Maternal treatment of cyclic glycine-proline improves memory, astrocyte plasticity, vascularization and GluR-1 expression of adult offspring in rats

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Keywords

Cyclic glycine-proline; Insulin-like growth factor-1; Morris water maze; Spatial memory; Astrocytic branching, Glutamate receptor-1, Cerebral vascular densities

Running title

cGP improves astrocytes-associated vascularization and GluR-1 expression

Data availability statements

The data that support the findings of this study are openly available in:

Fan D, Ann Clin Transl Neurol 6**:** 669-677. doi: 10.1002/acn3.743;

Fan D, Alzheimer's & dementia (Amsterdam, Netherlands) 12**:** e12025. doi: 10.1002/dad2.12025;

Guan J, Sci Rep 4**:** 4388. doi: 10.1038/srep04388; Guan J, J Biol Regul Homeost Agents 32**:** 465-478;

Li F, Nutr Metab Cardiovasc Dis. doi: 10.1016/j.numecd.2019.09.016;

Li F, Neuropeptides 76**:** 101935. doi: 10.1016/j.npep.2019.05.006;

Krishnamurthi R, British journal of pharmacology 156**:** 662-672;

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Abstract

Background and Purpose: Cyclic glycine-proline (cGP) regulates the function of insulin-like growth factor-1 (IGF-1), which is essential for post-natal brain development and adult cognitive function. We evaluated the efficacy of maternally administered cGP on spatial memory and the association with astrocytic plasticity, vascularization and synaptic expressions in the hippocampus of their adult offspring.

Experimental Approach: Either cGP or saline was orally administered to Sprague Dawley dams from post-natal days 8-22. Spatial memory was evaluated using Morris Water Maze tests between post-natal days 70-75. Using immunohistochemistry and stereological analysis, we evaluated capillary density, astrocytic processes and expression of synaptophysin and glutamate receptor-1 (GluR-1) in the CA1 stratum-radiatum of the hippocampus.

Key Results: Compared to the saline-treated offspring, cGP-treated offspring showed higher path efficiency of entry to the platform zone (p=0.03) and lower average heading errors to the platform zone (p=0.02). Astrocyte processes of cGP-treated offspring were longer and larger with more branches (p=0.03-0.0001) than saline-treated offspring. The density of capillaries (p=0.007) and GluR-1 (p=0.02) were also higher in cGP-treated offspring. The average heading error was negatively correlated with the length (r=-0.69), volume (r=-0.72) and number of astrocytic branches (r=-0.65). Independent of treatment, the changes of astrocyte processes were positively correlated with the density of capillaries (r=0.73-0.78) and expression of GluR-1 (r=0.66-0.68).

Conclusion and Implications: The improved spatial memory of cGP-treated offspring after post-natal maternal administration may be mediated via promoting astrocytic plasticity, vascularization and glutamate trafficking. Therefore, cGP may have a role in regulating IGF-1 function during brain development.

Authors contributions

G. S. M. conducted and analyzed the animal experiments, performed the immunohistochemical staining and wrote the manuscript. M. A. did morphological quantification and statistical analysis and manuscript preparation. K. S. and C.D.M. provided funding for experimental tests and helped in sample collection and manuscript revisions. C. M. provided fund to histological analysis and revised the manuscript. J. G. designed the experiment, provided funding for research and helped in sample collection and co-wrote and revised the manuscript.

Competing interests' statement

Dr Gagandeep Singh-Mallah is currently an employee of the AstraZeneca.

Introduction

Insulin-like growth factor-1 (IGF-1) plays a critical role in regulating post-natal brain development (Torres-Aleman, 2010), which influences brain function in adulthood. Sub-optimal function of IGF-1 during post-natal development, for example infants born prematurely (Hellström, Ley et al., 2016) can negatively impact the ability of learning and memory in their adult life (Synnes & Hicks, 2018). In addition to well-documented function of IGF-1 in brain tissues (Torres-Aleman, 2010), the function of IGF-1 in circulation is essential for cerebral vascular remodeling that also influences brain function (Lopez-Lopez, LeRoith et al., 2004).

The function of circulating IGF-1 is tightly regulated. Growth hormone (GH)-IGF-1 axis regulates hepatic production of IGF-1, which determines IGF-1 concentration in circulation (Bianchi, Locatelli et al., 2017). The majority of circulating IGF-1 is not bioavailable due to being bound to, mainly IGFBP-3 (Bianchi, Locatelli et al., 2017; Ranke, 2015). The binding of IGF-1 to IGFBP-3 is reversible, which regulates the amount of bioavailable IGF-1. The N-terminal tripeptide sequence of IGF-1 (Gly-Pro-Glu) is a major binding site for IGFBP-3. Following enzymatic cleavage the N-terminus of IGF-1 forms cyclic-glycine-proline (cGP) (Baker, Batchelor et al., 2005; Sara, Carlsson-Skwirut et al., 1993; Yamamoto & Murphy, 1994). The cGP retains the binding affinity and competes with IGF-1 to bind IGFBP-3 (Guan et al., 2014). An increase in cGP and/or a decrease in IGFBP-3 would increase the amount of bioavailable IGF-1 in circulation, thus the function of IGF-1 (Fan, Krishnamurthi et al., 2019; Fan, Pitcher et al., 2020; Guan, Singh-Mallah et al., 2018; Singh-Mallah, Singh et al., 2016).

The concentration of circulating IGF-1 is low in infant rats (Singh-Mallah, Singh et al., 2016). As an autocrine response to optimize IGF-1 function, the endogenous cGP increases

whereas IGFBP-3 decreases (Singh-Mallah, Singh et al., 2016). Administration of cGP to lactating dams increased concentrations of cGP in breast milk, and further elevated circulating cGP in suckling pups (Singh-Mallah, Singh et al., 2016). Such treatment improves the working memory of juvenile offspring (Singh-Mallah, Singh et al., 2016). The long-lasting effects of an cGP analogue on improving motor function has been reported in rats (Krishnamurthi, Mathai et al., 2008). Current study evaluated the long-term effects of cGP in memory of their adult offspring following maternal administration.

Astrocytes plays a critical role in vascular remodeling (Huang, Nakamura et al., 2019) and dynamics of synaptic neurotransmission (Nuriya & Hirase, 2016). For example, astrocytes regulate the homeostasis of synaptic trafficking of glutamate through glutamate-glutamine cycle in the hippocampus (Rouach, Koulakoff et al., 2008), which is critical for cognitive function (Guan, MacGibbon et al., 2015; Hatip-Al-Khatib, Iwasaki et al., 2007). The biological effects of cGP in promoting vascular remodeling (Guan et al., 2014) and synaptic expressions (Li, Liu et al., 2019b) have only been reported in separate studies. Other independent studies also show that higher expression of glutamate receptor-1 (GluR-1) and synaptophysin in the hippocampus are associated with improved learning and memory in rats (Guan, MacGibbon et al., 2015; Guillermo, Yang et al., 2015). To explore the biological changes underlying brain development we also examined the effects of cGP treatment in astrocyte plasticity, vascularization and synaptic expressions in CA1 of the hippocampus.

Methods

Animal experimental procedures

All animal experiments were approved by the Ruakura Animal Ethics Committee (Approval 12906, Hamilton, New Zealand). Aiming to evaluate the role for cGP in regulating developmental IGF-1 function, current study was the part of a longitudinal project that

131 examined the pharmacokinetics and dynamics of cGP in lactation/involution of mammary
132 glands and in the function of developing brains following maternal administration (Singh-
133 Mallah, McMahon, Guan, & Singh, 2017; Singh-Mallah et al., 2016). The part of
134 experimental procedures and maternal administration has been previous reported (Singh-
135 Mallah, Singh et al., 2016). Briefly, 32 Sprague-Dawley dams were randomly allocated to
136 two treatment groups (n = 16 dams per group). To reduce the experimental stress, the dams
137 were habituated to the experimenters with daily handling, including being held in an upright
138 position for gavage. The volume administered for gavage was less than 0.5ml and started 8
139 days after the dams gave birth. Either cGP dissolved in saline (3 mg/1.5 ml/kg body weight,
140 Bachem, Bubendorf, Switzerland; Cat# G-1720) or saline alone were administered once per
141 day to lactating dams using a gavage needle from post-natal (PN) day 8 to 22. Gavage was
142 conducted between 9 to 10 am and the dose was calculated daily based on the body weight of
143 the dams. The pups were then weaned at PN d22 and fed a standard laboratory chow (Diet
144 86; Sharps Grain and Seed, Carterton, New Zealand) with tap water being available *ad*
145 *libitum* throughout the trial. The offspring were housed in rooms maintained at 22 ^{o}C with a
146 12:12 (light:dark) photoperiod. The male offspring were randomly selected from 8 litters in
147 which the dams were treated with saline (saline-treated offspring, n = 21) and 10 litters in
148 which the dams were treated with cGP (cGP-treated offspring, n = 23). The litter effect on
149 brain function of the adult offspring has previously been shown to be minimal (Guillermo,
150 Yang et al., 2015; Li, Liu et al., 2019b). Twelve of the offspring in each group had been
151 previously used in novel object recognition tests from PN days 35 to 39, where it was found
152 that cGP-treated offspring had improved cognitive function (Singh-Mallah et al., 2016). To
153 evaluate whether this improved cognitive function would persist into adulthood, Morris water
154 maze (MWM) tests were carried out between PN days 70 to 75. Brain tissues were then
155 collected for histology analysis.

Morris water maze (MWM)

The apparatus and the procedure have been described previously (Guan et al., 2015a). Briefly the tests were conducted in a quiet room with lights dimmed to about 20 lux. The apparatus consisted of a black circular pool (0.6 meter deep × 2.2 meters in diameter) filled with water at a temperature maintained between 20 to 22 ºC. Multiple distal cues were mounted on the walls around the pool (e.g. a black colored triangle and a cross, a green colored shopping bag, a bright yellow box).

Acquisition trials: The daily procedures were carried out in the morning between 9 to 11am by an investigator who was blinded to the treatment groups. Rats underwent 4 trials on each day of the 4-day acquisition period, with an inter-trial interval of 6 minutes. The pool was virtually divided into four quadrants using the ANY-Maze software: north-east, north-west, south-east and south-west quadrants. A submerged transparent plastic platform (10 cm in diameter and 2 cm below the surface of water) was located in the north-east quadrant of the pool (20 cm from the side wall) in all the trials. Rats were released into the pool tail-first and facing the wall of a quadrant other than the north-east quadrant. The starting position for the task was changed for each trial and for each testing day, but remained the same for all rats in a particular trial. During each trial, rats were allowed to swim, locate and mount the platform for 120 seconds. If a rat was able to locate and mount the platform within 120 seconds, it was allowed to stay on the platform for an additional 15 seconds in order to reinforce spatial learning with respect to the distal cues. However, if a rat was unable to locate the platform within 120 seconds, it was gently guided towards the platform and allowed to remain on the platform for 15 seconds. The ANY-Maze tracking system was used to record the distance travelled before the first entry to the platform.

Probe trials: Two probe trials were conducted 24 and 48 hours after the last acquisition trial. During the probe trials, the platform was removed and the rat was allowed to swim for 30 seconds looking for the absent platform. The performance was recorded using the ANY-Maze tracking system.

Processing of brain tissue

Rats were deeply anesthetized using carbon dioxide. The brains were perfused via the transcardial route using normal saline (0.9%) for approximately 5 minutes until the outflow from the heart was clear. The brains were excised and separated into two hemispheres. The right hemisphere was used for the current experiment. The brain hemispheres were immersed in 4 % paraformaldehyde for 48 hours at 4 ^{o}C, and then transferred into 25 % sucrose solution at 4 ^{o}C until the tissue sank to the bottom.

Immunohistochemistry

The collection of brain sections and preparation for immunohistochemical analysis has been described previously (Guan et al., 2015b). Briefly, coronal sections of 25 µm thickness were cut using a freezing microtome (Microm, HM450, Microm international, Walldorf, Germany). Four series of sections containing the dorsal part of the hippocampus were collected based on a systematic sampling principle, with every 12th section collected. For each parameter of staining, one series of sections was used.

Primary antibodies against synaptophysin and GluR-1 were used to label synaptic vesicles and the GluR-1 sub-unit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, respectively, in the hippocampus. These markers were used to investigate expression at pre- and post-synaptic levels, respectively. Rat endothelial cell antibody (RECA)-1 and GFAP were also used to visualize the capillaries and astrocytes. All sections

were pretreated with 1% H_2O_2 in 50% methanol for 30 min to quench endogenous peroxidase activity. The sections were then incubated with 1.5% normal sheep serum diluted in phosphate-buffered saline with 0.2% Triton (PBST) at room temperature to block non-specific staining. The sections were incubated with the following primary antibodies: rabbit anti-GluR1 (Millipore, AB1504, MA, USA; 1:5000, mouse anti-synaptophysin (Sigma, S5768, NY, USA; 1:10000), mouse anti-RECA-1 (Abcam, ab9774, 1:5000) and mouse anti-GFAP (Sigma C9205, 1:10000) at 4 ^{o}C for 48 hours. Sections were incubated with biotin-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies accordingly (Sigma; 1:1000) at 4 ^{o}C overnight. Sections were then incubated with ExtrAvidin peroxidase tertiary antibody (Sigma; 1:1000) for 3 hours at room temperature. A brown reaction product was obtained by adding 0.05% 3, 3-diaminobenzidine substrate. Sections were washed with PBS (3 x 10 minutes) between each incubation. Stained sections were mounted onto gelatin-coated glass slides, dehydrated through an increasing gradient of ethanol to Safsolvent (ECP Ltd., Auckland, New Zealand), and a cover slip was then placed over DPX mountant.

The Hippocampal Image Acquisition and Delineation of CA1 Stratum Radiatum

Six offspring from each group were selected for 3D image analysis using the systematic sampling principle with an animal sampling fraction of 1 out of 4. Four sections of dorsal hippocampus from each rat were used for image acquisition and analysis. We analysed morphological changes of astrocytes and capillaries in the CA1 stratum radiatum (CA1 SR) sub-region of the hippocampus using established stereological techniques(Anzabi, Ardalan et al., 2018; Ardalan, Rafati et al., 2017). The delineation of the CA1 SR area was based on differentiating the CA1 pyramidal cell layer from the CA2/CA3 pyramidal cell layer (with bigger dense packed cells compared with CA1) and the subiculum (the region with lower cell density and bigger cellar size than CA1) (Ardalan, Wegener et al., 2016). We focused on the

CA1 SR area due to the high percentage of the excitatory synapses in this area (Megías, Emri et al., 2001). Image acquisition and analysis were performed in a blinded manner.

Acquisition of 3D Image, Reconstruction and Analysis of Astrocytes

A systematic set of Z-stacks of GFAP images were captured from CA1 SR sub-region of the hippocampus using a light microscope (x 60 objective lens) modified for stereology. Each reconstructed 3D image included more than one astrocytes. A total of 15 GFAP positive astrocytes from 4 dorsal hippocampal sections per rat were selected for 3D reconstruction and morphological analysis as previously described(Ardalan, Rafati et al., 2017). The selection criteria for morphological analyses were: (1) cell bodies must be in the middle of the section and have a clear border, (2) all branches are intact, (3) branches of the cell should be easily distinguishable from other cells or background staining. The images were analyzed by using the Filament Tracers algorithm in Imaris software (Version 8.4, Bitplane A.G., Zurich, Switzerland). Morphological parameters analyzed were; (1) total number of the processes, (2) total length of the processes, (3) the volume of convex hull of the longest process of each astrocyte and (4) the number of branches from the centre of the astrocyte in 10 μm increments (Figure 2B).

Length Density of Capillaries

The length density (Lv) estimated the length of capillaries per mm^3 of tissue in the CA1 SR sub-region of the hippocampus using the Global Spatial Sampling method(Larsen, Gundersen et al., 1998). The area of sampling fraction was at least 10% of the entire CA1 SR sub-region. The estimated length density of the capillaries was performed using a light microscope (Leica DFC 295, Germany, x 60 objective lens) modified for stereology with a digital camera (Leica DFC 295, Germany) and new CAST™ software (Visopharm, Hørsholm, Denmark) as described previously(Anzabi, Ardalan et al., 2018). Briefly, in a three-dimensional sampling box, isotropic virtual planes with a fixed-plane separation distance (d = 20 μm) were systematically and randomly projected onto the area of interest. The area of the average plane was 5748 μm^2 and the box height was 12 μm with a top guard zone of 2 μm. A capillary was defined as a vessel with a diameter of or below 10 μm. The length of capillaries was measured by the number of intersections between the virtual planes and the capillaries (Figure 3B). The length density of the capillaries was calculated using the following formula and presented as the length (mm) per mm^3 of tissue.

$$L_V = \frac{2 \cdot p(box)}{avg\,a(plane)} \cdot \frac{\sum Q}{\sum P}$$

Where L_v is the length density of the Reca-1 positive capillaries; ΣQ is the sum of intersections between the test lines and the capillaries; p (box) is the number of box corners (4); avg a (plane) is the average of the plane area; ΣP is the sum of the box corners hitting the area of interest.

Expression of GluR-1 and Synaptophysin

A total of 4 images of GluR-1 or Synaptophysin were acquired from 4 dorsal hippocampal sections of each rat using a light microscope (5x objective lens) modified for stereology. A

histogram of the mean linear intensity using a 300 bin interval was generated in an automated procedure independent of the background and threshold. The average intensity that measures the lightness of the image was then converted to a density for the darkness of the image (density = 255 – intensity) for statistical analysis and presentation of data.

Statistical analyses

The data from MWM tests were analyzed using either two-way repeated measure ANOVA if there were no missing values or mixed-effects analysis if there were missing values with the time points as the within-subjects factor and treatments as the between-subjects factor. The specific differences between time points or treatment groups were analyzed using Sidak's multiple comparisons or mixed-effects (GraphPad Prism version 8.2.1 (441), ©1992–2019 GraphPad Software, Inc.). Histological data were analyzed using independent t-test (SPSS, IBM Corp. Version 25.0. Armonk, NY). The effect size of cGP was measured by calculating Cohen's d and d = 0.20; d = 0.50 and d = 0.80 indicated small, medium and large effects respectively (SPSS). A two-tailed Pearson analysis was used to test the strength of the correlation (GraphPad Prism). Statistical significance was set at $p \leq 0.05$. The data are presented as mean ± SEM.

Results

Morris water maze

To test spatial reference learning the acquisition trials were conducted from PN day 70 to 73 in the adult offspring of cGP- or saline-treated dams. Mixed-effects analyses suggested a significant reduction in the distance travelled to first entry to the platform ($F_{(7.6, 299)} = 13.59$, $p < 0.0001$, Figure 1A) over the sixteen acquisition trials. There was no difference between

treatment groups and no interaction between trials and treatment groups. The data suggested that both groups had learnt to locate the platform.

To test the spatial reference memory two delayed probe trials were conducted 24 and 48 hours after the last acquisition trial. The mixed-effects analysis showed an overall increase in path efficiency in the cGP-treated offspring to the platform zone ($F_{(1, 37)} = 4.44$, $p = 0.04$, Figure 1B) and a decrease in path efficiency to the platform zone over time ($F_{(1,17)} = 6.0$, $p = 0.02$, Figure 1B). There was no interaction between the time points and treatment groups. Mixed-effects tests showed a significant increase in the path-efficiency of cGP-treated offspring at the 24-hour time-point compared with the saline-treated offspring ($p = 0.03$, Figure 1B).

The two-way repeated measure ANOVA showed no change in the average heading errors to the platform zone with time and treatment. However, a significant interaction was observed between the treatment and time ($F_{(1, 42)} = 3.97$, $p = 0.05$, Figure 1C). Sidak's multiple comparisons revealed a significant reduction in the average heading errors in cGP-treated offspring at the 48-hour time-point compared with the saline-treated offspring ($p = 0.03$, Figure 1C).

3D morphological changes of astrocytes in the CA1 SR sub-region of hippocampus

Figure 2A shows a representative image of GFAP positive astrocytes and the area of the CA1 SR sub-region of the hippocampus examined is delineated. Figures 2B shows representative photomicrographs of 3D reconstructions of astrocytes. Stereological analysis showed that the total length ($p = 0.0001$, Figure 2C), total numbers ($p = 0.0001$, Figure 2D) of processes of GFAP positive astrocytes were significantly increased in cGP-treated offspring compared with saline-treated offspring. The volume of the convex hull of astrocytes of cGP-treated offspring was significantly greater ($10195.39 \pm 1264.74\ \mu m^3$) compared with saline-treated

offspring (6674.61 ± 812.20 μm^3, p = 0.0001, Figure 2E). Sholl analysis showed a greater number of branching intersections from the cell soma at distances of 10 μm (p = 0.04), 20μm (p = 0.038), 30 μm (p = 0.001) and 40 μm (p = 0.006) in cGP-treated offspring compared to saline-treated offspring (Figure 2F). The results of Cohen's d calculation showed that the effects of cGP on the total length, number and convex hull volume of astrocytes processes were large (d = 1.84; d = 1.74; d = 1.67 respectively).

Changes in the length density of capillaries

Figure 3A shows a representative image of RECA-1 positive capillaries and the delineated area of the CA1 SR sub-region of hippocampus. Figure 3B shows the image of evaluating the length density (mm/mm^3) of RECA-1 positive capillaries. The results indicated that the length density of capillaries were significantly increased in cGP-treated offspring compared with saline-treated offspring (p = 0.007, Figure 3C). The results of Cohen's d calculation showed a large effect of cGP on the length density of capillaries (d = 1.40).

Changes of GluR-1 and synaptophysin

Figure 4A and B show representative images of GluR-1 and synaptophysin immunostaining respectively in the delineated area in the CA1 SR of the hippocampus. The density of GluR-1 staining in the CA1 SR sub-region of hippocampus was higher in cGP-treated offspring compared with saline-treated offspring (p = 0.02, Figure 4C). The effect size of cGP on GluR-1 density was large (d = 1.20). The expression of synaptophysin in the CA1 SR sub-region of hippocampus was similar between saline and cGP-treated offspring (Figure 4D).

Correlation between average heading errors and histological changes

The average heading errors 48-hours after acquisition was significantly correlated with the total length (p = 0.01, Figure 5A), the convex hull volume (p = 0.009, Figure 5B) and the

length density of the capillaries ($p = 0.02$, Figure 5C). However, a correlation between GluR-1 density and average heading errors 48 hours after acquisition was not statistically significant ($p = 0.09$).

Correlation between astrocytic morphological parameters, GluR-1 density and capillary length density

The density of GluR-1 was positively correlated with the total length ($p = 0.01$, Figure 6A) and convex hull volume of astrocytic processes ($p = 0.01$, Figure 6E), but not the number of astrocytic processes (p = 0.2, Figure 6C). The length density of capillaries was positively correlated with the length ($p = 0.006$, Figure 6B), numbers ($p = 0.004$, Figure 6D) and convex hull volume of astrocyte branches ($p = 0.002$, Figure 6F). There was no correlation between the density of GluR-1 and the length density of capillaries.

Discussion

We have previously shown that maternal administration of cGP during lactation increases the concentration of cGP in the plasma of the pups. Such treatment improved the working memory of these offspring when they were juvenile (Singh-Mallah, Singh et al., 2016) and spatial reference memory when they were young adults. Indeed, cGP-treated offspring had improved path efficiency of first entry to the platform zone 24 hours after acquisition, and made less heading errors to the platform zone 48 hours after acquisition. The changes in heading errors and path efficiency, particularly detected in such delayed trials have been suggested to be more sensitive parameters for identifying subtle improvements in experimental settings without brain injuries (Guan, MacGibbon et al., 2015; Vorhees & Williams, 2006; Vorhees & Williams, 2014). Our data support the notion that administration of cGP during early-life has a long-lasting effect on memory until early adulthood. Similarly administration of cG-2allyl-P, a structural analogue of cGP, improves motor function up to

10 weeks after treatment (Krishnamurthi, Mathai et al., 2008). The modest effects of cGP on memory might also be explained by the known action of normalizing, rather than stimulating IGF-1 function (Li, Liu et al., 2019a; Li, Liu et al., 2019b).

Astrocytes play a role in vascular remodeling and forming neuronal-vascular networks (Bernardinelli, Muller et al., 2014; Matute, Gutiérrez-Igarza et al., 1994). Morphological analysis showed that cGP-treated offspring have more, longer, and larger astrocyte processes with an increase of capillary density in the CA1 SR sub-regions of the hippocampus than saline-treated offspring. Correlation analysis suggested that the rats with more, larger and longer astrocyte processes also have more capillaries. The data suggested a role for astrocytic plasticity in cGP-mediated vascular remolding. The effects of cGP in improving vascularization have been shown to be mediated through regulating the amount of bioavailable IGF-1 in circulation (Guan, Gluckman et al., 2014), which is essential for supporting cerebral vascular remodeling and brain function (Lopez-Lopez, LeRoith et al., 2004). Low concentrations of circulating cGP are associated with the conditions of poor cerebral and systemic vascular health and function and occurs in patients with hypertension, during on-set of stroke and age-related dementia (Fan, Krishnamurthi et al., 2019; Fan, Pitcher et al., 2020; Guan, Singh-Mallah et al., 2018; Li, Liu et al., 2019a). In support, the administration of cGP reduces systolic blood pressure, protects against ischemic brain injury and improves memory in rats (Guan, Gluckman et al., 2014; Li, Liu et al., 2019a; Ostrovskaya, Gruden et al., 2007; Singh-Mallah, Singh et al., 2016).

Synaptic trafficking of glutamate AMPA receptors in the hippocampus play a role in spatial memory (Huganir & Nicoll, 2013). The density of GluR-1 staining, a sub-unit of AMPA receptors, was increased in the CA1 SR of the hippocampus of cGP-treated offspring compared with saline-treated offspring. An *in vitro* study reported that cGP modulates the activity of AMPA receptors (Gudasheva, Grigoriev et al., 2016). Administration of a cG-

2allyl-P improves spatial memory which is also associated with an increase of GluR-1 expression in the hippocampus of the rats with acute memory impairment (Guan, Zhang et al., 2010). GluR-1 is abundant in the end-feet of astrocytes processes (Matute, Gutiérrez-Igarza et al., 1994). Plasticity of astrocyte is known to play a role in regulating the dynamics of glutamate neurotransmission (Bernardinelli, Muller et al., 2014; Zhou, Zuo et al., 2019). For example, the glutamate-glutamine cycles (Sonnewald & Schousboe, 2016) in astrocytes and synapses of the hippocampus (Rouach, Koulakoff et al., 2008).

Independent of treatment, the average heading errors toward the escape platform was correlated to the length and volume of astrocytic processes, as well as capillary density. Thus, the astrocytic plasticity as the structural changes of cell processes and its associated vascularization could be the biology underlying spatial reference memory. The changes of astrocyte processes were also closely associated with the density of capillaries and GluR-1 expression. However, there was no correlation between density of capillaries and glutamate expression (data no show). The disassociation between capillary density and GluR-1 expression suggested a key role for astrocytic plasticity in developmental programming, leading to long-lasting biological changes in vascularization and GluR-1 expression. These results provided an initial evidence for cGP in regulating IGF-1 function during brain development of male rats. Given the potential developmental effects, the pharmacokinetics and dose response of cGP in developing rats need to be evaluated. The investigation into the potential negative influence of cGP in physiological function of IGF-1 during development is essential for its application in maximizing brain development of normal infants.

In conclusion, maternal administration of cGP during post-natal brain development improved the spatial memory of young adult offspring. This long-lasting effect of cGP on memory may be mediated via astrocytic plasticity and its associated vascularization and glutamate function in the CA1 SR sub-region of the hippocampus.

Figure legends

Figure 1 Maternal administration of cGP during lactation improved memory of adult offspring. The distance travelled to first entry to platform during 16 acquisition trials were similar between cGP-treated and saline-treated groups (A). Reference memory of rats was tested in two probe trials conducted 24 and 48 hours after the last acquisition trial by evaluating the path efficiency of entry (B) and average heading errors (C) to the platform zone. Data are presented as mean ± SEM, n = 21 (saline) and 23 (cGP). * shows significant differences between the treatment groups, with significance set at $p \leq 0.05$.

Figure 2 Maternal administration of cGP during lactation increases astrocytic processes in the hippocampus of adult offspring. Microscope image represents GFAP staining and the area used for analyzing the astrocytes in the CA1 SR subregion of the hippocampus (A, 1x objective lens). Figure 2B shows a 3D reconstruction of GFAP positive astrocyte, the points of process branching (red dots) and cell soma (blue dots). The total length of (C), total number of astrocyte processes (D), convex hull volume of the longest processes of astrocytes (E) and distal distances from cell soma (within 10µm, 20µm, 30µm and 40 µm, F) significantly increased in cGP-treated offspring compared to saline-treated offspring. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ shows significant differences between the treatment groups. Data are presented as mean ± SEM, n = 6, SR = Stratum Radiatum.

Figure 3 Administration of cGP during lactation increases the length density of capillaries in the hippocampus of adult offspring. Microscope image represents RECA-1 positive capillaries and the area used for analyzing the length density of the capillaries in the CA1 SR subregion of the hippocampus (1x objective lens, A). The Figure 3B shows a high magnification image of RECA-1 positive capillaries (x 63) and the methods used for estimating the length density. The four points at the corner of a box are used to estimate the

reference volume. The green line represents an intersection between isotropic virtual planes (blue lines) and the focal plane. The length of a capillary was measured by counting the number of intersections (x) between the focused capillary and the virtual planes. Scale bar = 30 μm. The length density of capillaries in the CA1 SR sub-region of hippocampus significantly increased in the cGP-treated offspring compared with the saline-treated offspring (C). $^{**}p<0.01$, Data are presented as mean ± SEM, n = 6. SR = Stratum Radiatum.

Figure 4 Maternal administration of cGP during lactation increased the expression of GluR-1 of adult offspring. The photo images show the area used for analyzing the expression of GluR-1 (A) and synaptophysin (B) in the hippocampus. The treatment with cGP increased the expression of GluR-1 (C), but not synaptophysin (D) in the CA1 SR subregion of hippocampus of adult offspring. Data are presented as mean ± SEM, n = 6, $^{*}p < 0.05$. SR = Stratum Radiatum.

Figure 5 The spatial memory was associated with the length and volume of astrocyte processes and capillary density. The average heading errors to the platform zone 48 hours after the acquisition was significantly correlated with the length (A) and convex hull volume (B) of astrocyte processes; and the length density of the capillaries (C). n = 12, SR = Stratum Radiatum.

Figure 6 Correlation of astrocyte processes with the density of capillary and GluR-1 expression. The GluR-1 density was positively correlated with the total length (A) and convex hull volume of astrocytes processes (E). The length density of capillaries was positively correlated with the total length (B), number (D) and convex hull volume of astrocyte processes (F) in the CA1 SR sub-region of hippocampus. n = 12, SR = Stratum Radiatum.

Graphic abstract. Maternal administration of cGP during lactation increases the amount of bioavailable IGF-1 in circulation, possibly in brain of infant pups by competing with IGFBP-3 to IGF-1 binding. Such treatment improved memory of their adult offspring likely due to improving IGF-1-mediated long-lasting vascularization and GluR-1 expression through astrocyte plasticity.

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