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Chroman-like cyclic prenylflavonoids promote neuronal differentiation and neurite outgrowth and are neuroprotective

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Abstract

Flavonoids target a variety of pathophysiological mechanisms and are therefore increasingly considered as compounds encompassed with therapeutic potentials in diseases such as cancer, diabetes, arteriosclerosis, and neurodegenerative diseases and mood disorders. Hops (*Humulus lupulus* L.) is rich in flavonoids such as the flavanone 8-prenylnaringenin, which is the most potent phytoestrogen identified so far, and the prenylchalcone xanthohumol, which has potent tumor-preventive, anti-inflammatory and antiviral activities. In the present study, we questioned whether hops-derived prenylflavonoids and synthetic derivatives thereof act on neuronal precursor cells and neuronal cell lines to induce neuronal differentiation, neurite outgrowth and neuroprotection. Therefore, mouse embryonic forebrain-derived neural precursors and Neuro2a neuroblastoma-derived cells were stimulated with the prenylflavonoids of interest, and their potential to activate the promoter of the neuronal fate-specific doublecortin gene and to stimulate neuronal differentiation and neurite outgrowth was analyzed. In this screening, we identified highly "neuroactive" compounds, which we termed "enhancement of neuronal differentiation factors" (ENDFs). The most potent molecule, ENDF1, was demonstrated to promote neuronal differentiation of neural stem cells and neurite outgrowth of cultured dorsal root ganglion neurons and protected neuronal PC12 cells from cobalt chloride-induced as well as cholinergic neurons of the nucleus basalis of Meynert from deafferentation-induced cell death. The results indicate that hops-derived prenylflavonoids such as ENDFs might be powerful molecules to promote neurogenesis, neuroregeneration and neuroprotection in cases of chronic neurodegenerative diseases, acute brain and spinal cord lesion and age-associated cognitive impairments.

Keywords: Prenylflavonoids; Neurite outgrowth; Neuroprotection; Neural stem cells; Doublecortin; Retinoic acid; NGF; Xanthohumol; *Humulus lupulus* L

1. Introduction

Age-related neurodegenerative diseases and acute lesions of the central nervous system (CNS) are characterized by an inexorable loss of neurons and axons resulting in functional deficits and ultimately in premature death. Moreover, the CNS is characterized by a poor spontaneous regenerative capacity, such as a limited capacity of CNS neurons to regrow injured axons [1]. The current demographic evolution in industrialized nations into "overaged" societies is associated with a yet-inescapable increase in age-related diseases. Since age is the main risk factor for many neurodegenerative diseases, such as Alzheimer's disease (AD), the incidence of the latter is undergoing worldwide an exponential increase, and yet, no effective therapies are available. Therefore, there is an urgent need to develop compounds protecting neurons against cell death [2]. In addition, compounds that stimulate neurogenesis and foster functional integration of new neurons might counteract neurodegeneration and must also be regarded as potential drug candidates [3].

Flavonoids target several molecular and cellular mechanisms in neural cells, since they have been shown to induce neurogenesis and promote neuronal differentiation in pluripotent stem cells and neural progenitors [4–12], to be neuroprotective [13–15] (for review, see Gutierrez-Merino et al. [16]), and to promote axonal outgrowth and nerve regeneration [17–19]. In vivo, there is growing evidence that flavonoid-rich diets promote cognitive functions and delay disease progression in AD [20–23] (for review, see Williams et al. [24] and

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Macready et al. [25]). Moreover, flavonoids are protective in certain age-related comorbidities. Hence, they lower the risk of acute cerebrovascular diseases such as stroke in humans [26] and are neuroprotective in animal models of stroke [27,28]. Also, flavonoids are anticonvulsive in animal models of epilepsy [29–31]. Finally, flavonoids have antidepressant activities in humans and in animal models of depression [32–34].

Hops (*Humulus lupulus* L.) are a rich source of prenylated flavonoids. For instance, hops contain the flavanone 8-prenylnaringenin, which is the most potent phytoestrogen known so far [35]. The most abundant flavonoid present in hops is xanthohumol, a prenylchalcone with antiproliferative, tumor-preventive, anti-inflammatory and antiviral activities [36–38] (for review, see Magalhaes et al. [39]). In addition to xanthohumol, other hops-derived prenylflavonoids have been reported to possess antiproliferative activities and to induce apoptosis in lymphoma cells [40,41].

Here, we identified hops-derived prenylflavonoids and synthetic derivatives thereof promoting neuronal differentiation, neurite outgrowth and neuroprotection. An initial screening was based on high-performance liquid chromatography-fractionated hops extracts rich in prenylflavonoids, which identifies in a luciferase assay substances that activate the human doublecortin (DCX) promoter, a gene associated with neuronal fate determination and neuronal differentiation [42]. Neuronal differentiation was further analyzed by marker gene expression in mouse embryonic forebrain (MEF) cells and in Neuro2a neuroblastoma cells, and neurite outgrowth was investigated in a dorsal root ganglion (DRG) neuron cultures. Finally, the potential of the substances to protect PC12 cells from cobalt chloride-induced cell death and to protect deafferentated cholinergic neurons of the basal nucleus of Meynert from cell death was tested.

2. Materials and methods

2.1. Compounds

Synthesis of flavonoid compounds is presented in detail in supplementary material. Other compounds, such as all-trans-retinoic acid (RA), valproic acid (VPA) and the flavonoids genistein, naringenin, liquritigenin, fisetin, oroxylinA and quercetin were obtained from Sigma-Aldrich (Steinheim, Germany). Epigallocatechin gallate, baicalin and baicalein were obtained from Selleck Chemicals (Houston, TX, USA), and osajin and wogonin were obtained from Chromadex (Irvine, CA, USA).

2.2. Cell culture

2.2.1. Primary MEF cultures

Pregnant NMRI mice (16 dpc) (Charles River Laboratories, Sulzfeld, Germany) were sacrificed, and the uteri promptly were removed and immersed in ice-cold Dulbecco's phosphate-buffered saline solution (DPBS). Eight embryos were released from the uteri; forebrains were taken out, separated from surrounding tissues and dissected using a razor blade. Following a 5-min centrifugation at 120×*g*, pellets were resuspended in 5 ml HBSS without Ca^{2+}/Mg^{2+} containing 0.01% Papain (Worthington, England, UK), 0.1% DispaseII (Roche, Basel, Switzerland), 0.01% DNaseI (Worthington) and 12.4 mM $MgSO_4$. The cell suspension was incubated 30 min at 37°C and triturated by pipetting every 10 min. The cell suspension was then centrifuged at 120×*g* for 5 min and washed three times in neurobasal (NB) medium (Gibco BRL, Karlsrahe, Germany) supplemented with 2% (vol/vol) B27 (Gibco BRL), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (hereafter NB-B27). Finally, the cell preparation was resuspended in 12 ml NB medium supplemented with 2% B27, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml epidermal growth factor (rhEGF), 20 ng/ml fibroblast growth factor (rhFGF; R&D Systems, Wiesbaden-Nordenstadt, Germany) and 2 μg/ml heparin (hereafter NB-B27-GF), seeded in one T75 flask and maintained in a humidified atmosphere at 37°C and 5% CO_2.

Within the first 2 days in culture, MEF cells had formed neurospheres. On day 3, half of the medium was refreshed, and on day 4, cells were passaged by using Accutase (PAA, Pasching, Austria). Cell number was determined by Trypan blue exclusion. For differentiation, the cells were resuspended in NB-B27 supplemented with 1% fetal calf serum (FCS; Lonza, Wuppertal, Germany) and seeded into poly-ornithine/laminin-coated 24-well plates (20,000 cells/well). One day later, cells had attached to the substrate. Medium was replaced every 3 days.

2.2.2. Adult rat neural progenitor cell cultures

Neural progenitor cells (NPCs) derived from rat hippocampus were generated as described [43,44]. Briefly, hippocampi from 2-month-old young female Fisher-344 rats (Charles River Deutschland GmbH) were aseptically removed and dissociated. Cells were resuspended in NB medium (Gibco BRL) supplemented with B27 (Gibco BRL), 2 mM L-glutamine (PAN, Aidenbach, Germany) and 100 U/ml penicillin/0.1 mg/l streptomycin (PAN), hereafter referred to as NB/B27. For maintenance and expansion of the cultures, the NB/B27 was further supplemented with 2 mg/ml heparin (Sigma, Germany), 20 ng/ml FGF-2 (R&D Systems) and 20 ng/ml EGF (R&D Systems). Cultures were maintained at 37°C in a humidified incubator with 5% CO_2. Neurosphere cultures from passage numbers 2 to 6 were used throughout this study and termed NPCs.

2.2.3. Mesenchymal stem cell cultures and mesenchymal stem cell-conditioned medium

Mesenchymal stem cell (MSC) cultures were as previously described [43]. Briefly, bone marrow (BM) plugs were harvested from femurs and tibias of 2-month-old Fisher-344 rats (Charles River Deutschland GmbH). Plugs were mechanically dissociated in αMEM (Gibco Invitrogen) and recovered by centrifugation. Cell pellets were resuspended in αMEM containing 10% FBS (PAA) and 1% penicillin/streptomycin (PAN Biotech GmbH) (αMEM-10% FBS) and seeded at 1×10^6 cells/cm^2. After 3 days, media was changed and nonadherent cells were removed. Adherent cells were incubated in fresh αMEM-10% FBS until a confluent layer of cells was achieved. Cells were trypsinized using 0.25% Trypsin (Gibco Invitrogen) and seeded in αMEM-10% FBS at 8000 cells/cm^2. After 3–5 days of culture, the resulting monolayer of cells, hereafter named rat BM-derived MSCs, was trypsinized and further cultured for experiments or frozen for later use. As demonstrated in our previous work, this cell culture preparation is highly enriched in multipotent MSCs with virtually no hematopoietic contamination [43]. MSC-CM was prepared as described [43]. MSCs were plated at 12,000 cells/cm^2 and incubated in MSC proliferation medium (αMEM-10% FBS). After 3 days, the conditioned medium was collected, filtered using a 0.22-μm pore filter and used for further experiments. MBP-promoter-reporter plasmid transfected NPCs (see further down) were treated with MSC-CM as previously described [43]. Briefly, NPCs were plated overnight onto poly-ornithine (250 μg/ml) and laminin (5 μg/ml)-coated glass coverslips at a density of 12,000 cells/cm^2 in αMEM-10% FBS. Next, media was replaced and cells were incubated with MSC-CM. NPCs were alternatively incubated in αMEM-10% FBS as a control condition or enhancement of neuronal differentiation factor (ENDF) 1. After 3 days in the luciferase experiments, cells were processed for bioluminescence analysis.

2.2.4. Neuro2a neuroblastoma cell cultures

Neuro2a cells (CCL-131; ATCC, Manassas, VA, USA) were grown in MEM with Earle's Salts (PAA) containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 mM natrium pyruvate and 10% FCS. Cells were passaged with trypsin/EDTA (PAA) twice a week.

2.2.5. Chicken embryonic day 15 DRG neurons (DRGs E15)

DRGs were prepared as described in Aigner et al. [45,46]. Briefly, fetuses were taken out of the egg, and 10 lumbar DRGs per fetus were dissected. One hundred DRGs were collected in 10 ml DPBS and centrifuged at 120×*g* for 3 min. Pelleted DRGs were then dissociated with 1 ml trypsin/EDTA for 15 min at 37°C. Then, 9 ml of DMEM, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS (hereafter DMEM-FCS) were added to inactivate the trypsin, and DRGs were centrifuged 120×*g* for 5 min. DRGs were resuspended and triturated in 5 ml of DMEM-FCS. Following centrifugation, cells were resuspended in 5 ml of DMEM-FCS and seeded into a 60-mm culture dish. Nonneuronal cells were allowed to adhere to the culture dish for 3 h at 37°C, and then the nonadherent fraction containing the DRG neurons was collected and centrifuged for 5 min at 120×*g*. The pellet was resuspended in 700 μl of DMEM-FCS and DRG suspension (20 μl/well) was seeded onto poly-L-ornithine/laminin-coated glass coverslip into a 24-well plate. Each well was filled with 400 μl of DMEM-FCS supplement with the different flavonoids compounds tested. Cultures were maintained at 37°C in a humidified incubator with 5% CO_2 for 24 h and processed for immunostainings.

2.2.6. Rat pheochromocytoma cell line (PC12 cells)

PC12 cells (CRL-1721; ATCC) were grown in RPMI supplemented with 10% horse serum (Sigma-Aldrich, Taufkirchen, Germany), 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine as adherent cultures on poly-L-ornithine-coated plates. The cells were maintained at 37°C in a humidified incubator with 5% CO_2. Cells were passaged once a week with Accutase.

2.2.7. Organotypic vibrosection cultures

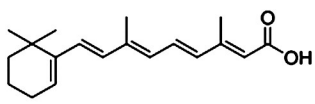
Organotypic vibrosection cultures of the nucleus basalis of Meynert (nBM) were performed as described in detail by Ullrich et al. [47]. Postnatal day 8, Sprague-Dawley rats were used. All experiments conformed to European and Austrian guidelines on the ethical use of animals. Following sacrifice, the brains were rapidly dissected and cut coronal at the level of the nBM. The brains were glued into a water-cooled vibratome (Leica VT1000A). Under aseptic conditions, 200-μm vibrosections were cut and collected in sterile medium. The organotypic vibrosections were carefully placed onto a 0.4-μm membrane inserts (Millipore PICM03050) within a 6-well plate (Greiner) and cultured at 37°C and 5% CO_2 with 1.2 ml/well of the following culture medium: 50% MEM/HEPES (Gibco), 25% heat-inactivated horse serum (Gibco/Lifetech, Austria), 25%

Hanks´ solution (Gibco), 2 mM $NaHCO_3$ (Merck, Wien, Austria), 6.5 mg/ml glucose (Merck, Darmstadt, Germany) and 2 mM glutamine (Merck, Darmstadt, Germany), pH 7.2. Vibrosections were incubated for 2 weeks, and 200 µl medium was changed twice a week. Vibrosections were cultured with or without 10 ng/ml nerve growth factor (NGF) or with or without equivalent volumes of DMSO or 10 µM ENDF. At the end of the experiment, vibrosections were fixed for 3 h at 4°C in 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS; pH 7.4) and then stored at 4°C in PBS until use.

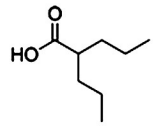
2.3. Plasmid transfections, cell treatments and luciferase assays

Luciferase assays were performed using a dual-luciferase enzyme assay as described in [48]. MEF cells were cotransfected with a plasmid encoding the firefly luciferase reporter gene driven by the DCX promoter [42] and a control plasmid encoding the renilla luciferase under control of the CMV promoter (Promega, Mannheim, Germany). In some experiments, a MBP promoter driving the firefly luciferase (kindly provided by Dr. Armin Schneider, Sygnis Heidelberg, Germany) was used to transfect adult rat NPCs. Plasmids were electroporated using an Amaxa Nucleofector II (program A-033) (Lonza, Köln, Germany). Transfected cells were resuspended in NB-B27 supplemented with 1% FCS and seeded into poly-ornithine/laminin-coated white 96-well plates. Twenty-four hours later, the medium was replaced with fresh media containing the flavonoid compounds at final concentrations of 1 and 10 µM, and cells were incubated for 3 days.

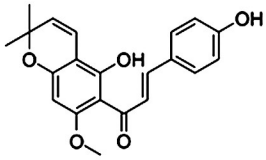
Prior to measurements, the medium was removed and cells were lysed with 25 µl/well of lysis solution consisting of 25 mM Tris-phosphate pH 7.8, 2 mM DTT, 1% Triton



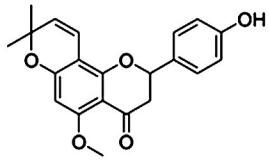
Retinoic acid (RA)
(2E,4E,6E,8E)-3,7-dimethyl-9-(6,6-dimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid



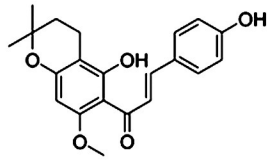
Valproic acid (VPA)
2-propylpentanoic acid



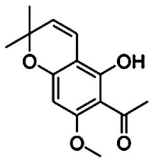
ENDF1
Xanthohumol-C (XNC)
(2E)-1-(5-hydroxy-7-methoxy-2,2-dimethyl-2H-chromen-6-yl)-3-(4-hydroxyphenyl)prop-2-en-1-one



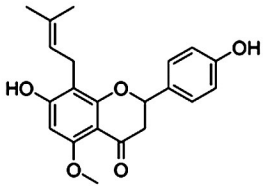
ENDF2
Isoxanthohumol-C (IXC)
2-(4-Hydroxy-phenyl)-5-methoxy-8,8-dimethyl-2,3-dihydro-8*H*-pyrano[2,3-f]chromen-4-one



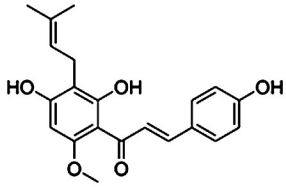
ENDF3
4´´,5´´-Dehydroxanthohumol-C (DHC)
(*E*)-1-(5-hydroxy-7-methoxy-2,2-dimethylchroman-6-yl)-3-(4-hydroxyphenyl)prop-2-en-1-one



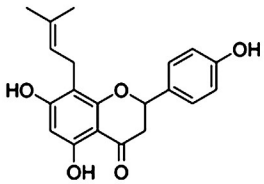
ENDF4
1-(5-hydroxy-7-methoxy-2,2-dimethyl-2H-chromen-6-yl)ethanone



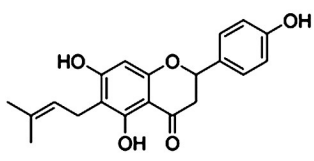
Isoxanthohumol (IX)
2,3-dihydro-7-hydroxy-2-(4-hydroxyphenyl)-5-methoxy-8-(3-methylbut-2-enyl)chromen-4-one



Xanthohumol (XN)
(E)-1-(2,4-dihydroxy-6-methoxy-3-(3-methylbut-2-enyl)phenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one



8-Prenylnaringenin (8PN)
5,7-Dihydroxy-2-(4-hydroxyphenyl)-8-(3-methyl-but-2-enyl)-chroman-4-one



6-Prenylnaringenin (6PN)
5,7-Dihydroxy-2-(4-hydroxyphenyl)-6-(3-methyl-but-2-enyl)-chroman-4-one

Fig. 1. Summary of compounds used in the present study. Nonscientific names and chemical names (IUPAC nomenclature) are mentioned.

X-100, 2 mM EDTA and 10% glycerol for 10 min. For analysis of the firefly luciferase activities, 80 µl of Reagent A (25 mM glycylglycine; Acros, Geel, Belgium), 15 mM KPO_4 (pH 8.0), 4 mM EGTA, 15 mM $MgSO_4$, 2 mM ATP, 1 mM DTT, 0.1 mM coenzyme A and 75 mM D-luciferin (Promega) was added and the light emission recorded. Then the renilla luciferase activities were measured following the addition of 100 µl of freshly prepared Reagent B [1.1 M NaCl, 2.2 mM Na_2EDTA, 0.22 M KPO_4 (pH 5.1), 0.44 mg/ml BSA, 1.3 mM NaN3, 1.43 mM coelenterazine (p.j.k., Kleinblittersdorf, Germany)] to each well. The bioluminescence was recorded with a TriStar Multimode Microplate Reader LB 941 (Berthold Technologies, Wien, Austria).

2.4. Determination of cell numbers

For measurement of cell expansion, 100,000 MEF cells/well were seeded in 6-well plates in NB-B27-GF and immediately stimulated with RA (10 µM)/VPA (50 µM), ENDF1, ENDF2, ENDF3 and ENDF4 (10 µM). After 24, 48 and 72 h, cell numbers were determined using a CASY Model TT (Roche Diagnostics Deutschland GmbH, Manheim, Germany).

2.5. Immunostainings

Cells were fixed with pH 7.5 phosphate-buffered 4% paraformaldehyde and blocked in fish skin gelatin buffer (FSGB), as described [49]. The specimens were incubated overnight at 4°C with the primary antibodies at the following dilutions: rabbit anti-DCX 1:500 (NEB, Frankfurt, Germany), mouse anti-Map 2a+2b 1:250 (Sigma-Aldrich, Taufkirchen, Germany), guinea pig anti-GFAP 1:500 (Progen Biotechnology, Heidelberg, Germany) and rabbit anti-GAP-43 1:500 (AbD Serotec, Oxford, UK). After washing three times with FSGB, cells were incubated with the fluorochromes-conjugated secondary antibodies for 2 h at room temperature. Cells were washed three times with PBS, and nuclear counterstaining was performed with 4′,6′-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) at 0.25 µg/µl (Sigma-Aldrich, Taufkirchen, Germany). Finally, cells were washed again three times with PBS and mounted on microscope slides using Prolong Antifade reagent (Invitrogen, Oregon, USA). The immunostainings were examined using Olympus IX81 inverted research microscope and the software Volocity 5.3.1.

Immunohistochemistry of brain slice cultures was performed as previously described [47]. Sections were washed 30 min with 0.1% Triton/PBS (T-PBS) at room temperature and pretreated 20 min with 20% methanol/1% H_2O_2/PBS. After thorough rinsing, the sections were blocked with 20% horse serum/0.2% BSA/T-PBS and then incubated for 2 days at 4°C with primary antibodies against choline acetyltransferase (ChAT; 1:750, Millipore AB144P). Then the sections were washed again with PBS and incubated with secondary biotinylated antigoat (ChAT) for 1 h at room temperature. Following further washing steps with PBS, sections were incubated in an avidin–biotin complex solution (ABC-Elite Vectastain reagent Vector Lab.) for 1 h. After being washed with 50 mM Tris-buffered saline (TBS), the signal was detected by using 0.5 mg/ml 3,3′-diaminobenzidine including 0.003% H_2O_2 as a substrate in TBS. The sections were mounted on glass slides, air dried and coverslipped with Entellan (Merck, Darmstadt, Germany).

2.6. Morphometric analysis of cultured neurons and quantitative analysis of survival of cholinergic neurons in brain slices

For analysis of neurite length, MEF, DRGs or Neuro2a cells were seeded on poly-L-ornithine/laminin-coated coverslips in a 24-well plate in their respective differentiation media, fixed and immunostained. The length of DCX or GAP43-positive neurites and arborization profiles were measured using the Volocity 5.4.1 software. The number of microscopically detectable ChAT-immunoreactive neurons in brain slice cultures was counted in the basal nucleus of Meynert using a 20× objective.

2.7. PC12 neuroprotection assay

PC12 cells were seeded in 96-well plates (10^4 cells/well in 100 µl) and further cultivated for 22 h in the presence of 300 µM $CoCl_2$ alone or together with ENDF1 (1 or 10 µM). Thereafter, an equal volume of Caspase-Glo 3/7-substrate-solution (Promega) was added, and cells were incubated for 2 h at room temperature. Then, caspase-3/7 activity was measured using a TriStar Multimode Microplate Reader LB 941 (Berthold Technologies, Wien, Austria). Luminescence signals measured from wells containing medium alone without cells were subtracted as background. NGF (50 ng/ml) was used as a positive control for neuroprotection.

2.8. Statistical analysis

Experiments were performed in three to four independent replicates. Data are presented as mean±SD. Statistical analyses were performed by one-way analysis of variance (ANOVA; Tukey or Newman–Keuls post hoc) or two-way ANOVA (Bonferroni post hoc) using PRISM5 (GraphPad, San Diego, CA, USA) and P values of $<.05$ were considered to be significant.

3. Results

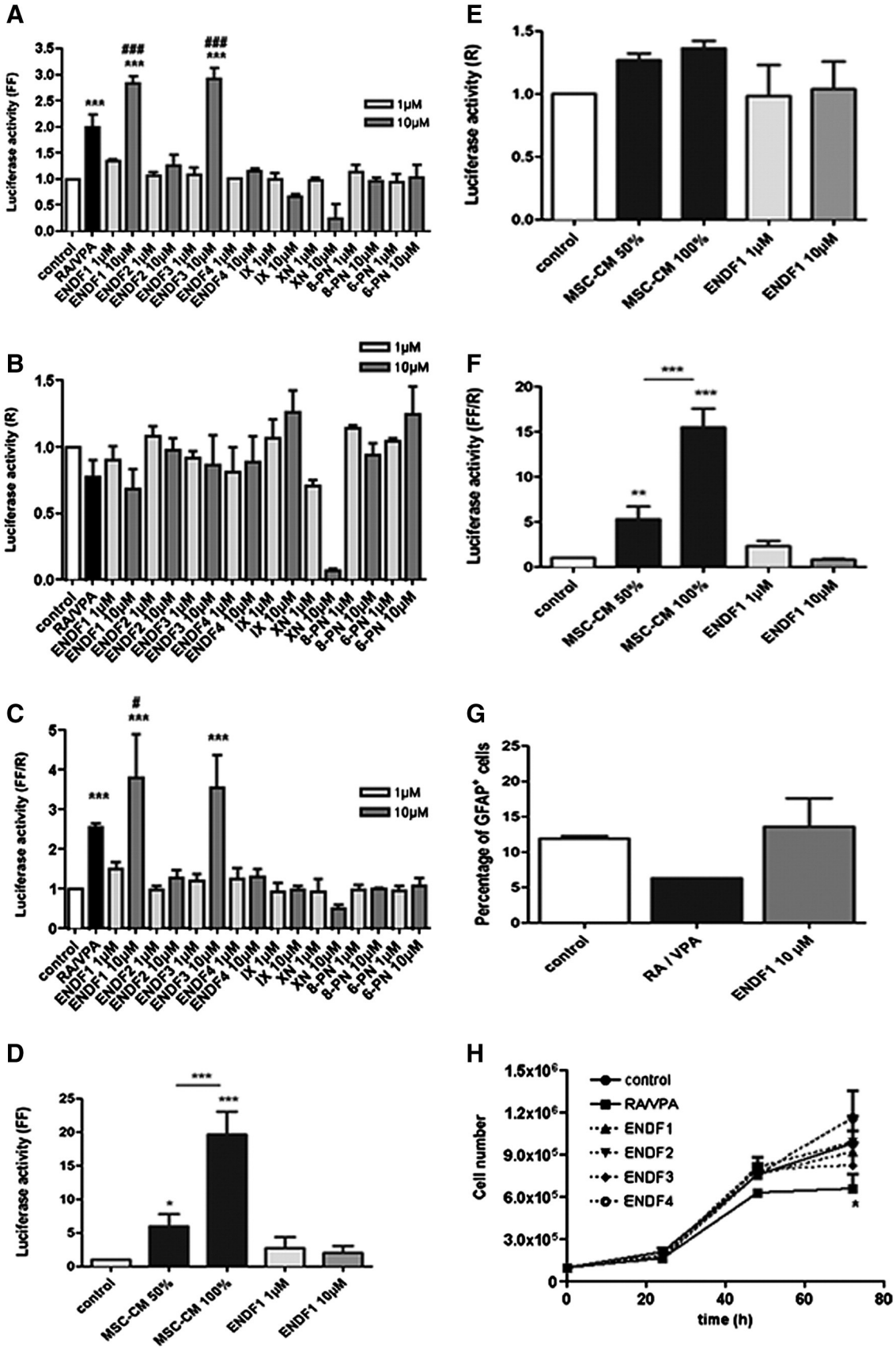
3.1. ENDFs induce the neuronal precursor specific DCX promoter

First, we screened a panel of hops-derived prenylflavonoids and derivatives for their capacity to activate the human DCX promoter in MEF cultures using a luciferase assay [42]. This culture model is mainly composed of neural precursors and neural stem cells, with some mature neurons, astrocytes and oligodendrocytes. Hence, together with the DCX promoter, which gets induced very early upon neuronal determination [49], this culture model is well suited to identify factors regulating neuronal differentiation, as we have previously shown [42,50]. MEF cells were transiently cotransfected with a plasmid encoding the DCX promoter driving the firefly luciferase gene and with a plasmid on which the expression of the renilla luciferase reporter gene was controlled by the constitutive CMV promoter. One day after transfection, cells were stimulated with 1 or 10 µM of the flavonoids compounds for 3 days. The most active compounds out of this initial screening, together with some previously reported prenylflavonoids, were selected for further investigation: ENDF1, ENDF2, ENDF3, ENDF4, isoxanthohumol (IX), xanthohumol (XN), 8-prenylnaringenin (8PN) and 6-prenylnaringenin (6PN) (Fig. 1). RA (10 µM) and VPA (50 µM) were used as a positive control for neuronal differentiation.

Among all substances tested, 10 µM ENDF1 and 10 µM ENDF3 were the most potent activators of the DCX promoter, being even stronger than the RA/VPA positive control (Fig. 2A–C). ENDF2 and ENDF4, as well as all other compounds tested, did not have significant effects on the DCX promoter activity. The low renilla luciferase value measured after XN treatment suggests that XN was most likely toxic to the cells. This was confirmed by phase-contrast microscopic observations (data not shown). To demonstrate the specificity of the ENDFs to induce a neuronal promoter, the oligodendroglial-specific MBP-promoter driving the luciferase reporter was used in parallel experiments. ENDF1 did not have any effect on the MBP promoter activity, suggesting that the effects of ENDFs were specific to the neuronal lineage (Fig. 2D–F). In contrast, conditioned medium derived from MSC cultures was used as a positive control to drive oligodendrocyte differentiation [43] and could strongly induce the MBP-promoter-driven reporter.

To further demonstrate the specific effects of the ENDFs on neuronal differentiation, we investigated the potential of the flavonoids to induce astroglial differentiation in MEF cultures. Therefore, MEF cultures were stimulated for 7 days with 10 µM of the flavonoid compounds and analyzed for the percentage of cells expressing the astroglial marker glial fibrillary acidic protein (GFAP). None of the substances tested modified the percentage of GFAP immunoreactive cells detected, thus arguing against an effect on lineage specification (Fig. 2G).

Fig. 2. ENDF1 and ENDF3 induce DCX promoter activity. (A–C) DCX promoter-firefly luciferase and CMV promoter-renilla firefly cotransfected MEF cells were stimulated for 3 days with the different substances and then processed for luciferase assay. (A) Firefly (FF)-luciferase data. (B) Renilla (R) luciferase data. (C) Normalized data (FF/R). Additionally, data were normalized against the values of the control. The effects of 10 µM ENDF1 and ENDF3 were significantly higher than that of the control condition ($P<.001$). (C) ENDF was superior compared to RA/VPA positive control in the normalized graph. (D–F) MBP promoter-firefly luciferase and CMV promoter-renilla firefly cotransfected rat NPCs were stimulated for 3 days with the different substances and then processed for luciferase assay. (A) firefly (FF)-luciferase data. (B) Renilla (R) luciferase data. (C) Normalized data (FF/R). While MSC-conditioned medium as a positive control induced MBP promoter activity, none of the flavonoids tested had any effect. (G) Effects on astroglial differentiation: MEF cultures treated for 7 days with ENDF1 and control substances. The percentage of GFAP-expressing cells is presented. (H) Effects of ENDFs on the cell proliferation. Note that only RA/VPA results in lower cell numbers than in the vehicle-treated cultures.


A
Luciferase activity (FF)
1μM
10μM
control
RA/VPA
ENDF1 1μM
ENDF1 10μM
ENDF2 1μM
ENDF2 10μM
ENDF3 1μM
ENDF3 10μM
ENDF4 1μM
ENDF4 10μM
IX 1μM
IX 10μM
XN 1μM
XN 10μM
8-PN 1μM
8-PN 10μM
6-PN 1μM
6-PN 10μM
B
Luciferase activity (R)
C
Luciferase activity (FF/R)
D
Luciferase activity (FF)
MSC-CM 50%
MSC-CM 100%
E
Luciferase activity (R)
F
Luciferase activity (FF/R)
G
Percentage of GFAP+ cells
RA / VPA
ENDF1 10 μM
H
Cell number
time (h)
control
RA/VPA
ENDF1
ENDF2
ENDF3
ENDF4

Finally, we examined if the flavonoid compounds would affect MEF progenitor cell proliferation. MEF cultures were stimulated for 24, 48 and 72 h with RA/VPA or ENDFs, and the total number of cells in the culture was thereafter determined. While RA/VPA significantly decreased the proliferation rate, none of the ENDFs tested affected cell proliferation as compared to vehicle MEF cultures (Fig. 2H). In summary, ENDF1 and ENDF3 were potent substances to induce neuronal determination in MEF cultures without affecting the cell lineage specification or proliferation rates.

Several flavonoids have been previously reported to promote neuronal differentiation. Hence, we compared the potency of ENDF1 to induce the DCX promoter with some of these already known flavonoids (i.e., genistein, epigallocatechin gallate, baicalein, liquiritigenin, oroxylinA, fisetin, osajin, naringenin, quercetin and wogonin). None of the flavonoids tested were as effective in activating the DCX promoter as the ENDF1 (Supplemental Figure 1A). Similarly, tocopherol and estradiol have been reported to promote neural stem cell proliferation and differentiation [51,52]. However, neither tocopherol nor estradiol was able to stimulate the DCX promoter in this culture model (Supplemental Figure 1B).

3.2. ENDFs promote neuronal differentiation and neurite outgrowth in fetal progenitor cells

Next, we explored the capacity of ENDFs to promote neuronal differentiation of MEF cells, as suggested by the induction of the DCX

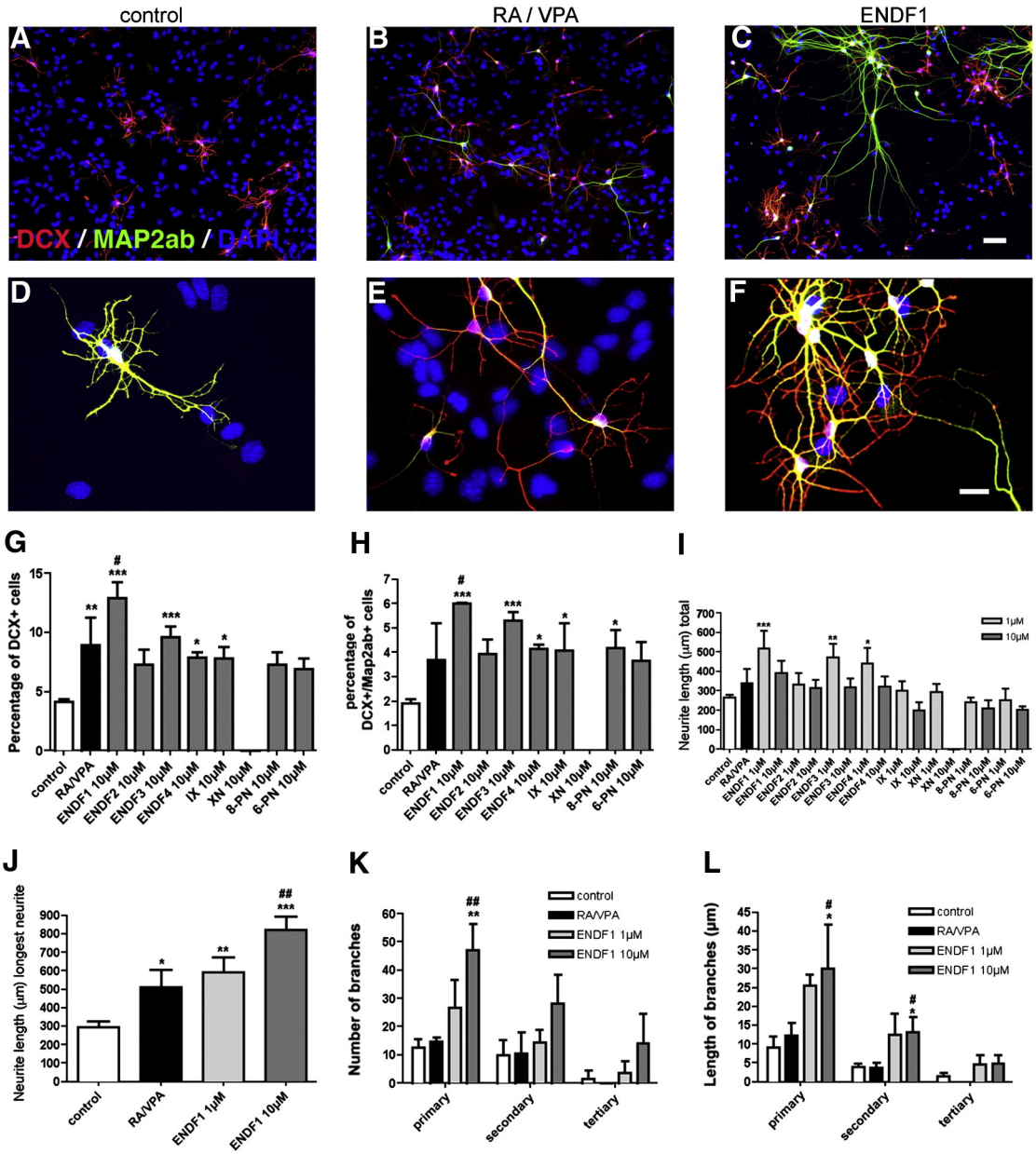


Fig. 3. ENDFs induce neuronal morphology and differentiation in MEF cells. MEF cells were treated for 7 days with control medium, RA/VPA, ENDFs and other flavonoids. (A–F) Representative micrographs of immunodetection of DCX (red), Map2ab (green) and DAPI (blue) of control cultures (A, D), after treatment with RA/VPA (B, E) and after treatment with ENDF1 (C, F) [scale bars: 100 µm (A–C) and 20 µm (D–F)]. The percentage of cells expressing DCX (G) and DCX+Map2ab (H) was determined. (I) Length of DCX-positive neurite profiles. Only neurites longer than 100 µm were taken into consideration. (J) Analysis of the longest neurite per cell. Only neurite longer that 200 µm were considered. (K) Number of primary, secondary and tertiary branches from primary neurites in a distance of 50 µm from the cell soma. (L) Length of the primary, secondary and tertiary branches started from the primary neurite in 50 µm. Experiments were performed at least in triplicate in three independent experiments. Data are shown as mean±SD. Statistical analysis: one-way ANOVA/Tukey post hoc and two-way ANOVA/Bonferroni post hoc. Asterisks indicate significant difference compared with control. Pound signs indicate significant difference compared to RA/VPA treatment.

promoter activity. Therefore, MEF cells were stimulated for 7 days with 10 μM of the flavonoid compounds and analyzed for the expression of DCX (young immature neuronal marker) and Map2ab (a neuronal marker appearing later than DCX during neuronal differentiation), as well as their morphology.

While following vehicle treatment, a few MEF cells were immunoreactive for DCX and some for Map2ab (Fig. 3A, D), RA/VPA promoted the generation of DCX and Map2ab-positive cells (Fig. 3B, E). Moreover, neurites in the DCX and Map2ab-positive cells were longer in the presence of RA/VPA (Fig. 3B, E). Morphological and gene expression analyses revealed that ENDF1 was the most potent compound to promote neuronal differentiation, followed by ENDF3 (Fig. 3C, F, G). ENDF1 generated the highest percentage of cells coexpressing DCX and Map2ab (Fig. 3H). In contrast, ENDF2 and 6PN had no significant effect on the percentage of DCX-expressing cells. Similarly, 8PN had no effect on the percentage of DCX-Map2ab coexpressing cells. ENDF4 and IX led to the generation of DCX-expressing and DCX-Map2ab coexpressing cells to a similar extend than RA/VPA (Fig. 3G, H).

Next, we analyzed neurite length and neurite complexity. We observed that 1 μM of ENDF1, ENDF3 and ENDF4 significantly enhanced neurite length as compared to vehicle (Fig. 3I). The effects of ENDF1 on neurite length were analyzed in more details by measuring the length of the longest neurite for each cell. The ENDF1 treatment led to an increase in the length of the longest neurite in a dose-dependent manner (Fig. 3J), and in addition, ENDF1 significantly promoted neurite branching (Fig. 3K). Hereto, the number of primary, secondary and tertiary branches emerging from primary neurites within a distance of 50 μm from the cell soma was quantified. As definition, the primary branches start from the primary neurite, which is emanating directly from the soma, the secondary branches start from the primary branches and the tertiary branches start from the secondary branches. Besides the number of branches, ENDF1 treatment also led to a significant increase in the length of primary and secondary branches compared to control and to RA/VPA treatment (Fig. 3L).

3.3. ENDFs promote neuronal differentiation and enhance neurite outgrowth in Neuro2a cells

We investigated whether ENDFs can also induce neuronal differentiation in neuronal cell lines in addition to primary neuronal cells. The neuroblastoma-derived cell line Neuro2a is a widely used cell culture model to study neuronal differentiation [42]. Therefore, Neuro2a cells were treated for 2 days with ENDFs or with RA/VPA as a positive control, before being fixed and processed for immunodetection of the axonal growth-associated protein GAP-43. The quantitative analysis of neurite length revealed that ENDF1, ENDF3, ENDF4 and RA/VPA significantly induced neurite growth in Neuro2a cells within 2 days. However, while RA/VPA induced only moderate neurite extension compared to the control conditions (Fig. 4A, B), ENDF1 strongly promoted elongation (Fig. 4C, D).

3.4. ENDF1 promotes neurite regeneration in DRG neurons

Cultures of DRG constitute a classical model to analyze neurite growth and neurite regeneration in vitro [45]. Embryonic day 15 (E15) chicken DRG neurons were incubated for 24 h with NGF 20 ng/ml and/or with 10 μM ENDF1 before being fixed and stained for GAP-43. While under control conditions, only a few neurons survived, NGF enhanced cell survival and neurite length (Fig. 5A, B, E). ENDF1 alone was almost as efficient as NGF in promoting neurite extension. The combination of ENDF with NGF did not further enhance neurite length (Fig. 5C, D, E).

3.5. ENDF1 promotes neuronal survival

We tested the potential of ENDF1 to promote survival of deafferentated cholinergic neurons of the basal nucleus of Meynert in organotypic brain slice cultures [47]. As previously described, application of 10 ng/ml NGF on the slice cultures for 2 weeks strongly

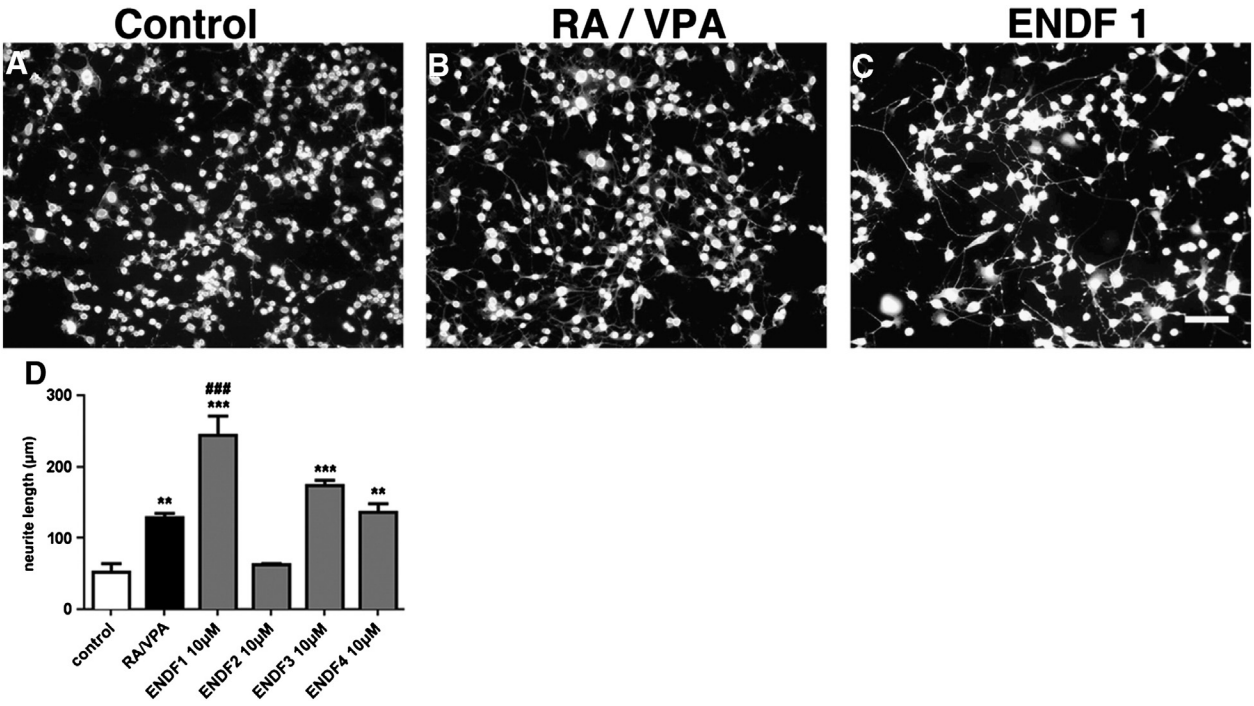


Fig. 4. ENDFs induce neurite outgrowth in Neuro-2a cells. Neuro-2a cells were treated for 2 days with control medium, RA/VPA or 10 μM ENDF1, ENDF2, ENDF3 and ENDF4, fixed and stained for GAP-43. (A–C) Representative micrographs of (A) control conditions, (B), RA/VPA treatment and (C) ENDF1 treatment. Scale bar: 50 μm. (D) The length of GAP-43-positive neurite profiles was measured. Note that ENDF1 treated cultures (C) showed longer and more branched neurites compared to vehicle-treated (A) or RA/VPA-treated (B) cultures. Statistical analysis showed a high significance with ENDF1 and ENDF3 ($P<.001$) as well as ENDF4 ($P<.01$) compared to control. Three independent experiments were performed in triplicate. Data are shown as means±SD. Statistical analysis was performed by one-way ANOVA/Tukey post hoc. Asterisks indicate significant difference compared with control.

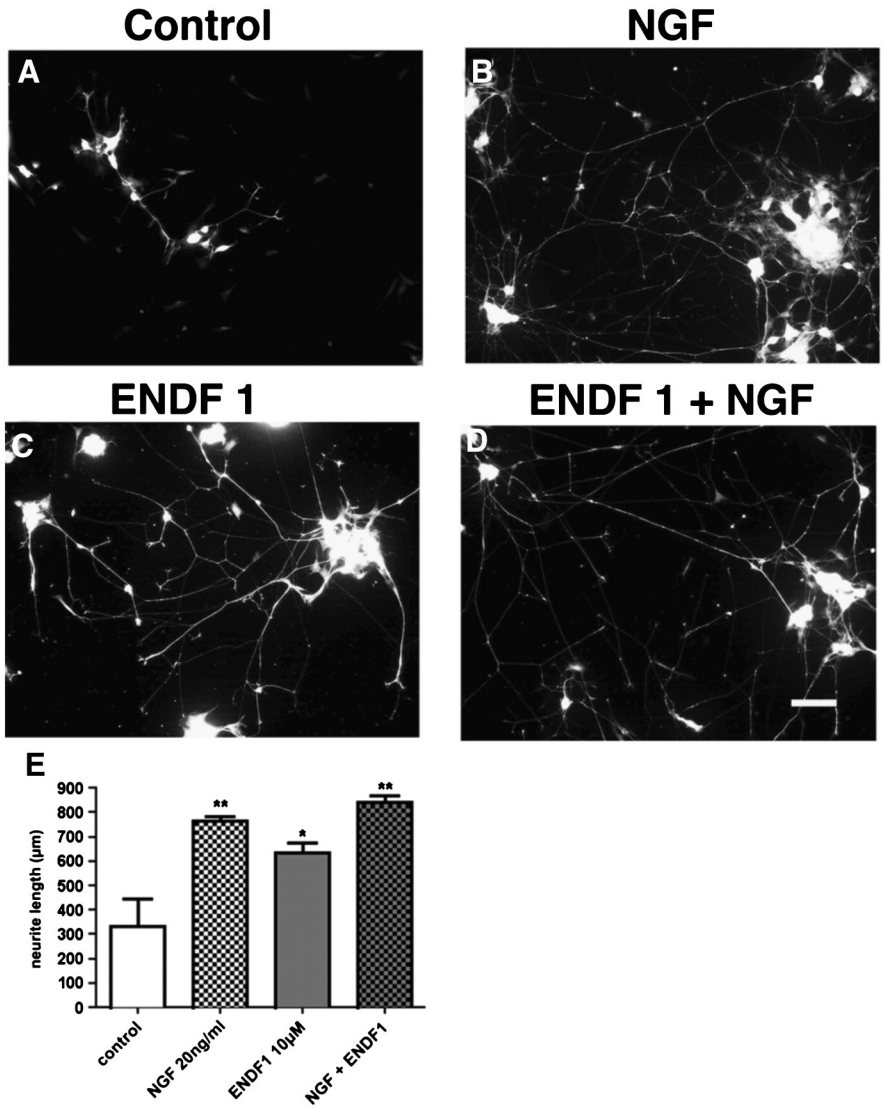


Fig. 5. ENDF1 promotes neurite extension in DRG cultures. DRG E15 neurons were treated overnight with control medium, NGF 20 ng/ml, ENDF1 10 μM or ENDF1 10 μM plus NGF 20 ng/ml, fixed and stained for GAP-43. (A–D) Illustrative fluorescent images for GAP-43 are shown for control condition (A) and after treatment with NGF 20 ng/ml (B), with ENDF1 10 μM (C) or with ENDF1 10 μM plus NGF 20 ng/ml (D). Scale: 50 μm. (E) The length of GAP-43-positive neurite profiles was measured. NGF ($P<.01$), ENDF1 ($P<.05$) and NGF plus ENDF1 ($P<.01$) strongly promote neurite extension compared to the control. Experiments were performed in triplicate in three independent experiments. Data are shown as mean±SD. Statistical analysis was performed by one-way ANOVA/Tukey post hoc. Asterisks indicate significant difference compared with control.

promoted the survival or ChAT-immunoreactive neurons in the basal nucleus of Meynert (Fig. 6A, C, D). Although to a lesser extent, 10 μM ENDF1 also promoted significantly the survival of these cholinergic neurons (Fig. 6A, B, D).

The neuroprotective activity of ENDFs was further tested in cell cultures of the noradrenergic pheochromocytoma PC12 cell line [53]. PC12 cells were challenged by exposure to 300 μM cobalt chloride for 24 h, and cell death was quantitatively analyzed according to the cell death effector caspase-3/7 activity, a widely used neurotoxicity assay [54]. NGF was used as a positive control for a neurotrophic and neuroprotective factor for PC12 cell. We observed that 50 ng/ml NGF and 10 μM ENDF1 significantly reduced the $CoCl_2$-induced caspase-3/7 activity, while 1 μM ENDF1 promoted only a mild, but not significant, cell survival (Fig. 6E).

4. Discussion

Here, we present a group of hops-derived prenylflavonoids as potent inducers of neurogenesis, neuronal differentiation and neurite outgrowth. Moreover, we report that ENDF1, the most potent compound identified in the present screening, also displays neuroprotective activity and could rescue neurons from apoptotic cell death. The differentiation-promoting activity was specific to the neuronal lineage, since neither oligodendroglial differentiation nor astroglial differentiation was induced, according to MBP promoter activation and the percentage of GFAP-expressing cells accordingly. Also, it is noteworthy that MEF cell proliferation was not affected by the application of ENDFs.

Flavonoids have previously been reported to be potent neuroactive molecules. For example, the flavonoids wogonin and liquiritigenin promote neuronal differentiation of precursor cells [6,55]. In addition, liquiritin, quercetin and the green tea polyphenol promote neurite outgrowth in PC12 cells [56–58]. In this study, we compared the induction of the DCX promoter activity in MEF cells resulting from ENDFs application with the induction obtained using previously reported neuroactive flavonoids, that is, genistein, epigallocatechin gallate, baicalein, liquiritigenin, oroxylinA, fisetin, osajin, naringenin, quercetin and wogonin, as well as other known potent neuronal

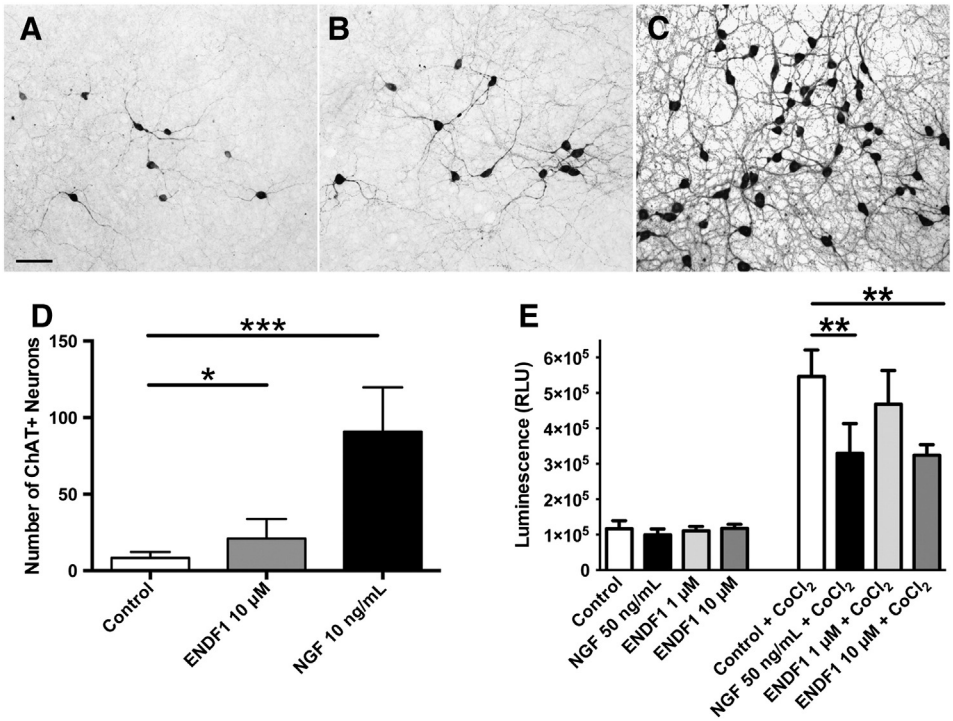


Fig. 6. ENDF1 prevents PC12 cells form cell death. (A–C) Representative micrograph of cholinergic neurons in the nucleus basalis of Meynert in slice cultures treated with (A) vehicle, (B) 10 µM ENDF1 or (C) NFG 10ng/ml. Scale bar in panel A=100 µM. (D) Quantification of the cholinergic neurons surviving in the slice cultures after 2 weeks revealed a significant neuroprotective effect of ENDF1 and NGF (*P<.05 and ***P<.001). (E) PC12 cells were stimulated for 24 h with the different substances before caspase-3/7 activation was measured. As expected. $CoCl_2$ strongly induced caspase-3/7 activation. Cells were protected from $CoCl_2$-induced cell death by NGF 50 ng/ml and by 10 µM ENDF1 All experiments were performed in triplicate in three independent experiments. Data are shown as mean±SD. Statistical analysis was performed by one-way ANOVA/Newman–Keuls post hoc. Asterisks indicate significant difference compared with control (*P<.05 and ***P<.001).

differentiation inducers like tocopherol and estradiol. This comparison revealed that ENDF1 was by far the most potent DCX promoter inducer and suggested that ENDF1 could promote neuronal differentiation effectively.

The effects of ENDFs on neural cells were not merely restricted to the induction of the DCX promoter activity. Indeed, ENDFs induced a broad spectrum of neuronal differentiation attributes. For example, stimulation of neural cells with ENDFs elevated the percentage of DCX or Map2-expressing cells and promoted neurite growth and neurite arborization complexity. Obviously, this might have enormous clinical implications since axonal sprouting and regeneration is a requirement for structural and functional repair after CNS lesions such as spinal cord injury [1]. Indeed, preliminary data suggest that ENDF1 partially overrides the axonal growth inhibitory activity of CNS extracellular matrix inhibitors (own preliminary results). In addition to the induction of neuronal differentiation and neurite outgrowth, flavonoids can promote neuronal survival (for review, see Mandel et al. [59] and Weinreb et al. [60]). In this report, we demonstrate that ENDF1 protected PC12 cells from $CoCl_2$-induced neuronal cell death. Moreover, ENDF1 moderately promoted the survival of deafferentated cholinergic neurons in brain slice cultures.

Based on previously reported investigations of various flavonoids, further neuroprotective activities for ENDFs can be presumed. For example, osajin was shown to be neuroprotective for cortical neurons against amyloid beta-induced neurotoxicity. Liquiritigenin inhibited the expression of amyloid beta in different brain regions in AD mice models [15,61]. In addition, resveratrol, quercetin, fisetin, luteolin and myricetin reduced inflammation and neuronal apoptosis induced by oxidative stress in in vitro models as well as following stroke [62,63].

The signaling pathways used by ENDFs to mediate their activities are currently unknown. However, the targets of some flavonoids have been identified, and their miscellany has recently been summarized [24]. For example, flavonoids can bind to several neuronal receptors such as the receptors for adenosine, $GABA_A$, δ-opioid, nicotine, BDNF, estrogen and testosterone [24]. In addition, downstream signaling of flavonoids involves the Map kinase and PI3-kinase pathways [64]. Identification of the signaling pathways activated by the ENDFs will certainly contribute to the understanding of the full potential of this new and potent class of neuroactive molecules.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2013.06.005.

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