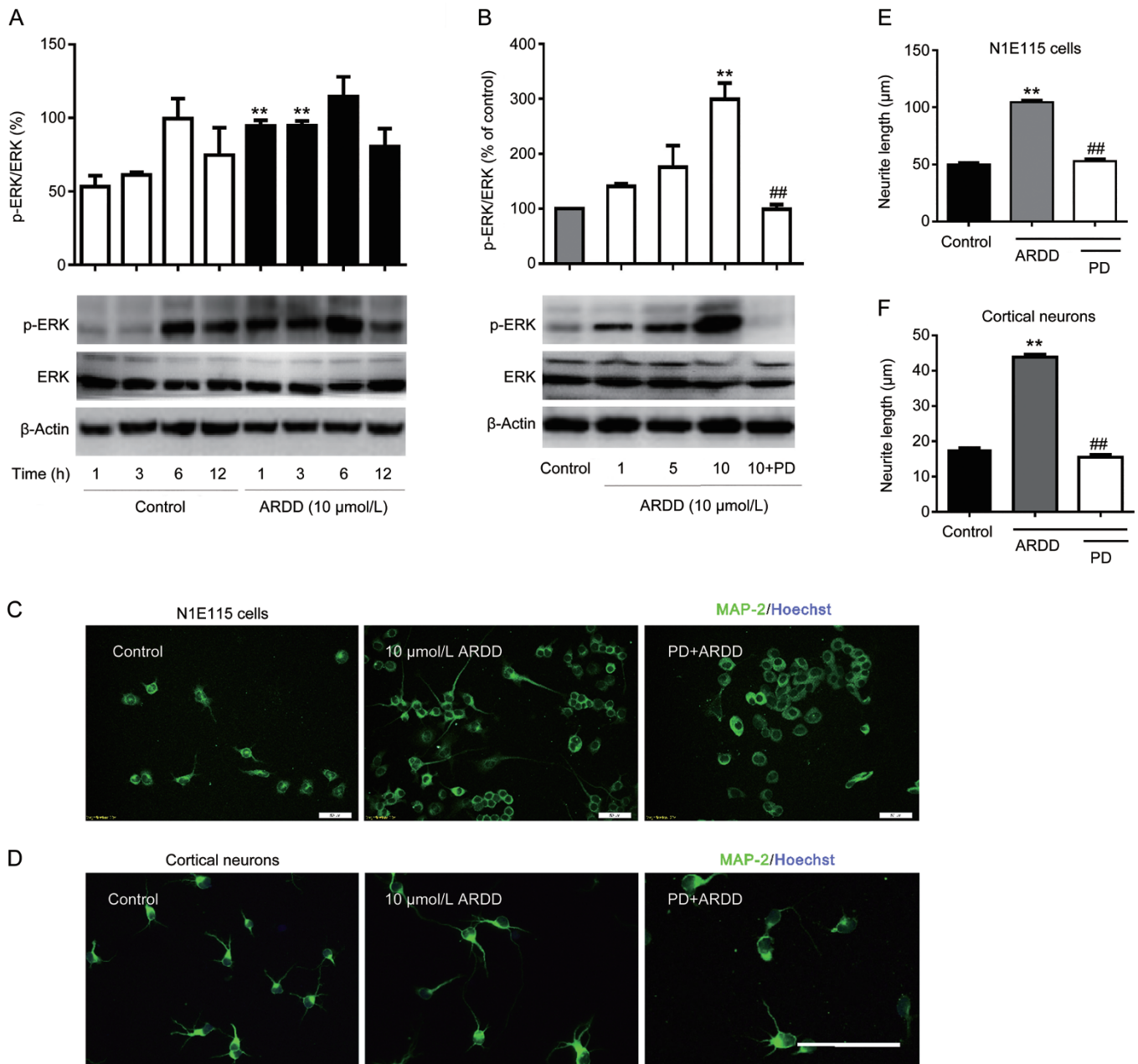


Figure 4. ARDD-induced neurite outgrowth is mediated by the ERK pathway in neuronal cells. (A, B) ARDD increased the expression level of ERK phosphorylation at different time or with different doses. The data are expressed as the mean±SEM ($n=3$). ** $P<0.01$ vs corresponding time point group or control group. ## $P<0.01$ vs ARDD-treated group. (C–F) PD98059 (10 μmol/L, PD) abolished the effects of ARDD on promoting neurite outgrowth of N1E115 cells and primary rat cortical neurons. The data are expressed as the mean±SEM ($n=60-80$). ** $P<0.01$ vs control group. ## $P<0.01$ vs ARDD-treated group.

rons even under OGD conditions.

Discussion

In the adult mammalian brain, neurogenesis is barely possible in both physiological and pathological conditions because of neurite growth inhibitory factors, such as the myelin-derived proteins MAG and Nogo, or the intrinsic frailty of neurons. Recent studies indicate that precursor cells in the subventricular zone (SVZ) adjacent to the lateral ventricles and the

subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus have the potential to generate new neurons^[22], which may be promising for dealing with neurodegenerative diseases. Neurogenesis involves several crucial steps of neural development, including neurite outgrowth. Here we used undifferentiated PC12 cells, N1E115 cells and primary cortical neurons to evaluate the effects of ARDD on neurite outgrowth^[23]. The NGF-induced differentiation of PC12 cells is a well-established *in vitro* model for neurite outgrowth research.

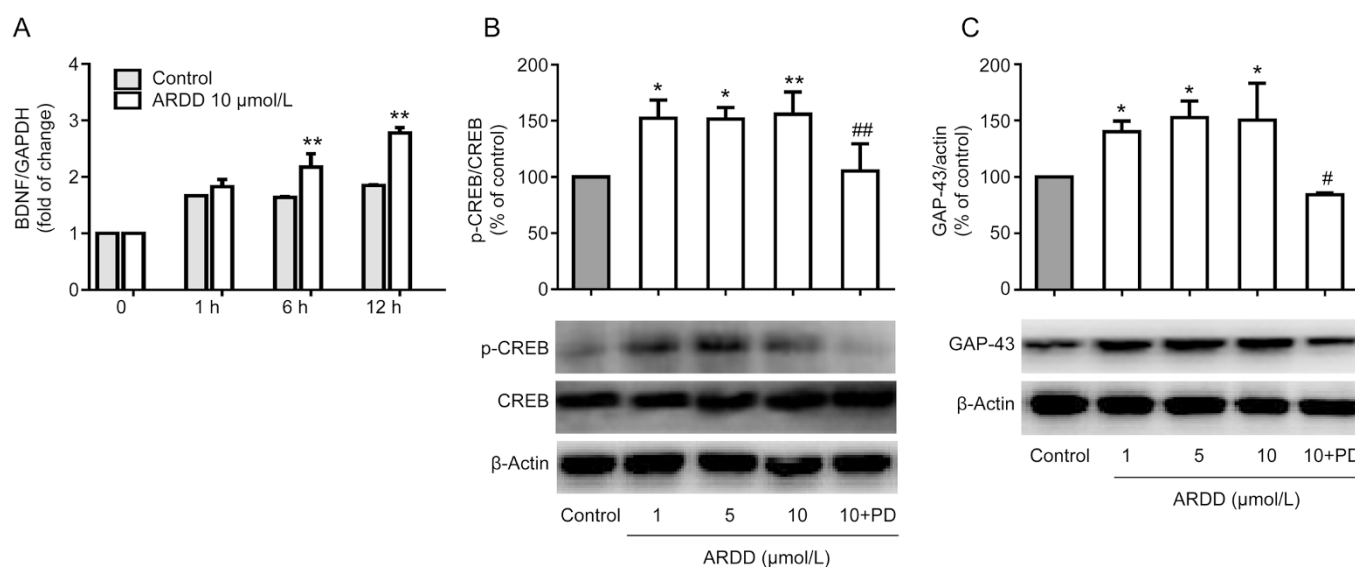


Figure 5. The BDNF/CREB pathway participates in ARDD-induced neurite outgrowth in neuronal cells. (A) BDNF gene expression was increased in the ARDD-treated group after 6 h or 12 h of ARDD incubation. The data are expressed as the mean±SEM ($n=3$) of at least three independent experiments. ** $P<0.01$ vs control group. (B) ARDD treatment enhanced the expression of CREB phosphorylation in N1E115 cells, but this effect was totally reversed by the ERK inhibitor PD98059 (10 µmol/L, PD). The data are expressed as the mean±SEM ($n=3$). * $P<0.05$, ** $P<0.01$ vs control group. ## $P<0.01$ vs ARDD-treated group. (C) The expression level of GAP-43 was increased in the ARDD-treated group, but this effect was inhibited by PD98059 (PD). The data are expressed as the mean±SEM ($n=3$). * $P<0.05$ vs control group. # $P<0.05$ vs ARDD-treated group.

Thus, the NGF-induced PC12 differentiation model was established for screening novel compounds that could promote neurite outgrowth from our in-house natural products compound library, and one novel natural product, ARDD, was discovered^[24]. To confirm this finding, the mouse neuroblastoma N1E115 cells and primary rat cortical neurons were also used to investigate the effects of ARDD on promoting neurite outgrowth by measuring average neurite length and other neuron differentiation criteria^[25]. Our data show that the effects of ARDD on promoting neurite outgrowth are consistent in these three cell culture models, indicating that ARDD may have the potential to promote neurogenesis and be applied to treat brain diseases.

To explore the underlying mechanisms for the effects of ARDD, the possible signaling pathways involved in the neurogenesis process were tested. The ERK signaling pathway was activated by ARDD, and the specific ERK inhibitor PD98059 totally reversed the ARDD effects, suggesting that the ARDD effects are mediated by activation of the ERK pathway. We also found that the Wnt/ β -catenin pathway inhibitor XAV partially reversed the effects of ARDD, indicating that the Wnt/ β -catenin pathway may also be involved in the effects of ARDD. Further studies show that ARDD increased inactive GSK-3 β phosphorylation, further enhanced β -catenin expression and up-regulated gene expression of Fzd1 and Wnt3a, but PD98059 reversed these effects, which indicates that the ERK/GSK-3 β / β -catenin signaling pathway may participate in the promoting effects of ARDD on neurite outgrowth. It has been reported that ERK is upstream of the GSK-3 β / β -catenin signaling pathway for neurogenesis in cortical neurons^[26]. Our

data demonstrate that ARDD stimulated the ERK pathway and its downstream GSK-3 β / β -catenin signaling pathway to mediate neurite outgrowth.

GAP-43 is one of the substrates of CREB, which can be produced by phospholipase C and diacylglycerol by triggering an undetectable calcium release in the neurite growth cone^[27, 28]. The interaction of GAP-43 with the growth cone regulates skeleton protein assembly and reduces neuronal injury^[29]. Our data show that GAP-43 protein and CREB phosphorylation levels were increased in neuronal cells exposed to ARDD, suggesting that the CREB/GAP-43 pathway is possibly involved in the effects of ARDD. However, PD98059 reversed these effects. It has been reported that CREB is a transcription factor with a neuroprotective role that enhances synaptic plasticity and regulates ERK activity^[30]. In addition, other studies show that phosphorylated ERK can translocate into the nucleus and further regulate CREB activity^[31]. However, the detailed mechanisms of the effect of ARDD on ERK activation to regulate CREB and GAP-43 proteins are not clear in this study and need to be further explored in the future.

Since ARDD has the ability to promote neurite outgrowth, it might have the potential to prevent neuronal injury under pathophysiological conditions such as ischemia. Therefore, the OGD pathological model in N1E115 neuronal cells was established in this study and our results demonstrate that ARDD prevented the OGD-induced reduction of neurite length through activation of the ERK pathway, indicating that ARDD has the potential to facilitate neurite outgrowth not only under physiological conditions but also in pathological conditions. Whether ARDD has the ability to promote neurite

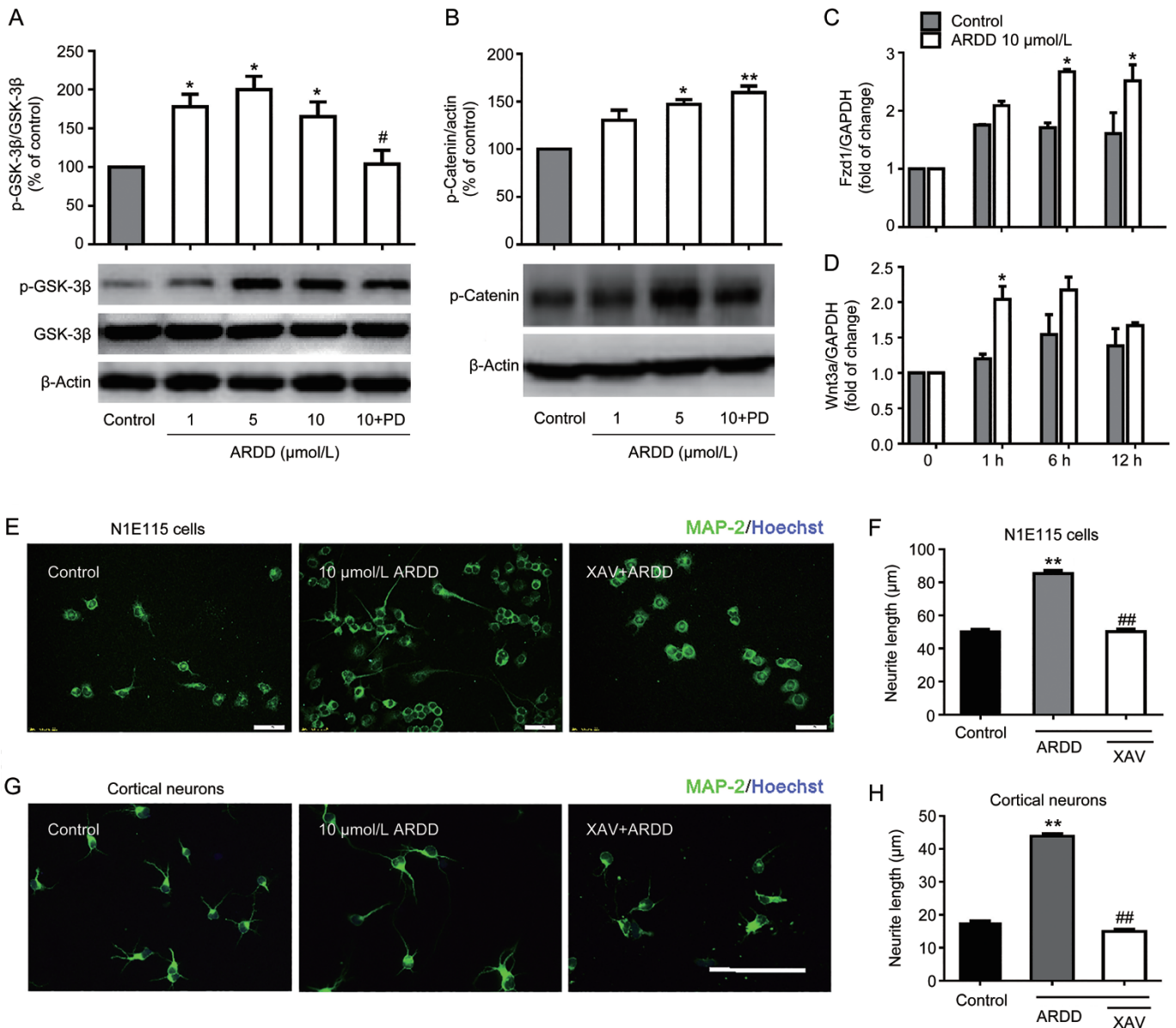


Figure 6. The Wnt/ β -catenin pathway is involved in ARDD-induced neurite outgrowth. (A) ARDD increased inactive GSK-3 β phosphorylation in N1E115 cells, but PD98059 (PD) reversed this effect. (B) The level of β -catenin was increased in the ARDD-treated group. (C, D) The gene expression levels of Fzd1 and Wnt3a were significantly increased by ARDD in N1E115 cells. The data are expressed as the mean \pm SEM ($n=3$) of at least three independent experiments. * $P<0.05$ vs corresponding time point group. (E–H) The Wnt/ β -catenin pathway inhibitor XAV939 (10 μ mol/L, XAV) abolished the effects of ARDD on neurite outgrowth in N1E115 cells or primary rat cortical neurons. The data are expressed as the mean \pm SEM ($n=60$ – 80). ** $P<0.01$ vs control group. # $P<0.05$, ### $P<0.01$ vs ARDD-treated group.

outgrowth in animal models, particularly the ischemic stroke model, will be determined in a future study.

In conclusion, we demonstrated that the novel natural product ARDD can promote neurite outgrowth in neuronal cells through activation of the ERK pathway. The involvement of the Wnt/ β -catenin and BDNF/CREB signaling pathways was found to play a role in the effects of ARDD, as illustrated in Figure 8. ARDD may have the potential to prevent and treat brain diseases by inducing neurite outgrowth or neurogenesis.

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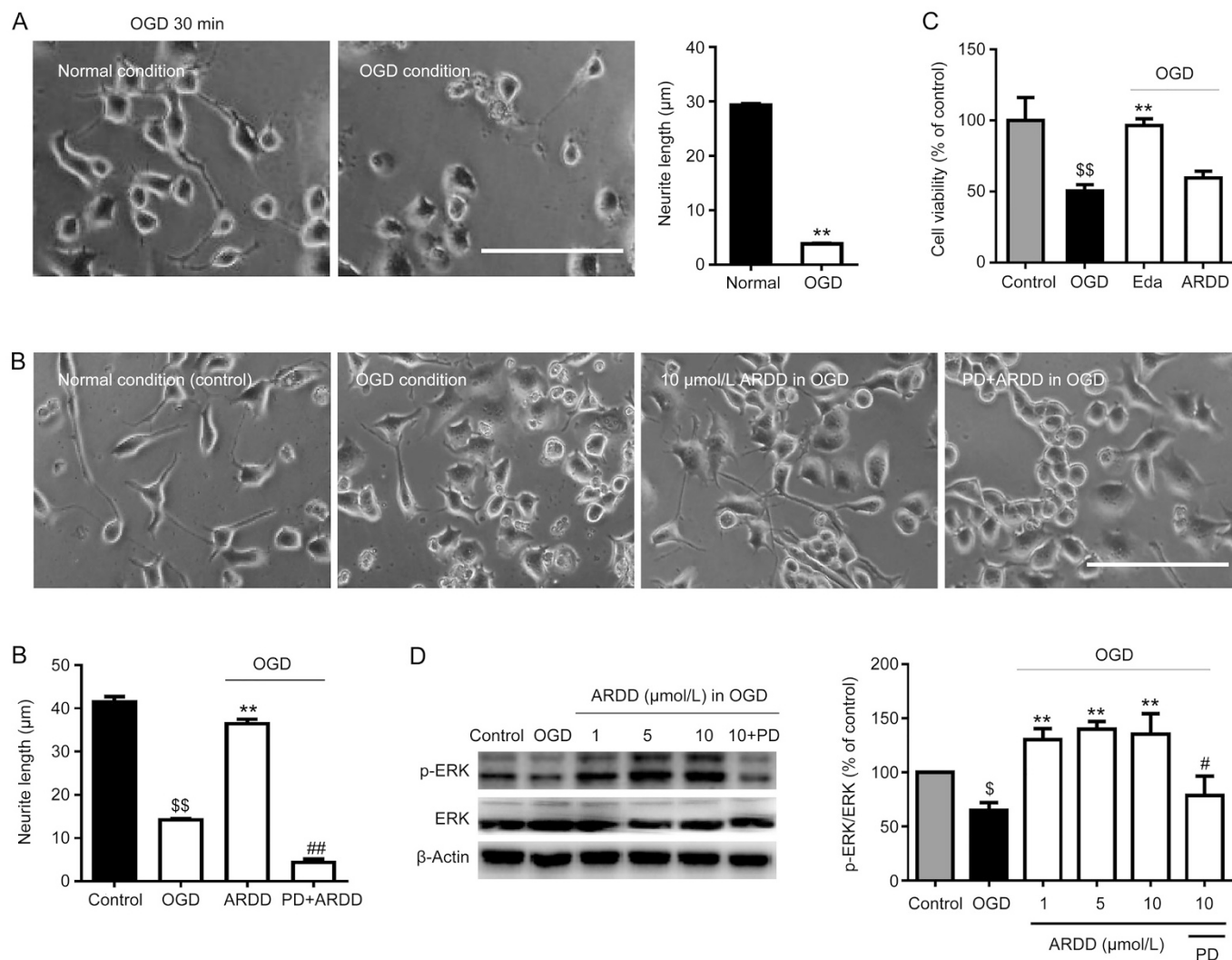


Figure 7. ARDD prevents OGD-induced neurite disruption in N1E115 cells. (A) Under 30 min of oxygen and glucose deprivation (OGD) conditions, neurites were destroyed. The data are expressed as the mean±SEM ($n=80-100$). $^{**}P<0.01$ vs normal group. (B) ARDD (10 μmol/L) incubation promoted neurite growth two days after 30 min of OGD in N1E115 cells. The data are expressed as the mean±SEM ($n=80-100$). $^{**}P<0.01$ vs control group (normal condition). $^{**}P<0.01$ vs OGD group. $^{##}P<0.01$ vs ARDD-treated group. (C) Cell viability of the ARDD-treated group and positive control edaravone (Eda)-treated group under OGD conditions. The data are expressed as the mean±SEM ($n=3$) of at least three independent experiments. $^{**}P<0.01$ vs control group. $^{**}P<0.01$ vs OGD group. (D) ARDD treatment reversed the OGD-induced reduction of ERK phosphorylation. The data are expressed as the mean±SEM ($n=3$) of at least three independent experiments. $^{*}P<0.05$ vs control group. $^{**}P<0.01$ vs OGD group. $^{#}P<0.05$ vs ARDD-treated group.

Author contribution

Sai CHANG, Tao PANG, and Hong LIAO designed the research; Sai CHANG, Wen-chen RUAN, Ya-zhou XU, Yun-jie WANG, and Jie PANG performed the research; Tao PANG, Hong LIAO, and Lu-yong ZHANG analyzed the data; Sai CHANG and Tao PANG wrote the paper.

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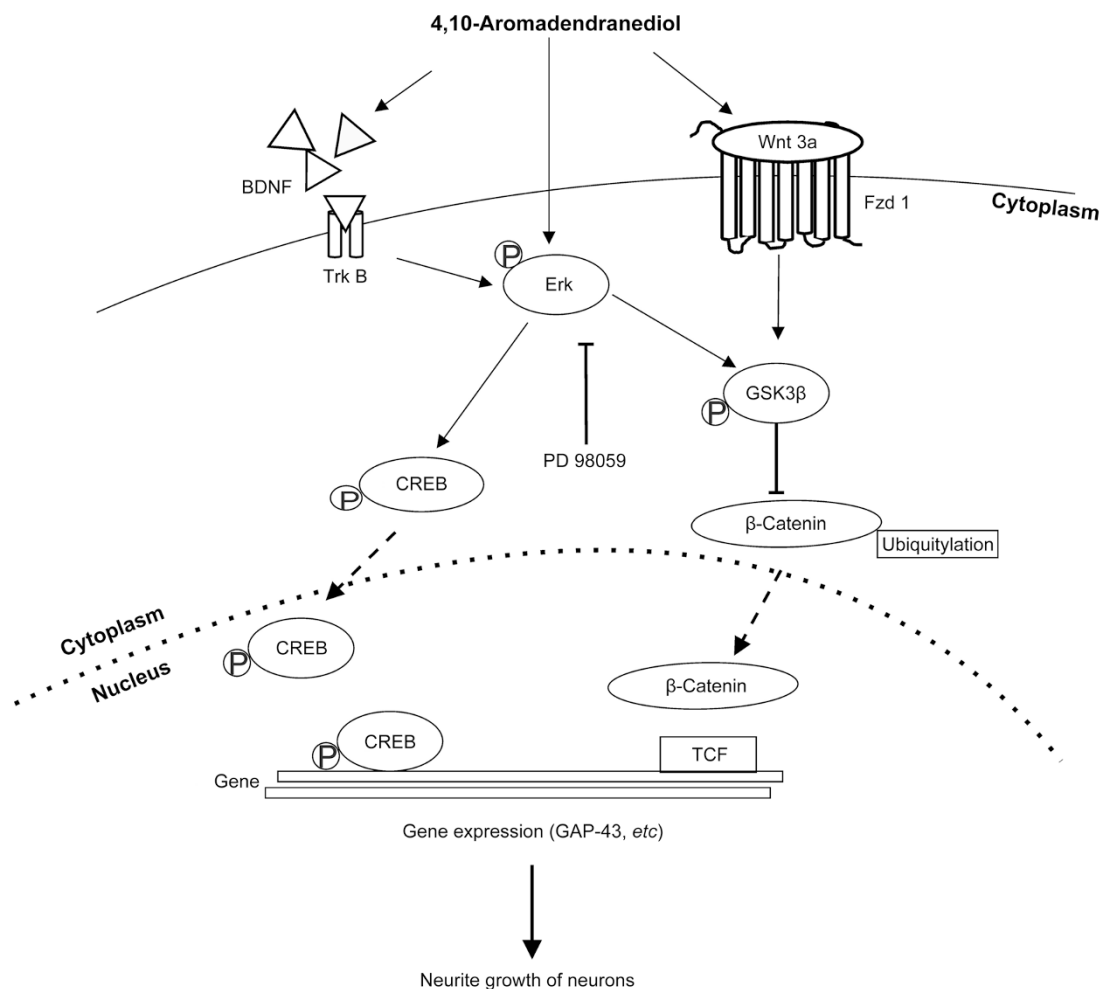


Figure 8. A scheme for the proposed mechanisms of ARDD on promoting neurite outgrowth in neuronal cells. The mechanisms involve activation of the ERK pathway and subsequently the GSK-3 β / β -catenin pathway and BDNF/CREB pathway to regulate gene expression related to neurite outgrowth of neurons.

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