Gelsemium Low doses Increases Bioenergetics and Neurite Outgrowth

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Gelsemium low doses increases bioenergetics and neurite outgrowth

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Abstract

Background: *Gelsemium sempervirens* (GS) is a traditional medicinal plant, described at ultra-low doses as a remedy for a variety of psychological and behavioral symptoms of anxiety and depression. Changes in neural plasticity have been shown to play a significant role in the onset and development of those mental illnesses. Mitochondria play an extremely important role in the central nervous system by being the main energy producer through the oxidative phosphorylation and being involved particularly in the regulation of cell survival or death, as well as synaptic plasticity. Neurite outgrowth is the differentiation process by which neurons establish synapses through the protrusion of neurons and their extension.

Methods: Because the effects of GS dilutions on mitochondrial function and neuroplasticity remain elusive, we aimed to investigate whether a treatment with GS at low doses (centesimal dilutions, C) improved bioenergetics parameters such as ATP production, mitochondrial respiration as well as cellular glycolysis before to characterize its effects on neurite outgrowth.

Nerve growth factor (NGF), which is known as a promotor of cell growth and survival, was used as a positive control.

Results: Our results demonstrate that GS dilutions (3C and 5C) efficiently ameliorated the bioenergetics of SH-SY5Y neuroblastoma cells by increasing cellular ATP level and mitochondrial respiration as well as promoting the cell survival. In addition, GS dilutions significantly improved neurite extension in the 2D as well as 3D culture model after 3 days of treatment. 3C and 5C dilutions showed similar functional effects than those obtained with the positive control nerve growth factor (NGF).

Conclusions: These findings indicate that GS dilutions modulate the mitochondrial bioenergetic phenotype and improve the neurite formation. The mitochondrial function improving properties of GS dilutions might represent one possible important pathway contributing to its neuroprotective effectiveness.

Key words: Gelsemium dilutions, mitochondria, bioenergetics, neurite outgrowth.

Background

Homeopathic Materia Medica suggested that *Gelsemium sempervirens* (GS) can be used as remedy of neurological and behavioural symptoms, including general prostration, drowsiness, tiredness, mental apathy, lack of muscular coordination, anxiety, depression (1). GS itself is originally known as a toxic plant (2). The toxic effects of the plant seem to be due to the high concentrations of alkaloids (2). Therefore, GS is currently used in homeopathic dilutions only (3). In contrast, there are no safety concerns for the use of ultra-low doses of GS (4). GS dilutions have been shown to act on the emotional reactivity of mice by exerting anxiolytic-like effects (5, 6). Basic evidence supported the existence of cellular effects of GS dilution 5C in the rat limbic system namely, hippocampus and amygdala, regions that are well-known to

pivotally modulate anxiety (7). The pharmacological mechanisms involved in the mediation of the action of the GS dilution 5C in hippocampus and amygdala were induced by the production of the neurosteroid allopregnanolone (3α,5α-THP) (7), an endogenous steroid that is able to rescue neuronal cells from oxidative stress-induced death through bioenergetic improvement (8).

Mitochondria are the main energy producer of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) and this energy is required for almost all cellular processes, from cell survival and death, to the regulation of synaptic plasticity and intracellular calcium homeostasis (9-11). Neurite outgrowth is an energy-consuming process where the neurons generate new projections as they grow in response to guidance cues. Neurotrophins, such as NGF, are one family of stimuli that modulate neurite growth (12). Marzotto and colleagues (13) have shown that in SH-SY5Y cells, a human neuronal cell line, the GS dilution 2C modulated the expression of genes involved in neuronal functions such as G-protein coupled receptor signaling pathways known to play key roles in synaptic plasticity by strengthening or weakening synapses and/or shaping dendritic spines (14). However, there is no evidence demonstrating that GS dilutions themselves can modulate mitochondrial function and/ or neuroplasticity. To gain more insights into the underlying cellular mechanisms of the mode of action of GS 3C and 5C dilutions, our first aim was to investigate its ability to modulate mitochondrial function via the determination of ATP levels, mitochondrial respiration and cellular glycolysis as well as cell survival. Then, we evaluated the effect of GS dilutions on several parameters of neuroplasticity.

Methods

Gelsemium sempervirens plant

GS plants also known as Yellow Jessamine were purchased from Herb's International Service SARL (France; Batch H140503595) in respect of the Good Agricultural and Collection Practices (GCAP) (15). A double identification of GS plant is realized by the supplier and Boiron's quality control. The GS plant is not present in the International Union for Conservation of Nature (IUCN) lists as an endangered species (16). In the present study, a GS batch sample used to manufacture the Mother Tincture is conserved at Boiron laboratories (Messimy, France).

Chemicals and reagents

Dulbecco's-modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin, Dimethylsulfoxid (DMSO) were from Sigma-Aldrich (St. Louis, MO, USA). Glutamax was from Gibco Invitrogen (Waltham, MA, USA). B27 supplement was from Gibco Invitrogen (Waltham, MA, USA). NGF was from Lubio (Zürich, Switzerland). GS dilutions were prepared by Boiron laboratory (Messimy, France) based on the 1.1.10 method of the European Pharmacopoeia (Ph. Eur.) guidelines for the production of homeopathic remedies (17). The Mother Tincture (MT) is produced by macerate for 10 days of dried *G. sempervirens* (L.) J.St.-Hil. plant roots with a 65% v/v ethanol solution. The ratio was 1g of plant for 9g of hydroalcoholic solution. Gelsemine is the major active principle of Gelsemium in the homeopathic monograph. The concentration of gelsemine estimated from the analyses of *G. sempervirens* mother tincture (batch number: M4090578, voucher number: 191138) was $6{,}49 \times 10^{-4}$M (0,023%). All the centesimal (C) dilutions tested were prepared in sterile water (OTEC, France) in order to avoid cell toxicity of alcohol. To obtain the first centesimal (1C) dilution, one volume of MT was diluted in 99 volumes of water and vigorously agitated using a mechanical shaker. The subsequent serial 100× dilutions, 3C and 5C GS dilutions were prepared by the same procedure with an final ethanol concentration largely below 0,0001%

(v/v). The vehicle control was prepared according to the same procedure described above using only sterile water (OTEC). All GS dilutions as well as vehicle or control solutions were stored at 4 °C before use.

Cell culture

Human SH-SY5Y neuroblastoma cells (ATCC CRL-2266) were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 2 mM Glutamax and 1% (v/v) penicillin/streptomycin and incubated at 37°C in a humidified incubator chamber under an atmosphere of 7.5% CO_2. The cells were grown in 10 cm^2 dishes and splitted twice a week. When they reached around 80% confluence the cells were plated, 2 days prior treatment.

In the 2D cell culture method, collagen type I (Rat tail BD Bioscience) at 0.05 mg/ml was used to coat the cell plates. In the 3D cell culture method, a BD PuraMatrix Peptide Hydrogel (BD Catalog #354250 packaged in one vial containing 1% solution (w/v) of purified synthetic peptide) was used. Cell plates were coated with 5mg/ml of PuraMatrix (0.5% diluted in sterile water, 50 µl for a 96-well plate) and gelation was induced by slowly and carefully adding medium to each well (100 µl for a 96-well plate). After 1 hour of incubation of the plates for a complete gelation, the medium was exchanged twice over a period of 1 hour to promote a physiological pH of the growth environment. Cells were seeded at a concentration of $5x10^3$ cells/ well (18).

Treatment paradigm

In accordance with the treatment protocol and findings that were recently described (19), the effects of the GS dilutions 3C and 5C were investigated in this study. One day after plating, SH-SY5Y cells were treated in DMEM + 10% FCS either with DMEM alone (untreated control condition, CTRL) or NGF (positive control at a final concentration of 50 ng/mL), or vehicle

control or the different dilutions of GS. Bioenergetic phenotype (OCR/ECAR profil) and effect on neurite outgrowth were investigated after treatment with CTRL or NGF (50 ng/ml), or 3C and 5C dilutions. Values were normalized to the untreated control group (CTRL).

MTT assays

To assess cell viability, MTT reduction assays were performed in accordance with the protocol from Mensah-Nyagan laboratory (20) and confirmed preliminary MTT assay readout data generated by the Mensah-Nyagan laboratory. Briefly, SH-SY5Y cells were seeded at $5x10^3$ cells/ well into 96-well plate in replicates and allowed to attach. 48 h after the plating, the treatments were initiated. After 24H treatments, cells were incubated with MTT (3-(4,5-dimethylthyazol-2-yl)-2,5- diphenyl-tetrazolium bromide) in DMEM for 3 hours. MTT is reduced to a violet formazan derivative by mitochondrial enzymatic activity. At the end of the reaction cells were dissolved in a MTT cell lysis buffer (DMSO). MTT absorbance was measured at 550 nm using the multi label plate reader Cytation3 (BioTek). MTT signal detected for the CTRL cells is arbitrary normalized to 100 %.

ATP levels

Total ATP content of SH-SY5Y cells was determined using a bioluminescence assay (ViaLighTM HT, Cambrex Bio Science, Walkersville, MD, USA) according to the instruction of the manufacturer, as previously described (21, 22). SH-SY5Y cells were seeded at $5x10^3$ cells cells/well into a white 96-well cell culture plate in 5 replicates (21, 22). The bioluminescent method measures the generation of light from ATP and luciferin by luciferase. The emitted light was linearly correlated to the ATP concentration and was dertermined using the Cytation 3 cell imaging multi-mode reader (21, 22).

Determination of oxygen consumption rate (mitochondrial respiration) and extracellular acidification rate (glycolysis):

The Seahorse Bioscience (North Billerica, MA, USA) XF24 Analyser was used to perform a simultaneous real-time measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). XF24 cell culture microplates (Seahorse Bioscience) were coated with 0.1% gelatine and SH-SY5Y cells were plated at a density of $2.5x10^4$ cells / well in 100 µl of the medium containing 10% FCS, 1 g/l glucose and 4 mM pyruvate and treated with CTRL or NGF or 3C and 5C dilutions After 24 h of treatment, all the cells were washed with PBS and incubated with 500 µl of assay medium (DMEM, without NaHCO3, without phenol red, with 1g/l glucose, 4 mM pyruvate, and 1% L-glutamine, pH 7.4) at 37°C in a CO_2-free incubator for 1 hour.

The OCR and ECAR values from the basal respiration state were recorded simultaneously. Data were extracted from the Seahorse XF-24 software, and the bioenergetics profils using the OCR and ECAR values were calculated according to the guideline of the company (21).

Neurite outgrowth

For the 2D or 3D cell culture, SH-SY5Y neuroblastoma cells were cultured in coated 96 well plates (black with clear bottom). The following day, cell differentiation was initiated by adding neurobasal medium containing 1 % fetal bovine serum and 10 µM retinoic acid (RA) for 3 days. Then, cells were treated either with CTRL, NGF (50 ng/ml) or with 3C and 5C dilutions. After 3 days of treatment, cells were fixed with 2% paraformaldehyde. All media were exchanged every 2 days to ensure the availability of growth factors and GS dilutions components in the culture (18).

Immunostaining

The protocol was used with 2D or 3D surface cultures of cells in plates. For 96-well black microplates with a clear bottom, it was possible to directly image the samples without transferring the gel to a glass slide. Immunolabeling of neurites was performed using an anti βIII-tubulin (R&D Systems, Biotechne, Minneapolis, MN, USA) and Alexa Fluor 488-conjugated secondary antibody (Thermofisher scientific, Waltham, MA, USA) (22). DraQ5 (Biostatus, Shepshed, Leicestershire, UK) or DAPI (Thermofisher scientific, Waltham, MA, USA) were used for the nucleus staining.

Microscopy and analysis (Software)

Images were obtained randomly using an inverted confocal microscope (Leica Microsystems TCS SPE DMI4000, 10x objective) connected to an external light source for enhanced fluorescence imaging (Leica EL6000). Axially, all the cells were entirely present within the confocal volume for the pinhole settings. One layer was taken for the 2D culture method. To visualize the whole 3D network, z-stacks were generated (3-4 layers). The maximum intensity projection was then used for 2D pictures analysis that was performed using ImageJ (Neurophology plugin) software to investigate parameters of neuroplasticity such as Neurite count, Total neurite length, attachment point (Number of branching points), endpoint (Number of contact points) (18).

Statistical analysis

Data are given as the mean ± SEM. Values were normalized to the untreated control group (=100%). Statistical analyses were done using the Graph Pad Prism software version 5.02. One-way ANOVA followed by Dunnett's multiple comparison tests *versus* the control group were used for statistical comparisons of more than two groups. Student unpaired *t*-test was used for statistical comparisons of two groups. The experimental data are investigated using the

GraphPad-Prism program (GraphPad-Prism, San Diego, CA, USA). P values<0.05 were considered statistically significant.

Results

GS dilutions increased ATP levels and cell viability

We first investigated the effects of the GS dilutions 3C and 5C on ATP production and cell survival in human neuroblastoma cells (SH-SY5Y) after 24h of treatment (**Figure 1**). The GS dilutions 3C and 5C significantly increased ATP levels compared to untreated control cells (**Figure 1a**) (3C: +7%; 5C: +8%) as well as to vehicle treated cells (3C: +10%; 5C: +11%). GS dilutions 3C and 5C raised the ATP level at the same range as the positive control NGF (+6% vs CTRL). The treatment with the vehicle solution was not different to the untreated control condition.

We next assessed whether a treatment with GS dilutions was able to improve cell survival in SH-SY5Y cells (**Figure 1b**). After 24h of treatment, the dilutions 3C and 5C significantly increased the viability of the SH-SY5Y cells when compared to untreated control cells (3C: +23%; 5C: +40%) as well as to vehicle treated cells (3C: +26%; 5C: +43%).

Because vehicle treatment was without any effect in both assays, we compared the effects of the GS dilutions to the untreated control condition in the following experiments.

GS dilutions activated the metabolic state of the human neuroblastoma cells

ATP molecules are mainly produced by the mitochondrial oxidative phosphorylation (OXPHOS) and the cellular glycolysis. Therefore, we evaluated the efficiency of 3C and 5C, and the positive control NGF to modulate one or both pathways. Seahorse Bioscience XF24 Analyzer was used to simultaneously monitor in real-time the OCR, an indicator of mitochondrial respiration and the ECAR, an indicator of glycolysis **(Figure 2)**. A treatment

with 3C and 5C induced a strong and significant improvement of the basal OCR compared to CTRL with +45% and +41% of increase respectively, while NGF induced a +58% increase compared to the control **(Figure 2a)**. The 3C dilution had a similar effect than NGF (+52%) in ameliorating significantly the glycolysis compared to CTRL, while 5C was more efficient by inducing an increase up to 85% **(Figure 2b)**. The bioenergetic phenotype of the cells **(Figure 2c)**, represented by OCR versus ECAR of the basal respiration, revealed that treatments with 3C and 5C were particularly efficient to improve both parameters, switching the cells to a metabolically more active state, with a comparable effect between NGF and 3C.

GS increased the neurite extension in 2D and 3D surface culture

To investigate the effect of GS dilutions on neurite outgrowth, 3C and 5C dilutions were tested on differentiated SH-SY5Y cells after 3 days of treatment. NGF (50 ng/ml) was again used as positive control because of its action as a promotor of survival and neuritic growth.

After 3 days of treatment, 3C and 5C were able to improve the neurite outgrowth when compared to the untreated control cells and with a higher effect than the positive control NGF itself (**Figures 3 and 4**).

In fact, after 3 days treatment with GS dilutions 3C and 5C increased significantly the neurite count (about +94.1% and +133.2% of increase respectively), total neurite length (about +173.3% and +214.4% of increase respectively), attachment point (up to 300% of increase) as well as the endpoint (up to 330% of increase) when compared to untreated control cells (**Figures 4**). NGF ameliorated significantly the neurite outgrowth of about +73.5% of increase for the neurite count, +130% of increase for the total neurite length, up to 176.9% of increase for attachment point as well as the endpoint with 187.7% of improvements compared to untreated control cells (**Figure 4**).

Based on the above results, we confirmed the beneficial effect of the GS dilutions on neurite extension using the 3D cell culture method to obtain a 3D view of the neuroplasticity. **Figure 5** displays a 3D view of the enhanced neurite length into the 3D-matrix after a treatment with 3C and 5C dilutions compared to the untreated control cells. We observed that GS dilutions treatment is able to ameliorate the neurite outgrowth by enhancing the formation of neurite extension (**Figure 5**).

Discussion

In the present study, we showed that the GS dilutions 3C and 5C were able to improve the mitochondrial bioenergetic metabolism, as well as to initiate the neurite outgrowth by: (1) ameliorating the cellular ATP levels; (2) promoting the cell survival; (3) stimulating the mitochondrial respiration and the cellular glycolysis; and (4) inducing neurite extension in the 2D, as well as 3D cell culture model.

In details, the 3C and 5C dilutions increased significantly the ATP levels, OCR as well as ECAR, switching the SH-SY5Y cells to a more metabolically active state. We highlighted a similar efficacy of the GS dilutions in comparison to the positive control NGF in modulating the OXPHOS-derived energy production and neurite outgrowth. In a recent study, we already demonstrated that NGF plays a crucial role in the stimulation of neurite outgrowth of SH-SY5Y cells by increasing the neurite count and length as well as the attachment point and the endpoint parameters (18). In line with this study, we showed that, after 3 days of incubation, 3C and 5C dilutions induced a significant increase of the neurite extension in neuroblastoma cells. These effects were comparable with those of the positive control NGF. GS dilutions-induced neurite outgrowth was confirmed in 3D-matrix of SH-SY5Y cultures. In accordance with the beneficial effect of GS dilutions reported in many studies as a treatment for several neuroses including anxiety and depression (2, 23, 24), we propose that the efficacy of 3C and 5C is

mediated by their ability to boost the mitochondrial activity, particularly the bioenergetics. It is an attractive hypothesis since mitochondrial function represents one of the important mechanisms involved in the neurite outgrowth (25). Indeed, the brain requires a considerable amount of energy in order to activate, sustain, and consolidate neuronal functions and plasticity (11). Nerve cells present a significant energy demands due to their postmitotic polarization state (11). In mitochondria, the energy in the form of ATP produced by OXPHOS is directed by the neurons into the development of interconnections, the synapses (10). Apart from the production of energy, other pathways could be implicated in the GS dilutions effect because mitochondria are the key modulators of brain cell survival and death by controlling redox equilibrium (which can in turn affects neuronal plasticity) and generating reactive oxygen species (ROS) (11). Furthermore, the regulation of mitochondrial dynamics plays an important role in neurite outgrowth via the mechanisms of extension, regeneration, and branching that require a continuous supply of energy (25-27).

Additional investigations will be necessary to characterize the specific mechanisms of action or cell signalling triggered by the GS dilutions 3C and 5C for the modulation of cellular bioenergetics and the cell survival as well as the stimulation of the neuroplasticity. A possible mechanisms of action would rely on the ability of GS dilution (5C) to induce the production of the neurosteroid allopregnanolone, as already shown in the rat hippocampus and amygdala (7). These effects might be mediated by the GS compound Gelsemine (7, 28, 29). Interestingly, allopregnanolone was also shown to rescue neuronal cells from oxidative stress-induced death through bioenergetic improvement (8). Besides, a recent study showed that another compound of GS, Koumine, an alkaloid, exerted cytoprotective effects against oxidative stress-induced apoptosis in a porcine intestinal epithelial cell line by suppressing the reactive oxygen species production, inhibiting the caspase-3 activity and influencing the expression of Bax and Bcl-2, regulators of mitochondrial function (30). These findings suggest that Gelsemine and Koumine

might act on pathways involved in the regulation of reactive oxygen species generation or the redox equilibrium, probably through the action of a single component or synergistic interaction with other still not identified constituents of GS dilutions.

Conclusions

The present study suggests that GS dilutions may markedly promote neurite outgrowth via the stimulation of the mitochondrial bioenergetics and the cell survival. The beneficial effect of GS dilutions through the modulation of the mitochondrial function lead to new working hypotheses on the anxiolytic and anti-depressant action of this plant as well as an appreciation of GS dilutions as a potential agent for neuroprotection.

List of abbreviation

3-(4,5-dimethylthyazol-2-yl)-2,5- diphenyl-tetrazolium bromide (MTT)

Adenosine triphosphate (ATP)

Control cells (CTRL)

Dimethylsulfoxid (DMSO)

Dulbecco's-modified Eagle's medium (DMEM)

Extracellular Acidification Rate (ECAR)

Fetal calf serum (FCS)

Gelsemium sempervirens (GS)

Human neuroblastoma cells (SH-SY5Y)

Nerve growth factors (NGF)

Oxidative phosphorylation (OXPHOS)

Oxygen Consumption Rate (OCR)

Retinoic acid (RA)

Standard error of the mean (SEM)

Three-Dimensional (3D)

Two-Dimensional (2D)

Volume/ volume (v/v)

Declaration

Ethics approval and consent to participate

Plant: GS as a traditional medicinal plant is no longer used because of its toxicity (3). It is currently used in homeopathic dilutions only (3). In the present paper, GS dilutions were prepared by Boiron laboratory (Messimy, France) based on the 1.1.10 method of the European Pharmacopoeia (Ph. Eur.) guidelines for the production of homeopathic remedies (17).

Source: It is not present in the International Union for Conservation of Nature (IUCN) lists as an endangered species (16).

Animals: not applicable.

Humans: There were no humans participating in this study and therefore there are no ethical issues that should be addressed. The biological materials that were used are anonymized and are excluded from the Human Research Act (HRA). Origin of the cells: Human SH-SY5Y neuroblastoma cells (ATCC CRL-2266, Virginia, USA, ATCC company).

Consent for publication.

Not applicable.

Availability of data and materials

All relevant data are within the manuscript and its supporting information files.

Competing interest

AE received an investigator research grant from Boiron Laboratory, France. NB and PT are employed by Boiron Laboratory, France. IL and AG declare that they have no competing interests.

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Author Contributions

Conceptualization, A.E. and I.L.; Formal analysis, I.L.; Funding acquisition, A.E.; Investigation, I.L.; Methodology, I.L.; Project administration, A.E.; Resources, N.B., P.T. and A.E.; Supervision, A.E. and I.L.; Writing original draft, I.L., A.G., N.B., P.T. and A.E. All authors have read and agreed to the published version of the manuscript.

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Figures

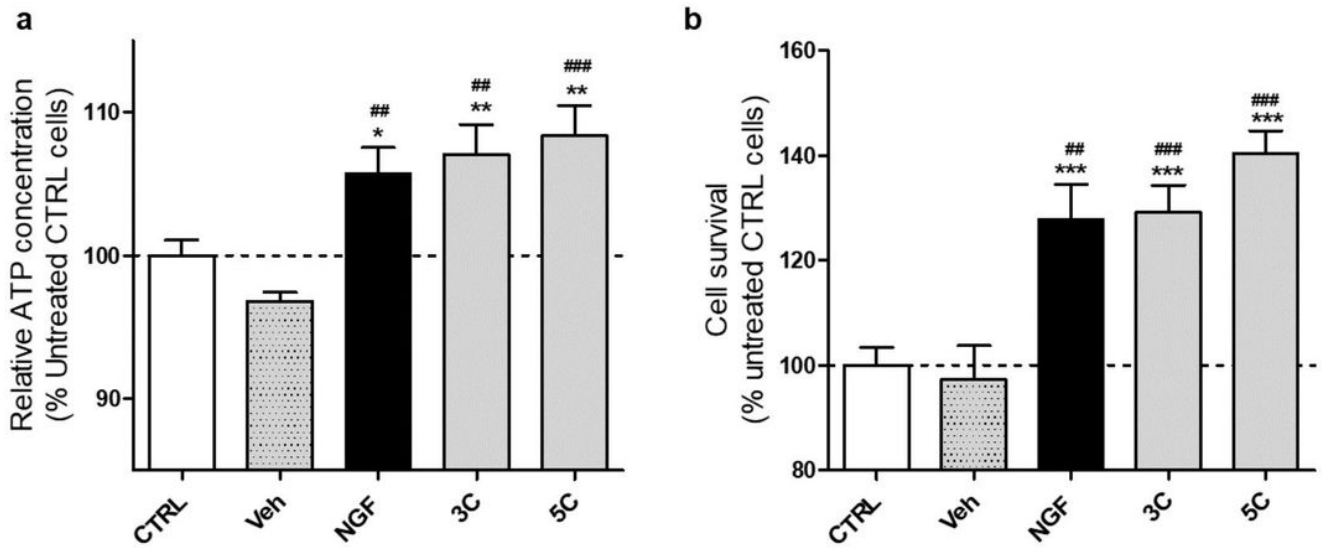

Figure 1

Effect of GS dilutions on ATP levels and cell viability after 24h treatment. 3C and 5C increased both parameters: (a) the ATP production and (b) the cell survival. Vehicle (Veh) treatment had no effect on ATP levels compared to CTRL cells. Values represent the mean± SEM (n = 13-18 replicates) of five independent experiments and were normalized to the untreated control group (CTRL, 100%, S1 Table). One-way ANOVA ((a, b): P□0.0001) and post hoc Dunnett's multiple comparison test versus untreated control cells, *P<0.05, **P<0.01, ***P<0.001. One-way ANOVA and post hoc Dunnett's multiple comparison test versus vehicle treated cells, ##P<0.01, ###P<0.001.

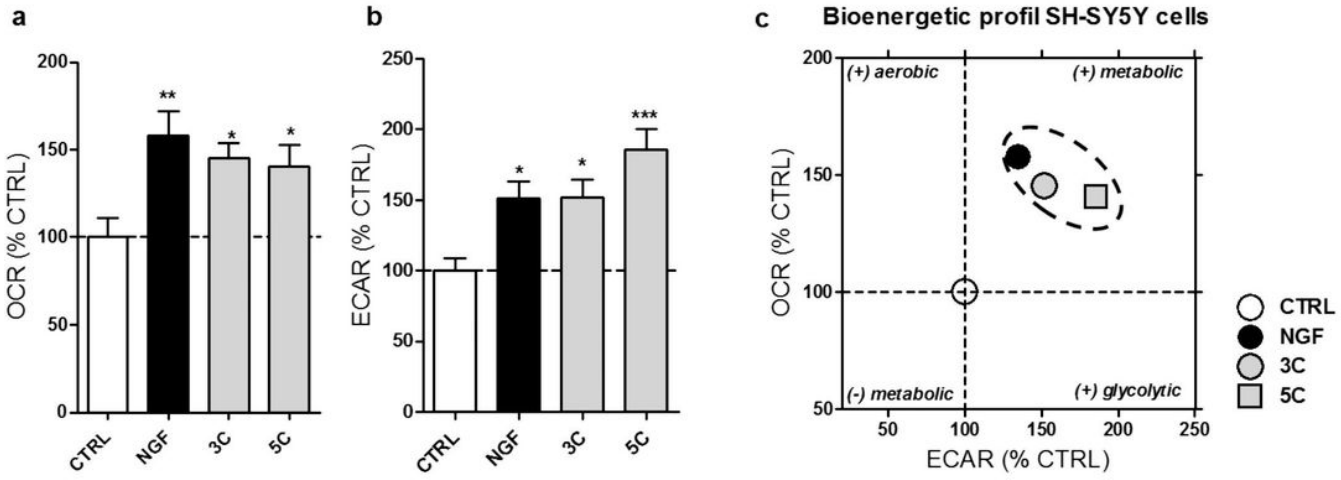

Figure 2

GS dilutions positively regulates bioenergetic activity in SH-SY5Y neuroblastoma cells. (a) Oxygen consumption rate (OCR) and (b) the extracellular acidification rate (ECAR) were measured simultaneously in SH-SY5Y cells after treatment (24h) with the GS dilutions 3C and 5C as well as the positive control NGF and compared to the untreated control cells after normalization using a XF24 Analyser (Seahorse Bioscience). Values represent the mean± SEM (n = 19-39 replicates) of four independent experiments (S2 Table). (c) Bioenergetic phenotype (OCR versus ECAR) of SH-SY5Y cells revealed an increased metabolic activity after treatment with 3C and 5C. Values represent the mean of each group (mean of the ECAR in abscissa/ mean of the OCR in ordinate) and were normalized to the untreated control group (CTRL, 100%). One-way ANOVA ((a): P=0.0031, (b) P=0.0001) and post hoc Dunnett's multiple comparison test versus untreated control cells *P<0.05, **P<0.01, ***P<0.001. OCR, Oxygen Consumption Rate (mitochondrial respiration); ECAR, Extracellular Acidification Rate (Glycolysis).

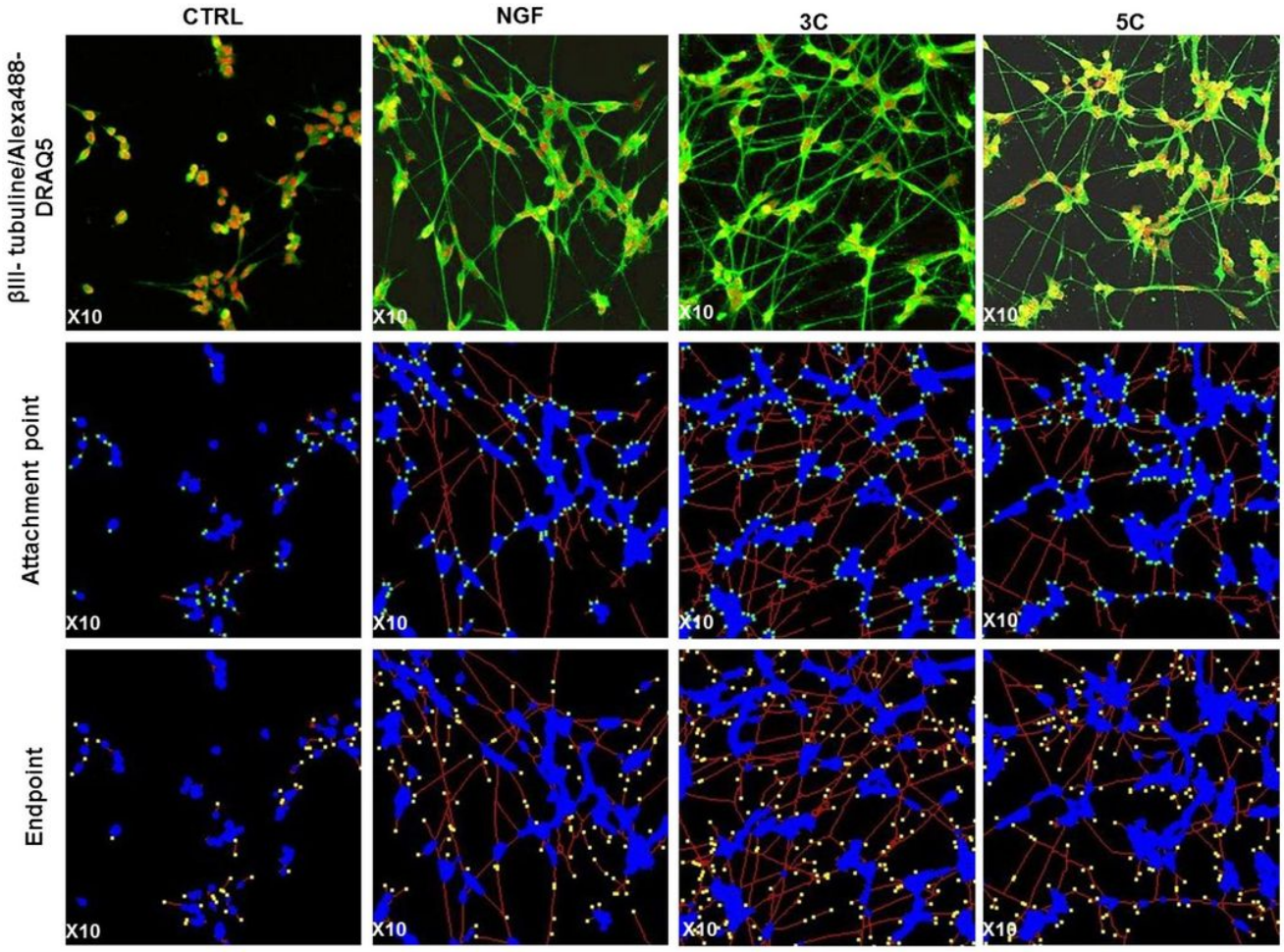

Figure 3

The GS dilutions 3C and 5C improved the neurite outgrowth of neuroblastoma cells after 3 days of treatment in a 2D cell culture. Pictures were taken using a confocal microscope (x10). Pictures in the

upper panel (S1 Figure) display neurite extension between the cells (βIII- tubuline/Alexa488, green) and DraQ5 (nucleus, red). Quantification of the neurite outgrowth parameters such as the attachment points (middle panels) and the endpoint numbers (lower panels), after NGF or GS treatment are shown in the middle and lower panel (Blue: soma, red: neurite, green point: attachment point, yellow point: endpoint). CTRL: untreated control cells.

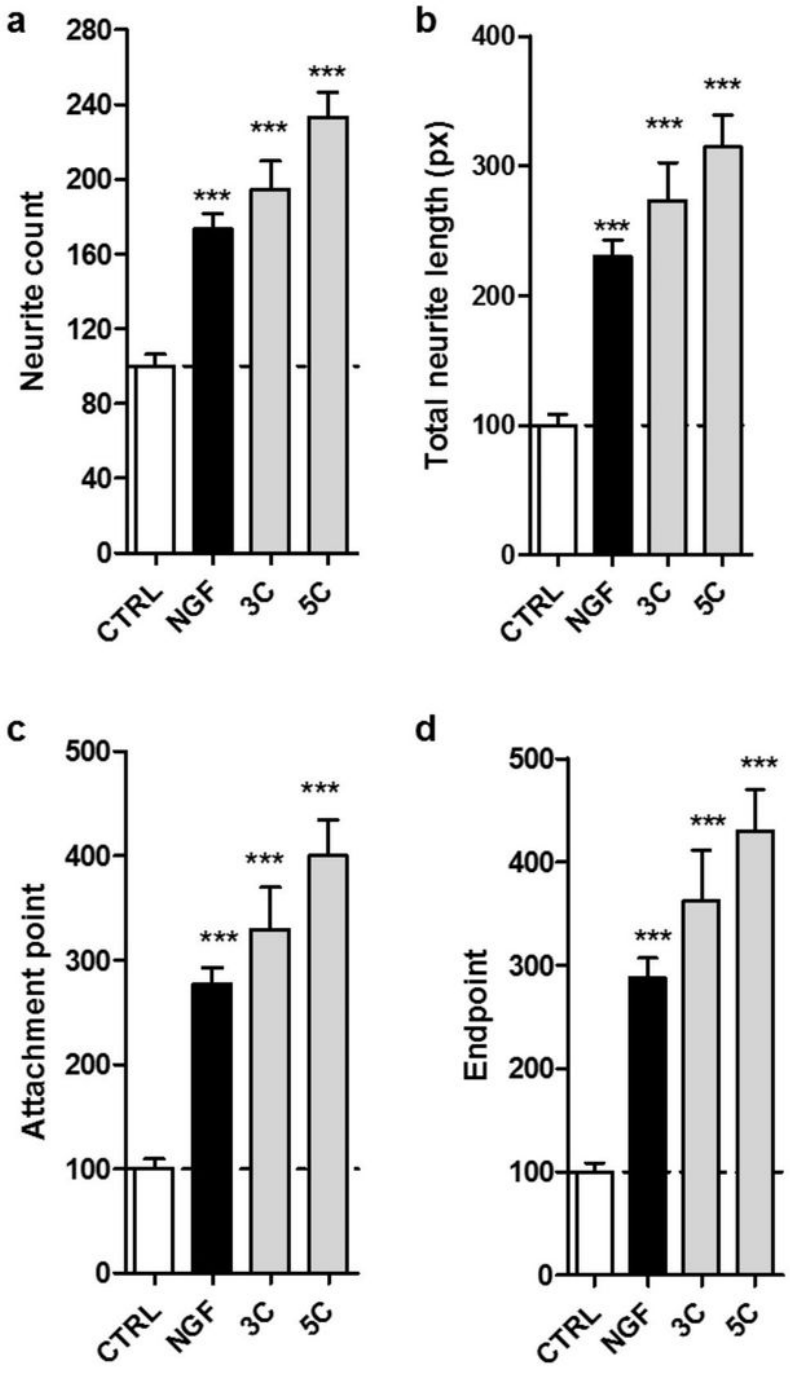

Figure 4

GS dilutions 3C and 5C increased the neurite outgrowth of neuroblastoma cells after 3 days of treatment in a 2D cell culture. Quantification of Figure 3 using NeurophologyJ (S3 Table). 3C and 5C significantly increased: (a) number of neurites (neurite count), (b) total neurite length, (c) number of attachment points and (d) number of endpoint. The effect of GS dilutions was similar than the positive control NGF when compared to the untreated cells. Values represent the mean± SEM of three independent experiments and were normalized to 100% of untreated control cells (CTRL). One way ANOVA (a-d: P□0.0001)) and post hoc Dunnett's multiple comparisons versus untreated control cells ***P<0.001.

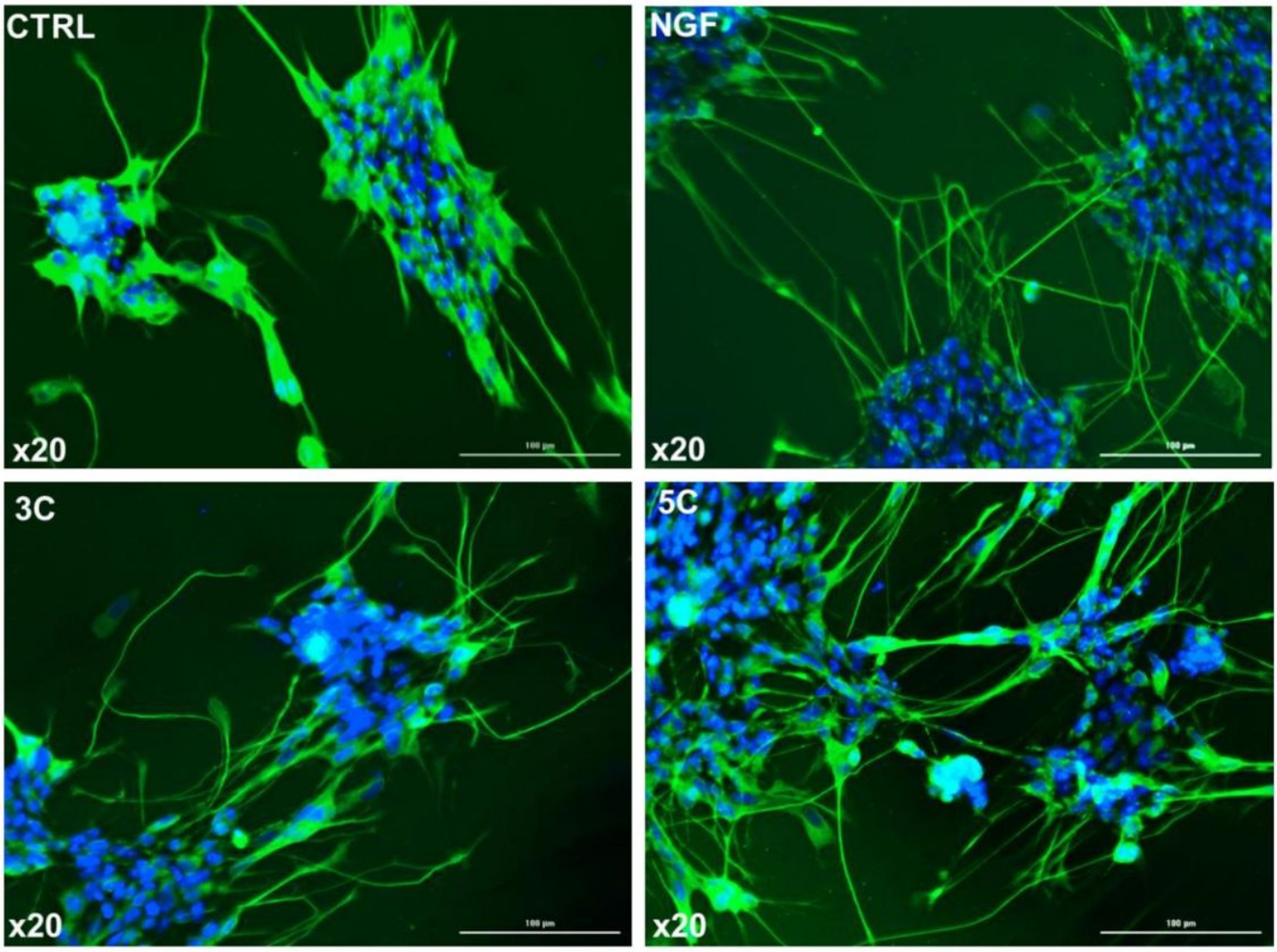

Figure 5

GS dilutions 3C and 5C induced neurite extension in a 3D-matrix by increasing the neurite outgrowth of neuroblastoma cells after 3 days of treatment. Pictures were obtained by merging 3–4 layers of cells (z-stack projection) on 3D-matrix using the multi-label plate reader Cytation3 (x20). Pictures display neurite extension between the cells (Immunostaining (IMS) with βIII- tubuline/Alexa488 (green) and DAPI (blue)). CTRL: untreated control cells.

Supplementary Files

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- Supplementarymaterialfile.docx