Differential neurogenic effects of casein-derived opioid peptides on neuronal stem cells: implications for redox-based epigenetic changes

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Abstract

Food-derived peptides, such as \(\beta\)-casomorphin BCM7, have potential to cross the gastrointestinal tract and blood–brain barrier and are associated with neurological disorders and neurodevelopmental disorders. We previously established a novel mechanism through which BCM7 affects the antioxidant levels in neuronal cells leading to inflammatory consequences. In the current study, we elucidated the effects of casein-derived peptides on neuronal development by using the neurogenesis of neural stem cells (NSCs) as an experimental model. First, the transient changes in intracellular thiol metabolites during NSC differentiation (neurogenesis) were investigated. Next, the neurogenic effects of food-derived opioid peptides were measured, along with changes in intracellular thiol metabolites, redox status and global DNA methylation levels. We observed that the neurogenesis of NSCs was promoted by human BCM7 to a greater extent, followed by A2-derived BCM9 in contrast to bovine BCM7, which induced increased astrocyte formation. The effect was most apparent when human BCM7 was administered for 1 day starting on 3 days postplating, consistent with immunocytochemistry. Furthermore, neurogenic changes regulated by bovine BCM7 and morphine were associated with an increase in the glutathione/glutathione disulfide ratio and a decrease in the S-adenosylhomocysteine/S-adenosylhomocysteine ratio, indicative of changes in the redox and the methylation states. Finally, bovine BCM7 and morphine decreased DNA methylation in differentiating NSCs. In conclusion, these results suggest that food-derived opioid peptides and morphine regulated neurogenesis and differentiation of NSCs through changes in the redox status and epigenetic regulation.

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1. Introduction

Proteins present in food undergo proteolysis to yield short peptides of varying length, as well as amino acids. Some food-derived peptides are able to cross the gastrointestinal epithelium and enter the systemic circulation [1,2], and some may also cross the blood–brain barrier [3,4]. Furthermore, some reports have described associations between food-derived peptides and neurological disorders, like autism [4–7], schizophrenia [8–12] and delayed psychomotor development in infants [13].

Abbreviations: BCM, \(\beta\)-casomorphin; bBCM7, bovine form of BCM7; bBCM9, bovine form of BCM9; hBCM7, human form of BCM7; bFGF, basic fibroblast growth factor; BrdU, bromodeoxyuridine; CYS, cysteine; CYS2, cystine; dpp, days postplating; EGF, epidermal growth factor; GSH, glutathione; GSSG, glutathione disulfide; HCY, homocysteine; HCY2, homocystine; HPLC, high-performance liquid chromatography; MET, methionine; NSC, neural stem cell; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

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One food-derived peptide, \(\beta\)-casomorphin BCM7, has attracted particular interest in recent years owing to its potential neurological affects, as well as other immune- and inflammation-related properties [14–17]. BCM7 is mainly produced by the proteolysis of A1 (\(\beta\)-casein) [18], a major component of cows’ milk that displays opioid activity [19]. Although associations between BCM7 and neurological disorders, especially neurodevelopmental disorders, have been identified, the underlying mechanism has not yet been evaluated. Therefore, we sought to investigate the potential neuro- logical effects of BCM7 and other related peptides on neurological development, using the neurogenesis of neural stem cells (NSCs) as a research model.

We have previously reported that proline-rich opioid peptides derived from milk or wheat reduced cysteine uptake in cultured human neuronal and gastrointestinal epithelial cells by activating opioid receptors [20]. Decreases in cysteine uptake were associated with alterations in intracellular glutathione (GSH) and S-adenosylmethionine (SAM). Moreover, bovine and human casein-derived opioid peptides increased genome-wide DNA methylation in transcription start sites, which affected the expression of genes involved in redox and methylation homeostasis. These results suggest that milk- and wheat-derived peptides exert antioxidant and epigenetic changes that may be important during the postnatal transition from placental to...
gastrointestinal nutrition and might also be relevant to comparisons of breastfed and formula-fed infants.

The redox state and epigenetic modifications are known to influence neural development [21]. For example, it was reported that inhibition of the eicosanoid signaling pathway promoted pluripotency and maintained levels of unsaturated fatty acids, while downstream oxidized metabolites (e.g., neuroprotectin D1) and substrates of prooxidative reactions (e.g., acyl-carnitines) promoted neuronal and cardiac differentiation in embryonic stem cells [22].

Regarding neural development, it has been reported that hypoxia promotes neurogenesis in embryos, newborns and adults in vivo and that oxygen and reactive oxygen species also play a role in neuronal differentiation in vitro [23]. Meanwhile, exposure to morphine inhibited the proliferation of neural progenitor cells and increased active caspase-3 expression in a dose-dependent manner in vitro [24]. Morphine also enhanced neuronal and glial differentiation. These effects were reversed by the opioid antagonist naloxone. These results highlight important roles of opioid receptors in the regulation of neural progenitor cell survival. In relation to these findings, it has been reported that maternal inflammation is associated with brain overgrowth and autism-associated behaviors, as a consequence of altered redox signaling in neural stem and progenitor cells [25].

Considering this background, and to elucidate the effects of food-derived peptides on neuronal development, we performed three experiments with the following objectives: Experiment 1 to investigate the transient changes in intracellular thiol metabolites during NSC differentiation; Experiment 2 to investigate the neurogenic effects of opioid peptides in differentiating NSCs; and Experiment 3 to measure the changes in intracellular thiol levels in differentiated NSCs exposed to opioid peptides at 3 days postplating (dpp).

2. Methods

2.1. NSC cultures

Previously isolated and frozen neuronal stem cell cultures were properly thawed, maintained and cultured for the current experiments as previously described [26]. Cell suspensions were grown in a defined medium (DF12) composed of DMEM/F12 (1:1), 2 mM l-glutamine, 1 mM sodium pyruvate, antibiotics/antimycotics (Invitrogen, Grand Island, NY), 0.6% glucose, 25 μg/ml insulin, 20 mM progesterone, 60 μM putrescine, 30 mM sodium selenite (all from Sigma, St. Louis, MO), 100 μg/ml human transferrin (Roche, Indianapolis, IN), 20 ng/ml human recombinant endothelial growth factor [epidermal growth factor (EGF); Roche or Invitrogen, Chicago, IL] and basic fibroblast growth factor (bFGF; Upstate Biotechnology, Lake Placid, NY). The cells grew as free-floating aggregates (neurospheres) and were passaged by mechanical dissociation every 3–4 days. After a minimum of four passages, the cells were plated at a density of 18,000 cells/cm² on eight-well glass slide chambers (Nalge Nunc International, Naperville, IL) coated with 15 μg/ml poly-l-lysine (Sigma). Cultures were maintained in DF12 and EGF or EGF plus bFGF for 3 days and then switched to DF12 without growth factors for longer culture periods. Immunocytochemical studies were performed at different time points between 3 and 10 dpp. To analyze the effects of opioid peptides, the cells were treated with 10 μM concentrations of morphine, human hBCM7, bovine hBCM7 and hBCM9 (American Peptide, Sunnyvale, CA). Peptides were reconstituted in sterile water and incubated at 37°C for 1 day or 3 days. Parallel wells were maintained in DF12 without the test peptides (untreated group). Immunocytochemical analyses were performed at 1, 3 or 10 days after treatment.

2.2. Isolation of intracellular thiol metabolites

Intracellular thiol metabolites were measured as previously described [27,28]. Briefly, neuronal stem cell cultures were grown to confluence in stem-cell–specific growth media as described above and were then incubated with the indicated drugs for specific times. The medium was aspirated and the cells were washed twice with 1 ml of ice-cold HBSS. Then, the HBSS was aspirated and 0.6 ml of ice-cold dH₂O was added to the cells and the cells were scraped from the flask/dish. The cell suspension was sonicated for 15 s on ice and 100 μl of the sonicate was used to determine protein content. The remaining lysate was added to a microcentrifuge tube with an equal volume of 0.4 N perchloric acid and incubated on ice for 5 min. Samples were centrifuged at 10,000g and the supernatant was transferred to new microcentrifuge tubes. Then, 100 μl of the sample was added to a conical microautosampler vial and kept at 4°C in the autosampler cooling tray. Finally, 10 μl of this sample was injected into a high-performance liquid chromatography (HPLC) system.

2.3. HPLC measurement of intracellular thiols

Concentrations of the following metabolites were measured: cysteine (CYS), cystine (CYS2), glutathione (GSH), glutathione disulfide (GSSG), homocysteine (Hcy), homocystine (Hcy2), methionine (MET), S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM). As previously described [25,26], the redox and methylation pathway metabolites were separated using an Agilent Eclipse XDB-C8 analytical column (3×150 mm; 3.5 μm) and an Agilent Eclipse XDB-C8 (4.6×12.5 mm; 5 μm) guard column. Two mobile phases were used. Mobile phase A comprised 0% acetonitrile, 25 mM sodium phosphate and 1.4 mM 1-octanesulfonic acid, adjusted to pH 2.65 with phosphoric acid. Mobile phase B was 50% acetonitrile. The flow rate was initially set at 0.6 ml/min and a step gradient was used, as follows: 0–9 min 0% B, 9–19 min 50% B and 19–30 min 50% B. The column was then equilibrated with 5% B for 12 min before the next run. The column temperature was maintained at 27°C. The electrochemical detector was an ESA CoulArray with BDD Analytical Cell Model 5040 and the operating potential was set at 1500 mV. Sample concentrations were determined from the peak area for each metabolite using standard operating potential was set at 1500 mV. Sample concentrations were determined from the peak area for each metabolite using standard calibration curves and ESA software and then normalized for protein concentration. Some samples were diluted in the mobile phase, as needed, or up to 50 μl of sample was injected to ensure the thiol concentration was within the range of the standard curve.

2.4. Assessment of cell proliferation and apoptosis

To identify proliferating cells, 100 μM bromodeoxyuridine (BrdU), an analog of thymidine, was added 24 h before cell fixation. After permeabilization with ethanol-acetic solution (19:1), cells were treated with 2 N HCl for 30 min at 4°C to denature DNA. A primary monoclonal antibody against BrdU (1:20; Dakopatts) was added for 1 h at room temperature and detected using AlexaFluor 488-labeled goat antimouse IgG (H+L). This method allowed us to identify cells that had duplicated their DNA in the last 24 h. Apoptotic cells were visualized with Hoechst 33342 (Life Technologies, MD) as fragmented pycnotic blue-stained nuclei and counted under the fluorescence microscope as previously described [26].

2.5. Indirect immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with an ethanol–acetic acid solution (19:1) at 20°C for 20 min, blocked with 10% fetal bovine serum and incubated with primary antibodies overnight at 4°C. Sister cultures served as negative controls and were similarly processed, except for incubation without the primary antibody in each case. Immunofluorescence was used for detection of all antigens. Monoclonal antinestin (clone Rat 401; 1:200) was obtained from DakoPatts (Glostrup, Denmark).
Monoclonal anti-β tubulin isotype III (1:2000) and polyclonal anti-β tubulin isotype III (1:2000) were purchased from Covance (Richmond, CA). Polyclonal anti-O1 (1:5) was obtained from a hybridoma purchased from American Type Culture Collection (Manassas, VA). Monoclonal anti-BrdU (1:50) was obtained from Dako (High Wycombe, UK), and monoclonal antineuronal nuclei (NeuN) was obtained from Chemicon (Temecula, CA). For single labeling of neural antigens, goat antimouse IgG (H+L) or goat antirabbit IgG (H+L) labeled with AlexaFluor 568 or AlexaFluor 488 was purchased from Molecular Probes (Eugene, OR).

2.6. Isolation of genomic DNA

Genomic DNA was isolated from cultured cells to measure global DNA methylation as previously described [27,28]. DNA was isolated from harvested cells using FitAmp Blood and Cultured Cell DNA Extraction Kits (Epigentek, Farmingdale, NY). The isolated DNA was cleaned for any contaminating RNA by treatment with RNase enzyme and quantified using ND-1000 NanoDrop spectrophotometer (Thermo Scientific).

2.7. Measurement of global DNA methylation

Global DNA methylation analysis was performed using MethylFlash Methylated DNA Quantification Kits according to the manufacturer’s instructions (Epigentek). Briefly, 100 ng of clean genomic DNA was used and DNA methylation was quantified using 5-methylcytosine monoclonal antibodies in an enzyme-linked immunosorbent assay–like reaction, as previously described [27,28]. The levels of methylated DNA were calculated based on the optical density of each well on a microplate reader at 450 nm. Results were normalized against a standard curve prepared using the kit’s methylated standards ranging from 0% to 100%.

2.8. Data analysis

Results are expressed as the mean±standard error of the mean (SEM) of direct counts of positive cells for each antibody from independent experiments done in triplicate or quadruplicate. Where indicated, the data were normalized relative to the relevant control group. In each culture, 25 predetermined visual fields were counted.
under a confocal microscope. The number of positive cells was corrected for the total number of cells in the same area, with Hoechst nuclear staining. Statistical analyses were performed using analysis of variance with the Bonferroni post hoc test or Student’s t test as appropriate. Differences were considered significant at $P < 0.05$. All statistical analyses were conducted using Prism 6.0 software (GraphPad Software, San Diego, CA).

3. Results

3.1. Transient changes in intracellular thiol metabolites during NSC differentiation

Previous reports have indicated changes in the intracellular concentrations of thiol metabolites during the differentiation of embryonic stem cells [20]; however, little is known about the changes in thiols during the differentiation of NSCs. Therefore, we first investigated the changes in thiol metabolites during the differentiation of NSCs. Fig. 1 shows the concentrations of 10 thiols measured at 0 (floating NSCs), 1, 3, 7 and 10 dpp. Of the 10 thiols measured, the concentrations of CYS and GSH were markedly greater than those of the other thiols in floating NSCs. The concentrations of both thiols decreased significantly with ongoing differentiation ($P < 0.05$ for both thiols at 1, 3, 7 and 10 dpp vs. 0 dpp). Significant reductions in the GSH/GSSG ratio (Fig. 1B) and SAM/SAH ratio (Fig. 1C) were also observed in differentiating NSCs. The GSH/GSSG ratio tended to show progressive reductions with time since plating. By contrast, after the initial reduction in the SAM/SAH ratio at day 1, it remained relatively constant thereafter.

3.2. Epigenetic changes during NSC differentiation

To examine whether the changes in thiol metabolites were associated with altered DNA methylation, we assessed the methylation state of CpG sites. In this experiment, we found that global DNA methylation levels increased slightly at 1 and 3 dpp compared with 0 dpp (Fig. 1D), but then it decreased at 7 and 10 dpp to values below the value at 0 dpp. It is noteworthy to mention that the assay employed in this study did not differentiate between the 5-methylcytosine and 5-hydroxymethylcytosine marks; however, we did see changes in the global DNA methylation levels.

3.3. Effects of opioid peptides on NSC differentiation

We next examined the effects of opioid peptides on NSC differentiation. Fig. 2A shows that administration of opioid peptides for 1 or 3 days starting on 3 dpp had a neurogenic effect (i.e., enhanced proliferation) compared with control cells. This effect was most apparent in cells treated with hBCM7 for 1 day starting on 3 dpp. Although extending administration to 3 days still had a neurogenic effect, this effect was muted compared with administration for 1 day. Starting administration of the peptides on 0 or 7 dpp did not appear to
have a neurogenic effect relative to the control conditions, irrespective of the duration of administration. The effects of these peptides on apoptosis (Fig. 2B) and the total number of cells (Fig. 2C) were highly variable with no consistent pattern, except for increases in apoptosis and decreases in the total cell counts during the later stages of differentiation; these effects were particularly evident in NSCs treated with morphine. These neurogenic effects of the opioid peptides were confirmed by immunocytochemistry of NSCs treated with the four peptides for 1 day after 3 dpp (Fig. 3).

3.4. Effects of opioid peptides on intracellular thiol levels of differentiating NSCs at 3 dpp

In our prior report, we showed that exposure to morphine, bBCM7 and hBCM7 for 4 and 24 h affected thiol metabolites including the GSH/GSSG and SAM/SAH ratios in SH-SY5Y neuroblastoma cells [20]. In the present study, we observed marked changes in thiol metabolite concentrations during differentiation (Fig. 1). Therefore, we investigated the combined effects of food-derived opioid peptides on thiol concentrations during differentiation. We treated the NSCs as described above and measured the thiol metabolites using HPLC coupled to an electrochemical gradient detector. The effects of opioid peptides (morphine, bBCM7, hBCM7 and bBCM9) on intracellular thiol levels in differentiating NSCs are presented in Fig. 4. The four peptides had varying effects on the thiol measured in this study. In particular, morphine, bBCM7 and bBCM9 significantly reduced CYS concentrations, while hBCM7 elicited a small, non-significant decrease in CYS concentrations, compared with the control conditions. hBCM7 and bBCM9 significantly reduced the GSH concentration, whereas morphine and bBCM7 significantly increased the GSH concentration (Fig. 4A). Administration of bBCM7 or morphine significantly increased the GSH/GSSG ratio (Fig. 4B) and significantly decreased the SAM/SAH ratio (Fig. 4C). Neither of these ratios was affected by hBCM7 or bBCM9.

3.5. Effects of opioid peptides on CpG methylation

In our prior reports, we found that exposure to morphine and food-derived opioid peptides influenced DNA methylation in SH-SY5Y neuroblastoma cells [20,28]. Here, we observed that the four opioid peptides under investigation tended to decrease global 5-methylcytosine levels as compared to control cells during differentiation phenomenon, although the changes were not statistically significant (Fig. 4D). It is intriguing to note that hBCM7 had a greater effect on CpG methylation at
3 dpp compared with either bBCM7 and morphine, which may be related to the diverging effects of these opioids on the redox state, especially the GSH/GSSG and SAM/SAH ratios as discussed above in this study and in our prior study [20].

4. Discussion

Plasma and brain GSH levels are decreased in a variety of neurodevelopmental, neurological/neurodegenerative and psychological disorders [29,30], indicative of a pivotal role of oxidative stress in these disorders [31]. Concurrent decreases in cerebrospinal fluid SAM concentrations and the SAM/SAH ratio have been reported in patients with Alzheimer's disease [32], and the cognitive function of patients with Parkinson's disease was positively associated with the SAM/SAH ratio [33]. In a postmortem brain study [34], we demonstrated a decrease in methionine synthase expression across the lifespan and proposed that progressive changes in redox state translate into epigenetic and metabolic adaptations.

There were three key findings of this study. First, the levels of cysteine and GSH as well as the GSH/GSSG ratio in NSCs progressively decreased in association with neuronal differentiation. Second, administration of hBCM7 promoted NSC neurogenesis to a greater extent than did administration of the other opioid peptides tested, including bBCM7, and this effect was most apparent when hBCM7 was administered for 1 day starting on 3 dpp. Third, administration of the opioid peptide bBCM7 and morphine significantly increased the GSH/GSSG ratio and decreased the SAM/SAH ratio compared with the ratios in control cells. Redox regulation as well as the intracellular levels of antioxidants such as GSH and the methyl donor SAM are important regulatory factors in NSC differentiation. Essentially, the NSC differentiation can also be viewed as an adaptive response to oxidative stress, under the influence of permissive epigenetic changes. SAM is the main methyl donor in transmethylation reactions, including DNA methylation and contributes to epigenetic regulation of gene transcription. The folate and vitamin B12-dependent enzyme methionine synthase controls SAM levels and exerts global control over the methylation state, including DNA methylation. Interestingly, methionine synthase is highly sensitive to the redox state and provides a mechanism by which a decrease in the GSH/GSSG ratio can promote epigenetic changes, which could contribute to the differentiation process. Similar to our previous observations, in the present study, we observed marked changes not only in the GSH/GSSG ratio but also in the SAM/SAH ratio in NSCs.

It can also be inferred from the current results that exposure to opioid peptides can alter neurogenesis, as well as GSH/GSSG and
underlying the differential effects of these two peptides are unclear, it is possible that bBCM7 has a stronger agonistic activity toward μ-opioid receptors expressed on NSCs, causing greater changes in the redox and methylation states (Fig. 5). Similar distinctive effects of hBCM7 and bBCM7 were observed in our prior study in SH-SY5Y neuroblastoma cells [20]. These differential effects of hBCM7 and bBCM7 might also contribute to the health benefits of breastfeeding relative to formula feeding in early infancy; however, we did not conduct any direct investigation for the same and it is an extrapolation. However, we have previously reported an association between the length of breastfeeding and incidence of autism in kids in Oman [35]. It is important to consider that BCM7 is primarily derived from the proteolysis of A1 β-casein, which is the predominant β-casein type in cows’ milk [18]. These study outcomes warrant further investigation; in particular, it will be necessary to examine the downstream cellular mechanisms involved in the differential effects of hBCM7 and bBCM7. Additionally, because the results obtained here were derived from NSCs in vitro, it will be necessary to verify these findings in vivo. Finally, considering the impact on methylation status, it would be informative to determine whether the methylation state of NSCs has global epigenetic effects or whether certain groups of genes (e.g., those involved in differentiation or redox reactions) are targeted in this process at their promoter region.

5. Conclusions

In conclusion, the results of this study reveal that the neurogenesis of NSCs is promoted by hBCM7 to a greater extent than by other opioid peptides. In fact, bBCM7 promotes higher astrocyte differentiation. The effect of hBCM7 was prominent when administered for 1 day starting on 3 dpp. The neurogenic changes regulated by bBCM7 and morphine were associated with an increase in the GSH/GSSG ratio, a decrease in the SAM/SAH ratio, and a decrease in CpG methylation. These results suggest that opioid peptides derived from β-casein can influence neurogenesis via redox-based epigenetic effects. Especially peptides like bBCM7 can skew the neurogenic potential of NSC.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2015.10.012.

References


