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GUANOSINE PROMOTES PROLIFERATION OF NEURAL STEM CELLS THROUGH cAMP-CREB PATHWAY

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In previous studies, we have found that extracellular guanosine can stimulate endogenous progenitor/stem cell proliferation in the spinal cord following chronic injury and in the subventricular zone of the brains of rats afflicted with Parkinson's Disease. In this study, using neural stem cells isolated from one-day old rats, we found that guanosine could stimulate neural stem cell proliferation, and that the proliferation was not due to the guanosine metabolism mechanism since guanine, which is interconverted by an ecto-purine nucleoside phosphorylase from guanosine, has no stimulating effect on the proliferation of neural stem cells. We determined that second messenger cAMP was involved in the pathway as results showed that 100 µM guanosine stimulated cAMP accumulation. Using western blot analysis, we found that 100 µM guanosine can activate the phosphorylation of CREB without changing the total amount of CREB. In conclusion, guanosine can stimulate neural stem cell proliferation, and the cAMP-CREB pathway is involved in this biological effect.

Extracellular guanosine (GUO), the natural purine nucleoside, has been shown to have a plethora of trophic and neuroprotective effects both *in vivo* and *in vitro* (1, 2). In previous studies, we have discovered that systemic administration of GUO promotes functional improvement and remyelination of the injured spinal cord by triggering the proliferation of adult oligodendroglial progenitor cells and their maturation into myelin-forming cells (3). Furthermore, using a proteasome inhibitor–induced Parkinson's disease model, we determined that GUO protects cells from apoptosis and stimulates the proliferation of the intrinsic adult progenitor/stem cells in the subventricular zone (SVZ) which may contribute to the regeneration of dopaminergic neurons in the *substantia nigra* of rats (4). These findings prompted us to investigate the mechanism through which GUO exerts its effect on cell proliferation in an *in vitro* stem cell model. In the current study, using neural stem cells isolated from the SVZ of one-day old rats, we tested the effect of GUO on cell proliferation.

Key words: Guanosine, guanine, cAMP, CREB, neural stem cells, cell proliferation

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

Studies have shown that GUO administered intraperitoneally can cross the blood brain barrier, and its concentration in the central nervous system (CNS) peaks at about 30 min after administration. Systemically administered GUO is actively metabolized, and after 30 min, its chief metabolic product in the CNS was guanine (GUA) (5). It is not known whether extracellular GUO acts directly on stem cells and/or whether GUO metabolizes extracellularly to a metabolic product that acts on cells. We do know, however, that the extracellular purine nucleoside phosphorylase (PNP) is the key enzyme in the conversion of GUO into GUA (6). To test the effect of GUO on cell proliferation, we questioned whether it was GUO itself or the metabolic product that acts directly on cells in the CNS. We reasoned that if guanosine can fulfill its proliferation stimulation function via PNP metabolism, then extracellular GUA should have a similar biological function. Thus, we also tested the effect of extracellular GUA on cell proliferation in the present study.

Evidence has shown that the cAMP/CREB (cAMP response element-binding protein) signaling pathway has been strongly involved in the regulation of a wide range of biological functions including cell proliferation and survival (7). We previously reported that neurite outgrowth in PC12 cells is enhanced by GUO predominantly through cAMP-dependent mechanisms (8). In the present study, we investigated further to determine whether the cAMP/CREB pathway is involved in the biological effect of GUO on neural stem cell proliferation.

MATERIALS AND METHODS

Neural stem cell isolation

Adult male and female Wistar rats (270-300 g, body weight, Charles River Laboratories, Inc.) were maintained in a temperature-controlled vivarium on a 12:12 hour light/dark cycle with food and tap water freely available. All experiments were performed in compliance with the requirements of the Animals for Research Act of Ontario and the Guidelines of the Canadian Council on Animal Care and had been approved by the Animal Research Ethics Board of McMaster University. Neural stem cell isolation was performed following the protocols described previously (9-12). Briefly, one-day old pups were killed by decapitation and brains were removed and placed into tissue dissection solution and transferred to the cell culture room. Brains were dissected along the sagittal plane to separate the hemispheres and permit isolation of the thin layer of tissue outside the ventricles which contains the SVZ (12).

The isolated tissues were minced up and placed into 15 ml tubes, and 2 ml of enzyme mix (see below) was added to each tube. The isolated tissue was dissociated with the mixture of enzymes for 20 min in a water bath set to 37°C. The sample was triturated with a Pasteur pipette thirty times every 5-6 min. The tissue was subsequently placed in 5.0 ml of serum free media (SFM) containing 250 µl of trypsin inhibitor (Roche Applied Science, Cat No: 10109878001, 1 mg/ml) to stop the digestion. The cells were centrifuged at 2000 rpm for 4 min, re-suspended in fresh SFM and centrifuged again. After that, all cells were placed into SFM media for further study.

Neural stem cell proliferation test

Immediately after isolation, cells were seeded in a 96 opaque well plate (Corning Incorporated, Costar 3610), bathed in SFM and maintained in a humidified 5% CO_2/95% air incubator at 37°C for 1 h before treatment. A dose ranging from 10-300 µM guanosine (GUO) or vehicle (VEH) was administered, with cell conditions as follows: 100 cells/µl, 100 µl/well. After treatment, cells were maintained in an incubator for three days. The CellTiter-Glo® Luminescent Cell Viability Assay, a high-throughput cell proliferation assay was then performed according to product instructions (Promega G7572) (13-14). Basically, the plate was brought to room temperature for 30 min, then 100 µl of lysis reagent was loaded to each well and mixed for 2 min to induce cell lysis. The plate was allowed to incubate at room temperature for 10 min to stabilize the luminescent signal. The luminescence was recorded using a microplate luminometer (Applied Biosystems, TR717®).

Effect of GUA on stem cell proliferation

Isolated neural stem cells were treated with GUA (25 µM, 50 µM or 75 µM) or corresponding VEH and, 72 h later, cell proliferation was detected by the presence of ATP with a Promega CellTiter-Glo luminescent cell viability assay kit as described above. The concentrations of GUA we chose are the most likely ones to be biologically active. Caciagli and his group found that GUA had an effect on primary cells including astrocytes and neurons at 25, 50, 75 µM (reaching a peak at 75 µM), but not at 100, 200 and 300 µM (unpublished data).

Second Messenger cAMP accumulation assay

Isolated neural stem cells were stabilized in an incubator for 1 h and then seeded at 500 cells/µl, 160 µl/well into 96 well microplates. Twenty µl of forskolin

(Sigma F6886), GUO or VEH were added to each well at final concentrations of 5 μM for forskolin, and 100 μM for both GUO and VEH. The treatment lasted for 10 min. Then 20 μl of lysis reagent was added to each well, and total cellular cAMP was measured using Amersham cAMP Biotrak enzyme immunoassay kit (GE Healthcare, RPN2251) following the non-acetylation EIA procedure provided by Amersham. Optical density was recorded at 450 nm using a Tecan Safire Fluorescence and Absorbance plate reader (Tecan Group Ltd.). GraphPad was used for data analysis.

Effect of GUO on CREB phosphorylation as analyzed by western blot

Isolated neural stem cells were incubated for 3 days before either 100 μM GUO or 100 μM VEH was administered. Cells were collected 20 min later, and were washed once with cold phosphate buffered saline (PBS), with cell pellet lysed by M-PER® Mammalian Protein Extraction Reagent (Thermo, 78503) with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo 789440) added. Protein samples then were analyzed by western blot for pho-CREB (Millipore-Upstate, 06519, dilution 1:1000), total CREB (Chemicon, MAB5432, dilution 1:1000) and ß-actin (Abcam, ab6276, dilution 1:3000). The ß-actin was used as an internal standard for normalization. Proteins were detected with an ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA)), scanned and quantified using AlphaImager software.

Medium composition

Tissue dissection solution: artificial cerebrospinal fluid (aCSF) containing 0.012 M NaCl, 0.005 M KCl, 0.03 M MgCl2, 0.026 M NaHCO3, 0.01 M glucose and 0.097 mM CaCl2 in dH2O with 4% antibiotics (Penicillin-Streptomycin, Gibco: 15140-122). Enzyme mix: 1.0 ml of aCSF containing 0.13 mg kynurenic acid (4-hydroxyquinoline-2-carboxylic acid, Sigma, St. Louis, MO), 0.66 mg type 1-S hyaluronidase (Sigma, St. Louis, MO) and 1.3 mg trypsin (Sigma, St. Louis, MO). Serum free media (SFM): DMEM/F12 (Life Technologies, Burlington, ON) with 0.03 M glucose, 0.005 M hepes buffer (Sigma, St. Louis, MO), 20 nM progesterone (4-pregnene-3, 20-dione, Sigma, St. Louis, MO), 60 μM putrescine (1,4-diaminobutane, tetra-methylenediamine, Sigma, St. Louis, MO), 0.1 ml insulin- transferrinsodium selenite (Roche), 2.0 ml B27 Growth supplement (Life Technologies, Burlington, ON) and 7.32 μl heparin (Sigma, St. Louis, MO) in a total of 100 ml of SFM. GUO solution: In all experiments, 10 mM GUO was freshly made just before each experiment. GUO (28 mg, Sigma-Aldrich, G6752, Canada) was pre-dissolved in 100 μl sodium hydroxide (1 N NaOH) and diluted with PBS and ddH2O (1:1, V/V) with final volume of 10 ml. Just before the final volume was reached, pH was adjusted to 7.4-7.7 by adding 1N HCl. Ten mM guanosine was further diluted to 100 μM using culture medium. GUA solution: GUA stock solution (1 mM) was made just before the experiment; 7.6 mg GUA (Sigma, G6779) was pre-dissolved into 150 μl sodium hydroxide (1 N NaOH) and diluted with PBS to a final volume of 50 ml. Just before the final volume was reached, the pH was adjusted to 7.4-7.7 by adding 1 N HCl. One mM guanine was further diluted with culture medium to reach the final concentration as required.

Statistical analysis

All data are presented as mean±SEM. Data from only two groups were analyzed by Student's *t*-test. Data from more than two groups were analyzed with the ANOVA test to determine whether there were any significant differences. If there were, post-hoc comparisons were then made using the Mann-Whitney U test. Data were considered statistically significant at $P<0.05$. Analyses were performed with SPSS 17.0.

RESULTS

GUO stimulates neural stem cell proliferation in a dose-dependent manner

Using corresponding concentrations of VEH as a control, guanosine stimulated cell proliferation in a dose-dependent manner at concentrations ranging from 25 μM to 100 μM. Within that range, GUO increased cell proliferation by 5% as indicated by ATP analysis, with doses of 25 μM and 100 μM GUO showing significant differences ($P<0.05$, Fig. 1).

GUA does not stimulate neural stem cell proliferation

Among the concentrations of GUA tested (25-75 μM), none stimulated stem cell proliferation (Fig. 2).

GUO stimulates neural stem cell cAMP accumulation

Compared with the VEH group, 100 μM GUO treatment significantly increased neural stem cell cAMP accumulation ($P<0.05$, Fig. 3). Forskolin, an activator of adenylate cyclase (15), was used as a positive control in this study for cAMP accumulation.

GUO-induced cAMP accumulation is associated with phosphorylation of CREB

Western blot analysis indicated that GUO treatment stimulates neural stem cell CREB phosphorylation as shown by antibody for Pho-

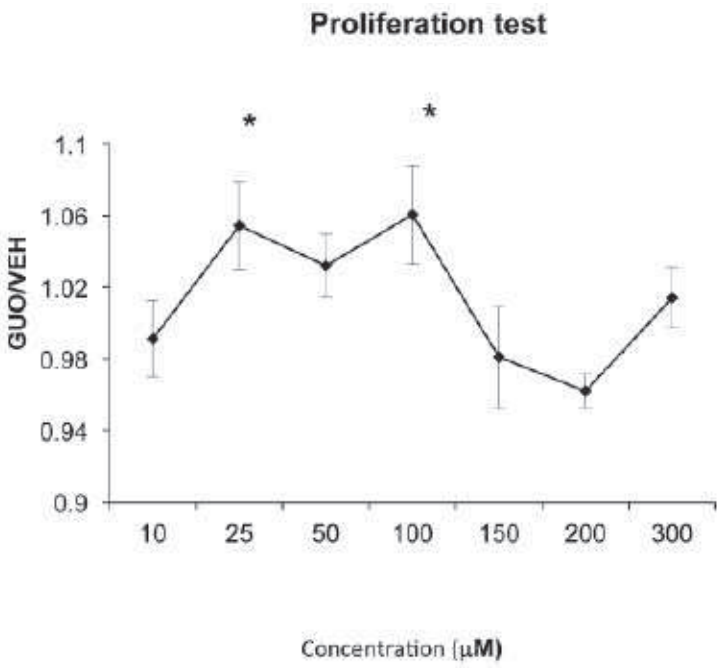

Fig. 1. *GUO stimulates neural stem cell proliferation. Neural stem cells were isolated from the SVZs of one-day old pups of rats and seeded into 96 well plates. Stem cells were immediately treated with either GUO or an equivalent amount of VEH. Seeding condition is 100 cells/μl, 100 μl/well. At least 12 wells were included in each treatment. Three days after treatment, cell proliferation was detected by presence of ATP using a PromegaCellTiter-Glo luminescent cell viability assay kit. Dosages of 25-100 μM GUO stimulated stem cell proliferation by 5% compared with vehicle control, with 25 and 100 μM doses showing significant differences (P<0.05). Data shown are means±SEM of at least 3 independent experiments.*

CREB (P<0.05); interestingly, total CREB was not significantly influenced by GUO under the test conditions (Fig. 4).

DISCUSSION

Evidence has shown that extracellular GUO exerts numerous neurotrophic effects and can cause the proliferation of a variety of cell types such as human brain capillary endothelial cells, fibroblasts, human astrocytoma and glial cells (16-19). We have reported that systemic administration of GUO to rats improves functional recovery following a chronic spinal cord injury that is associated with stimulating progenitor/stem cell proliferation (as measured by bromodeoxyuridine staining) (3). Furthermore, we have also discovered that in a Parkinson's disease model, GUO stimulated "intrinsic" adult progenitor/stem cells to become dopaminergic neurons in animal (4). Stimulating endogenous progenitor/stem cell proliferation might represent a new pharmacological strategy for CNS repair after injury. To elucidate the mechanisms and pathways involved in the effect of GUO on stem cell proliferation, we demonstrated that, in an *in vitro* rat neural stem cell model, GUO could stimulate cell proliferation at the concentrations of 25-100 μM.

Extracellular GUO and GUA are interconverted by PNP (1, 6). Studies have shown that GUA also has biological effects; for example, it can enhance memory by stimulating the generation of nitric oxide (NO) (2). We hypothesized that extracellular conversion of GUO to GUA through PNP metabolism may also contribute to the proliferative effect on cells. However, in this study, using a neural stem cell model, we did not observe such an effect of extracellular GUA. Therefore, we concluded that the mechanism by which GUO is metabolized to GUA is not involved in the proliferation of stem cells.

It has been hypothesized that the effects of GUO might be mediated through putative G-protein coupled receptors in the upstream pathways (20, 21).

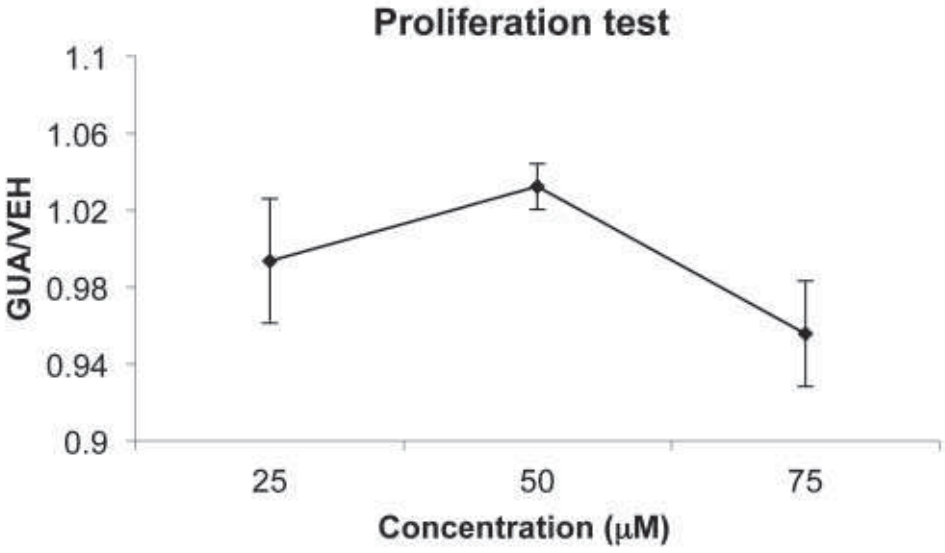

Fig. 2. *GUA shows no effect on neural stem cell proliferation. Neural stem cells were isolated from the SVZs of one-day old pups of rats and seeded into 96 well plates. Stem cells were immediately treated with different concentrations of GUA (25 μM, 50 μM, 75 μM) or an equivalent amount of VEH. Seeding condition was 100 cells/μl, 100 μl/well. At least 12 wells were included in each treatment. Three days after treatment, cell proliferation was detected by the presence of ATP using a PromegaCellTiter-Glo luminescent cell viability assay kit. GUA showed no effect on stem cell proliferation at any dose tested compared with vehicle control. Data shown are means±SEM of at least 3 independent experiments.*

Using a different model, it has been determined that multiple mechanisms are involved in the downstream pathways. For example, in both astrocytes and SH-SY5Y cells, the anti-apoptotic effect of GUO is mediated by activation of the PI3-Kinase/AKT/PKB pathway (22, 23). GUO treatment has also been shown to promote the accumulation of intracellular cAMP in both astrocyte (24) and PC12 cells (8). For PC12 cells, the effects of GUO are mediated through both cAMP-dependent and –independent mechanisms, with the former activating the MAPK cascade and potentially other protein kinases (8). In this study, we found that 100 μM GUO can activate second messenger cAMP accumulation in neural stem cells which indicates that the cAMP-dependent mechanism is involved in the cell signaling pathway.

Studies have demonstrated that the cAMP/CREB signaling pathway has been strongly implicated in the regulation of a wide range of biological functions, including growth factor-dependent cell proliferation and survival and synaptic plasticity (7, 25-27). While it is now known that CREB can be phosphorylated in response to various stimuli including Ca^{2+}/calmodulin-dependent kinase IV (28) and the mitogen/stress-activated kinase Msk1 (29), an overwhelming body of evidence has demonstrated that phosphorylation of CREB on Ser133 in kinase-inducible domain is crucial for activation of transcription in response to cAMP (7, 30). To determine whether CREB is the downstream effector of cAMP signaling, we examined total CREB and phosphorylated CREB after GUO administration. Results showed that, for neural stem cells treated with 100 μM GUO for 20 min, there is more phosphorylated CREB compared with control, but the total amount of CREB was not affected.

In conclusion, we determined that GUO stimulates neural stem cell proliferation and might also affect other downstream pathways. In an *in vitro* neural stem cell model, we established that the cAMP-CREB pathway is involved. However, GUA, previously reported to demonstrate a number of

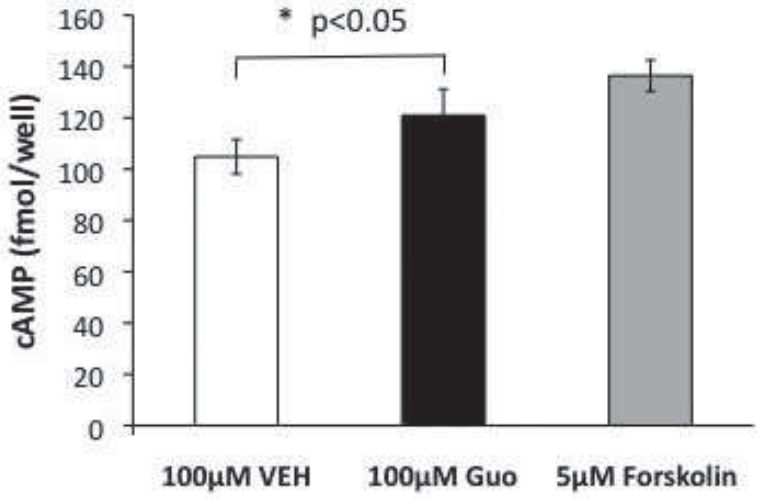

Fig. 3. *100 μM GUO stimulates neural stem cell cAMP accumulation. Neural stem cells were isolated from the SVZ of one-day old pups of rats and treated with either 100 μM GUO or VEH or 5 μM forskolin for 10 min. The amount of cAMP was determined using an Amersham cAMP Biotrak enzyme immunoassay kit. Compared with the control group, 100 μM GUO stimulated neural stem cell cAMP accumulation significantly (P<0.05). Data shown are means±SEM of at least 3 independent experiments.*

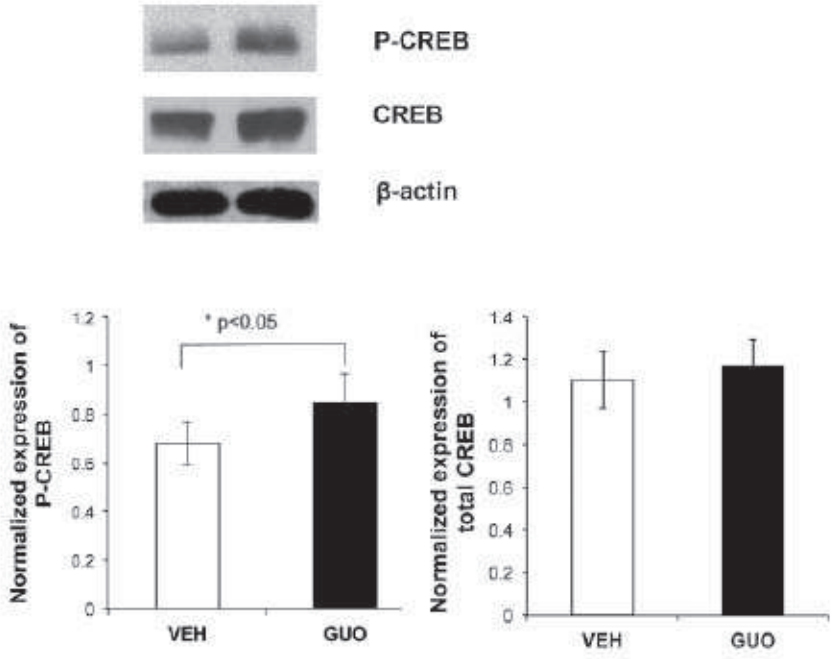

Fig. 4. *100 μM GUO stimulates neural stem cell CREB phosphorylation. Isolated neural stem cells were incubated for 3 days before treatment with either 100 μM GUO or an equivalent amount of VEH for 20 min. Proteins were extracted and analyzed by western blot for total CREB and phosphorylated CREB. Quantification shows that 100 μM GUO stimulated the phosphorylation of CREB (P<0.05), but did not affect the total amount of CREB, which indicates the activation of CREB by GUO. Data shown are means±SEM of at least 3 independent experiments.*

biological effects, did not appear to contribute to the effects of GUO on neural stem cell proliferation in this study, which indicated that the PNP mechanism was not involved in this activity.

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