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Role of cyclic-glycine-proline in neurodevelopmental programming

New insights into the physiological regulation of IGF-1 function

Gagandeep Singh-Mallah

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in Medicine, The University of Auckland, 2016.
ABSTRACT

Insulin-like growth factor-1 (IGF-1) is critical for postnatal brain development and adult cognition. As a metabolite of IGF-1, cyclic-glycine-proline (cGP) regulates the bioavailability of IGF-1, and thus normalizes IGF-1 function under pathophysiological conditions. The presence and changes in concentrations of endogenous cGP in rat milk and plasma were evaluated during lactation and postnatal development, respectively. The efficacy of maternal administration of cGP during lactation on normal brain development and function of offspring, and the lactational capacity of dams was also investigated.

Sprague-Dawley dams were gavaged with either cGP (3mg/kg) or saline daily from postnatal d8-22. Concentrations of cGP, IGF-1 and IGF binding protein-3 (IGFBP-3) were measured in dams’ milk, and in the plasma of dams and offspring. Neuroplasticity and memory of offspring were evaluated. The effect of cGP on milk production, and various morphological, cellular and molecular markers of post-lactational involution were investigated.

Concentrations of endogenous cGP were higher in milk during peak, than during late lactation. Concentrations of endogenous IGF-1 and IGFBP-3 were low, whereas concentrations of endogenous cGP were high in the plasma of pups. The reduced IGFBP-3 and increased cGP were a response to increase the bioavailability of IGF-1 during infancy. Exogenous cGP showed oral bioavailability in dams and effective maternal-infantile transfer through milk, which resulted in an increase in the bioavailability of IGF-1 in both dams and pups. Maternally transferred cGP improved learning and memory of offspring at adolescence and adulthood, which was associated with increased glutamatergic neuroplasticity. Milk production and composition of dams was not affected. However, cGP promoted the apoptosis of mammary cells, the loss of intact alveoli and the upregulation of cell survival factors during...
early-involution, and the reduction in phosphorylation of IGF-1 receptor during late-involution.

This is the first study to show the role of cGP in autocrine regulation of IGF-1 in vivo, and the efficacy of maternally-administered cGP in optimizing normal brain development and function of offspring. The role of cGP in promoting mammary gland involution, and the physiological decline in IGF-1 function are also novel findings. The positive modulation of neurodevelopmental programming supports clinical translation of cGP as maternal supplementation.
PUBLICATIONS ARISING FROM THIS THESIS

Original papers:


Review articles:


Co-authored publications:

1 in pregnancy related obesity, a potential biomarker for recovery. *Endocrinology (under review)*

**Conference presentations:**


DEDICATION

This thesis is dedicated to my family.
ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisors, senior scientists Drs Jian Guan, Kuljeet Singh and Christopher McMahon for considering me for this PhD project, and for their guidance, advice, encouragement and support. Your continual feedback made the write-up of this thesis possible. A special thanks to my primary supervisor, Jian, who has been a tremendous mentor to me since the time I first started working with her as a summer student. It was almost five years ago. Your critical analysis and interpretation of data is one of the many skills that I have gained during this time, and hope to use in my future research career. I and my wife are also indebted to you for your advices as a Pediatrician. A special thanks to Kuljeet and Chris, who despite very few face-to-face meetings, especially in the last two years of the PhD, maintained a very active contact with me and guided me through to the end of the thesis.

My thanks are extended to Robert Smith (Bobby), Ric Broadhurst and Genevieve Baildon of the Small Animal Colony at AgResearch (Ruakura, Hamilton) who trained me in animal handling, and assisted in sample collection for this project. I thank Joanne Dobson and Kim Oden of the Lactation Biology team (AgResearch, Hamilton) for training me in the immunohistochemistry and real time RT-PCR assays. Thanks for being so patient with me. I also thank members of my laboratory group at the Liggins Institute, both past and present: Karen Liu, Rong Zhang and Joyce Leung for help with sectioning the Brain tissue and immunohistochemistry; Stanley Yune, Marcus Svensson, Johan Karlsson and Johanna Milding for carrying out some of the immunohistochemistry assays for Chapter 4 of this thesis. I thank Panzao Yang for help with confocal imaging, and Kui Xie (Craig) for solving my Photoshop-related nightmares. I also thank Harold Henderson and Avinesh Pillai for assistance with the statistical analyses.

VIII
I probably cannot thank my family enough, without whom I would have not been able to finish this thesis. Big thanks to my lovely wife, Namleen Kaur, whom I met in the first year of the PhD, got married the next year and was blessed with two gorgeous twin girls, Karamleen Kaur and Keerat Kaur (9 months old now), by the last year. I am sorry for coming home late during the weekdays and being absent from home at most weekends in the last year. I promise to make up for it now. Thanks for helping with behavioral tests (and yes, for cleaning and drying the rats!). I wish I could put your picture here with those cute little rats. I am whole heartedly grateful to Mom and Dad, who encouraged and supported me (both financially and emotionally) to go to New Zealand for post-graduate studies, and themselves came and spent the past nine months here to help with taking care of the babies. Last but not the least, thanks to my dearest brother, Manpreet Singh, who has been tolerating my odd bad-mood days and keeping up the pleasant atmosphere at home. Thanks also for helping with formatting of the thesis.

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Finally, I bow down to Guru Granth Sahib Ji and the Sangat (the holy congregation) because there are absolutely no words to thank you for constantly reminding me the purpose of this life, in the good and the bad.
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**Chapter 4: Maternal cyclic-glycine-proline administration improves memory and neuroplasticity in adult offspring.**


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<tr>
<td>Marcus Svensson</td>
<td>performed GluR-1 immunohistochemistry assay on brains of pups</td>
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<tr>
<td>Johan Karlsson</td>
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<td>PI of the grant, designed the experiment, and helped in sample collection, and writing and revision of the manuscript</td>
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Chapter 5: Cyclic-glycine-proline reduces the phosphorylation of IGF-1R and promotes post-lactational involution in mammary glands of rats.


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### ABBREVIATIONS

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<tbody>
<tr>
<td>2’-FL</td>
<td>2’-Fucosyllactose</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BAD</td>
<td>B-cell lymphoma-2 associated D protein</td>
</tr>
<tr>
<td>Bax</td>
<td>B-cell lymphoma-2 associated X protein</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>B-cell lymphoma extra-long isoform</td>
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<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
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<td>CAMKII</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase</td>
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<td>cG-2allyl-P</td>
<td>Analogue of cGP with an allyl group added to the proline ring</td>
</tr>
<tr>
<td>cGP</td>
<td>Cyclic-L-glycine-L-proline</td>
</tr>
<tr>
<td>cGP-d(_2)</td>
<td>Cyclic-(2-d(_2)-glycine)-L-proline</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic adenosine monophosphate-responsive element-binding protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
</tbody>
</table>
G-2mPE  Analogue of GPE with a methyl group added to the proline ring
GluR-1  Glutamate receptor-1
GPE   Glycine-proline-glutamate
H₂O₂  Hydrogen peroxide
HI    Hypoxia-ischemia
HPLC-MS High-performance liquid chromatography-mass spectrometry
IGF-1  Insulin-like growth factor-1
IGF-1R  Insulin-like growth factor-1 receptor
IGFBP  Insulin-like growth factor binding protein
IR    Insulin receptor
LRP   Low density lipoprotein receptor-related protein
LTD   Long-term depression
LTP   Long-term potentiation
MAPK  Mitogen-activated protein kinase
MEC   Mammary epithelial cell
MFG   Milk fat globule
MMP   Matrix metalloproteinase
mTOR  Mammalian target of rapamycin
MWM   Morris water maze
NBQX  2, 3 – dihydroxy – 6 – nitro -7 –sulfamoyl – benzo (f) quinoxaline
NMDA  N-methyl-D-aspartate
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNZ2591</td>
<td>Commercial name of cG-2allyl-P</td>
</tr>
<tr>
<td>NORT</td>
<td>Novel object recognition test</td>
</tr>
<tr>
<td>OFT</td>
<td>Open field test</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphotidyl-inositol 3’-kinase</td>
</tr>
<tr>
<td>pIGF-1R</td>
<td>Phosphorylated insulin-like growth factor-1 receptor</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SYN</td>
<td>Synaptophysin</td>
</tr>
<tr>
<td>WAP</td>
<td>Whey acidic protein</td>
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</table>
THESIS OUTLINE

Chapter 1: Introduction

This chapter provides an introduction to the process of neurodevelopmental programming, with examples from early-life dietary intervention studies. This is followed by a review on the roles of IGF-1 in postnatal brain development, learning and memory, and post-lactational involution of mammary gland. The gaps in the current knowledge, especially the scarcity of research on cGP, and how this thesis aims to fill those gaps are identified.

Chapter 2: General materials and methods

This chapter describes in detail the general methods that were used in more than one results chapters of this thesis, including the experimental design. A brief description of all the remaining methods is provided, with details given in the specific results chapter.

Chapter 3: Maternally administered cyclic-glycine-proline increases insulin-like growth factor-1 bioavailability and novelty recognition in the developing offspring

Results presented in this chapter were recently published in Endocrinology. This chapter reports the qualitative and quantitative assessment of endogenous cGP in rat milk and plasma during lactation and postnatal development, respectively. In addition, the effects of maternal administration of cGP during lactation on the bioavailability of cGP in dams, the maternal-infantile transfer, and the recognition memory of adolescent offspring are presented.

Chapter 4: Maternal cyclic-glycine-proline administration improves memory and neuroplasticity in adult offspring
A manuscript based on this chapter is under review by Developmental Neuroscience. This chapter presents the effects of maternal administration of cGP during lactation on spatial learning and memory of adult offspring. The changes in protein expression of different markers of synaptic plasticity in brains of dams, pups and adult offspring are also presented.

Chapter 5: Cyclic-glycine-proline reduces the phosphorylation of IGF-1R and promotes post-lactational involution in mammary glands of rats

A manuscript based on this chapter is under review by The Journal of Physiology. This chapter shows the effect of maternal administration of cGP during lactation on the lactational capacity, in particular the milk production and the post-lactational involution, of mammary glands of rats. The effects of cGP on various morphological, cellular and molecular markers of involution are presented. The effect of cGP on the physiological decline in IGF-1 function is also reported.

Chapter 6: General discussion

This chapter combines the three results chapters to discuss if early life administration of cGP influences the neurodevelopmental programming of offspring. The physiological roles of endogenous cGP, and the implications of oral bioavailability of exogenous cGP in adult and infant rats are discussed. The role of cGP in mammary involution and the physiological regulation of IGF-1 function is also discussed. The strengths and limitations, the potential future research areas and the clinical implications of the current study are reported.
Chapter 1: Introduction

Early postnatal life represents a critical period of growth and development, such that alterations during this period can program the infants for life-long or permanent changes in metabolism, body composition and cognitive abilities such as learning and memory. This phenomenon is referred to as developmental programming. For instance, imbalanced maternal nutrition during lactation can lead to adolescent or adult onset of metabolic disorders, such as obesity and type 2 diabetes [1]. The structural and functional framework of nervous system are under rapid development during the early postnatal life, and the infant relies on an optimal supply of nutrients/bioactive factors from the maternal milk and/or formula for optimal brain development and function [2,3]. NUTRIMENTHE (http://www.nutrimenthe.eu/) is an international project investigating the effects of early life nutrition on cognitive performance in later life [4]. Neurodevelopmental programming in early life thus offers a tool to improve an individual’s long-term cognitive and social well-being and performance. This, in turn, has important implications on the development of public health policies, and could reduce the costs of health and social care, thus leading to economic growth [5].

Breastfeeding is consistently shown to have a positive effect on the cognitive development of the offspring. In a study published in 1929, the authors reported a positive association between breastfeeding and intelligence of children between the age of 7 to 13 years [6]. Since then several studies have followed which strengthen the importance of breastfeeding in cognitive development of children [for extensive reviews, see 7,8]. For instance, in the largest ever randomized trial conducted to study the influence of exclusive breastfeeding on cognitive development of children, Kramer and others [9] showed that prolonged and exclusive breastfeeding improved the performance of children in several measures of cognitive function,
Chapter 1: Introduction

including verbal, performance-based and the full-scale intelligence quotient (IQ), at 6.5 years of age. Furthermore, early life supplementation with dietary factors (either through mother or directly to the infant) is shown to cause long-lasting improvement in the cognition of the offspring, including the learning and memory function. For instance, maternal supplementation with docosahexaenoic acid (DHA), a long-chain polyunsaturated omega-3 fatty acid, during pregnancy and lactation is shown to improve the IQ of children at 4 years of age [10]. The prenatal (daily maternal oral supplementation throughout pregnancy) and postnatal (daily subcutaneous injections from birth to weaning) supplementation of rats with choline is shown to improve spatial memory of offspring at adulthood [11]. Recently, oral supplementation of infant rats with 2'-Fucosyllactose (2'-FL), an oligosaccharide, during lactation was found to improve learning and memory that persisted into adulthood [12].

The pro-cognitive effects of breastfeeding and some of the above mentioned specific nutrients are proposed to be mediated through neurotrophic factors, such as IGF-1 [13]. Considering the low production of endogenous IGF-1 in liver until weaning [14,15], the milk-borne IGF-1 is suggested to play a critical role during this period [16]. For instance, breastfed infants have higher plasma IGF-1 levels compared with those fed exclusively with the formula [17]. Early life administration of DHA is also proposed to improve learning and memory through IGF-1 mediated activation of phosphotidyl-inositol 3'-kinase (PI3K)/ protein kinase B (PKB or Akt)/mammalian target of rapamycin (mTOR) signaling pathway or the mitogen-activated protein kinase (MAPK)/cyclic AMP-responsive element-binding protein (CREB) pathway, which ultimately lead to the transcription of genes involved in synaptic plasticity, the neural mechanism underlying the processes of learning and memory [18]. Similarly, early life supplementation with choline is associated with activation of neurotrophic factors such as the
IGFs and the nerve growth factor [19,20], and involves the activation of MAPK/CREB signaling pathway in hippocampus [21].

It is well known that IGF-1 is crucial for early postnatal brain development, including synaptogenesis and myelination [22]. The role of IGF-1 in learning and memory has been primarily evaluated through the ability of IGF-1 in restoring learning and memory deficits associated with insults such as brain injury, neurodegenerative disorders and aging [23-25]. In fact, Stern and others claimed that no study has investigated the pro-cognitive effects of IGF-1 in a normal healthy brain, and showed that a single intracerebral injection of IGF-1 has no effect on the memory of normal rats [26]. The lack of research into the efficacy of early life supplementation of IGF-1 on neurodevelopmental programming of a normal brain is mainly due to the metabolic (e.g. hypoglycemia) and mitogenic side-effects of IGF-1, poor bioavailability of IGF-1 in offspring following maternal supplementation, and poor central uptake of peripheral IGF-1 [27,28]. Interestingly, these issues can be overcome through the use of IGF-1 derived small peptides, which show similar effects as IGF-1 [29].

The biological activity of IGF-1 is regulated through reversible binding with IGF binding proteins (IGFBP) [30]. Unbound IGF-1 either interacts with the receptors (e.g. IGF-1 receptor) to activate downstream signaling pathways, or is cleaved by an acid protease into an N-terminal tripeptide, glycine-proline-glutamate (GPE), and des-N-IGF-1 (des-IGF-1) (Figure 1.1) [31]. GPE is further metabolized to produce a cyclic end-product, cGP [32,33]. By altering the binding between IGF-1 and IGFBP, cGP regulates the bioavailability of IGF-1, and is shown to normalize IGF-1 function under pathophysiological conditions of altered IGF-1 bioactivity [34]. Furthermore, cGP is found to be neurobioactive [29,35], and has been identified as an endogenous nootropic peptide in the brain of adult rats [36]. Similar to IGF-1,
cGP is also shown to restore learning and memory deficits either induced through brain insults or associated with aging, via improvement in synaptic plasticity [29]. However, the role of cGP in neurodevelopmental programming of a normal brain has not been investigated, and therefore, forms the focus of this thesis (Chapters 3 and 4). The presence of endogenous cGP in the plasma and milk of rats is also investigated (Chapter 3).

Figure 1.1: Production of Cyclic-glycine-proline (cGP) from Insulin-like Growth Factor-1 (IGF-1).

The biological activity of IGF-1 is regulated through reversible binding with IGF binding proteins (IGFBP). Unbound IGF-1 either interacts with the receptors (e.g. IGF-1 receptor (IGF-1R) or insulin receptor (IR)) to activate downstream signaling pathways, or is cleaved by an acid protease into an N-terminal tripeptide, glycine-proline-glutamate (GPE), and des-N-IGF-1 (des-IGF-1). GPE is further metabolized to produce a cyclic end-product, cGP.
In early-life dietary intervention studies, maternal supplementation during lactation is preferred over direct infant supplementation because of the increased understanding of the benefits of exclusive breastfeeding among women, and the unwillingness of some mothers, especially vegetarians, to give any supplementation or pharmaceutical medicine to their infants [37]. Furthermore, because the concentrations of IGF-1 in milk change during the course of lactation [38,39], therefore, maternal supplementation of IGF-1 derived small peptides may have pharmacological implications for the infant. Consequently, in this thesis, the efficacy of cGP on neurodevelopmental programming of offspring is evaluated via maternal administration of cGP during lactation.

IGF-1 is shown to enhance the lactational capacity of mammary glands by delaying the process of post-lactational involution [40-44]. Enhanced lactational capacity, in turn, may benefit the cognitive development of the offspring [45,46]. Therefore, this thesis also investigates the effect of maternal administration of cGP during lactation on post-lactational involution of mammary glands (Chapter 5). Furthermore, IGF-1 may also influence the lactational capacity of mammary glands through central regulation of lactation. The effect of cGP on central regulation of lactation was investigated by Masters Student, Stanley Yune. The results from his study are discussed in Chapter 6 of this thesis. Overall, the current study is the first to investigate the role of cGP in post-lactational development of mammary gland.

The following sections of this chapter provide a critical review of the existing literature, identifying the gaps in the current knowledge and describing how this thesis aims to fill those gaps. The first section reviews the IGF family members, including the IGF-1 derived small peptides, such as cGP. A particular focus is given on the cGP-mediated regulation of IGF-1 bioavailability, and the role of IGF-1 in postnatal brain development. The second section
reviews the brain regions involved in learning and memory, and the neural mechanisms of learning and memory, with a particular focus on synaptic plasticity. The current knowledge of the roles of IGF-1 and cGP in learning and memory, which is primarily derived from the brain injury models, is also presented. The last section begins with a brief introduction to the process of post-lactational involution of mammary glands, followed by a review on the two stages of involution and the associated cell death pathways, and the role of IGF-1 in involution. The scarcity of research on cGP is highlighted throughout the literature review.

1.1 The IGF family

The IGF family includes IGF-1 and -2, IGF receptors (IGF-1R, IGF-2R), and IGFBP 1-6. IGF-1 is a pleiotropic growth factor with multiple actions in almost all major cell types of the body. IGF-1 is mainly produced by the liver, which contributes approximately 75% of the total circulating IGF-1 [47]. IGF-1 is also produced locally by several target organs, including brain, mammary gland, bone and muscle [48]. In the brain, IGF-1 is present in all cell types, and is found in a number of sub-regions including the hippocampus, hypothalamus, cortex, cerebellum and brain stem [48]. Local production of IGF-1 is highest during the perinatal period, with very low production in the adult brain [48]. In the mammary gland, IGF-1 is produced by the stromal cells [49].

IGF-1 signaling involves activation of IGF-1R, which is found in the same brain regions as IGF-1 and follows the same temporal expression pattern as IGF-1 [25]. In the mammary gland, IGF-1R is expressed in the epithelial cells, stromal cells, and the vascular endothelial cells [50]. In addition to IGF-1R, IGF-1 can also signal via the hybrid-receptor formed between IGF-1R and B-isoform of insulin receptor (IR-B), and the IGF-2R, albeit with low-affinity [48]. Both IGF-1R and IR are transmembrane hetero-tetrameric tyrosine kinases,
containing two polypeptide chains, each with an extracellular α-subunit and a cellular β-subunit. The extracellular subunit binds the ligand molecules while the cellular subunit possesses the tyrosine kinase activity [51]. IGF-1 binds to the extracellular α-subunit of IGF-1R, activating IGF-1R by inducing transphosphorylation of the cytoplasmic β-subunit. IGF-1R activation, in turn, activates a number of downstream signal transduction pathways, including but not limited to the insulin receptor substrate (IRS)/PI3K/Akt pathway, and the Ras/Raf/MAPK pathway. These pathways are discussed in detail in section 1.3.5 (Figure 1.10).

IGFBP have a greater affinity for IGF-1 than IGF-1R. Therefore, IGFBP tightly regulate the bioavailability of IGF-1, and thus play a key role in modulating IGF-1 function. For instance, in circulation, IGFBP-3 forms a complex with IGF-1 and acid labile sub-unit to increase the plasma half-life of IGF-1, and liberate IGF-1 in the tissues to allow interaction with the receptor molecules [51,52]. Alternatively, IGFBP may prevent the release of IGF-1 to inhibit the ligand-receptor interaction [51,52]. All six IGFBP are expressed in the brain and the mammary gland. IGFBP-5 is primarily produced by neurons, and IGFBP-2 by astroglia and epithelial cells of the choroid plexus [48]. IGFBP-5 is the dominant binding protein during mammary involution [53].

Due to the low production of central IGF-1 and the wide expression of IGF-1R at adulthood, the peripheral IGF-1 is suggested to play a crucial role in brain function [48]. Indeed, serum IGF-1 deficiency is shown to cause learning and memory impairment in mice [54]. Peripheral IGF-1 can enter brain either through the blood-CSF barrier (across the choroid plexus) or the blood-brain-barrier (BBB) (entering directly into the brain parenchyma) [48]. Transcytosis of peripheral IGF-1 across the epithelial cells of choroid plexus involves the IGF-1R and the
lipoprotein receptor-related protein 2 (LRP2) [48]. Blood-CSF transfer of peripheral IGF-1 is directly dependent on the concentrations of IGF-1 in blood [55]. After entering the CSF, peripheral IGF-1 can readily access the periventricular areas such as the hippocampus and the hypothalamus, however, entry into the deeper brain regions, such as the cerebellum, require transportation via IGFBP [48]. Transcytosis of IGF-1 across BBB is not completely understood but is thought to involve IGF-1R and LRP1, and is relatively independent of the concentrations of IGF-1 in blood [56]. The mode of transfer of peripheral IGF-1 into mammary gland is poorly understood [57], though it is known that IGF-1 enters the milk via a transcytotic pathway [58] (reviewed in section 1.3.1; Figure 1.7). Furthermore, local, rather than the peripheral, IGF-1 is shown to be more important for rapid branching of the rudimentary mammary gland in mice [59]. However, the role of peripheral versus local IGF-1 in post-lactational involution is not well understood.

Despite the different routes of entry to brain, peripheral IGF-1 does not easily enter the brain compared with the IGF-1 derived small peptides, for instance cGP, which show similar effects as IGF-1 [29]. The role of IGF-1 derived small peptides in mammary gland development has never been explored. The following section provides a brief review on the IGF-1 derived small peptides, with an emphasis on the mode of action of cGP.

1.1.1 IGF-1 derived small peptides

GPE forms the IGFBP-binding site of IGF-1, but shows no interaction with IGF-1R [60]. In contrast, des-IGF-1 shows poor binding to IGFBP but high affinity for IGF-1R [30]. IGF-1 is a well-known neuroprotective agent [for an extensive review, see 24]. For instance, IGF-1 prevents neuronal death in fetal sheep and adult and infant rats after hypoxic-ischemic (HI) brain injury, leading to long-term functional recovery [61-63]. However, the therapeutic
applications of IGF-1 are limited due to its poor central uptake and potential metabolic (e.g. hypoglycemia) and mitogenic side-effects [29]. In comparison to IGF-1, GPE is a smaller molecule and shows better central uptake [29]. However, due to its hydrophilic nature, peripheral GPE does not easily enter the brain under normal conditions, though the central uptake is greatly facilitated after breakdown of BBB (for instance, after HI injury) [35]. Similar to IGF-1, GPE is also neuroprotective following HI injury in adult and infant rats [64,65]. Additionally, the neuroprotective effects of GPE are shown in several animal models of neurodegenerative diseases. For instance, in an animal model of Parkinson’s disease, GPE was shown to prevent the 6-hydroxydopamine (6-OHDA)-induced depletion of dopamine neurons in substantia nigra by causing an upregulation of tyrosine hydroxylase (TH) immunoreactivity [66]. Despite the similar neuroprotective effects as IGF-1 and the prevention of mitogenic side-effects (because of poor interaction with IGF-1R), the clinical use of GPE is limited by its short plasma half-life (< 4 min), owing to the enzymatic instability [29]. GPE can, therefore, be further metabolized into single amino acids and dipeptides, such as cGP (Figure 1.1) [32,33]. Majority of GPE exists in trans isomeric configuration (cis:trans ratio 20:80). The enzymatic cleavage of glutamate leads to cyclization of glycine-proline binding, thus rendering cGP resistant to enzymatic degradation (plasma half-life ~ 2 h), and enhancing its lipophilicity for effective central uptake [29]. Similar to IGF-1 and GPE, cGP and its structural analogue cyclic-L-glycyl-L-2-allylproline (cG-2allyl-P), are also shown to be neuroprotective after HI injury [34,67]. Moreover, the central uptake of cGP is injury-independent, such that equivalent amounts of cG-2allyl-P are detected in the plasma and CSF of normal rats following systemic administration [67]. Using an in vitro albumin-binding assay it was shown that the pharmacokinetics of cGP involve binding and releasing from the plasma proteins, such that the neuroprotection conferred by systemically administered cG-2allyl-P is
dose-independent, whereas the central administration of cG-2allyl-P shows complex dose-dependency [67]. It was proposed that the amount of free cG-2allyl-P in plasma is stabilized owing to the sustained binding-release process mentioned above, whereas the constant turnover of CSF (approximately every 1 h) leads to the elimination of cG-2allyl-P from brain [67]. Nevertheless, cG-2allyl-P was still detectable in CSF 6 h after a bolus sub-cutaneous injection due to the sustained transfer from blood [67]. Interestingly, the beneficial effects of cG-2allyl-P are found to be long-lasting. For instance, in the Parkinsonian animal model (6-OHDA), systemic administration of cG-2allyl-P for 14 days (starting 2 weeks after the onset of lesion) improved motor deficits up to 12 weeks [68]. Similarly, chronic systemic administration of cG-2allyl-P for 5 days (starting 2 h post-injury) provided almost complete neuroprotection after 9 weeks of HI injury [67]. The long-lasting efficacy, and the ease of central uptake following peripheral administration makes cGP an ideal candidate for investigations into neurodevelopmental programming.

1.1.2 cGP normalizes IGF-1 function

The mechanism of action of cGP involves normalizing IGF-1 function via altering the binding of IGFBP to IGF-1 [34]. As the first evidence, Guan and co-workers showed that cGP-mediated neuroprotection following HI injury involved restoration of IGF-1R-associated angiogenic capillaries, suggesting that cGP actions may be mediated indirectly through IGF-1 [34]. In a series of experiments using the human endothelial cells, cGP was shown to both inhibit and stimulate IGF-1-induced cell growth depending on whether the IGF-1 bioactivity was elevated (e.g. through overexpression of IGF-1R or blockage of IGFBP-3) or diminished (e.g. through serum withdrawal) [34]. Through these experiments it was confirmed that cGP efficacy is a modulated IGF-1 function because the efficacious doses of cGP were only effective in modulating cell growth when given as a combined treatment with IGF-1, but not
when given alone [34]. In support, the knockdown of IGF-1R completely negated the effects of cGP [34]. Although there is no direct evidence against or in-favor of the interactions between cGP and IGF-1R, the facts that GPE does not interact with IGF-1R [60], and shares many biological features with cGP [67], suggest that cGP may also not interact directly with the IGF-1R. However, cGP may interact with IGFBP to modulate the bioavailability of IGF-1. For instance, depending on the molar ratio of cGP to IGF-1, cGP was shown to enhance (high cGP:IGF-1), maintain (equimolar cGP:IGF-1), or reduce (low cGP:IGF-1) the release of IGF-1 from IGFBP-3 [34]. These data could not explain the complex interactions between cGP, IGF-1 and IGFBP, however, it was suggested that GPE, being enzymatically unstable, may transform to a more stable isoform, cGP, which may interact with IGFBP to modulate the bioavailability of IGF-1 in order to maintain homeostatic IGF-1 function (Figure 1.2). To further confirm the role of cGP in normalizing IGF-1 function, cGP was shown to inhibit IGF-1-dependent tumor growth in a mouse model of lymphoma [34]. Taken together, these studies suggest that cGP is a significant factor in the auto-regulation of IGF-1 function, and normalizes IGF-1 function under pathophysiological conditions of excessively elevated (tumor) or diminished (HI injury) IGF-1 bioactivity (Figure 1.2). However, it is not known whether cGP can alter the physiological changes in IGF-1 function under normal conditions. Because the post-lactational involution of mammary glands is associated with a dramatic reduction in IGF-1 function (see section 1.3.6), therefore, chapter 5 of this thesis provided an opportunity to evaluate the role of cGP in modulating the physiological changes in IGF-1 function.
Chapter 1: Introduction

Figure 1.2: Exogenous Administration of cGP Normalizes IGF-1 Function under Pathophysiological Conditions of Elevated or Diminished IGF-1 Function.

The cGP forms the major IGFBP-binding site of IGF-1. Therefore, as a metabolite of IGF-1, cGP may retain its ability to bind IGFBP in similar affinity to that of IGF-1. Hence, it is proposed that the molar ratio of cGP to IGF-1 determines the competitive interactions of cGP and IGF-1 to IGFBP. (A) Flowchart showing balanced regulatory axis under normal physiological conditions, in which cGP to IGF-1 molar ratio dynamically regulates the amount of free, bioavailable IGF-1 (IGF-1 bioavailability). (B) Under pathophysiological conditions of IGF-1 deficiency, such as during the recovery from brain injury, exogenous cGP administration increases cGP to IGF-1 ratio, thus reducing the binding between IGF-1 and IGFBP-3, and increasing the bioavailability of IGF-1. (C) Pathophysiological states of elevated free IGF-1, such as tumorigenesis, may involve reduced enzymatic breakdown of free IGF-1, leading to reduced cGP to IGF-1 ratio. Exogenous cGP administration restores the physiological ratio between cGP and IGF-1, thus leading to an increase in the binding between IGF-1 and IGFBP-3, and normalization of IGF-1 bioavailability. The effect of exogenous administration of cGP on the physiological changes in IGF-1 function under normal conditions is not known, and is thus addressed in Chapter 5 of this thesis. Abbreviations: cGP: Cyclic-glycine-proline; IGF-1: Insulin-like growth factor-1; IGFBP: Insulin-like growth factor binding protein. Adapted with permission [29].

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1.1.3 IGF-1 and postnatal brain development

The postnatal period of brain development is dominated by cell maturation, synaptogenesis and myelination in both humans and rodents [69,70]. Postnatal days 1-3 in rodents (human gestation week 23-32; pre-term infant) are characterized by the formation of BBB, development of immune system, and increased proliferation of pre-oligodendrocytes. Postnatal days 7-10 (human gestation week 36-40; term infant) involve development of oligodendrocytes to an immature state, maximal gliogenesis, and increased growth of axons and dendrites. During postnatal days 10-20 (1-2 year old human), rapid increase in myelination occurs and growth rate of cells decreases. Between postnatal days 20-21 (2-3 year old human), 90-95 % of adult brain weight is reached, which is accompanied by maximal synaptogenesis (more than 50 % of adult levels) and myelination. Synaptic density reduces between postnatal days 35-49 (12-18 year old human) and reaches adult levels by postnatal day 60+ (20+ year old human), whereas myelination continues. Brain matures between postnatal days 90-100 in rodents [69], but continues to develop in humans up to 30 years of age [71].

IGF-1 is primarily involved in synaptogenesis, myelination and neurogenesis during the postnatal period of brain development. IGF-1 plays a critical role in synaptogenesis, which may involve the PI3K-Akt signaling pathway [48]. Compared with the control mice, overexpression of IGF-1 in transgenic mice caused a 42-105 % greater increase in synapse number in dentate gyrus from infancy to adulthood [72]. IGF-1 overexpression also caused an increase in the total number of neurons, however, calculations of the synapse-to-neuron ratio showed that the effect on synaptogenesis was much more profound than on neurogenesis [72]. In addition to synaptogenesis, IGF-1 also promotes the efficacy of synaptic transmission [73]. The role of IGF-1 in promoting synaptic plasticity, and therefore learning and memory, are
discussed in detail in section 1.2.4. IGF-1 is involved in myelin formation as it promotes the maturation of oligodendrocytes [74]. IGF-1 also promotes neurogenesis in the sub-ventricular zone (SVZ) and dentate gyrus (DG) sub-regions of hippocampus at adulthood. This is evident from reduced adult neurogenesis in the SVZ and DG regions of IGF-1 and IGF-1R knock-out mice [75,76], and in mice with low-serum IGF-1 [77]. The role of cGP in postnatal brain development has not been investigated, and therefore, forms one of the aims of this thesis (Chapter 4).

Both central and peripheral IGF-1 play crucial roles during postnatal brain development. However, the trophic support provided by IGF-1 decreases with advancing age [78]. For instance, in humans and rodents, the protein and mRNA expression of both central and peripheral IGF-1 are low during infancy, peak at puberty and then decline with advancing age [79]. Furthermore, the age-related decline in IGF-1 is associated with the hippocampal-dependent learning and memory deficits associated with aging [79,80], which can be prevented through central [81] or systemic [82] administration of IGF-1 in aged rodents. As an endogenous dipeptide, cGP has been identified in the adult rat brain [36]. However, the presence of endogenous cGP in circulation, and the developmental changes in the concentrations of peripheral and central cGP have not been investigated. Due to the technical difficulties in accurately determining cGP concentrations in tissue samples, this thesis only investigated the developmental changes in the peripheral concentrations of cGP in rats (Chapter 3).

1.1.4 Summary of IGF family

The IGF family includes IGF-1 and -2, IGF receptors (IGF-1R, IGF-2R), and IGFBP 1-6. IGF-1 is a well-known neuroprotective agent, and plays a crucial role in learning and memory.
However, the therapeutic applications of IGF-1 are limited due to its poor central uptake and potential metabolic (e.g. hypoglycemia) and mitogenic side-effects. Free IGF-1 (not bound to IGFBP) is enzymatically cleaved to GPE, which is comparatively a smaller molecule and shows better central uptake after brain injury. However, GPE is enzymatically unstable (plasma half-life < 4 min), and is cleaved to produce the final product, cGP. Owing to its cyclic structure and lipophilicity, cGP shows more enzymatic stability (plasma half-life ~ 2 h) than GPE, and effective central uptake even under normal conditions. By altering the binding between IGF-1 and IGFBP, cGP regulates the bioavailability of IGF-1, and is shown to normalize IGF-1 function under pathophysiological conditions of excessively elevated (tumor) or diminished (brain injury) IGF-1 bioactivity. IGF-1 plays a crucial role in early postnatal brain development, primarily synaptogenesis and myelination, and its concentration declines with advancing age. The developmental changes in the peripheral concentrations of endogenous cGP, and its role in early postnatal brain development needs to be investigated, and thus form the aims of this thesis.

1.2 Learning and memory

Memory systems can be classified into two types based on their anatomical and functional differences - the declarative or explicit memory system and the procedural or implicit memory system [83]. Declarative memory or the conscious memory for remembering facts and events, is contrasted with the procedural memory abilities that do not require conscious awareness for retrieval, for instance skills (e.g. motor sequence-based tasks) and habits [84]. Because this thesis investigates the effect of cGP on two forms of declarative memories, the recognition and the spatial memory, therefore the brain regions involved in this form of memory are reviewed in the following section.
1.2.1 Brain regions involved in learning and memory

Hippocampus and striatum (in conjunction with their associated cortical areas) are the major brain regions involved in the acquisition and consolidation (the process of transformation of fresh memories into more robust and enduring forms) of declarative memories. Behavioral tests such as the novel object recognition test (NORT) [85] and the morris water maze (MWM) test [86] are widely used to evaluate the recognition memory and the spatial memory in rodents, respectively. These tests are, therefore, used in the current thesis.

Hippocampus

Hippocampus is a folded structure situated in the temporal lobe of the brain. It is well established that hippocampus plays a key role in the acquisition and consolidation of declarative memories [87]. For instance, hippocampus-lesioned rats were severely impaired on the MWM task suggesting that hippocampus plays a critical role in spatial learning and memory. Furthermore, the volume of hippocampal damage directly correlated with the impairment in spatial learning [88,89]. Improved spatial memory in adult offspring of choline supplemented dams is shown to be associated with improved capacity for hippocampal plasticity [90,91]. Studies investigating the effect of early-life stress on cognitive dysfunction of adult brain have also shown that the cognitive impairment is associated with affected hippocampal structure [92]. For instance, poor performance in a MWM task was observed in adult offspring born to rats that were stressed either during gestation [93-95] or lactation [96,97]. These deficits in spatial learning were associated with altered hippocampal structure, which could be observed as early as postnatal day 1 [98] and lasted up to 22 months of age [99]. Similarly, in humans, childhood maltreatment has been shown to impair the development of hippocampus, resulting in cognitive deficits in later life [100].
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Hippocampal place cells help the animal to navigate through its environment by forming cognitive maps (such as during spatial navigational planning in a MWM task), because these cells become active in a location-specific manner [101-103]. Repeated replay of neuronal activity in these cells, especially during sleep, has been shown to result in the consolidation of declarative memories [for reviews see 104,105].

**Striatum**

Striatum forms a sub-set of basal ganglia, a group of subcortical nuclei located at the base of forebrain. Striatum receives several projections from hippocampus, prefrontal cortex and amygdala [106], and plays a crucial role in spatial learning and memory [107]. For instance, striatum is involved in the processing of spatial information acquired early during the MWM task [108], and in the consolidation of the procedural aspects of the task (i.e. executing the exploratory behaviors and acquiring spatial information) [109]. Furthermore, striatal α-amino-3-hydroxy-5methyl-4-isoxazole propionic acid (AMPA) receptors are required for spatial learning [107], and a reduction in striatal glutamate receptor-1 (GluR-1) expression, a sub-unit of AMPA receptors, is shown to be associated with learning and memory deficits in neurological disorders such as schizophrenia [110]. Similar to hippocampus, striatum is also involved in sleep-related memory consolidation. For example, striatum was found to be more activated in subjects in a retest session following overnight sleep as compared to similar periods of sleep deprivation [111,112].

Both the hippocampus and striatum are critically involved in spatial navigation. While the hippocampus acts as a filter system for selectively moving short term memory into long term storage, the striatum acts in consolidating memory for forming ‘habits’ of way to navigate [113]. The specific involvement of dorsal and ventral striatum in MWM has been an area of
some controversy. Some studies suggest that dorsal striatum is only involved in spatial learning and navigational strategies during MWM [108], and ventral striatum is essential for memory consolidation [109], while some others suggest that dorsal striatum is crucial for both spatial learning and memory retention [114]. The lack of well-defined anatomical or neurochemical boundaries between the dorsal and ventral striatum in rodents, and the differences in the regions of striatum examined as well as the manipulations used to examine these regions is the underlying cause of this controversy. Membrane trafficking of AMPA receptors in the hippocampus is crucial for spatial learning and memory [115]. For example, MWM training is associated with an increase in the expression of GluR-1 in the hippocampus [116]. Striatal GluR-1 expression is also essential for learning and memory [110], therefore, in this thesis we will examine the effect of cGP on GluR-1 and other markers of neuroplasticity in both striatum and hippocampus.

**Pre-frontal cortex**

Several anatomical connections link the prefrontal cortex to the hippocampus via the entorhinal cortex, which is essential for memory consolidation and retrieval [117,118]. For instance, the AMPA receptor-mediated glutamatergic neurotransmission in the prefrontal cortex is essential for the formation of immediate and long-term memory [119].

**1.2.2 Neuropharmacology of learning and memory**

Neurotransmitters are a group of endogenous chemicals that are required for transmitting a neural signal across a synapse to an adjacent neuron or a target cell [120]. Several neurotransmitters are required for cognitive functioning in humans and animals. This is evident from functional reductions in the activities of neurotransmitters in several degenerative conditions that are marked by cognitive impairments. For instance, several neurotransmitter
systems, including the glutamatergic, cholinergic, noradrenergic and serotonergic, are impaired in patients suffering from Alzheimer’s Disease [121]. Major neurotransmitters involved in learning and memory include: amino acids (both excitatory (e.g. glutamate, aspartate) and inhibitory (e.g. γ-aminobutyric acid, GABA)), monoamines and other biogenic amines (such as dopamine, norepinephrine, epinephrine and serotonin), peptides (such as opioid peptides and adenosine), and esters (e.g. acetylcholine) [87]. A detailed review of all these neurotransmitters and their role in learning and memory is beyond the scope of this review. The reader is referred to extensive reviews by several other authors on this topic [87,122-124]. Considering the well-studied effects of IGF-1 and cGP on glutamatergic neurotransmission, particularly the AMPA-mediated glutamatergic neurotransmission, the following section reviews the glutamate mediated neurotransmission with a focus on AMPA receptors.

Glutamate is the dominant excitatory neurotransmitter that plays a crucial role in learning and memory. Glutamate is produced either through the transamination of 2-oxoglutarate or via glutaminase-mediated degradation of glutamine. Glutamate released from the pre-synaptic vesicles binds to the glutamate receptors located on the post-synaptic membrane. Glutamate may bind to different types of glutamate receptors, which can be broadly categorized into two groups: ionotropic and metabotropic receptors. Ionotropic receptors are ligand-gated ion channels, which allow the exchange of ions (Na+, K+ and Ca2+), and thus cause rapid synaptic transmission compared with the metabotropic receptors which use G-protein mediated second messenger signaling. Ionotropic receptors include AMPA, N-methyl-D-aspartate (NMDA), kainate and ‘orphan’ receptors [125]. AMPA receptor complex is a tetramer consisting of subunits GluR-1 to GluR-4 (also known as GluR-A to GluR-D) [126]. All four receptor subunits are expressed in the hippocampus, striatum and pre-frontal cortex of rats [127,128].
Evidence for the role of AMPA receptors in learning and memory comes from studies in rodents where blockage of AMPA receptors, using either 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo (f) quinoxaline (NBQX) [129,130] or LY326325 [131], is shown to impair spatial learning and memory in a MWM task. Conversely, positive AMPA receptor modulators are shown to improve learning and memory of rats in several behavioral tasks [132-134]. Similarly, oral administration of CX516, an ampakine, is show to improve memory in healthy males of 20-35 years of age [135]. Transgenic GluR-1-knockout mice show severe impairments in spatial memory in a radial maze [136] and a T-maze task [137], which can be restored through genetic rescue of GluR-1 expression [138]. GluR-1 is also shown to be indispensable for long-term memory formation [116].

1.2.3 Neuroplasticity and memory

Neuroplasticity refers to the ability of the brain to reorganize its structure, function and circuitry in order to better adapt to changing demands and surrounding environments [139], and is critical for learning and memory. Neuroplasticity may involve re-circuiting of neural networks, either through the generation and integration of new synapses into the neural circuitry (synaptogenesis) or changes in the signaling between existing neurons (synaptic plasticity), generation of new neurons (neurogenesis), movement of newly generated neurons into specific locations within the cortex (neuronal migration), and/or a change in the neurotransmitter expression of existing neurons in the post-developmental stages (neurotransmitter switching) [120,140-142].

Of all forms of neuroplasticity, synaptic plasticity is the most widely studied in the processes of learning and memory. Synaptic plasticity refers to the strengthening or weakening of synapses over time, in response to the enhancement or the reduction in their activity,
respectively [143]. Therefore, synaptic plasticity forms one of the important neurochemical basis of learning and memory. Synaptic plasticity can be broadly categorized into short-term and long-term plasticity. Short-term plasticity refers to the short-lasting strengthening (facilitation) or weakening (depression) of a synapse which lasts for a few minutes or less [144]. For instance, synaptic facilitation occurs when two or more action potentials stimulate a pre-synaptic terminal within a few milliseconds, causing a build-up of Ca\(^{2+}\) in the pre-synaptic terminal, and thus causing a greater release of neurotransmitter following the subsequent action potential. Synaptic depression is associated with reduced release of neurotransmitter due to the ensuing depletion of synaptic vesicles after synaptic facilitation. Due to the short-lived changes in synaptic transmission, short-term plasticity is usually associated with only short-term memory function [144].

Long-term plasticity refers to the long-lasting strengthening (long-term potentiation (LTP)) or weakening (long-term depression (LTD)) of synaptic transmission, based on recent patterns of synaptic activity [145]. The expression of LTP/LTD, the experimental models of long-term plasticity, has been extensively studied in the hippocampus of rodents [146]. LTP/LTD occur over time scales of 30 min or more, and can lead to permanent changes in brain function [145]. The induction of LTP involves the inflow of Ca\(^{2+}\) into the post-synaptic terminal through the NMDA receptor (Figure 1.3) [for review, see 145]. However, this requires the removal of Mg\(^{2+}\) ions that block the pore of NMDA receptors under resting conditions. During the low-frequency synaptic transmission, the post-synaptic membrane is at the normal resting potential and the pore of the NMDAR is blocked by Mg\(^{2+}\). In this case, glutamate binds to both the NMDA and AMPA/kainite-type receptors, however, the exchange of ions (Na\(^{+}\) and K\(^{+}\)) and consequently the excitatory post-synaptic potential (EPSP), only occurs through the AMPA/kainite-type receptors (Figure 1.3). On the other hand, during the high-frequency
stimulation, the summation of EPSPs causes depolarization of the post-synaptic membrane that leads to the removal of the Mg$^{2+}$ block. Unblocking of the NMDA receptor pore causes the influx of Ca$^{2+}$ ions into the post-synaptic terminal which triggers LTP (Figure 1.3). This also implies that for LTP to occur the pairing of pre- and post-synaptic activities is necessary, i.e. the release of glutamate from the pre-synaptic terminal needs to coincide with the depolarization of the post-synaptic membrane in order to remove the Mg$^{2+}$ block from NMDA receptor. This process is sufficient for induction of LTP, however, the maintenance of LTP, or in other words, the strengthening of synaptic transmission during LTP, is dependent on downstream intracellular signaling which ultimately leads to the trafficking of AMPA receptors to the post-synaptic membrane (Figure 1.4). The downstream intracellular signaling cascade that leads to maintenance of LTP is currently one of the most active areas of research in cognitive neuroscience [115]. The influx of Ca$^{2+}$ activates a number of protein kinases, such as Ca$^{2+}$/calmodulin-dependent protein kinase (CaMKII) and protein kinase C (PKC), A (PKA) and G (PKG) (Figure 1.4). For a long-time the activation of CaMKII was believed to be both essential and sufficient for LTP, leading to the proposal of CaMKII as the ‘memory molecule’ [147]. However, this model was challenged with the discovery that the activation of CaMKII is a transient process during the induction of LTP, with activity returning to baseline within few minutes [148]. This, in turn, led to the hypothesis that the phosphorylation-dephosphorylation of downstream substrates by CaMKII and/or other protein kinases may be critical for the maintenance of LTP and LTD. It is now known that CaMKII and PKC cause phosphorylation of a number of downstream substrates, including the direct phosphorylation of GluR-1 subunit of AMPA receptors [149], which ultimately lead to the trafficking of AMPA receptors to the synapse (Figure 1.4). This dynamic membrane trafficking of AMPA receptors increases the postsynaptic response to the glutamate released from the pre-synaptic
terminal, leading to the strengthening of synaptic transmission. Conversely, the synaptic transmission can be weakened through increased lysosomal degradation of AMPA receptors within the post-synaptic terminal [150]. This requires the activation of protein phosphatases in response to a low-amplitude increase in post-synaptic $Ca^{2+}$ concentration.

As mentioned before, LTP can cause a permanent change in synaptic transmission. This involves the activation of certain transcription factors, notably cAMP response element-binding protein (CREB), by PKA which ultimately lead to the synthesis of new proteins, including more protein kinases, AMPA receptors and other transcriptional regulators (Figure 1.5) [151]. These new proteins create new synaptic contacts, thus making the synaptic potentiation permanent. LTP is shown to be essential for the formation of long-term memory, including spatial memory. For instance, deficits in spatial memory were observed through antagonist-mediated blocking of NMDA receptors [152], blocking of the CaMKII- and PKA-specific phosphorylation sites of GluR-1 [153], and the disruption of membrane trafficking of AMPA receptors [154].
Figure 1.3: Induction of Long-term Potentiation (LTP)

During the normal (low-frequency) synaptic transmission (left panel), the post-synaptic membrane is at the resting potential and the pore of NMDA receptor is blocked by Mg$^{2+}$. In this case, glutamate binds to both the NMDA and the AMPA/kainite-type receptors, however, the exchange of ions (Na$^+$ and K$^+$) and consequently the excitatory post-synaptic potential (EPSP), only occurs through the AMPA/kainite-type receptors. However, during the high-frequency stimulation (right panel), the summation of EPSPs causes depolarization of the post-synaptic membrane, that leads to the removal of Mg$^{2+}$ block. Unblocking of the NMDA receptor pore causes the influx of Ca$^{2+}$ ions into the post-synaptic terminal which triggers LTP. Adapted with permission [145].
Following the induction of LTP, the Ca$^{2+}$ ions cause activation of a number of protein kinases, including the Ca$^{2+}$/calmodulin-dependent protein kinase (CaMKII) and protein kinase C (PKC). These kinases cause phosphorylation of a number of substrates that ultimately lead to the trafficking of AMPA receptors to the post-synaptic membrane, resulting in an increased sensitivity of post-synaptic spine to glutamate. Adapted with permission [145].
Late phase of long-term potentiation (LTP) involves the activation of certain transcription factors, notably cAMP response element-binding protein (CREB), by PKA which ultimately lead to the synthesis of new proteins, including more protein kinases, AMPA receptors and other transcriptional regulators. These new proteins create new synaptic contacts, thus making the synaptic potentiation permanent. LTP is, therefore, essential for the formation of long-term memory, including spatial reference memory. Adapted with permission [145].

1.2.4 Roles of IGF-I and cGP in learning and memory

Both central and peripheral IGF-1 are indispensable for learning and memory function of brain [155,156]. For instance, the age-related decline in peripheral and central IGF-1 is associated with learning and memory deficits observed during aging [23,79,80], which can be prevented through central [81] or systemic [82] administration of IGF-1 in aged rodents. Furthermore, blocking the central production of IGF-1 [157], and the deficiency in peripheral IGF-1 [54] is shown to impair learning and memory in transgenic rodents. IGF-1 deficiency in childhood is also associated with impaired adult cognitive function. For instance, adults with childhood-
onset growth hormone deficiency have low growth hormone and IGF-1 levels in the serum, and show impaired learning and memory function [158]. Although the specific molecular mechanisms through which IGF-1 regulates learning and memory are largely unknown, recent studies point to a role of IGF-1 in mediating synaptic plasticity through AMPA receptors [78,159].

IGF-1 is shown to enhance the AMPA-dependent glutamatergic synaptic transmission in the hippocampus of juvenile [160], young adult and old rats [159]. Recently, IGF-1 was shown to persistently enhance LTP in a rat model of depression that was associated with an increase in GluR-1 expression in hippocampus, in an NMDA-independent fashion [161]. Conversely, IGF-1 is also shown to induce LTD in mice by reducing the glutamatergic neurotransmission through increased endocytosis of GluR-2 containing AMPA receptors [162]. Although evidence suggests that IGF-1 actions on synaptic plasticity may be NMDA-independent, some studies point to a role of IGF-1 in modulating the expression of NMDA receptor sub-units. For instance, IGF-1 administration prevented the cognitive decline in aged rodents [81] via restoration of NMDA expression [163,164]. Therefore, IGF-1 is a critical regulator of learning and memory, and may mediate its actions by modulating glutamatergic synaptic plasticity.

cGP belongs to the 2,5-diketopiperazine family of cyclic dipeptides, which are shown to possess nootropic properties, i.e. the ability to enhance learning and memory [165]. Indeed, cGP analogue, cG-2allyl-P, is shown to prevent the scopolamine-induced acute memory deficits in rats in a MWM task, through improvement in cholinergic neurotransmission [166]. Similarly, cG-2allyl-P is shown to prevent the memory deficits in aged rats in a MWM task, through an increase in the expression of GluR-1 in the hippocampus [167]. Furthermore, using a passive avoidance task, cGP is shown to reduce retrograde amnesia in rats following
maximal electroshock [36] and electroconvulsive shock [168], in which rats were given a 70 V electric shock for 300 msec. Despite being a potent nootropic agent, the anti-amnesic role of cGP has only been investigated in conditions with either induced memory deficits or as a prevention to neurological insults. Therefore, the evaluation of early life administration of cGP on neurodevelopmental programming of offspring provides an opportunity to investigate the pro-cognitive effects of cGP in a normal brain.

1.2.5  **Summary of Learning and Memory**

Hippocampus, striatum and pre-frontal cortex play crucial roles in declarative memory formation. Glutamate is the dominant excitatory neurotransmitter released from the pre-synaptic terminal, which binds to the glutamate receptors such as the NMDA, AMPA and/or kainate receptors on the post-synaptic membrane. Synaptic plasticity forms the underlying neurochemical basis of short- and long-term memory. The influx of Ca$^{2+}$ through the NMDA receptor, membrane trafficking of AMPA receptors, and the CREB-mediated transcription of synaptic growth proteins, including more AMPA receptors, is essential for the formation of long-lasting or permanent memory. Evidence from brain injury models suggest that both IGF-1 and cGP play important roles in learning and memory, possibly through the effects on AMPA-mediated synaptic plasticity. However, the role of cGP on neurodevelopmental programming of normal brain has not been investigated, and therefore, forms the major aim of this thesis.

1.3  **Post-lactational involution of mammary gland**

1.3.1  **Anatomy of mammary gland**

The parenchyma of a lactating mammary gland is composed of epithelial structures, including the lobulo-alveolar clusters and ducts, and the surrounding stromal connective tissue (Figure
An alveolus is the basic functional unit of the mammary gland where milk production and secretion occurs. Each alveolus has a hollow lumen lined by a single layer of about 300 polarized mammary epithelial cells (MEC). About 200 alveoli cluster together to form structures known as lobules, which group together to form a lobe. About 15-20 lobes are present in an adult female breast. Each alveolus in a lobule is drained by an intralobular duct, with each lobule having its own interlobular duct. Alveoli are surrounded by myoepithelial cells that contract in response to oxytocin, which is secreted by the posterior pituitary as part of a neuroendocrine reflex generated by an infant’s suckling stimulus. This causes the expulsion of milk from the alveolar lumen into the ductal system, which ultimately drains the milk into the teat (rodents and ruminants) or the nipple (humans and rabbits). Alveoli are further surrounded by a basement membrane which separates them from the stromal connective tissue sheath. The stroma contains fibroblasts, collagen and other connective tissue proteins, white adipose tissue (the fat pad) and blood vessels. Except lactation, the fat pad is highly prevalent during the early phases of fetal development, pregnancy and the late stages of involution.
Chapter 1: Introduction

Figure 1.6: Schematic Illustration of the Anatomy of a Mammary Gland.

The figure depicts the anatomy of a mammary gland with a single lobe illustrated. An alveolus is enlarged to show the lumen, milk secreting mammary epithelial cells, myoepithelial cells, basal membrane, blood capillaries and an intralobular duct. Modified from http://nydairyadmin.cce.cornell.edu/uploads/doc_113.pdf.

MEC are cuboidal in shape and possess a well polarised structure [58,170]. The apical cytoplasm of MEC is full of secretory vesicles containing casein micelles and lipid droplets. Figure 1.7 illustrates various milk secretion pathways that operate in a MEC during lactation, including the transcytotic pathway (III) through which IGF-1 enters the milk.
Figure 1.7: Schematic Representation of a Lobule, a Mammary Epithelial Cell (MEC), and the Milk Secretion Pathways.

The figure illustrates a lobule containing four alveoli and a duct (left panel), and the ultrastructure of a MEC as interpreted from electron micrographs (right panel). In the left panel, arrows inside the lobule show the direction of milk flow. In the right panel, the five milk secretion pathways operational in a MEC are numbered from I-V. Exocytotic or the Golgi pathway (I) is the primary pathway for the secretion of the aqueous fraction of milk, such as milk proteins (e.g. casein, whey proteins), oligosaccharides, lactose, calcium and phosphate. In the milk fat pathway (II), coalesced lipid droplets (LD) are secreted as milk fat globules (MFG), which are completely enveloped in the apical plasma membrane of MEC [190]. Examples include milk fat, lipid soluble hormones and drugs, and lipid-associated proteins. Through the transcytotic pathway (III), many proteins, hormones and growth factors (such as IGF-1) derived from serum or stromal cells find their place in milk. The transmembrane pathway (IV) is mainly used by the small molecules for transport across the apical plasma membrane (and the basal membrane for blood-borne substances) into the alveolar lumen. Examples include ions (such as Ca²⁺, K⁺, Na⁺, Cl⁻), glucose, amino acids and therapeutic drugs. The paracellular pathway (V) offers a direct, bi-directional exchange of both micro- and macro-molecular solutes between the interstitial fluid and the milk. Abbreviations: TJ, tight junction; GJ, gap junction; SV, secretory vesicle; LD, coalesced lipid droplet; AP, apical membrane; MFG, membrane bound milk fat globule; RER, rough endoplasmic reticulum; ME, myoepithelial cell; N, nucleus; BM, basement membrane; PC, plasma cell; FDA, fat depleted adipocyte. Adapted with permission [58].
1.3.2 Lactation and post-lactational involution

The developmental changes in mammary gland during lactation and post-lactational involution can be categorized into four phases. A “lactation curve” illustrates these changes through a difference in the daily milk yield as a function of time postpartum [171,172]. The lactation curves of different species vary quantitatively, but qualitatively they are similar. Figure 1.8 shows a typical lactation curve based on a logistic model of lactation data collected from dairy cows [171]. Phase I occurs immediately after parturition and is characterized by a rapid increase in milk production [173]. During phase II the milk production increases at a diminishing rate, reaching the peak level of milk production for the entire lactation cycle. Following peak lactation, there is a gradual decline in milk yield which can be observed in two distinct loss-of-function phases, phases III and IV. Increase in cell proliferation and/or secretory activity per cell is attributed to the increased milk output during phases I and II [174,175]. Conversely, increased cell apoptosis, loss of MEC biosynthetic capacity and the effect of maternal physiological factors, for instance pregnancy, causes the decline in milk production following peak lactation [175-178]. Phases III and IV are also described as the reversible and irreversible phases of mammary involution, respectively, and are discussed in detail in the next section.
Figure 1.8: The Lactation Curve.

The figure shows a typical lactation curve based on a logistic model of lactation data collected from dairy cows. It is represented by four distinct phases. The first two phases (I and II) are gain-of-function phases, characterized by increased milk production owing to increased cell proliferation and/or secretory activity per cell. Phases III and IV, also known as the reversible and the irreversible phase of mammary involution, respectively, are loss-of-function phases. These are characterized by a decline in milk production owing to epithelial cell apoptosis and tissue remodeling, and a sudden decline in the expression of IGF-1. Abbreviations: PL, peak lactation. The axes are not to scale. Adapted with permission [171].

1.3.3 Stages of involution

Involution is defined as the post-lactational regression of the mammary gland [179]. Involution occurs naturally following weaning, but can also be induced at any stage of lactation through complete cessation of milk removal in dairy animals, or by pup removal or teat sealing in rodents [176]. Natural involution occurs at about half the rate of the induced involution [41], and does not involve chronic milk stasis because of the autocrine inhibition of milk secretion, owing to irregular milk removal [180].

The experimental induction of involution has enabled the categorization of involution into two distinct phases, shown as phases III and IV in Figure 1.8. Different mechanisms govern the processes occurring during these two stages [179,181]. The first phase of involution (phase III
in Figure 1.8) follows peak lactation during natural weaning [182], or begins within 12 h of pup removal in rodents and lasts for about 72 h [183,184]. This phase is characterized by the engorgement of mammary gland due to milk accumulation within the alveoli and the ducts, and a subsequent fall in the level of galactopoietic hormones. The result is a rapid decline in milk synthesis and secretion, down-regulation of differentiated gene expression and the loss of MEC [177,183]. The loss of MEC is primarily through apoptosis, though autophagy [185,186], lysosomal-mediated pathway of cell death [187], and at least ten other genetically programmed cell death pathways have also been shown to occur in different situations [188]. The first phase of involution is reversible within 48 h of mammary engorgement as demonstrated through re-suckling of engorged glands in rats [184,189,190]. Local mammary-derived signals, rather than the circulating hormones, are implicated in the regulation of the first phase of involution [179]. For instance, teat-sealing, which causes mammary engorgement while maintaining the suckling stimulus and the level of galactopoietic hormones, is unable to prevent the loss of MEC through apoptosis [176,184,191]. This is further supported by the serial litter replacement experiments [184]. Similarly, artificial administration of galactopoietic hormones (such as glucocorticoids) does not prevent apoptosis, though it delays the entry into the second phase of involution [183,184,192].

The second phase of mammary involution (phase IV in Figure 1.8) begins after 72 h of mammary engorgement in rodents. It involves the activation of extracellular matrix (ECM)-degrading proteases, including matrix metalloproteinases (MMP) such as gelatinase A, stromelysin-1 and urokinase-type plasminogen activator [183,184], probably due to a fall in the level of systemic hormones [179]. Tissue inhibitors of metalloproteinases, which block MMP function during the first phase of involution, are downregulated during this stage [183,193]. MMP cause destruction of the basement membrane and the alveolar structures,
which is accompanied by an invasion of the mammary fat pad, recruitment of macrophages for clearing cellular debris and active plasma kallikrein that regulates fat cell differentiation and tissue remodeling [194,195]. Apoptosis continues, probably in response to anoikis, until about 50-80% of the MEC are cleared from the gland [196]. Therefore, this phase of involution is described as irreversible, with only limited recovery of lactation after 4 d of pup removal in rodents [183,190].

1.3.4 Cell death pathways during involution

As mentioned in the previous section, local mammary-derived signals are the trigger for the first, largely apoptotic, phase of involution [184]. These signals may include mechanical stretching of the alveolar epithelium [197], and/or the accumulation of pro-apoptotic factors within the alveolar lumen during milk stasis, leading to the activation of a number of cell death pathways. These pathways are required for the apoptosis of MEC, and includes the leukemia inhibitory factor (LIF)-signal transducer and activator of transcription 3 (STAT 3) axis, transforming growth factor beta3 (TGF-β3) axis, death receptor axis, integrin adhesion receptors and cell-cell adhesion receptors [198-200]. A detailed discussion of these signaling pathways is beyond the scope of this review, and the reader is referred to extensive reviews by others [201] [202] [203]. Using gene expression studies, it is shown that none of these pathways are sufficient to trigger apoptosis, and a crosstalk is required between these pathways for cell death to occur [179,201,202].

The cell death pathways act as the initiating events for apoptosis since they all lead to the activation of intracellular cysteine proteases known as caspases, which are the central executioners of apoptosis [201,202,204]. However, caspase-independent cell death pathways have also been described [205,206]. The activation of caspases is mediated either through a
direct (Extrinsic) or an indirect (Intrinsic) route. Both extrinsic and intrinsic pathways operate during the involution of mammary gland. For instance, initiator caspases (such as caspase 8 and 10) are activated through regulated protein-protein interactions using the extrinsic pathway [207]. The extrinsic or the death-receptor pathway involves binding of the ligands (e.g. Fas ligand) to their receptors (e.g. Fas receptor), resulting in receptor-trimerization and the subsequent formation of plasma membrane signaling complexes. These complexes then cause an aggregation and activation of several initiator caspases directly, which can further cause an activation of the downstream effector caspases, thereby causing cell death. The intrinsic pathway of caspase activation, which can be induced by intracellular stressors such as oxidants, employs a more complex activation mechanism, involving the release of multiple proteins, including cytochrome C and Smac/Diablo, from the mitochondrial intramembrane space into the cytosol (Figure 1.9) [208-210]. Once in the cytosol, diablo acts to get rid of the IAP (inhibitor-of-apoptosis protein) molecules, which prevent the spontaneous activation of procaspase 9 in a healthy cell [209,210]. Cytochrome C then oligomerizes the regulatory subunit of caspase-9 holoenzyme, Apaf-1 (apoptotic protease-activating factor-1) [208,211]. Procaspase-9 then binds to the Apaf-1 oligomers resulting in the formation of a large caspase-9 holoenzyme, also referred to as the apoptosome [212-214]. Once activated, the initiator caspases then activate the effector caspases (caspases 3, 6 and 7) proteolytically, which causes cell death owing to latter’s protease activity [201,202,215]. Caspase 3 is shown to be highly expressed during the involution of mammary gland, suggesting a major role for this protease in mammary cell destruction [216]. Cleaved caspase-3 is, therefore, widely used as a reliable marker of apoptosis during mammary gland involution [217], and is also used in Chapter 5 of this thesis.
The release of cytochrome C and diablo proteins from the mitochondrial intramembrane space into the cytosol is dependent on the formation of transmembrane channels within the outer mitochondrial membrane, a process which is regulated by the B-cell leukemia/lymphoma 2 (Bcl-2) family members (Figure 1.9). The Bcl-2 family consists of three groups [201,202,215,218]. Group 1 contain proteins that possess anti-apoptotic activity (examples include Bcl-2, Bcl-x, Bcl-w), whereas groups 2 and 3 proteins exhibit pro-apoptotic activity. Examples include, Bax (BCL2-associated X protein) and Bak (BCL2-associated K protein) in group 2, and Bcl-2-homology domain-3 (BH3)-containing proteins such as BAD (BCL2-associated D protein) in group 3. Bax and Bak homo-oligomerize to form the transmembrane channels during apoptosis (Figure 1.9) [219]. In a healthy cell, however, these proteins are either present outside the mitochondria (Bax) [220] or are present in a latent form due to binding with the anti-apoptotic protein Bcl-x (Bax and Bak) [221,222]. BH3-only proteins are also present in an inactive, phosphorylated form or are degraded through ubiquitination [201,223]. An apoptotic trigger dephosphorylates or upregulates the BH3-only protein expression, which then binds the anti-apoptotic proteins, Bcl-2 and Bcl-x [201,223]. BH3-only proteins also facilitate the translocation of Bax to the mitochondria, followed by its oligomerization and insertion in the outer mitochondrial membrane [221,224,225]. This leads to the formation of membrane pores that allow protein transport (cytochrome C, Smac/Diablo) into the cytoplasm (Figure 1.9). The next section reviews the signaling mechanisms by which IGF-1 phosphorylates BH3-containing proteins (e.g. BAD), and thus prevent apoptosis.
Figure 1.9: Intrinsic Apoptosis Mechanisms.

Schematic representation of the indirect/intrinsic mechanisms of caspase activation. (a) In healthy cells, caspases are present in a latent form because the IAP (inhibitor-of-apoptosis protein) molecules prevent their spontaneous activation. The proapoptotic proteins, Bax and Bak, are either present outside the mitochondria (Bax) or are inactivated due to binding with the anti-apoptotic protein Bclx (Bax and Bak). BH3-only proteins are also present in an inactive form or are degraded through ubiquitination. (b) An apoptotic trigger stimulates BH3-protein expression, which then binds the anti-apoptotic proteins Bcl-2 and Bclx. This enables the formation of mitochondrial trans-membrane channels by Bax and Bak that allow protein transport (cytochrome C, diablo) into the cytoplasm. Diablo acts to get rid of the IAP molecules, while cytochrome C activates the caspase-9 and apoptotic protease-activating factor-1 (Apaf-1) holoenzyme complex (also called as apoptosome). Apoptosome further activates the effector caspases, such as caspase 3, which causes cell death. Adapted with permission [201].

1.3.5 IGF-1 cell survival signaling

The cell survival effects of IGF-I are mediated through at least four different pathways, which begin with the activation of IGF-IR and end with the phosphorylation of pro-apoptotic protein BAD (Figure 1.10) [226,227]. The main pathway for the cell survival effects of IGF-I is the well-established IRS-1/PI3K/Akt pathway. This pathway begins with the activation of IGF-IR
by IGF-I, which then phosphorylates the adaptor protein IRS-1. IRS-1, in turn, activates PI3K [228], which leads to the phosphorylation of Akt protein (also known as PKB) [49,229,230]. The pathway terminates with the serine phosphorylation of pro-apoptotic protein BAD by Akt [231,232]. Phosphorylated BAD is unable to bind anti-apoptotic protein Bclx at the mitochondrial membrane (Figure 1.9) because it is sequestered by chaperone 14.3.3 in the cytosol, and is thus unable to impart its apoptotic activities [233].

In the absence of IRS-1, IGF-1R operates through alternative pathways [226], for instance, the Ras/Raf/MAPK pathway (Figure 1.10). This pathway involves the activation of substrate protein Shc (Src homology and collagen) by activated IGF-1R [234]. Shc is then bound by an adaptor protein Grb2 (growth factor receptor-bound protein 2), which can also bind IRS-1 protein, thus indicating a possible crosstalk between the PI3K and MAPK pathways. Grb2 then binds a guanine nucleotide-exchange protein Sos (son of sevenless) [235]. Sos loads guanine triphosphate molecules onto the small guanosine triphosphatase protein Ras, resulting in the activation of Ras/Raf/MAPK pathway (Figure 1.10) [226]. The third cell survival pathway involves the translocation of Raf proteins to the mitochondria and relies on the interaction between IGF-1R and 14.3.3 proteins [226]. This pathway also leads to the activation of MAPK pathway and terminates with BAD phosphorylation (Figure 1.10). Another IRS-1-independent signaling pathway involves activation of the MAPK pathway through transactivation of epidermal growth factor receptor (EGFR) by the activated IGF-1R (not shown) [236]. This pathway also plays an important role in the survival of MEC, which is evident from the loss of IGF-1-mediated survival upon treatment of MEC with an EGFR antagonist, ZD1839 [236]. It is believed that IGF-1 activates the MAPK pathway in mammary cells through EGFR transactivation, rather than through the activation of Shc proteins [237].
Chapter 1: Introduction

Schematic representation of the various cell survival/proliferation pathways employed by the insulin-like growth factor 1 (IGF-1) that involve activation of the insulin-like growth factor-1 receptor (IGF-IR). The main pathway is the well-established insulin receptor substrate 1 (IRS-1) / phosphatidylinositol 3’ kinase (PI3K) / protein kinase B (PKB) pathway. However, in the absence of IRS-1, IGF-IR employs alternative pathways which ultimately activate the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway. Importantly, all these pathways begin with IGF-IR activation and end with phosphorylation of pro-apoptotic protein BAD. BAD phosphorylation renders it unable to induce cell death, resulting in cell survival. **Akt**, another name for protein kinase B; **Shc**, Src homology and collagen protein; **Grb2**, growth factor receptor-bound protein 2; **Sos**, son of sevenless protein; **Ras** and **Raf**, serine/threonine protein kinases. Adapted with permission [226].

1.3.6 IGF-1 and involution

IGF-1 is essential for the maintenance of lactation because the loss of IGF-1 mediated survival signaling causes cell death during involution [198]. Boutinaud and co-workers [53] showed that the expression of IGF-1 mRNA is highest during the peak period of lactation in mice, and is rapidly downregulated within 2 days of the onset of involution. This corresponds with the changes in the milk concentrations of IGF-1, which is higher during peak lactation compared
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with late lactation [238]. The presence of endogenous cGP in mammary gland or milk of rodents, and its role in mammary involution has never been investigated. Therefore, this thesis aims to fill these gaps.

The evidence for the role of IGF-1 in mammary involution comes from studies in transgenic rodent models overexpressing IGF-1 under the control of a mammary specific promoter. These studies show that IGF-1 increases the lactational capacity of mammary glands by delaying involution [239]. For instance, Hadsell and co-workers [41,44] showed that over-expression of des-IGF-1 under the control of promoter for whey acidic protein gene (WAP-des) in mammary glands of mice resulted in delayed involution, with minimal loss of lobulo-alveolar structures and reduction in apoptosis. Similarly, Neuenschwander and co-workers [42] showed that mammary-specific overexpression of IGF-1 delayed involution in mice by reducing apoptosis. The IGF-1 mediated inhibition of mammary cell apoptosis involves signaling through the PI3K and MAPK pathways, resulting in the inactivation of pro-apoptotic protein BAD [239]. The mammary specific overexpression of IGF-1 in mice either did not influence [28] or only caused a slight improvement in milk production during lactation [240]. Therefore, IGF-1 may improve the lactational capacity of mammary glands primarily by delaying involution.

The activation of IGFBP during involution causes the loss of IGF-1 mediated survival signaling, leading to the apoptosis of MEC [241]. Indeed, in the study by Hadsell and co-workers [41], the delayed involution in WAP-des mice was found to be due to the increased bioavailability of IGF-1, via a reduction in the interaction between IGF-1 and IGFBP. This was evident through increased phosphorylation of IRS-1 and Akt. Similarly, the increased milk yield in WAP-des mice, determined through the gain in litter weight, during prolonged lactation was also due to the increased bioavailability of IGF-1 as evidenced through increased phosphorylation of IGF-1R [43]. Several IGFBP are upregulated during involution. For
instance, studies in rats have shown a 4-fold, a 6-fold and a 50-fold increase in both the mRNA and protein levels of IGFBP-2, -4 and -5, respectively, within 2 days of pup removal [236,242]. A dramatic 54-fold increase in IGFBP-5 mRNA level is also reported in the mammary glands of mice within 2 days of pup removal [53]. Thus, apoptosis of MEC is invariably associated with a corresponding increase in the levels of IGFBP, the most significant of which appears to be IGFBP-5. Indeed, transgenic mice over-expressing IGFBP-5 show increased caspase-3 cleavage and reduced expression of anti-apoptotic factors Bcl-2 and Bcl-xl (Bcl-x long isoform) [243]. Furthermore, the inhibition of phosphorylation of IGF-1R and Akt in the IGFBP-5 overexpressing mice shows that IGFBP-5 operates by disrupting the IGF-1 cell survival pathways [243]. This is corroborated by the reversal of defect in these mice through exogenous administration of IGF-1 analogue, R3-IGF-1, which shows weak binding affinity for IGFBP-5 [243]. A possible mechanism by which IGFBP may prevent IGF-1 action is through their ability to bind ECM proteins [244], which may sequester IGF-1 away from IGF-1R, thus preventing the activation of IGF-1-mediated survival pathways [245]. As a metabolite of IGF-1, cGP regulates the bioavailability of IGF-1 by altering the binding of IGFBP to IGF-1 [34]. However, the effect of exogenous administration of cGP on mammary involution has never been investigated, and is thus addressed in Chapter 5 of this thesis.

1.3.7 Lactational capacity and cognitive development

Enhanced lactational capacity can positively influence the cognitive development of the offspring. For instance, longer duration of breastfeeding is shown to correlate with higher IQ in children at 1 and 5 years of age, when compared with the shorter duration of breastfeeding [45]. In the same study, the authors also showed a linear relationship between the duration of breastfeeding and the mental developmental index of children, suggesting a dose-response relationship. Bernard and others [46] evaluated the effect of breastfeeding duration on the
overall neurological outcome of children at 3 years of age using the Ages and Stages Questionnaire (ASQ). For every additional month of breastfeeding an increase of 1.0 ASQ points in the exclusive-breastfeeding group and 0.60 ASQ points in the any-breastfeeding group was observed compared with the never-breastfed group. Furthermore, the milk composition also affects the cognitive development of offspring. For instance, a high-nutrient formula, containing higher protein and energy content compared with the standard formula, is shown to increase the IQ of infants at adolescence [246].

This thesis aims to investigate the effect of early life administration of cGP, via maternal supplementation during lactation, on the cognitive function of the offspring in rats. Considering that an increase in lactational capacity can improve the cognitive development of the offspring, therefore, the effect of cGP on lactational capacity of dams is also evaluated. More specifically, the effect on milk production, milk composition and post-lactational involution of mammary glands is investigated (Chapter 5). Because post-lactational involution is associated with a decline in IGF-1 function, therefore, Chapter 5 also provides an opportunity to determine the effect of cGP on the physiological decline in IGF-1 function.

1.3.8 Summary of post-lactational involution of mammary gland

Involution is defined as the post-lactational regression of mammary gland, which, in rodents, occurs naturally after peak-lactation, but can also be induced through teat-sealing or pup removal at any stage of lactation. Involution occurs in two phases. The first, reversible phase (within 48 h of mammary engorgement in rodents), involves a decline in milk synthesis and secretion, and loss of MEC through apoptosis. The second, irreversible phase (after 72 h of mammary engorgement), involves remodeling of the mammary gland to its pre-pregnant state via activation of MMPs. Both extrinsic and intrinsic cell death pathways are activated during
involution. However, intrinsic pathway is the most common pathway, and requires dephosphorylation of BH3-only proteins (e.g. BAD) and the activation of caspase-3 for the execution of apoptosis. IGF-1 is shown to prevent apoptosis via at least 4 different pathways, all of which begin with the activation of IGF-IR and end with the phosphorylation of BAD. The downregulation of IGF-1 function via upregulation of IGFBP, causes the onset of post-lactational involution. Importantly, mammary specific overexpression of IGF-1 is shown to enhance the lactational capacity of rodents by delaying involution, through an inhibition of apoptosis. Increased lactational capacity may enhance the cognitive development of the offspring. The effect of cGP on lactational capacity, in particular the post-lactational involution, of mammary gland is not known, and therefore, forms one of the aims of this thesis (Chapter 5).

1.4 Aims and objectives

Aims

The first aim of this thesis is to investigate the presence, the temporal changes in the concentrations, and the possible role of endogenous cGP in milk and plasma of rats during lactation and postnatal development, respectively. The second aim is to evaluate the effect of early life administration of cGP on neurodevelopmental programming of rats. The third and final aim is to determine if exogenous administration of cGP in rat dams affects the lactational capacity of mammary glands, in particular the process of post-lactational involution.

Objectives

In order to achieve these aims, either cGP dissolved in saline or saline alone (control group) will be gavaged in rat dams from postnatal d8-22. The milk samples will be collected
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throughout lactation from both treatment groups. Mammary glands will be either allowed to involute naturally or induced to involute through teat-sealing in order to study the different stages of involution. Dams and a sub-set of offspring from both groups will be killed at the end of the treatment, and plasma and brain samples will be collected. Involuting mammary glands will also be collected from the dams of both treatment groups. Out of the remaining litter, male offspring will be used for behavioral testing, including the recognition memory, locomotor function and anxiety-like behavior at adolescence, and spatial learning and memory at adulthood. The plasma and brain samples will be collected from the adult offspring. The concentrations of cGP, IGF-1 and IGFBP-3 will be measured in the milk and plasma samples using high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) and enzyme-linked immunosorbent assay (ELISA) in order to achieve the first aim of the thesis. Results from the behavioral tests, and the immunohistochemistry-based evaluation of markers of synaptic plasticity in the collected brains will help achieve the second aim. Lastly, using quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry, cGP mediated modulation of pro- and anti-apoptotic factors, including the changes in phosphorylation of IGF-1R, in involuting mammary glands will help achieve the last aim of the thesis.
Chapter 2: General materials and methods

This chapter contains description of the animal experiment, and the general materials and methods used in this thesis. The methods that were only used once in the thesis are briefly mentioned here, with details given in the specific results chapter.

2.1 Materials

2.1.1 Chemical and biological compounds

Manufacturers of brand products not mentioned specifically within the methods section of Chapters 3, 4 and 5 are detailed below. All other reagents used were of analytical grade, unless stated otherwise.

Table 2.1: List of General Materials used in this Thesis.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% hydrogen peroxide ( \text{H}_2\text{O}_2 )</td>
<td>BDH Laboratory Supplies, Poole, England</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)-fraction V</td>
<td>GIBCO Products, Invitrogen, New York, USA</td>
</tr>
<tr>
<td>Citric acid</td>
<td>Ajax Finechem, Auckland, New Zealand</td>
</tr>
<tr>
<td>di-Ethyl pyrocarbonate (DEPC)</td>
<td>Sigma Chemical Company, Missouri, USA</td>
</tr>
<tr>
<td>di-Sodium hydrogen phosphate</td>
<td>Scharlab, Sentmenat, Spain</td>
</tr>
<tr>
<td>Glycerol</td>
<td>BDH Laboratory Supplies, Poole, England</td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td>Ajax Finechem, Auckland, New Zealand</td>
</tr>
</tbody>
</table>
Chapter 2: Materials and methods

<table>
<thead>
<tr>
<th>Phosphate buffered saline (PBS)</th>
<th>Sigma Chemical Company, Missouri, USA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffered saline (TBS)</td>
<td>Sigma Chemical Company, Missouri, USA</td>
</tr>
<tr>
<td>Trizma Base (Tris)</td>
<td>Sigma Chemical Company, Missouri, USA</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Bio-Rad Laboratories, California, USA</td>
</tr>
</tbody>
</table>

2.2 Methods

2.2.1 Experimental design

Thirty-two female Sprague-Dawley rats weighing between 300-400 g were obtained from, and maintained in, the Small Animal Colony at AgResearch, Hamilton (New Zealand). All animal manipulations were conducted in accordance with the rules and guidelines of the Ruakura Animal Ethics Committee (Hamilton, New Zealand; AE application number 12906). All efforts were made to reduce animal suffering and sample size. The dams were fed a standard laboratory chow (Diet 86; Sharps Grain and Seed, Carterton, New Zealand) and provided water ad libitum throughout the trial. Animals were maintained in rooms at 25 °C with a 14 h light: 10 h dark daily photoperiod. Each female rat was mated at approximately 13 weeks of age, and the day of parturition was considered as postnatal (PN) d1. The litter sizes were adjusted to 9 pups between PN d2-d6. Dams were housed individually with their pups from PN d1 to d23, and allocated at random to two groups on PN d8 (n = 16 per group). From PN d8 to d22, dams from one of the groups were dosed with cGP (3 mg cGP/Kg body weight, Bachem, Bubendorf, Switzerland; Cat# G-1720) dissolved in saline using a gavage feeding needle once per day (Figure 2.1). Dams from the second group were dosed with an equivalent volume of saline for the same duration of time and served as the control group. The dose of cGP was determined based on the efficacious dose of cGP and its structural analogue, cG-
2allyl-P, in rats [67,68,166]. Dams and litter were weighed weekly during lactation (PN d1, d7, d14 and d21), and just before killing (PN d23).

The locomotor activity, recognition memory and anxiety-like behavior of male offspring from both treatment groups (n = 12 per group) were tested using the open field, novel object recognition and dark/light boxes tests, respectively. These tests were conducted between PN d35 to d39, when the offspring had reached adolescence (Figure 2.1). To re-test learning and memory at adulthood, morris water maze was carried out between PN d70-75 on the same offspring and 8 to 11 more male offspring in both treatment groups (n = 21 (control) and 23 (cGP)). To avoid litter effect, adolescent offspring were obtained from 6 (cGP) to 7 (control) different litters from a total of 16 litters in each treatment group. The additional cohort of offspring at adulthood was obtained from 1 (control) to 4 (cGP) additional litters. Due to the absence of separate equipment for male and female offspring, behavioral tests were conducted using male offspring only.

To investigate the effects of cGP on post-lactational involution, mammary glands of dams were either induced to involute through teat-sealing or allowed to involute naturally. Involution was induced by sealing the teats of cranial thoracic and caudal thoracic pair of mammary glands using an ethyl cyanoacrylate based adhesive glue (Selley’s® Quick FixTM Supa Glue, Wellington, New Zealand) on PN d20 and d22, respectively (Figure 2.1). All the other glands remained unsealed. After applying the glue, dams were restrained for further 10 seconds and a paper cap was placed on the teats to prevent the dams from licking off the glue. Dams were then housed in a separate cage away from the pups for 30 min. This practice helped in preventing the pups from licking off the glue or from removing the paper cap. At the time of tissue collection (i.e. PN d23), the unsealed glands, which were undergoing the gradual
process of natural involution, served as the early-involution time-point, and the glands sealed for 24 h and 72 h served as the mid- and late-involution time-points, respectively.

Post-mortem sample collection

On PN d23, the pups were weaned and all the dams plus one male and one female pup per litter were euthanized with carbon dioxide (Figure 2.1). The age of the dams was between 135 to 140 days at the time of killing. The adult offspring were euthanized on PN d82. Brain and blood samples were collected from dams, pups and adult offspring. Mammary glands were additionally collected from the dams.

Blood was collected via heart puncture into the heparin-coated vacutainer tubes (BD Vacutainer® Blood Collection Tubes, BD, Franklin lakes, NJ, USA). The samples were centrifuged at 1500 x g for 10 min at 4 °C in order to collect plasma (supernatant), which were stored at -20 °C for subsequent analysis (Chapter 3).

Transcardial perfusion using normal saline was performed until a clear outflow from heart could be seen. The brains were dissected out and split into two hemispheres. The left hemisphere was snap frozen in liquid nitrogen and stored at -80 °C. The right hemisphere was fixed in 4 % paraformaldehyde for 48 h at 4 °C, and then transferred into 25 % sucrose solution at 4 °C until the tissue sank to the bottom. The left hemisphere was collected for mRNA extraction, which does not form part of this thesis. The right hemisphere was used for immunohistochemistry assays (Chapter 4).

Pairs of sealed (cranial and caudal thoracic) and unsealed (abdominal) mammary glands were collected after separating from the skin and the surrounding tissue. Mammary glands collected from the left side of the body were fixed in 4 % paraformaldehyde for 48 h at 4 °C, and were
used for histology and immunohistochemistry assays (Chapter 5). Mammary glands collected from the right side of the body were snap frozen using liquid nitrogen and stored at -80 °C for subsequent mRNA extraction (Chapter 5).

**Milk collection**

Milk samples were collected on PN d7, d10, d15, d20 and d23 from each dam. One hour prior to milking, dams were separated from their litters. The period of separation was kept minimal as longer periods of separation are shown to affect the composition of milk [247]. Milking was carried out between 0900 and 1100 hours to avoid diurnal variations in the composition of milk [248]. The dams were anesthetized using Ketamine (7.5 ml Ketamine, 5 ml Xylazine, 7.5 ml sterile water; Phoenix Pharm, Auckland, New Zealand) injected subcutaneously at a dose of 0.2 ml/100 g body weight. Oxytocin (1 ml Oxytocin, 9 ml saline; Pharmaco Ltd., Auckland, New Zealand) was then injected subcutaneously at a dose of 0.15 ml/100 g body weight to release all the milk, which was collected in sterile capillary tubes by gentle hand stripping of the teats. Approximately 100-200 µl of milk was collected and stored at -80 °C for subsequent analysis.

![Figure 2.1: Experimental Design](image-url)
Female Sprague-Dawley rats were mated at approximately 13 weeks of age, and the day of parturition was considered as postnatal (PN) d1. Dams were orally gavaged with either cGP (3 mg/Kg body weight) or saline once per day from PN d8-d22. In both treatment groups, involution was induced by sealing the teats of cranial thoracic and caudal thoracic pairs of mammary glands on PN d20 and 22, respectively, using an adhesive glue. The remaining glands were allowed to involute naturally. Dams, and one male and one female pup per litter were euthanized on PN d23. Mammary glands were collected from dams, and brain and blood samples were collected from both dams and pups. Male offspring were obtained from the remaining litter, and tested for locomotor activity, recognition memory and anxiety-like behavior using the open field, novel object recognition and dark/light boxes tests, respectively. These tests were conducted between PN d35 to d39, when the offspring had reached adolescence. To re-test learning and memory at adulthood, morris water maze was conducted between PN d70-75. The adult offspring were euthanized on PN d82, and brain and blood samples were collected. n = 16 per treatment group PN d23, 12 per group PN d35-39, and 21 (control group) and 23 (cGP group) PN d70-75 and d82.

2.2.2 Behavioral tests

The details of three behavioral tests that were carried out on male adolescent offspring are provided in methods section of Chapter 3. Details of morris water maze test, which was carried out on adult male offspring, are provided in Chapter 4. Rats were acclimatized to handling by the experimenter two weeks prior to the start of the behavioral tests. Automated tracking software (ANY-Maze, v4.2, Stoelting Co., Wood Dale, IL, USA) was used to record and analyze the activity of animals during the behavioral tasks.

2.2.3 High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

Concentrations of cGP in milk and plasma were measured using HPLC-MS. The protocol is described in methods section of Chapter 3.

2.2.4 Enzyme Linked Immuno-sorbent Assay (ELISA)

Concentrations of IGF-1 and IGFBP-3 were measured in plasma using ELISA kits from Crystal Chem Inc. (Chicago, Illinois, USA; Cat# 80573 and 80581 respectively). More details are given in methods section of Chapter 3.
2.2.5 Milk composition

The total protein and lipid content of milk was measured using the infrared spectrometer Direct Detect™ (Merck Millipore, Darmstadt, Germany). Details are provided in methods section of Chapter 5.

2.2.6 Immunohistochemistry

Immunohistochemistry (IHC) was used for evaluating the protein expression of different markers of neuroplasticity in brains (Chapter 4), and cellular and molecular markers of involution in mammary glands (Chapter 5). Free-floating IHC procedures were adopted for semi-quantitative estimation of protein expression in brain tissue. The procedure is explained in detail in methods section of Chapter 4. Paraffin-based IHC procedures were used for mammary glands, details of which are provided in methods section of Chapter 5. Different IHC procedures were adopted because these procedures were routine practices in the laboratories of research groups that were collaborating in this project (Dr Kuljeet Singh – lactation biology, and Dr Jian Guan – neuroscience).

2.2.7 Histology

Mammary tissue were stained with hematoxylin and eosin for evaluating morphological changes associated with involution. The procedure is explained in methods section of Chapter 5.

2.2.8 Quantitative real-time reverse transcription PCR analysis

Quantitative real-time RT-PCR was performed on the frozen mammary gland samples for determining changes in the expression of genes during involution. The procedure is explained in methods section of Chapter 5.
2.2.9 Statistics

All statistical procedures were performed using SPSS (IBM SPSS Statistics 22, Chicago, IL, USA), and are detailed in the methods sections of Chapters 3, 4 and 5.
Chapter 3: Maternally administered cyclic-glycine-proline increases insulin-like growth factor-1 bioavailability and novelty recognition in developing offspring

3.1 Summary of chapter contents

This chapter investigates the presence, the changes in concentrations and the physiological role of endogenous cGP in rat milk and plasma during lactation and postnatal development, respectively. The maternal-infantile transfer of cGP following administration to rat dams during lactation is also investigated. Lastly, the efficacy of maternally administered cGP on novelty recognition, locomotor activity and anxiety-like behavior of adolescent offspring is evaluated. This chapter was recently published as an original research article in *Endocrinology* [249].

3.2 Introduction

IGF-1 plays an important role during postnatal development [250], including postnatal development of the brain [251,252]. IGF-1 is present in mammalian milk [38,238], and its concentration declines steadily over the course of lactation [39]. In both rats and humans, concentrations of IGF-1 in plasma are low at birth, peak during puberty and decrease during adulthood with age [253,254]. cGP is naturally produced from the cyclisation of N-terminal tripeptide of IGF-1 [reviewed in 29], and is shown to regulate the bioavailability of IGF-1 by altering the binding of IGFBP to IGF-1 [34]. The presence and the changes in the concentrations of endogenous cGP in rat milk and plasma have not been evaluated during lactation and postnatal development, respectively.
cGP is neurobioactive [29], and has been identified as an endogenous nootropic peptide in adult rat brain tissue [36]. cGP and its structural analogue cG-2allyl-P, improved memory in adult rats with acute memory deficits induced by either scopolamine [166] or maximal electroshock, an electric shock of 70 V given for 300 msec [36]. The neuroprotective actions of cGP are mediated through increased bioavailability of IGF-1 [29,34]. cGP is small, lipophilic, enzymatically stable and can be orally bioavailable with effective central uptake [27,29,35]. However, the pharmacokinetics of exogenous cGP following maternal administration during lactation, and its effect on the development and function of the brain of the offspring has not been evaluated.

Improved early postnatal brain development can have long-lasting effects on brain function, which can be associated with cognitive behaviors. Indeed, change in cognitive behavior has been used to assess brain function in relation to postnatal brain development [255,256]. Novel object recognition test (NORT) is commonly used to evaluate recognition memory in developing young rats [257]. Both anxiety and locomotor activity are confounding factors in this behavioral test because anxiety and locomotor function can influence the learning and memory abilities of the animal.

The current chapter investigates the presence and the changes in the concentrations of endogenous cGP in relation to endogenous IGF-1 and IGFBP-3 in rat milk and plasma during lactation and postnatal development, respectively. Maternal-infantile transmission and pharmacodynamics of maternal administration of cGP during lactation on novelty recognition of developing offspring are also evaluated.
3.3 Materials and Methods

3.3.1 Animal experiment

The animal experiment has been described in detail in section 2.2.1 of the thesis. Briefly, female Sprague-Dawley rats (n = 16 per group) were dosed with either cGP dissolved in saline (3 mg/Kg body weight) or saline alone using a gavage feeding needle once per day from PN d8 to d22. On PN d23, the pups were weaned and all the dams plus one male and one female pup per litter were euthanized with carbon dioxide, and blood samples were collected. The age of the dams was 135-140 days at the time of killing. Three behavioral tests were carried out on the male offspring from the remaining litter between PN d35 to d39, when the offspring had reached adolescence (n = 12 per treatment group). The male adolescents were euthanized on PN d82 when they reached adulthood, and blood samples were collected.

3.3.2 Milk collection

Milk samples were collected on PN d7, d10, d15, d20 and d23 from each dam. The methodology has been described in section 2.2.1.

3.3.3 Measurement of concentrations of cGP in plasma and milk using HPLC-MS

HPLC-MS was used to measure the concentrations of total cGP (i.e. bound and unbound forms) in the samples.

3.3.3.1 Extraction of cGP from plasma and milk

cGP-d₂ was used as the internal standard for the assay. 35 µl of 200 ng/ml cGP-d₂ was added to either 100 µl of plasma or milk diluted two-fold. The resulting solution was vortex mixed and transferred to a 1 ml Phree phospholipid removal cartridge (Phenomenex, Auckland, New Zealand) contained in a 4.5 ml Nunc CryoTubeTM vial (NuncTM Brand Products, Nalge
Nunc International). 500 µl of 1% formic acid in acetonitrile was then loaded into the cartridge and shaken gently. The resulting solution was centrifuged at 1000 rpm for 5 min at 4 °C for plasma and 1500 rpm for 10 min at 4 °C for milk. Another 500 µl of 1% formic acid in acetonitrile was added and centrifuged as before. Vials containing the filtrate were dried using a vacuum concentrator (Savant SC250 EXP Speed Vac Concentrator, Thermo Electron Corporation, San Jose, CA, USA) at room temperature with vacuum pressure initially set at 1.5 for one hour (Ramp set at 3) and then reduced to 0.6 for one hour for plasma and 4 hours for milk (Ramp set at 3). The dried extract was reconstituted in 80 µl of water:methanol (90:10 vol/vol) and transferred to a UPLC vial for injecting into the column. Extraction of cGP was done in the same way for both the standards (prepared in MQ-water) and the quality control samples.

### 3.3.3.2 HPLC-MS: assay conditions

An HPLC-MS system, consisting of an Accela MS pump and autosampler followed by an Ion Max APCI source on a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer were controlled by Finnigan Xcalibur software (Thermo Electron Corporation, San Jose, CA, USA). For separating cGP, a Synergy Hydro 2.5 µm column (dimensions: 100 x 2 mm; particle size: 2.5 µm; Phenomenex, Auckland, New Zealand) was used and operated at 35 °C. The initial mobile phase composition was 90% water: 10% methanol with a flow rate of 200 µl/minute. The optimized MS conditions consisted of heated electrospray ionization in positive mode with voltage set to 4500 V, sheath gas flow set to 35 psi, auxiliary gas flow set to 2 psi and capillary temperature set to 350 °C. Conditions for attaining fragmentation consisted of collision gas (argon) set to 1.2 mTorr and dissociation voltage set to 35 V. The MS analysis was performed in the selective reaction monitoring mode. Two transitions were used, respectively, for cGP and cGP-d2: 155.1 to 70.2 m/z and 157.1 to 70.2 m/z. Both the
peaks had a retention time of 3.6 min. The concentrations of cGP in the test samples were then calculated by comparing the peak area ratio of cGP/cGP-d2 with the standard curve of known concentrations.

3.3.4 Measurement of plasma concentrations of IGF-1 and IGFBP-3 using ELISA

Rat ELISA kits were used to measure the concentrations of total IGF-1 and IGFBP-3 in plasma (Cat# 80573 and 80581 respectively, Crystal Chem Inc., Chicago, Illinois, USA) as per the manufacturer’s instructions. The absorbance values were obtained using a plate reader (BioTek® Synergy™ 2 Multi-detection Microplate Reader, Gen5 software, Winooski, VT, USA) set at 450 nm for the excitation wavelength and at 630 nm for the emission wavelength. Concentrations were reported as ng/ml.

3.3.5 Behavioral tests

Three behavioral tests were carried out on the male adolescent offspring from both treatment groups between PN d35 to d39 in the following sequence (Figure 2.1): open field test (PN d35), NORT (PN d36 to d38) and dark/light boxes test (PN d39) (n = 12 per treatment group per test). Rats were acclimatized to handling by the experimenter two weeks prior to the start of the behavioral tests. Automated tracking software (ANY-Maze, v4.2, Stoelting Co., Wood Dale, IL, USA) was used to record and analyze the activity of animals during the behavioral tasks.

3.3.5.1 Open Field Test (OFT)

Locomotor activity and anxiety-like behavior was tested using OFT, a stress-sensitive behavioral task. A wooden open-top box (90 cm x 60 cm x 40 cm) served as the testing arena. The box was virtually divided into a central and a peripheral area using the ANY-Maze software. The central area measured 36 x 24 cm and was 27 cm away from the walls of the
box. Rats were placed in the central area and allowed to explore the entire box freely for 30 min. Total distance travelled, total resting time and ratio of time spent in the central and peripheral zones were recorded using the ANY-Maze tracking software. ANY-Maze calculated the resting time by detecting the immobility of rats, in which 65% of the rat’s body had to remain in the same place for at least 2 sec. The resting time included grooming, stretching and rearing.

### 3.3.5.2 Novel Object Recognition Test (NORT)

The NORT procedure is described below.

**Apparatus and habituation phase**

NORT was conducted one day after OFT. The same wooden open-top box was used as for the OFT, allowing the rats to become habituated to the new environment outside of their cage.

**Familiarization and test phases**

For the next three days, two identical objects were placed in two corners of the box 15 cm from the walls and remained at the same position throughout the trials. Two trials were conducted on each day with 2-3 hour intervals between the trials. In the familiarization phase, rats underwent five trials and were allowed to explore the objects for 3 minutes in each trial. To avoid coercing the rats to explore the objects, rats were always released into the box facing the wall opposite to the objects. During the test phase on the third day, one of the objects was replaced with a new object prior to the last trial. To ensure the odor cues did not influence the behavior of the rats, both the box and objects were cleaned with 70% ethanol after every trial. The ANY-Maze software recorded the time spent by each rat in exploring the objects. Exploration was defined when at least 3% of the rat’s body was in the zone containing the
object, and an exit from the zone was considered when < 1 % of the rat’s body remained in the zone. The ratio of time spent exploring the novel object to the familiar object was used as a measure for assessing the recognition memory of rats.

3.3.5.3 Dark-Light Boxes test

A black colored box was placed in one half of an open-top clear square plastic box (60 cm x 60 cm x 60 cm). The black box had a lid on top and a small opening on the side wall that allowed an easy commute to the light part of the box. The open and exposed light chamber provided a more aversive environment to the rats compared with the dark and sheltered chamber. A rat was placed in the dark chamber and was allowed 5 min to explore both the chambers. The time spent in the light box and the number of entries made to the light box were recorded using the ANY-Maze tracking software. An entry into the light box was considered to be made when 80% of the animal’s body was in the light box.

3.3.6 Statistics

Statistical analyses were carried out using the SPSS statistical software (IBM SPSS Statistics, Version 21, ©1989-2012 IBM® Corp., Chicago, IL, USA). One-way ANOVA was used for comparisons within the control group. Two-way ANOVA (age and treatment as factors), mixed ANOVA (treatment as between-subjects factor and time as within-subjects factor) and unpaired t-tests were used for comparisons between treatment groups. Bonferroni post hoc tests were chosen for multiple comparisons. Data were summarized as mean ± SEM. The significance level was set at P < 0.05.
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3.4 Results

3.4.1 Concentrations of cGP in plasma and milk

3.4.1.1 Plasma

One-way ANOVA within the control groups showed that the plasma concentrations of endogenous cGP changed significantly with age ($F_{(3, 63)} = 9.34, P < 0.001$, Figure 3.1). Bonferroni post hoc tests revealed that compared with the dams, the plasma concentrations of endogenous cGP were significantly higher in the male pups (PN d23, $P < 0.05$, Figure 3.1A) and the adult offspring (PN d82, $P < 0.001$, Figure 3.1A), but were not statistically different from the female pups (PN d23, $P = 0.1$, Figure 3.1A). There was no difference in the plasma concentrations of endogenous cGP in the pups and the adult offspring (Figure 3.1A).

Two-way ANOVA between treatment groups showed significant changes in the plasma concentrations of cGP with age ($F_{(3, 130)} = 8.07, P < 0.001$, Figure 3.1B) and treatment ($F_{(1, 130)} = 43.89, P < 0.001$, Figure 3.1B), and also showed significant interaction between age and treatment groups ($F_{(3, 130)} = 8.65, P < 0.001$, Figure 3.1B). Bonferroni post hoc tests revealed that the concentrations of cGP were significantly higher in the plasma of cGP-administered dams ($P < 0.05$) and their pups ($P < 0.001$) compared with the respective control groups (Figure 3.1B). There was no difference in the plasma concentrations of cGP in adult offspring from the two treatment groups (Figure 3.1B).
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Figure 3.1: Concentrations of Cyclic-glycine-proline (cGP) in Plasma of Dams, Pups and Adult Offspring following Oral administration of cGP in Dams during Lactation

Concentrations of cGP were measured in plasma of dams, pups and adult offspring using high performance liquid chromatography coupled with mass spectrometry. Dams were orally gavaged with either cGP (3 mg/Kg body weight, closed bars) or saline (open bars) once per day from postnatal (PN) d8-d22. (A) Comparisons of plasma concentrations of endogenous cGP between dams, pups and adult offspring from control group. (B) Comparisons of plasma concentrations of cGP between control and cGP-administered groups from dams, pups and adult offspring. Data are presented as mean ± SEM, n = 16 per group (dams and pups PN d23) and 21-23 per group (adult offspring PN d82). In A and B, significant differences compared with the dams are denoted by *P < 0.05, ****P < 0.001, and significant differences compared with the adult offspring are denoted by #P < 0.05. In B, significant differences between the control and treatment groups are denoted by *P < 0.05, ****P < 0.001.

3.4.1.2 Milk

One-way repeated measures ANOVA within the control group showed significant changes in the concentrations of endogenous cGP in milk from PN d7 to d23 (F (4, 36) = 9.72, P < 0.001, Figure 3.2A). Multiple comparisons showed that the concentrations of endogenous cGP in milk were 6-fold higher (P < 0.01) during the peak lactation period (PN d10 and d15) compared with the weaning period (PN d20 and d23, Figure 3.2A).

Mixed ANOVA showed significant changes in the concentrations of cGP in milk over time (F (4, 80) = 5.25, P < 0.01, Figure 3.2B) and a significant increase in the concentration of cGP in milk following maternal administration of cGP (F (1, 20) = 38.11, P < 0.001, Figure 3.2B). A significant time-by-treatment interaction was also observed (F (4, 80) = 5.12, P < 0.01, Figure 62.
3.2B). Bonferroni post hoc tests revealed that in comparison with the saline-administered dams, the concentrations of cGP in milk of cGP-administered dams were greater (P < 0.05) on PN d10 (85-fold), d15 (101-fold), d20 (6-fold) and d23 (7-fold) (Figure 3.2B).

![Figure 3.2: Concentrations of Cyclic-glycine-proline (cGP) in Milk following Oral administration of cGP in Dams during Lactation](image)

Concentrations of cGP were measured in milk of dams using high performance liquid chromatography coupled with mass spectrometry. Dams were orally gavaged with either cGP (3 mg/Kg body weight, closed circles) or saline (open circles) once per day from postnatal (PN) d8-d22. (A) Comparisons of concentrations of endogenous cGP in milk of control dams from PN d7-d23. (B) Comparisons of concentrations of cGP in milk of control and cGP-administered dams from PN d7-d23. Data are presented as mean ± SEM, n = 14 per group (PN d7), 16 per group (PN d10, d15, d20) and 13 per group (PN d23). In A, different letters denote significance at P < 0.001. In B, significant differences between the control and cGP-administered groups at the same time-point are denoted by *P < 0.05, ***P < 0.001.

### 3.4.2 Concentrations of total IGF-1 and IGFBP-3 in plasma

#### 3.4.2.1 IGF-1

In order to understand the temporal relationship between cGP, IGF-1 and IGFBP-3, and to calculate the ratio of cGP/IGF-1 as a measure of bioavailability of IGF-1 [34], the concentrations of total IGF-1 and IGFBP-3 were measured in the plasma of rats from different age groups.
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One-way ANOVA within the control groups showed that the concentrations of total IGF-1 in plasma changed significantly with age ($F_{(3, 55)} = 277.2, P < 0.001$, Figure 3.3A). Bonferroni post hoc tests revealed that in comparison with dams, the concentrations of total IGF-1 were significantly lower in the plasma of male and female pups (PN d23, $P < 0.001$, Figure 3.3A) but higher in that of the adult offspring (PN d82, $P < 0.001$, Figure 3.3A). Furthermore, concentrations of total IGF-1 were significantly higher in the plasma of adult offspring compared with that of the pups ($P < 0.001$, Figure 3.3A).

Concentrations of total IGF-1 were similar in the plasma of dams, pups and adult offspring from both treatment groups on PN d23 and d82 (Figure 3.3B).

One way ANOVA within the control groups showed that the molar ratios of cGP to IGF-1 in plasma changed significantly with age ($F_{(3, 55)} = 277.2, P < 0.001$, Figure 3.3C). Bonferroni post hoc tests revealed that in comparison with dams, the molar ratios of cGP to IGF-1 were significantly higher in the plasma of male and female pups (PN d23, $P < 0.001$, Figure 3.3C) but lower in that of the adult offspring (PN d82, $P < 0.001$, Figure 3.3C). Furthermore, molar ratios of cGP to IGF-1 were significantly lower in the plasma of adult offspring compared with that of the pups ($P < 0.001$, Figure 3.3C).

Two-way ANOVA between treatment groups showed significant changes in the molar ratios of cGP to IGF-1 with age ($F_{(3, 103)} = 35.89, P < 0.001$, Figure 3.3D) and treatment ($F_{(1, 103)} = 17.41, P < 0.001$, Figure 3.3D), and also showed significant interaction between age and treatment groups ($F_{(3, 103)} = 6.37, P < 0.001$, Figure 3.3D). Bonferroni post hoc tests revealed that although the molar ratios of cGP to IGF-1 were greater in the plasma of cGP-administered dams and pups compared with their respective control groups, the increase was statistically
significant only in the case of male pups (P < 0.01, Figure 3.3D). Molar ratios of cGP to IGF-1 were similar in the plasma of adult offspring from both treatment groups (Figure 3.3D).

Figure 3.3: Concentrations of Total Insulin-like Growth Factor-1 (IGF-1) and Molar Ratios of cGP to IGF-1 in Plasma of Dams, Pups and Adult Offspring following Oral administration of cGP in Dams during Lactation.

Concentrations of total IGF-1 were measured in plasma of dams, pups and adult offspring using enzyme linked immuno-sorbent assay. Dams were orally gavaged with either cGP (3 mg/Kg body weight; closed bars) or saline (open bars) once per day from postnatal (PN) d8-d22. (A) Comparisons of concentrations of total IGF-1 in plasma of dams, pups and adult offspring from control group. (B) Comparisons of plasma concentrations of IGF-1 between control and cGP-administered groups. (C) Comparisons of molar ratios of cGP to IGF-1 in plasma of dams, pups and adult offspring from control group. (D) Comparisons of molar ratios of cGP to IGF-1 between control and cGP-administered groups. Data are presented as mean ± SEM, n = 15 per group (dams and male pups, PN d23), 5 per group (female pups, PN d23), 21-23 per group (adult offspring, PN d82). In A and C, significant differences compared with the dams are denoted by **P < 0.01, ***P < 0.001, and significant
differences compared with the adult offspring are denoted by ##P < 0.01. In B and D, significant differences between the control and the cGP-administered groups are denoted by **P < 0.01.

3.4.2.2 IGFBP-3

Because cGP is capable of regulating the bioavailability of IGF-1 through competitive binding with IGFBP-3 [34,258], therefore, the concentrations of IGFBP-3 were determined in the plasma of rats from both the control and the cGP-administered group.

One-way ANOVA within the control groups showed that the concentrations of IGFBP-3 in plasma changed significantly with age (F(3, 67) = 215.7, P < 0.001, Figure 3.4A). Bonferroni post hoc tests revealed that in comparison with dams, the concentrations of IGFBP-3 were significantly lower in the plasma of male and female pups (PN d23, P < 0.001, Figure 3.4A) but higher in that of the adult offspring (PN d82, P < 0.001, Figure 3.4A). Furthermore, concentrations of IGFBP-3 were significantly higher in the plasma of adult offspring compared with that of the pups (P < 0.001, Figure 3.4A).

Concentrations of IGFBP-3 were similar in the plasma of dams, pups and adult offspring from both treatment groups on PN d23 and d82 (Figure 3.4B).

Figure 3.4: Concentrations of Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) in Plasma of Dams, Pups and Adult Offspring following Oral administration of cGP in Dams during Lactation
Concentrations of IGFBP-3 were measured in plasma of dams, pups and adult offspring using enzyme linked immuno-sorbent assay. Dams were orally gavaged with either cGP (3 mg/Kg body weight; closed bars) or saline (open bars) once per day from postnatal (PN) d8-d22. (A) Comparisons of concentrations of IGFBP-3 in plasma of dams, pups and adult offspring from control group. (B) Comparisons of plasma concentrations of IGFBP-3 between control and cGP-administered groups. Data are presented as mean ± SEM, n = 16 per group (dams and pups, PN d23), 21-23 per group (adult offspring, PN d82). In A, significant differences compared with the dams are denoted by **P < 0.01, ***P < 0.001, and significant differences compared with the adult offspring are denoted by ##P < 0.01.

3.4.3 Behavioral tests

3.4.3.1 OFT

Locomotor activity, indicated by the total distance travelled (Figure 3.5A) and the total resting time (Figure 3.5B) in the open field arena, was similar for adolescent offspring from saline- and cGP-administered dams. Similarly, anxiety-like behavior indicated by the ratio of time spent in the central zone to the peripheral zone was similar between the two treatment groups (Figure 3.5C).
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Figure 3.5: Maternal administration of Cyclic-glycine-proline (cGP) during Lactation does not Influence the Locomotor Activity or Anxiety-like Behavior of Adolescent Offspring in Open Field Test.

Performance of male adolescent offspring from cGP-administered (closed bars) and saline-administered (open bars) dams in open field test for (A) Total distance travelled, (B) Total resting time, and (C) Ratio of time spent in central zone/peripheral zone. Data are presented as mean ± SEM, n = 12 per group.

3.4.3.2 NORT

Mixed ANOVA showed a significant increase in total exploration time (i.e., time spent in exploring both objects) with trials ($F_{(5, 110)} = 24.59, P < 0.001$, Figure 3.6A), but no treatment
effect or an interaction between trials and treatment. Multiple comparisons showed that in both treatment groups, the total exploration time did not change during the familiarization trials, but increased significantly in the test trial (P < 0.001).

In comparison with the control group, the adolescent offspring from cGP-administered dams spent more time exploring the novel object than the familiar object (t (22) = 2.09, P < 0.05, Figure 3.6B).

![Figure 3.6: Maternal administration of Cyclic-glycine-proline (cGP) during Lactation enhances Novelty Recognition of the Adolescent Offspring in the Novel Object Recognition Test (NORT).](image)

Male adolescent offspring from cGP-administered (closed bars) and saline-administered (open bars) dams were tested for novelty recognition using NORT. Rats from both treatment groups showed significant increase in the total exploration time during the test phase; however, there was no difference in the total exploration time between the two treatment groups either during the familiarization or the test phase (A). Compared with the saline-administered dams, male adolescent offspring from cGP-administered dams spent more time exploring novel object than the familiar object during the test phase (B). Data are presented as mean ± SEM, n =12 per group. Significant differences between the treatment groups are shown by *P < 0.05, and significant differences between the consecutive trials are shown by #P < 0.001.
3.4.3.3 Dark-Light Boxes test

Anxiety-like behavior indicated by the time spent in the light box (Figure 3.7A) and number of entries made to the light box (Figure 8B) was similar for the adolescent offspring from saline- and cGP-administered dams.

![Graph A: Number of entries to light box](image1)

![Graph B: Time spent in light box](image2)

**Figure 3.7: Maternal administration of Cyclic-glycine-proline (cGP) during Lactation does not alter the Anxiety-like Behavior of Adolescent Offspring in Dark-Light Boxes Test.**

Performance of male adolescent offspring from cGP-administered (closed bars) and saline-administered (open bars) dams in dark-light boxes test for (A) Number of entries, and (B) Time spent in the light box. Data are presented as mean ± SEM, n = 12 per group.

3.5 Discussion

To the best of our knowledge, this is the first study to demonstrate the presence of endogenous cGP in rat milk and plasma during lactation and postnatal development, respectively. The lower endogenous IGFBP-3 and higher endogenous cGP concentrations in plasma were the responses to low endogenous IGF-1 concentrations during infancy, leading to improved bioavailability of IGF-1. Exogenously administered cGP showed oral bioavailability in dams, and demonstrated effective maternal-infantile transmission through breast milk, leading to an
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enhancement in the novelty recognition of the offspring at adolescence. Our data shows that cGP is an endogenous peptide present in rat milk and plasma, and may be involved in increasing the bioavailability of IGF-1 during infancy. Furthermore, maternal cGP supplementation offers an effective and physiological route of administration for improving novelty recognition in the developing offspring through increased bioavailability of IGF-1.

Results presented in this chapter showed that endogenous cGP is present in the plasma of dams, pups and adult offspring as well as in the breast milk of dams (Figure 3.1A and 3.2A). Concentrations of endogenous cGP in milk were higher during the peak lactation period (PN d10 and d15) compared with the late lactation period (PN d20 and d23, Figure 3.2A). This data suggests that cGP may be an essential component of maternal milk. An increase in milk production and mammary blood flow during peak lactation followed by a decline towards weaning may explain the decline in the transfer of cGP from blood to milk towards the end of lactation [259-261].

In terms of brain development, PN d23 and d82 in rats are comparable with human infants and adolescents, respectively, as the brain matures around 3 months of age in Sprague-Dawley rats [69,70]. On the other hand, dams at the age of 135 –140 days may be comparable with human adults if there is no lactation effect. Concentrations of endogenous cGP were significantly higher in the plasma of pups compared with the dams on PN d23, and remained higher in the pups at adulthood (PN d82, Figure 3.1A). High concentrations of endogenous cGP in the offspring may suggest a specific role for cGP during postnatal development.

In contrast, the concentrations of endogenous IGF-1 and IGFBP-3 were relatively lower in the plasma of pups compared with that of the dams and the adult offspring (Figure 3.3A and 3.4A). It is known that IGF-1 plays an important role during postnatal development [250],
including brain development [251,252]. However, concentrations of IGF-1 are lower in plasma of infants compared with adults in both humans [reviewed in 253] and rodents [254,262,263]. IGFBP-3 follows a similar developmental pattern as that of IGF-1 [264].

The majority of IGF-1 reported in plasma is not bioavailable due to its binding to the IGFBP [265]. The bioavailability of IGF-1 is tightly regulated through the reversible binding of IGF-1 to IGFBP. The reduction of IGFBP-3, the major IGFBP in plasma [266], increases the bioavailability of IGF-1 [267]. As a metabolite of IGF-1, cGP is capable of regulating the bioavailability of IGF-1 through competitive binding with IGFBP, such that a higher molar ratio of cGP/IGF-1 leads to an increase in the bioavailability of IGF-1 [34,258]. The molar ratio of cGP/IGF-1 was significantly higher in pups compared with dams and adult offspring (Figure 3.3C). Therefore, low endogenous IGFBP-3 and high endogenous cGP concentrations in plasma during infancy could be an autocrine response to low IGF-1 concentrations in order to increase the bioavailability of IGF-1, which is essential for supporting postnatal growth and development. The coherence between decreased concentrations of IGFBP-3 and an increased molar ratio of cGP/IGF-1 was recently proposed to be essential for weight loss in pregnancy-associated maternal obesity [258]. The autocrine regulation responsible for increasing the bioavailability of IGF-1 was not evident in the adult offspring and dams. This could simply be due to the high concentrations of total IGF-1 in their plasma (Figure 3.3A). With sufficient IGF-1 production during adulthood, the need for promoting bioavailability of IGF-1 through autocrine regulation may not be critical for maintaining homeostatic IGF-1 function.

Data presented in this chapter shows oral bioavailability of exogenous cGP in adult rats as indicated by an increase in the plasma concentrations of cGP in dams (Figure 3.1B). Furthermore, maternal-infantile transfer of cGP was evidenced through higher concentrations
of cGP in dams’ milk (Figure 3.2B) and pups’ plasma (Figure 3.1B) compared with the respective control groups, further suggesting the oral bioavailability of cGP in infant rats. The changes in the concentrations of cGP in milk and plasma following maternal administration also provided a pharmacological profile of maternal-infantile transfer of exogenous cGP (Figure 3.1B and 3.2B). Twenty-four hours after chronic maternal administration, the amounts of cGP transferred from dams’ plasma to milk and then to pups’ plasma were proportionally higher than that of endogenous cGP, with an approximate doubling in the concentrations of cGP. Pharmacokinetics of cGP involve binding and releasing from plasma proteins that act as a reservoir of cGP [34,67] and may contribute to the long-lasting increase in plasma cGP concentrations observed twenty-four hours after chronic treatment.

Changes in the concentrations of exogenous cGP in milk followed the same pattern as the endogenous cGP, with greater transfers of cGP occurring from blood to milk during peak lactation compared with late lactation (Figure 3.2B). The transfer of exogenous cGP from blood to milk during lactation may have pharmacological implications as it offers a ‘natural’ route of administration of cGP to the nursing offspring with ‘physiologically controlled’ pharmacokinetics. Concentrations of cGP in milk were more variable during peak lactation than late lactation (Figure 3.2B), which could be due to the differences in time of gavage and milk collection between dams.

The efficacy of maternal-infantile transfer of cGP was evident through the improved novelty recognition in the developing offspring. NORT is commonly used to test short-term memory in developing young rats [257]. Compared with the saline-administered dams, the adolescent offspring from cGP-administered dams spent more time exploring the novel object than the familiar object, suggesting improved novelty recognition (Figure 3.6B). Rats from both
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treatment groups showed similar total exploratory time during the familiarization phase, and demonstrated equivalent increase in total exploratory time during the test phase (Figure 3.6A), suggesting that the increased preference for novel object in the cGP-administered group was not due to a differential increase in the total level of exploration. Maternal separation does not affect the novelty recognition of male offspring of Sprague-Dawley rats [268,269]. Therefore, a control group for maternal separation was not included in the current study. However, both maternal separation [270] and human handling [271] during the first three weeks of life are known to affect the anxiety-like behavior of rats. Furthermore, both anxiety and locomotor activity are confounding factors that can influence the exploratory behavior of rats during NORT [257]. The data from behavioral tests shows that the improved novelty recognition was not associated with either the locomotor activity or the anxiety (Figure 3.5 and 3.7). The locomotor activity of adolescent rats was tested prior to NORT, and was similar between the two treatment groups (Figure 3.5A and B). The anxiety-like behavior was tested both before and after the NORT, in which the time spent in the central zone in the OFT (Figure 3.5C) and voluntary entry to the light box in the dark-light boxes test (Figure 3.7) was similar between the two treatment groups. The anti-amnesic effects of cGP have been reported by others in adult rats [36], however, this is the first study to demonstrate the cGP-mediated enhancement of recognition memory in developing rats.

Maternal cGP administration increased the bioavailability of IGF-1 in pups as indicated by an increase in the molar ratio of cGP/IGF-1 compared with the control group (Figure 3.3D), though cGP did not reduce the concentrations of IGFBP-3 in the plasma (Figure 3.4B). Similarly, maternal cGP administration did not alter the concentrations of total IGF-1 in the plasma (Figure 3.3B), which provides additional support for the efficacy of cGP which is not mediated through changing IGF-1 production rather modulating the homeostasis of IGF-1.
bioavailability [29,34]. Therefore, the efficacy of maternally administered cGP on novelty recognition of developing offspring may be interpreted as an IGF-1 effect [252,272].

The mode of transport of cGP from gut to blood and blood to milk is not known. Peptide transport systems in the enterocytes [273] and the mammary epithelial cells [274,275] might be involved in transporting cGP from gut to milk, however, this requires further investigation.

### 3.6 Summary

As a metabolite of IGF-1, cGP has been identified as an endogenous neuropeptide that restores memory deficits in adult rats. To accomplish the first aim of this thesis, the presence and the changes in the concentrations of endogenous cGP in rat milk and plasma were evaluated during lactation and postnatal development, respectively. Maternal-infantile transfer of cGP during lactation and its efficacy on memory of developing offspring were also investigated. This helped in partial accomplishment of the second aim of the thesis. Dams were gavaged with either cGP (3mg/Kg) or saline daily from postnatal d8-22. Concentrations of cGP were measured in dams’ milk, and concentrations of cGP, IGF-1 and IGFBP-3 were measured in plasma of dams, pups and adult offspring. The recognition memory, locomotor function and anxiety-like behavior of offspring were evaluated using behavioral tests. Endogenous cGP was detected in rat milk, and its concentration was higher during peak lactation compared with late lactation. Comparisons within control groups showed low endogenous IGF-1 and IGFBP-3 and high endogenous cGP concentrations in plasma of male pups. The reduced IGFBP-3 and increased cGP were proposed to be a response to increase the bioavailability of IGF-1 during infancy. Exogenous cGP showed oral bioavailability and effective maternal-infantile transfer through milk. Maternally transferred cGP improved recognition memory of the developing offspring, possibly by increasing IGF-1 bioavailability, with no effect on locomotor activity
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and anxiety-like behavior. These results show that cGP is an essential endogenous peptide during early postnatal development as it improves the bioavailability of IGF-1 during infancy. Further, maternal cGP supplementation offers an effective and natural route of administration for improving recognition memory of the developing offspring.
Chapter 4: Maternally administered cGP improves adult memory and neuroplasticity

4.1 Summary of chapter contents

The second aim of this thesis was to evaluate the effect of early life administration of cGP on neurodevelopmental programming of rats. Results presented in the previous chapter showed that early life administration of cGP improves the recognition memory of the offspring at adolescence. This chapter investigates the effect of cGP on brain development and adult memory function of the offspring.

Some of the data presented in this chapter was generated by the Masters students in the group. The details are provided in the co-authorship form attached in the beginning of this thesis. Briefly, the immunohistochemical staining and analysis of GluR-1 and synaptophysin in dams’ brains was carried out by Stanley Yune. The immunohistochemistry staining and analysis of GluR-1 and synaptophysin in pups’ brains was carried out by Johan Karlsson and Marcus Svensson, respectively. This chapter is under review by Brain Research as an original research article.

4.2 Introduction

Early stages of brain development have profound effects on neural function and behavior that persist into adulthood. This relationship originates from well-known association between breastfeeding and better cognitive function at adulthood [9,276]. However, rapid brain development during infancy is not only dependent on the optimal supply of nutrients but also on other highly bioactive factors from breast milk, including growth factors such as IGF-1 [3,13].
As an endogenous neurotrophic factor, IGF-1 is present in breast milk of both humans [277] and rats [278], and plays a critical role in postnatal brain development and function, including learning and memory [for review, see 156]. Sub-optimal IGF-1 levels, for example in offspring born to obese mothers [279], lead to learning and memory deficits in offspring that persist into adulthood [280,281]. However, exogenous administration of IGF-1 as a treatment for improving brain development can be problematic due to its large molecular size and potential metabolic (e.g. hypoglycemia) and mitogenic side-effects [27].

As a metabolite of IGF-1, cGP optimizes IGF-1 function by normalizing the bioavailability of IGF-1 [34]. cGP is a small, lipophilic diketopiperazine. It is potentially orally bioavailable with effective central uptake [29,35]. Neurotrophic effects of cGP have been shown following ischemic brain injury in rats [67]. Compared with adulthood, the circulating levels of IGF-1 are lower during infancy. Results presented in the previous chapter [Chapter 3, which is published as 249] showed for the first time that the circulating levels of cGP are increased during infancy, in a potential autocrine response to improve the bioavailability of IGF-1. It was also shown that cGP is present in rat milk, and maternal administration of cGP leads to effective maternal-infantile transfer through breast milk. The effectiveness of maternal administration of cGP on the offspring was demonstrated by an increase in the recognition memory of the offspring at adolescence (examined between PN d36-38). Considering that the mechanism of action of cGP involves normalizing IGF-1 function by modulating the bioavailability of IGF-1 [34], it is not known whether maternal administration of cGP can cause long-lasting improvement in brain function of offspring that persist into adulthood.

MWM test is widely used to evaluate spatial learning and reference memory of rats [for review, see 282]. MWM is a hippocampus-dependent task as hippocampal lesions impair spatial learning and memory of rats [146]. Striatum also plays a key role in processing of
spatial information acquired early during the MWM task [108]. Increased synaptic plasticity in these brain regions, for instance increased GluR-1 trafficking and synaptophysin immunoreactivity, are shown to be the underlying biological changes associated with the improvement in learning and memory [255,283].

The current chapter investigates the effects of maternally administered cGP on spatial learning and reference memory of adult offspring in a MWM task. The effects of maternal administration of cGP on synaptic plasticity in the hippocampus and striatum regions of brains collected from dams, pups and adult offspring are also evaluated.

4.3 Materials and Methods

4.3.1 Animal experiment

The animal experiment has been described in detail in section 2.2.1 of the thesis. Briefly, female Sprague-Dawley rats (n = 16 per group) were dosed with either cGP dissolved in saline (3 mg/Kg body weight) or saline alone using a gavage feeding needle once per day from PN d8 to d22. On PN d23, the pups were weaned, and all the dams plus one male and one female pup per litter were euthanized with carbon dioxide, and brain samples were collected. The dams were 135-140 days old at the time of tissue collection. MWM test was carried out on the remaining male offspring from both treatment groups (n = 21 (control) and 23 (cGP)) between PN d70-75. The adult offspring were euthanized on PN d82, and brain samples were collected.

4.3.2 Morris Water Maze

The apparatus and the procedure of MWM test is described below.
4.3.2.1 Apparatus

The test was conducted in a quiet room with dim lights. The intensity of light could not be measured because of lack of such equipment. A video camera was set up in the ceiling. To avoid lighting reflection to the water, three desk lamps were used for creating the dimmed lighting in the room. A computer was set up outside of testing room with a monitor used for observing the performance of the rat during the tests. Water maze apparatus consisted of a black circular pool (0.6 m deep x 2.2 m in diameter) filled with water (maintained at 20-22 °C). 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 colored shoe wrap). The water was cleaned off any rat feces after each trial.

4.3.2.2 Acquisition trials

Rats underwent 4 trials on each day of the 4-day acquisition period, with an inter-trial interval of 6 min. 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. The pool was further divided into an imaginary inner and outer circular area. The inner circle had a radius of 92 cm. The outer circle did not contain the platform and encompassed the area up to 18 cm from the side wall. Rats were released into the pool tail-first and facing the center of 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 same for all the rats in a particular trial. During each trial, rats were allowed to swim, locate and mount on the platform for 120 sec. If rat was able to locate and mount on the platform within 120 sec, it was allowed to stay on the platform for additional 15 sec in order to enforce spatial learning with respect to the distal
cues. However, if rat was unable to locate the platform within 120 sec, it was gently guided towards the platform and allowed to remain on the platform for 15 sec. ANY-Maze tracking system was used to record the escape latency (i.e., the time required to locate the platform), distance covered before first entry to the platform, time spent in the outer circle and distance travelled in the outer circle for each rat. Lower scores on the first two parameters indicated better spatial learning whereas lower scores in the last two parameters indicated better navigational strategy.

4.3.2.3 Probe trials

Two probe trials were conducted 24 and 48 h after the last acquisition trial. During the probe trials, the platform was removed and the rat was allowed to swim for 30 sec looking for the absent platform. Average heading errors and path efficiency of entry to the platform zone were recorded using the ANY-Maze tracking system. Both the average heading errors and path efficiency give an indication whether the animal had a focused search or not. Average heading errors report the average angle between the animal’s heading and a direct heading to the zone. The heading to the zone can be defined as either heading to the center of the zone or using the entire zone area. We calculated heading errors to the center of the platform zone. To calculate heading errors, the animal must have settled on a certain heading. It can either be the animal’s heading a certain period after the test starts or its heading after it has moved to a certain distance from its initial location. We calculated the heading errors 500 msec after the test had started. Path efficiency measures the efficiency of path taken by the animal to get from the first position in the test to the last position. A value of 1 indicates perfect efficiency i.e. the animal moved in a straight line. Values less than 1 indicate decreasing efficiency. Path efficiency is calculated by dividing the straight-line distance between the first position in the test and the last position in the test to the total distance travelled by the animal during the test.
Lower heading errors and higher path efficiency to the platform zone showed better spatial reference memory and a more focused search.

### 4.3.3 Immunohistochemistry

As described in section 2.2.1, the right hemisphere of the brain was collected for immunohistochemical analysis. Sequential coronal sections of either 25 µm (adult offspring) or 50 µm (dams and pups) thickness were collected using a frozen microtome, with every 12 or 6 sections pooled together, respectively (for instance, sections 1, 13, 25, 37 and so on, or sections 1, 7, 13, 20 and so on). For each parameter of staining, sections from the same sample pool were used, and staining was performed simultaneously across the treatment groups for each age group.

Primary antibodies against synaptophysin and GluR-1 were used to mark synaptic vesicles and GLuR-1 sub-unit of AMPA receptors, respectively, in the hippocampus and striatum regions of brain. These markers were chosen so as to investigate neuroplasticity at the pre- and post-synaptic level, respectively. Following the initial quenching of endogenous peroxidase activity using 1 % H$_2$O$_2$ in 50 % methanol for 30 min (in dark), the sections were incubated with the following primary antibody: rabbit anti-GluR1 (Millipore, MA, USA; 1:500 (pups), 1:5000 (adult offspring) and 1:2000 (dams)), or mouse anti-synaptophysin (Sigma, NY, USA; 1:10,000) at 4 oC for 48 h. Sections were incubated with biotin-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies accordingly (Sigma; 1:1000) at 4 oC overnight. Sections were then incubated with ExtrAvidin peroxidase tertiary antibody (Sigma; 1:1000) for 3 h at room temperature. A brown reaction product was obtained by adding 0.05 % 3, 3-diaminobenzidine substrate. A washing step (3 x 10 min) was included in-between the incubations. Stained sections were mounted on gelatin-coated glass slides, dehydrated through
increasing gradient of ethanol to Safsolvent (ECP Ltd., Auckland, New Zealand), and cover
slipped using DPX.

4.3.3.1 Image acquisition and analysis

Three images of hippocampus and striatum were taken from at least four sequential sections
using a microscope (Nikon Eclipse E800, Tokyo, Japan) fitted with a digital camera system
(Nikon DS Fi1, Tokyo, Japan) at 4x magnification. The average intensity of synaptophysin
and GluR-1 staining were measured in striatum and each sub-region of hippocampus using
ImageJ (v 1.49t, National Institutes of Health, Bethesda, MD, USA). Personnel conducting the
data acquisition and analyses were blinded to the treatment groups.

4.3.4 Statistical analyses

The data from acquisition and probe trials of MWM were analyzed using the mixed model
ANOVA (IBM SPSS Statistics 22, Chicago, IL, USA), with time as the within-subjects factor
and treatment as the between-subjects factor, followed by Bonferroni post hoc tests. For each
age group, intensity of synaptophysin and GluR-1 staining in hippocampus were analyzed
using two-way ANOVA (SPSS), with treatment and hippocampal sub-regions as the
independent variables, followed by Bonferroni post hoc tests. Parametric t-tests were used to
compare differences between treatment groups in striatum. Statistical significance was set at P
≤ 0.05. The data are presented as mean ± SEM.
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4.4 Results

4.4.1 Morris Water Maze

4.4.1.1 Acquisition trials

Acquisition trials were conducted from PN d70 to d73 to test the spatial learning of adult offspring from cGP- and saline-administered dams. Mixed ANOVA showed a significant reduction in distance travelled to first entry to platform (F (15, 285) = 8.0, P < 0.001, Figure 4.1A) and escape latency (F (15, 555) = 22.47, P < 0.001, Figure 4.1B) over the 16 acquisition trials, but no treatment effect or an interaction between trials and treatment. Comparisons relative to baseline performance, i.e. trial 1, revealed a significant reduction in distance travelled to platform in trial 6 for cGP offspring whereas control offspring did not show a significant reduction in any of the subsequent trials (Figure 4.1A). Compared with trial 1, cGP offspring showed a significant reduction in escape latency in trial 3 and control offspring showed a similar reduction in trial 4 (Figure 4.1B).

A significant reduction in the distance travelled (F (15, 570) = 20.72, P < 0.001, Figure 4.1C) and time spent (F (15, 570) = 28.41, P < 0.001, Figure 4.1D) in the outer circle was also observed over the 16 acquisition trials, but no treatment effect or an interaction between trials and treatment was observed. Comparisons relative to baseline performance, i.e. trial 1, showed a significant reduction in distance travelled in the outer circle in trial 6 for cGP offspring (P < 0.05) whereas control offspring did not show a significant reduction in any of the subsequent trials (Figure 4.1C). Compared with trial 1, cGP offspring showed a significant reduction in time spent in the outer circle in trial 2 and control offspring showed a similar reduction in trial 4 (P < 0.05, Figure 4.1D).
4.4.1.2 Probe trials

Two probe trials were conducted 24 and 48 h after the last acquisition trial to test the spatial reference memory of adult offspring from cGP- and saline-administered dams. Mixed ANOVA showed a significant reduction in path efficiency of entry to platform zone with time ($F_{(1, 17)} = 6.15, P < 0.05$, Figure 4.1E) and an overall increase in path efficiency with cGP treatment ($F_{(1, 17)} = 4.28, P = 0.054$, Figure 4.1E). No interaction was observed between the time points and the treatment groups. Bonferroni post hoc tests showed a significant increase in the path efficiency of cGP offspring at 24 h time-point compared with the control offspring ($P < 0.05$, Figure 4.1E).

No change in the average heading errors to the platform zone was observed with time and treatment, however, a significant interaction was observed between the time points and the treatment groups ($F_{(1, 42)} = 3.97, P = 0.053$, Figure 4.1F). Bonferroni post hoc tests revealed a significant reduction in the average heading errors of cGP offspring at the 48 h time-point compared with the control offspring ($P < 0.05$, Figure 4.1F).
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Figure 4.1: Maternal administration of Cyclic-glycine-proline (cGP) during Lactation improves Spatial Learning and Reference Memory of the Adult Offspring.

Dams were orally gavaged with either cGP (3 mg/Kg body weight) or saline once per day from postnatal (PN) d8-22. Spatial learning of adult offspring from cGP-administered (closed circles) and saline-administered (open circles) dams during 4 days of acquisition trials (4 trials per day) in morris water maze test (PN d70-73) were evaluated by analyzing the distance travelled to first entry to platform (A), escape latency (B), and distance travelled (C) and time spent (D) in the outer circle, a circular area that did not contain the platform and encompassed the area up to 18 cm from the side wall. Reference memory of rats was tested in two probe trials conducted 24 and 48 h after the last acquisition trial by evaluating the path efficiency of entry (E) and average heading errors (F) to the platform zone. Data are presented as mean ± SEM, n = 21 (Placebo) and 23 (cGP). *
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shows significant differences between the treatment groups, and # and + show significant differences compared with trial 1 for cGP-administered and saline-administered groups, respectively. Significance was set at P ≤ 0.05, mixed ANOVA.

4.4.2 Glutamate receptor-1

4.4.2.1 Hippocampus

Figure 4.2A shows the distribution of GluR-1 staining in the hippocampus. Differential intensity of staining for GluR-1 was observed in different sub-regions of hippocampus. Dentate gyrus (DG) and CA1-2 were intensely stained whereas CA3 and CA4 were weakly stained (Figure 4.2A). Separate intensity measurements for GluR-1 staining in stratum oriens (Or) and stratum radiatum-stratum lacunosum moleculare (SR-Mol) sub-regions of CA1-2 could be obtained due to the weaker staining in the neuronal cell layer that separates these two regions. Morphologically, GluR-1 positive staining was typically axonal with some neuronal cell staining observed in CA3 and CA4 sub-regions (data not shown).

Two-way ANOVA showed that the mean intensity of GluR-1 staining was significantly different in the hippocampal sub-regions of dams (F (4, 135) = 77.76, P < 0.001, Figure 4.2B), pups (F (4, 105) = 50.35, P < 0.001, Figure 4.2C) and adult offspring (F (4, 210) = 44.63, P < 0.001, Figure 4.2D). A significant treatment effect was observed in the adult offspring (F (1, 210) = 20.14, P < 0.001, Figure 4.2D) but not in the dams (Figure 4.2B) and the pups (Figure 4.2C). No interaction between the hippocampal sub-regions and treatment groups was observed in dams, pups and adult offspring. Bonferroni post hoc tests showed a significant increase in the intensity of GluR-1 staining in DG, Or and SR-Mol sub-regions of hippocampus of adult offspring from cGP-administered dams compared with the control group (P < 0.05, Figure 4.2D). Trends for increases in the intensity of GluR-1 staining were also observed in the CA3 (P = 0.07) and CA4 (P = 0.09) sub-regions (Figure 4.2D).
Figure 4.2: Maternal administration of Cyclic-glycine-proline (cGP) during Lactation increases the Intensity of Glutamate Receptor-1 (GluR-1) Staining in the Hippocampus of Adult Offspring.

Dams were orally gavaged with either cGP (3 mg/Kg body weight) or saline once per day from postnatal (PN) d8-22. Brain samples were collected from the dams and pups on PN d23, and from the adult offspring on PN d82. Representative grayscale image of GluR-1 staining in different sub-regions of hippocampus is shown (A). The mean intensity of GluR-1 staining in hippocampal sub-regions of dams (B), pups (C) and adult offspring (D) from control (open bars) and cGP (closed bars) groups is shown. Data are presented as mean ± SEM, n = 15 (placebo) and 14 (cGP), 13 (placebo) and 10 (cGP) and 21 (placebo) and 23 (cGP) for dams, pups and adult offspring, respectively. Significant differences between the treatment groups are shown by **P < 0.01 and *P < 0.05, two-way ANOVA. DG = Dentate gyrus, Or = Stratum oriens, SR-Mol = Stratum radiatum - Stratum lacunosum-moleculare.
4.4.2.2 Striatum

Characteristic striated pattern of GluR-1 staining was observed in the striatum, with uniform staining of grey matter connecting the caudate nucleus and the putamen, and the absence of staining in the white matter tracts of the internal capsule (Figure 4.3A). The mean intensity of GluR-1 staining in the striatum was higher in the cGP-administered dams (t (27) = 2.62, P = 0.01, Figure 4.3B) and the adult offspring (t (39) = 2.20, P = 0.03, Figure 4.3C) compared with their respective control groups.

A similar investigation into the striatum of pups’ brains could not be conducted because of the scarcity of tissue sections.
Figure 4.3: Maternal administration of Cyclic-glycine-proline (cGP) during Lactation increases the Intensity of Glutamate Receptor-1 (GluR-1) Staining in the Striatum of Dams and Adult Offspring.

Dams were orally gavaged with either cGP (3 mg/Kg body weight) or saline once per day from postnatal (PN) d8-22. Brain samples were collected from dams and adult offspring on PN d23 and PN d82, respectively. Representative grayscale image of GluR-1 staining in striatum is shown (A). The mean intensity of GluR-1 staining in striatum of dams (B) and adult offspring (C) from control (open bars) and cGP (closed bars) groups is shown. Data are presented as mean ± SEM, n = 15 (placebo) and 14 (cGP), and 20 (placebo) and 21 (cGP) for dams and adult offspring, respectively. *P < 0.05 shows significant differences between the treatment groups, unpaired t-tests.
4.4.3 Synaptophysin

4.4.3.1 Hippocampus

Sub-regions of hippocampus showed differential intensity of staining for synaptophysin. For instance, DG was the most intensely stained and CA4 the least intensely stained sub-region (Figure 4.4A). Different sub-regions within CA1-2 and CA3 were selected for intensity measurements. Stratum oriens (Or) and stratum radiatum (SR) sub-regions of CA1-2, and stratum oriens (SO) and stratum lucidum (SL) sub-regions of CA3 were selected owing to the absence of synaptophysin staining in the neuronal cell layer that separates these two regions, respectively. Stratum lacunsum-moleculare (Mol) and SR of CA1-2, and stratum lacunosum-moleculare (LM) and SL of CA3 were separable owing to different intensity of synaptophysin staining in these sub-regions, respectively.

Two-way ANOVA showed that the mean intensity of synaptophysin staining was significantly different in the hippocampal sub-regions of dams ($F_{(7, 216)} = 4.82, P < 0.001$, Figure 4.4B), pups ($F_{(7, 200)} = 5.99, P < 0.001$, Figure 4.4C) and adult offspring ($F_{(7, 325)} = 4.39, P < 0.001$, Figure 4.4D). The mean intensity of synaptophysin staining was significantly higher in the hippocampus of cGP-administered dams compared with the control dams ($F_{(1, 216)} = 10.92, P < 0.01$, Figure 4.4B). There was no difference between the two treatment groups of pups (Figure 4.4C) and adult offspring (Figure 4.4D). No interaction between the hippocampal sub-regions and treatment groups was observed in dams, pups and adult offspring. Bonferroni post hoc tests did not show any difference in the hippocampal sub-regions of cGP-administered dams compared with the control dams (Figure 4.4B).
Figure 4.4: Administration of Cyclic-glycine-proline (cGP) during Lactation increases the Intensity of Synaptophysin (SYN) Staining in the Hippocampus of Dams.
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Dams were orally gavaged with either cGP (3 mg/Kg body weight) or saline once per day from postnatal (PN) d8-22. Brains were collected from the dams and pups on PN d23, and from the adult offspring on PN d82. Representative grayscale image of SYN staining in different sub-regions of hippocampus is shown (A). The mean intensity of SYN staining in hippocampal sub-regions of dams (B), pups (C) and adult offspring (D) from control (open bars) and cGP (closed bars) groups is shown. Data are presented as mean ± SEM, n = 15 (placebo) and 14 (cGP), 16 (placebo) and 11 (cGP), and 21 (placebo) and 23 (cGP) for dams, pups and adult offspring, respectively. Significant differences between the treatment groups are shown by **P < 0.01, two-way ANOVA. DG = Dentate gyrus, SO = Stratum oriens, SL = Stratum lucidum, LM= Stratum lacunosum-moleculare, Or = Stratum oriens, SR = Stratum radiatum, Mol = Stratum lacunosum-moleculare.

4.4.3.2 Striatum

Characteristic striated pattern of synaptophysin staining was observed in the striatum, with uniform staining of grey matter, and the absence of staining in the white matter tracts of the internal capsule (Figure 4.5A). There was no difference between the two treatment groups of dams and adult offspring in the mean intensity of synaptophysin staining in the striatum (Figure 4.5 B and C).

A similar investigation into the striatum of pups’ brains could not be conducted because of the scarcity of tissue sections.
Figure 4.5: Maternal administration of Cyclic-glycine-proline (cGP) during Lactation does not alter the Expression of Synaptophysin (SYN) in the Striatum of Dams and Adult Offspring.

Dams were orally gavaged with either cGP (3 mg/Kg body weight) or saline once per day from postnatal (PN) d8-22. Brain samples were collected from dams and adult offspring on PN d23 and PN d82, respectively. Representative grayscale image of SYN staining in striatum is shown (A). The mean intensity of SYN staining in the striatum of dams (B) and adult offspring (C) from control (open bars) and cGP (closed bars) groups is shown. Data are presented as mean ± SEM, n = 14 (placebo) and 15 (cGP), and 21 (placebo) and 22 (cGP) for dams and adult offspring, respectively. Unpaired t-tests.
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4.5 Discussion

Results presented in this chapter show that early life administration of cGP, through maternal supplementation during lactation, improves the spatial learning and reference memory of offspring at adulthood. The long-term functional improvement was associated with increased glutamatergic neuroplasticity in the hippocampus and striatum. The efficacy of cGP in promoting neuroplasticity was also evident through the increased hippocampal expression of synaptophysin and striatal expression of GluR-1 in dams. This data may provide the evidence that promoting trophic support, for instance through IGF-1, during early stage of postnatal brain development can lead to better cognitive function at adulthood.

The performance in MWM test is commonly used to evaluate the spatial learning and reference memory abilities of rats, including the process and strategy of learning in the acquisition trials, and the retention of newly acquired reference information in the probe trials [282]. The ability of learning was similar between the two groups of offspring as the overall performances in escape latency and distance travelled to locate the platform were similar between the groups. In comparison with the baseline performance, i.e. trial 1, the escape latency was reduced in both groups suggesting both groups of offspring learnt to perform the tasks (Figure 4.1A and B). The cGP offspring, however, may have learnt faster and better than the control offspring because they showed an earlier reduction in escape latency (Figure 4.1B), and a significant reduction in distance travelled to platform (Figure 4.1A). It should be noted that the non-significant lower baseline distance travelled by the control offspring may have partially contributed to the non-significant decrease in swim distance over the acquisition trials for this group (Figure 4.1A).
The performance of animals in the outer circle of MWM (thigmotaxic behavior) has been used as an index of anxiety [284], and as a measure of navigational strategy [285,286]. The cGP offspring showed an earlier reduction in the thigmotaxic behavior compared with the control offspring by adopting the new escaping strategy (i.e. to locate the hidden platform rather than moving along the walls of the pool) at earlier time points (Figure 4.1 C and D). Because maternally administered cGP does not alter the anxiety-like behavior of offspring (Chapter 3), therefore, maternally administered cGP may have moderately improved the navigational strategy of the offspring.

The functional improvement associated with cGP was generally moderate. Given that the mechanism of action of cGP involves normalizing IGF-1 function [34], and a normal population of rats used in the current study, the further optimization of learning or memory would be small. Unlike the intervention studies in animals with obvious behavioral deficits, the efficacy of cGP on spatial learning of normal animals is expected to be moderate, and hence difficult to detect using the conventional parameters of performance analysis. Therefore, comparing the performance over time has provided an additional opportunity to detect the differences in spatial and strategic learning of normal animals.

Similarly, conducting the probe trials at delayed time-points helps in detecting moderate differences in memory retention between the treatment groups [283,287]. The probe trials are usually conducted 2 h and 24 h after the last acquisition trial to test the short-term and delayed memory functions, respectively. Because subtle differences were expected between the treatment groups in the current study, therefore, the two probe trials were conducted 24 and 48 h after the last acquisition trial. Out of several parameters analyzed for memory retention, two showed significant differences between the treatment groups. Though no difference was observed between the treatment groups at the 24 h time-point, cGP offspring made lower
average heading errors to the platform zone at the 48 h time-point (Figure 4.1F), possibly due to the trend towards an increase in heading error in the control offspring. Further, cGP offspring showed significant improvement in path efficiency of entry to the platform zone 24 h after training, but were unable to maintain their performance (Figure 4.1E). Lower average heading errors and better path efficiency compared with the control offspring show that cGP offspring had better spatial memory, likely due to a more focused search for the target. The long-lasting efficacy of cG-2allyl-P, a structural analogue of cGP, has been previously reported by showing the improved brain function 10 weeks after treatment [68].

Spatial learning and memory is known to be associated with neuroplasticity in the hippocampus, a brain region involved in navigation [131,283]. Specifically, membrane trafficking of AMPA receptors in the hippocampus is crucial for spatial learning and memory [for review, see 115, and section 1.2.3 of the current thesis]. For example, MWM training is associated with an increase in the expression of GluR-1, a sub-unit of AMPA receptors, in the hippocampus [116]. Maternal cGP administration increased the expression of GluR-1 in the hippocampus of adult offspring, with no effect in pups and dams (Figure 4.2 B-D). Considering that maternal cGP administration increases the plasma concentrations of cGP in dams and pups but not in the adult offspring (Chapter 3), we do not have a clear understanding of the different efficacy of cGP on glutamate trafficking between the age groups. It is possible that the maternal administration of cGP only ‘sensitized’ the glutamate response in the hippocampus of dams and pups at the time of treatment, and the intensive navigation training during 6 days of MWM tests stimulated the glutamatergic neurotransmission, leading to an increase in GluR-1 expression. A similar ‘sensitization’ of hippocampus for improved capacity for glutamatergic neurotransmission is reported by others. For instance, improved spatial memory in adult offspring of choline supplemented dams was associated with improved
capacity for glutamatergic neuroplasticity in the hippocampus [90,91]. The efficacy of cGP on GluR-1 expression has been reported previously. For instance, cG-2allyl-P was found to restore memory in rats with acute memory deficits via an increase in the expression of GluR-1 in hippocampus [167].

Striatum receives several projections from hippocampus, prefrontal cortex and amygdala [106,288], and plays an essential role in spatial learning and memory [107]. Furthermore, striatal AMPA receptors are required for spatial learning [110], and a reduction in striatal GluR-1 expression is shown to be associated with learning and memory deficits in neurological disorders such as schizophrenia [289]. Maternal cGP administration increased the expression of GluR-1 in the striatum of both dams and adult offspring (Figure 4.3 B and C). Striatum is shown to play a key role specifically in the processing of spatial information acquired early during the MWM task [108]. Therefore, increased striatal GluR-1 expression may associate with better learning in adult cGP offspring compared with the control offspring. However, the improved striatal GluR-1 expression in adult cGP offspring may not be associated with MWM training because GluR-1 expression was also increased in the striatum of cGP-administered dams.

Synaptophysin is an integral membrane glycoprotein of the pre-synaptic vesicles [290], and is essential for synaptic transmission [291]. It is widely used as a marker of non-specific changes in neuronal plasticity at the pre-synaptic locations [292], including the hippocampal plasticity associated with spatial learning and memory [293,294]. Maternal cGP administration caused a moderate increase in the expression of synaptophysin in the hippocampus of dams but not that of the offspring (Figure 4.4 B-D). The lack of an effect in adult offspring may suggest a transient effect of cGP on synaptophysin expression. The lack of effect in the pups could be due to the peak in synaptic density observed in weanling rats [295,296]. Given that cGP
normalizes IGF-1 mediated neuroplasticity, the chances to detect further improvement in already optimized developmental neuroplasticity will be minimal. Other studies have also shown that neuroplasticity triggered by placing the rats in an enriched environment is restricted to adult and aged rats with no change in the weanling rats [297].

The mechanism of action of cGP involves regulating the bioavailability of IGF-1 [34], therefore, the cGP efficacy is a regulated IGF-1 function [29]. The role of IGF-1 in brain development, neuroplasticity and brain function, including learning and memory has been well documented in the last 30 years [73,155]. Studies investigating the mechanisms of action have shown that IGF-1 mediates neuroplasticity through a number of ways, including the effects on GluR-1 [161] and synaptophysin [294] in the hippocampus. Exogenous IGF-1 supplementation enhances AMPA-receptor mediated synaptic transmission in juvenile, young adult and aged rats, and is proposed to be the mechanism through which IGF-1 ameliorates learning and memory deficits associated with aging [159,160]. A similar mechanism, owing to the cGP-mediated increase in the bioavailability of IGF-1, may be involved in the current study, and is further discussed in Chapter 6. In the current study, the role of better maternal care in improving the brain function of offspring [298] was not addressed, considering that there was an increase in neuroplasticity in cGP-administered dams. Therefore, this needs further investigation.

The clinical application of IGF-1 for improving learning and memory deficits is limited by its poor central uptake [299], and potential metabolic (e.g. hypoglycemia) and mitogenic side-effects [300]. Contrarily, cGP is lipophilic and enzymatically stable, and shows oral bioavailability with effective central uptake owing to its small molecular size [29,67]. Therefore, results presented in the current chapter validates future research in using cGP as a therapeutic agent for improving learning and memory deficits associated with sub-optimal
IGF-1 function. For example, maternal cGP supplementation may offer benefits to both the mother and the offspring in conditions with sub-optimal maternal and offspring IGF-1 levels, such as those associated with maternal obesity [258,279]. Recently, impaired IGF-1 signaling, including reduction in IGF-1 levels in hippocampus, was proposed as a possible mechanism underlying the learning and memory deficits observed in offspring born to obese mothers [279,281].

4.6 Summary

IGF-1 plays a crucial role in postnatal brain development and function. As a metabolite of IGF-1, cGP normalizes IGF-1 function via modulating the bioavailability of IGF-1. Results presented in Chapter 3 showed that maternally administered cGP improves the recognition memory of developing offspring by increasing the bioavailability of IGF-1. The current chapter evaluated the efficacy of maternally administered cGP on spatial memory of adult offspring. The effect on postnatal brain development was evaluated by analyzing the changes in neuroplasticity in brains of pups and adult offspring. Changes in neuroplasticity in dams’ brains provided an additional measure of transient effects of cGP treatment.

Either cGP or saline was orally given to dams from PN d8-22. Spatial memory of adult offspring was evaluated using MWM between PN d70-75. Changes in the expression of GluR-1 and synaptophysin in the brain sections of dams, pups and adult offspring was evaluated using immunohistochemistry.

The overall performance in acquisition trials was similar between the two treatment groups. However, in comparison with the baseline performance (i.e. acquisition trial 1), cGP offspring showed an earlier reduction in escape latency and distance to platform than the control offspring. Further, cGP offspring showed higher path efficiency of entry to platform zone 24h
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after acquisition and lower average heading errors 48h after acquisition compared with the control offspring. Expression of GluR-1 was increased in the hippocampus and striatum of adult cGP offspring, and the striatum of cGP-administered dams, which also showed an increase in the expression of synaptophysin in hippocampus.

In conclusion, maternal administration of cGP during the early stages of postnatal brain development improved spatial learning and memory of adult offspring, possibly by promoting glutamatergic neurotransmission. Therefore, cGP may be a trophic factor for optimizing brain development and adult cognition, possibly through mediating IGF-1 function. This data, together with the improvement in recognition memory of adolescent offspring (Chapter 3), suggests that maternally administered cGP positively influences neurodevelopmental programming of offspring. However, the effect of cGP on lactational capacity of dams needs to be investigated, which may also influence the cognitive performance of the offspring. This investigation is carried out in the next chapter.
Chapter 5: Cyclic-glycine-proline reduces the phosphorylation of IGF-1R and promotes post-lactational involution in mammary glands of rats

5.1 Summary of chapter contents
Maternal administration of cGP during lactation improves the recognition memory (Chapter 3) and the spatial memory (Chapter 4) of adolescent and adult offspring, respectively, which is associated with improved glutamatergic neuroplasticity in different brain regions (Chapter 4). An improvement in lactational capacity of dams may, however, also influence the cognitive function of the offspring. IGF-1 is well known to improve lactational capacity of rodents by delaying the onset of post-lactational involution in mammary glands. However, the role of cGP in mammary involution has never been investigated. This chapter, therefore, investigates the effect of maternal administration of cGP during lactation on the lactational capacity, more specifically the milk production and composition and the post-lactational involution of mammary glands, of dams. The effect of cGP on various morphological, cellular and molecular markers of involution is presented. Because involution is associated with a decline in IGF-1 function, therefore, this chapter also investigates the effect of cGP on the physiological decline in IGF-1 function. This chapter was recently published as an original research article in the Journal of Cellular Physiology [301].

5.2 Introduction
Post-lactational involution of mammary gland involves a coordinated process of removal of the MEC (primarily through apoptosis) and the lobulo-alveolar structures that ultimately lead to the remodeling of the gland to its pre-pregnant state [for review see 179, and section 1.3.3
of the current thesis]. Involution occurs naturally following weaning, but can also be induced through pup removal or teat-sealing in rodents [176]. The primary trigger for the induction of apoptosis and involution is the local engorgement of mammary alveoli due to milk stasis [184]. For instance, sealing teat of one gland does not trigger involution in the remaining glands [184].

Natural involution occurs at about half the rate of the induced involution [41], and does not involve chronic milk stasis because of the autocrine inhibition of milk secretion, owing to irregular milk removal [180]. Apoptosis of MEC occurs in both natural and induced involution. However, the induction of apoptosis is slower during natural involution compared with induced involution [176]. Experimental induction of involution has enabled the categorization of mammary involution into two distinct phases: the first phase is characterized by an increase in apoptosis of MEC to a peak rate, which is followed by a rapid decline in the expression of milk protein genes, milk synthesis and milk secretion [183]. This phase is, however, reversible within 48 h of mammary engorgement as demonstrated through re-suckling of engorged glands in rats [189]. The second, irreversible, phase of involution begins 72 h after mammary engorgement, and involves further apoptosis of MEC and extensive proteolytic degradation of the lobulo-alveolar structures [183,190].

IGF-1 is an important survival factor during post-lactational involution of mammary glands [302]. The increased apoptosis and intensive tissue remodeling during involution is associated with the reduced bioavailability of IGF-1, which is due to the increased binding of IGF-1 to IGFBP that are upregulated during involution [242]. The mammary specific overexpression of both IGF-1 and des-IGF-1 has been shown to delay involution in rodents by reducing apoptosis and preserving the secretory lobulo-alveolar structures [42,44]. Furthermore, des-
IGF-1 is shown to prolong lactation by increasing the bioactivity of IGF-1, as evidenced through increased phosphorylation of IGF-1 receptors [43].

cGP is produced from the cyclization of the N-terminal tripeptide of IGF-1, GPE, when IGF-1 is enzymatically broken down to GPE and des-IGF1 [reviewed in 29]. Results presented in Chapter 3 showed the presence of endogenous cGP in rat milk, with higher concentrations during peak lactation compared with late lactation/early involution. Furthermore, exogenous cGP was orally bioavailable in dams, and showed possible translocation to the mammary gland as evidenced through an increase in the concentrations of cGP in milk. By altering the binding of IGFBP to IGF-1, cGP regulates the bioavailability of IGF-1, and thus normalizes IGF-1 function [34]. For instance, cGP is shown to both inhibit and promote IGF-1 bioactivity when IGF-1 bioactivity is relatively increased and diminished, respectively [34]. The post-lactational involution in mammary glands of rodents is associated with a decline in the expression of IGF-1 [53]. Mammary-specific overexpression of IGF-1 delays involution [42], however, it is not known whether cGP can delay post-lactational involution by preventing or reducing the physiological decline in IGF-1 function.

Progressive weaning starts in rat pups from PN d16, accelerates between PN d18 and d25, and is shown to be complete by PN d29 when the pups start ingesting adult levels of solids [303]. Because involution can be precipitated through teat-sealing in rodents [176], in the current study, involution was induced by sealing the teats of a pair of mammary glands on PN d20 and d22 each. The sealed and unsealed glands were then collected on PN d23 to investigate the effect of maternal administration of cGP during lactation on early (unsealed glands)-, mid (d22 sealed glands)- and late (d20 sealed glands)- stages of involution. The effects of cGP on post-lactational involution were examined at the morphological level (preservation or loss of lobulo-alveolar structures), cellular level (changes in cell apoptosis and proliferation using
antibodies against the cleaved caspase-3 and Ki-67 antigens, respectively) and molecular level (changes in the expression of pro- and anti-apoptotic genes, and the phosphorylation of IGF-1R). Changes in milk production (determined through litter growth rate) and composition were also evaluated.

5.3 Materials and Methods

5.3.1 Experimental procedure

The experimental procedure has been described in section 2.2.1 of the thesis. Briefly, 32 female Sprague-Dawley rats (300–400 g) from the Small Animal Colony at AgResearch, Hamilton (New Zealand) were used for the experiment. Each female rat was mated at approximately 13 weeks of age, and the day of parturition was considered as PN d1. On PN d8, dams were randomly allocated to two groups (n = 16 per group). From PN d8 to d22, dams from one of the groups were gavaged daily with cGP (3 mg cGP/Kg body weight) dissolved in saline whereas the dams from the second group were gavaged daily with an equivalent volume of saline, and therefore served as the control group. Involution was induced by sealing the teats of cranial thoracic and caudal thoracic pair of mammary glands using an ethyl cyanoacrylate based adhesive glue on PN d20 and d22 respectively. After applying the glue, dams were restrained for further 10 seconds and a paper cap was placed on the teats to prevent the dams from licking off the glue. Dams were then housed in a separate cage away from the pups for 30 min. This practice helped in preventing the pups from licking off the glue or from removing the paper cap. On PN d23 the dams were euthanized using carbon dioxide, and pairs of sealed and unsealed (abdominal) mammary glands were collected after separating from the skin and the surrounding tissue. The unsealed glands, which were undergoing the gradual
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process of natural involution, served as the early-involution time-point, and the glands sealed for 24 h and 72 h served as the mid- and late-involution time-points, respectively.

5.3.2 Milk production

The weights of the dams and the litter were recorded on PN d1, d7, d14, d21 and d23. The gain in litter weight per week was used as an indirect indicator of milk production.

5.3.3 Milk composition

Milk samples were collected on PN d7, d10, d15, d20 and d23 from each dam between 0900 and 1100 hours as described in section 2.2.1. The total protein and lipid content of milk was measured using the infrared spectrometer Direct Detect™ (Merck Millipore, Darmstadt, Germany). Milk samples were diluted 80-fold in 30 mM solution of disodium hydrogen phosphate and sodium dihydrogen phosphate at pH 7.6. The total protein concentration was measured using the Analysis Method 1, and the relative absorbance values of lipids were determined using the Relative Absorbance mode of Direct Detect™ as per the manufacturer’s instructions. Each sample was assayed in triplicate.

5.3.4 Tissue preparation

Mammary glands collected from the left side of the body were used for histology and immunohistochemical analyses, and those collected from the right side of the body were used for mRNA extraction. Mammary glands for histology and immunohistochemical analyses were fixed in 4 % paraformaldehyde for 48 h at 4 °C, and then washed and stored in 70 % ethanol at 4 °C until use. Approximately one-half of the mammary gland was cut through the teat. A narrow transverse section (including the teat) was cut from one of these halves. Both the transverse section and the remaining half of mammary gland were then placed in Tissue-Tek III cassettes (Sakura-Fintek Europe BV, Alphen aan den Rijn, The Netherlands). These
mammary gland slices were processed automatically (Leica JUNGTP1050 Tissue Processor; Leica Microsystems, Auckland, New Zealand) through a range of alcohol washes (70 %, 95 %, 100 %), followed by clearing in xylene and infiltration with paraffin wax. The processed tissues were embedded in paraffin wax (BDH Laboratory Supplies, Dorset, England) and 7 µm thick serial sections of the embedded tissue were cut and mounted on the polysine-coated glass slides (LabServ, Auckland, New Zealand).

5.3.5 Histological analysis

One section per mammary gland sample (n = 16 per stage of involution per treatment group) was stained with hematoxylin and eosin for histological examination. Six representative photomicrographs were taken randomly at 200X magnification from each section using a light microscope (Nikon Eclipse E800, Tokyo, Japan), a digital camera (Nikon DS Fi1) and NIS Elements Documentation software (v 4.40, Nikon). The numbers of “intact” secretory, “engorged” and “regressed” alveoli were counted in each photomicrograph as per the guidelines from [304,305]. A total of 177-540 alveoli were counted per section to calculate the percentages of intact, engorged and regressed alveoli for each section.

5.3.6 Immunohistochemistry

Immunohistochemical analyses of cell proliferation and apoptosis was carried out using antibodies against the Ki-67 and caspase-3 (cleaved) antigens, respectively. Slides were deparaffinized by incubation at 60 °C for 30 min, followed by rehydration through Safsolvent (ECP Ltd., Auckland, New Zealand) and a graded series of ethanol at room temperature. Antigen retrieval was carried out in 2100-Retriever (Prestige Medical Ltd., Lancashire, England) using 0.01 M citrate buffer (0.1 M citric acid, 0.1 M trisodium citrate, pH 6.0) for 1 h. Slides were washed (3 x 10 min) between each step in either 0.01 M phosphate-buffered saline (pH 7.4) containing 0.2 % TritonX-100 (PBST) in the Caspase-3 assay or 0.01 M Tris-
buffered saline (pH 7.4) containing 0.1 % Tween 20 (TBST) in the Ki-67 assay. The sections were quenched in dark at room temperature (RT) for 30 min with either 1 % H$_2$O$_2$ (VWR International Ltd., Leicestershire, England) in 50 % (v/v) methanol and PBS for caspase-3 or the Dako Cytomation Biotin Blocking System (Dako Cytomation California Inc., Carpentaria, CA, USA) and the Dual Endogenous Enzyme Block (Dako) for Ki-67. Sections were then blocked with either 5 % normal horse serum (Sigma-Aldrich, Auckland, New Zealand) in PBS for 1 hour at RT for caspase-3 or 2.5 % bovine serum albumin (ChromatoPur™ Bovine Albumin, MP Biomedicals Ltd., Auckland, New Zealand) in TBST for 30 min at RT for Ki-67. Following pre-treatments, sections were incubated at 4 °C with either anti-Ki67 (sp6) rabbit monoclonal antibody (1:200, Abcam, ab16667, Cambridge, UK) diluted in antibody dilution buffer (StressMarq Biosciences Ltd., Victoria, Canada) for 24 h or cleaved caspase-3 (Asp 175) rabbit polyclonal antibody (1:100, Cell Signaling Technology, 9661L, MA, USA) diluted in 1 % normal horse serum (Sigma-Aldrich) in PBS for 48 h. The sections were then incubated with biotin-conjugated anti-rabbit secondary antibody (Sigma-Aldrich, B7389, St. Louis, MO, USA) at either 1:400 (Ki-67 assay) or 1:200 (Caspase-3 assay) dilutions overnight at 4 °C, followed by incubation with Extravidin-peroxidase (Sigma-Aldrich, E2886, St. Louis, MO, USA) in the same dilution as the secondary antibody for 3 h at RT. The staining was visualized using 3,3’-diaminobenzidine substrate (Sigma-Aldrich, D5637, Auckland, New Zealand) for 5 min. Slides were counterstained using hematoxylin, dehydrated through a graded series of ethanol followed by Safsolvent, mounted with DPX (Scharlab S. L., Sentmenat, Barcelona, Spain) and then cover slipped.

One section per mammary gland sample was used (n = 16 per stage of involution per treatment group) for semi-quantitative immunohistochemical analysis by taking 6 random photomicrographs at 200X magnification using the same microscope-digital camera system.
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described above. For the sections from the late-involution group, 4-8 photomicrographs of areas containing alveoli with engorged and/or regressed phenotypes were taken. These two types of alveoli were analyzed separately for the late-involution group (referred hereafter as late-involution (Eng) and late-involution (Reg) groups). The numbers of alveoli with one or more Ki-67 or caspase-3 positive nuclei were counted in each photomicrograph using the counter tool of ImageJ (National Institutes of Health, Bethesda, MD). An average of 214 (Ki-67 assay) and 227 (caspase-3 assay) alveoli were counted per section, and a percentage value was calculated for each section.

5.3.7 Immunofluorescence single- and double-labelling

Mammary sections were exposed to immunofluorescent staining for localizing the phosphorylated IGF-1 receptor (pIGF-1R) antigen. The pre-treatment procedure was same as for immunohistochemistry staining except the blocking was done using 10 % normal donkey serum (Jackson ImmunoResearch Laboratories Inc., 017-000-121, Westgrove, PA, USA) in TBS at RT for 1 h. Sections were incubated with anti-IGF1R (phospho Y1161) rabbit polyclonal antibody (1:500, Abcam, ab39398, Cambridge, UK) diluted in 1 % normal donkey serum (Jackson ImmunoResearch Laboratories Inc.) in TBS overnight at 4 °C. To visualize the staining, sections were incubated with Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody (1:400, Molecular Probes, A-21207, Eugene, Oregon, USA) for 3 h at RT in a dark and humidified chamber. Sections were washed with TBS and cover slipped using Prolong Gold antifade mountant (Molecular Probes, P36934, Eugene, Oregon, USA). Slides were stored at 4 °C overnight to allow Prolong Gold to “cure” and edges of coverslip were sealed using nail polish. One section per mammary gland sample was used (n = 16 per stage of involution per treatment group) for semi-quantitative immunofluorescence analysis by taking 10-14 random photomicrographs at 200X magnification using the Zeiss Axioskop 2 Plus
fluorescence microscope (Carl Zeiss GmbH, Jena, Germany), Zeiss Axiocam HRc camera, and Zeiss AxioVision software (v 3.0). As with the immunohistochemistry assay, the engorged and regressed alveoli from the late-involution group were analyzed separately. For each photomicrograph, the area and the mean intensity of pIGF1R staining was obtained using ImageJ. The corrected total fluorescence (CTF) value of pIGF1R staining was then calculated by multiplying (mean – background) intensity of staining with the area of staining.

Double-labelling immunofluorescence was performed to determine the co-localization of pIGF-1R and E-cadherin, an epithelial cell marker. The protocol was same as for single-labelling immunofluorescence except that the sections were incubated with both anti-IGF1R (phospho Y1161) rabbit polyclonal antibody (1:500) and the anti-E-Cadherin mouse monoclonal antibody (1:200, BD Biosciences, 610181, Erembodegem, Belgium), followed by co-incubation with Alexa Fluor 594-conjugated donkey anti-rabbit (1:400) and Alexa Fluor 488-conjugated donkey anti-mouse (1:400, Molecular Probes, A-21202, Eugene, Oregon, USA) secondary antibodies. The representative double-labelled images of intact, engorged and regressed alveoli were acquired using Zeiss LSM 510 confocal microscope.

5.3.8 Quantitative real-time RT-PCR

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from 100 mg of ground mammary tissue according to the manufacturer’s instructions. The concentration of total RNA was determined using a NanoDrop spectrophotometer (ND-1000 v3.7.1, NanoDrop Technologies Inc., Wilmington, DE, USA) by measuring the absorbance at 260 nm (A_{260}) such that A_{260} reading of 1 = 40 µg/ml. Total RNA (50 µg) was incubated with 1 U DNase I (Qiagen Sciences, MD, USA) to get rid of the contaminating DNA. DNase-treated RNA samples were then purified using the RNeasy gel columns (Qiagen Sciences, MD, USA) in accordance with the manufacturer’s instructions. The quality of RNeasy purified RNA was
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determined using the RNA Integrity Number (RIN) generated by Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, Agilent Technologies, Chatswood NSW, Australia). An RIN of 5 or better was considered adequate for real-time reverse transcription-polymerase chain reaction (qRT-PCR). The first strand cDNA was constructed from 1 µg of the RNeasy cleaned total RNA using the Superscript III First-Strand Synthesis System (Invitrogen) as per the manufacturer’s instructions. Oligonucleotide primer sequences for the detection of genes of interest (IGF-1, IGF-1R, IGFBP-3, IGFBP-5, Bcl-xl, Bax and β-casein) are listed in . Primer sequences were either obtained using the Primer3 Plus software [306] or the NCBI Primer-BLAST program [307]. For normalizing the qRT-PCR data, 10 housekeeping genes were tested but none of these genes showed stable expression across different stages of involution and therefore, the data was normalized to the concentration of cDNA in each RT sample as described below. The housekeeping genes that were tested included: Cyclophilin A, glyceraldehyde-3-phosphate, Ubiquitin, hypoxanthine phosphoribosyltransferase 1, IKAROS family zinc finger 2, NOBOX oogenesis homeobox, Zinc finger homeobox 2, Contactin 2 (axonal), zinc finger CCHC domain containing 8 and mitochondrial ribosomal protein S25. The real time qRT-PCR was performed using SYBR Premix ExTaq (Takara Bio Inc., Shiga, Japan) on a Rotor-Gene™ 6000 real-time rotary analyzer (Corbett, Sydney, NSW, Australia) according to the manufacturer’s instructions. The cDNA products were diluted 10-fold and samples (1 µl) were assayed in triplicates. Two control reactions were included in each assay; an RT negative control and a ‘no-template’ control (where cDNA was replaced with water). Each real-time reaction (10 µl) contained 5 µl SYBR using SYBR Premix ExTaq (Takara Bio Inc., Shiga, Japan), 3.8 µl Nuclease-free Ultrapure distilled water (Invitrogen), 1 µl cDNA template, 0.1 µl forward primer and 0.1 µl reverse primer. The following thermal cycling protocol was adopted: initial hot start activation of TaKaRa Ex Taq HS PCR enzyme at 95 °C
for 3 min, followed by 40 cycles of 15 sec at 95 °C (cDNA denaturation), 40 sec at 55-60 °C (annealing and extension reactions) and 5 sec at 80-86 °C (fluorescence data acquisition). To confirm the presence of a single target product, dissociation curve analysis was performed at the end of each run by raising the temperature by 1 °C per 5 sec from 72 to 99 °C. The relative abundance of mRNA for each gene of interest was calculated as described previously [308], but with following modifications: the threshold cycle (Ct) value was replaced with the take-off value, the amplification efficiencies were calculated using the comparative quantitation analysis feature of the Rotor-Gene Q Series software 2.1.0 (Build 9, Qiagen), and the values for each gene were normalized to the concentration of cDNA in each RT sample measured using the Quant-iT™ OliGreen ssDNA Assay Kit (Invitrogen) in accordance with Lundby, Nordsborg, Kusuhara, Kristensen, Neufer, Pilegaard (309). The normalized values for each gene were log10-transformed before statistical analysis. Means for each treatment group were back-transformed and results were expressed as fold-change relative to the lactating placebo group.
Table 5.1: Oligonucleotide Primer Pairs used for Quantitative Real-time RT-PCR Assays.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward 5' → 3'</th>
<th>Reverse 5' → 3'</th>
<th>Product size (bp)</th>
<th>Annealing temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>ggctgaaccttgtgagctgcctcg</td>
<td>ggcccgagatggaacga</td>
<td>204</td>
<td>60</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>gaaagtcgttgtgatagaaag</td>
<td>tctgggcacaagatggaagttg</td>
<td>134</td>
<td>58</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>cagaacacagactctctcctc</td>
<td>gcttgtagctctagtcatcagtcgccag</td>
<td>329</td>
<td>60</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>agaacaacctctcgacctcccaga</td>
<td>gcatggccagctgcgcgcgcgttgcgcgc</td>
<td>214</td>
<td>60</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>cccaggaaggaaaetctgaac</td>
<td>tcactgaatatgctctcggctagta</td>
<td>210</td>
<td>58</td>
</tr>
<tr>
<td>Bax</td>
<td>tgcaggaagggttgtctgac</td>
<td>gtacagcctgggctaccttttagta</td>
<td>172</td>
<td>60</td>
</tr>
<tr>
<td>β-casein</td>
<td>aggctctctctctgcttcctgcgttcggc</td>
<td>373</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

IGF-1, insulin-like growth factor-1; IGF-1R, insulin-like growth factor-1 receptor; IGFBP, insulin-like growth factor binding protein; Bcl-xl, B-cell lymphoma extra-long; Bax, Bcl-2-associated X protein; β-casein, beta casein.

5.3.9 Statistical analysis

Histology, immunohistochemistry, immunofluorescence and qRT-PCR data were analyzed using two-way ANOVA (IBM SPSS Statistics 22, Chicago, IL, USA) by treatment, stage of involution and their interaction, followed by Bonferroni post-hoc tests. Because engorged and regressed alveoli from the late-involution glands were assessed separately, therefore, the immunohistochemistry and immunofluorescence data were analyzed using two separate two-way ANOVA by including either the late-involution (Eng) or the late-involution (Reg) group. Differences between these groups within a treatment group were analyzed using the paired t-
test. Differences in the growth rate of litter were analyzed using the repeated measures ANOVA. Differences in milk composition were analyzed using ANCOVA with the pre-treatment composition (i.e. PN d7) as the covariate. Both the qRT-PCR and immunofluorescence data were log$_{10}$-transformed before statistical analyses. For qRT-PCR data, means for each treatment group were back-transformed and results were expressed as fold-change relative to the placebo early-involution group ± SEM. The pIGF1R data was expressed as back-transformed mean ± SEM. For milk composition analyses, means for each time-point were adjusted to the pre-treatment mean (i.e. PN d7), and the data was expressed as adjusted mean ± SEM. All the remaining data were expressed as treatment mean ± SEM. The significance level for differences between means was set at P ≤ 0.05.

5.4 Results

5.4.1 Milk production and composition

The gain in litter weight per week was used as an indirect indicator of milk production. A difference in gain in litter weight per week was observed with time (F (2, 30) = 22.26, P < 0.001), but no treatment effect or interaction between time and treatment was observed. Multiple comparisons revealed that the gain in litter weight was highest between PN d1-7 and lowest between PN d7-14 (Figure 5.1A) for both treatment groups.

The total concentration of protein in milk did not change throughout lactation in both treatment groups (Figure 5.1B). A 1.3-fold increase in the relative absorbance of lipids was observed on PN d10 (P < 0.05, Figure 5.1C), with a further 1.4-fold increase on PN d23 (P = 0.05, Figure 5.1C) in both treatment groups. Maternal administration of cGP had no effect on the milk protein concentration and the relative absorbance of lipids at any stage of lactation (Figure 5.1 B-C).
Figure 5.1: Changes in the Gain in Litter Weight per Week and the Composition of Milk of Rat Dams administered with either Cyclic-glycine-proline (cGP; 3 mg/kg Body Weight) or Saline from Postnatal d8-22.

Changes in the gain in litter weight per week (A), and milk protein concentration (B) and relative absorbance of lipids (C) is shown. Data are either expressed as mean ± SEM (A) or adjusted mean ± SEM (B and C). n = 16 per treatment group per time-point. Different letter denotes significant differences (P < 0.05) between all the groups in A, and between the placebo groups in B and C. No significant difference was observed between the treatment groups at any time-point in B and C.

5.4.2 Histology

Morphologically, the mammary tissue in the early-, mid- and late stages of involution from both treatment groups were heterogeneous, with lobules containing intact, engorged and regressed alveoli (Figure 5.2 A-F). Intact secretory alveoli, that are typically present during lactation, were characterized by the presence of open alveoli lined with MEC that had cuboidal appearance (Figure 5.2 A-B). The majority of the tissue area was covered by alveolar lumen with little inter-alveolar space. The mammary glands in the early stage of involution from both
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treatment groups were dominated by intact alveoli (62.3 ± 3.6 % (cGP), 79.24 ± 3.7 % (Placebo); Figure 5.2G). Engorged alveoli, that are typically present during the reversible phase of involution, were characterized by flattened MEC lining the alveolar lumen which appeared distended (or engorged) due to accumulation of milk (Figure 5.2 C-D). Both the MEC and the alveolar lumen were filled with large vacuole(s), and the lumen contained several sloughed MEC and/or other apoptotic bodies (Figure 5.2C-D). The mammary glands in the mid-involution stage (24h-engorgement) from both treatment groups were dominated by engorged alveoli (71.1 ± 5.1 % (cGP), 75.2 ± 5.3 % (Placebo); Figure 5.2H). Regressed alveoli, that are typically present during the late or irreversible phase of involution, were characterized by the presence of MEC that were irregular in shape (Figure 5.2 E-F). There were few remaining vacuoles, the alveoli had regressed with little or no apparent lumen, and the inter-alveolar space had increased which was primarily occupied by the stromal tissue (Figure 5.2E-F). The mammary glands in the late stage of involution (72h-engorgement) from both treatment groups were pre-dominantly occupied by the regressed alveoli (52.6 ± 5.0 % (cGP), 56.8 ± 4.9 % (Placebo); Figure 5.2I), but also retained a large proportion of the engorged alveoli (38.7 ± 5.3 % (cGP), 42.2 ± 5.1 % (Placebo); Figure 5.2H).

There was a significant interaction between the treatment groups and the stages of involution for the percentage of intact alveoli (F (2, 87) = 6.33, P < 0.01, Figure 5.2G). Multiple comparisons revealed a 16.9 % reduction (P < 0.01) in the percentage of intact alveoli in the early-involution glands of cGP-administered dams compared with those of the control dams (Figure 5.2G).
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- **Early Inv**: Placebo (A) vs. cGP (B)
- **Mid Inv**: Placebo (C) vs. cGP (D)
- **Late Inv**: Placebo (E) vs. cGP (F)
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**G**

![Graph showing percentage of intact alveoli at different stages of involution: Early, Mid, and Late. The graph indicates a significant difference at the ** level.](image)

**H**

![Graph showing percentage of engorged alveoli at different stages of involution: Early, Mid, and Late.](image)

**I**

![Graph showing percentage of regressed alveoli at different stages of involution: Early, Mid, and Late.](image)
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**Figure 5.2: Histological analysis of Mammary Tissue following the administration of either Cyclic-glycine-proline (cGP; 3 mg/kg Body Weight) or Saline in Rat Dams from Postnatal d8-d22.**

Representative grayscale photomicrographs of hematoxylin and eosin stained mammary tissue sections collected during early- (A and B), mid- (C and D) and late (E and F) stages of involution are shown. Insets in each photomicrograph show magnified view of the framed area. Insets in B show the intact and the regressed alveoli whereas the insets in E and F show the engorged and the regressed alveoli. Histological scoring was carried out by calculating the percentages of intact (G), engorged (H) and regressed (I) alveoli in mammary tissue from each stage of involution. Data are expressed as mean ± SEM, n = 15-16 per treatment group per stage of involution. Significant differences between treatment groups are denoted by **P < 0.01.

### 5.4.3 Immunohistochemistry

#### 5.4.3.1 Caspase-3

Caspase-3 positive nuclei were present in all stages of involution, and were located in the lumen and the epithelial lining of the alveoli and ducts as well as in the stromal connective tissue (Figure 5.3 A-H, ducts not shown). However, only the alveolar nuclei were counted for quantitative purposes (Figure 5.3I).

There was a reduction in the percentage of alveoli containing one or more caspase-3 positive nuclei with the stage of involution ($F_{(2, 87)} = 25.31$, $P < 0.001$, Figure 5.3I), and a treatment effect ($F_{(1, 87)} = 5.20$, $P < 0.05$), but no interaction between stage and treatment. Multiple comparisons revealed a decline in the percentage of alveoli containing caspase-3 nuclei from early- to mid- (18.6 ± 6.6 %, $P < 0.05$), and mid- to late (Reg) (17.9 ± 6.5 %, $P < 0.05$) stages of involution in the control group (Figure 5.3I). During the early stage of involution, the percentage of alveoli containing caspase-3 nuclei were lower in the cGP glands compared with the control glands (14.8 ± 6.5 %, $P < 0.05$, Figure 5.3I). However, similar to the control glands, cGP glands showed a reduction in the percentage of alveoli containing caspase-3 nuclei from mid- to late (Reg) (20.7 ± 6.5 %, $P < 0.01$) stage of involution.
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![Image of histological sections showing early, mid, and late involution stages under placebo and cGP conditions.](image-url)
Representative grayscale photomicrographs showing caspase-3 staining in paraformaldehyde-fixed, paraffin-embedded mammary tissue sections collected during early- (A and B), mid- (C and D) and late (E-H) stages of involution (inv). Engorged (E and F) and regressed (G and H) alveoli from the late-involution glands are shown separately as Late (Eng) and Late (Reg) groups, respectively. Insets in each photomicrograph show magnified view of the framed area. Arrows indicate positively stained nuclei. Percentage of alveoli containing one or more caspase-3 positive nuclei are shown (I). Data are expressed as mean ± SEM, n = 16 (early- and mid-inv), 13 (late (Eng)- inv) and 15 (late (Reg)- inv) for cGP, and 15 (early- and mid-inv) and 16 (late (Eng)- and late (Reg)-inv) for Placebo. Different letter denotes significant differences at P < 0.05. Inv = involution; Eng = engorged alveoli; Reg = regressed alveoli.

5.4.3.2 Ki-67

Ki-67 positive nuclei were present in all stages of involution, and were located in the epithelial lining of the alveoli and ducts as well as in the stromal connective tissue (Figure 5.4 A-H, ducts not shown). However, only the alveolar nuclei were counted for quantitative purposes (Figure 5.4I). One mammary tissue sample in the mid-involution (cGP group) and the late
(Reg)- stage of involution (control group) showed intra-lobular hyperplasia, characterized by stratification of proliferating MEC above the basement membrane into the alveolar lumen (data not shown). These two samples were excluded from the analysis.

There was an increase in the percentage of alveoli containing one or more Ki-67 positive nuclei with the stage of involution ($F_{(2, 86)} = 10.70$, $P < 0.001$, Figure 5.4I), but no treatment effect or stage by treatment interaction. In both treatment groups, an increase in the percentage of alveoli containing Ki-67 nuclei was observed from mid- to late (Eng)- (Control: $17.2 \pm 6.2 \%$, $P < 0.05$; cGP: $14.6 \pm 6.2 \%$, $P = 0.07$), and late (Eng)- to late (Reg) stage of involution (Placebo: $13.1 \pm 5.2 \%$, $t_{(15)} = 3.0$, $P < 0.05$; cGP: $8.9 \pm 3.4 \%$, $t_{(13)} = 2.7$, $P < 0.05$; Figure 5.4I).
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![Diagram](image)
Representative grayscale photomicrographs showing Ki-67 staining in paraformaldehyde-fixed, paraffin-embedded mammary tissue sections collected during early- (A and B), mid- (C and D) and late (E-H) stages of involution (inv). Engorged (E and F) and regressed (G and H) alveoli from the late-involution glands are shown separately as Late (Eng) and Late (Reg) groups, respectively. Insets in each photomicrograph show magnified view of the framed area. Arrows indicate positively stained nuclei. Percentages of alveoli containing one or more Ki-67 positive nuclei are shown (I). Data are expressed as mean ± SEM, n = 16 (early-inv), 15 (mid- and late (Eng)- inv) and 14 (late (Reg)- inv) for cGP, and 15 (early-, mid- and late (Reg)- inv) and 16 (late (Eng)- inv) for Placebo. Different letter denotes significant differences at P < 0.05. Inv = involution; Eng = engorged alveoli; Reg = regressed alveoli.

5.4.4 Immunofluorescence

Positive staining for pIGF1R was observed in the mammary tissue from both treatment groups in all stages of involution (Figure 5.5). In the early stage of involution, pIGF1R staining was observed along the epithelial lining of the intact alveoli and the ducts, with little or no luminal staining (Figure 5.5 A and B, ducts not shown). Double-labelling with E-cadherin, a marker
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for epithelial cells, confirmed the membranous staining of pIGF1R along the lining of epithelial cells (Figure 5.6A), and showed that the luminal staining of intact alveoli was also associated with the epithelial cells (Figure 5.6B). During the mid- and late (Eng)- stages of involution, strong pIGF1R staining was observed in both the lumen and the epithelial lining of the engorged alveoli and the ducts (Figure 5.5 C-F, ducts not shown). Double-labelling confirmed this (Figure 5.6 C and D), and further showed that the luminal staining of engorged alveoli was not associated with the epithelial cells (Figure 5.6C) or any other type of cells (Figure 5.6D). Compared with the early-, mid- and late (Eng) stages of involution, less intense staining for pIGF1R was observed in the late (Reg) stage of involution in both treatment groups, (Figure 5.5 G and H). Staining was present in the lumen and the epithelial lining of the regressed alveoli and the ducts, and could also be observed in the cytoplasm of the epithelial cells (Figure 5.6E, ducts not shown). Less intense pIGF1R staining was observed in the surrounding connective tissue and the stromal endothelial cells at all stages of involution (not shown in detail).

There was a reduction in the CTF of pIGF1R with the stage of involution (F (2, 83) = 44.88, P < 0.001, Figure 5.5I), and a treatment effect (F (1, 83) = 5.08, P < 0.05) but no interaction between stage and treatment. In both treatment groups, no change in CTF of pIGF1R was observed between the early-, mid- and late (Eng)- stages of involution, however, a dramatic reduction was observed from mid- to late (Reg)- (Placebo: 4-fold, P < 0.001; cGP: 4.8-fold, P < 0.001) and late (Eng)- to late (Reg)- stages of involution (Placebo: 4.5-fold, t (15) = 6.81, P < 0.001; cGP: 6.5-fold, t (13) = 7.21, P < 0.001; Figure 5.5I). Furthermore, the CTF of pIGF1R was lower in the cGP group compared with the control group during the late (Reg) stage of involution (1.6-fold, P < 0.01, Figure 5.5I).
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**Placebo**

A. Early Inv

B. Mid Inv

C. Late Inv (Eng)

D. Late Inv (Reg)

**cGP**

E. Early Inv

F. Mid Inv

G. Late Inv (Eng)

H. Late Inv (Reg)
Figure 5.5: Changes in the expression of Phosphorylated IGF-1 Receptor (pIGF1R) in Mammary Glands of Rat Dams Administered with either Cyclic-glycine-proline (cGP; 3 mg/kg Body Weight) or Saline from Postnatal d8-22.

Representative photomicrographs showing pIGF1R immunofluorescence staining in paraformaldehyde-fixed, paraffin-embedded mammary tissue sections collected during the early- (A and B), mid- (C and D) and late (E-H) stages of involution (inv). Engorged (E and F) and regressed (G and H) alveoli from the late-involution glands are shown separately as Late (Eng) and Late (Reg) groups, respectively. Insets in each image show magnified view of the framed area. The corrected total fluorescence values (CTF) of pIGF1R are shown (I). The data was log₁₀-transformed for statistical analysis, and is expressed as backtransformed mean ± SEM, n = 16 (early-inv), 14 (mid-inv), 15 (late (Eng)- inv) and 13 (late (Reg)- inv) for cGP, and 15 (early- and mid- inv) and 16 (late (Eng)- and late (Reg)- inv) for Placebo. Different letter and * denotes significant differences at P < 0.05. Inv = involution; Eng = engorged alveoli; Reg = regressed alveoli.
Figure 5.6: Immunofluorescence Double-Labelling of Phosphorylated IGF-1 Receptor (pIGF-1R) and E-cadherin.

Representative photomicrographs showing immunofluorescence double-labelling of pIGF-1R (red), E-cadherin (an epithelial cell marker, green) and Hoechst stain (nuclear stain, blue). (A-E) Photomicrographs showing the expression of pIGF-1R along the lining of epithelial cells (A) and the lumen (B) of intact alveoli, along the epithelial lining (C) and the lumen of engorged alveoli (D, E), and along the epithelial lining and the lumen of regressed alveoli with some cytoplasmic staining of the epithelial cells (F). Large vacuoles can be seen inside the epithelial cells and the lumen of engorged alveoli (C and D).

5.4.5 Quantitative real-time RT-PCR

Compared with the respective early-involution group, the mRNA levels of genes changed in the remaining involution groups of the control group as follows: IGF-1 increased at late-
involution (3.1-fold, \(P < 0.05\), Figure 5.7A), IGF-1R increased at mid-involution (2.6-fold, \(P < 0.05\), Figure 5.7B), IGFBP-3 did not change (Figure 5.7C), IGFBP-5 increased at mid- (2.8-fold, \(P < 0.01\)) and late-involution (6.6-fold, \(P < 0.001\), Figure 5.7D), Bcl-xl (1.9-fold, \(P < 0.05\), Figure 5.7E) and Bax (2.0-fold, \(P < 0.01\), Figure 5.7F) increased at mid-involution, and \(\beta\)-casein decreased at late-involution (0.5-fold, \(P < 0.01\), Figure 5.7H).

A treatment effect was observed for IGF-1R (\(F_{(1, 89)} = 4.5, P < 0.05\)) and Bcl-xl (\(F_{(1, 89)} = 4.4, P < 0.05\)), but no interaction between treatment and stage of involution was observed for any of the genes. Multiple comparisons revealed an increase in the mRNA levels of both IGF-1R (1.6-fold, \(P < 0.05\), Figure 5.7B) and Bcl-xl (1.6-fold, \(P < 0.05\), Figure 5.7E) in the early-involution glands of cGP dams compared with those of the control dams.
Figure 5.7: Changes in the expression of Genes in the Mammary Glands of Rat Dams administered with either Cyclic-glycine-proline (cGP; 3 mg/kg Body Weight) or Saline from Postnatal d8-22.

Changes in the mRNA levels of IGF-1 (A), IGF-1R (B), IGFBP-3 (C), IGFBP-5 (D), Bcl-xl (E), Bax (F), Bcl-xl/Bax (G) and β-casein (H) during the early-, mid- and late- stages of involution are shown. The data was log_{10}-transformed before statistical analyses. The means for each treatment group were then back-transformed, and the
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Results are expressed as fold-change relative to the placebo early-involuton group ± SEM. n = 16 per treatment group per stage of involution for each gene. Different letter denotes significant differences (P < 0.05) between the groups. IGF-1 = insulin-like growth factor-1; IGF-1R = insulin-like growth factor-1 receptor; IGFBP = insulin-like growth factor binding protein; Bcl-xl = B-cell lymphoma extra-long; Bax = Bcl-2-associated X protein.

5.5 Discussion

Results presented in this chapter show that administration of cGP to rat dams during lactation does not alter milk production and composition but promotes the onset of post-lactational involution in mammary glands. This is the first study to show the effect of cGP on mammary gland development during the post-lactation period. Maternal administration of cGP did not alter milk production (determined through change in litter growth rate) and milk composition during early-, peak- and/or late- stages of lactation. During the early stage of involution, cGP accelerated the loss of mammary cells through apoptosis, resulting in an early clearance of the intact secretory alveoli. This coincided with an earlier up-regulation of the cell survival factors, Bcl-xl and IGF-1 receptor, in the early-involuton glands of cGP dams compared with the control dams. During the late stage of involution, cGP reduced the bioactivity of IGF-1, which was evident through decreased phosphorylation of IGF1R in the regressed alveoli.

Litter weight gain before PN d16, the time when pups start ingesting solid food [310], is used as an indirect indicator of lactational capacity of rodents [43,311] when it is not associated with changes in milk composition [312]. It is shown to be a fairly reliable method of estimating milk production in rodents when compared with the other more direct and sophisticated methods, such as the mammary gland weight difference method and the tritiated water dilution method [261]. The gain in litter weight per week and the milk protein and lipid content was similar between the two treatment groups throughout lactation (Figure 5.1), suggesting that cGP had no effect on milk production and composition of dams. However,
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cGP accelerated the onset of post-lactational involution in mammary glands, which is discussed in the following sections.

As a measure of overall change to mammary glands during the progression of involution, a characteristic decline in mammary gland to dam body weight ratio was observed from early- and mid- to late-phase of involution in both treatment groups (data not shown). This is due to the accumulation of milk during the initial stages of involution, and the almost complete loss of epithelial cells and the lobulo-alveolar structures, along with diminished milk synthesis and secretion, during the late stage of involution [313]. Maternal administration of cGP had no effect on the ratio of mammary gland to dam body weight at any stage of involution.

Morphologically, lactating mammary glands are characterized by the presence of intact secretory lobulo-alveolar structures [314]. However, with the onset of milk stasis, both the MEC and the alveolar lumen become engorged with large vacuoles, which contain lipid droplets and protein material from the secretory vesicles [304,305]. The mid-involution glands from both treatment groups were dominated by the engorged alveoli (Figure 5.2H). However, the early-involution glands from both groups were still largely occupied by the intact alveoli (Figure 5.2G). The chronic milk stasis in the early-involution glands may have been prevented due to the autocrine inhibition of milk secretion, owing to irregular milk removal during natural involution [180]. Mammary glands of rodents enter an irreversible phase of involution after 72 h of induced milk stasis, which leads to the progressive regression of lobulo-alveolar structures owing to further apoptosis and activation of proteases [183,190]. The late-involution glands from both treatment groups were predominantly occupied by the regressed alveoli, but also retained a significant proportion of the engorged alveoli (Figure 5.2 H and I), which were bigger in size than the engorged alveoli of mid-involution glands (Figure 5.2 C-F). This reflects the non-homogenous nature of mammary gland involution, which involves continues
milk stasis in some alveoli and remodeling of the others [177]. The engorged and regressed alveoli of late-involution glands were assessed separately for the cellular and morphological markers of involution in the subsequent immunohistochemistry and immunofluorescence assays. The early-involution glands from the dams administered with cGP contained a significantly lower percentage of intact alveoli compared with the glands from control dams, suggesting that cGP causes an earlier induction of involution (Figure 5.2G).

Because loss of epithelial cells through apoptosis leads to the subsequent loss of lobulo-alveolar structures, the changes in apoptosis of mammary cells were analyzed through the immunohistochemistry-based detection of cleaved (activated) caspase-3, the executor of apoptosis [217]. Activation of caspases is a crucial step for the cleavage of intracellular substrates during the process of apoptosis [315]. Kinetics of apoptosis during the course of involution in control glands showed that the apoptotic cells were present in all stages of involution, but peaked during early involution (Figure 5.3). This is consistent with previous studies showing that peak apoptosis that occurs during induced involution, can be observed on PN d22-23 of natural involution [41,176]. There was a decline in apoptosis during mid- and late- involution, which is likely due to the loss of majority of cells in the early phase of involution (Figure 5.3I). In comparison with the control group, apoptosis was reduced in the cGP group during the early phase of involution (Figure 5.3I). However, similar to the control group, cGP group showed the characteristic reduction in apoptosis at late-involution (Figure 5.3I). These results may suggest that cGP accelerated post-lactational involution by increasing the loss of mammary cells through apoptosis prior to PN d23, an observation that is corroborated by the loss of intact alveoli in the early-involution cGP glands. Involvement of caspase-dependent apoptotic pathways has been previously reported in involuting rat [316], goat [317] and bovine [318] mammary glands. To the best of our knowledge, there are no
available data showing that cGP enhances apoptosis in mammary glands of rodents and/or dairy animals. However, cGP has been widely studied as a neurotrophic factor [for review, see 29], and the neuroprotective role of cGP analogue, cG-allyl-P, has been shown to involve modulation of caspase-3 mediated apoptosis [67].

Changes in cell proliferation in mammary glands was determined through immunohistochemical detection of nuclear proliferation antigen, Ki-67, a protein which is present in all stages of cell cycle except G0 [319]. Ki-67 provides a reliable measure of the proliferative state of cells under different physiological and pathophysiological conditions [320,321]. Proliferation of mammary cells did not change from early- to mid- phase of involution, but was increased at late-involution (Figure 5.4). This contrasts with the previous studies in non-pregnant lactating rodents, where a decline in proliferation of mammary cells is reported with the progression of involution [261,310,322]. In the current study, the Ki-67 positive cells present in the alveolar epithelium were counted. Therefore, the high numbers of proliferating cells may also represent the intraepithelial immune cells (e.g. macrophages) that are activated during the late stages of involution, for clearing the apoptotic cell debris through phagocytosis [196]. A similar observation was made by Kaur and others [323] in mammary glands from buffaloes. Administration of cGP to dams did not alter the proliferation of cells at any stage of involution, which suggests that the loss of intact alveoli in the early-involution cGP glands was not due to the reduction in proliferation of cells. Furthermore, the preservation of alveolar structure and the absence of any effect on cell proliferation suggests that cGP did not have any adverse effects on the structure and function of mammary glands.

The pro-apoptotic effects of cGP were investigated at the transcription level by measuring the changes in the mRNA levels of a number of pro- and anti-apoptotic genes. Expression of IGF-1, IGF-1R, IGFBP-3, IGFBP-5, Bax and Bcl-xl was upregulated during the mid- and late-
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phases of involution as has been shown previously in rodents [53,324,325]. The upregulation of anti-apoptotic Bcl-xl and IGF-1R is considered to be a response to promote cell survival during the initial stages of involution [325]. However, apoptosis becomes more frequent as the expression of Bax and other pro-apoptotic factors increase during the course of involution [325]. Elevated IGFBP also reduce the bioavailability of IGF-1 through increased binding, thus leading to enhanced apoptosis [241]. In comparison with the control group, the expression of IGF-1R and Bcl-xl was upregulated in the cGP group during the early phase of involution (Figure 5.7 B and E). This may be a response to an earlier induction of apoptosis, and thus, involution in the cGP glands because the mechanism of action of cGP involves modulating the bioavailability of IGF-1 by altering the binding of IGFBP to IGF-1 [34], and therefore, may not have any direct effect at the transcription level.

The molecular basis of pro-apoptotic effect of cGP was investigated by evaluating the changes in the phosphorylation of IGF-1R, which gives a measure of IGF-1 bioactivity. IGF-1 binding causes tyrosine phosphorylation of IGF-1R, which in turn triggers the downstream signaling cascade that leads to cell survival/proliferation [for review, see 226, and Figure 1.9 of the current thesis]. Membranous staining of pIGF1R was observed along the lining of epithelial cells in all types of alveoli (Figure 5.5 and 5.5). However, luminal staining was also observed, particularly in the engorged alveoli (Figure 5.5 and 5.5). Milk contains a heterogeneous population of somatic cells, including the MEC that are exfoliated from the mammary epithelium. To investigate whether the luminal staining of pIGF1R was associated with the MEC exfoliated into the alveolar lumen, immunofluorescence double-labelling was performed using antibodies against pIGF1R and E-cadherin, an epithelial cell marker. The luminal staining of pIGF1R was clearly associated with the MEC in the intact alveoli (Figure 5.6B), but not in the engorged alveoli (Figure 5.6 D-E). Milk fat is secreted by the MEC in a unique
apocrine fashion, in which the freshly synthesized lipid droplets coalesce together in the cytoplasm of MEC, fuse with the apical plasma membrane, and are then pinched off and secreted as membrane enveloped structures called milk fat globules (MFG) [326,327]. Many intracellular components (membranes and organelles) of MEC get trapped in the MFG except the nucleus [328]. The presence of cytoplasmic components of MEC in the MFG has offered a non-invasive tool to study gene expression using MFG isolated from milk of human [329] and dairy animals [330]. Because IGF-1R is expressed in both the cytoplasm and the plasma membrane of MEC [50, and Figure 5.6 of the current thesis], and IGF-1R transcripts have been previously isolated from MFG [329], it is suggested that the luminal staining of pIGF1R in the current study may represent the receptors associated with MFG in the lumen.

The initial, transient, non-significant increase in the expression of pIGF1R was probably a response to promote cell survival during the reversible phase of involution, which was followed by a dramatic decline, corresponding with the loss of majority of cells and the lobulo-alveolar structures, in the irreversible late (Reg) stage of involution. Compared with the control group, cGP consistently reduced the expression of pIGF1R in the mid- (1.3 fold), late (Eng)- (1.1-fold) and late (Reg)- (1.6-fold) stages of involution, though the difference reached statistical significance only in the late (Reg) stage (Figure 5.5I). There was no temporal correlation between the effects of cGP on the expression of pIGF1R and its pro-apoptotic effects. For the early involution group, this could probably because the changes in pIGF1R preceded the alteration in cell apoptosis, and for the late (Reg) involution group, this may be due to the loss of majority of cells in the earlier stages of involution. Nevertheless, a negative correlation between pIGF1R and caspase-3 data in the late (Reg) cGP group (r = -0.56, P < 0.05) (data not shown) suggests that downregulation of IGF-1 bioactivity may be involved in the pro-apoptotic effects of cGP. A similar analysis in the control group showed no correlation
(r = 0.31, P = 0.24). Previously, the neuroprotective effect of cGP following ischemic brain injury was shown to involve modulation of IGF-1 bioactivity [34].

As a regulator of IGF-1 bioavailability, cGP is found to either inhibit or stimulate IGF-1 function under pathophysiological conditions of elevated or diminished IGF-1 bioactivity, respectively [34]. This is the first study to investigate the effect of cGP on physiological changes in IGF-1 function, and shows that cGP does not prevent the physiological decline in IGF-1 function during involution, but, rather, promotes it. A similar investigation during peak lactation will determine if cGP promotes the physiological increase in IGF-1 function.

Results presented in this chapter showed that cGP facilitates mammary cell death during involution, likely by inhibiting IGF-1 mediated cell survival. This is the first study to show the effect of cGP on mammary involution. The cGP-mediated promotion of involution may also explain the drastic reduction in the concentrations of endogenous cGP in milk following peak lactation (shown in Chapter 3). Probably for a similar reason, exogenous administration of cGP to dams causes a much lower increase in milk cGP concentrations during the declining phase of lactation (PN d20, 6-7 fold) compared with the peak lactation (PN d10-15, 85-100 fold) (Chapter 3).

In agriculturally important species, post-lactational involution of mammary glands is critical for obtaining optimum milk production in the subsequent lactation [331,332]. For example, the lack of a dry period in dairy cows can reduce milk production by up to 20 % in the following lactation [333]. The current findings validate future research in using cGP for accelerating post-lactational involution in high-producing dairy cows.

The post-lactational mammary microenvironment resembles that of wound-healing, and promotes tumorigenesis [334]. Therefore, delayed or dysregulated post-lactational involution
increases the risk of developing breast cancer following pregnancy [335]. Furthermore, elevated IGF-1 is shown to delay post-lactational involution and increase the subsequent development of mammary tumors in mice [336]. IGF family members, including IGF-1 and IGF-1R, are also implicated in the malignant transformation of normal MEC [337]. Clinical studies have shown that elevated IGF-1 and increased IGF-1R staining in the terminal duct lobular units is an indicator of increased risk of developing breast cancer in women with benign breast disease [338,339]. Therapies aimed at targeting the IGF system are limited by potential side-effects of impaired IGF-1 function on other physiological processes of the body [51]. Results presented in this chapter suggest that cGP may play a useful role in the anti-IGF-1 targeted therapies against breast cancer, an area that needs further investigation. In support, cGP is shown to prevent IGF-1-dependent tumor growth in mice [34].

5.6 Summary

Administration of cGP to rat dams during lactation improves the recognition (Chapter 3) and spatial memory (Chapter 4) of adolescent and adult offspring, respectively, which is associated with improved glutamatergic neuroplasticity in different brain regions (Chapter 4). An improvement in lactational capacity of dams may, however, also influence the cognitive function of the offspring. This chapter investigated whether an improvement in lactational capacity of dams, following administration of cGP to dams during lactation, contributed to the cognitive improvement observed in the offspring (Chapters 3 and 4). More specifically, the effect on milk production and composition, and the post-lactational involution of mammary glands was investigated.

Rat dams were gavaged with either cGP or saline once per day from PN d8-22. Litter weight were recorded weekly throughout lactation. Milk samples were collected from each dam on
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PN d7, d10, d15, d20 and d23. Before collecting the tissue on PN d23, a pair of mammary glands were sealed on d20 (72h-engorgement, thus representative of late-involution) and d22 (24h-engorgement, thus representative of mid-involution), while the remaining glands were allowed to involute naturally (thus representative of early-involution). Histology, immunohistochemistry, immunofluorescence and qRT-PCR were used to evaluate the effect of cGP on various morphological, cellular and molecular markers of involution.

Administration of cGP to rat dams did not alter milk production (determined through change in litter growth rate) and milk composition during early-, peak- and/or late- stages of lactation. In comparison with the control treatment, cGP treatment accelerated the onset of involution as evident through a reduction in the percentage of intact alveoli and the upregulation of cell survival factors, IGF-1 receptor and Bcl-xl, in the early phase of involution. The accelerated onset of involution was due to an increase in the loss of mammary cells through apoptosis prior to PN d23, because early-involution cGP glands showed a reduced percentage of alveoli stained positive for cleaved caspase-3 compared with the control glands, followed by the characteristic reduction in staining at mid- and late- involution. At mid- and late-involution, there was no difference in tissue morphology, expression of cell survival factors or apoptosis between the cGP and the control group. At late-involution, however, a greater decrease in the phosphorylation of IGF-1R was observed in the regressed alveoli of cGP glands compared with those of the control glands. Taken together, these data show that maternal administration of cGP during lactation does not improve the lactational capacity of dams, and thus, may not contribute to the cognitive improvement in the offspring. Furthermore, this chapter shows that cGP promotes mammary involution, and the physiological decline in IGF-1 function.
Chapter 6: General discussion

The results presented in this thesis showed for the first time the presence and the temporal changes in the concentrations of endogenous cGP in rat milk and plasma during lactation and postnatal development, respectively. Furthermore, the current study demonstrated the physiological role of endogenous cGP in vivo. Specifically, endogenous cGP was found to increase the bioavailability of IGF-1 during infancy (Chapter 3). Because these are important new findings, therefore, the possible roles of milk- and blood-borne endogenous cGP and their potential association with the postnatal development of the offspring, including brain development and function, and the biological changes in mammary gland during the transition from lactation to involution are further discussed.

The second novel finding of this thesis was the oral bioavailability of exogenous cGP in dams, and maternal-infantile transmission through breast milk (Chapter 3). The pharmacodynamics of exogenous cGP in optimizing normal brain development and function (Chapters 3 and 4), and accelerating mammary gland involution (Chapter 5) were also demonstrated. The second section of this chapter discusses the implications of oral bioavailability of exogenous cGP in adult and infant rats, in comparison with those of IGF-1 and GPE. The potential routes of entry of cGP from gut to blood, and then to brain and mammary gland are briefly discussed.

Maternally administered cGP caused a long-lasting improvement in learning and memory of offspring, which was associated with improved neuroplasticity in different brain regions (Chapters 3 and 4). This data showed a positive influence of early life administration of cGP on neurodevelopmental programming of offspring. The cognitive improvement in the offspring was not caused by an increase in the lactational capacity of dams (discussed in Chapter 5). However, additional factors such as the effect of cGP on central regulation of
lactation and maternal care were not reported, and are, therefore, discussed in detail in this chapter. The possible intracellular mechanisms underlying the cGP-mediated improvement in neuroplasticity and memory are also briefly discussed.

In Chapter 5, cGP was found to promote post-lactational involution of mammary glands, possibly by downregulating IGF-1 function. This is an interesting new finding because the previous research has only investigated the roles of IGF-1 derived small peptides in brain. Therefore, the possible role of cGP in mammary involution is discussed together with the changes in the concentrations of cGP in milk during lactation and involution. Finally, the strengths and limitations, the potential future research areas and the clinical implications of the current study are described.

6.1 cGP is an endogenous bioactive peptide in rat milk and plasma

The first aim of this thesis was to investigate the presence, the changes in the concentrations, and the role of endogenous cGP in rat milk and plasma during lactation and postnatal development, respectively. It was demonstrated that endogenous cGP is present in the plasma and milk of rats (Chapter 3). The concentrations of endogenous cGP were higher in plasma of pups (23 days old) and adult offspring (82 days old) compared with the dams (135-140 days old), thus suggesting an increase in plasma concentrations of cGP during infancy and a decrease with advancing age. Studies in humans and rodents have shown a contrasting trend for developmental changes in plasma IGF-1 concentrations [253,254]. The plasma concentrations of IGF-1 are low during infancy, peak at puberty and then decline with advancing age. As an autocrine regulatory response to low IGF-1 during infancy, IGFBP-3, the major IGFBP in plasma, is also reduced to increase the bioavailability of IGF-1 [263]. The high plasma concentrations of endogenous cGP during infancy were suggested to be an
additional autocrine response to the low plasma IGF-1 concentrations, which may further increase the bioavailability of IGF-1. This was evident through an increase in the ratio of cGP/IGF-1 in plasma of pups. This is the first study to show the role of cGP in autocrine regulation of plasma IGF-1 in vivo.

The concentrations of endogenous cGP in milk were high during early and peak phases of lactation, and low during late lactation/weaning. The bioavailability of milk-borne cGP in infants (Chapter 3) suggests that milk-borne cGP may further increase the plasma concentrations of cGP during infancy. Taken together with the positive influence of early life administration of cGP on neurodevelopmental programming of offspring (Chapters 3 and 4), these data suggest that endogenous cGP may increase the trophic support provided by IGF-1 during the critical period of early postnatal brain development. Therefore, cGP may be an important bioactive component of milk and plasma, and deficiency of cGP may contribute to the pathogenesis of neurodevelopmental disorders that are associated with IGF-1 dysfunction in the early postnatal life, for instance, the autism spectrum disorders (ASD) (Chen et al., 2014). Therefore, this needs to be investigated in the future studies. In support, GPE analogues are shown to confer behavioral improvements and reverse some of the symptoms associated with ASD [340].

Milk-borne fraction of endogenous cGP may arise from blood or produced locally in the mammary gland. Evidence for the transfer of cGP from blood to milk comes from the increase in milk concentrations of cGP following exogenous cGP administration in dams (Chapter 3). Only the transcellular pathways are responsible for the transfer of solutes from blood to milk during lactation because the direct, bi-directional paracellular pathway is blocked due to the closing of tight-junctions between the MEC [341]. The transcellular pathways may involve both active transport, using the low-affinity and/or high-affinity peptide transporters that are
expressed throughout the mammary epithelia of rats [274,342], and passive transport, i.e. the
direct diffusion of cGP across the lipid membrane owing to its lipophilic properties [29,343].
Future studies may shed light on the specific pathways involved in transporting cGP from
blood to milk during lactation.

IGF-1 is locally produced in the mammary gland by stromal cells [49]. Therefore, in addition
to the transfer from blood, cGP may also be produced locally through the action of mammary
proteases, such as cathepsin-D [344], which is shown to cause extensive proteolysis of IGF-1
[31]. However, this needs to be investigated. Dramatic decline in milk concentrations of
endogenous cGP following peak lactation (Chapter 3) may suggest a specific role of milk-
borne cGP for the infant. Milk-borne bioactive peptides are shown to aid infant’s digestion
owing to the immature state of the gut [345]. With the maturation of gut over the course of
lactation, concentrations of milk-borne peptides decline [345]. It can be speculated that the
increased concentrations of cGP in milk up to peak lactation may play a role in increasing the
amount of free IGF-1 in milk, because free IGF-1 can be readily absorbed across the immature
gut of the infant compared with the bigger complexes of IGF-1 and IGFBP [16,346,347]. With
the maturation of gut, milk cGP concentrations may start to decline as observed during late
lactation/weaning (Chapter 3). The increased absorption of free IGF-1 across the gut may have
important implications on the growth, and the postnatal brain development and function of the
infant because the infant relies on milk-borne IGF-1 during the pre-weaning period [16], when
the production of endogenous IGF-1 by liver is low [14,15]. For instance, studies in rats, pigs
and cows have shown that offspring fed with growth factor-free milk have reduced serum
IGF-1 compared with the mother-fed animals [348-350]. In humans, the concentrations of
IGF-1 are higher in pre-term milk compared with the term milk, which also supports the
importance of milk-borne IGF-1 for the infant [346]. Future studies are needed to investigate
the presence and changes in concentrations of cGP in human milk during the course of lactation, and whether a similar difference exist in cGP concentrations between the pre-term and term human milk. In summary, milk-borne cGP may have two roles for the infant. First, to increase the plasma concentrations of cGP as part of the autocrine regulation of IGF-1 during infancy, and second, as a hypothesis, to facilitate the absorption of milk-borne IGF-1 across the gut.

In rodents, growth hormone stimulates IGF-1 production during lactation, and prolactin suppresses IGFBP production [241]. This results in an increase in the bioavailability of IGF-1 and consequently, the proliferative/cell survival effects of IGF-1 [241]. Conversely, the upregulation of IGFBP during involution, either due to the loss of prolactin and/or the activation of STAT-3, reduces the bioavailability of IGF-1, resulting in the suppression of IGF-1 function [241]. The reduction in milk concentrations of cGP during late lactation/weaning suggest that cGP may also be involved in reducing IGF-1 bioavailability during involution (through a reduction in cGP/IGF-1 ratio). The inverse may be true during peak lactation. It is worth mentioning that cGP/IGF-1 ratio could not be calculated for the mammary tissue or milk because of the technical limitations of HPLC-MS assay in accurately measuring the concentrations of cGP in tissue samples, and the insufficient volume of milk available for quantitative determination of IGF-1 (even after pooling samples within a treatment group).

6.2 Oral bioavailability of cGP in adult and infant rats

Oral administration of cGP to rat dams during lactation increased the concentrations of cGP in plasma of dams and pups (Chapter 3). This was associated with an increase in the neuroplasticity in the brains of dams and adult offspring, and an improvement in the learning
and memory of offspring at adolescence and adulthood (Chapters 3 and 4). Taken together, these data showed that oral gavage is an effective route of administering cGP in both adult and infant rats. In support of these findings, oral administration of cGP analogue, c-G-2allyl-P, is previously shown to prevent the scopolamine-induced memory deficits in adult rats [166].

Transport of cGP across the epithelium of gut into blood may involve both paracellular and transcellular pathways [273]. For instance, the glycyl-proline transport system present in enterocytes may play a role in the transcellular transport of cGP [273], but this needs to be investigated. Being a larger molecule than cGP, IGF-1 is not readily transported across the gut epithelium [16,347]. Data from the cGP-administered group further showed that plasma concentrations of cGP were significantly higher in the pups compared with the dams on PN d23. This could be simply due to the fact that exogenous administration of cGP in dams was terminated on PN d22, whereas the pups may have still suckled milk-borne cGP on PN d23. Nevertheless, other possibilities cannot be ruled out, for instance, the greater permeability of infant gut compared with the adult gut for the transport of peptides into circulation [345], and/or the loss of cGP from blood to milk in dams. The determination of complete pharmacokinetic profile of cGP, for instance the transfer to other body organs and excretion in urine, was beyond the scope of this thesis, and therefore, needs further investigation.

Peripheral IGF-1 shows poor central uptake due to its large molecular size, and requires the support of LRP for transportation into the brain [48]. On the other hand, small molecules such as cGP, may readily diffuse through the BBB without the need of carrier proteins [299]. Despite being smaller than IGF-1, the hydrophilic nature of GPE restricts its central uptake under normal conditions. The central uptake of GPE was shown to depend on an increase in the permeability of BBB. For instance, continues intravenous infusion of GPE for 4 h increased the CSF concentrations of GPE only in the HI injured rats but not in the control rats
Contrarily, cGP is lipophilic and the central uptake of cG-2allyl-P is shown to be injury-independent as evident through an increase in the CSF concentrations of cG-2allyl-P in normal control rats following a bolus sub-cutaneous injection [67]. The increased neuroplasticity in cGP-administered dams and their offspring in the current study further suggests that the central uptake of cGP is injury-independent, though the concentrations of cGP in CSF were not measured.

The maternal-infantile transfer of cGP through milk together with the long-lasting improvement in learning and memory of offspring suggests that maternal administration is an effective route of administering cGP to the offspring. This is highly desirable from a pharmacological point of view because of the increased understanding of the benefits of exclusive breastfeeding among women, and the unwillingness of some mothers, especially vegetarians, to give any supplementation or pharmaceutical medicine to their infants [37]. Furthermore, the increased transfer of cGP from blood to milk during peak lactation compared with late lactation, suggests that maternal administration may be more beneficial than the direct infant administration pertaining to its ‘physiologically controlled’ pharmacokinetics.

**6.3 Early life administration of cGP positively influences neurodevelopmental programming in rats**

This thesis investigated the effect of early life administration of cGP on neurodevelopmental programming of rats. Results presented in Chapters 3 and 4 showed that maternal administration of cGP during lactation improved postnatal brain development and learning and memory of offspring. Maternally administered cGP increased the bioavailability of IGF-1 in pups, which was associated with improved recognition memory at adolescence, and spatial learning and memory at adulthood, tested using NORT and MWM, respectively. In addition,
analysis of various markers of neuroplasticity suggested that adult offspring had increased capacity for glutamatergic neurotransmission in hippocampus and striatum.

Clinical application of IGF-1 for the CNS is limited due to its poor central uptake and potential mitogenic and metabolic side-effects. The mechanism of action of cGP involves normalizing IGF-1 function, therefore, it is considered to be safe [34]. There is no direct evidence for the interaction between cGP and IGF-1R, however, the facts that GPE does not interact with IGF-1R [60], and shares many biological features with cGP [67], suggest that cGP may also not interact directly with the IGF-1R. Therefore, unlike IGF-1, cGP may not have mitogenic side-effects. In fact, cGP is shown to suppress IGF-1-dependent tumor growth in a mouse model of lymphoma [34]. The lack of effect of exogenous cGP administration on growth of dams and litter provide an additional evidence (Chapter 5). In addition, no safety concerns have been reported for Trofinetide, a cGP analogue, in the FDA-approved toxicology studies conducted by Neuren Pharmaceuticals in USA [352]. The drug was given orally twice daily to patients with Rett syndrome at doses of 35 mg/Kg or 70 mg/Kg for 28 days.

The improved brain development and function of offspring following maternal cGP administration could be a direct effect of an increase in the bioavailability of IGF-1 during infancy, but may also be influenced by two other factors. Firstly, the increased neuroplasticity in dams could lead to better maternal care, which may influence the brain development and cognitive function of the offspring [353]. Secondly, cGP mediated increase in the bioavailability of IGF-1 in dams, may improve the lactational capacity of dams which may positively influence the cognitive development of the offspring [45,46]. Improvement in lactational capacity of dams may result from an increase in the milk production, delay in the post-lactational onset of involution in mammary gland, and/or the enhanced central regulation of lactation. Each of these additional factors are discussed in the following paragraphs.
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Maternal brain shows long-lasting increase in neuroplasticity and cognition during the postpartum period, which is considered essential for giving optimal care to the offspring [298,354]. Furthermore, it is shown in rats that better maternal care leads to better performance of offspring in MWM, which is associated with increased glutamatergic and cholinergic neurotransmission in the hippocampus of offspring [353]. Because maternal care (e.g. pup retrieval, licking/grooming, nursing [355]) was not quantified in the current study, therefore, the cGP mediated improvement in neuroplasticity of dams may have contributed to the cognitive improvements observed in the offspring, and thus need further investigation.

Increase in maternal lactational capacity is shown to improve the cognitive development of the offspring [45,46, and section 1.3.7 of the current thesis]. In the current study, the lactational capacity of dams was assessed by evaluating milk production, milk composition, onset of post-lactational involution in mammary glands and central regulation of lactation. As shown in Chapter 5, cGP did not improve milk production and composition of dams, and did not delay the onset of post-lactational involution in mammary glands, rather, promoted it. The effect of cGP on central regulation of lactation was investigated by Masters Student, Stanley Yune. The results presented by Stanley in his thesis [356] are discussed in the following paragraph, after a brief introduction to the central regulation of lactation.

Prolactin (PRL) is required for the differentiation of MEC into secretory cells, and thus play a crucial role in the initiation and maintenance of milk production during lactation [357]. PRL is produced in the anterior pituitary gland by cells called lactotrophs. Under normal conditions, PRL regulates its own production via an auto feedback inhibitory mechanism involving the dopaminergic neurons of hypothalamus [358]. Lactotrophs are innervated by a group of tuberoinfundibular dopamine neurons (TIDA), located in the arcuate nucleus region of hypothalamus [357]. TIDA neurons express PRL receptor (PRLR). PRL released by the
anterior pituitary enters the circulation, and is then believed to reach TIDA either through the fenestrated capillaries of median eminence, or via a carrier-mediated transport mechanism present in the choroid plexus across the blood-CSF barrier [358]. Binding of PRL to PRLR activates TIDA, which release dopamine (DA), ultimately resulting in the inhibition of additional PRL secretion by lactotrophs [358,359]. However, the suppression of this auto feedback inhibition mechanism is essential for the maintenance of high plasma PRL concentrations during lactation. Binding of PRL to PRLR activates downstream signaling cascade that involves activation of SH2 domain containing transducer proteins, including the STAT family [357]. STAT5 is the main transducer protein involved in PRL signaling. During lactation, the activation of STAT5 is significantly reduced, probably through the activation of suppressor of cytokine signaling proteins [360], resulting in the dephosphorylation of tyrosine hydroxylase (TH) at one its serine residues [361]. Because TH catalyzes the rate-limiting step in the biosynthesis of DA, therefore, dephosphorylation of TH results in the suppression of DA production [361]. There is a lack of direct evidence for the role of IGF-1 in central regulation of lactation. However, IGF-1 is involved in the regulation of secretion of PRL from anterior pituitary [43,362], and is shown to restore the loss of dopaminergic neurons associated with aging, via an increase in TH immunoreactivity [363]. Therefore, Stanley investigated the effect of cGP on the central regulation of lactation by analyzing the changes in the expression of PRLR and TH in the brains of dams collected in this thesis. He found that cGP did not alter the expression of TH and PRLR in striatum and hypothalamus, respectively, though there was an increase in the expression of synaptophysin in hypothalamus. It is unlikely that increased pre-synaptic plasticity in hypothalamus may have any influence on lactation, which was corroborated by a lack of correlation between synaptophysin and PRLR staining. Instead, it may represent a general increase in neuroplasticity as observed in the hippocampus of dams.
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(Chapter 4). Therefore, Stanley’s data corroborates the results presented in this thesis, and confirms that the improved brain development and function of cGP offspring was not due to an enhancement in the lactational capacity of dams, but likely a direct effect of increased bioavailability of IGF-1 during infancy.

IGF-1 is well known to improve learning and memory via modulation of glutamatergic synaptic plasticity [23, 48, 82]. For instance, IGF-1 administration caused a long-lasting improvement in spatial memory and AMPA-mediated neurotransmission in a mice model of depression [161]. Furthermore, the effects of early life administration of dietary factors, such as DHA, on long-lasting improvement in memory and neuroplasticity are mediated through neurotrophic factors, including IGF-1 [13]. The mechanisms by which cGP and IGF-1 may improve neuroplasticity and long-term memory are discussed in the next section.

6.4 Mechanism of action

Elucidating the intracellular mechanisms underlying the improvement in neuroplasticity in dams and offspring was beyond the scope of this thesis. However, increased IGF-1 bioavailability in dams and infants from cGP group suggests that IGF-1 mediated changes in neuroplasticity may be involved, which are briefly discussed in the following paragraphs.

Glutamate released by the excitatory synapses stimulates the surrounding neurons, astrocytes, pericytes and vascular endothelial cells to release various vasoactive mediators, such as ATP and prostaglandin E₂ (Figure 6.1) [56]. These vasoactive mediators increase the blood flow to the area, resulting in an increased influx of IGF-1-IGFBP-3 complexes, and also cause an activation of MMP-9, resulting in increased cleavage of IGFBP-3 and subsequent increase in the bioavailability of IGF-1 [56]. Increased free IGF-1 can then interact with the IGF-1R located on the endothelial cells. IGF-1R, together with LRP1, transcytose IGF-1 to the
adjoining neurons either directly or via the astrocytes [56]. This parallel activation of neurovascular and neurotrophic coupling is proposed to underlie the activity-dependent increase in IGF-1-mediated neuroplasticity [48]. The elevated concentrations of cGP in plasma may further increase the bioavailability of IGF-1 through competitive binding with IGFBP-3, resulting in increased influx of peripheral IGF-1 into the brain (Figure 6.1).

![Diagram of neuronal and astrocytic interactions](image)

**Figure 6.1**: Peripheral cGP may facilitate the uptake of Circulating IGF-1 into Brain as part of the Neurotrophic Coupling Mechanisms.

Glutamate released by the excitatory synapses stimulates the surrounding neurons, astrocytes, pericytes and the vascular endothelial cells to release various vasoactive mediators, such as ATP and prostaglandin E₂. These
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vasoactive mediators increase the blood flow to the area, resulting in an increased influx of IGF-1-IGFBP-3 complexes, and also cause an activation of MMP-9, resulting in increased cleavage of IGFBP-3 and subsequent increase in the bioavailability of IGF-1. Increased free IGF-1 can then interact with the IGF-1R located on the endothelial cells. IGF-1R, together with LRP1, transcytose IGF-1 to the adjoining neurons either directly or via the astrocytes. This parallel activation of neurovascular and neurotrophic coupling is proposed to underlie the activity-dependent increase in IGF-1-mediated neuroplasticity. The elevated concentrations of cGP in plasma may further increase the bioavailability of IGF-1, resulting in increased influx of peripheral IGF-1 into the brain.

*Abbreviations:* ATP: Adenosine triphosphate; cGP: cyclic-glycine-proline; IGF-1: Insulin-like growth factor-1; IGF-1R: Insulin-like growth factor-1 receptor; IGFBP-3: Insulin-like growth factor binding protein-3; MMP-9: Matrix metalloproteinase 9; LRP1: Low density lipoprotein receptor-related protein 1. Modified with permission [48].

Administration of cGP increased the expression of synaptophysin in hippocampus (Chapter 4), and GluR-1 in striatum (Chapter 4) and frontal cortex (data not shown) of dams. The increase in neuroplasticity in dam’s brain may represent the acute effects of cGP-mediated increase in IGF-1 bioavailability. Several IGF-1 signaling pathways that increase the expression of pre-synaptic proteins and promote trafficking of AMPA receptors to the post-synaptic membrane may be involved, which are briefly discussed below.

Synaptophysin is an integral membrane glycoprotein of synaptic vesicles, and is involved in the generation of vesicle fusion pore in the pre-synaptic membrane [364]. Synaptophysin also plays a key role in endocytosis and recycling of synaptic vesicles, and thus regulates the number of vesicles available for release at the synapse [365]. It is shown that IGF-1 may increase the expression of synaptophysin in hippocampus via the PI3K/Akt and CaMKII signaling pathways [294]. IGF-1 may promote AMPA receptor trafficking via several mechanisms. For instance, increased free IGF-1 may promote the phosphorylation of AMPA receptor sub-units, a process that is essential for the trafficking of receptors to the synapse [366], and/or the phosphorylation of proteins involved in targeting, translocation and anchorage of AMPA receptors to the synaptic membrane [160]. Alternatively, IGF-1 may suppress the endocytosis of AMPA receptors [160]. Furthermore, increase in free IGF-1 may
increase the activation of Ca\(^{2+}\) channels, leading to an increase in the influx of Ca\(^{2+}\) ions and the release of glutamate from the presynaptic terminal [367]. The specific downstream signaling pathways that lead to increased AMPA receptor trafficking are not known, however, it is proposed that the PI3K/Akt/mTOR and Ras/Raf/MAPK/ERK1/2 pathways may be involved [73].

The improved spatial memory and AMPAergic plasticity in brains of adult offspring may represent the long-lasting effects of an increase in IGF-1 bioavailability. The long-term effect of IGF-1 on learning and memory may result from the long-lasting facilitation of activity-dependent synaptic plasticity, which becomes rather independent of the presence of IGF-1 itself [161]. Importantly, the IGF-1 mediated long-term increase in synaptic plasticity is associated with an increase in GluR-1 density in the hippocampus, and involves a mechanism that is dependent on continued protein synthesis [161]. As reviewed in section 1.2.3 of this thesis, CREB-mediated transcription of new synapse growth proteins, including new AMPA receptors, is essential for the formation of permanent or long-lasting memory (Figure 1.5).

Interestingly, IGF-1 can activate CREB through various downstream signaling pathways [18,368,369]. For instance, the efficacy of early life administration of DHA on adult cognition is mediated through IGF-1-induced activation of CREB through the MAPK pathway, ultimately leading to the transcription of genes that are implicated in synaptic plasticity [18]. Another, rather peculiar, example is the Ames dwarf mice, which show negligible levels of systemic IGF-1 but exhibit normal learning and memory function, that is well maintained even during aging [370,371]. The normal cognitive function in Ames dwarf mice is shown to be due to the increase in protein levels of hippocampal IGF-1, and the subsequent activation of PI3K-Akt-CREB signaling pathway, leading to the synthesis of synapse growth proteins [369]. Therefore, as an acute effect, increase in free IGF-1 may increase the activation of CREB via
different pathways, which may ultimately lead to an increase in the pool of AMPA receptors through elevated protein synthesis. Increased number of AMPA receptors may contribute to an increase in the activity-dependent trafficking of these receptors to the synapses. This mechanism may underlie the increased AMPA receptor trafficking following MWM training in adult cGP offspring, however, this needs to be investigated. Of note, GPE is shown to increase the synthesis of synaptic growth proteins, such as the post-synaptic density protein 95 and synapsin 1, via the activation of PI3K/Akt pathway [372].

6.5 Role of cGP in mammary involution and the physiological regulation of IGF-1 function

IGF-1 plays a crucial role in mammary gland development, including the post-lactational involution. In rodents, mammary specific overexpression of IGF-1 is shown to delay involution by reducing the apoptosis of mammary cells and preservation of intact alveoli [42,44]. This is the first study to show the role of cGP in post-lactational involution of mammary gland. It was found that exogenous administration of cGP during lactation accelerates the onset of post-lactational involution by increasing the apoptosis of mammary cells and the loss of intact alveoli during early stages of involution. This was corroborated by an upregulation of cell survival factors, Bcl-xl and IGF-1R, during early-involution. As a regulator of IGF-1 bioavailability, cGP is shown to either inhibit or stimulate IGF-1 function under pathophysiological conditions of elevated or diminished IGF-1 bioactivity, respectively [34]. Involution is associated with a reduction in IGF-1 function. This was evident through a reduction in the phosphorylation of IGF-1R during late (Reg) stage of involution. Exogenous administration of cGP further reduced the phosphorylation of IGF-1R, suggesting that cGP does not prevent the physiological decline in IGF-1 function, but, rather, promotes it. We do not have a clear understanding of the cGP-mediated regulation of IGF-1 function under
physiological conditions. The autocrine regulatory mechanism described earlier is specific to the circulation, and therefore, may or may not apply to the mammary gland tissue as the autocrine regulation may be complicated by the local production of IGF-1 and IGFBP in the tissue (paracrine regulation). Therefore, we can only speculate that exogenous administration of cGP may have restored the decrease in cGP/IGF-1 ratio, resulting from the transient increase in IGF-1 during late (Eng) stage of involution. This may have resulted in the inhibition of IGF-1 function through an increase in the binding between IGF-1 and IGFBP (also refer to Figure 1.2 of Chapter 1, panel C). This is the very first study to show the role of cGP in physiological regulation of IGF-1 function. Previous studies have only investigated the brain-specific role of cGP. Results presented in the current study suggest that similar to IGF-1, cGP may also have pleiotropic effects in the body. Therefore, future studies under different physiological conditions may shed more light onto the cGP-mediated regulation of IGF-1 function.

6.6 Strengths and limitations

Opting maternal route of administration over direct infant administration helped achieve a number of objectives in a single animal experiment. For instance, determining the changes in the concentrations of endogenous cGP in milk during lactation, oral bioavailability of exogenous cGP in infant and adult rats, and the role of cGP in mammary involution. However, the potential effect of cGP on maternal behavior became a confounding factor, and should have been addressed through behavioral tests. Memory tests could not be conducted on dams because of the need to collect plasma and brain samples immediately after the treatment period. The possibility of a separate cohort of dams was limited by the sample size approved by the animal ethics committee. Nevertheless, maternal care could have been assessed by observing licking, grooming, nursing and pup-retrieval behaviors of dams [355]. Another
limitation of this thesis is the lack of behavioral evaluation in female offspring due to the absence of separate equipment. This needs to be addressed in the future studies for the clinical translation of current work. MWM and NORT are the most commonly used behavioral tests for evaluating ability of learning and memory in rodents. However, MWM involves forced learning whereas NORT involves voluntary learning. The performance in NORT is mostly dependent on the curiosity of the animal and their retention of short term memory. Therefore, young animals are good performers in NORT. Contrarily, performance in MWM requires more mature brain function for completing much complicated tasks during learning, for example, the changes in strategy of learning, using references for spatial navigation and knowledge consolidation which are beyond the function of developing brains. However, given its speed of administration, NORT could have been conducted in the older animals again. Although evidence suggests that IGF-1 actions on synaptic plasticity may be NMDA-independent, some studies point to a role of IGF-1 in modulating the expression of NMDA receptor sub-units [163,164]. Therefore, in addition to AMPA receptors, evaluating the expression of NMDA receptors may have proved beneficial, especially for determining neuroplasticity in pups’ brain because ‘silent’ synapses, the excitatory synapses that contain NMDA receptors but not AMPA receptors, are abundant during infancy [373]. Because cG-2allyl-P is shown to restore induced memory deficits in rats via modulation of cholinergic neurotransmission [166], therefore, expression of acetylcholine receptors could have been evaluated. Collecting brains from adolescent offspring would have helped correlate the behavioral data with the changes in neuroplasticity at adolescence. Milk production in dams could not be evaluated using alternative methods, such as the mammary gland weight difference method and the tritiated water dilution method, because of the limitations on approved sample size, and the potential influence on behavior of pups following the injection
of tritiated water [261]. An attempt was made to weigh individual pups, rather than the litter, in the beginning of the experiment, however, this had to be discontinued due to extensive cannibalism shown by dams. The total protein and lipid content of milk was measured using the infrared spectrometer Direct Detect™, which requires very low sample volumes per assay. While the protein concentration can be accurately determined, the assay can only measure the relative absorbance of lipids. Lastly, collecting mammary glands samples at peak lactation would have allowed investigations into the effect of cGP on mammary gland development during lactation.

6.7 Future directions

As mentioned before, investigations into the specific intracellular mechanisms that underlie improved neuroplasticity in dams and offspring was beyond the scope of this thesis, and therefore, need to be investigated in future studies. Nevertheless, an attempt was made to evaluate the expression of pIGF1R in hippocampus of adult offspring using both immunohistochemistry and immunofluorescence. However, the assays could not be optimized, primarily due to the loss of tissue at the antigen retrieval step. In future, alternative techniques of semi-quantitative protein quantification, such as western blotting, can be utilized. However, the disadvantage would be the inability to separately quantify different sub-regions of hippocampus.

Better neurodevelopmental programming may reduce the susceptibility of the offspring for age related cognitive decline. For instance, early life supplementation with choline promotes resilience of offspring to age-related memory decline [90]. Considering that cG-2allyl-P restores learning and memory deficits in aged rats [166], it becomes imperative to investigate
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whether early life administration of cGP can ameliorate cognitive deficits associated with aging.

While these research areas represent an extension of the work presented in this thesis, following sections describe the implications of current work for future research.

6.7.1 Infant formula

The efficacy of cGP in improving brain development and adult cognition provides potential translational opportunities for its clinical application as maternal supplementation or as a component of infant formula (IF). Because this thesis did not evaluate the effect of cGP on mammary gland development during lactation, therefore, more preclinical work is needed to address this issue.

Despite the well documented nutritional, health and psychological benefits of breastfeeding, and the recommendation by the World Health Organization for exclusive breastfeeding up to first 6 months of life, many women have to switch to IF much earlier (6-8 weeks after childbirth). There are a multitude of factors that contribute to this, for instance, insufficient milk supply, pre-term birth, caesarean birth, maternal obesity, and younger maternal age to name a few [374-377]. With our advancing knowledge of the complex composition of human breast milk, there is an increasingly global need for the reformulation of IF, which are primarily based on cow’s milk, and therefore, lack a number of bioactive components present in human breast milk. More recently, early life administration of 2’-FL in rats, an oligosaccharide which is present in human milk but not cow’s milk, was shown to cause long-lasting improvement in learning and memory of offspring [12]. Compared with the control group, the rat pups supplemented with 2’-FL before weaning, performed better on behavioral tasks, including the Y-maze test and the NORT, at adolescence and adulthood. Results presented in this thesis
show that early life administration of cGP causes long-lasting improvement in brain development and function of offspring that persist into adulthood. Furthermore, our recent preliminary experiments have shown that cGP is present in human milk but not cow’s milk. However, because IGF-1 is present in cow’s milk, therefore, protein hydrolysis, for instance during fermentation, may potentially lead to the production of cGP. Results from the current study have led to a research proposal which aims to identify dairy based ingredients that contain cGP, and their potential use for the enrichment of IF. The research proposal, which is in collaboration with Fonterra, New Zealand, is briefly outlined below.

There are four main objectives of the proposal:

1) Demonstrate the beneficial effects of supplementation of cGP in infant rats on brain development and adult cognitive function.

2) Measure the concentrations of cGP in at least 10 different dairy based ingredients. These dairy ingredients will be identified based on the known amino acid sequences and the outcomes of protein hydrolysis. Peptide sequences containing GPX (X = any amino acid) will be analyzed by liquid chromatography – mass spectrometry to confirm the molecular structure.

3) Determine the efficacy of these dairy ingredients in promoting brain development and adult cognition.

4) Measure cGP concentrations in human breast milk and existing IF\textsuperscript{1} to determine the optimal level of cGP supplementation needed for IF.

The outcome of this research will be the preclinical development of one evidence-based and IP protected new dairy ingredient, which will provide essential information for the clinical

\textsuperscript{1} The production of IF from dairy milk involves protein hydrolysis, therefore, low and variable amount of cGP may be present in some existing IF.
development and marketing of a novel IF. A new IF that is specifically designed for better brain development and long-term brain function would be a major asset for the high-end international market, and is thus expected to stimulate the economy of New Zealand by promoting dairy exports.

6.7.2 cGP as a biomarker of IGF-1 function

Depressed IGF-1 levels are implicated in the pathogenesis of several neurodevelopmental disorders that are associated with severe cognitive impairments. These include the ASD [378], progressive encephalopathy, hypsarrhythmia, and optic atrophy (PEHO) syndrome [379], symptomatic infantile spasms [380] and infantile neuronal ceroid lipofuscinosis [381]. Results from the current thesis show that cGP increases the trophic support provided by IGF-1 during early life. Therefore, in addition to IGF-1, cGP levels may also be altered in the neurodevelopmental disorders, and thus deserve investigation in future studies. Furthermore, cGP/IGF-1 molar ratio is proposed to be a more reliable biomarker of IGF-1 function compared with a) the IGF-1/IGFBP3 molar ratio, which may not be a reliable indicator of ‘free’ (bioactive) IGF-1 under different pathological conditions [258,382], and b) the IGF-1 concentration, which quantifies ‘total’ IGF-1. Recently, changes in cGP and cGP/IGF-1 molar ratio were proposed to be potential biomarkers in the disease progression and recovery from stroke and Parkinson’s disease (PD), conditions that are associated with impairments in IGF-1 function [383]. The plasma concentrations of cGP and cGP/IGF-1 molar ratio drastically decreased following the onset of stroke in human patients, and tended to increase during the recovery period². The 4-week blackcurrent anthocyanin supplementation in PD patients was associated with increase in CSF concentrations of cGP, which positively correlated with the changes in the concentrations of cGP and the cGP/IGF-1 molar ratio in plasma. In both of

² This is an ongoing clinical trial in our research group, and is being carried out by the PhD student, Dawei Fan.
these studies, no change in total IGF-1 concentration or IGF-1/IGFBP-3 molar ratio was observed. On a different note, weight loss in obese women is shown to be associated with a reduction in IGFBP-3 concentrations and an increase in cGP/IGF-1 molar ratio in plasma, with no changes in total IGF-1 concentration or IGF-1/IGFBP-3 molar ratio [258]. The reduced IGFBP-3 and increased cGP/IGF-1 molar ratio were proposed to be the autocrine responses for increasing IGF-1 bioavailability because IGF-1 dysfunction in obesity is characterized by a reduction in the plasma concentrations of IGF-1 [258,384]. No study has evaluated the changes in cGP concentrations in neurodevelopmental disorders. The results presented in the current thesis, therefore, validate future research into the measures of cGP and cGP/IGF-1 molar ratio as potential biomarkers of IGF-1 dysfunction in neurodevelopmental disorders.

The restoration of IGF-1/PI3K/Akt pathway is proposed as a therapeutic target for neurodevelopmental disorders [378]. The therapeutic applications of IGF-1 are limited due to its poor central uptake and potential metabolic (e.g. hypoglycemia) and mitogenic side-effects [29]. On the other hand, cGP has much more favorable pharmacokinetic profile compared with IGF-1 and other IGF-1 derived small peptides such as GPE, owing to its enzymatic stability and effective central uptake even under normal conditions. While GPE and its analogues have been extensively tested in various rodent models of neurodevelopmental disorders [385,386], the efficacy of cGP still need to be investigated. As an example, reduction in cortical GluR-1 expression is suggested to cause learning and memory impairments in Fragile X syndrome (FXS), a neurodevelopmental disorder associated with a reduction in CSF levels of IGF-1 [387]. Although GPE analogue, G-2m-PE, reverses some of the adverse phenotypes associated with FXS, it does not interact with the glutamate receptors [385]. In the current study, the efficacy of cGP in increasing GluR-1 expression in frontal cortex (data not shown) suggests
that cGP may be a potent therapeutic candidate in the treatment of FXS, and deserves investigation in the future studies.

While mammary specific overexpression of IGF-1 delays post-lactational involution by reducing the apoptosis of cells, it also promotes tumorigenesis [336]. Furthermore, serum IGF-1 levels are higher in breast cancer patients compared with normal controls [388]. Elevated serum IGF-1 concentrations are associated with reduced involution of terminal duct lobular units, leading to increased risk of breast cancer in women with benign breast disease [339]. Evaluation of serum IGF-1 levels is, therefore, proposed as a biomarker for breast cancer risk prediction [339]. Results from the current thesis show that cGP promotes post-lactational involution in mammary glands of rats by increasing the apoptosis of cells, possibly by reducing IGF-1 bioactivity. Therefore, future investigations into the measures of cGP and cGP/IGF-1 molar ratio as additional biomarkers of breast cancer risk-prediction are validated. Furthermore, cGP may prove to be an effective candidate in anti-IGF therapies against breast cancer. In support, cGP is shown to suppress IGF-1-mediated tumor growth in mice [34].

6.7.3 Energy homeostasis and cognition

Though this thesis has focused on the roles of IGF-1 in memory, synaptic plasticity and mammary involution, IGF-1 is also a crucial regulator of energy homeostasis, such that impaired IGF-1 signaling leads to disturbances in energy metabolism. In fact, the disturbances in maternal metabolism due to IGF-1 dysfunction may negatively affect learning and memory of the offspring [389]. For instance, maternal obesity is associated with a reduction in plasma IGF-1 levels [258,390], and causes long-lasting impairments in the learning and memory of offspring [280,391]. The learning and memory deficits in offspring are, in turn, associated with reduced synaptic plasticity [280] and impaired IGF-1 signaling in hippocampus [279,281]. Similarly, maternal diabetes induces learning and memory deficits in the offspring.
[392-394], which are associated with impaired IGF-1 signaling in the hippocampus [395],
cerebellum [396] and cerebellar cortex [397]. Intriguingly, increased bioavailability of IGF-1
reduces the risk of diabetes during pregnancy [398], and promotes weight loss in obese
mothers [258]. In the current thesis, administration of cGP to a normal population of rat dams
improved the bioavailability of IGF-1 in both dams and offspring, which further led to an
improvement in neuroplasticity. Future studies should investigate whether the administration
of cGP to diabetic or obese dams could restore the disturbances in maternal metabolism and
the corresponding deficits in learning and memory of offspring, via improvement in IGF-1
signaling.

6.8 Conclusions

IGF-1 plays a critical role in postnatal brain development and function, for instance, learning
and memory. As a metabolite of IGF-1, cGP is shown to regulate the bioavailability of IGF-1
and thus normalize IGF-1 function under pathophysiological conditions of excessive or
diminished IGF-1 bioactivity. Endogenous cGP is present in rat brain but its presence in rat
milk and plasma has never been investigated. Similar to IGF-1, cGP is also shown to possess
anti-amnesic properties as demonstrated through various animal models of induced memory
deficits. However, the role of cGP in neurodevelopmental programming of normal brain
remains unexplored. Mammary specific overexpression of IGF-1 delays the onset of post-
lactational in mammary glands, however, the effect of exogenous administration of cGP on
mammary involution is not known.

Results presented in the current thesis showed that endogenous cGP is present in rat milk and
plasma during lactation and postnatal development, respectively. The high plasma
concentrations of endogenous cGP were suggested to be an autocrine regulatory response to
low plasma concentrations of IGF-1 during infancy, resulting in an increase in the bioavailability of IGF-1. Exogenous cGP showed oral bioavailability in lactating rat dams, and effective maternal-infantile transfer through milk. Increase in plasma cGP concentrations led to an increase in the bioavailability of IGF-1 in both dams and pups. Maternally transferred cGP improved learning and memory of offspring when tested at adolescence and adulthood. This was associated with improved glutamatergic neuroplasticity in hippocampus and striatum of adult offspring. The cognitive improvement in the offspring was not influenced by the lactational capacity of dams because cGP had no effect on milk production and composition or the central regulation of lactation. Furthermore, cGP accelerated the onset of post-lactational involution in mammary glands by promoting the apoptosis of mammary cells and the loss of intact alveoli during early-involution, and by reducing the bioactivity of IGF-1 during late-involution.

This is the first study to show the role of cGP in autocrine regulation of IGF-1 in vivo, and the efficacy of maternally-administered cGP in optimizing normal brain development and function of offspring. The role of cGP in promoting mammary gland involution, and the physiological decline in IGF-1 function are also novel findings. Future studies are needed to investigate the intracellular mechanisms involved in cGP-mediated long-term and short-term improvements in neuroplasticity, and to understand the role of cGP in physiological regulation of IGF-1 function.
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**Title of New Work**: Cyclic glycine-proline improves memory of offspring: a pharmacological and nutritional investigation

**Publisher of New Work**: University of Auckland

**Expected publication date**: Jul 2016

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