Glycylglycine plays critical roles in the proliferation of spermatogonial stem cells

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Abstract. Glial cell line-derived neurotrophic factor (GDNF) is critical for the proliferation of spermatogonial stem cells (SSCs), but the underlying mechanisms remain poorly understood. In this study, an unbiased metabolomic analysis was performed to examine the metabolic modifications in SSCs following GDNF deprivation, and 11 metabolites were observed to decrease while three increased. Of the 11 decreased metabolites identified, glycylglycine was observed to significantly rescue the proliferation of the impaired SSCs, while no such effect was observed by adding sorbitol. However, the expression of self-renewal genes, including B-cell CLL/lymphoma 6 member B, ETS variant 5, GDNF family receptor α1 and early growth response protein 4 remained unaltered following glycylglycine treatment. This finding suggests that although glycylglycine serves an important role in the proliferation of SSCs, it is not required for the self-renewal of SSCs.

Introduction

Spermatogenesis is a complex developmental process that has spermatogonial stem cells (SSCs) at its foundation. The SSCs niche is in the basal compartment of the seminiferous tubules. SSCs undergo spermatogenesis to produce spermatozoa. Self-renewal and the potential to differentiate are two properties that distinguish stem cells from somatic cells, and SSCs are the only germline stem cells that can undergo self-renewal division (1). The balance between proliferation and differentiation is therefore essential to the normal function of SSCs and to maintain male fertility (2).

Glial cell line-derived neurotrophic factor (GDNF) is secreted by Sertoli cells, and is an important factor in the cell fate determination of SSCs, which was identified in the year 2000 (3). While *GDNF*$^{+/-}$ mice have depleted stem cell reserves (3), the overexpression of GDNF results in the accumulation of undifferentiated spermatogonia and in the development of testicular tumors (4), indicating that the right concentration of GDNF is critical for the proliferation of SSCs (5). Although GDNF has no significant effect on the activity of SSCs at concentrations in the range 1-100 ng/ml, GDNF at concentrations <1 ng/ml is insufficient to maintain the proliferation of SSCs *in vitro* over a period of 7 days (6). However, the mechanisms underlying the GDNF-dependent proliferation of SSCs remain elusive.

Metabolomics is the study of the final products of gene expression and, as a high-throughput analysis, is considered to be more reflective of the biological phenotype compared to genomic, transcriptomic and proteomic studies (7). Thus, metabolomics may be particularly well-suited to detect the dynamic modifications that occur during complex biological processes. Metabolomics has been used in the discovery of small molecules that are potential biomarkers of self-renewal (8), reprogramming (9) and differentiation (10), and it has also been previously used to reveal metabolic mechanisms related to spermatogenesis (11,12).

In the present study, metabolomics was used to evaluate the alterations in SSCs metabolites, including glycylglycine and sorbitol, following GDNF deprivation, in order to elucidate the underlying mechanisms of GDNF-dependent proliferation.

Materials and methods

Chemicals and reagents. The following chemicals and reagents were purchased from the respective chemical suppliers: Glycylglycine (cat. no. G1002; purity ≥99%; Sigma-Aldrich;

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Merck KGaA, Darmstadt, Germany); sorbitol (cat. no. S6021; purity ≥98%; Sigma-Aldrich; Merck KGaA); TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA); the Cell Counting Kit-8 (CCK-8) assay kit (Beyotime Institute of Biotechnology, Haimen, China); and the cDNA Synthesis kit and SYBR® Green Master Mix kit (Takara Bio, Inc., Otsu, Japan). The Cell Light™ EdU kit (cat. no. C10310) was purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China).

SSCs culture. SSCs were cultured *in vitro* following a previously described protocol (13), and the cells were characterized as in a previous study (14). All experiments involving mice were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (IACUC:1601247). The primary cells were isolated from 6-8 day old male C57BL/6 mice obtained from Nanjing Medical University. The mice were housed in groups in a polypropylene cages at 21±2˚C, a humidity of 50±10% and a 12 h light/dark cycle (lights on at 7:00 a.m.), and THY1-positive cells were enriched using magnetic-activated cell separation (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Cells were plated at a density of 1.5-2.0x10^5 cells/well on 12-well plates coated with mitotically inactivated SIM mouse embryo-derived thioguanine- and ouabain-resistant feeder layers (cat. no. SNLP76/7-4; American Type Culture Collection, Manassas, VA, USA). Long-term cultures of SSCs were supported in serum-free Minimum Essential Medium-α (Thermo Fisher Scientific, Inc.) and supplied with 20 ng/ml GDNF (R&D Systems China Co., Ltd., Shanghai, China), 1 ng/ml basic fibroblast growth factor 2 (BD Biosciences, San Jose, CA, USA), and 150 ng/ml GDNF family receptor α1 (GFRA1; R&D Systems China Co., Ltd.). In addition, 2 mM L-glutamine (Thermo Fisher Scientific, Inc.), 2% bovine serum albumin, 10 μg/ml transferrin, 50 μM free fatty acid mixture (5.6 mM linolenic acid, 13.4 mM oleic acid, 2.8 mM palmitoleic acid, 35.6 mM linoleic acid, 31 mM palmitic acid, 76.9 mM stearic acid;), 30 nM Na_2SeO_3, 50 μM 2-mercaptoethanol, 5 μg/ml insulin, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 60 μM putrescine, all purchased from Sigma-Aldrich; Merck KGaA, were added to the medium. Cells were maintained in a humidified atmosphere at 5% CO_2 and 37˚C. The medium was replaced every 2 days and cells were passaged every 5-6 days. For GDNF deprivation, the concentration of GDNF was reduced to 0.1 ng/ml for 12 h or 24 h when SSCs were ~50% confluent. For the rescue assay, two doses (1 and 10 μM) of each metabolite (glycylglycine and sorbitol) were chosen for optimization (according to http://www.hmdb.ca/metabolites/HMDB0000247). Given results from preliminary studies on the rescue effect, and the glycylglycine and sorbitol level in the body, 10 μM was selected for used in the subsequent experiments. After 5 days of treatment, the SSCs were imaged using a light microscope.

Metabolomic analysis. The SSCs sample preparation for metabolomic analysis was conducted according to a previous approach (8). Briefly, treated SSCs were washed five times with ice cold PBS, and ~6x10^5 SSCs/group were collected. Following the addition of 0.3 ml 50% methanol with internal standard, the cells were harvested by pipetting. Cells were sonicated for 3 min (frequency, 20 kHz; power, 60%; pulses, 6/4) and centrifuged at 16,099 x g at 4˚C for 15 min to remove cellular debris. The supernatant was subjected to metabolomic analysis. Quality control (QC) samples were prepared by mixing equal volumes of each SSCs sample.

The metabolomic analysis was performed on an UltiMate™ 3000 ultra high-performance liquid chromatography system (Dionex; Thermo Fisher Scientific, Inc.), coupled to an Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, Inc.) in both positive and negative modes simultaneously (15). The detailed operating procedures was conducted according to a previous study (16). Through this approach, >70% of differential metabolites observed in the QC sample had a percent relative standard deviation (%RSD) of <30%, and the internal standard had a %RSD of <20%, indicating the reliability of the metabolomic analysis (17).

Cell viability assay and proliferation. Cellular viability was evaluated using the CCK-8 Kit. Cells were plated at a density of 1.5x10^4 cells/well in 96-well plates and incubated overnight (37˚C, 5% CO_2). The SSCs treatment groups were: Complete medium; GDNF deprivation; GDNF deprivation with glycylglycine rescue; and GDNF deprivation with sorbitol rescue. On the 2nd, 3rd, 4 and 5th days, 10 μl CCK-8 solution was added to each well, and the cells were incubated for 2 h at 37˚C in 5% CO_2. The medium was replaced every 2 days. The absorbance was determined using a TECAN infinite M200 plate reader (Tecan Group, Ltd., Mannedorf, Switzerland) at 450 nm.

The Cell Light™ EdU kit was used to assess SSCs proliferation. SSCs (~8x10^3 cells/well) were seeded in a 96-well plate and treated for 5 days as aforementioned. EdU (25 μM) was added to the culture medium for an additional 10 h. The cells were fixed with 4% formaldehyde for 30 min at room temperature and neutralized using glycine (2 mg/ml) for 10 min at room temperature, according to the manufacturer's instructions. Then the cells were permeabilized using 0.5% Triton X-100 in PBS for 20 min, and staining with Hoechst 33342 was performed. Cell images and data were automatically obtained using High Content Screening (HCS; Cellomics ArrayScan VTI HCS Reader; Thermo Fisher Scientific, Inc.). Appropriate filter sets for the detection of two fluorophores were used, and different fluorescent signals were recorded in two different image collection channels. Channel 1 contained the blue nuclear images; channel 2 contained the red images indicating where EdU was incorporated and which cells were proliferating. For each treatment, three independent wells were measured. The x20 objective was used to collect images, and 48 fields/well were imaged. The analysis was performed after 5 days using the Thermo Scientific HCS Studio Cell Analysis Software 3.0 (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay of mRNA levels of self-renewal genes. Total RNA was isolated from SSCs using TRIzol® and the concentration was determined using a NanoDrop 2,000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Total RNA (1 μg) was used to synthesize cDNA using the following temperature protocol: 37˚C for 15 min and 85˚C for 5 sec. The mRNA levels of self-renewal genes, namely B-cell CLL/lymphoma 6 member B (*Bcl6b*), ETS variant *5*

Table I. Sequences of primers for reverse transcription-quantitative polymerase chain reaction.

Gene	Type of primer	Sequences
Bcl6b	Forward	5'-GGCTACGTCCGAGAGTTCAC-3'
	Reverse	5'-CTTGTGCGCTCTTAGGGGT-3'
Etv5	Forward	5'-CACCATGTATCGAGAGGGGC-3'
	Reverse	5'-GAGCAACCTCTTCCGGTTCT-3'
Gfra1	Forward	5'-CTCGGAATCCAGCCTACGTC-3'
	Reverse	5'-CACTTGTCCTCTCGTGTGCT-3'
Egr4	Forward	5'-GACGCGCTTCTCTCCAAG-3'
	Reverse	5'-CTCAAAGCCCAGCTCAAGAA-3'
GAPDH	Forward	5'-AGGTCGGTGTGAACGGATTTG-3'
	Reverse	5'-GGGGTCGTTGATGGCAACA-3'

Bcl6b, *B*-cell CLL/lymphoma 6 member B; *Etv5*, ETS variant 5; *Egr4*, early growth response protein 4; *Gfra1*, GDNF family receptor α1.

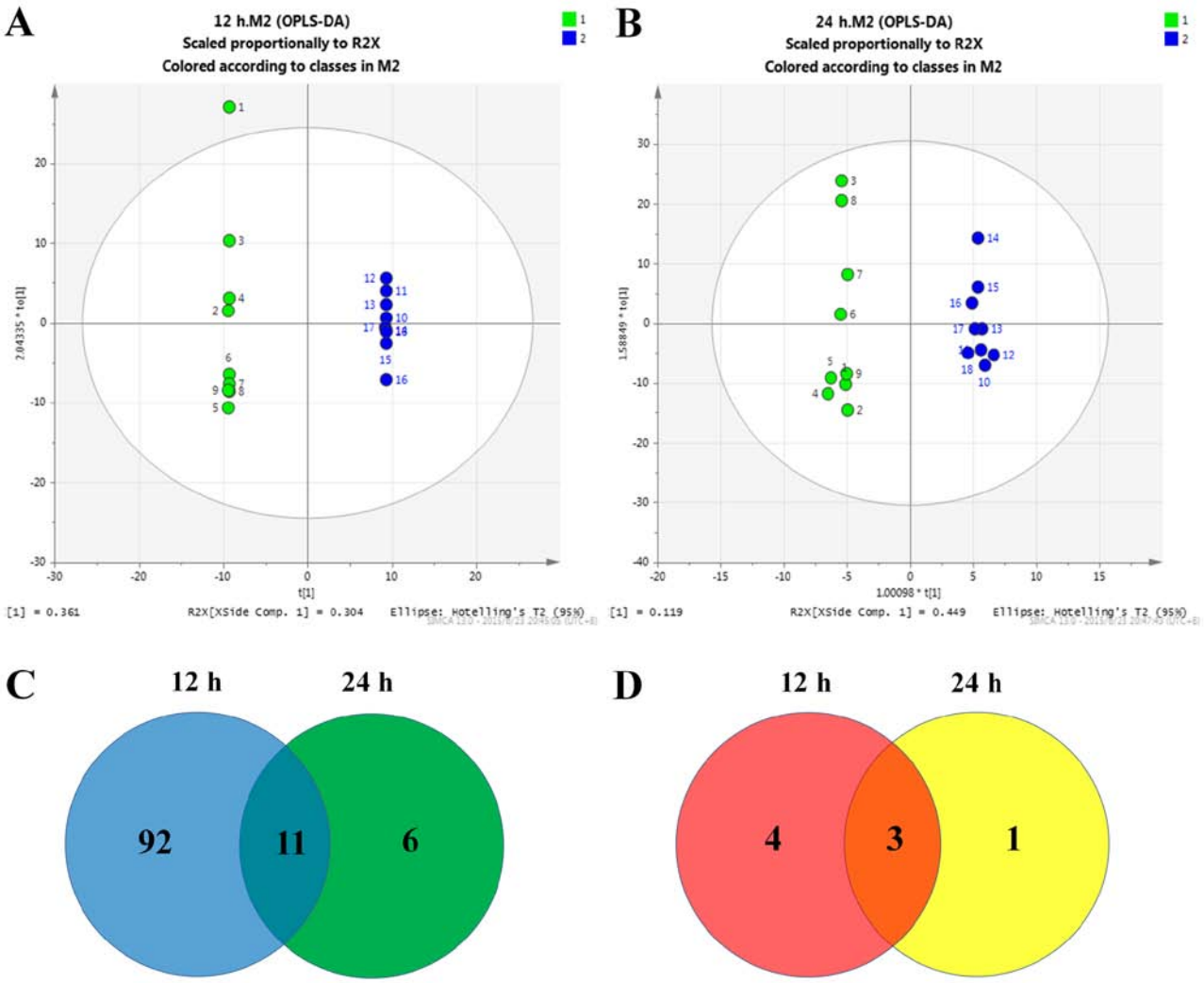

Figure 1. Metabolic modifications after GDNF deprivation. According to the OPLS-DA analysis, obvious separations between samples in the (A) GDNF deprivation for 12 h and (B) 24 h groups and the control were detected. (C) A total of 11 metabolites were consistently decreased in both the 12 and 24 h deprivation groups. (D) A total of three metabolites were increased in both the 12 and 24 h deprivation groups. OPLS-DA, orthogonal partial least square discriminate analysis; GDNF, glial cell line-derived neurotrophic factor.

(*Etv5*), *Gfra1* and early growth response protein 4 (*Egr4*) were analyzed using the SYBR® Green Master Mix kit, according to the manufacturer's instructions. *GAPDH* was used as a reference gene. Primer sequences synthesized by Invitrogen

Table II. Altered metabolites following glial cell line-derived neurotrophic factor deprivation for 12 h.

Peak	VIP	Fold change	P-value
Aminocaproic acid	1.366	0.176	$3.08x10^{-05}$
Taurine	1.339	0.164	$5.76x10^{-05}$
Theobromine	1.330	0.178	$7.06x10^{-05}$
Methylmalonic acid	1.306	0.193	$1.17x10^{-04}$
Melibiose	1.301	0.107	$4.86x10^{-05}$
Sucrose	1.301	0.107	$4.86x10^{-05}$
Trehalose	1.301	0.107	$4.86x10^{-05}$
Biotin	1.298	0.169	$1.38x10^{-04}$
Androstenedione	1.278	0.204	$2.00x10^{-04}$
Petroselinic acid	1.274	0.194	$2.15x10^{-04}$
Trans-Vaccenic acid	1.274	0.194	$2.15x10^{-04}$
Indoleacrylic acid	1.274	0.198	$2.17x10^{-04}$
Sorbitol	1.269	0.195	$2.37x10^{-04}$
D-Fructose 6-phosphate disodium salt hydrate	1.259	0.236	$2.85x10^{-04}$
2-Piperidinemethanol	1.251	0.198	$3.26x10^{-04}$
Guanine	1.250	0.275	$3.29x10^{-04}$
Cellobiose	1.250	0.107	$4.86x10^{-05}$
Formamide	1.248	0.212	$3.45x10^{-04}$
D-Mannose 6-phosphate sodium salt	1.242	0.393	$4.84x10^{-02}$
Cinnamaldehyde natural	1.241	0.178	$3.88x10^{-04}$
Cytosine	1.229	0.200	$4.70x10^{-04}$
Quinaldic acid	1.224	0.219	$5.10x10^{-04}$
16-Alpha-hydroxyestrone	1.221	0.201	$5.39x10^{-04}$
Androstenediol	1.221	0.201	$5.39x10^{-04}$
N-Acetyl-L-phenylalanine	1.221	0.201	$5.39x10^{-04}$
Progesterone	1.221	0.201	$5.39x10^{-04}$
Uracil	1.221	0.201	$5.39x10^{-04}$
2-Amino-1-Phenylethanol	1.221	0.201	$5.39x10^{-04}$
2-Deoxycytidine free base	1.221	0.201	$5.39x10^{-04}$
4-Hydroxyestrone	1.221	0.201	$5.39x10^{-04}$
Androsterone	1.221	0.201	$5.39x10^{-04}$
DL-alpha-Palmitin	1.221	0.201	$5.39x10^{-04}$
D-Plus-Neopterine	1.221	0.201	$5.39x10^{-04}$
L-A-Phosphatidylcholine	1.221	0.201	$5.39x10^{-04}$
Linoleic acid	1.221	0.201	$5.39x10^{-04}$
4-Hydroxycinnamic acid	1.221	0.201	$5.39x10^{-04}$
Acetyl-L-carnitine hydrochloride	1.221	0.201	$5.39x10^{-04}$
Gamma-Linolenic acid	1.221	0.201	$5.39x10^{-04}$
Indole-3-acetamide	1.221	0.201	$5.39x10^{-04}$
Estrone	1.221	0.201	$5.39x10^{-04}$
Theophylline	1.221	0.201	$5.39x10^{-04}$
Transtrans-farnesol	1.221	0.201	$5.39x10^{-04}$
Cytidine	1.221	0.417	$2.31x10^{-02}$
Riboflavin	1.218	0.202	$5.62x10^{-04}$
Suberic acid	1.217	0.133	$5.70x10^{-04}$
2-methoxycinnamic acid	1.216	0.203	$5.77x10^{-04}$
N-Acetylglutamine	1.216	0.203	$5.80x10^{-04}$
3-Methyladipic acid	1.215	0.203	$5.93x10^{-04}$
Tyramine	1.214	0.196	$5.95x10^{-04}$
Octadecanamide	1.214	0.296	$5.99x10^{-04}$
Guaiacol	1.213	0.204	$6.06x10^{-04}$
L-Allothreonine	1.211	0.205	$6.28x10^{-04}$

Table II. Continued.

Peak	VIP	Fold change	P-value
L-Homoserine	1.211	0.205	$6.28x10^{-04}$
Octadecanedioic acid	1.208	0.191	$6.54x10^{-04}$
Gluconolactone	1.208	0.206	$6.58x10^{-04}$
Sebacic acid	1.207	0.189	$6.70x10^{-04}$
N-Oleoylethanolamine	1.206	0.207	$6.78x10^{-04}$
Valeric acid	1.206	0.191	$6.78x10^{-04}$
Isovaleric acid	1.206	0.198	$2.73x10^{-04}$
345-Trimethoxycinnamic acid	1.204	0.208	$6.98x10^{-04}$
Benzocaine	1.204	0.211	$6.99x10^{-04}$
3-(2-Hydroxyethyl)indole	1.203	0.636	$1.74x10^{-04}$
N-Acetyl-L-alanine	1.198	0.210	$7.64x10^{-04}$
Folic acid	1.198	0.211	$7.69x10^{-04}$
D-Glutamic acid	1.191	0.381	$4.88x10^{-04}$
Hydrocinnamic acid	1.190	0.176	$8.58x10^{-04}$
1-Methyl-L-histidine	1.182	0.226	$9.68x10^{-04}$
2-Phenylacetamide	1.177	0.379	$4.75x10^{-05}$
Pentadecanoic acid	1.171	0.221	$1.13x10^{-03}$
Phthalic acid	1.169	0.081	$1.01x10^{-02}$
L-Pipecolic acid	1.169	0.267	$1.16x10^{-03}$
DL-O-Tyrosine	1.164	0.223	$1.24x10^{-03}$
Glycylglycine	1.157	0.252	$1.62x10^{-03}$
3-Ureidopropionic acid	1.157	0.314	$1.37x10^{-03}$
Thymidine	1.156	0.332	$1.64x10^{-02}$
Acetoacetic acid lithium	1.140	0.183	$1.72x10^{-03}$
Betaine	1.140	0.325	$1.72x10^{-03}$
5-Phenylvaleric acid	1.137	0.234	$1.79x10^{-03}$
Rhamnose	1.135	0.282	$1.83x10^{-03}$
Cinnamic acid	1.131	0.263	$1.93x10^{-03}$
Acetaminophen	1.125	0.122	$2.10x10^{-03}$
Acetylglycine	1.124	0.256	$2.12x10^{-03}$
Cholic acid	1.115	0.244	$2.37x10^{-03}$
Terephthalic acid	1.113	0.199	$2.42x10^{-03}$
L-Allo-isoleucine	1.113	0.188	$2.43x10^{-03}$
Indoleacetic acid	1.100	0.266	$2.85x10^{-03}$
Cytidine monophosphate	1.097	0.458	$2.97x10^{-03}$
Glucose 6-phosphate	1.092	0.220	$3.12x10^{-03}$
Dehydroepiandrosterone	1.078	0.258	$3.68x10^{-03}$
Mannitol	1.073	0.195	$2.37x10^{-04}$
Adenine	1.071	0.221	$3.98x10^{-03}$
Cuminaldehyde	1.071	0.264	$4.00x10^{-03}$
Trans-Cinnamic acid	1.067	0.274	$4.18x10^{-03}$
Salicin	1.063	0.264	$4.37x10^{-03}$
N-Acetylglutamic acid	1.061	0.373	$4.44x10^{-03}$
Serotonin hydrochloride	1.061	0.265	$4.46x10^{-03}$
N-Acetylleucine	1.056	0.097	$4.70x10^{-03}$
Tryptophanol	1.049	0.306	$5.10x10^{-03}$
Deoxyribose	1.039	0.507	$3.23x10^{-02}$
13-Dimethyluracil	1.037	0.287	$5.77x10^{-03}$
Eicosapentaenoic acid	1.030	0.287	$6.20x10^{-03}$
L-Aspartyl-L-phenylalanine	1.018	0.320	$7.04x10^{-03}$
Tryptamine	1.015	0.288	$7.26x10^{-03}$
Dodecanoic acid	1.284	1.715	$6.85x10^{-03}$

Table II. Continued.

Peak	VIP	Fold change	P-value
L-Phenylalanine	1.284	1.885	$5.32x10^{-04}$
L-Tryptophan	1.221	1.939	$5.68x10^{-04}$
L-Isoleucine	1.217	2.033	$1.80x10^{-04}$
L-Leucine	1.039	2.033	$1.80x10^{-04}$
Inosine	1.020	2.365	$7.24x10^{-03}$
Ursodeoxycholic acid	1.015	16.743	$5.65x10^{-03}$

VIP, variable importance in projection.

Table III. Altered metabolites following glial cell line-derived neurotrophic factor deprivation for 24 h.

Peak	VIP	Fold change	P-value
Glycylglycine	2.347	0.213	$7.17x10^{-04}$
Sorbitol	2.109	0.527	$7.00x10^{-04}$
Mannitol	2.109	0.527	$7.00x10^{-04}$
Biotin	2.051	0.337	$1.14x10^{-03}$
N-Acetylglutamic acid	1.913	0.430	$3.13x10^{-03}$
Proline	1.633	0.577	$1.57x10^{-02}$
Pyrrolidonecarboxylic acid	1.624	0.542	$1.65x10^{-02}$
Indoleacetic acid	1.607	0.475	$1.79x10^{-02}$
L-Allothreonine	1.515	0.613	$2.72x10^{-02}$
L-Homoserine	1.515	0.613	$2.72x10^{-02}$
D-Mannose 6-phosphate sodium salt	1.504	0.656	$2.85x10^{-02}$
D-Glutamic acid	1.498	0.531	$2.93x10^{-02}$
2-Phenylacetamide	1.498	0.425	$2.93x10^{-02}$
Methyl-L-histidine	1.477	0.475	$3.21x10^{-02}$
Octadecanedioic acid	1.149	0.488	$4.27x10^{-02}$
Gluconolactone	1.147	0.516	$4.39x10^{-02}$
Aminobenzoic acid	1.137	0.363	$4.98x10^{-02}$
Melibiose	1.137	34.339	$7.49x10^{-03}$
Sucrose	1.137	34.339	$7.49x10^{-03}$
Oxidized glutathione	1.104	1.922	$7.84x10^{-04}$
Inosine	1.466	2.239	$4.69x10^{-02}$

VIP, variable importance in projection.

(Thermo Fisher Scientific, Inc.) are presented in Table I. All RT-qPCR reactions were performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: 95˚C for 30 sec followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec.

Data analysis. For the metabolomics analysis, the data were imported into SIMCA-P software (Version 13.0; Umetrics; Sartorius AG, Göttingen, Germany) and were unit variance-scaled and auto log-transformed where appropriate. Orthogonal partial least square discriminate analysis (OPLS-DA) was applied to quantitatively assess the metabolites produced by the experimental and control treatments. The metabolites were subsequently indexed by their variable importance in projection (VIP). Data were validated using the leave one out cross-validation method and the quality of model was assessed by R2 and Q2 scores (18). The statistical significance of metabolites identified by OPLS-DA was then calculated via Student's t-test. VIP>1 and P<0.05 were considered to indicate a statistically significant difference in metabolite production (19).

The $2^{-\Delta\Delta Cq}$ method was used to analyze the results of the RT-qPCR (20). Differences among all the treatment groups and the control group were determined by one-way analysis of variance, followed by Dunnett's multiple comparison test. All the statistical analyses were performed using Stata 9.2 (StataCorp LP, College Station, TX, USA) and presented with GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). All analyses were two-sided and date are presented as the mean ± SD. P<0.05 was considered to indicate statistically significant difference.

Results

Metabolic modifications after GDNF deprivation. The OPLS-DA model demonstrated that GDNF deprivation for 12 or 24 h resulted in a distinct metabolic profile compared to the control (R2Y=99.4%, Q2=71.5% and R2Y=99.0%, Q2=53.0%, respectively; Fig. 1A and B. R2Y indicates the quality of fit, with an R2Y of 1 indicating a perfect description of the data by the model. Q2 indicates the predictive ability of the model, with a Q2 of 1 indicating complete predictability. A total of 103 metabolites had decreased and 7 had increased (VIP>1 and P<0.05) after 12 h of GDNF deprivation (Table II). After 24 h of GDNF deprivation the majority of those metabolites were restored to normal levels, and only a total of 17 metabolites were decreased (Fig. 1C) and 4 were increased (Fig. 1D; Table III). A comparison of these two lists demonstrated that there were 14 overlapping metabolites. Among these, based on the metabolic information in the HMDB database, glycylglycine and sorbitol were found to be the two most significantly decreased metabolites following GDNF deprivation. Therefore, the effects of glycylglycine and sorbitol on the proliferation of SSCs were further assessed.

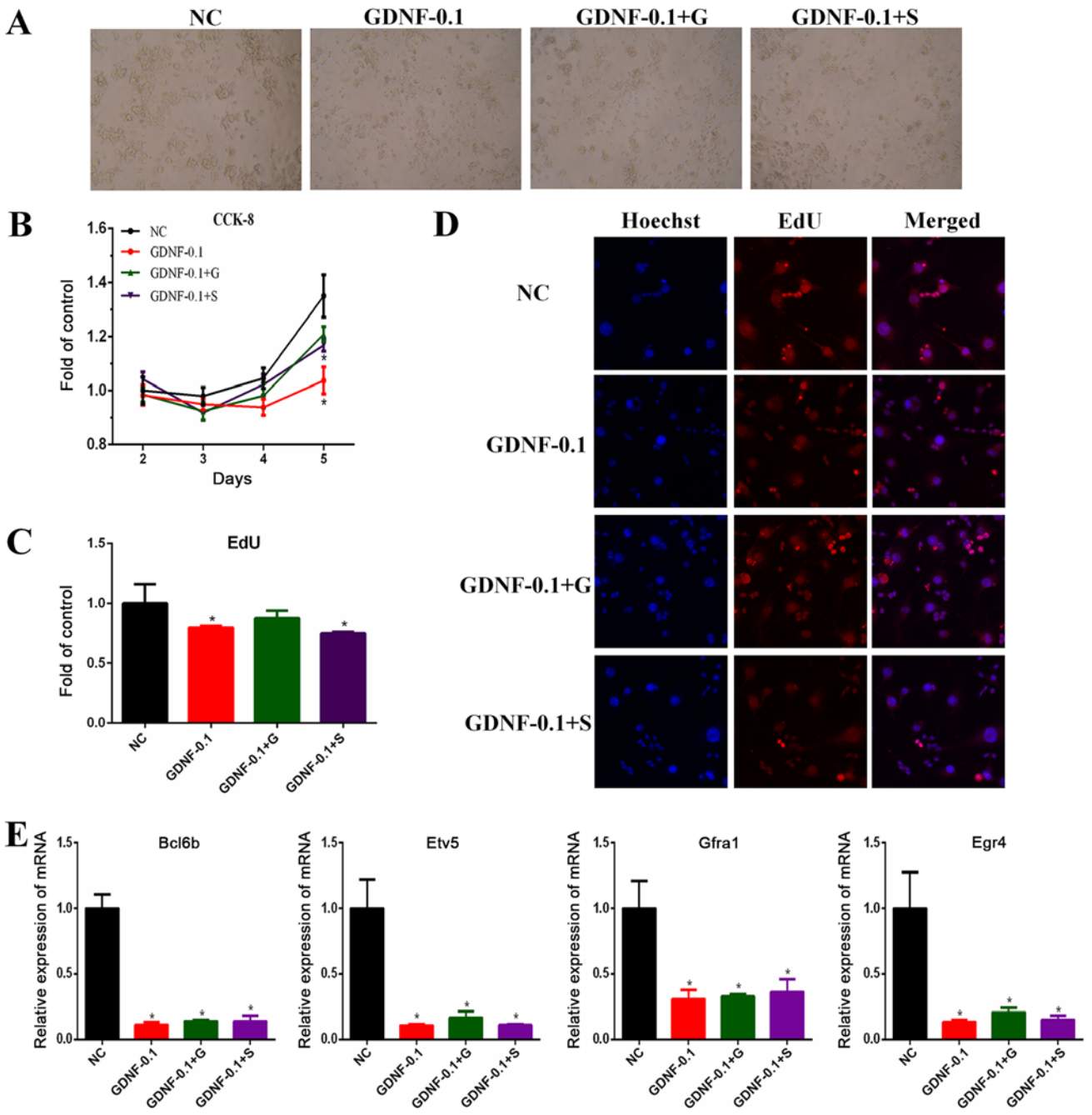

Figure 2. Effects of glycylglycine and sorbitol on the viability of SSCs. SSCs were exposed to complete medium (NC), GDNF (0.1 ng/ml), GDNF (0.1 ng/ml) and glycylglycine (10 μM), or GDNF (0.1 ng/ml) and sorbitol (10 μM). (A) Representative SSCs images under a light microscope on day 5. Magnification, x10 objective. (B) Cell viability was determined by CCK-8 assay. (C) Relative cell proliferation, as assessed by EdU assay. (D) Staining for nuclei (blue) and cell proliferation (red). Images were acquired with the ArrayScan HCS Reader with a x20 objective. (E) The mRNA expression levels of self-renewal genes (*Bcl6b*, *Etv5*, *Egr4* and *Gfra1*) were detected by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. respective NC. SSCs, spermatogonial stem cells; GDNF, glial cell line-derived neurotrophic factor; G, glycylglycine; S, sorbitol; CCK-8, Cell Counting Kit-8; NC, control; *Bcl6b*, B-cell CLL/lymphoma 6 member B; *Etv5*, ETS variant 5; *Egr4*, early growth response protein 4; *Gfra1*, GDNF family receptor α1.

Glycylglycine rescues the GDNF-deprivation-induced inhibition of SSCs proliferation. The effects of glycylglycine and sorbitol on the proliferation of SSCs were investigated using a CCK-8 and an EdU assay. SSCs were cultured in either complete medium or media without GDNF, and treated with either glycylglycine or sorbitol. After 5 days of treatment, the SSCs were imaged using a light microscope (Fig. 2A). The CCK-8 assay demonstrated that the viability of SSCs decreased significantly after GDNF deprivation. The addition of glycylglycine partially rescued the viability of the SSCs to levels similar to those observed in SSCs cultured in complete media, while sorbitol treatment failed to rescue their viability (Fig. 2B). Similar results were obtained with the EdU assay (Fig. 2C and D).

mRNA levels of self-renewal genes (Bcl6b, Etv5, Gfra1 and Egr4) remain unaltered following rescue with glycylglycine. The effects of glycylglycine and sorbitol on the expression of

SSCs self-renewal genes was assessed via RT-qPCR analysis of *Bcl6b*, *Etv5*, *Gfra1* and *Egr4*. The results demonstrated that GDNF deprivation downregulated the expression of the self-renewal genes of SSCs, and the addition of either glycylglycine or sorbitol did not restore the expression of these genes to normal levels (Fig. 2E).

Discussion

SSCs are responsible for accurately maintaining and transmitting parental genetic information (21). The proliferation and self-renewal of SSCs are precisely regulated by intrinsic and extrinsic signals (22). It is well known that GDNF serves a crucial role in regulating the proliferation of SSCs, and that the GDNF concentration is important for maintaining the function of SSCs (23). The present study demonstrated that GDNF deprivation decreased the viability of SSCs, and that this decrease in viability may be rescued by the addition of glycylglycine.

Glycylglycine is a dipeptide of glycine, which is the simplest amino acid. Glycine, a nonessential amino acid, is involved in the production of DNA, phospholipids and collagen, as well as the release of energy (24). It has also been demonstrated that glycine serves a key role in cell proliferation, potentially through the action of modifying enzymes (25,26). The synthesizing enzymes serine hydroxymethyltransferase, cytosolic, serine hydroxymethyltransferase, mitochondrial, C-1-tetrahydrofolate synthase, cytoplasmic, and monofunctional C1-tetrahydrofolate synthase, mitochondrial are responsible for glycine synthesis (27). Glycylglycine, which is used as a buffer, is a starting template for the preparation of more complex peptides and a substrate for the enzyme glycylglycine dipeptidase (28). Glycylglycine has been utilized in the purification and characterization of a fructose-6-phosphate aldolase from *Escherichia coli* and in the characterization of a poly(L-malate) hydrolase from a strain of *Comamonas acidovorans* (29,30). Glycylglycine has also been used in a [35S] guanosine triphosphate-γ-S binding assay to measure the functional coupling of G proteins with receptors (31). Sorbitol is a sugar alcohol that is gradually metabolized by the human body (32). Previous studies reported that sorbitol promotes proliferation in various cell types (33,34). However, the results of the present study indicated that glycylglycine, rather than sorbitol, may be a crucial molecule in the GDNF-dependent proliferation of SSCs.

GDNF-induced cell signaling also serves a central role in the self-renewal of SSCs (21). Previous studies demonstrated that GDNF regulates self-renewal through different signaling pathways, including but not limited to Ras/extracellular signal-regulated kinases 1/2 (35), mitogen-activated protein 2 kinase 1 (36) and phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (37). Meanwhile, a number of GDNF-associated genes, including *Gfra1*, *Bcl6b* and *Etv5,* have all been determined to be involved in the self-renewal of SSCs (36,38). The present study demonstrated that GDNF deprivation downregulated the expression of these self-renewal genes in cultured SSCs, and that the addition of glycylglycine or sorbitol did not rescue their expression. Unlike what was observed in the proliferation of SSCs, these data suggested that glycylglycine is not involved in the self-renewal of SSCs.

Although there are various shared factors and pathways, the proliferation and self-renewal of SSCs are regulated differently (39). Proliferation is an important feature of life that contributes to development and growth via division. The self-renewal of SSCs is a unique form of cell division in which SSCs proliferate and differentiate. This process requires control of the cell cycle and maintenance of the undifferentiated state (40).

In conclusion, through unbiased metabolomic analyses, the present study identified that the production of glycylglycine and sorbitol were significantly altered following GDNF deprivation. While the addition of glycylglycine restored the impaired proliferation of SSCs, the addition of sorbitol had no effect, which suggests that glycylglycine serves an important role in the proliferation of SSCs. This study also provided novel insights into the mechanism underlying the proliferation of SSCs.

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Availability of data and materials

The datasets generated and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

BX performed the EdU experiments and drafted the manuscript. XWe performed the experiments on spermatogonial stem cells. MC analyzed and interpreted the metabolomic data. ZH and TD performed the RT-qPCR experiments. KX and WH were involved in the design of the study and manuscript revisions. YZ, KZ and XH performed the Cell Counting Kit-8 assay, and participated in metabolomic preparation and table preparation. XWu and YX made substantial contributions to the study conception and design. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Animal Ethical and Welfare Committee of Nanjing Medical University (Nanjing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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