Supplementary information for

Quantifying orientational regeneration of injured neurons by natural products concentration gradients in a 3D microfluidic device

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This file includes: Table S1, Figure S1-S9, software simulation analysis

Table S1. Dimensions of chips used in this work

Inlets of side channels	Outlets of side channels	central gel channels	interconnecting grooves
width/height (μm)			length/width/height (μm)
200/100	1000/100	1000/100	250/100/100
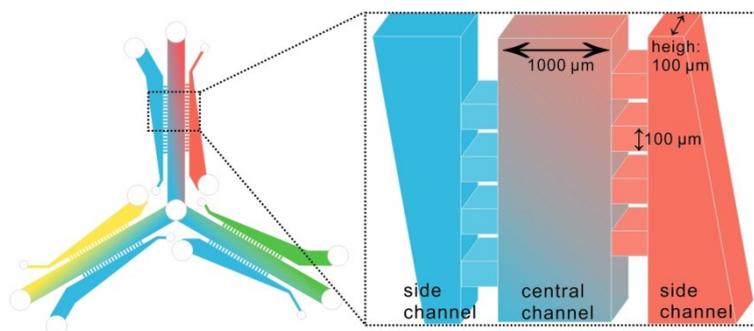
Figure S1. Schematic diagrams showing the dimensions of the microfluidic channels.
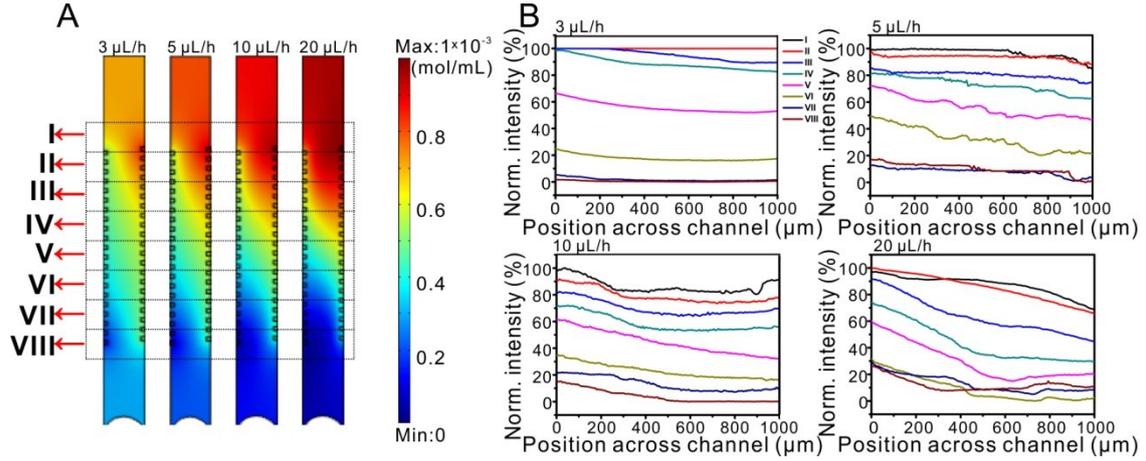
Figure S2. Computational and experimental characterizations of the gradients generated in the central gel channels. (A) The simulation of distributions on generating different gradients with various flow rates (from 3 µL/h to 20 µL/h), the width of interconnecting grooves is 100 µm. (B) Normalized fluorescence intensity profiles in the central gel channels, different colors represent the gradient formed in different regions (from 'I' to 'VIII').

Software simulation analysis

In the COMSOL Multiphysics simulation, 3D collagen hydrogel was treated as porous media to simulate the gradient distribution. This needs only two key parameters: the porosity of the porous media and the diffusion coefficient of those three molecules in fluid.

Referring to relevant literature, the porosity of the porous media was set at 0.85[1]. The diffusion coefficient was calculated with Wilke-Chang formula:

$$D_{AB} = 7.4 \times 10^{-15} \frac{(\phi M_B)^{\frac{1}{2}} T}{\mu V_A^{0.6}}.$$

Where D_{AB} is the diffusion coefficient of solute A in solvent B, m²/s; ϕ is the associated parameters of the solvent ($\phi = 2.6$ for water); M_B is the molar mass of solvent B, kg/kmol; T is the temperature of solution, K; μ is the viscosity of solvent B, $Pa \cdot s$; V_A is the molecular volume of A in the normal boiling point, cm³/mol) V_A can be estimated by Tyn-Calus method:

$V_A = 0.285 V_c^{1.048}$ (V_c is the critical volume of matter, cm³/mol). Because there is not much difference in the molar mass between the three molecules, the diffusion coefficients of these three molecules in fluid were calculated to be about 4×10⁻¹⁰ m²/s.
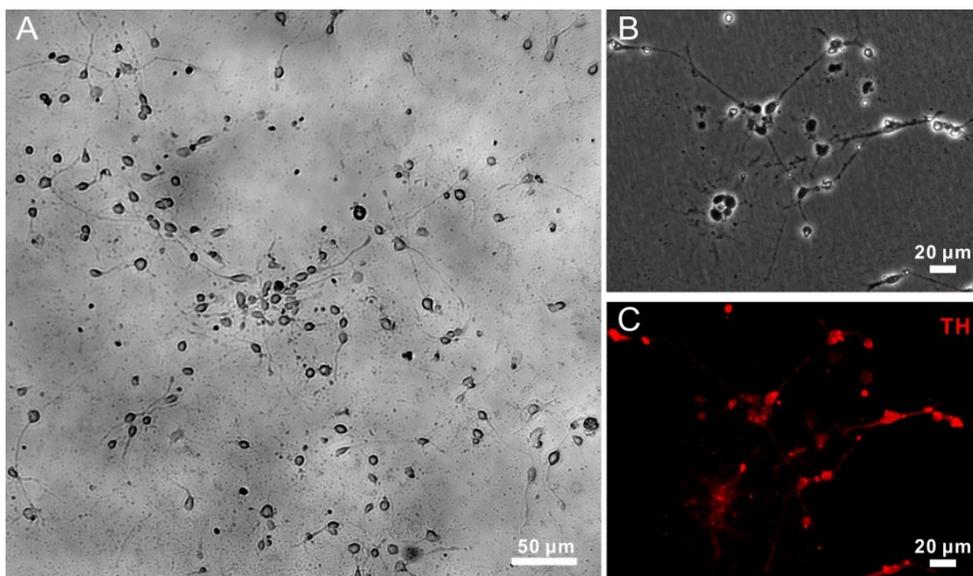
Figure S3. Photomicrographs of primary cultures of SD rat ventral tegmental area (VTA) dopaminergic neurons. (A) Micrograph of the VTA neurons at 7 DIV. (B) The enlarged micrograph of dopaminergic neurons, and (C) dopaminergic neurons were immunolabeled by the antibody against TH (red).

Catalpol   Gardenoside   Harpagide
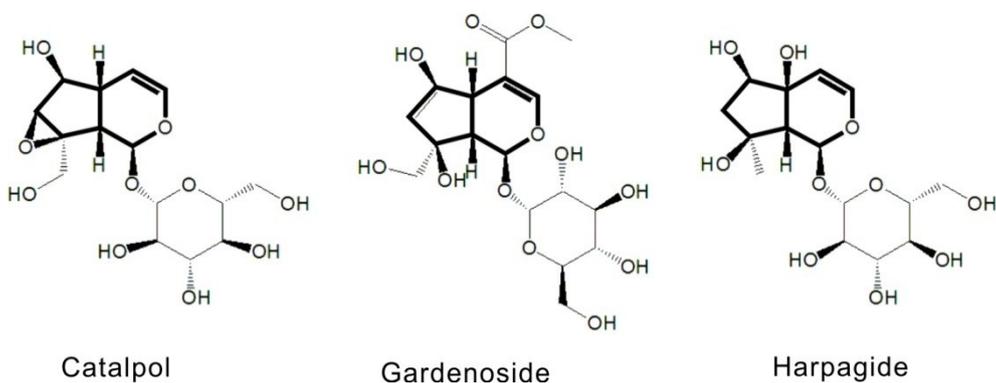
Figure S4. Molecular structures of three natural products of iridoid glycosides (catalpol, gardenoside and harpagide).
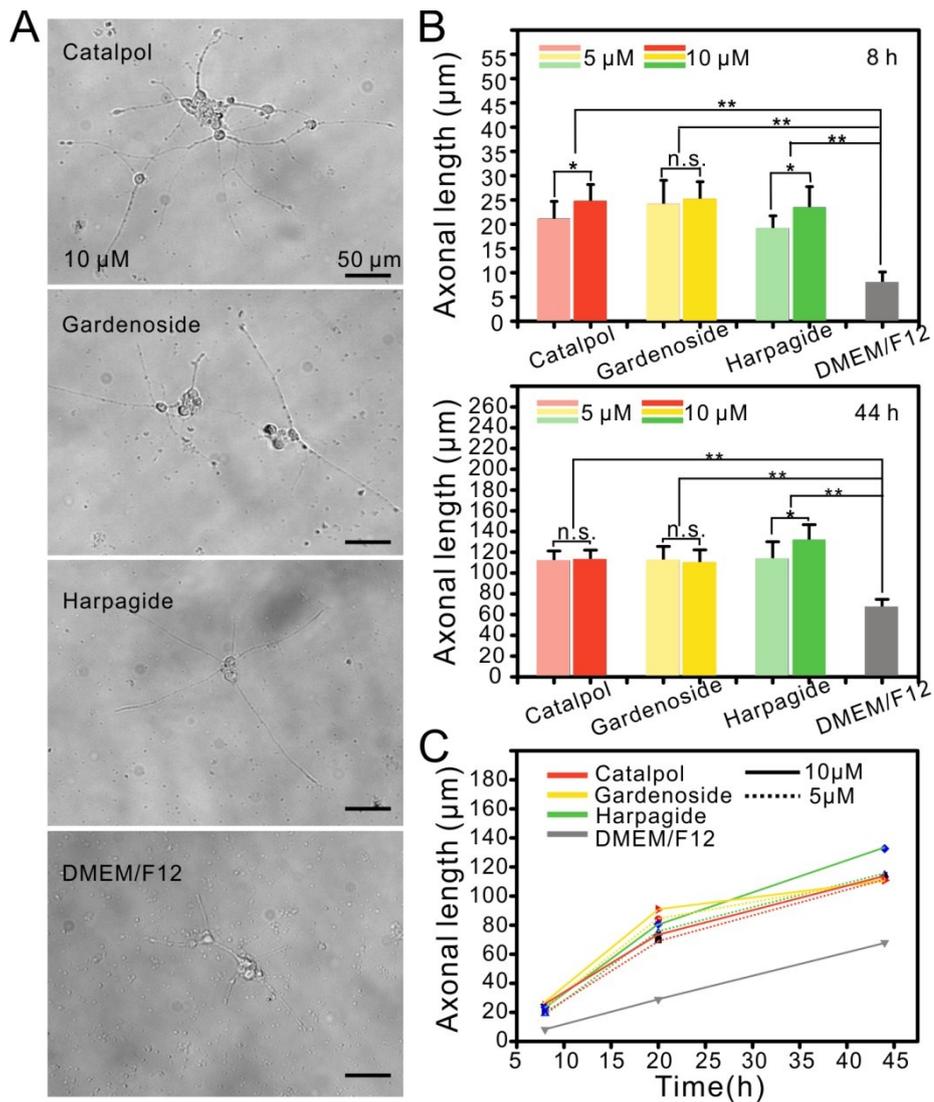
Figure S5. Axonal-outgrowth-promoting activity in primary cultured rat dopaminergic neurons and quantification of axonal length changes in 2D plastic substrates. (A) Representative phase contrast image of neurite-outgrowth-promoting by three kinds of natural products (10 μM) and basal medium (as a control) in 2D plastic substrates culture. (B) Comparative effect of three natural products with different concentrations (light lines represent 5 μM and dark lines present 10 μM) on the length of axons after being culture for 8 hours and 44 hours(one-way ANOVA; **p < 0.01; *p <0.05; n.s., not significant). (C) Statistics of the axonal length in response to different natural products as time increases (8 hours, 20 hours and 44 hours).
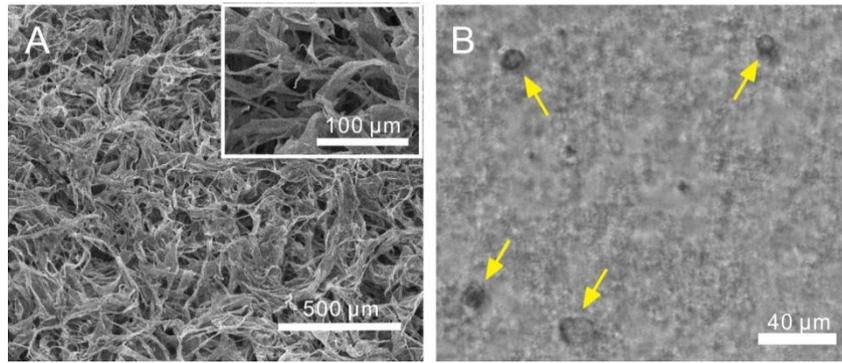
Figure S6. Collagen hydrogel for neurons culture. (A) SEM image of collagen with the concentration of 2.5 mg/mL. (B) Primary dopaminergic neurons cultured in collagen hydrogels with the concentration of 2.5 mg/mL. The yellow arrows refer to the undifferentiated DAergic neurons.
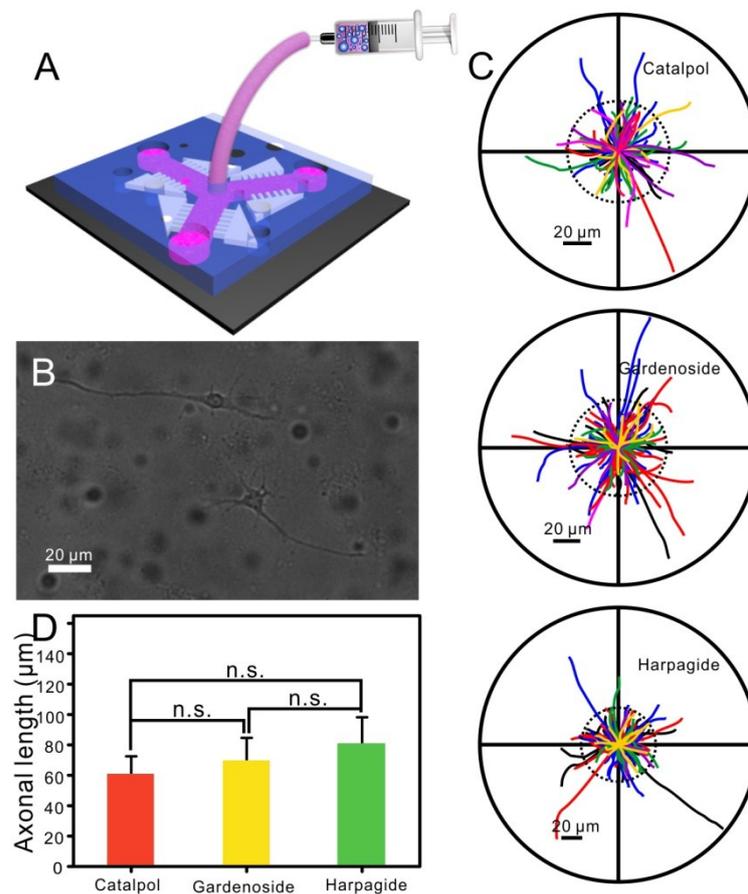
Figure S7. Response of axons to natural products in 3D microfluidic devices and statistical analysis of morphological changes. (A) Schematic representation of DA neurons perfused into the central channels of the chip. (B) Representative phase contrast image of DA neurons in 3D collagen hydrogel after being cultured for 5 d. (C) Typical tracks (starting at the origin) of neurons axonal growth promoted by three natural products without concentration gradients in a single chip. (D) Comparative effect of different natural products on the length of axons. (one-way ANOVA; n.s., not significant)
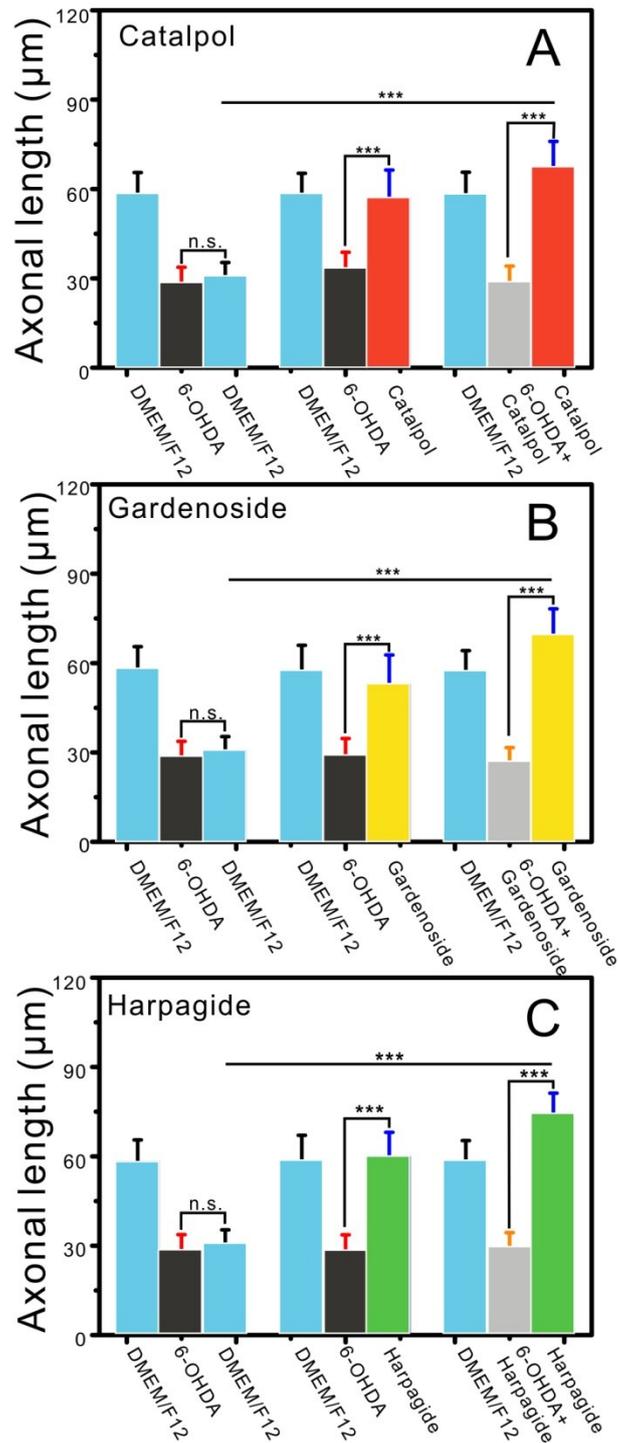
Figure S8. Statistics of axonal length during injury and regeneration by three natural products (10 μM) without gradients in 3D microfluidic chip. The black and gray columns represented DA neurons axonal length after treating with 100 μM of 6-OHDA in the absence or presence of natural products for 5 hours. The red, yellow and green columns represented the injured DA neurons axonal length after repairing with catalpol, gardenoside and harpagide (10 μM) for 3 days. (one-way ANOVA; ***$p < 0.001$; n.s., not significant)
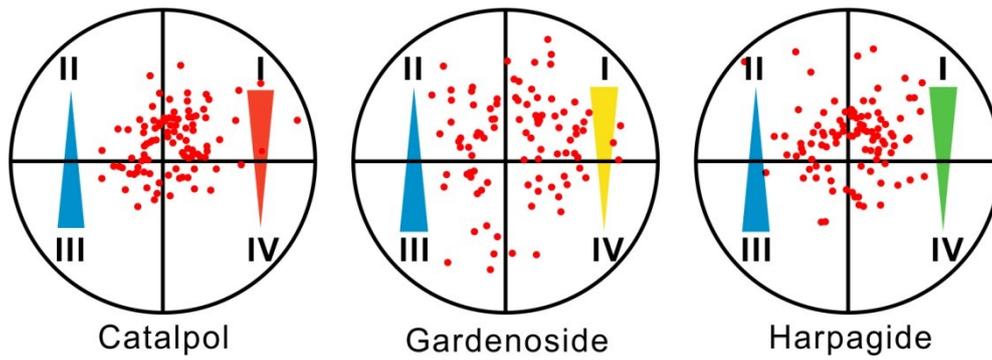
Figure S9. Statistics of the polarization direction of repaired neurons. Definition of the angle of axon polarized into four quadrants (I, II, III and IV). Red dots represent axonal tip of repaired neurons. Blue arrows represent the flow (from quadrant II to III) of basal medium, and red, yellow, green arrows represent the flow (from quadrant IV to I) of three natural products, respectively.

References:

1. N. Davidenko, J. J. Campbell, E.S. Thian, C. J. Watson, R.E. Cameron, *Acta Biomater.*, 2010, 6, 3957-3968.