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Berberine exposure triggers developmental effects on planarian regeneration

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The mechanisms of action underlying the pharmacological properties of the natural alkaloid berberine still need investigation. Planarian regeneration is instrumental in deciphering developmental responses following drug exposure. Here we report the effects of berberine on regeneration in the planarian *Dugesia japonica*. Our findings demonstrate that this compound perturbs the regenerative pattern. By real-time PCR screening for the effects of berberine exposure on gene expression, we identified alterations in the transcriptional profile of genes representative of different tissues, as well as of genes involved in extracellular matrix (ECM) remodeling. Although berberine does not influence cell proliferation/apoptosis, our experiments prove that this compound causes abnormal regeneration of the planarian visual system. Potential berberine-induced cytotoxic effects were noticed in the intestine. Although we were unable to detect abnormalities in other structures, our findings, sustained by RNAi-based investigations, support the possibility that berberine effects are critically linked to anomalous ECM remodeling in treated planarians.

Berberine (18,5,6-dihydro-9,10-dimethoxybenzo (g)-1,3-benzodioxolo (5,6-a) quinolizinium) is a natural alkaloid widely praised for its multiple pharmacological properties mediated by targeting molecules, which are involved in a wide range of biological processes, molecular functions and signaling pathways^{1,2}. This compound is present in various plants that show therapeutical properties, including *Hydrastis canadensis*, *Coptis chinensis*, *Berberis aquifolium*, *Berberis vulgaris* and *Berberis aristata*. Berberine is also one of the main alkaloids present in greater celandine (*Chelidonium majus*, Papaveraceae), an herb whose therapeutic properties and potential side effects still deserve testing in appropriate studies³. To promote understanding of the therapeutic actions, as well as the toxic potential of *C. majus*, our work focuses on the analysis of the effects produced *in vivo* by its main alkaloids⁴.

Berberine has been used in eye drops to treat some eye disorders⁵. The benefits of this compound have also been demonstrated in diabetes, cholesterol and other medical conditions^{3,6,7}. Consequently, berberine is a constituent of some food supplements and phytopreparations, that are used to control cholesterol and triglycerides blood levels in humans. It has been reported that oral preparations of berberine are well tolerated in rodents (100 to 300 mg/kg). In humans the typical oral dose of berberine associated with positive effects is 300 mg, taken three times a day. Higher doses appear to be non-toxic, although often associated with gastrointestinal discomfort⁸. However, because berberine hits multiple targets and can have interactions with a variety of molecular structures and different pathways², attention must be paid to control the efficacy and safety of berberine-based treatments and to avoid adverse interactions with other therapeutic agents. Due to a lack of available scientific evidence, limitations in the use of berberine are recommended in pregnant women and during lactation.

It is acknowledged in the pharmaceutical industry that more predictive tests need to be established in order to improve drug safety. This recognition is driving the development of reliable pharmacology and toxicology models. To evaluate the bioactivity of berberine on proliferating tissue and morphogenetic processes, herein we examine the effects of this compound on the planarian *Dugesia japonica*, an invertebrate animal model widely used in regenerative research to monitor drug responsiveness at the organismal level^{4,9-11}. In addition to regenerate any body tissues, including the nervous system, planarians do not show any physiological deterioration, as constitutively integrate new cells to replace all aged differentiated cell types for tissue maintenance. Planarian regeneration is a complex process that includes activation and migration of stem cells (neoblasts) to generate



outgrowths of non-dividing progeny cells (the regenerative blastema) at the wound site, as well as body remodeling to restore the normal morphology of the worm¹². During head regeneration differentiation of the two cephalic ganglia (the planarian brain) occurs early and two dorsal cerebral eyes are formed and connected by axons to the brain¹³. Finally, many genes and pathways associated to the remarkable regenerative abilities of planarians have been identified and functionally characterized by inhibiting their expression level through pharmacological treatments or RNA interference (RNAi)¹².

The purpose of our study is to use regeneration in planarians as a model to pinpoint developmental effects of exposure to this compound *in vivo*. Evolution of toxicological methodologies on the inexpensive planarian model is a suitable platform to assess a better level of developmental safety of a variety of drugs.

Results

Absorption of berberine in planarians. To determine whether berberine could enter cells, we observed its distribution in living animals and in dissociated planarian cells. After soaking of the animals in berberine, this compound, which emits yellowish-green fluorescence under ultraviolet light (365 nm), appeared intensively accumulated in the planarian body, especially at the intestine level (Figure 1A–E). Observation of dissociated cells demonstrated that all cells could be identified as berberine-positive cells, being the fluorescent signal distributed within the entire cell or specifically accumulated in the nucleus (Figure 1F–J).

As a prerequisite to investigate berberine bioactivities in planarians we performed a quantitative analysis of the content of this compound in our experimental samples by the analysis of fluorescence intensity on silica gel plates. Figure 1K illustrates the chemical structure of berberine, and Figure 1L shows the results obtained both for intact and regenerating planarians. Interestingly, it should be noted from the graph that, at the same berberine concentration in the soaking medium, intact planarians appear to absorb higher concentrations of the alkaloid than regenerating animals.

The simplest explanation for this result is that the planarian fragments - transiently unable to eat during regeneration - cannot absorb berberine from the pharynx (i.e. the planarian mouth), but only through the skin.

Effects of berberine on planarian regeneration. Following berberine exposure we observed that head fragments regenerated tails with morphology and macroscopic dynamics that were indistinguishable from those observed in control animals, while tail fragments redeveloped heads showing abnormalities in the visual system. Planarian eyes are two dorsal cephalic structures consisting of rhabdomeric photoreceptors and pigmented optic cup cells that are easily identifiable by light microscopy. The time taken for the eye differentiation is commonly used as a measure of the regeneration rate. Axonal projections of the photosensitive cells project both ipsilaterally and contralaterally to the visual area of the brain, forming an optic chiasm. Anti-arrestin (VC-1) immunostaining is commonly used to better visualize regeneration of the visual system¹⁴. Two asymmetric and smaller eyes or a single eye (cyclopic eye), as well as improper axonal projections unable to regenerate the stereotypical pattern of the optic chiasm found in the controls, were identified following 50 μ M, 400 μ M or 600 μ M berberine treatments (Figure 2A–E'). After 600 μ M berberine exposure heads regenerating without eyes were also detected (Figure 2E,E'). The graph shows the percentage of the different phenotypes observed (Figure 2F).

Phototactic behavior in regenerating fragments following 600 μ M berberine treatment. When exposed to light, planarians display negative phototaxis. This characteristic behavior has been analyzed

by a phototaxis test that consists in monitoring how quickly planarians move away in response to light¹⁴. Following 600 μ M berberine treatment most fragments regenerated a head with severe defects to the visual system and did not move or moved in a random way, while the controls moved away from a light source (Figure 2 G–H). Intriguingly, we observed that berberine treatment also impaired the movements in fragments regenerating a tail. These fragments did not show any defect in the visual system. In fact a slight, though significant, difference between berberine-treated specimens and controls in the time spent in the target area could be detected (Figure 2I). These data suggest that the reduced overall motility in berberine-treated planarians is probably the combined result of more general nervous system defects.

Effects of berberine on cell proliferation and apoptosis during planarian regeneration. To investigate the mechanism of action of berberine on planarian regeneration we first explored the possibility that the anomalous phenotypes were related to cell proliferation-inhibiting and/or apoptosis-inducing activity of this compound^{15,16}. We analyzed whether the basal levels of expression of molecular markers specific for proliferating cells were decreased by berberine treatments. Real-time RT-PCR assays were performed with the stem cell proliferation markers *Dj-mcm2*¹⁷ and *Dj-bruno*¹⁸, respectively. The results provide evidence that the expression level did not change significantly following the treatment (Figure 3A). As cell proliferation is essential for regeneration and growth of the blastema, as a further control we performed a morphometric analysis of the ratio between blastema area and the entire body. This analysis provided quantitative evidence that exposure to this compound did not cause any significant reduction in the relative size of the blastema with respect to the controls (Figure 3B). Further, as berberine promotes apoptosis via the mitochondrial/caspase pathway^{1,19}, we also evaluated the effects of this compound on activity of the caspase-3. The time-dependent spectrophotometric analysis of caspase-3 activity in 7-day old regenerant animals following 50 μ M, 400 μ M and 600 μ M berberine treatment was compared with that of untreated controls. As illustrated in Figure 3C no significant difference with the controls was observed up to 4 hours. As in normal conditions two apoptotic peaks characterize planarian regeneration at 4 hours and 3 days after amputation²⁰, we extended the analysis of activity of the caspase-3 to 4-hour and 3-day old regenerants (Supplementary Figure S1). On the whole these results indicate that berberine does not induce any significant effect on caspase-3 activity.

Transcriptional responses triggered by berberine exposure. To further dissect the molecular mechanisms of action of berberine we compared the expression of different categories of genes in berberine-treated planarians with untreated controls. We first considered genes that are activated during visual system regeneration in *D. japonica*^{21,22}. Our Real Time RT-PCR data demonstrated that early genes, such as *Dj-six1* and *Dj-eya*²³, show significant decrease of their expression level following berberine treatment. Under the same experimental conditions, we also observed that berberine has an inhibitory effect on the expression level of the medium-term gene *Dj-pax6A*²⁴, as well as on that of late genes, such as *Dj-ops* and *Dj-netB*^{22,23} (Figure 4A).

No gross morphogenetic defects in the regeneration of the central nervous system (CNS) could be detected with the pan-neural marker *Dj-syt*¹⁷ following 600 μ M berberine exposure (Figure 4B). Moreover, morphometric analysis showed that the brain of exposed planarians was similar in size and architecture to that of the controls (Figure 4C). Head fragments regenerating the whole posterior body also showed no defects. To acquire a more thorough understanding about the effect of 600 μ M berberine treatment on other neuronal cell types, we also compared gene transcription of other markers between berberine-treated planarians and controls. We selected

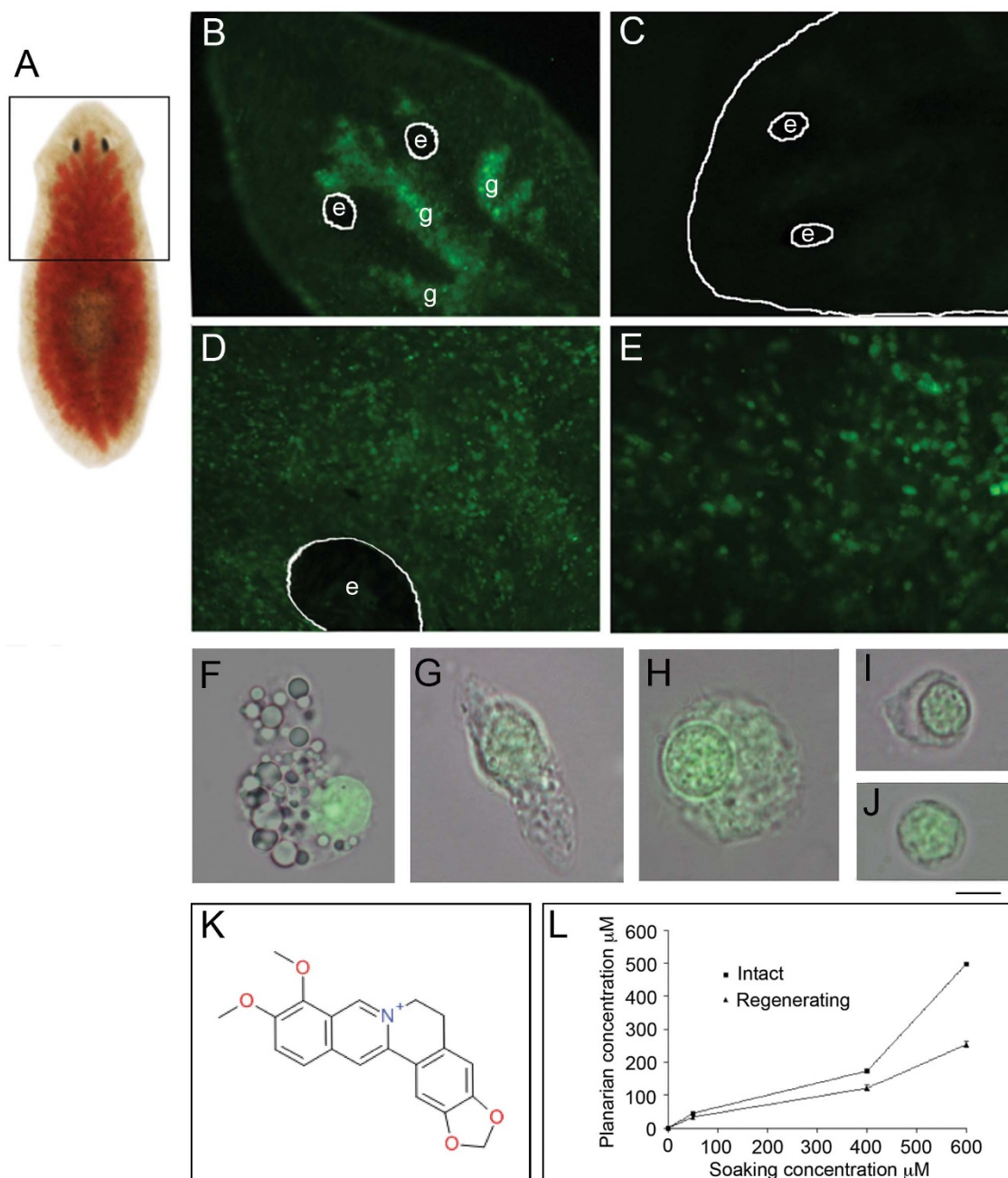


Figure 1 | Berberine absorption in planarians. (A) Dorsal view of a live planarian. The highly branched morphology of the intestine, consisting of a single anterior and two posterior primary branches³², is visualized by red food dye. Anterior is up. Black box indicates the region magnified in (B–E). (B–E) Head of an intact planarian, as visualized under UV light. Berberine shows a wide range of distribution and is mainly accumulated in the intestinal branches (g). (B) 400 μM berberine. (C) Control. The planarian body and eyes are highlighted by white lines. e: eye; g: gut branches. (D,E) Magnification of two regions in (B), localized at the gut branch level (D) and near the epidermis (E). (F–J) 600 μM berberine visualization in dissociated cells. (F) A gastrodermal cell showing a berberine-positive nucleus. The cytoplasm is filled with droplets and vacuoles. (G) A big elongated hypothetical muscle cell with a berberine-positive nucleus. (H) An oval, middle-sized cell, probably an acidophilic cell, shows both cytoplasmic and nuclear localization of berberine. (I) A middle-sized cell, probably a cell in the process of differentiation, shows a berberine-positive nucleus. (J) berberine stain in a neoblast-like cell. (K) Chemical structure of berberine. (L) Graph representing the concentration of berberine absorbed by planarians versus the berberine concentration in the soaking medium. Intact: intact planarians. Regenerating: regenerating planarians. Scale bar: 500 μm in (A); 150 μm in (B) and (C); 75 μm in (D); 30 μm in (E); 10 μm in (F–J).

Dj-gad, a marker of GABAergic neurons²⁵ and *Dj-rax*, a gene expressed in a subset of nerve cells²⁶. While no difference in *Dj-gad* expression level was observed, a significant reduction of *Dj-rax* transcripts was detected in berberine-treated planarians. *Dj-syt* also showed reduced expression following 600 μM berberine exposure (Figure 4D). Possible alterations in the brain branches, the region where sensory organs are located, were further evaluated with the

specific markers *Dj-otp* and *Dj-inx3*^{27,28}. Our data do not show any significant difference in gene expression profiles and organization of this structure following berberine treatment (Figure 4D,E,F).

Potential effects of this compound in other tissues were also assessed by comparing the expression profile of representative markers of postmitotic cells: *Dj-ahnak* (epithelial cells)²⁹, *Dj-inx1* (gastrodermal cells)²⁸, *Dj-mhca* (muscle cells)³⁰, *Dj-inx10* (secretory

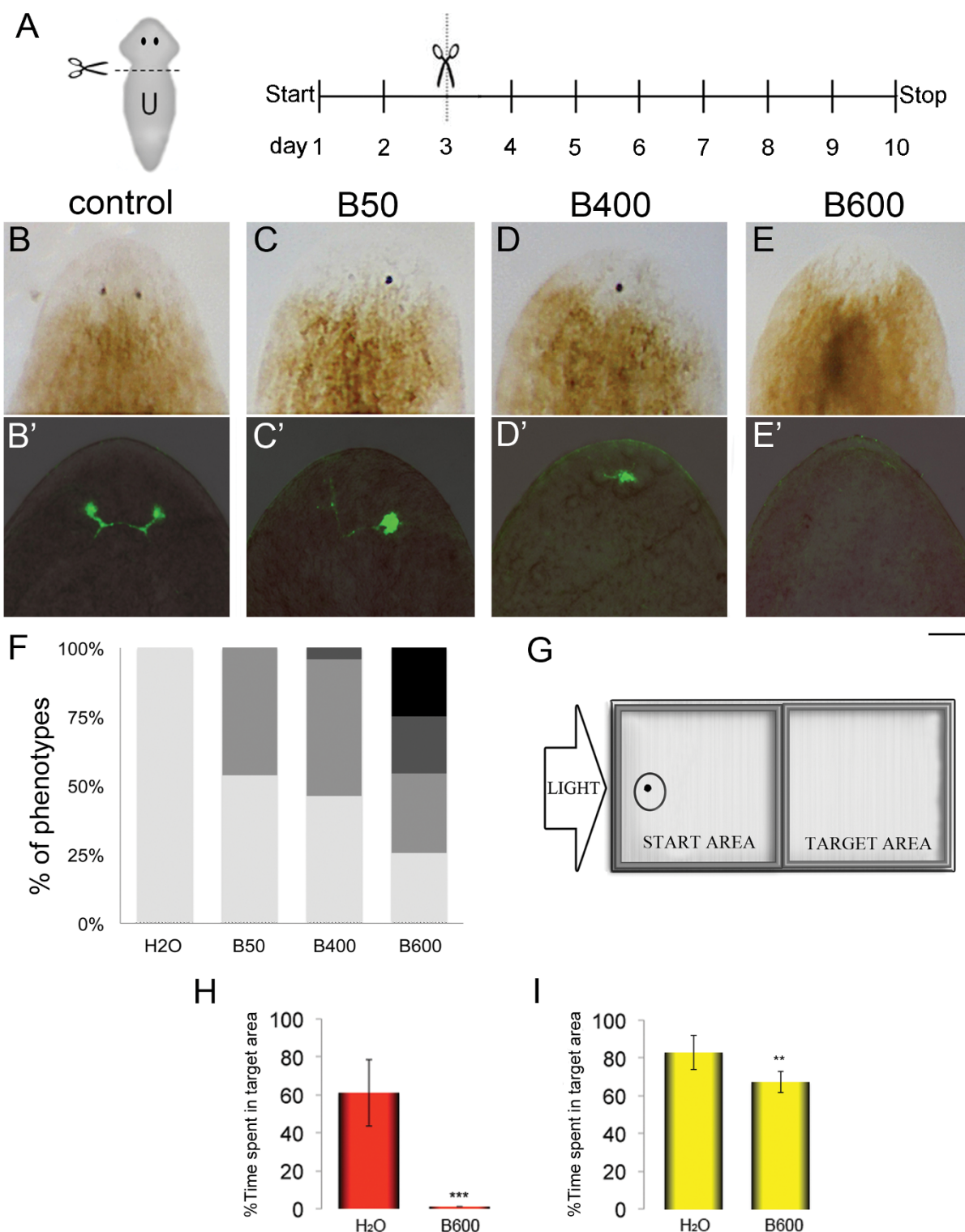


Figure 2 | Berberine treatment affects visual-system regeneration. (A) Schematic of the experimental procedure used to obtain regenerating fragments. (B–E) Representative brightfield images of planarians regenerating a head following berberine treatments (regeneration: 7 days). (B'–E') eyes and the optic chiasm are visualized by immunostaining with anti VC-1 antibody. (B) Control. (C) B50: berberine 50 μ M. (D) B400: berberine 400 μ M. (E) B600: berberine 600 μ M. (F) The graph shows the percentage of the different phenotypes. A number of 300 animals was analyzed for each berberine concentration. Light grey: normal eyes; grey: asymmetric and smaller eyes; dark grey: cyclopic eyes; black: no eyes. (G) Schematic of the phototactic test used to assess the behavior of regenerating planarians following 600 μ M berberine treatment (regeneration: 7 days). (H) The graph indicates the time spent in the target area by tail fragments regenerating a head. (I) The graph indicates the time spent in the target area by head fragments regenerating a tail. Each graph represents the mean \pm s.d. from 10 independent specimens. H₂O: control; B600: berberine 600 μ M. Scale bar: 100 μ m in (B–E) and 50 μ m in (B'–E').

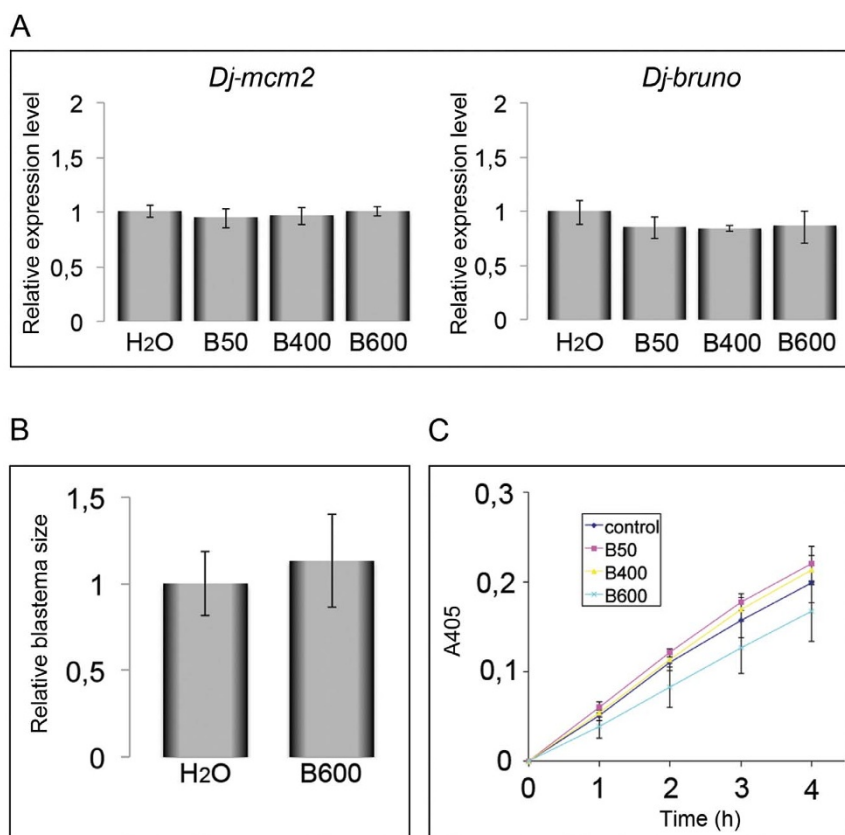


Figure 3 | Effect of berberine treatment on cell proliferation and apoptosis in regenerating planarians. (A) Expression level of *Dj-mcm2* and *Dj-bruno* in berberine-treated planarians and controls, analyzed by Real Time RT-PCR. H₂O: controls; B50: berberine 50 μ M; B400: berberine 400 μ M; B600: berberine 600 μ M. In Real Time RT-PCR experiments, the expression levels are indicated in relative units, assuming as unitary the value of the controls. Each value is the mean \pm s.d. of 3 independent experiments, carried out in 6 replicates (B) Morphometric analysis of blastema size in planarians regenerating a head following berberine 600 μ M (B600) treatment and in controls. Values are average \pm s.d. from 30 samples. (C) Effects of berberine on activation of caspase-3 in planarians after 7 days of regeneration. Berberine treatments: B50: 50 μ M berberine-treated planarians; B400: 400 μ M berberine-treated planarians; B600: 600 μ M berberine-treated planarians; control: planarians regenerating in water. Caspase-3 activation was monitored for 4 hours (h). A405: absorbance at 405 nm. Data represent mean values \pm s.d. from 3 independent samples.

cells²⁸, and *Dj-mmp1* (secretory cells)³¹ (Figure 5A). All berberine concentrations used in the present study induced expression of *Dj-ahnak*. *Dj-mhca* and *Dj-inx1* did not show any significant change in expression level with respect to the controls.

Transcription profiles of *Dj-inx10* and *Dj-mmp1* were significantly down regulated by berberine treatment. Although berberine appears to misregulate gene expression in many cell types, our in situ hybridizations did not reveal significant pattern defects in the corresponding tissues (Figure 5B). However, in about 60% of berberine-treated planarians some defects in the reorganization of intestinal branches could be detected after in situ hybridization with the intestine-specific riboprobe, *Dj-inx1* (Figure 5B). In addition, histological assessment by light microscopy of wax sections revealed that the typical morphology of this tissue³² was damaged (Supplementary Figure S2).

As *Dj-mmp1* encodes a matrix metalloproteinase - a metzincin enzyme considered a major executor of extracellular matrix remodeling in planarians³¹ - to assess whether berberine had any effect on extracellular matrix dynamics we compared the transcriptional regulation of other metzincin members. We noticed that the presence of berberine significantly inhibited the expression of *Dj-ast4*, an astacin-like gene involved in eye morphogenesis (Figure 5 and Supplementary Figure S3). Downregulation of *Dj-ast4* by RNAi also produced anomalous eye regeneration, similar to that caused by berberine (Figure 2 and Supplementary Figure S3). *Dj-mt-mmpA*, a gene that encodes a metalloproteinase modulating cell migration³¹

was also strongly downregulated by berberine treatment (Figure 5A). However, berberine-induced inhibition of *Dj-mt-mmpA* transcripts did not prevent regeneration nor formed blastemas of reduced size, as expected by the silencing of a gene that plays a key role in promoting cell migration. Although the complex mechanisms underlying the berberine-mediated transcriptional effects remain largely obscure, we sought to determine whether this discrepancy might be related to the functional inactivation of *Dj-timp*, a gene encoding a major cellular inhibitor of the matrix metalloproteinase sub-family³³ we recently identified in planarians (Supplementary Figure S4). We further explored whether berberine could interfere with the transcriptional profiling of *Dj-timp* and observed that its expression was strongly down regulated by berberine treatment (Figure 5). These results suggest the possibility that, without TIMP action, sufficient level of Dj-MT-MMPA protein is maintained, ensuring correct cell migration without the need of new gene expression. To test this hypothesis we reasoned that results consistent with those observed after berberine-mediated down regulation of *Dj-mt-mmpA* and *Dj-timp* might be obtained by simultaneous RNAi-mediated knockdown of *Dj-mt-mmpA* and *Dj-timp*.

Feeding RNAi to evaluate the long-term effects of *Dj-mt-mmpA*, *Dj-timp* and simultaneous *Dj-mt-mmpA*; *Dj-timp* knockdown in intact planarians and during regeneration. To evaluate the long-term effects of RNAi-mediated knockdown avoiding multiple series of injections, we chose to use feeding RNAi¹⁸ to abolish the

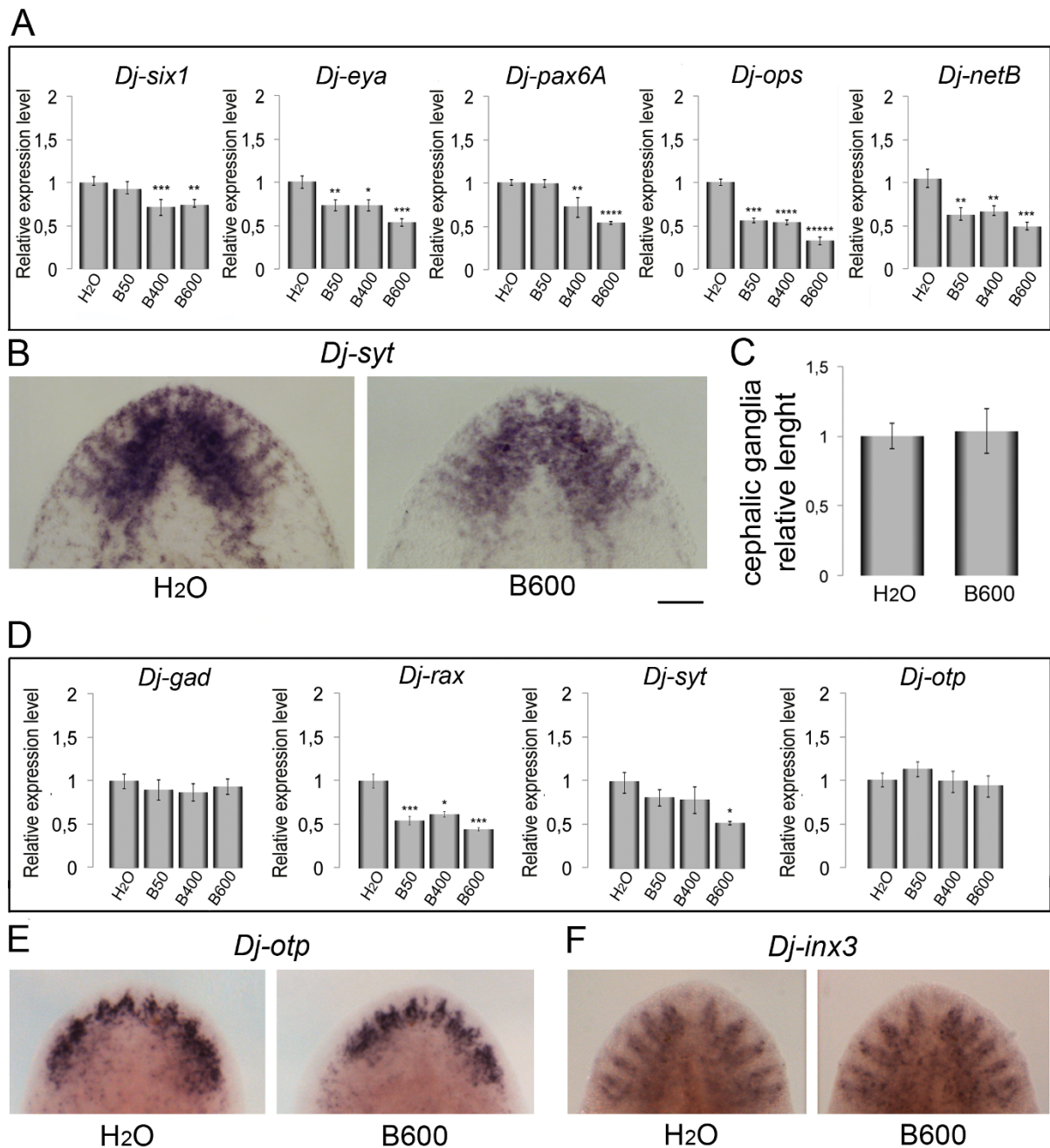


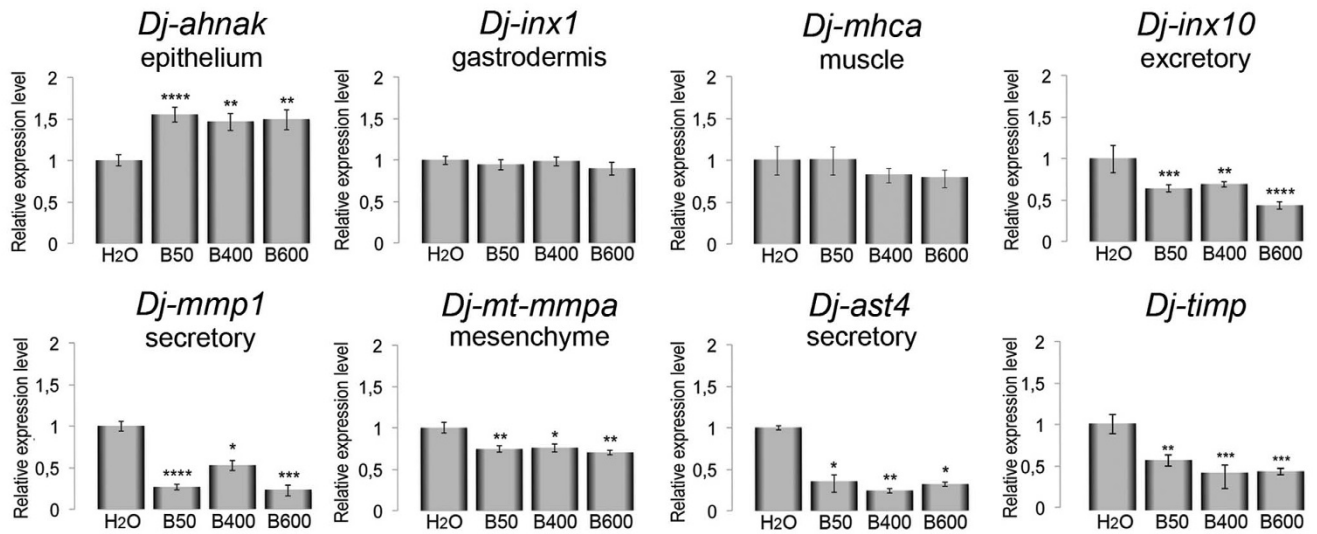
Figure 4 | Effects of berberine on the transcriptional profile of representative markers of nerve cells. (A) Comparison of mRNA levels of representative markers of the visual system: *Dj-six1*, *Dj-eya*, *Dj-pax6A*, *Dj-ops* and *Dj-netB* in berberine-treated planarians and in controls. H₂O: controls; B50: berberine 50 μ M; B400: berberine 400 μ M; B600: berberine 600 μ M. (B) Expression of *Dj-syt* is used to detect the CNS in a control planarian (H₂O) and following berberine 600 μ M (B600) treatment. (C) The graph shows quantification of the size of the regenerating cephalic ganglia in berberine 600 μ M (B600)-treated planarians and controls (H₂O), as measured after in situ hybridization with *Dj-syt*. Values are average \pm s.d. from 30 samples. (D) Comparison of mRNA levels of the neuronal markers *Dj-gad*, *Dj-rax*, *Dj-syt* and *Dj-otp* in berberine-treated planarians and in controls. H₂O: controls; B50: berberine 50 μ M; B400: berberine 400 μ M; B600: berberine 600 μ M. In Real Time RT-PCR experiments, the expression levels are indicated in relative units, assuming as unitary the value of the controls. Each value is the mean \pm s.d. of 3 independent experiments, carried out in 6 replicates. (E) Expression of *Dj-otp* is used to detect regeneration of the branches localized on the outer side of each lobe of the brain in a control planarian (H₂O) and following berberine 600 μ M (B600) treatment. (F) Expression of *Dj-inx3* is used to detect regeneration of the branches in a control planarian (H₂O) and following berberine 600 μ M (B600) treatment. Scale bars: 100 μ m in (B) and 200 μ m in (E,F).

expression of *Dj-mt-mmpA* and *Dj-timp* individually or simultaneously. Delivery of *Dj-mt-mmpA* dsRNA by feeding produced specific knockdown and phenotypes consistent with those observed after injection³¹. Planarians undergoing long-term *Dj-timp*(RNAi) also strongly reduced the level of the specific transcripts, but no

abnormal phenotype was detected. In the simultaneous RNAi both genes were efficiently knocked down (Supplementary Figure S5). Interestingly we observed that planarians that were simultaneously submitted to long term feeding of *Dj-mt-mmpA*; *Dj-timp* dsRNA appeared more similar to the controls than planarians fed with *Dj-*



A



B

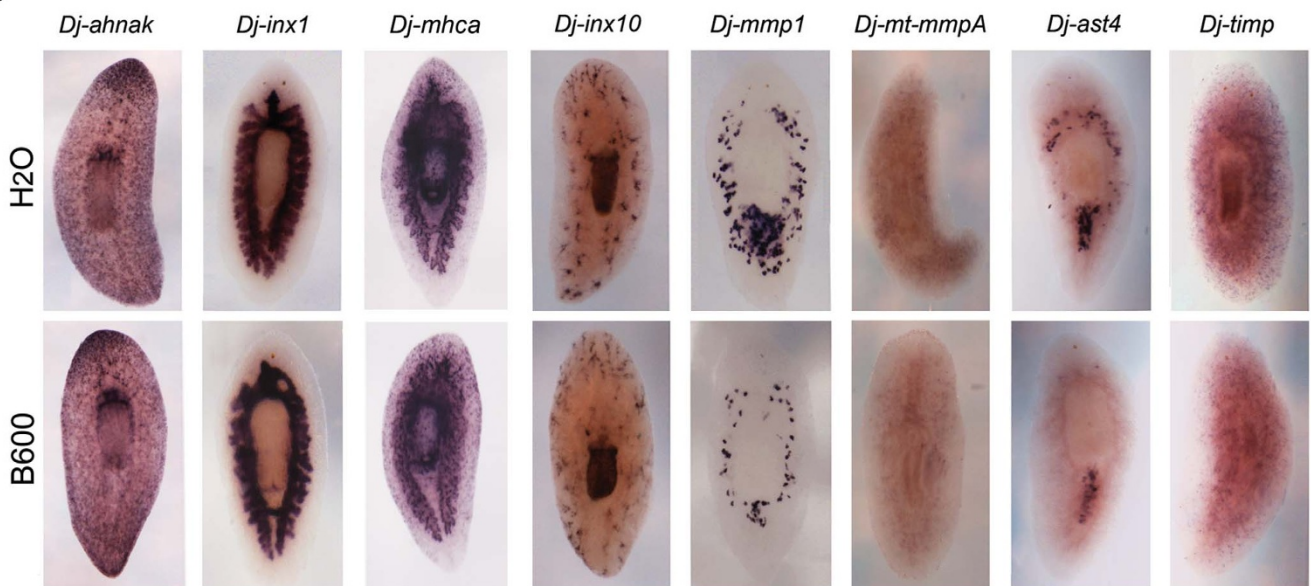


Figure 5 | Effects of berberine on the transcriptional profile of representative markers of other differentiated cell types. (A) Comparison of mRNA levels of representative markers expressed in different tissues: *Dj-ahnak*, *Dj-inx1*, *Dj-mhca*; *Dj-inx10*; *Dj-mmp1*; *Dj-mt-mmpA*, *Dj-ast4* and *Dj-timp* in berberine-treated planarians and in controls. H₂O: controls; B50: berberine 50 μ M; B400: berberine 400 μ M; B600: berberine 600 μ M. In Real Time RT-PCR experiments, the expression levels are indicated in relative units, assuming as unitary the value of the controls. Each value is the mean \pm s.d. of 3 independent experiments, carried out in 6 replicates. (B) Whole mount in situ hybridizations reveal the pattern of representative markers expressed in different tissues: *Dj-ahnak*, *Dj-inx1*, *Dj-mhca*, *Dj-inx10*; *Dj-mmp1*; *Dj-mt-mmpA*, *Dj-ast4* and *Dj-timp* in 600 μ M berberine-treated planarians (B600) and in controls (H₂O). Scale bar: 400 μ m.

mt-mmpA dsRNA, i.e. did not show the typical *Dj-mt-mmpA* dsRNA phenotype. Administration of BrdU provided evidence that the concurrent RNAi knock-down of *Dj-mt-mmpA* and *Dj-timp* did not impair cell migration in the most anterior area in front of the photoreceptors, that is devoid of neoblasts, during physiological cell turnover in intact planarians. Our experiments in fact demonstrated that no significant variation in the number of BrdU-positive cells was observed in *Dj-mt-mmpA*; *Dj-timp*(RNAi) planarians with respect to the controls, as well as in *Dj-timp*(RNAi) planarians. Conversely, the number of BrdU-positive cells migrating into this area was

significantly lower in *Dj-mt-mmpA*(RNAi) animals (Figure 6A,B). After 5 weeks of RNAi treatment some planarians of each experimental group were cut and left to regenerate for 5 days. As expected by our previous results³¹, *Dj-mt-mmpA*(RNAi) fragments regenerated slowly compared to the controls, while no significant delay in the blastema formation was observed both in *Dj-timp*(RNAi) and in *Dj-mt-mmpA*; *Dj-timp*(RNAi) fragments (Figure 6C). Mitotic cells were visualized by anti-H3P immunostaining. After *Dj-mt-mmpA*(RNAi) silencing we observed that a smaller number of anti-H3P-positive cells migrated into the blastema with respect to the

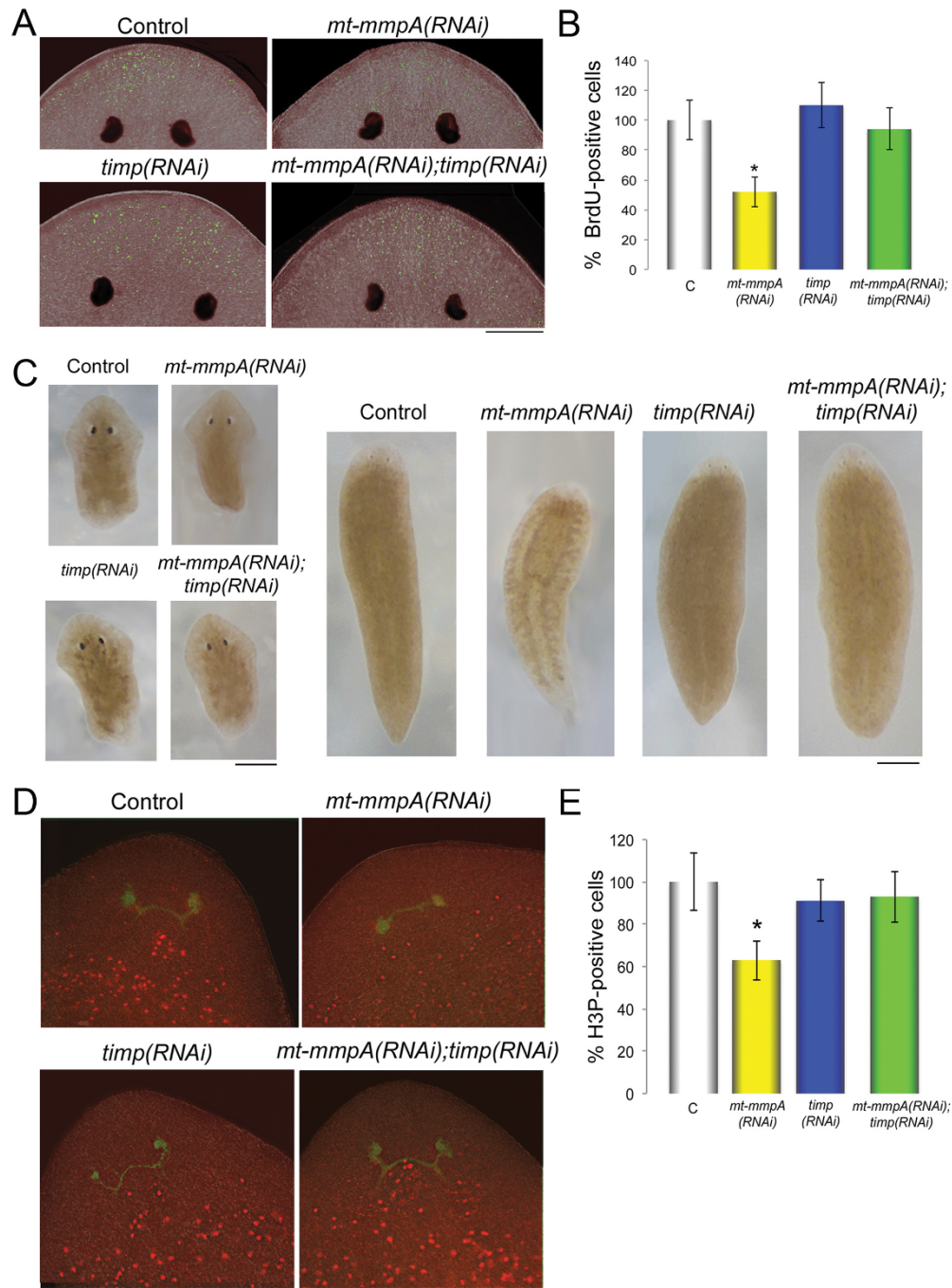


Figure 6 | Analysis of long-term effects of *Dj-mt-mmpA*, *Dj-timp* and simultaneous *Dj-mt-mmpA*; *Dj-timp* knockdown in intact and regenerating planarians. (A) Representative images of the region anterior to the photoreceptors in β -gal(RNAi) control, *mt-mmpA(RNAi)*, *timp(RNAi)* and *mt-mmpA(RNAi)* planarians. BrdU-labeling, used to monitor cell migration, is visualized in green and superposed upon a bright-field image (in dark red). Anterior is up. (B) The graph shows the significant decrease in the percentage of BrdU-positive cells in the region anterior to eyes of *mt-mmpA(RNAi)* phenotypes, compared to β -gal(RNAi) controls. No significant variation in the number of BrdU-positive cells with respect to the controls is detected after *timp(RNAi)* or simultaneous *mt-mmpA(RNAi); timp(RNAi)*. Results represent average \pm s.d. from 15 specimens assuming as 100% the value of control planarians. (unpaired t-test) * $P < 0.05$. (C) β -gal(RNAi); *mt-mmpA(RNAi)*; *mt-mmpA(RNAi); timp(RNAi)*; *timp(RNAi)*; *Dj-mt-mmpA(RNAi); timp(RNAi)*; *mt-mmpA(RNAi); timp(RNAi)*; simultaneous *Dj-mt-mmpA*; *Dj-timp(RNAi)*. (D) Immunostaining with anti-H3P antibody (red spots): 5 days of head regeneration after 5 weeks of feeding RNAi. VC-1 antibody (in green). H3P-positive cells and VC-1-positive photoreceptors are visualized superposed upon a bright-field image (in brown). Delayed morphogenesis of the visual system and a lower number of H3P-positive cells in the blastema area can be observed in *mt-mmpA(RNAi)* fragments. Control: β -gal(RNAi); *mt-mmpA(RNAi)*; *Dj-mt-mmpA(RNAi); timp(RNAi)*; *Dj-timp(RNAi)*; *mt-mmpA(RNAi); timp(RNAi)*; simultaneous *Dj-mt-mmpA*; *Dj-timp(RNAi)*. (E) Graph shows the significant decrease in the percentage of H3P-positive cells in the blastema of *mt-mmpA(RNAi)* fragments compared to controls. No significant variation in the number of H3P-positive cells was detected in the blastema of *timp(RNAi)* and *mt-mmpA(RNAi); Dj-timp(RNAi)* fragments. Results represent average \pm s.d. from 20 specimens assuming as 100% the value of control planarians. (unpaired t-test) * $P < 0.05$. (C): β -gal(RNAi); *mt-mmpA(RNAi)*; *Dj-mt-mmpA(RNAi); timp(RNAi)*; *timp(RNAi)*; *Dj-timp(RNAi)*; *mt-mmpA(RNAi); timp(RNAi)*; simultaneous *Dj-mt-mmpA*; *Dj-timp(RNAi)*. Scale bars: 100 μ m in (A,D); 500 μ m in (C).



controls. Conversely, no difference in the H3P-signal was detected both in *Dj-timp(RNAi)*, *Dj-mt-mmpA;Dj-timp(RNAi)* animals and in controls (Figure 6D,E).

Discussion

During planarian regeneration appropriate morphogenesis of the new body parts occurs in the blastema, while the old body fragment remodels and integrates with the new cells that are produced. This complex set of events requires accurate regulation of different cellular activities, all influenced by cell-ECM interactions, including stem cell proliferation, migration and differentiations. In the present investigation we have used the extraordinary regenerative potential of adult planarians as an informative model for assessing developmental responses to berberine *in vivo*. Although growing research has demonstrated absence of serious adverse effects of berberine in a variety of *in vitro* as well as *in vivo* models^{34,35}, it is almost unknown whether this alkaloid is equally safe when administered in a physiological developmental context. The molecular weight of this alkaloid is in fact low enough to cross the human placenta, with risk of foetal exposure. Here we show that berberine presents some degree of developmental toxicity when administered in the planarian regenerative model. Our data indicate that berberine enters the planarian body through the skin and the pharynx, all cells showing the fluorescent signal distributed within the entire cell or specifically accumulated in the nucleus. Accumulation of berberine in the planarian intestine is intriguing. Literature data also report abundant accumulation of this alkaloid in the intestine of mice. However it has poor absorption and its absolute bioavailability is low³⁶. Some proposals on how to interpret these data have been suggested, including the possibility that some ATP-binding cassette transporters recognize berberine as their transport substrate. This would support its efflux into the intestinal lumen. We do not know if similar efflux mechanisms are active in planarians, but it is interesting that a gene encoding an ABC transporter, such as the MDR1-like protein, is well conserved in the *S. mediterranea* genome. We also hypothesize that, following observation under light microscope, the high levels of fluorescent berberine that are accumulated in the planarian gastrovascular cavity may cause some cytotoxic effects on the intestinal tissue, as documented in Supplementary figure S2.

The morphogenesis of the visual system during head regeneration is a convenient end point for toxicological research in planarians. This process is in fact easy to follow, considering that *in vivo* two small eyespots are detectable in 3–4 days post amputation on each side of the cephalic blastema, which appears completely unpigmented. In addition, immunostaining with VC-1 antibody clearly delineates formation of the axonal projections that connect photoreceptors to the brain¹⁴. Pharmacological actions of berberine on the nervous system are reported since the 1970s³⁷. Since then a number of pre-clinical studies mainly contributed to provide insight into the mechanisms of neuroprotective action by which this alkaloid can act in various neurological disorders^{38,39}. Although recent data show high sensitivity of preimplantation mouse embryos to berberine⁴⁰ and a role in promoting axonal regeneration in injured nerves has been reported⁴¹, the safety and effectiveness of this compound in a neurological developmental context has not been extensively analyzed. We observed that berberine affects, in a dose-dependent manner, the regenerative process of the visual system without interfering with blastema growth. Accordingly, transcriptional profiling of genes involved in cell proliferation was not modified by the treatment. Given that recent studies provide evidence that berberine induces apoptosis in several tumor cell lines^{15,35,42}, we further assessed this possibility by the caspase-3 activity assay. No significant differences were found between berberine-treated and untreated planarians. Although we cannot completely exclude that berberine causes a certain level of caspase-independent cell death in these animals⁴², our results clearly demonstrate that berberine does not induce apoptosis.

This finding further supports the evidence that berberine may specifically inhibit proliferation and induce apoptosis in cancer cells, but not in normal cells^{15,35,43}. This observation is valuable for development of new therapies, with effective anti-cancer properties but limited side effects.

To further explore the possible mechanisms underlying the formation of abnormalities during regeneration of the visual system, we wondered whether berberine had an effect on the expression of genes involved in this process. It has been widely demonstrated that berberine forms complexes with DNA in living cells and that this association can effectively repress the transcription^{44–46}. Our analysis demonstrated that expression of key players of eye regeneration was significantly inhibited by berberine. We observed that the berberine-induced inhibitory effect was not generalized. Expression level of markers of other neuronal cell types, such as GABAergic neurons, was in fact not affected by the treatment. Furthermore, the structure of specific brain domains, such as the lateral branches - characterized by the expression of *Dj-otp* and *Dj-inx3* - appeared unmodified. Comparison of transcriptional profiles in berberine-treated specimens with untreated planarians using a number of markers that specifically characterize molecularly discrete cell populations in different planarian tissues further demonstrated how complex its range of activities is once this compound has been transported into a living cell. No alterations were observed in the expression level of gastrodermis and muscle markers. Conversely, *Dj-anhak*, whose expression characterizes epithelial cells, appeared upregulated by the treatment, while a clear suppressive effect on the transcription of markers of excretory and secretory cells was detected. With the exception of *Dj-inx1*, whose expression revealed defects in the reorganization of gut branches, *in situ* hybridizations with the other markers did not reveal significant alterations in the expression pattern of the analyzed tissues in berberine-exposed animals. Unfortunately, the molecular mechanisms of action that mediate the transcriptional responses produced by berberine are unknown and we actually know very little about what its long-term effects are on a living system. Recently, elegant experiments on a global scale have provided evidence for the first time that the suppressive effect of berberine on gene transcription occurs as the result of competitive association with TATA binding protein in the TATA box of eukaryotic promoters⁴⁷. In general, the effects on gene expression observed following berberine exposure could be the final result of intricate and complex interactions with many other regulatory mechanisms.

Most notably our work provides evidence, for the first time in a non-mammalian model system, that berberine may affect ECM remodeling. Analysis of the transcriptional profiling in fact produced a clear demonstration that berberine inhibits expression of representative genes encoding different metalloproteinases (*Dj-mmp1*; *Dj-mt-mmpA* and *Dj-ast4*) and an endogenous tissue inhibitor of metalloproteinases (*Dj-timp*), at a concentration as low as 50 μ M. It is well known that MMPs play a major role in the physiological remodeling of the ECM. The invasive and metastatic process of cancer cells also depends on their activity, making them valuable targets for pharmacotherapy⁴⁸. Berberine-mediated down-regulation effects on the expression of diverse MMPs and endogenous tissue inhibitors of metalloproteinases (TIMPs) have been demonstrated in mammalian cells *in vitro*^{49–53}. It is remarkable that similar inhibitory effects are conserved at the transcriptional level on molecules that are key modulators of the planarian ECM that is considered ancestral in many respects³¹. This finding raises the possibility that the mechanisms of action of berberine include a certain level of specificity.

We noticed that the inhibitory effects of berberine on *Dj-ast4* expression produced phenotypes that were very similar to those obtained by *Dj-ast4(RNAi)* (i.e. planarians regenerating cyclopic eyes). Conversely, berberine-induced downregulation of *Dj-mt-mmpA* transcripts did not form blastemas of reduced size³¹. We



reasoned that this difference might be connected to the inhibitory action that berberine has on *Dj-timp* expression. TIMPs are recognized as natural inhibitors of metalloproteinases, but no TIMP binding sites or significant activity against the astacins have been detected⁵⁴. It is known that, just following injury, planarians release large amounts of collagen-degrading MMPs that are stored in a catalytically inactive state in the intact animals, and this release does not require new gene expression⁵⁵. The simplest explanation for our results is therefore that, without TIMP action, the level of Dj-MT-MMPA protein is maintained, ensuring correct cell migration without the need of new gene expression. To test this hypothesis we performed gene silencing under long-term RNAi conditions and compared the effects of *Dj-mt-mmpA(RNAi)*, *Dj-timp(RNAi)* and simultaneous *Dj-mt-mmpA;Dj-timp(RNAi)* both in intact animals and during regeneration, 5 weeks after the first RNAi food. We observed a delayed regeneration response in *Dj-mt-mmpA(RNAi)* animals compared to controls. This result, confirmed by BrdU-labeling and immunohistochemistry with anti-H3P, reflects the key role of this gene in modulating cell migration during homeostasis and regeneration³¹. In our experimental conditions, *Dj-timp*, although efficiently knocked down, failed to produce any detectable phenotype. Remarkably, double silencing of *Dj-mt-mmpA* and *Dj-timp* rescued the phenotypic effects *Dj-mt-mmpA(RNAi)*, i.e. all fragments regenerated blastemas that were indistinguishable from the controls. The rescued animals displayed normal cell migration into the region anterior to the photoreceptors. In addition, during regeneration, anti-H3P-positive cells migrated into the blastema as in the control. These results support our prediction and raise the interesting possibility that, without TIMP gene activity, *Dj-mt-mmpA* protein product could still be present even if the mRNA is almost undetectable. Some proteins are stable over many days, so it is possible that, without the contribution of new transcripts, residual protein is sufficient for ensuring its activity. In addition it is known that MMPs are subject to extensive post-translational regulation by highly controlled processes of pro-enzyme activation and/or inhibition by TIMP and other inhibitors³¹. On the other hand, the identification of destabilization signals in the 3'UTR of *Dj-timp* (Supplementary Figure S4) implicates that finely regulated modulation of this inhibitor at the transcriptional level contributes to ensure the correct ratio of MMPs and TIMPs within the plastic planarian ECM. Further work is required to unravel the precise mechanisms by which berberine mediates its effects in planarians. It is likely that mechanistic understanding of these effects will contribute to a better understanding of its action in humans.

Methods

Animal model and treatments. Asexual specimens of *D. japonica* (Platyhelminthes, clonal strain GI) were used as animal model. The worms were maintained at 18°C in artificial planarian water (APW: 2.5 mM CaCl₂, 0.4 mM MgSO₄, 0.8 mM NaHCO₃, 77 μM KCl). Berberine chloride (C₂₀H₁₈ClNO₄, molecular weight 371.81; B3251, Sigma) solutions were freshly prepared in water for each experiment. Control groups were represented by planarians maintained in APW. For each berberine concentration, the animals were soaked in 25 ml of solution that was changed daily and maintained in the dark. Preliminary experiments where the worms were not pre-exposed to the drug until after decapitation produced phenotypes showing comparable morphology, transcriptional profiles and behavioral characteristics. However the incidence of the phenotypes was variable among the experiments. For this reason we decided to optimize the protocol pre-exposing the animals to berberine for 3 days before amputation (the minimal time for obtaining a reproducible number of phenotypes). Twenty planarians of similar body size were amputated at the prepharyngeal level and the fragments left to regenerate 7 days in presence of berberine before to be sacrificed for successive experiments. Worms pre-exposed to berberine for 3 days, but allowed to regenerate in its absence, were also sacrificed 7 days after cutting. After this treatment all animals regenerated without problems, with transcriptional profiles and behavioral characteristics comparable to those of the controls.

Toxicity of berberine on regenerating planarians. To evaluate toxic effects a wide range of berberine solutions were used (20 μM; 50 μM; 100 μM; 400 μM; 600 μM; 800 μM). At 800 μM, most of the amputated fragments died (about 90%). At 600 μM, the percentage of dead fragments decreased to about 10%. No dead

fragments were found after 400 μM, 100 μM or 50 μM berberine treatments. Both at 600 μM, 400 μM and 50 μM, morphological abnormalities in the regeneration of the eyes could be detected in fragments regenerating a head. At 20 μM all fragments regenerated as the controls. Consequently, to conduct the analysis in the most economical way, we selected for our study only three concentrations of berberine: 50 μM, 400 μM, 600 μM.

Determination of berberine content in *D. japonica* by the analysis of fluorescence intensity on silica gel plates. In order to verify whether berberine was actually taken by planarians and check whether berberine could be retrieved unmodified in planarian bodies after 10 days soaking, a qualitative thin-layer chromatography (TLC) was performed. To do this, samples from intact or regenerating planarians, following 50 μM berberine, 400 μM berberine or 600 μM berberine treatments, were submitted to TLC runs under different eluent conditions. In each sample, only one spot was detected under UV light ($\lambda = 365$ nm). These single spots were consistent with berberine according to both retention factor (R_f) and color and fluorescence characteristics. Upon soaking in media containing different concentrations of the alkaloid, concentrations of berberine within planarian bodies were quantified. In particular, the fluorescence intensities of berberine spots were measured after deposition of samples of homogenate planarians over silica gel TLC plates, visualization under UV light ($\lambda = 365$ nm), photograph capture and quantitative analyses of fluorescence intensity. In more detail, four samples in triplicate (planarians soaked in water (control), berberine 50 μM, berberine 400 μM and berberine 600 μM) were investigated. Each sample was made up of 5 intact or 5 regenerating (5 head + 5 tail fragments) animals. Animals from each triplicate sample were homogenated and sonicated in a volume of 100 μl of ethanol. Each homogenate was applied (1 μl) in duplicate over TLC plastic plates pre-coated with silica gel 60 F254 (Merck, Darmstadt, Germany). The samples were visualized under UV light ($\lambda = 365$ nm) and photographed with Canon power shot A 610. The quantitative analyses of fluorescence intensities were carried out with CpAtlas 2.0 software (<http://lazarsoftware.com>) by quantifying pixels related to the area of berberine spots in the green color channel. The protocol we used requires a preliminary calibration step in order to obtain calibration equations for berberine. Independent calibration curves were used whenever a quantification of berberine in homogenate planarian samples spotted over a silica gel TLC plate was desired. Calibration curves were obtained from stock solutions of berberine within the concentration range -5 to -3 logM, at concentration steps of 0.2 logM. Each concentration was used in triplicate. The results of the regression process indicate that berberine showed good linearity ($R_2 > 0.97$) within the concentration range -5 to -3 logM. The regression equations were achieved by linear regression of the fluorescence intensities of the green berberine spots, in terms of pixels related to the area of each spot versus the corresponding concentration expressed as log(M). Linear regression, as well as statistical analysis, was performed with GraphPad Prism 3.00 (GRAPHPAD, San Diego, CA, USA). The limit of quantification (LOQ) for berberine was determined at the $10 \times \text{Syx}/b$, where Syx is the standard deviation of y-intercept of the regression line, and b is the angular coefficient of the calibration. The LOQ, expressed as berberine concentration, resulted to be 2.8 μM. Berberine concentration was then calculated from a calibration equation considering a mean density of 1.03 mg/ml per planarian and a mean weight of 2 mg per planarian. Data are reported as mean \pm s.d. of three experiments.

Preparation of dissociated cells. Planarians were dissociated into a single cell suspension according to⁵⁶ and immediately analyzed under epifluorescence microscope (Nikon Eclipse E600). Cell types have been identified according to⁵⁷ classification.

RNA isolation, cDNA synthesis and real time PCR. Total RNA was isolated using the Eurogold total RNA kit (Euroclone), according to the manufacturer's instructions. Before cDNA synthesis, each extraction was tested for the absence of genomic DNA. cDNA was synthesized using QuantiTect[®] reverse transcription kit (Qiagen). For each experiment RNA extractions were performed with six independent experimental groups. Each RNA sample was obtained from an experimental group including 12 different regenerating fragments (6 head fragments + 6 tail fragments). Real time RT-PCR was performed at least three times for each examined gene, using three replicates for each cDNA. SYBR Green chemistry-based RT-PCR was carried out on a Rotor-Gene 6000 Real time-PCR (Corbett Research) with GoTaq[®] qPCR Master Mix (Promega). Details of the procedures are given in Supplementary Table I, according to the MIQE guidelines⁵⁸. Preliminary experiments were performed to evaluate the transcriptional stability of candidate endogenous reference genes. Under our experimental conditions, the stability of six planarian endogenous reference genes, *D. japonica elongation factor-2 (DjEF2)*, *D. japonica β-actin (DjACTB)*, *D. japonica elongation factor-1 (DjEF1)*, *D. japonica tubulin alpha chain (DjTUBA)*, *D. japonica 18S type I ribosomal RNA (Dj18S)*, *D. japonica glyceraldehyde 3-phosphate dehydrogenase (DjGAPDH)* were analyzed using NormFinder software. *DjACTB* was the most stable gene and for this reason it was selected as reference for comparative gene expression analysis.

In situ hybridization and RNAi experiments. Whole mount in situ hybridization was performed according to²⁷ with minor modifications²⁸. DIG-labeled riboprobes were synthesized as previously described³¹. RNAi by feeding in vitro-synthesized dsRNA was carried out according to [Rouhana et al., 2013]. Double-stranded RNA was obtained with TranscriptAid T7 High Yield Transcription Kit (Thermo



Scientific). In long-term RNAi experiments 100 intact planarians of similar body size were fed once per week with 1 $\mu\text{g}/10 \mu\text{l}$ *Dj-mt-mmpA;Dj-timp* dsRNA, or *Dj-mt-mmpA* dsRNA or *Dj-timp* dsRNA for 5 weeks. Throughout the experiments, the specimens were monitored for morphological and/or behavioral alterations. The efficiency and specificity of each RNAi was checked by Real Time RT-PCR (Supplementary Figure S5). After 5-week RNAi, planarians of similar body size were then amputated at the prepharyngeal level and the fragments left to regenerate. BrdU administration in intact planarians was performed as described by³¹. The scheduled experiments with both intact and regenerating RNAi specimens are summarized in Supplementary figure S5.

Immunofluorescence. Immunostaining was performed according to⁵⁹ with planarian anti-arrestin VC-1, a monoclonal antibody specific to the photoreceptors, 1:2000. Anti-phospho histone H3 antibody (anti-H3P) (Upstate) 1:500 was used to detect mitotic cells. These cells were counted and normalized as described by³¹. Alexa Fluor 488 or Alexa555 (Molecular Probes) 1:1000 were used as secondary antibodies. Whole mount images were captured using Nikon Eclipse Ti Inverted Microscope with Digital Sight Camera and NIS-Elements Microscope Imaging Software. Adobe Photoshop was used to orient, scale and adjust images and improve clarity.

Caspase -3 activity assay. Cell lysate proteins were obtained from 20 planarian fragments and caspase-3 activity was measured using the substrate Ac-DEVD-pNA (Enzo Life Sciences), as described by³¹.

Phototaxis assay. Phototaxis assay was performed as described¹⁴. Planarian negative phototactic behavior was recorded using a video camera (Panasonic Lumix DMC-FS11) for 120 seconds and SMART v2.0 behavior analysis software (Panlab, Spain).

Morphometric analysis. For morphometric analysis, the areas of the blastema and the stump were determined in regenerating planarians after berberine treatment and water controls, 7 days after the transection. The animals were photographed at the same magnification with a stereomicroscope after ethanol-fixation. The areas of the blastema and the stump were measured using Nikon ACT-2U imaging software. A number of 30 regenerating fragments were analyzed for each experimental condition. The size of the cephalic ganglia after in situ hybridization with *Dj-syt* was measured with the same procedure.

Statistics. Data are expressed as mean \pm standard deviation (s.d.) of at least three independent experiments with three or six replicates. Data tabulation and descriptive statistics were performed with GraphPad Prism Software. Normality and homoscedasticity were tested with Shapiro-Wilk, Kolmogorov-Smirnov test and F-test. Where necessary, the results were transformed to meet the requirements of normality and equal variance. Data were analyzed by Student's t-test and parametric ANOVA. Differences with P value less than 0.05 were considered statistically significant.

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Author contributions

L.B., A.M.B., M.E.I. and R.B. conceived and designed the experiments. L.B. and M.E.I. performed the experiments. L.B., A.B. and D.P. analysed the data and prepared the figures. L.B., P.D., A.M.B. and R.B. contributed with reagents, materials and analysis tools. R.B. revised the data and wrote the main manuscript text. All authors reviewed the manuscript.

Additional information

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